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

Novel inducers of human adipocyte browning;

Utilization of population scale cellular analysis

by Endre Károly Kristóf

Supervisor: Prof. László Fésüs

UNIVERSITY OF DEBRECEN

DOCTORAL SCHOOL OF MOLECULAR CELL AND IMMUNE BIOLOGY

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Supervisor: Prof. László Fésüs, MD, PhD, DSc, member of HAS

Doctoral School of Molecular Cell and Immune Biology, University of Debrecen

Head of the **Examination Committee**: Prof. Gábor Szabó, MD, PhD, DSc

Members of the Examination Committee: Prof. Gábor Bánhegyi, MD, PhD, DSc

Prof. Ferenc Erdődi, PhD, DSc

The Examination takes place at the discussion room of Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Debrecen at 11 am on December 1st, 2016.

Head of the **Defense Committee**: Prof. Gábor Szabó, MD, PhD, DSc

Reviewers: Krisztina Kovács, PhD, DSc

Prof. György Vereb, MD, PhD, DSc

Members of the Defense Committee: Prof. Gábor Bánhegyi, MD, PhD, DSc

Prof. Ferenc Erdődi, PhD, DSc

The PhD Defense takes place at the Lecture Hall of Department of Pediatrics, Faculty of Medicine, University of Debrecen at 1 pm on December 1st, 2016.

1. INTRODUCTION

1.1. The unique mammalian heat producing organ: Brown adipose tissue (BAT)

BAT was first described in marmots by Konrad Gessner in the 16th century. The fact that BAT is found in all mammals and its major function is heat production was identified 400 years later. In rodents maintained at thermoneutral conditions, BAT is mainly localized in the interscapular, subscapular, axillary and cervical areas.

Both white and brown adipocytes accumulate triglycerides in their cytoplasm. White adipocytes form a single large lipid vacuole (30-100 μm) *in vivo* and contain only a thin rim of cytoplasm around it. This appearance is often referred to as unilocular morphology. In contrast to white adipocytes, brown fat cells accumulate numerous smaller lipid droplets in a multilocular arrangement. Brown adipocytes have polygonal or ellipsoid shape and contain a larger amount of mitochondria-rich cytoplasm. The morphology of mitochondria visualized by electron microscopy in white and brown adipocytes is also markedly different. While the white cells contain small and elongated mitochondria; brown adipocytes have large, round-shaped mitochondria which are rich in cristae in an abundant number. In most of the cases clear anatomical borders between BAT and the surrounding white adipose tissue (WAT) depots do not exist. Furthermore, it should be emphasized that 20-50% of the cells in any fat depot are not adipocytes and constitute the so called stromal-vascular fraction (SVF), which contains vascular elements, adipose tissue-derived mesenchymal stem cells (ADMSCs), other progenitors, fibroblasts, macrophages (MΦs), lymphocytes, mast cells and nerves.

The mitochondrial protein which is responsible for the unique function of BAT, Ucp1 (Uncoupling protein 1, or known as Thermogenin) was discovered in 1978. Ucp1 is a member of the mitochondrial carrier protein family that uncouples mitochondrial ATP synthesis from the respiratory chain activity and therefore decreases the proton gradient generated through the internal mitochondrial membrane by the electron transfer system. In line with this, mitochondria in BAT contain high amounts of the mitochondrial respiratory chain enzymes but remarkably low amounts of the F1Fo-ATPase because of the low expression level of the nuclear ATP5G1 gene, which encodes the mitochondrial membrane-bound c subunit of the Fo oligomer. Ucp1 is activated by long-chain fatty acids which are cleaved by the hormone-sensitive lipase from triglycerides stored in the cytoplasmic lipid droplets of brown adipocytes as a result of β3-adrenergic stimulation. Fatty acids which are permanently associated with Ucp1 by hydrophobic interactions

carry protons within the Ucp1 protein through the inner mitochondrial membrane. Then, protons are released in the mitochondrial matrix but the fatty acid anion stays associated with Ucp1 and returns to initiate another H+ translocation cycle. This mechanism leads to the dissipation of energy mainly generated by β-oxidation of fatty acids as heat. Therefore, BAT plays a major role in maintaining the constant core body temperature of hibernating, small and newborn animals without shivering. The crucial role of Ucp1-dependent non-shivering thermogenesis (NST) mediated by BAT in rodents was further proven when the first UCP1-/- mice were generated which were unable to maintain their body temperature when transferred from normal animal house temperatures of approximately 23 to 5°C (acute cold exposure).

The possibility that the thermogenic activity of BAT might combat metabolic disturbances (and its functional disorders might cause severe metabolic inefficiency), e.g. weight gain was initially addressed in 1979, when the first induced thermogenic process, diet-induced thermogenesis (thermogenic capacity to combust excess energy in the diet) was described. Moreover, several other studies indicated that experimental increases in the amount or function of BAT in mice promote a lean and healthy phenotype.

The first experiments that linked the sympathetic nervous system (SNS) to the control of NST were conducted in the 1950s in rodents. The organ generating heat without shivering remained unrevealed for a long time after the major mediator, norepinephrine (NE) was identified. Later, it was also demonstrated that the blood flow to BAT, which is directly innervated by sympathetic nerves, surges following the injection of NE in rats. The β3-adrenergic signaling cascade is mediated via adenylyl cyclase activation by Gs proteins; then 3',5'-cyclic adenosine monophosphate (cAMP) and Protein Kinase A (PKA) transmit the thermogenic signal. That is the reason why PKA activators (e.g. forskolin) and cell permeable cAMP analogues (e.g. dibutyril-cAMP) are able to model natural thermogenic cues during the experiments. Moreover, NE has a major role not only in the acute induction of Ucp1-dependent NST but also in the long-term regulation of proliferation and differentiation processes in BAT.

1.2. Heat-generating fat depots: “Classical brown” and “beige” adipocyte development

Both white and brown adipocytes are able to store and liberate triglycerides, express a common set of genes and undergo a similar differentiation process controlled by PPARγ

and members of the C/EBP family of transcription factors. Because of these common features, brown cells were termed as adipocytes and they were assumed to be originated from the same precursors as white adipocytes for a very long period of time. Studies focusing on BAT development in rodents found that these "classical brown" adipose cells originate from a dermatomyotomal precursor and they are developmentally much closer to skeletal muscle cells than to white adipocytes. In response to certain stimuli, e.g. bone morphogenetic protein 7 (BMP7), the dermatomyotomal precursors are committed to differentiate into brown preadipocytes which are marked by the upregulation of early B cell factor-2 (EBF2).

The transcriptional cascades that control the process of "classical brown" adipocyte differentiation from the aforementioned preadipocytes were extensively studied in rodents. PRDM16 is one of the key mediators that specifies brown adipocyte identity and differentiation by direct interactions with several key adipogenic transcription factors (PPARα, PPARγ, PGC-1α, C/EBPβ, etc.) resulting in their transcriptional co-activation. The cold-induced, cAMP-dependent long-term thermogenic program which stimulate brown adipocyte differentiation and vascularization of BAT is potentiated by PRDM16 and FOXC2 resulting in the elevated expression of PPARγ Coactivator-1α (PGC-1α). PGC-1α is a transcriptional co-activator which interacts with IRF4 and nuclear hormone receptors including PPARγ and serves as a key regulator of adaptive NST due to the induction of genes involved in mitochondrial biogenesis and oxidative metabolic pathways.

More than 30 years ago, "brown adipocyte-like" cells were detected in rodent WAT depots as a result of cold- induced thermogenesis mediated by the SNS. These cells contained multilocular lipid droplets and high amount of Ucp1 expressing mitochondria. This phenomenon, which was assumed to be the reversible transdifferentiation of white adipocytes into brown fat cells, was associated with the improvement of obesity and insulin resistance. Later, experiments using mouse models (and human samples) suggested that the differentiation of these "brown adipocyte-like" cells arose from a distinct precursor as a result of several stimuli (e.g. cold, physical exercise, diet) at least partially regulated by the β3-adrenergic signaling pathway. These inducible cell populations which are generated mostly in subcutaneous WAT from preadipocytes (marked with PDGFRα and EBF2 expression) in a process called "browning" were termed as "beige" (or "brite") adipocytes.

