MOLECULAR REGULATION AND THERAPEUTIC POTENTIAL OF THERMOGENIC FAT CELLS

EDITED BY: Jun Wu
PUBLISHED IN: Frontiers in Endocrinology

frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88919-869-6
DOI 10.3389/978-2-88919-869-6

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view.
By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: **researchtopics@frontiersin.org**

MOLECULAR REGULATION AND THERAPEUTIC POTENTIAL OF THERMOGENIC FAT CELLS

Topic Editor:
Jun Wu, University of Michigan, Ann Arbor, USA

Obesity has emerged as a major threat to public health in both the western and developing world. Essentially a disorder of energy balance, obesity occurs when energy intake and storage exceeds expenditure. Much of energy homeostasis depends on the activity and function of adipose tissue. Adipocytes in mammals fall into two categories classified by their primary functions: white fat cells that mediate energy storage and thermogenic fat cells that counteract hypothermia and obesity through adaptive thermogenesis. Whereas white fat and its function as an energy reservoir and endocrine organ have been studied for decades and are relatively well understood, until recently many aspects of the thermogenic fat biology have remained elusive. Accumulating evidence supports the hypothesis that thermogenic fat cells arise from at least two different developmental origins: the ones of a skeletal muscle-like lineage are now called "classical" brown fat cells, and the rest of the thermogenic fat cells are normally referred to as the beige fat cells. The last decade has witnessed an explosion of interest and studies focusing on the regulation of thermogenic fat cells and potential therapeutics targeting these adipocytes. Here we summarize the recent advancements in our understanding of these metabolically active fat cells.

Citation: Wu, J., ed. (2016). Molecular Regulation and Therapeutic Potential of Thermogenic Fat Cells. Lausanne: Frontiers Media. doi: 10.3389/978-2-88919-869-6

Table of Contents

frontiers in Endocrinology

EDITORIAL
published: 29 March 2016
doi: 10.3389/fendo.2016.00026

Editorial: Molecular Regulation and Therapeutic Potential of Thermogenic Fat Cells

Jun Wu[1,2]*

[1] Life Sciences Institute, University of Michigan Medical School, Ann Arbor, MI, USA, [2] Department of Molecular and Integrative Physiology, University of Michigan Medical School, Ann Arbor, MI, USA

Keywords: brown fat, beige fat, obesity, adaptive thermogenesis, metabolic diseases

The Editorial on the Research Topic

Molecular Regulation and Therapeutic Potential of Thermogenic Fat Cells

Much of systemic metabolism depends on the activity and function of adipose tissue. Adipocytes in mammals fall into two categories classified by their primary functions: white fat cells that mediate energy storage and thermogenic fat cells that counteract hypothermia and obesity through adaptive thermogenesis. Whereas white fat and its function as an energy reservoir and endocrine organ have been studied for decades and are relatively well understood, until recently, many aspects of thermogenic fat biology have remained elusive.

It has long been appreciated that the thermogenic brown adipose tissue that exists in the interscapular depots of small rodents, hibernating mammals, and human infants provides significant evolutionary advantages against cold stress (1). The last decade has witnessed an explosion of interest and studies focusing on the regulation of thermogenic fat and potential therapeutics targeting these adipocytes. Some groundbreaking discoveries have led to our current understanding of thermogenic fat biology. Accumulating evidence supports the hypothesis that thermogenic fat cells arise from at least two different developmental origins: the ones of a skeletal muscle-like lineage are now called "classical" brown fat cells, and a new type of thermogenic fat cell that is interspersed within white adipose tissue, which is normally referred to as the beige/brite (brown in white) fat cell. Definitions of types and/or subtypes of thermogenic fat cells will continue to evolve as further studies reveal distinctive functions of these cells. The rediscovery of thermogenic fat cells in human adults ignited enthusiasm toward these cells serving as novel therapeutic targets against obesity and its associated metabolic disorders. One of the key questions is whether the average amount and activity of these cells in the adult human is sufficient to influence whole body metabolism. Emerging data are starting to address this as several recent reports have demonstrated that activated thermogenic fat cells help to improve glucose tolerance and insulin sensitivity in humans.

In this research topic, leading experts in the energy metabolism field provide an overview of our current understanding of these metabolically active fat cells. We begin our discussions with mechanistic insights on the transcriptional regulation of thermogenic fat cells [Park et al.; Mueller; Bayindir et al.]. Reviews and a perspective article next explore how mitochondrial function, including redox homeostasis, is regulated within these thermogenic adipocytes [Nam and Cooper; Jeanson et al.; Ro et al.]. Central control of adaptive thermogenesis and other functions of thermogenic adipocytes are also highlighted [Yang and Ruan; Zhang and Bi]. Importantly, several articles illustrate that interconnections exist between adipose tissue function and overall metabolic health, and how to increase the content and activity of these cells to ultimately fulfill their therapeutic potential in humans [Lester et al.; Peng et al.; Porter et al.]. As technology has advanced, novel interdisciplinary approaches have been developed to harvest the thermogenic capacity in fat tissue toward the control of obesity and metabolic diseases [Jiang et al.; Tharp and Stahl]. We anticipate that

Edited and Reviewed by:
Ralf Jockers, University of Paris, France

***Correspondence:**
Jun Wu
wujunz@umich.edu

Specialty section:
This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *01 March 2016*
Accepted: *14 March 2016*
Published: *29 March 2016*

Citation:
Wu J (2016) Editorial: Molecular Regulation and Therapeutic Potential of Thermogenic Fat Cells. Front. Endocrinol. 7:26. doi: 10.3389/fendo.2016.00026

all these articles will help the scientific community, both experts in the brown and beige fat area and newcomers entering the field to understand the molecular mechanisms of brown/beige fat development and induction, and appreciate the physiological impact of thermogenic fat activity. I would like to thank all the authors and reviewers for their contribution and discussion to put together this wonderful topic that may inspire further interest in this exciting new field.

AUTHOR CONTRIBUTIONS

JW conceived and wrote the manuscript.

REFERENCE

1. Cannon B, Nedergaard J. Brown adipose tissue: function and physiological significance. *Physiol Rev* (2004) **84**(1):277–359. doi:10.1152/physrev.00015.2003

Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

FUNDING

Work in the Wu laboratory is supported by R01DK107583 from the National Institute of Diabetes and Digestive and Kidney Diseases of the National Institutes of Health, Pilot and Feasibility Grants from the Michigan Diabetes Research Center (NIH Grant P30-DK020572), the Michigan Nutrition and Obesity Research Center (NIH/NIDDK Grant P30-DK089503), the Mallinckrodt Grant from the Edward Mallinckrodt Jr. Foundation, the McKay Grant from the Frankel Cardiovascular Center at the University of Michigan, and a Young Investigator Grant RGY0082/14 from the Human Frontier Science Program.

frontiers in Endocrinology

MINI REVIEW
published: 12 August 2015
doi: 10.3389/fendo.2015.00124

Intricate transcriptional networks of classical brown and beige fat cells

*Jun Hong Park, Wonhee Hur and Sean Bong Lee**

Department of Pathology and Laboratory Medicine, Tulane University School of Medicine, New Orleans, LA, USA

Brown adipocytes are a specialized cell type that is critical for adaptive thermogenesis, energy homeostasis, and metabolism. In response to cold, both classical brown fat and the newly identified "beige" or "brite" cells are activated by β-adrenergic signaling and catabolize stored lipids and carbohydrates to produce heat via UCP1. Once thought to be non-existent in adults, recent studies have discovered active classical brown and beige fat cells in humans, thus reinvigorating interest in brown and beige adipocytes. This review will focus on the newly discovered transcription factors and microRNAs that specify and orchestrate the classical brown and beige fat cell development.

Keywords: classical brown fat, beige, brite, transcriptional regulation, transcription factors, miRNAs, lincRNAs

Edited by:
Jun Wu,
University of Michigan, USA

Reviewed by:
Xingxing Kong,
Harvard Medical School, USA
Paul Cohen,
Rockefeller University, USA

***Correspondence:**
Sean Bong Lee,
Department of Pathology and Laboratory Medicine, Tulane University School of Medicine, 1700 Tulane Avenue Room 808, New Orleans, LA 70112, USA
slee30@tulane.edu

Specialty section:
This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *08 May 2015*
Accepted: *24 July 2015*
Published: *12 August 2015*

Citation:
Park JH, Hur W and Lee SB (2015) Intricate transcriptional networks of classical brown and beige fat cells. Front. Endocrinol. 6:124. doi: 10.3389/fendo.2015.00124

Two Types of Thermogenic Cells: Classical Brown and Beige Fat Cells

Non-shivering thermogenesis in mammals is carried out by a group of specialized fat cells known as brown adipocytes. Classical brown adipocytes are generated during embryogenesis in distinct brown adipose tissue (BAT) depots, such as the axillary, interscapular, and subscapular regions (1, 2). Classical BAT is abundant in rodents and hibernating mammals and it functions to maintain their body temperature in cold climate. Human infants are also born with classical BAT but it disappears over time and was considered to be non-existent in adults (3). However, PET-CT imaging studies with ^{18}F-fluorodeoxyglucose have discovered active BAT in the neck and supraclavicular regions in adult (4–8). These findings have revitalized the research on BAT and the efforts to utilize it as a potential therapy against obesity and other metabolic diseases.

Recent studies in rodents and humans have discovered a second type of brown fat cells known as the beige or brite (brown in white) cells (2, 9). Beige cells are generated postnatally within white adipose tissues (WAT) in response to cold or adrenergic stimulation. Both classical brown fat and beige cells are rich in mitochondria and uniquely express uncoupling protein 1 (UCP1), an inner mitochondria membrane protein that produces heat by uncoupling the proton gradient from ATP synthase. Although both brown and beige cells share the same thermogenic function, they arise from entirely different cell lineages (2, 10). Classical brown fat cells arise from myogenic progenitors that express Myf5 and Pax7 myogenic transcription factors (11, 12) in specific BAT depots during development. In contrast, beige cells are made postnatally in WAT depots and arise from Myf5-precursors that express platelet-derived growth factor receptor α (PDGFRα) (10, 13–15) or through transdifferentiation of mature white adipocytes (16–18) in response to cold or β-adrenergic stimulation. A recent study has also shown that beige cells can arise from smooth muscle cell (Myh11+) progenitors (19). Several mouse genetic lineage-tracing studies have led to discordant results regarding how beige cells are generated (13, 15, 17–19). The studies that permanently marked mature white adipocytes in subcutaneous (subQ) WAT showed that cold-induced beige cells are derived from mature white adipocytes in subQ WAT (17, 18), while another study showed that some

beige cells are generated *de novo* from progenitors (15). In contrast, studies that genetically marked PDGFRα+ progenitors in epididymal WAT (13) or smooth muscle progenitors (Myh11+) in subQ WAT (19) demonstrated that beige cells are derived from the respective progenitors. While further studies are needed to clarify these issues, these studies clearly demonstrate that different WAT depots have different "browning" capacity and might employ different mechanisms to generate beige cells.

The discovery of active BAT in humans has raised the issue of whether human BATs comprises classical brown or beige fat cells. Several studies have revealed that adult human BAT is more similar to the mouse beige cells (10, 20–22), while other studies showed that it is closer to the classical BAT (23, 24). Hence, similar to rodents, it is likely that adult humans possess both classical brown and beige fat cells, depending on different anatomical locations.

Preservation of Core Transcriptional Hierarchy in Brown and White Adipogenesis

During adipogenesis, external adipogenic signals activate a cascade of core transcription factors that are critical for both brown and white adipocyte differentiation. The sequential activation of these transcription factors has been elegantly worked out in 3T3-L1 cells (25), which were first established in Howard Green's lab from Swiss albino mouse embryonic fibroblasts (MEFs) (26) using a 3T3 protocol (27). Adipogenic stimulation of either white or brown preadipocytes leads to a sequential activation of core transcription factors (25, 28, 29) (**Figure 1**). One of the earliest activated transcription factors are CCAAT-enhancer-binding protein-β (CEBP-β) and CEBP-δ, which then form a heterodimer and transcriptionally activate peroxisome proliferator-activated receptor γ (PPARγ), along with another family member, CEBP-α. PPARγ is a member of the nuclear hormone receptor superfamily and is the master regulator of adipogenesis as its sole expression is sufficient to convert fibroblasts into adipocytes (30). Upon activation, PPARγ activates the transcription of CEBP-α and many other genes involved in fatty acid synthesis, lipid storage, and glucose metabolism (25). CEBP-α then reciprocally activates PPARγ as well as other adipogenic genes. While PPARγ and the CEBP-family proteins are the core transcriptional regulators of both BAT and WAT adipogenesis, auxiliary transcription factors, such as Kruppel-like factor 5 and 15 (KLF5 and KLF15), also modulate general adipogenesis (25). Interestingly, Zfp423, which contains 30 Kruppel-like zinc fingers and a SMAD-binding domain, was identified as a regulator of preadipocyte determination by activating the transcription of PPARγ (31) (**Figure 1**). Deletion of *Zfp423* in mice inhibits both brown and white adipogenesis.

Transcriptional Regulators of Brown and Beige Fat Cells

Several recent studies have revealed brown and beige fat-specific transcriptional regulators as well as microRNAs (miRNAs) and long intergenic non-coding RNAs (lincRNAs), which will be described in detail.

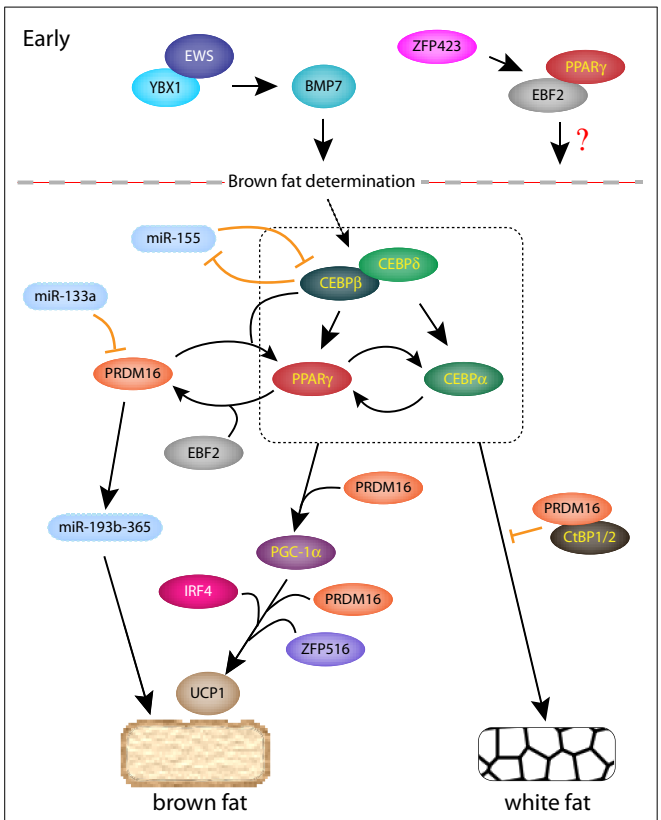


FIGURE 1 | A regulatory network of transcription factors and miRNAs in classical brown and white adipogenesis. Early transcription factors are shown above the dotted line and the box indicates the core transcription factors critical for brown and white adipogenesis.

PRD1-BF-1-RIZ1 Homologous-*Domain Member* 16

PRD1-BF-1-RIZ1 homologous-*Domain Member* 16 (PRDM16) and its close homolog PRDM3 were first identified as the histone 3-Lys 9-monomethyltransferases (H3K9me1) that are critical for heterochromatin organization (32). By examining transcription-related genes enriched in brown fat versus white fat, Seale et al. identified PRDM16 as a brown fat-specific transcription factor (33). Ectopic expression of PRDM16 in WAT results in increased beige cell formation in the mouse. Conversely, knockdown of PRDM16 blocks brown fat differentiation. Remarkably, knockdown of PRDM16 in primary brown preadipocytes leads to myocyte differentiation and ectopic PRDM16 expression in myoblasts turns them into brown fat cells upon adipogenic stimulation (11). These results suggest that PRDM16 controls a cell fate switch between brown fat and myocyte differentiation in bipotent progenitors. Interestingly, another H3K9 methyltransferase, EHMT1, interacts with PRDM16 and is required for BAT development (34). PRDM16 forms a complex with CEBPβ and together, these two factors are able to convert a naïve fibroblasts or myoblasts into brown fat cells (35). Additionally, PRDM16 interacts with C-terminal binding proteins, CtBP1 and CtBP2, and represses white fat gene expression program (36), but this

interaction can be displaced by PGC-1α or PGC-1β, which induces brown fat program upon binding to PRDM16 (33, 36). PRDM16 also interacts with PPARγ and enhances its transcriptional activity (11). In addition to cold and β3-agonists, PPARγ agonists can also induce beige cell differentiation in the mouse, which was shown to require PRDM16 (37). Addition of PPARγ agonists stabilizes PRDM16, likely through its interaction with PPARγ.

Initial description of global PRDM16 knockout (KO) mice, which was postnatal lethal, reported abnormal BAT morphology with reduced brown fat gene expression and ectopic myogenic gene expression (11). A recent study showed that specific deletion of PRDM16 in postnatal adipose tissues (BAT and WAT) using Adiponectin-Cre blocks cold- or β3-agonist-induced browning of subQ WAT, but has minimal effects on classical BAT and visceral (Vis) WAT (38), demonstrating that PRDM16 is essential for beige fat formation in subQ WAT (**Figure 2**). Furthermore, loss of PRDM16 induces subQ WAT to adopt Vis WAT gene expression profile and reduces its thermogenic capacity. These findings indicate that while PRDM16 is required during early brown cell fate determination (according to earlier studies), it is dispensable for mature BAT thermogenesis. Therefore, it was quite surprising when a specific deletion of PRDM16 in early myogenic progenitors using Myf5-Cre showed normal BAT development (39). In contrast to the Cohen et al. study which reported minimal effects on BAT (38), adult BATs derived from Myf5-specific deletion of PRDM16 shows increased white fat differentiation and reduced thermogenesis in aged animals, suggesting a role of PRDM16 in maintaining mature BAT function (39). A simultaneous deletion of both PRDM16 and its homolog PRDM3 shows much earlier and more prominent brown fat defect than the single PRDM16-KO, although the embryonic and early postnatal (2 weeks) BAT development are minimally affected. These studies show that in the absence of PRDM16, PRDM3 can serve a compensatory role.

FOXC2

A role of a winged helix/forkhead gene, *Foxc2*, in browning of white fat was demonstrated well before beige cell was recognized as a distinct cell type (40). Expression of *Foxc2* is highly restricted to both BAT and WAT, and adipose-specific expression of *Foxc2* using *aP2* (Fabp4) promoter in mice results in browning of WAT and hypertrophic BAT. Furthermore, Foxc2 transgenic mice are resistant to high-fat diet (HFD)-induced obesity and insulin and glucose resistance. This is at least partly due to increased mitochondria number and respiration of beige cells in WAT of transgenic mice. Intriguingly, expression of Foxc2 in 3T3-L1 cells blocks white fat differentiation by inhibiting the expression of certain PPARγ target genes (41).

EWS

Ewing sarcoma break point region 1 (*EWSR1*, herein termed *EWS*) encodes a highly abundant, multifunctional RNA/ssDNA binding protein (42). Originally presumed to play housekeeping roles in basic transcription and RNA splicing (43), generation of EWS-KO mouse and other studies have revealed a surprisingly diverse role of EWS in meiosis, B-cell development, prevention of cellular senescence, mitosis, DNA damage-induced alternative splicing, and miRNA regulation (44–49). More recently, it was discovered that classical BAT development was completed blocked in EWS-KO (50). Deletion of EWS results in a complete block in early

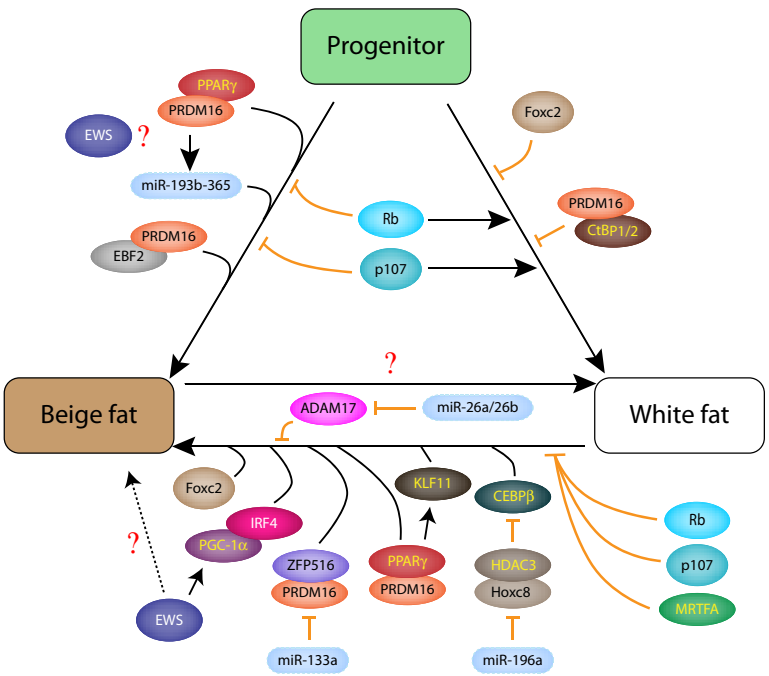


FIGURE 2 | Intricate networks of transcription factors and miRNAs in beige and white fat differentiation from progenitors or through transdifferentiation.

embryonic classical BAT development and loss of brown fat differentiation in preadipocytes. As a member of TGF-β superfamily, BMP7, bone morphogenic protein 7, plays a critical role in the commitment of early mesenchymal progenitors to brown fat (51). In the absence of EWS, BMP7 expression is lost in embryonic BAT and in brown preadipocytes undergoing adipogenesis (50). Following adipogenic stimulation, EWS forms a complex with Y box-binding protein 1 (YBX1) and activates BMP7 transcription. Depletion of YBX1 also results in loss of BMP7 expression and a block in brown fat differentiation. Notably, loss of EWS leads to ectopic myogenic expression in EWS-KO BAT, consistent with the idea that EWS determines the classical brown cell fate. Interestingly, EWS heterozygous mice show reduced beige cell recruitment in inguinal WAT in response to PPARγ agonist or β3-adrenergic stimulation (50). However, the definitive role of EWS in beige cell development will require further studies. As both brown fat and beige cells are rich in mitochondria, it is intriguing to note that EWS was recently shown to regulate mitochondria density and function by controlling PGC-1α protein stability (52). Finally, EWS may also have a role in white adipogenesis, at least *in vitro* (53).

EBF2

A search for brown fat-specific PPARγ-regulated promoters by ChIP-seq analysis identified an enrichment of early B-cell factor (EBF) binding sites in the PPARγ occupied DNA regions (54). It was subsequently shown that EBF2, one of the four EBF isoforms, is highly expressed in BAT compared to WAT or beige cells. Ectopic expression of EBF2 in C2C12 myoblasts or in stromal vascular fraction (SVF, which is known to contain adipocyte progenitors) leads to a strong induction of brown fat differentiation, while depletion of EBF2 blocks differentiation in brown preadipocytes. EBF2 recruits PPARγ to the PRDM16 promoter/enhancer region and synergistically activates its expression. EBF2 is expressed in early Myf5+/Pdgfrα+ brown progenitors as well as in Pdgfrα+ beige precursors from subQ WAT, serving as potential markers of these progenitors (55). However, classical BAT of EBF2 KO mouse shows normal levels of pan-adipocyte markers, PPARγ, adiponectin, and Fabp4, but loss of BAT-specific Ucp1, PRDM16, and Cidea expression, demonstrating that EBF2 is not required for general adipogenic process but specifically regulates BAT-specific gene expression (54, 55). Interestingly, a recent study showed that EBF2 forms a ribonucleoprotein complex with a long non-coding RNA (lncRNA) termed brown fat lncRNA 1 (Blnc1), which is transcriptionally regulated by EBF2 during brown adipogenesis, to promote adipogenesis in brown adipocytes (56).

KLF11, IRF4, and ZFP516

As aforementioned and reviewed in Ref. (25, 57), several KLF-family proteins play important roles in the common adipogenic differentiation of BAT and WAT. Notably, KLF11 was recently identified as an activator of beige cell differentiation of human adipose-derived stem cells (58). KLF11 is a direct target of PPARγ and activates the expression of beige-specific genes. Expression of interferon regulatory factor 4 (IRF4) is induced by cold in both BAT and WAT and overexpression of IRF4 in BAT and WAT leads to enhanced thermogenesis and resistance to HFD-induced obesity (59). Conversely, specific deletion of IRF4 in Ucp1+ cells (brown and beige cells) causes a reduction in energy expenditure and thermogenesis as well a block in beige cell formation in subQ WAT. Interestingly, PGC-1α interacts with IRF4 and this interaction appeared to be crucial for activation of Ucp1 expression. A search for transcription factors that directly activate Ucp1 led to an identification of Zfp516 containing ten C2H2 zinc finger protein (60). Ectopic expression or genetic deletion of Zfp516 results in browning of WAT or loss of classical BAT development. Though the exact mechanisms of Zfp516 are not clear, it interacts with PRDM16; however, since PRDM16 is dispensable for classical BAT development, how Zfp516 regulates BAT development remains unresolved.

Inhibitors of Brown and Beige Cell Differentiation: Rb Family Proteins and MRTFA

Retinoblastoma susceptibility (Rb) family proteins, Rb and p107, have important roles in determining white versus brown adipocyte differentiation (61, 62). Deletion of *Rb* in MEFs or embryonic stem (ES) cells results in brown fat differentiation (Ucp1+) upon adipogenic stimulation while control cells give rise to white adipocytes (61). Consistent with this, mesenchymal progenitor-specific Rb KO embryos show a significant increase in classical BAT mass (63). Intriguingly, while Rb is required for white adipocyte differentiation *in vitro* (64, 65), adipose-specific KO of Rb (66) or inactivation of Rb via SV40 T antigen in WAT (67) results in browning of WAT. Expression of p107 is abundant in the SVF from Vis WAT, lower in subQ WAT, and absent in BAT (68). Mature white adipocytes from any WAT depots do not express p107. Deletion of p107 in a congenic Balb/c background leads to an impairment of WAT development but not BAT, and causes extensive browning in various WAT depots (62). β-adrenergic stimulation reduces p107 expression in SVF and induces beige fat differentiation (68). Thus, Rb family proteins likely function as a negative regulator of beige cell differentiation. Similarly, genetic ablation of myocardin-related transcription factor A (MRTFA) results in browning of WAT depots without affecting BAT mass and function (69). MRTFA KO mice are protected from HFD-induced obesity and insulin resistance, demonstrating that MRTFA is a negative regulator of beige cell formation.

Regulation of Brown and Beige Cell by microRNAs

Recent studies have identified several miRNAs that specifically target the expression of critical brown or beige transcription factors described above. Accordingly, many miRNAs are expressed in BAT- or WAT-specific manner. One such miRNA, miR-193b-365, is activated by PRDM16 and is required for brown adipogenesis (70) (**Figure 1**). Forced expression of miR-193b-365 in myoblasts blocks myogenesis and upon adipogenic stimulation, induces brown fat differentiation. In contrast, miR-133a represses PRDM16 expression

and inhibits brown fat differentiation by targeting the 3′-UTR of PRDM16 transcripts (71, 72). Genetic inactivation of miR-133a has no effects on BAT development but increases beige cell development in WAT depots, which results in improved thermogenesis and glucose and insulin sensitivity. TGFβ, a potent inhibitor of adipogenesis (73), increases the expression of miR-155 and inhibits adipogenesis (74). Inhibition of adipogenesis by miR-155 overexpression suppresses CEBPβ expression, while CEBPβ represses miR-155 expression, forming a double negative loop in brown adipogenesis (**Figure 1**). Ectopic expression of miR-155 in the mouse reduces BAT size and function while miR-155 KO mice show improved BAT thermogenesis and enhanced beige cell formation in WAT. Expression of miR-196a is induced in subQ WAT following cold or β3-agonist stimulation and is required for Ucp1 expression (75). miR-196a represses the expression Hoxc8, homeobox c8, which is highly expressed in white fat cells and inhibits brown fat differentiation. Adipose-specific expression of miR-196a results in enhanced browning of WAT and protects mice from HFD-induced obesity and insulin resistance. Intriguingly, it was found that Hoxc8 represses CEBPβ expression by recruiting histone deacetylase, HDAC3. Thus, miR-196a regulates the expression of CEBPβ through Hoxc8 (**Figure 2**). miR-26a and miR-26b also have positive effects on converting human preadipocytes into beige cells (76). This is mediated by repressing ADAM17, ADAM metallopeptidase domain 17, expression and knockdown of ADAM17 recapitulates the increased beige adipogenesis. On the flip side, miR-27a/b negatively regulates multiple critical regulators of brown and beige adipogenesis, such as PRDM16, PPARγ, and PGC-1β, and its expression is repressed by cold exposure in BAT and subQ WAT (77).

In this short review, we highlighted the roles of the transcriptional regulators and miRNAs on brown and beige cell differentiation and function. While we have learned a great deal about brown and beige fat cells, there are still many unanswered questions. Recent studies suggest that cold- or β-adrenergic-stimulated induction of beige cells in WAT is transient and reversible (i.e., reversible transdifferentiation between mature white fat and beige cells) (17, 18). To achieve this plasticity, mature white fat and beige cells must have mechanisms to tightly and reciprocally regulate many of the beige-specific transcriptional regulators and miRNAs during this reversible process (**Figure 2**). As exemplified by the CEBPβ-miR-155 regulatory loop (75), delineating the intricate details of the interdependence and cross-regulation of the transcription factor and miRNA networks will provide a deeper understanding of brown and beige fat differentiation and facilitate the development of brown or beige cell-based therapy.

References

1. Frontini A, Cinti S. Distribution and development of brown adipocytes in the murine and human adipose organ. *Cell Metab* (2010) **11**:253–6. doi:10.1016/j.cmet.2010.03.004
2. Rosen ED, Spiegelman BM. What we talk about when we talk about fat. *Cell* (2014) **156**:20–44. doi:10.1016/j.cell.2013.12.012
3. Nedergaard J, Cannon B. The changed metabolic world with human brown adipose tissue: therapeutic visions. *Cell Metab* (2010) **11**:268–72. doi:10.1016/j.cmet.2010.03.007
4. Cypess AM, Lehman S, Williams G, Tal I, Rodman D, Goldfine AB, et al. Identification and importance of brown adipose tissue in adult humans. *N Engl J Med* (2009) **360**:1509–17. doi:10.1056/NEJMoa0810780
5. van Marken Lichtenbelt WD, Vanhommerig JW, Smulders NM, Drossaerts JM, Kemerink GJ, Bouvy ND, et al. Cold-activated brown adipose tissue in healthy men. *N Engl J Med* (2009) **360**:1500–8. doi:10.1056/NEJMoa0808718
6. Virtanen KA, Lidell ME, Orava J, Heglind M, Westergren R, Niemi T, et al. Functional brown adipose tissue in healthy adults. *N Engl J Med* (2009) **360**:1518–25. doi:10.1056/NEJMoa0808949
7. Zingaretti MC, Crosta F, Vitali A, Guerrieri M, Frontini A, Cannon B, et al. The presence of UCP1 demonstrates that metabolically active adipose tissue in the neck of adult humans truly represents brown adipose tissue. *FASEB J* (2009) **23**:3113–20. doi:10.1096/fj.09-133546
8. Saito M, Okamatsu-Ogura Y, Matsushita M, Watanabe K, Yoneshiro T, Nio-Kobayashi J, et al. High incidence of metabolically active brown adipose tissue in healthy adult humans: effects of cold exposure and adiposity. *Diabetes* (2009) **58**:1526–31. doi:10.2337/db09-0530
9. Kajimura S, Saito M. A new era in brown adipose tissue biology: molecular control of brown fat development and energy homeostasis. *Annu Rev Physiol* (2014) **76**:225–49. doi:10.1146/annurev-physiol-021113-170252
10. Wu J, Bostrom P, Sparks LM, Ye L, Choi JH, Giang AH, et al. Beige adipocytes are a distinct type of thermogenic fat cell in mouse and human. *Cell* (2012) **150**:366–76. doi:10.1016/j.cell.2012.05.016
11. Seale P, Bjork B, Yang W, Kajimura S, Chin S, Kuang S, et al. PRDM16 controls a brown fat/skeletal muscle switch. *Nature* (2008) **454**:961–7. doi:10.1038/nature07182
12. Lepper C, Fan CM. Inducible lineage tracing of Pax7-descendant cells reveals embryonic origin of adult satellite cells. *Genesis* (2010) **48**:424–36. doi:10.1002/dvg.20630
13. Lee YH, Petkova AP, Mottillo EP, Granneman JG. In vivo identification of bipotential adipocyte progenitors recruited by beta3-adrenoceptor activation and high-fat feeding. *Cell Metab* (2012) **15**:480–91. doi:10.1016/j.cmet.2012.03.009
14. Schulz TJ, Huang TL, Tran TT, Zhang H, Townsend KL, Shadrach JL, et al. Identification of inducible brown adipocyte progenitors residing in skeletal muscle and white fat. *Proc Natl Acad Sci U S A* (2011) **108**:143–8. doi:10.1073/pnas.1010929108
15. Wang QA, Tao C, Gupta RK, Scherer PE. Tracking adipogenesis during white adipose tissue development, expansion and regeneration. *Nat Med* (2013) **19**:1338–44. doi:10.1038/nm.3324
16. Barbatelli G, Murano I, Madsen L, Hao Q, Jimenez M, Kristiansen K, et al. The emergence of cold-induced brown adipocytes in mouse white fat depots is determined predominantly by white to brown adipocyte transdifferentiation. *Am J Physiol Endocrinol Metab* (2010) **298**:E1244–53. doi:10.1152/ajpendo.00600.2009
17. Lee YH, Petkova AP, Konkar AA, Granneman JG. Cellular origins of cold-induced brown adipocytes in adult mice. *FASEB J* (2015) **29**:286–99. doi:10.1096/fj.14-263038
18. Rosenwald M, Perdikari A, Rulicke T, Wolfrum C. Bi-directional interconversion of brite and white adipocytes. *Nat Cell Biol* (2013) **15**:659–67. doi:10.1038/ncb2740
19. Long JZ, Svensson KJ, Tsai L, Zeng X, Roh HC, Kong X, et al. A smooth muscle-like origin for beige adipocytes. *Cell Metab* (2014) **19**:810–20. doi:10.1016/j.cmet.2014.03.025
20. Lee P, Werner CD, Kebebew E, Celi FS. Functional thermogenic beige adipogenesis is inducible in human neck fat. *Int J Obes (Lond)* (2014) **38**:170–6. doi:10.1038/ijo.2013.82
21. Sharp LZ, Shinoda K, Ohno H, Scheel DW, Tomoda E, Ruiz L, et al. Human BAT possesses molecular signatures that resemble beige/brite cells. *PLoS One* (2012) **7**:e49452. doi:10.1371/journal.pone.0049452
22. Shinoda K, Luijten IH, Hasegawa Y, Hong H, Sonne SB, Kim M, et al. Genetic and functional characterization of clonally derived adult human brown adipocytes. *Nat Med* (2015) **21**:389–94. doi:10.1038/nm.3819
23. Jespersen NZ, Larsen TJ, Peijs L, Daugaard S, Homoe P, Loft A, et al. A classical brown adipose tissue mRNA signature partly overlaps with brite in the supraclavicular region of adult humans. *Cell Metab* (2013) **17**:798–805. doi:10.1016/j.cmet.2013.04.011

24. Cypess AM, White AP, Vernochet C, Schulz TJ, Xue R, Sass CA, et al. Anatomical localization, gene expression profiling and functional characterization of adult human neck brown fat. *Nat Med* (2013) **19**:635–9. doi:10.1038/nm.3112
25. Rosen ED, MacDougald OA. Adipocyte differentiation from the inside out. *Nat Rev Mol Cell Biol* (2006) **7**:885–96. doi:10.1038/nrm2066
26. Green H, Meuth M. An established pre-adipose cell line and its differentiation in culture. *Cell* (1974) **3**:127–33. doi:10.1016/0092-8674(74)90116-0
27. Todaro GJ, Green H. Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. *J Cell Biol* (1963) **17**:299–313. doi:10.1083/jcb.17.2.299
28. Farmer SR. Molecular determinants of brown adipocyte formation and function. *Genes Dev* (2008) **22**:1269–75. doi:10.1101/gad.1681308
29. Kajimura S, Seale P, Spiegelman BM. Transcriptional control of brown fat development. *Cell Metab* (2010) **11**:257–62. doi:10.1016/j.cmet.2010.03.005
30. Tontonoz P, Hu E, Spiegelman BM. Stimulation of adipogenesis in fibroblasts by PPAR gamma 2, a lipid-activated transcription factor. *Cell* (1994) **79**:1147–56. doi:10.1016/0092-8674(94)90006-X
31. Gupta RK, Arany Z, Seale P, Mepani RJ, Ye L, Conroe HM, et al. Transcriptional control of preadipocyte determination by Zfp423. *Nature* (2010) **464**:619–23. doi:10.1038/nature08816
32. Pinheiro I, Margueron R, Shukeir N, Eisold M, Fritzsch C, Richter FM, et al. Prdm3 and Prdm16 are H3K9me1 methyltransferases required for mammalian heterochromatin integrity. *Cell* (2012) **150**:948–60. doi:10.1016/j.cell.2012.06.048
33. Seale P, Kajimura S, Yang W, Chin S, Rohas LM, Uldry M, et al. Transcriptional control of brown fat determination by PRDM16. *Cell Metab* (2007) **6**:38–54. doi:10.1016/j.cmet.2007.06.001
34. Ohno H, Shinoda K, Ohyama K, Sharp LZ, Kajimura S. EHMT1 controls brown adipose cell fate and thermogenesis through the PRDM16 complex. *Nature* (2013) **504**:163–7. doi:10.1038/nature12652
35. Kajimura S, Seale P, Kubota K, Lunsford E, Frangioni JV, Gygi SP, et al. Initiation of myoblast to brown fat switch by a PRDM16-C/EBP-beta transcriptional complex. *Nature* (2009) **460**:1154–8. doi:10.1038/nature08262
36. Kajimura S, Seale P, Tomaru T, Erdjument-Bromage H, Cooper MP, Ruas JL, et al. Regulation of the brown and white fat gene programs through a PRDM16/CtBP transcriptional complex. *Genes Dev* (2008) **22**:1397–409. doi:10.1101/gad.1666108
37. Ohno H, Shinoda K, Spiegelman BM, Kajimura S. PPARgamma agonists induce a white-to-brown fat conversion through stabilization of PRDM16 protein. *Cell Metab* (2012) **15**:395–404. doi:10.1016/j.cmet.2012.01.019
38. Cohen P, Levy JD, Zhang Y, Frontini A, Kolodin DP, Svensson KJ, et al. Ablation of PRDM16 and beige adipose causes metabolic dysfunction and a subcutaneous to visceral fat switch. *Cell* (2014) **156**:304–16. doi:10.1016/j.cell.2013.12.021
39. Harms MJ, Ishibashi J, Wang W, Lim HW, Goyama S, Sato T, et al. Prdm16 is required for the maintenance of brown adipocyte identity and function in adult mice. *Cell Metab* (2014) **19**:593–604. doi:10.1016/j.cmet.2014.03.007
40. Cederberg A, Gronning LM, Ahren B, Tasken K, Carlsson P, Enerback S. FOXC2 is a winged helix gene that counteracts obesity, hypertriglyceridemia, and diet-induced insulin resistance. *Cell* (2001) **106**:563–73. doi:10.1016/S0092-8674(01)00474-3
41. Davis KE, Moldes M, Farmer SR. The forkhead transcription factor FoxC2 inhibits white adipocyte differentiation. *J Biol Chem* (2004) **279**:42453–61. doi:10.1074/jbc.M402197200
42. Paronetto MP. Ewing sarcoma protein: a key player in human cancer. *Int J Cell Biol* (2013) **2013**:642853. doi:10.1155/2013/642853
43. Tan AY, Manley JL. The TET family of proteins: functions and roles in disease. *J Mol Cell Biol* (2009) **1**:82–92. doi:10.1093/jmcb/mjp025
44. Cho J, Shen H, Yu H, Li H, Cheng T, Lee SB, et al. Ewing sarcoma gene Ews regulates hematopoietic stem cell senescence. *Blood* (2011) **117**:1156–66. doi:10.1182/blood-2010-04-279349
45. Li H, Watford W, Li C, Parmelee A, Bryant MA, Deng C, et al. Ewing sarcoma gene EWS is essential for meiosis and B lymphocyte development. *J Clin Invest* (2007) **117**:1314–23. doi:10.1172/JCI31222
46. Kim KY, Hwang YJ, Jung MK, Choe J, Kim Y, Kim S, et al. A multifunctional protein EWS regulates the expression of Drosha and microRNAs. *Cell Death Differ* (2013) **21**(1):136–45. doi:10.1038/cdd.2013.144
47. Azuma M, Embree LJ, Sabaawy H, Hickstein DD. Ewing sarcoma protein ewsr1 maintains mitotic integrity and proneural cell survival in the zebrafish embryo. *PLoS One* (2007) **2**:e979. doi:10.1371/journal.pone.0000979
48. Dutertre M, Sanchez G, De Cian MC, Barbier J, Dardenne E, Gratadou L, et al. Cotranscriptional exon skipping in the genotoxic stress response. *Nat Struct Mol Biol* (2010) **17**:1358–66. doi:10.1038/nsmb.1912
49. Paronetto MP, Minana B, Valcarcel J. The Ewing sarcoma protein regulates DNA damage-induced alternative splicing. *Mol Cell* (2011) **43**:353–68. doi:10.1016/j.molcel.2011.05.035
50. Park JH, Kang HJ, Kang SI, Lee JE, Hur J, Ge K, et al. A multifunctional protein, EWS, is essential for early brown fat lineage determination. *Dev Cell* (2013) **26**:393–404. doi:10.1016/j.devcel.2013.07.002
51. Tseng YH, Kokkotou E, Schulz TJ, Huang TL, Winnay JN, Taniguchi CM, et al. New role of bone morphogenetic protein 7 in brown adipogenesis and energy expenditure. *Nature* (2008) **454**:1000–4. doi:10.1038/nature07221
52. Park JH, Kang HJ, Lee YK, Kang H, Kim J, Chung JH, et al. Inactivation of EWS reduces PGC-1alpha protein stability and mitochondrial homeostasis. *Proc Natl Acad Sci U S A* (2015) **112**:6074–9. doi:10.1073/pnas.1504391112
53. Park JH, Lee SB. An essential role for Ewing sarcoma gene (EWS) in early white adipogenesis. *Obesity* (2015) **23**:138–44. doi:10.1002/oby.20934
54. Rajakumari S, Wu J, Ishibashi J, Lim HW, Giang AH, Won KJ, et al. EBF2 determines and maintains brown adipocyte identity. *Cell Metab* (2013) **17**:562–74. doi:10.1016/j.cmet.2013.01.015
55. Wang W, Kissig M, Rajakumari S, Huang L, Lim HW, Won KJ, et al. Ebf2 is a selective marker of brown and beige adipogenic precursor cells. *Proc Natl Acad Sci U S A* (2014) **111**:14466–71. doi:10.1073/pnas.1412685111
56. Zhao XY, Li S, Wang GX, Yu Q, Lin JD. A long noncoding RNA transcriptional regulatory circuit drives thermogenic adipocyte differentiation. *Mol Cell* (2014) **55**:372–82. doi:10.1016/j.molcel.2014.06.004
57. Wu Z, Wang S. Role of kruppel-like transcription factors in adipogenesis. *Dev Biol* (2013) **373**:235–43. doi:10.1016/j.ydbio.2012.10.031
58. Loft A, Forss I, Siersbaek MS, Schmidt SF, Larsen AS, Madsen JG, et al. Browning of human adipocytes requires KLF11 and reprogramming of PPARgamma superenhancers. *Genes Dev* (2015) **29**:7–22. doi:10.1101/gad.250829.114
59. Kong X, Banks A, Liu T, Kazak L, Rao RR, Cohen P, et al. IRF4 is a key thermogenic transcriptional partner of PGC-1alpha. *Cell* (2014) **158**:69–83. doi:10.1016/j.cell.2014.04.049
60. Dempersmier J, Sambeat A, Gulyaeva O, Paul SM, Hudak CS, Raposo HF, et al. Cold-inducible Zfp516 activates UCP1 transcription to promote browning of white fat and development of brown fat. *Mol Cell* (2015) **57**:235–46. doi:10.1016/j.molcel.2014.12.005
61. Hansen JB, Jorgensen C, Petersen RK, Hallenborg P, De Matteis R, Boye HA, et al. Retinoblastoma protein functions as a molecular switch determining white versus brown adipocyte differentiation. *Proc Natl Acad Sci U S A* (2004) **101**:4112–7. doi:10.1073/pnas.0301964101
62. Scime A, Grenier G, Huh MS, Gillespie MA, Bevilacqua L, Harper ME, et al. Rb and p107 regulate preadipocyte differentiation into white versus brown fat through repression of PGC-1alpha. *Cell Metab* (2005) **2**:283–95. doi:10.1016/j.cmet.2005.10.002
63. Calo E, Quintero-Estades JA, Danielian PS, Nedelcu S, Berman SD, Lees JA. Rb regulates fate choice and lineage commitment in vivo. *Nature* (2010) **466**:1110–4. doi:10.1038/nature09264
64. Higgins C, Chatterjee S, Cherington V. The block of adipocyte differentiation by a C-terminally truncated, but not by full-length, simian virus 40 large tumor antigen is dependent on an intact retinoblastoma susceptibility protein family binding domain. *J Virol* (1996) **70**:745–52.
65. Classon M, Kennedy BK, Mulloy R, Harlow E. Opposing roles of pRB and p107 in adipocyte differentiation. *Proc Natl Acad Sci U S A* (2000) **97**:10826–31. doi:10.1073/pnas.190343597
66. Dali-Youcef N, Mataki C, Coste A, Messaddeq N, Giroud S, Blanc S, et al. Adipose tissue-specific inactivation of the retinoblastoma protein protects against diabesity because of increased energy expenditure. *Proc Natl Acad Sci U S A* (2007) **104**:10703–8. doi:10.1073/pnas.0611568104
67. Ross SR, Choy L, Graves RA, Fox N, Solevjeva V, Klaus S, et al. Hibernoma formation in transgenic mice and isolation of a brown adipocyte cell line expressing the uncoupling protein gene. *Proc Natl Acad Sci U S A* (1992) **89**:7561–5. doi:10.1073/pnas.89.16.7561

68. De Sousa M, Porras DP, Perry CG, Seale P, Scime A. p107 is a crucial regulator for determining the adipocyte lineage fate choices of stem cells. *Stem Cells* (2014) **32**:1323–36. doi:10.1002/stem.1637
69. McDonald ME, Li C, Bian H, Smith BD, Layne MD, Farmer SR. Myocardin-related transcription factor A regulates conversion of progenitors to beige adipocytes. *Cell* (2015) **160**:105–18. doi:10.1016/j.cell.2014.12.005
70. Sun L, Xie H, Mori MA, Alexander R, Yuan B, Hattangadi SM, et al. Mir193b-365 is essential for brown fat differentiation. *Nat Cell Biol* (2011) **13**:958–65. doi:10.1038/ncb2286
71. Liu W, Bi P, Shan T, Yang X, Yin H, Wang YX, et al. miR-133a regulates adipocyte browning in vivo. *PLoS Genet* (2013) **9**:e1003626. doi:10.1371/journal.pgen.1003626
72. Trajkovski M, Ahmed K, Esau CC, Stoffel M. MyomiR-133 regulates brown fat differentiation through Prdm16. *Nat Cell Biol* (2012) **14**:1330–5. doi:10.1038/ncb2612
73. Ignotz RA, Massague J. Type beta transforming growth factor controls the adipogenic differentiation of 3T3 fibroblasts. *Proc Natl Acad Sci U S A* (1985) **82**:8530–4. doi:10.1073/pnas.82.24.8530
74. Chen Y, Siegel F, Kipschull S, Haas B, Frohlich H, Meister G, et al. miR-155 regulates differentiation of brown and beige adipocytes via a bistable circuit. *Nat Commun* (2013) **4**:1769. doi:10.1038/ncomms2742
75. Mori M, Nakagami H, Rodriguez-Araujo G, Nimura K, Kaneda Y. Essential role for miR-196a in brown adipogenesis of white fat progenitor cells. *PLoS Biol* (2012) **10**:e1001314. doi:10.1371/journal.pbio.1001314
76. Karbiener M, Pisani DF, Frontini A, Oberreiter LM, Lang E, Vegiopoulos A, et al. MicroRNA-26 family is required for human adipogenesis and drives characteristics of brown adipocytes. *Stem Cells* (2014) **32**:1578–90. doi:10.1002/stem.1603
77. Sun L, Trajkovski M. MiR-27 orchestrates the transcriptional regulation of brown adipogenesis. *Metabolism* (2014) **63**:272–82. doi:10.1016/j.metabol.2013.10.004

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

REVIEW
published: 02 March 2016
doi: 10.3389/fendo.2016.00019

Browning and Graying: Novel Transcriptional Regulators of Brown and Beige Fat Tissues and Aging

*Elisabetta Mueller**

Genetics of Development and Disease Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD, USA

Obesity represents a major risk factor for the development of a number of metabolic disorders, including cardiovascular disease and type 2 diabetes. Since the discovery that brown and beige fat cells exist in adult humans and contribute to energy expenditure, increasing interest has been devoted to the understanding of the molecular switches turning on calorie utilization. It has been reported that the ability of thermogenic tissues to burn energy declines during aging, possibly contributing to the development of metabolic dysfunction late in life. This review will focus on the recently identified transcriptional modulators of brown and beige cells and will discuss the potential impact of some of these thermogenic factors on age-associated metabolic disorders.

Keywords: age-associated metabolic dysfunction, brown fat thermogenesis, transcription factors, HSF1, FoxA3

OPEN ACCESS

Edited by:
Jun Wu,
University of Michigan, USA

Reviewed by:
Matthew J. Potthoff,
University of Iowa, USA
Robert O'Rourke,
University of Michigan Health Systems, USA

***Correspondence:**
Elisabetta Mueller
elisabettam@niddk.nih.gov

Specialty section:
This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *09 December 2015*
Accepted: *15 February 2016*
Published: *02 March 2016*

Citation:
Mueller E (2016) Browning and Graying: Novel Transcriptional Regulators of Brown and Beige Fat Tissues and Aging. Front. Endocrinol. 7:19. doi: 10.3389/fendo.2016.00019

INTRODUCTION

Obesity arises when the caloric input exceeds energy output. The principal organ that expands in response to nutritional overload is white fat, a tissue endowed with the critical evolutionary role to conserve and store energy, as triglycerides, to be deployed during periods of nutrients unavailability. White adipose depots located in diverse anatomical locations, such as in the visceral cavity and under the skin, have been shown to expand differentially in response to nutritional cues, hormonal signals, and during the aging process. In antithesis to white, brown fat, mainly present in the interscapular area in rodents and rich in mitochondria interspersed among multilocular lipid droplets, is entrusted with the function of burning chemical energy to generate the heat required to maintain core body temperatures during adverse atmospheric events, such as acute or prolonged exposure to cold (1, 2). For its thermogenic function, brown fat tissue relies on UCP1, a mitochondrial protein that uncouples oxidative phosphorylation from ATP synthesis, generating mitochondrial proton leak leading to energy dissipation (3). For a number of years, it had been suspected that an additional type of fat cells existed embedded within some of the white depots and thought to be responsible for the heterogeneous, mixed histological appearance observed after changes in ambient temperatures or in response to certain pharmacological stimuli; such elusive cells have been recently isolated in mice and called beige because of their intermediate functional characteristics between white and brown adipocytes (4). Beige cells have a smooth muscle-like developmental origin (5), deriving from precursors distinct from those known to give rise to white or brown fat, possess specific gene signatures, and are contradistinguished by unique markers, such as the developmental factor Tbx1 (4, 6). In response to cold temperatures or β-adrenergic stimuli, beige cells present in subcutaneous white tissue can promptly switch from energy storage functions to programs that initiate calorie burning through the induction of a futile cycle that involves creatine metabolism (7), in addition

to the activation of`UCP1-dependent thermogenic programs characteristic of brown fat cells. Because of the ability of these beige cells to mount a rapid thermogenic response, together with brown fat cells, they have been considered contributors to the overall ability of organisms to expend energy (8, 9).

TRANSCRIPTIONAL MODULATORS OF THERMOGENESIS

The functional importance of brown fat for heat generation to protect animals from the perils of exposure to low temperatures in the wilderness has been recognized for decades and has represented the focus of studies by morphologists and biochemists for a number of years. However, only in the last 15 years, there has been an improvement in our understanding of the molecular mechanisms that guide the development and govern the function of thermogenic tissues. Recent radiographic and molecular evidence establishing the presence of brown and beige tissues also in adult humans (10–14), in addition to neonates and rodents, has sparked an interest in the possibility of identifying molecular switches to be targeted for the induction of energy consumption as anti-obesity intervention (15).

PGC1α

One of the first advances in this field was made in the late 90s in the laboratory of Bruce Spiegelman, where Puigserver and colleagues performed a yeast two-hybrid screen to identify novel interactors of the master regulator of adipocyte differentiation, PPARγ, in brown fat tissue (16). This work led to the characterization of the peroxisome proliferator-activated receptor gamma coactivator 1α, PGC1α, as a novel coregulator of UCP1 expression, rapidly induced in brown fat upon cold exposure. It has now been recognized that PGC1α can modulate metabolism through its interaction with a number of transcription factors on promoters of genes encoding for proteins involved in metabolic functions, such as mitochondrial and peroxisomal remodeling and biogenesis, and β-oxidation in brown fat (17–19). Analysis of the *in vivo* requirements of PGC1α has demonstrated a direct involvement of PGC1α in thermogenesis and in beige fat biology as shown by the blunted response to cold challenge of mice with fat-specific deletion of PGC1α and by the decreased levels of thermogenic markers, such as UCP1, in the inguinal fat pads (iWAT) of Fat-PGC1α KO mice (20). In addition to the classic inducers of brown fat function, such as β-adrenergic stimuli and CREB signaling, known to regulate PGC1α mRNAs, it has been demonstrated that PGC1α protein levels can be regulated in an autocrine/paracrine fashion by factors such as FGF21, prompting PGC1α-dependent browning of iWAT (21). Given the role of PGC1α as a critical transcriptional coregulator of energy balance, a large emphasis has been placed on the identification of the upstream factors and signaling pathways that activate PGC1α and on the characterization of PGC1α downstream targets to ultimately enhance energy expenditure.

PRDM16

To further understand the mechanisms regulating brown fat physiology and to identify the key contributors to brown fat identity, a few years after the discovery of PGC1α, the Spiegelman laboratory carried out a systematic search for transcriptional regulators differentially expressed in brown fat tissue in comparison to epididymal white depots. Through this analysis, Seale and colleagues identified the zinc finger protein PR domain containing 16, PRDM16, as a brown fat selective cofactor able to activate brown fat gene programs (22). Mechanistically, it was demonstrated that PRDM16 can modulate UCP1 expression *via* its direct interaction with PGC1α and β. Studies of putative brown fat depots of WT and PRDM16 global knockout mice at embryonic day 17 showed reduced expression of thermogenic genes and elevation of muscle-specific genes supporting a role for PRDM16 as an early determinant of brown fat lineage and as a negative regulator of muscle development (23). Analysis of transgenic mice with conditional expression of PRDM16 in white fat driven by the promoter of the fatty acid binding protein, aP2, demonstrated that PRDM16 is also involved in the development of beige adipocytes in subcutaneous fat and that it induces thermogenic genes, such as Ucp1, Cidea, Cox8b, and Elovl3, in these cells. The molecular changes induced by *in vivo* overexpression of PRDM16 in fat tissue were associated with increased whole body energy expenditure and protection from the weight gain induced by high-fat diet, indicating a selective role for PRDM16 in adaptive thermogenic responses mediated by beige cells (24). Conversely, analysis of mice with ablation of PRDM16 selectively in adipose tissues demonstrated that the absence of PRDM16 is associated with a switch in the molecular and morphological characteristics of inguinal fat into those of epididymal WAT. Mice with fat selective ablation of PRDM16 exposed to high-fat and -carbohydrate diet for 16 weeks maintained at room temperature developed obesity and insulin resistance (25). Of note, the effects of PRDM16 ablation in fat tissues driven by the adiponectin promoter appeared to be inguinal fat specific, causing the depletion in beige cells but not altering brown fat tissue functionality. Overall these gain- and loss-of-function studies performed both *in vitro* and *in vivo* have provided evidence for a role of PRDM16 in the regulation of brown and beige fat tissues maintenance and in restricting muscle developmental programs.

PRDM3

Recently, Harms and colleagues in the laboratory of Seale demonstrated that PRDM3, a factor closely related to PRDM16, plays a role in establishing brown fat identity (26). PRDM3 induces UCP1 and PGC1α when overexpressed in C2C12 cells. PRDM3 levels appear to be highly regulated and are shown to decline in brown fat tissue as the mice age, with the highest levels observed at embryonic stage 18. These data suggest that PRDM3 may complement the function of PRDM16 during early developmental phases. This possibility is supported by the evidence that double knockout mice for both PRDM3 and PRDM16 have marked decrease in brown fat formation. Recent molecular data have demonstrated that, similarly to PRDM16, PRDM3 can alter chromatin structure at brown fat gene-specific promoters *via* its interaction with MED1, thereby enhancing target gene expression (27). Given that PRDM3 has been reported to play broad fundamental functions in heterochromatin maintenance (28), future studies may reveal how PRDM3 regulates gene

transcription in a depot-selective manner and whether it may rely on additional – yet to be identified – tissue-specific interacting partners to achieve brown fat tissue effects.

TAF7L

Another important molecular determinant of brown fat was recently discovered in the laboratory of Robert Tijan. Zhou and colleagues reported that the TATA-binding protein-associated factor 7L (TAF7L), previously shown to be a critical regulator of white adipose tissue differentiation, also functions as a molecular switch between brown fat and muscle lineages (29). Through studies involving histological and molecular analysis, Zhou and collaborators showed that TAF7LKO mice have decreased brown fat tissue and increased muscle mass. These effects are associated with upregulation of genes involved in skeletal muscle development and function, and a decrease in brown fat gene expression programs. Gain- and loss-of-TAF7L-function studies performed in cell lines, such as 10T1/2 and C2C12, further demonstrated that TAF7L modulates mesenchymal cells fate. To determine the mechanisms through which TAFL4 may tip the balance toward the brown adipose lineage, the authors performed immune-precipitation studies and identified a fat-specific complex containing only a subset of canonical TFIID-TAF subunits co-purifying with TAF and PPARγ. These TAF7L-containing TFIID complexes appear to mediate DNA looping between distal enhancers and core promoter elements in adipose tissues, suggesting that TAF7L is a tissue-specific subunit of TFIID involved in the coordination of long-range chromatin interactions to specify brown fat lineage. These studies suggest the possibility of the existence of novel additional fat depot-selective TFIID complexes each involved in the determination of a distinct fat cell type.

Zfp516

In search for novel regulators of thermogenic tissue function, Dempersmier and colleagues in the group of Hei Sook Sul performed a high-throughput screen of factors binding to the UCP1 promoter. Through this analysis, it was shown that the Kruppel-like zinc finger protein Zfp516 directly binds to a proximal region of the UCP1 promoter present at −70 to −45 bp from the start site (30). More detailed molecular analysis revealed that Zfp516 is induced upon cold exposure and that it regulates UCP1 gene expression in complex with the coactivator PRDM16. Analysis of promoter sequences upstream of a number of brown fat genes revealed the presence of sequence similarities with the CCACT DNA stretch identified in the UCP1 promoter, and bound by Zfp516, and ChiP studies confirmed the ability of Zfp516 to occupy a similar motif also in the promoter of PGC1α and Cox8b. Analysis of inguinal fat depots of mice with overexpression of Zfp516 driven by the −5.4 aP2 promoter kept at room temperatures showed increased clusters of cells containing multilocular lipid droplets, elevated UCP1 staining, consistent with increased amounts of beige fat cells. Furthermore, it was shown that these mutant mice have higher oxygen consumption levels. Assessment of the specific tissues contributing to increased respiratory activity in mice overexpressing Zfp516 revealed a selective effect of Zfp516 in inguinal fat, while no changes in respiration were observed in brown fat tissue. Consistent with the effects resulting from increased number of beige fat cells in iWAT, Zfp516 transgenic mice showed elevation in their core basal temperatures, had improved cold tolerance, and were protected from diet induced obesity. Global ablation of Zfp516, although lethal, allowed the analysis of the effects of ablation of Zfp516 on the early developmental phases of brown fat formation. Embryos lacking Zfp516 at day 20.5 showed reduced brown fat mass and decreased brown fat gene expression in presumptive BAT. Overall, the data obtained in the two genetic models described highlight a dual role for Zfp516 in controlling the development of brown tissue and also in the modulation of beige fat cells function in adult mice.

P107

To characterize novel transcriptional regulators involved in the commitment of stem cells toward the adipocyte lineage, Scimè and colleagues (31) investigated the role of the Rb family member p107, previously shown to modulate white adipocyte differentiation through its repression of PGC1α (32). The expression of p107 appeared to be tightly controlled given that it was found to be expressed selectively in white fat stem cells but completely absent from those giving rise to brown fat. Loss-of-function studies demonstrated that p107 ablation is permissive for the formation of brown fat adipocytes and required for PRDM16-mediated brown fat programing of mesenchymal stem cells. Additional studies will determine the specific mechanisms through which this transcriptional corepressor ultimately influences brown versus white fate choices.

Ewing Sarcoma

The group of Sean Lee recently demonstrated an unexpected, novel role for the Ewing Sarcoma (EWS) factor in brown fat biology (33). Park and colleagues showed that in the absence of EWS, pups die within 24 h after their birth and that the only pathological abnormality they manifest is a marked reduction in brown fat tissue. Histological analysis of the presumptive BAT present in these embryos revealed reduced UCP1 staining, loss of the characteristic multilocular lipid droplet phenotype and molecular studies demonstrated decreased levels of factors such as PGC1α and PRDM16. Analysis of brown adipocyte differentiation in immortalized preadipocytes generated from EWS WT and null newborn pups showed that EWS null cells are unable to differentiate when treated with a differentiation-inducing cocktail. To gain further clues to mechanistically define how EWS affects differentiation, the authors studied the patterns of gene expression in WT and EWS null cells and observed that EWS deficiency affected the mRNA levels of the gene encoding for the bone morphogenic protein 7 (BMP7), as well as for CEBPβ during the initial stages of differentiation. Specific analysis of the effects of EWS on the expression of this cell fate determination factor revealed that EWS occupies the BMP7 promoter when in a complex with YBX1, a multifunctional protein involved in both transcription and translation. Complementation studies employing exogenous BMP7 restored differentiation in EWS null cells demonstrating that lack of BMP7 constituted their differentiation block. To assess whether lack of EWS would affect beige cells function, the authors studied EWS heterozygous mice and showed that they have significantly reduced beige gene activation

in response to rosiglitazone and β-adrenergic stimulation. These studies demonstrate novel tissue-specific functions of EWS as a brown fat determination factor through the control of the early cell fate steps involving the activation of BMP7 and suggest a possible role in EWS in the activation of beige cells in response to pharmacological treatment. Studies of fat tissue conditional animal models will permit the assessment of the contribution of EWS on mature brown fat functionality.

Foxa3

To specifically identify novel transcriptional regulators involved in early events regulating fat differentiation, Xu and colleagues performed a genetic screen using a si-RNA library and assessed the effects of knocking down the expression of each forkhead protein on adipogenesis (34). This systematic analysis led to the demonstration that the winged helix factor Foxa3 is a novel modulator of adipocyte differentiation and of its function. Analysis of WT and Foxa3 null mice revealed that the absence of Foxa3 *in vivo* can decrease the expansion of the visceral adipose tissue compartment in response to HFD but does not reduce the enlargement of the subcutaneous fat tissue. This depot-selective protection from the development of intra-abdominal obesity during high-fat diet regimens (34) was associated with improved insulin sensitivity. Analysis of the long-term effects of Foxa3 deficiency on late onset metabolic dysfunction revealed that mice lacking Foxa3 kept on a normal chow diet for more than a year have increased browning of subcutaneous tissue, as indicated by increased UCP1 staining in iWAT and elevated expression of a number of classic thermogenic and lipid oxidation genes in addition to beige specific markers such as Tbx1. These morphological and gene expression changes in iWAT, consistent with the acquisition of a beige phenotype, were associated with increased oxygen consumption (35). Analysis of Foxa3's mode of action indicated that Foxa3 controls calorie hoarding through the upregulation of PPARγ levels *via* transcriptional cooperation with CEBPs, as revealed by coimmunoprecipitation and ChIP studies demonstrating that CEBPs interact with Foxa3 at the PPARγ promoter. In addition, Foxa3 suppresses thermogenesis in BAT and iWAT through interference with CREB-mediated induction of PGC1α expression. Analysis of the changes in Foxa3 mRNA levels in response to HFD and during the course of aging revealed that Foxa3 is fat depot-selectively induced in response to nutritional and developmental cues. Specifically, while diets rich in fat lead to increased Foxa3 mRNA levels only in the visceral fat depot, and not in subcutaneous adipose tissue, the aging process is associated with increased Foxa3 levels most prominently in subcutaneous fat and in BAT. Given the role that other Foxa family members have been shown to play as pioneering factors on chromatin remodeling (36), it is plausible that Foxa3 may exert its calorie hoarding function through a number of other mechanisms in addition to those involving the control of PPARγ and PGC1α and their downstream programs. Ongoing studies in the laboratory are characterizing novel Foxa3's gene targets in distinct fat subtypes and unraveling the signals regulating the unique pattern of expression of Foxa3 in selected depots.

HSF1

To identify novel modulators of fat tissue browning, Ma and colleagues used PGC1α expression as a read-out and performed an *in silico* screen of possible functional binding sites located upstream of the PGC1α promoter (37). This analysis led to the identification of a putative heat shock motif (HSE) and studies involving overexpression of HSF1, or HSF1 activation *via* its agonists, in isolated cells and *in vivo*, demonstrated that HSF1 induces PGC1α and that it can turn on a cascade of mitochondrial PGC1α-dependent gene programs. ChIP assays revealed that upon cold exposure, HSF1 occupies the heat shock element present in the PGC1α promoter in both BAT and iWAT, and coimmunoprecipitation studies demonstrated that HSF1 physically interacts and functionally cooperates with PGC1α in a feed-forward regulatory loop on the PGC1α promoter. Given the *in vitro* evidence of HSF1 involvement in PGC1α activation, it was hypothesized that absence of HSF1 *in vivo* would bring about abnormal metabolic functions. Analysis of the effects of ablation of HSF1 on a number of parameters demonstrated that HSF1 KO mice are more sensitive to low temperatures. Furthermore, morphological and molecular analysis of adipose tissues of WT and HSF1 KO mice revealed increased lipid deposition in BAT and iWAT of mice lacking HSF1, reduced UCP1 staining, decreased UCP1 protein levels in inguinal fat tissues, and reduced thermogenic and β-oxidation expression programs, indicating overall reduced brown and beige tissues functionality. Genetic experiments involving gain-of-HSF1-function selectively in iWAT and in muscle enabled through adenoviral-mediated expression of HSF1 provided further confirmatory evidence that HSF1 increases mitochondrial and thermogenic gene programs and leads to elevation in energy consumption. Analysis of the consequences of pharmacological activation of HSF1 during high-fat diet *via* celastrol, a natural HSF1 agonist present in herbal extracts used in Chinese medicine, revealed enhanced subcutaneous fat browning, increased thermogenesis and energy consumption, and reduced adipose tissue expansion, providing a proof of concept that HSF1 may represent a possible target in obesity prevention. Of note, it has been recently reported that celastrol is also efficacious in inducing weight loss in obese mice through a mechanisms involving appetite suppression *via* leptin sensitization (38). Although it appears that celastrol prevents and treats obesity through distinct pathways, the evidence that obese mice treated with celastrol lose weight even when their food intake is equalized to that of controls suggest that celastrol may activate the HSF1 pathway and increase energy expenditure also in obese states. Ongoing studies are dissecting genetically the contribution of distinct organs to HSF1-mediated modulation of energy expenditure and discriminating between HSF1 effects on central regulatory signals and those on peripheral organs.

KLF11

Loft and colleagues in the Mandrup laboratory have recently demonstrated a novel role for the Kruppel-like factor, KLF11, in the control of browning of human adipocytes (39). KLF11, also known as Mody 1, had been previously shown to regulate pancreatic β-cell function and variants of this gene have been

shown to be associated with diabetes. The Mandrup group has now provided novel evidence that KLF11 is highly expressed in beige cells and that it is a direct target of agonist liganded PPARγ. This factor induces reprograming of beige cells in response to TZDs through functional cooperation with PPARγ and selective activation of brown fat super-enhancers, suggesting a novel role for KLF11 in the stabilization of the expression of beige gene targets in human cells.

IRF4

Kong and colleagues in the Rosen group recently characterized the interferon regulatory factor 4 (IRF4) as a transcriptional regulator of adaptive thermogenesis (40). IRF4 had been previously studied in the context of white adipocytes and shown to have an anti-lipogenic activity through the control of genes such as adipocyte triglyceride lipase and hormone-sensitive lipase (41). In the new analysis, Kong and colleagues show that IRF4 is induced by cold exposure in brown fat cells. This pattern of expression prompted the assessment of the function of IRF4 also in BAT, in addition to that previously described in white fat. Analysis of brown fat-specific IRF4 transgenic mice demonstrated that IRF4 is sufficient to increase energy expenditure and to protect from diet-induced obesity. IRF4 overexpressing mice show smaller brown fat size, reduced lipid stores, and increased expression of a selected number of genes involved in thermogenesis. Of note, IRF4 overexpression induced browning in epididymal WAT but not in iWAT. Conversely, selective ablation of IRF4 driven by the UCP1 promoter was associated with reduced energy expenditure, increased predisposition to diet induced obesity and cold intolerance. Interestingly, in contrast to the selective "beigeing" effects in epididymal WAT of IRF4 overexpressing mice, UCP1 promoter-driven ablation of IRF4 reduced beige cells in iWAT of mice chronically exposed to low temperatures. Mechanistically, IRF4 cooperates with PGC1α, *via* reciprocal gene expression regulation, direct protein–protein interaction and transcriptional cooperation in the induction of thermogenic genes. These data suggest that IRF4 coordinates heat generation by modulating lipolysis in white fat and thermogenic gene expression programs in brown and beige fat depots.

Zic 1

It has been recently suggested that Zic 1, a member of a family of zinc finger factors of the cerebellum previously involved in a variety of developmental processes, may also play a role in brown fat development and function. Zic 1 involvement in BAT biology was initially suggested when it was identified as one of the top genes differentially expressed in brown fat in comparison to white fat (22). A number of studies have now provided evidence that Zic 1 expression is restricted to brown fat cells, even in undifferentiated states, and that its levels are independent of changes in temperatures and dietary states, proposing Zic 1 as a selective marker of brown adipose tissue. *In vitro* analyses have only recently started to address the function of this transcriptional regulator. Studies performed by Nedergaard's group have demonstrated that Zic 1 downregulation *via* knockdown is associated with blunted UCP1 induction in response to norepinephrine stimulation (42). Additional functional and physiological studies possibly involving genetic mouse models with conditional modulation of Zic 1 levels in brown fat will define more precisely whether Zic 1 is strictly required for BAT development or for its functionality in adulthood or for both.

Zbtb16

To identify novel regulators of adaptive thermogenesis, Plaisier and colleagues analyzed transcriptomes coordinately regulated upon cold exposure in both BAT and muscle and identified Zbtb16 among the most robustly expressed genes in these conditions (43). Zbtb16 (also called PLZF) had previously emerged from a genome-wide screen of genes modulating adipocyte differentiation (44) and shown to suppress differentiation in L1 cells; however, no studies linked Zbtb16 to BAT function. In the studies by Plaisier and colleagues, overexpression of Zbtb16 was associated with increased expression of brown fat makers, such as UCP1, PGC1α, PPARα, and mitochondrial and β-oxidation genes. Detailed analysis of mitochondrial energetics revealed increased respiration in cells, such as primary adipocytes and C2C12 myoblasts, with overexpression of Zbtb16 and showed increased mitochondrial biogenesis. Survey of the expression of Zbtb16 in tissues such as white fat and heart of 100 different mouse strains indicated that Zbtb16 levels inversely correlate to metabolic traits, such as increased body weight and fat mass. The generation and the detailed characterization of conditional genetic models of this factor will aid in the definition of the physiological function of Zbtb16 in brown and/or beige fat physiology.

