Final report of NKFIH FK_19 131424 project

Analysis of human heat producing adipocytes to reveal novel mechanisms of thermogenic stimulation for combating obesity

Objectives

Brown adipose tissue (BAT) plays a major role in maintaining the constant core body temperature of humans. Active BAT can be found in specific adipose depots in adult humans, amounts to 1.5% of total body mass and is mostly enriched in the supraclavicular, deep neck (DN), and paravertebral regions. Brown and beige adipocytes accumulate numerous small lipid droplets in a multilocular arrangement, contain a large amount of mitochondria-rich cytoplasm and convert glucose, fatty acids, and amino acids into heat. The heat production is mainly mediated by uncoupling protein (UCP) 1, a mitochondrial carrier protein that uncouples ATP synthesis from the respiratory chain activity. The ratio of 'anti-obesity' beige and 'pro-obesity' white precursors and adipocytes is strongly influenced by genetic predisposition; the *FTO* rs1421085 T-to-C single nucleotide polymorphism shifts the differentiation program towards white adipocytes in subcutaneous (SC) fat. Cold induced non-shivering thermogenesis (NST) and adipocyte browning are primarily regulated by the activation of sympathetic nervous system (SNS) which densely innervates BAT. The released norepinephrine binds to β-adrenergic receptors, which induce a signaling cascade mediated via adenylyl cyclase activation by Gs proteins, then 3',5'-cyclic adenosine monophosphate (cAMP) and Protein Kinase A (PKA) transmit the thermogenic signal. Irisin was discovered as a myokine which induced a beige program of SC white adipose tissue (WAT) in mouse models. BMP7, a paracrine/autocrine mediator, induces the preadipocytes to differentiate into classical brown adipocytes but has no effect on the white adipogenesis during embryonic development. When the thermogenic stimulus subsides, selective autophagy is activated for mitochondrial clearance (mitophagy) and dormant/inactive beige adipocytes persist that, however, have a white adipocyte-like morphology *in vivo*.

The project intended to learn more about human adipocyte browning including three specific aims. This report summarizes the results achieved on these three aims:

1. Identification of novel molecular elements of the browning machinery in different human adipose tissue depots

a) Primarily, we screened and compared global gene expression patterns by RNA sequencing of human adipose-derived stromal cells (hASCs)-derived white and brown differentiated adipocytes. Brown differentiation was initiated in response to sustained Peroxisome proliferator-activated receptor γ (PPARγ) stimulation. The hASCs were isolated from paired DN and cervical SC adipose tissue samples of nine donors, three of each *FTO* rs1421085 genotype: T/T-risk-free, T/C-heterozygous, and C/C-obesity-risk. The rs1421085 *FTO* risk allele results in a loss of ARID5B-mediated repression of IRX3 and IRX5 which promotes excess WAT formation *(Claussnitzer et al., 2015, PMID: 26287746)*. The presence of the C risk-allele of the *FTO* rs1421085 locus was tested using specific PCR probes. We found that DN and SC-derived hASCs had similar adipogenic differentiation potential but differed in 1420 differentially expressed genes (DEGs). Adipocytes derived from DN hASCs displayed higher browning features and characteristic DEG patterns revealing associated pathways which were highly expressed

(thermogenesis, interferon, cytokine, retinoic acid) or downregulated (particularly extracellular matrix remodeling) as compared to those that were originated from SC. Part of the DEGs in either DN or SC browning were PPARγ-dependent. However, the gene expression pattern of the differentiated adipocytes was determined at a greater extent by the anatomical origin of the hASCs than the applied differentiation protocol. We also reported that the presence of the *FTO* obesity-risk allele suppressed the expression of mitochondrial and thermogenesis-related genes in both SC and DN-derived adipocytes. To our knowledge, the influence of this genetic background in the browning process of human cervical area-derived adipocytes has not been recognized so far *(Tóth et al., 2020, PMID: 32316277)*.

