Brief Communication

MOLECULAR METABOLISM

TGF-β receptor 1 regulates progenitors that promote browning of white fat

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ABSTRACT

Objective: Beige/brite adipose tissue displays morphological characteristics and beneficial metabolic traits of brown adipose tissue. Previously, we showed that TGF-β signaling regulates the browning of white adipose tissue. Here, we inquired whether TGF-β signals regulated presumptive beige progenitors in white fat and investigated the TGF-β regulated mechanisms involved in beige adipogenesis.

Methods: We deleted TGF-β receptor 1 (TβRI) in adipose tissue ($T\beta RI^{AdKO}$ mice) and, using flow-cytometry based assays, identified and isolated presumptive beige progenitors located in the stromal vascular cells of white fat. These cells were molecularly characterized to examine beige/brown marker expression and to investigate TGF-β dependent mechanisms. Further, the cells were transplanted into athymic nude mice to examine their adipogenesis potential.

Results: Deletion of TβRI promotes beige adipogenesis while reducing the detrimental effects of high fat diet feeding. Interaction of TGF-β signaling with the prostaglandin pathway regulated the appearance of beige adipocytes in white fat. Using flow cytometry techniques and stromal vascular fraction from white fat, we isolated presumptive beige stem/progenitor cells (iBSCs). Upon genetic or pharmacologic inhibition of TGF-β signaling, these cells express high levels of predominantly beige markers. Transplantation of TβRI-deficient stromal vascular cells or iBSCs into athymic nude mice followed by high fat diet feeding and stimulation of β-adrenergic signaling via CL316,243 injection or cold exposure promoted robust beige adipogenesis *in vivo*.

Conclusions: TβRI signals target the prostaglandin network to regulate presumptive beige progenitors in white fat capable of developing into beige adipocytes with functional attributes. Controlled inhibition of TβRI signaling and concomitant PGE2 stimulation has the potential to promote beige adipogenesis and improve metabolism.

Keywords Beige/brite adipogenesis; Progenitors; Metabolism; Diabetes; Obesity; TGF-beta; Prostaglandin E2; Cyclooxygenase 2

1. INTRODUCTION

Adipose tissue dysfunction is at the core of the obesity and diabetes epidemic [16,28,46,53]. White adipose tissue (WAT) stores fat while brown adipose tissue (BAT) dissipates it via thermogenesis [7], driven primarily by the inner mitochondrial membrane protein UCP1. Landmark findings that metabolically active BAT exists in humans [10,38] renewed interest in its therapeutic potential to combat metabolic diseases [13,39]. In addition, beige or brite adipocytes appear in response to cold exposure or upon stimulation by β-adrenergic pathways [9,18]. Importantly, like brown adipocytes, presence of beige adipocytes is correlated with improvements in glucose and energy homeostasis, providing a strong therapeutic rationale for their study.

Brown and beige adipocytes may have a distinct origin [43,62] with evidence favoring the existence of specialized progenitors [5,49,58,61], although a role for trans-differentiation has also been proposed [3,8,22,31,47]. A bi-potential progenitor that differentiates towards white or brown adipocytes has been identified [32], although its ability to promote beige adipocyte differentiation is unknown. Given our limited understanding into molecular control of brown and beige cell fate and function [24], further research into understanding beige adipogenesis is needed [4,20,46].

The TGF-β ligand transmits its signals via dual serine/threonine kinase receptors and transcription factors called Smads [36]. TGF-β receptor I (TβRI) phosphorylates Smad3, the main signal transducer of TGF-β signals, at key serine/threonine residues. We previously reported that TGF-β signaling regulates the browning of white fat, associated with

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improvements in glucose homeostasis [63]. Here, we describe an important role of TβRI in regulating glucose and energy homeostasis via targeting of putative beige stem cells in white fat. These progenitor cells express key beige/brown signature markers and upon transplantation demonstrate the capacity to undergo beige adipogenesis in response to β-adrenergic pathway stimulation. Finally, we demonstrate a mechanistic interaction between the TGF-β and prostaglandin signaling pathways in regulation of beige adipogenesis.

2. MATERIAL AND METHODS

2.1. Mouse experimentation

C57BL6 mice were purchased from Jackson Laboratory (Bar Harbor, ME). Six-week-old male mice (n = 5) were fed either normal chow (NC) or 60% high fat diet (HFD; Research Diets) for 8 weeks, 16 weeks or 24 weeks. Adipose tissue-specific TβRI knockout ($T\beta RI^{AdKO}$) mice and littermate control TβRI wild type ($T\beta RI^{AdWT}$) mice were generated by breeding TβRI floxed mice [29] with aP2-Cre mice [21]. All mice were maintained in 12 h light and dark cycle with *ad libitum* access to food. Body weights, food intake, and glucose tolerance tests were performed as previously described [63]. The NIDDK/NIH Animal Care and Use Committee approved all animal studies.

2.2. RNA isolation and real time quantitative PCR

RNA extraction, cDNA synthesis, and RT-PCR were performed as described previously [63] using gene specific primers (Table S3) by using Applied Biosystems 7500 Fast Real Time PCR System and using Fast SYBR® Green Master Mix.

