Brief Communication

Beta-hydroxybutyrate dampens adipose progenitors’ profibrotic activation through canonical Tgfβ signaling and non-canonical ZFP36-dependent mechanisms

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ABSTRACT

Background/Purpose: Adipose tissue contains progenitor cells that contribute to beneficial tissue expansion when needed by de novo adipocyte formation (classical white or beige fat cells with thermogenic potential). However, in chronic obesity, they can exhibit an activated pro-fibrotic, extracellular matrix (ECM)-depositing phenotype that highly aggravates obesity-related adipose tissue dysfunction.

Methods: Given that progenitors’ fibrotic activation and fat cell browning appear to be antagonistic cell fates, we have examined the anti-fibrotic potential of pro-browning agents in an obesogenic condition.

Results: In obese mice fed a high fat diet, thermoneutral housing, which induces brown fat cell dormancy, increases the expression of ECM gene programs compared to conventionally raised animals, indicating aggravation of obesity-related tissue fibrosis at thermoneutrality. In a model of primary cultured murine adipose progenitors, we found that exposure to β-hydroxybutyrate selectively reduced Tgfβ-dependent profibrotic responses of ECM genes like Ctgf, Lox2 and Fn1. This effect is observed in both subcutaneous and visceral-derived adipose progenitors, as well as in 3T3-L1 fibroblasts. In 30 patients with obesity eligible for bariatric surgery, those with higher circulating β-hydroxybutyrate levels have lower subcutaneous adipose tissue fibrotic scores. Mechanistically, β-hydroxybutyrate limits Tgfβ-dependent collagen accumulation and reduces Smad2-3 protein expression and phosphorylation in visceral progenitors. Moreover, β-hydroxybutyrate induces the expression of the ZFP36 gene, encoding a post-transcriptional regulator that promotes the degradation of mRNA by binding to AU-rich sites within 3’UTRs. Importantly, complete ZFP36 deficiency in a mouse embryonic fibroblast line from null mice, or siRNA knock-down in primary progenitors, indicate that ZFP36 is required for β-hydroxybutyrate anti-fibrotic effects.

Conclusion: These data unravel the potential of β-hydroxybutyrate to limit adipose tissue matrix deposition, a finding that might exploited in an obesogenic context.

Keywords Adipocyte; Progenitors; Fibrosis; Extracellular matrix

1 INTRODUCTION

Increasing fat mass over time favours progression to metabolic diseases and type-II diabetes. Maladaptive metabolism in obesity relies on chronic low-grade inflammation and subsequent disruption of insulin sensitivity [1]. It is linked to metabolic inflexibility of the expanded fat pads, which acquire features of a stiff, extracellular matrix-embedded fibrotic tissue, unable to sustain efficient lipid sequestration [2]. Adipose tissue (AT) fibrosis has strong deleterious impacts on glucose homeostasis, demonstrated in obese subjects [3,4] and in mouse models [5,6]. Conversely, thermogenic AT (brown or beige) is highly efficient in glucose uptake and lipid utilization [7,8], therefore a beneficial actor in blood glucose control [9,10]. Pathways controlling acquisition of a thermogenic fat cell phenotype are linked to the induction of a specific mitochondrial program dependent on PPARγ-associated transcriptional regulators like PRDM16 and PGC1α [11]. The recruitment of a pool of thermogenic adipocytes to target obesity-associated metabolic impairment has now become a therapeutic strategy under active research.

The source of brown-like and white adipocytes is a bulk of progenitors residing within the AT stroma-vascular fraction. The global adipose progenitor population has different propensities for beige/white adipogenesis, and can also develop into a pro-fibrotic fibroblastic phenotype, underlining a dual fibro/adipogenic potential highly

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Received February 28, 2022 • Revision received May 2, 2022 • Accepted May 3, 2022 • Available online 9 May 2022

https://doi.org/10.1016/j.molmet.2022.101512
dependent on the tissue microenvironment. Although highly heterogeneous and yet incompletely characterized, the pool of AT progenitors (CD31+, CD45-) shares PDGFRα expression as a common cell surface marker [12]. Using this marker for functional progenitor targeting, we observed that fibrogenic responses to high fat diet, and fat browning potential of mouse subcutaneous adipose tissue, were inversely related pathways [6]. In line, another report demonstrated an inverse relationship between mouse fat browning and AT fibrosis, mediated by a PRDM16-dependent signal released from the adipocytes [13]. Thus, progenitors’ fibrotic transformation and brown-like adipogenesis may involve antagonistic cell fates, associated with opposite consequences on whole body metabolic regulation.