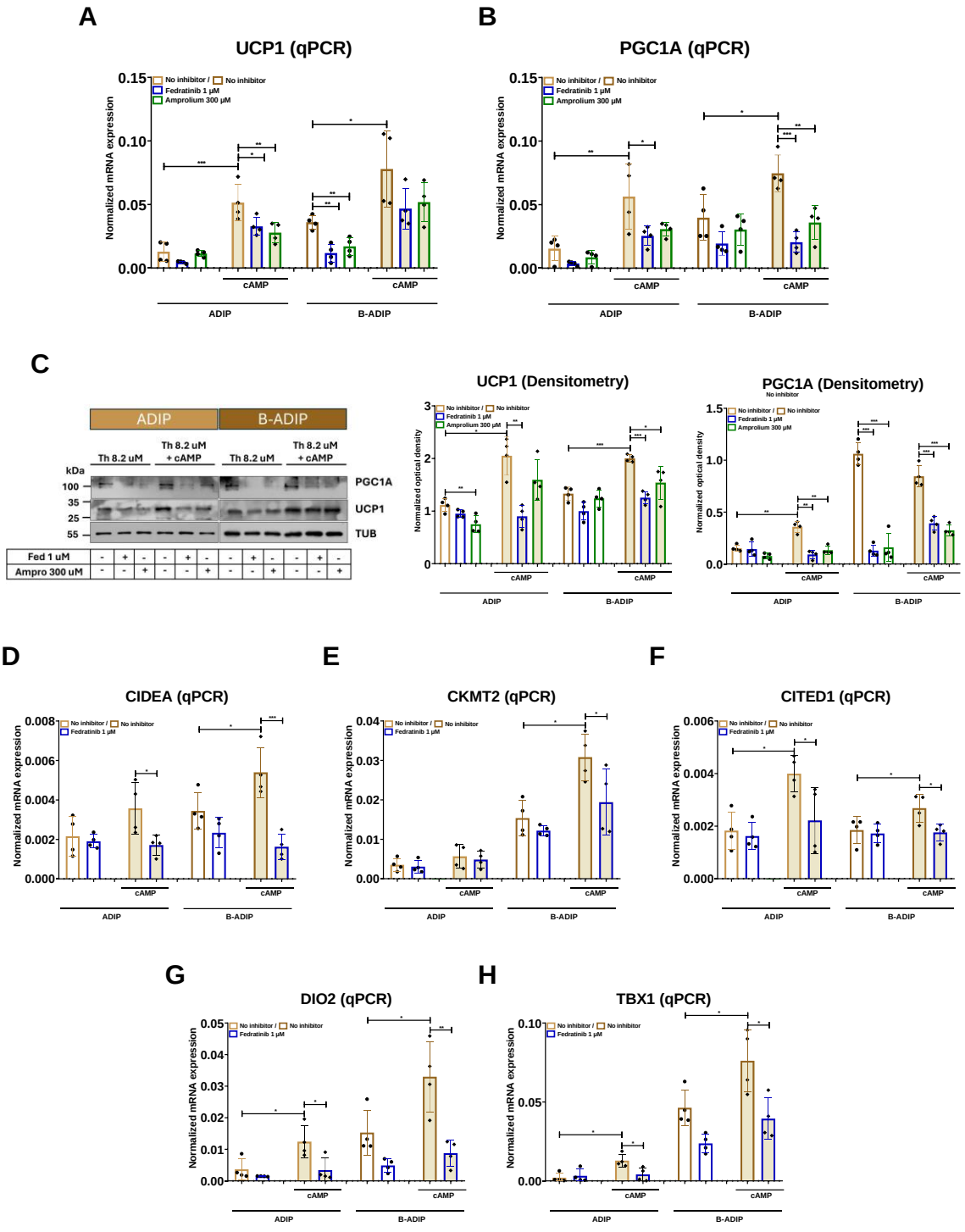
b) The Asc-1 amino acid transporter, encoded by *SLC7A10* gene, provides access to several amino acids (e.g., Gly, Ser, Ala, Cys) into cells in a Na^+-independent manner. Based on RNA sequencing data *(1/a)*, the *SLC7A10* gene was more expressed in DN-derived brown adipocytes compared to SC-derived white-like ones. As a next step, in accordance with the work plan, the direct effect of pharmacological inhibition of Asc-1 on heat production was investigated. Dibutyryl-cAMP stimulation of differentiated adipocytes led to elevated uptake of Ser, Cys, and Gly, in parallel with increased oxygen consumption, augmented UCP1-dependent proton leak, increased creatine-driven substrate cycle-coupled respiration, and upregulation of thermogenesis marker genes and several respiratory complex subunits; these outcomes were impeded in the presence of the specific Asc-1 inhibitor, BMS-466442. In these experiments, the effect of the compound was tested on primary cervical and Simpson–Golabi–Behmel syndrome (SGBS) adipocytes that were introduced previously by our research group as a model system to investigate browning of human SC adipocytes *(Klusóczki et al., 2019, PMID: 30967578)*. Our data suggest that Asc-1-dependent consumption of Ser, Cys, and Gly is required for efficient thermogenic stimulation of human adipocytes *(Arianti et al., 2021, PMID: 34197627)*.

