SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Human SGBS cells can serve for modelling of adipocyte browning

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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, 11 a.m., 6th of February, 2020

1. INTRODUCTION

1.1.Obesity

Obesity and overweight are determined as a systemic disease that displays abnormal and excessive accumulation of body fat leading to harmful health effects. In spite of significant attempts to increase awareness, the epidemic of obesity persists in at a frightening rate (Arroyo et al., 2016). Obesity is connected with higher rates of mortality driven by chronic disorders such as metabolic syndrome, type 2 diabetes mellitus (T2DM), certain types of cancers, hypertension, dyslipidemia, infertility and gastroesophageal reflux (Haslam et al., 2005). There are three possible measures of obesity often used in several studies; however, the most commonly used is body mass index (BMI) which equals the ratio of body weight in kilograms divided by height in meters squared (kg/m^2). The classes are the followings: normal (BMI: 18.5-24.9 kg/m^2), overweight (BMI: 25-29.9 kg/m^2) and obese (BMI: >30 kg/m^2) determined by WHO. In Europe, more than half of the population is overweight and up to 30% is obese; moreover, the prevalence of obesity has doubled since 1980 (GBD Obesity Collaborators, 2015). In Hungary, these numbers are very similar, 60% of the population is overweight, and half of them are obese. It is a well-known fact that in many cases obesity is preventable, although the pathogenesis of obesity is an exceedingly complex and is far from being revealed. The key modulator of obesity is, however, the long-term dysregulation of energy balance, containing reduced energy expenditure and increased energy intake. In spite of continuous research and developments in the understanding of the regulators of energy balance, there is only limited data and limited number of drugs that can be useful for the effective treatment of obesity. Studies, targeting specific components of the neuroendocrine regulation, such as leptin or neuropeptides, have been unsuccessful yet. However, new alternatives focusing on adipose tissue functions e.g. heat production as a result of thermogenesis, can potentially have therapeutic relevance in the future. Adipose tissue is a complex organ with profound effects on

the physiology and pathophysiology of the organism, serving as a calorie storage after feeding and as the source of circulating free fatty acids during fasting. This is the first tissue that is affected by several changes in response to obesity. Traditionally, two types of adipose tissue have been described: white adipose tissue (WAT) and brown adipose tissue (BAT). WAT functions as an energy store, mainly in the form of triglycerides and releases fatty acids upon fasting. On the other hand, BAT contains highly specialized cells with high number of mitochondria which can dissipate energy in the form of heat through activation of Uncoupling Protein-1 (UCP1).

1.1.1. White adipose tissue

WAT is a heterogeneous tissue which contains various cell types in the stromal vascular fraction (SVF): endothelial cells, fibroblasts, pre-adipocytes, macrophages and histiocytes. In addition to its function as an energy store, WAT has an important role as an active endocrine organ, plays a role in the modulation of physiological functions such as energy expenditure, immunity, inflammation, appetite or insulin sensitivity. Moreover, several important proteins have been described to be secreted by WAT, including: leptin, adiponectin, resistin, tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), angiotensinogen as well as others. WAT is widely dispersed in humans and divided into different depots, mainly subcutaneous and visceral adipose tissue. Subcutaneous WAT is located under the skin and plays an important role as a barrier against dermal infection; it also prevents loss of heat and protects the body against external mechanical stress

1.1.2. Brown adipose tissue

The number of studies focusing on the therapeutic potential of BAT against obesity has increased over the last decade. Two types of thermogenic adipocytes exist in mammals: the classical brown and beige/brite adipocytes. Evolutionary, brown adipocytes appear only in

placental mammals. Human infants have significant brown fat depots, in order to provide heat in the cold environment in the post-natal period. Previously, it was proposed that adult humans are mostly lacking of brown fat, unless they are exposed to chronic cold or to state of catecholaminergic excess. The existence of significant classical brown fat in adult humans was proven by ^{18}F-FDG radiolabeled metabolic substrates. Such scans for cancer diagnosis, like PET/CT revealed positive depots of BAT mostly in the supraclavicular and spinal regions of patients. As a result of the positive PET/CT scans, these regions were tested for immunohistochemical analysis of UCP1

Studies focusing on this thermogenic fat depots showed that BAT activity has been forecasted for 2,7-5% of BMR in humans, which can result in more than 4 kg of weight loss per year. The benefits of BAT activation could potentially be a therapeutic approach to reduce elevated triglyceride concentrations and fighting against obesity in humans.

1.2. The development of brown and beige adipocytes

It was presumed for a long time that brown and white adipocytes have a common precursor, because a large number of similarities exist between the two cell types. Nonetheless, data gained from the last decade have shown that classical brown fat is considered to be related to myocytes and originates from a common myogenic factor 5 (Myf5+) positive precursor. Meanwhile, inducible beige adiopcytes together with white adipocytes are believed to arise from a separate lineage (Myf5-). However, tracing studies found some Myf5+ presursors also in WAT, which indicates that white and beige cells may be derived from both lineages. Beige adipocytes can be acivated by cold or other inducers, but after the cold challenge is removed, these cells return to the resting state and assume the morphology of white adipocytes.

1.2.1. The origin of classical brown adipocytes

In spite of the differences in the developmental origins of white and brown adipocytes, both differentiation pathways have similar transcriptional cascades. Firstly, Peroxisome Proliferator Activated Receptor Gamma (PPARɣ) plays an important role in the differentiation of both white and brown adipocytes. Brown adipocyte differentiation requires PPARɣ, but this factor alone is not sufficient to regulate the program of mesenchymal cells to brown adipocytes. Secondly, members of the C/EBP family of transcription factors also take part in activating and maintaining the expression of adipogenic genes.

Studies focusing on PRDM16 revealed that it acts primarily through binding to, then modulating the activity of several transcriptional factors like PPARɣ Coactivator-1α (PGC1-α), C/EBPß, PPARα or PPARɣ. Additionally, it converts white adipocyte precursor cells and myoblasts into thermogenic, UCP1-containing adipocytes. The expression of PRDM16 in white adipocytes not only activates the browning program, but also represses the white or muscle-directed gene program. Cold or ß3-adrenergic stimulation results in brown adipocyte differentiation and vascularization of BAT which is promoted by PRDM16 and FOXC2. Together PRDM16 and FOXC2 drive an increased expression of PGC1α. As a result of cold or ß3-adrenergic stimulation, PGC1α is a key regulator of the browning process, it interacts with IRF4.