A better understanding of how adipose progenitors orient cell choices towards fibrogenesis is essential for the development of new approaches to limit metabolic deterioration in obesity. A switch in the abundance of CD9-expressing visceral progenitors has been reported to promote pro-fibrotic activation [4]. It is also likely that the obese AT pro-inflammatory microenvironment triggers fibrogenesis at the expense of adipogenesis, linked to disruption in Tgfβ (Transforming growth factor) family members balance [14]. Moreover, the high demand for lipid storage in obesity might lead to preadipocytes premature exhaustion and senescence, precluding an adequate supply of new adipocytes [15].

In this study, we have explored the paradigm of pro-fibrotic versus adipogenic development of progenitors in the context of the obese AT. In particular, we demonstrate aggravated fibrosis in obese high fat diet-fed mice in which brown/beige fat dormancy is shaped by raising at thermoneutrality. We also demonstrate the anti-fibrotic properties of the fat browning factor β-hydroxybutyrate in primary Tgfβ-induced cultured progenitors from subcutaneous or visceral tissues. In addition to blunting of Smad/Tgfβ signalling by β-hydroxybutyrate in visceral progenitors, we unravel a novel mechanism linked to ZFP36/Trimetantrapol, a post-transcriptional regulator of mRNA stability, which is induced by β-hydroxybutyrate, and restrains fibrotic activation of subcutaneous progenitors.

2. MATERIALS AND METHODS

2.1. Materials

Collagenase A (R11088793001) was purchased from Roche, DMEM/ Glutamax (61,965-026) from Gibco and foetal calf serum (CVFSVF000-01) from Eurobio Scientific. Sodium 3-β-Hydroxybutyrate (54,965) and L-(+)-lactic acid (L1750) were obtained from Sigma. Tgfβ1 (human recombinant, ref 130-126-724) was from Myltenyi Biotech, Bmp4 (Human recombinant, ref PHC9534) from Invitrogen.

2.2. Patient studies

Human samples used were obtained from patients with obesity (N = 30, age 42.5 ± 14.5, 10 men, 20 women, body mass index 44.0 ± 6.5 kg/m²) involved in the bariatric surgery program at the Nutrition Department of Pitié-Salpêtrière hospital, France. Ten patients (7 women, 3 men) had normal glucose control, while the 20 others were either diabetic or glucose intolerant. Among diabetics, 4 were treated by GLP1 analogs, 2 by insulin, 5 by metformin. Six patients (3 men, 3 women) received statins to treat hyperlipemia. Eleven (7 women, 4 men) were on anti-hypertensive drugs. Blood samples were collected during preoperative hospital examination after an overnight fast. All patients received nutritional information and are encouraged to healthy diets. No specific diet pattern is recommended before surgery. Patients were part of a follow-up cohort with tissue bio banking during the surgery and benefited fibrosis scoring of subcutaneous AT based on Picrosirius Red labelling of paraffin-embedded samples as described previously [16]. Briefly, label detection thresholds were adjusted with an image-analysis module using Calpox software (TRIBUN) and manual delimitation of red-stained AT, avoiding blood vessels and staining artifacts was performed. Label quantification was expressed as the ratio of red—stained to total tissue area. A combined semi-quantitative SCAT fibrosis score (FAT score) was then attributed to each patient according to the following rules: stage 0: no peribiliary staining (thickness less than the diameter of average adipocyte) and no pericellular fibrosis (no accumulation of collagen around adipocytes localized in the depth of the AT lobules). Stage 3: severe paracellular fibrosis (thickness similar to the diameter of two average adipocytes) and severe paracellular fibrosis (thick labeling around adipocytes and presence of within label-trapped adipocytes). Ethical approval was obtained from the Research Ethics Committee of Hôtel-Dieu Hospital (CPP Ile-de-France N°1). Informed written consent was obtained from all subjects and the protocol was registered on http://www.clinicaltrials.gov (NCT01655017, NCT00476658). β-Hydroxybutyrate was measured on serum aliquots (5 μl) stored at −80°C with a colorimetric assay kit (Abnova KA0854).

2.3. Mouse studies

Animal studies were conducted in agreement with ARRIVE guidelines, approved by local ethical committee (n°31,719). 4–6 week old C57Bl6/J mice were fed a high fat diet (Research Diet, D12492) with free access to drinking water. They were housed (maximum of 5 mice per cage) at room temperature (22 °C) or at thermoneutrality (30 °C) in a climate chamber (TSE, PhenoMaster), with matched day–night light cycles and controlled humidity. Body composition was analyzed by nuclear magnetic resonance (Bruker Mouse Minispec, LF90). AT from visceral (gonadal), subcutaneous (inguinal) and interscapular brown fat (BAT) were frozen in liquid nitrogen or incubated with collagenase as described [6].