c) We also investigated the association of *ENPP1* (encoding Ectonucleotide pyrophosphatase/phosphodiesterase 1) SNP K121Q (rs1044498) with insulin resistance and *ADIPOQ* (encoding adiponectin) SNP + 267G>T (rs1501299) with circulating adiponectin levels in a case–control study. The presence of the Q121 allele of *ENPP1* resulted in significantly higher fasting glucose, fasting insulin levels, and HOMA-IR, as compared to homozygous K121 carriers. The risk of insulin resistance was elevated in individuals with obesity carrying Q121 instead of homozygous K121. Individuals with obesity carrying homozygous protective alleles (TT) of *ADIPOQ* tended to have lower adiponectin levels as compared to GT and GG carriers, however, we did not find statistically significant effects of the +276G>T SNP of the *ADIPOQ* gene on the plasma adiponectin levels or on the development of obesity *(Arianti et al., 2021, PMID: 34208364)*.

d) According to newly obtained RNA sequencing data, irisin upregulated genes belonging to various cytokine signaling pathways in both DN and SC-derived adipocytes. Out of the several upregulated cytokines, CXCL1, the highest upregulated, was released throughout the entire differentiation period, and predominantly by differentiated adipocytes. DN area tissue biopsies also showed a significant release of CXCL1 upon irisin treatment. Continuous blocking of the NFκB pathway, using a cell permeable inhibitor of NFκB nuclear translocation, significantly reduced CXCL1 release. The released CXCL1 exerted a positive effect on the adhesion of endothelial cells. Together, our findings demonstrate that irisin stimulates the release of a novel adipokine, CXCL1, via upregulation of NFκB pathway in cervical area-derived adipocytes, which might play an important role in improving tissue vascularization *(Shaw et al., 2021, PMID: 34708041)*.