THERMOGENESIS AND AGING

It has been reported that the aging process is associated with a selective expansion and redistribution of white fat stores specifically in the visceral compartment and with progressive metabolic decline (45). Evidence indicates that not only the amount of thermogenic fat tissues decreases during the aging process due to apoptosis but that its thermogenic functionality is reduced in old animals and in aged humans due, at least in part, to decreased sensitivity to β-adrenergic stimulation (46). Specifically in mice, it has been observed that as they approach mid age, their brown adipose tissue gradually involutes. It has been hypothesized that the progressive atrophy observed may be endogenously controlled and determined by the decrease in gonadal hormones, known to support the viability and functionality of BAT in maturity, and by the tight negative control exerted by cortisol (46). Given the impact of brown fat and beige cells on energy balance, preservation, and/or restoration of functional brown and beige tissues may be an attractive strategy to protect from obesity and metabolic disorders that arise at an old age (47); however, to date, the main transcriptional switches that slow down, or accelerate, the involution of this tissue during late life stages have not been identified. Although the majority of the factors reviewed here have been shown to affect either the development of thermogenic tissues or their function in adult mice, only a few of them have been fully characterized in mid age or older mice. Specifically, it has been analyzed the long-term impact of the ablation PRDM16 specifically in the brown adipose lineage (Myf5-ΔPRDM16) (26). These studies revealed that 11-month-old Myf-ΔPRDM16 KO

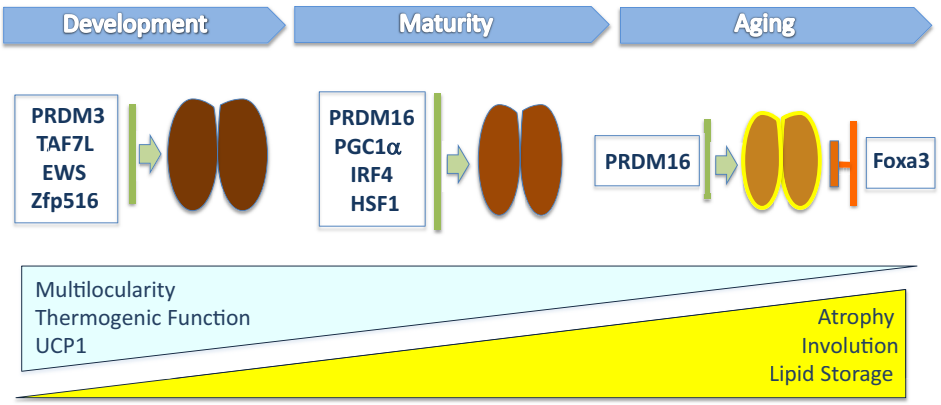


FIGURE 1 | This illustration depicts the transcriptional regulators that have been shown to modulate *in vivo* brown fat tissue during organogenesis, maturation, and involution. The blue triangle indicates the progressive loss of multilocular lipid content, thermogenic function, and UCP1 levels during aging, while the yellow one indicates the increased whitening of BAT, composed of cells containing large lipid deposits, and atrophy observed in old mice.

mice have increased whitening of brown fat tissue and reduced oxygen consumption levels; however, despite their decreased energy output and dysfunctional BAT, they do not show increased predisposition to obesity compared to age-matched WT controls. It remains to be determined whether the absence of an obese phenotype in these mice is animal model-dependent or whether compensatory mechanisms counteract the accretion of white fat stores in these mice. Indeed, given that PRDM3 offsets the effects of PRDM16 ablation during early development, it is conceivable that other factors, or other PRDM family members, may partly complement and compensate for the loss of PRDM16 late in life. Alternatively, it is possible that obesity would become evident only at later stages in life due to small, cumulative effects on fat stores. Nonetheless, metabolic studies of Myf-ΔPRDM16 mice older than 1 year or of old PRDM16 fat KO mice with impoverished beige cell contingent and measurement of longevity in diverse PRDM16 KO and transgenic mice will better define the role of PRDM16 in age-associated metabolic dysfunction and lifespan extension.

Analysis of the metabolic profiles of WT and Foxa3 null mice have provided supportive evidence that increasing beige fat cells in aging may be beneficial in preventing metabolic dysfunction and even extending life. *In vivo* ablation of Foxa3 was shown to be associated with a lean phenotype, increased energy expenditure, and improved insulin sensitivity in mice older than 1 year fed a normal chow diet (35). The metabolic beneficial effects of Foxa3 ablation appeared to be associated with increased browning of iWAT in 14-month-old mice. In addition, it was observed that ablation of Foxa3 positively impacted longevity contributing to lifespan extension. Analysis of gene expression revealed that Foxa3 levels are robustly upregulated selectively in inguinal and brown fat depots in 14-month-old mice compared to levels observed in young 2-month-old mice. Overall, the analysis of the consequences of Foxa3 ablation in late life stages suggests that Foxa3 may constitute a potential target to counteract aging and its associated metabolic pathologies (**Figure 1**). These studies support the idea that systematic metabolic profiling of knockout or transgenic mice with altered expression of transcriptional regulators of brown and beige fat during the aging process will permit a more detailed mechanistic understanding of the impact of thermogenic tissues on metabolic disorders that occur late in life.

It has been demonstrated that brown and beige thermogenic cells increase energy expenditure and suggested that turning on these cells to boost consumption may represent a successful approach to combat the rampant obesity epidemic (8, 15). As we improve our understanding of the key molecular switches that can modulate thermogenesis, a number of questions remain to be answered to fully exploit the therapeutic potential of these cells in counteracting metabolic dysfunction at different life stages. For example, it needs to be clarified how changes in hormonal signals occurring during the aging process can contribute to the progressive involution of brown fat tissue. Given some of the reports suggesting that cortisol levels increase during aging (48), assessing how steroid hormones may regulate the expression of thermogenic effectors late in life could provide clues on some of the mechanisms through which endogenous signals unable or disable the functionality of brown and beige cells. Furthermore, the generation of new animal models to allow the modulation of the expression of specific transcriptional regulators in a spatiotemporal-restricted manner – fat depot-selectively at an old age – will be beneficial in determining which thermogenic factors may be targeted to prevent the functional decay of brown and beige fat cells shown to occur during the aging process.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

ACKNOWLEDGMENTS

I am thankful to Pasha Sarraf, Xinran Ma, and Lingyan Xu for helpful discussions and to Camilla Sarraf-Mueller for support during the preparation of this manuscript. The work performed in my laboratory cited in this review was funded by the Intramural Research Program of the National Institutes of Health and the National Institute of Diabetes and Digestive and Kidney Diseases.

REFERENCES

1. Farmer SR. Transcriptional control of adipocyte formation. *Cell Metab* (2006) **4**:263–73. doi:10.1016/j.cmet.2006.07.001
2. Mueller E. Understanding the variegation of fat: novel regulators of adipocyte differentiation and fat tissue biology. *Biochim Biophys Acta* (2014) **1842**:352–7. doi:10.1016/j.bbadis.2013.05.031
3. Farmer SR. Molecular determinants of brown adipocyte formation and function. *Genes Dev* (2008) **22**:1269–75. doi:10.1101/gad.1681308
4. Wu J, Bostrom P, Sparks LM, Ye L, Choi JH, Giang AH, et al. Beige adipocytes are a distinct type of thermogenic fat cell in mouse and human. *Cell* (2012) **150**:366–76. doi:10.1016/j.cell.2012.05.016
5. Long JZ, Svensson KJ, Tsai L, Zeng X, Roh HC, Kong X, et al. A smooth muscle-like origin for beige adipocytes. *Cell Metab* (2014) **19**:810–20. doi:10.1016/j.cmet.2014.03.025
6. de Jong JM, Larsson O, Cannon B, Nedergaard J. A stringent validation of mouse adipose tissue identity markers. *Am J Physiol Endocrinol Metab* (2015) **308**:E1085–105. doi:10.1152/ajpendo.00023.2015
7. Kazak L, Chouchani ET, Jedrychowski MP, Erickson BK, Shinoda K, Cohen P, et al. Creatine-driven substrate cycle enhances energy expenditure and thermogenesis in beige fat. *Cell* (2015) **163**:643–55. doi:10.1016/j.cell.2015.09.035
8. Boss O, Farmer SR. Recruitment of brown adipose tissue as a therapy for obesity-associated diseases. *Front Endocrinol* (2012) **3**:14. doi:10.3389/fendo.2012.00014
9. Wu J, Cohen P, Spiegelman BM. Adaptive thermogenesis in adipocytes: is beige the new brown? *Genes Dev* (2013) **27**:234–50. doi:10.1101/gad.211649.112
10. Cypess AM, Lehman S, Williams G, Tal I, Rodman D, Goldfine AB, et al. Identification and importance of brown adipose tissue in adult humans. *N Engl J Med* (2009) **360**:1509–17. doi:10.1056/NEJMoa0810780
11. van Marken Lichtenbelt WD, Vanhommerig JW, Smulders NM, Drossaerts JM, Kemerink GJ, Bouvy ND, et al. Cold-activated brown adipose tissue in healthy men. *N Engl J Med* (2009) **360**:1500–8. doi:10.1056/NEJMoa0808718
12. Virtanen KA, Lidell ME, Orava J, Heglind M, Westergren R, Niemi T, et al. Functional brown adipose tissue in healthy adults. *N Engl J Med* (2009) **360**:1518–25. doi:10.1056/NEJMoa0808949
13. Skarulis MC, Celi FS, Mueller E, Zemskova M, Malek R, Hugendubler L, et al. Thyroid hormone induced brown adipose tissue and amelioration of diabetes in a patient with extreme insulin resistance. *J Clin Endocrinol Metab* (2010) **95**:256–62. doi:10.1210/jc.2009-0543
14. Jespersen NZ, Larsen TJ, Peijs L, Daugaard S, Homoe P, Loft A, et al. A classical brown adipose tissue mRNA signature partly overlaps with brite in the supraclavicular region of adult humans. *Cell Metab* (2013) **17**:798–805. doi:10.1016/j.cmet.2013.04.011
15. Cypess AM, Kahn CR. Brown fat as a therapy for obesity and diabetes. *Curr Opin Endocrinol Diabetes Obes* (2010) **17**:143–9. doi:10.1097/MED.0b013e328337a81f
16. Puigserver P, Wu Z, Park CW, Graves R, Wright M, Spiegelman BM. A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. *Cell* (1998) **92**:829–39. doi:10.1016/S0092-8674(00)81410-5
17. Wu Z, Puigserver P, Andersson U, Zhang C, Adelmant G, Mootha V, et al. Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. *Cell* (1999) **98**:115–24. doi:10.1016/S0092-8674(00)80611-X
18. Bagattin A, Hugendubler L, Mueller E. Transcriptional coactivator PGC-1alpha promotes peroxisomal remodeling and biogenesis. *Proc Natl Acad Sci U S A* (2010) **107**:20376–81. doi:10.1073/pnas.1009176107
19. Puigserver P, Spiegelman BM. Peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1 alpha): transcriptional coactivator and metabolic regulator. *Endocr Rev* (2003) **24**:78–90. doi:10.1210/er.2002-0012
20. Kleiner S, Mepani RJ, Laznik D, Ye L, Jurczak MJ, Jornayvaz FR, et al. Development of insulin resistance in mice lacking PGC-1α in adipose tissues. *Proc Natl Acad Sci U S A* (2012) **109**:9635–40. doi:10.1073/pnas.1207287109
21. Fisher FM, Kleiner S, Douris N, Fox EC, Mepani RJ, Verdeguer F, et al. FGF21 regulates PGC-1α and browning of white adipose tissues in adaptive thermogenesis. *Genes Dev* (2012) **26**:271–81. doi:10.1101/gad.177857.111
22. Seale P, Kajimura S, Yang W, Chin S, Rohas LM, Uldry M, et al. Transcriptional control of brown fat determination by PRDM16. *Cell Metab* (2007) **6**:38–54. doi:10.1016/j.cmet.2007.06.001
23. Seale P, Bjork B, Yang W, Kajimura S, Chin S, Kuang S, et al. PRDM16 controls a brown fat/skeletal muscle switch. *Nature* (2008) **454**:961–7. doi:10.1038/nature07182
24. Seale P, Conroe HM, Estall J, Kajimura S, Frontini A, Ishibashi J, et al. Prdm16 determines the thermogenic program of subcutaneous white adipose tissue in mice. *J Clin Invest* (2011) **121**:96–105. doi:10.1172/JCI44271
25. Cohen P, Levy JD, Zhang Y, Frontini A, Kolodin DP, Svensson KJ, et al. Ablation of PRDM16 and beige adipose causes metabolic dysfunction and a subcutaneous to visceral fat switch. *Cell* (2014) **156**:304–16. doi:10.1016/j.cell.2013.12.021
26. Harms MJ, Ishibashi J, Wang W, Lim HW, Goyama S, Sato T, et al. Prdm16 is required for the maintenance of brown adipocyte identity and function in adult mice. *Cell Metab* (2014) **19**:593–604. doi:10.1016/j.cmet.2014.03.007
27. Harms MJ, Lim HW, Ho Y, Shapira SN, Ishibashi J, Rajakumari S, et al. PRDM16 binds MED1 and controls chromatin architecture to determine a brown fat transcriptional program. *Genes Dev* (2015) **29**:298–307. doi:10.1101/gad.252734.114
28. Pinheiro I, Margueron R, Shukeir N, Eisold M, Fritzsch C, Richter FM, et al. Prdm3 and Prdm16 are H3K9me1 methyltransferases required for mammalian heterochromatin integrity. *Cell* (2012) **150**:948–60. doi:10.1016/j.cell.2012.06.048
29. Zhou H, Wan B, Grubisic I, Kaplan T, Tjian R. TAF7L modulates brown adipose tissue formation. *Elife* (2014) **3**:e02811. doi:10.7554/eLife.02811
30. Dempersmier J, Sambeat A, Gulyaeva O, Paul SM, Hudak CS, Raposo HF, et al. Cold-inducible Zfp516 activates UCP1 transcription to promote browning of white fat and development of brown fat. *Mol Cell* (2015) **57**:235–46. doi:10.1016/j.molcel.2014.12.005
31. De Sousa M, Porras DP, Perry CG, Seale P, Scimè A. p107 is a crucial regulator for determining the adipocyte lineage fate choices of stem cells. *Stem Cells* (2014) **32**:1323–36. doi:10.1002/stem.1637
32. Scime A, Grenier G, Huh MS, Gillespie MA, Bevilacqua L, Harper ME, et al. Rb and p107 regulate preadipocyte differentiation into white versus brown fat through repression of PGC-1alpha. *Cell Metab* (2005) **2**:283–95. doi:10.1016/j.cmet.2005.10.002
33. Park JH, Kang HJ, Kang SI, Lee JE, Hur J, Ge K, et al. A multifunctional protein, EWS, is essential for early brown fat lineage determination. *Dev Cell* (2013) **26**:393–404. doi:10.1016/j.devcel.2013.07.002
34. Xu L, Panel V, Ma X, Du C, Hugendubler L, Gavrilova O, et al. The winged helix transcription factor Foxa3 regulates adipocyte differentiation and depot-selective fat tissue expansion. *Mol Cell Biol* (2013) **33**:3392–9. doi:10.1128/MCB.00244-13
35. Ma X, Xu L, Gavrilova O, Mueller E. Role of forkhead box protein A3 in age-associated metabolic decline. *Proc Natl Acad Sci U S A* (2014) **111**:14289–94. doi:10.1073/pnas.1407640111
36. Friedman JR, Kaestner KH. The Foxa family of transcription factors in development and metabolism. *Cell Mol Life Sci* (2006) **63**:2317–28. doi:10.1007/s00018-006-6095-6
37. Ma X, Xu L, Alberobello AT, Gavrilova O, Bagattin A, Skarulis M, et al. Celastrol protects against obesity and metabolic dysfunction through activation of a HSF1-PGC1α transcriptional axis. *Cell Metab* (2015) **22**:695–708. doi:10.1016/j.cmet.2015.08.005
38. Liu J, Lee J, Salazar Hernandez MA, Mazitschek R, Ozcan U. Treatment of obesity with celastrol. *Cell* (2015) **161**:999–1011. doi:10.1016/j.cell.2015.05.011
39. Loft A, Forss I, Siersbaek MS, Schmidt SF, Larsen AS, Madsen JG, et al. Browning of human adipocytes requires KLF11 and reprogramming of PPARgamma superenhancers. *Genes Dev* (2015) **29**:7–22. doi:10.1101/gad.250829.114
40. Kong X, Banks A, Liu T, Kazak L, Rao RR, Cohen P, et al. IRF4 is a key thermogenic transcriptional partner of PGC-1alpha. *Cell* (2014) **158**:69–83. doi:10.1016/j.cell.2014.04.049
41. Eguchi J, Wang X, Yu S, Kershaw EE, Chiu PC, Dushay J, et al. Transcriptional control of adipose lipid handling by IRF4. *Cell Metab* (2011) **13**:249–59. doi:10.1016/j.cmet.2011.02.005
42. de Jong J, Wald TB, Timmons JA, Petrovic N, Hansen IR, Cannon B, et al. Expression of zinc finger of the cerebellum 1 suggests a possible role in development and/or function of brown adipose tissue. *Proceedings of The Physiological Society* (2012) **29**:C02.

43. Plaisier CL, Bennett BJ, He A, Guan B, Lusis AJ, Reue K, et al. Zbtb16 has a role in brown adipocyte bioenergetics. *Nutr Diabetes* (2012) **2**:e46. doi:10.1038/nutd.2012.21
44. Mikkelsen TS, Xu Z, Zhang Z, Wang L, Gimble JM, Lander E, et al. Comparative epigenomic analysis of murine and human adipogenesis. *Cell* (2010) **143**:156–69.
45. Barzilai N, Huffman DM, Muzumdar RH, Bartke A. The critical role of metabolic pathways in aging. *Diabetes* (2012) **61**:315–22. doi:10.2337/db11-1300
46. Nedergaard J, Cannon B. The changed metabolic world with human brown adipose tissue: therapeutic visions. *Cell Metab* (2010) **11**:268–72. doi:10.1016/j.cmet.2010.03.007
47. Lecoultre V, Ravussin E. Brown adipose tissue and aging. *Curr Opin Clin Nutr Metab Care* (2011) **14**:1–6. doi:10.1097/MCO.0b013e328341221e
48. Lupien SJ, de Leon M, de Santi S, Convit A, Tarshish C, Nair NPV, et al. Cortisol levels during human aging predict hippocampal atrophy and memory deficits. *Nat Neurosci* (1998) **1**:69–73. doi:10.1038/1149

Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 17 August 2015
doi: 10.3389/fendo.2015.00129

Transcriptional pathways in cPGI$_2$-induced adipocyte progenitor activation for browning

Irem Bayindir[1], Rohollah Babaeikelishomi[1], Silvia Kocanova[2,3], Isabel Sofia Sousa[1,4], Sarah Lerch[1], Olaf Hardt[5], Stefan Wild[5], Andreas Bosio[5], Kerstin Bystricky[2,3], Stephan Herzig[6,7†] and Alexandros Vegiopoulos[1†]*

[1] DKFZ Junior Group Metabolism and Stem Cell Plasticity, German Cancer Research Center, Heidelberg, Germany, [2] University of Toulouse (UPS), Toulouse, France, [3] Laboratoire de Biologie Moléculaire des Eucaryotes, Centre national de la recherche scientifique, Toulouse, France, [4] Department of Life Sciences, University of Coimbra, Coimbra, Portugal, [5] Miltenyi Biotec GmbH, Bergisch Gladbach, Germany, [6] Helmholtz Center Munich, Institute for Diabetes and Cancer IDC, Neuherberg, Germany, [7] Joint Heidelberg-IDC Translational Diabetes Program, Heidelberg University Hospital, Heidelberg, Germany

OPEN ACCESS

Edited by:
Maximilian Bielohuby, Sanofi, Germany

Reviewed by:
Elahu Gosney Sustarsic, University of Copenhagen, Denmark
Annie Moisan, Roche, Switzerland

***Correspondence:**
Alexandros Vegiopoulos, DKFZ Junior Group Metabolism and Stem Cell Plasticity, German Cancer Research Center, Im Neuenheimer Feld 280, Heidelberg D-69120, Germany
a.vegiopoulos@dkfz.de

†Stephan Herzig and Alexandros Vegiopoulos have contributed equally to this work.

Specialty section:
This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *24 April 2015*
Accepted: *03 August 2015*
Published: *17 August 2015*

Citation:
Bayindir I, Babaeikelishomi R, Kocanova S, Sousa IS, Lerch S, Hardt O, Wild S, Bosio A, Bystricky K, Herzig S and Vegiopoulos A (2015) Transcriptional pathways in cPGI$_2$-induced adipocyte progenitor activation for browning. Front. Endocrinol. 6:129. doi: 10.3389/fendo.2015.00129

De novo formation of beige/brite adipocytes from progenitor cells contributes to the thermogenic adaptation of adipose tissue and holds great potential for the therapeutic remodeling of fat as a treatment for obesity. Despite the recent identification of several factors regulating browning of white fat, there is a lack of physiological cell models for the mechanistic investigation of progenitor-mediated beige/brite differentiation. We have previously revealed prostacyclin (PGI$_2$) as one of the few known endogenous extracellular mediators promoting *de novo* beige/brite formation by relaying β-adrenergic stimulation to the progenitor level. Here, we present a cell model based on murine primary progenitor cells defined by markers previously shown to be relevant for *in vivo* browning, including a simplified isolation procedure. We demonstrate the specific and broad induction of thermogenic gene expression by PGI$_2$ signaling in the absence of lineage conversion, and reveal the previously unidentified nuclear relocalization of the *Ucp1* gene locus in association with transcriptional activation. By profiling the time course of the progenitor response, we show that PGI$_2$ signaling promoted progenitor cell activation through cell cycle and adhesion pathways prior to metabolic maturation toward an oxidative cell phenotype. Our results highlight the importance of core progenitor activation pathways for the recruitment of thermogenic cells and provide a resource for further mechanistic investigation.

Keywords: beige/brite differentiation, adipocyte progenitors, prostacyclin, PGI$_2$, adipocyte cell model, adipose tissue remodeling, nuclear localization

Introduction

The abundance and activation of thermogenic adipocytes are associated with improved metabolic health and protection from obesity, impaired glucose tolerance and dyslipidemia, at least as proven in diverse mouse models (1). Along with the discovery of functional thermogenic adipocytes in humans, this fact has potentiated research efforts toward understanding the biology of thermogenic adipocytes (2, 3). Beyond classical brown adipose tissue (BAT) depots, thermogenic adipocytes can be recruited and activated in other fat depots of rodents in the context of a tissue remodeling process

from a lipid storing to an oxidative/thermogenic phenotype. The recruitment of these so-called beige or brite adipocytes occurs under conditions of prolonged cold exposure, β3-adrenoreceptor agonist treatment, and possibly physical exercise and environmental enrichment (2). The degree of recruitment has been shown to depend on the anatomical location of the fat depots as well as the genetic background.

The cellular origin of multilocular beige/brite adipocytes expressing uncoupling protein-1 (Ucp1) has not been fully determined. However, different mechanisms appear to occur in parallel (1, 2). On the one hand, multiple reports described the derivation of beige/brite adipocytes from unilocular "white-appearing" adipocytes, implying a metabolic conversion (4–6). On the other hand, a substantial proportion of beige/brite adipocytes were shown to be recruited through adipogenic differentiation of immature progenitor cells *in vivo* and in primary cultures (7–9).

The master signal driving thermogenic adipose tissue remodeling is provided by sympathetic nerves via norepinephrine (NE) and β-adrenergic signaling (1, 10). We have previously described cyclooxygenase (COX)-2-derived prostaglandins as some of the very few endogenous mediators reported to act on progenitor cells to promote beige/brite differentiation during β-adrenergic stimulation (8). We demonstrated that prostaglandin synthesis was acutely increased in β-adrenergically stimulated adipose tissue, and importantly, COX-2 function was required for browning of white adipose tissue, a finding confirmed in an independent report (11). Furthermore, we identified prostacyclin (PGI_2) as a key prostaglandin downstream of COX-2. We could show that signaling induced by the stable analog carbaprostacyclin ($cPGI_2$) promoted beige/brite differentiation in mouse and human primary progenitor cells from white fat (8). PGI_2 can signal through the Ptgir G-protein-coupled receptor as well as through direct activation of all three members of the peroxisome proliferator-activated receptor (Ppar) family (12, 13). We could show that the full activation of the thermogenic program in progenitor cells as well as *in vivo* was dependent on signaling through both the Ptgir and Pparg receptors (8).

Despite the identification of a number of key regulatory nodes required for browning (1), we are far from understanding the signaling and transcriptional pathways regulating beige/brite differentiation downstream of extracellular mediators. This is partly due to the paucity of physiological cell models. Here, we describe a cell model for beige/brite differentiation based on adipogenic progenitors with defined surface markers and present a simplified method for their prospective isolation. Furthermore, we profile the cascade of progenitor cell responses to $cPGI_2$ throughout differentiation and show that progenitor activation by $cPGI_2$ via cell cycle and adhesion pathways precedes and synergizes with $cPGI_2$-induced metabolic maturation of beige/brite adipocytes.

Materials and Methods

Mice

Female NMRI mice (Charles River WIGA GmbH, Sulzfeld, Germany) or C57BL/6N mice from bred in the internal facility were housed at ambient temperature with 12-h light–dark cycle on chow (Kliba Nafag #3437, Provimi Kliba, Kaiseraugst, Switzerland). Stromal-vascular fraction (SVF) FACS profiles were not significantly different and beige/brite differentiation capacity was comparable between the two strains across numerous-independent experiments (data not shown). The RNA expression profiling data were obtained from NMRI cells. Animal handling and experimentation were performed in accordance with the European Union directives and the German animal welfare act (Tierschutzgesetz) and approved by local authorities (Regierungspräsidium Karlsruhe).

Adipose Tissue Digestion and SVF Preparation

Posterior subcutaneous adipose tissue (gluteal + inguinal) or the brown part of the interscapular fat was dissected, minced with scissors, and digested with 0.1 w.u./ml purified collagenase (LS005273, Worthington Biochemical, Lakewood, NJ, USA) and 2.4 U/ml Neutral Protease (LS02104, Worthington Biochemical) in Hank's balanced salt solution (HBSS, Sigma-Aldrich, Munich, Germany) containing 4 mM calcium chloride and 0.05 mg/ml DNase I (1284932001, Roche Diagnostics, Grenzach-Wyhlen, Germany) for 50 min at 37°C in a shaker. The suspensions were strained through a 300 μ mesh (4-1411, Neolab, Heidelberg, Germany). Floating mature adipocytes and SVF were separated by centrifugation at 145 × *g* for 10 min at 20°C. SVF cells were washed, and centrifuged at 300 × *g* for 5 min at 20°C.

FACS Analysis/Sorting of SVF Cells

Stromal-vascular fraction single cell suspensions in D-PBS (Life Technologies, Darmstadt, Germany) supplemented with 0.5% BSA and 1 mM EDTA (Sigma-Aldrich) were preincubated with FcBlock [anti-CD16/32 (93, eBioscience), Frankfurt, Germany] for 10 min on ice. Cells were then incubated with Anti-Ter119 MicroBeads (130-049-901, Miltenyi Biotec, Bergisch Gladbach, Germany) on ice for 15 min, to perform erythrocyte depletion by magnetic-activated cell sorting (MACS®) with an OctoMACS Separator according to the manufacturer's instructions. The flow-through was collected and stained with CD45-FITC (30-F11, eBioscience), CD31-eFluor 450 (390, eBioscience), CD29-PerCP-eFluor 710 (HMb1-1, eBioscience), CD34-Alexa Fluor 647 (RAM34, BD Biosciences, Heidelberg, Germany), Sca-1-Alexa Fluor 700 (D7, eBioscience), and CD140a(Pdgfrα)-biotin (APA5, eBioscience) for 30 min on ice, followed by staining with streptavidin-PE-Cy7 (eBioscience). After antibody staining, samples were washed and sorted with a BD FACS Aria (BD Biosciences). Unstained cells as well as FMO stainings were used as negative controls. Single-stained controls were used for compensation. Data were analyzed using FlowJo software (FlowJo, Ashland, OR, USA). Sorted cells were centrifuged at 300 × *g* for 5 min for further processing.

MACS® Cell Isolation

Stromal-vascular fraction single cell suspensions in D-PBS (Life Technologies) supplemented with 0.5% BSA and 1 mM EDTA (Sigma-Aldrich) were preincubated with FcBlock (anti-CD16/32 (93, eBioscience, Frankfurt, Germany) for 10 min on ice. Cells were then stained with biotin-conjugated Ter119 (TER-119), CD31 (390), and CD45 (30-F11) antibodies (eBioscience) for 30 min on ice, washed and incubated with Streptavidin

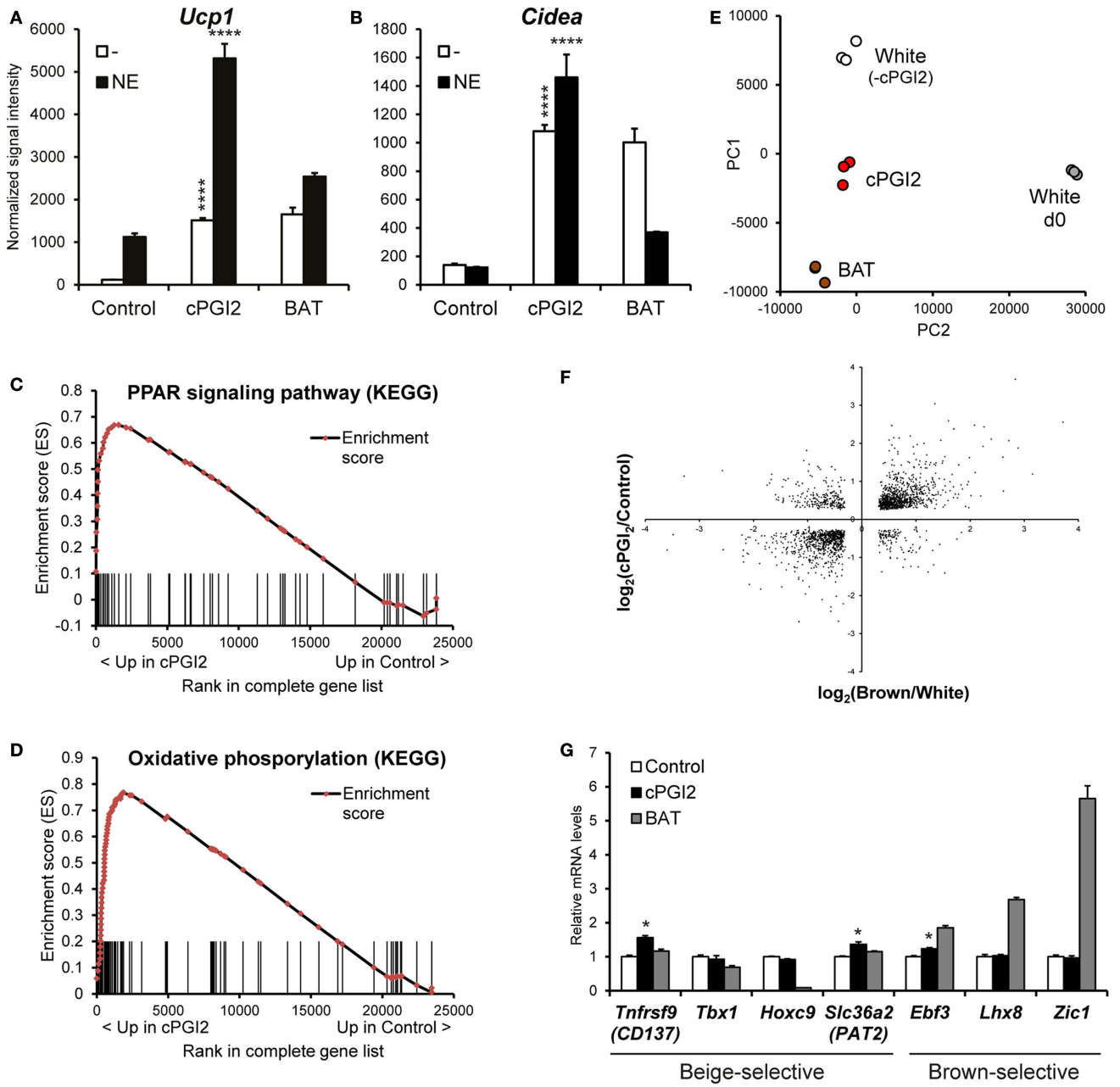


FIGURE 1 | cPGI$_2$ specifically induces a broad thermogenic gene expression program in adipocyte progenitors without lineage conversion. Lin$^-$CD29$^+$CD34$^+$Sca-1$^+$ cells from posterior subcutaneous fat were cultured in adipogenic media ± cPGI$_2$ for 8 days. Lin$^-$CD29$^+$CD34$^+$Sca-1$^+$ cells from interscapular brown fat (BAT) were cultured in adipogenic media for 8 days and used as a reference. Three hours before harvest, cells were cultured ± norepinephrine (NE). RNA expression profiling was performed with Illumina beadchip arrays ($n = 3$). **(A,B)** Normalized signal intensities for the indicated genes/probes are shown (asterisks indicate Bonferroni cPGI$_2$ vs. Control ****$p < 0.0001$, *$p < 0.05$, $n = 3$). **(C,D)** Enrichment plots of the OxPhos **(C)** and PPAR **(D)** gene sets obtained by GSEA (cPGI2 vs. Control) with the KEGG pathway gene set collection (FDR $q \leq 0.0001$, see **Table 1**). Vertical bars represent the individual genes of the gene set/pathway ranked according to their regulation by cPGI$_2$ (based on signal-to-noise ratio, see Materials and Methods). *X*-axis values represent the rank within the complete ranked gene list (transcriptome). The enrichment score (ES) reflects the degree to which a gene set is overrepresented at the top or bottom of the complete ranked gene list. **(E)** Principal component analysis was performed on RNA expression profiles from day 8 differentiated cells as indicated (White, cPGI2, BAT) including undifferentiated subcutaneous Lin$^-$CD29$^+$CD34$^+$Sca-1$^+$ cells (White d0). The sample coordinates for principal component (PC) 1 and 2 are shown. PC1 and PC2 captured 80% of the overall variability. **(F)** 1793 genes were selected with significant differential expression ($p < 0.05$) in both the cPGI$_2$ vs. Control and the BAT vs. "white" (equivalent to Control, i.e., minus cPGI$_2$) comparisons. The log2-ratios of the corresponding expression levels in the two comparisons were plotted. **(G)** Normalized signal intensities of the indicated genes are plotted (relative to Control). (* indicates Tukey cPGI$_2$ vs. Control $p < 0.01$, $n = 3$).

MicroBeads (130-048-102, Miltenyi Biotec) for magnetic separation with an OctoMACS Separator according to the manufacturer's instructions. The flow-through (Lin^- cells) was stained with Sca1-PE-Cy7 (D7, eBioscience) for 30 min on ice, washed and incubated with Anti-Cy7 MicroBeads (130-091-652, Milenyi Biotec) for magnetic separation according to the manufacturer's instructions. The flow-through ($Lin^-Sca\text{-}1^+$ cells) was centrifuged at 300 × *g* for 5 min for further processing. Flow cytometry for the assessment of recovery/purity was performed on a FACScalibur (BD Biosciences). For the isolation of Lin^-Sca^+ cells without post-isolation flow cytometric analysis, Anti-Sca-1 MicroBeads (130-106-641) were used following the isolation of Lin^- cells or alternatively, the Adipose Tissue Progenitor Isolation Kit (130-106-639) on SVF isolated using the Adipose Tissue Dissociation Kit (130-105-808) in combination with the gentleMACS Octo Dissociator (130-096-427) (all Miltenyi Biotec).

Cell Culture and Adipogenic Differentiation

Media and supplements were purchased from Life Technologies (Darmstadt, DE) unless stated otherwise. Sorted (FACS or MACS) cells were plated out on BIOCOAT Laminin-coated plates (BD Biosciences) and maintained in DMEM plus 10% FCS, 1% penicillin/streptomycin, and 10 ng/ml murine bFGF (R&D Systems, Wiesbaden, DE) without passaging. Adipogenic differentiation of confluent cells was performed in the presence of 1 μM $cPGI_2$ (BIOZOL, Eching, DE) throughout the course of differentiation (unless otherwise indicated) or corresponding concentration of ethanol as control ("white" cells). Media ± $cPGI_2$ were replaced daily with fresh. Differentiation was induced with medium consisting of DMEM, 10% FCS, 1% penicillin/streptomycin, 1 μg/ml insulin, 500 nM dexamethasone, 3 nM triiodothyronine (T3) (Sigma-Aldrich) for 2 days. Subsequently, cells were cultured in differentiation medium with 5% FCS lacking dexamethasone for 5 days, and DMEM, 5% FCS, 1% penicillin/streptomycin for 1 day. Wherever indicated, 3 h before harvest of cultures, cells received fresh medium with and without 0.5 μM NE (Sigma-Aldrich) but omitting $cPGI_2$.

RNA Isolation and qRT-PCR Analysis

Cell lysis was performed in QIAZOL (QIAGEN, Hilden, Germany), and RNA was prepared using the RNeasy micro kit (QIAGEN) including DNase treatment. Reverse transcription was performed with 0.1–1 μg total RNA and oligo(dT) primers using Superscript II (Life Technologies). Quantitative PCR was performed with the TaqMan Universal Master Mix II and gene-specific Taqman probes on a StepOnePlus machine (Life Technologies, Darmstadt, DE). Relative mRNA expression levels were calculated with the ΔΔCt method and TATA-binding protein (*Tbp*) as a reference.

Microarray Expression Profiling

Preparation of biotin-labeled cRNA samples from 500 ng total RNA, and hybridization on Illumina Mouse Sentrix-6 BeadChip arrays (Illumina, San Diego, CA, USA) were performed according to the manufacturer's instructions. Scanning was performed on a Beadstation array scanner (Illumina). The raw microarray data are available in the ArrayExpress database[1] under accession number E-MTAB-3693. Beads with a value >20 (bead level) were selected, and outliers with values >2.5 median absolute deviation (MAD) were removed. All remaining data points were used for the calculation of the mean average signal for a given probe. Intensity values were normalized by quantile normalization using Chipster Software (CSC, Espoo, Finland). Probe annotation was according to MouseWG-6_V2_0_R1_11278593_A. Principal component analysis was performed with TM4 MeV software on log2-transformed values using median centering (14). Two-group significance tests as indicated were performed in Chipster without pre-filtering of probes on log2-transformed values (empirical Bayes with Bonferroni-Holm *p*-value adjustment). Gene set enrichment analysis [GSEA (15)] was performed on the complete probe dataset based on the MouseWG-6_V1_1_R4_11234304_A annotation. The following gene set collections from the Molecular Signatures Database (MSigDB)[2] were used: The c2.cp.kegg.v3.0.symbols.gmt gene set collection, derived from http://www.genome.jp/kegg/pathway.html, and the c3.tft.v3.0.symbols.gmt gene set collection, in with gene sets contain genes that share a transcription factor binding site defined in the TRANSFAC database[3]. The following key parameters were applied: permutation type = phenotype (1000×), enrichment statistic = weighted, metric for ranking genes = Signal2Noise, normalization mode = meandiv. Gene Sets were ranked by the false discovery rate.

DNA–Fluorescence *In situ* Hybridization

Glass cover slips (HECH1001/12, Karl Hecht, Sondheim, Germany) were coated with 4 μg/cm^2 laminin (Santa Cruz Biotechnology, Heidelberg, Germany) for 1 h at 37°C and washed with DMEM. Freshly isolated progenitor cells (see above) were plated on glass cover slips in growth and differentiation media, as described above. Cells were fixed at the indicated time points with fresh 4% paraformaldehyde. DNA–fluorescence *in situ* hybridization (FISH) experiments were performed, as previously described (16). DNA from BAC clones MSG01-182C14 for *Ucp1* and RP24-238M20 for *Pum1* purchased from DNA Bank, RIKEN and BACPAC C.H.O.R.I. Center, (USA), respectively, was directly labeled using nick translation (BioPrime DNA Labeling System, Life Technologies, Saint Aubin, France) by incorporation of fluorochrome-conjugated nucleotidesChromaTide® AlexaFluor® 488-5-dUTP (Life Technologies) for *Ucp1* and Atto647N-dUTP-NT (Jena Biosciences, Jena, Germany) for *Pum1*. One hundred nanogram of each labeled DNA probe together with 7 μg Cot-1 mouse DNA and 5 μg sonicated salmon sperm DNA were used per coverslip. Cells were examined by Nikon Ti-E/B epifluorescence microscope, equipped with a HG Intensilight® illumination source, a CCD Orca R2 camera (Hamamatsu®) and imaged through an NIKON oil-immersion objective 60× (Plan APO 1.4). The devices were controlled by NIS-elements® 3.2. Three-dimensional images were captured at 200 nm intervals in the *z*-axis, using an objective fitted with a piezo nano Z100 Ti.

[1] www.ebi.ac.uk/arrayexpress
[2] http://www.broadinstitute.org/gsea/msigdb/index.jsp
[3] http://www.gene-regulation.com/

Analysis of nuclear position of the detected fluorescent signals was performed using NEMO software (17). The radial localizations of loci were then calculated in Microsoft Excel. Three shells of equal area eroded from the center (shell 1) to the periphery (shell 3) of the nucleus were used. Images from 30 to 50 nuclei were analyzed in each experiment. Finally, the images were processed using Adobe Photoshop.

EdU Incorporation Analysis

Cells were incubated with 10 μM 5-ethynyl-2-deoxyuridine (EdU) in their normal medium for 1 h, trypsinized and washed. Fixation (4% paraformaldehyde, 15 min), permeabilization and the Click-it reaction for AlexaFluor647 labeling were performed using the Click-iT® Plus EdU Flow Cytometry Assay Kit (Life Technologies, Darmstadt, Germany). Cells were treated with FxCycle™PI/RNase Staining Solution (Life Technologies) and analyzed on a FACSCalibur (BD Biosciences, Heidelberg, Germany).

Statistical Analysis

Plots depict means and SEM unless otherwise indicated. The corresponding test and significance level are indicated in the figure legends. Gene expression data were tested in the log-scale for approximation of normality. Two-way ANOVA was applied with Bonferroni *post hoc* pairwise test. One-way ANOVA was applied with Tukey *post hoc* pairwise test. Two-sided *t*-test was applied for two-group experiments. The statistical significance of differences in nuclear radial localization (FISH) was assessed using the chi-square (χ^2) test to examine the null hypothesis that the foci exhibit the same radial distribution in both treatments ($cPGI_2$ vs. Control). A p-value ≤ 0.05 was considered statistically significant.

Results

$cPGI_2$ Broadly and Specifically Induces the Thermogenic Gene Expression Program in $Lin^-CD29^+CD34^+Sca\text{-}1^+Pdgfra^+$ Progenitors Without Lineage Conversion

We have previously demonstrated that beige/brite differentiation can be efficiently induced by $cPGI_2$, a stable analog of PGI_2, in $Lin(Ter119/CD31/CD45)^-CD29^+CD34^+Sca\text{-}1^+$ cells isolated by FACS from subcutaneous tissue (8). This population has been shown to contain all adipogenic cells in the SVF (18). Genetic lineage tracing *in vivo* has revealed that expression of Platelet-derived growth factor receptor a (Pdgfra) marks progenitors with both beige/brite and white adipogenic potential (7, 19). Importantly, it is the only marker proven so far by genetic lineage tracing to be broadly expressed in beige/brite progenitors *in vivo*. In accordance with Berry et al., we could confirm that the majority of the $Lin^-CD29^+CD34^+Sca\text{-}1^+$ population (>90%) was positive for Pdgfra expression (Figures S1A–C in Supplementary Material) (19), implying that it is likely to include most immature beige/brite progenitor cells.

In order to obtain a global picture of the differentiation phenotype induced by $cPGI_2$ in progenitor cells, we performed time course expression profiling of $Lin^-CD29^+CD34^+Sca\text{-}1^+$ cells stimulated with $cPGI_2$ under adipogenic conditions. As shown previously, $cPGI_2$ robustly induced the thermogenic/brown adipocyte marker genes *Ucp1* and *Cidea* after 8 days of differentiation (**Figures 1A,B**) (8). *Ucp1* expression could be super-activated by NE, demonstrating the responsiveness of $cPGI_2$-treated cells to this thermogenic inducer. Notably, expression levels of *Ucp1* and *Cidea* were comparable to adipocytes differentiated from $Lin^-CD29^+CD34^+Sca\text{-}1^+$ cells from interscapular BAT (**Figures 1A,B**). $cPGI_2$ has been proposed to promote adipogenic differentiation (20). However, in our primary cell model, most adipogenic marker genes include Adiponectin (*Adipoq*) and Resistin (*Retn*) were not or only modestly and inconsistently induced by $cPGI_2$ (Figures S2A,B in Supplementary Material, and data not shown).

We next performed GSEA (15) to examine the biological pathways induced in $cPGI_2$-treated cells at 8 days of differentiation in an unbiased fashion. **Figures 1C,D** illustrate the enrichment of genes involved in oxidative phosphorylation and the PPAR signaling pathway, respectively, in the fraction of genes up-regulated by $cPGI_2$. The oxidative phosphorylation gene set contains all the subunit genes of the respiratory chain complexes, and their upregulation by $cPGI_2$ is consistent with mitochondrial biogenesis and thermogenic differentiation. The upregulation of PPAR signaling pathway genes confirms the essential function of PPAR nuclear receptors downstream of $cPGI_2$ (8). The top 10 most significantly enriched pathways from 159 KEGG pathways in the $cPGI_2$-up-regulated gene fraction included oxidative phosphorylation, TCA cycle, fatty acid metabolism, and other metabolic pathways (**Table 1**). This result demonstrates that differentiation toward an oxidative adipocyte phenotype is the main and specific response of progenitors to prolonged $cPGI_2$ treatment.

The question remained as to the extent to which the $cPGI_2$-induced cell phenotype resembles a classical brown adipocyte phenotype. To this end, we performed principal component

TABLE 1 | Top 10 most significantly enriched gene sets in the $cPGI_2$-up-regulated gene fraction following 8 days of progenitor differentiation.

Gene set name[a]	Size	Enrichment score[b]	FDR *q*-value
KEGG_PARKINSONS_DISEASE[c]	87	0.77	<0.0001
KEGG_OXIDATIVE_PHOSPHORYLATION	89	0.77	<0.0001
KEGG_HUNTINGTONS_DISEASE[c]	129	0.67	<0.0001
KEGG_PEROXISOME	57	0.74	<0.0001
KEGG_ALZHEIMERS_DISEASE[c]	121	0.64	<0.0001
KEGG_CITRATE_CYCLE_TCA_CYCLE	25	0.82	<0.0001
KEGG_FATTY_ACID_METABOLISM	35	0.72	0.0001
KEGG_PPAR_SIGNALING_PATHWAY	54	0.67	0.0001
KEGG_VALINE_LEUCINE_AND_ISOLEUCINE_DEGRADATION	40	0.65	0.001
KEGG_FRUCTOSE_AND_MANNOSE_METABOLISM	22	0.72	0.0026

[a]$Lin^-CD29^+CD34^+Sca\text{-}1^+$ cells were cultured in adipogenic media ± $cPGI_2$ for 8 days. RNA expression profiling was performed with Illumina beadchip arrays (n = 3). Global expression profiles ($cPGI_2$ vs. Control) were subjected to gene set enrichment analysis (GSEA) with the KEGG pathway gene set collection. Gene sets were ranked by the False Discovery Rate (FDR) q-value reflecting significance.

[b]Maximum of the corresponding enrichment score curve.

[c]The indicated gene sets are overlapping >50% with the KEGG_OXIDATIVE_PHOSPHORYLATION gene set.

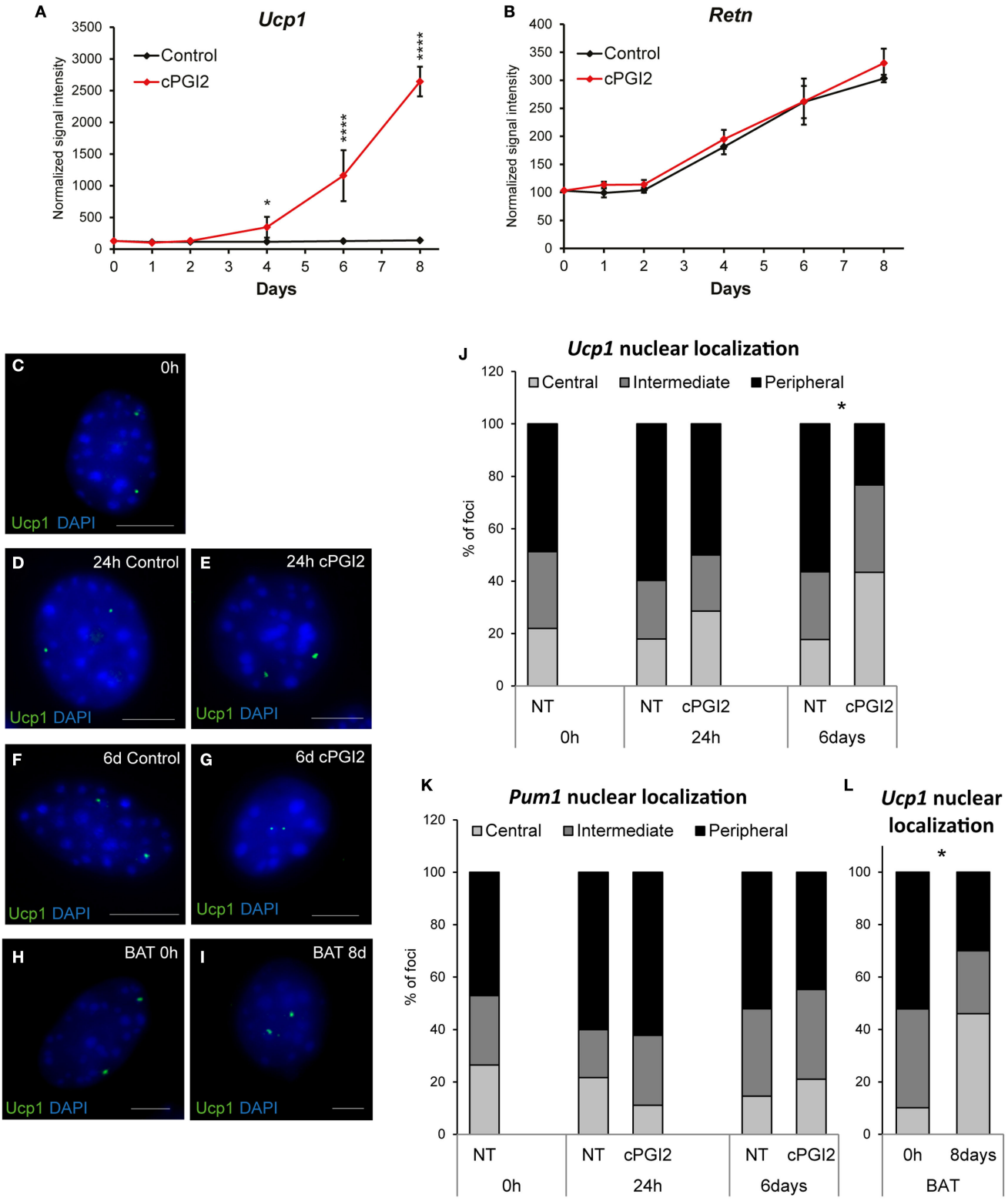


FIGURE 2 | Late non-linear upregulation of *Ucp1* expression by cPGI$_2$ and associated late nuclear relocalization of the *Ucp1* gene locus.

(Continued)

FIGURE 2 | Continued
Lin$^-$CD29$^+$CD34$^+$Sca-1$^+$ cells were cultured in adipogenic media ± $cPGI_2$. **(A,B)** RNA was obtained at the indicated time points for expression profiling with Illumina beadchip arrays ($n = 3$). Normalized signal intensities for the indicated genes/probes are shown (asterisks indicate Bonferroni $cPGI_2$ vs. Control $^*p < 0.05$, $^{****}p < 0.0001$). **(C–L)** Cells were fixed for 3D nuclear architecture-preserving DNA–FISH analysis in the undifferentiated state (0 h) **(C)** and at 24 h **(D,E)** or 6 days **(F,G)** of differentiation with **(E,G)** or without **(D,F)** $cPGI_2$. Cells from interscapular BAT (without $cPGI_2$) were analyzed at 0 h or 8 days **(H,I)**. The *Ucp1* gene locus **(C–I)** was detected using an AlexaFluor® 488-5-dUTP-labeled probe (green) and the cells were stained with DAPI (blue). Representative images of progenitor and adipocyte nuclei are shown (scale bar 10 μm). **(J)** For quantitative analysis of *Ucp1* localization, the nuclei were segmented into three shells (Central/Intermediate/Peripheral). The distribution of *Ucp1* loci under the indicated conditions is shown (* indicates χ^2 test $cPGI_2$ vs. Control $p = 2.4 \times 10^{-8}$, $n = 30–50$). **(K)** Distribution of the *Pum1* locus as detected in Figures S4B,C in Supplementary Material. **(L)** Distribution of the *Ucp1* locus in cells from interscapular BAT (* indicates χ^2 test 8 days vs. 0 h $p = 4.8 \times 10^{-16}$, $n = 30–50$).

analysis to compare global gene expression in $cPGI_2$-treated and control cells at day 8 as well as adipocytes from classical BAT-derived progenitors. As a reference we included undifferentiated progenitors from posterior subcutaneous fat (White d0). The three types of mature adipocytes aligned with each other and separately from undifferentiated cells on principal component 1 (PC1), likely reflecting similar degrees of adipogenesis (**Figure 1E**). PC2 mainly represents the differences between the adipocyte types and reveals an intermediate phenotype of $cPGI_2$-treated cells between the white and brown cell phenotypes. To further delineate this, we examined the expression pattern of $cPGI_2$-regulated genes in the comparison of classical brown vs. control white adipocytes (without $cPGI_2$ treatment). One thousand seven hundred ninety-three of 3589 $cPGI_2$-regulated genes ($cPGI_2$ vs. Control, $p < 0.05$ at day 8) were also differentially expressed in brown vs. white/control adipocytes ($p < 0.05$). Remarkably, the expression patterns were highly concordant in the two comparisons (**Figure 1F**), and this was not due to varying differentiation efficiencies compared to white control cultures (Figure S3A in Supplementary Material). In addition, there was no concordance of $cPGI_2$-dependent expression with the general adipogenic differentiation program (day 8 vs. 0, Figure S3B in Supplementary Material). Taken together, these results suggest that $cPGI_2$ broadly promotes a brown-like gene expression program independently of any effects on adipogenic differentiation.

To test whether $cPGI_2$ influences developmental lineage commitment, we analyzed the expression of genes known to be brown lineage-selective, but could not detect a consistent $cPGI_2$ effect on *Ebf3*, *Lhx8*, or *Zic1* (**Figure 1G**) (21, 22). In addition, $cPGI_2$ did not consistently alter the expression of beige-selective genes including *Tnfrsf9* (CD137), *Tbx1*, *Hoxc9*, and *Slc36a2* (Pat2) (22–24). Taken together, our findings suggest that $cPGI_2$ promotes oxidative brown-like differentiation without inducing a major lineage switch or enrichment.

Synergism of $cPGI_2$ Signaling During Progenitor Activation and Beige/Brite Adipocyte Maturation Results in the Late Activation of *Ucp1* Transcription

Based on our results so far, it was not clear at which differentiation stage $cPGI_2$ triggers progenitor browning. To address this, we first examined the time course of induction of brown marker genes in relation to the progress of adipogenesis. Whereas $cPGI_2$ did not influence the linear upregulation of the adipogenic marker resistin (*Retn*), the induction of *Ucp1* by $cPGI_2$ began between day 2 and 4 of treatment, but displayed exponential kinetics indicating the involvement of synergistic $cPGI_2$ effects (**Figures 2A,B**).

The relocalization of gene loci away from the nuclear lamina and toward the center of the nucleus has been shown to be associated with their transcriptional activation during differentiation in mammalian cells (25–27). To gain more insight into the mode and kinetics of transcriptional regulation of thermogenic genes by $cPGI_2$, we asked whether nuclear localization of *Ucp1* correlates with the induction of *Ucp1* transcription by $cPGI_2$. Nuclear structure-preserving 3D-FISH revealed that in the undifferentiated state (0 h) the two *Ucp1* alleles had peripheral or intermediate localization in the majority of progenitor nuclei (**Figures 2C,J**). Induction of differentiation and $cPGI_2$ treatment did not acutely alter this localization pattern (24 h, **Figures 2D,E,J**). Within the subsequent 5 days of $cPGI_2$ treatment, the number and density of DAPI^{bright} chromocenters decreased, and remarkably, *Ucp1* loci significantly shifted toward the interior of the nuclei (**Figures 2F,G,J**), correlating with the late transcriptional activation by $cPGI_2$ (**Figure 2A**). To control the specificity of this finding, we examined the nuclear localization of the Pumilio 1 (*Pum1*) gene locus, the expression of which was not affected by differentiation or $cPGI_2$ treatment (Figure S4A in Supplementary Material). In accordance with the expression pattern, the nuclear localization of the *Pum1* locus was not altered by $cPGI_2$ (**Figure 2K**; Figures S4B,C in Supplementary Material). In addition, we tested the nuclear localization of the *Ucp1* locus in adipocytes derived from interscapular brown fat progenitor cells, which express high levels of *Ucp1* mRNA without $cPGI_2$ stimulation. Whereas undifferentiated BAT progenitors displayed mainly intermediate/peripheral nuclear localization of *Ucp1*, mature brown adipocytes had 46% central localization, which is comparable to 43.3% in $cPGI_2$-treated adipocytes from subcutaneous white fat (**Figures 2H,I,L**). Taken together, these results are in line with a late transcriptional activation of *Ucp1* by $cPGI_2$.

Given the late upregulation of *Ucp1* expression by $cPGI_2$, we sought to define the role of late vs. early $cPGI_2$ signaling. To define the differentiation time window in which $cPGI_2$ stimulation promotes beige/brite differentiation, we restricted the duration of $cPGI_2$ treatment (**Figures 3A,B**). Neither the early nor the late $cPGI_2$ stimulation was sufficient to induce the full activation of *Ucp1* and *Cidea* expression. Intriguingly, early $cPGI_2$ signaling during progenitor commitment (day 0–2) synergized with late $cPGI_2$ stimulation during maturation (day 3–8) in a non-additive manner, highlighting an important role during progenitor activation.

$cPGI_2$ Induces Progenitor Activation Through Cell Cycle and Adhesion Pathways Prior to Metabolic Maturation

We next sought to determine the transcriptional pathways underlying the early progenitor response to $cPGI_2$. GSEA at 24 h of

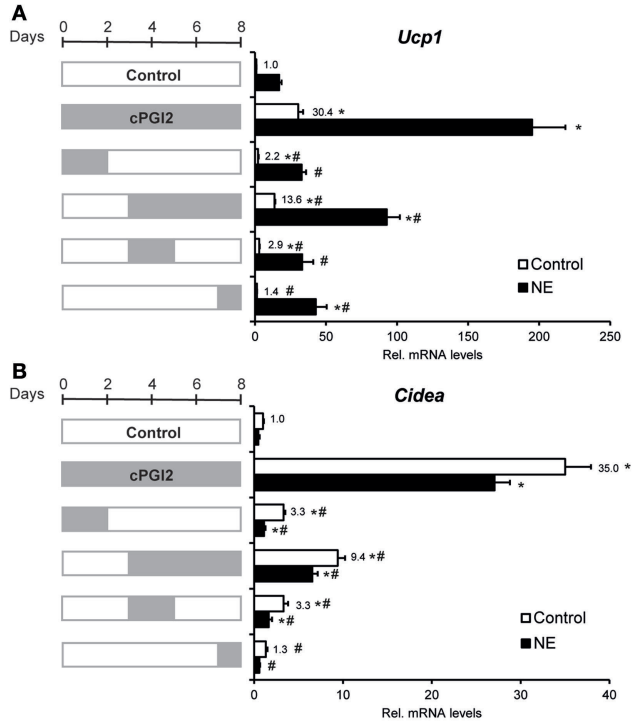


FIGURE 3 | Synergism of cPGI$_2$ signaling during progenitor activation and adipocyte maturation for the induction of thermogenic marker genes. Lin$^-$CD29$^+$CD34$^+$Sca-1$^+$ cells were cultured in adipogenic media for 8 days. cPGI$_2$ was included in the media as indicated by the gray bars. Three hours before harvest, cells were cultured ± NE. RNA expression of *Ucp1* **(A)** and *Cidea* **(B)** was analyzed by qRT-PCR [* and # indicate Tukey $p < 0.05$ for the respective group vs. Control (0–8 days) and vs. cPGI$_2$ (0–8 days), respectively, $n = 3$].

cPGI$_2$ treatment using the KEGG pathway collection revealed the marked enrichment of cell cycle and proliferation pathways in the gene fraction up-regulated by cPGI$_2$ (**Table 2**; **Figure 4A**).

Furthermore, we applied GSEA with gene sets consisting of genes which share specific conserved transcription factor motifs in their promoter regions (15). In this way, we found that genes with E2F1 motifs were highly up-regulated by cPGI$_2$ at 24 h (**Figure 4B**). The transcription factor E2F1 is known to promote G1–S cell cycle transition and S phase progression (28), and thus, cPGI$_2$ may be inducing S phase gene expression, including DNA replication genes, through modulation of E2F1 activity. To test whether these cPGI$_2$-mediated gene expression changes resulted in transient progenitor proliferation at the onset of differentiation, we pulse-labeled cells at 24 h of cPGI$_2$ treatment with the nucleotide analog EdU and measured incorporation into genomic DNA by flow cytometry (**Figures 4D–F**). Indeed, cPGI$_2$ caused a significant increase in the proportion of EdU$^+$ cycling cells (from 5.1 to 9% within 1 h of labeling). Interestingly, the top 10 gene sets from the KEGG pathway collection (GSEA) in the cPGI$_2$-down-regulated gene fraction at 24 h were dominated by cell adhesion and cytoskeletal pathways (**Table 2**; **Figure 4C**), indicating that cPGI$_2$ promotes changes in cell adhesion and morphology associated with progenitor activation in parallel to cell cycle effects.

TABLE 2 | Top 10 most significantly enriched gene sets in the cPGI$_2$-up- and down-regulated gene fraction at 24 h of progenitor differentiation.

Gene set name[a]	Size	Enrichment score	FDR *q*-value
ENRICHED IN THE cPGI$_2$-UP-REGULATED GENE FRACTION			
KEGG_DNA_REPLICATION	24	0.88	<0.0001
KEGG_HOMOLOGOUS_RECOMBINATION	22	0.86	<0.0001
KEGG_CELL_CYCLE	98	0.61	<0.0001
KEGG_SPLICEOSOME	71	0.65	<0.0001
KEGG_NUCLEOTIDE_EXCISION_REPAIR	31	0.73	<0.0001
KEGG_MISMATCH_REPAIR	15	0.85	<0.0001
KEGG_PYRIMIDINE_METABOLISM	73	0.6	0.0001
KEGG_BASE_EXCISION_REPAIR	25	0.72	0.0002
KEGG_NON_HOMOLOGOUS_END_JOINING	12	0.74	0.0196
KEGG_AUTOIMMUNE_THYROID_DISEASE	22	0.61	0.0276
ENRICHED IN THE cPGI$_2$-DOWNREGULATED GENE FRACTION			
KEGG_FOCAL_ADHESION	157	−0.48	0.0032
KEGG_VASCULAR_SMOOTH_MUSCLE_CONTRACTION	89	−0.51	0.0054
KEGG_TIGHT_JUNCTION	88	−0.52	0.0043
KEGG_AXON_GUIDANCE	105	−0.46	0.0247
KEGG_REGULATION_OF_ACTIN_CYTOSKELETON	164	−0.43	0.0321
KEGG_LONG_TERM_DEPRESSION	58	−0.51	0.0288
KEGG_LEUKOCYTE_TRANSENDOTHELIAL_MIGRATION	85	−0.46	0.0284
KEGG_ADHERENS_JUNCTION	58	−0.51	0.0327
KEGG_ENDOCYTOSIS	117	−0.44	0.0371
KEGG_ECM_RECEPTOR_INTERACTION	69	−0.47	0.0386

[a]*Lin$^-$CD29$^+$CD34$^+$Sca-1$^+$ cells were cultured in adipogenic media ± cPGI$_2$ for 24 h. RNA expression profiling was performed with Illumina beadchip arrays (n = 3). Global expression profiles (cPGI$_2$ vs. Control) were subjected to GSEA with the KEGG pathway gene set collection. Gene sets were ranked by the False Discovery Rate (FDR) q-value reflecting significance.*

In order to determine the temporal order of progenitor responses to cPGI$_2$ and their relation to the metabolic differentiation toward the oxidative thermogenic phenotype, we performed GSEA across the time course of cPGI$_2$ treatment, i.e., cPGI$_2$ vs. Control at each time point (**Figure 5A**). Intriguingly, the cytoskeletal and adhesion changes as well as the transient cell cycle activation by cPGI$_2$ preceded the upregulation of oxidative pathways including Ppar target genes. Taken together, our findings suggest that the synergism between early and late cPGI$_2$ signaling results from the early activation of progenitors and priming for the later induction of oxidative/thermogenic genes by cPGI$_2$ (**Figure 5B**).

A Simplified Method for the Prospective Isolation of Defined Progenitors for Beige/Brite Differentiation

Finally, we aimed at developing a more accessible method for the isolation of progenitors with beige/brite potential based on

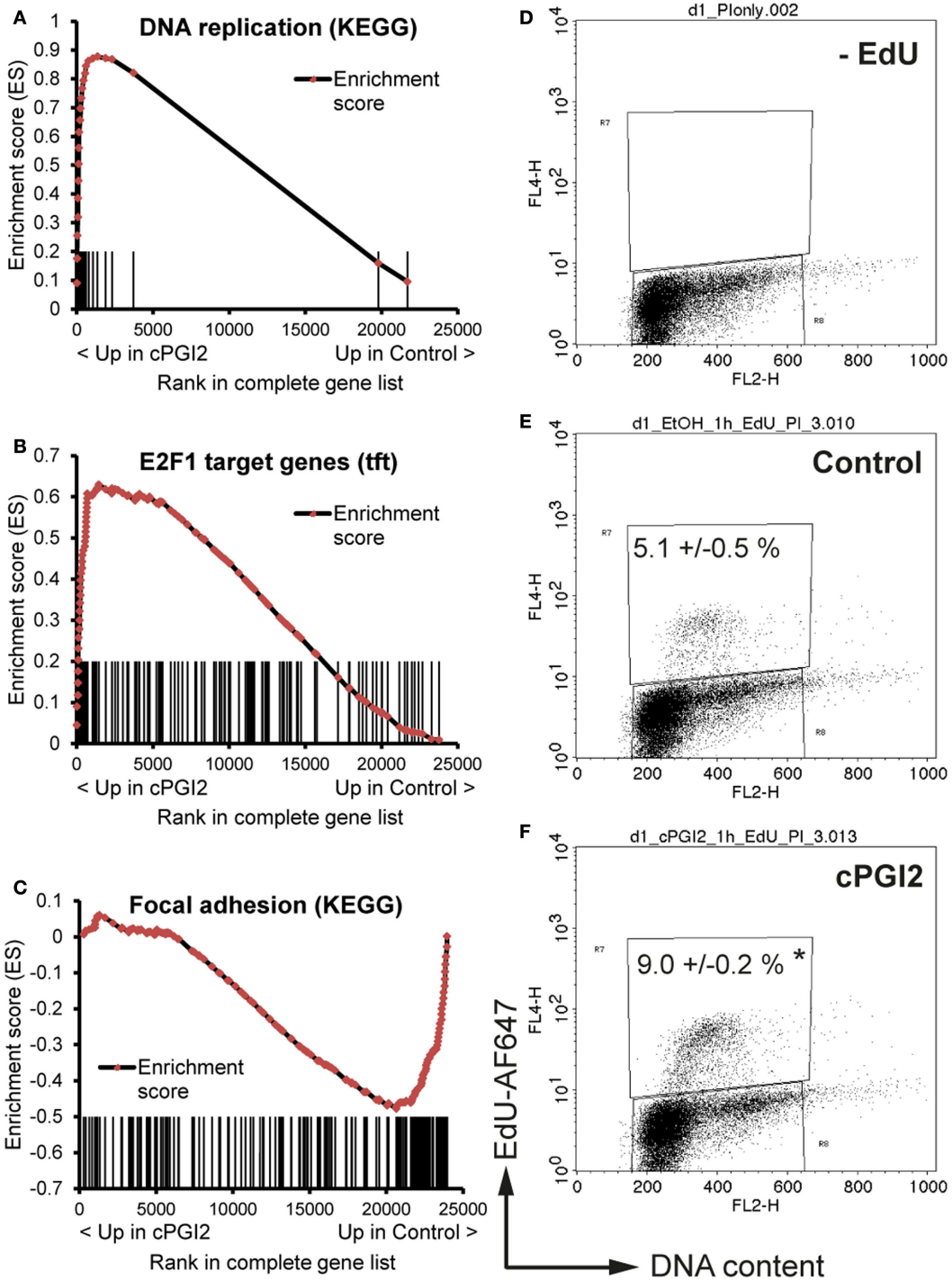


FIGURE 4 | $cPGI_2$ induces progenitor activation through cell cycle and adhesion pathways. $Lin^-CD29^+CD34^+Sca\text{-}1^+$ cells were cultured in adipogenic media ± $cPGI_2$ for 24 h. **(A–C)** RNA was obtained for expression profiling with Illumina beadchip arrays ($n = 3$). Enrichment plots of the indicated gene sets were obtained by GSEA ($cPGI_2$ vs. Control) with the KEGG pathway [**(A,C)**, see **Table 2**] or the transcription factor motif **(B)** gene set collections. Vertical bars represent the individual genes of the gene set/pathway ranked according to their regulation by $cPGI_2$. *X*-axis values represent the rank within the complete ranked gene list (transcriptome). The enrichment score (ES) reflects the degree to which a gene set is overrepresented at the top or bottom of the complete ranked gene list. **(E,F)** Cells were pulse-labeled with EdU for 1 h, processed for EdU-AlexaFluor647 and propidium iodide staining, and analyzed by flow cytometry ($n = 3$). Cells cultured without EdU served as negative control **(D)**. The mean percentage of EdU^+ cells is shown (* indicates *t*-test $cPGI_2$ vs. Control $p = 0.003$).

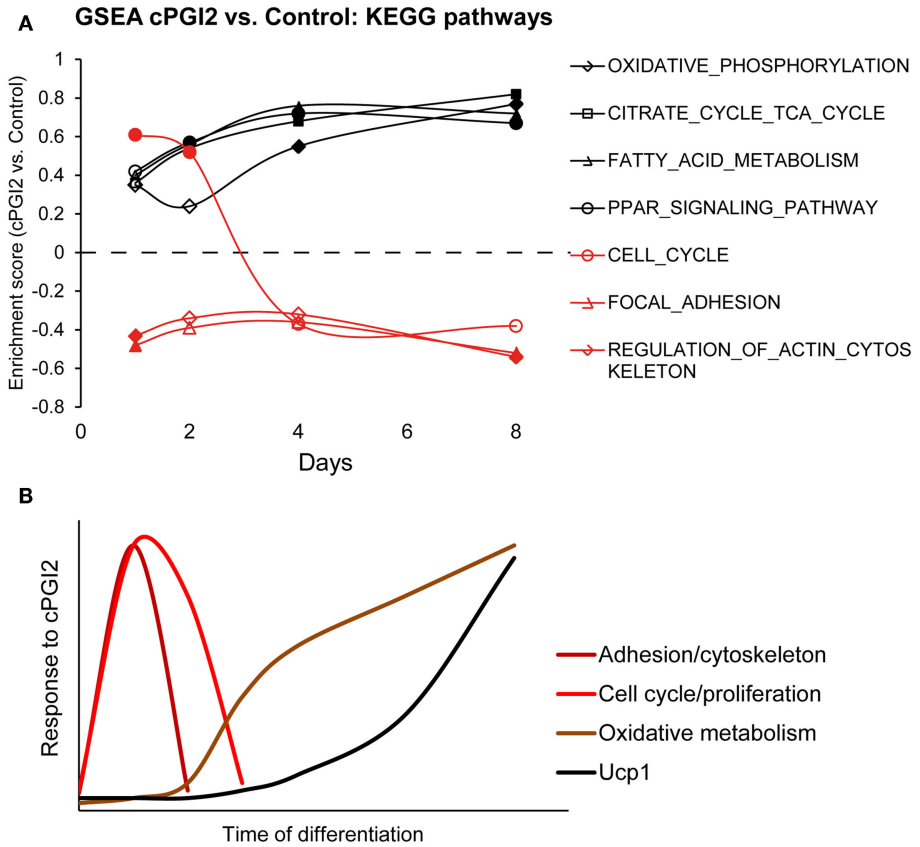


FIGURE 5 | Progenitor activation by cPGI$_2$ precedes metabolic differentiation. **(A)** Lin$^-$CD29$^+$CD34$^+$Sca-1$^+$ cells were cultured in adipogenic media ± cPGI$_2$ for the indicated times, at which RNA was obtained for expression profiling with Illumina beadchip arrays ($n = 3$). GSEA was performed at each time point (cPGI$_2$ vs. Control) with the KEGG pathway gene set collection. The enrichment scores of the indicated gene sets related to progenitor activation (red) or metabolic maturation (black) are plotted. Closed markers indicate significance (FDR $q < 0.05$). **(B)** Schematic summary of the kinetics of progenitor cell responses to cPGI$_2$ as detected by gene expression assays, GSEA and EdU incorporation analysis.

magnetic bead separation with few specific markers. Since Pdgfra is the only currently known marker of beige/brite progenitors proven by genetic lineage tracing, we assessed its potential as a single marker for the prospective isolation of beige/brite progenitors. As reported by others, Pdgfra expression was detectable in a fraction of Lin$^+$ cells, in particular, CD45$^+$ leukocytes (**Figures 6A,B**) (19). Nevertheless, 76% of Pdgfra$^+$ cells were Lin$^-$. Importantly, though, only 36% of Lin$^-$Pdgfra$^+$ cells were CD34$^+$Sca-1$^+$ and thereby adipogenic (**Figure 6C**). This expression pattern suggests that Pdgfra-based cell isolation would not result in highly specific enrichment of adipogenic/beige/brite progenitors.