“Beige” adipocyte development can be highly enhanced by several neuro-endocrine or paracrine factors and share numerous common regulatory transcriptional mechanisms with “classical brown” adipocyte differentiation. The ratio of energy-dissipating “beige” and energy-storing white adipocytes is determined, at least partially, during early differentiation of mesenchymal progenitors into adipocyte subtypes. A crucial mechanism regulating this process was described recently. When a repressor binds to a mesenchymal super enhancer in one of the intronic regions of the FTO gene, resulting in the downregulation of IRX3 and IRX5 expression, this drives the progenitors toward a “beige” cell fate, elevated thermogenesis and reduced lipid storage.

In contrast to “classical brown” adipocytes, “mature beige” cells express thermogenic genes at low levels under basal unstimulated conditions. In addition, when a thermogenic stimulus subsides, “masked beige” cells persist that have a white adipocyte-like morphology *in vivo*. Both “mature” and “masked beige” adipocytes are able to strongly activate UCP1 expression and their thermogenic capacity in response to recurring β-adrenergic stimuli. In summary, “beige” adipocytes are able to switch on and off their UCP1-dependent thermogenic program in response to external cues.

Although many similarities and differences between the two thermogenic fat cell types have been elucidated recently, there is no clear consensus (especially in human studies) on the criteria of classification of “browning” adipocytes into “classical brown” or “beige”. Higher expression of thermogenic genes (e.g. UCP1, PGC-1α, CIDEA, ELOVL3, PRDM16) and mitochondrial markers (e.g. CYC1, COX2, COX5B, ATP5B) compared to white adipocytes can be found in both heat producing cell types, especially when they are stimulated for thermogenesis. In mice, “classical brown” adipocytes express ZIC1, LHX8, MYLPF, PDK4, etc. at elevated levels compared to white or “beige” adipocytes. Among these genes only ZIC1 and LHX8 were validated in human samples as “classical brown” markers so far. Of note, the constitutive expression of UCP1 is always higher in classical BAT than in “beige” fat depots. To recognize “beige” adipocytes in mice, the most accepted gene expression markers are TBX1, TMEM26, HOXC9, CD137, CITED1 and TNFRSF9. However, to date, widely accepted surface markers that can discriminate between these adipose cell types have not been described, which limit the possibility to specifically identify and sort out different types of thermogenic adipocytes in a heterogeneous cell population. Furthermore, to our knowledge, “masked beige” cells cannot be detected among white adipocytes by specific molecular markers.

Isolated mitochondria of murine classical BAT and "beige" fat depots were both able to efficiently perform UCP1-dependent thermogenesis connected with a limited capacity to produce ATP through oxidative phosphorylation. It is becoming clear, that thermogenic adipocytes contain other, UCP1-independent energy dissipating pathways. Recent findings revealed a novel futile cycle of creatine metabolism which enhances energy expenditure in the mitochondria of "beige" (and in a smaller extent in "classical brown") adipocytes. Mitochondrial creatine kinase 1 or 2 catalyzes the conversion of creatine and utilizes ATP to create phosphocreatine (PCr) and ADP, before Phospho1 liberates the high-energy phosphate group from PCr. However, how these energy dissipating pathways relatively contribute to heat production in different fat depots and which regulatory mechanisms switch on and off the aforementioned substrate cycles, especially in humans, remain elusive.

1.3. Implications of "browning" for metabolic health in humans

Obesity is one of the major risk factors of metabolic syndrome, coronary heart disease and cancer which are leading causes of morbidity and mortality today. In Europe, approximately four million people die each year as a result of cardiovascular diseases. However, the rate of cardiovascular mortality in Hungary (640/10000 inhabitants) has been continuously decreasing in the recent years, it is still more than two-fold higher than the mean cardiovascular mortality of the EU-25 countries. In our country, the prevalence of overweight people (BMI: 25-29.9 kg/m2) is 58%. Approximately half of them are obese (BMI: >30 kg/m2). Obese patients have 2-3 times higher relative morbidity risk than people with normal body weight (BMI: 18-24.9 kg/m2) for the other components of metabolic syndrome. In 2012, the public expenditure in regard to obesity was more than 200 billion HUF (700 million USD) in Hungary. The idea to increase mitochondrial uncoupling to burn off the excess fat as heat is dated back for a long time. Therefore, 2,4-dinitrophenol was administered broadly to obese patients in order to increase their metabolic rate. However, 2,4-dinitrophenol treatment was later withdrawn because of severe side-effects.

Studies using positron or gamma radiation emitting radiolabelled metabolic substrates (^{18}F-FDG, ^{123}I-MIBG) in nuclear medicine detected high incidence of metabolically active BAT, which can dissipate energy directly into heat as a result of a cold challenge, in healthy adult humans of different ethnical groups. These thermogenic fat depots can be found interspersed in the human body and are mostly enriched in the supraclavicular,

“deep neck” and paravertebral regions. Furthermore, independent trials proved the strong negative correlation between obesity or glucose intolerance and the amount of metabolically active BAT in humans. The activity of these heat producing, anti-obesity fat depots was predicted to account for up to 5% of basal metabolic rate in adult humans, which could cumulatively support more than 4 kg of fat loss per year.

Although adipocytes which express “classical brown” markers were found in the “deep neck” fat of adult humans, most of the obtained data suggest that the energy expenditure of thermogenic adipose depots in the entire body is less pronouncedly mediated by the ab ovo differentiated “classical brown” adipocytes. The development of “beige” cells which can be found interspersed in WAT and generated in a process called “browning” seems to play a more significant role. Several studies suggest that a large proportion of the thermogenic fat depots in adult humans is mostly composed of “beige” cells. However, there is only limited information about the origin of “beige” adipocytes and the regulators of “beige” adipogenesis in humans.

This “browning” process which results in unstimulated, slightly active “beige” cells would determine a “beige” potential or the “thermogenic competency” of each individual. The “beige” potential is fundamentally defined by the proportion of the differentiated energy-dissipating “beige” and energy-storing white adipocytes in each person. Those who carry the risk-allele of the FTO locus fail to drive the adipocyte progenitors toward a “beige” cell fate and harbor a strong genetic association with obesity. This recent discovery proposed to date the first strong genetic link in humans between obesity and thermogenic competency generated by “beige” fat development. The thermogenic competency might be increased pharmacologically by “browning-inducers” or by implants of “masked” or activated “beige” adipocyte depots. After a complete “browning”, both mature and “masked beige” adipocytes are able to enhance UCP1 expression and heat production (both in a UCP1 dependent and independent manner) in response to anti-obesity cues, such as an adrenergic stimulus, in a process called “thermogenic activation”. As a natural thermogenic stimulus, cold exposure causes the SNS to release NE and induce heat production of brown and “beige” fat in humans through consumption of fatty acids and glucose. Since non-specific β-adrenergic agonists have striking effects on the cardiovascular system, selective induction of the β3-receptor mediated pathway (e.g. by mirabegron) can be a promising future therapeutic target that boost “thermogenic activation”.

It is also becoming clear that brown and "beige" adipocytes are not only heat-generating cells. Recent results suggest that "beige" adipocytes contribute significantly to the regulation of whole body energy expenditure and systemic metabolic homeostasis not exclusively by thermogenesis and mitochondrial uncoupling. For example, BAT-derived Interleukin-6 (IL-6) is required for the profound effects of BAT on glucose homeostasis and insulin sensitivity in mice. There is, however, only limited information about the secreted factors by brown and "beige" adipocytes („batokines") in humans.