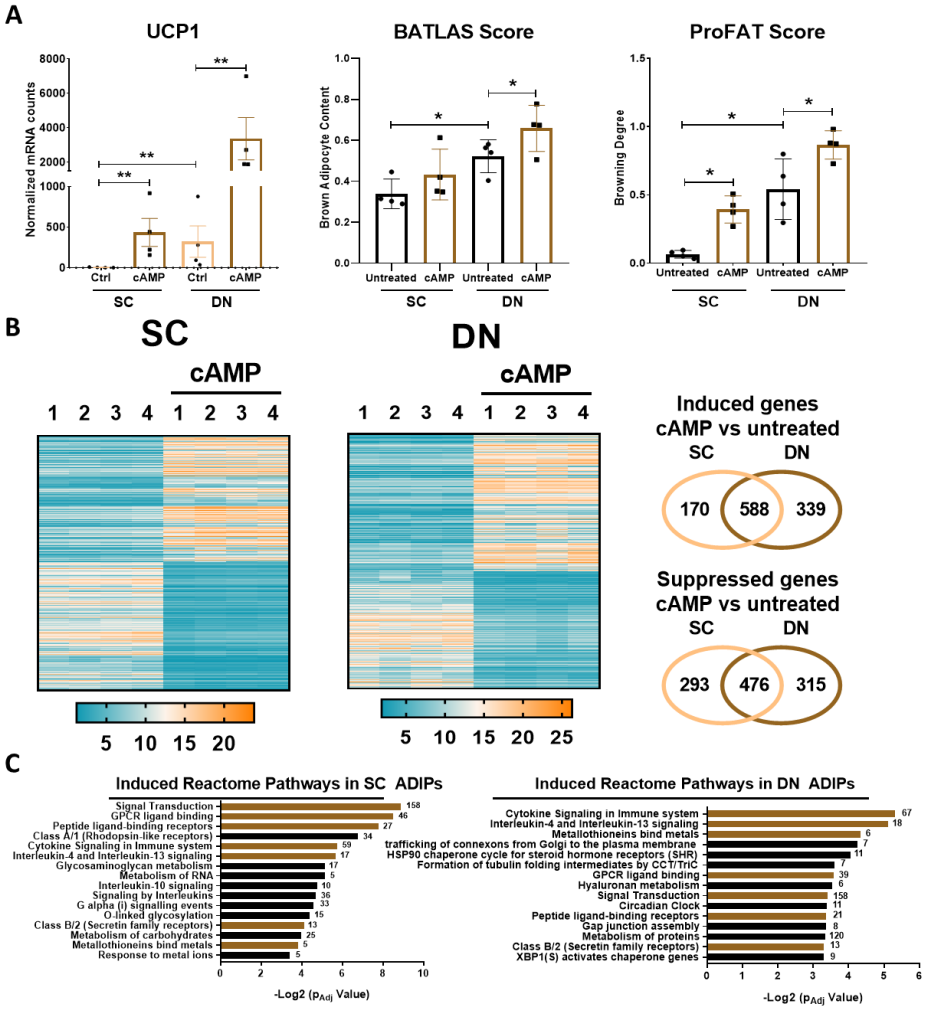
e) Based on RNA sequencing *(1/d)*, we found that BMP7 did not influence differentiation but upregulated browning markers in SC and DN-derived adipocytes. BMP7 also enhanced mitochondrial DNA content, levels of oxidative phosphorylation complex subunits, along with PGC1α and p-CREB upregulation, and fragmentation of mitochondria. Furthermore, both UCP1-dependent proton leak and UCP1-independent, creatine-driven substrate cycle coupled thermogenesis were augmented upon BMP7 addition. The gene expression analysis also shed light on the possible role of genes unrelated to NST thus far, including *ACAN*, *CRYAB*, and *ID1*, which were among the highest upregulated ones by BMP7 treatment. This shows that BMP7 strongly upregulates NST in human cervical area-derived derived adipocytes, along with genes, which might have a supporting role in energy expenditure *(Shaw et al., 2021, PMID: 34832860)*.

f) Thiamine (vitamin B1) is an essential cofactor of mitochondrial enzyme complexes that catalyze key steps in the catabolism of nutrients. We found that UCP1 enriched adipocytes differentiated from precursors of DN depot highly expressed ThTr2 transporter of thiamine (encoded by *SLC19A3* gene) *(1/a)* and consumed higher amounts of thiamine during thermogenic activation of these adipocytes by SNS. Inhibition of ThTr2 led to lower thiamine consumption with decreased proton leak respiration reflecting reduced uncoupling. In the absence of thiamine, cAMP-induced uncoupling was diminished but restored by thiamine addition reaching the highest levels at thiamine concentrations larger than present in human blood plasma. Thiamine is converted to thiamine pyrophosphate (TPP) in cells; the addition of TPP to permeabilized adipocytes increased uncoupling fueled by TPP-dependent pyruvate dehydrogenase. ThTr2 inhibition also hampered cAMP-dependent induction of UCP1, PGC1a, and other browning marker genes and proteins, and the upregulation of these markers was potentiated by thiamine in a concentration-dependent manner *(Arianti et al., 2023, PMID: 37230255)*. Next, we differentiated primary human ASCs that were cultivated from SC or DN adipose tissues in the presence of gradually increasing thiamine concentrations during their 14 day differentiation program. Higher thiamine levels resulted in increased expression of ThTr1 and 2 both at mRNA and protein levels in human cervical area-derived adipocytes. Gradually increasing concentrations of thiamine led to increased basal, cAMP-stimulated, and proton leak respiration along with elevated mitochondrial biogenesis of the differentiated adipocytes. The extracellular thiamine availability during adipogenesis determined the expression levels of UCP1, PGC1a, CKMT2, and other browning-related genes and proteins in primary SC and DN-derived adipocytes in a concentration-dependent manner. Providing abundant amounts of thiamine further increased the thermogenic competency of the adipocytes *(Vinnai et al., 2023, PMID: 37781121)*. These revealed the importance of amply supplied thiamine during adipogenesis and thermogenic activation in human adipocytes which provides TPP for TPP-dependent enzymes probably not fully saturated with this cofactor and by potentiating the induction of thermogenic genes. Our study raises the possibility of a novel strategy with thiamine supplementation, which can enhance NST in cervical-derived adipocytes for preventing or combating obesity. The pharmacological inhibition of ThTrs also hampered the SNS-driven induction of thermogenic markers in SGBS adipocytes which suggests that thiamine availability is an important factor for efficient NST by each adipose depot of the human body.