We went on to assess the suitability of Sca-1 as a marker for the enrichment of adipogenic cells includes beige/brite progenitors, given that the majority of Lin$^-$CD29$^+$CD34$^+$Sca1$^+$ cells were Pdgfra$^+$ (Figure S1 in Supplementary Material). Seventy percent of Sca-1$^+$ cells were Lin$^-$, and the Lin$^-$Sca-1$^+$ population contained mainly Pdgfra$^+$ cells, which were CD34$^+$ to a high proportion (54% of the Lin$^-$Sca-1$^+$ population) (**Figures 6D–F**).

On this basis, we went on to develop a two-step magnetic separation procedure with a negative selection of Lin$^+$ cells (erythroid Ter119$^+$, CD31$^+$ and CD45$^+$) followed by positive selection of Sca-1$^+$ cells. This approach led to quantitative recovery of Sca-1$^+$ cells with a purity of approximately 75% (**Figure 6G**). To confirm that the Lin$^-$Sca-1$^+$ MACS-purified cells retain the beige/brite adipogenic potential compared to the Lin$^-$CD29$^+$CD34$^+$Sca1$^+$ population, we cultured both cell isolates under adipogenic conditions in the presence of cPGI$_2$. We examined the expression of *Ucp1*, *Cidea*, *Cpt1b*, and *Cox8b*, known thermogenic adipocyte markers ranking within the 10 most significantly regulated genes in the expression profiles (day 8 cPGI$_2$ vs. Control, adjusted $p < 10^{-6}$). The expression of all markers increased markedly and to a similar extent by cPGI$_2$ in both cell types (**Figure 6H**). Taken together, we demonstrate the MACS-separated Lin$^-$Sca-1$^+$ cells represent a good approximation of the Lin$^-$CD29$^+$CD34$^+$Sca1$^+$Pdgfra$^+$ population retaining the capacity for the induction of beige/brite differentiation.

Discussion

An increasing number of molecular factors have been established as regulators of adipose tissue browning through the use of

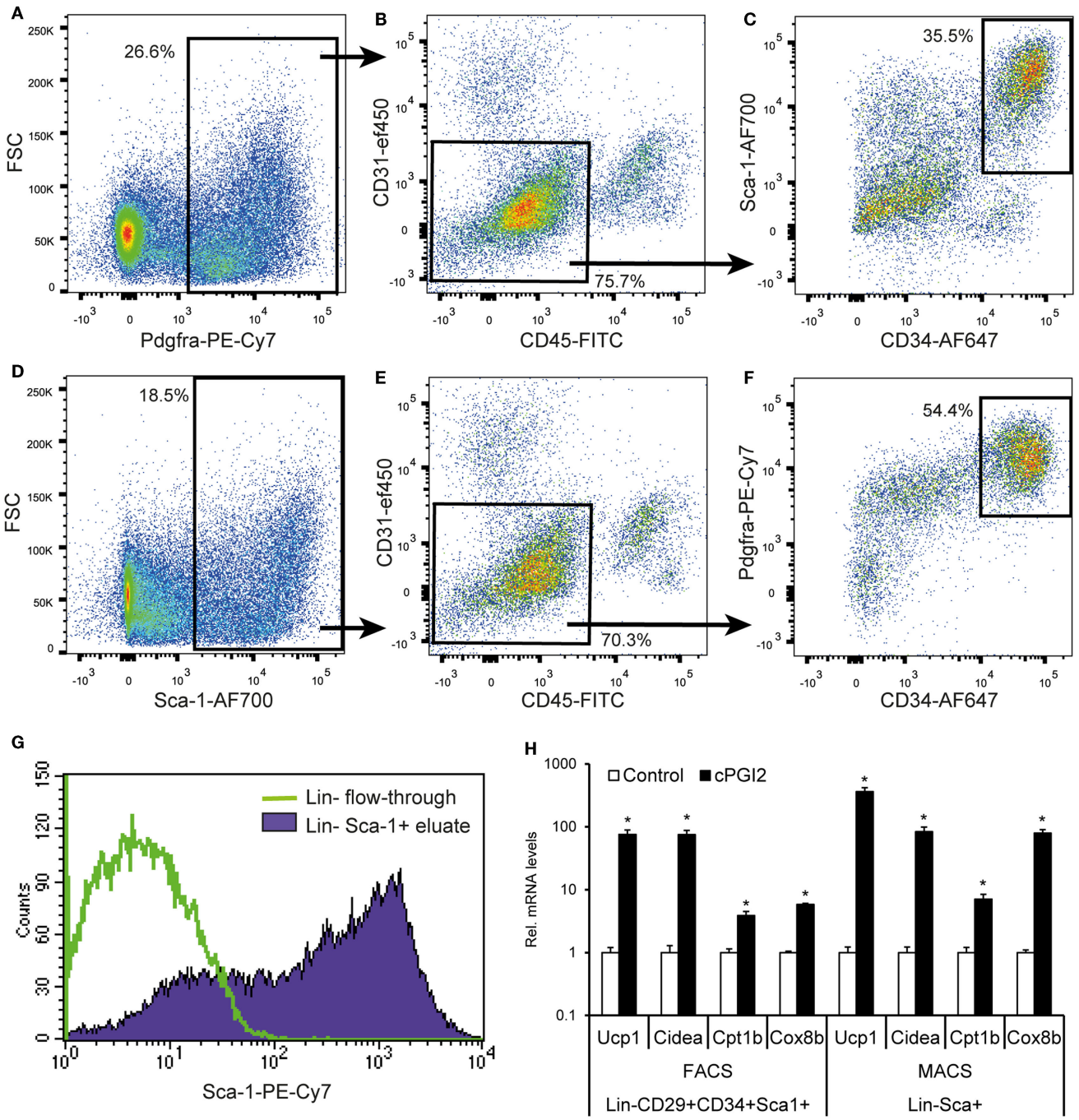


FIGURE 6 | A MACS selection procedure for the prospective isolation of progenitor cells for beige/brite differentiation. **(A–F)** Single cell suspensions were obtained by collagenase digestion of subcutaneous fat, and stained for six-color FACS with the indicated antibodies after removal of adipocytes by centrifugation and erythrocytes by TER119-MACS® depletion. Debris and singlets were excluded and selected through FSC/SSC and FSC-A/H, respectively. **(A–C)** and **(D–F)** represent independent gating schemes. Values (%) indicate cells % of parent plot. Representative plots from multiple independent experiments are shown. **(G)** Erythrocytes, leukocytes, and endothelial cells were removed from single cell suspensions in a first MACS step with biotinylated Ter119, CD45, and CD31 antibodies and streptavidin-conjugated microbeads. Sca-1$^+$ cells were enriched in a second MACS step with Sca-1-PE-Cy7 antibody and anti-PE-Cy7 microbeads. The resulting cell population (Lin$^-$Sca-1$^+$ eluate) as well as the Lin$^-$ flow-through were subjected to flow cytometry. Comparable purities were obtained with directly conjugated antibody-bead combinations (data not shown). **(H)** MACS- and FACS-purified cells were cultured and differentiated for 8 days in the presence or absence of $cPGI_2$ (see Materials and Methods) before subjected to RNA expression analysis by qRT-PCR (* indicates *t*-test $p < 0.003$ $cPGI_2$ vs. Control, $n = 3$).

genetic mouse models (1). However, the investigation of the signaling and transcriptional pathways downstream of physiological mediators of progenitor-dependent beige/brite differentiation has been hampered, partly by the paucity of appropriate cell models. Our study contributes to this challenge in two ways. (a) We present a cell model of beige/brite differentiation based on defined primary adipose tissue progenitor cells and the physiological inducer PGI_2. (b) Using time course expression profiling, we dissect the cascade of progenitor cell responses during beige/brite differentiation, and show that early progenitor activation by $cPGI_2$ through cell cycle and morphology pathways precedes and synergizes with the late upregulation of thermogenic gene expression.

Although certain cell surface markers have been shown to be preferentially expressed in beige progenitor cells and used for their enrichment, we focused on markers based on previous lineage tracing evidence (7, 19, 22). So far, Pdgfra has been the only marker shown by lineage tracing to be expressed in beige/brite progenitors. However, we and others could show that expression of Pdgfra is not restricted to adipogenic cells (**Figures 6A–C**) (19). The $Lin^-CD29^+CD34^+Sca\text{-}1^+$ population, which was shown to contain the majority of adipogenic cells, homogeneously expressed Pdgfra (Figures S1A–C in Supplementary Material) (18, 19). On this basis, we used this marker combination for FACS-isolation, and furthermore, developed a simplified magnetic enrichment procedure yielding cells with beige/brite potential comparable to FACS-isolated cells (**Figure 6**). Of note, the simplified isolation method could also be applicable for addressing questions related to general ("white") adipogenesis from defined progenitors, if $cPGI_2$ treatment is omitted.

Wu et al. provided evidence for the existence of distinct committed progenitors for white vs. beige/brite adipocytes (22). Alternatively, bipotential white/beige progenitors have been implicated in browning (7). Our cell model for the investigation of beige/brite adipocyte recruitment from progenitors is applicable to both hypotheses, as it is likely to include all adipogenic cells including beige/brite progenitors. Notably, our data do not indicate enrichment of beige/brite lineage cells or a lineage switch by $cPGI_2$ (**Figure 1G**).

An emerging concept on the induction of progenitor responses and adipose tissue browning suggests a key role of transient inflammatory signals at the onset of the remodeling process (29–31). In this light, the function of COX-2 and prostaglandins in the recruitment of beige/brite adipocytes becomes evident. In addition, the suitability of $cPGI_2$ as a physiological inducer of beige/brite differentiation is supported by the following facts. (i) $cPGI_2$ caused broad and specific induction of the thermogenic expression program without strong effects on general adipogenesis (**Figure 1**; Figures S2 and S3 in Supplementary Material; **Table 1**). (ii) It is able to induce thermogenic marker genes in primary human adipose tissue progenitors (8) as well as in the human hMADS model (Ez-Amri, personal communication). (iii) $cPGI_2$ relays NE signaling to the progenitor level and activates Ptgir-cAMP as well as Ppar pathways, which are central to thermogenic differentiation (2, 8, 12, 13).

The induction of beige/brite differentiation by the Pparg agonist rosiglitazone has been used for the initial definition of brite adipogenesis (32). However, this inducer causes supra-physiological activation of Pparg and potently promotes general adipogenesis, which is the reason for its broad usage in adipogenic differentiation media (33, 34). An alternative robust model for beige/brite differentiation involves treatment of progenitors from white fat with bone morphogenetic protein 7 (Bmp7), even though the function of Bmp7 in browning *in vivo* remains to be solidified (35).

Time course profiling of the progenitor response to $cPGI_2$ revealed that the upregulation of thermogenic genes occurred late in the differentiation process (**Figures 2** and **5**). The late induction was accompanied by a profound reorganization of the nuclear architecture and relocalization of the *Ucp1* gene locus from the nuclear periphery to central territories, which to our knowledge has not been reported previously. This relocalization was also observed during the differentiation of adipocyte progenitors from brown fat, implying a general functional relevance. Thus, our cell model could serve the exploration of this novel potential level of regulation of *Ucp1* expression.

Despite the late upregulation of thermogenic markers, we could show that the full induction of these genes required the synergistic action of early and late $cPGI_2$ signaling. Focusing on the early $cPGI_2$-mediated transcriptional pathways, we detected changes indicative of transient $cPGI_2$-induced cell cycling at 24 h, which was confirmed by EdU incorporation analysis (**Figures 4** and **5**; **Table 2**). Whereas mitotic clonal expansion has been shown to play a role in adipogenesis, a link to beige/brite differentiation has not been reported (36). Importantly, increased cell cycling was not associated with increased adipogenesis in $cPGI_2$-treated cells (**Figures 1** and **2**; Figures S2 and S3 in Supplementary Material). The mechanistic link between progenitor cycling and the commitment to beige/brite differentiation is currently under investigation. A connecting node could be the retinoblastoma protein, the inactivation of which has been shown to promote G1–S progression as well as thermogenic adipocyte differentiation (37, 38). In addition to cell cycling, $cPGI_2$ affected additional pathways related to progenitor activation, namely, the early downregulation of cell adhesion and cytoskeletal pathways (**Figures 4** and **5**; **Table 2**). Recently, the MRTF/SRF transcription factors were implicated in the regulation of beige/brite differentiation downstream of cytoskeletal changes (39). It is tempting to speculate that $cPGI_2$-mediated morphological responses are causally related to the priming of thermogenic gene expression. Overall, our results highlight the importance of core progenitor activation and commitment pathways for the recruitment of thermogenic cells.

According to current theory, beige/brite thermogenic adipocytes can be recruited from immature progenitors as well as from mature cells (2). Independently of the degree of contribution of each path to physiological browning in rodents, the proliferation capacity and plasticity of progenitor cells highlight their potential for the therapeutic recruitment of thermogenic cells in the context of metabolic disease. Understanding the biology of primary adipocyte progenitor cells is a prerequisite in this direction.

Acknowledgments

We thank Julia Marx and all current members of the Metabolism and Stem Cell Plasticity lab, as well as Steffen Schmitt, Klaus Hexel, and Ann Atzberger (DKFZ Flow Cytometry Unit), and Sabine Henze and Oliver Heil (DKFZ GPCF). We are grateful to Janina Kuhl (Miltenyi Biotec) for excellent technical assistance and the TRI-Imaging facility at the IBCG, Toulouse. This work was supported by grants from the Deutsche Forschungsgemeinschaft (HE 3260/8-1), the EU FP7 project DIABAT (HEALTH-F2-2011-278373), the Human Frontier Science Program (RGY0082/2014), the ANR (Agence Nationale de la Recherche) ANDY project, and the Région Midi-Pyrenées TAMISE project.

Supplementary Material

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fendo.2015.00129

References

1. Harms M, Seale P. Brown and beige fat: development, function and therapeutic potential. *Nat Med* (2013) **19**(10):1252–63. doi:10.1038/nm.3361
2. Diaz MB, Herzig S, Vegiopoulos A. Thermogenic adipocytes: from cells to physiology and medicine. *Metabolism* (2014) **63**(10):1238–49. doi:10.1016/j.metabol.2014.07.002
3. Sidossis L, Kajimura S. Brown and beige fat in humans: thermogenic adipocytes that control energy and glucose homeostasis. *J Clin Invest* (2015) **125**(2):478–86. doi:10.1172/JCI78362
4. Barbatelli G, Murano I, Madsen L, Hao Q, Jimenez M, Kristiansen K, et al. The emergence of cold-induced brown adipocytes in mouse white fat depots is determined predominantly by white to brown adipocyte transdifferentiation. *Am J Physiol Endocrinol Metab* (2010) **298**(6):E1244–53. doi:10.1152/ajpendo.00600.2009
5. Lee YH, Petkova AP, Konkar AA, Granneman JG. Cellular origins of cold-induced brown adipocytes in adult mice. *FASEB J* (2015) **29**(1):286–99. doi:10.1096/fj.14-263038
6. Rosenwald M, Perdikari A, Rulicke T, Wolfrum C. Bi-directional interconversion of brite and white adipocytes. *Nat Cell Biol* (2013) **15**(6):659–67. doi:10.1038/ncb2740
7. Lee YH, Petkova AP, Mottillo EP, Granneman JG. In vivo identification of bipotential adipocyte progenitors recruited by beta3-adrenoceptor activation and high-fat feeding. *Cell Metab* (2012) **15**(4):480–91. doi:10.1016/j.cmet.2012.03.009
8. Vegiopoulos A, Muller-Decker K, Strzoda D, Schmitt I, Chichelnitskiy E, Ostertag A, et al. Cyclooxygenase-2 controls energy homeostasis in mice by de novo recruitment of brown adipocytes. *Science* (2010) **328**(5982):1158–61. doi:10.1126/science.1186034
9. Wang QA, Tao C, Gupta RK, Scherer PE. Tracking adipogenesis during white adipose tissue development, expansion and regeneration. *Nat Med* (2013) **19**(10):1338–44. doi:10.1038/nm.3324
10. Jimenez M, Barbatelli G, Allevi R, Cinti S, Seydoux J, Giacobino JP, et al. Beta 3-adrenoceptor knockout in C57BL/6J mice depresses the occurrence of brown adipocytes in white fat. *Eur J Biochem* (2003) **270**(4):699–705. doi:10.1046/j.1432-1033.2003.03422.x
11. Madsen L, Pedersen LM, Lillefosse HH, Fjaere E, Bronstad I, Hao Q, et al. UCP1 induction during recruitment of brown adipocytes in white adipose tissue is dependent on cyclooxygenase activity. *PLoS One* (2010) **5**(6):e11391. doi:10.1371/journal.pone.0011391
12. Brun RP, Tontonoz P, Forman BM, Ellis R, Chen J, Evans RM, et al. Differential activation of adipogenesis by multiple PPAR isoforms. *Genes Dev* (1996) **10**(8):974–84. doi:10.1101/gad.10.8.974
13. Narumiya S, Sugimoto Y, Ushikubi F. Prostanoid receptors: structures, properties, and functions. *Physiol Rev* (1999) **79**(4):1193–226.
14. Saeed AI, Sharov V, White J, Li J, Liang W, Bhagabati N, et al. TM4: a free, open-source system for microarray data management and analysis. *Biotechniques* (2003) **34**(2):374–8.
15. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A* (2005) **102**(43):15545–50. doi:10.1073/pnas.0506580102
16. Kocanova S, Kerr EA, Rafique S, Boyle S, Katz E, Caze-Subra S, et al. Activation of estrogen-responsive genes does not require their nuclear co-localization. *PLoS Genet* (2010) **6**(4):e1000922. doi:10.1371/journal.pgen.1000922
17. Iannuccelli E, Mompart F, Gellin J, Lahbib-Mansais Y, Yerle M, Boudier T. NEMO: a tool for analyzing gene and chromosome territory distributions from 3D-FISH experiments. *Bioinformatics* (2010) **26**(5):696–7. doi:10.1093/bioinformatics/btq013
18. Rodeheffer MS, Birsoy K, Friedman JM. Identification of white adipocyte progenitor cells in vivo. *Cell* (2008) **135**(2):240–9. doi:10.1016/j.cell.2008.09.036
19. Berry R, Rodeheffer MS. Characterization of the adipocyte cellular lineage in vivo. *Nat Cell Biol* (2013) **15**(3):302–8. doi:10.1038/ncb2696
20. Negrel R, Gaillard D, Ailhaud G. Prostacyclin as a potent effector of adipose-cell differentiation. *Biochem J* (1989) **257**(2):399–405.
21. Timmons JA, Wennmalm K, Larsson O, Walden TB, Lassmann T, Petrovic N, et al. Myogenic gene expression signature establishes that brown and white adipocytes originate from distinct cell lineages. *Proc Natl Acad Sci U S A* (2007) **104**(11):4401–6. doi:10.1073/pnas.0610615104
22. Wu J, Bostrom P, Sparks LM, Ye L, Choi JH, Giang AH, et al. Beige adipocytes are a distinct type of thermogenic fat cell in mouse and human. *Cell* (2012) **150**(2):366–76. doi:10.1016/j.cell.2012.05.016
23. Ussar S, Lee KY, Dankel SN, Boucher J, Haering MF, Kleinridders A, et al. ASC-1, PAT2, and P2RX5 are cell surface markers for white, beige, and brown adipocytes. *Sci Transl Med* (2014) **6**(247):247ra103. doi:10.1126/scitranslmed.3008490
24. Walden TB, Hansen IR, Timmons JA, Cannon B, Nedergaard J. Recruited vs. nonrecruited molecular signatures of brown, "brite," and white adipose tissues. *Am J Physiol Endocrinol Metab* (2012) **302**(1):E19–31. doi:10.1152/ajpendo.00249.2011
25. Meister P, Taddei A. Building silent compartments at the nuclear periphery: a recurrent theme. *Curr Opin Genet Dev* (2013) **23**(2):96–103. doi:10.1016/j.gde.2012.12.001
26. Therizols P, Illingworth RS, Courilleau C, Boyle S, Wood AJ, Bickmore WA. Chromatin decondensation is sufficient to alter nuclear organization in embryonic stem cells. *Science* (2014) **346**(6214):1238–42. doi:10.1126/science.1259587
27. Volpi EV, Chevret E, Jones T, Vatcheva R, Williamson J, Beck S, et al. Large-scale chromatin organization of the major histocompatibility complex and other regions of human chromosome 6 and its response to interferon in interphase nuclei. *J Cell Sci* (2000) **113**(Pt 9):1565–76.
28. Helin K. Regulation of cell proliferation by the E2F transcription factors. *Curr Opin Genet Dev* (1998) **8**(1):28–35. doi:10.1016/S0959-437X(98)80058-0
29. Lee YH, Petkova AP, Granneman JG. Identification of an adipogenic niche for adipose tissue remodeling and restoration. *Cell Metab* (2013) **18**(3):355–67. doi:10.1016/j.cmet.2013.08.003
30. Qiu Y, Nguyen KD, Odegaard JI, Cui X, Tian X, Locksley RM, et al. Eosinophils and type 2 cytokine signaling in macrophages orchestrate development of functional beige fat. *Cell* (2014) **157**(6):1292–308. doi:10.1016/j.cell.2014.03.066
31. Wernstedt Asterholm I, Tao C, Morley TS, Wang QA, Delgado-Lopez F, Wang ZV, et al. Adipocyte inflammation is essential for healthy adipose tissue expansion and remodeling. *Cell Metab* (2014) **20**(1):103–18. doi:10.1016/j.cmet.2014.05.005
32. Petrovic N, Walden TB, Shabalina IG, Timmons JA, Cannon B, Nedergaard J. Chronic peroxisome proliferator-activated receptor gamma (PPARgamma) activation of epididymally derived white adipocyte cultures reveals a population of thermogenically competent, UCP1-containing adipocytes molecularly distinct from classic brown adipocytes. *J Biol Chem* (2010) **285**(10):7153–64. doi:10.1074/jbc.M109.053942

33. Lehmann JM, Moore LB, Smitholiver TA, Wilkison WO, Willson TM, Kliewer SA. An antidiabetic thiazolidinedione is a high-affinity ligand for peroxisome proliferator-activated receptor gamma(PPAR-gamma). *J Biol Chem* (1995) **270**(22):12953–6. doi:10.1074/jbc.270.22.12953
34. Samarasinghe SP, Sutanto MM, Danos AM, Johnson DN, Brady MJ, Cohen RN. Altering PPARgamma ligand selectivity impairs adipogenesis by thiazolidinediones but not hormonal inducers. *Obesity* (2009) **17**(5):965–72. doi:10.1038/oby.2008.629
35. Schulz TJ, Huang TL, Tran TT, Zhang H, Townsend KL, Shadrach JL, et al. Identification of inducible brown adipocyte progenitors residing in skeletal muscle and white fat. *Proc Natl Acad Sci U S A* (2011) **108**(1):143–8. doi:10.1073/pnas.1010929108
36. Tang QQ, Otto TC, Lane MD. Mitotic clonal expansion: a synchronous process required for adipogenesis. *Proc Natl Acad Sci U S A* (2003) **100**(1):44–9. doi:10.1073/pnas.0137044100
37. Hansen JB, Jorgensen C, Petersen RK, Hallenborg P, De Matteis R, Boye HA, et al. Retinoblastoma protein functions as a molecular switch determining white versus brown adipocyte differentiation. *Proc Natl Acad Sci U S A* (2004) **101**(12):4112–7. doi:10.1073/pnas.0301964101
38. Scime A, Grenier G, Huh MS, Gillespie MA, Bevilacqua L, Harper ME, et al. Rb and p107 regulate preadipocyte differentiation into white versus brown fat through repression of PGC-1alpha. *Cell Metab* (2005) **2**(5):283–95. doi:10.1016/j.cmet.2005.10.002
39. McDonald ME, Li C, Bian H, Smith BD, Layne MD, Farmer SR. Myocardin-related transcription factor A regulates conversion of progenitors to beige adipocytes. *Cell* (2015) **160**(1–2):105–18. doi:10.1016/j.cell.2014.12.005

Conflict of Interest Statement: Stefan Wild, Andreas Bosio, and Olaf Hardt are full time employees of Miltenyi Biotec GmbH. The remaining co-authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

MINI REVIEW
published: 30 June 2015
doi: 10.3389/fendo.2015.00104

Role of energy metabolism in the brown fat gene program

*Minwoo Nam and Marcus P. Cooper**

Division of Cardiovascular Medicine, Department of Medicine, University of Massachusetts Medical School, Worcester, MA, USA

In murine and human brown adipose tissue (BAT), mitochondria are powerful generators of heat that safely metabolize fat, a feature that has great promise in the fight against obesity and diabetes. Recent studies suggest that the actions of mitochondria extend beyond their conventional role as generators of heat. There is mounting evidence that impaired mitochondrial respiratory capacity is accompanied by attenuated expression of *Ucp1* and other BAT-selective genes, implying that mitochondria exert transcriptional control over the brown fat gene program. In this review, we discuss the current understanding of brown fat mitochondria, their potential role in transcriptional control of the brown fat gene program, and potential strategies to treat obesity in humans by leveraging thermogenesis in brown adipocytes.

Keywords: brown fat, mitochondria, respiratory capacity, thermogenesis, brown fat gene program

OPEN ACCESS

Edited by:
Jun Wu,
University of Michigan, USA

Reviewed by:
Anthony Scime,
York University, Canada
Lin Chang,
University of Michigan, USA

****Correspondence:***
Marcus P. Cooper,
Division of Cardiovascular Medicine, Department of Medicine, University of Massachusetts Medical School, 368 Plantation Street, Albert Sherman Center, 7th Floor West, AS7-1053, Worcester, MA 01605, USA
marcus.cooper@umassmed.edu

Specialty section:
This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *08 May 2015*
Accepted: *15 June 2015*
Published: *30 June 2015*

Citation:
Nam M and Cooper MP (2015) Role of energy metabolism in the brown fat gene program. Front. Endocrinol. 6:104. doi: 10.3389/fendo.2015.00104

Introduction

Brown fat is composed of thermogenic adipocytes that convert chemical energy to heat. Found in homeothermic animals such as mammals, brown fat protects against cold stress. In neonates, brown fat plays a critical role in thermoregulation. The thermogenic capacity of brown fat is attributed to abundant mitochondria, which dissipates heat via uncoupling (1).

There are two types of thermogenic adipocytes: classical brown adipocytes and beige/brite adipocytes. Classical brown adipocytes exhibit constitutive thermogenic capacity with large numbers of mitochondria. Developmentally programed brown fat, such as the rodent interscapular depot, consists of these cells. Another type of thermogenic adipocytes is found in white fat. These cells are recruited by thermogenic stimuli and in turn display comparable thermogenic capacity to classical brown adipocytes. Due to these properties, they have been named beige/brite (brown-in-white) adipocytes (1, 2).

Brown fat has unique genetic signatures that support its thermogenic function. Uncoupling protein 1 (*Ucp1*), a key thermogenic protein, is highly expressed in brown fat. Cell death-inducing DFFA-like effector (*Cidea*), a modulator of UCP1, and Type II iodothyronine deiodinase (*Dio2*), an enzyme that converts T4 to active T3 within the tissue locally, are among major thermogenic genes (3–5). Fatty acid oxidation (FAO) genes and electron transport chain (ETC) subunit genes are also enriched (6). This transcriptional signature is in part determined by PR domain containing 16 (*Prdm16*), a determinant of brown fat, and peroxisome proliferator-activated receptor gamma coactivator *1*-alpha (*Ppargc1a*), a key coactivator of peroxisome proliferator-activated receptor alpha (*Ppara*) and gamma (*Pparg*) (7, 8). The transcriptional network of the brown fat genes have been reviewed elsewhere (9). Since many of the brown fat-selective genes regulate thermogenesis, hereafter "the thermogenic gene program" and "the brown fat gene program" are reciprocally used.

Mitochondria play a central role in providing an energetic basis for thermogenesis in brown fat. Brown fat mitochondria fulfill their duty as cellular powerhouses in the non-stimulated state.

In sympathetically stimulated brown fat, UCP1 is rapidly activated and uncouples electron transit and ATP production. Instead, the chemical energy stored in the proton gradient is dissipated as heat (10). Interestingly, several studies have demonstrated that impaired mitochondrial respiratory capacity is accompanied by attenuated expression of *Ucp1* and other brown adipose tissue (BAT)-selective genes in classical brown adipocytes (11, 12). In addition to a conventional role as generators of heat, this suggests that brown fat mitochondria have an unappreciated role in regulating genes involved in thermogenesis depending on the status of respiratory capacity. Critical evaluation of these studies will broaden our thoughts on mitochondria in brown fat. This review summarizes the features of brown fat mitochondria and discusses their potential role in transcriptional control of the brown fat gene program and its therapeutic implications in humans.

Features of Brown Fat Mitochondria

Morphological Features

Brown fat mitochondria are dynamic organelles that meet the thermogenic needs of the organism by regulating their number and networking as well as their biochemical and ultrastructural profile. Acute cold exposure (or activation with norepinephrine) immediately promotes mitochondrial fission, an event that precedes and augments thermogenesis (13). Brown fat mitochondria exhibit unique morphological features. Notably, brown fat mitochondria are enlarged and densely packed with respiratory units, resulting in dense cristae (14). The morphological features of brown fat also differ across gender with females having larger mitochondria and denser cristae (15). The density of brown fat mitochondria is among the highest of any tissue (16). Even so, chronic cold exposure increases mitochondrial mass even further. This is mediated by catecholamines via β-adrenergic signaling, which increases PGC-1α, a transcriptional coactivator that induces ERRα and NRF-1, culminating in increased mitochondrial mass (17–19).

Biochemical Features

Early biochemical and functional studies on brown fat mitochondria in rodents revealed high cellular respiration but low ATP synthase activity (20). This implied that pathway involving proton leakage must underlie the basis of thermogenesis. Biochemical studies identified that UCP1 constitutes the molecular basis for enhanced proton leak (21). Interestingly, in larger mammals such as lambs the abundance of ATP synthase is higher (22), presumably because larger animals are less dependent on non-shivering thermogenesis due to their smaller surface-to-volume ratio and due to their increased capacity for shivering.

Long-chain free fatty acids (LCFAs) activate UCP1, while purine nucleotides inhibit UCP1 (10). Intuitively, mobilization of free fatty acids via β-adrenergic activation is coupled to UCP1 activation and heat production. Fedorenko et al. (23) reported that UCP1 does not exhibit constitutive proton transport activity. Instead, there is obligatory binding of LCFAs to UCP1, a process that transfers protons associated with LCFA into the matrix via a conformational change of UCP1. LCFAs can also overcome inhibition by purine nucleotides. This is important because in the basal state, there is no lipolysis and inhibition of UCP1 by purine nucleotides will promote coupled ATP-generating respiration.

In theory, uncoupling should de-energize brown fat mitochondria, culminating in an ATP crisis. ATP, however, is required for the activation of fatty acids during uncoupling. Brown fat mitochondria circumvent this by increasing glycolysis as well as the TCA cycle. Arsenite, an inhibitor of pyruvate dehydrogenase complex (PDC) and α-ketoglutarate dehydrogenase, depleted ATP in norepinephrine-stimulated brown adipocytes, implying that the TCA cycle is critical for maintaining ATP during thermogenesis (24). Although succinyl-CoA synthetase primarily generates GTP, this TCA enzyme complex can also generate ATP, a process which may explain how the TCA cycle is critical for maintaining ATP in brown fat (discussed further in Proteomical Features). Glycolysis is an important source of ATP, too. Notably, hexokinase activity increases fourfold in cold acclimated rats, achieving glycolytic activity similar to liver (25).

Proteomical Features

Mass spectrometric analysis of brown fat mitochondria has revealed striking proteomic difference compared with white fat mitochondria (26). In fact, the proteomic profile of brown fat mitochondria was most similar to that of skeletal muscle. Compared with white fat mitochondria, there was an enrichment of catabolic pathways including ETC, TCA cycle, and fatty acid metabolism in brown fat mitochondria. Complexes I–IV are present at higher levels, whereas complex V is present at lower levels, a pattern favorable for thermogenesis. There is robust expression of enzymes involved in the TCA cycle–ADP-forming succinyl-CoA synthetase β subunit (A-SCS-β), pyruvate dehydrogenase kinase 4 (PDK4), and pyruvate dehydrogenase phosphatase regulatory subunit (PDPr). SCS converts succinyl-CoA to succinate. This reaction is coupled to the formation of ATP or GTP, which is determined by two different β subunits, ADP-forming and GDP-forming. In mouse, rat, and human, metabolically active tissues, such as brain and heart, express high levels of ADP-forming subunits compared with GDP-forming subunits (27). Likewise, brown fat mitochondria may preferentially use A-SCS-β to supply ATP, a feature that matches a role of substrate-level phosphorylation in stimulated brown adipocytes. During cold exposure, lipid uptake and lipogenesis replenish fat stores that have been oxidized (28). Control of lipogenesis during cold exposure is complex and partly regulated by pyruvate metabolism (29, 30). Pyruvate can be targeted for complete oxidation by converting it into acetyl-CoA via the enzymatic action of PDC (31). Alternatively, inhibition of PDC by PDK4 diverts pyruvate into glycerol, which is the backbone for free fatty acid (FFA) esterification (31). An enzymatic complex consisting of PDPr counteracts the action of PDK4, and thus, targets pyruvate for complete oxidation (32, 33). In summary, opposing regulation by PDK4 and PDP may play a critical role in whether or not the brown adipocyte uses pyruvate for lipogenesis (PDK4-mediated) or complete oxidation (PDPr-mediated).

Fatty acids serve as major substrates for thermogenesis and they activate UCP1 (10). In brown fat, there is a high expression of enzymes involved in FAO, including short-, medium-, long-chain

acyl-CoA dehydrogenases and 3-ketoacyl-CoA thiolase (26). Long-chain fatty acids require a carnitine palmitoyltransferase 1B (CPT1B)-mediated carnitine shuttle for oxidation in mitochondria. Supporting a role for robust oxidative capacity in brown fat, CPT1B is 50-fold higher in brown fat compared with white fat (26). Brown fat may also exhibit metabolic flexibility in fuel utilization. Highly expressed in brown fat, acetyl-CoA synthetase 2-like (ACSS1) permits oxidation of ketone bodies during starvation (26, 34, 35). Indeed, activity of ketone body oxidizing enzymes in brown fat parallels that of the heart (36).

Role of Energy Metabolism in the Brown Fat Gene Program

Although manipulating regulators of mitochondrial biogenesis, such as PGC-1 coactivators and ERRα, affects *Ucp1* expression, those factors are also known to directly regulate transcription of respiratory subunits (37), making it challenging to delineate a direct relationship between respiratory capacity and the thermogenic gene program *per se*. Here, we discuss approaches to directly manipulate mitochondrial respiratory capacity and the attendant effects on the brown fat genes, which are summarized in **Table 1**.

Leucine-Rich Pentatricopeptide Repeat Containing Motif (LRPPRC; also called Leucine-Rich Protein 130 kDa, LRP130)

A potential role of mitochondrial respiratory capacity in the brown fat gene program was reported in a study using LRPPRC-deficient brown adipocytes (11). LRPPRC was originally identified as a causal protein in a rare neurological disorder called Leigh Syndrome French Canadian variant (42). Initial studies using human fibroblasts identified defects in cytochrome *c* oxidase deficiency; however, later studies using mouse models revealed that LRPPRC affects the expression of all mitochondrially encoded subunits of the ETC but their differential effects on respiratory complex activity related to cell type (43–46). Brown adipocytes with depleted LRPPRC were notable for impaired oxygen consumption but intact mitochondrial density and PGC-1 coactivators, indicating a specific impairment of respiratory capacity without altering mitochondrial biogenesis (11).

LRPPRC-deficient brown adipocytes had a marked reduction in brown fat-selective genes, including *Ucp1* and *Cidea*, suggesting a link between respiratory capacity and a basal expression of certain brown fat genes (11). While LRRPRC is weakly expressed in the nucleus, recent data show that the majority of LRPPRC is localized to mitochondria and regulates mtDNA-encoded transcripts across various species, suggesting that a nuclear role of LRPPRC in the regulation of brown fat genes may be modest and that the predominant effect is mediated by impaired cellular respiration (44, 47–49). In addition, cAMP-mediated induction of *Ucp1* was unaffected in LRPPRC-deficient brown adipocytes (11). Given that PGC-1α is responsible for this cAMP effect, it is less likely that LRPPRC is an essential part of PGC-1α coactivator complexes necessary for *Ucp1* expression. Furthermore, in human

TABLE 1 | Effects of manipulating mitochondrial respiratory capacity on the brown fat gene program.

	Model	Respiratory capacity	Mitochondrial mass	Brown fat morphology	Brown fat genes	Other	Reference
Mouse	COX7RP KO	↓10–20% in O_2 consumption	n/a	Hypertrophic Pale brown	↓75% in *Ucp1*; ↓*Dio2*, *Elovl3*	Normal *Erra*, *Nrf1*, *Tfam*	Ikeda et al. (12)
	TFAM FKO (Fabp4-Cre)	↓40–60% in complex I and IV; ↑70% in complex II (BAT); ↑30% in O_2 consumption (BAT); ↑80% in state3 OCR (BAT mt)	↑20% in citrate synthase activity	Normal	Normal	↓DIO ↓Insulin resistance	Vernochet et al. (38)
	TFAM FKO (Adipoq-Cre)	↓40–80% in complex I and IV (BAT)	↑80% in citrate synthase activity	Whitening	Normal	↓DIO ↑Insulin resistance Lipodystrophy and inflammation in WAT	Vernochet et al. (39)
	CRIF1 FKO (Fabp4-Cre)	↓OXPHOS subunits protein	↓Mitochondrial abundance ↑Mitochondrial size	Smaller size	Unaltered UCP1	Defective WAT postnatal death at week 3	Ryu et al. (40)
	LSD1 Tg (iWAT)	↑*Nrf1*, *Cpt1b*, *Cox8b* complex II and IV	↑Mitochondrial abundance	↑Beige/brite adipocytes in IWAT	↑*Prdm16*, *Ppargc1a*, *Ucp1*	↓DIO ↓Insulin resistance	Duteil et al. (41)
Cell	LRPPRC KD in brown adipocytes	↓20% in O_2 consumption			↓40–75% in *Ucp1*, *Cidea*, *Cox7a1*		Cooper et al. 2008 (11)

↓, *Decrease;* ↑, *increase; KO, knockout; Tg, transgenic; FKO, fat-specific KO; KD, knockdown; mt, mitochondria; BAT, brown adipose tissue; IWAT, inguinal white adipose tissue; DIO, diet-induced obesity; n/a, not assessed.*

fibroblasts, deficiency of LRPPRC did not affect expression of PGC-1α target genes (50), implying that signals secondary to impaired cellular respiration, not reduced nuclear expression of LRPPRC, are likely important.

Cytochrome C Oxidase Subunit VIIa Polypeptide 2-Like (COX7RP)

Recent work using COX7RP knockout mice provides direct evidence for the role of respiratory capacity in the brown fat gene program (12). In this study, COX7RP was identified as a novel assembly factor for respiratory chain supercomplexes in mitochondria. ETC complexes are known to form supercomplexes, consisting mainly of complex I, III, and IV (so-called respirasome), which enhances respiratory activity (51). With reduced oxygen consumption at a whole-body level, COX7RP KO mice revealed hypertrophic and pale brown fat, generally indicative of defective brown fat. More importantly, *Ucp1* was severely reduced in this dysmorphic brown fat. Microarray analysis also showed a downregulation of several brown fat-enriched genes including *Dio2* and *Elovl3*. Expression of PGC-1 coactivators was decreased but their downstream targets, such as *Erra*, *Nrf1*, and *Tfam*, were unaltered, implying no significant impact on the PGC-1 coactivator network. All together, this study strongly suggests that respiratory capacity dictates a retrograde signaling from mitochondria to the nucleus regulating the brown fat gene program.

Transcription Factor A, Mitochondrial (TFAM)

One approach to genetically manipulate respiratory capacity is to target components of the basal transcriptional machinery of mitochondrial DNA (mtDNA). Among them is TFAM, a key player in mtDNA transcription and maintenance (52). Fabp4-Cre-driven loss of TFAM led to diminished respiratory activity and a drop in mtDNA copy number in brown and white fat (38). Paradoxically, in those mice, brown fat showed enhanced respiratory capacity as evidenced by increased oxygen consumption, FAO, and citrate synthase activity. Although reduced in weight, this brown fat had normal expression of brown fat genes. Similarly, brown fat markers were intact in brown fat with Adipoq-Cre-driven loss of TFAM, which was accompanied by increased citrate synthase activity (39). Therefore, it is likely that the unaltered brown fat gene program in TFAM-deficient fat is ascribed to a compensatory increase in respiratory capacity. Although not decisive, observations from adipose-specific TFAM knockout mice imply that whole-cell respiratory capacity is monitored by an innate sensor to dictate the brown fat gene program.

CR6-Interacting Factor 1 (CRIF1)

CRIF1 is a mitochondrial protein that controls the translation and insertion of mitochondrially encoded respiratory subunits into the inner membrane (53). The activities of Complexes I, III, and IV are abrogated by CRIF1 deficiency in mouse embryonic fibroblasts (53). The severity of impaired respiratory capacity by ablation of CRIF1 is evident in brain-specific and cardiac muscle-specific knockout mice in which severe neurodegeneration and premature death develop, respectively (53, 54). Adipose-specific CRIF1 knockout mice (driven by Fabp4-Cre) show a developmental defect in white fat, reduced body weight, and postnatal death at week 3 (40). Brown fat in these mice is smaller in size; however, histology is unremarkable and UCP1 expression is normal. Because mice die by 3 weeks of age, it was not possible to assess the chronic effect of respiratory defects in brown fat. Although mice with Adipoq-Cre-driven deletion of CRIF1 were viable, data regarding the brown fat genes were not shown (40). Even so, these data could suggest that impaired mitochondrial function does not influence brown adipocyte development. Future studies will be necessary to address if mitochondrial function is critical for the maintenance of the brown fat program. Finally, as mentioned for TFAM, the method by which cellular respiration is disrupted may have differential effects on mitochondrial signaling and subsequent transcriptional events in the nucleus.

LSD1 (Lysine-Specific Demethylase 1)

LSD1 demethylates mono- and di-methylated lysines (particularly lysine 4 and 9 of histone H3) via the cofactor flavin adenosine dinucleotide (FAD) (55). Ubiquitously expressed, LSD1 is essential for embryogenesis and tissue-specific differentiation (56). In the study by Duteil et al. (41), LSD1 was newly identified as a cold-, and β3-adrenergic signaling-inducible protein in mouse white fat. Ectopic expression of LSD1 further revealed that it was sufficient to induce respiratory capacity through nuclear respiratory factor 1 (NRF1) in white adipose cell lines. In addition, there was an activation of the brown fat gene program including *Prdm16*, *Ppargc1a,* and *Ucp1* in LSD1-overexpressing white adipocytes. LSD1 transgenic mice confirmed these *in vitro* findings. Interestingly, this browning effect was more robust in subcutaneous white fat where beige adipocytes reside. However, in brown fat of LSD1 transgenic mice, respiratory activity was modestly increased and there were no significant changes in the brown fat markers. These data suggest that augmented respiratory capacity may promote browning by stimulating beige adipocytes.

Therapeutic Implications

There is an association between impaired brown fat function and various animal models of obesity and diabetes, including *ob/ob* mice, *db/db* mice, SHR/N-*cp* rats, and high-fat/high sucrose (HFHS)-fed mice (57–61). A similar association has been reported for human obesity (62–64). In the aforementioned murine models of obesity and diabetes, there is "de-browning" of brown fat, characterized by concomitant reduction in UCP1 protein, and respiratory complex activities (40, 53–56). Similar to classical brown adipocytes, beige adipocytes exhibit this type of de-browning in mice fed a high-fat diet, implying a shared mechanism for all types of thermogenic adipocytes (65). As detailed earlier, several models and systems have established a causal relationship between mitochondrial respiratory capacity and transcription of genes involved in thermogenesis. Impaired respiration in certain models of obesity and diabetes may explain impaired transcription of the thermogenic genes. In the future, altering respiratory capacity may prove promising in restoring brown fat function in certain forms of obesity and diabetes. Even if there are no effects on transcriptional programs involved in thermogenesis, augmenting respiratory capacity *per se* could still effectively protect against

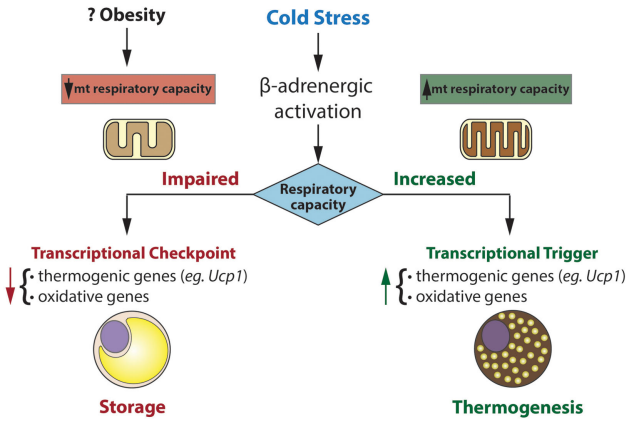


FIGURE 1 | Mitochondria exert transcriptional control over gene programs involved in oxidative metabolism and thermogenesis. Transcriptional control is dependent on mitochondrial respiratory capacity. In this model, impaired respiratory capacity acts as a transcriptional checkpoint, whereas augmented respiratory capacity acts as a transcriptional trigger. In humans and mice, obesity is associated with reduced brown fat function. Our model may explain the inverse association between respiratory capacity and thermogenic gene expression in brown fat of certain obese mice (eg. *ob/ob*) and perhaps obese humans.

obesity and diabetes by way of increased respiratory capacity, which in turn would increase thermogenic capacity.

In humans and mice, cold exposure and β3-adrenergic agonists activate brown fat to promote energy expenditure (10, 66–68). Many adrenergic receptor (AR) agonists, including pan-adrenergic (ephedrine) and β3-adrenergic (CL-316,243, etc.) agonists, have been unsuccessful in humans due to either undesirable cardiovascular effects, poor oral availability, or in the case of CL 316,243, weak agonism for the human β3-AR (69). Recently, a clinical study using a new class of β3-AR agonist increased energy expenditure via brown fat-meditated thermogenesis (70). Based on our review of the literature, the status of mitochondrial respiratory capacity may powerfully influence the outcome of β3-adrenergic agents and should be considered as adjunctive therapies to either restore or enhance the brown fat gene program.

Concluding Remarks

The putative role of mitochondrial respiratory capacity in transcriptional programs that regulate thermogenesis raises an intriguing question (**Figure 1**). Why is respiratory capacity *per se* linked to the regulation of the thermogenic gene program in brown fat? And what are the signals from mitochondria that govern this control? From a teleological perspective, impaired respiratory capacity would impair thermogenesis, making it a futile process. There seems to be interesting retrograde signaling from the mitochondrion to the nucleus, signaling, which may inactivate programs that expend energy and may promote storing that energy as lipid. Upon recovery of respiratory capacity, such signaling is turned off and energy expending programs are re-activated.

Control of nuclear genes based on the status of mitochondrial function (or stress) has been termed "mitohormesis" and is readily apparent in yeast and mammalian cells (71). In brown fat, a highly specialized tissue for thermogenesis, such mitohormesis may be crucial in matching functional and metabolic capacity to genetic programs that influence them.

In the future, it will be of great interest to unravel the signaling pathways by which respiratory capacity regulates the thermogenic gene program. Elucidating these signals and downstream pathways may inform the search for diagnostic and therapeutic interventions important for obesity and diabetes.

Acknowledgments

We thank Thomas E. Akie for critical reading and helpful comments. This work was supported by NIDDK grant 5R01DK089185 (MC) and by a predoctoral fellowship (13PRE13700002) from the American Heart Association (MN).

References

1. Kajimura S, Saito M. A new era in brown adipose tissue biology: homeostasis. *Annu Rev Physiol* (2014) **76**:225–49. doi:10.1146/annurev-physiol-021113-170252
2. Wu J, Cohen P, Spiegelman BM. Adaptive thermogenesis in adipocytes: is beige the new brown? *Genes Dev* (2013) **27**(3):234–50. doi:10.1101/gad.211649.112
3. Ricquier D. Uncoupling protein 1 of brown adipocytes, the only uncoupler: a historical perspective. *Front Endocrinol* (2011) **2**:85. doi:10.3389/fendo.2011.00085
4. Zhou Z, Yon TS, Chen Z, Guo K, Ng CP, Ponniah S, et al. Cidea-deficient mice have lean phenotype and are resistant to obesity. *Nat Genet* (2003) **35**(1):49–56. doi:10.1038/ng1225
5. de Jesus LA, Carvalho SD, Ribeiro MO, Schneider M, Kim SW, Harney JW, et al. The type 2 iodothyronine deiodinase is essential for adaptive thermogenesis in brown adipose tissue. *J Clin Invest* (2001) **108**(9):1379–85. doi:10.1172/JCI13803
6. Shinoda K, Luijten IH, Hasegawa Y, Hong H, Sonne SB, Kim M, et al. Genetic and functional characterization of clonally derived adult human brown adipocytes. *Nat Med* (2015) **21**(4):389–94. doi:10.1038/nm.3819
7. Seale P, Kajimura S, Yang W, Chin S, Rohas LM, Uldry M, et al. Transcriptional control of brown fat determination by PRDM16. *Cell Metab* (2007) **6**(1):38–54. doi:10.1016/j.cmet.2007.06.001
8. Uldry M, Yang W, St-Pierre J, Lin J, Seale P, Spiegelman BM. Complementary action of the PGC-1 coactivators in mitochondrial biogenesis and brown fat differentiation. *Cell Metab* (2006) **3**(5):333–41. doi:10.1016/j.cmet.2006.04.002
9. Kajimura S, Seale P, Spiegelman BM. Transcriptional control of brown fat development. *Cell Metab* (2010) **11**(4):257–62. doi:10.1016/j.cmet.2010.03.005
10. Cannon B, Nedergaard J. Brown adipose tissue: function and physiological significance. *Physiol Rev* (2004) **84**(1):277–359. doi:10.1152/physrev.00015.2003
11. Cooper MP, Uldry M, Kajimura S, Arany Z, Spiegelman BM. Modulation of PGC-1 coactivator pathways in brown fat differentiation through LRP130. *J Biol Chem* (2008) **283**(46):31960–7. doi:10.1074/jbc.M805431200
12. Ikeda K, Shiba S, Horie-Inoue K, Shimokata K, Inoue S. A stabilizing factor for mitochondrial respiratory supercomplex assembly regulates energy metabolism in muscle. *Nat Commun* (2013) **4**:2147. doi:10.1038/ncomms3147
13. Wikstrom JD, Mahdaviani K, Liesa M, Sereda SB, Si Y, Las G, et al. Hormone-induced mitochondrial fission is utilized by brown adipocytes as an amplification pathway for energy expenditure. *EMBO J* (2014) **33**(5):418–36. doi:10.1002/embj.201385014

14. Justo R, Oliver J, Gianotti M. Brown adipose tissue mitochondrial subpopulations show different morphological and thermogenic characteristics. *Mitochondrion* (2005) **5**(1):45–53. doi:10.1016/j.mito.2004.09.003
15. Rodriguez-Cuenca S, Pujol E, Justo R, Frontera M, Oliver J, Gianotti M, et al. Sex-dependent thermogenesis, differences in mitochondrial morphology and function, and adrenergic response in brown adipose tissue. *J Biol Chem* (2002) **277**(45):42958–63. doi:10.1074/jbc.M207229200
16. Loncar D. Convertible adipose tissue in mice. *Cell Tissue Res* (1991) **266**(1):149–61. doi:10.1007/BF00678721
17. Puigserver P, Wu Z, Park CW, Graves R, Wright M, Spiegelman BM. A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. *Cell* (1998) **92**(6):829–39. doi:10.1016/S0092-8674(00)81410-5
18. Schreiber SN, Knutti D, Brogli K, Uhlmann T, Kralli A. The transcriptional coactivator PGC-1 regulates the expression and activity of the orphan nuclear receptor estrogen-related receptor alpha (ERRalpha). *J Biol Chem* (2003) **278**(11):9013–8. doi:10.1074/jbc.M212923200
19. Wu Z, Puigserver P, Andersson U, Zhang C, Adelmant G, Mootha V, et al. Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. *Cell* (1999) **98**(1):115–24. doi:10.1016/S0092-8674(00)80611-X
20. Nicholls DG. The bioenergetics of brown adipose tissue mitochondria. *FEBS Lett* (1976) **61**(2):103–10. doi:10.1016/0014-5793(76)81014-9
21. Nicholls DG, Bernson VS, Heaton GM. The identification of the component in the inner membrane of brown adipose tissue mitochondria responsible for regulating energy dissipation. *Experientia Suppl* (1978) **32**:89–93.
22. Cannon B, Romert L, Sundin U, Barnard T. Morphology and biochemical properties of perirenal adipose tissue from lamb (*Ovis aries*). a comparison with brown adipose tissue. *Comp Biochem Physiol B* (1977) **56**(1):87–99.
23. Fedorenko A, Lishko PV, Kirichok Y. Mechanism of fatty-acid-dependent UCP1 uncoupling in brown fat mitochondria. *Cell* (2012) **151**(2):400–13. doi:10.1016/j.cell.2012.09.010
24. Williamson JR. Control of energy metabolism in hamster brown adipose tissue. *J Biol Chem* (1970) **245**(8):2043–50.
25. Cooney GJ, Newsholme EA. The maximum capacity of glycolysis in brown adipose tissue and its relationship to control of the blood glucose concentration. *FEBS Lett* (1982) **148**(2):198–200. doi:10.1016/0014-5793(82)80807-7
26. Forner F, Kumar C, Luber CA, Fromme T, Klingenspor M, Mann M. Proteome differences between brown and white fat mitochondria reveal specialized metabolic functions. *Cell Metab* (2009) **10**(4):324–35. doi:10.1016/j.cmet.2009.08.014
27. Lambeth DO, Tews KN, Adkins S, Frohlich D, Milavetz BI. Expression of two succinyl-CoA synthetases with different nucleotide specificities in mammalian tissues. *J Biol Chem* (2004) **279**(35):36621–4. doi:10.1074/jbc.M406884200
28. Christoffolete MA, Linardi CC, de JL, Ebina KN, Carvalho SD, Ribeiro MO, et al. Mice with targeted disruption of the Dio2 gene have cold-induced overexpression of the uncoupling protein 1 gene but fail to increase brown adipose tissue lipogenesis and adaptive thermogenesis. *Diabetes* (2004) **53**(3):577–84. doi:10.2337/diabetes.53.3.577
29. McCormack JG, Denton RM. Evidence that fatty acid synthesis in the interscapular brown adipose tissue of cold-adapted rats is increased *in vivo* by insulin by mechanisms involving parallel activation of pyruvate dehydrogenase and acetyl-coenzyme A carboxylase. *Biochem J* (1977) **166**(3):627–30.
30. Yu XX, Lewin DA, Forrest W, Adams SH. Cold elicits the simultaneous induction of fatty acid synthesis and beta-oxidation in murine brown adipose tissue: prediction from differential gene expression and confirmation *in vivo*. *FASEB J* (2002) **16**(2):155–68. doi:10.1096/fj.01-0568com
31. Cadoudal T, Distel E, Durant S, Fouque F, Blouin JM, Collinet M, et al. Pyruvate dehydrogenase kinase 4: regulation by thiazolidinediones and implication in glyceroneogenesis in adipose tissue. *Diabetes* (2008) **57**(9):2272–9. doi:10.2337/db08-0477
32. Yan J, Lawson JE, Reed LJ. Role of the regulatory subunit of bovine pyruvate dehydrogenase phosphatase. *Proc Natl Acad Sci U S A* (1996) **93**(10):4953–6. doi:10.1073/pnas.93.10.4953
33. Huang B, Gudi R, Wu P, Harris RA, Hamilton J, Popov KM. Isoenzymes of pyruvate dehydrogenase phosphatase. DNA-derived amino acid sequences, expression, and regulation. *J Biol Chem* (1998) **273**(28):17680–8. doi:10.1074/jbc.273.28.17680
34. Sakakibara I, Fujino T, Ishii M, Tanaka T, Shimosawa T, Miura S, et al. Fasting-induced hypothermia and reduced energy production in mice lacking acetyl-CoA synthetase 2. *Cell Metab* (2009) **9**(2):191–202. doi:10.1016/j.cmet.2008.12.008
35. Fujino T, Kondo J, Ishikawa M, Morikawa K, Yamamoto TT. Acetyl-CoA synthetase 2, a mitochondrial matrix enzyme involved in the oxidation of acetate. *J Biol Chem* (2001) **276**(14):11420–6. doi:10.1074/jbc.M008782200
36. Agius L, Williamson DH. The utilization of ketone bodies by the interscapular brown adipose tissue of the rat. *Biochim Biophys Acta* (1981) **666**(1):127–32. doi:10.1016/0005-2760(81)90098-9
37. Finck BN, Kelly DP. PGC-1 coactivators: inducible regulators of energy metabolism in health and disease. *J Clin Invest* (2006) **116**(3):615–22. doi:10.1172/JCI27794
38. Vernochet C, Mourier A, Bezy O, Macotela Y, Boucher J, Rardin MJ, et al. Adipose-specific deletion of TFAM increases mitochondrial oxidation and protects mice against obesity and insulin resistance. *Cell Metab* (2012) **16**(6):765–76. doi:10.1016/j.cmet.2012.10.016
39. Vernochet C, Damilano F, Mourier A, Bezy O, Mori MA, Smyth G, et al. Adipose tissue mitochondrial dysfunction triggers a lipodystrophic syndrome with insulin resistance, hepatosteatosis, and cardiovascular complications. *FASEB J* (2014) **28**(10):4408–19. doi:10.1096/fj.14-253971
40. Ryu MJ, Kim SJ, Kim YK, Choi MJ, Tadi S, Lee MH, et al. Crif1 deficiency reduces adipose OXPHOS capacity and triggers inflammation and insulin resistance in mice. *PLoS Genet* (2013) **9**(3):e1003356. doi:10.1371/journal.pgen.1003356
41. Duteil D, Metzger E, Willmann D, Karagianni P, Friedrichs N, Greschik H, et al. LSD1 promotes oxidative metabolism of white adipose tissue. *Nat Commun* (2014) **5**:4093. doi:10.1038/ncomms5093
42. Mootha VK, Lepage P, Miller K, Bunkenborg J, Reich M, Hjerrild M, et al. Identification of a gene causing human cytochrome c oxidase deficiency by integrative genomics. *Proc Natl Acad Sci U S A* (2003) **100**(2):605–10. doi:10.1073/pnas.242716699
43. Merante F, Petrova-Benedict R, MacKay N, Mitchell G, Lambert M, Morin C, et al. A biochemically distinct form of cytochrome oxidase (COX) deficiency in the Saguenay-Lac-Saint-Jean region of Quebec. *Am J Hum Genet* (1993) **53**(2):481–7.
44. Liu L, Sanosaka M, Lei S, Bestwick ML, Frey JH Jr, Surovtseva YV, et al. LRP130 protein remodels mitochondria and stimulates fatty acid oxidation. *J Biol Chem* (2011) **286**(48):41253–64. doi:10.1074/jbc.M111.276121
45. Ruzzenente B, Metodiev MD, Wredenberg A, Bratic A, Park CB, Camara Y, et al. LRPPRC is necessary for polyadenylation and coordination of translation of mitochondrial mRNAs. *EMBO J* (2012) **31**(2):443–56. doi:10.1038/emboj.2011.392
46. Mourier A, Ruzzenente B, Brandt T, Kuhlbrandt W, Larsson NG. Loss of LRPPRC causes ATP synthase deficiency. *Hum Mol Genet* (2014) **23**(10):2580–92. doi:10.1093/hmg/ddt652
47. Bratic A, Wredenberg A, Gronke S, Stewart JB, Mourier A, Ruzzenente B, et al. The bicoid stability factor controls polyadenylation and expression of specific mitochondrial mRNAs in *Drosophila melanogaster*. *PLoS Genet* (2011) **7**(10):e1002324. doi:10.1371/journal.pgen.1002324
48. Barkan A, Rojas M, Fujii S, Yap A, Chong YS, Bond CS, et al. A combinatorial amino acid code for RNA recognition by pentatricopeptide repeat proteins. *PLoS Genet* (2012) **8**(8):e1002910. doi:10.1371/journal.pgen.1002910
49. Sterky FH, Ruzzenente B, Gustafsson CM, Samuelsson T, Larsson NG. LRPPRC is a mitochondrial matrix protein that is conserved in metazoans. *Biochem Biophys Res Commun* (2010) **398**(4):759–64. doi:10.1016/j.bbrc.2010.07.019
50. Gohil VM, Nilsson R, Belcher-Timme CA, Luo B, Root DE, Mootha VK. Mitochondrial and nuclear genomic responses to loss of LRPPRC expression. *J Biol Chem* (2010) **285**(18):13742–7. doi:10.1074/jbc.M109.098400
51. Vartak R, Porras CA, Bai Y. Respiratory supercomplexes: structure, function and assembly. *Protein Cell* (2013) **4**(8):582–90. doi:10.1007/s13238-013-3032-y
52. Scarpulla RC. Transcriptional paradigms in mammalian mitochondrial biogenesis and function. *Physiol Rev* (2008) **88**(2):611–38. doi:10.1152/physrev.00025.2007
53. Kim SJ, Kwon MC, Ryu MJ, Chung HK, Tadi S, Kim YK, et al. CRIF1 is essential for the synthesis and insertion of oxidative phosphorylation polypeptides in the mammalian mitochondrial membrane. *Cell Metab* (2012) **16**(2):274–83. doi:10.1016/j.cmet.2012.06.012
54. Shin J, Lee SH, Kwon MC, Yang DK, Seo HR, Kim J, et al. Cardiomyocyte specific deletion of Crif1 causes mitochondrial cardiomyopathy in mice. *PLoS One* (2013) **8**(1):e53577. doi:10.1371/journal.pone.0053577

55. Shi Y, Lan F, Matson C, Mulligan P, Whetstine JR, Cole PA, et al. Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. *Cell* (2004) **119**(7):941–53. doi:10.1016/j.cell.2004.12.012
56. Pedersen MT, Helin K. Histone demethylases in development and disease. *Trends Cell Biol* (2010) **20**(11):662–71. doi:10.1016/j.tcb.2010.08.011
57. Valerio A, Cardile A, Cozzi V, Bracale R, Tedesco L, Pisconti A, et al. TNF-alpha downregulates eNOS expression and mitochondrial biogenesis in fat and muscle of obese rodents. *J Clin Invest* (2006) **116**(10):2791–8. doi:10.1172/JCI28570
58. Shimizu I, Aprahamian T, Kikuchi R, Shimizu A, Papanicolaou KN, MacLauchlan S, et al. Vascular rarefaction mediates whitening of brown fat in obesity. *J Clin Invest* (2014) **124**(5):2099–112. doi:10.1172/JCI71643
59. Goodbody AE, Trayhurn P. GDP binding to brown-adipose-tissue mitochondria of diabetic – obese (db/db) mice. decreased binding in both the obese and pre-obese states. *Biochem J* (1981) **194**(3):1019–22.
60. Masaki T, Yoshimatsu H, Chiba S, Sakata T. Impaired response of UCP family to cold exposure in diabetic (db/db) mice. *Am J Physiol Regul Integr Comp Physiol* (2000) **279**(4):R1305–9.
61. Atgie C, Marette A, Desautels M, Tulp O, Bukowiecki LJ. Specific decrease of mitochondrial thermogenic capacity in brown adipose tissue of obese SHR/N-cp rats. *Am J Physiol* (1993) **265**(6 Pt 1):C1674–80.
62. van Marken Lichtenbelt WD, Vanhommerig JW, Smulders NM, Drossaerts JM, Kemerink GJ, Bouvy ND, et al. Cold-activated brown adipose tissue in healthy men. *N Engl J Med* (2009) **360**(15):1500–8. doi:10.1056/NEJMoa0808718
63. Vijgen GH, Bouvy ND, Teule GJ, Brans B, Schrauwen P, van Marken Lichtenbelt WD. Brown adipose tissue in morbidly obese subjects. *PLoS One* (2011) **6**(2):e17247. doi:10.1371/journal.pone.0017247
64. Cypess AM, Lehman S, Williams G, Tal I, Rodman D, Goldfine AB, et al. Identification and importance of brown adipose tissue in adult humans. *N Engl J Med* (2009) **360**(15):1509–17. doi:10.1056/NEJMoa0810780
65. Rong JX, Qiu Y, Hansen MK, Zhu L, Zhang V, Xie M, et al. Adipose mitochondrial biogenesis is suppressed in db/db and high-fat diet-fed mice and improved by rosiglitazone. *Diabetes* (2007) **56**(7):1751–60. doi:10.2337/db06-1135
66. Cypess AM, Chen YC, Sze C, Wang K, English J, Chan O, et al. Cold but not sympathomimetics activates human brown adipose tissue *in vivo*. *Proc Natl Acad Sci U S A* (2012) **109**(25):10001–5. doi:10.1073/pnas.1207911109
67. van der Lans AA, Hoeks J, Brans B, Vijgen GH, Visser MG, Vosselman MJ, et al. Cold acclimation recruits human brown fat and increases nonshivering thermogenesis. *J Clin Invest* (2013) **123**(8):3395–403. doi:10.1172/JCI68993
68. Carey AL, Formosa MF, Van EB, Bertovic D, Eikelis N, Lambert GW, et al. Ephedrine activates brown adipose tissue in lean but not obese humans. *Diabetologia* (2013) **56**(1):147–55. doi:10.1007/s00125-012-2748-1
69. Arch JR. Challenges in β (3)-adrenoceptor agonist drug development. *Ther Adv Endocrinol Metab* (2011) **2**(2):59–64. doi:10.1177/2042018811398517
70. Cypess AM, Weiner LS, Roberts-Toler C, Elia EF, Kessler SH, Kahn PA, et al. Activation of human brown adipose tissue by a beta3-adrenergic receptor agonist. *Cell Metab* (2015) **21**(1):33–8. doi:10.1016/j.cmet.2014.12.009
71. Yun J, Finkel T. Mitohormesis. *Cell Metab* (2014) **19**(5):757–66. doi:10.1016/j.cmet.2014.01.011

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

REVIEW
published: 09 October 2015
doi: 10.3389/fendo.2015.00158

A new role for browning as a redox and stress adaptive mechanism?

*Yannick Jeanson, Audrey Carrière and Louis Casteilla**

UMR STROMALab, CNRS 5273, INSERM U1031, Université Toulouse III – Paul Sabatier, Toulouse, France

The worldwide epidemic of obesity and metabolic disorders is focusing the attention of the scientific community on white adipose tissue (WAT) and its biology. This tissue is characterized not only by its capability to change in size and shape but also by its heterogeneity and versatility. WAT can be converted into brown fat-like tissue according to different physiological and pathophysiological situations. The expression of uncoupling protein-1 in brown-like adipocytes changes their function from energy storage to energy dissipation. This plasticity, named browning, was recently rediscovered and convergent recent accounts, including in humans, have revived the idea of using these oxidative cells to fight against metabolic diseases. Furthermore, recent reports suggest that, beside the increased energy dissipation and thermogenesis that may have adverse effects in situations such as cancer-associated cachexia and massive burns, browning could be also considered as an adaptive stress response to high redox pressure and to major stress that could help to maintain tissue homeostasis and integrity. The aim of this review is to summarize the current knowledge concerning brown adipocytes and the browning process and also to explore unexpected putative role(s) for these cells. While it is important to find new browning inducers to limit energy stores and metabolic diseases, it also appears crucial to develop new browning inhibitors to limit adverse energy dissipation in wasting-associated syndromes.

Keywords: adipose tissue plasticity, adipocytes, redox, uncoupling protein-1, browning, stress

OPEN ACCESS

Edited by:
Jun Wu,
University of Michigan, USA

Reviewed by:
Tae-Hwa Chun,
University of Michigan, USA
Jonathan R. Brestoff,
Perelman School of Medicine at the University of Pennsylvania, USA

***Correspondence:**
Louis Casteilla
louis.casteilla@inserm.fr

Specialty section:
This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *23 June 2015*
Accepted: *24 September 2015*
Published: *09 October 2015*

Citation:
Jeanson Y, Carrière A and Casteilla L (2015) A new role for browning as a redox and stress adaptive mechanism? Front. Endocrinol. 6:158. doi: 10.3389/fendo.2015.00158

In the past, brown and white adipocytes were described as classically located in brown and white fat, respectively. Although these cells shared the name of adipocytes, their functions were traditionally opposed and those of brown adipocytes were strongly associated with the thermoregulation process and energy dissipation. The first discoveries that brown adipocytes could be detected in white fat were made in the 1980–1990s and revealed an unexpected plasticity of white adipose tissue (WAT), largely driven by β3-agonist, at least in rodents (1–3) and dogs (4). Unfortunately, the lack of efficiency of β3-agonist treatment in humans caused interest in this field to wane. However, in recent years, the rediscovery of uncoupling protein-1 (UCP1)-expressing cells in white fat together with the simultaneous demonstration of the presence of functional brown adipocytes in human adults have been accompanied by very new insights into the developmental origins of the different types of adipocytes. These ground breaking discoveries have completely changed our view on adipose tissue plasticity and generated great enthusiasm associated with a huge number of publications in the field, reporting unexpected results. The present review tries to summarize these recent findings and discusses new perspectives concerning unexpected roles and functions of UCP1-expressing cells.

WHITE ADIPOCYTES, BROWN ADIPOCYTES, AND ADIPOSE TISSUE PLASTICITY: A HISTORICAL PERSPECTIVE

White Adipocytes

Mature white adipocytes play a fundamental role in energy homeostasis and constitute the main stores of energy in the form of triglycerides (5). They display variable size (25–200 μm) and a unilocular morphology. In addition to their metabolic functions, white adipocytes have strong endocrine activity and secrete a variety of molecules commonly called adipokines, highlighting the fact that adipose tissue has interactions with other organs in the body (6). These cells are mainly localized in WAT, which are found at distinct anatomical sites. Subcutaneous and visceral fat pads (mesenteric, perigonadal, perirenal, etc.) are classically distinguished because the subcutaneous WAT has a protective action whereas the visceral fat pad is thought to be more deleterious in the development of obesity and related metabolic diseases (type II diabetes, cardiovascular disease, etc.) (7–9).

Brown Adipocytes and UCP1 Expression

Brown adipose tissue (BAT) contains brown adipocytes, which are smaller (15–60 μm) than white adipocytes and have a multilocular morphology (5). Brown adipocytes are highly oxidative cells that release energy as heat (10, 11) thanks to their high mitochondrial content and to the expression of UCP1. This protein, inserted in the inner mitochondrial membrane, acts as a proton channel and uncouples the production of ATP by ATP synthase from respiratory chain functioning. This leads to the activation of catabolism and subsequent heat production. This non-shivering thermogenesis plays a key role in body temperature homeostasis (10, 11) as definitively demonstrated by UCP1-deficient mice, which are cold sensitive (12). Moreover, the presence of large amounts of BAT in species like sheep or hibernates, which require active thermogenesis to protect the new-born animals from cold exposure on a snow-covered pasture in spring or to emerge from hibernation, also argues that BAT evolved primarily to function as a thermogenic system to maintain body temperature (11, 13). BAT is more vascularized than WAT and is probably among the most vascularized tissues of the body (14). Such vascularization is required for the integrity of BAT. It was recently demonstrated that reducing the vasculature specifically in adipose tissues induced the whitening of BAT (15). In a temperate environment, UCP1 is inhibited by di- and triphosphate nucleotides. During cold exposure, UCP1 is strongly and quickly activated by the sympathetic nervous system (SNS) (16). The local release of noradrenaline in BAT activates lipolysis, which induces the release of free fatty acids. These constitute not only oxidative substrates for brown adipocytes but also potent activators of UCP1 (17–19). The same adrenergic stimulus up-regulates the expression of UCP1 at the transcriptional level via activation of the PKA-p38 mitogen-activated protein kinase signaling pathway that induces the phosphorylation and activation of molecular transactivators of UCP1 (19, 20). With increasing time of cold exposure, SNS triggers a trophic response through activation of mitochondriogenesis that also contributes to the increase of the global thermogenic capacity of brown adipocytes (21). Recently, noradrenaline has also been shown to induce the phosphorylation of dynamin-related protein-1, which induces the fragmentation of brown adipocyte mitochondria. This fragmentation promotes uncoupling and mitochondrial depolarization (22). Lastly, in addition to the activation of the thermogenic program of mature brown adipocytes, noradrenaline also induces the proliferation of brown progenitors through the stimulation of β1-adrenergic receptors and their differentiation (23).

In addition to their role in maintaining body temperature, brown fat cells possess strong oxidative potential and can thus also burn excessive calories (11, 24–26). Transplantation of BAT into the visceral cavity of mice fed with a high-fat diet or having the ob/ob genetic background increases glucose tolerance and improves insulin sensitivity (26, 27). However, UCP1-deficient mice are not obese when kept at room temperature (21°C) (12). The onset of obesity is only observed when these mice are housed at thermal neutrality, exempt from any thermal stress (28). These findings underscore the importance of thermogenesis in the energy balance of homeothermic animals and can explain the different relative importance of diet-induced thermogenesis related to BAT activity according to the developmental status or the species (29). Because of their small size, rodents and many new-born mammals are permanently in thermogenic deficit, which leads to low grade but permanent BAT activation. Despite these limitations, when brown fat cells are stimulated, they exhibit properties that offer hope of new therapies as the cells constitute putative targets in the fight against metabolic diseases such as diabetes and obesity (30).

