Original Article

MOLECULAR METABOLISM

Latent TGFβ-binding proteins regulate UCP1 expression and function via TGFβ2

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ABSTRACT

Objective: Activation of brown adipose tissue (BAT) in humans has been proposed as a new treatment approach for combating obesity and its associated diseases, as BAT participates in the regulation of energy homeostasis as well as glucose and lipid metabolism. Genetic contributors driving brown adipogenesis in humans have not been fully understood.

Methods: Profiling the gene expression of progenitor cells from subcutaneous and deep neck adipose tissue, we discovered new secreted factors with potential regulatory roles in white and brown adipogenesis. Among these, members of the latent transforming growth factor beta-binding protein (LTBP) family were highly expressed in brown compared to white adipocyte progenitor cells, suggesting that these proteins are capable of promoting brown adipogenesis. To investigate this potential, we used CRISPR/Cas9 to generate LTBP-deficient human preadipocytes.

Results: We demonstrate that LTBP2 and LTBP3 deficiency does not affect adipogenic differentiation, but diminishes UCP1 expression and function in the obtained mature adipocytes. We further show that these effects are dependent on TGFβ2 but not TGFβ1 signaling: TGFβ2 deficiency decreases adipocyte UCP1 expression, whereas TGFβ2 treatment increases it. The activity of the LTBP3—TGFβ2 axis that we delineate herein also significantly correlates with UCP1 expression in human white adipose tissue (WAT), suggesting an important role in regulating WAT browning as well.

Conclusions: These results provide evidence that LTBP3, via TGFβ2, plays an important role in promoting brown adipogenesis by modulating UCP1 expression and mitochondrial oxygen consumption.

Keywords Obesity; Adipose tissue; Adipogenesis; Browning; TGF beta

1. INTRODUCTION

Brown adipose tissue (BAT) has recently been recognized as a potential therapeutic target for addressing obesity and its comorbidities. The thermogenic activity of BAT is crucial for the survival of neonates, but active BAT has also been detected in adults, suggesting relevant functions in adulthood as well [1—4]. A number of studies has established BAT as a key regulator of human energy homeostasis with crucial roles in glucose and lipid metabolism (for review see [5]).

Thermogenesis within brown adipocytes relies on uncoupling protein 1 (UCP1), which upon activation by fatty acids or beta-adrenergic receptor agonists dissociates cellular respiration from ATP generation [6]. UCP1, however, is also found in thermogenically active beige or brite adipocytes, which arise in white adipose tissue (WAT) upon chronic cold exposure, a process usually referred to as browning [7—9]. While such browning improves the glucose and lipid metabolism of rodents [10], the occurrence and metabolic contributions of browning in humans have not yet been fully elucidated. Of note, though, studies using the β3-adrenergic agonist Mirabegron have demonstrated the induction of a browning-associated gene expression pattern in human WAT [11]. Due to the exceptionally high energy demand of thermogenically active adipocytes, not only activation of present BAT but also induction of browning in WAT may yield metabolic benefits [12]. The identification of factors involved in brown and beige adipocyte differentiation and activation thus might also open new avenues for obesity treatment.

We have recently demonstrated that adipose-derived stromal cells (ASCs) isolated from subcutaneous (sc) adipose tissue display a distinct gene expression pattern compared to cells isolated from deep neck (dn) adipose tissue, a depot where brown adipocytes are frequently found in humans [13]. Upon adipogenic differentiation *ex vivo*, cells from the sc depot differentiated into adipocytes with low UCP1 expression, whereas cells from the dn depot acquired a phenotype resembling that of brown adipocytes, with a high

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expression of UCP1 and other markers of brown adipocytes, such as PRDM16 and LHX8 [13]. This suggests that two different pools of progenitor cells exist within these depots, giving rise to either white or brown adipocytes [13]. To identify genes relevant to adipocyte browning, we compared the gene expression patterns of the corresponding ASCs by array analysis.

Focusing on potentially secreted factors, we found members of the latent transforming growth factor-binding proteins (LTBPs) family to be more highly expressed in ASCs isolated from the deep neck depot, and in particular LTBP1 [13]. LTBPs constitute a family of extracellular matrix (ECM) proteins that are important regulators of transforming growth factor beta (TGFβ) bioavailability and ECM modulation [14]. They form intracellular complexes with the TGFβ pro-peptide (latency-associated peptide, LAP). Upon secretion, this large latent complex (LLC) binds to fibronectin and/or fibrillin in the ECM, where it keeps TGFβ in an inactive state [14] until it is released by proteolytic cleavage, pH changes, or integrin binding [14–16].

Our study as well as recently published data [13,17] demonstrate that members of the LTBP family are differentially expressed between cells of white and brown adipose origin. We thus hypothesized that LTBPs released from cells within adipose tissue in a TGFβ-dependent or -independent manner steer adipogenesis towards a brown adipocyte phenotype. To this end, we generated LTBP- as well as TGFβ-specific knockouts in a cell model of human adipogenesis using CRISPR/Cas9. We found that knockout of LTBP2 and LTBP3 diminishes expression and function of UCP1, which is mediated by TGFβ2 signaling.

2. MATERIALS AND METHODS

2.1. Human primary adipose tissue samples

All procedures were performed according to the Declaration of Helsinki guidelines and authorized by the ethics committee of Ulm University. Written informed consent was obtained from all patients in advance. Expression of latent TGFβ-binding proteins was analyzed in cells derived from subcutaneous and deep neck adipose tissue biopsies taken from 6 patients undergoing neck surgery. Isolation and *in vitro* differentiation of progenitor cells were performed as described previously [13]. To evaluate expression of LTBPs, TGFβ, and UCP1 in another cohort of WAT, we used adipose tissue from 28 patients undergoing elective mamma surgery. Primary ASCs were isolated from subcutaneous adipose tissue of 3 children undergoing hernia correction using collagenase digestion as described before [18].