1.2.2. The origin of beige adipocytes

Beige adipocytes do not derive from the same precursor cells (Pax7+, Myf5+) like classical brown adipocytes, as they do not express *Myf5*. Even though several studies suggest that a large percentage of the thermogenic fat in adult humans mostly consist of beige cells, there is only limited data about the origin and regulators of beige adipocytes in the literature. It

seems that the mechanism for beige adipocyte induction in inguinal WAT is different from that seen in epididymal WAT.

It is still an important question whether beige adipocytes come from white adipocytes via transdifferentiation program or arise from the differentiation of precursor cells. A number of mouse studies indicate that beige cells have a distinct precursor from classical brown adipocytes, and the differentiaition program is induced as a result of several stimuli. These inducible cells are mostly differentiated from preadipocytes in subcutaneous WAT, during a so-called "browning" process. When the termogenic stimuli stops, "masked" beige adipocytes resemble a white adipocyte-like morphology *in vivo*. These results indicate that the thermogenic capacity of beige adipocytes is reversible and adrenergic stimulus is required for maintaining of the thermogenic profile. The balance between the energy-storing white and energy-dissipating beige adipocytes is determined during the initial differentiation of mesenchymal precursor cells into adipocyte subtypes. The mechanism regulating this process was described in the recent years and contributes to the better understanding of beige adipocyte commitment. Genomewide association studies can be used to identify disease-relevant genomic regions; Obesity-Associated gene (*Fto*) was investigated by this method in humans. There can be a T-to-C conversion at Rs1421085 position, which has an effect on a mesenchymal superenhancer site. Individuals carrying the risk-allele of the FTO locus demonstrate a diminished rate of beige cell differentiation from adipocyte progenitors – therefore, this SNP has a strong genetic association with obesity. When carrying a healthy genotype, a repressor binds to a super enchancer in one of the intronic region of *Fto* gene, resulting in decreased *Irx5* and *Irx3* expression levels during the early differentiation. Meanwhile, a developmental shift may occur towards more energy-dissipating beige adipocytes.

1.3. Activators of brown and beige adipocyte development & function

The sympathetic nervous system (SNS) is involved in regulating both the development and the thermogenic functions of brown adipocytes. It has also been documented that the presence of sensory neurons in BAT are likely to participate in regulating the functions of brown adipocytes in which heat production is under the control of hypothalamus, where the control of temperature and feeding status are integrated. Via the SNS, the outgoing signal is transmitted to the direction of BAT. In response to stimuli (for example cold exposure), norepinephrine (NE) is released from the SNS, which acts primarily on the ß3-adrenergic receptor. ß3- adrenergic receptors are coupled to adenylyl cyclase (AC), which induces the production of a secondary messenger, cyclic adenosine monophosphate (cAMP). During the last decade it was described that cAMP stimulates protein kinase A (PKA) and hormone-sensitive lipase. ß-adrenergic signaling cascade is contolled via AC activation, then cAMP and PKA transmit the thermogenic signal. PKA activation directly stimulates lipolysis and leads to gene expression changes (e.g. UCP1 upregulation). Several rodent studies showed increased activity of BAT and browning of WAT during exercise. An exercise-induced mediator, irisin, has been discovered in mice in the last decade. Irisin is a myokine, which is released by skeletal muscles, whose levels increase following exercise, when it is released, it can be a potent inducer of browning.

1.4. UCP1

In BAT, brown adipocytes are filled with mitochondria that contain UCP1, which uncouples ATP synthesis from the respiratory chain acivity and reduces the proton gradient through the inner mitochondrial membrane (IMM) produced by the electron transfer system. In line with this, mitochondria in BAT contain high amounts of the mitochondrial respiratory chain enzymes but remarkably low amount of the F_1F_0-ATPase. The latter is probably caused by the low expression level of the nuclear ATP5G1 gene, which encodes the mitochondrial

membrane-bound c subunit of Fo oligomer.UCP1 increases IMM conductance for H^+ to dissipate the mitochondrial H^+ gradient and convert the energy of substrate oxidation into heat. When thermogenesis is physiologically required, NE is released by surrounding sympathetic fibers that activates lipolysis, which in turn increases the level of free fatty acids in brown adipocyte mitochondria. Long-chain free fatty acids (LCFAs) are not only substrates for oxidation but also activate UCP1, and they are cleaved by the hormone-sensitive lipase from triglycerides in the cytoplasmic lipid droplets upon ß3-adrenergic stimulation of BAT. LCFA is associated with UCP1, while UCP1 effectively operates as an H+ carrier through the IMM, then protons are released in the mitochondrial matrix. This mechanism leads to the dissipation of energy generated by ß-oxidation of fatty acids mainly as heat.

1.5. Browning inducers which directly target adipose tissue

As a result of certain stimuli, e.g. Bone Morphogenic Protein-7 (BMP7), which was shown to play a crucial role in BAT development, drives both classical brown adipogenesis and recruit beige adipocytes in mice. Additionally, liver also plays a crucial role in the activation of BAT. An important hepatic factor, fibroblast growth factor-21 (FGF21), was recently identified as one of the regulators of BAT, then inducing mitochondrial uncoupled respiration. In neonates, FGF21 directly activates the heat production by BAT, while in adults, FGF21 promotes the browning of WAT depots. Heart also plays a significant role in the activation of BAT, by producing natriuretic peptides (NPs) in mouse and human adipocytes, which induce thermogenesis. IL-6 as a myokine has been shown to activate beige adipocyte development and is also required for exercise-induced WAT beige-ing in mice. IL-6 targets several tissues like the liver, pancreas, brain, WAT and BAT; moreover, it balances between catabolic pathways to mediate glycemic control. However, the effect of IL-6 on the differentiation of human adipocytes is still unclear.

1.6. UCP1 independent mechanisms of thermogenesis

UCP1-independent heat-producing mechanisms were described as a beige specific feature, besides the UCP1 dependent thermogenesis. Several years ago, a creatine-phosphate futile cycle was recognized, which requires coupled ATP synthesis to enhance energy expenditure through stimulation of mitochondrial ATP turnover. Mitochondrial creatine kinase 1 or 2 (CKMT1/2) catalyzes the phosphorylation of creatine, while using ATP to create phospho-creatine (PCr) and ADP. Immediately, PCr is dephosphorylated which leads to heat production.