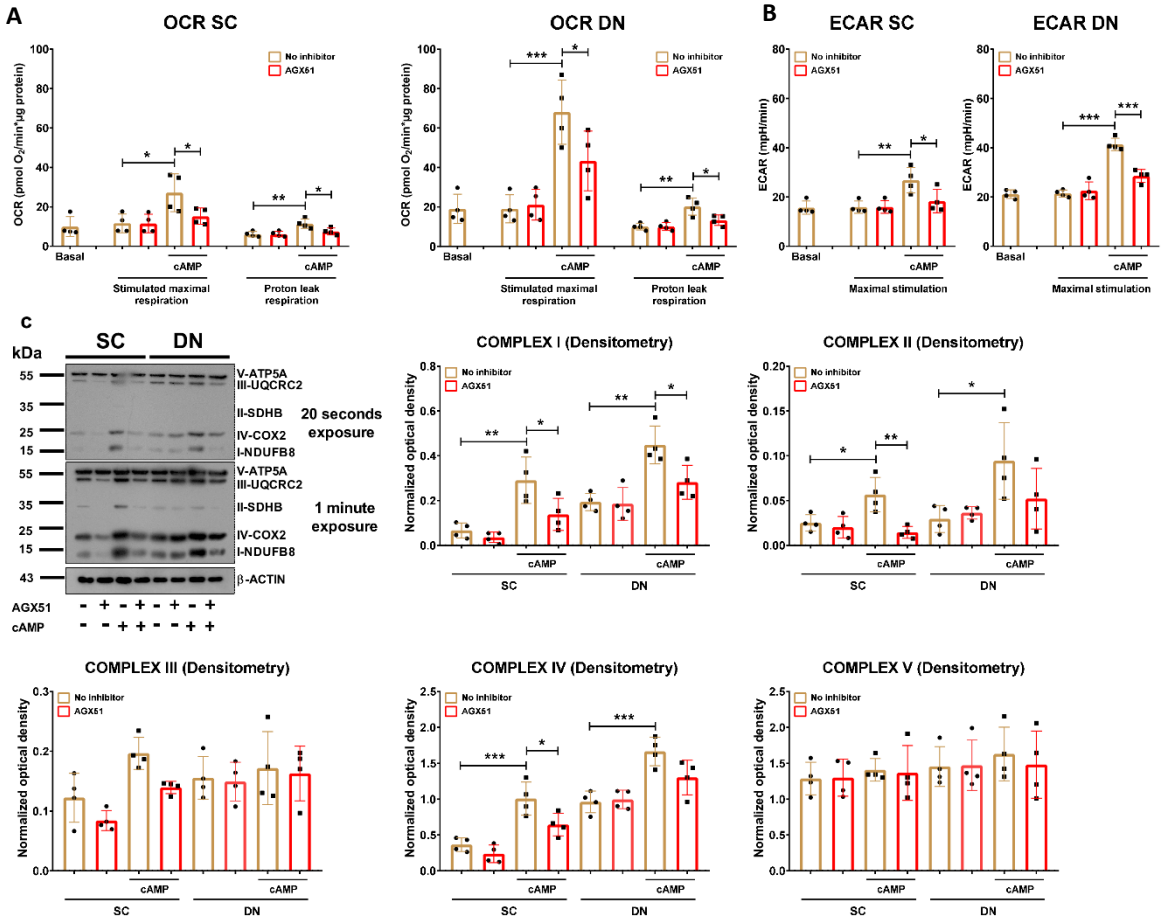
Effect of thiamine (Th) transporter inhibitors (Fedratinib and Amprolium) on the basal and cAMP-induced expression of thermogenic markers in SGBS adipocytes (ADIPs) and brown differentiated adipocytes (B-ADIPs)*. After 14 days differentiation at regular culture conditions (8.2 µM Th), ADIPs and B-ADIPs were treated with 500 µM dibutyryl-cAMP (cAMP, brown bars) in the presence or absence of Th transporter inhibitors for 10 h. (A and B) mRNA expression of UCP1 and PGC1a assessed by RT-qPCR. (C) Protein expression of UCP1 and PCG1a detected by immunoblotting. (D–H) mRNA expression of CIDEA, CKMT2, CITED1, DIO2, and TBX1 assessed by RT-qPCR, n=4. Statistical analysis was performed by one-way ANOVA, *p<0.05, **p<0.01, ***p<0.001.*

