

Genome-wide analysis of gene expression during adipogenesis in human adipose-derived stromal cells reveals novel patterns of gene expression during adipocyte differentiation



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ABSTRACT

We have undertaken an in-depth transcriptome analysis of adipogenesis in human adipose-derived stromal cells (ASCs) induced to differentiate into adipocytes in vitro. Gene expression was assessed on days 1, 7, 14 and 21 post-induction and genes differentially expressed numbered 128, 218, 253 and 240 respectively. Up-regulated genes were associated with blood vessel development, leukocyte migration, as well as tumor growth, invasion and metastasis. They also shared common pathways with certain obesity-related pathophysiological conditions. Down-regulated genes were enriched for immune response processes. KLF15, LMO3, FOXO1 and ZBTB16 transcription factors were up-regulated throughout the differentiation process. *CEBPA*, *PPARG*, *ZNF117*, *MLXIP1*, *MMP3* and *RORB* were up-regulated only on days 14 and 21, which coincide with the maturation of adipocytes and could possibly serve as candidates for controlling fat accumulation and the size of mature adipocytes. In summary, we have identified genes that were up-regulated only on days 1 and 7 or days 14 and 21 that could serve as potential early and late-stage differentiation markers.

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1. Background

Adipose tissue plays an important role in energy homeostasis and the regulation of insulin sensitivity in the muscle, and is an endocrine organ that secretes factors that regulate energy metabolism (Spiegelman and Flier, 2001; Rosen and Spiegelman, 2006). White adipose tissue (WAT) constitutes the predominant type of fat present in adult humans and is mainly involved in energy storage, while brown adipose tissue (BAT) which has thermogenic properties is abundant in newborns with small depots located in the supraclavicular regions and neck in adults (Merklín, 1974; Cypess et al., 2009). A third class of adipose tissue known as “beige” or “brite” adipose tissue is found within certain WAT depots and has similar thermogenic functions to those of BAT (Petrovic et al., 2010; Schulz et al., 2011; Cinti, 2012). Excessive accumulation of WAT, which is constituted of adipocytes, results in obesity. Obesity is a major risk factor for the development of type 2 diabetes mellitus (T2DM), cancer and cardiovascular disease (CVD) (Stephens,

2012). Recent WHO reports have stated that in 2012, of all new cancer cases, half a million were due to overweight and obesity with the proportion of obesity-related cancers being higher in women than in men (WHO, 2014). This trend was reported to be higher in richer countries with a similar trend being observed in some developing countries. Furthermore, the number of overweight and obesity related diseases is set to increase in virtually every country in the world, thus making it an issue of international public health concern.

The regulation of many genes such as the retinoblastoma gene and the E2F family of transcription factors has been reported to occur in a differentiation dependent manner during adipogenic differentiation in 3T3-L1 cells (Richon et al., 1997). Other transcription factors (TFs) like peroxisome proliferator activated receptor-gamma (PPARG) and CCAAT-enhancer binding protein-alpha (CEBPA) are activated during adipogenesis, as well as many other genes involved in lipid metabolism, which altogether play a role in the proper functioning of the mature adipocyte (Darlington et al., 1998; Rosen et al., 2000; MacDougald and Lane, 1995). To better understand the scale of gene expression changes that occur during the formation of mature adipocytes from preadipocytes, we compared and characterised the transcriptome profile of stromal cells derived from human adipose tissue, otherwise known as adipose-derived stromal cells (ASCs), undergoing adipocyte differentiation in vitro on days 1, 7, 14 and 21 (which represent the early to late stage processes of adipogenesis).

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Microarray technology has been used successfully to study gene expression in the 3T3-L1 preadipocyte cell line during adipogenesis and in adipocytes from mouse both *in vitro* and *in vivo* (Guo and Liao, 2000; Soukas et al., 2001). This technique was systematically employed to study gene expression in human ASCs during adipogenic differentiation over a 21 day period to identify genes that are important in driving adipogenesis *in vitro*. The results could contribute to our understanding of adipogenesis, related biological processes, and the development of obesity and its co-morbidities. These genes could potentially be manipulated to control adipogenesis at different stages, with an overall goal of reducing excess accumulation of fat. This may open up new avenues for the control of adipogenesis to combat obesity and its related diseases.

2. Material and methods

2.1. Ethics statement

All subjects participating in this study provided written informed consent and the study was approved by the Faculty of Health Sciences Research and Ethics committee, University of Pretoria, South Africa (ethics reference no.: 421/2013). This study was conducted according to the Declaration of Helsinki.

2.2. ASC culture, immunophenotypic characterisation and induction of adipogenic differentiation

ASCs were isolated from human abdominal subcutaneous adipose tissue from four individual donors undergoing liposuction as previously described (Zuk et al., 2001), with some modifications. Samples were labelled A, B, C and D (A; female of age 19, B; male of age 32, C; female of age 28 and D; female of age 20). Each individual constituted one of the four biological replicates referred to herein. Cells from donors A, B, C and D were cultured in complete culture medium consisting of α -MEM (GIBCO, Life Technologies™, New York, USA) supplemented with 10% foetal bovine serum (FBS; Biochrom) and 2% (v/v) penicillin [10,000 U/mL]–streptomycin [10,000 μ g/mL] (p/s; GIBCO, Life Technologies™, New York, USA) in 80 cm² culture flasks (NUNC™, Roskilde Site, Kamstrupvej, Denmark) at 37 °C in a 5% CO₂ incubator (Thermo Forma CO₂ water jacketed incubator, 3111TF). The cells used in this study had been cryopreserved and were thawed just prior to use. When the cultures reached 80% confluence, they were washed twice with 4 mL PBS containing 2% (v/v) p/s and detached from the bottom of culture flask by adding 3 mL trypsin (GIBCO, Life Technologies™, New York, USA) followed by incubation at 37 °C in 5% CO₂ for about 7–10 min to ensure that the cells were dislodged. Trypsin was neutralised by adding equal amounts of complete medium and the suspension was centrifuged at 184 g for 5 min to pellet the cells. Immunophenotypic characterisation had previously been performed using flow cytometry on fresh cells at each passage and on frozen cells prior to adipogenic induction. Briefly, each sample was incubated for 15 min at 37 °C in the dark with a panel of monoclonal antibodies (mouse anti-human CD73-APC (BioLegend, San Diego, USA)/-FITC (eBioscience, San Diego, USA), CD105-PE, CD90-FITC/-PC5, CD34-PC7/-ECD/-PE/-FITC, and CD45-PC5/-PC7/-ECD (Beckman Coulter, Miami, USA). Data was acquired on either a FC500 MCL or Gallios (10 colours, 3 lasers) flow cytometer (Beckman Coulter, Miami, USA). All data sets were analysed using Kaluza Flow Cytometry analysis software 1.2 (Beckman Coulter, Miami, USA). For adipogenic differentiation, cells from donors A, B, C and D at passages 15, 6, 12 and 12 respectively were seeded at a density of 5000 cells/cm² in complete culture medium and grown to confluence. The cell cultures were washed twice with 4 mL PBS containing 2% (v/v) p/s and differentiation was induced by adding adipogenic induction cocktail to the cell cultures as previously described (Ogawa et al., 2004), with slight modifications, while control cultures (non-induced) received only complete culture medium.