1.7. Secreted factors by the adipose tissue

The number of proteins found to be produced and released from adipose tissue and the list of secreted factors by WAT consist of more than one hundred products. Leptin is a circulating hormone exclusively secreted by adipocytes, which serves as an essential regulator of body weight. Additionally, it increases BAT thermogenesis via hypothalamic pathways. Many of the WAT-secreted adipokines and inflammatory cytokines are moderately expressed in BAT of mammals; however, the secretory profile of BAT is quite distinct. Brown and beige adipocytes are able to secrete several autocrine factors, which leads to inhibited (sLR11) or increased (FGF21, BMP8b, endothelin-1, IL-6, LPGDS) thermogenic activity. Thermogenic activation of BAT induces the expression and release of FGF21, which is regulated by noradrenergic, cAMP-mediated mechanisms. BMP8b is produced mainly by mature brown adipocytes and its expression is enhanced as a result of thermogenic and nutritional factors, like cold exposure or high-fat diet (HFD). The expression of Angiopoietin-like 8 (ANGPTL8) or lipasin, is induced as a result of cold in BAT. Lastly, it has been shown recently that Neuregulin 4 (NRG4), which is a member of the Epidermal growth factor (EGF) family, is secreted by brown adipocytes and is induced during adipocyte differentiation.

1.8. SGBS cells

Contrary to detailed studies in rodents, there is only limited data about regulatory networks that drive human brown or beige adipocyte differentiation. Therefore, human cell line models are needed in order to investigate key molecular elements of browning and to find targets of novel pharmacological treatments that can enhance browning. A non-immortalized human preadipocyte cell line, namely the Simpson-Golabi-Behmel syndrome (SGBS) cell strain was described in the last decade by our collaborating partners (Wabitsch et al., 2001). The SGBS is an X-linked, rare, congenital overgrowth syndrome, caused by point mutations or deletions in the glypican-3 (*Gpc-3*) gene. SGBS cell line was found to be a proper model for white adipocyte differentiation. Wabitsch et al. found that SGBS cells behave very similarly to human primary preadipocytes without any differentiation; moreover, *in vitro* differentiated SGBS adipocytes are functionally indistinguishable from human adipocytes.

1.9. Transglutaminase 2

A multifunctional protein, TG2, has different cellular localization and plays a role in several physiological (regulation of cell survival - cell death processes, migration, cell adhesion, proliferation, signal transduction) and pathological processes (neurodegenerative disorders, coeliac disease, inflammatory diseases, metabolic diseases, cancer, fibrosis). Moreover, TG2 is known to take part in the differentiation processes of several cell types. TG2 is expressed in almost all cell compartments, including the mitochondria, cytoplasm, nucleus and recycling endosomes. It can be found on the cell surface, as well as being secreted into the extracellular matrix via non-classical mechanisms. Previously, a TG2 KO mouse model was generated to uncover the complex biological functions of TG2. TG2 participated in a crosstalk between phagocytic and dying cells in order to maintain tissue integrity. Moreover, it is also required for the differentiation and bacterial killing of neutrophils. Researchers have done more detailed examinations which revealed several abnormalities and important changes that were studied

under stress and pathological conditions. The lack of TG2 led to diabetes impaired wound healing and autoimmunity.

2. AIMS OF THE STUDY

1. To investigate whether brown or beige adipocyte differentiation can be induced in SGBS cells, by

- gene expression profile analysis
- analysis of the cell morphology by laser scanning cytometry
- functional Seahorse measurements

2. To learn the effect of irisin and BMP7 on SGBS cells

3. To test the involvement of the newly identified creatin-kinase/phosphatase substrate cycle in the heat production of SGBS derived adipocytes (ß-GPA treatment during Seahorse measurements)

4. To clarify whether beige differentiation can be reversed to white adipcoytes or they maintain their beige morphology

5. To quantify the secretion of cytokines ("batokines") by SGBS adipocytes

6. To examine the effect of the secreted IL-6 on the beige adipocyte differentiation of primary cells

7. To examine TG2 gene and protein expression both in white and browning SGBS adipocytes

3. MATERIALS AND METHODS

3.1. Ethics statement

Before the surgical procedure, written informed consent from all participants was obtained. The study protocol was approved by the Medical Research Council of Hungary (20571-2/2017/EKU). All experiments were carried out according to the approved ethical guidelines and regulations.

3.2. Isolation and cultivation of hADMSCs

Adipose tissue specimens were dissected from fibrous materials and blood vessles, minced into small pieces and digested in Phosphate Buffered Saline (PBS) with 120 U/ml collagenase for 60 min in a 37 °C water bath. Then, the completely disassembled tissue was filtered to remove any remaining tissue, by a filter with pore size of 140 μm. As a next step, cell suspension was centrifugated at 1300 rpm for 10 minutes. The pellet of hADMSCs was re-suspended in DMEM-F12 medium supplemented with 10% FBS, 100 U/ml penicillin-streptomycin, 33 μM biotin and 17 μM pantothenic acid.

hADMSCs were seeded into Ibidi eight-well μ-slides or 6-well plates at 37°C in 5% CO_2 to attach, at a density of $3x10^4$ cells/cm^2 and cultured in the same medium. After 24 hours, floating cells were washed away with PBS and the remaining hADMSCs were cultured until they became confluent. The absence of mycoplasma was tested by polymerase chain reaction (PCR) analysis.

3.3. Flow cytometry: characterization of adipose tissue-derived stem cells

To investigate the phenotype of the undifferentiated SGBS preadipocytes, a multiparametric analysis of surface antigen expression was performed by three-color flow cytometry using fluorochrome-conjugated antibodies with isotype matching controls. After harvesting the cells with 0,025% trypsin-EDTA, they were washed once with normal medium. Then, cells were

washed two more times with FACS buffer. Cells were incubated with FACS buffer and fixed in 1% PFA/PBS. Analysis was carried out within one day. Samples were measured using FACS Calibur BD flow cytometer and data were analyzed using BD Multiset software.

3.4. Differentiation of human SGBS preadipocytes into mature white and brown adipocytes

For our experiments hADMSCs and Simpson Golabi Behmel Syndrome (SGBS) preadipocytes have been used as adipocyte progenitors. SGBS human cell line was kindly provided by our collaborating partners, Pamela Fischer-Posovszky and Martin Wabitsch who previously developed it.

