REGULAR ARTICLE

Differentiation of human adipose stromal cells in vitro into insulin-sensitive adipocytes

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Abstract Adipose tissue-related diseases such as obesity and type 2 diabetes are worldwide epidemics. In order to develop adipose tissue cultures in vitro that mimic more faithfully the in vivo physiology, new well-characterized and publicly accepted differentiation methods of human adipose stem cells are needed. The aims of this study are (1) to improve the existing natural adipose tissue extract (ATE)-based induction method and (2) to study the effects of a differentiation method on insulin responsiveness of the resulting adipocytes. Different induction media were applied on human adipose stromal cell (hASC) monocultures to study the differentiation capacity of the induction media and the functionality of the differentiated adipocytes. Cells were differentiated for 14 days to assess triglyceride accumulation per cell and adipocyte-specific gene expression ($PPAR\gamma$, $adiponectin$, $AP2$, $leptin$, $Glut4$, $Prdm16$, $CIDEA$, $PGC1$-α, $RIP140$, UCP and $ADCY5$). Insulin response was studied by measuring glucose uptake and inhibition of lipolysis after incubation with 100 or 500 nM insulin. The selected differentiation method included a 3-day induction with ATE, 6 days in serum-free medium supplemented with 1.15 μM insulin and 9.06 μM Troglitazone, followed by 4 days in a defined serum- and insulin-free stimulation medium. This protocol induced prominent general adipocyte gene expression, including markers for both brown and white adipocytes and triglyceride accumulation. Moreover, the cells were sensitive to insulin as observed from increased glucose uptake and inhibition of lipolysis. This differentiation protocol provides a promising approach for the induction of hASC adipogenesis to obtain functional and mature human adipocytes.

Keywords Adipocyte · Adipogenesis · In vitro differentiation · Maturity · Adipose stromal cells

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Introduction

The prevalence of adipose tissue-related diseases, such as obesity and type 2 diabetes, has reached worldwide epidemic proportions. In addition to type 2 diabetes, obesity and overweight are associated with an increased incidence of other comorbidities including several cancer types, sleep apnea, asthma, degenerative joint disease, hypertension, renal failure, stroke and cardiovascular disease (Switzer et al. 2013; van Baak 2013). As the prevalence of obesity has increased, so has the need to study adipose tissue and find treatments to related diseases. The growing interest creates the need to develop better methods to study the process of adipogenesis and adipose tissue function.

Adipose tissue is not only a depot for energy storage but also a dynamic endocrine organ secreting bioactive factors that control systemic insulin sensitivity, energy metabolism, immune responses and cardiovascular homeostasis (Gu and Xu 2013; Choi et al. 2010a). Adipose tissue can expand via

adipocyte hypertrophy, where existing cells grow in size, or by hyperplasia, an increase in adipocyte number, which requires progenitor cells to differentiate into adipocytes (Spalding et al. 2008). New adipocytes develop through adipogenesis, which is typically described as a two-phase process that includes commitment and terminal differentiation (Cristancho and Lazar 2011; Rosen and MacDougald 2006). In the determination phase, stem cells transform into committed preadipocytes (Rosen and MacDougald 2006). Commitment is followed by a growth arrest after which an appropriate mixture of adipogenic and mitogenic signals is needed for terminal differentiation (Gregoire 2001). In terminal differentiation, preadipocytes transform into insulin-sensitive, lipid synthesizing and transporting mature adipocytes that secrete adipocyte-specific secretory products (Rosen and MacDougald 2006; Gregoire et al. 1998). Differentiation into a mature adipocyte phenotype is typically characterized by chronological changes in the expression of the early, intermediate and late mRNA/ protein markers (Gregoire et al. 1998).

Insulin is an important regulator of adipocyte metabolism. It promotes the synthesis and storage of triglycerides and inhibits their catabolism (Rutkowski et al. 2015). In the high fed state, insulin binds to its receptor on adipocytes and causes the translocation of glucose transporter 4 (Glut4) from the cytosol to the cell surface, thus allowing effective glucose influx into the adipocytes (Rutkowski et al. 2015; Watson and Pessin 2007). The influx of glucose is not merely necessary for ATP production as glucose is also needed for effective adipocyte lipid packaging (Bederman et al. 2009; Guan et al. 2002). Insulin also inhibits lipolysis by activating phosphodiesterase 3B, which inactivates the function of cAMP (Choi et al. 2010b). Thus, the downstream activation of protein kinase A (PKA), adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL) is inhibited (Choi et al. 2010b).

The complex pathways of lipid metabolism are highly species-specific (Bergen and Mersmann 2005). Thus, new human cell-based methods offer biologically relevant tools to study human adipose tissue-related diseases. Currently available cell models include preadipocyte cell lines that are already committed to the adipocyte lineage and multipotent stem cells that are able to commit to different lineages, including adipocyte, muscle and bone (Armani et al. 2010). Multipotent stem cells used in adipogenesis assays include embryonic stem cells and adipose stromal cells (ASC) obtained from adipose tissue (Armani et al. 2010). ASC can be obtained from various species (including humans) and from different fat depots, which enables the study of differences between species and depots (Gregoire et al. 1998; Armani et al. 2010). ASC have been proved as a feasible source for adipose tissue engineering as they possess high proliferative and differentiation capacity (Girandon et al. 2011). However, due to the fact that ASC are primary cells, the heterogeneity of ASC may be a problem as well as cultures having a limited life span, which restrains their time window for experimental procedures (Gregoire et al. 1998; Scroyen et al. 2013).