2.4. Primary cell culture

Six female mice fed on a standard Chow diet were used for each cell preparation. Minced AT were incubated in collagenase dissociation medium for 30 min (visceral AT) or 1 h (Subcutaneous AT) under lateral shaking (100 rpm) at 37 °C. After filtration through a 100 μm cell strainer, medium was adjusted to 30 ml with PBS, cells were pelleted (10 min at 1500rpm), and suspended into 12 ml of DMEM/ 10% fetal calf serum. 0.5 ml was plated into 12-well culture plates in fresh medium. The next day, all cultures were more than 80% confluent, and β-hydroxybutyrate (βOHBA) (or other browning agents) was added 24 h prior to TGFβ was introduced and maintained for additional 3 days. This combinatory treatment was defined to mimic the transition from a healthy pro-browning to a pro-fibrotic AT microenvironment.

2.5. Transfection with siRNA

Cells in DMEM 10% fetal calf serum were incubated with 20 nM siRNA duplexes (SR426376) or control scrambled sequences (SR0003) from Origene, using the lipofectamine RNAiMax transfection reagent (ref 13,778– Invitrogen). Cell treatment with effectors was performed on the next day in fresh medium.

2.6. ZFP36-deficient mouse fibroblasts

Cell line and its matched WT control line were originally derived from ZFP36−/− and +/+ embryos [17], and were cultured in DMEM high
glucose medium containing 1% penicillin/streptomycin and 10% fetal calf serum.

2.7 Gene and protein expression
Total RNA was extracted using RNeasy Mini kit (Qiagen, 74,104), and 500 μg was used for reverse transcription, followed by Quantitative real-time PCR using SyBr Green Master Mix (Applied Biosystems). All primer pairs were validated with cDNA calibration curves and a unique amplification product. For protein analysis, lysates in RIPA buffer (Sigma Aldrich, R0278) containing proteinase inhibitors (Complete Mini, Roche) were prepared (Bertin Technologies, Precellys 24) and cleared for 10 min at 10,000 rpm. Protein concentration was measured (ThermoFisher Scientific, BCA assay). Western blotting was performed as described [18].

2.8 Bioinformatics
We downloaded six data sets from publicly available reports [19–21] detailing information from chow-fed mice of different sexes, developmental states or adipose tissue anatomical locations. To keep a global overview of all datasets, we did not remove any genes from any of the six datasets, and kept all mitochondrial genes. In order to combine several scRNAseq experiments from different mouse studies while minimizing batch effects, we used Batch balanced K nearest Neighbours method (BBKNN) [22] available in the Scanpy Package to convert the distance between members of the same cluster into projectable connectivity. We then performed a Leiden Clustering (https://www.nature.com/articles/s41598-019-41695-z), which defined groups with similarity relationships. For each cluster, a ranking of genes defining the cluster was established. Based on a list of genes, we looked at the normalized expression of these genes in each of the clusters and performed stacked violin plots.

2.9 Statistical analysis
Cell culture experiments were performed in triplicate wells, repeated at least three times with independent cell preparations. Mean values from independent experiments were compared by Student’s t test with a significance threshold at p < 0.05. In mouse experiments, differences in mean values from individual mice (6–10 per group) were evaluated by Student’s t test. Spearman correlations were considered significant when p < 0.05.

3. RESULTS

3.1 Thermogenic fat dormancy by thermoneutral housing aggravates obesity-dependent extracellular matrix (ECM) gene expression in mouse AT
Cold exposure that activates beige adipocyte biogenesis reduces adipose tissue fibrosis and improves systemic glucose homeostasis in mice [5,23]. To examine if shutting down of thermogenic activity could reciprocally increase the severity of high fat diet (HFD)-induced AT fibrosis we compared HFD mice (both sexes) housed at either thermoneutrality (TN, 30°C) or room temperature (RT, 20–22°C). While pre- or post-HFD mean body weights did not significantly differ between groups (Suppl Fig. 1A), TN females gained more weight than RT females, close to growth rates of RT males (Suppl Fig. 1B). Moreover, 9 weeks on HFD at TN instead of RT significantly increased fat mass in female mice (Suppl Fig. 1C). Thus, TN housing combined with HFD amplified fat accretion, more markedly in females than in males. Compared to their conventionally raised counterparts fed HFD, TN mice expressed lower levels of BAT Ucp1 mRNA (Figure. 1A), and developed more inflamed BAT, with higher expression of Il6, Tnfa and Cd68 (Figure. 1B). Moreover, a number of genes involved in ECM development were overexpressed in TN compared to RT. Specifically, TN enhanced genes encoding fibrillary collagens (Col1a1, and Col3a1), ECM modifying enzymes such as Lox12 (a collagen/elastin crosslinker), and the matrix metalloproteinase regulator Timp1 (Figure. 1C) which are implicated in the development of AT fibrosis [24]. Interestingly, BAT Ucp1 negatively correlated with Col1a1 and Col3a1 expression (Figure. 1D). Comparison of BAT histology showed bigger lipid droplets in TN mice, as expected (Figure. 1E). At low magnification, we noticed the presence of prominent white spans formed with a-cellular material indicative of deposited extracellular matrix delimiting lobules in TN BAT. Semi-quantitative analysis showed significantly increased number of lobules in TN compared to RT samples (Figure. 1F). Thus, TN housing amplified the HFD-dependent ECM gene fibrotic program and matrix deposition in BAT.