2.2. Cell culture

Simpson-Golabi-Behmel syndrome (SGBS) cells and primary ASCs were cultured and differentiated into adipocytes using an established protocol [19]. Briefly, cells were seeded into cell culture vessels in DMEM:F12 containing 33 μM biotin, 17 μM pantothenate, and 10% FCS. Adipogenic differentiation was induced for 14 days in serum-free DMEM:F12 supplemented with 10 μg/mL apo-transferrin, 20 nM insulin, 200 pM T3, and 100 nM cortisol. For the first 4 days, 2 μM rosiglitazone, 250 μM isobutyl methylxanthine (IBMX), and 25 nM dexamethasone were added. Where indicated, recombinant TGFβ2 (Cell Signaling Technologies, #8406) or SB431442 (biotechne, #1614) was included in the differentiation medium. Human multipotent adipose-derived stem cells (hMADS) were cultured and differentiated into UCP1-negative or -positive adipocytes as described before [20]. Briefly, cells were grown in DMEM:F12 containing 33 μM biotin, 17 μM pantothenate, 10% FCS, and 2.5 ng/mL fibroblast growth factor 2 (FGF2). Differentiation was induced on the second day post–confluence (designated as day 0) in DMEM/Ham's F12 media supplemented with 10 μg/mL apo-transferrin, 10 nM insulin, 0.2 nM T3, 1 μM dexamethasone, and 500 μM IBMX. After two days, the medium was refreshed with IBMX, dexamethasone was removed, and 100 nM rosiglitazone was added. At day 9, rosiglitazone was withdrawn to enable white adipocyte differentiation. To promote white-to-brite adipocyte conversion, 100 nM rosiglitazone was added on day 14. Differentiated cells were used at day 18. Adipogenic differentiation was quantified by counting differentiated and undifferentiated cells using a net micrometer.

2.3. Generation of LTBP- and TGFβ-deficient preadipocytes

Knockout SGBS and hMADS preadipocytes were generated using a CRISPR/Cas9 system. sgRNA duplexes for *LTBP1*, *LTBP2*, *LTBP3*, *LTBP4*, *TGFB1*, *TGFB2*, and a non-targeting control (sequences are given in Table S1) were designed [21] and inserted into the pMuLE ENTR U6 stuffer sgRNA scaffold L1-R5 plasmid (Multiple Lentiviral Expression Kit, Addgene #1000000060, kindly provided by Ian Frew) [22]. Sanger sequencing was employed to verify correct insertion. Using LR Clonase II Plus (ThermoFisher Scientific), the specific pMuLE ENTR U6-sgRNA and pMuLE ENTR CMV-hCas9 L5-L2 plasmids were recombined with SleepingBeauty transposon plasmid pMuSE eGFP-P2A-PuroR DEST, which had been generated earlier by our laboratory [23]. SGBS preadipocytes and hMADS cells were co-transfected with the resulting pMuSE U6-sgRNA+CMV-hCas9+RPBSA-eGFP-P2A-PuroR plasmids and the SleepingBeauty-expressing pCMV(CAT) T7-SB100 plasmid (Addgene #34879, kindly provided by Zsuzsanna Izsvak) at a mass ratio of 19:1 using a Neon Transfection System (Thermo Fisher Scientific) with 3 × 10 ms pulses of 1400 V. Stable bulk cultures were obtained by puromycin selection at 5 μg/mL.

2.4. siRNA-mediated knockdown of SMAD4

SGBS preadipocytes were transfected either with a non-targeting control siRNA pool (NTC, 20 nM) or a siRNA pool targeting SMAD4 (20 nM) (all Dharmacon) using Lipofectamine2000 (Thermo Fisher Scientific) 48 h before the induction of adipogenic differentiation. Sequences of siRNAs are given in Table S2.

2.5. Expression analysis

mRNA was isolated with the Direct-zol RNA kit (Zymo Research, Irvine, USA) and up to 1 μg of total RNA was transcribed into cDNA using SuperScript II Reverse Transcriptase (Thermo Fisher Scientific). Relative expression of target genes was analyzed by quantitative real-time PCR using the ssoAdvanced Universal SYBR Green Supermix on a CFX Real Time PCR Detection System (BioRad) using specific primers (sequences are given in Table S3). Expression values were calculated using the ddCt method with hypoxanthine-guanine phosphoribosyl transferase (*HPRT*) as a reference gene.

Cellular protein was extracted and protein expression analyzed by Western blot analysis as described before [23]. The following antibodies were used: mouse anti-UCP1 (R&D Systems, MAB6158), rabbit anti-PPARγ (Cell Signaling Technologies, #2443), rabbit anti-TGFβ1 (abcam, ab179696), mouse anti-TGFβ2 (abcam, ab36495), mouse anti-OXPHOS antibody cocktail (abcam, ab110411), hFAB Rhodamine anti-GAPDH (BioRad, #12004168), and hFAB Rhodamine anti-α-tubulin (BioRad, #12004165).

2.6. Citrate synthase activity assay

Citric acid synthase activity was assayed as a measure of mitochondrial content and activity as described before [24]. In brief, 5 μg total protein was added to the reaction buffer (100 mM Tris HCl pH 8.1, 100 μM 5,5' dithiobis-2-nitrobenzoic acid (DTNB), 300 μM acetyl-CoA,