In order to achieve maximal adipogenic differentiation in vitro, cell culture models treated with adipogenic cocktails, typically including supraphysiological concentrations of insulin, dexamethasone (DEX) and isobuthylmethylxanthine (IBMX), have been developed (Gregoire 2001; Armani et al. 2010; Ntambi and Young-Cheul 2000). Other adipogenic factors, such as indomethacin, glucocorticoids, troglitazone and triiodothyronine, are also commonly used (Armani et al. 2010). Improved versions of induction medium have been developed for enhanced adipogenesis. Among those, Lequeux et al. (2009) composed an improved adipogenic differentiation medium in which they replaced indomethacin by adding rosiglitazone, hydrocortisone and triiodothyronine to the basic adipogenic ingredients isobutyl-methylxanthine and dexamethasone. Also, new ways of adipogenesis induction have been studied. We have previously developed a novel cell-free angiogenesis- and adipogenesis-inducing agent, adipose tissue extract (ATE), from mature human adipose tissue. ATE contains a wide number of promoters of, e.g., adipogenesis and it has been shown to induce dose-dependent adipogenesis and angiogenesis in vitro (Sarkanen et al. 2012a). ATE is produced from mature human adipose tissue without affecting cell viability, the final mixture containing a majority of growth factors, cytokines and chemokines present in mature adipose tissue (Sarkanen et al. 2012a). The advantage of ATE is that it can be used to induce natural adipogenesis in vitro and that the majority of adipose stromal cells are committed towards adipocytes (Sarkanen et al. 2012a). However, due to the content of ATE, as seen in its dual role as adipogenesis and angiogenesis-inducing agent, the differentiation may not be driven into fully differentiated adipocytes.

The aim of the present study was to find the most effective adipocyte in vitro differentiation protocol mimicking natural adipogenesis. The adipogenic effect of ATE was further studied and improved and the additional components and combinations needed for optimal adipocyte differentiation and maturation were investigated. To assess the functionality of the differentiated adipocytes, their insulin responsiveness was monitored.

Materials and methods

This study conforms to the principles outlined in the Declaration of Helsinki. The human adipose tissue samples were obtained from waste material of surgical operations and human umbilical cords were received from caesarean sections with informed consents at Tampere University Hospital, Tampere, Finland. The use of human adipose stromal cells (hASC) was approved by the Ethics Committee of the

Pirkanmaa Hospital District, Tampere, Finland, with permit number R03058.

Isolation and culture of human adipose stromal cells

hASC were isolated from human adipose tissue by using mechanical and enzymatic procedures as described previously (Sarkanen et al. 2012b). Briefly, human adipose tissue specimens were mechanically cut into small pieces and enzymatically digested with 0.15 % collagenase I (Invitrogen, Paisley, UK) in Dulbecco's Modified Eagle's Medium Nutrient Mixture F-12 (DMEM/F12; Gibco, Carlsbad, CA, USA). The cells were tested for mycoplasma contamination (MycoAlert® Mycoplasma Detection Kit; Lonza, Basel, Switzerland) following the isolation and characterized for markers CD73, CD90 and CD105 (BD, Biosciences, Erembodegem, Belgium) with flow cytometer FACSCAnto II (BD) according to Huttala et al. (2015), before experimental use.

Adipose tissue extract

ATE was produced as described previously (Sarkanen et al. 2012a) with a slight modification to the ratio of medium to fat. Briefly, the human adipose tissue sample was manually cut into small pieces. The sample was then incubated in DMEM/F12 (Gibco) at a ratio of 2:3, i.e., 2 parts manually dissected fat and 3 parts DMEM/F12 (Gibco), for 24 h at 37 °C in 5 % CO_2 humified atmosphere. This was then centrifuged at $200g$ for 3 min and the liquid was filtered through a 0.2-μm filter. To determine the protein content of the extract, a Pierce™ BCA Protein Assay Kit (Thermo Scientific, Waltham, MA, USA) was used according to the manufacturer's instructions using bovine serum albumine (BSA) as a standard. Results were measured after 30 min incubation at 37 °C at 562 nm with a Varioskan™ Flash Multimode Reader (Thermo Scientific). The ATE was stored at −20 °C until used. Patches were not pooled.

Cell culture set up for differentiation protocols

To initiate adipogenic differentiation, hASC were plated at passage 2 at a density of 20,000 cells/cm^2 in hASC medium (Table 1). The next day, different differentiation media (Table 1) were applied on hASCs according to the scheme seen in Table 2. Troglitazone (Trogl; Sigma-Aldrich, St. Louis, MO, USA) was used at a concentration of 4 μg/ml (9.06 μM). A combination of hASC medium and serum-free medium was used as the negative and ATE as the positive control for adipocyte differentiation (Table 2). The culture time for all the treatments was 14 days.

Triglyceride accumulation

The triglyceride accumulation was determined as the relative amount of triglycerides per cell. This was done by first determining the relative cell number by using Cell Proliferation Reagent WST-1 (Roche Life Science, Indianapolis, IN, USA) according to the manufacturer's instructions. Briefly, 50 μl per 48-well plate of WST-1 reagent was added in the culture medium on day 14. After 1.5 h incubation at 37 °C in 5 % CO_2, the absorbances were measured at 450 nm with the Varioskan™ Flash Multimode Reader (Thermo Scientific). The accumulation of triglycerides was analyzed from the same wells from which the WST-1 analysis was done by using AdipoRed Assay reagent (Lonza) according to the manufacturer's instructions. Briefly, at the end point of cultivations, the culture was washed with DPBS. Then, AdipoRed reagent diluted with PBS was added on the plate and after 10 min incubation at room temperature, absorbances were measured with the Varioskan™ Flash Multimode Reader (Thermo Scientific) with excitation at 485 nm and emission at 572 nm.