1.4. Activators of brown and "beige" fat development and function

It has been known for a long time from rodent experiments, and was later proven in humans, that cold exposure facilitates NE release from the SNS which not only induces a rapid thermogenic program but also enhances BAT development. Recently it was discovered that alternatively activated (or M2) MΦs which are recruited during "browning" secrete also catecholamines to sustain NST. Insulin was also described in rodents as meal-induced, centrally acting, acute thermogenic cues decades ago. Then, it was demonstrated that insulin signaling, which stimulates the upregulation and transfer of GLUT4 to the plasma membrane, plays a crucial role in mediating the uptake of glucose and storage of lipid droplets not only in white but also in brown adipocytes.

Leptin, which reduces appetite as a circulating hormone secreted by adipocytes, also enhances BAT thermogenesis by inducing SNS via the release of melanocyte-stimulating hormone in the hypothalamus. In contrast to centrally acting glucocorticoids which decreased the effect of leptin *in vivo*, dexamethasone treatment on primary cultures of murine brown adipocytes resulted in the upregulation of ELOVL Fatty Acid Elongase 3 (ELOVL3) and promoted their differentiation. Previously, the metabolic effects of thyroid hormones including elevated heat production have been suggested to be peripherally mediated. However, recent studies revealed that T3 inhibits AMPK in the ventromedial hypothalamus resulting in the activation of SNS that leads to increased "thermogenic competency" and induction in mice.

Central serotonin (5HT) is known to regulate energy balance by decreasing appetite and increasing BAT thermogenesis through effects on the nervous system. These actions were exploited by appetite-suppressing drugs, e.g fenfluramine or sibutarmine, in the treatment of obesity. However, these drugs were later withdrawn, because of frequent cardiovascular side effects. Recently two groups reported independently that peripheral 5HT has an opposing effect. In mice, 5HT reduced the "beige" potential and the

sensitivity of brown and “beige” adipocytes to thermogenic induction in a cell autonomous manner. Second-generation antipsychotic drugs (SGAs), including clozapine bind to different 5HT, muscarinic and histamine receptors and antagonizes adrenergic α1 and 2 or various dopamine receptors. SGAs, especially clozapine, olanzapine, risperidone and quetiapine increase the incidence of weight gain and metabolic syndrome in patients with severe mental illnesses (SMI) with diverse but not completely revealed molecular mechanisms.

Physical exercise has well-known beneficial metabolic effects and protects against several pathological conditions, such as metabolic syndrome, neurodegenerative disorders, or cancer. Skeletal and cardiac muscle cells secrete various hormones, which were termed as “myokines”, in response to physical activity. These factors significantly contribute to the crosstalk between the brain, muscle and adipose tissue, by which “browning” is also regulated.

Irisin was discovered as a “myokine” which is cleaved from the Fndc5 transmembrane protein and induced a “beige” program of subcutaneous WAT in mouse models. Physical exercise (as well as shivering) induced the upregulation of Fndc5 and the subsequent secretion of irisin in skeletal myocytes driven by PGC-1α. The production of irisin by cardiac muscle was also demonstrated in rats. Then, irisin acts as a “browning-inducer” in a cell-autonomous manner, presumably through an unknown selective receptor, via the p38 MAPK and ERK pathways. In humans, the gene which encodes Fndc5 carries a G to A mutation in the ATG start codon. This change probably results in a shorter Fndc5 protein lacking the part from which irisin is generated. However, several studies have demonstrated the presence of irisin in human blood plasma, using mass spectrometry or many different antibodies, as a function of exercise or other metabolic parameters. Furthermore, more than 10-fold higher irisin plasma concentrations were detected in rodents (>100 ng/μl) than in humans (<10 ng/μl) by mass spectrometry. Fndc5 is expressed in distinct areas of the brain and central effects of circulating irisin were described recently as well.

Recent findings suggest that IL-6 not only acts as a crucial mediator of inflammatory processes but also serve as an endocrine modulator of metabolism for the entire body. As a “myokine”, IL-6, which target several tissues including liver, skeletal muscle, pancreas, brain, WAT and BAT, seems to balance exercise-associated catabolic pathways in order to mediate glycemic control during recovery. Furthermore, IL-6 can mediate some of the long-term systemic beneficial effects of physical training. In

accordance with the homeostatic roles of IL-6, it can contribute to the exercise induced alternative activation of MΦs and the induction of "thermogenic competency" in mice.

Another key endocrine factor, atrial natriuretic peptide, which is produced by cardiomyocytes and switches on p38 MAPK signaling and phosphorylation of ATF2, directly increasing UCP1 transcription, promotes "thermogenic activation" and mitochondrial biogenesis in murine "beige" fat and in human adipocytes. p38 MAPK signaling is also induced by distinct BMPs which are key endocrine-paracrine regulators of "thermogenic competency and activation". BMP7 was described earlier as a locally acting mediator in mice that both drives "classical brown" adipogenesis and recruits "beige" adipocytes. Then, the effect of BMP8b and BMP4 was connected to increased "thermogenic induction" of BAT and accelerated "beige" adipocyte differentiation, respectively.

Fibroblast growth factor 21 (FGF21) is secreted by hepatocytes and "beige" adipocytes and binds to a receptor complex in which βKlotho interacts with FGF receptors 1c and 4. Then, FRS2α docking protein and ERK1/2 are phosphorylated which improves insulin sensitivity and induces "browning" in WAT. Because of the beneficial effects of FGF21 on the liver and adipose tissue, which results in decreased blood sugar levels and increased energy expenditure, its analogues are being tested in preclinical and clinical trials for the management of diabetes and obesity.

Adenosine, a locally acting purine nucleoside secreted by adipocytes or generated from the released ATP by SNS, activates ADORA2A receptor, leading also to increased cAMP- and PGC-1α-dependent signaling, that enhances thermogenesis and lipolysis in murine and human brown and "beige" adipocytes. As discussed above, there is growing evidence, that "browning" is regulated by several factors which might not affect the central nervous system (CNS). Some of these mediators are released by "browning" adipocytes and act in a paracrine-autocrine manner. These might open up better strategies to stimulate "browning" specifically or to establish an *in vitro* "engineered BAT" that helps the treatment of obese or diabetic patients more effectively. However, most of these factors were tested only in rodent models and their effects on human adipocyte "browning" are unrevealed. Therefore, we aimed to quantify human brown and "beige" adipocyte differentiation in response to different "browning-inducers" *ex vivo* at a single cell level in a highly replicative manner by using a slide-based image cytometry approach.

2. AIMS OF THE STUDY

- To quantify human brown and "beige" adipocyte differentiation *ex vivo* at a single cell level in a highly replicative manner by using a slide-based image cytometry approach.

- To clarify the direct effect of irisin and BMP7, two potent browning-inducers described in mice, on the induction of browning in our *ex vivo* human model system.

- To identify novel effects of drugs on human browning by complementing gene expression and oxygen consumption measurements (OC) with the laser-scanning cytometry (LSC) based population scale analysis of *ex vivo* brown adipogenic differentiation.

- To learn the molecular mechanism that can explain the unexpected browning effect of clozapine.

- To investigate the secretion of cytokines ("batokines") by primary human brown and "beige" adipocytes.

3. MATERIALS AND METHODS

3.1. Ethics statement

Human adipose-derived mesenchymal stem cells (hADMSCs) were isolated from subcutaneous abdominal adipose tissue of healthy volunteers (body mass index < 29.9) aged 20–65 years who underwent a planned surgical treatment (herniotomy). Written informed consent from all participants was obtained before the surgical procedure. The study protocol was approved by the Ethics Committee of the University of Debrecen, Hungary (No. 3186-2010/DEOEC RKEB/IKEB). All experiments were carried out in accordance with the approved ethical guidelines and regulations.