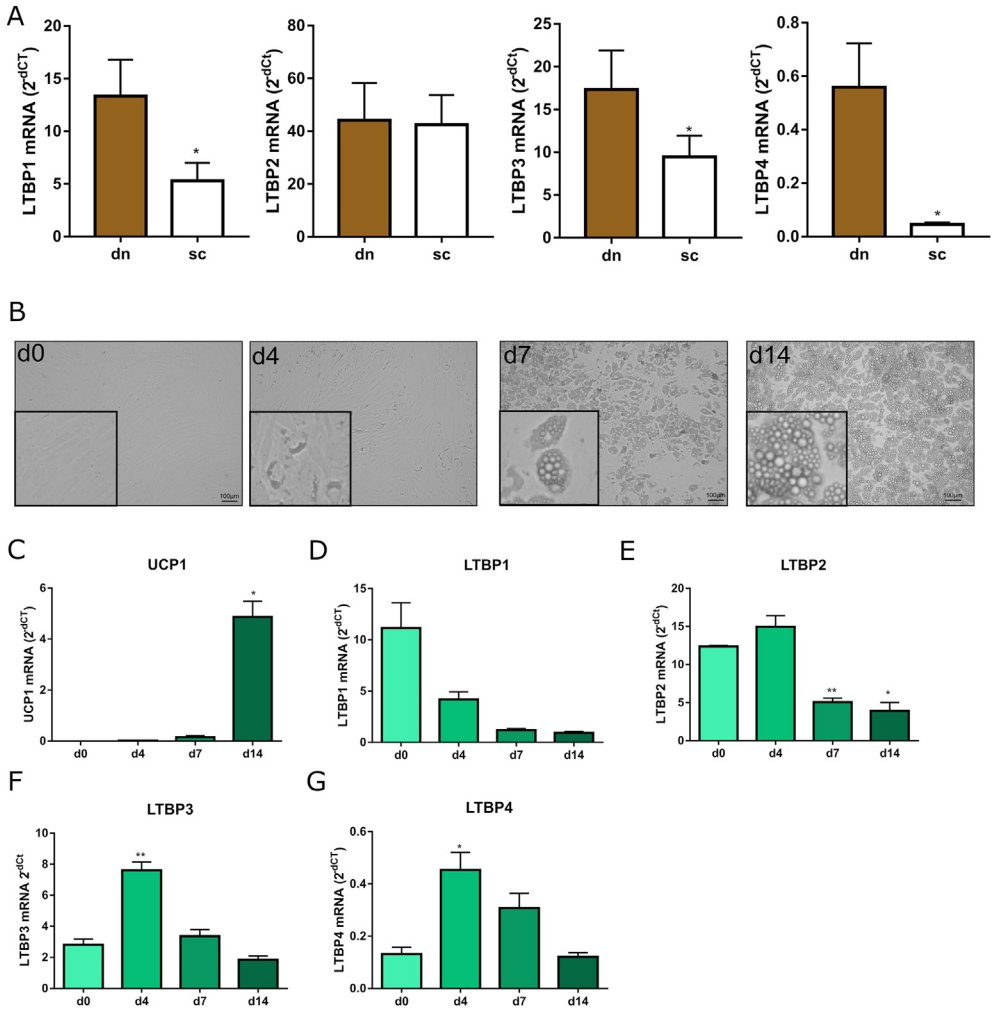

Figure 1: Expression of LTBP family members in isolated ASCs from human subcutaneous and deep neck adipose tissue and in SGBS cells during adipogenesis. (A) mRNA expression of all 4 members of the LTBP family was determined in human ASCs derived from deep neck (dn) and subcutaneous (sc) adipose tissue. *HPRT* was used as a reference gene. Mean +SEM of 6 paired patient samples are shown. *$p < 0.05$, paired t-test. **(B–G)** SGBS preadipocytes (d0) were subjected to adipogenic differentiation for 14 days. **(B)** Microphotographs of SGBS cells at different timepoints during adipogenesis (bar = 100 μm, inlets 6-fold enlarged). **(C–G)** mRNA expression of **(C)** *UCP1*, **(D)** *LTBP1*, **(E)** *LTBP2*, **(F)** *LTBP3*, and **(G)** *LTBP4* during adipogenic differentiation. *HPRT* was used as a reference gene. Mean +SEM of 3 independent experiments are shown; *$p < 0.05$; **$p < 0.01$; two-way ANOVA vs. d0 followed by Tukey test.

0.1% Triton X 100) and specific activity was determined by measuring conversion of DNTB into 2-nitro-5-benzoic acid (TNB) at a wavelength of 405 nm after adding 500 μM oxaloacetate as substrate.

2.7. Extracellular flux analysis

Cells were plated in 96-well cell culture microplates (XFe96, Agilent Technologies) and differentiated for 14 days into adipocytes. One day before measurement, the culture medium was changed to insulin-free medium. On the day of measurement, the cells were incubated for 1 h in bicarbonate-free DMEM containing 5 mM HEPES, 10 mM glucose, 1 mM pyruvate, 2 mM GlutaMAX, and 1% bovine serum albumin. Oxygen consumption and extracellular acidification rates (OCRs and ECARs) were measured simultaneously using a Seahorse XFe96 Flux Analyzer (Agilent Technologies). To mimic thermogenic activation, the cells were treated with 500 μM dibutyryl cyclic adenosine monophosphate (cAMP). Uncoupled (proton leak) respiration was profiled by injecting 2 μM oligomycin (an ATP synthase inhibitor) and full substrate oxidation capacity was determined by injecting 4 μM carbonylcyanide-p-trifluoromethoxyphenylhydrazone (FCCP, a chemical uncoupler). Non-mitochondrial respiration was determined by injecting 1.5 μM antimycin A and 1.5 μM rotenone (ETC inhibitors). Data were normalized to lipid content by NileRed or cell number by JanusGreen staining.

2.8. ELISA

TGFβ1, TGFβ2, and TGFβ3 concentrations in cell culture supernatants were determined with DuoSET ELISA kits (R&D Systems, #DY007, #DY008, #DY240, #DY302, and #DY243). Samples were heated for 5 min at 80 °C to activate TGFβ.

2.9. Statistics

If not stated otherwise, data from at least 3 independent experiments are expressed as mean +standard error of the mean (SEM). For statistical comparison, analysis of variance (ANOVA) or t-tests were used as indicated. A $p < 0.05$ was considered statistically significant. GraphPad Prism version 7.01 (GraphPad Software) was used for all analyses.