To induce a white adipogenic differentiation, cells were incubated in serum-free medium for 4 days, supplemented with 100 U/ml penicillin/streptomycin, 33 μM biotin, 17 μM pantothenic acid, 2 μM rosiglitazone, 20 nM human insulin, 25 nM dexamethasone, 10 μg/ml human apo-transferrin, 500 μM 3-isobutyl-1-methylxantine, 100 nM cortisol and 200 pM triiodothyronine. The medium was changed after the fourth day; and rosiglitazone, dexamethasone and 3-isobutyl-1-methylxantine were removed from the differentiation media during the remaining 10 days of differentiation. In every fourth day, the differentiation cockatil was replaced (Fischer-Posovszky et al., 2008).

PPARγ-driven browning differentiation was induced for four days using serum-free DMEM/F12 medium supplemented with 100 U/ml penicillin/streptomycin, 33 μM biotin, 17 μM pantothenic acid, 0,85 mM human insulin, 1 μM dexamethasone, 10 μg/ml human apo-transferrin, 500 μM 3-isobutyl-1-methylxantine and 200 pM triiodothyronine. From the fifth day, cells were futher cultured in DMEM/F12 serum-free medium, for the following ten days adding 500 nM rosiglitazone while dexamethasone and 3-isobutyl-1-methylxantine were omitted (Elabd et al., 2009). In long-term experiments either the same culture conditions were

applied for 21 and 28 days or it was replaced by the white differentiation medium without rosiglitazone, 3-isobutyl-1-methylxantine and dexamethasone. In every fourth days, the differentiation cockatil was changed.

Human recombinant irisin was administered at 250 ng/ml concentration, and human recombinant BMP7 was used at 50 ng/ml concentration (Raschke et al., 2013; Kristóf et al., 2015). 12-hours-long irisin or BMP7 treatment was applied after the two weeks-long differentiation program to examine the short term effect of them. Where indicated, the browning inducers mentioned above were added to the differentiating cells in increasing concentrations.

Where indicated, primary adipocytes were treated with human IL-6R alpha or IgG Isotype control antibody at 5 μg/ml every day during the whole differentiation.

To examine the short-term effect of NC9, 9 hours treatment was applied after the two weeks-long (14 days) white and PPARγ-driven browning differentiation, then the cells were collected. Paralelly, we were curious about the long-term effect of NC9. During the whole differentiation (21 days) NC9 was administered in every fourth day, when the media was replaced and after 21 days cells were collected.

3.5. RNA isolation, RT-PCR, qPCR

Differentiated SGBS adipocytes were collected and total RNA was extracted from the samples by using Trizol reagent. RNA concentrations were quantified by spectrophotometry. To generate cDNA, high-capacity TaqMan™ reverse transcription reagents kit was used according to the manufacturer's instructions. Applied Biosystems designed the gene primers and probes. Quantitative real-time PCR (qPCR) was performed in a LightCycler 480 with the program of 10 seconds at 94ºC, followed by 40 cycles of 12 seconds at 94ºC and 30 seconds at 60ºC. All samples were measured in triplicates. Normalized gene expressions values were calculated by

the comparative delta cycle threshold (ΔCt) method. This method is able to correct the differences between RNA samples in which the Ct of target genes were normalized to Ct of *Gapdh,* which was used as an endogenous control. Normalized gene expression levels equal $2^{-\Delta Ct}$.

3.6. Total DNA isolation and mitochondrial DNA quantification

Total DNA was isolated from differentiated SGBS adipocytes by the phenol-chloroform extraction, using Trizol reagent. During the qPCR we were using diluted samples (100 ng DNA), 10μM from each primer; human mitochondrial DNA specific primers:

forward 5'CTATGTCGCAGTATCTGTCTTTG-3'
reverse 5'-GTTATGATGTCTGTGTGGAAAG-3'

nuclear specific primers (SIRT1 gene):

forward 5'CTTTGTGTGCTATAGATGATATGGTAAATTG-3'
reverse 5'GATTAAACAGTGTACAAAAGTAG-3'

and Maxima SYBR Green/ROX qPCR Master Mix. LightCycler 480 was used with the program of 20 minutes at 95ºC, and 50 cycles of 15 seconds at 95ºC, 20 seconds at 58ºC, 20 seconds at 72ºC. Relative mitochondrial DNA content was determined from the difference between the threshold cycle (Ct) values for mitochondrial DNA and nuclear specific amplification. Results show mitochondrial genomes per diploid nuclei.

3.7. Antibodies and immunoblotting

Differentiated SGBS adipocytes and undifferentiated control cells were collected and washed once with PBS, and then homogenized in 5x Laemmli loading buffer and boiled for 10 min at

100ºC. Equal amounts of protein were loaded onto a 12 %-SDS-polyacrylamide gel. Proteins were transferred onto a PVDF Immobilon-P Transfer Membrane. Then the membranes were blocked in Tris-buffered saline containing 0,05% Tween-20 (TBS-T) and 5% skimmed milk for 1 hour.

The membranes were then probed at 4ºC with primary antibodies overnight: polyclonal anti-UCP1 (1:500, Sigma, U6382; 1:500, Thermo Scientific, PA1-24894), monoclonal anti-UCP1 (1:1000, R&D Systems, MAB6158), anti-OXPHOS (1:1000, Abcam, UK, ab110411) and anti-TG2 (1:1000; Zedira). All of them were diluted into TBS-T containing 1 % non-fat skimmed milk, followed by the incubation with horseradish-peroxidase (HRP)-conjugated species-specific secondary antibodies for 1 hour at room temperature. For loading control, rabbit polyclonal antibody against ß-actin (1:10000, Sigma, A2066) was used overnight at 4°C in TBS-T containing 1% skimmed milk. Immunoreactive proteins were visualized by Immobilion western chemiluminescence substrate. ImageJ software was used to carry out the densitometry measurements.

3.8. Immunfluorescence staining

SGBS cells and hADMSCs were plated on eight-well ibidi micro slides and differentiated as described in 4.2.4. On the day of measurement, cells were washed once with PBS, then fresh medium was added. Cells were stained with Hoechst 33342 (50µg/ml) for 20 minutes. Next, cells were washed with PBS and fixed in 4% paraformaldehyde for 5 minutes. Then, blocking step was carried out with 5% skimmed milk for 2 hours and staining with anti-UCP1 (Sigma, U6382; 1:500) for 6 hours at room temperature. As a secondary antibody, Alexa 488 goat anti-rabbit IgG was applied. Between and after antibody usage, washing steps were carried out in the presence of 0.1% saponin in PBS, to make cells more permeable.