g) To explore the gene expression profile of adipocytes derived from human SC and DN upon stimulation by cAMP (which mimics *in vivo* adrenergic stimulation of NST), we performed another RNA sequencing analysis and found 1527 (758 induced and 769 suppressed genes) and 1718 (927 induced and 791 suppressed genes) DEGs in SC and DN-derived adipocytes, respectively. A total of 588 genes were commonly induced whereas 476 genes were commonly suppressed in SC and DN-derived adipocytes upon cAMP stimulation in comparison to untreated ones. The expression of thermogenic genes, such as *UCP1*, *DIO2*, and *CITED1*, was more expressed in cAMP-treated adipocytes derived from both SC and DN. We further obtained functional data, with respect to the role of several SNS-induced genes, e.g. transglutaminase 2 (encoded by *TGM2* gene) which importance was revealed in murine adipocytes (*Lénárt et al., 2022, PMID: 35563567*), in thermogenic activation.



Gene expression pattern of subcutaneous (SC) and deep neck (DN)-derived adipocytes after 10 hours of thermogenic activation. *(A) mRNA expression of UCP1 (left), brown adipocyte content quantified by BATLAS (middle), browning capacity quantified by ProFAT (right), n=4, *p<0.05, **p<0.01, statistical analysis by one-way ANOVA. (B) Heatmaps displaying the expression values of differentially expressed genes (DEGs) in SC (left) or DN (right)-derived adipocytes. Venn diagrams displaying the numbers of more (top) or less expressed (bottom) DEGs in comparison of cAMP vs untreated. (C) Overrepresented Reactome pathways which are upregulated in cAMP-treated SC (left) or DN (right)-derived adipocytes. Brown bars indicate common overrepresented Reactome pathways in both types of adipocytes, numbers indicate the involved DEGs in the particular pathway.*

Inhibitors of DNA binding and cell differentiation (ID) proteins 1-4 are classified within the helix-loop-helix transcription factor family. The cell-permeable cAMP analog not only increased NST, but also the expression of the *ID1* and *ID3* genes (see *1/e*). Therefore, we intended to investigate whether pharmacological inhibition of IDs influences the activation of human adipocyte thermogenesis by SNS stimulus *ex vivo*. We observed that the cAMP-stimulated elevation of proton leak respiration, extracellular acidification, mitochondrial complex I content, and expression of thermogenic genes and proteins (UCP1, PGC1A, DIO2, CITED1) was hampered by the ID antagonist, which promoted the degradation of ID1 and ID3 proteins. Our preliminary results suggested that ID proteins, especially ID1 and ID3, play important roles for efficient thermogenic response during SNS stimulation in human cervical-derived adipocytes.



