Microarray analysis during adipogenesis identifies new genes altered by antiretroviral drugs

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Objective: To elucidate the pathogenesis of HAART-associated lipodystrophy, by investigating the effects of antiretroviral drugs on adipocyte differentiation and gene expression profile.

Design and methods: Analysis of gene expression profile by DNA microarrays and quantitative RT–PCR of 3T3-L1 preadipocytes treated with the nucleoside reverse transcriptase inhibitors (NRTI) lamivudine, zidovudine, stavudine, and zalcitabine, and with the protease inhibitors (PI) indinavir, saquinavir, and lopinavir during maturation into adipocytes.

Results: Under standard adipogenic differentiation protocols, PI significantly inhibited adipocyte differentiation, as demonstrated by cell viability assay and Oil Red O staining and quantification, whereas NRTI had mild effects on adipogenesis. Gene expression profile analysis showed that treatment with NRTI modulated the expression of transcription factors, such as *Aebp1*, *Pou5f1* and *Phf6*, which could play a key role in the determination of the adipocyte phenotype. PI also modulated gene expression toward inhibition of adipocyte differentiation, with up-regulation of the Wnt signaling gene *Wnt10a* and down-regulation of the expression of genes encoding master adipogenic transcription factors (e.g., C/EBPα and PPARγ), oestrogen receptor β, and adipocyte-specific markers (e.g., Adiponectin, Leptin, Mrap, Cd36, S100A8).

Conclusions: This study identifies new genes modulated by PI and NRTI in differentiating adipocytes. Abnormal expression of these genes, which include master adipogenic transcription factors and genes involved in lipid metabolism and cell cycle control, could contribute to the understanding of the pathogenesis of HAART-associated lipodystrophy.
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Keywords: HAART, lipodystrophy, adipocytes, Wnt pathway, microarray analysis, nucleoside reverse transcriptase inhibitors, protease inhibitors

Introduction

HAART, a treatment consisting of a combination of protease inhibitors (PI), nucleoside analogue reverse transcriptase inhibitors (NRTI), and non-NRTI, is associated with severe metabolic side-effects, including lipodystrophy [1]. The mechanism at the basis of HAART-associated lipodystrophy seems to involve dysregulation of fat cell growth and differentiation by both PI and NRTI [1–4]. In this study, we used

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microarray analysis to identify genes that are modulated by PI and NRTI in differentiating 3T3-L1 preadipocytes, a widely used *in vitro* model of adipogenesis [5].

Methods

3T3-L1 cells (clone 173, American Type Culture Collection, Rockville, Maryland, USA) were grown and differentiated into mature adipocytes according standard protocols and treated with NRTI and PI during differentiation (see Figs 1 and 2). Adipogenic differentiation and lipid content were measured by lipid-specific Oil-Red O staining. Gene expression profile of treated versus untreated 3T3-L1 cells was evaluated by DNA microarray analysis and quantitative real-time RT–PCR (See Fig. 2). For microarray analysis, polyA$^+$ RNA was isolated from 3T3-L1 cells using Micro-Fast Track^{TM} 2.0 Kit (Invitrogen, Milan, Italy) according to the manufacturer's instructions. For each sample, 3 μg polyA$^+$ RNA was reverse transcribed and labelled using SuperScript II reverse transcriptase (Invitrogen), oligodT primers, and Cy3-dCTP or Cy5-dCTP dyes (Amersham Biosciences, Milan, Italy). Labelled cDNA probes were hybridized to microarray glass slides produced by CRIBI core facility (University of Padova, Italy), which contained 1 344 370 mer oligonucleotides (Operon version 1.1, designed on Mouse Unigene clusters). After hybridization, slides were washed and scanned on a Affymetrix 428^{TM} Array Scanner (MWG-Biotech AG, Ebersberg, Germany) and the images analyzed by ImaGene 5.6 and GeneSight 4.1.6 softwares (BioDiscovery, El Segundo, California, USA). Data analysis included hierarchical clustering to group experimental conditions according to expression profile, differential regulation analysis to determine the significance of average change in expression (ratio of drug-treatment/control), and *t* test to determine the significance of differences between different experimental conditions. $P < 0.005$ was taken as significant. Microarray experiments were performed in duplicate and repeated two times. Each sample was analyzed in duplicate, including reciprocal labeling of fluorochromes.

Results and discussion

Differentiation and proliferation analysis

Treatment of 3T3-L1 cells during differentiation with the NRTI zidovudine (ZDV) 10 μM and lamivudine (3TC) 20 μM, either alone or in combination, did not significantly alter cell viability and adipogenesis, as evaluated by counting the number of cells and the number adipocytes upon lipid staining (Fig. 1). At variance, treatment with the PI indinavir (IDV) 20 μM and saquinavir (SQV) 20 μM, either alone or in combination with NRTI, significantly reduced the number of differentiated cells, without any evident toxic effect, assayed by total cell counting. This effect was more evident with SQV than with IDV and in late phases of differentiation. In fact, on day 6 and 10, treatment with SQV and IDV reduced by 40% and 30%, respectively, the number of differentiated cells, which were characterized by the presence of smaller lipid droplets than control cells (Fig. 1a). A significant reduction in lipid accumulation of PI-treated 3T3-L1 cells was confirmed by spectrophotometric measurement upon Oil Red O staining (Fig. 1b). The stronger effect of PI than NRTI on adipocyte differentiation could in part be explained by the strong protein binding of PI *in vivo*, at variance with NRTI, which are not highly protein bound [6], and therefore the higher concentration of active PI drug *in vitro*.

Analysis of gene expression profile during adipocyte differentiation

To identify genes that were modulated in the process of adipocyte differentiation, the gene expression profile of 3T3-L1 cells grown in the presence of the differentiation medium was compared with 3T3-L1 preadipocytes grown in medium without supplementation of adipogenic factors. On day 3 of differentiation (early adipogenesis), a total of 1091 genes (8.1% of the expressed genes) were significantly altered as compared to 3T3-L1 preadipocytes. Up-regulated genes encoded adipocyte-specific transcription factors, enzymes involved in lipid and carbohydrate metabolism and adipocytokines, whereas down-regulated genes encoded mainly proteins of the extracellular matrix and cytoskeleton. On day 10 of differentiation (late adipogenesis), a total of 1084 genes were up- or down-regulated with respect to preadipocytes. Over-expressed genes included those encoding adipose tissue-specific marker and adipocytokines, such as aP2, angiotensinogen, haptoglobin, adipsin, adiponectin, CD36 antigen, and fat-specific gene 27. Under-expressed genes included those encoding Wnt signaling molecules, extracellular matrix proteins, and proteins expressed in fibroblasts (e.g., Wnt1 inducible signaling pathway proteins 1 and 2, high mobility group proteins, alpha actin, tissue inhibitor of metalloproteinase, stromal cell derived factor 1). A list of genes modulated during adipogenesis is given in Table 1.

Gene expression profile of 3T3-L1 cells treated with antiretroviral drugs during adipogenic differentiation

Microarray analysis was subsequently performed to investigate the effect of antiretroviral drug combinations on gene expression profile of differentiating 3T3-L1 cells. Since the *in vitro* effect of antiretroviral drugs on gene expression of 3T3-L1 cells has been shown to occur mainly in the early phases of differentiation [2,7], the time point corresponding to day 3 of differentiation was chosen for microarray experiments. To analyse the effect of NRTI, 3T3-L1 cells were induced to differentiate in the presence or absence of ZDV 10 μM and 3TC 20 μM,