The beneficial effects of brown adipocytes on energy metabolism are also due to their endocrine/paracrine activity (31). It has long been known that brown adipocytes release triiodothyronine from thyroxine by their deiodase activity, thus stimulating their thermogenic capacity (32). More recently, the secretion of fibroblast growth factor-21 (FGF21), besides vascular endothelial growth factor, fibroblast growth factor-2, interleukin-6, and neural growth factor (31, 33–35), has been attracting much attention. High levels of FGF21, a protein originally known to be produced by the liver in response to fasting, are produced and secreted into the bloodstream by BAT, especially during thermogenic activation (36). FGF21 acts on many tissues, including WAT, the liver, pancreas, heart, or central nervous system (31). Furthermore, some adipokines, such as leptin, adiponectin, and resistin, formerly recognized as specifically secreted by white adipocytes (37, 38), are also secreted by brown adipocytes. By analogy with the term "adipokines" for proteins secreted by white adipocytes, molecules secreted by brown adipocytes have sometimes been referred to as "batokines" (26, 31).

Until the discovery of a third type of adipocyte, things were very simple and focused on UCP1 only: the absence or presence of UCP1 in an adipocyte defined the white or brown phenotype, respectively. Thus, many studies investigated the control of UCP1 gene expression and first focused on catecholamines and thiiodothyronine pathways (19, 39). In addition to cAMP-dependent responsive element binding protein and thyroid hormone receptor, other molecules, including CCAAT/enhancer

binding protein-β transcription factor, retinoic acid receptor, activating transcription factor-2, and peroxisome proliferator-activated receptors gamma and alpha (PPARγ and PPARα), strongly up-regulate the expression of UCP1 at transcriptional level (40, 41). Among all these regulatory proteins, PPARγ plays a pivotal role and mice having non-functional PPARγ exhibit brown adipocytes with a unilocular lipid droplet (looking like white adipocytes!) and a decrease in UCP1 expression after cold exposure (42). The presence of PPARγ agonists, such as the anti-diabetic agent rosiglitazone, is required for the expression of UCP1 in adipocytes differentiated in primary culture (43). Various co-regulators, such as peroxisome proliferator-activated receptor coactivator-1α (PGC-1α) and the positive regulatory domain containing-16 protein (PRDM16) are also crucial. The expression of PGC-1α is regulated by the PKA pathway (44) and, *in vitro* as well as *in vivo*, overexpression of PGC-1α induces the expression of UCP1 along with other mitochondrial genes (45–47). In the same manner, specific overexpression of PRDM16 in adipose tissue is sufficient to induce browning of visceral adipose tissue (48). Furthermore, beside phosphorylation events that tightly control the activity of transcription factors and co-regulators, NAD^+-dependent deacetylase sirtuin-1 deacetylates PPARγ on two lysine residues, enabling PRDM16 recruitment and the subsequent activation of UCP1, in a rosiglitazone-dependent manner (49). It should be noted that none of these molecular regulators are specific to brown adipocytes and UCP1 expression. In particular, PPARγ, which appears crucial for the expression of UCP1, also has the great disadvantage of promoting white adipocyte differentiation (50).

Adipose Tissue Plasticity: From Brown to Brite

As early as 1984, Young et al. described the presence of UCP1-expressing adipocytes in murine parametrial fat (1). This observation was more precisely described in later studies by Loncar (2) and Cousin et al. (3). Such apparent tissue plasticity was found to be strongly promoted after cold exposure or β3-adrenergic agonist, including in dogs (4). The relative numbers of these cells varies according to the fat pad. Subcutaneous WAT is more likely to develop UCP1-expressing cells than other depots such as mesenteric and perigonadic WAT. We therefore proposed a gradient in the plasticity of the different white fat pads similar to that described for skeletal muscles, which are classified as glycolytic, oxidative, and mixed fiber (3). Two non-exclusive hypotheses could explain the emergence of UCP1-expressing adipocytes in WAT: *de novo* differentiation from progenitors and/or transdifferentiation of mature white into brown adipocytes (51). The hypothesis of transdifferentiation is supported by several findings demonstrating that there is neither an increase in DNA amount nor a process of preadipocyte proliferation during acute cold exposure in the peri-ovarian adipose tissue (52).

It is noteworthy that, for decades, UCP1-expressing cells in white fat were considered as comparable to the classical brown adipocytes present in a typical BAT depot, i.e., the interscapular BAT. However, recent findings have strongly challenged this view and demonstrated that these cells have distinct gene expression profiles (53, 54) and different developmental origins (55–58). UCP1-expressing adipocytes in white fat thus constitute a third type of adipocytes, which have recently been named beige or brite (brown in white) adipocytes (43, 54, 59, 60). The development of these cells in WAT has been termed the browning process.

A NEW LOOK ON WAT HETEROGENEITY AND PLASTICITY: ADIPOCYTES OF ALL COLORS AND ORIGINS

It was long thought that white and brown adipocytes had a common progenitor but recent works have challenged this idea. A first step came with the proposal that classical brown adipocytes, and not brite adipocytes, derived from the paraxial mesoderm, particularly the dermomyotome, and that they originated from a precursor expressing myogenic transcription factor-5 (Myf5) (55). The commitment of the bipotent progenitor to the brown phenotype is controlled by members of the TGF-β superfamily, such as BMP7 and the PRDM16-C/EBPβ transcriptional complex, which induce the expression of PPARγ and PGC-1α (41, 61, 62). The common developmental origin of muscle cells and typical brown adipocytes is also supported by their very similar transcriptional signature (53, 54) and the old observation of the presence of glycogen stocks in brown adipocytes (63, 64). Furthermore, the demonstration that human skeletal muscle contains CD34 positive progenitor cells that can easily differentiate into brown adipocytes is another argument in favor of the link between brown adipocytes and skeletal muscle cells (65). The common origin of both types of cells could also be related to their metabolic activities, which are very close from a bioenergetics point of view. Shivering thermogenesis in muscle corresponds to the implementation of a futile cycle with a high rate of ATP synthesis associated with a high rate of ATP consumption by contraction processes. The resulting high oxidative catabolism dissipates a large amount of energy as heat. In this case, the functioning of ATP synthase resembles that of a mitochondrial proton channel such as UCP1. From a somewhat provocative point of view, brown adipocytes could be presented more as specialized muscle cells than as specialized adipocytes. Whatever the fate of such a hypothesis, this rather simplistic portrayal was quickly complicated and the developmental origin of the different types of adipocytes does not appear to be as simple as initially proposed. While brown adipocytes present in the interscapular BAT are thought to derive from Myf5-positive progenitors, those present in the brown perirenal and peri-aortic fat pads would originate from a Myf5-negative progenitor (56). Furthermore, some white and brite adipocytes could also emerge from Myf5-positive cells, according to the nature of fat deposits (56, 58). To increase the complexity further, it has been proposed that a subpopulation of brite adipocytes derives from smooth muscle cell (66).

Like the developmental origin, the cell mechanism(s) at the origin of the browning process in adults are not yet clear and are still widely debated. Consistently with the transdifferentiation hypothesis, a recent study using a transgenic mouse model with permanent or temporary staining of UCP1-expressing cells, has shown that some "phenotypically white" adipocytes can acquire

a brite phenotype (i.e., displaying multilocular droplets together with UCP1 expression) after cold exposure (67). Conversely, when these mice were returned to 21°C, the brite adipocytes returned to a white phenotype (i.e., unilocular droplet), reacquiring a brite phenotype after re-exposure to cold (67). In apparent opposition to this conclusion, another study has recently demonstrated that beige adipocytes arise from *de novo* differentiation of precursors (68). The browning mechanism would also rely on different cell mechanisms according to the nature of the fat pad. While UCP1-expressing adipocytes within subcutaneous WAT would arise from the transdifferentiation phenomenon, those from the epididymal fat pad would originate from the proliferation and the differentiation of progenitor cells (69, 70). Adding to this complexity, it should not be forgotten that non-stimulated brite adipocytes could have a phenotype very close to that of the white adipocyte. This phenotype homology could lead to a misinterpretation of browning as a white adipocyte transdifferentiation when it could simply correspond to the activation of inactive brite adipocytes. It is noteworthy that, whatever the intensity of the stimuli, some unilocular white cells are totally refractory to cell plasticity. This would also mean that different types of unilocular cells lacking UCP1 expression could co-exist inside the same fat pad.

The compilation of these findings shows the current confusion, which is probably due to the underestimated complexity of the system. It seems that there are several pathways and developmental origins that give rise to unilocular UCP1 negative adipocyte(s) and to multilocular UCP1 positive adipocyte(s), without anyone knowing if cells having a similar apparent phenotype have the same functions and carry out the same regulations. This also emphasizes the lack of relevant, robust tools to identify and distinguish the different types of adipocytes because the white adipocyte phenotype is defined by its lack of brite or brown adipocyte markers, which is clearly insufficient. Although a large number of markers specifically expressed in each type of adipocytes or precursors have been proposed from gene expression profiling of isolated white, brown, and brite adipocytes and/or from studies on whole adipose tissues, their validity is currently under investigation (71). This kind of approach does not really take account of the heterogeneity of cells inside the tissues. Even in conditions of browning such as cold exposure, brite adipocytes represent only a small proportion of the tissue cells and this can lead to a biased view grouping white and brite adipocytes together. It is therefore necessary to characterize the different adipocytes in homogeneous cell populations. Also, we cannot exclude the possibility that typical brown adipocytes could co-exist with brite adipocytes in the same white fat pad since brite adipocytes can co-exist with typical brown adipocytes in typical brown fat pad.

UNEXPECTED BROWNING INDUCERS REVEAL PUTATIVE NEW ROLES FOR UCP1-EXPRESSING ADIPOCYTES

After the old failure of β3-agonist treatment in humans, the discovery of functional brown fat in adult humans has revived the research. Besides the adrenergic pathway that represents the ancestral and best described activator of UCP1 expression and in addition to thyroid hormones, several peptides have recently been identified as browning inducers or inhibitors that correspond to as many therapeutic targets. Several recent reviews summarize the different regulators of the browning process (41, 72–74). We decided to focus on the unexpected physiological or physio-pathological conditions recently found to regulate the browning process and that highlight putative new functions for these cells (**Figure 1**).

Browning as an Adaptive Element in Redox Metabolism?

While an increasing number of agents have been reported to stimulate browning as discussed, very few studies have investigated the role of the metabolic environment in the phenomenon. We recently demonstrated the unexpected role of redox metabolites, such as lactate, in the browning process. Lactate, which is a major product of anaerobic metabolism, promotes WAT browning through its transport by the monocarboxylate transporters (75). Lactate-induced browning is mediated by a change in the intracellular redox state (**Figure 2**). The transformation of lactate into pyruvate by lactate dehydrogenase B induces a higher $NADH/NAD^+$ ratio and modulation of this ratio by different strategies modulates the expression of UCP1 (75). As, in turn, uncoupling the ATP production from the respiratory chain by high levels of UCP1 accelerates the oxidation of NADH by the complex I, we postulate that browning constitutes an adaptive mechanism to alleviate a strong redox pressure (75) (**Figure 2**). The increased expression of the lactate-importing transporter during activation of UCP1-expressing adipocytes, e.g., after cold exposure (75) suggests that lactate could also be used as a substrate by these cells. We postulate that, in the same way as for glucose and triglyceride homeostasis, UCP1-expressing cells could regulate plasma lactate levels and lactate metabolism. We propose that there is a redox regulatory loop where lactate induces a metabolic reprograming of adipocytes toward an uncoupled oxidative phenotype that, in turn, favors lactate consumption and dissipation.

This points to a totally new function for brite adipocytes and extends the role of lactate as a signaling molecule in the browning field. This signaling pathway could also be involved in the emergence of UCP1-expressing adipocytes in white fat during obesity. WAT is an important producer of lactate in physiological conditions (76, 77). It could be postulated that, in an obesity context where large adipocytes are hypoxic, they could locally produce large amounts of lactate that would stimulate the browning of neighboring white adipocytes and/or the recruitment of brite progenitors that promote energy dissipation (77). This link between browning and redox state is also emphasized by two recent studies, demonstrating that UCP1 expression is strongly controlled by different redox elements such as oxidative stress and antioxidant defenses. Sestrins, stress-inducible proteins that limit oxidative stress, inhibit UCP1 expression (78) while glutathione deficit, one of the key elements of the cytosolic antioxidant

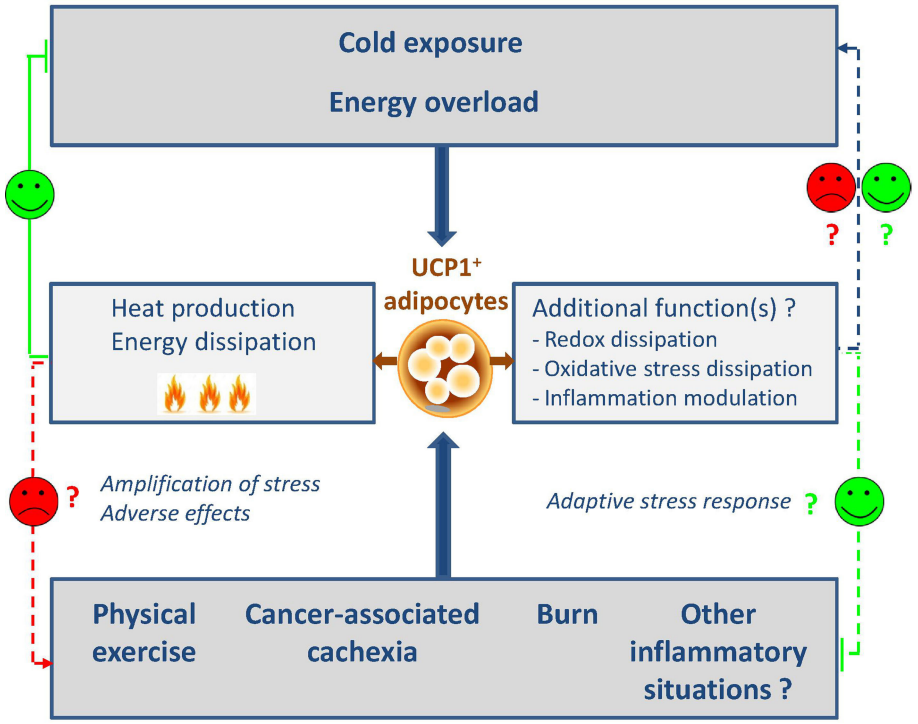


FIGURE 1 | Classic and new regulators of browning. The induction of browning is classically described after cold exposure or high-fat diet and leads to the generation of heat and energy dissipation. In this context, browning represents a relevant target to fight against metabolic diseases. More recently, unexpected physiological or physio-pathological situations were also described as new browning inducers. These findings shed the light on putative and unexpected roles of browning such as redox and oxidative stress dissipation and inflammation modulation. On the other hand, the automatic association with heat and energy dissipation could appear as adverse side effects that will be necessary to control. The design of browning antagonists will be then relevant.

defenses, increases it (79). Oxidative stress and redox state thus constitute new and important regulators of UCP1 expression.

Browning as a Non-Specific Stress Response?

Three unexpected physiological or pathophysiological situations were found to be associated with browning: exercise, cancer-associated cachexia, and burns. Before our findings, physical exercise had been shown to activate BAT and WAT browning (80, 81). In addition to IL-6 (82), two muscle-derived hormones, irisin and meteorin-like, are believed to mediate such effects (80, 83). The expression and the impact of irisin in humans have been strongly debated but its existence has been unequivocally demonstrated by a recent study using tandem mass spectrometry (84). Irisin is detectable in the blood of untrained persons during exercise or exposure to low temperatures (84, 85). While some studies have shown that irisin has no effect on the differentiation of human preadipocytes toward the brite phenotype (86), others have demonstrated that irisin activates the thermogenic activity of preadipocytes extracted from human neck (87). Additional signals, including lactate, whose plasma levels rise strongly during physical activity, could also contribute to exercise-induced UCP1 expression (75). The second situation, cachexia, is related to physiopathology associated with wasting syndromes, such as diabetes, sepsis, and cancer (88). It results in chronic inflammation and severe weight loss associated with atrophy of fat deposits and muscles. It is often associated with high plasma levels of lactate and usually precedes the death of the patient (89). Several studies have demonstrated that cachexia induced by the injection of cancer cells is associated with browning of WAT and an increase in energy expenditure (90–92). Inflammation and, especially, the production of interleukin-6 play a key role in this phenomenon and the lack of interleukin-6 receptor blocks browning induced by cancer cells (90). Parathyroid hormone-related protein may also mediate cancer-cell-induced browning of WAT (91). Lastly, in addition to exercise and cachexia, severe burns are associated with hypermetabolic response, hyperthermia, and browning in rodents and in humans (93, 94).

The role(s) of UCP1-expressing cells in these different conditions still remain to be unraveled. The fact that muscle contraction during exercise promotes the emergence and activation of energy-dissipating and thermogenic cells does not seem physiologically consistent with the energy conservation theory. This discrepancy would be explained if muscle-derived signals that induce browning could be shown to have evolved from shivering-related muscle contraction (87). In this scenario, WAT browning would represent a coherent and additive mechanism in thermoregulation promoted by shivering. For cachexia, associated browning could constitute an adaptive thermoregulatory

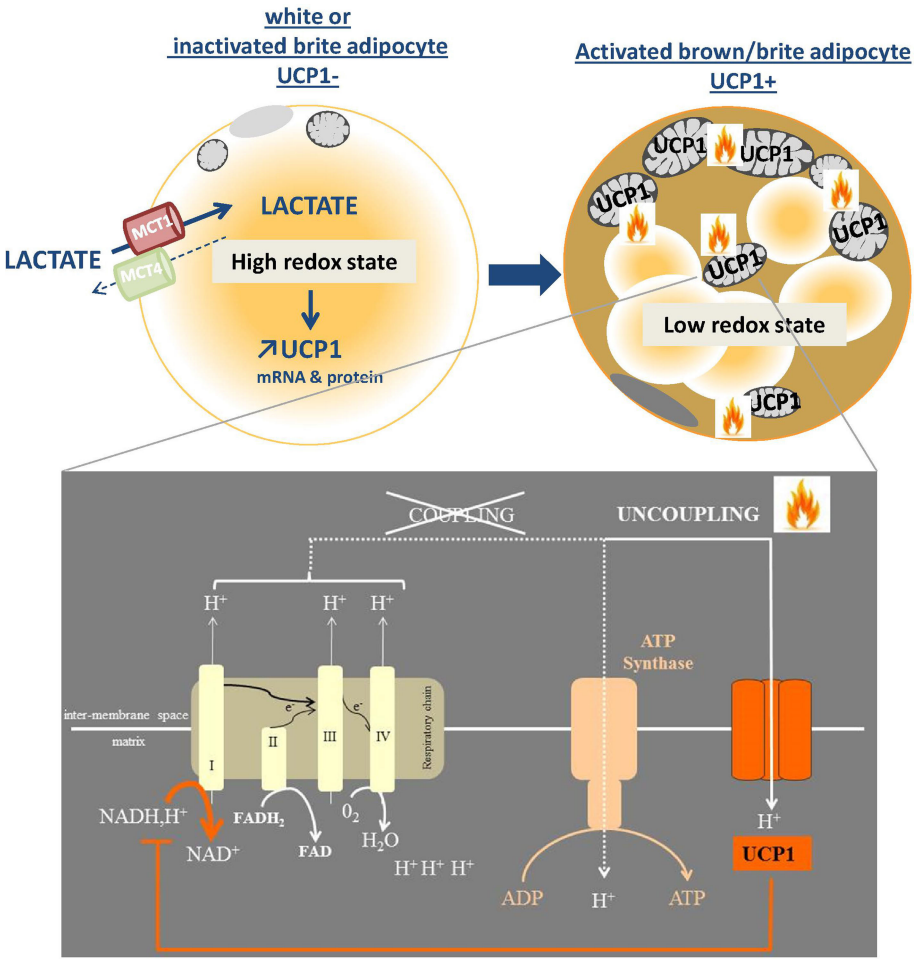


FIGURE 2 | Browning as a redox responsive mechanism. The import of lactate via the monocarboxylate transporter MCT1 is associated with a redox state (NADH/NAD) increase which, in turn, triggers the expression of browning genes including UCP1. The effect of activated UCP1, which is inserted into the inner mitochondrial membrane, is to uncouple the respiratory chain function from the ATP synthesis. The subsequent increase in oxidative metabolism generates heat and oxidizes reduced equivalents such as NADH, which was at the origin of the up-regulated UCP1 expression.

response to fight against the hypothermia associated with the decrease of both fat insulation and heat-related muscle mass observed during cachexia. Addressing this issue would require these results to be reproduced at thermoneutrality, as illustrated by the study conducted by Champigny et al. in starving conditions (95). In this study, the authors found an up-regulation of UCP1 expression and brown adipocyte activation during starvation, which are physiologically inconsistent (95). In fact, starvation-induced UCP1 expression disappeared when mice were kept at thermoneutrality, suggesting that UCP1 up-regulation was an adaptive thermogenic response of rats to fight against the temperature deficit associated with starvation-induced torpor (95). Browning is always associated with thermoregulation responses in these situations but could have adverse metabolic effects that need to be controlled. For massive burn injury, browning would be triggered by the large stress release of catecholamine and inflammatory signals and would participate in the increase of the whole body metabolic rate (94). In fact, the role of inflammatory signals and cytokines on the browning process was first reported during cold exposure (96). Cyclooxygenase-2, whose expression is increased in WAT during cold exposure, is thought to trigger the browning process through the synthesis of prostaglandins E2 and I2 (97, 98). The link between prostaglandins and UCP1 is not new and it has been proposed that the natural ligand of PPARγ is prostaglandin PGJ2 (99). However, these results have been questioned following the recent publication of the opposite effect of prostaglandins E2 and F2α on the expression of UCP1 (100). Surprisingly, immune cells are involved in cold-induced browning as eosinophils express and secrete interleukin-4 and interleukin-13, which recruit alternative M2 macrophages in the subcutaneous WAT during cold exposure (101). These macrophages secrete catecholamines that induce browning. The meteorin-like hormone secreted by both WAT and BAT during cold exposure or during physical exercise has the same

effect on eosinophils (83). At the same time, another group has demonstrated that the activation by interleukin-33 of Group 2 innate lymphoid cells (ILC2s), known for their role in tissue remodeling, could also induce the recruitment of eosinophils via the release of interleukin-13 (102). ILC2s may also, in response to interleukin-33, produce and secrete methionine-enkephalin, a new browning inducer (103). Although all these mechanisms were discovered in the context of cold exposure, it is reasonable to speculate that any events able to activate ILC2s or eosinophils are able to promote browning. Together with the fact that interferon regulatory factor-4 controls UCP1 expression (104), these observations bring to light an undiscovered link between browning and inflammation/immunity.

The consequences of the apparition of UCP1-expressing cells in such inflammation- and injury-related scenarios currently remain poorly understood but it is noteworthy that the common point between all the situations is the notion of intense stress and inflammation. For this reason, with the limits concerning thermoregulation discussed previously and although these fields are evolving rapidly, we can already assume that browning could appear in any severe stress conditions. Whether these cells have positive or negative effects on such stress and loss of tissue homeostasis conditions is unknown (**Figure 1**). They could have adverse effects through increased energy dissipation in wasting syndromes and injury conditions but also could play positive roles through unexpected functions such as redox and oxidative stress dissipation, metabolite consumption, or even anti-inflammatory actions. This is an underexplored scientific area that will remain very open for the coming years.

PERSPECTIVES IN HUMANS

In humans, it has long been considered that BAT is only present in neonates, with some exceptions such as patients suffering from pheochromocytoma (105, 106) or workers exposed to cold temperatures (107). Mainly localized in the interscapular region like the main murine BAT depot, the BAT present in new-born babies enables heat to be produced to combat the thermal stress associated with birth. While this fat pad disappears rapidly and is absent in human adults like in adult sheep (13), some histological studies performed in the 1970s claimed that human adults possessed some brown fat deposits in additional zones, including around the kidney, heart, and adrenal gland, and along the aorta and carotid (108). However, these studies were unable to change the general opinion that human adults did not possess BAT. Recently, this view has been contradicted by medical imaging based on the measurement of glucose uptake (using 18-fluorodesoxyglucose associated with positron emission tomography–computed tomography) This approach, widely used in oncology to detect tumors, when associated with histological and immunocytochemistry studies, highlighted the presence of BAT in healthy human adults (109–112). The activity of BAT is inversely correlated with body mass index, suggesting that it could play a role in energy homeostasis (109–112). Given their high oxidative potential and their ability to consume nutrients such as glucose and triglycerides, brown adipocytes in humans possess very attractive properties for fighting against metabolic diseases, including type II diabetes and obesity (30). While molecular profiling studies have shown that human brown adipocytes are very similar to brite adipocytes described in mice (59, 113, 114), some others claim that brown fat deposits in humans are made of a mixture of brown and brite adipocytes, the differential distributions of which appear to depend on their anatomical location, consistently with our previous remarks. In the neck region, brown adipocytes would be located deep within the tissues and brite adipocytes rather at the surface (115, 116). The search for brite progenitors in human WAT led to the discovery of bipotent (white and brite) progenitors that expressed the tissue non-specific alkaline phosphatase marker (117), while CD29 enabled the isolation of brown progenitors present in the deep regions of the human neck (118). Among the putative targets (72), we cannot ignore the β3-adrenergic pathway. Although many efforts failed to find suitable compounds (119), new pharmacological and genetic studies have revealed that targeting this pathway could be efficient (120–122). While PPARγ is a very potent inducer of UCP1 expression, it also causes many side effects, including body weight gain and cardiovascular issues, that strongly impair its therapeutic use (50). One way to bypass this issue would be to design a selective PPAR modulator (123, 124). Recently, oral supplementation of the bile acid chenodeoxycholic acid has been shown to activate BAT and to increase whole body energy expenditure in humans, making bile acids attractive candidates for counteracting obesity and related metabolic diseases (125).

By contrast, recent works on inflammation and burns have also revealed the bad and the ugly of browning as previously described. While it is important to find new browning inducers to limit energy stores and metabolic diseases, it also appears crucial to develop new browning inhibitors to limit adverse energy dissipation in wasting-associated syndromes.

CONCLUSION

All these recent discoveries constitute a very active field attracting groups of scientists who are expert not only in metabolism but also in immunology, development, cancer, etc. This has shed new and unexpected light on brown and brite adipocytes and has already given original insights into the putative role of these cells. For the future, it is reasonable to speculate that the story is not finished because this field is now wide open and raises new questions and hypotheses, keeping in mind the key ancestral role of UCP1 in thermogenesis and bioenergetics.

ACKNOWLEDGMENTS

This work was supported by the EU FP7 project DIABAT (HEALTH-F2-2011-278373) and the EU FP7 project METABOSTEM (PCIG9-GA-2011-293720).

REFERENCES

1. Young P, Arch JR, Ashwell M. Brown adipose tissue in the parametrial fat pad of the mouse. *FEBS Lett* (1984) **167**(1):10–4. doi:10.1016/0014-5793(84)80822-4
2. Loncar D. Convertible adipose tissue in mice. *Cell Tissue Res* (1991) **266**(1):149–61. doi:10.1007/BF00678721
3. Cousin B, Cinti S, Morroni M, Raimbault S, Ricquier D, Penicaud L, et al. Occurrence of brown adipocytes in rat white adipose tissue: molecular and morphological characterization. *J Cell Sci* (1992) **103**:931–42.
4. Champigny O, Ricquier D, Blondel O, Mayers RM, Briscoe MG, Holloway BR. Beta 3-adrenergic receptor stimulation restores message and expression of brown-fat mitochondrial uncoupling protein in adult dogs. *Proc Natl Acad Sci U S A* (1991) **88**(23):10774–7. doi:10.1073/pnas.88.23.10774
5. Rosen ED, Spiegelman BM. What we talk about when we talk about fat. *Cell* (2014) **156**(1–2):20–44. doi:10.1016/j.cell.2013.12.012
6. Fasshauer M, Bluher M. Adipokines in health and disease. *Trends Pharmacol Sci* (2015) **36**(7):461–70. doi:10.1016/j.tips.2015.04.014
7. Barrera C, Gatica A, Morgan C. Obese visceral adipose tissue grafted in lean mice can alter glucose homeostasis and energy efficiency. *J Biol Regul Homeost Agents* (2012) **26**(3):411–7.
8. Karelis AD, St-Pierre DH, Conus F, Rabasa-Lhoret R, Poehlman ET. Metabolic and body composition factors in subgroups of obesity: what do we know? *J Clin Endocrinol Metab* (2004) **89**(6):2569–75. doi:10.1210/jc.2004-0165
9. Tran TT, Yamamoto Y, Gesta S, Kahn CR. Beneficial effects of subcutaneous fat transplantation on metabolism. *Cell Metab* (2008) **7**(5):410–20. doi:10.1016/j.cmet.2008.04.004
10. Nicholls DG, Locke RM. Thermogenic mechanisms in brown fat. *Physiol Rev* (1984) **64**(1):1–64.
11. Himms-Hagen J. Brown adipose tissue thermogenesis: interdisciplinary studies. *FASEB J* (1990) **4**(11):2890–8.
12. Enerback S, Jacobsson A, Simpson EM, Guerra C, Yamashita H, Harper ME, et al. Mice lacking mitochondrial uncoupling protein are cold-sensitive but not obese. *Nature* (1997) **387**(6628):90–4. doi:10.1038/387090a0
13. Casteilla L, Champigny O, Bouillaud F, Robelin J, Ricquier D. Sequential changes in the expression of mitochondrial protein mRNA during the development of brown adipose tissue in bovine and ovine species. Sudden occurrence of uncoupling protein mRNA during embryogenesis and its disappearance after birth. *Biochem J* (1989) **257**(3):665–71. doi:10.1042/bj2570665
14. Cao Y. Angiogenesis and vascular functions in modulation of obesity, adipose metabolism, and insulin sensitivity. *Cell Metab* (2013) **18**(4):478–89. doi:10.1016/j.cmet.2013.08.008
15. Shimizu I, Aprahamian T, Kikuchi R, Shimizu A, Papanicolaou KN, MacLauchlan S, et al. Vascular rarefaction mediates whitening of brown fat in obesity. *J Clin Invest* (2014) **124**(5):2099–112. doi:10.1172/JCI71643
16. Tupone D, Madden CJ, Morrison SF. Autonomic regulation of brown adipose tissue thermogenesis in health and disease: potential clinical applications for altering BAT thermogenesis. *Front Neurosci* (2014) **8**:14. doi:10.3389/fnins.2014.00014
17. Ricquier D. Uncoupling protein 1 of brown adipocytes, the only uncoupler: a historical perspective. *Front Endocrinol* (2011) **2**:85. doi:10.3389/fendo.2011.00085
18. Fedorenko A, Lishko PV, Kirichok Y. Mechanism of fatty-acid-dependent UCP1 uncoupling in brown fat mitochondria. *Cell* (2012) **151**(2):400–13. doi:10.1016/j.cell.2012.09.010
19. Collins S. Beta-adrenoceptor signaling networks in adipocytes for recruiting stored fat and energy expenditure. *Front Endocrinol* (2011) **2**:102. doi:10.3389/fendo.2011.00102
20. Cao W, Daniel KW, Robidoux J, Puigserver P, Medvedev AV, Bai X, et al. p38 mitogen-activated protein kinase is the central regulator of cyclic AMP-dependent transcription of the brown fat uncoupling protein 1 gene. *Mol Cell Biol* (2004) **24**(7):3057–67. doi:10.1128/MCB.24.7.3057-3067.2004
21. Nechad M, Nedergaard J, Cannon B. Noradrenergic stimulation of mitochondriogenesis in brown adipocytes differentiating in culture. *Am J Physiol* (1987) **253**(6 Pt 1):C889–94.
22. Wikstrom JD, Mahdaviani K, Liesa M, Sereda SB, Si Y, Las G, et al. Hormone-induced mitochondrial fission is utilized by brown adipocytes as an amplification pathway for energy expenditure. *EMBO J* (2014) **33**(5):418–36. doi:10.1002/embj.201385014
23. Bronnikov G, Houstek J, Nedergaard J. Beta-adrenergic, cAMP-mediated stimulation of proliferation of brown fat cells in primary culture. Mediation via beta 1 but not via beta 3 adrenoceptors. *J Biol Chem* (1992) **267**(3):2006–13.
24. Rothwell NJ, Stock MJ. A role for brown adipose tissue in diet-induced thermogenesis. *Obes Res* (1997) **5**(6):650–6. doi:10.1002/j.1550-8528.1997.tb00591.x
25. Bartelt A, Bruns OT, Reimer R, Hohenberg H, Ittrich H, Peldschus K, et al. Brown adipose tissue activity controls triglyceride clearance. *Nat Med* (2011) **17**(2):200–5. doi:10.1038/nm.2297
26. Stanford KI, Middelbeek RJ, Townsend KL, An D, Nygaard EB, Hitchcox KM, et al. Brown adipose tissue regulates glucose homeostasis and insulin sensitivity. *J Clin Invest* (2013) **123**(1):215–23. doi:10.1172/JCI62308
27. Liu X, Wang S, You Y, Meng M, Zheng Z, Dong M, et al. Brown adipose tissue transplantation reverses obesity in Ob/Ob mice. *Endocrinology* (2015) **156**(7):2461–9. doi:10.1210/en.2014-1598
28. Feldmann HM, Golozoubova V, Cannon B, Nedergaard J. UCP1 ablation induces obesity and abolishes diet-induced thermogenesis in mice exempt from thermal stress by living at thermoneutrality. *Cell Metab* (2009) **9**(2):203–9. doi:10.1016/j.cmet.2008.12.014
29. Kozak LP. Brown fat and the myth of diet-induced thermogenesis. *Cell Metab* (2010) **11**(4):263–7. doi:10.1016/j.cmet.2010.03.009
30. Algire C, Medrikova D, Herzig S. White and brown adipose stem cells: from signaling to clinical implications. *Biochim Biophys Acta* (2013) **1831**(5):896–904. doi:10.1016/j.bbalip.2012.10.001
31. Villarroya J, Cereijo R, Villarroya F. An endocrine role for brown adipose tissue? *Am J Physiol Endocrinol Metab* (2013) **305**(5):E567–72. doi:10.1152/ajpendo.00250.2013
32. de Jesus LA, Carvalho SD, Ribeiro MO, Schneider M, Kim SW, Harney JW, et al. The type 2 iodothyronine deiodinase is essential for adaptive thermogenesis in brown adipose tissue. *J Clin Invest* (2001) **108**(9):1379–85. doi:10.1172/JCI200113803
33. Bagchi M, Kim LA, Boucher J, Walshe TE, Kahn CR, D'Amore PA. Vascular endothelial growth factor is important for brown adipose tissue development and maintenance. *FASEB J* (2013) **27**(8):3257–71. doi:10.1096/fj.12-221812
34. Yamashita H, Sato N, Kizaki T, Oh-Ishi S, Segawa M, Saitoh D, et al. Norepinephrine stimulates the expression of fibroblast growth factor 2 in rat brown adipocyte primary culture. *Cell Growth Differ* (1995) **6**(11):1457–62.
35. Hansen-Algenstaedt N, Algenstaedt P, Schaefer C, Hamann A, Wolfram L, Cingoz G, et al. Neural driven angiogenesis by overexpression of nerve growth factor. *Histochem Cell Biol* (2006) **125**(6):637–49. doi:10.1007/s00418-005-0111-z
36. Hondares E, Iglesias R, Giralt A, Gonzalez FJ, Giralt M, Mampel T, et al. Thermogenic activation induces FGF21 expression and release in brown adipose tissue. *J Biol Chem* (2011) **286**(15):12983–90. doi:10.1074/jbc.M110.215889
37. Buyse M, Viengchareun S, Bado A, Lombes M. Insulin and glucocorticoids differentially regulate leptin transcription and secretion in brown adipocytes. *FASEB J* (2001) **15**(8):1357–66. doi:10.1096/fj.00-0669com
38. Viengchareun S, Zennaro MC, Pascual-Le Tallec L, Lombes M. Brown adipocytes are novel sites of expression and regulation of adiponectin and resistin. *FEBS Lett* (2002) **532**(3):345–50. doi:10.1016/S0014-5793(02)03697-9
39. Bianco AC, McAninch EA. The role of thyroid hormone and brown adipose tissue in energy homoeostasis. *Lancet Diabetes Endocrinol* (2013) **1**(3):250–8. doi:10.1016/S2213-8587(13)70069-X
40. Collins S, Yehuda-Shnaidman E, Wang H. Positive and negative control of Ucp1 gene transcription and the role of beta-adrenergic signaling networks. *Int J Obes (Lond)* (2010) **34**(Suppl 1):S28–33. doi:10.1038/ijo.2010.180
41. Kajimura S, Seale P, Spiegelman BM. Transcriptional control of brown fat development. *Cell Metab* (2010) **11**(4):257–62. doi:10.1016/j.cmet.2010.03.005
42. Gray SL, Dalla Nora E, Backlund EC, Manieri M, Virtue S, Noland RC, et al. Decreased brown adipocyte recruitment and thermogenic capacity in mice with impaired peroxisome proliferator-activated receptor (P465L PPARgamma) function. *Endocrinology* (2006) **147**(12):5708–14. doi:10.1210/en.2006-0684
43. Petrovic N, Walden TB, Shabalina IG, Timmons JA, Cannon B, Nedergaard J. Chronic peroxisome proliferator-activated receptor gamma (PPARgamma) activation of epididymally derived white adipocyte cultures reveals a population of thermogenically competent, UCP1-containing adipocytes

molecularly distinct from classic brown adipocytes. *J Biol Chem* (2010) **285**(10):7153–64. doi:10.1074/jbc.M109.053942
44. Herzig S, Long F, Jhala US, Hedrick S, Quinn R, Bauer A, et al. CREB regulates hepatic gluconeogenesis through the coactivator PGC-1. *Nature* (2001) **413**(6852):179–83. doi:10.1038/35093131
45. Puigserver P, Wu Z, Park CW, Graves R, Wright M, Spiegelman BM. A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. *Cell* (1998) **92**(6):829–39. doi:10.1016/S0092-8674(00)81410-5
46. Tiraby C, Tavernier G, Lefort C, Larrouy D, Bouillaud F, Ricquier D, et al. Acquirement of brown fat cell features by human white adipocytes. *J Biol Chem* (2003) **278**(35):33370–6. doi:10.1074/jbc.M305235200
47. Puigserver P, Spiegelman BM. Peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1 alpha): transcriptional coactivator and metabolic regulator. *Endocr Rev* (2003) **24**(1):78–90. doi:10.1210/er.2002-0012
48. Seale P, Kajimura S, Yang W, Chin S, Rohas LM, Uldry M, et al. Transcriptional control of brown fat determination by PRDM16. *Cell Metab* (2007) **6**(1):38–54. doi:10.1016/j.cmet.2007.06.001
49. Qiang L, Wang L, Kon N, Zhao W, Lee S, Zhang Y, et al. Brown remodeling of white adipose tissue by SirT1-dependent deacetylation of Ppargamma. *Cell* (2012) **150**(3):620–32. doi:10.1016/j.cell.2012.06.027
50. Cariou B, Charbonnel B, Staels B. Thiazolidinediones and PPARgamma agonists: time for a reassessment. *Trends Endocrinol Metab* (2012) **23**(5):205–15. doi:10.1016/j.tem.2012.03.001
51. Smorlesi A, Frontini A, Giordano A, Cinti S. The adipose organ: white-brown adipocyte plasticity and metabolic inflammation. *Obes Rev* (2012) **13**(Suppl 2):83–96. doi:10.1111/j.1467-789X.2012.01039.x
52. Cousin B, Bascands-Viguerie N, Kassis N, Nibbelink M, Ambid L, Casteilla L, et al. Cellular changes during cold acclimatation in adipose tissues. *J Cell Physiol* (1996) **167**(2):285–9. doi:10.1002/(SICI)1097-4652(199605)167:2<285::AID-JCP12>3.0.CO;2-7
53. Timmons JA, Wennmalm K, Larsson O, Walden TB, Lassmann T, Petrovic N, et al. Myogenic gene expression signature establishes that brown and white adipocytes originate from distinct cell lineages. *Proc Natl Acad Sci U S A* (2007) **104**(11):4401–6. doi:10.1073/pnas.0610615104
54. Walden TB, Hansen IR, Timmons JA, Cannon B, Nedergaard J. Recruited vs. nonrecruited molecular signatures of brown, "brite," and white adipose tissues. *Am J Physiol Endocrinol Metab* (2012) **302**(1):E19–31. doi:10.1152/ajpendo.00249.2011
55. Seale P, Bjork B, Yang W, Kajimura S, Chin S, Kuang S, et al. PRDM16 controls a brown fat/skeletal muscle switch. *Nature* (2008) **454**(7207):961–7. doi:10.1038/nature07182
56. Sanchez-Gurmaches J, Guertin DA. Adipocytes arise from multiple lineages that are heterogeneously and dynamically distributed. *Nat Commun* (2014) **5**:4099. doi:10.1038/ncomms5099
57. Sanchez-Gurmaches J, Guertin DA. Adipocyte lineages: tracing back the origins of fat. *Biochim Biophys Acta* (2014) **1842**(3):340–51. doi:10.1016/j.bbadis.2013.05.027
58. Sanchez-Gurmaches J, Hung CM, Sparks CA, Tang Y, Li H, Guertin DA. PTEN loss in the Myf5 lineage redistributes body fat and reveals subsets of white adipocytes that arise from Myf5 precursors. *Cell Metab* (2012) **16**(3):348–62. doi:10.1016/j.cmet.2012.08.003
59. Wu J, Bostrom P, Sparks LM, Ye L, Choi JH, Giang AH, et al. Beige adipocytes are a distinct type of thermogenic fat cell in mouse and human. *Cell* (2012) **150**(2):366–76. doi:10.1016/j.cell.2012.05.016
60. Wu J, Cohen P, Spiegelman BM. Adaptive thermogenesis in adipocytes: is beige the new brown? *Genes Dev* (2013) **27**(3):234–50. doi:10.1101/gad.211649.112
61. Kajimura S, Seale P, Tomaru T, Erdjument-Bromage H, Cooper MP, Ruas JL, et al. Regulation of the brown and white fat gene programs through a PRDM16/CtBP transcriptional complex. *Genes Dev* (2008) **22**(10):1397–409. doi:10.1101/gad.1666108
62. Kajimura S, Seale P, Kubota K, Lunsford E, Frangioni JV, Gygi SP, et al. Initiation of myoblast to brown fat switch by a PRDM16-C/EBP-beta transcriptional complex. *Nature* (2009) **460**(7259):1154–8. doi:10.1038/nature08262
63. Thomsom JF, Habeck DA, Nance SL, Beetham KL. Ultrastructural and biochemical changes in brown fat in cold-exposed rats. *J Cell Biol* (1969) **41**(1):312–34. doi:10.1083/jcb.41.1.312
64. Creasey NH, Gray CH. Enzymes concerned in the synthesis of glycogen from glucose in the brown adipose tissue. *Biochem J* (1951) **50**(1):74–81. doi:10.1042/bj0500074
65. Crisan M, Casteilla L, Lehr L, Carmona M, Paoloni-Giacobino A, Yap S, et al. A reservoir of brown adipocyte progenitors in human skeletal muscle. *Stem Cells* (2008) **26**(9):2425–33. doi:10.1634/stemcells.2008-0325
66. Long JZ, Svensson KJ, Tsai L, Zeng X, Roh HC, Kong X, et al. A smooth muscle-like origin for beige adipocytes. *Cell Metab* (2014) **19**(5):810–20. doi:10.1016/j.cmet.2014.03.025
67. Rosenwald M, Perdikari A, Rulicke T, Wolfrum C. Bi-directional interconversion of brite and white adipocytes. *Nat Cell Biol* (2013) **15**(6):659–67. doi:10.1038/ncb2740
68. Wang QA, Tao C, Gupta RK, Scherer PE. Tracking adipogenesis during white adipose tissue development, expansion and regeneration. *Nat Med* (2013) **19**(10):1338–44. doi:10.1038/nm.3324
69. Lee YH, Petkova AP, Mottillo EP, Granneman JG. In vivo identification of bipotential adipocyte progenitors recruited by beta3-adrenoceptor activation and high-fat feeding. *Cell Metab* (2012) **15**(4):480–91. doi:10.1016/j.cmet.2012.03.009
70. Lee YH, Petkova AP, Konkar AA, Granneman JG. Cellular origins of cold-induced brown adipocytes in adult mice. *FASEB J* (2015) **29**(1):286–99. doi:10.1096/fj.14-263038
71. de Jong JM, Larsson O, Cannon B, Nedergaard J. A stringent validation of mouse adipose tissue identity markers. *Am J Physiol Endocrinol Metab* (2015) **308**(12):E1085–105. doi:10.1152/ajpendo.00023.2015
72. Poher AL, Altirriba J, Veyrat-Durebex C, Rohner-Jeanrenaud F. Brown adipose tissue activity as a target for the treatment of obesity/insulin resistance. *Front Physiol* (2015) **6**:4. doi:10.3389/fphys.2015.00004
73. Bartelt A, Heeren J. Adipose tissue browning and metabolic health. *Nat Rev Endocrinol* (2014) **10**(1):24–36. doi:10.1038/nrendo.2013.204
74. Sidossis L, Kajimura S. Brown and beige fat in humans: thermogenic adipocytes that control energy and glucose homeostasis. *J Clin Invest* (2015) **125**(2):478–86. doi:10.1172/JCI78362
75. Carriere A, Jeanson Y, Berger-Muller S, Andre M, Chenouard V, Arnaud E, et al. Browning of white adipose cells by intermediate metabolites: an adaptive mechanism to alleviate redox pressure. *Diabetes* (2014) **63**(10):3253–65. doi:10.2337/db13-1885
76. DiGirolamo M, Newby FD, Lovejoy J. Lactate production in adipose tissue: a regulated function with extra-adipose implications. *FASEB J* (1992) **6**(7):2405–12.
77. Trayhurn P, Alomar SY. Oxygen deprivation and the cellular response to hypoxia in adipocytes – perspectives on white and brown adipose tissues in obesity. *Front Endocrinol* (2015) **6**:19. doi:10.3389/fendo.2015.00019
78. Ro SH, Nam M, Jang I, Park HW, Park H, Semple IA, et al. Sestrin2 inhibits uncoupling protein 1 expression through suppressing reactive oxygen species. *Proc Natl Acad Sci U S A* (2014) **111**(21):7849–54. doi:10.1073/pnas.1401787111
79. Lettieri Barbato D, Tatulli G, Maria Cannata S, Bernardini S, Aquilano K, Ciriolo MR. Glutathione decrement drives thermogenic program in adipose cells. *Sci Rep* (2015) **5**:13091. doi:10.1038/srep13091
80. Bostrom P, Wu J, Jedrychowski MP, Korde A, Ye L, Lo JC, et al. A PGC1-alpha-dependent myokine that drives brown-fat-like development of white fat and thermogenesis. *Nature* (2012) **481**(7382):463–8. doi:10.1038/nature10777
81. De Matteis R, Lucertini F, Guescini M, Polidori E, Zeppa S, Stocchi V, et al. Exercise as a new physiological stimulus for brown adipose tissue activity. *Nutr Metab Cardiovasc Dis* (2013) **23**(6):582–90. doi:10.1016/j.numecd.2012.01.013
82. Knudsen JG, Murholm M, Carey AL, Bienso RS, Basse AL, Allen TL, et al. Role of IL-6 in exercise training- and cold-induced UCP1 expression in subcutaneous white adipose tissue. *PLoS One* (2014) **9**(1):e84910. doi:10.1371/journal.pone.0084910
83. Rao RR, Long JZ, White JP, Svensson KJ, Lou J, Lokurkar I, et al. Meteorin-like is a hormone that regulates immune-adipose interactions to increase beige fat thermogenesis. *Cell* (2014) **157**(6):1279–91. doi:10.1016/j.cell.2014.03.065
84. Jedrychowski MP, Wrann CD, Paulo JA, Gerber KK, Szpyt J, Robinson MM, et al. Detection and quantitation of circulating human irisin by tandem mass spectrometry. *Cell Metab* (2015). doi:10.1016/j.cmet.2015.08.001

85. Hofmann T, Elbelt U, Stengel A. Irisin as a muscle-derived hormone stimulating thermogenesis – a critical update. *Peptides* (2014) **54**:89–100. doi:10.1016/j.peptides.2014.01.016
86. Raschke S, Elsen M, Gassenhuber H, Sommerfeld M, Schwahn U, Brockmann B, et al. Evidence against a beneficial effect of irisin in humans. *PLoS One* (2013) **8**(9):e73680. doi:10.1371/journal.pone.0073680
87. Lee P, Linderman JD, Smith S, Brychta RJ, Wang J, Idelson C, et al. Irisin and FGF21 are cold-induced endocrine activators of brown fat function in humans. *Cell Metab* (2014) **19**(2):302–9. doi:10.1016/j.cmet.2013.12.017
88. Evans WJ, Morley JE, Argiles J, Bales C, Baracos V, Guttridge D, et al. Cachexia: a new definition. *Clin Nutr* (2008) **27**(6):793–9. doi:10.1016/j.clnu.2008.06.013
89. Argiles JM, Lopez-Soriano FJ, Toledo M, Betancourt A, Serpe R, Busquets S. The cachexia score (CASCO): a new tool for staging cachectic cancer patients. *J Cachexia Sarcopenia Muscle* (2011) **2**(2):87–93. doi:10.1007/s13539-011-0027-5
90. Petruzzelli M, Schweiger M, Schreiber R, Campos-Olivas R, Tsoli M, Allen J, et al. A switch from white to brown fat increases energy expenditure in cancer-associated cachexia. *Cell Metab* (2014) **20**(3):433–47. doi:10.1016/j.cmet.2014.06.011
91. Kir S, White JP, Kleiner S, Kazak L, Cohen P, Baracos VE, et al. Tumour-derived PTH-related protein triggers adipose tissue browning and cancer cachexia. *Nature* (2014) **513**(7516):100–4. doi:10.1038/nature13528
92. Tsoli M, Moore M, Burg D, Painter A, Taylor R, Lockie SH, et al. Activation of thermogenesis in brown adipose tissue and dysregulated lipid metabolism associated with cancer cachexia in mice. *Cancer Res* (2012) **72**(17):4372–82. doi:10.1158/0008-5472.CAN-11-3536
93. Porter C, Herndon DN, Bhattarai N, Ogunbileje JO, Szczesny B, Szabo C, et al. Severe burn injury induces thermogenically functional mitochondria in murine white adipose tissue. *Shock* (2015) **44**(3):258–64. doi:10.1097/SHK.0000000000000410
94. Sidossis LS, Porter C, Saraf MK, Borsheim E, Radhakrishnan RS, Chao T, et al. Browning of subcutaneous white adipose tissue in humans after severe adrenergic stress. *Cell Metab* (2015) **22**(2):219–27. doi:10.1016/j.cmet.2015.06.022
95. Champigny O, Ricquier D. Effects of fasting and refeeding on the level of uncoupling protein mRNA in rat brown adipose tissue: evidence for diet-induced and cold-induced responses. *J Nutr* (1990) **120**(12):1730–6.
96. Brestoff JR, Artis D. Immune regulation of metabolic homeostasis in health and disease. *Cell* (2015) **161**(1):146–60. doi:10.1016/j.cell.2015.02.022
97. Vegiopoulos A, Muller-Decker K, Strzoda D, Schmitt I, Chichelnitskiy E, Ostertag A, et al. Cyclooxygenase-2 controls energy homeostasis in mice by de novo recruitment of brown adipocytes. *Science* (2010) **328**(5982):1158–61. doi:10.1126/science.1186034
98. Madsen L, Pedersen LM, Lillefosse HH, Fjaere E, Bronstad I, Hao Q, et al. UCP1 induction during recruitment of brown adipocytes in white adipose tissue is dependent on cyclooxygenase activity. *PLoS One* (2010) **5**(6):e11391. doi:10.1371/journal.pone.0011391
99. Forman BM, Tontonoz P, Chen J, Brun RP, Spiegelman BM, Evans RM. 15-Deoxy-delta 12, 14-prostaglandin J2 is a ligand for the adipocyte determination factor PPAR gamma. *Cell* (1995) **83**(5):803–12. doi:10.1016/0092-8674(95)90193-0
100. Pisani DF, Ghandour RA, Beranger GE, Le Faouder P, Chambard JC, Giroud M, et al. The omega6-fatty acid, arachidonic acid, regulates the conversion of white to brite adipocyte through a prostaglandin/calcium mediated pathway. *Mol Metab* (2014) **3**(9):834–47. doi:10.1016/j.molmet.2014.09.003
101. Qiu Y, Nguyen KD, Odegaard JI, Cui X, Tian X, Locksley RM, et al. Eosinophils and type 2 cytokine signaling in macrophages orchestrate development of functional beige fat. *Cell* (2014) **157**(6):1292–308. doi:10.1016/j.cell.2014.03.066
102. Lee MW, Odegaard JI, Mukundan L, Qiu Y, Molofsky AB, Nussbaum JC, et al. Activated type 2 innate lymphoid cells regulate beige fat biogenesis. *Cell* (2015) **160**(1–2):74–87. doi:10.1016/j.cell.2014.12.011
103. Brestoff JR, Kim BS, Saenz SA, Stine RR, Monticelli LA, Sonnenberg GF, et al. Group 2 innate lymphoid cells promote beiging of white adipose tissue and limit obesity. *Nature* (2015) **519**(7542):242–6. doi:10.1038/nature14115
104. Kong X, Banks A, Liu T, Kazak L, Rao RR, Cohen P, et al. IRF4 is a key thermogenic transcriptional partner of PGC-1alpha. *Cell* (2014) **158**(1):69–83. doi:10.1016/j.cell.2014.04.049
105. Ricquier D, Nechad M, Mory G. Ultrastructural and biochemical characterization of human brown adipose tissue in pheochromocytoma. *J Clin Endocrinol Metab* (1982) **54**(4):803–7.
106. Bouillaud F, Villarroya F, Hentz E, Raimbault S, Cassard AM, Ricquier D. Detection of brown adipose tissue uncoupling protein mRNA in adult patients by a human genomic probe. *Clin Sci (Lond)* (1988) **75**(1):21–7. doi:10.1042/cs0750021
107. Huttunen P, Hirvonen J, Kinnula V. The occurrence of brown adipose tissue in outdoor workers. *Eur J Appl Physiol Occup Physiol* (1981) **46**(4):339–45. doi:10.1007/BF00422121
108. Heaton JM. The distribution of brown adipose tissue in the human. *J Anat* (1972) **112**(Pt 1):35–9.
109. Cypess AM, Lehman S, Williams G, Tal I, Rodman D, Goldfine AB, et al. Identification and importance of brown adipose tissue in adult humans. *N Engl J Med* (2009) **360**(15):1509–17. doi:10.1056/NEJMoa0810780
110. Virtanen KA, Lidell ME, Orava J, Heglind M, Westergren R, Niemi T, et al. Functional brown adipose tissue in healthy adults. *N Engl J Med* (2009) **360**(15):1518–25. doi:10.1056/NEJMoa0808949
111. van Marken Lichtenbelt WD, Vanhommerig JW, Smulders NM, Drossaerts JM, Kemerink GJ, Bouvy ND, et al. Cold-activated brown adipose tissue in healthy men. *N Engl J Med* (2009) **360**(15):1500–8. doi:10.1056/NEJMoa0808718
112. Saito M, Okamatsu-Ogura Y, Matsushita M, Watanabe K, Yoneshiro T, Nio-Kobayashi J, et al. High incidence of metabolically active brown adipose tissue in healthy adult humans: effects of cold exposure and adiposity. *Diabetes* (2009) **58**(7):1526–31. doi:10.2337/db09-0530
113. Shinoda K, Luijten IH, Hasegawa Y, Hong H, Sonne SB, Kim M, et al. Genetic and functional characterization of clonally derived adult human brown adipocytes. *Nat Med* (2015) **21**(4):389–94. doi:10.1038/nm.3819
114. Sharp LZ, Shinoda K, Ohno H, Scheel DW, Tomoda E, Ruiz L, et al. Human BAT possesses molecular signatures that resemble beige/brite cells. *PLoS One* (2012) **7**(11):e49452. doi:10.1371/journal.pone.0049452
115. Cypess AM, White AP, Vernochet C, Schulz TJ, Xue R, Sass CA, et al. Anatomical localization, gene expression profiling and functional characterization of adult human neck brown fat. *Nat Med* (2013) **19**(5):635–9. doi:10.1038/nm.3112
116. Jespersen NZ, Larsen TJ, Peijs L, Daugaard S, Homoe P, Loft A, et al. A classical brown adipose tissue mRNA signature partly overlaps with brite in the supraclavicular region of adult humans. *Cell Metab* (2013) **17**(5):798–805. doi:10.1016/j.cmet.2013.04.011
117. Esteve D, Boulet N, Volat F, Zakaroff-Girard A, Ledoux S, Coupaye M, et al. Human white and brite adipogenesis is supported by MSCA1 and is impaired by immune cells. *Stem Cells* (2015) **33**(4):1277–91. doi:10.1002/stem.1916
118. Xue R, Lynes MD, Dreyfuss JM, Shamsi F, Schulz TJ, Zhang H, et al. Clonal analyses and gene profiling identify genetic biomarkers of the thermogenic potential of human brown and white preadipocytes. *Nat Med* (2015) **21**(7):760–8. doi:10.1038/nm.3881
119. Mund RA, Frishman WH. Brown adipose tissue thermogenesis: beta3-adrenoreceptors as a potential target for the treatment of obesity in humans. *Cardiol Rev* (2013) **21**(6):265–9. doi:10.1097/CRD.0b013e31829cabff
120. Bordicchia M, Pocognoli A, D'Anzeo M, Siquini W, Minardi D, Muzzonigro G, et al. Nebivolol induces, via beta3 adrenergic receptor, lipolysis, uncoupling protein 1, and reduction of lipid droplet size in human adipocytes. *J Hypertens* (2014) **32**(2):389–96. doi:10.1097/HJH.0000000000000024
121. Yoneshiro T, Ogawa T, Okamoto N, Matsushita M, Aita S, Kameya T, et al. Impact of UCP1 and beta3AR gene polymorphisms on age-related changes in brown adipose tissue and adiposity in humans. *Int J Obes (Lond)* (2013) **37**(7):993–8. doi:10.1038/ijo.2012.161
122. Cypess AM, Weiner LS, Roberts-Toler C, Franquet Elia E, Kessler SH, Kahn PA, et al. Activation of human brown adipose tissue by a beta3-adrenergic receptor agonist. *Cell Metab* (2015) **21**(1):33–8. doi:10.1016/j.cmet.2014.12.009

123. Fruchart JC. Selective peroxisome proliferator-activated receptor alpha modulators (SPPARMalpha): the next generation of peroxisome proliferator-activated receptor alpha-agonists. *Cardiovasc Diabetol* (2013) **12**:82. doi:10.1186/1475-2840-12-82
124. Blackburn GL. From bench to bedside: novel mechanisms and therapeutic advances through the development of selective peroxisome proliferator-activated receptor gamma modulators. *Am J Clin Nutr* (2010) **91**(1):251S–3S. doi:10.3945/ajcn.2009.28449A
125. Broeders EP, Nascimento EB, Havekes B, Brans B, Roumans KH, Tailleux A, et al. The bile acid chenodeoxycholic acid increases human brown adipose tissue activity. *Cell Metab* (2015) **22**(3):418–26. doi:10.1016/j.cmet.2015.07.002

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

PERSPECTIVE
published: 24 July 2015
doi: 10.3389/fendo.2015.00114

Sestrin2, a regulator of thermogenesis and mitohormesis in brown adipose tissue

*Seung-Hyun Ro[1], Ian Semple[1], Allison Ho[1], Hwan-Woo Park[2] and Jun Hee Lee[1]**

[1] Department of Molecular and Integrative Physiology, University of Michigan, Ann Arbor, MI, USA, [2] Department of Cell Biology, College of Medicine, Konyang University, Daejeon, South Korea

Sestrin2 is a stress-inducible protein that functions as an antioxidant and inhibitor of mTOR complex 1. In a recent study, we found that Sestrin2 overexpression in brown adipocytes interfered with normal metabolism by reducing mitochondrial respiration through the suppression of uncoupling protein 1 (UCP1) expression. The metabolic effects of Sestrin2 in brown adipocytes were dependent on its antioxidant activity, and chemical antioxidants produced similar effects in inhibiting UCP1-dependent thermogenesis. These observations suggest that low levels of reactive oxygen species (ROS) in brown adipocytes can actually be beneficial and necessary for proper metabolic homeostasis. In addition, considering that Sestrins are ROS inducible and perform ROS detoxifying as well as other metabolism-controlling functions, they are potential regulators of mitohormesis. This is a concept in which overall beneficial effects result from low-level oxidative stress stimuli, such as the ones induced by caloric restriction or physical exercise. In this perspective, we incorporate our recent insight obtained from the Sestrin2 study toward a better understanding of the relationship between ROS, Sestrin2, and mitochondrial metabolism in the context of brown adipocyte physiology.

Keywords: Sestrin2, brown adipose tissue, mitochondria metabolism, thermogenesis, reactive oxygen species, antioxidants, mitohormesis, aging

OPEN ACCESS

Edited by:
Gary Sweeney, York University, Canada

Reviewed by:
Jae B. Kim, Seoul National University, South Korea
William T. Festuccia, University of São Paulo, Brazil

****Correspondence:***
Jun Hee Lee, Institute of Gerontology, Department of Molecular and Integrative Physiology, University of Michigan Medical School, 109 Zina Pitcher Place, 3019 BSRB, Ann Arbor, MI 48109, USA
leeju@umich.edu

Specialty section:
This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *27 May 2015*
Accepted: *11 July 2015*
Published: *24 July 2015*

Citation:
Ro S-H, Semple I, Ho A, Park H-W and Lee JH (2015) Sestrin2, a regulator of thermogenesis and mitohormesis in brown adipose tissue. Front. Endocrinol. 6:114. doi: 10.3389/fendo.2015.00114

Introduction: Sestrins

Sestrins are a group of stress-inducible proteins, which are highly conserved across species and have two distinct biologically active functions (1). First, they function as an antioxidant that suppresses reactive oxygen species (ROS) accumulation (2, 3) through a poorly characterized biochemical mechanism (4), which may involve regulation of antioxidant transcription factors (5). Independently of this redox function, Sestrins also act as feedback inhibitors of mechanistic target of rapamycin complex 1 (mTORC1) through activation of AMP-activated protein kinase (AMPK) (6, 7) or through inhibition of Rag GTPases (8–11). Through these functions, Sestrins have been shown to attenuate multiple age- and obesity-associated metabolic pathologies, such as fat accumulation, glucose intolerance, insulin resistance, mitochondrial dysfunction, muscle degeneration, and cardiac malfunction (5–7, 12–15). Because the expression of Sestrins is induced upon a variety of environmental stresses, such as DNA damage, oxidative stress, and hypoxia (2), Sestrins are considered a mechanistic link between stress and aging (16).

ROS as Signaling Molecules

Reactive oxygen species are a group of free oxygen radicals and reactive non-radicals, such as superoxide $(O_2^{\cdot -})$, hydroxyl radical ($OH^{\cdot}$), nitric oxide ($NO^{\cdot}$), and hydrogen peroxide (H_2O_2) (17–19). In mammalian cells, ROS can be generated from various sources, such as mitochondria, peroxisomes, and ROS-producing cytosolic enzymes, such as NADPH oxidases (20–23). Under normal physiological conditions, the intracellular levels of ROS are homeostatically controlled. However, aberrantly increased ROS levels can damage intracellular organelles and critical macromolecules, such as proteins, DNA, and lipids. The ROS-induced oxidative damage can contribute to the development of numerous pathological disorders, such as cardiovascular disease, neurodegenerative diseases, mitochondrial disease, obesity, diabetes, cancer, and aging (24–30).

Although ROS were initially considered to be undesired byproducts of metabolism (31), a modernized view of ROS has emerged; ROS are now considered to be important signaling molecules (32–35). ROS can control diverse signaling pathways, including MAPK/ERK1/2 pathway (36, 37), PI3K/Akt pathway (38, 39), IKK/NF-kB pathway (40–42), and p38 MAPK pathway (43, 44), which are critically involved in cell growth, differentiation, metabolism, and inflammation. Through these pathways, ROS contribute to the maintenance of physiological homeostasis in cells and tissues (45–49). These new findings support the concept of mitohormesis, which explains how physiological ROS can be beneficial to the cells and organism; ROS serve as sub-lethal stressors that act as signaling molecules to induce endogenous defense mechanisms, which ultimately improve mitochondrial metabolism and promote stress resistance, metabolic health, and longevity (50–52).

Role of ROS in BAT Metabolism

BAT is the organ mainly responsible for non-shivering thermogenesis, which is mediated by uncoupling protein 1 (UCP1) (53–55). UCP1 is the key protein for thermogenesis, which is specifically induced in BAT upon exposure to cold temperature. Low temperatures activate p38 MAPK by stimulating sympathetic neurons and inducing cAMP accumulation in BAT (56–58). Activated p38 MAPK subsequently activates several transcription factors, such as ATF-2 and PGC-1α, which induces UCP1 expression in BAT (58, 59). By translocating into the mitochondrial inner membrane and dissipating the proton gradient, UCP1 uncouples mitochondrial respiration from ATP synthesis and generates heat (53, 60). UCP1-dependent thermogenesis in BAT increases energy expenditure reduces body fat and improves metabolic homeostasis (60, 61).

Chronic accumulation of ROS produced by dysfunctional mitochondria may deteriorate the BAT metabolism (62, 63), which can be one of the mechanisms of how aging and obesity interferes with BAT metabolism (64–66). In this respect, suppressing excessive ROS might be key to reinforcing BAT metabolism against aging. Chemical antioxidants, artificial or naturally occurring substances, serve to scavenge ROS, many of which are byproducts of cellular metabolism, which when in excess can cause oxidative damage and promote disease development and aging (26, 32). Most cells utilize various antioxidant enzymes, such as catalases, superoxide dismutases, glutathione peroxidases, peroxiredoxins, and Sestrins, which serve to reduce the negative consequences of ROS accumulation (3, 33, 67). We have recently investigated the role of ROS in BAT metabolism through the use of chemical antioxidants, such as butylated hydroxyanisole (BHA) and *N*-acetylcysteine (NAC), as well as forced overexpression of Sestrin2 (68).

Surprisingly, our recent results indicated that physiological levels of ROS could also play a critical role in thermogenic processes (68). BHA and NAC, as well as Sestrin2 overexpression, resulted in a strong reduction of UCP1 expression in BAT. Subsequent *in vitro* and *in vivo* studies showed that physiological ROS in BAT potentiate cAMP-induced p38 MAPK activation, which mediates cold-induced UCP1 expression. Correspondingly, Sestrin2-overexpressing mice were unable to upregulate UCP1 or generate heat in response to cold exposure. These results demonstrate that ROS are critical for proper BAT metabolism. Therefore, prolonged antioxidant treatment may interfere with thermogenesis and possibly other ROS-dependent physiological processes. This idea is consistent with recent reports where administered antioxidants had neutral or negative effects on health and life span in animals and humans (69, 70).

Caloric Restriction and Physical Exercise as Potential Inducers of ROS and Mitohormesis

Caloric restriction, being defined as a reduction in *ad libitum* calorie uptake, has been shown to extend life span in a variety of organisms (71). Notably, emerging evidence indicates that caloric restriction is capable of increasing mitochondrial metabolism, such as oxidative respiration, in yeast (72), *Caenorhabditis elegans* (73) and *Drosophila* (74, 75). Increased production of ROS as a consequence of increased mitochondrial respiration has been suggested to be a critical regulator of life span during caloric restriction (73, 76–79). Caloric restriction-induced ROS can stimulate antioxidant defense mechanisms, such as radical-scavenging enzymes, that mediate various mitohormetic responses (5, 73, 80–82). Carbohydrate-deficient diets also increase oxidative metabolism, subsequently resulting in enhanced ROS defense (83). Therefore, these recent studies suggest that caloric restriction increases longevity at least partially by inducing oxidative metabolism and a mitohormetic defense system.

Physical exercise undoubtedly provides positive effects on diverse diseases, such as obesity, type 2 diabetes, cardiovascular disease, cancer, and general aging (84–88). Similar to caloric restriction, exercise is capable of increasing mitochondrial biogenesis, oxidative metabolism, and mitochondrial ROS production (89–92). Many studies propose that exercise-induced ROS contributes to mitohormesis, which increases health span and mean life span (93–97). Because co-treatment with antioxidants prevented the beneficial mitohormetic response of physical exercise (85, 98), physiological ROS produced by exercise was considered

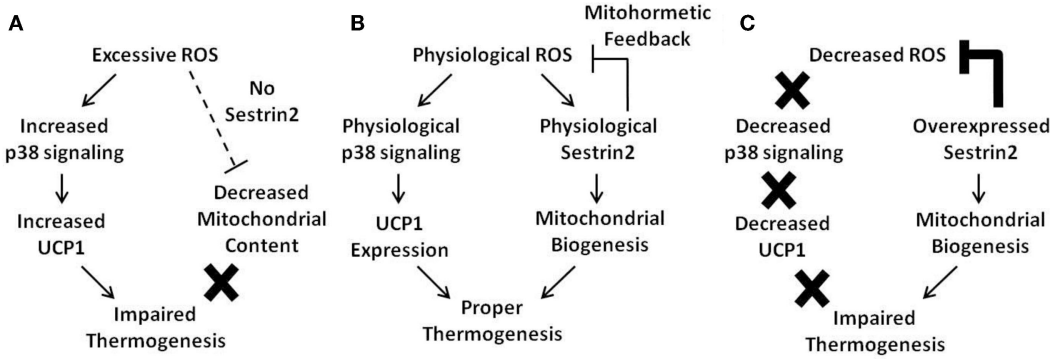


FIGURE 1 | Current model of the mitohormetic relationship between ROS, Sestrin2, and mitochondrial thermogenesis. Under conditions of excessive or decreased ROS, BAT thermogenesis is impaired. However, a physiological level of ROS is able to maintain proper BAT metabolism. **(A)** In cases where antioxidant activity is low, such as in the absence of Sestrin2, ROS levels are upregulated. Excessive ROS may directly impair mitochondrial homeostasis by direct damage to the organelle. In addition, Sestrin2 is critical for mitochondrial biogenesis. Therefore, in the absence of Sestrin2, BAT mitochondrial content is decreased as well as its thermogenic capacity. **(B)** When Sestrin2 is physiologically expressed, an adequate level of ROS is produced to ensure proper p38 MAPK activation and UCP1 expression. Physiological Sestrin2 expression promotes mitochondrial biogenesis, and the mitohormetic mechanism conferred by Sestrin2 is necessary to maintain proper BAT metabolism such as thermogenesis. **(C)** When Sestrin2 is overexpressed, ROS are dramatically suppressed. Because ROS are critical for UCP1 expression in BAT, Sestrin2 overexpression decreases UCP1 expression. Although mitochondrial biogenesis is enhanced by Sestrin2 overexpression, decreased UCP1 expression prohibits proper thermogenesis in BAT.

critical for the benefits of exercise. Therefore, both caloric restriction and exercise seem to utilize physiological ROS induction as a means to induce antioxidant defense and promote the life and health span of an organism.

Sestrins as Potential Regulators of Mitohormesis

Sestrins have been identified as important regulators of age- and obesity-associated pathologies in diverse tissues including liver, adipose, and muscle (5–7, 12–15, 68). Sestrins are thought to attenuate tissue aging through their dual biological activities in reducing ROS and inhibiting mTORC1 (1, 2, 16). Excessive accumulation of ROS and chronic activation of mTORC1 signaling are well-known promoters of tissue aging. Considering that Sestrins are transcriptionally activated upon oxidative stress, it is highly likely that Sestrins may be regulators of mitohormesis. For example, upon a non-toxic level of ROS stimuli, Sestrins may be induced to perform antioxidant and mTORC1-suppressive functions to defend against oxidative damage and attenuate tissue aging.

It should also be noted that, although critical for attenuating tissue aging, the loss of Sestrins *per se* does not substantially reduce life span of *Drosophila* (1) or *C. elegans* (15). This could be because there are unknown compensatory mechanisms instigated by the loss of Sestrins, which are capable of maintaining life span, but are not sufficient to restore health span. However, Sestrin-deficient *C. elegans* is hypersensitive to oxidative stress (15), suggesting that Sestrin is indeed critical for the stress adaptation of an organism. It has been shown in the same organism that low levels of oxidative stress (conferred by a chemical that induces mitochondrial ROS) can increase the life span of *C. elegans* (99, 100). Caloric restriction in *C. elegans* extends life span partially by inducing mitochondrial production of ROS (73). Therefore, it would be very interesting to investigate if Sestrins are indeed regulators of the mitohormetic effect in these aging models.

Role of Sestrin2 in BAT Metabolism

Although the beneficial role of Sestrins in attenuating tissue aging and obesity-associated metabolic pathologies has been clear in several tissues, such as liver and skeletal/cardiac muscle (6, 7, 12, 13), the role of Sestrin2 in BAT metabolism seems to be more complicated. As discussed above, Sestrin2 overexpression in BAT interferes with proper UCP1 expression and mitochondrial uncoupling (68). Even though Sestrin2 overexpression activated AMPK and subsequently promoted mitochondrial biogenesis, these beneficial effects were nullified by the drastic effect of UCP1 loss. As a result, the Sestrin2-overexpressing mice were defective in BAT thermogenesis and exhibited increased fat accumulation (68).