3.2. Isolation and cultivation of hADMSCs

Adipose tissue specimens were dissected from fibrous material and blood vessels, minced into small pieces and digested in PBS with 120 U/ml collagenase for 60 min in a 37 ^{0}C water bath with gentle agitation. The cell suspension was centrifuged for 10 min at 200 g, and the pellet of SVF was re-suspended in DMEM-F12 medium (Sigma-Aldrich) containing 10% FBS (Gibco), 100 U/ml penicillin-streptomycin (Sigma-Aldrich), 33 μM biotin (Sigma-Aldrich) and 17 μM pantothenic acid (Sigma-Aldrich). hADMSCs were seeded into 6-well plates or Ibidi eight-well μ-slides at a density of 15000 cells/cm^2 and cultured in the same medium at 37 ^{0}C in 5% CO2 for 24 h to attach. The absence of mycoplasma was checked by polymerase chain reaction (PCR) analysis (PromoKine).

3.3. Induction of white and beige adipocyte differentiation *ex vivo*

After the cell culture became confluent, adipogenic differentiation was initiated. White adipocyte differentiation was induced for four days using the following medium: DMEM-F12 supplemented with 33 μM biotin, 17 μM panthothenic acid, 10 μg/ml human apo-transferrin (Sigma-Aldrich), 20 nM human insulin (Sigma-Aldrich), 100 nM hydrocortisone (Sigma-Aldrich), 200 pM T3, 2 μM rosiglitazone (Cayman Chemicals), 25 nM dexamethasone (Sigma-Aldrich) and 500 μM IBMX (Sigma-Aldrich). After four days rosiglitazone, dexamethasone and IBMX were omitted from the differentiation medium. Beige adipogenic differentiation was carried out for three days using the following medium: DMEM-F12 containing 33 μM biotin, 17 μM panthothenic acid, 10 μg/ml apo-transferrin, 0.85 μM human insulin, 200 pM T3, 1 μM dexamethasone and

500 μM IBMX. Three days later, the medium was changed (dexamethasone and IBMX were omitted) and 500 nM rosiglitazone was added. From this point on media were changed every other day and cells were assayed after 14 days of differentiation.

3.4. Treatments of hADMSCs and differentiated white and "beige" adipocytes with browning-inducers or activators

Where indicated, white or browning adipocytes were differentiated in the presence of potent browning-inducers: 250 ng/ml human recombinant irisin (Cayman Chemicals) or 50 ng/ml human recombinant BMP7 (R&D Systems). Irisin or BMP7 was administered during the whole differentiation procedure, or in the last 4 days of the differentiation. Cells were treated with clozapine (Sigma-Aldrich) every day at 100 ng/mL concentration on the last 2 and 4 days or during the whole adipogenic differentiation process. Where indicated, cells were treated with 5HT (Sigma-Aldrich) every day at 10 μM concentration during the whole adipocyte differentiation. To investigate the response of differentiated adipocytes to thermogenic induction, cells received a single bolus of dibutyryl-cAMP (Sigma-Aldrich) at 500 μM final concentration for 4 hours.

3.5. RNA Preparation and TaqMan reverse transcription-coupled quantitative PCR (RT-qPCR)

Total cellular RNA was isolated from hADMSCs and differentiated adipocytes using TRI Reagent. TaqMan reverse transcription reagent (Applied Biosystems) was applied for generating cDNA according to manufacturer's instructions. An ABI Prism 7700 sequence detection system (Applied Biosystems) or a LightCycler 480 (Roche Diagnostics) was used to determine relative gene expression of "classical brown", "beige", white and general adipocyte markers. Gene primers and probes were designed and supplied by Applied Biosystems. Human GAPDH was used as endogenous control. Gene expression values were calculated by the comparative Ct method. ΔCt represents the threshold cycle (Ct) of the target minus that of GAPDH. Normalized gene expression levels equal $2^{-\Delta Ct}$.

3.6. Quantification of mitochondrial (mt) DNA by quantitative PCR

Total cellular DNA was isolated by a conventional phenol-chloroform method from hADMSCs and differentiated white or browning adipocytes using TRI Reagent. Quantitative PCR was carried out in triplicates on diluted DNA using 10 μM each

primer and Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific) in a LightCycler 480 with a program of 20 min at 95 ^{0}C, followed by 50 cycles of 15 sec at 95 ^{0}C, 20 sec at 58 ^{0}C and 20 sec at 72 ^{0}C. Results were calculated from the difference in Ct values for mtDNA and nuclear specific amplification.

3.7. Western blotting

hADMSCs and differentiated adipocytes were washed with ice cold PBS and collected followed by lysing in 50 mMTris–HCl; 0.1% Triton X-100; 1 mM EDTA; 15 mM 2-MEA and protease inhibitors. Then, the lysates were mixed with 5× Laemmli loading buffer, boiled for 10 min and loaded onto a 10% SDS polyacrylamide gel. Proteins were transferred onto PVDF Immobilon-P Transfer Membranes followed by blocking in 5% skimmed milk for 1h. Membranes were probed by polyclonal anti-Ucp1 (Sigma-Aldrich) and monoclonal anti-GAPDH (Merck-Millipore) antibodies overnight at 4°C, followed by incubation with horseradish-peroxidase-conjugated species-corresponding secondary antibodies (Covalab) for 1 h at room temperature. Immunoblots were developed with Immobilon Western chemiluminescent substrate.

3.8. Vital and immunofluorescence staining of differentiated adipocytes

hADMSCs were plated on Ibidi eight-well μ-slides and differentiated as described above. On the day of measurement, cells were washed once with PBS and then kept in fresh medium for sub vital scanning with 50 μg/ml Hoechst 33342 (Thermo Fisher Scientific) for 60 minutes and 750 μg/ml Nile Blue (Sigma-Aldrich) for 20 minutes. Next, cells were washed and fixed in 4% paraformaldehyde for 5 min followed by blocking in 5% skimmed milk for 2h and staining with anti-Ucp1 or anti-Cidea (Covalab) primary antibodies for 6h at room temperature. Alexa 488 goat anti-rabbit IgG (Thermo Fisher Scientific) was applied as a secondary antibody. Antibodies were used and additional washing steps between and after Ab usage were carried out in the presence of 0.1% saponin (Sigma-Aldrich) in PBS for effective cell permeabilization.

3.9. Image acquisition by laser-scanning cytometry (LSC), recognition of cellular objects

Images were collected with an iCys Research Imaging Cytometer (iCys, Thorlabs Imaging Systems) following the protocol of Doan-Xuan et al. Sample slides were attached on a computer-controlled stepper-motor driven stage. An area with optimal

confluence was defined in low-resolution scout scan with a ×10 magnification objective and a 10-µm scanning step. As a next step, high-resolution images were consequently obtained by using a ×40 objective and a 0.25-µm scanning step. The size of a pixel was set to 0.25 µm × 0.245 µm at ×40 magnification.

Laser lines were separately operated, namely a 405-nm diode laser was used to excite Hoechst 33342, a solid-state 488-nm laser was used for Alexa 488 goat anti-rabbit IgG and a 633-nm HeNe gas laser for Nile Blue. Emissions were collected by three photomultiplier tubes; Hoechst was detected at 450 ± 20 nm, Alexa 488 at 530 ± 15 nm and Nile Blue at above 650 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).

Hoechst-stained nuclei were first identified and defined as primary objects. Based on parent nuclei, the secondary objects, a whole cell, were then identified according to its Nile Blue fluorescence.

3.10. Texture analysis, quantification of Ucp1 and Cidea protein content of single cells and population scale analysis of human adipocyte browning

2000-3000 cells per sample were collected for image analysis. Clustered, detached or dead cells were omitted and 1000-2000 cells were quantified per data set. Textural pattern of light absorption and light scatter signal per identified objects was analyzed with built-in modules in CellProfiler. Parameter variance measured the difference between intensity of the central pixel and its neighbourhood; “sum variance” roughly depicted the size of lipid droplets. Beside texture parameters, the other major profiles that were also extracted are: Integral, which is the sum of the pixel intensities for a given event that provides information about the expression level of the labelled protein; and Area, which is the area enclosed by the boundary contour of the object, in square micrometers. From these parameters, Ucp1 or Cidea immunofluorescence intensity per cell (Ucp1 or Cidea protein content of each adipocyte) could be evaluated as the value of the Integrated intensity relative to the Area of each event. Ucp1 or Cidea intensities and texture “sum variance” of each differentiated adipocyte were then plotted. Adipocytes with morphological characteristics of browning were recognized as the ones

that contained small lipid droplets (their texture "sum variance" was low) and high levels of Ucp1 or Cidea protein, in contrast to white adipocytes, which accumulated large lipid droplets (their texture "sum variance" was high) and expressed low amount of Ucp1.