Morphological analysis

For the microscopic inspections, cultures were washed with DPBS and fixed using cold 70 % EtOH, left in DPBS and stored at +4 °C until imaging. Microscopic imaging was done with a Nikon Eclipse Ti-S inverted fluorescence microscope (Nikon, Tokyo, Japan) and a Nikon digital sight DS-U2 − camera (Nikon) and automated imaging with Cell-IQ (Chipman Tech., Tampere, Finland) with a ×10 objective and 5 × 5 grid. Confocal imaging was done with LSM710 and with a Zeiss Axio Observer Z1 inverted microscope (Carl Zeiss, Oberkochen, Germany). Images were further processed with NIS Elements (Nikon), ZEN 2012 software (Carl Zeiss) and Adobe Photoshop CS3-software (Adobe Systems, San Jose, CA, USA).

RNA isolation and gene expression analysis

At day 14, the cell cultures were washed with PBS and total RNA was isolated with a PureLink® RNA Mini kit (Ambion/ Life Technologies). Five–six wells/treatment were combined to form each total RNA sample. Samples were obtained from three independent cell culture experiments. Reverse transcription was carried out by using a VILO® kit (Invitrogen). Messenger RNAs were quantified by quantitative reverse transcriptase PCR (qPCR) with the gene-specific primer pairs for *PPARγ*, *adiponectin*, *AP2*, *leptin* and *Glut*, by using *36B4* (acidic ribosomal phosphoprotein) and *SDHA* (succinate dehydrogenase complex, subunit A) as housekeeping references (primer sequences, see Electronic Supplementary Material, Table S1). Relative gene expression results were calculated

Table 1 Media used in the differentiation protocols, their abbreviations and content

	Medium	Abbreviation	Content	Manufacturer
1	hASC medium	hASC medium	DMEM/F12	Gibco
			10 % human serum (HS)	PAA
			2 mM L-glutamine (L-glut)	Gibco
2	Serum-free medium	SFM	DMEM/F12	Gibco
			2,56 mM L-glut	Gibco
			0,1 nM 3,3',5-Triiodo-L-thyronine sodium salt (T3)	Sigma
			ITS™ premix:	BD
			6,65 µg/ml (1,15 µM) insulin	PAA
			6,65 µg/ml transferrin	Gibco
			6,65 ng/ml seleniuous acid	
			1 % bovine serum albumin (BSA)	
			2.8 mM sodium puryvate (NaP)	
3	ATE medium	ATEm	1800 µg/ml adipose tissue extract (ATE)	-
			DMEM/F12	Gibco
			10 % HS	PAA
			2 mM L-glut	GIBCO
			100 IU/ml penicillin/0.1 mg/ml streptomycin	GIBCO
4	Stimulation medium	SM	Serum-free medium (see medium 2 above)	Sigma
			supplemented with	Sigma
			200 µg/ml AA	Sigma
			0,5 µg/ml heparin (HE)	Sigma
			5,5 µM: 2 µg/ml Hydrocortisone (HY)	R&D systems
			10 ng/ml Vascular endothelial growth factor (VEGF)	R&DSystems
			1 ng/ml fibroblast growth factor (FGF-2)	
5	Stimulation medium w/o insulin	SM -ins	Same as stimulation medium except for ITS (BD) that has been replaced with:	Sigma
			6,65 µg/ml Transferrin	Sigma
			6,65 ng/ml Seleniuous acid	
6	ATE + ASM	A + A	Stimulation medium	
			ATE medium	

by normalizing the Ct values with the geometric mean of the two housekeeping genes.

For the analysis of brown and white adipocyte-typical mRNAs primer pairs for *Prdm16*, *CIDEA*, *PGC1-α*, *UCP*, *RIP140*, *leptin* and *adenylate cyclase* (*ADCY5*) were used (see Electronic Supplementary Material, Table S1). These were analyzed from two independent cell culture experiments, two cDNA syntheses and qPCR analyses (each in triplicate wells) carried out from each experiment ($n = 4$). Relative gene expression levels were calculated by normalizing the Ct values with the geometric mean of the two housekeeping genes.

Assays for insulin responsiveness

Glucose uptake test

At day 14, DMEM/F12 (Gibco) was changed on the cells and incubated for 2 h at 37 °C in 5 % CO_2. Cells were then exposed to 100 nM or 500 nM insulin and incubated at 37 °C in 5 % CO_2 for 30 min and [^{3}H]-2-deoxy-D-glucose (0.2 µCi/well; Perkin Elmer, Waltham, MA, USA) was added for another 20 min. The cells were washed twice with ice-cold PBS and then lysed in 0.1 % sodium dodecyl sulfate (SDS). The radioactivity of the samples was measured by a liquid scintillation counter Wallac1410 (Perkin Elmer) using OptiPhase HiSafe 2 oscillation liquid (Perkin Elmer). Total protein content of the samples was analyzed with a Pierce™ BCA Protein Assay Kit (Thermo Scientific) as described above and total protein content was used to normalize the results.

Inhibition of lipolysis

At day 14, the medium was removed, cells were washed with PBS and DMEM/F12 (Gibco) was added on the cells. After 2 h, 2 µM isoproterenol (Sigma) was added and incubated for

Table 2 Differentiation protocols; culture settings and medium changes

Protocol	Day 0	Day 1	Days 3–4	Days 6–7	Day 10	Day 14
1 (undifferentiated control)	Cell seeding in hASC medium on 48-well plate at 20 000 cells/ cm² SM	hASCm	SFM	SFM	SFM	End point analysis
2 (ATE induction)		ATEm	ATEm	ATEm	ATEm	
3		ATEm	SM	SM	SM	
4		ATEm	ATEm	SM	SM	
5		ATEm	SM	SM	SM-ins	
6		ATEm	SM -ins + 9 μM Trogl	SM -ins + 9 μM Trogl	SM -ins + 9 μM Trogl	
7		ATEm	SM + 9 μM Trogl	—— (no medium change)	SM -ins	
8		ATEm	ATE			
+9 μM Trogl		SM -ins				
9		ATEm	SM	SM-ins	SM -ins	
10		ATEm	SM	SM	SM -ins	
11		A + A	A + A	A + A	A + A	

1.5 h after which 100 nM or 500 nM insulin (Sigma) was added. After 15 min, 30 min or 45 min incubation, the medium was collected and the amount of glycerol released was assessed with an EnzyChrom Adipolysis Assay Kit (BioAssay Systems, Hayward, CA, USA) according to the manufacturer's instructions. The fluorescence was measured with the Varioskan™ Flash Multimode Reader (Thermo Scientific) at I_{ex} 530 nm and I_{em} 585 nm. Following the collection of the medium, cells were lysed with 0.1 % SDS and the protein content was measured with the BCA kit as described above. Protein content was used to normalize the results.