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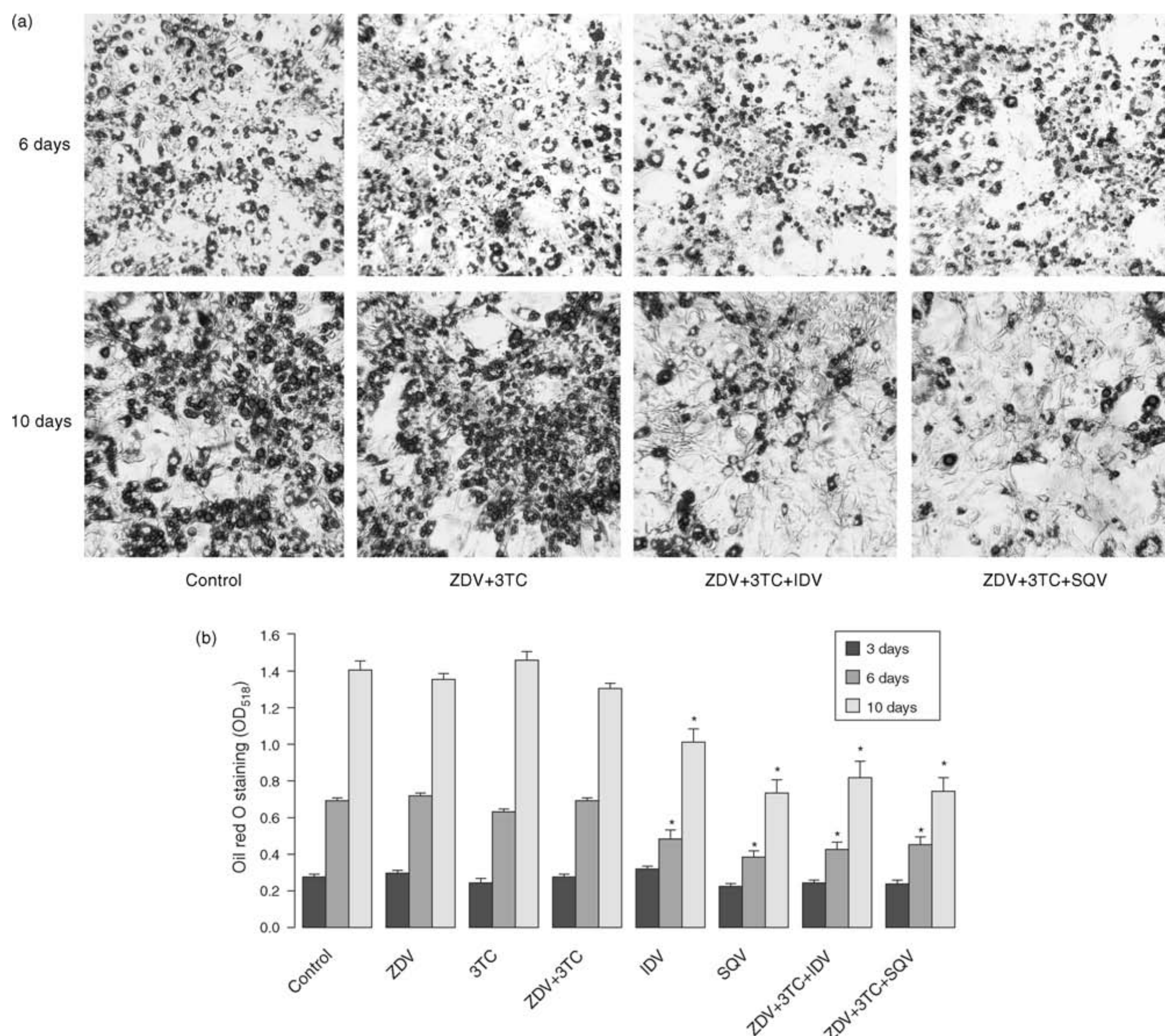

Fig. 1. Effect of antiretroviral drugs on 3T3-L1 adipogenic differentiation. In all experiments, 3T3-L1 cells were grown in Dulbecco's modified Eagle medium supplemented with 10% foetal bovine serum, 2 mM glutamine, 200 U/ml penicillin, and 150 μg/ml streptomycin (all from Invitrogen S.R.L., Milan, Italy). Differentiation was induced by incubating 1-day post-confluence 3T3-L1 cells (day 0) with the above medium supplemented with 2 μM insulin (Eli Lilly, Indianapolis, Indiana, USA), 1 μM dexamethasone, and 0.5 mM isobutylmethylxanthine (Sigma-Aldrich Srl, Milan, Italy) for 3 days. On day 3, the medium was replaced with medium without isobutylmethylxanthine and re-fed every 3 days until day 10. To evaluate the effect of antiretroviral drugs on adipogenic differentiation, 3T3-L1 preadipocytes were grown and differentiated in 12-well dishes on glass coverslips in the presence or absence of ZDV (GlaxoSmithKline, Brentford, Middlesex, UK) 10 μM, 3TC (GlaxoSmithKline) 20 μM, IDV (Merk Sharp & Dohme Laboratories, Harlow, Essex, UK) 20 μM, and SQV (Roche SpA) 20 μM, alone or in combination, as indicated in the figure. On day 0 of the differentiation protocol, antiretroviral drugs were added to the medium and replenished every 2 days. Adipogenic differentiation and lipid content were monitored by microscopy analysis and by lipid-specific Oil-Red O staining on days 3, 6, and 10. Cell viability was determined by Trypan-blue exclusion dye test (Sigma-Aldrich). In particular, after 3, 6, and 10 days from induction of adipocyte differentiation, cells were fixed in 10% formalin for 1 h at 4°C, washed in water and stained with a solution 60% in water of 0.5% Oil-Red-O in isopropanol for 15 min at room temperature and then photographed. Representative images at days 6 and 10 of differentiation are shown in (a) (200 × magnifications). The lipid content was measured by isopropanol extraction of dye and optical density quantification at 518 nm with a BioPhotometer (Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany). Results are expressed as mean ± SD in (b). Experiments were performed three times in duplicate. $*P<0.05$ *vs* control (unpaired t test).

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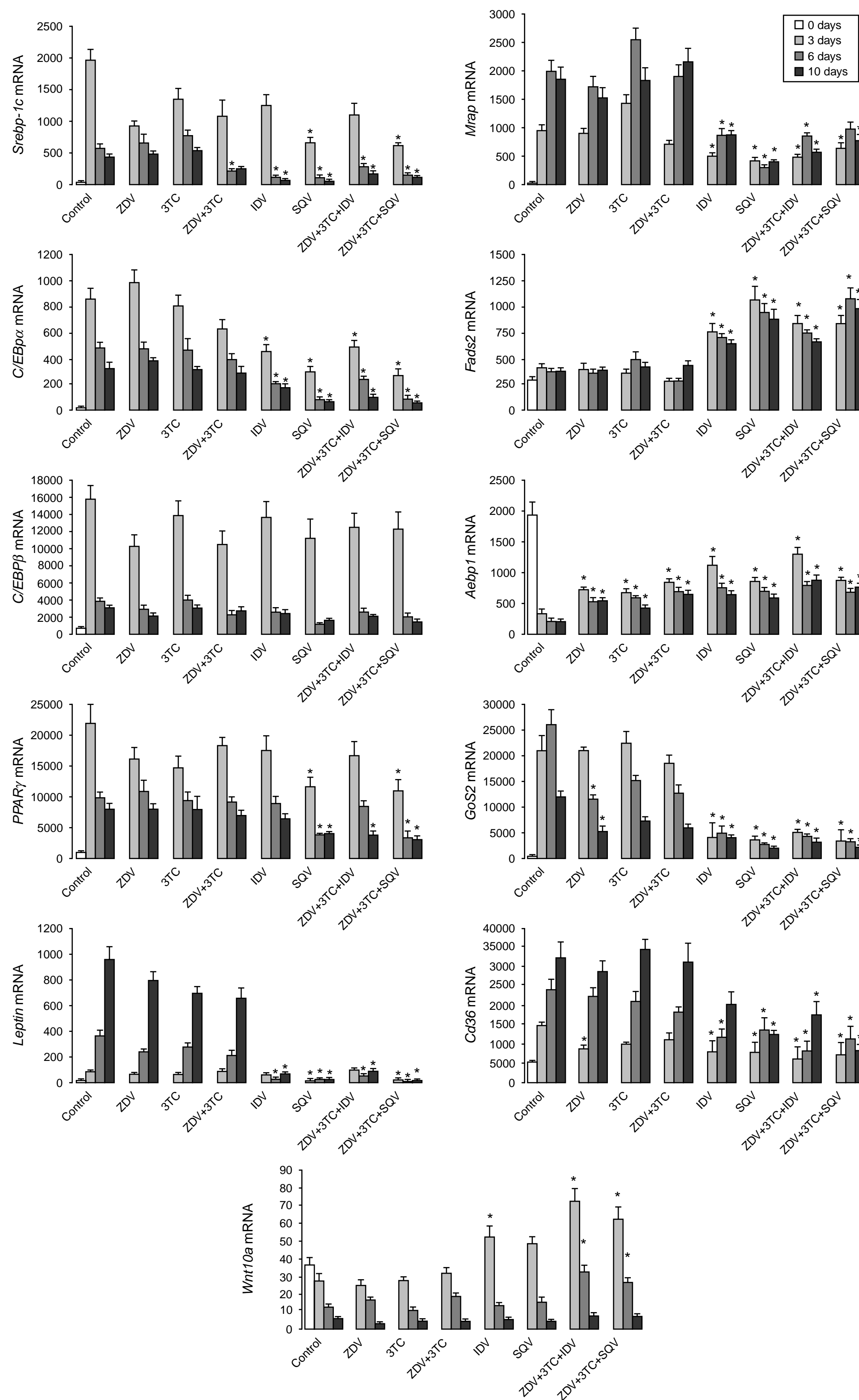