In subcutaneous fat (SCAT), we found increased collagen1 protein content in TN versus RT mice (Figure. 1G). Although the topology of collagen accumulation in TN mice SCAT was not determined in the present study, we previously observed that HFD mice at RT preferentially accumulated collagen as perilobular fibrosis [6]. SCAT is a depot in which conventional white and brown-like fat cells are mixed, resulting in highly variable Ucp1 mRNA expression among individual mice. A lower proportion of TN mice expressed high (above median) Ucp1 mRNA, indicating a reduced propensity for SCAT browning at TN (Figure. 1H). Among SCAT expressing Ucp1 above the median, Ucp1 mRNA negatively associated with Col3a1 and Fndt (Figure. 1I). Thus, thermoneural housing of mice showed an Ucp1-dependent inverse link to ECM gene patterns, which is consistent with antagonistic regulation of fibrotic and browning programs.

3.2 Reciprocal browning and fibrotic responses of progenitors in a simplified culture system
As AT resident progenitors are a common source of both beige fat cells or profibrotic fibroblasts, we aimed to establish a simplified in vitro system to investigate fibrotic versus beige fat development. We used the stroma-vascular cell fraction of mouse AT from subcutaneous (SCAT) or visceral (VAT) fat depots, known to exhibit common susceptibility but distinct efficiencies for browning or fibrotic development in vivo. Considering high heterogeneity of adipose progenitor cell population revealed by single cell sequencing [19–21], we aimed to minimize any drift in relative abundance of progenitor subsets that might occur during in vitro propagation. Cells recovered from collagenase digestion were plated at high density (80% confluence after 4-hour attachment), which ensured minimal proliferation and limited composition shifts from potential differences in proliferative rates among progenitor subtypes. Moreover, only primary first-passage cells were used, and culture duration did not exceed 5 days.

In this system, we tested cell responses to the canonical pro-fibrotic agent Tgfβ, as well as to previously characterized browning factors, i.e., β-hydroxybutyrate (BHB) and Bone Morphogenic Protein 4 (Bmp4) [13,25]. All progenitors responded to Tgfβ exposure by marked morphological changes, with elongated cell shapes and dense cell–cell contacts (Suppl Fig. 2A). As expected, pro-fibrotic Tgfβ treatment upregulated the expression of ECM marker genes (i.e. Col1a1, Ctgf, Lox2) in progenitors derived from either visceral (Suppl Fig. 2B) or subcutaneous (Suppl Fig. 2C) fat. Fnt1 (encoding the abundant matrix protein fibronectin) and Col3a1 mRNAs were up-regulated by Tgfβ in visceral progenitors only. We also observed that lactate addition in the culture medium stimulated ECM gene expression, with synergistic effect to Tgfβ
Figure 1: Thermoneutral housing aggravates HFD-induced adipose tissue ECM gene program in mice. Mice were housed for 9 weeks at room temperature (RT, 22 °C) or, thermoneutrality (TN, 30 °C) and received ad libitum HFD feeding. A-D: Brown adipose tissue (BAT) gene expression after 9 weeks on HFD was evaluated by Real time Q-PCR and normalized to 18 S. Values from individual mice are plotted, parentheses indicate significant differences between groups assessed by Student’s t test. E: Hematoxylin/eosin BAT slices from RT and TN mice at different focus level (see scale bar in each image). Left panels show increased lipid droplet size in TN compared to RT (left panels). White spans consisting of acellular material (vertical image) were identified in whole tissue scans, delimiting lobules. F: Semi quantitative analysis of lobule number. Each point is from individual mice. G: Western blot analysis of SCAT Collagen1 content in RT and TN mice. Four tissues from individual mice are shown in each group, signals are normalized to Caveolin 1 for quantification. Parenthesis indicates significant difference (p < 0.05) by t test. H: Proportion of mice with Ucp1 positive SCAT (defined as above median Ucp1 mRNA value) in RT and TN groups. P value = 0.035 by Chi2 Test. I: Correlation between Ucp1 expression in SCAT and ECM genes (Fn1: upper panel, Col3a1: lower panel). Significant correlation (p < 0.05) is found in Ucp1+ SCAT (black) but not Ucp1- SCAT (grey). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

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As expected, progenitor response to browning agents depended on fat tissue origin. Indeed, visceral progenitors (Suppl Fig. 2D) responded to Bmp4 by inducing Ppargc1a mRNA, a master regulator of the mitochondrial program, as well as Zeb1, Zfp423, Ebf1 and Ucp1. βOHB treatment had limited effects on visceral progenitors and only slightly increased Zeb1 mRNA. Subcutaneous progenitors (Suppl Fig. 2E)
induced Prdm16, Ppargc1a and Zfp423 when exposed to either βOHB or Bmp4, but displayed marginal responses in other markers. Noteworthy, in this experiment, no adipocyte differentiation cocktail was applied and progenitors were maintained for only 5 days, which might explain partial up-regulation of the browning gene program.