Statistical analysis

Statistical analyses were performed and graphs processed with GraphPadPrism 5.0 (GraphPad Software, San Diego, CA, USA). The results concerning triglyceride accumulation were subjected to one-way ANOVA followed by Dunnett's post-test. The results concerning gene analysis, glucose uptake and lipolysis were subjected to two-way ANOVA followed by Tukey's post-test. The results were reported as mean ± SD and differences were considered significant when $p < 0.05$.

Results

Effects of the differentiation protocols on triglyceride accumulation

Eleven different differentiation protocols (Table 2) for hASC were tested to find the most effective differentiation protocol for mature in vivo-like adipocytes. The first phenotypic criterion assessed was triglyceride accumulation, as determined by AdipoRed staining at day 14. When comparing the accumulation of triglycerides per relative cell number, the cells grown by protocols 2, 6, 7 and 11 differed significantly from the undifferentiated cells (Table 2; Fig. 1). Morphological examination of cultures stained with AdipoRed revealed that the pattern of lipid accumulation was morphologically most different between protocol 2 (ATE induction) and protocol 7 (3-day induction with ATE, 6 days in serum-free medium supplemented with 1.15 μM insulin and 9.06 μM Troglitazone, followed by 4 days in a defined serum- and insulin-free stimulation medium) (Fig. 1). ATE induced triglyceride accumulation with many small cytoplasmic lipid droplets in nearly all of the cells in the culture but the amount of the accumulated lipid per cell was modest compared to protocol 7 cells. Protocol 7 induced accumulation of lipid in fewer cells than plain ATE induction but the size of the lipid storage in individual cells was greater. The outcome of protocol 6 resembled protocol 7, while that of protocol 11 resembled protocol 2 (Fig. 1).

Effect of differentiation protocols on gene expression

The expression of adipocyte marker mRNAs *PPARγ*, *adiponectin*, *leptin*, *AP2* and *Glut4* were studied in the differentiated cells at day 14. Adipose stromal cells differentiated with protocols 3, 6, 7, 10 and 11 showed the most elevated expression of the gene markers (see Electronic Supplementary Material, Fig. S1). Based on the results of the triglyceride accumulation, three of the new protocols, 6, 7 and 11, were chosen for a closer inspection at gene expression level (Fig. 2). Protocol 7 consistently yielded the highest and most even expression of *PPARγ*, *Glut4*, *AP2* and *adiponectin*. Expression of *leptin* mRNA in all cell specimens, except for those from protocol 11, remained low. Protocols 2 and 6 did not yield significant upregulation of the studied genes compared to undifferentiated control.

To investigate whether the tested adipocyte differentiation protocols selectively induce a brown or a white adipocyte-like phenotype, we performed qPCR analysis of mRNAs typical of brown adipocytes, *PRDM16*, *CIDEA*, *UCP1* and *PGC1α*

and white adipocytes, *ADCY5*, *RIP140* and *leptin* with protocols 2, 6, 7 and 11 with undifferentiated cells (protocol 1) as control (Fig. 3). The protocols 2, 6 and 7 resulted in elevated expression of the brown adipocyte markers *PRDM16*, *CIDEA* and *UCP1*, while *PGC1α* expression was induced only under conditions 6 and 7. Of the white adipocyte markers, *ADCY5* was induced under conditions 6 and 7, while *RIP140* was only marginally affected. Of note, *leptin* was induced most prominently by protocol 11 but not by protocol 7.

Insulin responsiveness of the adipocytes

Glucose uptake

Glucose uptake by undifferentiated control cells and the most promising differentiation method, protocol 7 adipocytes, was studied by employing [^{3}H]deoxyglucose (Fig. 4). The cells were treated for 30 min with 100 or 500 nM insulin prior to the uptake assays. The differentiated cells showed a significant insulin-induced enhancement of glucose uptake at 100 nM concentration of insulin, when compared to the undifferentiated control.
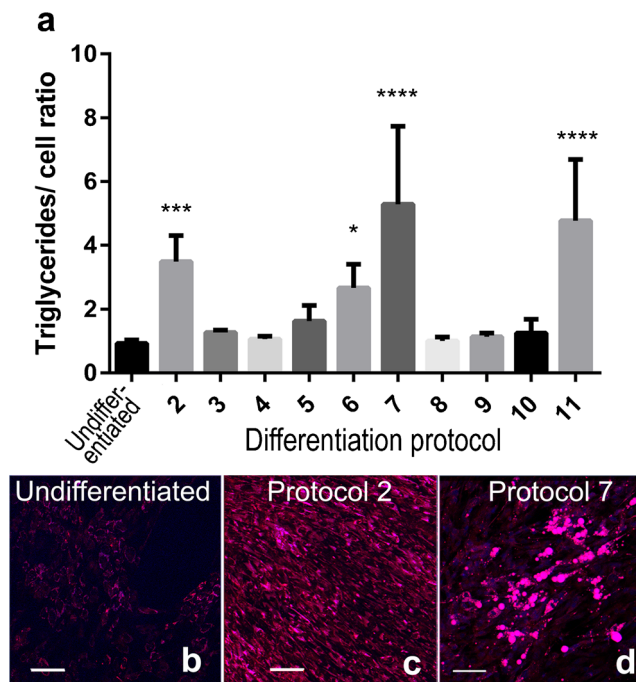
Fig. 1 Cellular lipid accumulation in the differentiation protocols tested. **a** Means ± SD for triglyceride accumulation in 11 different differentiation schemes. Protocols 2 (ATE induction), 6, 7 and 11 showed a statistically significant difference when compared to the negative control (=undifferentiated cells) in one-way ANOVA with Dunnett test, $n \geq 6$; *$p < 0.05$; ***$p < 0.001$; ****$p < 0.0001$. In the confocal images: **b** the undifferentiated cells, **c** adipocytes from ATE induction and **d** protocol 7 adipocytes stained with AdipoRed and DAPI at day 14. Scale bar 100 μm