Fig. 2. Effect of antiretroviral drugs on expression of adipogenic marker genes. 3T3-L1 preadipocytes were induced to differentiate into adipocytes as reported in Fig. 1 in the presence or absence of antiretroviral drugs at the following concentrations: ZDV 10 μM, 3TC 20 μM, IDV 20 μM, SQV 20 μM. On day 0, 3, 6, and 10 of differentiation, total RNA was purified from treated and untreated (control) 3T3-L1 cells. mRNA levels of *Srebp-1c, C/EBPα, C/EBPβ, PPARγ, Leptin, Mrap, Aebp1, Fads2, Cd36, G0s2*, and *Wnt10a* were measured by quantitative real-time RT–PCR and normalized against *β2-microglobulin* mRNA. In

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whereas to analyse the effect of the combination of two NRTI with a PI, 3T3-L1 cells were grown in the presence of ZDV 10 μM and 3TC 20 μM, associated with either IDV 20 μM or SQV 20 μM. For each treatment protocol, the gene expression profile of drug-treated 3T3-L1 cells was compared with that of untreated (i.e., cultured in the same differentiation medium without antiretroviral drugs) 3T3-L1 cells at day 3 of differentiation. To analyse the global cellular genetic responses after the various treatments, microarray data were subjected to hierarchical clustering analysis, which showed that gene expression profiles of cells treated with the two combinations of PI–NRTI were quite similar, but markedly distinct from that of treatment with only NRTI. Moreover, drug combinations including a PI had a greater impact on cellular gene expression profile than treatment with NRTI, as suggested by the higher number of up- and down-regulated genes. In fact, when 3T3-L1 cells were treated with the combination of NRTI, a total of 58 genes were differentially regulated at 99% confidence level, whereas when cells were treated with the NRTI and IDV or SQV, the number of differentially regulated genes increased to 98 and 132, respectively. Of these genes, 44 were differently regulated and had the same expression pattern in all three treatment conditions and 26 were differently regulated in the two PI-including treatment conditions, but not in the treatment condition including only NRTI.

Genes modulated by NRTI during 3T3-L1 early adipogenic differentiation

Of the 44 genes significantly modulated by NRTI, both alone and in combination with a PI, 32 were up-regulated by treatment, whereas 12 were inhibited (Table 2). Induced genes included transcription regulators and genes involved in signal transduction. Some of these genes showed increased expression during adipogenesis and were further enhanced by antiretroviral drugs, but others, such as *Aebp-1* (adipocyte enhancer-binding protein 1) and *Timp-2* (tissue inhibitor of metalloproteinase 2), were found to be repressed upon adipocyte differentiation, but markedly induced by treatment with NRTI. Aebp1 was originally characterized as a repressor of transcription of the adipocyte fatty acid binding protein gene *aP2* [8], an important marker of adipocyte late differentiation. Aebp1 seems to correspond to aortic carboxypeptidase-like protein, i.e, a secreted protein associated with the extracellular matrix whose expression is induced during smooth muscle differentiation [9]. Over-expression of this protein in preadipocytes has been reported to inhibit adipogenesis [10,11] and to promote preadipocyte transdifferentiation into smooth muscle-like cells [10], even though this finding has not been confirmed by other authors [12]. Tissue inhibitors of metalloproteinase are a family of four secreted proteins (TIMP-1 to TIMP-4) that selectively inhibit matrix metalloproteinases. The proteolytic activity of matrix metalloproteinases has been hypothesized to play a critical role in the early step of adipocyte differentiation, as suggested by their differential regulation in adipose tissue and by the demonstration that inhibitors of matrix metalloproteinases decrease C/EBPβ expression and adipocyte differentiation [13].

Down-regulated genes included *Faim* (fas apoptotic inhibitory molecule), a negative regulator of apoptosis [14]; genes encoding transcription factors, such as *Pou5f1* and *Phf6*; and genes encoding lipid metabolizing enzymes up-regulated in mature adipocytes, such as *Hadh* and *Elovl3*. *Pou5f1* encodes a POU transcription factor expressed by early embryo cells and germ cells, whose activity is essential for maintaining pluripotency in embryonic stem cells [15]. *Phf6* encodes a novel zinc finger gene of unknown function. Mutations of the corresponding human gene are responsible of the Borjeson–Forssman–Lehmann syndrome (BFLS; OMIM 301900), a X-linked disorder of intellectual disability, characteristic craniofacial features, hypogonadism, hypometabolism, obesity with marked gynecomastia, swelling of subcutaneous tissue of the face, short stature and epilepsy [16]. *Hadh*, which encodes hydroxylacyl–coenzyme A dehydrogenase, is a mitochondrial enzyme that catalyses a step of fatty acid β-oxidation. Over-expression of this enzyme allows the mitochondria to maintain a high rate of oxidative phosphorylation and production of ATP, whereas its down-regulation may determine cell death [17]. The *Elovl3* gene belongs to the *Elovl* gene family coding for microsomal enzymes involved in elongation of very long chain fatty acids. Its expression is dramatically increased in mouse brown adipose tissue upon cold stimulation and this induction is under the control of PPARα, whereas it is inhibited by LXR agonists and SREBP-1 activation [18].

Some genes were modulated only by NRTI treatment, but not by treatment conditions including PI. Up-regulated genes included *Bid3*, an important inductor of apoptosis through the regulation of mitochondrial

Fig. 2 (*continued*)
particular, total RNA was isolated using the RNeasy kit (Qiagen S.p.A., Milan, Italy). Random primed cDNA was synthesized from 3 μg total RNA using MuLV reverse transcriptase (Applied Biosystems, Foster City, California, USA). Real-time RT–PCR was performed with SYBR Green in the ABI PRISM 7900 sequence detection system (Applied Biosystems). Absolute quantification of transcripts was performed against standard curves obtained by amplification of serially diluted solutions of pCR2.1 plasmids (Invitrogen), in which target sequences were subcloned. Each sample was normalized to its *β2-microglobulin* mRNA content, as measured by quantitative RT–PCR. Primer sequences used are available on request. mRNA values are reported as $\times 10^3$ copies/μg total RNA and data represent mean and SD of three experiments performed in duplicate. $*P < 0.05$ versus control (unpaired t test).

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Table 1. Genes significantly up- and down- regulated during 3T3-L1 preadipocyte differentiation[a].