3.11. Determination of cellular oxygen consumption (OC)

OC was determined using an XF96 oximeter (Seahorse Biosciences). Cells were seeded and differentiated in 96-well XF96 assay plates. On the day of measurement, after recording the baseline OC for 30 min, adipocytes received a single bolus dose of dibutyryl-cAMP (at 500 μM final concentration) modelling adrenergic stimulation. Then, stimulated OC was measured every 30 minutes. Adipocytes were treated with 5 μM etomoxir (Sigma-Aldrich) or with 2 mM β-GPA (Sigma-Aldrich) to block beta-oxidation and creatine-driven substrate cycle. Next, proton leak respiration was recorded after adding oligomycin (Enzo Life Sciences) at 2 μM concentration to block ATP synthase activity. As a last step, cells received a single bolus dose of Antimycin A (10 μM final concentration) (Sigma-Aldrich) for baseline correction. The oxygen consumption rate (OCR) was normalized to protein content and normalized readings were shown.

3.12. Determination of cytokine release

During regular replacement of differentiation media, culture supernatants were harvested and stored for cytokine measurements. Then, conditioned differentiation media from the same donor and differentiated sample were pooled. Where indicated, media were changed and culture supernatants were collected every day. The concentration of IL-6, IL-1β, IL-8, TNFα and MCP-1 was measured from the collected cell culture media using ELISA DuoSet Kit (R&D Systems).

3.13. Statistical analysis

Each experiment was repeated 3-10 times with SVFs from independent healthy donors. For multiple comparisons of groups statistical significance was calculated and evaluated by one-way ANOVA followed by Tukey post-hoc test. In comparison of two groups two-tailed Student's t-test was used.

4. RESULTS

4.1. A previously described brown adipogenic protocol induced a "beige"-like gene expression pattern in differentiating human adipocytes

hADMSCs were isolated from the SVF of abdominal subcutaneous WAT and previously described differentiation regimens were applied to induce white and brown adipocyte differentiation. We selected to examine the expression of a core set of BAT-specific genes (UCP1, CIDEA, PGC-1α and ELOVL3) and a marker of mitochondrial enrichment (CYC1). The expression of PRDM16 and C/EBPβ which are key transcriptional regulators of browning was also measured. A "beige"-selective (TBX1) and a "classical brown" adipocyte marker gene (ZIC1) were also investigated. Finally, key drivers of the adipogenic program (C/EBPα, PPARγ) and general (LEPTIN, FABP4) adipocyte marker genes were assessed. The expression of UCP1 was not detectable in undifferentiated hADMSCs and showed a basal level in adipocytes differentiated by the white adipogenic cocktail for two weeks. Significantly higher expression of UCP1, both at mRNA and protein level, and increased mitochondrial DNA amount were found in whole cell lysates of adipocytes differentiated in the presence of the brown compared to the white protocol.

In line with the increased UCP1 expression, we found upregulated browning marker genes (CIDEA, ELOVL3, CYC1 and PGC-1α) in response to the brown differentiation cocktail. The expression of ZIC1 remained at a low level after 14 days of brown adipogenic differentiation excluding that this protocol induces "classical brown" adipocyte phenotype. Contrarily, the significant upregulation of TBX1, "beige" marker gene, suggests that human primary adipocytes from abdominal subcutaneous fat follow the „beige" pathway when differentiated according to the protocol developed by Elabd et al. From this point forward, we are referring to these cells as "beige" adipocytes.

4.2. Laser-scanning cytometry (LSC) can quantify human adipocyte browning

To date, white, "classical brown" or "beige" adipocyte differentiation has mostly been evaluated based on the detection of mRNA or protein expression in whole cell lysates. However, up to 50% of precursor cells remain undifferentiated in human cellular models of adipocyte development. In line with other studies, depending on individual donors, 40-60% of the hADMSCs were able to accumulate lipid droplets as a result of 14 days long adipocyte differentiation. Since the cell cultures of both white and "beige"

adipocytes were heterogeneous, we intended to quantify adipocyte browning *ex vivo* at a single cell level in a highly replicative manner by the abovementioned slide-based image cytometry approach.

We found significantly lower texture "sum variance" along with the accumulation of smaller lipid droplets as a result of "beige" differentiation. Immunofluorescent staining showed that Ucp1 was mostly distributed between the lipid droplets of "beige" adipocytes, while Cidea accumulated highly in the perinuclear lipid-free region of these cells. When images, which captured 1000-2000 cells per donor, were quantified, we found that "beige" differentiation compared to white lead to a two-fold higher Ucp1 and Cidea protein content in single human adipocytes.

Next, we plotted Ucp1 or Cidea protein content and texture "sum variance" for each differentiated adipocyte (undifferentiated progenitors were omitted from the analysis). Adipocytes with morphological characteristics of browning were recognized as the ones that contained small lipid droplets and high levels of Ucp1/Cidea protein, contrary to white adipocytes, which accumulated large lipid droplets and expressed low amount of browning marker protein. Our results show that the population of differentiated adipocytes remains heterogeneous regardless of whether white or "beige" program was induced. Furthermore, we could detect a significant amount of cells (15-30% of the adipocytes) with the characteristic morphological features of browning even in response to the white adipogenic cocktail. However, when adipocytes were differentiated in the presence of the aforementioned "beige" regimen, the rate of browning cells increased strongly, depending on individual donors, by approximately 3-fold.

4.3. Irisin and BMP7 induces browning of human adipocytes

Our next aim was to clarify whether human recombinant irisin and BMP7, two potent endogenous browning-inducers described in mice, were able to shift the adipocyte differentiation towards browning. Using the LSC approach described above, we determined that irisin and BMP7 treated differentiating white adipocytes contained smaller lipid droplets and higher amount of Ucp1 and Cidea protein than the untreated cells. When we analyzed Ucp1 immunofluorescence intensity and texture "sum variance" of 2000 differentiated cells in 3 different donors, we found that 30-60% of adipocytes had the characteristic morphological features of browning in response to irisin or BMP7 treatment. Thus, by using a slide-based image cytometry approach we

could validate mouse data in human samples demonstrating the effectiveness of irisin and BMP7 to induce a browning program in human subcutaneous adipocytes.

4.4 Clozapine enhances browning of human adipocytes detected by LSC

Next, we aimed to apply our slide-based image cytometry method to test the effect of exogenous drugs on the induction of human adipocyte browning. Only a few studies investigated the direct effect of SGAs on differentiating human adipocytes so far. To fill this hiatus and to follow-up the previous study by Sárvári et al. where expression of selected adipogenic and pro-inflammatory genes in SGA-treated human white adipocytes were investigated, we examined how the propensity of hADMSCs to differentiate into heat-generating browning cells is influenced by clozapine. Surprisingly, we found that the long-term clozapine administration on top of the white adipogenic cocktail resulted in the occurrence of more and smaller lipid droplets in the differentiated adipocytes, similarly but less extensively than in the case of "beige" adipocytes. In addition, elevated Ucp1 protein content was observed simultaneously in single adipocytes. Following the practice shown above, we demonstrated that, depending on individual donors, 30-40% of differentiating adipocytes had the characteristic morphological features of browning cells in response to clozapine treatment. The rate of browning adipocytes was increased by approximately 1.5-fold compared to white fat cells.

4.5. Irisin and BMP7 administration during adipocyte differentiation results in different gene expression patterns

As a next step, irisin or BMP7 was administered on the last 4 days or during the whole white or "beige" differentiation process. Then, whole cell lysates were collected and the expression of a panel of marker genes was determined by RT-qPCR. We found that, both irisin and BMP7 treatment during white adipocyte differentiation significantly upregulated UCP1, CIDEA, ELOVL3, CYC1 and PGC-1α genes. Furthermore, elevated expression of C/EBPβ, PRDM16, C/EBPα and PPARγ was detected in whole cell lysates of BMP7 treated adipocytes.