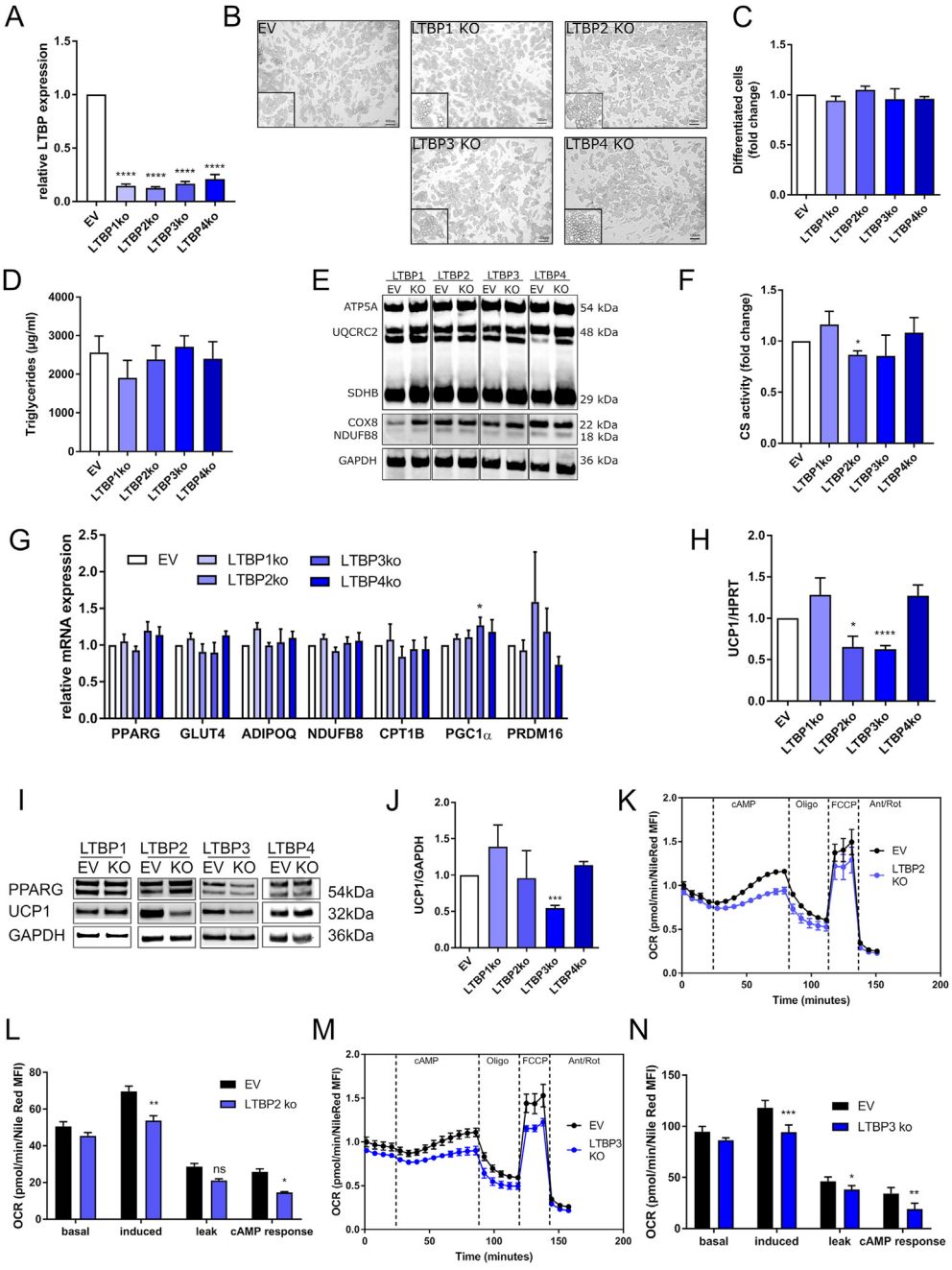

Figure 2: LTBP2 and LTBP3 deficiency decrease adipocyte UCP1 expression and curb response to cAMP. (A) *LTBP* mRNA expression in LTBP-deficient and corresponding empty vector control (EV) cells. HPRT was used as a reference gene. **(B–N)** Cells were subjected to adipogenic differentiation for 14 days. **(B)** Microphotographs of differentiated cells (bar = 100 µm, inlets 2.5-fold enlarged). **(C)** Differentiation rate was determined by counting the number of undifferentiated and differentiated cells using a net micrometer. Fold change compared to EV is shown. **(D)** Triglyceride content of differentiated cells. **(E)** Representative Western Blot of OXPHOS proteins. **(F)** Determination of citrate synthase activity. **(G)** mRNA expression of key adipogenic and mitochondrial marker genes and **(H)** *UCP1*. *HPRT* was used as a reference gene. Fold change compared to EV control is shown. **(I)** Representative Western blot and **(J)** densitometric analysis of PPARγ and UCP1 protein expression. GAPDH was used as a loading control. Fold change to EV is shown. **(K–N)** Oxygen consumption rates (OCRs) of **(K)** LTBP2- and **(M)** LTBP3-deficient (blue color) and control adipocytes (EV, black color) in media containing 1% BSA. To induce UCP1 activity, cells were stimulated with 500 µM dibutyryl-cAMP (cAMP). **(L and N)** From the OCR data, basal, proton leak, and cAMP-induced values as well as response to cAMP were determined. Data were normalized to cellular lipid content by Nile Red staining (MFI mean fluorescence intensity). Mean +SEM of 3–5 independent experiments are shown; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.001$, Student's t-test vs. EV control.

3. RESULTS

3.1. LTBP1, LTBP3, and LTBP4 are highly expressed in ASCs from deep neck adipose tissue

In a previous study, we compared the gene expression profiles of adipose-derived stromal cells (ASCs) isolated from subcutaneous and deep neck adipose tissue [13]. Within the list of differentially expressed genes, we focused on LTBP1, which was more highly expressed in ASCs isolated from the deep neck depot (2.05-fold). Interestingly, members of the LTBP family were also proposed to constitute brown-enriched factors in human ASCs in another study [17]. Assuming that LTBP family members may regulate different aspects of adipose tissue function, we analyzed the expression of all known LTBP family members in the patient ASCs used for the original transcriptome analyses [13]. Aside from LTBP2, which displayed no differential expression between deep neck and subcutaneous ASCs, the expression of all other LTBPs was significantly higher in ASCs derived from deep neck adipose tissue (LTBP1: 2.5-fold, LTBP3: 1.8-fold, and LTBP4: 14-fold; Figure 1A).

3.2. Expression of LTBPs is regulated during adipogenic differentiation

To study the role of LTBPs in adipogenesis *in vitro*, we used cells with Simpson-Golabi-Behmel syndrome (SGBS), an established human preadipocyte model that was derived from subcutaneous white adipose tissue [25] and is exceptionally well-suited for investigating the process of adipocyte browning [19,24,26,27]. Upon induction using a chemically-defined hormonal cocktail, SGBS cells differentiate efficiently into lipid-laden adipocytes (Figure 1B). As described earlier, mature SGBS adipocytes express basal levels of UCP1 (Figure 1C). In line with the data from primary samples and consistent with the white adipose origin of SGBS cells, expression levels of all LTBPs before induction of adipogenesis were comparable to those observed in primary cells (Figure 1D–G). Upon adipogenic differentiation, *LTBP1* and *LTBP2* mRNA levels decreased, reaching their minimum on day 14 (92% and 68% reduction, respectively; Figure 1D,E), whereas *LTBP3* and *LTBP4* mRNA levels increased transiently until day 4 and then returned to near-basal levels by day 14 (Figure 1F,G). These data suggest that LTBP isoforms respond differently to adipogenic inducers during early stages of differentiation and might point to distinct functions of specific LTBP isoforms in human preadipocytes during adipogenesis.