2.3. Histology, immunohistochemistry, immunoprecipitation, western blot analyses

Histology and immunohistochemistry and western blot methods were performed as previously described [63]. For immunoprecipitation (IP), FLAG-Ubiquitin was transiently expressed in 3T3-L1 cells. After 48 h, the cells were treated with TGF-β1, SB431542 with or without MG132 for additional 24 h and pre-cleared lysates were immunoprecipitated overnight at 4 °C with antibody against the FLAG-tag. Non-immune IgG was included as a negative control. The immune complex was then subjected to SDS—PAGE followed by immunoblotting (IB). Antibody information is listed in Table S4.

2.4. Isolation of primary preadipocytes, adipogenesis and cell culture assay

Primary white preadipocytes were isolated from EWAT of 14—16-week HFD fed $T\beta RI^{AdWT}$ and $T\beta RI^{AdKO}$ mice. Mice were fed HFD for 8 weeks for preadipocyte, SVCs and presumptive progenitor cells unless otherwise mentioned. To harvest optimum amount of adipose tissue, three mice on HFD for 14—16 weeks and of same genotype were pooled for cell isolation. SVCs and 3T3L1 cells were treated overnight with TGFβ (10 ng/ml), SB431542 (10 uM) and MG132 (20 uM). To measure the PGE2 production, SVCs were treated overnight with TGFβ (2 ng/ml), SB431542 (10 uM) and Celecoxib (10 uM). For iBSCs, $T\beta RI^{AdWT}$ and $T\beta RI^{AdKO}$ iBSCs were cultured overnight and supernatant media was collected to measure the PGE2 production by ELISA Assay (Cayman Chemicals). To measure oxygen consumption rate (OCR), SVCs and iBSCs were treated overnight with TGFβ (2 ng/ml), SB431542 (10 uM). Next morning, OCR was measured using the Seahorse X24 analyzer (Seahorse Bioscience Inc.). Oxygen consumption and extracellular acidification rate were measured in basal conditions and after the addition of oligomycin (0.5 μM), FCCP (1 μM) and antimycin A (0.25 μM).

2.5. Isolation of presumptive progenitor cell (iBSC) population

Epididymal adipose tissue depots were excised from three mice of the same genotype that were on a HFD for 14—16 weeks. The tissues were minced and digested with buffered Collagenase-I at 1 mg/ml (Worthington) for 45 min at 37 °C in shaking water bath. After digestion, the slurry was filtered through a 100um filter followed by centrifugation of the filtered portion at 250gX5 for 5 min. The cell pellet was washed with ACK buffer (NH_4Cl 150 mM, $KHCO_3$ 10 mM, Na_2EDTA 0.1 mM) to remove red blood cells. Collected cells were washed two times with FACS buffer (PBS supplemented with 1% BSA and 0.25 mM EDTA). Collected cells were stained with antibodies listed in Table S4 for 45 min on ice. Stained samples were washed twice and sorted on FACS-Aria sorter (BD Biosciences, USA) equipped with 407, 488, 532, and 633 LASER lines using DIVA v6.1.3 software. Populations were identified and sorted as per the gating strategy displayed in Figure 2C.

2.6. Presumptive progenitor iBSC and SVC transplantation

SVCs (1.5×10^6 cells) and iBSCs (3×10^5 cells) were subcutaneously injected into both flank sites in 8-week old male nude mice (*Foxn1*^{nu} (nude) mice n = 6 mice, 3 per treatment i.e. CL316,243 and cold exposure) (The Jackson Laboratories) under anesthesia. Mice were fed HFD before and after cells transplantation. Mice were anaesthetized and surgically transplanted with an IPTT-300 transponder (Biomedic Data Systems). Before the cell transplantation, mice could recover from the procedure for 2 weeks. SVCs and iBSCs were injected in left and right flank ($T\beta RI^{AdWT}$ and $T\beta RI^{AdKO}$ respectively) and grafts could grow for 8 weeks. During the 9th week, mice were challenged with either cold exposure (4 °C, 90 min/day for seven consecutive days) or administered CL316,243 (1 mg/kg bw per day for seven consecutive days) injection. During cold exposure the local and core body temperature of the mice was monitored. Surface temperature was monitored with IPTT-300 transponder and DAS-7006/7s wand (Biomedic Data Systems) and core body temperature was monitored by rectal probe (TH-5 Thermalert Monitoring Thermometer). The transplant experiments were conducted two separate times on two separate groups of donor and recipient mice followed by treatments and analyses as described.

2.7. Microarray analyses

Microarray analyses protocols were described previously [63].

2.8. Statistical analysis

Data are expressed, as mean ± SEM. Statistical significance between two groups was determined using two-tailed Student's t test. Multiple groups statistical significance was tested by one-way ANOVA and group wise differences were determined by post hoc Tukey HSD test by using statistical and visualization software R. A p value of <0.05 was considered statistically significant, and all tests were two sided. See Table S5 for details. Error Bars are expressed as ± Standard Errors, *$p < 0.05$; **$p < 0.005$; ***$p < 0.001$.

3. RESULTS

3.1. Adipose-tissue specific deletion of TβRI promotes beige adipogenesis that is associated with improved metabolism

We observed a time-dependent and depot-specific increase in Smad3 and TβRI expression in the stromal vascular cells (SVC) of epididymal (EWAT) and anterior-subcutaneous (AWAT) white fat depots (Figure 1A—D) from high fat diet (HFD) mice. To investigate the role of TGF-β signaling, we generated adipose tissue-specific TβRI knockout ($T\beta RI^{AdKO}$) mice by breeding TβRI floxed mice [29] with aP2-Cre mice