3.9. Laser scanning cytometry

Scanning was done by an iCys Research Imaging Cytometer (iCys, Thorlabs Imaging Systems, Sterling, VA, USA). Imaging Cytometer equipped with 405-nm, 488-nm and 633-nm solid-state lasers, photodiode forward scatter detectors and photomultiplier tubes with three filters in front. The images were processed and analyzed (n=3, 2000 cells per SGBS sample / hADMSCs) by our high-throughput automatic cell-recognition protocol, which was developed by Doan-Xuan et al. (2013) with some modifications using the iCys companion software (iNovator Application Development Toolkit version 7.0, CompuCyte Corporation, Westwood, MA, USA). Cells were identified in accordance with their Hoeschst 33342 nuclear staining. Sample slides were mounted on a computer-controlled stepper motor-driven stage. An area with optimal confluence was selected in low-resolution scout scan with 10x magnification objective and 10 µm scanning step. Subsequently, high-resolution images were taken by using 40x objective (NA 0.75) and a 0.25-µm scanning step. The size of a pixel was regulated to 0.25µm x 0.245µm at 40x magnification. Laser lines were separately operated, namely a 405-nm diode laser was used to excite Hoechst 33342 and a solid-state 488-nm laser was used for Alexa 488 goat anti-rabbit IgG. Emissions of Hoechst was detected at 450 ± 20 nm and Alexa 488 at 530 ± 15 nm. Transmitted laser light was captured by diode photodetectors in which light loss and shaded relief signals were measured to gain information about light absorption, light scattering and texture of the objects. Then, images were processed and analyzed by our high throughput automatic cell recognition protocol using the iCys companion software (iNovator Application Development Toolkit, CompuCyte Corporation) and CellProfiler (The Broad Institute of MIT).

The hereunder process was used to determine adipocytes in the mixed cultures. Hoechst-stained nuclei were identified first and marked as primary objects. To characterize the lipid droplet content of the cells, texture feature was used, therefore cells which contained lipids above a present threshold value were considered as adipocytes and they were included in further

analysis. On texture "sum variance" (SV) vs. UCP1 expression plots undifferentiated progenitor segregated from the rest of the cells and were narrowly confined around the (0, 0) coordinates. Image regions occupied by these cells were excluded from further analysis.

Next, the fluorescence signal intensity of the UCP1 immunostaining and the texture sum variance of the light scatter signal of lipid droplets were quantified in each cell within the 30-pixel immediate outward vicinity of the nucleus contour by the Cell Profiler software. Afterwards, based on these fluorescence and light scatter signal of single cells, a semi-automated classification and enumeration of the differentiated white and brown adipocytes and undifferentiated preadipocytes was carried out applying the trained classification "Fast Gentle Boosting" of the Cell Profiler Analyst software.

3.10. Oxygen consumption and extracellular acidification measurements

Real-time oxygen consumption and extracellular acidification rates were measured by using an XF96 oximeter (Seahorse Biosciences, North Billerica, MA, USA). SGBS cells and hADMSCs were seeded onto 96-well XF96 cell culture microplates. Cells were kept in growth medium at the longest for 24 hours and then the differentiation process started as described in 4.2.4. On the days of measurement, after recording the baseline oxygen consumption (OC) and extracellular acidification rate (ECAR) for 30 minutes, cells received a single bolus of dibutyril-cAMP at 500 μM concentration to induce adrenergic stimulation. Then, stimulated OC was measured in every 30 minutes. The final reading took place at 6 h post-treatment. Differentiated adipocytes were treated with 2 mM ß-guanidinopropionic acid to block the creatine-driven substrate cycle. In addition, proton leak respiration was measured by oligomycin treatment at 2 μM concentration, which blocks the ATP synthase. As a last step, for baseline correction, cells received a single bolus of Antimycin A treatment at 10μM concentration. After the measurements, oxygen consumption rate (OCR) and ECAR was normalized to protein content.

For statistical analysis, the fold change of OC and ECAR levels were determined comparing basal, cAMP stimulated and oligomycin inhibited (both in unstimulated and stimulated cells) OCRs/ECARs of each sample to the basal OCR/ECAR of untreated white adipocytes.

3.11. Determination of cytokine release

In SGBS cell experiment, supernatants were harvested every fourth day during regular replacement of differentiation media and stored for cytokine measurements. The concentration of IL-6, IL-1β, IL-8, TNFα and MCP-1 was measured from the collected media using ELISA DuoSet Kit.

3.12. Genotyping

Genomic DNA was purified with GeneJET Genomic DNA Purification Kit according to the manufacturer's protocol. Rs1421085 SNP (single nucleotide polymorphism) was genotyped by qPCR using TaqMan genotyping assays and by DNA sequencing. To amplify the corresponding genomic region, we designed the following primer pair:

Forward: 5'GATGACACACACCATGAGCC
Reverse: 5'TAACAGTGGAGGTCAGCACA

Following PCR amplification, we purified the PCR product with NucleoSpin® Gel and PCR Clean-up kit. Then the quality of the product was investigated by 2% agarose gel electrophoresis. DNA was sequenced by Sanger sequencing method.

3.13. Statistical analysis

Results are expressed as the mean ± SD for the number of assays indicated. The normality of distribution of the data was tested by Kolmogorov-Smirnov test. For multiple comparisons of

groups statistical significance was calculated and evaluated by one-way ANOVA followed by Tukey post-hoc test. To compare two groups, two-tailed paired Student's t-test was used.

4. RESULTS

4.1. SGBS cells express surface markers similarly to primary preadipocytes and are heterozygous for the FTO risk allele Rs1421085.

A multiparametric analysis of surface antigen expression was performed by three-color flow cytometry. We found that hematopoietic/monocyte markers (CD34, CD47), endothelial markers (CD54), fibroblast markers (CD73, CD90), integrins and CAMs (integrin ß1, CD44, CD325) were expressed on the surface of undifferentiated SGBS preadipocytes. As a next step, we made a comparison in the surface antigen expression pattern of SGBS preadipocytes to hADMSC cells isolated from human abdominal subcutaneous fat. CD34, CD44, CD146 and HLA-DR expression levels were higher in SGBS preadipocytes, while CD105, CD49a and CD31 antigens were expressed at a lower level as compared to primary preadipocytes. Next, we tested the presence of the C risk-allele of the rs1421085 locus; DNA sequencing and qPCR-based genotyping analysis determined that SGBS cells are heterozygous for the C risk allele.

4.2. SGBS preadipocytes respond to sustained PPARγ ligand and irisin or BMP7 treatment by inducing either beige or classical brown marker genes.