The expression of ZIC1 remained at a low level after irisin administration excluding that irisin induces "classical brown" adipocyte differentiation. However, expression of the "beige" marker, TBX1 increased selectively as a result of irisin treatment. When, on the other hand, we applied BMP7 on top of both the white and "beige" adipogenic

protocol, we found that BMP7 resulted in the upregulation of the "classical brown"-specific ZIC1. In summary, our results suggest that irisin is able to induce a "beige" program in differentiating human primary subcutaneous white adipocytes, while BMP7 induces a "classical brown" adipocyte-like phenotype.

4.6. Clozapine enhances "beige" potential of human adipocytes via inhibiting 5HT-receptor mediated signaling

As a next step, we intended to examine the gene expression changes underlying the browning effect of clozapine by RT-qPCR. Therefore, differentiating white adipocytes were treated with clozapine on the last 2 and 4 days or during the whole differentiation process at a dose comparable to its therapeutic plasma concentration. We found that UCP1 was expressed 5-fold higher at mRNA level as a result of clozapine administration in each case. In line with the upregulation of UCP1 gene, significantly elevated expression of CIDEA, CYC1, ELOVL3 and PGC-1α was found compared to white adipocytes. Furthermore, clozapine treatment moderately induced the expression of beta-oxidation related mitochondrial genes. The expression of ZIC1 remained at a low level after clozapine administration excluding that clozapine induces a classical brown adipocyte phenotype. Contrarily, the "beige" indicator TBX1 upregulated when we applied clozapine on top of the white adipogenic protocol suggesting that the drug could shift the adipocyte differentiation towards browning, with gene expression changes indicating the "beige" program.

Next, we wanted to learn if Ucp1 was upregulated at protein level in adipocytes that were differentiated in the presence of clozapine. In the collected whole cell lysates we found a 3-fold elevated Ucp1 protein level compared to the untreated cells.

To obtain mechanistic data, we tested if browning induced by clozapine can be explained by its known pharmacological effect of antagonizing 5HT receptors. The expression of Tryptophan Hydroxylase 1 (TPH1), encoding the enzyme which catalyzes the rate-limiting step of 5HT synthesis, was detectable in hADMSCs and did not change as a result of white adipocyte differentiation, suggesting that these cells are capable of autonomously generating and secreting 5HT during adipogenesis. However, we found reduced TPH1 expression in clozapine treated and in "beige" adipocytes. In addition, we found that browning cells expressed 5HT receptors 2A, 1D, 7 at mRNA level and the up-regulation of browning markers by clozapine was diminished in the presence of exogenous 5HT.

Out of the 5HT-receptors, HTR2A was expressed at the highest level in hADMSCs and in differentiated adipocytes. Interestingly, long-term clozapine administration resulted in the down-regulation of HTR2A gene. Our data suggest that the disturbance of 5HT-production and 5HT-receptor-mediated signaling by clozapine might, at least partially, explain the browning effect of the drug described in the present dissertation.

4.7. The "beige" adipogenic cocktail, irisin and BMP7 induces a functional browning program while clozapine treated adipocytes are less capable to respond to thermogenic cues.

As a next step, we intended to analyze the functional capacity of human primary adipocytes differentiated in the presence of the aforementioned browning-inducers. Irisin or BMP7 treated white adipocytes contained higher amount of mitochondrial DNA, to a similar extent as "beige" adipocytes. Clozapine treated primary adipocytes had a moderately increased mitochondrial DNA content compared to white cells but less than the adipocytes differentiated by the "beige" regimen.

In accordance with the gene expression and morphological changes, *ex vivo* differentiated "beige" adipocytes had higher basal OCR than white adipocytes. Basal mitochondrial respiration of irisin and clozapine treated white adipocytes was also elevated as compared to the untreated white cells. After the cells received a single bolus dose of cell permeable dibutyryl-cAMP mimicking adrenergic stimulation, we found that adipocytes differentiated in the presence of irisin, BMP7 or the "beige" adipogenic cocktail had significantly increased stimulated mitochondrial respiration compared to white adipocytes. However, adipocytes that were differentiated in the presence of clozapine were less capable than the untreated cells to induce their respiration.

ATP synthase activity was inhibited after adding oligomycin to detect proton leak respiration. Both basal and cAMP stimulated proton leak OCRs were significantly higher in "beige" than in white adipocytes. In the case of clozapine induced browning adipocytes elevated proton leak respiration could be only detected in basal conditions.

We also tested the involvement of fatty acid beta-oxidation and the recently described creatine-driven substrate cycle in the metabolism of *ex vivo* differentiated adipocytes. When "beige" cells and adipocytes differentiated in the presence of clozapine were treated with etomoxir or β-GPA, basal and cAMP stimulated OCRs were decreased at a higher extent than in the case of white adipocytes. Our results show that the clozapine induced browning cells, similarly to "beige" adipocytes, consume more fatty acids by

beta-oxidation compared to white adipocytes and further enhance their energy expenditure by activating the futile cycle of creatine metabolism.

We also analyzed the changes in UCP1 and PGC-1α expression in response to a 4 h long dibutyryl-cAMP treatment that serves as an accepted model of thermogenic induction mimicking natural anti-obesity cues. UCP1 and PGC-1α expression showed a robust upregulation in response to cAMP treatment of white and "beige" adipocytes differentiated in the absence of clozapine. This effect of thermogenic induction was less manifested in clozapine generated "beige" adipocytes, with regard to UCP1.

Our results suggest that browning induced by irisin, BMP7 or the "beige" adipogenic cocktail is coupled with enhanced mitochondrial biogenesis and elevated energy expenditure in response to thermogenic stimuli. Clozapine treatment induced the "beige" program in differentiating white adipocytes, these "masked beige" cells, however, were less capable to respond to β-adrenergic induction of thermogenesis.

4.8. Differentiating human browning adipocytes secrete cytokines ("batokines")

Finally, we investigated the secretion of "batokines" by primary human white, brown and "beige" adipocytes. Conditioned differentiation media were collected during the regular replacement of the adipogenic cocktails and secreted IL-6, IL-8, TNFα, MCP-1 and IL-1ß were measured by ELISA after samples stored on the 1st and 2nd week of differentiation were pooled. Neither hADMSCs nor differentiating adipocytes secreted TNFα and IL-1β. Interestingly, IL-6, MCP-1 and IL-8 secretion was significantly higher by "beige" compared to white adipocytes. In contrast to BMP7 administration (when "classical brown" adipocyte differentiation occurs), irisin treatment (which induced "beige" adipocyte differentiation) resulted in an increased total IL-6, MCP-1 and IL-8 production.

When we examined the time frame of cytokine production during adipocyte differentiation and therefore collected daily replaced media, we found that MCP-1 and IL-8 secretion was induced at the end of the first week of "beige" adipogenic differentiation then declined. On the other hand, IL-6 seems to be continuously secreted by differentiating human "beige" adipocytes in a regulated way, since pooled and daily replaced samples of conditioned differentiation media contained the same amount of produced IL-6. This suggests that browning adipocytes adjusted their production of the cytokine to reach an optimal level in the medium.

5. DISCUSSION

5.1. Methodological overview of analysis of *ex vivo* browning – an unsolved problem

Although, a protocol to induce human brown adipocyte differentiation in cell cultures was established several years ago, there is only limited data about regulatory networks that drive, or mediators that regulate "classical brown" or "beige" adipocyte differentiation in humans. Primarily, we aimed to optimize the aforementioned adipogenic differentiation protocol to induce browning of hADMSCs cultivated from abdominal subcutaneous fat. The long-term administration of some thiazolidinediones (TZDs), including rosiglitazone which is the key component of this regimen, is not only capable of inducing browning of adipocytes *ex vivo* but also of increasing the thermogenic competency of WAT in rodents.