Decription	Day 3 adipocyte/ preadipocyte ratio	Day 10 adipocyte/ preadipocyte ratio
015P16_Eosinophil-associated ribonuclease 1	59.45	1798.32
016P08_Eosinophil-associated ribonuclease 3	44.29	423.48
005G21_Adipsin	33.00	50.40
034J13_RIKEN cDNA 9030611O19 gene	25.51	23.94
003P17_G0/G1 switch gene 2	19.99	227.98
003E03_Glutamine synthetase	16.81	13.00
009J12_Complement component 3	15.49	14.49
002L24_Pyruvate decarboxylase	14.97	62.16
009B08_CD36 antigen (fatty acid translocase)	12.81	22.40
017I05_Carbonic anhydrase 5b	12.81	22.40
007N22_Lipocalin 2	12.49	36.34
011I12_Haptoglobin	12.08	35.84
003E08_Angiogenin	11.89	1072.62
007B19_Fat specific gene 27	11.49	17.00
022F04_RIKEN cDNA 2310001A20 gene	11.29	11.84
004J24_Solute carrier family 25, member 10	10.74	5.97
014D06_Breast cancer anti-oestrogen resistance 3	10.10	4.12
012N09_Wnt inhibitory factor 1	9.99	1.00
009B10_Acyl-Coenzyme A dehydrogenase, very long chain	9.65	4.99
010L18_Diacylglycerol acyltransferase	9.54	10.20
007F14_Angiotensinogen	9.46	24.75
004I24_Fatty acid synthase	8.77	4.12
021G14_melanocortin 2 receptor accessory protein	8.75	89.74
004J02_Adiponectin	8.66	49.40
009N13_Peroxisomal biogenesis factor 11a	7.94	13.39
007P23_Solute carrier family 2, member 4	7.88	70.48
035K06_RIKEN cDNA 0610010B06 gene	7.84	7.04
005N20_Orosomucoid 1	7.56	79.79
027A21_RIKEN cDNA 5730438N18 gene	7.21	4.32
009C18_Sulfotransferase family 1A, phenol-preferring, member 1	6.97	322.97
008H13_Aldehyde dehydrogenase family 1, subfamily A4	6.67	107.03
014F05_Uridine monophosphate kinase, pseudogene	6.60	4.47
003P03_Annexin A8	6.52	1.36
012I09_Fatty acid Coenzyme A ligase, long chain 2	6.24	15.56
001M06_Adipocyte protein aP2	6.23	14.30
011J17_Regulator of G-protein signaling 2	6.13	18.23
016K01_Killer cell lectin-like receptor, subfamily A, member 3	5.92	30.47
011E13_Angiogenin related protein 2	5.91	148.41
001H04_Fatty acid binding protein 5, epidermal	5.85	1.70
023H10_Thyroid hormone responsive SPOT14 homolog	5.78	1036.81
014O01_Calcium binding protein Kip 2	5.56	37.45
017B10_Proviral integration site 1	5.46	4.17
017K08_Integrin alpha 7	5.46	334.84
003N01_Glycerol kinase	5.34	1.47
010I24_Peroxisomal membrane protein 2, 22 kDa	5.26	275.40
017I24_Stearoyl-Coenzyme A desaturase 1	5.01	6.52
005A24_Cytochrome P450, 2f2	4.94	244.15
003P20_Peroxisome proliferator activated receptor gamma	4.86	7.33
011K22_Carbonyl reductase 1	4.60	9.13
018M13_MAP kinase-interacting serine/threonine kinase 1	4.60	11.62
008A03_Glycerol phosphate dehydrogenase 1, cytoplasmic adult	4.59	39.77
016N21_ARL-6 interacting protein-2	4.51	5.62
021I08_RIKEN cDNA 2310004B05 gene	4.40	3.99
018A09_CCAAT/enhancer binding protein (C/EBP), alpha	4.39	2.78
018M09_FK506 binding protein 5 (51 kDa)	4.36	9.04
027D17_RIKEN cDNA 2310004G06 gene	4.32	3.97
018O13_Integrin alpha 6	4.26	2.30
002P06_Cyclin-dependent kinase inhibitor 2C (p18)	4.08	7.68
008A20_ADP-ribosylation-like 4	4.06	30.74
012M11_Glyceronephosphate O-acyltransferase	4.02	6.55
033E07_RIKEN cDNA 3110007F17 gene	3.99	0.45
027A18_Glyoxylase 2	3.71	4.12
010G02_S100 calcium binding protein A8 (calgranulin A)	3.52	11.63
015F05_Glycerol-3-phosphate acyltransferase, mitochondrial	2.93	4.89
018M05_Insulin-like growth factor binding protein 4	2.82	119.64
019E21_Fatty acid binding protein 4, adipocyte	2.76	3.71
010E12_Carbonyl reductase 2	2.76	4.77
014J11_Leptin	2.60	568.75
002K03_Peroxisome proliferator activator receptor delta	2.46	5.54
005H07_CCAAT/enhancer binding protein (C/EBP), beta	2.41	0.95
013C07_Insulin receptor substrate 2	2.37	3.32

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Table 1 (*continued*)

Decription	Day 3 adipocyte/ preadipocyte ratio	Day 10 adipocyte/ preadipocyte ratio
026N10_Kruppel-like factor 15	2.24	505.90
010I10_Elongation of very long chain fatty acids-like 3	2.13	65.60
002O05_Epidermal growth factor	2.00	14.30
013H03_Melanocortin 2 receptor	1.93	449.52
006N02_Growth hormone releasing hormone	1.82	89.24
002A10_Urokinase plasminogen activator receptor	1.77	0.13
007C08_Melanocortin 5 receptor	1.73	10.17
002H08_Lipase, hormone sensitive	1.65	15.20
005E13_Adrenergic receptor, beta 3	1.64	31.37
003C06_Angiopoietin	1.08	29.66
003C11_Transcription factor EB	0.95	10.12
012J15_Monoglyceride lipase	0.79	177.86
006B12_Adrenergic receptor, beta 2	0.77	11.38
004O12_Insulin-like growth factor 2	0.56	130.91
016I06_Delta-like homolog (Pref-1)	0.38	0.31
004I05_Extracellular matrix protein 1	0.33	0.10
023N18_Junction cell adhesion molecule 2	0.28	0.07
009H01_Mus musculus XMP (Xmp) mRNA, complete cds	0.27	0.01
013C18_Lymphocyte antigen 84	0.24	0.02
015H14_P21 (CDKN1A)-activated kinase 1	0.24	0.13
007J15_WNT1 inducible signaling pathway protein 1	0.22	0.19
007L06_Phospholipase A2 group VII	0.22	0.01
010J03_Differential display and activated by p53	0.21	0.01
003N05_Transforming growth factor beta 1 induced transcript 1	0.20	0.29
016C05_Testis derived transcript	0.19	0.01
004F07_Platelet derived growth factor receptor, beta polypeptide	0.19	0.28
002G02_Myosin VIIa	0.19	0.08
014A17_CDNA sequence AB023418	0.19	0.02
005G19_Matrix metalloproteinase 9	0.19	0.35
015D01_Toll-like receptor 2	0.19	0.05
010C14_Zinc finger protein 57	0.18	0.07
006P03_Necdin	0.18	0.67
005H17_Tissue inhibitor of metalloproteinase 3	0.18	0.01
015C20_Lymphocyte antigen 6 complex, locus F	0.17	0.29
002J03_Apolipoprotein D	0.17	10.16
020B09_Polydomain protein	0.17	0.01
026H20_RIKEN cDNA 1200009O22 gene	0.17	0.02
004H08_High mobility group protein I, isoform C	0.15	0.16
017P15_Procollagen, type VIII, alpha 1	0.15	0.01
009P04_Matrix metalloproteinase 14 (membrane-inserted)	0.15	0.89
022I04_RIKEN cDNA 0610037B21 gene	0.15	0.13
003D05_Serine protease inhibitor 4	0.15	0.12
012E02_Matrix metalloproteinase 23	0.15	0.36
001H07_Vascular cell adhesion molecule 1	0.14	0.46
008F16_WNT1 inducible signaling pathway protein 2	0.14	0.04
007M18_Dihydropyrimidinase-like 3	0.14	0.02
013G17_MAD homolog 7 (Drosophila)	0.14	0.05
008F01_Transforming growth factor, beta induced, 68 kDa	0.14	0.08
009O04_Interferon activated gene 203	0.13	0.06
029D06_Ribonuclease/angiogenin inhibitor 2	0.13	2.32
008N01_Serine protease inhibitor 2-1	0.11	0.96
021P01_Complement component 1, r subcomponent	0.11	0.69
003L15_Argininosuccinate synthetase 1	0.11	0.12
013K03_Slit homolog 2 (Drosophila)	0.11	0.01
015C16_Fibroblast growth factor 7	0.11	0.23
001B17_Interferon-induced protein with tetratricopeptide repeats 3	0.10	0.04
012F02_Epithelial membrane protein 1	0.10	0.06
009D22_Procollagen C-proteinase enhancer protein	0.10	0.44
019O10_Thymosin, beta 10	0.09	0.13
003N14_Glycoprotein 38	0.09	0.00
006H17_Four and a half LIM domains 2	0.09	0.19
015H05_Matrix gamma-carboxyglutamate (gla) protein	0.08	0.65
010L08_Procollagen, type I, alpha 1	0.08	0.48
001I15_Lysyl oxidase	0.08	0.48
005B14_AE-binding protein 1	0.07	0.08
004D16_Enolase 2, gamma neuronal	0.07	0.02
027K10_RIKEN cDNA 1810026B04 gene	0.04	0.01
009K09_Actin, alpha, vascular smooth muscle	0.02	0.08

[a]Gene whose function is unknown or with very low expression levels are not reported in the table. A complete list of modulated genes is available upon request.