Lipolysis

The ability of insulin to inhibit lipolysis in undifferentiated control cells and protocol 7 adipocytes was monitored by measuring glycerol released in the growth medium after exposure of the cells to 0, 100 or 500 nM insulin at time points of 15, 30 and 45 min (Fig. 4) at day 14. In the control cells, treatment with 500nM insulin enhanced, rather than inhibited, the release of glycerol, whereas a marked (50–80 %) inhibition of lipolysis was evident in protocol 7-differentiated cells upon treatment with 500 nM insulin at all the studied time points.

Discussion

Differentiation of pre-adipocytes into adipocytes is defined by the acquisition of a lipid-filled morphology and appropriate hormone responsiveness, signaling pathways and metabolism (Novakofski 2004). In the last phase of adipocyte differentiation, preadipocytes transform into insulin-sensitive, lipid synthesizing and transporting mature adipocytes that secrete adipocyte-specific secretory products (Stephens 2012; Rosen and MacDougald 2006; Gregoire 2001; Gregoire et al. 1998). In order to develop a new, native-like human adipocyte in vitro model, 11 different in vitro differentiation protocols were studied using hASC (Tables 1, 2). The outcome was analyzed by using triglyceride accumulation, morphology of lipid deposits and adipocyte gene expression as criteria. Of the tested differentiation strategies, the most promising (protocol 7) was chosen for further studies of insulin responsiveness. This protocol consisted in culture of the hASC (1) for 3 days in adipose tissue extract (ATE) medium, (2) for 6 days in serum-free medium with 1,15 μM insulin and 9,06 μM Troglitazone, a thiazolidinedione (TZD) compound and (3) for 4 days in serum-free medium in the absence of insulin. The expression of adipocyte marker genes (*adiponectin*, *Glut4*, *AP2* and *PPARγ*) was significantly higher in the selected differentiation protocol 7 than in the other protocols tested. Moreover, the resulting adipocytes were responsive to insulin as judged from glucose uptake and inhibition of lipolysis at insulin concentrations of 100 or 500 nM, respectively. According to the criteria set for the differentiated adipocytes, protocol 7 produced mature native-like adipocytes.

In complicated 3D cell cultures, the major challenge is to create a culture environment that allows the growth and differentiation of multiple cell types. We have previously developed an angiogenesis stimulation medium for creating mature vascular structures from adipose stromal cells and endothelial cells in vitro (Huttala et al. 2015). This stimulation medium (SM), consisting of 5.5 μM hydrocortisone, 0.1 nM 3,3',5-Triiodo-L-thyronine sodium salt (T3), 1.15 μM insulin, 1 % BSA, 10 ng/
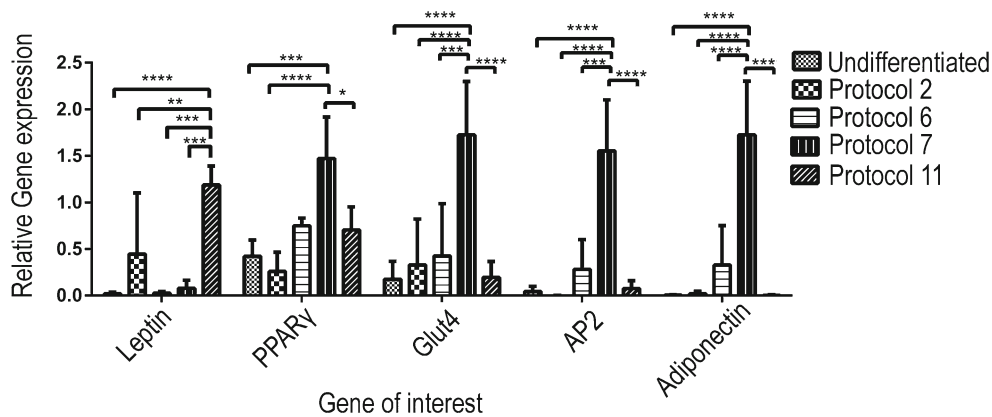
Fig. 2 Expression of *Leptin*, *PPARγ*, *Glut4*, *AP2* and *Adiponectin* mRNAs in adipocytes of selected protocols: undifferentiated control, 2 (ATE induction), 6, 7 and 11. Expressions of *PPARγ*, *Glut4*, *AP2* and *Adiponectin* were significantly higher in protocol 7 adipocytes than in other adipocytes. *Leptin* was expressed at the highest level in protocol 11 adipocytes. *Bars* represent mean ± SD. Statistics calculated with Two-way ANOVA and Tukey's test, $n = 5$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$