Since populations of *ex vivo* differentiated adipocytes are heterogeneous regardless of whether white or brown adipogenic differentiation was induced, we wished to examine human adipocyte browning not only by determining the expression of "classical brown", "beige", white or general adipocyte markers at the mRNA and protein levels using total cell lysates but also to specifically identify browning cells in mixed adipocyte populations according to their morphological parameters. On one hand, investigators have already successfully used flow cytometry to assess surface protein expression of human primary adipocytes or to sort out floating adipocytes from SVF cells including undifferentiated progenitors. In spite of its analytical power, however, no concise protocol is available to identify browning adipocytes in a large population of cells by flow cytometry due to the fact that widely accepted surface markers have not been identified, and the collection of accurate morphological data (e.g. determination of the size or number of lipid droplets in single adipocytes) and the possibility to inspect adipocyte differentiation at consecutive time points are sacrificed under the experimental conditions it requires.

On the other hand, LSC, which combines scanning lasers, a microscope and automated image acquisition and inherits both the cytometric attributes of flow cytometry and the photographing operation of a microscope, allows automated examination of large population of cells with negligible perturbation. Thus, LSC is not limited to analyzing cells in flowing fluids and it can perform high-content analysis of adherent cells cultured in chamber slides. Considering the limitations of flow cytometry and the

advantages of LSC, we decided to quantify *ex vivo* browning at a single cell level in a highly replicative manner by using the slide-based image cytometry approach.

5.2. Combined analysis of texture and Ucp1 by laser-scanning cytometry (LSC) effectively identifies browning adipocytes

Browning adipocytes have small lipid droplets in multilocular arrangement and the highest number of mitochondria in mammalian organisms. Lipid droplets can be quantified and analyzed directly by segmentation of a fluorescent signal. However, this method showed its disadvantage in the fact that the segmentation highly depends on the threshold algorithm as well as on the consistency of the staining. Moreover, Doan-Xuan et al. showed that texture parameters were more sensitive to interpret adipocyte differentiation or at least gave similar results compared to signals of fluorescent lipid staining. Due to possibilities of inconsistent sample preparations, different fluorescent signals among samples and errors in the segmentation of small objects we have chosen texture analysis as a reliable method to reflect the size of lipid droplets.

In our model, not only texture parameters but also the expression of major browning marker proteins was detected at the same time in single adipocytes following the immunostaining against Ucp1 or Cidea. Then we plotted Ucp1 or Cidea immunofluorescence intensity and texture "sum variance" for each differentiated adipocyte. Browning adipocytes were identified as the ones that contained small lipid droplets and high levels of Ucp1 or Cidea protein, in contrast to white adipocytes, which accumulated larger lipid droplets and expressed low amount of browning marker proteins.

With these combined approaches, we found that human ADMSCs from abdominal subcutaneous fat can be differentiated into "beige" adipocytes *ex vivo*. On one hand, we observed that after two weeks of differentiation 30-50% of the cells accumulated lipids and out of those 15-30%, depending on individual donors, had the characteristic morphological features of browning even in response to the white adipogenic cocktail. Most probably already committed "beige" precursors, which could not be differentiated into white adipocytes even in the presence of a white protocol, can be found in a significant amount in the SVFs isolated from subcutaneous WAT. Moreover, 30-70% of the differentiated adipocytes appeared as browning cells when they were differentiated by the browning cocktail. This regimen developed by Elabd et al. was described originally as a brown adipocyte differentiation protocol and indeed resulted in a high

expression level of browning markers, more mitochondrial DNA and elevated OC. On the other hand, we showed that these changes correspond more to a “beige” rather than a “classical brown” program, in as much as the “beige”-selective TBX1 was induced without the up-regulation of ZIC1.

The observed shift in the mixed adipocyte populations indicates that the browning cocktail, most probably, induces the commitment of multi- or bipotent human mesenchymal progenitor cells to “beige” adipocytes at some point during the differentiation. The proportion of anti-obesity “beige” and lipid-storing white adipocytes is determined, at least partially, when mesenchymal progenitors are committed into white or “beige” preadipocyte subtypes from which mature fat cells are differentiated. Furthermore these results strengthen the hypothesis that “beige” adipocytes originate form distinct precursors. Another study showed that “beige” adipocyte progenitors can be found in association with expanding capillary networks in human fat biopsies and proliferate rapidly in response to pro-angiogenic factors. We believe that these cells exist in the SVF isolated from subcutaneous WAT specimens and their differentiation can be followed in a time dependent manner by LSC.

In summary, we showed that LSC is a tool, which in combination with gene expression measurements of widely accepted adipocyte markers makes effective population scale analysis of *ex vivo* human brown or „beige” adipogenic differentiation possible. This method can help researchers to clarify how endogenous mediators or exogenous drugs affect human adipocyte browning directly. In our experiments, we validated mouse data in human samples demonstrating the effectiveness of irisin and BMP7 to induce browning of subcutaneous white adipocytes. Using this method we also demonstrated that clozapine could shift the adipogenic differentiation program towards browning. In the future, this technique might allow to test the propensity of hADMSCs of each individual to differentiate into heat-generating brown or “beige” cells (thermogenic competency) or to sort out homogenously differentiated hADMSC populations not only providing the possibility to understand the differences between “classical brown” or “beige” differentiation pathways in humans but also to “engineer” thermogenically active, transplantable browning adipocytes, as it was proposed by Min et al. recently, to aid weight reduction in obese individuals.

5.3. Gene expression pattern and "batokine" secretion distinguish irisin and BMP7 induced browning

When human adipocytes were differentiated either in the presence of irisin, BMP7 or clozapine on top of the white adipogenic cocktail, similar morphological alterations were found. The simultaneous gene expression changes and functional consequences, however, were markedly different. In line with previous results obtained in mice, irisin induced a gene expression pattern which indicated the "beige" program. Irisin treated cells had more mitochondrial DNA and higher basal mitochondrial respiration than white adipocytes and responded robustly to dibutyryl-cAMP administration. Moreover, irisin treatment had an enhancing effect on IL-6, MCP-1 and IL-8 production during adipogenesis. "Beige" adipocytes differentiated following the regimen of Elabd et al. also behaved similarly as irisin induced "beige" cells. Contrarily, BMP7 treatment enhanced the expression of C/EBPβ, PRDM16, PPARγ and ZIC1 genes in human adipocytes suggesting that this mediator rather induced a "classical brown"-like differentiation. This program, similarly to irisin administration, resulted in increased mitochondrial DNA and Ucp1 levels and elevated OC both at basal and at cAMP stimulated conditions. On the other hand, BMP7 treatment decreased the secretion of IL-6, MCP-1 and IL-8 cytokines.

Our results suggest that human SVFs isolated from subcutaneous WAT consist of a mixture of progenitors which have the potential to differentiate into white, "classical brown" or "beige" adipocytes in response to natural cues. In our experiments, the *ex vivo* differentiated thermogenic adipocytes, irrespectively of whether "classical brown" or "beige" gene expression program was induced, were active in a sense that they could prominently respond to an anti-obesity thermogenic cue with robustly enhanced energy expenditure. However, "classical brown" and "beige" adipocytes might have distinct non-thermogenic functions, with regard to the elevated secretion of IL-6, MCP-1 and IL-8 "batokines" by "beige" cells. Of note, the secretion of inflammatory mediators or the recruitment of MΦs and eosinophil granulocytes is not only linked to the remodeling of WAT during obesity but also to the differentiation and activation of browning adipocytes in special conditions.