Interestingly, loss of Sestrin2 also interfered with proper BAT metabolism. Although UCP1 expression was relatively increased in BAT of Sestrin2-deficient mice, the whitening of BAT due to fat accumulation is markedly increased upon Sestrin2 deficiency (68). Analysis of mRNA markers for mitochondrial biogenesis and quantification of mitochondrial DNA suggests that BAT from Sestrin2-deficient mice exhibited decreased mitochondrial contents (68). This could be because Sestrin2 plays a critical role in producing sufficient amounts of mitochondria in BAT, which would be necessary for proper energy dissipation and homeostatic BAT metabolism. Sestrin2 may promote mitochondrial biogenesis through activation of AMPK (7, 12, 101, 102) and subsequent upregulation of PGC-1α activity (103, 104). Therefore, although overexpression of Sestrin2 can interfere with the physiological level of ROS necessary for thermogenesis control, endogenous Sestrin2 still plays a critical role in maintaining mitochondrial homeostasis.

Conclusion

Upon diverse environmental stresses including oxidative stress, Sestrin-family proteins are transcriptionally upregulated to reduce pathogenic levels of ROS and suppress chronic activation of mTORC1 signaling. As chronic ROS accumulation and prolonged mTORC1 activation are both detrimental for metabolic homeostasis, Sestrins are potential regulators of mitohormesis, which is a beneficial metabolic effect of low-level ROS production. Sestrins may also play a critical metabolism-controlling role in BAT. However, because ROS are also critical for UCP1 expression and subsequent mitochondrial uncoupling, artificial overexpression of Sestrin2 and subsequent elimination of ROS interfered with non-shivering thermogenesis, which is one of the most critical physiological functions of BAT. Therefore, it is highly likely that ROS levels, Sestrin2 expression, and mitochondrial metabolism are connected to each other through a complicated and finely coordinated network, and a delicate balance between these components seems to be critical for proper BAT homeostasis (**Figure 1**).

Acknowledgments

This work was supported by grants from the American Diabetes Association (Grant 1-13-BS-106) and Ellison Medical Foundation (Grant AG-NS-0932-12).

References

1. Lee JH, Budanov AV, Karin M. Sestrins orchestrate cellular metabolism to attenuate aging. *Cell Metab* (2013) **18**(6):792–801. doi:10.1016/j.cmet.2013.08.018
2. Budanov AV, Lee JH, Karin M. Stressin' Sestrins take an aging fight. *EMBO Mol Med* (2010) **2**(10):388–400. doi:10.1002/emmm.201000097
3. Budanov AV, Sablina AA, Feinstein E, Koonin EV, Chumakov PM. Regeneration of peroxiredoxins by p53-regulated sestrins, homologs of bacterial AhpD. *Science* (2004) **304**(5670):596–600. doi:10.1126/science.1095569
4. Woo HA, Bae SH, Park S, Rhee SG. Sestrin 2 is not a reductase for cysteine sulfinic acid of peroxiredoxins. *Antioxid Redox Signal* (2009) **11**(4):739–45. doi:10.1089/ARS.2008.2360
5. Bae SH, Sung SH, Oh SY, Lim JM, Lee SK, Park YN, et al. Sestrins activate Nrf2 by promoting p62-dependent autophagic degradation of Keap1 and prevent oxidative liver damage. *Cell Metab* (2013) **17**(1):73–84. doi:10.1016/j.cmet.2012.12.002
6. Lee JH, Budanov AV, Park EJ, Birse R, Kim TE, Perkins GA, et al. Sestrin as a feedback inhibitor of TOR that prevents age-related pathologies. *Science* (2010) **327**(5970):1223–8. doi:10.1126/science.1182228
7. Lee JH, Budanov AV, Talukdar S, Park EJ, Park HL, Park HW, et al. Maintenance of metabolic homeostasis by Sestrin2 and Sestrin3. *Cell Metab* (2012) **16**(3):311–21. doi:10.1016/j.cmet.2012.08.004
8. Peng M, Yin N, Li MO. Sestrins function as guanine nucleotide dissociation inhibitors for Rag GTPases to control mTORC1 signaling. *Cell* (2014) **159**(1):122–33. doi:10.1016/j.cell.2014.08.038
9. Chantranupong L, Wolfson RL, Orozco JM, Saxton RA, Scaria SM, Bar-Peled L, et al. The Sestrins interact with GATOR2 to negatively regulate the amino-acid-sensing pathway upstream of mTORC1. *Cell Rep* (2014) **9**(1):1–8. doi:10.1016/j.celrep.2014.09.014
10. Parmigiani A, Nourbakhsh A, Ding B, Wang W, Kim YC, Akopiants K, et al. Sestrins inhibit mTORC1 kinase activation through the GATOR complex. *Cell Rep* (2014) **9**(4):1281–91. doi:10.1016/j.celrep.2014.10.019
11. Kim JS, Ro SH, Kim M, Park HW, Semple IA, Park H, et al. Sestrin2 inhibits mTORC1 through modulation of GATOR complexes. *Sci Rep* (2015) **5**:9502. doi:10.1038/srep09502
12. Morrison A, Chen L, Wang J, Zhang M, Yang H, Ma Y, et al. Sestrin2 promotes LKB1-mediated AMPK activation in the ischemic heart. *FASEB J* (2014) **29**:408–17. doi:10.1096/fj.14-258814
13. Park HW, Park H, Ro SH, Jang I, Semple IA, Kim DN, et al. Hepatoprotective role of Sestrin2 against chronic ER stress. *Nat Commun* (2014) **5**:4233. doi:10.1038/ncomms5233
14. Tao R, Xiong X, Liangpunsakul S, Dong XC. Sestrin 3 protein enhances hepatic insulin sensitivity by direct activation of the mTORC2-Akt signaling. *Diabetes* (2015) **64**(4):1211–23. doi:10.2337/db14-0539
15. Yang YL, Loh KS, Liou BY, Chu IH, Kuo CJ, Chen HD, et al. SESN-1 is a positive regulator of lifespan in *Caenorhabditis elegans*. *Exp Gerontol* (2013) **48**(3):371–9. doi:10.1016/j.exger.2012.12.011
16. Lee JH, Bodmer R, Bier E, Karin M. Sestrins at the crossroad between stress and aging. *Aging (Albany NY)* (2010) **2**(6):369–74.
17. Murphy MP, Holmgren A, Larsson NG, Halliwell B, Chang CJ, Kalyanaraman B, et al. Unraveling the biological roles of reactive oxygen species. *Cell Metab* (2011) **13**(4):361–6. doi:10.1016/j.cmet.2011.03.010
18. Dickinson BC, Chang CJ. Chemistry and biology of reactive oxygen species in signaling or stress responses. *Nat Chem Biol* (2011) **7**(8):504–11. doi:10.1038/nchembio.607
19. Halliwell B, Whiteman M. Measuring reactive species and oxidative damage in vivo and in cell culture: how should you do it and what do the results mean? *Br J Pharmacol* (2004) **142**(2):231–55. doi:10.1038/sj.bjp.0705776
20. Babior BM. NADPH oxidase: an update. *Blood* (1999) **93**(5):1464–76.
21. Shabalina IG, Vrbacký M, Pecinová A, Kalinovich AV, Drahota Z, Houštek J, et al. ROS production in brown adipose tissue mitochondria: the question of UCP1-dependence. *Biochim Biophys Acta* (2014) **1837**(12):2017–30. doi:10.1016/j.bbabio.2014.04.005
22. Sandalio LM, Romero-Puertas MC. Peroxisomes sense and respond to environmental cues by regulating ROS and RNS signalling networks. *Ann Bot* (2015). doi:10.1093/aob/mcv074
23. Wang B, Van Veldhoven PP, Brees C, Rubio N, Nordgren M, Apanasets O, et al. Mitochondria are targets for peroxisome-derived oxidative stress in cultured mammalian cells. *Free Radic Biol Med* (2013) **65**:882–94. doi:10.1016/j.freeradbiomed.2013.08.173
24. Phaniendra A, Jestadi DB, Periyasamy L. Free radicals: properties, sources, targets, and their implication in various diseases. *Indian J Clin Biochem* (2015) **30**(1):11–26. doi:10.1007/s12291-014-0446-0
25. Gupta RK, Patel AK, Shah N, Chaudhary AK, Jha UK, Yadav UC, et al. Oxidative stress and antioxidants in disease and cancer: a review. *Asian Pac J Cancer Prev* (2014) **15**(11):4405–9. doi:10.7314/APJCP.2014.15.11.4405
26. Alfadda AA, Sallam RM. Reactive oxygen species in health and disease. *J Biomed Biotechnol* (2012) **2012**:936486. doi:10.1155/2012/936486
27. El Assar M, Angulo J, Rodriguez-Manas L. Oxidative stress and vascular inflammation in aging. *Free Radic Biol Med* (2013) **65**:380–401. doi:10.1016/j.freeradbiomed.2013.07.003
28. Maiese K. New insights for oxidative stress and diabetes mellitus. *Oxid Med Cell Longev* (2015) **2015**:875961. doi:10.1155/2015/875961
29. Hayashi G, Cortopassi G. Oxidative stress in inherited mitochondrial diseases. *Free Radic Biol Med* (2015). doi:10.1016/j.freeradbiomed.2015.05.039
30. Cobb CA, Cole MP. Oxidative and nitrative stress in neurodegeneration. *Neurobiol Dis* (2015). doi:10.1016/j.nbd.2015.04.020
31. Harman D. Aging: a theory based on free radical and radiation chemistry. *J Gerontol* (1956) **11**(3):298–300. doi:10.1093/geronj/11.3.298
32. Halliwell B, Gutteridge JMC. *Free Radicals in Biology and Medicine*. 4th ed. (Vol. xxxvi). Oxford: Oxford University Press (2007). 851 p.
33. Rhee SG, Woo HA. Multiple functions of peroxiredoxins: peroxidases, sensors and regulators of the intracellular messenger H(2)O(2), and protein chaperones. *Antioxid Redox Signal* (2011) **15**(3):781–94. doi:10.1089/ars.2010.3393
34. Jiang F, Zhang Y, Dusting GJ. NADPH oxidase-mediated redox signaling: roles in cellular stress response, stress tolerance, and tissue repair. *Pharmacol Rev* (2011) **63**(1):218–42. doi:10.1124/pr.110.002980
35. D'Autreaux B, Toledano MB. ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis. *Nat Rev Mol Cell Biol* (2007) **8**(10):813–24. doi:10.1038/nrm2256

36. Chan DW, Liu VW, Tsao GS, Yao KM, Furukawa T, Chan KK, et al. Loss of MKP3 mediated by oxidative stress enhances tumorigenicity and chemoresistance of ovarian cancer cells. *Carcinogenesis* (2008) **29**(9):1742–50. doi:10.1093/carcin/bgn167
37. McCubrey JA, Steelman LS, Chappell WH, Abrams SL, Wong EW, Chang F, et al. Roles of the Raf/MEK/ERK pathway in cell growth, malignant transformation and drug resistance. *Biochim Biophys Acta* (2007) **1773**(8):1263–84. doi:10.1016/j.bbamcr.2006.10.001
38. Liu LZ, Hu XW, Xia C, He J, Zhou Q, Shi X, et al. Reactive oxygen species regulate epidermal growth factor-induced vascular endothelial growth factor and hypoxia-inducible factor-1alpha expression through activation of AKT and P70S6K1 in human ovarian cancer cells. *Free Radic Biol Med* (2006) **41**(10):1521–33. doi:10.1016/j.freeradbiomed.2006.08.003
39. Higaki Y, Mikami T, Fujii N, Hirshman MF, Koyama K, Seino T, et al. Oxidative stress stimulates skeletal muscle glucose uptake through a phosphatidylinositol 3-kinase-dependent pathway. *Am J Physiol Endocrinol Metab* (2008) **294**(5):E889–97. doi:10.1152/ajpendo.00150.2007
40. Li Q, Engelhardt JF. Interleukin-1beta induction of NFkappaB is partially regulated by H2O2-mediated activation of NFkappaB-inducing kinase. *J Biol Chem* (2006) **281**(3):1495–505. doi:10.1074/jbc.M511153200
41. Wang Y, Huang X, Cang H, Gao F, Yamamoto T, Osaki T, et al. The endogenous reactive oxygen species promote NF-kappaB activation by targeting on activation of NF-kappaB-inducing kinase in oral squamous carcinoma cells. *Free Radic Res* (2007) **41**(9):963–71. doi:10.1080/10715760701445045
42. Ruiz-Ramos R, Lopez-Carrillo L, Rios-Perez AD, De Vizcaya-Ruíz A, Cebrian ME. Sodium arsenite induces ROS generation, DNA oxidative damage, HO-1 and c-Myc proteins, NF-kappaB activation and cell proliferation in human breast cancer MCF-7 cells. *Mutat Res* (2009) **674**(1–2):109–15. doi:10.1016/j.mrgentox.2008.09.021
43. Dolado I, Swat A, Ajenjo N, De Vita G, Cuadrado A, Nebreda AR. p38alpha MAP kinase as a sensor of reactive oxygen species in tumorigenesis. *Cancer Cell* (2007) **11**(2):191–205. doi:10.1016/j.ccr.2006.12.013
44. Ushio-Fukai M, Alexander RW, Akers M, Griendling KK. p38 mitogen-activated protein kinase is a critical component of the redox-sensitive signaling pathways activated by angiotensin II. Role in vascular smooth muscle cell hypertrophy. *J Biol Chem* (1998) **273**(24):15022–9. doi:10.1074/jbc.273.24.15022
45. Ray PD, Huang BW, Tsuji Y. Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. *Cell Signal* (2012) **24**(5):981–90. doi:10.1016/j.cellsig.2012.01.008
46. Liou GY, Storz P. Reactive oxygen species in cancer. *Free Radic Res* (2010) **44**(5):479–96. doi:10.3109/10715761003667554
47. Barazzoni R, Zanetti M, Gortan Cappellari G, Semolic A, Boschelle M, Codarin E, et al. Fatty acids acutely enhance insulin-induced oxidative stress and cause insulin resistance by increasing mitochondrial reactive oxygen species (ROS) generation and nuclear factor-kappaB inhibitor (IkappaB)-nuclear factor-kappaB (NFkappaB) activation in rat muscle, in the absence of mitochondrial dysfunction. *Diabetologia* (2012) **55**(3):773–82. doi:10.1007/s00125-011-2396-x
48. Dozio E, Vianello E, Briganti S, Fink B, Malavazos AE, Scognamiglio ET, et al. Increased reactive oxygen species production in epicardial adipose tissues from coronary artery disease patients is associated with brown-to-white adipocyte trans-differentiation. *Int J Cardiol* (2014) **174**(2):413–4. doi:10.1016/j.ijcard.2014.04.045
49. Gómez-Hernández A, Otero YF, de las Heras N, Escribano O, Cachofeiro V, Lahera V, et al. Brown fat lipoatrophy and increased visceral adiposity through a concerted adipocytokines overexpression induces vascular insulin resistance and dysfunction. *Endocrinology* (2012) **153**(3):1242–55. doi:10.1210/en.2011-1765
50. Ristow M. Unraveling the truth about antioxidants: mitohormesis explains ROS-induced health benefits. *Nat Med* (2014) **20**(7):709–11. doi:10.1038/nm.3624
51. Tapia PC. Sublethal mitochondrial stress with an attendant stoichiometric augmentation of reactive oxygen species may precipitate many of the beneficial alterations in cellular physiology produced by caloric restriction, intermittent fasting, exercise and dietary phytonutrients: "mitohormesis" for health and vitality. *Med Hypotheses* (2006) **66**(4):832–43. doi:10.1016/j.mehy.2005.09.009
52. Sharma K. Mitochondrial hormesis and diabetic complications. *Diabetes* (2015) **64**(3):663–72. doi:10.2337/db14-0874
53. Tews D, Wabitsch M. Renaissance of brown adipose tissue. *Horm Res Paediatr* (2011) **75**(4):231–9. doi:10.1159/000324806
54. Poher AL, Altirriba J, Veyrat-Durebex C, Rohner-Jeanrenaud F. Brown adipose tissue activity as a target for the treatment of obesity/insulin resistance. *Front Physiol* (2015) **6**:4. doi:10.3389/fphys.2015.00004
55. Enerback S. The origins of brown adipose tissue. *N Engl J Med* (2009) **360**(19):2021–3. doi:10.1056/NEJMcibr0809610
56. Robidoux J, Cao W, Quan H, Daniel KW, Moukdar F, Bai X, et al. Selective activation of mitogen-activated protein (MAP) kinase kinase 3 and p38alpha MAP kinase is essential for cyclic AMP-dependent UCP1 expression in adipocytes. *Mol Cell Biol* (2005) **25**(13):5466–79. doi:10.1128/MCB.25.13.5466-5479.2005
57. Cao W, Medvedev AV, Daniel KW, Collins S. beta-Adrenergic activation of p38 MAP kinase in adipocytes: cAMP induction of the uncoupling protein 1 (UCP1) gene requires p38 MAP kinase. *J Biol Chem* (2001) **276**(29):27077–82. doi:10.1074/jbc.M101049200
58. Cao W, Daniel KW, Robidoux J, Puigserver P, Medvedev AV, Bai X, et al. p38 mitogen-activated protein kinase is the central regulator of cyclic AMP-dependent transcription of the brown fat uncoupling protein 1 gene. *Mol Cell Biol* (2004) **24**(7):3057–67. doi:10.1128/MCB.24.7.3057-3067.2004
59. Silva JE, Rabelo R. Regulation of the uncoupling protein gene expression. *Eur J Endocrinol* (1997) **136**(3):251–64. doi:10.1530/eje.0.1360251
60. Christian M, Parker MG. The engineering of brown fat. *J Mol Cell Biol* (2010) **2**(1):23–5. doi:10.1093/jmcb/mjp035
61. Enerbäck S, Jacobsson A, Simpson EM, Guerra C, Yamashita H, Harper ME, et al. Mice lacking mitochondrial uncoupling protein are cold-sensitive but not obese. *Nature* (1997) **387**(6628):90–4. doi:10.1038/387090a0
62. Marchi S, Giorgi C, Suski JM, Agnoletto C, Bononi A, Bonora M, et al. Mitochondria-ros crosstalk in the control of cell death and aging. *J Signal Transduct* (2012) **2012**:329635. doi:10.1155/2012/329635
63. Wallace DC. A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: a dawn for evolutionary medicine. *Annu Rev Genet* (2005) **39**:359–407. doi:10.1146/annurev.genet.39.110304.095751
64. Lecoultre V, Ravussin E. Brown adipose tissue and aging. *Curr Opin Clin Nutr Metab Care* (2011) **14**(1):1–6. doi:10.1097/MCO.0b013e328341221e
65. Graja A, Schulz TJ. Mechanisms of aging-related impairment of brown adipocyte development and function. *Gerontology* (2015) **61**(3):211–7. doi:10.1159/000366557
66. Shimizu I, Aprahamian T, Kikuchi R, Shimizu A, Papanicolaou KN, MacLauchlan S, et al. Vascular rarefaction mediates whitening of brown fat in obesity. *J Clin Invest* (2014) **124**(5):2099–112. doi:10.1172/JCI71643
67. Mates JM, Sanchez-Jimenez F. Antioxidant enzymes and their implications in pathophysiologic processes. *Front Biosci* (1999) **4**:D339–45. doi:10.2741/Mates
68. Ro SH, Nam M, Jang I, Park HW, Park H, Semple IA, et al. Sestrin2 inhibits uncoupling protein 1 expression through suppressing reactive oxygen species. *Proc Natl Acad Sci U S A* (2014) **111**(21):7849–54. doi:10.1073/pnas.1401787111
69. Fusco D, Colloca G, Lo Monaco MR, Cesari M. Effects of antioxidant supplementation on the aging process. *Clin Interv Aging* (2007) **2**(3):377–87.
70. Bjelakovic G, Nikolova D, Gluud C. Antioxidant supplements and mortality. *Curr Opin Clin Nutr Metab Care* (2014) **17**(1):40–4. doi:10.1097/MCO.0000000000000009
71. Fontana L, Partridge L, Longo VD. Extending healthy life span – from yeast to humans. *Science* (2010) **328**(5976):321–6. doi:10.1126/science.1172539
72. Lin SJ, Kaeberlein M, Andalis AA, Sturtz LA, Defossez PA, Culotta VC, et al. Calorie restriction extends *Saccharomyces cerevisiae* lifespan by increasing respiration. *Nature* (2002) **418**(6895):344–8. doi:10.1038/nature00829
73. Schulz TJ, Zarse K, Voigt A, Urban N, Birringer M, Ristow M. Glucose restriction extends *Caenorhabditis elegans* life span by inducing mitochondrial respiration and increasing oxidative stress. *Cell Metab* (2007) **6**(4):280–93. doi:10.1016/j.cmet.2007.08.011
74. Magwere T, Goodall S, Skepper J, Mair W, Brand MD, Partridge L. The effect of dietary restriction on mitochondrial protein density and flight muscle mitochondrial morphology in *Drosophila*. *J Gerontol A Biol Sci Med Sci* (2006) **61**(1):36–47. doi:10.1093/gerona/61.1.36
75. Piper MD, Skorupa D, Partridge L. Diet, metabolism and lifespan in *Drosophila*. *Exp Gerontol* (2005) **40**(11):857–62. doi:10.1016/j.exger.2005.06.013

76. Masoro EJ. Hormesis and the antiaging action of dietary restriction. *Exp Gerontol* (1998) **33**(1–2):61–6. doi:10.1016/S0531-5565(97)00071-5
77. Agarwal S, Sharma S, Agrawal V, Roy N. Caloric restriction augments ROS defense in *S. cerevisiae*, by a Sir2p independent mechanism. *Free Radic Res* (2005) **39**(1):55–62. doi:10.1080/10715760400022343
78. Sharma PK, Agrawal V, Roy N. Mitochondria-mediated hormetic response in life span extension of calorie-restricted *Saccharomyces cerevisiae*. *Age (Dordr)* (2011) **33**(2):143–54. doi:10.1007/s11357-010-9169-1
79. Zuin A, Carmona M, Morales-Ivorra I, Gabrielli N, Vivancos AP, Ayté J, et al. Lifespan extension by calorie restriction relies on the Sty1 MAP kinase stress pathway. *EMBO J* (2010) **29**(5):981–91. doi:10.1038/emboj.2009.407
80. Mahlke MA, Cortez LA, Ortiz MA, Rodriguez M, Uchida K, Shigenaga MK, et al. The anti-tumor effects of calorie restriction are correlated with reduced oxidative stress in ENU-induced gliomas. *Pathobiol Aging Age Relat Dis* (2011) **1**:7189. doi:10.3402/pba.v1i0.7189
81. Schmeisser S, Priebe S, Groth M, Monajembashi S, Hemmerich P, Guthke R, et al. Neuronal ROS signaling rather than AMPK/sirtuin-mediated energy sensing links dietary restriction to lifespan extension. *Mol Metab* (2013) **2**(2):92–102. doi:10.1016/j.molmet.2013.02.002
82. Zarse K, Schmeisser S, Groth M, Priebe S, Beuster G, Kuhlow D, et al. Impaired insulin/IGF1 signaling extends life span by promoting mitochondrial L-proline catabolism to induce a transient ROS signal. *Cell Metab* (2012) **15**(4):451–65. doi:10.1016/j.cmet.2012.02.013
83. Nazarewicz RR, Ziolkowski W, Vaccaro PS, Ghafourifar P. Effect of short-term ketogenic diet on redox status of human blood. *Rejuvenation Res* (2007) **10**(4):435–40. doi:10.1089/rej.2007.0540
84. Hu FB, Manson JE, Stampfer MJ, Colditz G, Liu S, Solomon CG, et al. Diet, lifestyle, and the risk of type 2 diabetes mellitus in women. *N Engl J Med* (2001) **345**(11):790–7. doi:10.1056/NEJMoa010492
85. Lanza IR, Short DK, Short KR, Raghavakaimal S, Basu R, Joyner MJ, et al. Endurance exercise as a countermeasure for aging. *Diabetes* (2008) **57**(11):2933–42. doi:10.2337/db08-0349
86. Manini TM, Everhart JE, Patel KV, Schoeller DA, Colbert LH, Visser M et al. Daily activity energy expenditure and mortality among older adults. *JAMA* (2006) **296**(2):171–9. doi:10.1001/jama.296.2.171
87. Powers SK, Nelson WB, Hudson MB. Exercise-induced oxidative stress in humans: cause and consequences. *Free Radic Biol Med* (2011) **51**(5):942–50. doi:10.1016/j.freeradbiomed.2010.12.009
88. Warburton DE, Nicol CW, Bredin SS. Health benefits of physical activity: the evidence. *CMAJ* (2006) **174**(6):801–9. doi:10.1503/cmaj.051351
89. Alessio HM, Goldfarb AH. Lipid peroxidation and scavenger enzymes during exercise: adaptive response to training. *J Appl Physiol (1985)* (1988) **64**(4):1333–6.
90. Chevion S, Moran DS, Heled Y, Shani Y, Regev G, Abbou B, et al. Plasma antioxidant status and cell injury after severe physical exercise. *Proc Natl Acad Sci U S A* (2003) **100**(9):5119–23. doi:10.1073/pnas.0831097100
91. Davies KJ, Quintanilha AT, Brooks GA, Packer L. Free radicals and tissue damage produced by exercise. *Biochem Biophys Res Commun* (1982) **107**(4):1198–205. doi:10.1016/S0006-291X(82)80124-1
92. Powers SK, Jackson MJ. Exercise-induced oxidative stress: cellular mechanisms and impact on muscle force production. *Physiol Rev* (2008) **88**(4):1243–76. doi:10.1152/physrev.00031.2007
93. Ristow M, Zarse K. How increased oxidative stress promotes longevity and metabolic health: the concept of mitochondrial hormesis (mitohormesis). *Exp Gerontol* (2010) **45**(6):410–8. doi:10.1016/j.exger.2010.03.014
94. Boveris A, Navarro A. Systemic and mitochondrial adaptive responses to moderate exercise in rodents. *Free Radic Biol Med* (2008) **44**(2):224–9. doi:10.1016/j.freeradbiomed.2007.08.015
95. Crawford DR, Davies KJ. Adaptive response and oxidative stress. *Environ Health Perspect* (1994) **102**(Suppl 10):25–8. doi:10.2307/3432208
96. Davies KJ. Intracellular proteolytic systems may function as secondary antioxidant defenses: an hypothesis. *J Free Radic Biol Med* (1986) **2**(3):155–73. doi:10.1016/S0748-5514(86)80066-6
97. Gomez-Cabrera MC, Domenech E, Vina J. Moderate exercise is an antioxidant: upregulation of antioxidant genes by training. *Free Radic Biol Med* (2008) **44**(2):126–31. doi:10.1016/j.freeradbiomed.2007.02.001
98. Ristow M, Zarse K, Oberbach A, Klöting N, Birringer M, Kiehntopf M, et al. Antioxidants prevent health-promoting effects of physical exercise in humans. *Proc Natl Acad Sci U S A* (2009) **106**(21):8665–70. doi:10.1073/pnas.0903485106
99. Lee SJ, Hwang AB, Kenyon C. Inhibition of respiration extends *C. elegans* life span via reactive oxygen species that increase HIF-1 activity. *Curr Biol* (2010) **20**(23):2131–6. doi:10.1016/j.cub.2010.10.057
100. Hwang AB, Ryu EA, Artan M, Chang HW, Kabir MH, Nam HJ, et al. Feedback regulation via AMPK and HIF-1 mediates ROS-dependent longevity in *Caenorhabditis elegans*. *Proc Natl Acad Sci U S A* (2014) **111**(42):E4458–67. doi:10.1073/pnas.1411199111
101. Budanov AV, Karin M. p53 target genes sestrin1 and sestrin2 connect genotoxic stress and mTOR signaling. *Cell* (2008) **134**(3):451–60. doi:10.1016/j.cell.2008.06.028
102. Sanli T, Linher-Melville K, Tsakiridis T, Singh G. Sestrin2 modulates AMPK subunit expression and its response to ionizing radiation in breast cancer cells. *PLoS One* (2012) **7**(2):e32035. doi:10.1371/journal.pone.0032035
103. Atherton PJ, Babraj J, Smith K, Singh J, Rennie MJ, Wackerhage H. Selective activation of AMPK-PGC-1alpha or PKB-TSC2-mTOR signaling can explain specific adaptive responses to endurance or resistance training-like electrical muscle stimulation. *FASEB J* (2005) **19**(7):786–8. doi:10.1096/fj.04-2179fje
104. Jornayvaz FR, Shulman GI. Regulation of mitochondrial biogenesis. *Essays Biochem* (2010) **47**:69–84. doi:10.1042/bse0470069

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

REVIEW
published: 25 September 2015
doi: 10.3389/fendo.2015.00149

Neuronal control of adaptive thermogenesis

Xiaoyong Yang[1,2,3] and Hai-Bin Ruan[1,2]*

[1] Program in Integrative Cell Signaling and Neurobiology of Metabolism, Yale University School of Medicine, New Haven, CT, USA, [2] Section of Comparative Medicine, Yale University School of Medicine, New Haven, CT, USA, [3] Department of Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, CT, USA

The obesity epidemic continues rising as a global health challenge, despite the increasing public awareness and the use of lifestyle and medical interventions. The biomedical community is urged to develop new treatments to obesity. Excess energy is stored as fat in white adipose tissue (WAT), dysfunction of which lies at the core of obesity and associated metabolic disorders. By contrast, brown adipose tissue (BAT) burns fat and dissipates chemical energy as heat. The development and activation of "brown-like" adipocytes, also known as beige cells, result in WAT browning and thermogenesis. The recent discovery of brown and beige adipocytes in adult humans has sparked the exploration of the development, regulation, and function of these thermogenic adipocytes. The central nervous system drives the sympathetic nerve activity in BAT and WAT to control heat production and energy homeostasis. This review provides an overview of the integration of thermal, hormonal, and nutritional information on hypothalamic circuits in thermoregulation.

Keywords: brown adipose tissue, white adipose tissue, beige fat, thermogenesis, hypothalamus, sympathetic nervous system, Ucp1, obesity

OPEN ACCESS

Edited by:
Jun Wu,
University of Michigan, USA

Reviewed by:
Lei Sun,
Duke-NUS Graduate Medical School, Singapore
Yun-Hee Lee,
Yonsei University, Korea

****Correspondence:***
Xiaoyong Yang,
Section of Comparative Medicine, Yale University School of Medicine, P.O. Box 208016, New Haven, CT 06520-8016, USA
xiaoyong.yang@yale.edu

Specialty section:
This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *28 July 2015*
Accepted: *07 September 2015*
Published: *25 September 2015*

Citation:
Yang X and Ruan H-B (2015) Neuronal control of adaptive thermogenesis. Front. Endocrinol. 6:149. doi: 10.3389/fendo.2015.00149

A Trio of Fat

Obesity is a rising global epidemic. Excess adiposity is a major risk factor for type 2 diabetes, cardiovascular disease, and hypertension. Overweight and obesity arise when energy intake exceeds energy expenditure and consequently excessive calories are stored in the adipose tissue. The adipose organ comprises white adipose tissue (WAT) and brown adipose tissue (BAT). WAT primarily stores energy as triglycerides, whereas BAT dissipates chemical energy as heat, a process mediated by uncoupling protein 1 (UCP1) (1–3). WAT excess and dysfunction lie at the core of obesity and associated metabolic disorders. By contrast, BAT-mediated adaptive thermogenesis serves to maintain body temperature during cold, contributes to fever during infection, as well as counteracts obesity and related metabolic dysfunction (4). Here, adaptive thermogenesis is defined as non-shivering heat production in response to changes in environmental and physiological settings, such as cold, diet, fever, and stress. Adaptive thermogenesis in BAT is believed to be solely dependent on UCP1 (1–6). However, UCP1-independent thermogenesis in other tissues such as WAT has also been suggested (7, 8).

In 2009, metabolically active BAT was "re-discovered" in adult humans (9–13). The activity of BAT in humans responds to cold challenge and is inversely associated with body mass index (BMI) and age (14). Yet in its infancy, increasing the mass and activity of BAT is considered as a therapeutic option for human obesity.

A "brown-like" type of adipocytes, called beige cells, has been recently discovered in specific WAT depots. Although different from classic brown adipocytes in their origin and molecular identity, beige adipocytes express *Ucp1*, contribute to thermoregulation, and prevent metabolic dysfunction in mice (15, 16). The process of recruiting and activating beige adipocytes is referred to as "browning." Although classical brown adipocytes are present in adult humans (9, 17, 18), growing evidence suggests that adult human BAT is mainly composed of beige adipocytes (19–21). Considering the fact that interscapular BAT in human infants consists of *bona fide* brown adipocytes and that BAT depots in newborns and adults are located at different sites (22, 23), we speculate that classic brown adipocytes degenerate whereas beige adipocytes gradually prevail with age in humans. Understanding the molecular mechanisms under this transition is important for future therapeutics designed to boost BAT function. It is also intriguing and important to determine the existence and identity of beige adipocytes in other WAT depots, such as subcutaneous fat in humans.

The potential of brown and beige adipocytes as anti-obesity targets attracts extensive interest. The last decade has seen an explosion in our understanding of the development, regulation, and pathophysiology of these distinct adipocytes. Chronic cold exposure, by stimulating the sympathetic nervous system (SNS), is a major and potent activator of BAT thermogenesis and WAT browning. Agonists to β3-adrenergic receptors (AR) that are selectively expressed in brown and beige adipocytes stimulate thermogenesis in both rodents and humans (24). In addition, numerous intrinsic proteins and secreted factors have been shown to affect the development and function of brown and/or beige adipocytes (15, 16, 25). The role of the central nervous system (CNS) in controlling adipose tissue thermogenesis has been an area of intense investigation (26–31). In this review, we summarize the recent progress in our understanding of the central regulation of thermogenesis in brown and beige cells.

Hypothalamus Orchestrates Metabolism

The hypothalamus acts as to orchestrate homeostatic functions such as food intake, energy expenditure, glucose metabolism, and circadian rhythm. Homeostasis is achieved through the complex crosstalk between the hypothalamus and peripheral tissues in response to environmental cues. The arcuate nucleus (ARC) of the hypothalamus is considered to be a primary integrator of peripheral signals, including hormones and nutrients. Two extensively studied populations of neurons in the ARC are orexigenic neurons expressing agouti-related protein (AgRP)/neuropeptide Y (NPY) and anorexigenic neurons expressing proopiomelanocortin (POMC). These neurons are sensitive, mostly in opposite ways, to hormones such as leptin, insulin, and ghrelin, as well as nutrients such as glucose, amino acids, and fatty acids (32–34). Melanocortin peptides produced by POMC neurons are agonists whereas AgRP is an antagonist of melanocortin-3 and -4 receptors (MC3R and MC4R) that are expressed on the second-order neurons. Located largely in the lateral hypothalamus (LH) and the paraventricular nucleus (PVN) of the hypothalamus, these downstream neurons receive projections from the ARC as well as direct inputs from peripheral signals. Together with the ARC, the LH and PVN function as a metabolic integrator and regulator by projecting to high-order neurons in the CNS and secreting various neuropeptides, for example orexin, melanin concentrating hormone (MCH), cocaine- and amphetamine-regulated transcript (CART), and corticotropin-releasing hormone (CRH).

In addition to their neuroendocrine role, hypothalamic neurons also project to the SNS to control peripheral metabolism (**Figure 1**). Both BAT and WAT are extensively innervated by the sympathetic fibers that can be tracked back to the hypothalamus (35). By using the neurotropic pseudorabies virus (PRV), a number of studies have described the neuroanatomy of the sympathetic control of adipose tissues in rodents. Although BAT and WAT are anatomically and functionally distinct, common brain areas with efferent projections to both adipose tissues have been identified, including the ARC, LH, and PVN of the hypothalamus and other neuronal sites discussed below (35). Release of catecholamine, particularly norepinephrine (NE), from the sympathetic fibers and subsequent activation of β-AR signaling in adipocytes are necessary for the initiation of lipolysis and the activation of thermogenesis (36). It is thus reasonable to speculate that a "command" neural network dictates these two processes in BAT and WAT (35). It should be noted that only a small portion (about 5–15%) of individual neurons in these common brain regions projects both to BAT and WAT and distinct sympathetic circuits project to different WAT depots (35, 36). It is conceivable that the anatomic architecture of neuronal projections to adipose tissues is evolved to allow differential sympathetic drive across fat depots in response to different lipolytic/thermogenic stimuli (37). Generally speaking, sympathetic drive to BAT is more intense than that to WAT depots, demonstrated by more sympathetic nerve endings on adipocytes, higher NE levels and NE turnover rates, and increased expression levels of tyrosine hydroxylase (37–39). Moreover, there are differential sympathetic activities between various WAT depots at the basal, cold-induced, and fasting-induced conditions (37, 39). It will be interesting to determine what factors control such differential effects of the SNS on adipose tissues.

Along with well-characterized leptin resistance and insulin resistance, catecholamine resistance develops in obesity, which is characterized by impaired catecholamine synthesis and/or sensitivity (40–42). Catecholamine-induced lipolysis and thermogenesis are compromised in WAT of obese animals and patients through several proposed mechanisms, including leptin resistance, α2-AR expression, and inflammation (43, 44). Yet the contribution of catecholamine resistance to the defects in thermoregulation awaits future investigation.

The Preoptic Area Integrates Thermal Information

Cold exposure and subsequent SNS activation have a profound effect on brown and beige adipocytes. Upon cold exposure, cutaneous transient receptor potential (TRP) cation channels, e.g. TRPM8, sense skin temperature and transmit signals to primary sensory neurons in the dorsal root ganglia (28). This thermal information is delivered to third-order sensory neurons in the

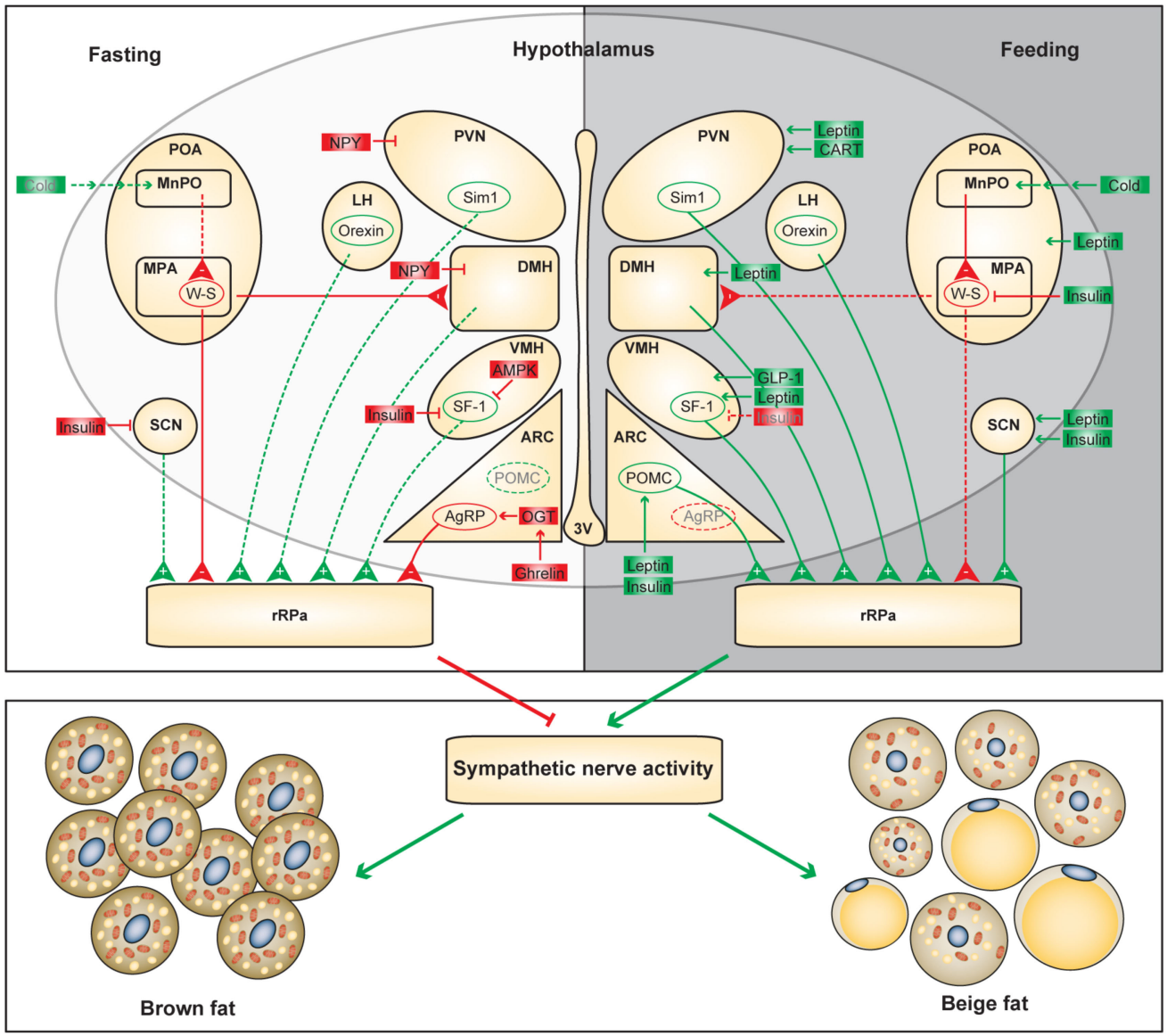


FIGURE 1 | Functional neuroanatomical model of the hypothalamic control of adaptive thermogenesis. Differential regulation of the hypothalamic circuits during fasting and feeding is shown. Positive and negative regulators of the adaptive thermogenesis are shown in green and red, respectively. Dashed lines and gray letters indicate inactive or diminished signals.

lateral parabrachial nucleus (LPB) and then finally to the median preoptic (MnPO) subnucleus of the hypothalamic preoptic area (POA). The medial preoptic area (MPA) of the POA contains warm-sensitive neurons, which receive inhibitory projections from the MnPO (26, 30). Cooling of both distal skin and local hypothalamus reduces the discharge of these warm-sensitive neurons, suggesting that the POA is a central temperature sensor.

Neurons in the POA control SNS activity and thermogenesis by projecting to the dorsomedial hypothalamic area (DMH) and rostral raphe pallidus (rRPa) in the rostral ventromedial medulla, and finally to the sympathetic preganglionic neurons in the intermediolateral nucleus (IML). The neuroanatomical blueprint and neurotransmitters involved in POA regulation of thermogenesis have been reviewed in detail elsewhere (26, 28, 30). Direct cooling of the POA elicits BAT activation (45). Glutamatergic activation of the MnPO and lesions in the inhibitory MPA region both evoke thermogenesis in BAT (46, 47). Conversely, inhibition of MnPO blocks BAT thermogenesis induced by skin cooling (47).

These data demonstrate that the POA integrates thermal information to regulate cold-induced thermogenesis. Febrile responses are also mediated by the POA, which will not be discussed in this review (26, 30). The POA provides efferent signals to WAT depots as well (35); however, roles of the POA in the regulation of WAT browning have not been characterized.

Hypothalamic Hormone Sensing in Thermoregulation

In the late 1970s, seminal experiments performed by Rothwell and Stock demonstrated that cafeteria diet increased the activity of SNS and BAT (48). Diet-induced thermogenesis has since been considered as an important compensatory mechanism that offsets energy surplus. A series of diets such as cafeteria diet and high-fat diet (HFD) induce metabolic inefficiency and BAT recruitment, thus being called as "recruiting diets" (1). Several factors have been proposed to be important for the thermogenic effect of recruiting diets, including the protein-dilution effect, adipocyte-derived leptin, and hypothalamic neurons (1). Parasagittal hypothalamic knife-cuts and medial hypothalamic lesions in rats impaired cafeteria diet-induced thermogenesis in BAT (49, 50). The PVN was later demonstrated as one of the important hypothalamic nuclei mediating diet-induced thermogenesis (51).

In addition, caloric restriction and daily feeding–fasting cycles also control body temperature and thermogenesis in BAT and beige fat (52, 53, 39). Importantly, cold-induced thermogenesis induces and requires food consumption (39, 54). These data suggest that feeding or sufficient nutrient supply provides permissive signals to adaptive thermogenesis. Leptin secreted by adipocytes in proportion to the fat mass and insulin secreted by pancreatic β cells in response to blood glucose have been proposed as these permissive signals.

Preoptic Area

The POA, as a temperature central sensor, is also under the hormonal regulation. Leptin-responsive neurons are abundant in the POA (55). Cold induces the activity of the leptin receptor (LepR)-expressing neurons in the POA, which project to rRPa to regulate sympathetic BAT inputs (56). The insulin receptor is also detectable in the POA. Insulin injection into the POA decreases the firing rate of warm-sensitive neurons, which results in BAT thermogenesis and elevated core body temperature (57). To understand the molecular mechanisms underlying the integration of various thermogenic cues in the POA will be a promising avenue for future research.

Arcuate Nucleus

The ARC is considered as the primary mediator of leptin signaling in the regulation of energy balance and glucose metabolism (33). Injection of leptin into the ARC increases sympathetic drive to BAT (58), and GABAergic RIP-Cre neurons in the ARC may mediate the ability of leptin to stimulate thermogenesis (59). Orexigenic neuropeptides AgRP and NPY inhibit BAT function, while anorexigenic α-MSH increases SNS activity and BAT function (60, 61).

However, it was not known whether neuronal circuits in the ARC also control WAT browning until recently. Acute activation of hunger-promoting AgRP neurons in the hypothalamus suppresses the browning process. O-linked β-*N*-acetylglucosamine (O-GlcNAc) modification of cytoplasmic and nuclear proteins is a nutrient-sensitive pathway (62, 63). The levels of O-GlcNAc modification and O-GlcNAc transferase (OGT) are enriched in AgRP neurons and are elevated by fasting and the hunger hormone Ghrelin (39). Genetic ablation of OGT in AgRP neurons inhibits neuronal excitability, promotes WAT browning, and protects mice against diet-induced obesity and insulin resistance (39). On the other hand, insulin and leptin act synergistically on POMC neurons to promote WAT browning and prevent diet-induced obesity (64). Phosphatases PTP1B and TCPTP attenuate leptin and insulin signaling in POMC neurons, and double knockout of PTP1B and TCPTP in POMC neurons promotes WAT browning (64). These two complimentary stories demonstrate that the hunger and satiety neurons in the ARC control browning of fat depending on the body's energy state. Activation of POMC neurons during caloric intake protects against diet-induced obesity whereas activation of AgRP neurons informs the body to store energy during fasting.

Dorsomedial Hypothalamic Area

Neurons in the DMH receive GABAergic inputs from warm-sensitive neurons in the MPA (28). Derepression of DMH neurons by infusing an antagonist to the $GABA_A$ receptor rapidly and profoundly increases BAT and core body temperature (65, 66), which is dependent on the activation of downstream rRPa neurons (67, 68).

A population of neurons in the DMH expresses the LepR. The intra-DMH injection of leptin increases BAT temperature, in a β3-AR-dependent manner (56, 69). Moreover, blockage of leptin signaling in the DMH blunts the increase in BAT temperature elicited by intraperitoneal injection of leptin (69). Recently, Rezai-Zadeh and colleagues demonstrated that selective activation of DMH neurons by DREADD technique promotes BAT thermogenesis and decreases body weight (70). Conversely, the deficiency of the LepR in the DMH reduces thermogenesis and promotes weight gain (70).

Neuropeptide Y is also expressed by neurons within the DMH, besides the ARC of the hypothalamus. *Npy* expression in the DMH is induced in the conditions where animals demand more energy, for example, chronic food restriction and exercise (71, 72). Similar to that in the ARC, NPY in the DMH promotes food intake and body weight (73). Using viral-mediated knockdown approach in rat, the same group recently showed that DMH knockdown of *Npy* increases BAT activation and the browning of WAT through the SNS (74). Collectively, these data suggest that the DMH is another hypothalamic locus where orexigenic and anorexigenic signals converge to regulate thermogenesis in fat tissue.

Ventromedial Hypothalamic Area

The ventromedial hypothalamic area (VMH) is one of the first hypothalamic sites that have been identified to regulate thermogenesis. Although the anatomical linkage between the VMH and adipose tissue is controversial (35), electrical and glutamate stimulation of the VMH has been extensively shown to activate BAT thermogenesis via the SNS (30, 75, 76). Moreover, genetic manipulation of steroidogenic factor-1 (SF-1) neurons, the major population in the VMH, has also demonstrated the importance of the VMH in thermoregulation (77, 78).

Leptin microinjection into the VMH increases glucose uptake in BAT, although thermogenic effect was not tested in the study (79). Selective deletion of the LepR in SF-1 neurons reduces *Ucp1* expression in BAT, suppresses thermogenesis, and produces obesity (80, 81). It has been suggested that the action of leptin on BAT thermogenesis is preferentially mediated by the PI3K/AKT/FOXOI pathway (82, 83). By contrast, insulin suppresses the firing frequency of SF-1 neurons in the VMH (84). Microinjection of insulin to the VMH suppresses BAT thermogenesis in response to cold and glutamate stimulation (85, 86). More interestingly, the magnitude of the suppression shows diurnal rhythm, which is greater at noon than at night in rats (87).

Fatty acid metabolism is essential for neuronal function (88). AMPK-regulated lipogenesis in the VMH is an important regulator of BAT thermogenesis and controller of body weight (89). Recently, Lopez and colleagues further showed that various hormones, including thyroid hormones, estradiol, and glucagon-like peptide-1 (GLP-1), activate BAT thermogenesis through relieving the suppression on lipogenic genes by AMPK in the VMH (89–91). These studies strongly argue that the VMH senses hormonal cues to regulate BAT function; however, the involvement of the VMH in the regulation of WAT browning has not been explored.

Paraventricular Nucleus

The PVN is strongly susceptible to trans-synaptic infection by RPV from BAT (35); however, its effect on thermogenesis has been controversial. Electrical stimulation of the PVN does not affect BAT function (92). Microinjection of *N*-methyl-D-aspartate (NMDA) or bicuculline to activate neurons in the PVN blocks sympathetic drive to BAT induced by cold, indicating that the PVN negatively regulates thermogenesis (93). Paradoxically, stimulation of the PVN with glutamate is shown to activate BAT thermogenesis (94).

There is a large population of MC4R-expressing neurons in the PVN, and the MC4R agonist melanotan II (MTII) induces BAT thermogenesis (95). Single-minded 1 (Sim1) is necessary for development of the PVN, and ablation of Sim1 neurons reduces *Ucp1* expression, BAT temperature, and energy expenditure (96). Restoration of MC4R in the Sim1 neurons fails to rescue reduced energy expenditure in *Mc4r* knockout mice, suggesting that MC4R elsewhere controls energy expenditure (97). However, a recent study of *Mc4r* deletion in Sim1 neurons showed that Sim1 neurons are important in the regulation of energy expenditure (98).

Leptin regulates the synaptic activity of neurons in the PVN that project to BAT (99). Viral administration of leptin in the PVN stimulates the expression of *Ucp1* in BAT (100). The orexigenic peptide NPY reduces energy expenditure; however, its site of action was not identified until recently (101). Shi et al. demonstrated that ARC-derived NPY decreases tyrosine hydroxinase (TH) expression in the PVN to down-regulate BAT *Ucp1* expression and energy expenditure (61). On the other hand, the anorexigenic peptide CART, when injected in the PVN, induces the *Ucp1* expression in both BAT and WAT (102).

Suprachiasmatic Nucleus

Core body temperature, like all vital aspects of physiology and metabolism, shows circadian rhythm (103). Thermogenic plasticity in BAT is also rhythmic and under the control of cell-autonomous clock machinery (104–107). Directly entrained by light, the suprachiasmatic nucleus (SCN) in the hypothalamus has been established as a circadian pacemaker that synchronizes peripheral clocks across the body to adjust behavior and physiology in accordance with the day/night cycle (108, 109). Both BAT and WAT receive sympathetic flow that can be tracked back to the SCN (35, 110). Glutamate injection to activate the neurons in the SCN stimulates BAT thermogenesis, and this effect is greater during the dark phase (111). Nevertheless, it is still unknown whether the SCN controls the circadian rhythm of thermogenesis and if WAT browning is also under circadian regulation.

LepR-expressing neurons exist in the SCN, and leptin phase-advances the circadian rhythm of SCN on brain slices (112). *In vivo* analysis revealed that leptin modulates clock gene expression in the SCN and the sleep/wake cycle (113). It will be of interest to determine whether leptin in the SCN regulates the circadian rhythm of thermogenesis. In addition, intra-SCN injection of insulin to rats decreases the sympathetic activity on BAT at noon (fasted state) and increases the activity at night (fed state), indicating a circadian control of BAT function by insulin action on the SCN (87). These data suggest that the SCN may receive peripheral satiety cues to modulate the circadian rhythm of energy expenditure to maintain homeostasis.

Psychological Regulation of Thermogenesis

Psychological fever, one of the most common psychological diseases, is characterized by acute or persistent increase in body temperature when patients are psychologically stressed (101). It was suggested that SNS-activated BAT thermogenesis is responsible for stress-induced hyperthermia (114, 115). Orexin neurons in the hypothalamus control multiple physiological processes, including arousal, wakefulness, and appetite. The orexin neurons are considered important for inflammatory fever and the defense against cold (116). It has also been shown that the orexin neurons are indispensable for the stress-induced *Ucp1* expression and thermogenesis in BAT and resultant hyperthermia (117). Recently, a functional neuroanatomical study using an optogenetic approach has identified a DMH-medullary raphe circuit that drives psychological stress-activated BAT thermogenesis and hyperthermia (118).

Environmental enrichment is the stimulation of the brain by physical and social surroundings. This regimen supports neurogenesis and could aid the treatment of neurodegenerative diseases (119). Intriguingly, environmental enrichment in rodents induces WAT browning and decreases adiposity (120). Hypothalamic brain-derived neurotrophic factor (BDNF) has been shown to increase thermogenesis and energy expenditure by acting on neurons in the PVN and VMH (121, 122). Environmental enrichment up-regulates hypothalamic BDNF, and the inhibition

of BDNF blocks the environmental enrichment-induced WAT browning (120).

Summary and Perspective

Yet to be fully defined, the developmental and functional identities of brown and beige adipocytes are distinct (16). Separate populations of neurons in common areas of the hypothalamus projecting to BAT and different WAT depots may contribute to the functional and regulatory differences between fat depots (35). The blueprint of the neuroanatomical regulation in BAT thermogenesis is becoming apparent; however, we just begin to explore the central regulation of the browning process in WAT (39, 64, 123).

It is not surprising that the hypothalamic areas that control food intake also modulate adaptive thermogenesis. In the hunger state, orexigenic neurons attenuate SNS-mediated heat production in brown and beige fat to preserve energy; conversely, in the satiety state, anorexigenic neurons promote energy expenditure to maintain homeostasis (**Figure 1**). However, adaptive thermogenesis is not efficacious to offset long-term energy imbalance, which partially explains the susceptibility to diet-induced obesity and the difficulty of weight loss by dietary interventions in humans. It is important to unravel the biological logic of distinct hypothalamic efferent outputs. In addition to mapping the neuronal circuits controlling thermogenesis in BAT and WAT, there is an urgent need to identify intracellular mechanisms by which neuronal activities are dictated by thermogenic cues. Understanding the molecular and cellular basis of neuronal regulation of adaptive thermogenesis will lay a foundation for future therapeutics against obesity. For example, GLP-1 receptor agonists, which have been widely used to treat diabetes, can be administrated centrally to stimulate brown adipose thermogenesis (90). ARC-specific administration of OGT inhibitors and PTP1B inhibitors suppress AgRP neurons and stimulate POMC neurons, respectively, to promote energy expenditure in brown and beige adipocytes (39, 64).

Hypothalamic circuits respond to both thermal information and nutritional cues to modulate cold- and diet-induced thermogenesis. It has been demonstrated that feeding provides permissive signals for the activation of brown and beige adipocytes upon cold challenge (39, 54). It is conceivable that yet-to-be-found neuronal circuits exist in the hypothalamus serve as an integrator of various thermogenic stimuli. Characterization of such integration sites will help us understand how mammals, including human beings, tradeoff between life-history variables such as hunger and cold during the evolution.

Adaptive thermogenesis is thought to evolve slowly (in days) to adjust to changes in temperature and food availability/composition; however, the activation or silencing of CNS and SNS respond immediately (in minutes) to stimuli, followed by delayed (in hours) changes in *Ucp1* expression. Appropriate tools to manipulate neurons should be utilized to better characterize neuronal networks in thermoregulation. Studies using the chemical/electrical stimulation and anatomical lesions are difficult to control efficacy and often lead to off-target effects. Genetically modified animal models can continue to be used to reveal neuron population-specific function. However, the caution should be taken because genetically modified animals may exhibit developmental defects due to non-specific Cre expression and observed phenotypes are often the results of the long-term and/or secondary effects. Recent advances in optogenetics and chemogenetics produce powerful tools to acutely and precisely control neuronal activity (124). These tools in combination with classical transgenic approaches will accelerate the progress in defining the functional neuronal architecture in thermoregulation.

Acknowledgments

This work was supported by National Institutes of Health R01DK089098, National Institutes of Health P01 DK057751, State of Connecticut Department of Public Health 2014-0139, and Ellison Medical Foundation to XY, and American Heart Association 14SDG20120052 to H-BR.

References

1. Cannon B, Nedergaard J. Brown adipose tissue: function and physiological significance. *Physiol Rev* (2004) **84**:277–359. doi:10.1152/physrev.00015.2003
2. Cinti S. The adipose organ at a glance. *Dis Model Mech* (2012) **5**:588–94. doi:10.1242/dmm.009662
3. Tchkonia T, Thomou T, Zhu Y, Karagiannides I, Pothoulakis C, Jensen MD, et al. Mechanisms and metabolic implications of regional differences among fat depots. *Cell Metab* (2013) **17**:644–56. doi:10.1016/j.cmet.2013.03.008
4. Schulz TJ, Tseng YH. Brown adipose tissue: development, metabolism and beyond. *Biochem J* (2013) **453**:167–78. doi:10.1042/BJ20130457
5. Nedergaard J, Golozoubova V, Matthias A, Asadi A, Jacobsson A, Cannon B. UCP1: the only protein able to mediate adaptive non-shivering thermogenesis and metabolic inefficiency. *Biochim Biophys Acta* (2001) **1504**:82–106. doi:10.1016/S0005-2728(00)00247-4
6. Feldmann HM, Golozoubova V, Cannon B, Nedergaard J. UCP1 ablation induces obesity and abolishes diet-induced thermogenesis in mice exempt from thermal stress by living at thermoneutrality. *Cell Metab* (2009) **9**:203–9. doi:10.1016/j.cmet.2008.12.014
7. Granneman JG, Burnazi M, Zhu Z, Schwamb LA. White adipose tissue contributes to UCP1-independent thermogenesis. *Am J Physiol Endocrinol Metab* (2003) **285**:E1230–6. doi:10.1152/ajpendo.00197.2003
8. Ukropec J, Anunciado RP, Ravussin Y, Hulver MW, Kozak LP. UCP1-independent thermogenesis in white adipose tissue of cold-acclimated Ucp1-/- mice. *J Biol Chem* (2006) **281**:31894–908. doi:10.1074/jbc.M606114200
9. Cypess AM, Lehman S, Williams G, Tal I, Rodman D, Goldfine AB, et al. Identification and importance of brown adipose tissue in adult humans. *N Engl J Med* (2009) **360**:1509–17. doi:10.1056/NEJMoa0810780
10. Saito M, Okamatsu-Ogura Y, Matsushita M, Watanabe K, Yoneshiro T, Nio-Kobayashi J, et al. High incidence of metabolically active brown adipose tissue in healthy adult humans: effects of cold exposure and adiposity. *Diabetes* (2009) **58**:1526–31. doi:10.2337/db09-0530
11. van Marken Lichtenbelt WD, Vanhommerig JW, Smulders NM, Drossaerts JM, Kemerink GJ, Bouvy ND, et al. Cold-activated brown adipose tissue in healthy men. *N Engl J Med* (2009) **360**:1500–8. doi:10.1056/NEJMoa0808718
12. Virtanen KA, Lidell ME, Orava J, Heglind M, Westergren R, Niemi T, et al. Functional brown adipose tissue in healthy adults. *N Engl J Med* (2009) **360**:1518–25. doi:10.1056/NEJMoa0808949
13. Zingaretti MC, Crosta F, Vitali A, Guerrieri M, Frontini A, Cannon B, et al. The presence of UCP1 demonstrates that metabolically active adipose tissue in the neck of adult humans truly represents brown adipose tissue. *FASEB J* (2009) **23**:3113–20. doi:10.1096/fj.09-133546
14. Tam CS, Lecoultre V, Ravussin E. Brown adipose tissue: mechanisms and potential therapeutic targets. *Circulation* (2012) **125**:2782–91. doi:10.1161/CIRCULATIONAHA.111.042929
15. Harms M, Seale P. Brown and beige fat: development, function and therapeutic potential. *Nat Med* (2013) **19**:1252–63. doi:10.1038/nm.3361

16. Wu J, Cohen P, Spiegelman BM. Adaptive thermogenesis in adipocytes: is beige the new brown? *Genes Dev* (2013) **27**:234–50. doi:10.1101/gad.211649.112
17. Cypess AM, White AP, Vernochet C, Schulz TJ, Xue R, Sass CA, et al. Anatomical localization, gene expression profiling and functional characterization of adult human neck brown fat. *Nat Med* (2013) **19**:635–9. doi:10.1038/nm.3112
18. Jespersen NZ, Larsen TJ, Peijs L, Daugaard S, Homoe P, Loft A, et al. A classical brown adipose tissue mRNA signature partly overlaps with brite in the supraclavicular region of adult humans. *Cell Metab* (2013) **17**:798–805. doi:10.1016/j.cmet.2013.04.011
19. Sharp LZ, Shinoda K, Ohno H, Scheel DW, Tomoda E, Ruiz L, et al. Human BAT possesses molecular signatures that resemble beige/brite cells. *PLoS One* (2012) **7**:e49452. doi:10.1371/journal.pone.0049452
20. Wu J, Bostrom P, Sparks LM, Ye L, Choi JH, Giang AH, et al. Beige adipocytes are a distinct type of thermogenic fat cell in mouse and human. *Cell* (2012) **150**:366–76. doi:10.1016/j.cell.2012.05.016
21. Shinoda K, Luijten IH, Hasegawa Y, Hong H, Sonne SB, Kim M, et al. Genetic and functional characterization of clonally derived adult human brown adipocytes. *Nat Med* (2015) **21**:389–94. doi:10.1038/nm.3819
22. van Marken Lichtenbelt W. Human brown fat and obesity: methodological aspects. *Front Endocrinol* (2011) **2**:52. doi:10.3389/fendo.2011.00052
23. Lidell ME, Betz MJ, Dahlqvist Leinhard O, Heglind M, Elander L, Slawik M, et al. Evidence for two types of brown adipose tissue in humans. *Nat Med* (2013) **19**:631–4. doi:10.1038/nm.3017
24. Cypess AM, Weiner LS, Roberts-Toler C, Franquet Elia E, Kessler SH, Kahn PA, et al. Activation of human brown adipose tissue by a beta3-adrenergic receptor agonist. *Cell Metab* (2015) **21**:33–8. doi:10.1016/j.cmet.2014.12.009
25. Sidossis L, Kajimura S. Brown and beige fat in humans: thermogenic adipocytes that control energy and glucose homeostasis. *J Clin Invest* (2015) **125**:478–86. doi:10.1172/JCI78362
26. Morrison SF, Nakamura K. Central neural pathways for thermoregulation. *Front Biosci (Landmark Ed)* (2011) **16**:74–104. doi:10.2741/3677
27. Clapham JC. Central control of thermogenesis. *Neuropharmacology* (2012) **63**:111–23. doi:10.1016/j.neuropharm.2011.10.014
28. Morrison SF, Madden CJ, Tupone D. Central neural regulation of brown adipose tissue thermogenesis and energy expenditure. *Cell Metab* (2014) **19**:741–56. doi:10.1016/j.cmet.2014.02.007
29. Celi FS, Le TN, Ni B. Physiology and relevance of human adaptive thermogenesis response. *Trends Endocrinol Metab* (2015) **26**:238–47. doi:10.1016/j.tem.2015.03.003
30. Contreras C, Gonzalez F, Ferno J, Dieguez C, Rahmouni K, Nogueiras R, et al. The brain and brown fat. *Ann Med* (2015) **47**:150–68. doi:10.3109/07853890.2014.919727
31. Richard D. Cognitive and autonomic determinants of energy homeostasis in obesity. *Nat Rev Endocrinol* (2015) **11**:489–501. doi:10.1038/nrendo.2015.103
32. Lenard NR, Berthoud HR. Central and peripheral regulation of food intake and physical activity: pathways and genes. *Obesity (Silver Spring)* (2008) **16**(Suppl 3):S11–22. doi:10.1038/oby.2008.511
33. Belgardt BF, Okamura T, Bruning JC. Hormone and glucose signalling in POMC and AgRP neurons. *J Physiol* (2009) **587**:5305–14. doi:10.1113/jphysiol.2009.179192
34. Dietrich MO, Horvath TL. Hypothalamic control of energy balance: insights into the role of synaptic plasticity. *Trends Neurosci* (2013) **36**:65–73. doi:10.1016/j.tins.2012.12.005
35. Stefanidis A, Wiedmann NM, Adler ES, Oldfield BJ. Hypothalamic control of adipose tissue. *Best Pract Res Clin Endocrinol Metab* (2014) **28**:685–701. doi:10.1016/j.beem.2014.08.001
36. Bartness TJ, Liu Y, Shrestha YB, Ryu V. Neural innervation of white adipose tissue and the control of lipolysis. *Front Neuroendocrinol* (2014) **35**:473–93. doi:10.1016/j.yfrne.2014.04.001
37. Brito NA, Brito MN, Bartness TJ. Differential sympathetic drive to adipose tissues after food deprivation, cold exposure or glucoprivation. *Am J Physiol Regul Integr Comp Physiol* (2008) **294**:R1445–52. doi:10.1152/ajpregu.00068.2008
38. Trayhurn P, Ashwell M. Control of white and brown adipose tissues by the autonomic nervous system. *Proc Nutr Soc* (1987) **46**:135–42. doi:10.1079/PNS19870017
39. Ruan HB, Dietrich MO, Liu ZW, Zimmer MR, Li MD, Singh JP, et al. O-GlcNAc transferase enables AgRP neurons to suppress browning of white fat. *Cell* (2014) **159**:306–17. doi:10.1016/j.cell.2014.09.010
40. Connacher AA, Bennet WM, Jung RT, Bier DM, Smith CC, Scrimgeour CM, et al. Effect of adrenaline infusion on fatty acid and glucose turnover in lean and obese human subjects in the post-absorptive and fed states. *Clin Sci (Lond)* (1991) **81**:635–44. doi:10.1042/cs0810635
41. Horowitz JF, Klein S. Whole body and abdominal lipolytic sensitivity to epinephrine is suppressed in upper body obese women. *Am J Physiol Endocrinol Metab* (2000) **278**:E1144–52.
42. Jocken JW, Blaak EE. Catecholamine-induced lipolysis in adipose tissue and skeletal muscle in obesity. *Physiol Behav* (2008) **94**:219–30. doi:10.1016/j.physbeh.2008.01.002
43. Yehuda-Shnaidman E, Buehrer B, Pi J, Kumar N, Collins S. Acute stimulation of white adipocyte respiration by PKA-induced lipolysis. *Diabetes* (2010) **59**:2474–83. doi:10.2337/db10-0245
44. Mowers J, Uhm M, Reilly SM, Simon J, Leto D, Chiang SH, et al. Inflammation produces catecholamine resistance in obesity via activation of PDE3B by the protein kinases IKKepsilon and TBK1. *Elife* (2013) **2**:e01119. doi:10.7554/eLife.01119
45. Imai-Matsumura K, Matsumura K, Nakayama T. Involvement of ventromedial hypothalamus in brown adipose tissue thermogenesis induced by preoptic cooling in rats. *Jpn J Physiol* (1984) **34**:939–43. doi:10.2170/jjphysiol.34.939
46. Szymusiak R, Satinoff E. Acute thermoregulatory effects of unilateral electrolytic lesions of the medial and lateral preoptic area in rats. *Physiol Behav* (1982) **28**:161–70. doi:10.1016/0031-9384(82)90118-4
47. Nakamura K, Morrison SF. Preoptic mechanism for cold-defensive responses to skin cooling. *J Physiol* (2008) **586**:2611–20. doi:10.1113/jphysiol.2008.152686
48. Rothwell NJ, Stock MJ. A role for brown adipose tissue in diet-induced thermogenesis. *Nature* (1979) **281**:31–5. doi:10.1038/281031a0
49. Coscina DV, Chambers JW, Park I, Hogan S, Himms-Hagen J. Impaired diet-induced thermogenesis in brown adipose tissue from rats made obese with parasagittal hypothalamic knife-cuts. *Brain Res Bull* (1985) **14**:585–93. doi:10.1016/0361-9230(85)90108-X
50. Hogan S, Himms-Hagen J, Coscina DV. Lack of diet-induced thermogenesis in brown adipose tissue of obese medial hypothalamic-lesioned rats. *Physiol Behav* (1985) **35**:287–94. doi:10.1016/0031-9384(85)90351-8
51. De Luca B, Monda M, Amaro S, Pellicano MP, Cioffi LA. Lack of diet-induced thermogenesis following lesions of paraventricular nucleus in rats. *Physiol Behav* (1989) **46**:685–91. doi:10.1016/0031-9384(89)90352-1
52. Apfelbaum M, Bostsarron J, Lacatis D. Effect of caloric restriction and excessive caloric intake on energy expenditure. *Am J Clin Nutr* (1971) **24**:1405–9.
53. Shibata H, Bukowiecki LJ. Regulatory alterations of daily energy expenditure induced by fasting or overfeeding in unrestrained rats. *J Appl Physiol (1985)* (1987) **63**:465–70.
54. Cannon B, Nedergaard J. Nonshivering thermogenesis and its adequate measurement in metabolic studies. *J Exp Biol* (2011) **214**:242–53. doi:10.1242/jeb.050989
55. Frontini A, Giordano A. Leptin-sensitive neurons in mouse preoptic area express alpha 1A- and alpha 2A-adrenergic receptor isoforms. *Neurosci Lett* (2010) **471**:83–8. doi:10.1016/j.neulet.2010.01.016
56. Zhang Y, Kerman IA, Laque A, Nguyen P, Faouzi M, Louis GW, et al. Leptin-receptor-expressing neurons in the dorsomedial hypothalamus and median preoptic area regulate sympathetic brown adipose tissue circuits. *J Neurosci* (2011) **31**:1873–84. doi:10.1523/JNEUROSCI.3223-10.2011
57. Sanchez-Alavez M, Tabarean IV, Osborn O, Mitsukawa K, Schaefer J, Dubins J, et al. Insulin causes hyperthermia by direct inhibition of warm-sensitive neurons. *Diabetes* (2010) **59**:43–50. doi:10.2337/db09-1128
58. Harlan SM, Morgan DA, Agassandian K, Guo DF, Cassell MD, Sigmund CD, et al. Ablation of the leptin receptor in the hypothalamic arcuate nucleus abrogates leptin-induced sympathetic activation. *Circ Res* (2011) **108**:808–12. doi:10.1161/CIRCRESAHA.111.240226
59. Kong D, Tong Q, Ye C, Koda S, Fuller PM, Krashes MJ, et al. GABAergic RIP-Cre neurons in the arcuate nucleus selectively regulate energy expenditure. *Cell* (2012) **151**:645–57. doi:10.1016/j.cell.2012.09.020