The adipogenic induction cocktail used consisted of Dulbecco's Modified Eagle's Medium (GIBCO, Life Technologies™, New York, USA) supplemented with 10% FBS, 2% (v/v) p/s, insulin (10 μ g/mL), dexamethasone (1 μ M), isobutylmethylxanthine (0.5 M) and indomethacin (200 μ M). Differentiation was allowed to proceed over a 21 day period and cells from both adipogenic induced cultures and their respective controls were harvested on days 1, 7, 14 and 21 (as illustrated in the study design in Supplementary Fig. S1) and used for RNA isolation. To confirm lipid droplet formation during the process of adipogenesis, both the non-induced and induced cells were stained with 4', 6-diamino-2-phenylindole, dihydrochloride [2.5 μ g/mL] (DAPI, Life Technologies, Oregon, USA) and Nile Red [50 ng/mL] (Life Technologies, Oregon, USA). Images were captured at 20 \times magnification using an AxioVert A1 inverted fluorescence microscope (Carl Zeiss, Gottingen, Germany) equipped with an AxioCam Cm1 camera (Carl Zeiss, Gottingen, Germany).

2.3. RNA isolation and RT-qPCR

Total cellular RNA was isolated from non-treated and treated cells on days 1, 7, 14 and 21 following adipogenic induction using the RNeasy Minikit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Prior to the generation of cDNA, the quality and integrity of the RNA were assessed using the Agilent Tape station 2000 and corresponding kit (Agilent Technologies, California, USA) according to the manufacturer's instructions. cDNA was synthesised using the iScript™ Reverse Transcription Supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA). PCR reactions were performed in 10 μ L using the LightCycler® 480 SYBR Green I Master Mix (Roche, Basel, Switzerland), with primer concentrations of 400 nM and a cDNA concentration of 20 ng/ μ L. RT-qPCR was performed on a LightCycler® 480 II (Roche, Basel, Switzerland) under the following conditions: denaturation at 95 °C for 5 min and 45 cycles of amplification at 95 °C for 30 s, 62 °C for 30 s, and 72 °C for 30 s. After amplification a melt curve was performed at 95 °C for 30 s and 40 °C for 30 s, and ramped at 0.1 °C/s. The primers (IDT, Coralville, IA, USA) and their sequences for the genes of interest and the reference genes (internal controls) used are shown in Supplementary Table S1.

2.4. Microarray gene expression experiment

RNA (200 ng) isolated on D1, D7, D14 and D21 from non-induced and adipogenic induced ASC cultures from donors A, B, C, and D at passages 15, 6, 12 and 12 respectively was used for first and second strand cDNA syntheses. This was followed by the synthesis and amplification of complementary RNA (cRNA) by *in vitro* transcription using an Affymetrix GeneChip® WT PLUS Reagent Kit according to the manufacturer's protocol. Magnetic purification beads supplied with the kit were used to purify amplified cRNA product and 15 μ g of the purified cRNA product was used to synthesise second cycle single stranded cDNA (ss-cDNA). This was followed by another purification step using magnetic purification beads as described in the manufacturer's protocol. Purified ss-cDNA (5.5 μ g) was fragmented, labelled and used to prepare a hybridisation cocktail as described in the Affymetrix GeneChip® WT PLUS Reagent Kit manual. Hybridisation was performed using the Affymetrix GeneChip® Hybridisation Wash and Stain Kit as described in the manufacturer's protocol. Hybridisation cocktail was hybridised to Affymetrix GeneChip® Human Gene 2.0 ST arrays. The array was placed in an Affymetrix GeneChip® Hybridisation Oven-645 rotating at 60 rpm at 45 °C for 17 h, after which the chip was washed and stained in an Affymetrix GeneChip® Fluidics Station-450Dx before scanning using an Affymetrix GeneChip® Scanner-7G. The output Affymetrix CEL files which have intensity values for all probes present on the scanned chips were used for further analysis. The Robust Multiarray Analysis algorithm (Irizarry et al., 2003) in Affymetrix Expression Console™ was used to perform background correction, summarisation,

normalisation and calculation of probe set expression values from CEL files, giving the resultant output in a CHP file format. Affymetrix Transcription Analysis Console™ was used to calculate fold change of each probe set or transcript cluster identifier number and mapped onto the corresponding gene. We applied very stringent criteria of fold-change of ≥ 4 and ≤ -4 with $p < 0.05$ and $FDR < 0.5$ for selecting differentially expressed genes which were used for further analysis. The fold change value of each gene represents how much the expression of that particular gene has changed from the control to the induced cells based on the signal measured. The microarray data files of this study have been deposited in NCBI GEO (Gene Expression Omnibus) with assigned GEO accession number [GSE77532](#).

2.5. Bioinformatics analyses

The principal component analysis (PCA) tool provided by Affymetrix Expression Console™ software was used to assess relatedness of samples from all four donors used in this study based on the microarray data. Considering the genes from microarray data as variables, PCA creates a cluster of principal components which best explains the experimental response of each sample based on the genes' features. The PCA plot has three components namely PCA1, PCA2 and PCA3. Clustering of samples along PCA1 axis occurs such that it retains as much variation of the data as possible making it the most important component on a PCA plot. PCA2 captures the majority of the variation not captured in PCA1 while PCA3 captures the majority of the variation not captured in PCA2. The AmiGO 2 Gene ontology tool provided by BIOBASE TRANSFAC® (Matys et al., 2006) license software was used to classify differentially expressed genes into biological processes that they were enriched for. STRING v10 (Szklarczyk et al., 2015) was used to determine known and predicted functional interactions among genes at the protein level and also to predict other functional proteins interacting with these gene products in *Homo sapiens*. Molecular network and pathway analysis were performed using QIAGEN's Ingenuity® Pathway Analysis (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity). IPA uses computational algorithms to identify canonical pathways and molecular interaction networks that are enriched for the input gene set in the Ingenuity Pathways Knowledge Base. It then uses the Fisher's exact test to test for the significance of each identified pathway and the interaction network linked to the input gene compared to the whole Ingenuity Pathways Knowledge Base.

3. Results and discussion

3.1. ASC characterisation

ASCs were isolated from four individual donors. These cells were positive for CD 73, 90 and 105 and were negative for CD 34 and 45 as shown by Durandt et al. (2016). The induced ASCs successfully underwent adipogenesis over the 21 day induction period, while the non-induced ASCs were devoid of characteristic adipogenic lipid droplet formation (Fig. 1) (Durandt et al., 2016). Intracellular accumulation of lipid droplets is a key characteristic of adipocyte differentiation and quantification of intracellular lipid content by flow cytometry (Nile Red staining) as described in Durandt et al. (2016) showed a gradual increase in the percentage of cells with increased intracellular lipid content over the 21-day induction period: $2.80 \pm 4.42\%$ 24 h post-induction, $7.50 \pm 9.08\%$ at 7 days post-induction, $15.18 \pm 3.70\%$ at 14 days post-induction and $20.48 \pm 13.49\%$ at 21 days post-induction. However, since this study did not investigate the effect of cell passage number on the differentiation potential of ASCs, it is possible that the relatively low differentiation rates observed here could have been due to the relatively late passage number of some of the cells used as it has been shown that ASC adipogenic potential decreases with increased passage number (Safwani et al., 2014).