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Table 2. Genes modulated by nucleoside reverse transcriptase inhibitors (NRTI) and protease inhibitors (PI) in differentiating 3T3-L1 adipocytes.

GB Accession no.	Gene	Description	Function	Day 3 adipocyte/ preadipocyte ratio[a]	Day 10 adipocyte/ preadipocyte ratio[a]	Day 3 ZDV + 3TC-treated/ untreated ratio[b]	Day 3 ZDV + 3TC + IDV-treated/ untreated ratio[c]	Day 3 ZDV + 3TC + SQV-treated/ untreated ratio[d]
Genes modulated by NRTI								
Up-regulated genes								
NM_026243	*9130411I17Rik*	UDP-N-acetylglucosamine: alpha-1,3-D-mannoside beta-1, 4-N-acetylglucosaminyltransferase IV	Transferase	**3.11**	**19.48**	**11.05**	**17.11**	**18.41**
NM_027170	*Krtap13-1*	032K20_RIKEN cDNA 2310057N15 gene	Unknown	**2.88**	**18.59**	**10.45**	**8.50**	**14.47**
AK002950	*Arpp21*	Cyclic AMP-regulated phosphoprotein, 21	Unknown	**2.12**	**4.87**	**10.29**	**4.04**	**5.63**
AK013278	*Btbd5*	BTB (POZ) domain containing 5	Protein binding	1.92	**9.70**	**9.18**	**9.94**	**25.57**
AB041556	*Nap1l5*	Nucleosome assembly protein 1-like 5	Nucleosome assembly	**5.26**	0.55	**9.11**	**5.14**	**3.19**
AK006077	*1700018A14Rik*	RIKEN cDNA 1700018A14 gene	Unknown	**3.68**	**3.92**	**7.54**	**6.88**	**12.08**
AK019654	*Trim42*	Tripartite motif-containing 42	Protein ubiquitination	**2.36**	**2.42**	**7.38**	**4.63**	**8.76**
AF113751	*Pom210*	Nucleoporin 210	Porin	1.18	0.86	**6.86**	**2.31**	**3.59**
AB017615	*Znfn1a4*	Zinc finger protein, subfamily 1A, 4	Transcription regulation	**2.74**	**5.18**	**6.52**	**6.28**	**7.09**
AK016321	*4930579G24Rik*	RIKEN cDNA 4930579G24 gene	Unknown	0.80	0.59	5.94	5.60	4.38
NM_023115	*Pcdh15*	Protocadherin 15	Calcium ion binding	1.79	**6.88**	**5.55**	**3.14**	**4.06**
AF053757	*C3ar1*	Complement component 3a receptor 1	Signal transduction	**2.72**	0.19	**5.40**	**3.81**	**6.96**
NM_017469	*Gucy1b3*	Guanylate cyclase 1, soluble, beta 3	Intracellular signaling cascade	**4.95**	**4.77**	**5.36**	**22.64**	**2.03**
AF282286	*Olfr970*	Mus musculus odorant receptor K30 gene	G-protein coupled receptor	**2.08**	**5.45**	**5.35**	**5.09**	**3.98**
M75721	*Spi1-1*	Serine protease inhibitor 1-1	Protease inhibitor	1.25	**5.03**	**4.53**	**5.52**	**5.31**
AF028737	*Hcn1*	Hyperpolarization-activated, cyclic nucleotide-gated K+ 1	Cation channel	0.76	0.69	**4.39**	**5.93**	**16.77**
AK015848	*4930519P11Rik*	Hypothetical protein LOC74721	Unknown	**2.05**	**21.75**	**4.27**	**9.39**	**10.77**
AF121352	*Dcl1*	C-type lectin DCL1	Transmembrane receptor	**2.30**	**9.65**	**4.09**	**2.10**	**4.67**
AF053943	*Aebp1*	AE-binding protein 1	Transcription corepressor, carboxypeptidase	0.19	0.11	**4.00**	**4.23**	**4.53**
AK006335	*1700025F22Rik*	RIKEN cDNA 1700025F22 gene	Unknown	1.28	1.86	**3.87**	**4.11**	**4.67**
Y07915	*Etv6*	Ets variant gene 6 (TEL oncogene)	Transcription factor activity.	0.89	**3.40**	**3.83**	**3.17**	**2.36**
AB052617	*Sval2*	Seminal vesicle antigen-like 2	Secreted protein		**4.33**	**3.66**	**3.50**	**6.01**
AK008734	*Dusp3*	Dual specificity phosphatase 3	Phosphoprotein phosphatase	0.77	**2.14**	**3.47**	**4.40**	**8.11**
AK015893	*4930525F21Rik*	RIKEN cDNA 4930525F21 gene	Unknown	1.62	**8.60**	**3.41**	**4.29**	**13.36**
AK004002	*Alox5ap*	Arachidonate 5-lipoxygenase activating protein	Leukotriene biosynthesis	1.68	**4.40**	**3.19**	**8.50**	**9.63**
AK010042	*Spink5*	Serine protease inhibitor, Kazal type 5	Protease inhibitor	1.35	**10.14**	**3.03**	**2.30**	**2.45**
U80892	*D0Kist5*	DNA segment, KIST 5	Transcription-regulatory protein	0.93	1.50	**2.95**	**3.85**	**2.16**
X62622	*Timp2*	Tissue inhibitor of metalloproteinase 2	Inhibitor of cell proliferation	0.16	0.50	**2.77**	**2.32**	**3.02**
AF231406	*Ly6i*	Lymphocyte antigen 6 complex, locus I	Lymphocyte antigen	0.18	0.33	**2.54**	**5.26**	**4.87**
AB032605	*Piwil1*	Miwi like, piwi like homolog 1	Unknown	**3.21**	**3.28**	**2.30**	**4.29**	**10.19**
NM_026205	*Rnf151*	Ring finger protein 151	Unknown	1.42	**3.12**	**2.11**	**3.63**	**7.19**
M84918	*Myod1*	Myogenic differentiation 1	Myoblast differentiation	1.07	**5.00**	**4.74**	**7.93**	**4.40**
Down-regulated genes								
AF183960	*Ccrn4l*	CCR4 carbon catabolite repression 4-like	Circadian expression	**6.88**	**2.48**	*0.49*	*0.31*	*0.16*
X78874	*Clcn3*	Chloride channel 3	Chloride transport	1.05	1.17	*0.47*	*0.42*	*0.47*
AK017628	*Mcp*	Mitochondrial carrier protein	Oxidative phosphorylation	**7.21**	**4.32**	*0.44*	*0.44*	*0.45*
AB039933	*Dp1l1*	Deleted in polyposis 1-like 1	Intracellular membrane trafficking	**2.04**	**3.71**	*0.39*	*0.43*	*0.45*
AK017994	*Ganc*	Glucosidase, alpha; neutral C	Carbohydrate metabolism	1.23	**9.07**	*0.37*	*0.42*	*0.47*
D29639	*Hadh*	Hydroxylacyl-Coenzyme A dehydrogenase	Fatty acid metabolism	2.65	2.58	*0.36*	*0.47*	*0.29*