3.3. CRISPR/Cas9-mediated knockouts of LTBP2 and LTBP3 reduce UCP1 expression and function

To study the impact of LTBPs on adipogenic differentiation and elucidate whether they are involved in adipocyte browning, we generated LTBP-specific SGBS-knockout preadipocytes using a CRISPR/Cas9 system. Successful targeting of the LTBP coding regions was confirmed by cleavage assays (Fig. S1). The CRISPR/Cas9 strategy resulted in an over 80% reduction of *LTBP* mRNA expression in bulk cultures (Figure 2A). Upon induction of adipogenesis, more than 80% of the cells of all lines differentiated into mature, lipid-laden adipocytes (Figure 2B). Of note, the rate of adipogenesis was comparable between control and LTBP-deficient cells, as judged by microscopic determination of the differentiation rate, triglyceride content, and mRNA expression of key adipogenic marker genes *PPARG*, *GLUT4*, and *ADIPOQ* (Figure 2C,D,G). These data clearly demonstrate that a loss of LTBP function does not interfere with the process of adipogenic differentiation. We next addressed whether LTBP deficiency leads to alterations in mitochondrial content, as mitochondrial abundance is a common feature of brown compared to white adipocytes [28]. As judged by the expression of mitochondrial marker genes *NDUFB8* and *CPT1B*, mitochondrial protein expression as well as citrate synthase activity, mitochondrial density was not affected by LTBP deficiency (Figure 2E–G).

Next, we studied the expression of the key brown adipocyte marker UCP1 in control and LTBP-deficient adipocytes. We expected that if LTBPs promote a brown adipocyte differentiation program, the absence of LTBPs would lead to a reduction of UCP1 expression in adipocytes. Interestingly, we found that *UCP1* mRNA levels were reduced by 35% and 38% in LTBP2- and LTBP3-deficient adipocytes, respectively (Figure 2H), whereas LTBP1 and LTBP4 deficiency did not affect *UCP1* mRNA expression. On the protein level, UCP1 was significantly reduced in LTBP3-deficient cells only (Figure 2I–J)

To address whether alterations in UCP1 expression are also reflected on a functional level, we assessed the mitochondrial respiration of LTBP2- and LTBP3-deficient adipocytes using a plate-based respirometer (Figure 2K-N). To trigger free fatty acid release and stimulate UCP1 activity, cells were treated with dibutyryl-cAMP [23]. This treatment resulted in a robust increase in respiration in control cells (Figure 2K-N). While basal respiration in LTBP2- and LTBP3-deficient cells did not differ from control cells, cAMP-induced respiration was significantly reduced, reaching only 77% and 83% of control cell activity, respectively (Figure 2K-N). In LTBP4-deficient cells, however, we did not observe any decrease in lipolysis-driven respiration, while in LTBP1-deficient cells, respiration increased (Fig. S2).

To validate this finding in another independent cell model, we performed CRISPR/Cas9 knockouts of LTBP2 and LTBP3 in hMADS cells (Fig. S3A–B). These cells are frequently used as a human cell model for adipocyte browning and can be differentiated into UCP1-negative ("white") and -positive ("brite") cells using distinct differentiation conditions [20,29]. As expected, differentiation using the "brite" adipogenic protocol induced UCP1 mRNA and protein expression, whereas for differentiation using the white adipogenic protocol, UCP1 was barely detectable (Fig. S3C–F). In line with the data from SGBS cells, knockout of LTBP2 and LTBP3 resulted in a decrease in UCP1 mRNA levels by 67% and 54%, respectively (Fig. S3C–F).

Taken together, these results demonstrate that LTBP2 and LTBP3 deficiency in human preadipocytes directs their differentiation towards an adipocyte phenotype with reduced expression and function of UCP1.

3.4. TGFβ2 signaling promotes brown-like adipogenesis in human preadipocytes

As LTBPs are crucial for TGFβ bioavailability [14,30], we sought to investigate whether blocking the TFGβ signaling cascade during adipogenesis affects UCP1 expression in mature adipocytes. Inhibition of TGFβ signaling during adipogenic differentiation using the TGFβ receptor 1 inhibitor SB431542 led to a robust reduction of UCP1 mRNA expression in adipocytes derived from SGBS cells and hMADS cells (Fig. S3G–I) as well as primary human ASCs (Fig. S4) We next aimed to target the TGFβ signaling pathway further downstream. SMAD proteins are core components of the TGFβ signaling cascade, regulating the expression of central TGFβ target genes [31]. We thus used siRNAs to knock down *SMAD4* in SGBS preadipocytes prior to the induction of adipogenesis, which also resulted in a significant decrease in UCP1 expression and function in mature adipocytes (Fig. S5).

To further delineate which isoform of TGFβ might be involved in the regulation of adipocyte UCP1 expression, we assessed the production of different TGFβ isoforms in LTBP-deficient SGBS preadipocytes and adipocytes.

All TGFβ isoforms as well as TGFβ receptors were expressed in all SGBS-derived cell lines, both in the undifferentiated and differentiated