As a whole, these data confirm the dual potential of primary cultured progenitors to respond to browning and pro-fibrotic agents, mirroring the *in vivo* situation. Notably, although constitutive stimulation of the PDGF receptor was shown to activate fibrotic development of progenitors in multiple mouse tissues *in vivo* [26], we could not detect pro-fibrotic responses to PDGF-A in cell culture (Suppl. Fig. 2F-G), likely because PDGF receptor expression/function might be compromised by collagenase digestion or *in vitro* cell maintenance.

### 3.3. βOHB butyrate counteracts Tgfβ stimulation of ECM genes and is anti-fibrotic

Next, progenitors were exposed to βOHB or Bmp4 alone for 24 h prior to addition of Tgfβ in combination with the browning agent for the following three days. We found that Tgfβ diminished expression of beige adipocyte markers in subcutaneous progenitors, alone or with βOHB or Bmp4 co-exposure (Figure 2A). The anti-browning effect of Tgfβ over βOHB was also seen in visceral progenitors, although less marked and mostly restricted to Tbx1 and Ppargc1a mRNAs (Figure 2B). By contrast, Ppargc1a and Zfp423 mRNAs were not suppressed by Tgfβ in Bmp4-stimulated visceral cells. Thus, Tgfβ selectively inhibits βOHB-dependent browning.

We then examined if browning agents had the ability to counteract Tgfβ-dependent fibrotic response. βOHB (but not Bmp4) abolished Ctgf and Loxl2 mRNA induction by Tgfβ in subcutaneous and visceral cells (Fig. 2CD-FG). Fn1 gene induction by Tgfβ in visceral progenitors was also attenuated by βOHB specifically (Figure 2E), and the βOHB/Tgfβ combination significantly lowered Fn1 in subcutaneous progenitors while Tgfβ alone did not produce stimulation (Figure 2H). In *vivo*, βOHB circulating concentrations change from a physiological range (0.1–0.5 mM) to more than 1 mM in fasting subjects, and rise up to 10 mM or more in diabetes. βOHB blunted Tgfβ-stimulated Ctgf, Loxl2 and Fn1 mRNA expression of primary progenitors in a dose dependent manner (Figure 2I). Noticeably, in the fibroblastic ST3-L1 cell line committed to adipogenesis, we also observed attenuated Ctgf mRNA induction in the combined presence of Tgfβ and βOHB compared to Tgfβ alone (Suppl. Fig. 3A). Thus, βOHB can counteract pro-fibrotic action of Tgfβ in progenitors, independent of their tissue origin. Attenuation by βOHB of Tgfβ-dependent gene expression translated into lower Collagen1 protein contents, an effect shared with acetooacetate, a closely βOHB-related ketone body (Figure 2J).

To examine if the anti-fibrotic action of βOHB might have some relevance *in vivo*, we examined serum βOHB concentrations in a group of 30 patients with obesity involved in a bariatric surgery program, in which SCAT fibrosis was graded according to a previously defined “Fat score” integrating pericellular and perilobular tissue fibrosis, as described in [16]. Despite a similar degree of obesity in this small-sized cohort, a trend towards lower serum βOHB was found in those with higher Fat scores (Figure 3A). After stratification on βOHB levels (median = 0.067 mM), equal distribution among highly fibrotic or non-fibrotic AT was observed in the low βOHB group, a proportion that fell to 25/75 in patients with high βOHB (Figure 3B). Quantitative picrosirius red labelling indicated negative correlation with serum βOHB concentrations (Figure 3C), consistent with an anti-fibrotic role of βOHB *in vivo*. Serum βOHB regulation obviously extends beyond AT fibrosis. In agreement, we observed that βOHB levels positively

