MOLECULAR
METABOLISM

EBF2 promotes the recruitment of beige adipocytes in white adipose tissue

Rachel R. Stine [1,2], Suzanne N. Shapira [1,2], Hee-Woong Lim [1,3], Jeff Ishibashi [1,2], Matthew Harms [1,2], Kyoung-Jae Won [1,3], Patrick Seale [1,2,*]

ABSTRACT

Objective: The induction of beige/brite adipose cells in white adipose tissue (WAT) is associated with protection against high fat diet-induced obesity and insulin resistance in animals. The helix-loop-helix transcription factor Early B-Cell Factor-2 (EBF2) regulates brown adipose tissue development. Here, we asked if EBF2 regulates beige fat cell biogenesis and protects animals against obesity.
Methods: In addition to primary cell culture studies, we used *Ebf2* knockout mice and mice overexpressing EBF2 in the adipose tissue to study the necessity and sufficiency of EBF2 to induce beiging *in vivo*.
Results: We found that EBF2 is required for beige adipocyte development in mice. Subcutaneous WAT or primary adipose cell cultures from *Ebf2* knockout mice did not induce Uncoupling Protein 1 (UCP1) or a thermogenic program following adrenergic stimulation. Conversely, over-expression of EBF2 in adipocyte cultures induced UCP1 expression and a brown-like/beige fat-selective differentiation program. Transgenic expression of *Ebf2* in adipose tissues robustly stimulated beige adipocyte development in the WAT of mice, even while housed at thermoneutrality. EBF2 overexpression was sufficient to increase mitochondrial function in WAT and protect animals against high fat diet-induced weight gain.
Conclusions: Taken together, our results demonstrate that EBF2 controls the beiging process and suggest that activation of EBF2 in WAT could be used to reduce obesity.

Keywords EBF2; Beige fat; Brown fat; Obesity; Thermogenesis

1. INTRODUCTION

Obesity is a major contributor to chronic illness and premature death in many countries. Unfortunately, there are limited therapeutic options to help people lose weight. A promising avenue to counteract weight gain is through increasing the activity of thermogenic brown and beige adipocytes [1]. Both of these cell types express high levels of Uncoupling Protein 1 (UCP1) in their mitochondria. When activated, UCP1 induces high rates of substrate oxidation with significant amounts of energy being expended in the form of heat [1]. Thermogenesis in brown and beige adipocytes is activated in response to cold-exposure and protects animals against hypothermia [2]. Additionally, UCP1 activity in brown and/or beige adipocytes regulates energy balance. Loss of UCP1 promotes weight gain in mice maintained at thermoneutrality and thus exempt from cold stress [3]. Conversely, animals with increased brown and beige fat activity are lean and have a healthy metabolic profile [4—9]. Importantly, brown/beige fat activity levels in people are also correlated with leanness [10—13].

Brown adipocytes develop in dedicated depots of brown adipose tissue (BAT) and express relatively high levels of UCP1 under a variety of environmental conditions. By contrast, beige adipocytes, which develop in WAT, are highly inducible [1]. In animals kept at normal vivarium conditions (~ 22 °C), there are relatively few beige adipocytes. However, upon cold-exposure or chronic stimulation with β-adrenergic agonists such as CL 316, 243, there is a dramatic increase in beige fat cell recruitment and UCP1 levels. In mice, the cold-stimulated induction of beige adipocytes is particularly prominent in the inguinal WAT (ingWAT), a major subcutaneous fat depot. Importantly, recent studies have indicated that beige adipocyte activity affects systemic metabolism and contributes in a significant way to whole body insulin sensitivity [14,15].

[1]Institute for Diabetes, Obesity & Metabolism, Smilow Center for Translational Research, 3400 Civic Center Blvd, Rm. 12-105, Philadelphia, PA, 19104, USA [2]Department of Cell and Developmental Biology, Smilow Center for Translational Research, 3400 Civic Center Blvd, Rm. 12-105, Philadelphia, PA, 19104, USA [3]Department of Genetics, Perelman School of Medicine at the University of Pennsylvania, Smilow Center for Translational Research, 3400 Civic Center Blvd, Rm. 12-111, Philadelphia, PA, 19104, USA

*Corresponding author. Perelman School of Medicine at the University of Pennsylvania, Smilow Center for Translational Research, 3400 Civic Center Blvd, Rm. 12-105, Philadelphia, PA, 19104, USA. Tel.: +1 215 573 8856; fax: +1 215 898 5408. E-mail: sealep@upenn.edu (P. Seale).

Brief communication

Brown adipocytes are distinct from the beige adipocytes found in ingWAT; they arise from separate lineages [16], but many studies have suggested that the differentiation and development of both cell types are controlled by a shared transcriptional program. Work by our lab and others has shown that the transcription factor PRDM16 is required for "beiging" of ingWAT and for the maintenance of BAT [6,15,17,18]. PGC-1α, a transcriptional co-activator also plays important roles in both brown and beige fat cells [19,20]. We recently identified Early B-cell factor-2 (EBF2) as a factor required for the BAT development [21]. Through an independent line of investigation, we also identified EBF2 as a selective marker of both brown and beige preadipose cells in embryonic and adult adipose depots [22]. This raised the question of whether EBF2 plays a critical role in beige fat development and function.

In this study, we report that EBF2 controls beige adipocyte development in WAT. We show that genetic loss of *Ebf2* in mice blocks beige fat development and function without having any other obvious effects on WAT. EBF2 is required for the re-programming of primary ingWAT-derived preadipose cells into thermogenically competent beige/brown-like adipocytes *in vitro*. Finally, transgenic expression of *Ebf2* in adipose tissue using the *Fabp4* promoter/enhancer powerfully stimulates beige fat development and protects animals against high fat-diet-induced weight/fat gain. RNAseq analysis demonstrates that EBF2 robustly induces a mitochondrial gene program similar to that of cold-induced beige fat. Overall, our results demonstrate that EBF2 plays a central role in regulating beige fat function and could potentially be manipulated to improve overall systemic metabolism in mice.