The effect of the Inhibitor of DNA Binding (ID) antagonist, AGX51 on the oxygen consumption and extracellular acidification rates (OCR and ECAR) and expression of mitochondrial complex subunits in subcutaneous (SC) and deep neck (DN)-derived adipocytes after 10 h of dibutyryl-cAMP (cAMP)-driven thermogenic activation. *Basal, cAMP stimulated maximal, and proton leak OCR (A) and basal and stimulated maximal ECAR (B) were quantified by Seahorse extracellular flux analysis. (C) Protein expression of mitochondrial complex subunits detected by immunoblotting. n=4, statistical analysis by one-way ANOVA followed by Tukey's post-hoc test, *p<0.05, **p<0.01, ***p<0.001.*

2. Characterization of active to dormant beige adipocyte conversion in humans

a) A lot of our brownable WAT (WAT in areas with thermogenically active adipocytes) may contain dormant brown/beige adipocytes. In accordance with the work plan, we intended to find novel

molecular regulators that characterize active to dormant beige adipocyte conversion which might open up novel strategies to prevent the entry into dormancy and subsequently to keep the energy dissipation high. Primarily, we observed that abdominal SC-derived white-differentiated adipocytes have already high autophagic flux and mitophagy rates. In this case, the cell-permeable cAMP analog promptly increased the thermogenic potential of white adipocytes similarly to the beige ones. The activation not only upregulated thermogenesis-related genes but also quickly downregulated mitophagy via PKA in both white and beige adipocytes, resulting in more mitochondria and increased UCP1 levels. We reported that mitophagy was repressed in response to a short-term adrenergic cue, as a fast regulatory mechanism, which prevents the entry into dormancy, to provide high mitochondrial content for thermogenesis (*Szatmári-Tóth et al., 2020, PMID: 32316277*).

b) To investigate active to dormant beige adipocyte conversion in a cell culture model, primary abdominal SC hASCs were differentiated to beige for 14 days, then either the beige culture conditions were applied for an additional 14 days or it was replaced by a white medium. Control white adipocytes were differentiated by their specific cocktail for 28 days. PPARγ-driven beige differentiation resulted in increased mitochondrial biogenesis, UCP1 expression, and respiration as compared to white. Morphology, UCP1 content, and basal respiration of the adipocytes that underwent transition, along with the induction of mitophagy, were similar to control white adipocytes. However, white converted beige adipocytes had a stronger responsiveness to SNS stimulus, than the control white ones. Expression of general autophagy markers showed a decreasing trend following PPARγ activation and were increased upon beige to white transition. Gene expression patterns showed that the removal of mitochondria in transitioning adipocytes may involve both parkin-dependent and independent pathways. CALCOCO2/NDP52 expression was increased during white adipogenesis and transition compared with fully differentiated beige adipocytes; this suggests the possibility of enhanced removal of the mitochondrial mass by NDP52-dependent mechanism (*Vámos et al., 2022, PMID: 35337160*).

c) To investigate the effect of the *FTO* rs1421085 SNP on abdominal SC adipocyte differentiation, thermogenic function, and active to dormant beige conversion, we further optimized the active beige differentiation protocol, described in *2/b*, which was supplemented with a 4-hour dibutyryl-cAMP treatment on the 14th day of differentiation. With this modification, we intended to increase the thermogenic capacity of beige adipocytes. RNA sequencing was performed to investigate the gene expression pattern of adipocytes carrying different *FTO* alleles and found that active beige adipocytes had higher brown adipocyte content and browning capacity compared to white or inactive beige ones when the cells were obtained from risk-free TT but not from obesity-risk CC genotype carriers. Active beige adipocytes carrying *FTO* CC had lower thermogenic gene (e.g. *UCP1*, *PM20D1*, *CIDEA*) expression and thermogenesis measured by proton leak respiration as compared to TT carriers. In addition, active beige adipocytes with CC alleles exerted lower expression of Asc-1 neutral amino acid transporter (see *1/b*) and less consumption of Ala, Ser, Cys, and Gly as compared to risk-free carriers. We also found that active beige adipocytes had higher expression of serine hydroxymethyltransferase (SHMT) 1 as compared to white or inactive beige adipocytes in the presence of the *FTO* risk-free variant, but this difference was not observed in obesity-risk carrier samples. We did not observe any influence of the *FTO* rs1421085 SNP on white and dormant beige adipocytes highlighting its exclusive and critical effect when adipocytes were activated for thermogenesis *(Vámos et al., 2023, PMID: 37416800)*.