3.2. Differentially expressed genes during adipogenesis

Gene expression was studied over a 21 day period in a total of 32 samples from four biological replicates each consisting of non-induced and induced ASC cultures using Affymetrix GeneChip® Human Gene 2.0 ST arrays containing a 48,226 probe set. Microarray analysis revealed that 61, 124, 138 and 149 genes were significantly up-regulated while 67, 94, 115 and 91 genes were significantly down-regulated on days 1, 7, 14 and 21 respectively. A complete list of all genes that were differentially expressed during the differentiation process is provided in the supplementary data (Tables S2_1–S2_4).

PCA plots showed two large independent clusters along principal component 1 axis (PCA1) (Fig. S2). The first cluster which lies close to the origin of the PCA1 axis consists of all the control samples from all four donors, while the other cluster which lies further up the PCA1 axis consists of all induced samples from all four donors. Furthermore, D1 induced samples for all four donors formed a minor cluster which is closer to all the induced samples on D7–D21 in the same plane (PCA1). The clustering of all induced samples away from all the control samples confirms consistency in gene expression during ASC adipogenic differentiation for all four donors (Fig. S2).

3.3. Gene expression profile of ASCs during adipogenesis

Induction of adipogenesis in ASCs resulted in 61 genes being significantly up-regulated within 24 h. Of the 61 genes, 32 (*AOX1*, *CHRDL1*, *CIDEA*, *CNR1*, *CPM*, *CRISPLD2*, *EDNRB*, *FASN*, *FOXO1*, *GALNT15*, *GLUL*, *GREB1L*, *HSD11B1*, *KLF15*, *LMO3*, *MAOA*, *METTL7A*, *MT1F*, *MT1M*, *MT1X*, *NEFL*, *NID1*, *NRCAM*, *PDK4*, *PRUNE2*, *RASD1*, *RASSF4*, *RGCC*, *SAMHD1*, *SLC19A2*, *WNT5A* and *ZBTB16*) remained significantly up-regulated throughout the 21 day differentiation period. The constant up-regulation of these genes suggests that they play an important role in mediating adipogenic differentiation in ASCs.

Four of the 32 consistently up-regulated genes (*KLF15*, *LMO3*, *FOXO1* and *ZBTB16*) encode TFs which are likely to be important in driving the adipogenic differentiation process by regulating the expression of downstream target genes. Silencing of the *LMO3* TF has been shown to suppress adipogenesis (Lindroos et al., 2013), which is in line with its identification in our study as an important TF in adipogenesis and its constant up-regulation during this process. *ZBTB16*, reported to have a role in brown adipocyte bioenergetics (Plaisier et al., 2012), remains to be characterised functionally with respect to its precise role in adipogenesis. *FOXO1* and *KLF15* have been shown to play crucial roles in adipocyte differentiation (Munekata and Sakamoto, 2009; Wu and Wang, 2013). Consequently, the identification of these 4 important TFs validates our study methodology which is aimed at identifying important genes with functional roles during adipogenesis. *RASSF4*, an oncogene that has been shown to promote cell cycle progression, senescence evasion, and tumorigenesis in alveolar rhabdomyosarcoma (Croze et al., 2014), with no previously reported function in adipogenesis, was found to be expressed in this study. However, this observation requires further investigation to determine its role in adipogenesis and other pathophysiological disorders such as obesity related cancer.

A unique gene signature pattern was identified for ASCs at different time points during the differentiation process as shown in Fig. 2. Genes that were expressed specifically on different days may play a pivotal role at that particular time point. However, their exact role remains to be functionally determined in vivo. The gene expression profile reported in this study is likely to be specific to the differentiation and accumulation of human adipocytes in the subcutaneous fat depot. This expression profile would most likely differ from ASCs isolated from other fat depots due to the inherent properties of depot-specific ASCs, which may contribute to the overall depot-specific changes in fat tissue that occur at different rates and with increasing age (Kirkland et al., 2002; Cartwright et al., 2007). In addition, subcutaneously derived ASCs have been shown to exhibit higher levels of lipid accumulation,

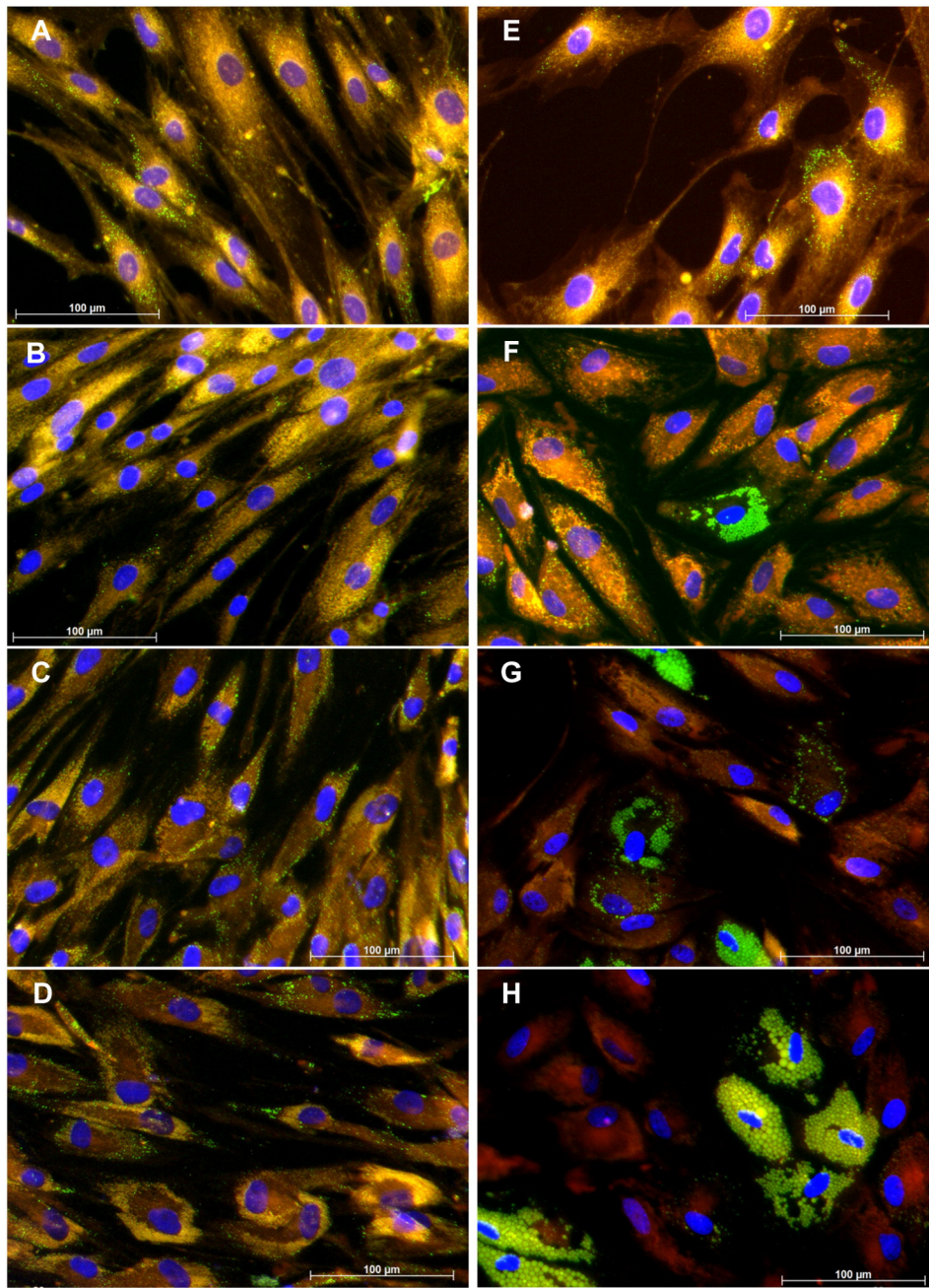


Fig. 1. Fluorescence microscopy images of ASCs induced to differentiate into adipocytes. Images were captured 24 h post-induction (A & E), 7 days post-induction (B & F), 14 days post-induction (C & G) and 21 days post-induction (D & H). A–D: Representative images of ASCs not induced to undergo adipogenesis (controls). E–H: Representative images of ASCs that were induced to differentiate into adipocytes. All images were captured at 20 \times magnification.