ml vascular endothelial growth factor (VEGF) and 1 ng/ml fibroblast growth factor 2 (FGF-2) as active components, was further used in the current study for adipogenesis induction and adipocyte maturation together with ATE (Sarkanen et al. 2012a). ATE is a cell-free angiogenesis- and adipogenesis-inducing agent from human adipose tissue. ATE contains a majority of growth factors, cytokines and chemokines present in mature adipose tissue, e.g., leptin, adiponectin, FGF-2, IL-6, IGF-1, VEGF (-A) and angiogenin, among others and has the potential to induce natural adipogenesis in vitro (Sarkanen et al. 2012a). ATE has been shown to induce a homogenous adipogenic differentiation of hASC and to induce triglyceride accumulation comparable to treatment with a commonly used adipogenic cocktail, with dexamethasone (DEX), insulin, indomethacin and isobutylmethylxanthine (IBMX) (Sarkanen et al. 2012a; Verseijden et al. 2009; Ghoniem et al. 2015; Foley et

al. 2015; Lequeux et al. 2009; Rubin et al. 1978). ATE has been shown to be an essential component for triggering adipogenesis, as none of the tested media induced adipogenesis on their own (data not shown). ATE seemed to cause cell commitment towards adipocytes but also cell proliferation. Therefore, constant addition of ATE was not beneficial for adipogenesis. The explanation for this may come from the multiple cytokines in ATE contributing to the adipose stromal cell commitment (Sarkanen et al. 2012a). Due to the multiple factors in the plain ATE induction, adipogenesis may not be the dominant event in cell culture, which was also seen in the current study as relatively small lipid droplet size and fairly low level of adipocyte marker gene expression. The balance of contradictory signals experienced by preadipocytes in vivo influences whether cells undergo adipogenesis (MacDougald and Mandrup 2002). It is known that, e.g., macrophage-derived
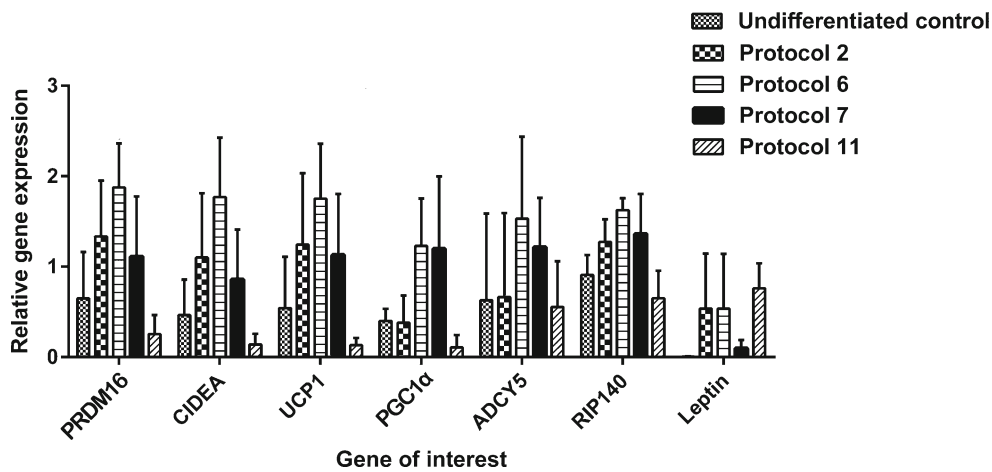
Fig. 3 Expression of brown (*PRDM16*, *CIDEA*, *UCP1* and *PGC1α*) or white (*ADCY5*, *RIP140* and *leptin*) adipocyte-typical mRNAs in the adipocytes differentiated with protocols 2, 6, 7 and 11. The results are from two independent cell culture experiments, two cDNA syntheses and qPCR analyses (each in triplicate wells) carried out from each experiment ($n = 4$). *Bars* represent mean ± SD. Statistics calculated with two-way ANOVA and Tukey's test; *$p < 0.05$; **$p < 0.01$
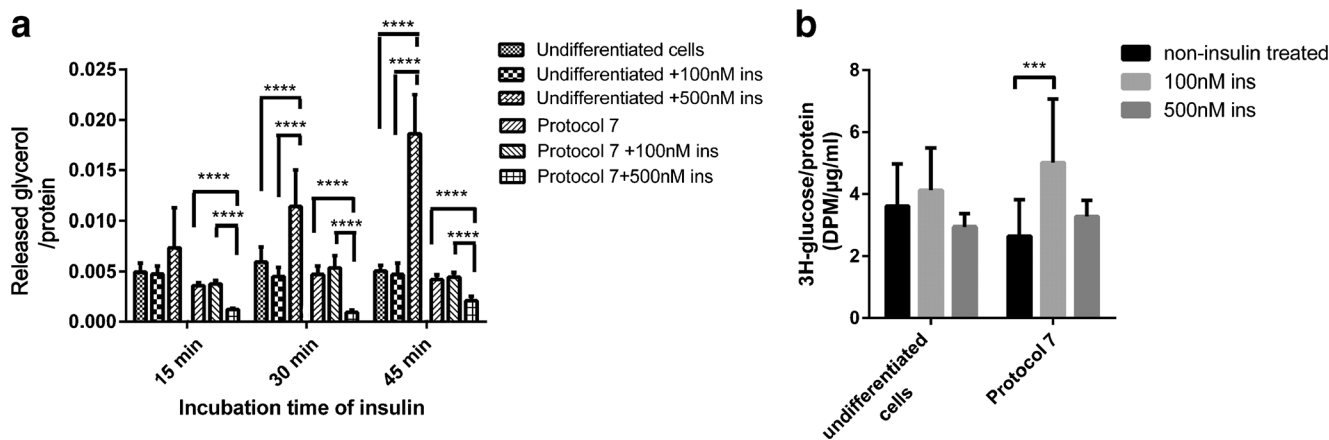
Fig. 4 Responses to 100 or 500 nM insulin at day 14 in undifferentiated control and protocol 7 adipocytes. **a** Released glycerol per total protein after insulin exposure. The cells were incubated with or without insulin for 15, 30 or 45 min. Protocol 7 responded to insulin at all studied time points. **b** Glucose uptake, which was significantly higher in 100-nM insulin-treated protocol 7 adipocytes than in the adipocytes without insulin treatment. *Bars* represent mean ± SD. The results were analyzed by two-way ANOVA with Tukey's post-test, $n \geq 6$; ***$p < 0.001$; ****$p < 0.0001$

proinflammatory factors impair adipogenesis (MacDougald and Mandrup 2002; Lacasa et al. 2007).