5.4. Clozapine, an unexpected novel browning-inducer

When we unexpectedly observed that clozapine treated adipocytes had more and smaller lipid droplets and increased mitochondrial DNA amount than white fat cells, we

found that the drug significantly up-regulated ELOVL3, CIDEA, CYC1, PGC-1α and TBX1 genes but not ZIC1, suggesting the induction of the "beige" and not the "classical brown" phenotype. In line with our observations in regard to elevated cytokine production during "beige" adipogenesis, Sárvári et al. demonstrated that clozapine administration enhanced the secretion of MCP-1 and IL-8 by differentiating adipocytes. Moreover, a functional deficit of clozapine induced "beige" cells was detected by an XF96 oxymeter. Basal mitochondrial respiration (even basal proton leak OC) of clozapine treated cells was significantly elevated as compared to white adipocytes; however, the generated "masked beige" cells could not be efficiently stimulated by the cell permeable cAMP agonist. Additional gene expression results suggest that adipocytes differentiated in the presence of clozapine are less sensitive to cAMP activation. Previous studies, in which altered cAMP signaling was reported in the brain of rodents and humans in response to SGA treatment, further support these findings. On the other hand, clozapine could facilitate the energy expenditure of adipocytes by the induction of the recently identified substrate cycle of creatine-metabolism. The importance of this pathway in the metabolism of "beige" adipocytes was shown formerly in mice and in human cell lines; however, as far as we are aware, this is the first study in which a functional creatine-driven futile cycle was detected in primary human adipocytes.

The prevalence rate of obesity and its co-morbidities is at least two times higher in patients suffering in schizophrenia or in other SMIs compared to the general population. Clozapine induced browning in our *ex vivo* experiments in spite of its well documented effect to promote obesity in patients. A similar phenomenon was precedented by certain TZDs. Different central and peripheral mechanisms were suggested which might elucidate the molecular background of SGA-induced weight gain. For example, Kim et al. demonstrated that SGAs up-surged the appetite by the activation of hypothalamic AMP kinase via histamine H1 receptors in rodents.

Finally, we aimed to learn the molecular mechanism which can elucidate the browning effect of clozapine. To obtain mechanistic data, we tested if browning induced by clozapine can be explained by its known pharmacological effect of antagonizing 5HT receptors. 14 different 5HT-receptor (HTR1-7) types have been already identified and most of them are expressed by white and brown adipocytes in rodents. The release of 5HT by 3T3-L1 adipocytes was also reported formerly. In human adipocytes, the expression of HTR1D, 2A, 3A and 7 were reported in different studies. In our

experiments, the expression of TPH1, the gene encoding the enzyme which catalyzes the rate-limiting step of 5HT synthesis outside the CNS, was detectable in hADMSCs of subcutaneous origin by RT-qPCR and did not change as a result of white adipocyte differentiation, suggesting that these cells are capable of autonomously generating and secreting 5HT during adipogenesis. However, we found decreased TPH1 expression in clozapine induced and in "beige" adipocytes. In addition, we showed that primary cells expressed HTR2A, 1D and 7 and the up-regulation of browning markers by clozapine was diminished in the presence of exogenous 5HT. Recently, two groups reported independently that peripheral 5HT reduced the "beige" potential and the sensitivity of browning adipocytes to thermogenic induction in a cell autonomous manner in mice. Other studies showed that increased levels of peripheral 5HT and polymorphisms in TPH1 gene were associated with obesity. In our experiments, out of the 5HT-receptors, HTR2A to which clozapine can be bound with the strongest affinity, was expressed at the highest level in hADMSCs and in differentiated adipocytes. Gq signaling, which is otherwise initiated by HTR2A, was recently reported to abolish browning in mice and in human adipocytes. Our results suggest that the disturbance of 5HT-receptor-mediated signaling by clozapine might, at least partially, explain the browning phenomenon described above.

In summary, we found that clozapine modified the differentiation program of human adipocyte progenitors; presumably via the inhibition of 5HT receptor mediated signaling, leading to generation of "beige" adipocytes with masked and not fully responsive thermogenic potential. The detected incomplete acute thermogenic response to cAMP can be one of the reasons why these "masked beige" adipocytes function ineffectively also *in vivo*. Our data suggest that novel pharmacological stimulation of these "masked beige" adipocytes can be a future therapeutic target for treatment of SGA-induced obesity. We hope that our results initiate studies which identify the "masked beige" cells differentiated in response to clozapine treatment *in vivo* and find ways to induce their thermogenic function independently from the SNS.

6. KEYWORDS

adipocyte, beige, browning, clozapine, irisin, laser-scanning cytometry, obesity, serotonin, thermogenesis, UCP1

7. SUMMARY

• By complementing measurements of gene expression changes from total cell lysates, Laser-scanning cytometry (LSC) was presented as a tool that made the population scale analysis of *ex vivo* browning possible. Our approach combined texture analysis which reflected the size and number of lipid droplets and detection of Ucp1 and Cidea protein content in single browning adipocytes of mixed cell populations.

• Irisin administration during white adipogenic differentiation resulted in a significant upregulation of several brown and "beige" adipocyte marker genes. Irisin treated cells had smaller lipid droplets, more mitochondrial DNA, higher mitochondrial respiration and contained more Ucp1 and Cidea protein than the untreated white adipocytes.

• On the contrary, BMP7 treatment resulted in a functional browning in parallel with the gene expression pattern which indicated the "classical brown" program.

• We unexpectedly observed that clozapine reprogrammed the gene expression pattern of differentiating human adipocytes *ex vivo*, leading to an elevated expression of the browning marker gene UCP1, more and smaller lipid droplets and more mitochondrial DNA than in the untreated white adipocytes. Furthermore, clozapine significantly up-regulated TBX1 gene but not ZIC1 suggesting induction of the "beige"-like and not the "classical brown" phenotype. The clozapine induced "beige" cells displayed increased basal and oligomycin inhibited (proton leak) oxygen consumption but these cells showed a lower response to cAMP stimulus as compared to control "beige" adipocytes.

• When we tested if browning induced by clozapine can be explained by its known pharmacological effect of antagonizing serotonin (5HT) receptors it was found that browning cells expressed 5HT receptors 2A, 1D, 7 and the up-regulation of browning markers was diminished in the presence of exogenous 5HT. Our results suggest that the disturbance of 5HT-receptor-mediated signaling by clozapine might, at least partially, explain the browning effect of the drug.

• When conditioned differentiation media were collected during the replacement of the adipogenic cocktails, we found that IL-6, MCP-1 and IL-8 secretion was significantly higher by "beige" compared to white adipocytes. Media replaced daily or in three day periods contained the same steady-state amount of IL-6 depending only on the phase of differentiation. This suggests that adipocytes adjust their production of the cytokine to reach an optimal level in the medium.

8. PUBLICATIONS

Registry number: DEENK/283/2016.PL
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Candidate: Endre Kristóf
Neptun ID: N64U4X
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List of publications related to the dissertation

1. **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.
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7. **Kristóf, E.**, Zahuczky, G., Katona, K., Doró, Z., Nagy, É., Fésüs, L.: Novel role of ICAM3 and LFA-1 in the clearance of apoptotic neutrophils by human macrophages.
Apoptosis. 18 (10), 1235-1251, 2013.
DOI: http://dx.doi.org/10.1007/s10495-013-0873-z
IF: 3.614

8. Zahuczky, G., **Kristóf, E.**, Majai, G., Fésüs, L.: Differentiation and Glucocorticoid Regulated Apopto-Phagocytic Gene Expression Patterns in Human Macrophages: role of Mertk in Enhanced Phagocytosis.
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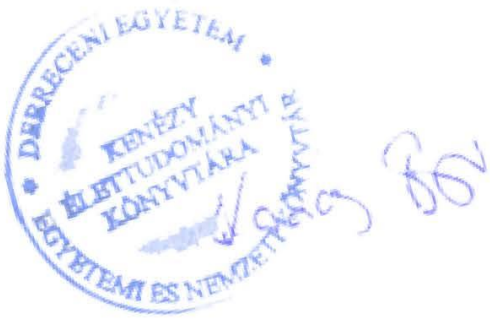
DOI: http://dx.doi.org/10.1371/journal.pone.0021349
IF: 4.092

Total IF of journals (all publications): 37,368
Total IF of journals (publications related to the dissertation): 10,766

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

26 October, 2016



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