adipogenic transcription factor expression, and replicative potential and less TNF- α induced apoptosis than ASCs from other fat depots (Cartwright et al., 2007).

STRING v10 analysis of the four TFs (KLF15, LMO3, FOXO1 and ZBTB16) that were significantly up-regulated throughout the differentiation process identified sirtuin 1 (SIRT1), v-akt murine thymoma viral oncogene homolog 1 (AKT1), histone deacetylase 1 (HDAC1) and nuclear receptor co-repressor 2 (NCOR2) as functional partners that strongly interact with KLF15, FOXO1 and ZBTB16 but not with LMO3 (Fig. 3, panel A), thereby suggesting their functional role in adipogenesis. SIRT1 has been shown to promote lipolysis and loss of fat in differentiating 3T3-L1 adipocytes (Picard et al., 2004). RNA interference of protein kinase B/Akt 1 has been shown to block adipogenic differentiation in 3T3-L1 preadipocytes (Xu and Liao, 2004). Conversely, HDAC1 knock-down has been shown to promote adipogenesis, while its

overexpression in 3T3-L1 preadipocyte cell lines attenuates the process (Yoo et al., 2006). NCOR2 inhibits adipogenic differentiation by binding to PPARG and represses the expression of PPARG target genes (Schmitt et al., 2011). The identification of genes involved in adipogenesis through this methodology suggests that they play a fundamental role in adipogenesis through their interactions, which should therefore be studied further in appropriate models in vivo.

Six genes (*CEBPA*, *ZNF117*, *MLXIPL*, *MMP3*, *RORB*, *PPARG*) encoding for TFs were identified to be significantly up-regulated from D14 onwards during the differentiation process (Fig. 2). *CEBPA* and *PPARG* have been studied extensively and have been demonstrated to play crucial roles in determining adipocyte fate (Lefterova and Lazar, 2009). Identification of *MLXIPL*, *ZNF117*, *MMP3* and *RORB* together with *CEBPA* and *PPARG* suggests that they may also play a crucial role in terminal differentiation and maturation, and proper functioning of mature

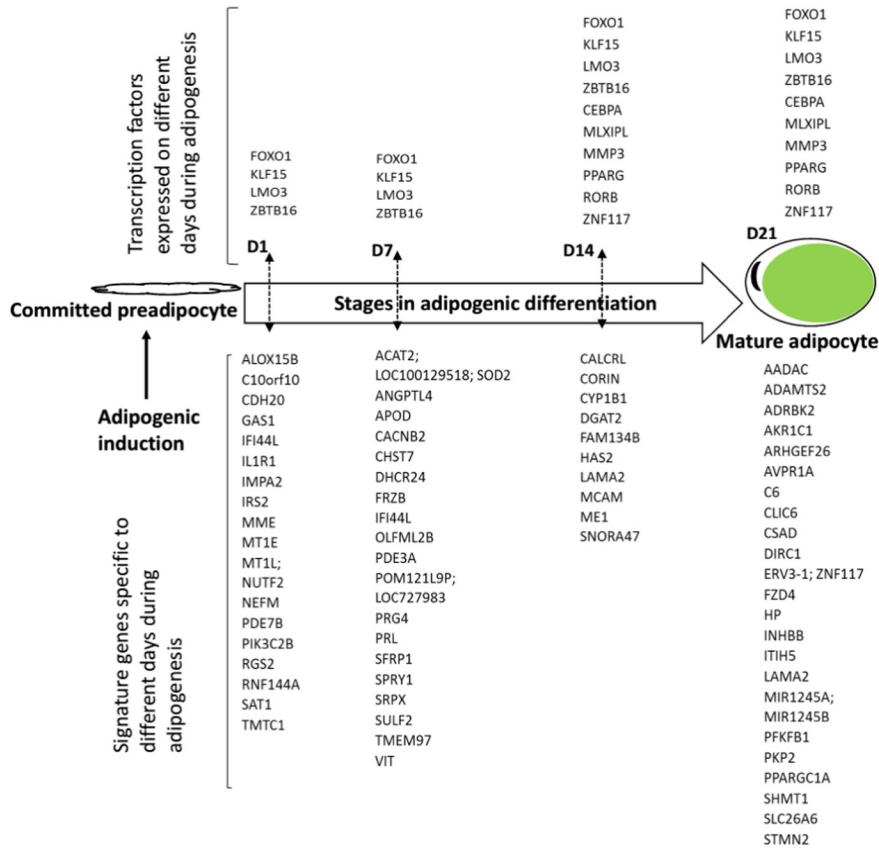


Fig. 2. Gene expression on different days during adipogenesis in ASCs. Transcription factors expressed on the different days (D1, D7, D14 and D21) are shown in the top half of the panel while unique gene signatures at the same time points are shown in the lower half of the panel.

adipocytes, either directly or indirectly by regulating the expression of their downstream target genes. The early expression (D1 and D7) of the circadian core clock gene *PER1* and the late expression of *RORB* (D14 and D21) with its role in circadian behaviour (André et al., 1998) suggest that *PER1* and *RORB* are two important elements controlling the internal clock mechanism in adipogenesis/adipocyte functioning in response to anticipated environmental changes such as circulating levels of insulin, adrenaline, glucose, fatty acids and triglycerides. *PER1* and *RORB* could therefore serve as potential targets for correcting disorders arising from an abnormal circadian rhythm in

adipose tissue functioning. *MLXIPL* and *ZNF117* were identified in this study but have no previously reported role in adipogenesis. Based on our gene expression studies, we therefore propose that *MLXIPL* and *ZNF117* may play a functional role in adipocyte differentiation which will require validation in vivo.