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**Figure 3:** Serum βOHB levels negatively associate with subcutaneous adipose tissue fibrosis in patients with obesity. A: Serum βOHB levels were assessed in a group of 29 obese patients eligible for bariatric surgery, in which the intensity of subcutaneous fat fibrosis had been scored as previously described. Men are represented as open symbols. B: Patient stratification according to serum βOHB (relative to median value) indicates preferential clustering of patients with low Fat score in the high βOHB group. Numbers represent the total number of patients in stratum. C: Negative association (Spearman correlation) of serum βOHB levels with subcutaneous AT fibrosis. D: Correlations between serum βOHB and selected clinical parameters. Spearman r and p value are shown. E: Distribution of patients according to Fat score and biopsy-proven liver status. Numbers represent the total number of patients in stratum, with the number of men in parenthesis. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
Figure 4: Mechanisms of fibrotic attenuation by βOHb. A: Smads mRNA expression in the presence of βOHb or chronic Tgfβ. Bars are mean values ± sem from 4 independent cell preparations. B: A representative western blot probed with antibodies against Smad2-3 (Cell signalling, ref 8685), Phospho Smad2 (Ser465/467)/Smad3 (Ser423/425) (Cell signalling, ref 8828 and Caveolin 1 (BD Transduction Laboratory, ref 610,060). βOHb concentration range is as in Figure 1, in the presence or absence of a chronic Tgfβ stimulation. C-D: Quantitative analysis of protein signal intensity after normalization with Caveolin-1 as a loading control. Bars are mean values from 3 independent cell preparations. Parentheses indicate significant differences between groups by t test. E: ZFP36 mRNA upregulation by βOHb. F: Expression of ZFP36 and related transcripts by βOHb and Tgfβ in subcutaneous and visceral progenitors. Bars are mean values ± sem from 3 to 5 independent cell preparations. * indicate significant differences compared to basal by Student t test. G–H: Gene expression in fibroblast cell lines from Wild Type (WT) or ZFP36 KO mice. Note that in KO mice, ZFP36 mRNA is transcribed but contains an insertion in exon 2 which prevents protein production. I-J: Knock-down of ZFP36 with siRNA in primary progenitors maintained with Tgfβ with or without βOHb. Bars are mean values from 3 independent experiments. Parentheses indicate significant differences between groups by t test.
correlated with serum free fatty acids or systolic blood pressure, and negatively associated to triglyceridemia and thyroid stimulating hormone (Figure. 3D). The results were not influenced by gender. Regarding liver status that could be documented from a liver biopsy in 25 patients of the cohort, no link was found between Fat score and liver fibrosis. An equal proportion of adipose tissue samples were scored for high or low fat fibrosis among patients with normal liver histology or non-alcoholic fatty liver disease (NAFLD). Even, in 10 patients with biopsy-proven non-alcoholic steato-hepatitis (NASH), only 2 had elevated Fat score, whereas 8 had non-fibrotic fat tissue (Figure. 3E).

3.4. Mechanisms of βOHButyrate anti-fibrotic activity by canonical SMAD signalling and non-canonical ZFP36 induction

3.4.1. βOHButyrate reduces smad activation in visceral progenitors

βOHB is metabolized into acetylCoA that enter the TCA cycle. We reasoned that if βOHB metabolism was involved, its impact should be stronger in conditions of carbon sources limitation. Progenitors were cultured in Gin High, a high glucose, glutamine-supplemented medium, or in Gin Low, a high glucose, glutamine-depleted medium, in which glutamine was only available from serum. Such reduced glutamine/high glucose conditions the AT microenvironment found in obese diabetic subjects, as described [27]. We observed that βOHB equally attenuated Tgfβ-dependent elevation of Ctgf mRNA in low or high Gin (Suppl Fig. 3B), suggesting that modulation of carbon entry in the TCA cycle is not a major contributor of βOHB anti-fibrotic effect. Tgfβ regulates the expression of ECM genes by activation of SMAD-2/3 downstream of Tgfβ receptor [28]. We first considered that anti-fibrotic βOHB might act at the pre-receptor level by production of extracellular modulators able to restrain ligand binding or presentation [29]. However, exposure to a bona fide exogenous Tgfβ antagonist, follistatin, alone or in combination with βOHB, had no effect on visceral progenitors ECM gene expression (Suppl Fig. 3C). We next examined SMAD-2/3 regulation, whose phosphorylation on serine residues 423–426 is induced downstream of Tgfβ receptor. We observed that Smad2 and Smad3 mRNAs were slightly but significantly reduced by βOHB treatment in, whereas Smad4 mRNA did not change (Figure. 4A). Western blots confirmed a dose-dependent decrease (30–40%) of Smad-2/3 protein contents upon βOHB exposure, in both unstimulated cells or after chronic Tgfβ stimulation (Figure 4B,C). Moreover, SMAD-2/3 serine phosphorylation was reduced in chronically Tgfβ treated visceral progenitors (Figure. 4D). Thus, blunting of Smad-2/3 dependent Tgfβ signalling by βOHB likely contributes its anti-fibrotic response. However, in similar experiments with subcutaneous progenitors, Smad-dependent Tgfβ response was not significantly affected by βOHB (data not shown), suggesting additional mechanisms might operate depending on cell origin.