60. Yasuda T, Masaki T, Kakuma T, Yoshimatsu H. Hypothalamic melanocortin system regulates sympathetic nerve activity in brown adipose tissue. *Exp Biol Med (Maywood)* (2004) **229**:235–9.
61. Shi YC, Lau J, Lin Z, Zhang H, Zhai L, Sperk G, et al. Arcuate NPY controls sympathetic output and BAT function via a relay of tyrosine hydroxylase neurons in the PVN. *Cell Metab* (2013) **17**:236–48. doi:10.1016/j.cmet.2013.01.006
62. Hart GW, Housley MP, Slawson C. Cycling of O-linked beta-N-acetylglucosamine on nucleocytoplasmic proteins. *Nature* (2007) **446**:1017–22. doi:10.1038/nature05815
63. Ruan HB, Singh JP, Li MD, Wu J, Yang X. Cracking the O-GlcNAc code in metabolism. *Trends Endocrinol Metab* (2013) **24**:301–9. doi:10.1016/j.tem.2013.02.002
64. Dodd GT, Decherf S, Loh K, Simonds SE, Wiede F, Balland E, et al. Leptin and insulin act on POMC neurons to promote the browning of white fat. *Cell* (2015) **160**:88–104. doi:10.1016/j.cell.2014.12.022
65. Zaretskaia MV, Zaretsky DV, Shekhar A, Dimicco JA. Chemical stimulation of the dorsomedial hypothalamus evokes non-shivering thermogenesis in anesthetized rats. *Brain Res* (2002) **928**:113–25. doi:10.1016/S0006-8993(01)03369-8
66. Dimicco JA, Zaretsky DV. The dorsomedial hypothalamus: a new player in thermoregulation. *Am J Physiol Regul Integr Comp Physiol* (2007) **292**:R47–63. doi:10.1152/ajpregu.00498.2006
67. Cao WH, Fan W, Morrison SF. Medullary pathways mediating specific sympathetic responses to activation of dorsomedial hypothalamus. *Neuroscience* (2004) **126**:229–40. doi:10.1016/j.neuroscience.2004.03.013
68. Cao WH, Morrison SF. Glutamate receptors in the raphe pallidus mediate brown adipose tissue thermogenesis evoked by activation of dorsomedial hypothalamic neurons. *Neuropharmacology* (2006) **51**:426–37. doi:10.1016/j.neuropharm.2006.03.031
69. Enriori PJ, Sinnayah P, Simonds SE, Garcia Rudaz C, Cowley MA. Leptin action in the dorsomedial hypothalamus increases sympathetic tone to brown adipose tissue in spite of systemic leptin resistance. *J Neurosci* (2011) **31**:12189–97. doi:10.1523/JNEUROSCI.2336-11.2011
70. Rezai-Zadeh K, Yu S, Jiang Y, Laque A, Schwartzenburg C, Morrison CD, et al. Leptin receptor neurons in the dorsomedial hypothalamus are key regulators of energy expenditure and body weight, but not food intake. *Mol Metab* (2014) **3**:681–93. doi:10.1016/j.molmet.2014.07.008
71. Bi S, Robinson BM, Moran TH. Acute food deprivation and chronic food restriction differentially affect hypothalamic NPY mRNA expression. *Am J Physiol Regul Integr Comp Physiol* (2003) **285**:R1030–6. doi:10.1152/ajpregu.00734.2002
72. Kawaguchi M, Scott KA, Moran TH, Bi S. Dorsomedial hypothalamic corticotropin-releasing factor mediation of exercise-induced anorexia. *Am J Physiol Regul Integr Comp Physiol* (2005) **288**:R1800–5. doi:10.1152/ajpregu.00805.2004
73. Yang L, Scott KA, Hyun J, Tamashiro KL, Tray N, Moran TH, et al. Role of dorsomedial hypothalamic neuropeptide Y in modulating food intake and energy balance. *J Neurosci* (2009) **29**:179–90. doi:10.1523/JNEUROSCI.4379-08.2009
74. Chao PT, Yang L, Aja S, Moran TH, Bi S. Knockdown of NPY expression in the dorsomedial hypothalamus promotes development of brown adipocytes and prevents diet-induced obesity. *Cell Metab* (2011) **13**:573–83. doi:10.1016/j.cmet.2011.02.019
75. Perkins MN, Rothwell NJ, Stock MJ, Stone TW. Activation of brown adipose tissue thermogenesis by the ventromedial hypothalamus. *Nature* (1981) **289**:401–2. doi:10.1038/289401a0
76. Minokoshi Y, Saito M, Shimazu T. Sympathetic denervation impairs responses of brown adipose tissue to VMH stimulation. *Am J Physiol* (1986) **251**:R1005–8.
77. Musatov S, Chen W, Pfaff DW, Mobbs CV, Yang XJ, Clegg DJ, et al. Silencing of estrogen receptor alpha in the ventromedial nucleus of hypothalamus leads to metabolic syndrome. *Proc Natl Acad Sci U S A* (2007) **104**:2501–6. doi:10.1073/pnas.0610787104
78. Kim KW, Zhao L, Donato J Jr, Kohno D, Xu Y, Elias CF, et al. Steroidogenic factor 1 directs programs regulating diet-induced thermogenesis and leptin action in the ventral medial hypothalamic nucleus. *Proc Natl Acad Sci U S A* (2011) **108**:10673–8. doi:10.1073/pnas.1102364108
79. Minokoshi Y, Haque MS, Shimazu T. Microinjection of leptin into the ventromedial hypothalamus increases glucose uptake in peripheral tissues in rats. *Diabetes* (1999) **48**:287–91. doi:10.2337/diabetes.48.2.287
80. Dhillon H, Zigman JM, Ye C, Lee CE, Mcgovern RA, Tang V, et al. Leptin directly activates SF1 neurons in the VMH, and this action by leptin is required for normal body-weight homeostasis. *Neuron* (2006) **49**:191–203. doi:10.1016/j.neuron.2005.12.021
81. Bingham NC, Anderson KK, Reuter AL, Stallings NR, Parker KL. Selective loss of leptin receptors in the ventromedial hypothalamic nucleus results in increased adiposity and a metabolic syndrome. *Endocrinology* (2008) **149**:2138–48. doi:10.1210/en.2007-1200
82. Xu Y, Hill JW, Fukuda M, Gautron L, Sohn JW, Kim KW, et al. PI3K signaling in the ventromedial hypothalamic nucleus is required for normal energy homeostasis. *Cell Metab* (2010) **12**:88–95. doi:10.1016/j.cmet.2010.05.002
83. Kim KW, Donato J Jr, Berglund ED, Choi YH, Kohno D, Elias CF, et al. FOXO1 in the ventromedial hypothalamus regulates energy balance. *J Clin Invest* (2012) **122**:2578–89. doi:10.1172/JCI62848
84. Klockener T, Hess S, Belgardt BF, Paeger L, Verhagen LA, Husch A, et al. High-fat feeding promotes obesity via insulin receptor/PI3K-dependent inhibition of SF-1 VMH neurons. *Nat Neurosci* (2011) **14**:911–8. doi:10.1038/nn.2847
85. Amir S, Lagiorgia M, Pollock R. Intra-ventromedial hypothalamic injection of insulin suppresses brown fat thermogenesis in the anaesthetized rat. *Brain Res* (1989) **480**:340–3. doi:10.1016/0006-8993(89)90204-7
86. Amir S, Schiavetto A, Pollock R. Insulin co-injection suppresses the thermogenic response to glutamate microinjection into the VMH in rats. *Brain Res* (1990) **527**:326–9. doi:10.1016/0006-8993(90)91153-8
87. Sakaguchi T, Takahashi M, Bray GA. Diurnal changes in sympathetic activity. Relation to food intake and to insulin injected into the ventromedial or suprachiasmatic nucleus. *J Clin Invest* (1988) **82**:282–6. doi:10.1172/JCI113584
88. Panov A, Orynbayeva Z, Vavilin V, Lyakhovich V. Fatty acids in energy metabolism of the central nervous system. *Biomed Res Int* (2014) **2014**:472459. doi:10.1155/2014/472459
89. Lopez M, Varela L, Vazquez MJ, Rodriguez-Cuenca S, Gonzalez CR, Velagapudi VR, et al. Hypothalamic AMPK and fatty acid metabolism mediate thyroid regulation of energy balance. *Nat Med* (2010) **16**:1001–8. doi:10.1038/nm.2207
90. Beiroa D, Imbernon M, Gallego R, Senra A, Herranz D, Villarroya F, et al. GLP-1 agonism stimulates brown adipose tissue thermogenesis and browning through hypothalamic AMPK. *Diabetes* (2014) **63**:3346–58. doi:10.2337/db14-0302
91. Martinez de Morentin PB, Gonzalez-Garcia I, Martins L, Lage R, Fernandez-Mallo D, Martinez-Sanchez N, et al. Estradiol regulates brown adipose tissue thermogenesis via hypothalamic AMPK. *Cell Metab* (2014) **20**:41–53. doi:10.1016/j.cmet.2014.03.031
92. Holt SJ, Wheal HV, York DA. Response of brown adipose tissue to electrical stimulation of hypothalamic centres in intact and adrenalectomized Zucker rats. *Neurosci Lett* (1988) **84**:63–7. doi:10.1016/0304-3940(88)90338-2
93. Madden CJ, Morrison SF. Neurons in the paraventricular nucleus of the hypothalamus inhibit sympathetic outflow to brown adipose tissue. *Am J Physiol Regul Integr Comp Physiol* (2009) **296**:R831–43. doi:10.1152/ajpregu.91007.2008
94. Amir S. Stimulation of the paraventricular nucleus with glutamate activates interscapular brown adipose tissue thermogenesis in rats. *Brain Res* (1990) **508**:152–5. doi:10.1016/0006-8993(90)91129-5
95. Song CK, Vaughan CH, Keen-Rhinehart E, Harris RB, Richard D, Bartness TJ. Melanocortin-4 receptor mRNA expressed in sympathetic outflow neurons to brown adipose tissue: neuroanatomical and functional evidence. *Am J Physiol Regul Integr Comp Physiol* (2008) **295**:R417–28. doi:10.1152/ajpregu.00174.2008
96. Xi D, Gandhi N, Lai M, Kublaoui BM. Ablation of Sim1 neurons causes obesity through hyperphagia and reduced energy expenditure. *PLoS One* (2012) **7**:e36453. doi:10.1371/journal.pone.0036453
97. Balthasar N, Dalgaard LT, Lee CE, Yu J, Funahashi H, Williams T, et al. Divergence of melanocortin pathways in the control of food intake and energy expenditure. *Cell* (2005) **123**:493–505. doi:10.1016/j.cell.2005.08.035
98. Xu Y, Wu Z, Sun H, Zhu Y, Kim ER, Lowell BB, et al. Glutamate mediates the function of melanocortin receptor 4 on Sim1 neurons in body weight regulation. *Cell Metab* (2013) **18**:860–70. doi:10.1016/j.cmet.2013.11.003

99. Jiang Y, Munzberg H, Derbenev A, Zsombok A. Leptin regulates synaptic activity of brown adipose tissue-related pre-sympathetic neurons in the paraventricular nucleus of the mice. *FASEB J* (2015) **29**:1057.1.
100. Bagnasco M, Dube MG, Katz A, Kalra PS, Kalra SP. Leptin expression in hypothalamic PVN reverses dietary obesity and hyperinsulinemia but stimulates ghrelin. *Obes Res* (2003) **11**:1463–70. doi:10.1038/oby.2003.196
101. Oka T, Oka K, Hori T. Mechanisms and mediators of psychological stress-induced rise in core temperature. *Psychosom Med* (2001) **63**:476–86. doi:10.1097/00006842-200105000-00018
102. Wang C, Billington CJ, Levine AS, Kotz CM. Effect of CART in the hypothalamic paraventricular nucleus on feeding and uncoupling protein gene expression. *Neuroreport* (2000) **11**:3251–5. doi:10.1097/00001756-200009280-00040
103. Refinetti R, Menaker M. The circadian rhythm of body temperature. *Physiol Behav* (1992) **51**:613–37. doi:10.1016/0031-9384(92)90188-8
104. Redlin U, Nuesslein B, Schmidt I. Circadian changes of brown adipose tissue thermogenesis in juvenile rats. *Am J Physiol* (1992) **262**:R504–8.
105. Chappuis S, Ripperger JA, Schnell A, Rando G, Jud C, Wahli W, et al. Role of the circadian clock gene per2 in adaptation to cold temperature. *Mol Metab* (2013) **2**:184–93. doi:10.1016/j.molmet.2013.05.002
106. Gerhart-Hines Z, Feng D, Emmett MJ, Everett LJ, Loro E, Briggs ER, et al. The nuclear receptor Rev-erbalpha controls circadian thermogenic plasticity. *Nature* (2013) **503**:410–3. doi:10.1038/nature12642
107. Nam D, Guo B, Chatterjee S, Chen MH, Nelson D, Yechoor VK, et al. The adipocyte clock controls brown adipogenesis through the TGF-beta and BMP signaling pathways. *J Cell Sci* (2015) **128**:1835–47. doi:10.1242/jcs.167643
108. Welsh DK, Takahashi JS, Kay SA. Suprachiasmatic nucleus: cell autonomy and network properties. *Annu Rev Physiol* (2010) **72**:551–77. doi:10.1146/annurev-physiol-021909-135919
109. Partch CL, Green CB, Takahashi JS. Molecular architecture of the mammalian circadian clock. *Trends Cell Biol* (2014) **24**:90–9. doi:10.1016/j.tcb.2013.07.002
110. Bartness TJ, Song CK, Demas GE. SCN efferents to peripheral tissues: implications for biological rhythms. *J Biol Rhythms* (2001) **16**:196–204. doi:10.1177/074873040101600302
111. Amir S, Shizgal P, Rompre PP. Glutamate injection into the suprachiasmatic nucleus stimulates brown fat thermogenesis in the rat. *Brain Res* (1989) **498**:140–4. doi:10.1016/0006-8993(89)90409-5
112. Prosser RA, Bergeron HE. Leptin phase-advances the rat suprachiasmatic circadian clock in vitro. *Neurosci Lett* (2003) **336**:139–42. doi:10.1016/S0304-3940(02)01234-X
113. Mendoza J, Lopez-Lopez C, Revel FG, Jeanneau K, Delerue F, Prinssen E, et al. Dimorphic effects of leptin on the circadian and hypocretinergic systems of mice. *J Neuroendocrinol* (2011) **23**:28–38. doi:10.1111/j.1365-2826.2010.02072.x
114. Hayashida S, Oka T, Mera T, Tsuji S. Repeated social defeat stress induces chronic hyperthermia in rats. *Physiol Behav* (2010) **101**:124–31. doi:10.1016/j.physbeh.2010.04.027
115. Lkhagvasuren B, Nakamura Y, Oka T, Sudo N, Nakamura K. Social defeat stress induces hyperthermia through activation of thermoregulatory sympathetic premotor neurons in the medullary raphe region. *Eur J Neurosci* (2011) **34**:1442–52. doi:10.1111/j.1460-9568.2011.07863.x
116. Takahashi Y, Zhang W, Sameshima K, Kuroki C, Matsumoto A, Sunanaga J, et al. Orexin neurons are indispensable for prostaglandin E2-induced fever and defence against environmental cooling in mice. *J Physiol* (2013) **591**:5623–43. doi:10.1113/jphysiol.2013.261271
117. Zhang W, Sunanaga J, Takahashi Y, Mori T, Sakurai T, Kanmura Y, et al. Orexin neurons are indispensable for stress-induced thermogenesis in mice. *J Physiol* (2010) **588**:4117–29. doi:10.1113/jphysiol.2010.195099
118. Kataoka N, Hioki H, Kaneko T, Nakamura K. Psychological stress activates a dorsomedial hypothalamus-medullary raphe circuit driving brown adipose tissue thermogenesis and hyperthermia. *Cell Metab* (2014) **20**:346–58. doi:10.1016/j.cmet.2014.05.018
119. Baroncelli L, Braschi C, Spolidoro M, Begenisic T, Sale A, Maffei L. Nurturing brain plasticity: impact of environmental enrichment. *Cell Death Differ* (2010) **17**:1092–103. doi:10.1038/cdd.2009.193
120. Cao L, Choi EY, Liu X, Martin A, Wang C, Xu X, et al. White to brown fat phenotypic switch induced by genetic and environmental activation of a hypothalamic-adipocyte axis. *Cell Metab* (2011) **14**:324–38. doi:10.1016/j.cmet.2011.06.020
121. Wang C, Bomberg E, Billington C, Levine A, Kotz CM. Brain-derived neurotrophic factor in the hypothalamic paraventricular nucleus increases energy expenditure by elevating metabolic rate. *Am J Physiol Regul Integr Comp Physiol* (2007) **293**:R992–1002. doi:10.1152/ajpregu.00516.2006
122. Wang C, Bomberg E, Billington CJ, Levine AS, Kotz CM. Brain-derived neurotrophic factor (BDNF) in the hypothalamic ventromedial nucleus increases energy expenditure. *Brain Res* (2010) **1336**:66–77. doi:10.1016/j.brainres.2010.04.013
123. McGlashon JM, Gorecki MC, Kozlowski AE, Thirnbeck CK, Markan KR, Leslie KL, et al. Central serotonergic neurons activate and recruit thermogenic brown and beige fat and regulate glucose and lipid homeostasis. *Cell Metab* (2015) **21**:692–705. doi:10.1016/j.cmet.2015.04.008
124. Aston-Jones G, Deisseroth K. Recent advances in optogenetics and pharmacogenetics. *Brain Res* (2013) **1511**:1–5. doi:10.1016/j.brainres.2013.01.026

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

REVIEW
published: 31 August 2015
doi: 10.3389/fendo.2015.00136

Hypothalamic regulation of brown adipose tissue thermogenesis and energy homeostasis

Wei Zhang and Sheng Bi*

Laboratory of Psychiatry and Behavioral Sciences, Department of Psychiatry and Behavioral Sciences, Johns Hopkins University School of Medicine, Baltimore, MD, USA

Obesity and diabetes are increasing at an alarming rate worldwide, but the strategies for the prevention and treatment of these disorders remain inadequate. Brown adipose tissue (BAT) is important for cold protection by producing heat using lipids and glucose as metabolic fuels. This thermogenic action causes increased energy expenditure and significant lipid/glucose disposal. In addition, BAT in white adipose tissue (WAT) or beige cells have been found and they also exhibit the thermogenic action similar to BAT. These data provide evidence indicating BAT/beige cells as a potential target for combating obesity and diabetes. Recent discoveries of active BAT and beige cells in adult humans have further highlighted this potential. Growing studies have also shown the importance of central nervous system in the control of BAT thermogenesis and WAT browning using animal models. This review is focused on central neural thermoregulation, particularly addressing our current understanding of the importance of hypothalamic neural signaling in the regulation of BAT/beige thermogenesis and energy homeostasis.

Keywords: central nervous system, brown adipose tissue, thermogenesis, energy homeostasis, sympathetic nervous system

OPEN ACCESS

Edited by:
Jun Wu,
University of Michigan, USA

Reviewed by:
Qingchun Tong,
The University of Texas Health Science Center at Houston, USA
Jacob M. McGlashon,
University of Iowa, USA

****Correspondence:***
Wei Zhang,
Laboratory of Psychiatry and Behavioral Sciences, Department of Psychiatry and Behavioral Sciences, Johns Hopkins University School of Medicine, 720 Rutland Avenue, Baltimore, MD 21205, USA
wzhang63@jhmi.edu

Specialty section:
This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *08 June 2015*
Accepted: *17 August 2015*
Published: *31 August 2015*

Citation:
Zhang W and Bi S (2015) Hypothalamic regulation of brown adipose tissue thermogenesis and energy homeostasis. Front. Endocrinol. 6:136. doi: 10.3389/fendo.2015.00136

Introduction

Brown adipose tissue (BAT) is a thermogenic organ that protects the body from cold environment via dissipating chemical energy (lipids and glucose) as heat. For a long period of time, BAT was thought to be only present in certain species of mammals, including rodents, hibernating animals, and newborn humans. Recently, active BAT has been found in adult humans at a cold environment (1–6). These findings have prompted investigation into the potential action of BAT in fighting against obesity and its associated metabolic disorders in humans (2, 4, 7). For instance, recent reports have shown that cold exposure (at 15–16°C for 6 h daily for 10 days or 17°C for 2 h daily for 6 weeks) can recruit human BAT and increase non-shivering thermogenesis (8) and lower body fat mass (9), although whether such effects could last for prolonged periods (months to years) is unclear. Animal studies have shown that subcutaneous transplants of embryonic BAT can reverse type I diabetes in streptozotocin-treated mice (10). The magnitude of BAT thermogenesis depends on the amount of

Abbreviations: ARC, arcuate nucleus; BAT, brown adipose tissue; CNS, central nervous system; DMH, dorsomedial hypothalamus; LH, lateral hypothalamus; POA, preoptic area; PVN, paraventricular hypothalamus; rRPa, rostral raphe pallidus; SNS, sympathetic nervous system; VMH, ventromedial hypothalamus; WAT, white adipose tissue.

recruitment of active BAT (11), which is influenced by various factors (3, 11, 12). Recently, several central neural modulators have been identified to reduce body weight and adiposity through promoting thermogenic activity of BAT and/or browning of white adipose tissue (WAT) to combust energy. This review will focus on the role of central nervous system (CNS), particularly hypothalamic neural signals, in the regulation of BAT thermogenesis and energy homeostasis.

Distribution of BAT

There are two types of brown adipocytes. The classic brown adipocytes, such as the ones that reside in the interscapular region in rodents, are originated from myf5-positive cells (13). The other is the beige cells that are discretely distributed in the WAT, such as inguinal WAT in rodents (14). Beige cells are originated from the lineage of myf5-negative cells and also known as inducible or recruitable brown adipocytes in WAT (13). The amount of classic BAT in mouse strains is genetically invariant, whereas the amount of inducible brown adipocytes in WAT is more variable among strains (15). Thus, the capacity for the potential of BAT against obesogenic influence may also depend upon the genetic variability of inducible BAT in addition to classic BAT. In rodents, BAT has been found in multiple locations, including interscapular, cervical, peri-aortic, pericardiac, perirenal, and axillary regions (16, 17). In humans, BAT exists more abundantly in new born babies corresponding to the high level of non-shivering thermogenesis. The amount of BAT significantly decreases with aging as BAT mass in pediatric populations is about 10-fold higher than that of adult populations (18). Intriguingly and also most importantly, data have shown that BAT is recruitable in adult humans as cold acclimation results in great increases in BAT mass and non-shivering thermogenesis in adult humans who do not possess detectable BAT before treatment (8, 9). Human BAT is primarily distributed in the supraclavicular and neck regions, as well as in paravertebral, mediastinal, para-aortic, and suprarenal areas, but less in the interscapular area (1–5). Adult human BAT appears to contain both functional brown and beige cells. Cypess et al. have reported that although the properties of human neck fat vary substantially between individuals, human brown adipocytes share many similarities with classical rodent BAT (2). Shinoda and colleagues have further reported that adult human BAT possesses the genetic and functional features more similar to those of rodent beige cells (19).

Function of BAT

Brown adipose tissue contains a large number of mitochondria that act as a heat generator through uncoupling protein 1 (UCP1). During BAT activation, UCP1 uncouples the oxidation of fatty acids from ATP synthesis to dissipate energy as heat. This function is very important for small mammals to maintain body temperature, especially during cold environment (11). Fatty acid oxidation (or energy combustion) in BAT could account for a great amount of total energy intake. For instance, a mouse at 5°C will have food intake approximately 3–4 times that at 30°C (20). This enormous effect on energy utilization highlights BAT as an appealing target for the prevention and treatment of obesity and other metabolic disorders.

Brown adipose tissue thermogenesis is predominately governed by the sympathetic nervous system (SNS) via the adrenergic receptor signaling pathways. Upon stimulation, sympathetic nerve releases norepinephrine that binds to β3 adrenergic receptors in the membrane of brown adipocytes to activate a cascade of signaling pathways, leading to increases in fatty acid β-oxidation and heat production (11). Recently, three subtypes of β adrenergic receptors have been found in BAT (21). Data have shown that mice lacking all three β-adrenergic receptors failed to induce BAT thermogenesis and led to rapid drop of core body temperature in response to cold exposure (22), supporting the importance of β-adrenergic receptors in thermogenesis. Particularly, during brown adipocyte development, β1-adrenergic receptors act to regulate proliferation of brown preadipocytes, while β3-adrenergic receptors mainly affect differentiation of mature brown adipocytes through a cAMP-dependent pathway (11, 23). Deficits in either of them alter BAT functions. β1-adrenergic receptor knockout mice develop hypothermia during cold exposure and exhibit impaired interscapular BAT (iBAT) thermogenesis. These mice become more susceptible to diet-induced obesity and fail to develop diet-induced thermogenesis relative to wild-type mice (21). Intriguingly, while cold induces brown adipocyte development in WAT of wild-type mice, this effect is significantly attenuated in β3-adrenergic receptor knockout mice (24), suggesting that β3 adrenergic receptors are also likely to mediate browning of WAT.

We have appreciated that BAT uses lipids as substrates for thermogenesis and activation of BAT promotes oxidative metabolism and heat production, leading to a great increase in energy utilization (11). In support of this view, recent reports have demonstrated an enormous role for BAT in triglyceride and glucose clearance. Bartelt et al. have reported that cold exposure drastically accelerated plasma clearance of triglycerides as a result of increased uptake into BAT of mice, and that in pathophysiological settings, cold exposure corrected hyperlipidemia and improved deleterious effects of insulin resistance using mouse models (25). Human studies have also shown cold-induced activation of oxidative metabolism in BAT coupled with increased uptake of non-esterified fatty acid (26). Fatty acid transport protein 1 (FATP1) likely contributes to the uptake of fatty acids in BAT as FATP1 knockout animals display smaller lipid droplets in BAT and fail to defend their core body temperature at 4°C, despite elevated levels of serum free fatty acid (27). In addition, BAT has been considered as a sink of glucose disposal. Both *in vivo* and *in vitro* studies have suggested that BAT-associated glucose uptake is regulated by norepinephrine and insulin (28–31). BAT transplantation into age- and sex-matched recipient mice increased insulin-stimulated glucose uptake (32). While glucose transporter isoform 4 (GLUT4) mediates insulin-stimulated glucose uptake in BAT in the way similar to WAT and muscle, the mechanism for norepinephrine-mediated glucose uptake is still unclear.

Browning of WAT

Brown adipose tissue in WAT depots was initially reported by Young et al. (33), who observed that cold acclimation led to the accumulation of BAT in the parametrial fat pad. Although the intertransdifferentiation between brown and white adipocytes might be a case in fat browning (34), the identification of beige

(or brite) adipocytes in WAT in both mice and humans (14, 35–37) has significantly advanced our understanding of BAT in WAT. Importantly, increased recruitment of active brown and/or beige cells in WAT has been shown to promote energy utilization/expenditure and improve glucose tolerance and insulin sensitivity. We now appreciate that browning of WAT is under the control of various key transcription factors, such as PGC-1α, C/EBPα, PPARγ, and PRDM16 (38). Growing data have also indicated the importance of CNS in fat browning (39, 40). This review will update our understanding of hypothalamic regulation of BAT thermogenesis and fat browning since the last report showing that knockdown of neuropeptide Y (NPY) in the dorsomedial hypothalamus (DMH) promotes brown adipocyte development in WAT, increases BAT activity, and elevates energy expenditure (41).

Hypothalamic Regulation of BAT Thermogenesis

Although a role for the CNS in the regulation of BAT thermogenesis has long been known (11), neural circuits and chemicals underlying the central thermoregulation remain incompletely understood. Recently developed techniques for brain research have made it possible to unravel the neural mechanisms of the control of BAT thermogenesis. Using pseudorabies virus as a polysynaptic retrograde tracer, several labs have investigated potential brain areas and pathways in the modulation of sympathetic innervation of BAT and WAT. Injection of pseudorabies virus into the iBAT has revealed the sympathetic outputs from the brain to iBAT (42–44). Within the hypothalamus, viral-infected neurons were detected in the paraventricular hypothalamus (PVN), lateral hypothalamus (LH), DMH, arcuate nucleus (ARC), and preoptic area (POA), but very few or absent in the ventromedial hypothalamus (VMH) (42–45). Similarly, viral-infected neurons were found in the PVN, DMH, and POA of animals receiving pseudorabies virus injection into WAT (46). These findings suggest that the descending signals from hypothalamic areas modulate sympathetic innervation of BAT and WAT to affect BAT thermogenesis and WAT browning. The results from functional studies have provided support for this view. Using innovative approaches to manipulate specific gene and/or neuron activities in animal models, we and other investigators have identified distinct roles of hypothalamic peptides and neurons in the control of BAT thermogenesis and WAT browning. The following is primarily focused on the recent understanding of hypothalamic signaling in thermoregulation.

POA and BAT Thermogenesis

The POA is located in the rostral hypothalamus and acts as a coordinate that receives and integrates the inputs of changes in temperature from local brain and periphery system to restore thermal homeostasis in the body (47). The POA contains both warm-sensitive and cold-sensitive neurons that relay the peripheral and central thermal signals (48). The warm-sensitive neurons control both heat production and heat loss through independent pathways (49). Both shivering and non-shivering thermogenesis are affected by the signals from the warm-sensitive neurons (50, 51), which is mainly innervated by GABAergic neurons (52). Two subnucleus are identified in the POA: the median preoptic nucleus (MnPO) and the medial preoptic area (MPO) (53). For instance, inhibition of MnPO neurons completely blocks the activation of BAT thermogenesis (54), while stimulation of MPO neurons by infusion of the α-melanocyte-stimulating hormone analog melanotan II (MTII) into the MPO evokes iBAT thermogenesis (55). The POA is also activated by pyrogens, such as prostaglandin E2 (PGE2) during fever. Ablation of the PGE receptor subtype EP3 in the MnPO dramatically attenuates fever (56). Neuroanatomically, POA neuronal modulation of BAT thermogenesis and body temperature is mediated through DMH and rostral raphe pallidus (rRPa) neurons (57–59). POA neurons project to the DMH where neurons further innervate the rRPa (57, 58), and some of POA neurons also directly project to the rRPa (59). The rRPa contains sympathetic premotor neurons that serve as an important output of brain to regulate the sympathetic activity of iBAT (57–59). In support of this view, a recent report has shown that loss of portions (serotonin neurons) of this descending pathway greatly reduces BAT thermogenesis as well as WAT browning (40). Thus, the system of POA–DMH–rRPa–iBAT thermoregulation exhibits a distinct role in the control of thermal homeostasis.

DMH and BAT Thermogenesis

Earlier investigation of hypothalamic sites in thermogenesis has revealed the importance of the DMH in thermoregulation, especially by showing that the dorsal subregion of the DMH contains neurons that project to the rRPa to affect sympathetic innervation of iBAT to modulate BAT and body core temperature (60, 61). Studies have demonstrated that disinhibition of DMH neurons with the GABA receptor antagonist bicuculline methiodidide (BMI) results in increases in both core body temperature and iBAT temperature and such effects are abolished by systemic pretreatment with propranolol, a β-adrenergic receptor blocker (62). Moreover, thermogenesis evoked by activation of DMH neurons is reversed by injection of glutamate receptor antagonists in the rRPa (63). These data indicate that DMH neurons are tonically inhibited by GABAergic inputs and disinhibition of DMH neurons (resulting in activation of glutamatergic neurons) promotes glutamatergic descending signals to the rRPa to elevate subsequent sympathetic activity to iBAT. Recent studies have further demonstrated a specific role for leptin in thermoregulation through acting on dorsal DMH neurons in mice. Activation of LepRb neurons in the DMH/DHA promotes BAT thermogenesis, and intra-DMH/DHA injections of leptin normalize hypothermia and reduce body weight gain in *ob/ob* mice (64). Furthermore, prolactin-releasing peptide (PrRP) neurons in the DMH have been shown to mediate the thermogenic effect of leptin. Disruption of LepRb selectively in PrRP neurons blocks thermogenic responses to leptin and causes obesity in mice (65). Data have also shown that leptin can directly act on NPY-expressing neurons in the DMH (specifically in the non-compact subregion) in diet-induced obese mice, but it is puzzling that these NPY neurons do not contain LepRb (66). In contrast to mice, lepRb-expressing neurons are found in the ventral subregion of the DMH, but undetectable in the dorsal area in rats (67), implying that the nature of dorsal DMH neurons in modulating BAT thermogenesis likely differs between mice and rats or other species. We have recently found that NPY-expressing neurons in the compact subregion of the DMH play a significant

role in energy homeostasis in rats via affecting both food intake and energy expenditure. Overexpression of NPY in the DMH increases food intake and body weight, leading to obesity and type 2 diabetes in the Otsuka Long Evans Tokushima fatty (OLETF) rats. These phenotypes can be rescued by NPY knockdown in the DMH via adeno-associated virus (AAV)-mediated NPY-specific RNAi (68). Furthermore, DMH NPY knockdown increases iBAT thermogenesis and results in browning of WAT in subcutaneous inguinal fat in Sprague-Dawley rats and prevents diet-induced obesity (41). A neuroanatomical analysis of DMH NPY and LepRb-expressing neurons has revealed that DMH NPY neurons do not contain lepRb and that *Npy* expression in the DMH is not affected by alterations in circulating leptin levels in rats (67), indicating that DMH NPY is not under the control of leptin in rats. Given that the dorsal DMH contains NPY Y1 and Y5 receptors, we speculate that DMH NPY may modulate neuronal signaling in the dorsal DMH neurons to affect BAT thermogenesis.

VMH and BAT Thermogenesis

Several lines of evidence have suggested the involvement of the VMH in BAT thermogenesis. Lesion studies have shown that the rats with VMH lesions fail to respond to both cold and prostaglandin E1 (PGE1)-induced thermogenesis in iBAT (69, 70). However, data from the studies of the effects of electrical or chemical stimulation of the VMH on BAT thermogenesis provide controversial results. Some groups have shown that electrical stimulation of the VMH causes an increase in iBAT thermogenesis (71) and elevates blood flow to the BAT, which is an important contributor to its thermogenesis (72). However, Halvorson and colleagues have found that both electrical and chemical stimulation of the VMH increase iBAT thermogenesis only in rats acclimated to 4°C but not 21°C (73). DiMicco's group has further reported that chemical stimulation of the DMH, but not the PVN and VMH, evokes non-shivering thermogenesis in rats (62).

Despite these discrepancies, recent studies at molecular levels provide support for the role of the VMH in thermoregulation. One report has shown that estradiol inhibits AMP-activated protein kinase (AMPK) through the estrogen receptor α (ERα) in the VMH and results in activation of BAT thermogenesis through the SNS (74). Studies have also shown that stimulation of the glucagon-like peptide-1 (GLP-1) system with GLP-1 analogs in the VMH increases BAT thermogenesis and WAT browning, leading to decreased body weight in a feeding-independent manner (75). Such effects can be normalized by activation of AMPK signaling using viral-mediated constitutive active AMPKα (75). Bone morphogenetic proteins (BMPs) have been found to regulate both differentiation of BAT progenitor cells and physiological function of mature brown adipocytes. BMP8 upregulates expression of UCP1 and other genes associated with mitochondriogenesis and β-oxidation of fatty acids through BMP8b receptors. BMP8b receptors are highly expressed in the VMH (76). BMP8B activate BAT thermogenesis through increasing BAT response to adrenergic stimulation as well as brain sympathetic outflow to BAT via deactivation of hypothalamic AMPKα likely mediated by VMH BMP8b receptors (76). Together, these findings suggest that the VMH also contributes to the regulation of BAT thermogenesis. Since viral-infected neurons are scarcely detected in the VMH of animals receiving pseudorabies virus injection into iBAT as descried above, one possibility for the action of VMH neurons in modulating BAT thermogenesis is through a neuroendocrine reflex system.

ARC and BAT Thermogenesis

The ARC has been well identified as one of the most important hypothalamic nuclei that affect energy homeostasis. It contains both anorexigenic neurons (POMC and CART) and orexigenic neurons (NPY and AGRP). Food restriction causes significantly increased expression of NPY and AgRP in the ARC, while food restriction also decreases adaptive thermogenesis (77), implying ARC NPY and AgRP in thermoregulation. In support of this view, recent studies have found that ARC NPY suppresses sympathetic outflow to iBAT and lowers BAT thermogenesis via Y1 receptor-mediated reduction of tyrosine hydroxylase expression in the PVN (78). These effects are independent of changes in body weight and physical activity (78). Recent evidence has also demonstrated the importance of AgRP neurons in thermoregulation and specifically, data have shown that inactivation of AgRP neurons promotes retroperitoneal WAT browning and protects mice against diet-induced obesity and insulin resistance (79). This effect is mediated by a key enzyme called *O*-linked β-*N*-acetylglucosamine (*O*-GLcNAc) transferase (79). *O*-GLcNAc levels in AgRP neurons are elevated in response to fasting, at a condition when thermogenesis is suppressed, whereas genetic ablation of *O*-GlcNAc transferase in AgRP neurons limits fasting-induced suppression of thermogenesis, suggesting that *O*-GlcNAc signaling in AgRP neurons is essential for suppressing thermogenesis to conserve energy in response to fasting (79). Since NPY and AgRP are co-localized in ARC neurons and data have shown that the PVN mediates the thermogenic effect of ARC NPY (78), ARC AgRP regulation of BAT thermogenesis is also likely to be mediated through the PVN. On the other hand, POMC neurons in the ARC receive the adiposity signals leptin and insulin corresponding to energy status and exhibit an anorectic effect on energy balance. Dodd et al. recently report that a combined action of leptin and insulin on POMC neurons promotes WAT browning and energy expenditure and prevents diet-induced obesity through protein tyrosine phosphatase 1B (PTP1B) and T cell protein tyrosine phosphatase (TCPTP) signaling as deletion of the phosphatases PTP1B and TCPTP enhanced insulin and leptin signaling in POMC neurons (80). Given that ARC POMC and NPY/AgRP neurons are functionally antagonistic, the next logic step would be of interest to determine whether the ARC–PVN neural pathway contributes to this POMC neuron-mediated thermogenic action.

LH and BAT Thermogenesis

The neurochemical characterization of the hypothalamic projections to the BAT in rats identifies the presence of melanin-concentrating hormone (MCH) and orexin (hypocretins) neurons in the caudal aspect of LH (43), implicating MCH and orexin neurons in thermoregulation. In support of this view, MCH knockout mice have elevated levels of UCP1 protein by threefold along with an increase in energy expenditure as compared to control mice (81). The next question would be

important to evaluate whether MCH-containing neurons in the LH contribute to this effect and its underlying neural circuits. Orexin neurons in the LH play a critical role in both arousal and energy balance. A recent study has demonstrated that orexin-null mice displayed impaired BAT differentiation and function, which can be normalized by the injection of orexin to the orexin-null dams (82). Moreover, data have shown that orexin neurons in the perifornical lateral hypothalamus (PeF/LH) are positively correlated with sympathetic outflow to iBAT and BAT thermogenesis (83), although such effects appear unexpected as the orexigenic peptide orexin would produce a negative action on BAT thermogenesis and energy expenditure. Nevertheless, recent evidence indicates the importance of LH signaling in overall thermoregulation.

PVN and BAT Thermogenesis

The PVN plays a critical role in the modulation of energy balance. As discussed above, PVN neurons serve as an important mediator of ARC neural regulation of energy expenditure. Shi and colleagues have found that tyrosine hydroxylase expressing neurons in the PVN mediate ARC NPY-induced decrease in BAT thermogenesis (78). Data from pharmacological studies have also provided support for a role of PVN melanocortin in thermogenesis. Intra-PVN injection of melanocortin receptor agonist MTII significantly increased energy expenditure (oxygen consumption) in mice (84). Song et al. have shown that melanocortin-induced activation of PVN neurons affects sympathetic outflow to iBAT and BAT thermogenesis (85). Acute parenchymal microinjection of MTII into the PVN increases iBAT temperature in hamsters (85). These results indicate that PVN melanocortin affects BAT thermogenesis. By contrast, genetic studies provide mixed results. Using PVN-selected single-minded 1 (Sim1)-Cre transgenic mice, Balthasar et al. have reported that genetic restoration of MC4R expression in the PVN of *Mc4r* null mice reversed hyperphagia, but did not affect energy expenditure (86), whereas Xu and colleagues have found that MC4Rs on Sim1 neurons in the PVN regulate both energy expenditure and food intake (87). Particularly, they have shown that while the restoration of MC4Rs in Sim1 neurons in the PVN dramatically reduced obesity of Mc4r-null mice, selective disruption of glutamate release from these MC4R neurons prevented this reversal effect by affecting both energy expenditure and food intake, further indicating that glutamate mediates the function of MC4Rs on PVN Sim1 neurons in thermoregulation as well as feeding control (87). In addition, previous work has suggested that ARC POMC and NPY/AGRP neurons project to GABA interneurons in the PVN to coordinately regulate GABA release and thereby affect food intake and energy expenditure (84). Xu and colleagues have found that MC4Rs are largely co-localized with vesicular glutamate transporter 2 (VGLUT2), but few with vesicular GABA transporter (VGAT) in PVN Sim1 neurons, suggesting that most MC4R neurons in these PVN regions are glutamatergic (87). Nevertheless, one explanation is that the PVN contains both populations of neurons (glutamatergic and GABAergic) that integrate the inputs of melanocorin agonist and antagonist directly or indirectly to control food intake and energy expenditure.

Hypothalamic Thermoregulation in Coping with Environmental Challenges

Cold-Induced BAT Thermogenesis

Physiologically, when the ambient temperature falls below the thermoneutral zone (cold environment), BAT is activated to produce heat to maintain body temperature in mammals. As discussed above, studies on animal models have shown that the hypothalamus serves as an essential site in this thermoregulation. Particularly, the POA/DMH/rRPa neural system is excited to promote the sympathetic activity in BAT to increase BAT and body temperature in response to cold exposure (47, 61). Using c-Fos as a marker of neuronal activation, other hypothalamic areas, such as the PVN, ARC, and LH, and extra-hypothalamic regions, such as the nucleus of the solitary tract (NTS), have also been identified in the rodent brain in response to cold (59), indicating that cold exposure causes neuronal activation at multiple brain sites, suggesting that these areas may also contribute to thermoregulation due to cold environment. Data have shown that animals with cold exposure become hyperphagic (20), indicating that animals increase their energy intake to meet energy demands derived from increased energy expenditure, i.e., cold exposure actually elevates both thermal sensory (directly) and feeding regulatory (secondarily) neuronal activities. For instance, hypothalamic orexigenic peptides (such as ARC AgRP/NPY) would be elevated while anorectic peptides (such as POMC) would be decreased to promote energy intake to restore energy balance. Thus, although ARC AgRP/NPY and POMC neurons play an important role in the regulation of energy expenditure, these neural systems do not appear to contribute to cold-induced thermogenesis physiologically. Similarly, we have found that knockdown of NPY in the DMH enhanced cold-induced thermogenesis via increased BAT thermogenesis, but NPY gene expression is actually increased in the DMH of intact rats in response to cold exposure. Thus, these data support the view that physiologically, the hypothalamic systems are organized in the way to integrate central and peripheral signals (derived from changes in energy status) to maintain energy homeostasis. Recently, cold exposure has been shown to promote recruitment of active brown and/or beige adipocytes in fat depots of adult humans (1–6). Lean subjects have increased BAT thermogenesis much greater than obesity subjects by exploring to a cold environment, suggesting that loss of active BAT in obese individuals may become a contribute factor to obesity pathologically. Do the hypothalamic neural systems in the overall control of energy balance also go awry in these individuals? In other words, while thinking of cold-induced recruitment of active BAT for fighting against obesity, adding additional activation of SNS outflow to BAT through manipulating hypothalamic neural activities, such as knockdown of DMH NPY, could provide an important route for the effective control of body weight.

Diet-Induced BAT Thermogenesis

Diet-induced thermogenesis in BAT was initially reported in 1979 by Rothwell and Stock who showed that rats fed high energy cafeteria diet (containing high fat and sugar) had increased body core and BAT temperature (88). This effect appears diet selective as the thermogenic capacity of BAT was reduced in rats fed a high

protein, carbohydrate-free diet (89). Similar to the SNS mediation of cold-induced BAT thermogenesis, β-adrenergic receptors are necessary for diet-induced thermogenesis because mice lacking β-adrenergic receptors developed massive obesity that was due entirely to a failure of diet-induced thermogenesis (22). In contrast to well-studied cold-induced thermogenesis (61), the central neuromodulation of diet-induced thermogenesis remains less understood. Feeding studies have shown that signals arisen from peripheral metabolic changes can be relayed to the CNS through vagal afferent signaling pathways during the control of food intake. Recent studies have demonstrated the importance of this vagal signaling in BAT thermogenesis. Blouet and Schwartz reported that duodenal lipid sensing activates vagal afferents to regulate BAT thermogenesis in rats (90). This effect can be blocked by systemic administration of the cholecystokinin (CCK)-1 receptor antagonist or parenchymal administration of the glutamate *N*-methyl-D-aspartate receptor blocker MK-801 directly into the caudomedial nucleus of the solitary tract (NTS), indicating that the CCK–NTS signaling also mediates diet-induced BAT thermogenesis. Intriguingly, peripheral administration of CCK activates neurons in the NTS, as well as within the PVN and the DMH using c-Fos as an activation marker (91), suggesting that neurons in the PVN and the DMH may modulate diet-induced thermogenesis as well as control food intake.

Stress-Induced BAT Thermogenesis

Stress has long been recognized to cause thermogenesis or hyperthermia. Forced immobilization stress results in increased heat production in BAT and this effect is prevented by sympathetic denervation of BAT (92), indicating that stress-induced BAT

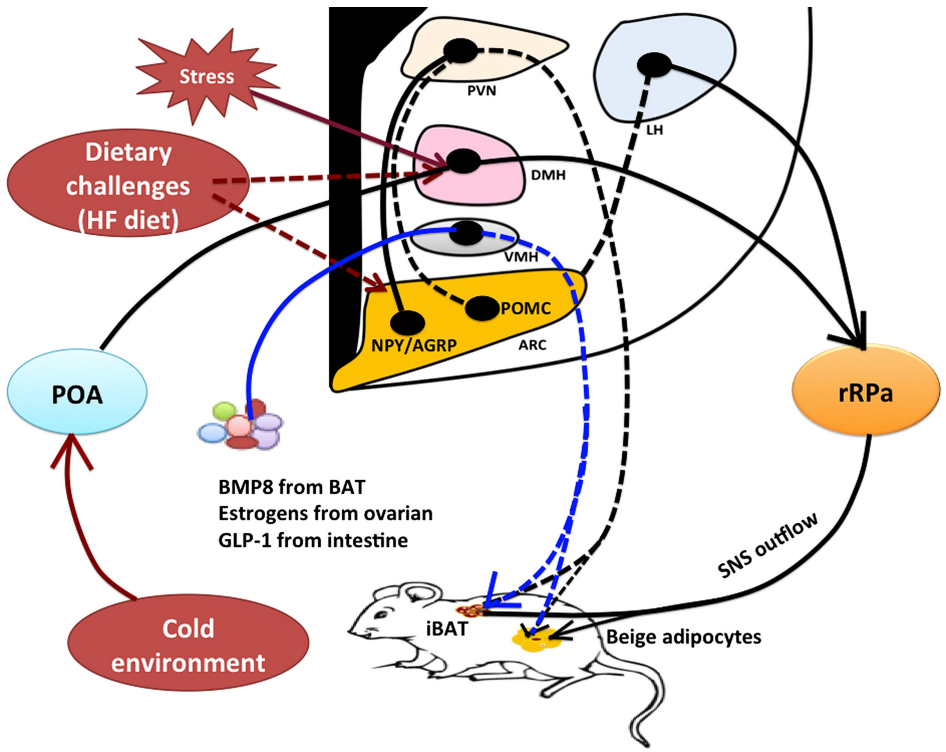


FIGURE 1 | Model of hypothalamic neural regulation of BAT thermogenesis. Within the hypothalamus, the POA contains thermal-sensitive neurons that receive and integrate thermal sensory signals from cold exposure to promote BAT thermogenesis through the POA–DMH–rRPa pathway. The rRPa contains sympathetic premotor neurons that relay central thermal signals from the POA and DMH to affect sympathetic activity of BAT to produce heat. The DMH and rRPa pathway also contributes to the central regulation of stress-induced BAT thermogenesis and hyperthermia. Stress stimulation activates thermoregulatory neurons within the DMH thereby affecting sympathetic input to the rRPa to cause thermogenesis, but the upstream neural pathway from the DMH remains unclear. The hypothalamus contains both orexigenic and anorexigenic neurons, such as NPY neurons in the DMH, NPY/AGRP neurons and POMC neurons in the ARC, and orexin and melanin-concentrating hormone (MCH) neurons in the LH. These neurons are also involved in thermoregulation. Excessive high-fat diet likely results in alterations to these neuronal activities to increase energy expenditure (BAT thermogenesis) in order to restore energy balance, but neural circuits contributing to diet-induced thermogenesis have yet to be well characterized. Furthermore, the hypothalamus can mediate the effects of peripheral signals on BAT thermogenesis likely through a neuroendocrine fashion. For instance, BMP8 from BAT, estrogens from ovaries, and GLP-1 from intestine all have a significant impact on BAT thermogenic action through the VMH. Both classical interscapular brown adipose tissue (iBAT) and beige/brite cells in the white adipose tissue (WAT) are under the control of sympathetic nervous system (SNS). *Solid lines or arrows indicate the known pathways, and dash lines or arrows indicate unknown pathways. BAT, brown adipose tissue; iBAT, interscapular brown adipose tissue; POA, preoptic area; DMH, dorsomedial hypothalamus; rRPa, rostral raphe pallidus; NPY, neuropeptide Y; AGRP, agouti-related peptide; POMC, proopiomelanocortin; BMP8, bone morphogenetic protein 8; GLP-1, glucagon-like peptide-1; PVN, paraventricular hypothalamus; LH, lateral hypothalamus; VMH, ventromedial hypothalamus; ARC, arcuate nucleus; HF diet, high-fat diet.

thermogenesis is mediated through the SNS. Further studies have shown a cross adaptive thermogenesis between cold and stress. Repetitive immobilization stress improves cold tolerance. This improvement is likely through enhancing the capacity of BAT thermogenesis because noradrenaline (NA) turnover of BAT was greatly increased by both stress and cold challenges (93). Despite these observations, the central neural mechanism underlying the effect of stress on BAT thermogenesis has yet to be explored till recently. Given that the DMH is an important site for autonomic responses to stress stimuli (94), Kataoka and colleagues have demonstrated that the DMH–rRPa neural pathway also mediates a psychosocial stress-induced thermogenesis in BAT (95). Inactivation of DMH neurons via muscimol prevents stress-induced increases in BAT and body temperature (95). Within the rRPa, both glutamatergic and serotonergic neurons are involved in psychosocial stress-induced BAT thermogenesis and hyperthermia as injection of glutamate receptor antagonists or a 5-HT_{1A} receptor agonist eliminated these thermogenic effects induced by social defeat stress (95). Moreover, in response to repetitive insertion of a temperature probe into their rectum (handling stress), prepro-orexin knockout mice showed a normal temperature change as compared to that of wild-type littermates (WT), while orexin neuron-ablated mice showed an attenuated response, suggesting that neurotransmitters other than orexin in orexin neurons play an important role in stress-induced non-shivering thermogenesis (96). Furthermore, data have shown that in addition to its feeding effect, NPY plays a pivotal role in modulating various stress responses (97), but a particular role for hypothalamic NPY in the regulation of stress-induced hyperthermia is undetermined. We have recently found that knockdown of NPY in the DMH promotes BAT thermogenesis, elevates energy expenditure, and enhances cold stress response (98). Thus, DMH NPY signaling might also contribute to stress-induced thermogenesis.

Summary and Perspective

The findings of active BAT (brown and beige cells) in adult humans have provided the potential for BAT in combating obesity and associated comorbidities. Using animal models, we now appreciate that the brain regulates sympathetic outflows to BAT to modulate BAT thermogenesis and body temperature. Especially, recent studies have revealed that hypothalamic peptide signaling plays an important role in the control of BAT thermogenesis and WAT browning in rodents (**Figure 1**). Physiologically, the hypothalamic neural system integrates central and peripheral signals of energy status to regulate both food intake and energy expenditure to maintain energy homeostasis. Since obese individuals have lost or have very low levels of active BAT, the critical question in the aspect of the relationship between inactive BAT and hypothalamic thermoregulation under the obesity condition needs to be addressed. For example, how does impaired hypothalamic signaling cause ineffective recruitment of active BAT in obese individuals? Can pathological changes in metabolic syndrome result in dysfunction of hypothalamic control of BAT recruitment? Dysfunctional BAT thermogenesis leads to loss of the capacity of BAT to regulate body weight. Thus, the complete characterization of the physiological and pathological roles of the hypothalamus in the overall control of food intake and energy expenditure will significantly advance our understanding of the hypothalamic-mediated BAT thermoregulation system, promoting the strategies for the better development of pharmaceutical drugs for the treatment of obesity and metabolic disorders.

Acknowledgments

This work was supported by US National Institute of Diabetes and Digestive and Kidney Diseases Grants DK074269 and DK087888.

References

1. Nedergaard J, Bengtsson T, Cannon B. Unexpected evidence for active brown adipose tissue in adult humans. *Am J Physiol Endocrinol Metab* (2007) **293**:E444–52. doi:10.1152/ajpendo.00691.2006
2. Cypess AM, White AP, Vernochet C, Schulz TJ, Xue R, Sass CA, et al. Anatomical localization, gene expression profiling and functional characterization of adult human neck brown fat. *Nat Med* (2013) **19**:635–9. doi:10.1056/NEJMoa0810780
3. Saito M, Okamatsu-Ogura Y, Matsushita M, Watanabe K, Yoneshiro T, Nio-Kobayashi J, et al. High incidence of metabolically active brown adipose tissue in healthy adult humans: effects of cold exposure and adiposity. *Diabetes* (2009) **58**:1526–31. doi:10.2337/db09-0530
4. van Marken Lichtenbelt WD, Vanhommerig JW, Smulders NM, Drossaerts JM, Kemerink GJ, Bouvy ND, et al. Cold-activated brown adipose tissue in healthy men. *N Engl J Med* (2009) **360**:1500–8. doi:10.1056/NEJMoa0808718
5. Virtanen KA, Lidell ME, Orava J, Heglind M, Westergren R, Niemi T, et al. Functional brown adipose tissue in healthy adults. *N Engl J Med* (2009) **360**:1518–25. doi:10.1056/NEJMoa0808949
6. Zingaretti MC, Crosta F, Vitali A, Guerrieri M, Frontini A, Cannon B, et al. The presence of UCP1 demonstrates that metabolically active adipose tissue in the neck of adult humans truly represents brown adipose tissue. *FASEB J* (2009) **23**:3113–20. doi:10.1096/fj.09-133546
7. Timmons JA, Pedersen BK. The importance of brown adipose tissue. *N Engl J Med* (2009) **361**:415–6; author reply 418-421. doi:10.1056/NEJMc091009
8. van der Lans AA, Hoeks J, Brans B, Vijgen GH, Visser MG, Vosselman MJ, et al. Cold acclimation recruits human brown fat and increases nonshivering thermogenesis. *J Clin Invest* (2013) **123**:3395–403. doi:10.1172/JCI68993
9. Yoneshiro T, Aita S, Matsushita M, Kayahara T, Kameya T, Kawai Y, et al. Recruited brown adipose tissue as an antiobesity agent in humans. *J Clin Invest* (2013) **123**:3404–8. doi:10.1172/JCI67803
10. Gunawardana SC, Piston DW. Reversal of type 1 diabetes in mice by brown adipose tissue transplant. *Diabetes* (2012) **61**:674–82. doi:10.2337/db11-0510
11. Cannon B, Nedergaard J. Brown adipose tissue: function and physiological significance. *Physiol Rev* (2004) **84**:277–359. doi:10.1152/physrev.00015.2003
12. Chechi K, Nedergaard J, Richard D. Brown adipose tissue as an anti-obesity tissue in humans. *Obes Rev* (2014) **15**:92–106. doi:10.1111/obr.12116
13. Harms M, Seale P. Brown and beige fat: development, function and therapeutic potential. *Nat Med* (2013) **19**:1252–63. doi:10.1038/nm.3361
14. Wu J, Bostrom P, Sparks LM, Ye L, Choi JH, Giang AH, et al. Beige adipocytes are a distinct type of thermogenic fat cell in mouse and human. *Cell* (2012) **150**:366–76. doi:10.1016/j.cell.2012.05.016
15. Xue B, Rim JS, Hogan JC, Coulter AA, Koza RA, Kozak LP. Genetic variability affects the development of brown adipocytes in white fat but not in interscapular brown fat. *J Lipid Res* (2007) **48**:41–51. doi:10.1194/jlr.M600287-JLR200
16. Giordano A, Frontini A, Castellucci M, Cinti S. Presence and distribution of cholinergic nerves in rat mediastinal brown adipose tissue. *J Histochem Cytochem* (2004) **52**:923–30. doi:10.1369/jhc.3A6246.2004
17. Bastard J-P, Fe`Ve B; Association Francise D'etude Et De Recherche Sur L'obéSité. *Physiology and Physiopathology of Adipose Tissue*. Paris, NY: Springer (2013).
18. Ponrartana S, Hu HH, Gilsanz V. On the relevance of brown adipose tissue in children. *Ann N Y Acad Sci* (2013) **1302**:24–9. doi:10.1111/nyas.12195
19. Shinoda k, Luijten IH, Hasegawa Y, Hong H, Sonne SB, Kim M, et al. Genetic and functional characterization of clonally derived adult human brown adipocytes. *Nat Med* (2015) **21**:389–94.

20. Cannon B, Nedergaard J. Nonshivering thermogenesis and its adequate measurement in metabolic studies. *J Exp Biol* (2011) **214**:242–53. doi:10.1242/jeb.050989
21. Ueta CB, Fernandes GW, Capelo LP, Fonseca TL, Maculan FD, Gouveia CH, et al. beta(1) Adrenergic receptor is key to cold- and diet-induced thermogenesis in mice. *J Endocrinol* (2012) **214**:359–65. doi:10.1530/JOE-12-0155
22. Bachman ES, Dhillon H, Zhang CY, Cinti S, Bianco AC, Kobilka BK, et al. betaAR signaling required for diet-induced thermogenesis and obesity resistance. *Science* (2002) **297**:843–5. doi:10.1126/science.1073160
23. Bronnikov G, Bengtsson T, Kramarova L, Golozoubova V, Cannon B, Nedergaard J. beta1 to beta3 switch in control of cyclic adenosine monophosphate during brown adipocyte development explains distinct beta-adrenoceptor subtype mediation of proliferation and differentiation. *Endocrinology* (1999) **140**:4185–97. doi:10.1210/endo.140.9.6972
24. Jimenez M, Barbatelli G, Allevi R, Cinti S, Seydoux J, Giacobino JP, et al. Beta 3-adrenoceptor knockout in C57BL/6J mice depresses the occurrence of brown adipocytes in white fat. *Eur J Biochem* (2003) **270**:699–705. doi:10.1046/j.1432-1033.2003.03422.x
25. Bartelt A, Bruns OT, Reimer R, Hohenberg H, Ittrich H, Peldschus K, et al. Brown adipose tissue activity controls triglyceride clearance. *Nat Med* (2011) **17**:200–5.
26. Ouellet V, Labbe SM, Blondin DP, Phoenix S, Guerin B, Haman F, et al. Brown adipose tissue oxidative metabolism contributes to energy expenditure during acute cold exposure in humans. *J Clin Invest* (2012) **122**:545–52. doi:10.1172/JCI60433
27. Wu Q, Kazantzis M, Doege H, Ortegon AM, Tsang B, Falcon A, et al. Fatty acid transport protein 1 is required for nonshivering thermogenesis in brown adipose tissue. *Diabetes* (2006) **55**:3229–37. doi:10.2337/db06-0749
28. Cooney GJ, Caterson ID, Newsholme EA. The effect of insulin and noradrenaline on the uptake of 2-[1-14C]deoxyglucose in vivo by brown adipose tissue and other glucose-utilising tissues of the mouse. *FEBS Lett* (1985) **188**:257–61. doi:10.1016/0014-5793(85)80383-5
29. Marette A, Bukowiecki LJ. Stimulation of glucose transport by insulin and norepinephrine in isolated rat brown adipocytes. *Am J Physiol* (1989) **257**:C714–21.
30. Marette A, Bukowiecki LJ. Noradrenaline stimulates glucose transport in rat brown adipocytes by activating thermogenesis. Evidence that fatty acid activation of mitochondrial respiration enhances glucose transport. *Biochem J* (1991) **277**(Pt 1):119–24. doi:10.1042/bj2770119
31. Liu X, Perusse F, Bukowiecki LJ. Chronic norepinephrine infusion stimulates glucose uptake in white and brown adipose tissues. *Am J Physiol* (1994) **266**:R914–20.
32. Stanford KI, Middelbeek RJ, Townsend KL, An D, Nygaard EB, Hitchcox KM, et al. Brown adipose tissue regulates glucose homeostasis and insulin sensitivity. *J Clin Invest* (2013) **123**:215–23. doi:10.1172/JCI62308
33. Young P, Arch JR, Ashwell M. Brown adipose tissue in the parametrial fat pad of the mouse. *FEBS Lett* (1984) **167**:10–4. doi:10.1016/0014-5793(84)80822-4
34. Cinti S. Between brown and white: novel aspects of adipocyte differentiation. *Ann Med* (2011) **43**:104–15. doi:10.3109/07853890.2010.535557
35. Beranger GE, Karbiener M, Barquissau V, Pisani DF, Scheideler M, Langin D, et al. In vitro brown and "brite"/"beige" adipogenesis: human cellular models and molecular aspects. *Biochim Biophys Acta* (2013) **1831**:905–14. doi:10.1016/j.bbalip.2012.11.001
36. Carey AL, Vorlander C, Reddy-Luthmoodoo M, Natoli AK, Formosa MF, Bertovic DA, et al. Reduced UCP-1 content in in vitro differentiated beige/brite adipocytes derived from preadipocytes of human subcutaneous white adipose tissues in obesity. *PLoS One* (2014) **9**:e91997. doi:10.1371/journal.pone.0091997
37. Sidossis L, Kajimura S. Brown and beige fat in humans: thermogenic adipocytes that control energy and glucose homeostasis. *J Clin Invest* (2015) **125**:478–86. doi:10.1172/JCI78362
38. Seale P, Kajimura S, Spiegelman BM. Transcriptional control of brown adipocyte development and physiological function – of mice and men. *Genes Dev* (2009) **23**:788–97. doi:10.1101/gad.1779209
39. Bi S, Li L. Browning of white adipose tissue: role of hypothalamic signaling. *Ann N Y Acad Sci* (2013) **1302**:30–4. doi:10.1111/nyas.12258
40. McGlashon JM, Gorecki MC, Kozlowski AE, Thirnbeck CK, Markan KR, Leslie KL, et al. Central serotonergic neurons activate and recruit thermogenic brown and beige fat and regulate glucose and lipid homeostasis. *Cell Metab* (2015) **21**:692–705. doi:10.1016/j.cmet.2015.04.008
41. Chao PT, Yang L, Aja S, Moran TH, Bi S. Knockdown of NPY expression in the dorsomedial hypothalamus promotes development of brown adipocytes and prevents diet-induced obesity. *Cell Metab* (2011) **13**:573–83. doi:10.1016/j.cmet.2011.02.019
42. Bamshad M, Song CK, Bartness TJ. CNS origins of the sympathetic nervous system outflow to brown adipose tissue. *Am J Physiol* (1999) **276**:R1569–78.
43. Oldfield BJ, Giles ME, Watson A, Anderson C, Colvill LM, Mckinley MJ. The neurochemical characterisation of hypothalamic pathways projecting polysynaptically to brown adipose tissue in the rat. *Neuroscience* (2002) **110**:515–26. doi:10.1016/S0306-4522(01)00555-3
44. Ryu V, Garretson JT, Liu Y, Vaughan CH, Bartness TJ. Brown adipose tissue has sympathetic-sensory feedback circuits. *J Neurosci* (2015) **35**:2181–90. doi:10.1523/JNEUROSCI.3306-14.2015
45. Cao WH, Fan W, Morrison SF. Medullary pathways mediating specific sympathetic responses to activation of dorsomedial hypothalamus. *Neuroscience* (2004) **126**:229–40. doi:10.1016/j.neuroscience.2004.03.013
46. Bamshad M, Aoki VT, Adkison MG, Warren WS, Bartness TJ. Central nervous system origins of the sympathetic nervous system outflow to white adipose tissue. *Am J Physiol* (1998) **275**:R291–9.
47. Boulant JA. Role of the preoptic-anterior hypothalamus in thermoregulation and fever. *Clin Infect Dis* (2000) **31**(Suppl 5):S157–61. doi:10.1086/317521
48. Boulant JA. The effect of firing rate on preoptic neuronal thermosensitivity. *J Physiol* (1974) **240**:661–9. doi:10.1113/jphysiol.1974.sp010628
49. Nagashima K, Nakai S, Tanaka M, Kanosue K. Neuronal circuitries involved in thermoregulation. *Auton Neurosci* (2000) **85**:18–25. doi:10.1016/S1566-0702(00)00216-2
50. Zhang YH, Yanase-Fujiwara M, Hosono T, Kanosue K. Warm and cold signals from the preoptic area: which contribute more to the control of shivering in rats? *J Physiol* (1995) **485**(Pt 1):195–202. doi:10.1113/jphysiol.1995.sp020723
51. Chen XM, Hosono T, Yoda T, Fukuda Y, Kanosue K. Efferent projection from the preoptic area for the control of non-shivering thermogenesis in rats. *J Physiol* (1998) **512**(Pt 3):883–92. doi:10.1111/j.1469-7793.1998.883bd.x
52. Lundius EG, Sanchez-Alavez M, Ghochani Y, Klaus J, Tabarean IV. Histamine influences body temperature by acting at H1 and H3 receptors on distinct populations of preoptic neurons. *J Neurosci* (2010) **30**:4369–81. doi:10.1523/JNEUROSCI.0378-10.2010
53. Morrison SF, Madden CJ, Tupone D. Central control of brown adipose tissue thermogenesis. *Front Endocrinol* (2012) **3**:00005. doi:10.3389/fendo.2012.00005
54. Nakamura K, Morrison SF. A thermosensory pathway that controls body temperature. *Nat Neurosci* (2008) **11**:62–71. doi:10.1038/nn2027
55. Monge-Roffarello B, Labbe SM, Lenglos C, Caron A, Lanfray D, Samson P, et al. The medial preoptic nucleus as a site of the thermogenic and metabolic actions of melanotan II in male rats. *Am J Physiol Regul Integr Comp Physiol* (2014) **307**:R158–66. doi:10.1152/ajpregu.00059.2014
56. Lazarus M, Yoshida K, Coppari R, Bass CE, Mochizuki T, Lowell BB, et al. EP3 prostaglandin receptors in the median preoptic nucleus are critical for fever responses. *Nat Neurosci* (2007) **10**:1131–3. doi:10.1038/nn1949
57. Cano G, Passerin AM, Schiltz JC, Card JP, Morrison SF, Sved AF. Anatomical substrates for the central control of sympathetic outflow to interscapular adipose tissue during cold exposure. *J Comp Neurol* (2003) **460**:303–26. doi:10.1002/cne.10643
58. Morrison SF, Cao WH, Madden CJ. Dorsomedial hypothalamic and brainstem pathways controlling thermogenesis in brown adipose tissue. *J Therm Biol* (2004) **29**:333–7. doi:10.1016/j.jtherbio.2004.08.006
59. Yoshida K, Konishi M, Nagashima K, Saper CB, Kanosue K. Fos activation in hypothalamic neurons during cold or warm exposure: projections to periaqueductal gray matter. *Neuroscience* (2005) **133**:1039–46. doi:10.1016/j.neuroscience.2005.03.044
60. Dimicco JA, Zaretsky DV. The dorsomedial hypothalamus: a new player in thermoregulation. *Am J Physiol Regul Integr Comp Physiol* (2007) **292**:R47–63. doi:10.1152/ajpregu.00498.2006
61. Morrison SF, Madden CJ. Central nervous system regulation of brown adipose tissue. *Compr Physiol* (2014) **4**:1677–713. doi:10.1002/cphy.c140013
62. Zaretskaia MV, Zaretsky DV, Shekhar A, Dimicco JA. Chemical stimulation of the dorsomedial hypothalamus evokes non-shivering

thermogenesis in anesthetized rats. *Brain Res* (2002) **928**:113–25. doi:10.1016/S0006-8993(01)03369-8
63. Cao WH, Morrison SF. Glutamate receptors in the raphe pallidus mediate brown adipose tissue thermogenesis evoked by activation of dorsomedial hypothalamic neurons. *Neuropharmacology* (2006) **51**:426–37. doi:10.1016/j.neuropharm.2006.03.031
64. Rezai-Zadeh K, Yu S, Jiang Y, Laque A, Schwartzenburg C, Morrison CD, et al. Leptin receptor neurons in the dorsomedial hypothalamus are key regulators of energy expenditure and body weight, but not food intake. *Mol Metab* (2014) **3**:681–93. doi:10.1016/j.molmet.2014.07.008
65. Dodd GT, Worth AA, Nunn N, Korpal AK, Bechtold DA, Allison MB, et al. The thermogenic effect of leptin is dependent on a distinct population of prolactin-releasing peptide neurons in the dorsomedial hypothalamus. *Cell Metab* (2014) **20**:639–49. doi:10.1016/j.cmet.2014.07.022
66. Lee SJ, Verma S, Simonds SE, Kirigiti MA, Kievit P, Lindsley SR, et al. Leptin stimulates neuropeptide Y and cocaine amphetamine-regulated transcript coexpressing neuronal activity in the dorsomedial hypothalamus in diet-induced obese mice. *J Neurosci* (2013) **33**:15306–17. doi:10.1523/JNEUROSCI.0837-13.2013
67. Bi S, Robinson BM, Moran TH. Acute food deprivation and chronic food restriction differentially affect hypothalamic NPY mRNA expression. *Am J Physiol Regul Integr Comp Physiol* (2003) **285**:R1030–6. doi:10.1152/ajpregu.00734.2002
68. Yang L, Scott KA, Hyun J, Tamashiro KL, Tray N, Moran TH, et al. Role of dorsomedial hypothalamic neuropeptide Y in modulating food intake and energy balance. *J Neurosci* (2009) **29**:179–90. doi:10.1523/JNEUROSCI.4379-08.2009
69. Niijima A, Rohner-Jeanrenaud F, Jeanrenaud B. Role of ventromedial hypothalamus on sympathetic efferents of brown adipose tissue. *Am J Physiol* (1984) **247**:R650–4.
70. Monda M, Sullo A, De Luca V, Viggiano A, Pellicano MP. Acute lesions of the ventromedial hypothalamus reduce sympathetic activation and thermogenic changes induced by PGE1. *J Physiol Paris* (1997) **91**:285–90. doi:10.1016/S0928-4257(97)82408-4
71. Perkins MN, Rothwell NJ, Stock MJ, Stone TW. Activation of brown adipose tissue thermogenesis by the ventromedial hypothalamus. *Nature* (1981) **289**:401–2. doi:10.1038/289401a0
72. Iwai M, Hell NS, Shimazu T. Effect of ventromedial hypothalamic stimulation on blood flow of brown adipose tissue in rats. *Pflugers Arch* (1987) **410**:44–7. doi:10.1007/BF00581894
73. Halvorson I, Gregor L, Thornhill JA. Brown adipose tissue thermogenesis is activated by electrical and chemical (L-glutamate) stimulation of the ventromedial hypothalamic nucleus in cold-acclimated rats. *Brain Res* (1990) **522**:76–82. doi:10.1016/0006-8993(90)91579-6
74. Martinez de Morentin PB, Gonzalez-Garcia I, Martins L, Lage R, Fernandez-Mallo D, Martinez-Sanchez N, et al. Estradiol regulates brown adipose tissue thermogenesis via hypothalamic AMPK. *Cell Metab* (2014) **20**:41–53. doi:10.1016/j.cmet.2014.03.031
75. Beiroa D, Imbernon M, Gallego R, Senra A, Herranz D, Villarroya F, et al. GLP-1 agonism stimulates brown adipose tissue thermogenesis and browning through hypothalamic AMPK. *Diabetes* (2014) **63**:3346–58. doi:10.2337/db14-0302
76. Whittle AJ, Carobbio S, Martins L, Slawik M, Hondares E, Vazquez MJ, et al. BMP8B increases brown adipose tissue thermogenesis through both central and peripheral actions. *Cell* (2012) **149**:871–85. doi:10.1016/j.cell.2012.02.066
77. Major GC, Doucet E, Trayhurn P, Astrup A, Tremblay A. Clinical significance of adaptive thermogenesis. *Int J Obes (Lond)* (2007) **31**:204–12. doi:10.1038/sj.ijo.0803523
78. Shi YC, Lau J, Lin Z, Zhang H, Zhai L, Sperk G, et al. Arcuate NPY controls sympathetic output and BAT function via a relay of tyrosine hydroxylase neurons in the PVN. *Cell Metab* (2013) **17**:236–48. doi:10.1016/j.cmet.2013.01.006
79. Ruan HB, Dietrich MO, Liu ZW, Zimmer MR, Li MD, Singh JP, et al. O-GlcNAc transferase enables AgRP neurons to suppress browning of white fat. *Cell* (2014) **159**:306–17. doi:10.1016/j.cell.2014.09.010
80. Dodd GT, Decherf S, Loh K, Simonds SE, Wiede F, Balland E, et al. Leptin and insulin act on POMC neurons to promote the browning of white fat. *Cell* (2015) **160**:88–104. doi:10.1016/j.cell.2014.12.022
81. Segal-Lieberman G, Bradley RL, Kokkotou E, Carlson M, Trombly DJ, Wang X, et al. Melanin-concentrating hormone is a critical mediator of the leptin-deficient phenotype. *Proc Natl Acad Sci U S A* (2003) **100**:10085–90. doi:10.1073/pnas.1633636100
82. Sellayah D, Bharaj P, Sikder D. Orexin is required for brown adipose tissue development, differentiation, and function. *Cell Metab* (2011) **14**:478–90. doi:10.1016/j.cmet.2011.08.010
83. Tupone D, Madden CJ, Cano G, Morrison SF. An orexinergic projection from perifornical hypothalamus to raphe pallidus increases rat brown adipose tissue thermogenesis. *J Neurosci* (2011) **31**:15944–55. doi:10.1523/JNEUROSCI.3909-11.2011
84. Cowley MA, Pronchuk N, Fan W, Dinulescu DM, Colmers WF, Cone RD. Integration of NPY, AGRP, and melanocortin signals in the hypothalamic paraventricular nucleus: evidence of a cellular basis for the adipostat. *Neuron* (1999) **24**:155–63. doi:10.1016/S0896-6273(00)80829-6
85. Song CK, Vaughan CH, Keen-Rhinehart E, Harris RB, Richard D, Bartness TJ. Melanocortin-4 receptor mRNA expressed in sympathetic outflow neurons to brown adipose tissue: neuroanatomical and functional evidence. *Am J Physiol Regul Integr Comp Physiol* (2008) **295**:R417–28. doi:10.1152/ajpregu.00174.2008
86. Balthasar N, Dalgaard LT, Lee CE, Yu J, Funahashi H, Williams T, et al. Divergence of melanocortin pathways in the control of food intake and energy expenditure. *Cell* (2005) **123**:493–505. doi:10.1016/j.cell.2005.08.035
87. Xu Y, Wu Z, Sun H, Zhu Y, Kim ER, Lowell BB, et al. Glutamate mediates the function of melanocortin receptor 4 on Sim1 neurons in body weight regulation. *Cell Metab* (2013) **18**:860–70. doi:10.1016/j.cmet.2013.11.003
88. Rothwell NJ, Stock MJ. A role for brown adipose tissue in diet-induced thermogenesis. *Nature* (1979) **281**:31–5. doi:10.1038/281031a0
89. Brito MN, Brito NA, Migliorini RH. Thermogenic capacity of brown adipose tissue is reduced in rats fed a high protein, carbohydrate-free diet. *J Nutr* (1992) **122**:2081–6.
90. Blouet C, Schwartz GJ. Duodenal lipid sensing activates vagal afferents to regulate non-shivering brown fat thermogenesis in rats. *PLoS One* (2012) **7**:e51898. doi:10.1371/journal.pone.0051898
91. Chen J, Scott KA, Zhao Z, Moran TH, Bi S. Characterization of the feeding inhibition and neural activation produced by dorsomedial hypothalamic cholecystokinin administration. *Neuroscience* (2008) **152**:178–88. doi:10.1016/j.neuroscience.2007.12.004
92. Shibata H, Nagasaka T. Contribution of nonshivering thermogenesis to stress-induced hyperthermia in rats. *Jpn J Physiol* (1982) **32**:991–5. doi:10.2170/jjphysiol.32.991
93. Murazumi K. [Effects of cold acclimation and repetitive stress on stress-induced neuroendocrine response]. *Hokkaido Igaku Zasshi* (1988) **63**:85–96.
94. DiMicco JA, Samuels BC, Zaretskaia MV, Zaretsky DV. The dorsomedial hypothalamus and the response to stress: part renaissance, part revolution. *Pharmacol Biochem Behav* (2002) **71**:469–80. doi:10.1016/S0091-3057(01)00689-X
95. Kataoka N, Hioki H, Kaneko T, Nakamura K. Psychological stress activates a dorsomedial hypothalamus-medullary raphe circuit driving brown adipose tissue thermogenesis and hyperthermia. *Cell Metab* (2014) **20**:346–58. doi:10.1016/j.cmet.2014.05.018
96. Zhang W, Sunanaga J, Takahashi Y, Mori T, Sakurai T, Kanmura Y, et al. Orexin neurons are indispensable for stress-induced thermogenesis in mice. *J Physiol* (2010) **588**:4117–29. doi:10.1113/jphysiol.2010.195099
97. Hirsch D, Zukowska Z. NPY and stress 30 years later: the peripheral view. *Cell Mol Neurobiol* (2012) **32**:645–59. doi:10.1007/s10571-011-9793-z
98. Bi S, Kim YJ, Zheng F. Dorsomedial hypothalamic NPY and energy balance control. *Neuropeptides* (2012) **46**:309–14. doi:10.1016/j.npep.2012.09.002

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 03 August 2015
doi: 10.3389/fendo.2015.00116

Hepatic CEACAM1 over-expression protects against diet-induced fibrosis and inflammation in white adipose tissue

*Sumona G. Lester[1,2], Lucia Russo[1,2], Simona S. Ghanem[1,2], Saja S. Khuder[1,2], Anthony M. DeAngelis[1,2], Emily L. Esakov[3], Thomas A. Bowman[1,2], Garrett Heinrich[1,2], Qusai Y. Al-Share[1,2], Marcia F. McInerney[1,3], William M. Philbrick[4] and Sonia M. Najjar[1,2]**

[1] Center for Diabetes and Endocrine Research, College of Medicine and Life Sciences, University of Toledo, Toledo, OH, USA, [2] Department of Physiology and Pharmacology, College of Medicine and Life Sciences, University of Toledo, Toledo, OH, USA, [3] Department of Medicinal and Biological Chemistry, College of Pharmacy and Pharmaceutical Sciences, Toledo, OH, USA, [4] Section of Endocrinology and Metabolism, Department of Internal Medicine, Yale University School of Medicine, New Haven, CT, USA

OPEN ACCESS

Edited by:
Jun Wu,
University of Michigan, USA

Reviewed by:
Zachary Gerhart-Hines,
University of Copenhagen, Denmark
Sandra Kleiner,
Regeneron Pharmaceutical, USA

***Correspondence:**
Sonia M. Najjar,
College of Medicine and Life Sciences, University of Toledo, Health Science Campus, 3000 Arlington Avenue, Mail Stop 1009, Toledo, OH 43614, USA
sonia.najjar@utoledo.edu

Specialty section:
This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology

Received: 19 May 2015
Accepted: 13 July 2015
Published: 03 August 2015

Citation:
Lester SG, Russo L, Ghanem SS, Khuder SS, DeAngelis AM, Esakov EL, Bowman TA, Heinrich G, Al-Share QY, McInerney MF, Philbrick WM and Najjar SM (2015) Hepatic CEACAM1 over-expression protects against diet-induced fibrosis and inflammation in white adipose tissue. Front. Endocrinol. 6:116. doi: 10.3389/fendo.2015.00116

CEACAM1 promotes insulin extraction, an event that occurs mainly in liver. Phenocopying global *Ceacam1* null mice (*Cc1*$^{-/-}$), C57/BL6J mice fed a high-fat (HF) diet exhibited reduced hepatic CEACAM1 levels and impaired insulin clearance, followed by hyperinsulinemia, insulin resistance, and visceral obesity. Conversely, forced liver-specific expression of CEACAM1 protected insulin sensitivity and energy expenditure, and limited gain in total fat mass by HF diet in L-CC1 mice. Because CEACAM1 protein is barely detectable in white adipose tissue (WAT), we herein investigated whether hepatic CEACAM1-dependent insulin clearance pathways regulate adipose tissue biology in response to dietary fat. While HF diet caused a similar body weight gain in L-CC1, this effect was delayed and less intense relative to wild-type (WT) mice. Histological examination revealed less expansion of adipocytes in L-CC1 than WT by HF intake. Immunofluorescence analysis demonstrated a more limited recruitment of crown-like structures, and qRT-PCR analysis showed no significant rise in TNFα mRNA levels in response to HF intake in L-CC1 than WT mice. Unlike WT, HF diet did not activate TGF-β in WAT of L-CC1 mice, as assessed by Western analysis of Smad2/3 phosphorylation. Consistently, HF diet caused relatively less collagen deposition in L-CC1 than WT mice, as shown by Trichrome staining. Coupled with reduced lipid redistribution from liver to visceral fat, lower inflammation and fibrosis could contribute to protected energy expenditure against HF diet in L-CC1 mice. The data underscore the important role of hepatic insulin clearance in the regulation of adipose tissue inflammation and fibrosis.

