White Primary Human Preadipocytes Can Be Induced to Express UCP-1 and Transdifferentiate into Brown-like Adipocytes *In vitro*

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Introduction

White adipose tissue (WAT) in subcutaneous and visceral body compartments stores excess energy in the form of triglycerides. Excess accumulated fat, particularly in visceral adipose tissue, can lead to a number of disorders such as dyslipidemia, fatty liver disease, insulin resistance, Type 2 diabetes, hypertension, and cardiovascular diseases that are associated with other metabolic disturbances called metabolic syndrome. On the other hand, brown adipose tissue (BAT) aids to regulate body temperature in hibernating mammals and newborn humans. Within the brown adipocytes, accumulated fatty acids are degraded in the mitochondria and free energy is released as heat. This process, called thermogenesis, works by uncoupling the mitochondrial electron transport chain from the production of ATP. Expressed in the mitochondria’s inner membrane, uncoupling protein 1 (UCP-1) bypasses the proton import path of the ATP synthase, thus releasing the energy of the proton gradient as heat. The discovery of active BAT in adult humans has raised great interest in the scientific community to expand research with BAT for new therapeutic strategies in the treatment of obesity and related disorders. Activating and expanding BAT *in vivo* may be one such strategy. BAT can be expanded through exposure to cold, or through pharmacological agents. For researchers and drug developers, an *in vitro* system of human brown adipocytes would be of great value to gain a better understanding of the key factors involved in transdifferentiation (see Figure 1 for an overview of the development of different types of adipocytes).1

As the supply of adult human BAT is limited, alternative robust sources would be advantageous. The aim of this study was to gain an understanding to which extent human preadipocytes can express a BAT phenotype. We demonstrate that primary human white preadipocytes of subcutaneous and visceral origin, treated with certain pharmacological factors *in vitro*, can give rise to a brown-like phenotype indicated by accumulated intracellular lipid droplets and expression of the marker UCP-1. In addition, we show that through treatment with forskolin, the UCP-1 marker can be induced in undifferentiated preadipocytes to various levels, depending on the donor. Taken together, such an *in vitro* system may provide results of greater relevance than those obtained with animal-derived cells.

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**Figure 1.** Origin and development of different types of adipocytes. Mesenchymal stem cells, triggered by the action of PPARγ, give rise to Myf5 negative and positive precursor cells. The latter ones give rise to brown adipocytes and muscle cells, the former to white and beige adipocytes (intermediates of brown and white adipocytes). Beige adipocytes differentiate to brown adipocytes upon treatment with β-adrenergic agonists. PROM16 inhibits the induction of myoblast-specific genes and the expression of white adipocyte-specific genes.1 (PPARγ: Peroxisome Proliferator-activated Receptor γ, master regulator of adipogenesis; Myf5: Myogenic Factor 5; BMP: bone morphogenic protein; PGC1α: PR domain containing protein; PGC1α: PPARγ coactivator 1α)
**Materials and Methods**

**Cell Culture**

Human subcutaneous and visceral preadipocytes [Lonza] were thawed according to manufacturer’s protocol and seeded in flat bottom well plates. Culture medium was DMEM [high glucose] with 10% FCS, 1% L-Glutamine, penicillin/streptomycin [all from Lonza]. The cells were grown to confluence for 48 hours. Differentiation was started after confluence was reached.

**Differentiating Preadipocytes to Brown-like Phenotype**

The differentiation was performed according to two different published protocols which differed in the various factors added to the cell culture and the regimens of incubation times and media changes. Differentiation was started by replacing culture medium with differentiation medium containing the following factors (for actual concentrations see references listed at the end).

**Protocol 1:**

Differentiation medium was supplemented with IBMX (isobutyl-methylxanthine), dexamethasone, T3 [triiodo-L-thyronine], rosiglitazone, human insulin, and indomethacin. After 7 days, medium was replaced by medium containing only rosiglitazone and insulin for 10 days.

**Protocol 2:**

Cells were seeded after thawing in proliferation medium (culture medium, see above, containing insulin, T3, isoprenaline). After reaching confluence, proliferation medium was replaced by differentiation medium containing additionally IBMX, dexamethasone, rosiglitazone, transferrin, pantothenate, biotin, human growth hormone, human insulin-like growth factor 1 for 7 days.

**Visualizing and Quantifying Intracellular Lipid Droplets**

The lipid droplets were stained using the AdipoRed™ Assay [Lonza] according to supplier’s protocol, subsequently followed by fluorescence-based imaging using a ZEISS microscope (AXIO Observer.Z1). Fluorescence was quantitated as relative fluorescence units (RFU) in a plate reader (FluoroSkan Ascent FL, Labsystems) after micrographs had been taken.

**Quantitation of Cell Viability**

The luminescence-based ViaLight™ Plus Assay [Lonza] was used according to manufacturer’s protocol to quantify the cellular ATP concentration in individual wells as a measure of cell viability. Luminescence was quantified in a plate reader [FluoroSkan Ascent FL, Labsystems]. The AdipoRed™ Assay and ViaLight™ Plus Assay could be multiplexed, i.e., subsequently be carried out in the same well.

**Quantitative RT-PCR**

Cells were lysed in the well using lysis buffer provided in the Power SYBR® Green Cells-to-CT™ Kit (Life Technologies, Inc.). Cell lysates were used to generate cDNA from cellular mRNA using the specified reagents for reverse transcription supplied in the kit. For carrying out the PCR, again components of the kit were used. Primers for UCP-1 and for human \( \beta \)-2-microglobulin (\( \beta 2\)-m) were from Qiagen [RT² qPCR Primer Assay].