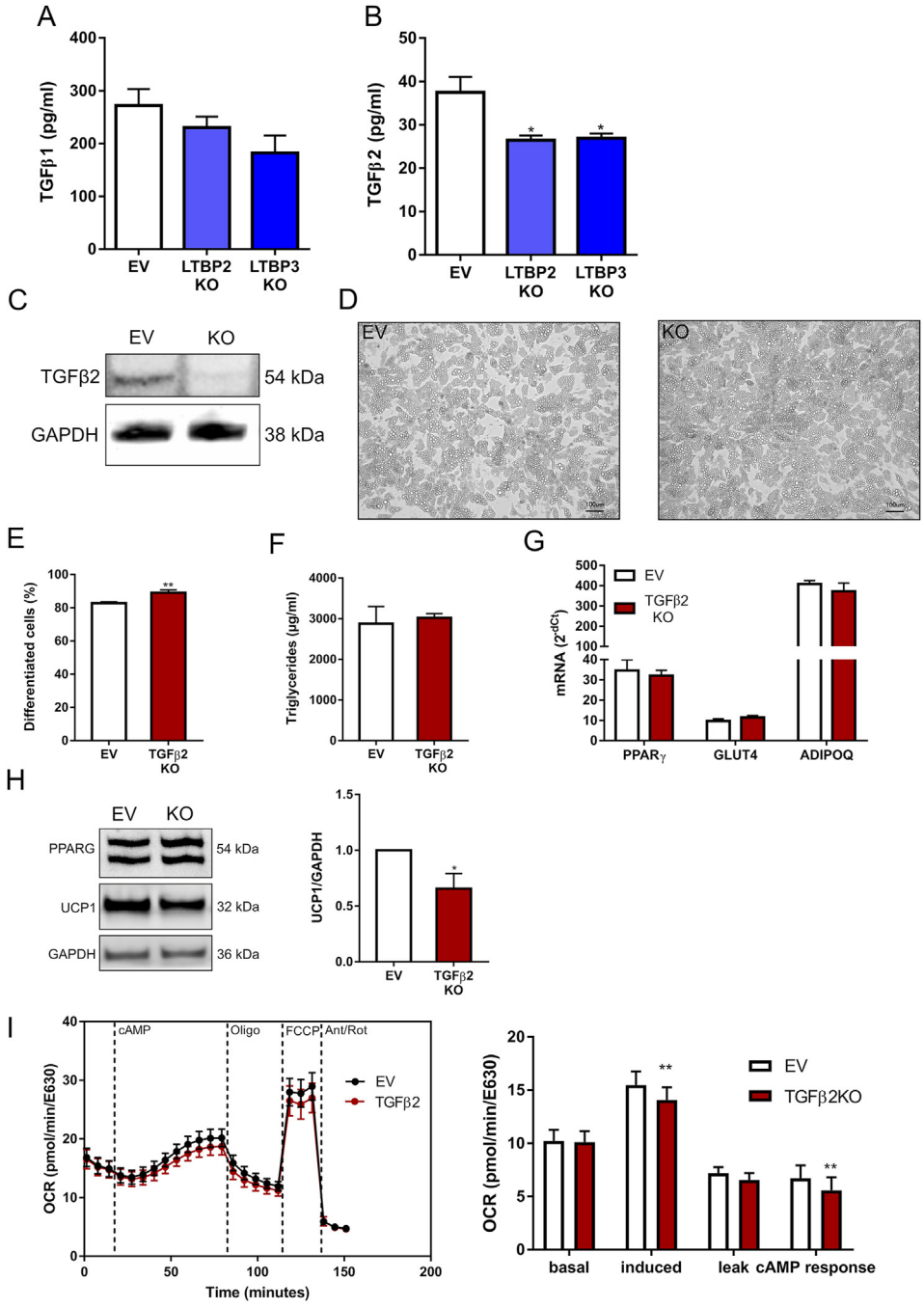

Figure 3: TGFβ2 deficiency decreases adipocyte UCP1 expression and curbs response to cAMP. (A and B) TGFβ1 and TGFβ2 concentrations were determined in supernatants of LTBP-deficient and empty vector control (EV) cells using an enzyme-linked immunosorbent assay. TGFβ3 concentrations were below the detection limit. Mean +SEM of 3 independent experiments are shown; *p < 0.05, Student's t-test vs. EV control. **(C–I)** TGFβ2-deficient SGBS cells were generated using a CRISPR/Cas9 system. **(C)** Western blot of TGFβ2 protein expression. GAPDH was used as loading control. Representative of 4 independent experiments. **(D–I)** Cells were subjected to adipogenic differentiation for 14 days. **(D)** Microphotographs of differentiated cells (bar = 100 µm). **(E)** Differentiation rate was determined by counting the number of undifferentiated and differentiated cells using a net micrometer. **(F)** Triglyceride content and **(G)** mRNA expression of key adipogenic marker genes. *HPRT* was used as a reference gene. **(H)** Representative Western blot and densitometry of UCP1 protein expression. GAPDH was used as a loading control. Fold change to EV is shown. **(I)** Oxygen consumption rates (OCRs) of TGFβ2-deficient (red color) and control adipocytes (EV, black color) in media containing 1% BSA. To induce UCP1 activity, cells were stimulated with 500 µM dibutyryl-cAMP (cAMP). From the OCR data, basal and cAMP-induced values as well as responses to cAMP were determined. Data were normalized to cell number by Janus Green staining. Mean +SEM of 4 independent experiments are shown; *p < 0.05, **p < 0.01, Student's t-test vs. EV control.

state (Fig. S6). In supernatants of preadipocytes, we could detect TGFβ1 and TGFβ2, while TGFβ3 was below the detection limit of the assay (Figure 3). Interestingly, TGFβ2 was reduced in the supernatants of both LTBP2- and LTBP3-deficient cells (Figure 3B). There was also a trend towards reduction of TGFβ1, but this did not reach statistical significance (Figure 3A). To elucidate whether an absence of TGFβ evokes effects comparable to the knockout of LTBP2 and LTBP3, we generated TGFβ1-and TGFβ2-deficient SGBS preadipocytes. Successful TGFβ