Keywords: insulin clearance, liver-adipose tissue axis, insulin resistance, steatosis, CEACAM1, fibrosis, inflammation

Abbreviations: α-Sma, alpha-smooth muscle actin; ApoB48/ApoB100, apolipoprotein B; Col6α3; alpha 3 chain of collagen 6-also known as endotrophin; *Cc1*$^{-/-}$, global *Ceacam1* null mouse; CEACAM1, carcinoembryonic antigen-related cell adhesion molecule 1; *Ceacam1*, gene encoding CEACAM1 protein; Fasn, fatty acid synthase; FAT/CD36, fatty acid translocase/cluster of differentiation protein 36; FATP, fatty acid transporter protein; HF, high-calorie high-fat diet; Hsl, hormone-sensitive lipase; L-CC1, mice with liver-specific over-expression of wild-type CEACAM1; Lpl, lipoprotein lipase; L-SACC1, liver-specific S503A CEACAM1 mutant mouse; NEFA, non-esterified fatty acids; RD, regular diet; SREBP-1c, sterol regulatory element-binding protein; TNFα, tumor necrosis factor alpha; TGF-β transforming growth factor beta.

Introduction

Carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) promotes insulin extraction in liver to regulate insulin action. This finding is bolstered by the observation that mice with liver-specific inactivation of CEACAM1 (L-SACC1) or with global null deletion of Ceacam1 gene ($Cc1^{-/-}$) manifest hyperinsulinemia secondary to impaired insulin clearance. This causes insulin resistance in addition to hepatic steatosis (1–3). The latter is mediated by the transcriptional activation of lipogenic genes by sterol regulatory element-binding protein (SREBP-1c) in response to chronically elevated levels of insulin (4). It can also be caused by blunted CEACAM1-mediated downregulation of fatty acid synthase (Fasn) activity under hyperinsulinemic conditions (5).

Both L-SACC1 and $Cc1^{-/-}$ mutant mice also display visceral obesity (1–3). Contributing to this metabolic anomaly, is increased redistribution of hepatic triacylglycerol to white adipose tissue (WAT), in particular that mice are propagated on the C57/BL6J genetic background, which favors redistribution to adipose tissue (6).

We have recently shown that high-fat (HF) intake causes insulin resistance in part by reducing CEACAM1 mRNA and protein levels in liver (7). Conversely, transgenically protecting hepatic CEACAM1 in L-CC1 mice prevented diet-induced insulin resistance and limited hepatic steatosis in response to HF diet (7). Additionally, it blunted the negative effect of HF diet on energy expenditure and spontaneous locomotor activity. This protective effect of hepatic CEACAM1 gain-of-function could be mediated, at least in part, by inducing FGF21, which in turn, enhances fatty acid oxidation in liver (8, 9) and increases brown adipogenesis (10, 11).

L-CC1 transgenic mice with liver-specific gain-of-function also accumulated less total fat mass than their wild-type (WT) counterparts in response to 4 months of HF intake (7). Because CEACAM1 is barely detectable at the protein level in adipose tissue (12), we hypothesized that extra-adipocytic factors brought about by overexpressing CEACAM1 in liver are involved. To test this hypothesis, we investigated whether altered CEACAM1-dependent insulin clearance pathways regulate adipose tissue biology in response to HF diet.

Materials and Methods

Mice Generation

As recently described (7), L-CC1 transgenic mice with liver-specific over-expression of FLAG-tagged WT rat CEACAM1 were generated using human apolipoprotein A1 (APOA1) promoter/enhancer element (13), and propagated on the C57/BL6J background (Jackson Laboratories), as described (7). The "minigene" construct was obtained by subcloning the proximal APOA1 promoter 5′ of a *Ceacam1* rat minigene-containing intron 1 into pCMV-3Tag-3A plasmid at *Not1* site.

Starting at 2 months of age, male mice were fed *ad libitum* either a standard diet (12:66:22% calories from fat:carbohydrate:protein) or a HF diet (45:35:20% calories from fat:carbohydrate:protein) (Catalog #D12451, Research Diets). Mice were kept in a 12-h-dark/light cycle. This study was carried out in accordance with the recommendations of the Institutional Animal Care and Utilization Committee (IACUC), which approved all procedures.

Metabolic Parameters

Mice were fasted overnight and their retro-orbital venous blood drawn at 11:00 hours the following day. Plasma was stored at −80°C until levels of insulin (Linco Research), FFA (NEFA C, Wako), and triacylglycerol (Pointe Scientific Triglyceride, Canton) were assessed. Hepatic triacylglycerol content was measured as previously described (1, 3).

Body Composition

Whole body composition was assessed by nuclear magnetic resonance (NMR; Bruker Optics).

Immunofluorescence of Visceral White Adipose Tissue

Visceral WAT was formalin-fixed, cut into 2–3 mm sections, and transferred to Dulbecco's phosphate buffered saline (PBS, Sigma Aldrich) for 48 h at 4°C. Adipose tissue was permeabilized in 1% Triton X-100 (Fisher) in PBS for 10 min before being stained with rat anti-mouse F4/80 (Invitrogen, Carlsbad) to mark macrophages and detected with donkey anti-rat IgG conjugated to Alexa Fluor488 (Invitrogen). Tissues were incubated with primary antibodies overnight at room temperature (RT) in the dark and washed 3× with staining buffer before being subjected to secondary stain for 2 h and wash (3×) in PBS. Stained samples were then counterstained for 15 min at RT with 5 μM BODIPY 558/568 (Molecular Probes, Inc.) to visualize lipid. Adipose tissue samples were placed directly on a coverslip with buffer and visualized.

Laser-Scanning Confocal Microscopy

Samples were imaged using a Leica TCS SP5 laser-scanning microscope (Leica Microsystems) equipped with conventional solid state and a Ti-sapphire tunable multi-photon laser (Coherent). Images were acquired in the 3D XYZ plane in 2.5 μm steps with a 20× objective (NA 0.70) using the sequential scan mode to eliminate any spectral overlap in the individual fluorophores. Specifically, AlexaFluor488 (Invitrogen) was excited at 488 nm with collection at 500–558 nm. The BODIPY 558/568 dye (Molecular Probes, Inc.) was excited at 561 nm and collected at 567–609 nm. Selected images are a 2D representation of the 3D laser-scanning confocal microscopy (LSCM) image *z*-stack, as labeled.

Gomori's Trichrome Staining

Adipose tissue ($n = 5$ per mouse group) was fixed in 10% formalin and replaced by 70% ethanol before undergoing blocking in paraffin. Sections were deparaffinized at 60°C and hydrated in deionized water. Antigens were unmasked in Bouin's Fluid at 56°C for 45 min. Upon rinsing in deionized water, nuclei were stained with Working Weigert's Iron Hematoxylin at RT for 10 min. Trichrome stain was performed using the Thermo Scientific Richard-Allan Scientific Chromaview-advanced Testing (Cat. No. 87020). In brief, rinsed slides were trichrome stained at RT for 15 min and dehydrated sequentially in 1% acetic acid solution for 1 min, 95% ethanol for 30 s, and 100% ethanol for 1 min (twice). Sections were

then cleared in three changes of clearing reagent for 1 min each and mounted.

Western Analysis

Tissues were lysed and proteins analyzed by SDS-PAGE followed by immunoprobing with polyclonal antibodies against Fasn (α-Fasn) (Assay Designs), fatty acid translocase/cluster of differentiation protein 36 (FAT/CD36) (Santa Cruz Biotechnology), apolipoprotein B (Chemicon International), and total and phospho-Smad 2 and Smad 3 (Cell Signaling). For normalization, monoclonal antibodies against α-actin (Santa Cruz) were used. Blots were incubated with horseradish peroxidase-conjugated anti-goat IgG (Santa Cruz Biotechnology), anti-mouse or anti-rabbit IgG (Amersham) antibodies, and proteins detected by enhanced chemiluminescence (ECL; Amersham) prior to quantification by densitometry (Image J software).

Semi-Quantitative Real-Time RT-PCR

Total hepatic RNA was isolated with PerfectPure RNA Tissue Kit (5′), and total adipose tissue RNA was isolated with RNeasy Lipid Tissue Mini Kit (Qiagen) according to the manufacturer's protocol. cDNA was synthesized by ImProm-II™ Reverse Transcriptase (Promega), using 1 μg of total RNA and oligo dT primers (**Table 1**). cDNA was evaluated with real-time quantitative PCR (Step One Plus, Applied Biosystems). The relative amounts of mRNA were calculated by comparison to the corresponding standards and normalized relative to 18S.

Statistical Analysis

Data were analyzed with SPSS software by two-way analysis of variance (ANOVA) or two-tailed Student's *t*-test with GraphPad Prism 4 software. $P < 0.05$ was statistically significant.

TABLE 1 | Real-time PCR primer sequences from mouse genes.

Primer	Forward sequence (5′–3′)	Reverse sequence (5′–3′)
Cd36	TCTTGGCTACAGCAAGGCC AGATA	AGCTATGCATGGAACATGACG
Fatp-1	TCACTGGCGCTGCTTTGGTT	GGACGTGGCTGTGTATGG
Fatp-4	GTGAGATGGCCTCAGCTATC	GAAGAGGGTCCAGATGCTCT
Lpl	AAGGTCAGAGCCAAGAGAA GCA	CCAGAAAAGTGAATCTTGACT TGGT
Srebp-1c	GGAGCCATGGATTGCACATT	GCTTCCAGAGAGGAGGCCA
Hsl	GGCTTACTGGGCACAGATACCT	CTGAAGGCTCTGAGTTGCTCAA
F4/80	CAAGGAGGACAGAGTTTATCGTG	CTTTGGCTATGGGCTTCCAGTC
TNFα	CCACCACGCTCTTCTGTCTAC	AGGGTCTGGGCCATAGAACT
Smad7	GTTGCTGTGAATCTTACGGG	ATCTGGACAGCCTGCA
Col6α3	ACCTAGAGAACGTTACCTCACT	GTCAGCTGAGTCTTGTGCTGT
α-Sma	CGTGGCTATTCCTTCGTTAC	TGCCAGCAGACTCCATCC
18S	TTCGAACGTCTGCCCTATCAA	ATGGTAGGCACGGCGACTA

Results

Lipid Metabolism

As expected, HF diet caused hyperinsulinemia and fed hyperglycemia (**Table 2**), markers of insulin resistance, in WT mice. In support of the positive effect of hyperinsulinemia on lipogenesis (4), qRT-PCR (**Table 3**) and Western blot analysis showed increased mRNA and protein levels of Fasn, respectively, in the liver of HF-fed WT mice relative to mice fed a regular diet (RD) (**Figure 1A**). HF diet also induced hepatic mRNA (**Table 3**) and protein levels of CD36 fatty acid translocase (**Figure 1A**), in addition to inducing the mRNA level of fatty acid transport protein-1 (Fatp-1) (**Table 3**). Together with increased lipogenesis, elevated lipid transport could contribute to increased hepatic triacylglycerol content in HF-fed WT mice (**Table 2**).

Increased production of hepatic triacylglycerol drives output, as supported by elevated plasma apolipoprotein B (ApoB48/ApoB100) protein levels in WT mice (**Figure 1B**). With fasting plasma triacylglycerol levels being intact (**Table 2**), it is likely that triacylglycerol was redistributed to the WAT, as expected from mice on the C57/BL6J genetic background that favors substrates partitioning to the adipose tissue (6). Supporting this notion, mRNA levels of Fatp-4 and lipoprotein lipase (Lpl) that are critical in lipid transport to the adipocyte were elevated in HF-fed relative to RD-fed WT mice (**Table 4**). This appears to drive adipogenesis, as suggested by higher Srebp-1c mRNA levels (**Table 4**), and ultimately, increased visceral obesity (**Table 2**). Elevated mRNA levels of hormone sensitive lipase (Hsl) (**Table 4**) and the rise in fasting plasma NEFA levels (**Table 2**) are consistent with increased lipolysis in HF-fed WT mice.

In L-CC1 mice, however, hepatic triacylglycerol content was neither modified by HF intake (**Table 2**) nor were mRNA levels of Fasn, Fatp-1, and Cd36 (**Table 3**). Consistent with normal hepatic lipogenesis, plasma ApoB48/ApoB100 protein levels in L-CC1 mice were not significantly altered by HF diet (**Figure 1B**). Moreover, HF did not modulate the mRNA level of genes involved in lipid metabolism in the adipocyte (Lpl, Fatp-4, and Srebp-1c) (**Table 4**). Consistent with normal fasting plasma NEFA (**Table 2**), HF did not alter Hsl mRNA levels in the WAT of L-CC1, as it did to WT mice (**Table 4**).

Visceral Obesity

High-fat feeding time dependently increased body weight gain in WT mice (**Figure 2A**). In contrast, it took ≥6 weeks before HF induced a statistically significant body weight gain in L-CC1 (**Figure 2A**). NMR analysis (**Figure 2B**) revealed persistently lower fat mass in L-CC1 relative to WT mice under normal feeding conditions until ~5 months of age, at which point this difference became statistically insignificant (**Figure 2B**, starting at about 11 weeks of experimental feeding). In contrast to total fat mass, visceral (mostly gonadal) and subcutaneous fat mass remained lower in RD-fed L-CC1 than RD-fed WT mice even until 6 months of age (**Table 2**).

While HF feeding caused an increase in total fat mass relative to RD feeding in both mouse groups, this effect was delayed (at 5 vs. 3 weeks in L-CC1 vs. WT) (**Figure 2B**). Furthermore, fat accumulation in L-CC1 mice did not become significantly higher than RD-fed WT until after 7 weeks of HF feeding but remained lower than HF-fed WT even after 4 months of HF, as shown by NMR (**Figure 2B**), and by ^{1}H-magnetic resonance spectroscopy (7). After 4 months of HF diet, visceral fat mass remained lower in L-CC1 than WT mice, while subcutaneous fat mass became comparable in both groups of mice (**Table 2**).

TABLE 2 | Effect of high-fat diet for 4 months on plasma and tissue biochemistry.

	WT		L-CC1	
	RD	HF	RD	HF
Body weight (BW) (g)	26.0 ± 0.80	34.0 ± 1.12*	24.0 ± 1.03	34.0 ± 2.32*
Body length (cm)	9.73 ± 1.52	10.5 ± 2.21	9.70 ± 1.00	10.4 ± 1.77
Visceral adipose tissue (% BW)	2.45 ± 0.25	7.55 ± 0.26*	1.36 ± 0.26†	5.76 ± 0.48*†
Brown adipose tissue (% BW)	0.39 ± 0.05	0.39 ± 0.03	0.34 ± 0.03	0.36 ± 0.04
Subcutaneous fat (% BW)	1.76 ± 0.12	6.23 ± 0.51*	1.18 ± 0.13†	5.02 ± 0.65*
Fasting plasma insulin (pM)	60.0 ± 1.43	162.0 ± 8.15*	58.2 ± 2.14	78.3 ± 3.34*†
Fed blood glucose (mg/dl)	120.0 ± 1.42	150.0 ± 3.52*	124.0 ± 3.25	130.0 ± 2.82†
Hepatic triacylglycerol (μg/mg protein)	122.0 ± 5.00	505.0 ± 20.4*	135.0 ± 3.40	152.0 ± 14.0†
Fasting plasma triacylglycerol (mg/dl)	65.4 ± 3.82	75.3 ± 4.60	50.3 ± 3.22†	58.4 ± 1.42†
Fasting plasma NEFA (mEq/l)	0.62 ± 0.04	0.92 ± 0.03*	0.58 ± 0.02	0.64 ± 0.04†

Mice ($n > 8$/feeding group/genotype) were fed an HF diet for 4 months starting at 2 months of age. Fat depots were excised and weighed. Intra-abdominal visceral adipose tissue fat refers to combined weight of mesenteric and gonadal fat. The relative mass of fat depots was expressed as percentage of body weight (% BW).
Values are expressed as mean ± SEM.
**$P < 0.05$ HF vs. RD per genotype.*
†$P < 0.05$ L-CC1 vs. WT per feeding group.

TABLE 3 | Effect of high-fat diet on mRNA levels of lipid metabolism genes in liver.

	WT		L-CC1	
	RD	HF	RD	HF
Fasn	1.25 ± 0.12	3.60 ± 0.40*	1.00 ± 0.20	1.45 ± 0.25†
Cd36	1.23 ± 0.11	2.00 ± 0.30*	0.67 ± 0.12	0.86 ± 0.12†
Fatp-1	1.19 ± 0.15	2.33 ± 0.10*	1.18 ± 0.05	0.87 ± 0.08†

Tissue mRNA levels were analyzed in duplicate per mouse ($n \geq 6$ mice/feeding group/genotype).
Values were normalized to 18S and expressed as mean ± SEM.
**$P < 0.05$ HF vs. RD per genotype.*
†$P < 0.05$ L-CC1 vs. WT per feeding group.

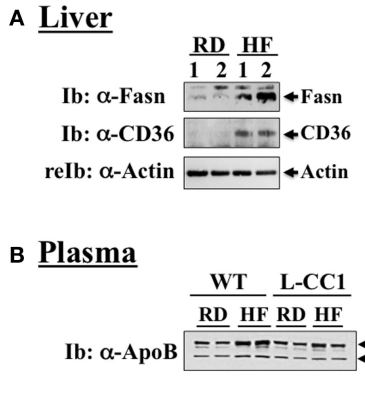


FIGURE 1 | Western analysis of proteins involved in hepatic lipid homeostasis. **(A)** Liver lysates from wild-type mice fed a regular (RD) or a high-fat diet (HF) for 4 months were analyzed by immunoblotting with α-Fasn and α-CD36 antibodies, followed by reprobing with α-actin for normalization. A representative gel of three different experiments performed on two mice per feeding group is included. **(B)** Plasma from both WT and L-CC1 mice was diluted and analyzed by 4–10% gradient SDS-PAGE and immunoblotting with an antibody against apolipoprotein B (ApoB), which recognizes both ApoB48 and ApoB100. A representative gel of two independent experiments performed on two different pairs of mice per feeding group per genotype is included.

TABLE 4 | Effect of 4 months of high-fat diet on mRNA of genes in white adipose tissue.

	WT		L-CC1	
	RD	HF	RD	HF
Fatp-4	0.80 ± 0.10	1.80 ± 0.22*	0.96 ± 0.24	0.55 ± 0.11†
Lpl	1.20 ± 0.21	3.51 ± 0.30*	1.12 ± 0.12	1.25 ± 0.40†
Srebp-1c	1.09 ± 0.13	4.03 ± 0.70*	1.26 ± 0.51	0.94 ± 0.32†
Hsl	0.49 ± 0.10	0.80 ± 0.11*	0.51 ± 0.13	0.27 ± 0.14†
F4/80	8.85 ± 4.50	21.8 ± 2.61*	5.45 ± 1.22	9.08 ± 3.01*†
TNFα	2.75 ± 1.37	6.56 ± 0.78*	3.55 ± 0.78	3.01 ± 1.05†
Smad7	11.3 ± 2.56	1.72 ± 0.40*	15.2 ± 2.05	9.40 ± 2.04†
Col6α3	0.67 ± 0.11	1.58 ± 0.25*	0.58 ± 0.17	0.48 ± 0.13†
α-Sma	0.03 ± 0.01	1.05 ± 0.33*	0.06 ± 0.01	0.19 ± 0.05†

Mice ($n > 5$ per feeding group per genotype) were fed an HF diet for 4 months starting at 2 months of age.
Tissue mRNA levels were analyzed in duplicate per each mouse and normalized to 18S.
Values are expressed as mean ± SEM.
**$P < 0.05$ HF vs. RD per genotype.*
†$P < 0.05$ L-CC1 vs. WT per feeding group.

Increased Macrophage Recruitment to White Adipose Tissue in Response to High-Fat Diet

Histological analysis of H&E stained sections from WAT showed a significant expansion of adipocytes in both groups of mice in response to 4 months of HF intake, but to a lower extent in L-CC1 mice (**Figure 3**).

Moreover, immunofluorescence analysis of F4/80, a macrophage marker, showed multiple crown-like structures (CLS) containing macrophages in WAT from HF-fed WT mice (**Figures 4A,A'**, green). As previously shown (14), moving through the individual *z*-stack sections shows numerous small pieces of lipid inside the macrophages in CLS (**Figures 4A,A'**), indicating adipocyte degradation in HF-fed WT mice. In contrast, HF-fed L-CC1 mice exhibited minimal CLS formation with little evidence of lipids inside the macrophages in these structures. Consistently, HF diet-induced F4/80 mRNA levels by ~2.5-fold in WT as compared to ~1.5-fold in WAT of L-CC1 mice, and mRNA levels of TNFα adipokine in WT mice (by ~2-fold), but

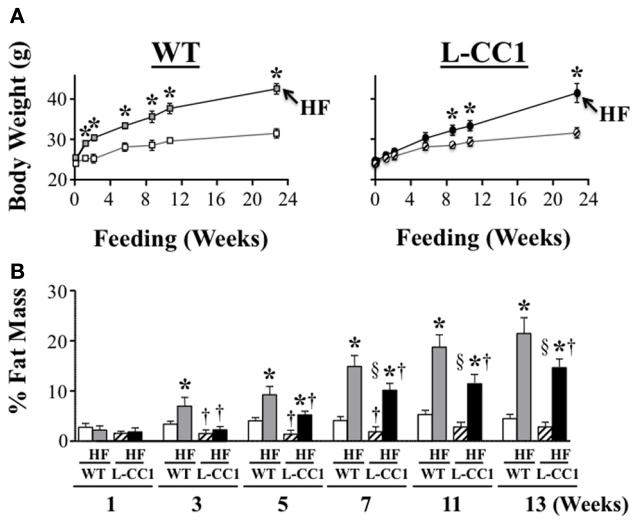


FIGURE 2 | Time-dependent effect of high-fat diet on fat distribution. **(A)** Mice ($n > 10$ mice/feeding group/genotype) were fed a regular (RD) or a high-fat (HF) diet and their body weight (grams) was weighed for 0–24 weeks. Values expressed as mean ± SEM. $^{*}P < 0.05$ HF vs. RD and $^{\dagger}P < 0.05$ L-CC1 vs. WT per feeding group. **(B)** Whole body fat mass was determined using NMR and expressed as percentage of total body weight ($n > 10$ mice/feeding group/genotype). Values expressed as mean ± SEM. $^{*}P < 0.05$ HF vs. RD, $^{\dagger}P < 0.05$ L-CC1 vs. WT per feeding group and $^{\S}P < 0.05$ HF-fed L-CC1 vs. RD-fed WT mice.

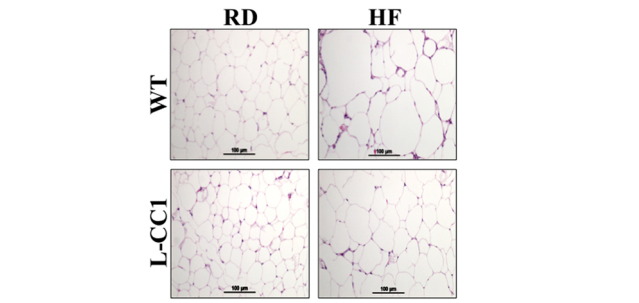


FIGURE 3 | Histological analysis of white adipose tissue. White adipose tissue histology was assessed in H&E stained sections ($n > 4$ mice/feeding group/genotype). Lipid droplets indicate adipocytes' expansion in response to high-fat (HF) relative to regular diet (RD) in both genotypes. Of note, the expansion in L-CC1 was relatively weaker than in WT mice. Representative images from three sections per mouse are shown.

not in L-CC1 mice (**Table 4**). Together, this demonstrates that protecting hepatic CEACAM1 levels against HF diet limited the development of inflammation in the WAT of L-CC1 mice.

Induction of Fibrosis and TGF-β Activation in White Adipose Tissue by High-Fat Diet

In agreement with reports showing TNFα inhibition of the expression of Smad 7 (15), a negative regulator of TGF-β–Smad2/3 signaling pathway (16, 17), HF markedly repressed

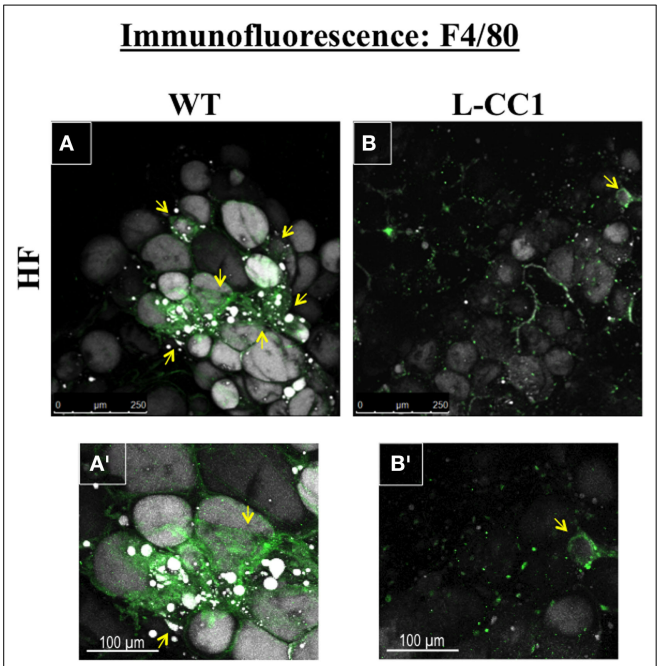


FIGURE 4 | Immunofluorescence analysis of white adipose tissue. Whole white adipose tissue from HF-fed WT and L-CC1 mice was stained with BODIPY 558/568 to detect lipid (gray) and with anti-F4/80 to detect macrophages (green). All images were captured using LSCM with a 20× objective and are 2D projections of a 3D image *z*-stack. **(A)** Representative picture from HF-fed WT mice. Yellow arrows show multiple areas with crown-like structures (CLS). **(A')**. A blow up of the area around the bottom left yellow arrow in (A) showing multiple layers of CLS containing macrophages (green) surrounding adipocytes (gray). Also visible are small pieces of lipid inside the macrophages. **(B)** Representative projection from HF-fed L-CC1 mice. One yellow arrow points out the single CLS detected in the image. **(B')**. A blow up of the single CLS in (B) (yellow arrow) surrounding one adipocyte from HF-fed L-CC1 mice. No small pieces of lipid are visible inside of macrophages. Representative images from three sections per mouse are shown.

(by ~5-fold) the mRNA level of Smad 7 in WT, but not L-CC1 mice (**Table 4**). Subsequently, HF induced Smad 2 and Smad 3 activation in the WAT of WT, but not L-CC1 mice, as assessed by Western analysis of Smad 2 and Smad 3 phosphorylation (**Figure 5A**).

In adipose tissue derived from WT, but not L-CC1 mice, HF induced significantly the transcript levels of two main players in fibrosis (**Table 4**): α-smooth muscle actin (α-Sma) (by ≥10-fold) and endotrophin/Col6α3 (by ~2-fold), a protein that promotes metabolic derangement and fibrosis in adipose tissue (18). This translated into a higher induction of collagen deposition in the WAT of WT than L-CC1 mice by HF feeding, as shown by trichrome staining (**Figure 5B**).

Discussion

Phenocopying L-SACC1 mice with liver-specific inactivation of CEACAM1 and global *Cc1*$^{-/-}$ null mice (1–3), HF diet represses hepatic CEACAM1 levels to impair insulin clearance and cause hyperinsulinemia, which in turn, activates *de novo* lipogenic

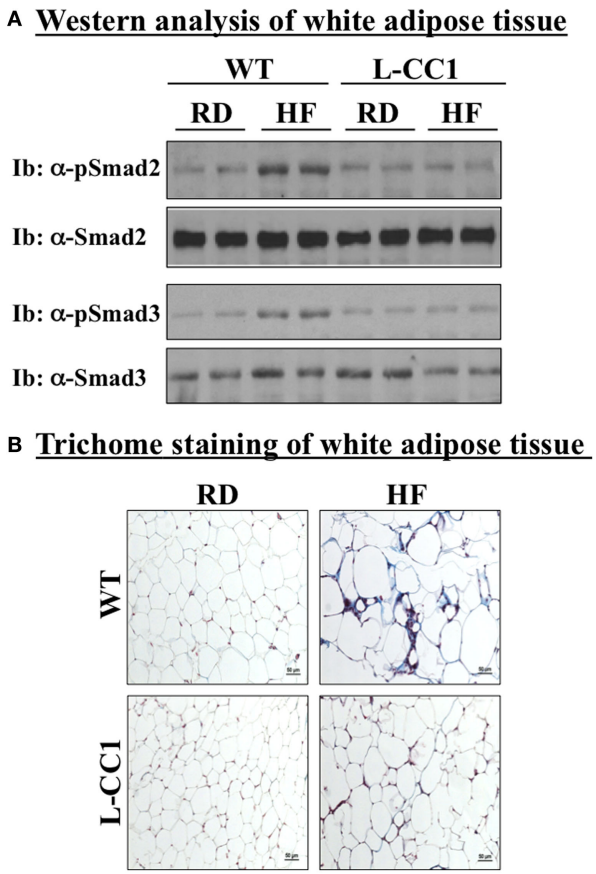


FIGURE 5 | Fibrosis in white adipose tissue. **(A)** Lysates from white adipose tissue of wild-type (WT) and L-CC1 mice fed a regular (RD) or a high-fat diet (HF) for 4 months were analyzed by immunoblotting with α-phospho-Smad 2 and phospho-Smad 3 antibodies followed by reimmunoprobing (relb) with antibodies against total Smad 2 and 3, respectively, for normalization. A representative gel of three experiments performed on two different mice per feeding group is included. **(B)** White adipose tissue from five mice/feeding group/genotype was analyzed by trichrome staining to detect collagen deposition. Representative images from three sections per mouse are shown.

pathways and elevates lipid production in liver (7). The current studies show that this drives lipid output (manifested by a rise in plasma ApoB100/ApoB48) and redistribution to WAT to provoke visceral obesity. This is consistent with a positive correlation between liver steatosis, hyperinsulinemia, and high plasma ApoB levels in humans and rodents (19–23). For instance, mice with conditional null mutation of the insulin receptor in liver exhibit impairment of insulin clearance and hepatic insulin resistance with elevated lipogenesis, and a rise in plasma ApoB100/ApoB48 levels in parallel to low plasma triacylglycerol levels and obesity (24).

In support of the important role that reducing hepatic CEACAM1 level plays in the pathogenesis of diet-induced metabolic derangement, protecting hepatic CEACAM1 levels by means of transgenic induction preserves insulin clearance and prevents insulin resistance and hepatic steatosis in response to HF feeding (7). The current studies show that it also causes a delay in the progression of fat accumulation and limits the expansion of adipocytes in response to HF diet. Together, this supports an important role for altered CEACAM1-dependent insulin clearance pathways in the pathogenesis of diet-induced hepatic steatosis and visceral obesity. These findings lend a mechanistic underpinning for the marked reduction of hepatic CEACAM1 observed in insulin-resistant obese subjects (25).

Immunofluorescence analysis showed less recruitment of CLS-containing macrophages to the WAT of L-CC1 and less adipocyte degradation inside these macrophages compared to WT mice. In addition to normal mRNA levels of TNFα, an adipokine that blunts insulin action (26), this could explain the protected insulin sensitivity along the liver-WAT axis (7).

In addition to inflammation (26), visceral obesity and insulin resistance are associated with increased fibrosis and activation of TGF-β signaling pathways in WAT in rodents (18, 27) and humans (28). Consistently, HF feeding induced more collagen deposition and activation of the TGF-β–Smad 2/3 pathway (16, 17) in WT than L-CC1 mice. This could prevent adipose tissue remodeling and contribute to the more metabolic derangement in HF-fed WT mice (29).

In summary, the current studies demonstrate that gain-of-function of CEACAM1 in liver restricts visceral obesity caused by HF diet, and that this is mediated, at least in part, by reducing lipid output from liver and limiting inflammation and fibrosis in WAT. Together with increased FGF21 production (7), this could mediate in part, preserved energy expenditure in L-CC1 mice (7, 28, 30). Because CEACAM1 protein is not produced to a significant extent in WAT (12), preserving adipose tissue biology could be attributed to extra-adipocytic factors caused by inducing CEACAM1 expression in liver. Although the underlying mechanisms are not fully delineated, the current studies provide a proof-of-principle of the importance of hepatic CEACAM1-dependent insulin clearance in the regulation of visceral obesity, and adipose tissue inflammation and fibrosis in response to HF diet.

Author Contributions

SL researched data, designed experiments, and wrote a first draft of the manuscript, LR, SG, SK, EE, TB, GH, and QA researched data. AD designed and generated the targeting vector for the generation of the L-CC1 mouse line, screened and propagated the mouse line, and reviewed the manuscript. WP generated the L-CC1 mouse. MM participated in scientific discussions, in detecting CLS, and in the revision of the manuscript. SN was responsible for study design, conceptualization, data analysis and results interpretation, reviewing, and revising the manuscript. SN had full access to all the data of the study and takes responsibility for the integrity and accuracy of data analysis and the decision to submit and publish the manuscript.

Acknowledgments

This work was supported by grants from the NIH [R01 DK054254, R01 DK083850, and R01 HL112248 (to SN) and R15 DK103196 (to MM)]. The work was also supported in part by the Wolfe Innovation Fund (University of Toledo Foundation). SG is supported by the Middle East Diabetes Research Center.

References

1. Park SY, Cho YR, Kim HJ, Hong EG, Higashimori T, Lee SJ, et al. Mechanism of glucose intolerance in mice with dominant negative mutation of CEACAM1. *Am J Physiol Endocrinol Metab* (2006) **291**:E517–24. doi:10.1152/ajpendo.00077.2006
2. Poy MN, Yang Y, Rezaei K, Fernstrom MA, Lee AD, Kido Y, et al. CEACAM1 regulates insulin clearance in liver. *Nat Genet* (2002) **30**:270–6. doi:10.1038/ng840
3. DeAngelis AM, Heinrich G, Dai T, Bowman TA, Patel PR, Lee SJ, et al. Carcinoembryonic antigen-related cell adhesion molecule 1: a link between insulin and lipid metabolism. *Diabetes* (2008) **57**:2296–303. doi:10.2337/db08-0379
4. Osborne TF. Sterol regulatory element-binding proteins (SREBPs): key regulators of nutritional homeostasis and insulin action. *J Biol Chem* (2000) **275**:32379–82. doi:10.1074/jbc.R000017200
5. Najjar SM, Yang Y, Fernstrom MA, Lee SJ, Deangelis AM, Rjaily GA, et al. Insulin acutely decreases hepatic fatty acid synthase activity. *Cell Metab* (2005) **2**:43–53. doi:10.1016/j.cmet.2005.06.001
6. Haluzik M, Colombo C, Gavrilova O, Chua S, Wolf N, Chen M, et al. Genetic background (C57BL/6J versus FVB/N) strongly influences the severity of diabetes and insulin resistance in ob/ob mice. *Endocrinology* (2004) **145**:3258–64. doi:10.1210/en.2004-0219
7. Al-Share QY, DeAngelis AM, Lester SG, Bowman TA, Ramakrishnan SK, Abdallah SL, et al. Forced hepatic over-expression of CEACAM1 curtails diet-induced insulin resistance. *Diabetes* (2015) **64**:2780–90. doi:10.2337/db14-1772
8. Inagaki T, Dutchak P, Zhao G, Ding X, Gautron L, Parameswara V, et al. Endocrine regulation of the fasting response by PPARalpha-mediated induction of fibroblast growth factor 21. *Cell Metab* (2007) **5**:415–25. doi:10.1016/j.cmet.2007.05.003
9. Fisher FM, Chui PC, Nasser IA, Popov Y, Cunniff JC, Lundasen T, et al. Fibroblast growth factor 21 limits lipotoxicity by promoting hepatic fatty acid activation in mice on methionine and choline-deficient diets. *Gastroenterology* (2014) **147**(1073–1083):e1076. doi:10.1053/j.gastro.2014.07.044
10. Emanuelli B, Vienberg SG, Smyth G, Cheng C, Stanford KI, Arumugam M, et al. Interplay between FGF21 and insulin action in the liver regulates metabolism. *J Clin Invest* (2014) **124**:515–27. doi:10.1172/JCI67353
11. Owen BM, Ding X, Morgan DA, Coate KC, Bookout AL, Rahmouni K, et al. FGF21 acts centrally to induce sympathetic nerve activity, energy expenditure, and weight loss. *Cell Metab* (2014) **S1550-4131**(1514):320–9. doi:10.1016/j.cmet.2014.07.012
12. Najjar SM. Regulation of insulin action by CEACAM1. *Trends Endocrinol Metab* (2002) **13**:240–5. doi:10.1016/S1043-2760(02)00608-2
13. Walsh A, Ito Y, Breslow JL. High levels of human apolipoprotein A-I in transgenic mice result in increased plasma levels of small high density lipoprotein (HDL) particles comparable to human HDL3. *J Biol Chem* (1989) **264**:6488–94.
14. Ebke LA, Nestor-Kalinoski AL, Slotterbeck BD, Al-Dieri AG, Lester SG, Russo L, et al. Tight association between macrophages and adipocytes in obesity: implications for adipocyte preparation. *Obesity* (2014) **22**:1246–55. doi:10.1002/oby.20634
15. Nagarajan RP, Chen F, Li W, Vig E, Harrington MA, Nakshatri H, et al. Repression of transforming-growth-factor-beta-mediated transcription by nuclear factor kappaB. *Biochem J* (2000) **348**(Pt 3):591–6. doi:10.1042/0264-6021:3480591
16. Nakao A, Afrakhte M, Moren A, Nakayama T, Christian JL, Heuchel R, et al. Identification of Smad7, a TGFbeta-inducible antagonist of TGF-beta signalling. *Nature* (1997) **389**:631–5. doi:10.1038/39369
17. Bitzer M, von Gersdorff G, Liang D, Dominguez-Rosales A, Beg AA, Rojkind M, et al. A mechanism of suppression of TGF-beta/SMAD signaling by NF-kappa B/RelA. *Genes Dev* (2000) **14**:187–97. doi:10.1101/gad.14.2.187
18. Sun K, Park J, Gupta OT, Holland WL, Auerbach P, Zhang N, et al. Endotrophin triggers adipose tissue fibrosis and metabolic dysfunction. *Nat Commun* (2014) **5**:3485–96. doi:10.1038/ncomms4485
19. Elam MB, Wilcox HG, Cagen LM, Deng X, Raghow R, Kumar P, et al. Increased hepatic VLDL secretion, lipogenesis, and SREBP-1 expression in the corpulent JCR:LA-cp rat. *J Lipid Res* (2001) **42**:2039–48.
20. Horton JD, Goldstein JL, Brown MS. SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. *J Clin Invest* (2002) **109**:1125–31. doi:10.1172/JCI0215593
21. Ginsberg HN, Zhang YL, Hernandez-Ono A. Regulation of plasma triglycerides in insulin resistance and diabetes. *Arch Med Res* (2005) **36**:232–40. doi:10.1016/j.arcmed.2005.01.005
22. Matikainen N, Manttari S, Westerbacka J, Vehkavaara S, Lundbom N, Yki-Jarvinen H, et al. Postprandial lipemia associates with liver fat content. *J Clin Endocrinol Metab* (2007) **92**:3052–9. doi:10.1210/jc.2007-0187
23. Vine DF, Takechi R, Russell JC, Proctor SD. Impaired postprandial apolipoprotein-B48 metabolism in the obese, insulin-resistant JCR:LA-cp rat: increased atherogenicity for the metabolic syndrome. *Atherosclerosis* (2007) **190**:282–90. doi:10.1016/j.atherosclerosis.2006.03.013
24. Biddinger SB, Hernandez-Ono A, Rask-Madsen C, Haas JT, Aleman JO, Suzuki R, et al. Hepatic insulin resistance is sufficient to produce dyslipidemia and susceptibility to atherosclerosis. *Cell Metab* (2008) **7**:125–34. doi:10.1016/j.cmet.2007.11.013
25. Lee W. The CEACAM1 expression is decreased in the liver of severely obese patients with or without diabetes. *Diagn Pathol* (2011) **6**:40. doi:10.1186/1746-1596-6-40
26. Najjar SM, Russo L. CEACAM1 loss links inflammation to insulin resistance in obesity and non-alcoholic steatohepatitis (NASH). *Semin Immunopathol* (2014) **36**:55–71. doi:10.1007/s00281-013-0407-3
27. Yadav H, Rane SG. TGF-beta/Smad3 signaling regulates brown adipocyte induction in white adipose tissue. *Front Endocrinol* (2012) **3**:35. doi:10.3389/fendo.2012.00035
28. Divoux A, Tordjman J, Lacasa D, Veyrie N, Hugol D, Aissat A, et al. Fibrosis in human adipose tissue: composition, distribution, and link with lipid metabolism and fat mass loss. *Diabetes* (2010) **59**:2817–25. doi:10.2337/db10-0585
29. Sun K, Kusminski CM, Scherer PE. Adipose tissue remodeling and obesity. *J Clin Invest* (2011) **121**:2094–101. doi:10.1172/JCI45887
30. Choi MS, Kim YJ, Kwon EY, Ryoo JY, Kim SR, Jung UJ. High-fat diet decreases energy expenditure and expression of genes controlling lipid metabolism, mitochondrial function and skeletal system development in the adipose tissue, along with increased expression of extracellular matrix remodelling- and inflammation-related genes. *Br J Nutr* (2015) **113**:867–77. doi:10.1017/S0007114515000100

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

REVIEW
published: 19 November 2015
doi: 10.3389/fendo.2015.00174

Unlock the Thermogenic Potential of Adipose Tissue: Pharmacological Modulation and Implications for Treatment of Diabetes and Obesity

Xiao-Rong Peng[1], Peter Gennemark[2], Gavin O'Mahony[3] and Stefano Bartesaghi[1]*

[1] Cardiovascular and Metabolic Diseases IMED Biotech Unit, Diabetes Bioscience Department, AstraZeneca R&D, Mölndal, Sweden, [2] Cardiovascular and Metabolic Diseases IMED Biotech Unit, Drug Metabolism and Pharmacokinetics Department, AstraZeneca R&D, Mölndal, Sweden, [3] Cardiovascular and Metabolic Diseases IMED Biotech Unit, Medicinal Chemistry Department, AstraZeneca R&D, Mölndal, Sweden

Brown adipose tissue (BAT) is considered an interesting target organ for the treatment of metabolic disease due to its high metabolic capacity. Non-shivering thermogenesis, once activated, can lead to enhanced partitioning and oxidation of fuels in adipose tissues, and reduce the burden of glucose and lipids on other metabolic organs such as liver, pancreas, and skeletal muscle. Sustained long-term activation of BAT may also lead to meaningful bodyweight loss. In this review, we discuss three different drug classes [the thiazolidinedione (TZD) class of PPARγ agonists, β_3-adrenergic receptor agonists, and fibroblast growth factor 21 (FGF21) analogs] that have been proposed to regulate BAT and beige recruitment or activation, or both, and which have been tested in both rodent and human. The learnings from these classes suggest that restoration of functional BAT and beige mass as well as improved activation might be required to fully realize the metabolic potential of these tissues. Whether this can be achieved without the undesired cardiovascular side effects exhibited by the TZD PPARγ agonists and β_3-adrenergic receptor agonists remains to be resolved.

Keywords: brown adipose tissue, thermogenesis, uncoupling protein 1, drug discovery, PPARγ agonists, thiazolidinediones, β_3-adrenergic receptor agonists, FGF21 analogs

OPEN ACCESS

Edited by:
Jun Wu,
University of Michigan, USA

Reviewed by:
Bin Xu,
Virginia Tech, USA
Qin Yang,
University of California Irvine, USA

****Correspondence:***
Xiao-Rong Peng
xiao-rong.peng@astrazeneca.com

Specialty section:
This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *26 September 2015*
Accepted: *28 October 2015*
Published: *19 November 2015*

Citation:
Peng X-R, Gennemark P, O'Mahony G and Bartesaghi S (2015) Unlock the Thermogenic Potential of Adipose Tissue: Pharmacological Modulation and Implications for Treatment of Diabetes and Obesity. Front. Endocrinol. 6:174. doi: 10.3389/fendo.2015.00174

INTRODUCTION

According to the International Diabetes Federation (IDF), 8.3% of adults worldwide – 370 million people – have type-2 diabetes (T2D), and the number of people with the disease is set to rise beyond 592 million in under 25 years (1). Although there are many drugs available for diabetes, none of them safely and durably prevent or reverse disease progress and its associated comorbidities. Poor diet, sedentary lifestyle, and obesity are considered major risk factors for diabetes. Inappropriate fuel handling by adipose tissue, liver, and skeletal muscle, combined with ectopic lipid deposition in key metabolic organs (such as liver, pancreas, muscle, and heart) have been hypothesized to play a significant role in the development of insulin resistance. Insulin resistance increases the overall burden on β-cells, which over time leads to β-cell failure and development of T2D.

Adipose tissue can be grossly divided into two major depots, white adipose tissue (WAT) and brown adipose tissue (BAT). WAT stores excess energy as triglycerides (TGs), which can be mobilized by lipolysis to generate free fatty acids (FFAs) for use by other tissues. BAT, on the other hand, is the main site of non-shivering thermogenesis (NST), which requires a brown adipocyte-specific protein called uncoupling protein 1 (UCP1).

Non-shivering thermogenesis by BAT is an interesting target for the treatment of metabolic disease due to the high metabolic capacity of BAT. BAT is highly vascularized and richly innervated by sympathetic nerves, and its activation is predominantly regulated by the sympathetic nerve system via β-adrenergic receptors (β-ARs). Enhancing energy expenditure (EE) through activation of NST by β_3-adrenergic receptor (β_3-AR) agonists has been investigated as an alternative to inhibition of food intake for bodyweight loss. This has, however, been unsuccessful in human clinical trials. This lack of effect on EE was partly attributed to negligible BAT function in adult humans compared to the situation in rodents.

The rediscovery of BAT in the adult human in 2007, and the subsequent demonstration of functional involvement of human BAT in NST have revitalized this area (2–5). In addition, the presence of brown-like adipocytes in WAT [referred to as beige or brown-in-white (brite) adipocytes] further increased the interest in brown adipocyte biology, as WAT mass is relatively large and any increase in cellular energetics in this tissue may have a significant impact on whole-body metabolism and EE. The beige nomenclature will be used for this review. Utilization of FFAs during NST could lead to depletion of brown and or beige adipocytes' lipid stores, which may result in redistribution of fuels [including glucose and non-esterified fatty acids (NEFA)] toward brown and beige adipocytes. In turn, this could lead to a reduced fuel over-supply to other metabolic organs (heart, skeletal muscle, and liver) and, thus, improved insulin sensitivity (**Figure 1**).

Transcriptional and hormonal regulation of the "browning" program of adipose stem cells and characterization of the molecular signature have been reviewed extensively elsewhere (6–12). This paper focuses on the metabolic potential of BAT and beige adipocytes, how these systems can be manipulated by pharmacological means, and how to assess if a brown adipocyte phenotype has been achieved by pharmacological intervention. Finally, we discuss the challenges of drug discovery in this area by reviewing three classes of clinically investigated pharmacological agents that regulate various aspects of BAT and beige adipocyte function: thiazolidinedione (TZD) PPARγ agonists, β_3-AR agonists, and fibroblast growth factor 21 (FGF21) analogs.

BROWN ADIPOSE TISSUE IN ADULT HUMANS

Typically, BAT is located in the interscapular (iBAT), cervical, auxiliary, perirenal and paraaortic areas of animals or human infants (13–15). In adult humans, BAT depots have a diffuse anatomic distribution, with mixtures of white and beige adipocytes, seeming to coexist in close proximity. Beige adipocytes have also been reported to arise in what are normally considered WAT depots (such as inguinal adipose in rodents) in response to various stimuli such as cold, TZDs, and β-AR agonists (9). In humans, beige adipocytes were found in the WAT of pheochromocytoma patients due to the presence of catecholamine-secreting tumors (16, 17) and in the subcutaneous adipose of severely burned patients where heat loss is increased and who experience prolonged adrenergic stress (18). Both lineage-tracing studies and transcriptional profiling of classical brown and beige adipocytes indicate that these two cell types seem to originate developmentally from distinct cell lineages. Classical brown adipocytes in interscapular BAT arise from precursors that are *myf5*$^+$, a gene known to be

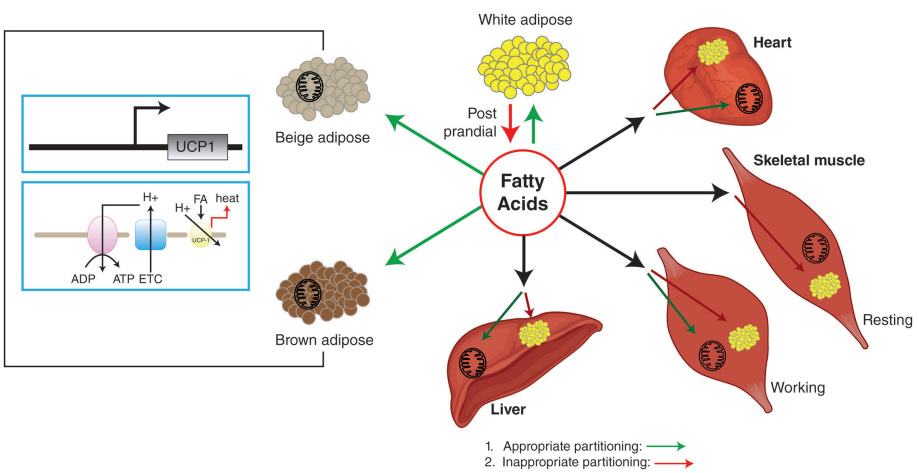


FIGURE 1 | White adipose tissue stores excess energy as triglycerides that can be mobilized by lipolysis to generate FFA for use by other tissues. BAT is the main site of NST, which is carried out by UCP1. Beige adipocytes have uncoupling capabilities similar to brown adipocytes, but are found in what is normally considered WAT. Appropriate partitioning and oxidation of fatty acids into BAT, WAT, beige adipocytes, and other metabolic organs can reduce ectopic fat deposition in metabolic organs, resulting in improved insulin sensitivity. Green arrows indicate appropriate partitioning and red arrows indicate inappropriate partitioning.

also expressed in committed skeletal muscle precursors (19, 20). The developmental origin of the beige adipocyte remains to be elucidated. The whole-body NST will have contributions from both classical brown and beige adipocytes.

It is now beyond doubt that BAT is present in adult humans and plays a role in NST (3). BAT activity can be detected by ^{18}F-fluorodeoxyglucose (^{18}F-FDG) uptake using PET–CT (2, 4, 5). Retrospective analysis of populations that have undergone PET–CT examination indicates that the prevalence of BAT varies between 1 and 5%. For example, one study on 4011 asymptomatic individuals (<5% obese subjects) showed that BAT prevalence is 5% in female and 1.3% in male (21). The BAT-positive subjects had lower body mass index (BMI), less visceral and subcutaneous fat areas, lower fasting glucose and TG levels, and increased HDL cholesterol concentrations compared to the BAT-negative subjects. Similar results were reported for 56 healthy volunteers (22), and for cancer patients (2, 23). The inverse correlation between BAT activity and BMI was further confirmed in non-diabetic subjects over a wide range of body compositions (BMI ranging from 22 to 48 kg/m^2) (4, 24–27). A recent retrospective analysis of ^{18}F-FDG uptake data (analysis of the neck regions of two relatively large cohorts of individuals) reports that the average body weight of BAT-positive individuals is approximately 5 kg lower compared to that of BAT-negative subjects (21, 23).

Using a combination of MRI and molecular analysis, Enerbäck's group clearly demonstrated that iBAT in human infants consists of classical brown adipocytes (15). However, the molecular signature of brown adipocytes isolated from the neck regions of adult humans resembles that of the rodent beige phenotype rather than classical brown adipocytes (15, 28–31). What causes the BAT phenotype transformation between infant and adult humans is not understood. It is conceptually important to unravel the function, regulation, and differentiation of beige and classical brown adipocytes in order to be able to pharmacologically enhance thermogenesis in humans. For example, if the adipocytes with various white and beige appearances in adult human neck region are, in fact, simply dormant brown adipocytes, they may be readily re-activated by cold or sympathomimetics. In addition, it is important to understand the translational aspects of BAT biology, i.e., whether the same or different pharmacological agent(s) will show desirable effects in preclinical animal species and man?

BAT AND METABOLIC SIGNIFICANCE

The contribution of BAT to whole-body metabolism in rodents has recently been examined using tools such as radioactive tracers and PET–CT imaging. Bartelt et al. showed that BAT is the major site of triglyceride-rich lipoprotein (TRL) clearance during acute cold exposure (32). Cold exposure also dramatically increased the glucose disposal to BAT tissue. The remarkable capacity of BAT to take up substrates is illustrated by the ratio of BAT mass to the total glucose and TG uptake by BAT compared to that of other major organs in mice under cold challenge. Labbe et al. extended this observation through PET–CT analysis of the rate of substrate flux and oxidation in the iBAT of both warm- and cold-adapted rats (33). The rate of glucose uptake into iBAT was relatively low at 27°C but increased 10-fold upon acute cold exposure and increased 46-fold after cold acclimation at 10°C. Similarly, NEFA levels rose 6-fold upon acute cold exposure, and ~100-fold after cold acclimation. The metabolic activity of the iBAT reached levels similar to that of heart and liver after 6 h of cold exposure. In spite of these results, it should be kept in mind that although BAT glucose uptake per unit volume of tissue is important, the bulk of glucose turnover during cold exposure is mediated by skeletal muscle metabolic activation even when shivering is minimized (7).

It is more challenging to determine the specific contribution of beige adipocytes to whole-body metabolism. Bartelt et al. showed that acute cold exposure also increases TRL uptake in inguinal WAT (iWAT), but to a smaller extent compared to iBAT (32, 34). Seale's group reported an aP2-PRDM16 transgenic mouse that exhibited a highly favorable metabolic phenotype, in which iBAT remained unchanged but with extensive browning in iWAT. This suggests that beige adipocytes may contribute to the overall metabolic phenotype observed in this mouse (35).

On the whole-body level, Reitman's group dissected the relative contributions of cold-induced, diet-induced, and physical activity-associated EE in mice in relation to the basal metabolic rate (BMR) at various temperatures (36). This work clearly illustrated that at 22°C, the temperature at which most reported metabolic studies have been conducted, mice expend a relatively large amount of energy to generate heat (120% of BMR). Adult humans, on the other hand, live in or near their thermoneutral zone, with a relatively small contribution from adaptive thermogenesis to EE (5% of BMR) (11). Interestingly, in mice housed at thermoneutrality (30–32°C), the relative contributions of BMR, diet, physical activity, and adaptive thermogenesis in mice to overall EE are reported to be ~60, 12, 25, and 0%, respectively (36). These figures are very similar to the relative contributions seen in humans with low activity levels, and might represent experimental conditions more suitable for translational research in this field. The EE increase in relation to external temperature is conceptually depicted in **Figure 2** (not scaled to real data).

Numerous studies that address the role of human BAT activation and its quantitative impact on whole-body metabolism have been published. Based on the heat production capacity of mouse BAT, Rothwell and Stock proposed in the 1980s that 40–50 g of BAT, if maximally activated, could account for 20% of daily EE in human (37). More recently PET–CT measurements estimated the average active BAT volume in healthy humans to be 137 cm^3, corresponding to a conservative estimate of around 50 g BAT mass (11). Virtanen et al. estimated the EE of human BAT to be 55 W/kg (5) based on the rate of glucose uptake during cold exposure, as measured by dynamic PET–CT. These conservative estimates suggest that the EE of fully activated BAT could amount to 2–5% of BMR. Recent cold exposure experiments confirmed that the cold-induced NST-associated increase in EE accounts for 0–15% of BMR (11). Using a human body-composition model (38), we further extrapolated that a 4% increase of BMR could lead to a 3% bodyweight reduction per year, assuming that the effects are sustained. A key assumption in such an extrapolation is that functional tolerance can be avoided (39). In support of this assumption, some pieces of evidence suggest that increased thermogenesis is not always fully compensated for and negated by an increase in food intake, for reasons that are not fully understood (40).

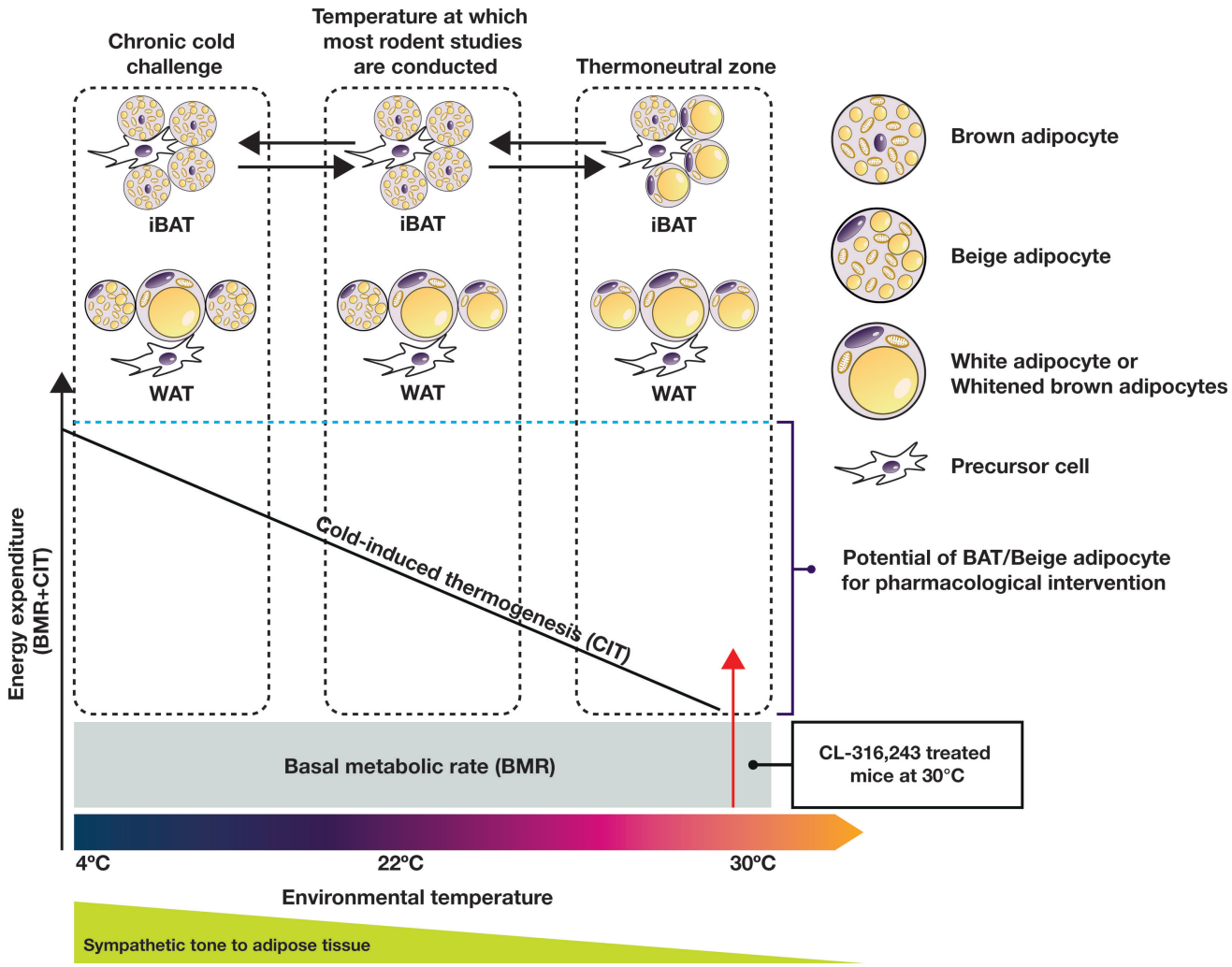


FIGURE 2 | Adaptation of EE and adipose tissue morphology and function to the changes of environmental temperature in the mouse. Sympathetic tone to adipose tissue plays an important role in this process. Most current published rodent studies are conducted at temperatures below the thermoneutral zone, while humans typically live near the thermoneutral zone. For adequate translation between species, rodent studies should be performed under thermoneutral conditions.

Cypess et al. recently reported a 13% increase in BMR as a result of BAT activation upon administration of an acute dose of the β_3-AR agonist mirabegron (41). Again, assuming that this BMR increase could be sustained upon chronic treatment, modeling suggests that this could potentially lead to an 8% bodyweight loss per year in BAT-positive healthy men. Besides potential functional tolerance, it remains uncertain if chronic β-AR-mediated stimulation of BAT is possible without encountering the side effects often associated with β-AR agonists.

Bodyweight reduction has been shown to have positive effects not only on preventing the progression of pre-diabetes to diabetes, but also leads to a reduction of hemoglobin A1c (HbA1c) (42). More recently, Hanssen et al. showed that 10-day cold acclimation in obese T2D patients could increase BAT activity, which was in turn associated with a reduction of BAT TG content and increased EE (43). One very encouraging observation is the 43% improvement of glucose infusion rate during a clamp study. Importantly, the improvement in insulin sensitivity of both adipose and skeletal muscle appeared before any bodyweight change could be seen (43). As indicated by Hanssen et al., the cold-induced improvement in insulin sensitivity exceeds that which was observed after long-term exercise training. To place this in a pharmacological context, this improvement in insulin sensitivity is similar in extent to that seen after 2-week treatment with dapagliflozin (Farxiga/Forxiga), which affords an 18% improvement in tissue glucose disposal (44).

FUNCTIONAL BROWN/BEIGE ADIPOCYTES

The remarkable metabolic capacity of classical brown adipocytes in the activated state has been well characterized using rodent

brown adipocytes. Functional characterization of beige adipocytes from the inguinal adipose tissue of mice or differentiated beige adipocytes from human adipose stem cells suggests that these cells are functionally similar to classical brown adipocytes (45, 46). The developmental origin of brown and beige adipocytes is currently an area of intense research and has been extensively reviewed elsewhere (47, 48).

Uncoupling of the mitochondrial transmembrane proton gradient in brown adipocytes in order to generate heat in response to cold is a complicated but well-orchestrated event. The key metabolic and signaling pathways in the brown or beige adipocyte are summarized in **Figure 3**. To be able to carry out this function, brown adipocytes need to be equipped with a complex machinery that is able to (i) signal through norepinephrine (NE) or other catecholamines via β-ARs, (ii) generate intracellular FFAs through hydrolysis of TGs from lipid droplets (lipolysis), and (iii) uncouple the ATP-generating process via UCP1 activation (13, 49). Chronically, cold challenge leads to a recruitment process in which cell proliferation, mitochondrial biogenesis, and angiogenesis are enhanced. Given the broad range of cellular processes that are involved in these events, it is not surprising that large numbers of genes/factors have been described to regulate the differentiation and function of brown and beige adipocytes (9, 34).

The β_3-AR has emerged as a leading molecular target for the activation of brown or beige adipocytes (18, 41). Rodent and human adipose tissue display different β-AR expression profiles. (50–53). In mouse, the β_3-AR is highly expressed in both BAT and WAT with several-fold higher abundance than the β_1-AR, whereas in the adult human, the β_3-AR is only expressed in BAT. In human WAT, β_1-AR has been reported to be 50-fold more abundant than β_3-AR (52). The β_3-AR subtypes also differ in their potency toward various ligands, G-protein coupling, and desensitization. The binding affinity of NE to β_3-AR was reported to be in the low micromolar range, while the potency for cAMP accumulation is in the low nanomolar range when measured in intact cells, suggesting that the coupling efficacy to adenylyl cyclase of β_3-AR was higher than that of β_1-AR (52, 54). In addition, β_1-AR desensitizes more rapidly than β_3-AR upon exposure to agonists. This led to the hypothesis that β_1-AR may mediate the NE response to low levels of sympathetic stimulation. On the other hand, the activation of β_3-AR may require higher levels of sympathetic stimulation, but once activated this receptor is likely to deliver a more sustained effect (52). It is important to note that circulating NE levels are generally in the low nanomolar concentration range, and it is likely that NE concentrations at the synaptic clefts will be much higher during cold response, providing sufficiently high local concentrations in BAT to enable local activation of β_3-AR in spite of the low plasma concentration. Recently, several studies have shown that systemic administration of adrenergic activators, such as isoproterenol (ISO) and ephedrine, fail to elicit BAT activation in man (55–57). To properly interpret these clinical data, it is crucial to understand if the plasma concentrations of the adrenergic agonists used reached a sufficiently high concentration to enable activation of lipolysis and thermogenesis in human BAT.

Norepinephrine-mediated β-AR activation results in an increased intracellular cAMP concentration, which in turn stimulates lipolysis in brown adipocytes via activation of the protein kinase A pathway. Lipolysis is a stepwise process with different enzymes acting at each step: TGs are hydrolyzed by desnutrin/adipose triglyceride lipase (ATGL) to form diacylglycerol (DAG). DAG is then hydrolyzed by hormone-sensitive lipase (HSL) to monoacylglycerol and, subsequently glycerol, with a fatty acid released at each stage. Intracellular FFAs are the direct activators of UCP1 (58). In humans, the BAT radiodensity (which is indicative of intracellular TG stores) is inversely correlated with NST, strongly suggesting that depletion of intracellular TG occurs during cold exposure. In rats, inhibition of lipolysis by nicotinic acid-mediated GPR109a agonism significantly reduced the oxidative capacity of iBAT in response to cold, again suggesting a key role for lipolysis in UCP1-mediated thermogenesis (33). Additionally, adipose tissue-specific knockout of ATGL led to the formation of "whitened" brown adipocytes and resulted in impaired lipolysis and defective thermogenesis in BAT (59–62).

The brown adipocytes' cold-depleted energy stores are replenished by *de novo* lipogenesis and glycogen synthesis, which requires cellular uptake of circulating glucose and FFAs (derived either from TRLs or from lipolysis in WAT). In brown adipocytes, glucose uptake is mostly mediated by glucose transporter 1 (GLUT1) and glucose transporter 4 (GLUT4) and subsequently stored as glycogen or converted to lactate through anaerobic glycolysis. As shown in **Figure 3**, FFAs are transported into the cell by cluster of differentiation 36 (CD36) and TGs are subsequently synthesized through re-esterification by a series of enzymes, including glycerol-3-phosphate acyltransferase (GPAT) and diacylglycerol O-acyltransferases (DGATs) (49). The process of recruitment of new brown or beige adipocytes and the maintenance of mature adipocyte function during acute and chronic cold acclimation are subject to complex transcriptional and hormonal regulation, for example, by PPARγ, bone morphogenic proteins (BMPs), the thyroid axis, and FGF21. These regulatory mechanisms have been extensively reviewed elsewhere (8, 47, 48, 63).

The functional activity of BAT is reduced by chronic warm acclimation, old age, obesity, and diabetes. Rodent models of genetic deficiency of leptin (*ob/ob*, *db/db*, and obese *fa/fa* rats) are cold sensitive, and their brown adipocytes have a white adipocyte appearance with reduced expression of UCP1 protein (64, 65). Old age and lack of cold challenge also reduce the thermogenic activity of the brown adipocytes (66). Brown adipocytes isolated from guinea pigs housed at 30°C appear unilocular, although these cells appear to retain their brown adipocyte identity and are able to respond to NE with a robust increase of thermogenesis, which is not the case for adipocytes isolated from WAT (67). In humans, BAT activity is inversely correlated with age, fat mass, and BMI. Insulin-stimulated glucose uptake is also compromised in the BAT of diabetic individuals (68). Recent reports that both chronic cold acclimation and weight loss can enhance BAT activity in humans are encouraging (27, 69, 70), indicating that reduced BAT function may be restored. In this respect, adipocytes or adipose precursor cells are highly plastic and able to adapt to the functional needs, as has been shown in rodent using lineage tracing experiments (71).