Based on STRING v10 analysis, CEBPA, MMP3, RORB and PPARG but not *MLXIPL* and *ZNF117* TFs strongly interact with each other at the protein level with peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (PPARGC1A), nuclear receptor coactivator 1 (NCOA1), mediator complex subunit 1 (MED1) and E1A binding protein

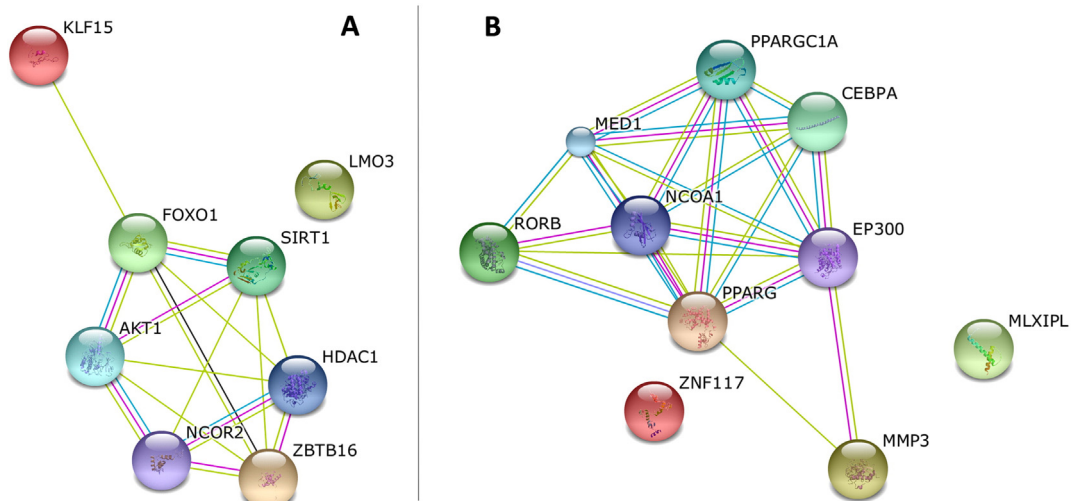


Fig. 3. STRING v10 prediction of functional interacting partners of TF gene product during adipogenesis. Panel A represents the functional interaction of TFs up-regulated throughout adipogenesis while panel B represents the functional interaction of TFs up-regulated on D14 and D21 only with new functional partners.

p300 (EP300) being identified as functional proteins that interact with these TFs (Fig. 3, panel B). This interaction suggests their involvement in common biological functions/processes, in this case, terminal differentiation, maturation, size determination and fatty acid metabolism in adipocytes. This makes them attractive targets for controlling these and other functions related to mature adipocytes.

3.4. Functional analysis by gene ontology classification reveals that adipogenesis is associated with other important biological processes

Functional classification of differentially expressed genes (DEGs) by gene ontology (GO) enrichment revealed that gene sets that were significantly up-regulated on different days (D1–D21) during ASC differentiation were significantly enriched for biological processes such as the response to glucocorticoids (*ACACB*, *ADIPOQ*, *GLUL*, *GPD1*, *MAOA*, *PDK4*, *RASD1* and *TSC22D3*), the response to insulin (*ACACB*, *ADIPOQ*, *CIDE-3*, *FOXO1*, *INHBB*, *KLF15*, *LIPE*, *LPL*, *PDK4*, *PLIN4*, *PNPLA3* and *RBP4*), and the fatty acid metabolic process (*ABCD2*, *ACSF2*, *ACSL1*, *ACSL5*, *ADIPOQ*, *FABP4*, *FASN*, *GPAM*, *LIPE*, *LPL* and *PDK4*). Down-regulated genes from D1 to 21 were significantly enriched for GO biological process such as collagen fibril organisation (GO:0030199). Furthermore, *C3*, *CFB*, *COLEC12*, *CXCL12*, *DPP4*, *ICAM1*, *IFI30*, *IL6*, *LOXL4*, *PDCD1LG2*, *IER3* and *SEMA7A* which are associated with immune responses (GO:0006955) and with positive regulation of immunoglobulin secretion (GO:0051024) were significantly enriched in down-regulated gene sets. *DRAM1*, *ABCA9*, *MET*, *CXCL12*, *IL6*, *DOK5*, *DPP4*, *NT5E*, *IFI30* and *CFB* which are associated with positive regulation of neutrophil differentiation, monocyte differentiation, positive regulation of T-cell proliferation and activation, B-cell activation and regulation of antigen processing and presentation of peptide antigens via MHC class II GO biological processes were also significantly enriched in down-regulated gene sets during ASC differentiation. This suggests that adipogenesis is associated with down-regulation of immune function.

Genes that were associated with GO biological processes such as vasculogenesis and angiogenesis (*EDNRB*, *CHRD1*, *EDNRB*, *RGCC*, *ANGPT1*, *ANGPTL1*, *PDE3B*, *DAMTS15*, *ANGPTL4*, *FOXO1*, *PRL*, *F3*, *HAS2*, *MCAM*, *CALCRL*, *COMP*, *HP* and *FZD4*) and neural development (*NEFL*, *MME*, *APOD*, *FAM134B*, *MTIF*, *MTIX* and *SLC19A3*) were significantly enriched in up-regulated gene sets during ASC differentiation. This suggests that adipogenesis is associated with neural and blood vessel development and supports previous findings by other authors (Billon et al., 2010).

3.5. Adipogenesis revealed as a concurrent multi-step process

Based on GO biological process terms searched, genes that were significantly up-regulated up to D7 of adipogenic induction were enriched for lipid and fatty acid biosynthesis, metabolism, transport and storage processes. These biological processes increased exponentially from D1 to D14 before levelling out from D14 to D21 (Fig. 4). Significant up-regulation of *CEBPA*, *GPD1* and *PPARG*, which are actively involved in the positive regulation of fat cell differentiation, was observed from D14 onwards, suggesting that adipogenic differentiation occurs in ASCs after D7 of induction as shown in Fig. 4. Lipid and fatty acid formation as well as fat cell differentiation occur simultaneously during this time period (D7–D14) and level out from D14 to 21 (Fig. 4). This suggests that terminal differentiation of ASCs from D14 onwards is accompanied by the maturation of functional adipocytes. Conversely, GO terms enriched for cell proliferation remain fairly constant throughout the differentiation process. From our gene expression studies, adipogenesis in ASCs could be described as follows; adipogenic induction of preadipocytes causes an initial increase in lipid and fatty acid biological processes in the cell within one week of induction (D1–D7). This process increases exponentially (D1–D14) and occurs simultaneously with the differentiation of preadipocytes into adipocytes (post-D7 of induction), which is then followed by terminal differentiation and

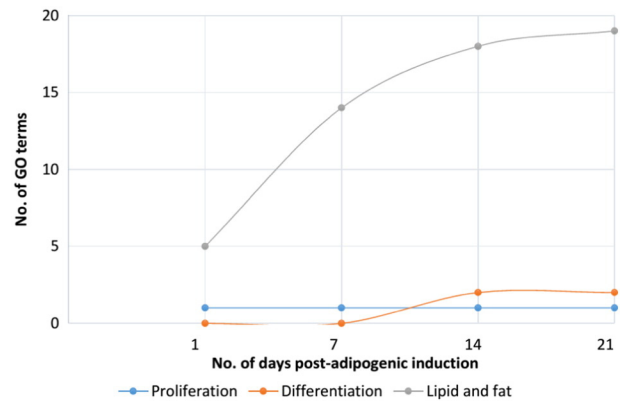


Fig. 4. Multi-step process of adipogenesis in ASCs. This gene expression study reveals that adipogenesis in ASCs is a multi-step process with lipid and fatty acid cellular and biochemical processes dominating one week post-adipogenic induction followed by terminal differentiation and maturation of adipocytes.

maturation of adipocytes (D14–21). The initial increase in lipid and fatty acid metabolism, biosynthesis, transport and storage processes may be very important in that it prepares the cells to differentiate into adipocytes post-D7 of induction.