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AK012211	*Phf6*	PHD finger protein 6	Transcription factor	**0.56**	**0.32**	*0.47*	*0.30*	*4.26*
AK010289	*2400006N03Rik*	RIKEN cDNA 2400006N03 gene	Unknown	0.68	0.51	*0.27*	*0.22*	*0.46*
AK017253	*5430401F13Rik*	RIKEN cDNA 5430401F13 gene	Unknown	1.57	**4.10**	*0.24*	*0.41*	*0.42*
U97107	*Elovl3*	Elongation of very long chain fatty acids-like 3	Fatty acid biosynthesis	2.13	**65.60**	*0.23*	*0.12*	*0.21*
NM_013633	*Pou5f1*	POU domain, class 5, transcription factor 1	Transcription factor	0.72	0.69	*0.15*	*0.45*	*0.32*
NM_011810	*Faim*	Fas apoptotic inhibitory molecule	Negative regulation of apoptosis	1.57	1.25	*0.08*	*0.09*	*0.13*
Genes modulated only by NRTI								
D49545	*Kifc2*	Kinesin family member C2	Microtubule-based movement	1.36	**5.67**	*0.33*	0.86	0.70
AF134119	*Vps4b*	Vacuolar protein sorting 4b	Protein transport	0.98	0.92	*0.39*	0.86	0.59
AK002633	*Tcfcp2l3*	Transcription factor CP2-like 3	Transcription factor	1.21	1.02	*0.45*	0.82	1.00
AF181685	*Tde1l*	Tumor differentially expressed 1, like	Unknown	0.59	1.45	*0.43*	0.68	0.89
AB045293	*Tmem6*	Transmembrane protein 6	Unknown	0.52	1.56	**3.07**	1.22	1.16
AK015847	*4930519N16Rik*	Nucleoredoxin	Electron transport	0.98	**2.83**	**2.55**	1.13	1.89
D83698	*Bid3*	BH3 interacting domain, apoptosis agonist	Regulation of apoptosis	1.20	*0.34*	**5.56**	1.55	0.89
U00445	*G6pc*	Glucose-6-phosphatase, catalytic	Glycogen biosynthesis	*0.33*	*0.32*	**2.37**	1.23	0.65
AK016612	*4933402D24Rik*	RIKEN cDNA 4933402D24 gene	Unknown	1.57		**5.13**	1.63	1.00
J03750	*Rpo2tc1*	RNA polymerase II transcriptional coactivator	Transcription coactivator	0.56		**3.25**	0.75	0.75
AF043276	*Oprl*	Opioid receptor-like	Signal transduction	1.05	0.72	**3.75**	1.54	0.93
Genes modulated by PIs								
U08185	*Prdm1*	PR domain containing 1, with ZNF domain	Transcriptional repressor	**2.40**	1.70	1.61	**10.60**	**10.06**
NM_008530	*Ly6f*	Lymphocyte antigen 6 complex, locus F	Lymphocyte antigen	*0.17*	*0.29*	0.82	**9.16**	**14.89**
U61969	*Wnt10a*	Wingless related MMTV integration site 10a	Wnt receptor signaling pathway	0.71	*0.30*	1.33	**6.68**	**4.67**
AB016275	*Oaz3*	Ornithine decarboxylase antizyme 3	Ornithine decarboxylase inhibitor	**2.10**	**7.50**	1.89	**5.67**	**7.07**
AF126798	*Fads2*	Delta-6 fatty acid desaturase (fatty acid desaturase 2)	Lipid metabolism	*0.47*	1.24	1.42	**5.16**	**6.50**
NM_022654	*Lrdd*	P53 protein induced, with death domain (leucine-rich and death domain containing; Pidd)	p53-mediated apoptosis	0.89	*0.35*	1.97	**4.40**	**6.61**
X53929	*Dcn*	Decorin	Extracellular matrix protein	*0.10*	0.98	1.57	**3.78**	**2.62**
Y14634	*Accn1*	Amiloride-sensitive cation channel 1	Sodium channel	1.70	**3.89**	1.96	**3.07**	**4.79**
NM_023143	*C1r*	Complement component 1r	Proteolysis and peptidolysis	*0.11*	0.69	1.78	**3.07**	**3.21**
AB036749	*Ppn*	Porcupine homolog	Glycoprotein metabolism	0.52	**3.09**	1.00	**2.95**	**3.85**
U43327	*Lamc2*	Laminin, gamma 2	Extracellular matrix protein	1.18	1.93	1.37	**2.30**	**6.42**
K03235	*Plf2*	Proliferin 2	Hormone activity	1.08	*0.30*	0.85	**2.27**	**2.14**
NM_031191	*Plf*	Proliferin	Hormone activity	0.72	*0.28*	0.97	**2.26**	**2.52**
X16009	*Mrpplf3*	Mitogen regulated protein, proliferin 3	Hormone activity	0.95	*0.28*	1.13	**2.22**	**2.32**
Down-regulated genes								
D16503	*Lef1*	Lymphoid enhancer binding factor 1	Wnt receptor signaling pathway	**2.68**	**3.67**	0.91	0.47	*0.21*
M61737	*Fsp27*	Fat specific gene 27	Lipid metabolism	**11.49**	**17.00**	0.98	*0.42*	*0.29*
U18812	*Lep*	Leptin	Lipid metabolism	**2.60**	**568.75**	1.72	*0.40*	*0.41*
L23108	*Cd36/FAT*	CD36 antigen/fatty acid translocase	Lipid metabolism	**12.81**	**22.40**	0.64	*0.39*	*0.33*
X04673	*Adn*	Adipsin	Proteolysis and peptidolysis	**33.00**	**50.40**	1.12	0.35	*0.27*
U37222	*Adipoq*	Adiponectin	Lipid and glucose metabolism	**8.66**	**49.40**	0.81	0.31	*0.13*
AK011560	*Hist1h4i*	Histone 1, H4i	Nucleosome assembly	1.12	**5.19**	0.95	*0.25*	*0.45*
X95280	*G0s2*	G0/G1 switch gene 2	Cell grow/cycle regulator	**19.99**	**227.98**	0.81	*0.21*	*0.45*
NM_010157	*Esr2*	Estrogen receptor 2 (beta)	Steroid hormone receptor	0.61	0.71	0.84	*0.20*	*0.25*
X87966	*S100a8*	S100 calcium binding protein A8 (calgranulin A)	Fatty acid carrier	**3.52**	**11.63**	0.91	*0.17*	*0.20*

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Table 2 (*continued*)

GB Accession no.	Gene	Description	Function	Day 3 adipocyte/ preadipocyte ratio[a]	Day 10 adipocyte/ preadipocyte ratio[a]	Day 3 ZDV + 3TC-treated/ untreated ratio[b]	Day 3 ZDV + 3TC + IDV-treated/ untreated ratio[c]	Day 3 ZDV + 3TC + SQV-treated/ untreated ratio[d]
AK003912	*Mrap*	Melanocortin 2 receptor accessory protein	Membrane protein	**8.75**	**89.74**	0.85	*0.10*	*0.23*
AF294617	*Pfkfb3*	Inducible 6-phosphofructo-2-kinase	Fructose metabolism	**2.98**	0.76	0.64	*0.04*	*0.09*

[a]Ratio of mean expression level in 3T3-L1 adipocytes at day 3 or 10 of differentiation over mean expression level in 3T3-L1 preadipocytes.
[b]Ratio of mean expression level at day 3 of differentiation in 3T3-L1 adipocytes treated by zidovudine (ZDV) 10 μM and lamivudine (3TC) 20 μM over mean expression level in untreated 3T3-L1 adipocytes.
[c]Ratio of mean expression level at day 3 of differentiation in 3T3-L1 adipocytes treated by ZDV 10 μM, 3TC 20 μM, and indinavir (IDV) 20 μM over mean expression level in untreated 3T3-L1 adipocytes.
[d]Ratio of mean expression level at day 3 of differentiation in 3T3-L1 adipocytes treated by ZDV 10 μM, 3TC 20 μM, and SQV 20 μM over mean expression level in untreated 3T3-L1 adipocytes. Gene expression was defined as significantly altered between experimental and control condition by differential regulation analysis at 99% confidence level using the GeneSight software (BioDiscovery Inc., Santa Clara, California, USA). Ratio of over-expressed genes is in boldface, whereas ratio of under-expressed genes is in boldface and italic.

function and caspase-3 activation [19], whereas inhibited genes included the transcription factor *Tcfcp2l3*. An inactivating mutation of the corresponding human gene *TFCP2L3* was identified in a large family with an autosomal dominant form of progressive non-syndromic sensorineural hearing loss [20].