2. MATERIALS AND METHODS

2.1. Animals

All animal experiments were performed in accordance with an approved University of Pennsylvania Institutional Animal Care and Use Committee protocol. Mice were fed standard chow unless otherwise specified and kept on a 12 h light/dark cycle. *Ebf2* whole body knockout animals were obtained from R. Reed (Johns Hopkins, Baltimore, MD, USA) and have been described previously [23]. *Fabp4-Ebf2* transgenic mice were generated by cloning full-length *Ebf2* cDNA downstream of the 5.4 kb *Fabp4* promoter/enhancer. A bovine growth hormone polyadenylation site was inserted 3′ to the *Ebf2* cDNA. The construct was injected into FVB mouse oocytes by the University of Pennsylvania Transgenic and Chimeric Mouse Facility. For CL 316,243 treatment, *Ebf2* knockout mice and wild type (WT) littermate controls were raised at thermoneutrality (30 °C) and injected with 1 mg/kg CL 316,243 diluted in PBS every day for 6 days. Mice were sacrificed for analysis on day 7. For weight gain analysis, *Fabp4-Ebf2* mice were housed at thermoneutrality and fed a 45% high-fat diet (Research Diets).

2.2. Cell culture

Primary adipogenic precursor cells were isolated from mouse ingWAT as described previously [24]. For adipocyte differentiation assays, cells were grown to confluence and then treated with differentiation induction medium (DMEM/F12 with 10% FBS, 1 μM dexamethasone, 0.5 mM isobutylmethylxanthine, 125 nM indomethacin, 20 nM insulin, and 1 nM T3). Induction medium was removed after 48 h and cells were then grown in DMEM/F12 medium containing 10% FBS, 1 nM T3 and 20 nM insulin until they were harvested. To stimulate a thermogenic program, cells were incubated either with 10 μM isoproterenol for 3 h or with 1 μM rosiglitazone for the entire differentiation time course. For Oil Red O staining, cell cultures were washed with PBS, fixed with 4% paraformaldehyde for 15 min and stained with Oil Red O solution for 30 min. All primary cell experiments were done using three technical replicates.

2.3. Western blot analysis

Cells or tissues were lysed in RIPA buffer containing 0.5% NP-40, 0.1% sodium deoxycholate, 150 mM NaCl, 50 mM Tris-Cl, pH 7.5, protease inhibitor cocktail (Complete; Roche) and 1 mM phenyl-methylsulfonyl fluoride (PMSF). Protein concentrations were quantified using detergent-compatible (DC) protein assay kit (Bio-Rad). Lysates or nuclear fractions were run on Bis-Tris NuPAGE gels (Invitrogen), transferred to PVDF membrane (Millipore), and probed with primary antibody. Antibodies used were sheep anti-Ebf2 (R&D systems, AF7006), mouse anti-Ucp1 (R&D systems, MAB6158), rabbit anti-VDAC (Cell Signaling, 4866), mouse anti-actin (clone C4, Millipore, MAB1501) and mouse anti-tubulin (Sigma, T6199).

2.4. RT-qPCR analysis

Total RNA was isolated from cultured cells or whole tissue using TRIzol extraction (Invitrogen). RNA was then purified using PureLink RNA columns (Life Technologies). RNA was reverse transcribed to generate cDNA using a High-Capacity cDNA Synthesis kit (Applied Biosystems). Real-time PCR was used to quantify transcript levels using SYBR Green master mix (Applied Biosystems) on a 7900 Fast HT real time PCR machine (Applied Biosystems). For all real-time PCR, *Tata-binding protein* (*Tbp*) was used as an internal normalization control. P-values were determined in Excel using a two-tailed T-test. Primer sequences are in Table S1.

2.5. Histology

For immunohistochemistry, whole inguinal fat pads were fixed in 4% PFA overnight, dehydrated, and embedded in paraffin for sectioning. Sections were stained with hematoxylin and eosin using standard methods. For whole mount immunofluorescence staining of fat pads, mice were transcardially perfused with 2% paraformaldehyde in PBS. Inguinal fat pads were removed and 6 mm punch biopsies were taken from the fat pad region immediately adjacent to the lymph node. Tissues were incubated in 4% paraformaldehyde overnight, washed with PBS and blocked in PBS with 5% Normal Donkey Serum, 0.5% Triton X at 4 °C overnight. Tissues were incubated in primary antibody diluted in blocking solution overnight and secondary antibody diluted in block for 2 h at room temperature. Tissues were mounted on concavity slides in Prolong Gold antifade mounting medium. Slides were imaged on a Leica TCS Sp8 confocal microscope. Primary antibodies used were rabbit anti-UCP1 (1:1000, AZ) and sheep anti-EBF2 (1:200, R&D Systems). Secondary Alexa fluor-conjugated antibodies were from Life Technologies.

2.6. Tissue O$_2$ consumption

A 6 mm punch biopsy was taken from each ingWAT sample directly below the lymph node. For BAT, one lobe was used. Tissues were weighed and minced in a respiration buffer (2% BSA, 1.1 mM sodium pyruvate, and 25 mM glucose in PBS). Oxygen consumption was measured for each sample in an MT200A Respirometer Cell (Strathkelvin) until oxygen consumption rate was stable (for approximately 5 min). Oxygen consumption rates were normalized to tissue weight. Two measurements were taken from each mouse.

2.7. RNAseq

An RNAseq library was prepared using isolated RNA from whole ingWAT pads with lymph nodes removed using the Illumina TrueSeq