The qPCR was run on a Bio-Rad C1000™ Thermal Cycler equipped with a CFX96® Real-time System. Relative levels of UCP-1 mRNA were correlated with the expression level of the housekeeping gene \( \beta 2\)-m by the \( \Delta \Delta \)CT method, fold induction of UCP-1 normalized to a non-induced control by the \( \Delta \Delta \)CT method [2\( \Delta \Delta \)CT]. Data shown represent means of two independent samples with standard deviations of 5% or below.

**Immunofluorescent Staining**

Cells were seeded in 24-well plates and treated with forskolin after reaching confluence. Forskolin was exchanged for culture medium after 24 hours and the cells incubated for another 24 hours. Cells were fixed with paraformaldehyde and permeabilized with Triton™ X-100. UCP-1 was stained with a rabbit polyclonal antibody [Novus Biologicals NB2-20796] and with Cy3-conjugated goat anti-rabbit secondary antibody [Jackson ImmunoResearch Laboratories]. Nuclei were stained using Hoechst 33342. Micrographs were taken on a Zeiss AxioObserver.Z1 microscope. As a staining control, forskolin induced cells were incubated with the secondary antibody only. Micrographs were taken at the same exposure time as the properly stained images. No fluorescence was detected (not shown).

**Results**

1. **Differentiation of Preadipocytes to a Brown-like Phenotype In vitro**

Preadipocytes derived from either subcutaneous or visceral WAT were put in culture and grown to confluence. Differentiation conditions were applied by changing media on Day 0. The two differentiation procedures were carried out according to published protocols (see Materials and Methods section). Cells were stained for accumulated lipid droplets using the Nile-red based AdipoRed™ Assay [Lonza]. Staining of cells was documented by fluorescence microscopy (Figure 2). By semi-quantitative estimation, roughly 50% of the cells were differentiated, i.e., had accumulated intracellular lipid droplets.
The overall fluorescence was quantified in a plate reader. Viability of the cells was subsequently determined in the same wells through the luminescence-based ViaLight™ Plus Assay. Both protocols led to an accumulation of intracellular lipid droplets shown in an example from Protocol 1 in Figure 2. The quantitation results shown in Figure 3A for Protocol 1 reveal a steady lipid accumulation over the time course where subcutaneous and visceral cells differentiate at about the same rate and intensity.

Protocol 2, in contrast, facilitated differentiation and lipid accumulation at a comparable level in a much shorter time interval. In these cases, subcutaneous and visceral preadipocytes responded very differently to the differentiation stimuli. Lipid storage in subcutaneous adipocytes was much higher than in visceral cells.

The differentiating cells expressed the brown adipocyte marker UCP-1 (quantified mRNA expression shown in Figure 4). The expression level of UCP-1 increases over the time course, roughly in correlation with the accumulation of intracellular lipids, except for the subcutaneous adipocytes in Protocol 2. Here, expression of UCP-1 mRNA was already significantly expressed on Day 2 in subcutaneous cells. This might be attributed to the fact that proliferation medium and differentiation medium of Protocol 2 is supplemented with isoprenaline, an adrenergic agonist also acting on β3 adrenergic receptors, thus enhancing transdifferentiation of intermediate to brown adipocytes [see Figure 1].

Inducibility of UCP-1 is not very high at the latest time point as the cells express already high amounts of UCP-1 [compare Figure 4A].

In this context, forskolin treatment of the cells was used to trigger UCP-1 expression at specific time points during the course of Protocol 1 differentiation. Forskolin is acting in the adrenergic signaling pathway by increasing intracellular levels of the second messenger cAMP through direct activation of the adenylyl cyclase. Figure 5 shows the fold increase of UCP-1 mRNA expression after forskolin treatment, based on the expression level of non-induced samples. Induction under the chosen conditions is strongest on Days 11 and 14 for subcutaneous adipocytes, and at low levels on Days 0 and 18. For visceral adipocytes, there is one expression peak on Day 14.
2. Inducibility of UCP-1 in Undifferentiated Preadipocytes

In order to assess the ability of preadipocytes to express UCP-1, cells were grown in plain culture medium to confluence for 72 hours, then treated with forskolin for 24 hours. Expression level of UCP-1 was determined by qPCR.

Figure 6 very clearly shows that in different preadipocyte lots, UCP-1 is expressed at varying levels, obviously depending on the tissue origin and the donor variability. This may reflect that only a portion of the cells is actually capable of giving rise to the brown or brown-like phenotype, so-called intermediate adipocytes, as depicted in Figure 1.

The expression of UCP-1 protein after induction with forskolin could be demonstrated by immunofluorescence staining (Figure 7). Exemplarily, the expression of UCP-1 protein in Lot F is shown, comparing cells stimulated by forskolin with unstimulated cells. The expression of UCP-1 is strongly induced by treatment with forskolin.

Conclusion

Subcutaneous and visceral human preadipocytes can be differentiated to brown-like adipocytes, accumulating intracellular lipid droplets and expressing the marker UCP-1. Expression of UCP-1 mRNA and protein could be induced by treatment with forskolin in undifferentiated preadipocytes. Taken together, these results suggest that primary white preadipocytes may be utilized to develop useful BAT model systems in vitro.

References

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