3.4.2. Identification of ZFP36 as a mediator of βOHB anti-fibrotic action

To identify other βOHB-driven mechanisms, we explored data generated from single cell RNA sequencing studies, which document specific AT stromal gene expression in progenitor subgroups. From publicly available reports [19-21], data sets detailing information from chow fed mice at different developmental states, AT anatomical locations or sex were downloaded, integrated using Batch Balanced K-nearest Neighborhood algorithm (https://doi.org/10.1093/bioinformatics/bzv625), and processed by Leiden clustering for community detection (https://doi.org/10.1038/s41598-019-41695-z), which defined groups with similarity relationships. We found that Fabp4, Cd36, Ppara and Cav1 genes, associated with adipogenesis, top ranked in three clusters, likely preadipocyte subsets at different stages (Suppl Fig. 4A). Using the ranked gene list to define group identity, we preselected a cluster comprising top ranking expression of ECM related genes (Col1a1, Col3a1, Gsn, Dcn) and the progenitor gene marker Pdgfra. This progenitor cluster with high potential for ECM deposition also contained Tgfβ receptor genes, suggesting responsiveness to pro-fibrotic factors. A top ranked gene in this cluster was ZFP36, which encodes a protein known as Tristetraprolin (TTP), a member of the "early response gene" family closely related to ZFP36L1 and ZFP36L2. ZFP36 proteins belong to a family of RNA binding proteins known to destabilize AU—rich element (ARE)—containing mRNAs by promoting the removal of their polyA tails [30]. Indeed, ZFP36 was found to decrease the stability of anti-adipogenic Tnfα mRNA, and ZFP36L1 could target inflammatory transcripts of numerous components of senescence-associated secretory phenotype. Search in ARED-Plus (http://brop.kfshrc.edu.sa/ared), the AU-Rich Element Database [31] revealed hits in the 3’UTRs of Ctgf and Fn1 mRNAs, making ZFP36 an attractive candidate regulator of ECM transcript stability. Moreover, a survey of ZFP36 expression patterns from public databases indicated preferential expression of ZFP36 mRNA, and related ZFP36L1 and ZFP36L2 in BAT versus white fat (GSE 8044), while in GSE40486, BAT ZFP36 was induced by cold (Suppl Fig. 4B). High-throughput studies in T cells highly expressing ZFP36, to identify direct targets by sequencing UV crosslinked immunoprecipitated transcripts, revealed hundreds of mRNAs to which ZFP36 could bind [32], among which Glutaminase (encoded by Gls gene). Functionally, glutaminolysis is a pathway that feeds proline synthesis, a highly abundant amino acid in collagen. Thus, links from glutaminase to collagen deposition through ZFP36 has functional relevance. We found that Tgfβ stimulated Gls expression more than 3-fold in 3T3-L1 preadipocytes, an effect that was completely blunted by the [βOHB/ Tgfβ] combination (Suppl Fig. 3D). Such a similarity in the regulation of Gls, a bona fide ZFP36 target, with that of Ctgf and Fn1, bearing potential ZFP36 binding sequences, reinforced the possibility that these might be under ZFP36 post-transcriptional control. Consistent with a role for ZFP36 in βOHB anti-fibrogenic effects, we found that primary subcutaneous progenitors ZFP36 mRNA dose-dependently increased (2-fold) upon βOHB exposure (Figure. 4E). βOHB also stimulated the expression of the closely related ZFP36L1 and ZFP36L2 (Figure. 4F). Conversely, pro-fibrotic stimulation with Tgfβ, in which optimal stability of ECM transcripts is expected, led to a significant drop in ZFP36 and related ZFP36L1 and 2 (Figure. 4G). Comparable regulation by βOHB and Tgfβ was observed in visceral progenitors, although with lesser magnitude (Figure. 4F).

We next investigated mouse fibroblast cell lines derived from ZFP36 null (KO) or control (WT) mice [17]. Knock-out of ZFP36 expression [33] was achieved by insertion of a neomycin cassette into the second exon of the ZFP36 gene, leading to the synthesis of a non-functional ZFP36 fusion mRNA detected at high levels in KO compared to WT cells (Figure. 4G), in accordance with previously reported ZFP36 autoregulation. In favour of a direct control of Fn1 mRNA abundance by ZFP36, Fn1 was upregulated in KO versus WT cells (Figure. 4H). However, Ctgf and Lox2 mRNAs were strongly suppressed in the KO fibroblasts (Figure. 4H).

We then performed ZFP36 knock-down in primary subcutaneous progenitors using siRNAs which also down-regulated ZFP36L1 (Figure. 4I), βOHB was no longer able to restrain Tgfβ—mediated stimulation of Ctgf, Lox2 and Fn1 in ZFP36/ZFP36L1 deficient progenitors (Figure. 4J). Thus, ZFP36 expression is required for βOHB anti-fibrotic action. However, the ability of βOHB to stimulate genes associated with
beige adipogenesis was not affected in ZFP36 deficient progenitors (Data not shown).