The white and browning differentiation regimes were applied to differentiate SGBS preadipocytes. We selected to examine the expression of core set of BAT-specific genes (*Ucp1, Cidea, Elovl3 and Pgc1α*), the beige selective (*Tbx1, Cited1*) and "classical brown' adipocye marker genes (*Zic1, Pdk4*), expression of *C/ebpß*, mitochondrial enrichment *Cyc1*, key drivers of the adipogenic program *(Pparγ*) and general (*Lep, Fabp4*) adipocyte marker genes were investigated. The browning cocktail highly induced *Ucp1* mRNA expression. Similarly, the

presence of human recombinant irisin or BMP7 resulted in increased *Ucp1* mRNA expression on the top of the white differentiation protocol. We found that mRNA of brown-fat specific genes, like *Cidea, Elovl3 and Pgc1α* were also enriched during the administration of the browning cocktail and when irisin was added to the white differentiation cocktail. On the contrary, we observed decreased expression of the white adipocyte marker gene, *Lep*, in response to the browning differentiation. The expression level of the mitochondrial enrichment marker, *Cyc1*, was significantly higher in browned adipocytes compared to white ones. Moreover, irisin treatment resulted in similar effect. In line with the increased *Ucp1* expression, we found that irisin or the browning protocol resulted in marked upregulation of *Tbx1* and *Cited1* but no *Zic1* induction. BMP7, on the other hand, only upregulated *Zic1* markedly and even prevented *Tbx1* induction when it was combined with the browning differentiation cocktail. Interestingly, *Pdk4*, originally described as a classical brown marker in mice, showed a similar expression pattern as the beige-selective *Tbx1* and *Cited1*, in SGBS adipocytes

4.3. Laser scanning cytometry can quantify SGBS adipocyte browning.

Next, we investigated the morphological characteristics of the white and browned SGBS cells by assessing the textural parameters and UCP1 protein content of the individual adipocytes using laser scanning cytometry (LSC). Applying image analysis, single cells were identified based on their nuclei and classified according to their UCP1 content and lipid droplet structure. As a result of the 14-day-long PPARγ-driven browning differentiation, we found higher UCP1 content either in single SGBS adipocytes or in total cell lysates, as compared to white. In addition, we detected enhanced UCP1 protein content in response to irisin or BMP7 supplementation. Next, we plotted texture sum variance and UCP1 protein content for each differentiated adipocyte. Our results indicate that the population of differentiated adipocytes remains heterogeneous regardless of whether white or browning protocol was applied. Furthermore, we could detect a significant amount of adipocytes with the characteristic

morphological features of browning even in response to the white differentiation. In contrast to to primary adipocytes the texture sum variance did not decrease significantly during the induction of browning marked by elevated UCP1 expression

4.4. Differentiated SGBS adipocytes respond to activation as functional beige cells using the creatine phosphate cycle.

Next, we intended to investigate the functional capacity of human SGBS adipocytes. Cells were differentiated in the presence of browning-inducers and mitochondrial oxygen consumption rate (OCR) was measured. In accordance with the gene expression and morphological changes, we found higher basal OCR after the browning was induced by either PPARγ ligand, irisin or BMP7. We stimulated the adipocytes by a single bolus dose of cell permeable dibutyryl-cAMP. OCR of browning adipocytes was elevated more robustly as compared to the white adipocytes. In parallel, we detected significantly increased extracellular acidification rate (ECAR) both in untreated and cAMP-stimulated browned adipocytes. We examined a characteristic feature of beige adipocytes, utilizing ß-guanidinopropionic acid (β-GPA) which is an inhibitor of this cycle. In case of the browned SGBS cells, the creatine-cycle related OCR was appreciably elevated in contrast to untreated white adipocytes, and the same trend was observed as a result of irisin or BMP7 treatment on white adipocytes. This suggests that the creatine phosphate futile cycle is present and active in browned SGBS adipocytes

4.5. The brown/beige adipocyte phenotype of differentiated SGBS cells is maintained in the absence of the PPARγ-ligand.

In order to determine whether the PPARγ-driven differentiation could maintain a beige phenotype in a longer time frame, we performed a long-term experiment after 2 weeks altogether for 21 or 28 days; and in parallel samples the browning cocktail was replaced by the white differentiation cocktail for the third (B14,W7) and the fourth week (B14,W14).

We could detect the highest expression of *Ucp1* at day 28, at the end of the long-term browning differentiation program. When we replaced the browning cocktail to white, *Ucp1* mRNA level was increased at least until day 21, then dropped to the level of day 14. We found that UCP1 protein was slightly expressed in browned cells after 14 days of differentiation. Long-term rosiglitazone induction led to further robust upregulation of UCP1 at protein level. When the PPARγ stimulus was omitted after 2 weeks of browning differentiation program, the expression level of UCP1 protein was slightly increased by the end of the third week, and it was still detectable one week later at a lower level. Mitochondrial oxidative phosphorylation (OXPHOS) plays a fundamental role in the energy production of brown/beige adipocytes. We detected higher expression of respiratory chain proteins in browned adipocytes. The omitted PPARγ agonist resulted in only a slight reduction in the level of these proteins, suggesting the clearance of a small fraction of the mitochondria. These results suggest that the signaling pathways driving the beige phenotype in SGBS cells can be maintained at least for two consecutive weeks, even when rosiglitazone is eliminated from the differentiation media.

4.6. Browning cocktail resulted in increased expression of IL-8, Il-6 and MCP1 cytokines compared to white adipocytes.

Additionally, we investigated the secretion of cytokines by human SGBS cells: IL-8, IL-6, IL1ß, TNFα and MCP-1 were measured by ELISA. Neither undifferentiated nor differentiated adipocytes secreted TNFα and IL-1ß. In undifferentiated preadipocytes, IL-8, IL-6 and monocyte chemoattractant protein-1 (MCP1) were detected at a comparatively high level. Interestingly, the secretion of these cytokines was significantly higher by browned adipocytes as compared to white adipocytes. In contrast to BMP7 administration, irisin treatment resulted in an increased total IL-8 production.

4.7. Continuous inhibition of the IL-6 receptor resulted in a downregulation of UCP1 and extracellular acidification during browning differentiation.