3.6. Gene expression profile reveals expression patterns characterising different stages of adipocyte differentiation in ASCs

Analysis of gene expression patterns during adipogenesis revealed that the levels of known general adipogenic differentiation marker genes such as *FABP4*, *ADIPOQ*, *LPL*, and *PLIN1* increased during adipogenic differentiation (D7–D21), which is in accordance with previous reports (Ullah et al., 2013). This implies that genes that were significantly up-regulated only from D7 to 21 will most likely also serve as potential general adipogenic differentiation markers (Fig. 5). The increase in fold change of known adipogenic differentiation markers with ASC differentiation indicates that the cell culture system used in this study represents a fully functional system to study adipogenesis in vitro. Furthermore, genes with increasing fold change in a differentiation dependent manner may play an increasingly important role in driving the differentiation process in ASCs forward. From the 61 genes significantly up-regulated within 24 h of induction of adipogenesis, five, namely *CCDC69*, *PER1*, *REV3L*, *SLC7A8* and *TMEM158* were uniquely up-regulated up to D7 and thereafter dropped below the cut-off point ($FC \geq 4$) (Fig. 6A). Studies have shown that knock down of *PER1* in 3T3 fibroblasts, 3T3-L1 preadipocytes, and MMH-D3 hepatocytes gives rise to a cell type-specific clock phenotype and also shows strong behavioral effects in knockout mice (Ramanathan et al., 2014). It will therefore be important to investigate the role played by the early expression of this circadian clock gene in the process of adipogenic differentiation in ASCs. The role of *SLC7A8*, *CCDC69* and *TMEM158* in adipogenesis has not been well described and requires functional studies to establish their role in preadipocyte differentiation. Overall, these five genes that were significantly expressed only up to 7 days of differentiation may play an important role in defining the initial steps in adipogenesis and could therefore serve as potential early adipogenic differentiation markers in preadipocytes (genes expressed by preadipocytes primed towards adipogenic differentiation).

We identified 33 genes (*ACSF2*, *AOC2*, *C7*, *CEBPA*, *CFD*, *CLMN*, *CNTFR*, *COL4A1*, *COL4A2*, *COMP*, *CRLF1*, *CYP7B1*, *DUSP4*, *F3*, *FBN2*, *GYG2*, *KLB*, *LIPE*, *MLXIPL*, *MMP3*, *OLFM2*, *PCDH9*, *PCK1*, *PNPLA2*, *PNPLA3*, *PPARG*, *PPP1R1A*, *RORB*, *SLC38A4*, *SMOC2*, *SORBS1*; *KIAA0894*, *TUSC5* and *ZNF117*) that were significantly up-regulated specifically on D14 and D21 (Fig. 6B) coinciding with the adipocyte maturation phase. Some of these genes, which include *CEBPA* and *PPARG*, are known to be

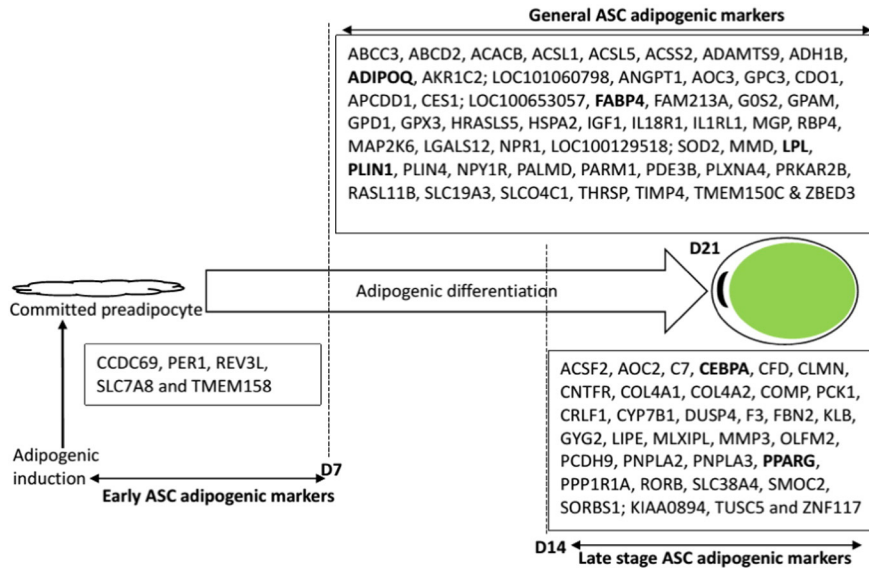


Fig. 5. Possible markers characterising adipocyte differentiation in ASCs. Gene expression patterns during adipogenic differentiation in ASCs reveal the possible marker genes indicative of different stages in adipogenesis. Genes in bold represent well characterised genes for the different stages in adipogenesis.

expressed by mature adipocytes (Lefterova and Lazar, 2009), and may serve as potential late stage adipogenic differentiation markers (genes expressed by terminally differentiated and mature adipocytes) and possibly markers representing a characteristic phenotype of a mature

adipocyte. Of these 33 genes, *AOC2*, *CNTFR*, *COMP*, *F3*, *FBN2*, *GYG2*, *KLB*, *PCDH9*, *PPP1R1A* and *SLC38A4* encode functional proteins, whose role in adipogenesis has not yet been well described. The late expression (D14 and D21) of these 10 genes during this adipogenic time-course