4. DISCUSSION

In response to nutritional and environmental cues, fat tissue is constantly remodeled, a process involving adipose progenitors dialog with mature adipocytes and other immune cell actrors, to modulate adipogenesis or tissue fibro-inflammation. Our study documents a reciprocal cross talk between brown-like adipogenesis and fibrogenic activation of progenitors. We show that metabolites promoting brown-like fat cell development might also alleviate AT responses to profibrotic Tgfβ, suggesting potential targeted approaches to reduce AT fibrosis development. First, mirroring previously reported improvement of metabolic health by adipose tissue thermogenic activation in rodents [5,6], we show here that raising mice at thermoneutrality to induce thermogenic dormancy further aggravates AT fibrotic response to HFD. We demonstrate that β-hydroxybutyrate, a metabolite able to activate brown-like adipogenesis, can also counteract Tgfβ-dependent profibrotic responses in primary cultured progenitors. We identified different pathways that underlie this cross talk, governed by tissue origin of adipose progenitors (visceral or subcutaneous). In visceral progenitors, βOHB downregulates the expression and phosphorylation of Smads downstream of Tgfβ receptor activation, whereas in subcutaneous preadipocytes, βOHB is found to promote post-transcriptional regulation of Tgfβ gene targets notably through inhibition of ZFP36/Tristetraprolin, a protein regulating mRNA destabilisation. The mechanism by which BOHB exerts its anti-fibrotic effect might be accounted for its property as a signalling molecule or a local mediator. Other studies showed that βOHB inhibits TGFβ-induced expression of ECM genes. This suggests that raising βOHB in the obese AT context might have some interest against fibrosis development. Interestingly, a new class of anti-diabetic drugs designed to promote glucose renal excretion, referred to as “SGLT2 inhibitors” were shown to increase β-hydroxybutyrate levels in mouse models [36]. The possibility that beneficial metabolic effects of these drugs could be partly mediated by beta-hydroxybutyrate-dependent remodelling of adipose tissue is an interesting perspective that remains to be explored.

We identified the RNA binding protein ZFP36/Tristetraprolin as a mediator of the anti-fibrotic effect of β-hydroxybutyrate. ZFP36 is considered a broad anti-inflammatory molecule, acting on post-transcriptional regulation of many immune-related genes by decreasing the stability of corresponding mRNAs [37–39]. ZFP36/Tristetraprolin null mice suffer from hyper-inflammation associated with cachexia, arthritis and dermatitis, which can be prevented by treatment with anti-TNFα antibodies [33]. Conversely, it was also demonstrated that increased Tristetraprolin expression could protect mice against immune-mediated inflammatory pathologies [40]. We report the induction of ZFP36 gene expression by βOHB, and its downregulation by Tgfβ, along with changes in Tgfβ- or βOHB-mediated fibrotic responses of progenitors. The present study extends the spectrum of ZFP36 targets to several Tgfβ-regulated mRNAs. Among those, Ctgf/induced by the canonical Tgfβ-Smad pathway [41], is a crucial downstream effector in mediating Tgfβ-related fibrogenesis [42]. Its inhibition might not only prevent but also reverse fibrosis progression [43]. Based on its broad actions, ZFP36 has great potential to impact the progenitor niche towards attenuation of ECM gene program and concomitant reduction in inflammation [44]. In accordance with the present findings that ZFP36 expression dampens adipose progenitor response to profibrotic Tgfβ, several reports have associated ZFP36/Tristetraprolin deficiency with liver fibrosis aggravation [45], or its over-expression to protection against lung fibrosis following ischemia/reperfusion [46]. In the colonic epithelium, ZFP36 was also shown to target iNos mRNA [47], an important mediator in fibro-inflammatory regulated by Tnfα. Recently, the turnover of the peptide hormone FGF21 was found to be directly regulated by ZFP36 family members in the context of alcoholic [48] or non-alcoholic hepatic steatosis [49], which extends the spectrum of ZFP36 targets to metabolic regulation. Thus, the ZFP36 family has great potential as a multi-faced post-transcriptional regulator coordinating inflammation, extracellular matrix composition and metabolic responses.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. Florence Marchelli (Centre de recherche Nutrition Humaine, CRNH, Ile de France and Assistance Publique Hôpitaux de Paris), who contributed to clinical and biological data collections in patients and data base constitution. The authors thank Prof Jean-Michel Oppert, Christine Poitou and Judith Aron-Wisneswky for his contribution to patient recruitment and the paramedic staff from the Nutrition department, Pitié-Salpêtrière hospital. The investigation was performed at Clinical Center of Human Nutrition (Paris-Ile de France). This work was supported by National Agency of Research (Capitor), Fondation pour la Recherche Medicale, and EFSD/Lilly research grant. WSL and PJB are supported in part by the Intramural Research Program of the NEHS, NIH.

CONFLICT OF INTERESTS

None declared.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molmet.2022.101512.

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