Thermogenic, noradrenergic activation of brown adipocytes is in line with increased IL-6 expression. Recently we examined the cytokine secretion of human adipose tissue samples and primary adipocytes and we found the IL-6 secretion was maintinaed until the end of the differentiation program. we investigated the effect of a continuous blocking of IL-6 receptor alpha (encoded by IL6A gene) with an antibody.With LSC method we could detect the texture and UCP1 protein content in single adipocytes. When the blocking antibody was applied on top of the PPARɣ-driven browning protocol, it resulted in increased texture sum variance, which associated with larger lipid droplets, meanwile the mean Ucp1 intensity decreased. These results suggest that the differentiation shifted towards producing more white adipocytes.

4.8. TG2 is expressed at a higher level in browned adipocytes and as a result of its inhibition both TG2 and UCP1 expression decreased.

We also analyzed the gene and protein expression of TG2 during the long-term experiment. Our results show that *Tg2* is expressed at a higher level in browned SGBS adipocytes. Furthermore, TG2 expression is correlating with UCP1 expression at mRNA and protein level as well. NC9 is a penetrating, irreversible, site-specific inhibitor of TG2. As a result of NC9 treament, either for 9 hours or 21 days in differentiated brown SGBS adipocytes, that both *Tg2* and *Pm20d1* gene expression were significantly decreased. 21-day-long NC9 treatment on browned adipocytes led to significantly lower expression of *Ucp1*. In case of white adipocytes we did not observe any significant difference between the NC9 treated and untreated cells. We also tested the changes of protein expression in TG2 and UCP1 in response to the white and browning differentiation cocktail in the presence or absence of NC9 treatment for 9 hours or 21 days, respectively. 21-day-long NC9 administration resulted in significantly decreased TG2

protein expression on differentiated browned adipocytes. A similar trend was found in the case of UCP1 protein expression.

5. DISCUSSION

1. Distribution of BAT in the human body and studying browning of hADMSCs.

BAT allows mammals to maintain their body temperature by non-shivering thermogenesis. The key mediator of heat production of brown and beige adipocytes is UCP1, which is located in the IMM, where it dissociates cellular respiration from the ATP generation. As a result of certain stimuli, such as cold exposure, NE activates ß3-adrenergic signaling on brown adipocytes, which triggers the release of FFA and subsequent activation of UCP1 (Cannon et al., 2004; Fedorenko et al., 2012). In the mitochondria of beige adipocytes an effective UCP1-independent thermogenic mechanism was described recently, which is mediated by a futile cycle of creatine metabolism. ^{18}F-FDG-PET/CT has been used to image BAT activity, especially in human adults, which clearly demonstrates cold-activated BAT. Having a better understanding of human adipocyte browning, hADMSCs isolated from SVFs are the most widely used *ex vivo* system. Recently, we showed that hADMSCs isolated from abdominal subcutaneous fat could be differentiated into beige adipocytes, by laser-scanning cytometry. In the current study, we applied this tool to quantify the amount of browned SGBS adipocytes as well. However, there are many limitations of using hADMSCs, due to the limited availability of biopsy materials and the huge variability between donors, which can make it difficult to reproduce experiments.

2. Human preadipocyte cell lines exist and have some features consistent with having browning capacity.

PAZ6 cells, which were obtained from SVF of human infant BAT, and transformed with SV40 T and t antigens were able to differentiate into brown adipocytes *in vitro*Another research

group, Xue et al. generated human preadipocyte cell lines by immortalization process, from the supraclavicular region of four healthy patients. Preadipocyte clones from the deep neck area were capable of differentiating to functional thermogenic adipocytes and responded to BMP7 administration. In another study, Shinoda et al. compared the differentiation of 65 clonal preadipocyte lines originated from supraclavicular fat biopsies. They found that the majority of the adipocytes derived from single cell clones of SVF from supraclavicular regions exhibited gene expression signatures which resembled the beige type of thermogenic adipocytes.

3. Interpretation of SGBS adipocyte browning observed by other groups.

The SGBS human preadipocyte cell line is often used as a representative model of studying human adipocyte biology and previously it was described as a representative model of white adipocyte differentiation. The first results were described in 2013, showing the possibility that SGBS cells can differentiate into thermogenic adipocytes. 2-oxoglutarate dependent dioxygenase (encoded by the proposed *Fto* gene) deficient SGBS preadipocytes differentiated to white adipocytes had increased UCP1 expression and uncoupled respiration, without any changes in mitochondrial mass or structure. Later, Tews et al. found that TENM2 (teneurin-2), which may inhibit the classical brown marker, *Zic1*, is enriched in white adipocyte progenitor cells. They examined SGBS cells with TENM2 knockdown by siRNA, which led to increased UCP1 at mRNA and protein level as well. Moreover, basal and proton leak mitochondrial respiration were enhanced. Contrary to these studies and to the results presented here, Guennoun et al. found that the SGBS adipocytes switch their phenotype during a four weeks-long differentiation program. Within two weeks, they demonstrated high UCP1 expression and a thermogenic phenotype even as a result of the white differentiation protocol without adding any browning inducer or stimulus. Then, the expression of UCP1 greatly declined as a result of the continuation of the white differentiation for additional two weeks. Another study seemingly strengthened these results by comparing the gene expression pattern of the differentiated SGBS

and primary hADMSC-derived subcutaneous adipocytes. They also observed that UCP1 protein expression was high in SGBS cells differentiated for 12 days even when either rosiglitazone or T3 were omitted from the media.

4. SGBS cells are able to differentiate into brown or beige adipocytes, and respond to either rosiglitazone, irisin or BMP7.

Using laser-scanning cytometry and applying functional assays we aimed to examine whether classical brown or beige adipocyte differentiation (browning) can be induced in SGBS cells. Our experiments clearly shows that the PPARγ-driven browning protocol (including rosiglitazone) and irisin treatment could be successfully used to induce browning of SGBS adipocytes, which resulted in a beige phenotype. Our data imply that irisin induces browning of already committed "beige" preadipocytes or multipotent progenitors. administration of BMP7 had a more moderate effect and induced a distinct gene expression program without the upregulation of the beige-selective markers, *Tbx1* and *Cited1*. Both in primary subcutaneous and in SGBS adipocytes, BMP7 treatment results in an increased gene expression of *Pparg* and *Zic1* suggesting that this mediator induces a classic brown-like differentiation instead. The functional studies detected UCP1-dependent proton leak (heat production) and high extracellular acidification in the case of browned adipocytes. UCP1-independent heat-producing mechanisms were described as a beige specific feature. In our experiments, the involvement of the creatine-phosphate futile cycle was investigated. We demonstrated the induction of this futile cycle in response to a β-adrenergic cue in beige SGBS adipocytes.