Genes modulated by PI during 3T3-L1 early adipogenic differentiation

Of the 26 genes significantly modulated by both treatment conditions including NRTI and a PI, but not by NRTI alone, 14 were up-regulated by treatment, whereas 12 were inhibited (Table 2). In particular, treatment with PI markedly induced the transcription factor *Prdm1/Blimp1*. *Prdm1/Blimp1* is a transcriptional repressor with Krüppel-type zinc fingers which has been demonstrated to be essential for B-cell development [21] and, recently, also to play a critical role in the specification of mouse primordial germ cells and in the regulation of cell fate specification and morphogenetic processes [22,23]. Other genes induced by PI included those encoding extracellular matrix proteins (i.e., laminin gamma 2 and decorin), proteases (i.e., complement component 1r), and proliferins 1–3, which were found to be repressed in differentiated adipocytes. PI also induced expression of Δ-6 fatty acid desaturase (*Fad2*), a key enzyme in the biosynthesis of polyunsaturated fatty acids, such as arachidonic acid and decosahexaenoic acid, which is expressed in nearly all human tissues [24]. Transcription of *Fad2* is induced by SREBP-1c and PPARα ligand activators [25,26], but suppressed by polyunsaturated fatty acids via inhibition of SREBP-1c [25,26] and by PPARγ agonists [27]. Increased Δ-6 desaturase activity has been associated with insulin resistance [27]. Of note is that PI dramatically induced expression of *Wnt10a* (wingless related MMTV integration site 10a) and repressed *Lef1* (lymphoid enhancer factor 1), which are both involved in Wnt signaling pathway. Activation of the Wnt signaling pathway has been shown to inhibit the differentiation of 3T3-L1 preadipocytes by preventing the induction of C/EBPα and PPARγ [28], to block the development of white and brown adipose tissue and, in differentiated brown adipocytes, to promote their conversion to white adipocytes [29]. *Wnt10a* encodes a secreted signaling protein and has been demonstrated to be over-expressed in preadipocytes with decreased ability to differentiate into mature brown adipocytes [30]. The effect of Wnt signaling on adipogenesis has been shown to be mediated by both β-catenin-dependent and β-catenin-independent mechanisms [31]. LEF1 and the other members of this family of nuclear transcription factors, in response to Wnt signals, associate with β-catenin and activate Wnt-responsive target genes [32]. Analysis of *Lef1*-deficient mice indicated that LEF1 may have a function in epithelium-to-mesenchyme signaling networks. In fact, targeted inactivation of *Lef1* resulted in a complete block of development of multiple ectodermal appendages, such as teeth, vibrissae, hair, and mammary

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glands [33,34]. Our results show that *Lef1* expression is induced during adipogenesis and inhibited by PI, thus suggesting a role in adipocyte differentiation. In the *Lef1*-deficient mouse model, *Wnt10a* is expressed independently of *Lef1* in the dental epithelium [34], in agreement with the discordant expression pattern observed in our study in adipocytes. In addition to *Lef1*, PI down-regulated expression of several other genes typically expressed in differentiated adipocytes and mainly involved in lipid metabolism, such as *Fsp27* (fat specific gene 27), *Lep* (leptin), *Adn* (adipsin), *Adipoq* (adiponectin), and *Pfkfb3* (inducible 6-phosphofructo-2-kinase), and already known to be inhibited by PI [2,7], as well as other genes induced during adipogenesis, but less well characterized, such as *Mrap, Cd36/FAT, Hist1h4i, G0s2,* and *S100a8*. *Mrap* encodes melanocortin 2 receptor accessory protein, which has been recently identified as an interacting partner of the ACTH receptor MC2R and has been supposed to have a role in the trafficking MC2R from the endoplasmic reticulum to the cell surface [35]. MRAP was first identified as a protein that is up-regulated upon differentiation of 3T3-L1 cells into adipocytes [36]. Interestingly, *Mc2r* is also up-regulated in 3T3-L1 cells during differentiation via PPARγ and mediates the lipolytic effects of ACTH [37]. The G_0/G_1 switch gene (*G0s2*) is involved in cell cycle regulation and has a temporal pattern of expression similar to that of *Mrap*, being induced during adipogenesis and further increased by PPARγ agonists [38]. *CD36/FAT* expression is also induced by PPARγ during adipocyte differentiation [39]. CD36/FAT mediates the uptake and accumulation of lipids in macrophages, adipose tissue and skeletal muscle [40] and its deficiency has been associated with dyslipidaemia and insulin resistance [41,42]. The role of CD36 in the pathogenesis of HAART-associated dyslipidaemia has already been investigated but with opposing findings. In fact, CD36 expression in circulating monocytes of HIV-infected patients treated with antiretroviral therapy including a PI has been reported to be reduced in a study [43], but increased in a more recent investigation in a larger population of patients [44]. Similarly, PI have been demonstrated to both inhibit [43] and induce [45] CD36 expression in different human cell lines *in vitro*. S100A8 belongs to the S100 family of calcium-binding proteins and, together with S100A9, is expressed in cells of the myeloid lineage where its is predominantly localized to the cytoplasm [46]. The secreted S100A8/S100A9 complex specifically binds polyunsaturated fatty acids (such as arachidonic acid) in a calcium-dependent manner [47] and interacts with CD36/FAT to facilitate cellular uptake of fatty acids [48]. Thus, PI could inhibit fatty acid accumulation and adipogenesis by down-regulation of both CD36/FAT and S100A8 expression. Indeed, polyunsaturated fatty acids, and in particular arachidonic acid, have been shown to stimulate adipogenesis probably by acting as PPARγ agonists [49]. In this context, up-regulation of Fads2 (Δ6-fatty acid desaturase) by PI could be the consequence of reduced intracellular polyunsaturated fatty acids. Finally, treatment with PI significantly inhibited expression of *Esr2*, which encodes oestrogen receptor β (ERβ). This result is in agreement with our findings in HIV-positive patients receiving antiretroviral therapy [50]. Our study demonstrated reduced ERβ mRNA levels in the subcutaneous adipose tissue of lipodystrophy patients, the down-regulation of ERβ expression in the adipose tissue of HIV-positive patients receiving antiretroviral therapy containing PI, and the restoration of ERβ mRNA levels after switching from PI [50]. Thus, ERβ could represent another nuclear transcription factor involved in the cascade of events triggered by PI that lead to impairment of adipocyte differentiation and metabolism. Overall, our observations with microarray experiments are consistent with previous reports which demonstrated that PI down-regulate the expression of lipogenesis genes, such as *Fsp27*, *Lep*, *Adn*, *Pfkfb3,* and *Adipoq*, which were reported to be repressed both in *in vitro* studies [2,7,51–53] and *in vivo* in the subcutaneous adipose tissue from lipodystrophic HIV-infected patients [7,50,54–56]. Moreover, our microarray study identifies new genes, such as *Mrap*, *Cd36/FAT*, and *S100a8*, that are inhibited *in vitro* by PI and that are also involved in adipogenesis and adipocyte function. Our results are thus in agreement with the presence of peripheral lipoatrophy in HIV-infected patients treated with PI [57]. Peripheral lipoatrophy, central fat accumulation, and lipomatosis are common problems in adult patients with HIV-1 infection on antiretroviral drugs. Many of the adverse metabolic effects associated with PI therapy, including hypertriglyceridaemia and insulin resistance, resemble those seen in patients with the rare congenital and acquired lipodystrophy syndromes [57]. Thus it has been proposed that peripheral lipoatrophy may be the primary effect caused by PI therapy, which subsequently leads to other adverse effects such as insulin resistance and many other endocrine disturbances ultimately leading to fat redistribution with an increase of the visceral fat and of lipids stored also inside the muscle fibres [58]. Therefore, the generation of the ‘hypertrophic phase’ in fat distribution must be seen as a secondary event linked to the alteration of the endocrine-metabolic milieu and the loss of the capacity by subcutaneous fat cells to store adequately the flux of free fatty acids within the cell and thus preferentially channelling these substrates towards other targets, which are the fat cells of the visceral area and other cells which get ‘fatty’, as is the case of muscle cells and hepatocytes [59]. Although what happens *in vivo* should be distinguished from what may be seen in *in vitro* models, our findings, together with the observations by others [7], support the general hypothesis of a primary damage of the subcutaneous adipose cell.

Quantitative RT–PCR analysis of genes modulated by antiretroviral drugs

To confirm microarray results, the expression of a subset of genes modulated during differentiation and in response to