Due to the improper maturation of adipocytes in plain ATE induction, this natural adipogenesis induction was modified and improved by adding 9.06 μM troglitazone in serum-free stimulation medium and depleting both insulin and troglitazone from the culture for the last days of the culture period. Although the SM is serum-free, it contains albumin that acts in vivo as a carrier of fatty acids that is needed for their internalization into cells (Walker et al. 2014). This novel treatment, protocol 7, a combination of ATE and other adipogenesis stimulators, resulted in a smaller percentage of cells accumulating triglyceride droplets than plain ATE induction but the lipid accumulation per cell was greater and the differentiated cells resembled fully differentiated native adipocytes in morphology. Another tested protocol, 11 (incubation for the entire 14 days in ATE + serum-free SM), also resulted in prominent triglyceride accumulation; however, gene expression analysis demonstrated under these conditions only induction of *leptin* and *PPARγ*, while the markers of more mature adipocytes, *adiponectin* and *AP2*, were not induced. Of note, protocol 11 produced the most homogenious lipid accumulation among cells and could thus represent a useful model for the study of the early stages of adipogenesis.

The new differentiation method, protocol 7, included incubation of the cells with TZD compound troglitazone and T3. TZDs are PPAR agonists, which stimulate adipogenesis and the redistribution of lipids from liver and muscle into adipose tissue (Greenberg and Obin 2006). Moreover, they activate AMP-activated protein kinase (AMPK), a central sensor for nutrient status, resulting in enhanced glucose uptake into adipose tissues and muscle (Fryer et al. 2002). TZDs have also been shown to decrease *11βHSD-1* and increase *adiponectin* (Greenberg 2003). Work on preadipocytes among human

adipose-derived stem cells or ones developed in vitro from other cell lineages suggests that integration of T3 and TZD signaling enhances the adipogenic differentiation potential (Ortega et al. 2009; Gerhold et al. 2002). T3, on its behalf, interacts with the two primary thyroid receptor isoforms, thyroid receptor a1 (TRa1) and its antagonist receptor a2 (TRa2), expressed in fat tissue. The binding of T3 to TRa1 induces adipogenesis while TRa2 negatively regulates the activity of T3 (Ortega et al. 2009). These observations provide plausible explanations for the observed beneficial effects of the troglitazone and T3-containing media on the adipogenic differentiation in the new differentiation protocol.

The glucocorticoid hydrocortisone used in the SM has been shown to increase the secretion of leptin by cultured rat adipose tissue (Mick et al. 2000) and the synthetic glucocorticoid DEX is commonly used to induce adipogenesis as it increases the expression of *c/EBP* and *PPARγ* (Wu et al. 1996). Clinical cases of glucocorticoid excess are characterized by increased fat mass and obesity through the accumulation of white adipocytes (Hochberg et al. 2015; Barclay et al. 2015). Hydrocortisone has also been shown to increase the secretion of leptin by cultured rat adipose tissue (Mick et al. 2000); however, this was not seen in our study. Previously, Lequeux et al. (2009) also composed an improved adipogenic differentiation medium that was further studied by Ghoniem et al. (2015). Lequeux et al. replaced indomethacin with 1 μM rosiglitazone (another TZD), 10nM hydrocortisone and 2 nM triiodothyronine (T3) and added the basic adipogenic ingredients 0.15 UI/ml insulin, IBMX and DEX. Our serum-free SM, which contains 5.5 μM hydrocortisone, 0.1nM T3 and 9.06 μM TZD troglitazone, is in part similar to their differentiation medium. Although our concentrations are higher, we have no serum, IBMX and DEX, which could explain the need for higher concentrations of the active components we used.

Angiogenic factors are also known to promote adipose tissue growth and expansion (Cao 2014). Angiogenic factors FGF-2 (Xiao et al. 2010; Marra et al. 2008; Tabata et al. 2000) and VEGF (Girandon et al. 2011), among others, have been used when creating new adipocytes (Lowe et al. 2011). In addition, obesity is associated with elevated circulating levels of VEGF, particularly in the context of visceral adipose tissue expansion (Miyazawa-Hoshimoto et al. 2003; Fain et al. 2004). These findings prompted the use of VEGF and FGF-2 in our SM medium. As ATE also contains several angiogenic inducers including VEGF and FGF-2 (Sarkanen et al. 2012a), it further enables the adipogenesis but could also be the reason for the lack of maturation in protocol 11, as a large quantity of angiogenic factors may drive the differentiation towards angiogenesis hence preventing adipogenesis.

A number of well-studied genes are characteristically expressed during adipocyte differentiation or in mature adipocytes. Peroxisome proliferator-activated receptor γ (*PPARγ*) induces adipocyte differentiation by regulating several genes critical for adipogenesis, lipid uptake and lipid metabolism (Schoonjans et al. 1996; Rosen et al. 1999) and is regarded as a mid-differentiation marker (Flynn and Woodhouse 2008). Its induction was detected in most of the differentiation protocols tested in the present study and thus proved that all the protocols were directing the cells towards adipogenesis. Expression of the glucose transporter *Glut4* has been shown to rise in the terminal stage of differentiation (Gregoire et al. 1998). *Glut4* is activated and built into the plasma membrane upon insulin stimulation (Berenguer et al. 2010) and its effect is enhanced by TDZ rosiglitazone (Martinez et al. 2010). In the present study, a strong induction of *Glut4* was only observed in protocol 7, suggesting that this protocol produces a somewhat more mature adipocyte phenotype than the related protocol 6 that lacks troglitazone. The adipocyte hormone leptin is mainly produced and secreted by mature adipocytes (Gregoire 2001). Its main function is the regulation of the body energy balance (Coelho et al. 2013; Kershaw and Flier 2004; Gregoire 2001). Among the present adipogenesis schemes, *leptin* was prominently induced only in protocol 11 and mildly in protocol 2. One reason for low *leptin* expression could be a low energy supply in the serum-free medium at day 14. Both protocols 2 and 11 contain human serum in addition to the ATE. They may thus support the cellular energy status better than the other tested conditions, potentially resulting in an induction of *leptin*.

