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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*, *MLXIPL*, *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.

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 (Merklin, 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 andmethods

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 μg/mL] (p/s; GIBCO, Life Technologies™, New York, USA) in 80 cm^2 culture flasks (NUNC™, Roskilde Site, Kamstrupvej, Denmark) at 37 °C in a 5% CO_2 incubator (Thermo Forma CO_2 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% CO_2 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/cm^2 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 μg/mL), dexamethasone (1 μM), isobutylmethylxanthine (0.5 M) and indomethacin (200 μM). 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 μg/mL] (DAPI, Life Technologies, Oregon, USA) and Nile Red [50 ng/mL] (Life Technologies, Oregon, USA). Images were captured at 20× 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 μL 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/μL. 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 μg 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 μg) 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,