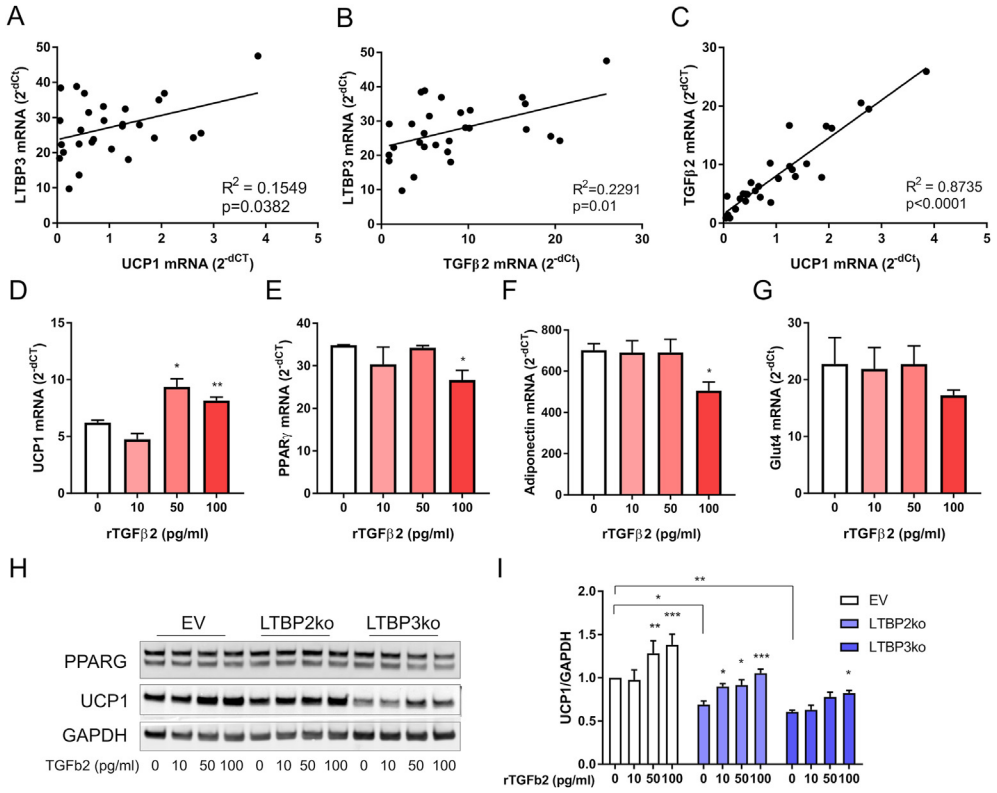

Figure 4: LTBP3 and TGFβ2 expression correlate positively with UCP1 expression in human subcutaneous adipose tissue samples, and TGFβ2 induces UCP1 expression in SGBS adipocytes. (A–C) mRNA expression of *LTBP3*, *TGFβ2*, and *UCP1* in human subcutaneous adipose tissue samples derived from mamma tissues of 28 patients undergoing plastic surgery. Correlation of mRNA expression between **(A)** *LTBP3* and *UCP1*, **(B)** *LTBP3* and *TGFβ2*, and **(C)** *TGFB2* and *UCP1*. Mean age ±SD: 45 ± 15 years and mean BMI ±SD: 28.2 ± 4.9 kg/m^2 $p < 0.05$ was considered statistically significant. **(D–G)** SGBS preadipocytes were subjected to 14-day adipogenic differentiation, which included treatment with recombinant TGFβ2 (rTGFβ2) from day 0 to day 4. **(D)** UCP1, **(E)** PPARG, **(F)** ADIPOQ, and **(G)** GLUT4 mRNA expression. Mean +SEM of 3 independent experiments are shown; *$p < 0.05$, **$p < 0.01$, Student's t-test. **(H and I)** Control (EV) and LTBP2- or LTBP3-deficient cells were subjected to adipogenic differentiation in the presence of different concentrations of recombinant TGFβ2 (rTGFβ2). Protein expression of PPARG and UCP1 was determined by Western Blot. Alpha-Tubulin (TUB) was used as loading control. One representative blot of three is shown. **(I)** Densitometric analysis, means +SEM of three experiments are shown, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (ANOVA).

knockout was confirmed by Western blot (Figure 3C, Fig. S7). TGFβ2 deficiency resulted in a slight increase in adipogenic differentiation (Figure 3D–E); however, this was neither reflected in changes of triglyceride levels nor in adipogenic marker gene expression (Figure 3F–G). Of note, in accordance with our observations in LTBP2- and LTBP3-deficient cells, the absence of TGFβ2 led to a 35% reduction of adipocyte UCP1 protein expression (Figure 3H). This was paralleled by a 10% reduction of cAMP-stimulated respiration (Figure 3I), indicating that TGFβ2 deficiency impairs UCP1 expression and function. In contrast, knocking out TGFβ1 did not interfere with either UCP1 expression or function in this model system (Suppl. Fig. S7).

We next studied the relationship between LTBP and TGFβ mRNA expression in human primary adipose tissue samples from 28 patients undergoing plastic surgery. In line with our hypothesis that the LTBP–TGFβ2 axis affects human adipocyte browning, linear regression analysis of the mRNA expression of *LTBP3*, *TGFB2*, and *UCP1* revealed significant positive correlations between *LTBP3* and *TGFB2*, *LTBP3* and *UCP1*, and *TGFB2* and *UCP1* in this cohort (Figure 4A–C).

The detrimental effect of the TGFβ2 signaling blockade on UCP1 expression, as well as the strong correlation between *TGFB2* and *UCP1* expression in adipose tissue, indicates that TGFβ2 is a key regulator of adipocyte browning. We thus investigated whether treatment with recombinant TGFβ2 during adipogenic differentiation impacts the expression of UCP1. Indeed, addition of 50 pg/mL recombinant TGFβ2 during the first 4 days of adipogenic induction significantly induced UCP1 expression in SGBS (Figure 4D), and in hMADS cells, it differentiated in the presence of rosiglitazone (Figs. S7J–K). Treatment with a higher dose (100 pg/mL) induced UCP1 expression even further, but was paralleled by decreased expression of adipogenic marker genes *PPARG*, *GLUT4*, and *ADIPOQ* (Figure 4E–G). Furthermore, treatment with recombinant TGFβ2 during adipogenesis was able to rescue the negative effects of LTBP2 and LTBP3 deficiency on UCP1 protein expression (Figure 4H–I).

Taken together, we delineate an LTBP3–TGFβ2 axis that exerts control over the induction of UCP1 expression during human adipocyte formation.

4. DISCUSSION

Brown adipose tissue activity in humans is associated with increased energy expenditure and improved metabolic health [32–34]. Inducing thermogenesis has thus frequently been discussed as a promising approach for treating obesity and associated metabolic disorders [12,35–37]. Browning white adipose tissue has been extensively studied in animal models [7,8,10], but the exact mechanisms of this process in humans have not yet been resolved in detail. The identification of new factors that are involved in human WAT browning may thus also reveal therapeutic targets to combat obesity.

To elucidate the cellular origins of white and brown adipocytes, we previously compared the gene expression patterns of human ASCs derived from subcutaneous and deep neck adipose tissue, where brown adipocytes are usually found. Among the differentially expressed genes, we discovered members of the LTBP family whose expression was elevated in cells derived from the deep neck depot, suggesting a potential role of these LTBPs in promoting brown adipogenesis. Interestingly, members of the LTBP family were also found differentially regulated in another study comparing progenitor cells originating from white and brown adipose tissue [17]. Here, we establish a causal relationship between the presence of LTBPs in preadipocytes and the expression and function of UCP1 in adipocytes arising from them.

The influence of LTBPs on TGFβ bioavailability and secretion has been intensively studied in animal models as well as clinical settings [30,38–44]. In mice, inhibition of LTBP–TGFβ binding by mutation of the respective binding site in TGFβ leads to a phenotype similar to that of a TGFβ knockout [30]. Deletion of different exons in the LTBP1 gene in mice results in cardiac defects and embryonic lethality due to decreased TGFB levels [38,39]. Furthermore, LTBP3-knockout mice are characterized by osteopetrosis and premature ossification of the skull, indicating reduced TGFβ signaling [40,41]. Similarly, silencing of LTBP3 in zebrafish causes cardiovascular defects, which can be rescued by expression of a constitutively active form of the TGFβ receptor [42]. In human subjects with LTBP4 deficiency, a reduced deposition of TGFβ occurs, resulting in decreased phospho-SMAD levels in different tissues [43]. Interaction studies revealed that LTBP1 and LTBP3 are binding partners of all TGFβ isoforms, whereas LTBP4 only binds TGFβ1. In contrast, LTBP2 is not able to bind any TGFβ isoform and probably fulfills functions in the ECM other than modulation of TGFβ signaling [45]. In line with this, deleting LTBP2 in mice has only minor effects [44].