Adipocyte lipid binding protein 2 (AP2, also known as FABP4) is a cytoplasmic fatty acid chaperone expressed in adipocytes (Yang and Smith 2007) and considered to be a critical link between lipid metabolism, hormone action and cellular functions in adipocytes (Maeda et al. 2001). It triggers ubiquitination and subsequent proteosomal degradation of PPARγ in the terminal stage of adipocyte differentiation (Yang and Smith 2007; Rodriguez et al. 2007; van Beek et

al. 2007; Gregoire et al. 1998). *AP2* was found to be elevated in the hASC-based obesogen screening model (Foley et al. 2015), as well as in our new differentiation method, protocol 7. Another adipocyte marker representing terminal differentiation is adiponectin (Flynn and Woodhouse 2008). It is a hormone that decreases free fatty acids (FFA) in serum, glucose and triacylglycerol concentrations (Fruebis et al. 2001) and is secreted exclusively by adipose tissue (Kershaw and Flier 2004). *Adiponectin* regulates the energy balance of the body via activation of AMPK in the hypothalamus (Coelho et al. 2013). Strong induction of both *AP2* and adiponectin upon the present protocol 7 suggests that this differentiation protocol produced relatively mature, terminally differentiated adipocytes.

To investigate whether the adipocyte differentiation protocols 2, 6, 7 and 11 selectively induce a brown or a white adipocyte-like phenotype, we carried out qPCR analyses of mRNAs predominantly expressed in either one of these adipocyte types. As mRNAs are abundant in brown adipocytes, we employed *PRDM16*, *CIDEA*, *UCP1* and *PGC1α* (Shinoda et al. 2015; Seale et al. 2007), while in white adipocytes we used *ADCY5*, *RIP140* and *leptin* (Knigge et al. 2015; Sawada et al. 2010; Maffei et al. 1995; Zhang et al. 1994). The previously published ATE treatment (Sarkanen et al. 2012a) and conditions 6 and 7 resulted in elevated expression of the brown adipocyte markers *PRDM16*, *CIDEA* and *UCP1*, while *PGC1α* expression was induced only under conditions 6 and 7. The induction of brown adipocyte-typical messages was most prominent with protocol 6. Of the white adipocyte-typical messages, *ADCY5* was induced under conditions 6 and 7, while RIP140 was only marginally affected. To conclude, none of the protocols tested selectively induces either the brown or the white adipocyte phenotype but overall induction of adipocytic genes is observed.

The role of insulin in adipocyte differentiation is evident, as the regulation of adipose tissue metabolism is one of the central physiologic functions of insulin (Cohen 2006). Insulin stimulates the uptake of amino acids and glucose into adipocytes and initiates signaling via effectors like phosphatidylinositol-3 kinase and AKT1/2 with central roles in adipogenesis (Kim and Chen 2004; Garofalo et al. 2003) and represses lipolysis in adipocytes (Watson and Pessin 2007; Choi et al. 2010b). It promotes the synthesis and storage of triglycerides and inhibits their catabolism (Rutkowski et al. 2015). When developing cell models for the mechanisms underlying adipose insulin sensitivity/resistance in metabolic disease, it would be essential to generate adipocyte cultures in which the insulin responsiveness characteristics of the donor tissue are preserved. Since adipocytes generally lose a lot of their insulin responsiveness in culture (Gerrits et al. 1993), this requirement forms a major challenge. In the present study, we showed that protocol 7 adipocytes do respond to insulin in terms of two central parameters: glucose uptake (100 nM

insulin) and inhibition of lipolysis (500 nM insulin), further supporting the view that this optimized protocol yields insulin-responsive adipocytes with patterns of lipid accumulation and gene expression resembling those of native human adipocytes. The lack of accelerated glucose uptake with 500 nM insulin (Fig. 4b) is most likely due to a negative feedback response to excessive insulin hyperstimulation (Ma et al. 2013). Further optimization of the differentiation protocol is therefore necessary. Moreover, the insulin responsiveness of adipocyte cultures could be increased by selecting well-responsive hASC through a more profound characterization.

Conclusion

In this study, a novel adipocyte differentiation protocol mimicking natural adipogenesis was developed. The new protocol employed a natural inducer of adipogenesis, adipose tissue extract, our previously developed serum-free angiogenesis stimulation medium and the thiazolidinedione troglitazone. The results showed that the new protocol produces adipocytes that accumulate triglycerides, express general adipocyte marker mRNAs and respond to insulin as judged by glucose uptake and inhibition of lipolysis. Our qPCR analyses suggested that the protocol does not selectively induce either the brown or the white adipocyte phenotype. The cells showed no regression of adipocyte morphology and remained viable and attached to substratum for at least 10 weeks when the differentiation medium was removed.

A functional model for human adipogenesis is a desirable tool that would enormously benefit adipose tissue and obesity research. This can be achieved by use of well-characterized cells of human origin and a differentiation protocol that produces mature and functional in vivo-like adipocytes. The new differentiation protocol developed in this study and the adipocyte model generated with it from hASC represent a crucial step towards this goal. By developing it further, it will be possible to create accurate, reliable and efficient test systems for the research of obesity and metabolic disease.

Compliance with ethical standards

Conflict of interest Patent issued in USA (WO2010026299A1), pending elsewhere.

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