3. Development of a novel imaging platform to discriminate adipocyte types and evaluate their function

a) Mitochondria are dynamic organelles which change their morphology between larger networks of elongated mitochondria and small, fragmented units, respectively. In adipocytes, mitochondrial fragmentation enhances uncoupling and energy expenditure. Primarily, immunostaining for UCP1 and/or Translocase of Outer Mitochondrial Membrane 20 (TOMM20) was performed. This allowed us to visualize heat producing mitochondria in adipocytes and to classify them into subpopulations, e.g. elongated or fragmented, respectively. SNS-driven thermogenic activation resulted in mitochondrial fragmentation in white and beige differentiated abdominal SC adipocytes (*Szatmári-Tóth et al., 2020, PMID: 32316277*). BMP7 administration resulted in similar effects on the mitochondrial morphology in cervical-area derived adipocytes (*Shaw et al., 2021, PMID: 34832860*). We also found that mitochondrial fragmentation was elevated following the active beige differentiation and subsided upon beige to white inactivation in abdominal SC adipocytes (*Vámos et al., 2022, PMID: 35337160*; and *2023, PMID: 37416800*). Detecting fragmented and functional thermogenic mitochondria aids our imaging platform to discriminate the active heat producing adipocyte subpopulation.

b) When TOMM20 and Microtubule-associated protein 1A/1B-light chain 3 (LC3) co-immunostaining was carried out, the visualization of the subcellular distribution of LC3 and its co-localization with TOMM20 made the quantification of autophagosome formation and the delivery of mitochondria for degradation possible. We found that abdominal SC-derived white adipocytes contained more LC3 punctae per cell than the beige ones. In addition, the beige to white transition significantly increased the number of LC3 punctae, as compared to the fully differentiated beige adipocytes which suggested that general autophagy was induced in a cell-autonomous manner during the *ex vivo* beige to white transition of human adipocytes. In parallel, we observed elevated LC3-TOMM20 co-localization during white adipogenesis compared with the beige adipocytes. The co-localization was stronger in adipocytes that underwent transition than in fully differentiated beige cells (*Vámos et al., 2022, PMID: 35337160*; and *2023, PMID: 37416800*). In case of adrenergic-driven activation of thermogenesis and browning, we found the opposite, which proved the suppression of autophagy in adipocytes stimulated for thermogenesis (*Szatmári-Tóth et al., 2020, PMID: 32316277*).

c) BAT and skeletal muscle have shared metabolic features and embryonic origins. Genetic fate mapping experiments in mice demonstrate that the dermomyotome regions of the somites, marked by the expression of transcription factors including Pax3, Pax7, Meox1, and Myf5, give rise to most fat cells within the interscapular and retroperitoneal adipose depots. The fact that these lineages are traced to dorsal-anterior-located muscle, brown and white adipocytes suggests that they are location markers, rather than identity markers. Tbx1 has been known as beige adipocytes marker. However, recent findings showed that supraclavicular BAT adipocytes arise from Tbx1+ progenitors. In addition, *TBX1* also marked the human DN-derived adipocytes, which possess high thermogenic capacity. In summary, the identification of Tbx1+ lineage cells as progenitors of supraclavicular BAT brown adipocytes reveals location-specific myoprogenitors for different BAT depots in rodents and possibly humans *(Huang et al., 2023, PMID: 38048357)*.

Impact

In summary, the experiments planned for project were successfully carried out. Based on our results, thirteen articles were published and the experimental data was presented and disseminated at national and international conferences in this period.

The project leader was invited to edit an e-book:

Cereijo R., Kristóf E.: Novel regulatory mechanisms behind thermogenesis of brown and beige adipocytes. Frontiers Media, Lausanne, 2023. ISBN: 9782832534007

and four special issues:

https://www.frontiersin.org/research-topics/31618/novel-regulatory-mechanisms-behind-thermogenesis-of-brown-and-beige-adipocytes/magazine

https://www.frontiersin.org/research-topics/57638/novel-regulatory-mechanisms-behind-thermogenesis-of-brown-and-beige-adipocytes---volume-ii

https://www.mdpi.com/journal/life/special_issues/Adipo_Life

https://www.mdpi.com/journal/life/special_issues/BDQ1HTRE87

aiming to disseminate adipose tissue research to the international scientific community.