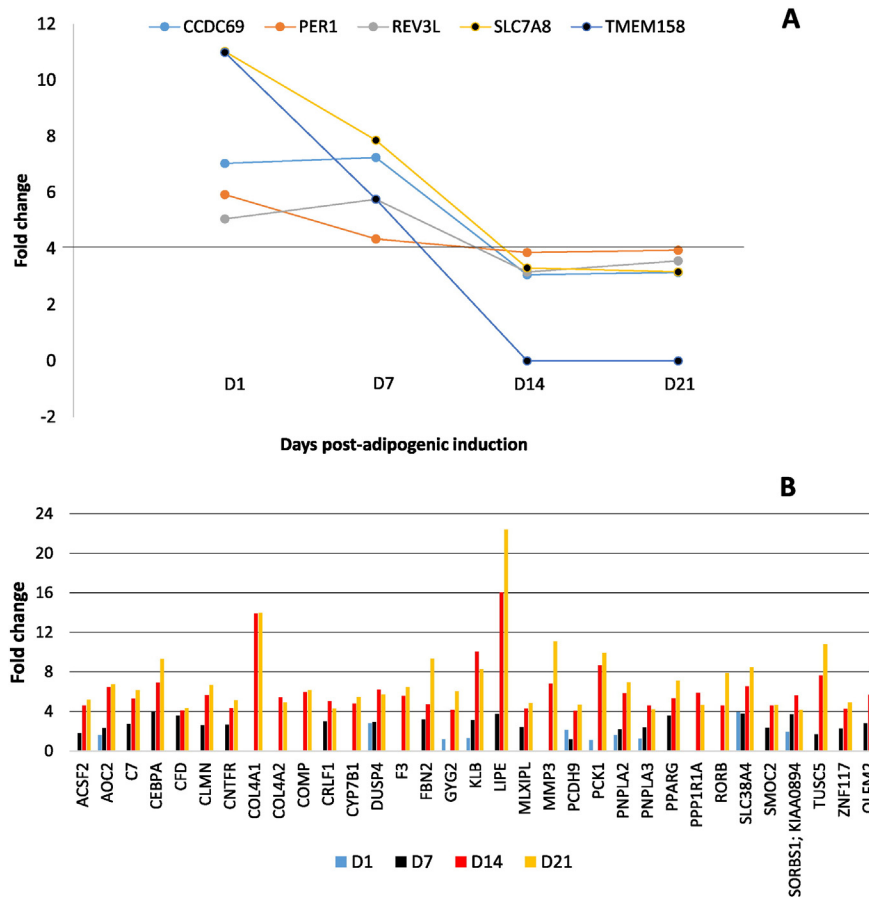


Fig. 6. Expression profile for early and late stage markers of adipocyte differentiation. The early stage marker genes are expressed in ASCs immediately upon adipogenic stimulation and their expression drops significantly after D7 (A), while the late stage genes are expressed in ASCs only from D14 representing terminally differentiated and mature ASCs (B). The horizontal line at 4-fold change represents the fold change threshold used in this study. The value plotted for each gene is the average fold change value obtained for the four biological replicates computed by TAC using unpaired One-Way Analysis of Variance (ANOVA) at $p < 0.05$ and Benjamini–Hochberg Step-Up FDR p -value < 0.5 .

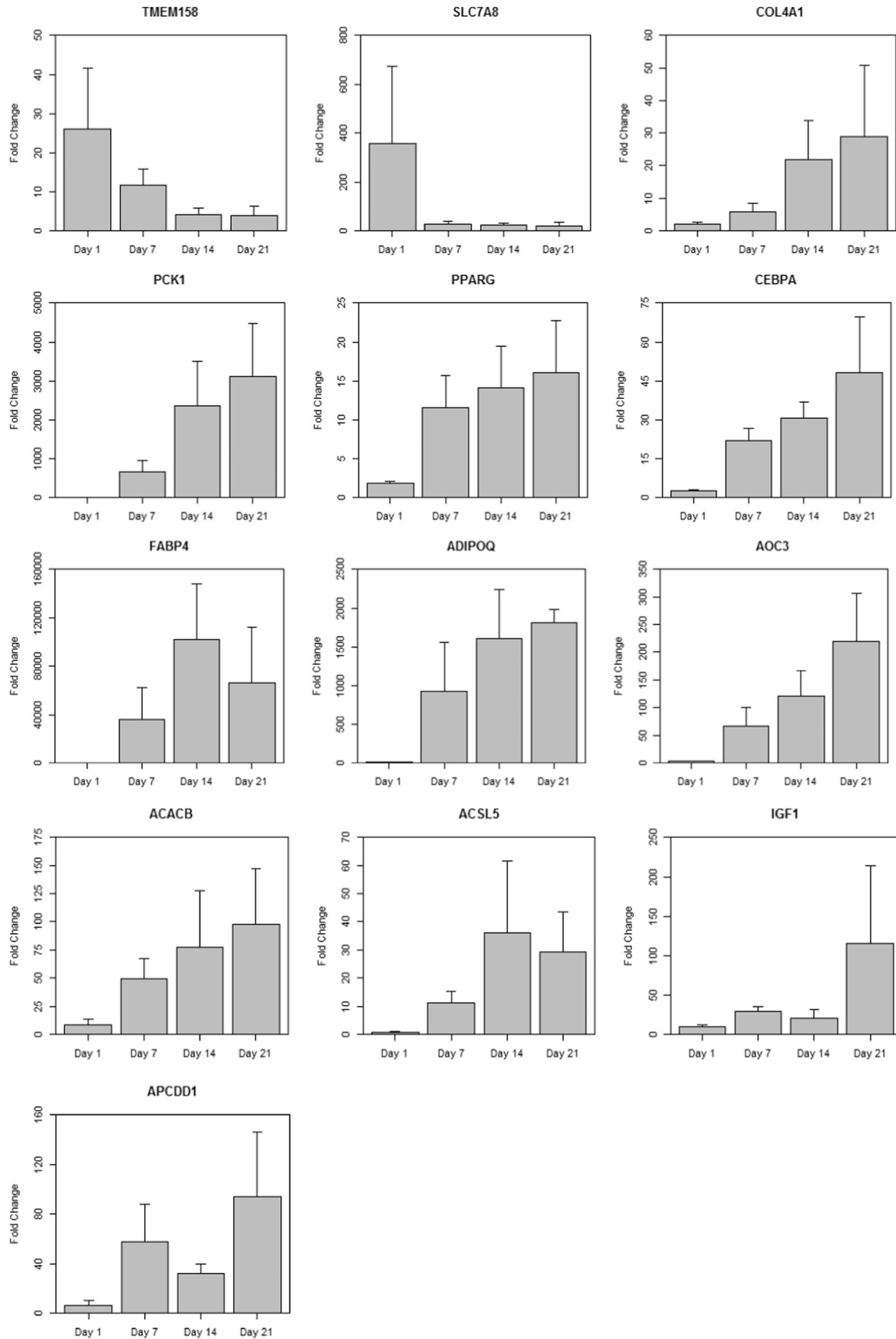


Fig. 7. RT-qPCR validation of gene expression profiles obtained by microarray. RT-qPCR data of genes representing general, early and late stage differentiation showed similar trends in gene expression profile to those obtained by microarray. Expression values plotted were mean and SEM for four biological replicates.

differentiation study suggests that they may be actively involved in adipocyte maturation, lipid formation, accumulation and metabolic processes or size determination of mature adipocytes. This remains to be tested in functional studies.

We validated gene expression using RT-qPCR for some of the genes representing general adipocyte differentiation markers as well as genes characterised from the microarray experiments in this study representing early and late stage differentiation. Similar pattern in gene expression profiles was observed using RT-qPCR and the microarray gene expression data, and this is shown in Fig. 7.

3.7. Relevant canonical pathways and molecular networks in ASCs during adipogenic differentiation

Molecular network analysis using QIAGEN's Ingenuity® Pathway Analysis (IPA) software showed that *HSD11B1*, *ZBTB16*, *FOXO1*, *LMO3*, *WNT5A*, *IRS2*, *RGS2*, *FRZB*, *IGF1*, *LGALS12*, *PIK3R1*, *SFRP1*, *ADIPOQ*, *FABP4*, *MMP3*, *PPARG*, *CEBPA* and *PPARGC1A* are up-regulated and are involved in adipocyte differentiation on different days with *HSD11B1*, *ZBTB16*, *FOXO1*, *LMO3*, and *WNT5A* being significantly up-regulated from D1 to D21. The observed increase in the expression of *WNT5A* during adipocyte differentiation was inconsistent with the IPA prediction, which reported that human *WNT5A* decreases with adipocyte differentiation. Molecular network analysis revealed that adipocyte differentiation is linked to other functional and disease processes such as cell movement and migration of leukocytes, cancer and cardiovascular disease. This is due to genes that are shared amongst their networks and that are also involved in adipocyte differentiation. *ZBTB16*, *FOXO1*, *WNT5A*, *IRS2*, *IGF1*, *PIK3R1*, *SFRP1*, *ADIPOQ*, *FABP4*, *PPARG*, *CEBPA*, *CNR1*, *NPR1*, *CES1*, *CD36*, *AOC3*, *MCAM*, *ANGPT*, *F3*, *C6* and *HP* are up-regulated and are involved in cell movement and leukocyte migration. *LGALS12*, *FOXO1*, *WNT5A*, *MMP3*, *IGF1*, *SFRP1*, *ADIPOQ*, *FASN*, *GPC3*, *IL1RL1*, *TIMP4*, *PRL*, *APCDD1* and *MCAM* are up-regulated and are involved in the activation of different cancer related processes like tumor growth (benign and malignant) including mammary tumor growth, and tumor cell invasion and metastasis. *HSD11B1*, *FABP4*, *ADIPOQ*, *IGF1*, *EDNRB*, *IL1R1*, *CD36*, *ACAT2*, *LPL* and *CORIN* are involved in the activation of different CVD related processes such as cardiac contraction, heart rate, blood pressure, atherosclerosis and dysfunction of the heart. This data provides a potential link between adipocyte differentiation and leukocyte migration, tumor growth, invasion and metastasis and CVD.