We show here that LTBP2 and LTBP3 deficiency reduces UCP1 expression in SGBS and hMADS adipocytes, paralleled by a reduced oxidative response when lipolysis is induced. The role of LTBPs in the context of WAT browning has not been addressed so far. LTBPs are highly expressed in cells of mesenchymal origin [46]. In line with this, we observed a high expression of LTBPs in SGBS preadipocytes, with levels decreasing throughout adipogenic differentiation. Interestingly, LTBP3 was shown to be significantly more highly expressed in ASCs isolated from BAT compared to those from WAT in a study that utilized RNA sequencing to compare the molecular signatures of these cells [17], further highlighting the potential role of LTBP3 in regulating brown and possibly beige adipogenesis.

LTBP3 has been shown to regulate TGFβ bioavailability [47]. We indeed observed lower levels of TGFβ2 in the supernatants of LTBP3-deficient SGBS preadipocytes, whereas TGFβ1 was not altered. Unexpectedly, we also observed lower TGFβ2 levels in the supernatants of LTBP2-deficient cells. It has been demonstrated that LTBP2 is not able to bind any TGFβ isoform [45]. We have not yet addressed whether this is due to other mechanisms, such as changes in the expression of other LTBP members that compensate for the loss of LTBP2. Upon further investigation, we revealed that the effects of LTBP2 and LTBP3 deficiency on adipocyte UCP1 expression depend on this lowering of TGFβ2 levels.

Animal models of TGFβ loss are either perinatally lethal or cause severe developmental disorders, which hampers a detailed characterization of their function *in vivo* [48]. While information on the role of LTBPs in adipocyte biology is scarce, the TGFβ pathway has been shown to play a fundamental role in adipogenesis and adipocyte metabolism. Clinical studies demonstrate a link between high TGFβ1 serum levels and high body mass index (BMI) [49], hypertension, and cardiovascular disease [50]. Moreover, TGFβ levels in adipose tissue are associated with BMI in morbid obesity [51]. The TGFβ pathway has furthermore been shown to influence white adipocyte browning both *in vitro* and *in vivo* [49,52,53], partially contradicting our findings. For instance, a knockdown of SMAD3 in murine 3T3-L1 adipocytes increases UCP1 expression [49], and its knockout in mice promotes WAT browning [49]. *In vitro* studies using SB431542 during adipogenic differentiation demonstrated an increase in UCP1 expression in adipocytes derived from murine ASCs and human-induced pluripotent stem cells (iPSCs) [53,54]. However, it was also shown that SB431542 treatment leads to a higher number of differentiated cells, which in itself may explain the higher UCP1 expression observed in this study [54]. In our hands, SB431542 treatment of SGBS cells and human ASCs resulted in significantly lower UCP1 levels compared to the vehicle controls. This is in line with a study by Takeda et al. describing a similar detrimental effect of SB431542 treatment on UCP1 expression [55]. They stimulated dermal fibroblasts with an "optimized chemical cocktail" containing *inter alia* SB431542 to differentiate these cells into chemically-induced brown adipocytes (ciBAs). These mature ciBAs expressed basal levels of UCP1. However, when SB431542 was omitted from the differentiation cocktail, UCP1 expression was significantly higher (5-fold). Strikingly, this effect was only seen in the absence of serum in the differentiation cocktail, suggesting that factors of the circulation further modulate the effect of TGFβ isoforms on adipocyte browning. Since SGBS cells are differentiated under serum-free conditions, any involvement of serum factors can be excluded. Strengthening our data on the TGFβ signaling blockade, an siRNA-mediated *SMAD4* knockdown in SGBS preadipocytes also reduced the expression of UCP1 by over 90% for mRNA and over 70% for protein level, with respective functional consequences. Although we are aware that SMAD4 is a central integrator for all TGFβ superfamily members, including bone morphogenetic proteins (BMPs), and we cannot exclude involvement of BMP signaling, our results further indicate that loss of TGFβ signaling in particular inhibits adipocyte browning.

Among the different TGFβ isoforms, TGFβ2 seems to play a distinct role, as the phenotype of TGFβ2-knockout mice displays no similarities to that of either TGFβ1-or TGFβ3-knockout mice [56]. Importantly, TGFβ2 requires the additional coreceptor TGFβR2/betaglycan to establish full binding to the TGFβR1/2 heterodimer [31]. This indicates that the intracellular signaling of TGFβ2 might be distinct from that of TGFβ1. TGFβ2 has recently also been described as an exercise-induced adipokine and mediator of the beneficial effects of physical exercise on glucose metabolism in both mice and humans [57]. Mice challenged with high fat diet (HFD) and infused with recombinant TGFβ2 by an osmotic pump exhibited metabolic improvements, including a better glucose tolerance and insulin sensitivity compared to controls. TGFβ2 has moreover been shown to induce UCP1 expression in murine brown adipocytes *in vitro* [57] as well as in murine BAT *in vivo*.

Here, we generated TGFβ2-deficient SGBS cells. When we subjected these cells to adipogenesis, we observed similar changes to those stemming from LTBP2 and LTBP3 deficiency—specifically, a significant reduction in UCP1 expression and function. TGFβ1 deficiency, in contrast, had no effect on either aspect. In turn, TGFβ2 treatment elevated UCP1 expression in SGBS adipocytes. Thus our data further support the notion that TGFβ2, but not other TGFβ isoforms, exerts beneficial metabolic effects.

Our study remains limited in some respects, however. As most of the data were obtained in a one-dimensional *in vitro* system, the study

lacks tissue and *in vivo* context. Indeed, recent mouse studies demonstrate that TGFβ2 has more pleiotropic effects on metabolism than just inducing UCP1 expression in adipocytes [57], adding additional layers of complexity to TGFβ2 action. Interestingly, TGFβ2 also exerts anti-inflammatory properties by regulating macrophage infiltration in mice fed an HFD. This may have further implications for adipocyte metabolism—even more so because macrophages themselves are an important source of TGFβ.

In conclusion, we show that LTBP2 and LTBP3 play important roles in brown and beige adipogenesis by modulating TGFβ2 bioavailability and signaling. Our data suggest that a blockade of the TGFβ signaling pathway suppresses a brown phenotype, while treatment with recombinant TGFβ2 fosters it. Importantly, we demonstrate that this pathway may also play a role in human WAT browning. The LTBP3—TGFβ2 axis should also be further investigated as a potential therapeutic target to promote WAT browning and raise overall energy expenditure in humans.

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CONFLICT OF INTEREST

None declared.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molmet.2021.101336.

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