5. FTO locus is connected to beige or "masked beige" potential of adipocytes.

Each individual has different life-style, dietary and genetic background - for example the presence of the risk-allele of the FTO locus which has an effect of adipocyte browning and represents genetic association with obesity. The potential of mitochondrial thermogenesis is

reduced in those individuals who carry the C risk allele which occurs at a high frequency (approx. 44%) in the European population. In this study, we described that the SGBS cell line carries one copy of the C risk allele; however, these preadipocytes were able to differentiate into functional beige adipocytes if browning stimuli (e.g. rosiglitazone or irisin treatment) were continuously present during their differentiation.

6. Cytokine secretion of SGBS cells and hADMSCs.

In line with SGBS cells, our laboratory also investigated the effect of browning inducers to hADMCSs. Comparing the action of Irisin on hADMSCs vs. SGBS cells, the treatment had an increasing effect on IL-6, MCP-1 and IL-8 production on hADMSCs (Kristóf et al., 2019), and on IL-8 production of SGBS cells as compared to white control adipocytes. Only IL-6 was produced and maintained till the end of the differentiation by the adipocytes. In patients, the pharmacological blockade of IL-6 resulted in weight gain, which can be partially explained with the reduced browning, enchanced by auto/paracrine IL-6 signaling. We investigated the inhibition of the auto/paracrine effect of IL-6 receptor and our findings suggest that the inhibition led to a decreased beige phenotype of human adipocytes.

7. The role of transglutaminase 2 and its inhibitor in SGBS adipocyte differentiation.

Studies focusing on gene expression profile of human adipose tissues revealed that in BAT *Tg2* expression is 6-fold higher than in WAT, presumably it suggests that *Tg2* is involved in thermogenic functions of adipose tissue. Earlier, researchers in our laboratory investigated the gene expression profile of *Tg2* in human deep neck (DN) and subcutaneous neck (SCN) adipose tissue. They found that *Tg2* gene expression is higher in deep neck than in subcutaneous neck adipose tissue (unpublished data). During our long-term experiment we investigated the expression of TG2 as well. Our data show that TG2 is expressed at a higher level in browned SGBS adipocytes either at mRNA and protein level as well.

We tested the effect of NC9 (a-N-carbobenzyloxy-c-N-acryloyl-L-lysine(2-(2-dansylaminoethoxy)ethoxy)ethanamide), which is an irreversible inhibitor of TG2. The importance of this pathway in the metabolism of beige adipocytes is still unclear; however, our data clearly shows that TG2 plays an important role in browned adipocytes, and NC9 has an effect on them.

6. SUMMARY

- SGBS preadipocytes represent an uncommitted preadipocyte stage with one FTO risk allele, which is able to differentiate either white or browned adipocytes.

- Our findings show that SGBS cells can be used as an easily applicable and valuable model for studying human adipocyte biology *in vitro.*

- We could follow SGBS adipocyte differentiation at different time points and detect single browning adipocytes in a high-throughput manner. Our measurement combined texture analysis with the size and number of lipid droplets and detection of UCP1 protein content.

- As a result of rosiglitazone or irisin treatment, the induction of browning was successful in SGBS cells, which followed the beige pathway. The induction of beige phenomenon could be detected by the enhanced expression of brown and beige marker genes (*Ucp1, Cidea, Elovl3, Pparɣ, Cyc1, Tbx1, Pm20d1*), enrichment of mitochondria and multilocular morphology, and these features are strongly associated with high oxygen consumption as a result of cAMP treatment.

- Administration of BMP7 during white adipocyte differentiation led to upregulation of *Ucp1, Cidea, Pgc1α, PparɣY* and *Zic1* gene expression suggesting appearance of classical brown adipocyte features.
- We could observe significant involvement of the creatine-phosphate futile cycle in heat production of browned SGBS adipocytes, induced either by rosiglitazone, irisin or BMP7; detected by appreciably elevated OCR in response to ß-adrenergic stimulus.
- In long-term experiments we could demonstrate that when the browning-inducer, rosiglitazone was omitted from the differentiation media, the expression of UCP1 and mitochondrial enrichment could be partially maintained.
- Beige (browned) SGBS adipocytes secrete increased levels of IL-6, IL-8 and MCP1 cytokines.
- Continuous inhibition of the IL-6 receptor resulted in decreased beige phenotype of human primary adipocytes (downregulation of the browning marker genes). Basal, stimulated or proton-leak oxygen consumption did not decrease markedly, but the extracellular acidification was significantly lower in the inhibited cells.
- Transglutaminase 2 is expressed at a higher level in browned SGBS adipocytes, which correlates with UCP1 expression at gene and protein level as well. Transglutaminase inhibitor treatment for short or long-term resulted in decreased *Tg2* gene expression. Long-term inhibition of TG2 led to significantly lower expression of *Ucp1* in browned SGBS cells. In line with the results of gene expression, significantly decreased TG2 and UCP1 protein expression were observed in response to long-term administration of the inhibitor. However, in browning functional test we could not observe significant effect of transglutaminase inhibition.

7. KEYWORDS

SGBS adipocyte, human preadipocyte, rosiglitazone, irisin, browning, beige, thermogenesis differentiation, obesity, cytokine

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List of publications related to the dissertation

1. **Klusóczki, Á.**, Veréb, Z., Vámos, A., Fischer-Posovszky, P., Wabitsch, M., Bacsó, Z., Fésüs, L., Kristóf, E.: Differentiating SGBS adipocytes respond to PPARγ stimulation, irisin and BMP7 by functional browning and beige characteristics.
   Sci. Rep. 9 (1), 1-35, 2019.
   DOI: http://dx.doi.org/10.1038/s41598-019-42256-0
   IF: 4.011 (2018)
2. Kristóf, E., **Klusóczki, Á.**, Veress, R., Shaw, A., Combi, Z., Varga, K., Győry, F., Balajthy, Z., Bai, P., Bacsó, Z., Fésüs, L.: Interleukin-6 released from differentiating human beige adipocytes improves browning.
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   DOI: http://dx.doi.org/10.1016/j.yexcr.2019.02.015
   IF: 3.329 (2018)

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List of other publications

3. Kristóf, E., Doan-Xuan, Q. M., Sárvári, A. K., **Klusóczki, Á.**, Fischer-Posovszky, P., Wabitsch, M., Bacsó, Z., Bai, P., Balajthy, Z., Fésüs, L.: Clozapine modifies the differentiation program of human adipocytes inducing browning.
Transl. Psychiatry. 6 (11), 1-12, 2016.
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