A comparative analysis of canonical pathways enriched for up-regulated gene sets on D1–D21 was performed and the Fisher's exact test was used to score the significance of each pathway at the different time points. This analysis revealed up-regulated genes on D1 that were significantly enriched for signaling pathways such as ErbB2–ErbB3, JAK1 and JAK3 in γ c cytokine signaling, VEGF and others (Table S3) suggest that these pathways might play an important role in the initial phase of adipocyte differentiation. Genes involved in PPAR signaling, IL-17 signaling and some cancer pathways were significantly enriched in D21 up-regulated gene sets (Table S3), suggesting not only the importance this set in the proper functioning of the mature adipocyte, but also the possible association of these genes with disease processes like cancer. Furthermore, up-regulated genes from D7 to 21 were significantly enriched for pathways such as T2DM, adipogenesis and PPAR α /RXR α activation. Surprisingly, only D14 up-regulated gene sets were significantly enriched for triacylglycerol biosynthesis metabolic pathways, suggesting the importance of genes expressed on D14 in determining the characteristic phenotype of mature white adipocytes, since triglyceride is the main storage form of fat in white adipose tissue (Dawkins and Stevens, 1966; Ahmadian et al., 2007).

Based on the calculated activation z-score in IPA, the NFAT canonical pathway that plays a role in cardiac hypertrophy was predicted to be activated (z-score = 2) on D7 and D21, while the colorectal cancer metastasis signaling pathway was predicted to be activated (z-score > 2) only on D21.

Further analysis in IPA for diseases and disorders significantly associated with genes up-regulated on different days revealed that genes that were up-regulated from D1 to 21 were significantly enriched for certain pathophysiological conditions classified in IPA as belonging to cancer, CVD, metabolic disease, reproductive system disease, nutritional disease, gastrointestinal disease, hepatic system disease, neurological disease, haematological disease, developmental disorders, endocrine system disorders, connective tissue disorders, inflammatory disease, skeletal and muscular disorders, psychological disorders, hereditary disorders and immunological disease. Some of the identified conditions here have previously been reported to be obesity related such as cancer, CVD and metabolic disease (Stephens, 2012; WHO, 2014). The enrichment therefore of up-regulated gene sets representative of these pathophysiological conditions suggests a possible association with obesity. In addition, the up-regulated genes strongly associated with the different pathophysiological conditions in this study might serve as potential prognostic markers for the different obesity related conditions. Detailed analysis of the cancer and cardiovascular signaling pathways enriched in up-regulated gene sets on each day during adipocyte differentiation revealed that genes associated with ovarian cancer signaling, colorectal cancer metastasis signaling, acute myeloid leukaemia signaling, cardiac β -adrenergic signaling and eNOS signaling pathways were significantly enriched on D21 only. Genes associated with prostate cancer signaling, HER-2 signaling breast cancer and NFAT signaling in cardiac hypertrophy were specifically enriched only in D1 up-regulated gene sets. This observation further supports the positive correlation between excess adipocyte accumulation and an increased risk of developing cancer and CVD (Stephens, 2012; WHO, 2014). In addition, IPA predicted genes that were up-regulated on D21 formed a functional interacting network identified as being operative in cellular movement, nervous system development and function and cancer. Similarly, interactions among D14 up-regulated genes formed an interactive network identified to be operative in cardiovascular disease, cancer, organismal injury and abnormalities. This suggests that genes up-regulated during adipogenic differentiation play an important functional role in interactive networks that occur in different biological processes and reveals their potential link to some of the obesity-related diseases like cancer and CVDs.

4. Conclusions

Here we report a comprehensive and unique transcriptomic study of the kinetics of gene expression during ASC adipogenesis in vitro. Despite the limitation of the study design including the use of relatively late passage ASCs and the lack of BMI measurements for all four donors, the study nonetheless identified genes that could potentially be used to distinguish adipocytes at the early (*SLC7A8* and *TMEM158*) and late (*COL4A1*, *PCK1*, *RORB*, *CEBPA* and *PPARG*) stages of differentiation, while others (*FABP4*, *PLIN1*, *ADIPOQ*, *AOC3*, *IGF1*, *ACSL5* and *APCDD1*) were identified to be general adipocyte markers. ASCs were also found to express unique gene signatures on different days during in vitro adipogenesis. The identification of *FOXO1* and its functional interacting partners such as *SIRT1*, whose role in adipogenesis has been well described in US patent US20050136429 A1 (Guarente and Picard, 2005), validates our hypothesis and the methodology that could be used to identify potential anti-obesity therapeutic targets. The identification of *MED1*, *EP300* and *NCOA1* as functional partners interacting strongly with the anti-diabetic thiazolidinedione (TZD) drug target *PPARG* (Lehrke and Lazar, 2005) warrants further investigation in vivo to establish their suitability as potential drug targets in combating obesity and its associated diseases. *PER1* and *RORB* are two important circadian clock genes identified in this study, which could play a role in controlling the internal circadian clock driving adipogenesis/adipocyte formation. *MLXIPL* and *ZNF117* TFs, which have not previously been reported to play a role in adipogenesis, were identified to be significantly up-regulated from D14 onwards. Functional studies of these TFs will

provide greater insight into their role in adipogenesis and might serve as potential targets for regulating this process. Functional network analysis revealed that ASC adipogenesis leads to significant enrichment of genes associated with different pathophysiological conditions including various cancers and cardiovascular signaling pathways. For example, adipocyte maturation correlates with the activation of colorectal cancer metastasis signaling, strengthening the link between obesity and the risk of cancer development. Overall, this study provides a unique resource for many functional studies of genes with no previously reported role in adipogenesis.

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Competing interests

No conflicts of interest, financial or otherwise are declared by the authors.

Authors' contributions

MAA performed all microarray experiments, did the bioinformatics data analysis and wrote the first draft of the manuscript.

CaDe designed and performed all cell culture experiments, did the RNA isolation and RT-qPCR experiments and analysed the data therefrom.

ChDu performed immunophenotyping of all cell cultures and extensive validation of adipogenesis in these cells.

MSP conceived, planned and co-ordinated the study, raised the funding and edited the manuscript.

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