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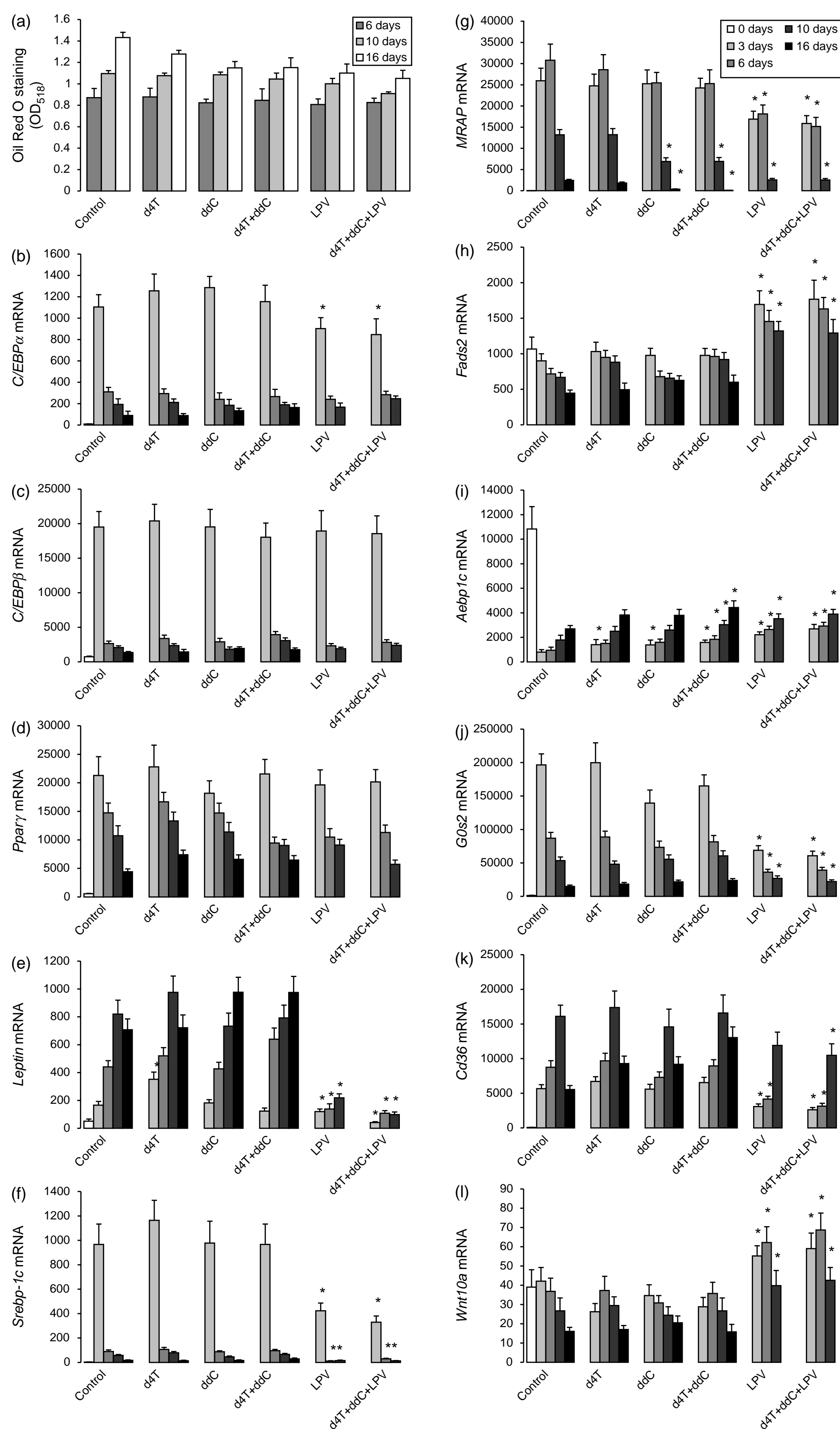

Fig. 3. Effect of NRTI and PI on adipogenic differentiation and expression of adipogenic marker genes in 3T3-L1 cells. 3T3-L1 preadipocytes were induced to differentiate into adipocytes as reported in Fig. 1 in the presence or absence of antiretroviral drugs at the following concentrations: ddC (Roche SpA, Milan, Italy) 0.2 μM, d4T (Bristol-Myer Squibb, Princeton, New Jersey, USA) 10 μM, LPV (Abbott Diagnostics, Illinois, USA) 10 μM. On day 6, 10, and 16 of differentiation, lipid content was determined by Oil Red O staining and measurement (a), as reported in Fig. 1, and mRNA expression of adipogenic marker genes was determined

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treatment with NRTI and PI was further investigated using quantitative RT–PCR in a time-course experiment (Fig. 2). Overall, quantitative RT–PCR evaluation confirmed results obtained with microarray analysis. Expression of *Srebp-1c*, *C/EBPα*, *C/EBPβ*, and *Pparγ* mRNA was evaluated as early marker of adipogenesis, whereas expression of *Leptin* represented a marker of fully differentiated adipocytes. Among these genes, only *Leptin* expression was shown to be significantly modulated by antiretroviral drugs at microarray analysis and *Srebp-1c* probes were not represented in our microarray slides. As expected, in 3T3-L1 cells, *Srebp-1c*, *C/EBPα*, *C/EBPβ*, and *Pparγ* were rapidly and transiently induced during the early phase of adipocyte differentiation, whereas *Leptin* mRNA levels increased in the late phase of adipogenesis. Treatment with the NRTI ZDV and 3TC did not significantly modify expression of these genes at all time points of adipocyte differentiation, even though a slight decrease of *Srebp-1c* and *Leptin* mRNA as compared with untreated control cells could be observed during early and late adipocyte differentiation, respectively. These results are in agreement with Oil Red O staining which did not show a significant inhibition of adipocyte differentiation. At variance with NRTI, the PI SQV and IDV determined a significant inhibition of *Srebp-1c*, *C/EBPα*, *Pparγ*, and *Leptin* expression, and this inhibition was more evident with SQV than with IDV (Fig. 2). PI also determined a decrease, although not significant, of *C/EBPβ* transcript levels. The combination of a PI with two NRTI had the same effect on gene expression as treatment with only a PI.

Among the novel genes that microarray analysis demonstrated to be modulated by antiretroviral drugs, *Mrap*, *Fads2*, *Aebp1*, *G0s2*, *Cd36* and *Wnt10a* were selected for further investigation using quantitative RT–PCR. These genes had different expression profiles during adipogenesis, since *G0s2* was transiently induced during the early phase of adipogenesis, *Mrap* and *Cd36* were induced during the late phase of adipogenesis, *Aebp1* and *Wnt10a* were rapidly and markedly reduced during adipogenesis, whereas *Fads2* expression remained unchanged during adipocyte differentiation. RT–PCR analysis showed that treatment with NRTI significantly increased *Aebp1* mRNA levels as compared to untreated control cells, but had no effect on transcript levels of the other marker genes, thus confirming microarray results. Treatment with PI determined a marked reduction of *Mrap*, *G0s2*, and *Cd36* mRNA levels and an increase of *Wnt10a* and *Fads2* expression, both at early and late phases of differentiation. Even in this case, the association of a PI with two NRTI did not seem to modify the effect observed with the single drug treatment. The milder effect of NRTI than PI on adipocyte differentiation could in part be accounted to the relatively lower concentration of NRTI than PI used in our study. Although NRTI altered the gene expression profile in differentiating adipocytes, these effects were probably not so marked to be detectable at morphological examination and Oil Red O quantification.

Effect of other drug combinations on adipogenesis

The time-course experiments were replicated with different combinations of anti-retroviral drugs, including the NRTI zalcitabine (ddC) 0.2 μM and stavudine (d4T) 10 μM, which have been more strongly linked to lipoatrophy [60], and the PI lopinavir (LPV) 10 μM. Moreover, since the effects of NRTI on mitochondria and thus their presumed toxicity is time dependent, the time-course experiment was prolonged to 16 days (Fig. 3). Also with these combinations of NRTI, no significant alterations of cell proliferation or adipogenesis were observed, even after prolonged (16 days') treatment, whereas the number of differentiated adipocytes was reduced by LPV treatment. This effect of LPV on adipocyte differentiation was paralleled by abnormal expression of genes involved in adipogenesis (Fig. 3).

Conclusions

In this study, by microarray gene expression analysis, we identified genes modulated by NRTI and PI during early adipogenesis. We hypothesize that up-regulation of master transcription factors and modulation of the Wnt signaling pathway by PI could represent a key event leading to inhibition of adipocyte differentiation and down-regulation of expression of adipocyte-specific markers, such as adiponectin, leptin, MRAP, Cd36/FAT, and S100A8. With respect to PI, the effect of NRTI on adipocyte differentiation and gene expression profile was milder, even though NRTI modulated the expression of tissue inhibitors of metalloproteinases and transcription factors, such as *Aebp1*, which could play an important role in the determination of the adipocyte phenotype. As already demonstrated for ERβ [50], abnormal expression of these genes could be at the basis of HAART-associated lipodystrophy and could represent a potential target for the treatment of this syndrome.

Acknowledgments

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Fig. 3 (*continued*)
by real-time quantitative RT–PCR (b–n), as reported in Fig. 2. In (a) results represent mean and SD of three experiments performed in duplicate. In (b–n), mRNA values are reported as $\times 10^3$ copies/μg total RNA and data represent mean and SD of three experiments performed in duplicate. $*P < 0.05$ versus control (unpaired t test).

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