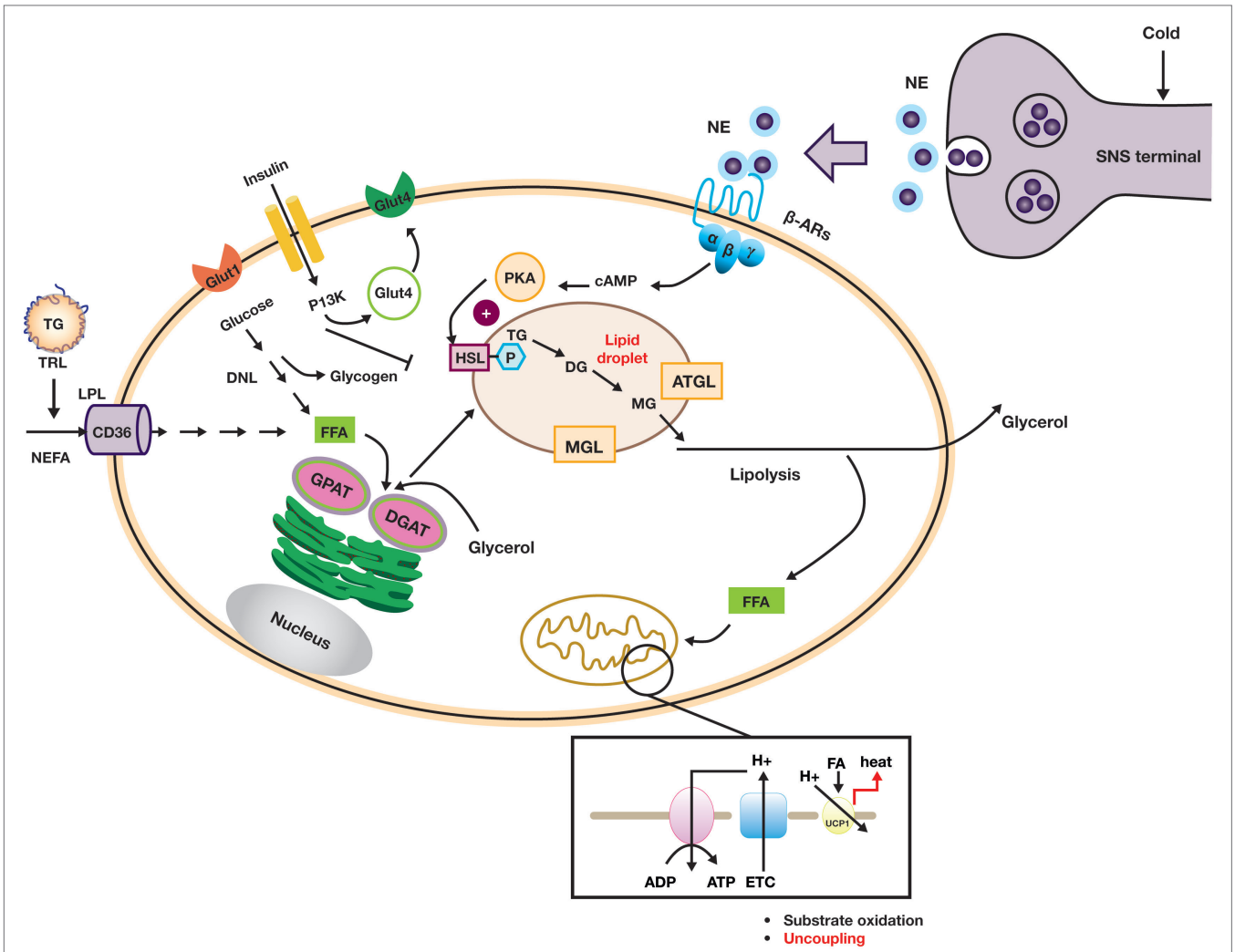


FIGURE 3 | Diagram showing key metabolic and signaling pathways in brown or beige adipocytes.

THIAZOLIDINEDIONES

The TZDs [represented by rosiglitazone (Avandia) and pioglitazone (Actos)] are a chemical class of PPARγ agonists used as insulin sensitizers for the treatment of T2D (72). The primary site of action of the TZDs is adipose tissue, where they improve several functional aspects, including the uptake and storage of plasma NEFA. They also increase FFA mobilization under fasting conditions and enhance postprandial suppression of FFA mobilization by insulin (73).

Thiazolidinediones have been shown to increase UCP1 expression and BAT mass in rodents. Rosiglitazone has been shown *in vitro* by several laboratories to induce UCP1 in rodent brown adipocytes and differentiated adipose stem cells (74–77). In addition, chronic treatment of human subcutaneous adipose stem cells with rosiglitazone upregulated several components of the mitochondrial electron transport chain, which is consistent with what has been observed in human (78). The UCP1 protein in human adipocytes differentiated *in vitro* in the presence of rosiglitazone is only functional when cells have been allowed to differentiate for a longer time than is typically reported in mouse studies (45). Although TZDs have the ability to recruit the "browning program" in both mouse and human adipocytes, the thermogenic capacity of UCP1-expressing cells cannot be unleashed without subsequent activation (e.g., by β-AR agonists). An increase in the oxygen consumption rate (OCR) of human adipocytes differentiated *in vitro* in the presence of rosiglitazone could only be observed when the cells were stimulated with isoproterenol or in the presence of exogenously provided FFAs. The increase of OCR in these beige cells is completely UCP1 dependent, as UCP1 knock-down abolishes the effect (45).

Various *in vivo* rodent models of insulin resistance have been used to show that the TZDs increase UCP1 mRNA in iBAT and overall iBAT weight. However, this is not associated with a subsequent increase in thermogenesis or whole-body EE. In addition, TZD treatment leads to the brown adipocytes becoming lipid filled (79–81). This led to the hypothesis that obese and diabetic animals or humans could first be primed by a PPARγ

agonist to expand the BAT capacity, followed by its activation via a β-AR agonist. This concept was tested pre-clinically in *ob/ob* mice by pre-treating with a PPARγ agonist (COOH, a non-TZD PPARγ agonist) followed by treatment with the β_3-AR agonist CL-316,243. Synergistic effects on EE and bodyweight reduction were indeed observed in this single study (82). However, a later study using rosiglitazone followed by acute cold exposure failed to reproduce these effects (83). A possible explanation for the lack of increased EE in spite of increased TG storage, UCP1 expression and BAT mass induced upon PPARγ activation by rosiglitazone (84) may be the downregulation of β_3-AR and iodothyronine deiodinase type II (DIO2) expression caused by TZD treatment (85, 86). In addition, as COOH belongs to a different structural class of PPARγ agonists to the TZDs, it is not unlikely that different outcomes could be observed between the two compounds. In humans, 12 weeks of pioglitazone treatment has been shown to generate a small increase in UCP1 mRNA levels in subcutaneous adipose tissue (87). However, combined pioglitazone and ephedrine treatment for 12 weeks in obese human subjects failed to deliver significant bodyweight reduction (88).

When comparing the observed outcomes of studies employing PPARγ agonists, it must be kept in mind that even structurally closely related compounds may exhibit very different PPARγ-dependent pharmacodynamic profiles. This is, in part, due to ligand-dependent modulation of the PPARγ protein that leads to recruitment of different coactivator and corepressor proteins, in turn resulting in a unique transcriptional profile for each compound (89). Since the identification of PPARγ as the molecular target of the TZDs (90), significant effort has been invested by the pharmaceutical industry in PPARγ agonist drug discovery (91–96) albeit largely without commercial success (97). The availability and high throughput of PPARγ LBD-based ligand binding assays and chimeric PPARγ–GAL4 reporter gene/transactivation assays enabled the generation of many highly potent, structurally diverse, selective PPARγ full and partial agonists. Despite this, since the launch of pioglitazone and rosiglitazone, no new selective PPARγ agonists have survived clinical testing and remain on the market, mainly due to preclinical and clinical safety issues (98–101). The central role of PPARγ in adipose biology has, however, not diminished due to these failures and PPARγ remains an attractive but challenging drug target.

The lack of translation between *in vitro* receptor binding and the functional (both *in vitro* and *in vivo*) effects of the compounds and inter-species differences in PPARγ biology are considered major obstacles in this field. This is due to a number of factors, including the combinatorial nature of the activation of the PPARγ:RXR heterodimer (102, 103), subtle differences in coactivator/corepressor recruitment between superficially related compounds (104–107), ligand effects on the extent of posttranslational modifications (108, 109), and non-transcriptional effects of the ligands (72). This suggests that a reductionist approach to PPARγ agonist discovery based on the use of isolated protein domains and chimeric reporter gene assays is unlikely to provide compounds with the desired functional or clinical outcome.

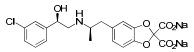
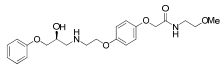
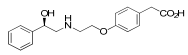
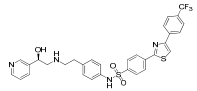
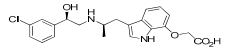
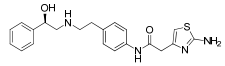
Since the "traditional" approach of optimizing receptor binding and agonist potency has not borne fruit, a radical change in the preclinical approach to PPARγ drug discovery is needed in order for the functional potential (for example, browning of WAT) of small-molecule PPARγ activation to be realized. For example, the application of phenotypic screening in relevant cell systems [i.e. primary human cells (110)] is one approach to front-load the functional assessment of compounds, with traditional *in vitro* assessment of PPARγ activity included in a secondary wave of assays. In addition, the recent widespread availability of omics techniques (such as *RNA*omics and proteomics) makes the preclinical identification of PPARγ agonists with a desirable functional profile a realistic prospect. A combination of such approaches avoids focus on a single receptor-dependent pathway or mechanism and allows pleiotropy to be accounted for. However, whether the perceived target-related risk associated with PPARγ agonism is considered acceptable in proportion to the potential commercial viability of a safe PPARγ agonist remains to be seen.

BETA-3 ADRENERGIC RECEPTOR

Several sympathomimetic β_3-AR agonists that selectively stimulate rodent brown and white adipocyte lipolysis were discovered from the mid-1980s onward (e.g., BRL-37344, CL-316,243, and CGP-12177A) (50, 51, 111). Early optimization of these compounds was mostly performed in rodent tissue or cell models, as the human β_3-AR was not cloned until 1989 (112). The compounds showed potent anti-obesity and anti-diabetic effects in rodent models of obesity and diabetes, but none of these compounds advanced beyond the clinical phase II due to lack of efficacy. Specifically, compounds optimized using rodent β_3-AR did not effectively translate to human. Several β_3-AR agonists were synthesized and evaluated after the cloning of human β_3-AR cDNA (113). A summary of studies investigating the effect of β_3-AR agonism on EE in man is given in **Table 1**. Note that many binding and adenylyl cyclase activity assays were performed using isolated membrane preparations, returning β_3-AR binding affinities in the micromolar range for the compounds tested. Lower potencies (in the nanomolar range) have been reported for the same compounds when measured in whole-cell assays (cAMP, lipolysis, or respiration) (52), suggesting a G-protein coupling efficiency for the β_3-AR that is only captured in a whole-cell context.

Most early human trials of β_3-AR agonists used bodyweight reduction as a clinical endpoint, to be achieved through increased EE. Data on BAT activation and metabolic parameters are scarce, with the exception being for the CL-316,243 study (124). In this study, treatment of lean healthy men for 4 weeks did not increase EE, but resulted in a 45% increase of insulin-mediated glucose disposal and a reduced 24-h respiratory quotient (24-h RQ), indicating enhanced fat oxidation. Intriguingly, CL-316,243 significantly increased fasting FFA levels in parallel with improved insulin action. These data are in line with recently published improved glucose infusion rates in T2D patients after 10 days of mild cold exposure (43). In both cases, improved insulin action preceded any significant weight loss. This improved action of insulin may be due to partitioning of FFAs toward BAT, which in turn reduces the fatty acid burden on other metabolic tissues (see **Figure 1**). Indeed, improved glucose uptake was seen in the skeletal muscle of T2D patients after cold exposure (43).

TABLE 1 | *In vitro* properties of β_3-adrenergic receptor agonists that have been tested in humans, and their effect on energy expenditure in man.

PHYSICAL AND PHARMACOKINETIC PROPERTIES						
Compound name(s) and chemical structure	**CL-316,243** 	**ZD7114** 	**ZD2079 (Talibegron)** 	**L-796568** 	**TAK-677 (Rafabegron)** 	**Mirabegron (YM-178)** 
MW (g/mol)	466.8 (Na_2 salt), 421.8 (free acid)	418.5	315.4	624.7	402.9 (free acid)	396.5
clogP	−1.4	2.1	−0.3	5.3	0.7	1.3
Human β_3 potency/TE	EC_{50} 1.15 μM/0.63[a] (114)	K_{act} 20 nM (for ZD201651, the major active metabolite) (115)	K_{act} 191 nM/0.91[a] (116)[b]	3.6 nM/0.94[a] (117)	EC_{50} 0.062 nM/1.16[a] (118)	22 nM/0.8[a] (119)
Rodent β_3 potency/TE	Rat K_i 1 μM, K_{act} 0.71 nM/1[a] in β_3 overexpressing CHO cells (111)	–	–	–	0.016 nM/1.1[a] (rat cAMP)	–
Fold selectivity β_3:β_2:β_1	1:228:96 Based on CHO data (114). Reported as an antagonist @ β_1 and β_2 (120)	–	–	1:667 (partial):1333 (partial)	1:209 (partial):1032 (partial)	1:>446 (partial):>446 (partial)
$T_{1/2}$/%F	16 h/10% (human)[c]	–	–	>8 h/17% (rat)	–	–
PHARMACODYNAMICS						
Population	Healthy young lean males (124)	Obese men and women with BMI 27–39 kg/m^2 (115)	Obese men and women with BMI 27–39 kg/m^2 (115)	Healthy overweight to obese men (121)	Obese men and women, mean BMI 33.9 kg/m^2 (122)	Healthy male subjects with detectable BAT (41)
Administration	1.5 g/day for 8 weeks (n = 10)	150 mg/day for 2 weeks (n = 5)	1.2 g/day for 2 weeks (n = 9)	375 mg/day for 28 days (n = 10)	0.5 mg BID for 29 days (n = 22)	200 mg acutely (n = 12; crossover study)
	Placebo (n = 4)	300 mg/day for 2 weeks (n = 8) Placebo (n = 22)	Placebo (n = 8)	Placebo (n = 10)	Placebo (n = 4)	
Exposure	30 ± 11 nM (steady-state C_{min})	–	–	77 ± 30 nM (steady-state C_{min})	24 ± 13 nM (at t = 2 h)	781 ± 184 nM (C_{max})
Fold *in vitro* EC_{50}	<0.1	–	–	>20	>1000	>30
BAT activity	–	–	–	–	–	Significant increase in BAT glucose uptake, from 1 to 130 mL × SUV_{mean} × g/mL
Energy expenditure	24-h EE after 8 weeks did not differ from baseline	No effect on 24 h EE	Trend for stimulatory effect on 24-h EE (2.4%)	Mean change in 24-h EE upon treatment did not differ significantly between treated and placebo	Slight increase (~50 kcal/day) in 24-h EE at the highest dose	Increased resting metabolic rate by 203 ± 40 kcal/day (+13%; p = 0.001)

(Continued)

TABLE 1 | Continued

Fasting plasma insulin	Trend toward decrease	–	–	No significant change	No significant change	Significant increase from 5.06 (3.56–6.19) to 7.61 (6.90–8.66) (μU/mL)
Other observations	No change in body weight or body composition. Increase in insulin action, decrease in 24-h RQ implying 23% increase in fat oxidation. No β_1 or β_2 side effects (bp/heart rate)	Acid metabolite also a β_3 partial agonist. Increases UCP1 expression in dogs (2-week study 10 mg/kg BID) (123)	Trend for increase in spontaneous physical activity	No major lipolytic or thermogenic effect but lowered triacylglycerol concentrations. No β_1/β_2 side effects (heart rate or tremor)	No effect on 24-h RQ or fat oxidation	BAT metabolic activity was a significant predictor of the changes in RMR. Heart rate and systolic BP increased

MW, molecular weight; TE, agonist top effect; EE, energy expenditure; RQ, respiratory quotient.

[a]*Isoproterenol response defined as 100%.*

[b]*Primary reference refers to an, at that time, yet unpublished paper from ICI.*

[c]*Claimed to be a full agonist, but only 80% TE in in vitro assay.*

Similarly to CL-316,243 treatment, chronic dosing of the β_3-AR agonists ZD7114 and ZD2079 did not significantly increase EE, although the latter compound showed a tendency toward a non-statistically significant increase of 2% (115).

Data have been reported for both acute and chronic dosing of β_3-AR agonist L-796568 in overweight to obese men. Acutely, van Baak et al. (125) reported a significant increase (8%) in EE after 4 h for the highest dose used. However, no average increase was observed over the period of observation, 0–4 h. Chronic dosing of L-796568 for 4 weeks did not result in any significant change in EE (121). The authors discuss possible reasons for this lack of effect, e.g., declining plasma exposure levels over time, insufficient agonist-induced BAT proliferative capability of β_3-AR-responsive tissues in humans during chronic stimulation, potential downregulation of β_3-AR receptors, or other functional feedback. We believe that the small effect on EE observed in the acute study is another reason.

A chronic β_3-AR agonism study (using TAK-677) in obese humans with no observed effect on EE or diabetic parameters was reported by Redman et al. (122). Whether BAT activation truly occurred after administration of compound was not carefully assessed in this study, which makes data interpretation challenging.

In contrast to the above-mentioned β_3-AR agonism studies, Cypess et al. recently reported a ~13% increase in resting metabolic rate (RMR) upon acute treatment with high doses of β_3-AR agonist mirabegron (41). In this study, healthy young male subjects with detectable BAT were selected in order to provide proof of concept (PoC) for β_3-AR agonist-mediated BAT activation. Importantly, it was found that BAT metabolic activity was a significant predictor of the changes in RMR. This study also reports a weak correlation between cold- and drug-induced detectable BAT activities. Generally, such data from the same individuals are important to help put the large quantity of cold-induced BAT activation data into a drug-discovery context. It remains to be investigated if the reported energy-expenditure effect is sustained upon chronic dosing and if the efficacy of mirabegron will persist in females and other patient subpopulations, such as those with different ages and BMIs.

The failure of β_3-AR agonists to show clinical effects on weight loss decreased interest in the mechanism as a means of treating the metabolic syndrome. However, the recent rediscovery of BAT in adult humans as well as the demonstration of functional activation of BAT by a β_3-AR agonist may lead to a resurgent interest in β_3-AR agonists for the treatment of metabolic disorders. The observation that improved insulin action preceded any significant weight loss upon β_3-AR agonist treatment is particularly encouraging.

Clearly, important questions remain unanswered with respect to the role of the β_3-AR and the clinical profile of β_3-AR agonists. In order to make significant progress in β_3-AR drug discovery, the lack of translation from rodent to human (i.e., receptor expression and functional differences) and from *in vitro* to *in vivo* for the human setting needs to be resolved. The clinically assessed β_3-AR agonists exhibit structural and physicochemical property diversity; however, clinical plasma exposures relative to EC_{50} are similar for the chronic and acute studies (**Table 1**), and activation

of BAT would therefore be expected in the chronic studies. The lack of effect on EE observed in the chronic studies may either be due to not measuring a mechanism-relevant clinical endpoint or that the patients recruited lacked sufficient BAT to lead to an effect on overall EE. The activation of pre-existing BAT by β_3-AR agonists may require a personalized healthcare approach, unless combined with a compound or mechanism to expand the BAT depot.

Finally, cardiovascular side effects have been associated with β_3-AR agonist treatment, usually attributed to insufficient selectivity toward the β_1- and β_2-ARs. However, the *in vitro* functional activity of many of the clinical compounds assessed does not explain the increase in heart rate observed – several of the compounds are functional β_1-AR antagonists (β_1-AR blockers), and as such could be expected to have the opposite effect to that clinically observed. The complex nature of AR biology needs to be further investigated if this mechanism is to be reconsidered as a means of activating BAT.

FIBROBLAST GROWTH FACTOR 21

Fibroblast growth factor 21 (FGF21) is a member of the endocrine FGF 19/21/23 family. FGF21 protein is expressed in liver, pancreas, and adipose tissue and is regulated by fasting, ketogenic diet, low protein diet, PPARγ, and PPARα activation as well as glucagon action (126). It acts through binding to a cell-surface receptor complex composed of conventional FGF receptors (FGFR1c/2c/3c) and the co-factor β-Klotho, leading to activation of FGF receptor substrate 2α and ERK1/2 phosphorylation. While FGF receptors are ubiquitously expressed, β-Klotho expression is restricted to a few tissues, including BAT, WAT, and liver (127, 128), which are the main sites of FGF21 action. When administered pharmacologically, FGF21 enhances EE and insulin sensitivity, reduces bodyweight, glucose, and lipids, and thus has the potential to be used for treatment of the metabolic syndrome via multiple mechanisms (129–131).

Functional enhancement of existing BAT and recruitment of beige adipocytes have been hypothesized to be the mechanism behind the EE increase, bodyweight loss, and improved glucose and lipid homeostasis induced by FGF21. FGF21 expression is increased in BAT upon cold challenge (132, 133). Adipose-derived FGF21 acts in an autocrine/paracrine manner to increase expression of UCP1 and other thermogenic genes, such as *PGC1*α, *PRDM16*, *BMP8b*, and *DIO2* in iBAT and iWAT (134, 135). In neonates, FGF21 expression in liver is increased by suckling, which occurs via activation of PPARα, leading to induced BAT thermogenesis (136).

Attempts have been made to directly assess the contribution of BAT and beige adipocytes to FGF21-mediated effects. Surgical resection of iBAT in two different mouse models showed that FGF21-mediated EE increase and bodyweight reduction were retained (137, 138). However, one of the studies showed that several beige adipocyte markers, including *PPARγ*, *PRDM16*, *PGC1α*, and *CIDEA* tended toward upregulation in subcutaneous adipose tissue, and perigonadal adipose weight also was reduced (138). The authors propose that residual BAT and beige adipocytes may have compensated for the loss of iBAT. Recently, the role of UCP1 in mediating the pharmacological effects of FGF21 was assessed by treating UCP1 knockout (UCP1 KO) mice with either recombinant FGF21 or FGF21 fused with fragment crystallizable region (Fc-FGF21) (139, 140). Surprisingly, several FGF21-mediated effects were largely retained in the absence of UCP1. Again, compensatory mechanisms seem to be activated in UCP1 KO mice treated with recombinant FGF21. In FGF21-treated UCP1 KO mice, reduced food intake offset the decrease in EE and resulted in a similar bodyweight reduction to that observed in FGF21-treated wild-type mice. In addition, genes regulating fatty acid metabolism were upregulated in liver and epididymal adipose tissue, suggesting that FGF21 recruits UCP1-independent pathways in these tissues to compensate for the lack of UCP1. An intriguing observation was a two- to threefold increase in FGF21 secretion in iBAT when UCP1 KO mice were challenged by cold, suggesting that FGF21 may be one of the factors that recruit alternative thermogenic mechanisms when iBAT/UCP1 fails to generate heat. It should be noted that many previous publications have showed remodeling of the WAT in UCP1 KO mice, and that alternative thermogenic mechanisms have been discussed (141, 142).

In spite of the attractive metabolic effects of FGF21, development of FGF21-based therapeutics has encountered several technical challenges, including short *in vivo* half-life and poor biophysical properties of FGF21 protein (e.g., it is prone to aggregation). To date, three different analogs of FGF21 have advanced to concept testing in human: (130, 143, 144) (i) LY2405319 (an aggregation-resistant FGF21 analog), (ii) PF-05231023 (FGF21 linked to a Fab fragment of a scaffold antibody), and (iii) ARX-618 (PEGylated FGF21). For both LY2405319 and PF-05231023, the effects on TG (close to 50% reduction in 28 days), LDL cholesterol, and HDL cholesterol were substantial and bodyweight reduction was significant. However, the failure of FGF21 analogs to achieve clinically meaningful glucose-lowering effects was unexpected, and the mechanism behind this remains to be understood. Recently, Genentech reported a bispecific monoclonal antibody agonist that binds to both FGFR1c and β-Klotho, with sustained effects on NST, EE, and bodyweight over a 35-day period after a single administration to diet-induced obese mice (145). The effects on glucose and lipids were similar to those observed after administration of recombinant FGF21, and mostly attributed to peripheral FGF21 action as CNS exposure of the antibody was reported to be minimal. Together, these data further support the importance of BAT function in FGF21 action. As for the β_3-AR agonist story, it may be important for future clinical concept testing to assess BAT recruitment/activation in order to understand if sufficiently high FGF21 levels have been achieved in patients. In addition, it is critical to constantly monitor potential safety concerns of this treatment principle in all studies, including bone density, growth hormone resistance, and female fertility.

PERSPECTIVES

The understanding of BAT physiology has increased rapidly in recent years. Data generated in both rodents and humans in

cold/warm acclimation studies suggest that both BAT and beige adipocytes demonstrate recruitability and plasticity, which opens the door to pharmacological intervention. As obese and T2D patients are likely to have reduced BAT function, in order to realize the full potential of NST, it may be necessary to first restore the function of "whitened" brown or beige adipocytes before activation (**Figure 2**). In this respect, future studies using rodent models require consideration of external temperature in order to more closely mimic human physiology. Cold acclimation for a short duration in obese and diabetic humans indicates that it is possible to enhance BAT activity and achieve an improved metabolic profile before significant bodyweight reduction occurs. The improved insulin action in the absence of bodyweight change seen in the 4-week treatment of healthy individuals by CL-316,243 (124) suggests that restoration of BAT function may at least lead to improved metabolic control in patients with T2D. Acute treatment of BAT-positive human subjects with the β_3-AR agonist mirabegron resulted in a significant increase in EE. If this effect is sustained upon chronic dosing and without eliciting cardiovascular side effects, this mechanism may finally lead to significant weight loss.

PPARγ remains an interesting target for the recruitment of BAT or beige adipocytes, although discovery of a compound that does not impair the adrenergic sensitivity or thyroid function of the adipocyte is extremely challenging. Phenotypic screening, for example a screen based on human brown adipocyte function, may however facilitate the discovery of such a compound.

Finally, if an FGF21 analog with appropriate half-life and potency could be developed, it would enable PoC testing in humans in order to understand if the potential beneficial metabolic effects of this hormone can be realized.

AUTHOR CONTRIBUTIONS

XP: conception, structure and content of the text, including the figures. PG: mathematical modeling of the literature data and critical examination and interpretation of pharmacokinetic and pharmacodynamic data on the β_3-AR agonists in clinical studies. Major contribution to the Table 1. GO'M critical review and interpretation of PPARγ and β_3-AR agonists structure, physiochemical properties and assays used for generating the data of all compounds included in Table 1. SB: critical review of bioenergetic of cell biology, general contribution of bioscience-related text, and critical reading and editing of review.

ACKNOWLEDGMENTS

The authors would like to thank Nick Oakes (AstraZeneca) for his contribution to **Figure 1**.

REFERENCES

1. IDF DIABETES ATLAS Sixth edition (2013). Available from: https://www.idf.org/sites/default/files/EN_6E_Atlas_Full_0.pdf
2. Cypess AM, Lehman S, Williams G, Tal I, Rodman D, Goldfine AB, et al. Identification and importance of brown adipose tissue in adult humans. *N Engl J Med* (2009) **360**:1509–17. doi:10.1056/NEJMoa0810780
3. Nedergaard J, Bengtsson T, Cannon B. Unexpected evidence for active brown adipose tissue in adult humans. *Am J Physiol Endocrinol Metab* (2007) **293**:E444–52. doi:10.1152/ajpendo.00691.2006
4. van Marken Lichtenbelt WD, Vanhommerig JW, Smulders NM, Drossaerts JM, Kemerink GJ, Bouvy ND, et al. Cold-activated brown adipose tissue in healthy men. *N Engl J Med* (2009) **360**:1500–8. doi:10.1056/NEJMoa0808718
5. Virtanen KA, Lidell ME, Orava J, Heglind M, Westergren R, Niemi T, et al. Functional brown adipose tissue in healthy adults. *N Engl J Med* (2009) **360**:1518–25. doi:10.1056/NEJMoa0808949
6. Betz MJ, Enerback S. Human brown adipose tissue: what we have learned so far. *Diabetes* (2015) **64**:2352–60. doi:10.2337/db15-0146
7. Blondin DP, Labbe SM, Phoenix S, Guerin B, Turcotte EE, Richard D, et al. Contributions of white and brown adipose tissues and skeletal muscles to acute cold-induced metabolic responses in healthy men. *J Physiol* (2015) **593**:701–14. doi:10.1113/jphysiol.2014.283598
8. Chechi K, Carpentier AC, Richard D. Understanding the brown adipocyte as a contributor to energy homeostasis. *Trends Endocrinol Metab* (2013) **24**:408–20. doi:10.1016/j.tem.2013.04.002
9. Harms M, Seale P. Brown and beige fat: development, function and therapeutic potential. *Nat Med* (2013) **19**:1252–63. doi:10.1038/nm.3361
10. Nedergaard J, Cannon B. The browning of white adipose tissue: some burning issues. *Cell Metab* (2014) **20**:396–407. doi:10.1016/j.cmet.2014.07.005
11. van Marken Lichtenbelt WD, Schrauwen P. Implications of nonshivering thermogenesis for energy balance regulation in humans. *Am J Physiol Regul Integr Comp Physiol* (2011) **301**:R285–96. doi:10.1152/ajpregu.00652.2010
12. Vosselman MJ, van Marken Lichtenbelt WD, Schrauwen P. Energy dissipation in brown adipose tissue: from mice to men. *Mol Cell Endocrinol* (2013) **379**:43–50. doi:10.1016/j.mce.2013.04.017
13. Cannon B, Nedergaard J. Brown adipose tissue: function and physiological significance. *Physiol Rev* (2004) **84**:277–359. doi:10.1152/physrev.00015.2003
14. Enerback S. Human brown adipose tissue. *Cell Metab* (2010) **11**:248–52. doi:10.1016/j.cmet.2010.03.008
15. Lidell ME, Betz MJ, Dahlqvist Leinhard O, Heglind M, Elander L, Slawik M, et al. Evidence for two types of brown adipose tissue in humans. *Nat Med* (2013) **19**:631–4. doi:10.1038/nm.3017
16. Frontini A, Vitali A, Perugini J, Murano I, Romiti C, Ricquier D, et al. White-to-brown transdifferentiation of omental adipocytes in patients affected by pheochromocytoma. *Biochim Biophys Acta* (2013) **1831**:950–9. doi:10.1016/j.bbalip.2013.02.005
17. Ricquier D, Nechad M, Mory G. Ultrastructural and biochemical characterization of human brown adipose tissue in pheochromocytoma. *J Clin Endocrinol Metab* (1982) **54**:803–7.
18. Sidossis LS, Porter C, Saraf MK, Borsheim E, Radhakrishnan RS, Chao T, et al. Browning of subcutaneous white adipose tissue in humans after severe adrenergic stress. *Cell Metab* (2015) **22**:219–27. doi:10.1016/j.cmet.2015.06.022
19. Seale P, Bjork B, Yang W, Kajimura S, Chin S, Kuang S, et al. PRDM16 controls a brown fat/skeletal muscle switch. *Nature* (2008) **454**:961–7. doi:10.1038/nature07182
20. Timmons JA, Wennmalm K, Larsson O, Walden TB, Lassmann T, Petrovic N, et al. Myogenic gene expression signature establishes that brown and white adipocytes originate from distinct cell lineages. *Proc Natl Acad Sci U S A* (2007) **104**:4401–6. doi:10.1073/pnas.0610615104
21. Wang Q, Zhang M, Xu M, Gu W, Xi Y, Qi L, et al. Brown adipose tissue activation is inversely related to central obesity and metabolic parameters in adult human. *PLoS One* (2015) **10**:e0123795. doi:10.1371/journal.pone.0123795
22. Saito M, Okamatsu-Ogura Y, Matsushita M, Watanabe K, Yoneshiro T, Nio-Kobayashi J, et al. High incidence of metabolically active brown adipose

tissue in healthy adult humans: effects of cold exposure and adiposity. *Diabetes* (2009) **58**:1526–31. doi:10.2337/db09-0530
23. Persichetti A, Sciuto R, Rea S, Basciani S, Lubrano C, Mariani S, et al. Prevalence, mass, and glucose-uptake activity of (1)(8)F-FDG-detected brown adipose tissue in humans living in a temperate zone of Italy. *PLoS One* (2013) **8**:e63391. doi:10.1371/journal.pone.0063391
24. Lee P, Greenfield JR, Ho KK, Fulham MJ. A critical appraisal of the prevalence and metabolic significance of brown adipose tissue in adult humans. *Am J Physiol Endocrinol Metab* (2010) **299**:E601–6. doi:10.1152/ajpendo.00298.2010
25. Lee P, Swarbrick MM, Zhao JT, Ho KK. Inducible brown adipogenesis of supraclavicular fat in adult humans. *Endocrinology* (2011) **152**:3597–602. doi:10.1210/en.2011-1349
26. Ouellet V, Labbe SM, Blondin DP, Phoenix S, Guerin B, Haman F, et al. Brown adipose tissue oxidative metabolism contributes to energy expenditure during acute cold exposure in humans. *J Clin Invest* (2012) **122**:545–52. doi:10.1172/JCI60433
27. Vijgen GH, Bouvy ND, Teule GJ, Brans B, Hoeks J, Schrauwen P, et al. Increase in brown adipose tissue activity after weight loss in morbidly obese subjects. *J Clin Endocrinol Metab* (2012) **97**:E1229–33. doi:10.1210/jc.2012-1289
28. Cypess AM, White AP, Vernochet C, Schulz TJ, Xue R, Sass CA, et al. Anatomical localization, gene expression profiling and functional characterization of adult human neck brown fat. *Nat Med* (2013) **19**:635–9. doi:10.1038/nm.3112
29. Jespersen NZ, Larsen TJ, Peijs L, Daugaard S, Homoe P, Loft A, et al. A classical brown adipose tissue mRNA signature partly overlaps with brite in the supraclavicular region of adult humans. *Cell Metab* (2013) **17**:798–805. doi:10.1016/j.cmet.2013.04.011
30. Sharp LZ, Shinoda K, Ohno H, Scheel DW, Tomoda E, Ruiz L, et al. Human BAT possesses molecular signatures that resemble beige/brite cells. *PLoS One* (2012) **7**:e49452. doi:10.1371/journal.pone.0049452
31. Wu J, Bostrom P, Sparks LM, Ye L, Choi JH, Giang AH, et al. Beige adipocytes are a distinct type of thermogenic fat cell in mouse and human. *Cell* (2012) **150**:366–76. doi:10.1016/j.cell.2012.05.016
32. Bartelt A, Bruns OT, Reimer R, Hohenberg H, Ittrich H, Peldschus K, et al. Brown adipose tissue activity controls triglyceride clearance. *Nat Med* (2011) **17**:200–5. doi:10.1038/nm.2297
33. Labbe SM, Caron A, Bakan I, Laplante M, Carpentier AC, Lecomte R, et al. In vivo measurement of energy substrate contribution to cold-induced brown adipose tissue thermogenesis. *FASEB J* (2015) **29**:2046–58. doi:10.1096/fj.14-266247
34. Bartelt A, Heeren J. Adipose tissue browning and metabolic health. *Nat Rev Endocrinol* (2014) **10**:24–36. doi:10.1038/nrendo.2013.204
35. Seale P, Conroe HM, Estall J, Kajimura S, Frontini A, Ishibashi J, et al. Prdm16 determines the thermogenic program of subcutaneous white adipose tissue in mice. *J Clin Invest* (2011) **121**:96–105. doi:10.1172/JCI44271
36. Abreu-Vieira G, Xiao C, Gavrilova O, Reitman ML. Integration of body temperature into the analysis of energy expenditure in the mouse. *Mol Metab* (2015) **4**:461–70. doi:10.1016/j.molmet.2015.03.001
37. Rothwell NJ, Stock MJ. Whither brown fat? *Biosci Rep* (1986) **6**:3–18. doi:10.1007/BF01145174
38. Hall KD, Sacks G, Chandramohan D, Chow CC, Wang YC, Gortmaker SL, et al. Quantification of the effect of energy imbalance on bodyweight. *Lancet* (2011) **378**:826–37. doi:10.1016/S0140-6736(11)60812-X
39. Gennemark P, Hjorth S, Gabrielsson J. Modeling energy intake by adding homeostatic feedback and drug intervention. *J Pharmacokinet Pharmacodyn* (2015) **42**:79–96. doi:10.1007/s10928-014-9399-4
40. Cannon B, Nedergaard J. Thermogenesis challenges the adipostat hypothesis for body-weight control. *Proc Nutr Soc* (2009) **68**:401–7. doi:10.1017/S0029665109990255
41. Cypess AM, Weiner LS, Roberts-Toler C, Franquet Elia E, Kessler SH, Kahn PA, et al. Activation of human brown adipose tissue by a beta3-adrenergic receptor agonist. *Cell Metab* (2015) **21**:33–8. doi:10.1016/j.cmet.2014.12.009
42. McAdam-Marx C, Mukherjee J, Bellows BK, Unni S, Ye X, Iloeje U, et al. Evaluation of the relationship between weight change and glycemic control after initiation of antidiabetic therapy in patients with type 2 diabetes using electronic medical record data. *Diabetes Res Clin Pract* (2014) **103**:402–11. doi:10.1016/j.diabres.2013.12.038
43. Hanssen MJ, Hoeks J, Brans B, van der Lans AA, Schaart G, van den Driessche JJ, et al. Short-term cold acclimation improves insulin sensitivity in patients with type 2 diabetes mellitus. *Nat Med* (2015) **21**:863–5. doi:10.1038/nm.3891
44. Merovci A, Solis-Herrera C, Daniele G, Eldor R, Fiorentino TV, Tripathy D, et al. Dapagliflozin improves muscle insulin sensitivity but enhances endogenous glucose production. *J Clin Invest* (2014) **124**:509–14. doi:10.1172/JCI70704
45. Bartesaghi S, Hallen S, Huang L, Svensson PA, Momo RA, Wallin S, et al. Thermogenic activity of UCP1 in human white fat-derived beige adipocytes. *Mol Endocrinol* (2015) **29**:130–9. doi:10.1210/me.2014-1295
46. Shabalina IG, Petrovic N, de Jong JM, Kalinovich AV, Cannon B, Nedergaard J. UCP1 in brite/beige adipose tissue mitochondria is functionally thermogenic. *Cell Rep* (2013) **5**:1196–203. doi:10.1016/j.celrep.2013.10.044
47. Seale P. Transcriptional regulatory circuits controlling brown fat development and activation. *Diabetes* (2015) **64**:2369–75. doi:10.2337/db15-0203
48. Wu J, Jun H, McDermott JR. Formation and activation of thermogenic fat. *Trends Genet* (2015) **31**:232–8. doi:10.1016/j.tig.2015.03.003
49. Festuccia WT, Blanchard PG, Deshaies Y. Control of brown adipose tissue glucose and lipid metabolism by PPARgamma. *Front Endocrinol* (2011) **2**:84. doi:10.3389/fendo.2011.00084
50. Arch JR. The discovery of drugs for obesity, the metabolic effects of leptin and variable receptor pharmacology: perspectives from beta3-adrenoceptor agonists. *Naunyn Schmiedebergs Arch Pharmacol* (2008) **378**:225–40. doi:10.1007/s00210-008-0271-1
51. Atgie C, D'Allaire F, Bukowiecki LJ. Role of beta1- and beta3-adrenoceptors in the regulation of lipolysis and thermogenesis in rat brown adipocytes. *Am J Physiol* (1997) **273**:C1136–42.
52. Granneman JG. Why do adipocytes make the beta 3 adrenergic receptor? *Cell Signal* (1995) **7**:9–15. doi:10.1016/0898-6568(94)00066-K
53. Lowell BB, Flier JS. Brown adipose tissue, beta 3-adrenergic receptors, and obesity. *Annu Rev Med* (1997) **48**:307–16. doi:10.1146/annurev.med.48.1.307
54. Muzzin P, Revelli JP, Kuhne F, Gocayne JD, McCombie WR, Venter JC, et al. An adipose tissue-specific beta-adrenergic receptor. Molecular cloning and down-regulation in obesity. *J Biol Chem* (1991) **266**:24053–8.
55. Carey AL, Formosa MF, Van Every B, Bertovic D, Eikelis N, Lambert GW, et al. Ephedrine activates brown adipose tissue in lean but not obese humans. *Diabetologia* (2013) **56**:147–55. doi:10.1007/s00125-012-2748-1
56. Cypess AM, Chen YC, Sze C, Wang K, English J, Chan O, et al. Cold but not sympathomimetics activates human brown adipose tissue in vivo. *Proc Natl Acad Sci U S A* (2012) **109**:10001–5. doi:10.1073/pnas.1207911109
57. Vosselman MJ, van der Lans AA, Brans B, Wierts R, van Baak MA, Schrauwen P, et al. Systemic beta-adrenergic stimulation of thermogenesis is not accompanied by brown adipose tissue activity in humans. *Diabetes* (2012) **61**:3106–13. doi:10.2337/db12-0288
58. Nicholls DG. A history of UCP1. *Biochem Soc Trans* (2001) **29**:751–5. doi:10.1042/bst0290751
59. Ahmadian M, Abbott MJ, Tang T, Hudak CS, Kim Y, Bruss M, et al. Desnutrin/ATGL is regulated by AMPK and is required for a brown adipose phenotype. *Cell Metab* (2011) **13**:739–48. doi:10.1016/j.cmet.2011.05.002
60. Li Y, Fromme T, Schweizer S, Schottl T, Klingenspor M. Taking control over intracellular fatty acid levels is essential for the analysis of thermogenic function in cultured primary brown and brite/beige adipocytes. *EMBO Rep* (2014) **15**:1069–76. doi:10.15252/embr.201438775
61. Mottillo EP, Bloch AE, Leff T, Granneman JG. Lipolytic products activate peroxisome proliferator-activated receptor (PPAR) alpha and delta in brown adipocytes to match fatty acid oxidation with supply. *J Biol Chem* (2012) **287**:25038–48. doi:10.1074/jbc.M112.374041
62. Zechner R. FLUX FAT: enzymes, regulators, and pathophysiology of intracellular lipolysis. *EMBO Mol Med* (2015) **7**:359–62. doi:10.15252/emmm.201404846
63. Kajimura S, Saito M. A new era in brown adipose tissue biology: molecular control of brown fat development and energy homeostasis. *Annu Rev Physiol* (2014) **76**:225–49. doi:10.1146/annurev-physiol-021113-170252

64. Collins S, Daniel KW, Rohlfs EM, Ramkumar V, Taylor IL, Gettys TW. Impaired expression and functional activity of the beta 3- and beta 1-adrenergic receptors in adipose tissue of congenitally obese (C57BL/6J ob/ob) mice. *Mol Endocrinol* (1994) **8**:518–27. doi:10.1210/me.8.4.518
65. Triandafillou J, Himms-Hagen J. Brown adipose tissue in genetically obese (fa/fa) rats: response to cold and diet. *Am J Physiol* (1983) **244**:E145–50.
66. Rogers NH, Landa A, Park S, Smith RG. Aging leads to a programmed loss of brown adipocytes in murine subcutaneous white adipose tissue. *Aging Cell* (2012) **11**:1074–83. doi:10.1111/acel.12010
67. Rafael J, Fesser W, Nicholls DG. Cold adaptation in guinea pig at level of isolated brown adipocyte. *Am J Physiol* (1986) **250**:C228–35.
68. Blondin DP, Labbe SM, Noll C, Kunach M, Phoenix S, Guerin B, et al. Selective impairment of glucose but not fatty acid or oxidative metabolism in brown adipose tissue of subjects with type 2 diabetes. *Diabetes* (2015) **64**:2388–97. doi:10.2337/db14-1651
69. Blondin DP, Labbe SM, Tingelstad HC, Noll C, Kunach M, Phoenix S, et al. Increased brown adipose tissue oxidative capacity in cold-acclimated humans. *J Clin Endocrinol Metab* (2014) **99**:E438–46. doi:10.1210/jc.2013-3901
70. Lee P, Smith S, Linderman J, Courville AB, Brychta RJ, Dieckmann W, et al. Temperature-acclimated brown adipose tissue modulates insulin sensitivity in humans. *Diabetes* (2014) **63**:3686–98. doi:10.2337/db14-0513
71. Rosenwald M, Perdikari A, Rulicke T, Wolfrum C. Bi-directional interconversion of brite and white adipocytes. *Nat Cell Biol* (2013) **15**:659–67. doi:10.1038/ncb2740
72. Soccio RE, Chen ER, Lazar MA. Thiazolidinediones and the promise of insulin sensitization in type 2 diabetes. *Cell Metab* (2014) **20**:573–91. doi:10.1016/j.cmet.2014.08.005
73. Oakes ND, Thalen PG, Jacinto SM, Ljung B. Thiazolidinediones increase plasma-adipose tissue FFA exchange capacity and enhance insulin-mediated control of systemic FFA availability. *Diabetes* (2001) **50**:1158–65. doi:10.2337/diabetes.50.5.1158
74. Digby JE, Montague CT, Sewter CP, Sanders L, Wilkison WO, O'Rahilly S, et al. Thiazolidinedione exposure increases the expression of uncoupling protein 1 in cultured human preadipocytes. *Diabetes* (1998) **47**:138–41. doi:10.2337/diab.47.1.138
75. Elabd C, Chiellini C, Carmona M, Galitzky J, Cochet O, Petersen R, et al. Human multipotent adipose-derived stem cells differentiate into functional brown adipocytes. *Stem Cells* (2009) **27**:2753–60. doi:10.1002/stem.200
76. Ohno H, Shinoda K, Spiegelman BM, Kajimura S. PPARgamma agonists induce a white-to-brown fat conversion through stabilization of PRDM16 protein. *Cell Metab* (2012) **15**:395–404. doi:10.1016/j.cmet.2012.01.019
77. Petrovic N, Shabalina IG, Timmons JA, Cannon B, Nedergaard J. Thermogenically competent nonadrenergic recruitment in brown preadipocytes by a PPARgamma agonist. *Am J Physiol Endocrinol Metab* (2008) **295**:E287–96. doi:10.1152/ajpendo.00035.2008
78. Sears DD, Hsiao G, Hsiao A, Yu JG, Courtney CH, Ofrecio JM, et al. Mechanisms of human insulin resistance and thiazolidinedione-mediated insulin sensitization. *Proc Natl Acad Sci U S A* (2009) **106**:18745–50. doi:10.1073/pnas.0903032106
79. Burkey BF, Dong M, Gagen K, Eckhardt M, Dragonas N, Chen W, et al. Effects of pioglitazone on promoting energy storage, not expenditure, in brown adipose tissue of obese fa/fa Zucker rats: comparison to CL 316,243. *Metabolism* (2000) **49**:1301–8. doi:10.1053/meta.2000.9524
80. Kelly LJ, Vicario PP, Thompson GM, Candelore MR, Doebber TW, Ventre J, et al. Peroxisome proliferator-activated receptors gamma and alpha mediate in vivo regulation of uncoupling protein (UCP-1, UCP-2, UCP-3) gene expression. *Endocrinology* (1998) **139**:4920–7. doi:10.1210/en.139.12.4920
81. Smith SR, De Jonge L, Volaufova J, Li Y, Xie H, Bray GA. Effect of pioglitazone on body composition and energy expenditure: a randomized controlled trial. *Metabolism* (2005) **54**:24–32. doi:10.1016/j.metabol.2004.07.008
82. Sell H, Berger JP, Samson P, Castriota G, Lalonde J, Deshaies Y, et al. Peroxisome proliferator-activated receptor gamma agonism increases the capacity for sympathetically mediated thermogenesis in lean and ob/ob mice. *Endocrinology* (2004) **145**:3925–34. doi:10.1210/en.2004-0321
83. Festuccia WT, Blanchard PG, Oliveira TB, Magdalon J, Paschoal VA, Richard D, et al. PPARgamma activation attenuates cold-induced upregulation of thyroid status and brown adipose tissue PGC-1alpha and D2. *Am J Physiol Regul Integr Comp Physiol* (2012) **303**:R1277–85. doi:10.1152/ajpregu.00299.2012
84. Festuccia WT, Blanchard PG, Turcotte V, Laplante M, Sariahmetoglu M, Brindley DN, et al. The PPARgamma agonist rosiglitazone enhances rat brown adipose tissue lipogenesis from glucose without altering glucose uptake. *Am J Physiol Regul Integr Comp Physiol* (2009) **296**:R1327–35. doi:10.1152/ajpregu.91012.2008
85. Bakopanos E, Silva JE. Thiazolidinediones inhibit the expression of beta3-adrenergic receptors at a transcriptional level. *Diabetes* (2000) **49**:2108–15. doi:10.2337/diabetes.49.12.2108
86. Festuccia WT, Oztezcan S, Laplante M, Berthiaume M, Michel C, Dohgu S, et al. Peroxisome proliferator-activated receptor-gamma-mediated positive energy balance in the rat is associated with reduced sympathetic drive to adipose tissues and thyroid status. *Endocrinology* (2008) **149**:2121–30. doi:10.1210/en.2007-1553
87. Bogacka I, Ukropcova B, McNeil M, Gimble JM, Smith SR. Structural and functional consequences of mitochondrial biogenesis in human adipocytes in vitro. *J Clin Endocrinol Metab* (2005) **90**:6650–6. doi:10.1210/jc.2005-1024
88. Bogacka I, Gettys TW, de Jonge L, Nguyen T, Smith JM, Xie H, et al. The effect of beta-adrenergic and peroxisome proliferator-activated receptor-gamma stimulation on target genes related to lipid metabolism in human subcutaneous adipose tissue. *Diabetes Care* (2007) **30**:1179–86. doi:10.2337/dc06-1962
89. Nettles KW, Greene GL. Ligand control of coregulator recruitment to nuclear receptors. *Annu Rev Physiol* (2005) **67**:309–33. doi:10.1146/annurev.physiol.66.032802.154710
90. Lehmann JM, Moore LB, Smith-Oliver TA, Wilkison WO, Willson TM, Kliewer SA. An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor gamma (PPAR gamma). *J Biol Chem* (1995) **270**:12953–6. doi:10.1074/jbc.270.22.12953
91. Cho N, Momose Y. Peroxisome proliferator-activated receptor gamma agonists as insulin sensitizers: from the discovery to recent progress. *Curr Top Med Chem* (2008) **8**:1483–507. doi:10.2174/156802608786413474
92. Henke BR. 1. Peroxisome proliferator-activated receptor gamma (PPARgamma) ligands and their therapeutic utility. *Prog Med Chem* (2004) **42**:1–53. doi:10.1016/S0079-6468(04)42001-3
93. Nevin DK, Lloyd DG, Fayne D. Rational targeting of peroxisome proliferating activated receptor subtypes. *Curr Med Chem* (2011) **18**:5598–623. doi:10.2174/092986711798347243
94. Savkur RS, Miller AR. Investigational PPAR-gamma agonists for the treatment of Type 2 diabetes. *Expert Opin Investig Drugs* (2006) **15**:763–78. doi:10.1517/13543784.15.7.763
95. Takada I, Makishima M. PPARgamma ligands and their therapeutic applications: a patent review (2008-2014). *Expert Opin Ther Pat* (2015) **25**:175–91. doi:10.1517/13543776.2014.985206
96. Willson TM, Brown PJ, Sternbach DD, Henke BR. The PPARs: from orphan receptors to drug discovery. *J Med Chem* (2000) **43**:527–50. doi:10.1021/jm990554g
97. Garcia-Vallve S, Guasch L, Tomas-Hernandez S, Del Bas JM, Ollendorff V, Arola L, et al. Peroxisome proliferator-activated receptor gamma (PPARgamma) and ligand choreography: newcomers take the stage. *J Med Chem* (2015) **58**:5381–94. doi:10.1021/jm501155f
98. Abbas A, Blandon J, Rude J, Elfar A, Mukherjee D. PPAR-gamma agonist in treatment of diabetes: cardiovascular safety considerations. *Cardiovasc Hematol Agents Med Chem* (2012) **10**:124–34. doi:10.2174/18715251280038948
99. Bortolini M, Wright MB, Bopst M, Balas B. Examining the safety of PPAR agonists – current trends and future prospects. *Expert Opin Drug Saf* (2013) **12**:65–79. doi:10.1517/14740338.2013.741585
100. Rubenstrunk A, Hanf R, Hum DW, Fruchart JC, Staels B. Safety issues and prospects for future generations of PPAR modulators. *Biochim Biophys Acta* (2007) **1771**:1065–81. doi:10.1016/j.bbalip.2007.02.003
101. Tang WH, Maroo A. PPARgamma agonists: safety issues in heart failure. *Diabetes Obes Metab* (2007) **9**:447–54. doi:10.1111/j.1463-1326.2006.00616.x
102. Germain P, Iyer J, Zechel C, Gronemeyer H. Co-regulator recruitment and the mechanism of retinoic acid receptor synergy. *Nature* (2002) **415**:187–92. doi:10.1038/415187a
103. Hamza MS, Pott S, Vega VB, Thomsen JS, Kandhadayar GS, Ng PW, et al. De-novo identification of PPARgamma/RXR binding sites and direct targets during adipogenesis. *PLoS One* (2009) **4**:e4907. doi:10.1371/journal.pone.0004907
104. Bulynko YA, O'Malley BW. Nuclear receptor coactivators: structural and functional biochemistry. *Biochemistry* (2011) **50**:313–28. doi:10.1021/bi101762x

105. Lonard DM, O'Malley BW. Nuclear receptor coregulators: judges, juries, and executioners of cellular regulation. *Mol Cell* (2007) **27**:691–700. doi:10.1016/j.molcel.2007.08.012
106. Millard CJ, Watson PJ, Fairall L, Schwabe JW. An evolving understanding of nuclear receptor coregulator proteins. *J Mol Endocrinol* (2013) **51**:T23–36. doi:10.1530/JME-13-0227
107. Nolte RT, Wisely GB, Westin S, Cobb JE, Lambert MH, Kurokawa R, et al. Ligand binding and co-activator assembly of the peroxisome proliferator-activated receptor-gamma. *Nature* (1998) **395**:137–43. doi:10.1038/25931
108. Ahmadian M, Suh JM, Hah N, Liddle C, Atkins AR, Downes M, et al. PPARgamma signaling and metabolism: the good, the bad and the future. *Nat Med* (2013) **19**:557–66. doi:10.1038/nm.3159
109. Banks AS, McAllister FE, Camporez JP, Zushin PJ, Jurczak MJ, Laznik-Bogoslavski D, et al. An ERK/Cdk5 axis controls the diabetogenic actions of PPARgamma. *Nature* (2015) **517**:391–5. doi:10.1038/nature13887
110. Moisan A, Lee YK, Zhang JD, Hudak CS, Meyer CA, Prummer M, et al. White-to-brown metabolic conversion of human adipocytes by JAK inhibition. *Nat Cell Biol* (2015) **17**:57–67. doi:10.1038/ncb3075
111. Dolan JA, Muenkel HA, Burns MG, Pellegrino SM, Fraser CM, Pietri F, et al. Beta-3 adrenoceptor selectivity of the dioxolane dicarboxylate phenethanolamines. *J Pharmacol Exp Ther* (1994) **269**:1000–6.
112. Emorine LJ, Marullo S, Briend-Sutren MM, Patey G, Tate K, Delavier-Klutchko C, et al. Molecular characterization of the human beta 3-adrenergic receptor. *Science* (1989) **245**:1118–21. doi:10.1126/science.2570461
113. Lelias JM, Kaghad M, Rodriguez M, Chalon P, Bonnin J, Dupre I, et al. Molecular cloning of a human beta 3-adrenergic receptor cDNA. *FEBS Lett* (1993) **324**:127–30. doi:10.1016/0014-5793(93)81377-C
114. Hu B, Ellingboe J, Han S, Largis E, Lim K, Malamas M, et al. Novel (4-piperidin-1-yl)-phenyl sulfonamides as potent and selective human beta(3) agonists. *Bioorg Med Chem* (2001) **9**:2045–59. doi:10.1016/S0968-0896(01)00114-6
115. Buemann B, Toubro S, Astrup A. Effects of the two beta3-agonists, ZD7114 and ZD2079 on 24 hour energy expenditure and respiratory quotient in obese subjects. *Int J Obes Relat Metab Disord* (2000) **24**:1553–60. doi:10.1038/sj.ijo.0801452
116. Pietri-Rouxel F, Strosberg AD. Pharmacological characteristics and species-related variations of beta 3-adrenergic receptors. *Fundam Clin Pharmacol* (1995) **9**:211–8. doi:10.1111/j.1472-8206.1995.tb00288.x
117. Mathvink RJ, Tolman JS, Chitty D, Candelore MR, Cascieri MA, Colwell LF Jr, et al. Discovery of a potent, orally bioavailable beta(3) adrenergic receptor agonist, (R)-N-[4-[2-[[2-hydroxy-2-(3-pyridinyl)ethyl]amino]ethyl]phenyl]-4-[4 -[4-(trifluoromethyl)phenyl]thiazol-2-yl]benzenesulfonamide. *J Med Chem* (2000) **43**:3832–6. doi:10.1021/jm000286i
118. Harada H, Hirokawa Y, Suzuki K, Hiyama Y, Oue M, Kawashima H, et al. Discovery of a novel and potent human and rat beta3-adrenergic receptor agonist, [3-[(2R)-[[(2R)-(3-chlorophenyl)-2-hydroxyethyl]amino]propyl]-1H-indol-7-yloxy]acetic acid. *Chem Pharm Bull* (2005) **53**:184–98. doi:10.1248/cpb.53.184
119. Takasu T, Ukai M, Sato S, Matsui T, Nagase I, Maruyama T, et al. Effect of (R)-2-(2-aminothiazol-4-yl)-4'-{2-[(2-hydroxy-2-phenylethyl)amino]ethyl} acetanilide (YM178), a novel selective beta3-adrenoceptor agonist, on bladder function. *J Pharmacol Exp Ther* (2007) **321**:642–7. doi:10.1124/jpet.106.115840
120. Blin N, Nahmias C, Drumare MF, Strosberg AD. Mediation of most atypical effects by species homologues of the beta 3-adrenoceptor. *Br J Pharmacol* (1994) **112**:911–9. doi:10.1111/j.1476-5381.1994.tb13167.x
121. Larsen TM, Toubro S, van Baak MA, Gottesdiener KM, Larson P, Saris WH, et al. Effect of a 28-d treatment with L-796568, a novel beta(3)-adrenergic receptor agonist, on energy expenditure and body composition in obese men. *Am J Clin Nutr* (2002) **76**:780–8.
122. Redman LM, de Jonge L, Fang X, Gamlin B, Recker D, Greenway FL, et al. Lack of an effect of a novel beta3-adrenoceptor agonist, TAK-677, on energy metabolism in obese individuals: a double-blind, placebo-controlled randomized study. *J Clin Endocrinol Metab* (2007) **92**:527–31. doi:10.1210/jc.2006-1740
123. Champigny O, Ricquier D, Blondel O, Mayers RM, Briscoe MG, Holloway BR. Beta 3-adrenergic receptor stimulation restores message and expression of brown-fat mitochondrial uncoupling protein in adult dogs. *Proc Natl Acad Sci U S A* (1991) **88**:10774–7. doi:10.1073/pnas.88.23.10774
124. Weyer C, Tataranni PA, Snitker S, Danforth E Jr, Ravussin E. Increase in insulin action and fat oxidation after treatment with CL 316,243, a highly selective beta3-adrenoceptor agonist in humans. *Diabetes* (1998) **47**:1555–61. doi:10.2337/diabetes.47.10.1555
125. van Baak MA, Hul GB, Toubro S, Astrup A, Gottesdiener KM, DeSmet M, et al. Acute effect of L-796568, a novel beta 3-adrenergic receptor agonist, on energy expenditure in obese men. *Clin Pharmacol Ther* (2002) **71**:272–9. doi:10.1067/mcp.2002.122527
126. Laeger T, Henagan TM, Albarado DC, Redman LM, Bray GA, Noland RC, et al. FGF21 is an endocrine signal of protein restriction. *J Clin Invest* (2014) **124**:3913–22. doi:10.1172/JCI74915
127. Adams AC, Cheng CC, Coskun T, Kharitonenkov A. FGF21 requires betaklotho to act in vivo. *PLoS One* (2012) **7**:e49977. doi:10.1371/journal.pone.0049977
128. Ding X, Boney-Montoya J, Owen BM, Bookout AL, Coate KC, Mangelsdorf DJ, et al. betaKlotho is required for fibroblast growth factor 21 effects on growth and metabolism. *Cell Metab* (2012) **16**:387–93. doi:10.1016/j.cmet.2012.08.002
129. Angelin B, Larsson TE, Rudling M. Circulating fibroblast growth factors as metabolic regulators – a critical appraisal. *Cell Metab* (2012) **16**:693–705. doi:10.1016/j.cmet.2012.11.001
130. Gimeno RE, Moller DE. FGF21-based pharmacotherapy – potential utility for metabolic disorders. *Trends Endocrinol Metab* (2014) **25**:303–11. doi:10.1016/j.tem.2014.03.001
131. Owen BM, Ding X, Morgan DA, Coate KC, Bookout AL, Rahmouni K, et al. FGF21 acts centrally to induce sympathetic nerve activity, energy expenditure, and weight loss. *Cell Metab* (2014) **20**:670–7. doi:10.1016/j.cmet.2014.07.012
132. Chartoumpekis DV, Habeos IG, Ziros PG, Psyrogiannis AI, Kyriazopoulou VE, Papavassiliou AG. Brown adipose tissue responds to cold and adrenergic stimulation by induction of FGF21. *Mol Med* (2011) **17**:736–40. doi:10.2119/molmed.2011.00075
133. Hanssen MJ, Broeders E, Samms RJ, Vosselman MJ, van der Lans AA, Cheng CC, et al. Serum FGF21 levels are associated with brown adipose tissue activity in humans. *Sci Rep* (2015) **5**:10275. doi:10.1038/srep10275
134. Fisher FM, Kleiner S, Douris N, Fox EC, Mepani RJ, Verdeguer F, et al. FGF21 regulates PGC-1alpha and browning of white adipose tissues in adaptive thermogenesis. *Genes Dev* (2012) **26**:271–81. doi:10.1101/gad.177857.111
135. Lee P, Linderman JD, Smith S, Brychta RJ, Wang J, Idelson C, et al. Irisin and FGF21 are cold-induced endocrine activators of brown fat function in humans. *Cell Metab* (2014) **19**:302–9. doi:10.1016/j.cmet.2013.12.017
136. Hondares E, Gallego-Escuredo JM, Flachs P, Frontini A, Cereijo R, Goday A, et al. Fibroblast growth factor-21 is expressed in neonatal and pheochromocytoma-induced adult human brown adipose tissue. *Metabolism* (2014) **63**:312–7. doi:10.1016/j.metabol.2013.11.014
137. Camporez JP, Jornayvaz FR, Petersen MC, Pesta D, Guigni BA, Serr J, et al. Cellular mechanisms by which FGF21 improves insulin sensitivity in male mice. *Endocrinology* (2013) **154**:3099–109. doi:10.1210/en.2013-1191
138. Emanuelli B, Vienberg SG, Smyth G, Cheng C, Stanford KI, Arumugam M, et al. Interplay between FGF21 and insulin action in the liver regulates metabolism. *J Clin Invest* (2015) **125**:458. doi:10.1172/JCI80223
139. Samms RJ, Smith DP, Cheng CC, Antonellis PP, Perfield JW II, Kharitonenkov A, et al. Discrete aspects of FGF21 in vivo pharmacology do not require UCP1. *Cell Rep* (2015) **11**:991–9. doi:10.1016/j.celrep.2015.04.046
140. Veniant MM, Sivits G, Helmering J, Komorowski R, Lee J, Fan W, et al. Pharmacologic effects of FGF21 are independent of the "browning" of white adipose tissue. *Cell Metab* (2015) **21**:731–8. doi:10.1016/j.cmet.2015.04.019
141. Keipert S, Kutschke M, Lamp D, Brachthauser L, Neff F, Meyer CW, et al. Genetic disruption of uncoupling protein 1 in mice renders brown adipose tissue a significant source of FGF21 secretion. *Mol Metab* (2015) **4**:537–42. doi:10.1016/j.molmet.2015.04.006
142. Ukropec J, Anunciado RP, Ravussin Y, Hulver MW, Kozak LP. UCP1-independent thermogenesis in white adipose tissue of cold-acclimated Ucp1-/- mice. *J Biol Chem* (2006) **281**:31894–908. doi:10.1074/jbc.M606114200

143. Dong JQ, Rossulek M, Somayaji VR, Baltrukonis D, Liang Y, Hudson K, et al. Pharmacokinetics and pharmacodynamics of PF-05231023, a novel long-acting FGF21 mimetic, in a first-in-human study. *Br J Clin Pharmacol* (2015) **80**(5):1051–63. doi:10.1111/bcp.12676
144. Gaich G, Chien JY, Fu H, Glass LC, Deeg MA, Holland WL, et al. The effects of LY2405319, an FGF21 analog, in obese human subjects with type 2 diabetes. *Cell Metab* (2013) **18**:333–40. doi:10.1016/j.cmet.2013.08.005
145. Kolumam G, Chen MZ, Tong R, Zavala-Solorio J, Kates L, van Bruggen N, et al. Sustained brown fat stimulation and insulin sensitization by a humanized bispecific antibody agonist for fibroblast growth factor receptor 1/betaKlotho complex. *EBioMedicine* (2015) **2**:730–43. doi:10.1016/j.ebiom.2015.05.028

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

REVIEW
published: 13 October 2015
doi: 10.3389/fendo.2015.00156

The therapeutic potential of brown adipocytes in humans

Craig Porter[1,2], Maria Chondronikola[1,3] and Labros S. Sidossis[1,2,4,5]**

[1] *Metabolism Unit, Shriners Hospitals for Children-Galveston, Galveston, TX, USA,* [2] *Department of Surgery, University of Texas Medical Branch, Galveston, TX, USA,* [3] *Department of Preventive Medicine and Community Health, University of Texas Medical Branch, Galveston, TX, USA,* [4] *Department of Nutrition and Dietetics, Harokopio University of Athens, Athens, Greece,* [5] *Department of Internal Medicine, University of Texas Medical Branch, Galveston, TX, USA*

Obesity and its metabolic consequences represent a significant clinical problem. From a thermodynamic standpoint, obesity results from a discord in energy intake and expenditure. To date, lifestyle interventions based on reducing energy intake and/or increasing energy expenditure have proved ineffective in the prevention and/or treatment of obesity, owing to poor long-term adherence to such interventions. Thus, an effective strategy to prevent or correct obesity is currently lacking. As the combustion engines of our cells, mitochondria play a critical role in energy expenditure. At a whole-body level, approximately 80% of mitochondrial membrane potential generated by fuel oxidation is used to produce ATP, and the remaining 20% is lost through heat-producing uncoupling reactions. The coupling of mitochondrial respiration to ATP production represents an important component in whole-body energy expenditure. Brown adipose tissue (BAT) is densely populated with mitochondria containing the inner mitochondrial proton carrier uncoupling protein 1 (UCP1). UCP1 uncouples oxidative phosphorylation, meaning that mitochondrial membrane potential is dissipated as heat. The recent rediscovery of BAT depots in adult humans has rekindled scientific interest in the manipulation of mitochondrial uncoupling reactions as a means to increase metabolic rate, thereby counteracting obesity and its associated metabolic phenotype. In this article, we discuss the evidence for the role BAT plays in metabolic rate and glucose and lipid metabolism in humans and the potential for UCP1 recruitment in the white adipose tissue of humans. While the future holds much promise for a therapeutic role of UCP1 expressing adipocytes in human energy metabolism, particularly in the context of obesity, tissue-specific strategies that activate or recruit UCP1 in human adipocytes represent an obligatory translational step for this early promise to be realized.

Keywords: adipose tissue, mitochondria, uncoupling protein 1, thermogenesis, obesity

OPEN ACCESS

Edited by:
Jun Wu,
University of Michigan, USA

Reviewed by:
Elahu Gosney Sustarsic,
University of Copenhagen, Denmark
Sona Kang,
Beth Israel Deaconess Medical Center, USA

****Correspondence:***
Craig Porter and Labros S. Sidossis,
University of Texas Medical Branch, 301 University Boulevard, Galveston, TX 77550, USA
cr2porte@utmb.edu; lasidoss@utmb.edu

Specialty section:
This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *30 June 2015*
Accepted: *18 September 2015*
Published: *13 October 2015*

Citation:
Porter C, Chondronikola M and Sidossis LS (2015) The therapeutic potential of brown adipocytes in humans. Front. Endocrinol. 6:156. doi: 10.3389/fendo.2015.00156

Introduction

Obesity and its associated metabolic complications (hyperlipidemia, insulin resistance, and glucose intolerance) have become significantly more prevalent in the United States and around the world in recent years (1, 2). One third of Americans are either obese (1) or exhibit symptoms of pre-diabetes (3). Worldwide, excessive adiposity is responsible for more than three million

deaths and is a significant cause of disability (4). Increasing adiposity is associated with derangements in glucose and lipid metabolism and in the development of insulin resistance (5, 6). Therapeutic strategies aimed at preventing obesity or treating its metabolic complications typically target dietary intake and/or physical activity, but to date do not seem to exhibit a great deal of long-term efficacy. Mitochondrial proton leaks, i.e., respiration uncoupled from ATP production, accounts for around 20% of total mitochondrial respiration (7). The physiological role ascribed to this uncoupling of oxidative phosphorylation is heat production (thermogenesis). While thermogenesis represents a significant portion (~20%) of whole-body energy expenditure (7, 8), its therapeutic role in energy metabolism has only recently begun to receive attention.

Brown adipose tissue (BAT) is the major tissue responsible for non-shivering thermogenesis in mammals (9). The recent (re)discovery of BAT in human adults (10–12) has triggered scientific interest in the role of BAT in human energy metabolism. Unique features of BAT include an abundance of mitochondria that contain the transmembrane carrier protein thermogenin, most commonly referred to as uncoupling protein 1 (UCP1) (13). Upon activation by long-chain fatty acids, UCP1 acts as an inner mitochondrial proton carrier (14), allowing protons to reenter the mitochondrial matrix independently of ATP synthase, thus uncoupling mitochondrial respiration from ATP production (9). This process alters mitochondrial energy transduction, where mitochondrial membrane potential is lost as heat (**Figure 1**). The purpose of this review is to summarize the current evidence regarding the role of the human adipose tissue as a therapeutic target against obesity and its related metabolic complications.

The Role of Brown Adipose Tissue in Energy Balance and Obesity

Although the role of BAT in energy metabolism and obesity has been studied in detail in rodents (15–17), our understanding of the role BAT plays in human energy metabolism remains in its infancy. For obvious reasons, it is more difficult to manipulate and study BAT in humans. Retrospective reviews of medical records and positron emission tomography–computed tomography (PET/CT) scans, originally performed for diagnostic purposes, demonstrate an association between BAT and body mass index (BMI) (10, 18, 19) and with non-alcoholic fatty liver disease (20). The retrospective nature of these studies along with the fact that the PET/CT scans were performed under non-standardized conditions limit the generalizability of these data. However, these findings are bolstered by prospective studies which report a correlation between BAT volume and activity with BMI (11, 21), total fat mass (11, 21), and both abdominal subcutaneous and visceral adiposity (21). Moreover, weight loss via bariatric surgery has been linked to increased BAT activity (22), further suggesting that BAT may contribute to energy balance in human beings.

Acute (non-shivering) cold exposure studies have been recently performed in an attempt to estimate the contribution of BAT to cold-induced energy expenditure. However, the reported results have been highly variable. These studies reported that acute cold exposure (2–4 h at 16–19°C) induces BAT activation, resulting in a 13–27% increase in resting energy expenditure (REE) (11, 23, 24). In contrast, Ouellet et al. reported an 80% increase in REE with acute cold exposure (for 2 h at ~18°C) (25), a result over threefold higher than those of other studies, possibly attributable to muscle shivering. Muzik et al. used oxygen 15 (^{15}O)-PET/CT in an attempt to more directly quantify the contribution of BAT to energy expenditure (26, 27). In contrast to previous studies, these investigators found that BAT minimally contributed to the reported increase in energy expenditure (15–25 kcal/day). Although these two studies shed doubt on the role of BAT in energy expenditure in humans, the rather short cold stimulation protocol (30 min at 18°C) may not have been adequate to fully activate BAT. Indeed, BAT recruitment via daily mild cold exposure (17°C for 6 weeks) was associated with increased thermogenesis and decreased body fat, supporting a relationship between BAT activity and adiposity (28). Similarly, Lee et al. performed a crossover cold acclimation study in five healthy participants. These researchers showed that BAT recruitment was reversibly associated with increased post-prandial energy expenditure (diet-induced thermogenesis) after cold exposure, further supporting the role of BAT in energy expenditure (29).

When interpreting the results of these studies, one important point to consider is that chronic weight gain can result from even a small discordance in daily energy balance (e.g., an energy surplus of as little as 25 kcal/day in humans can result in a weight gain of 1 kg/year). Thus, even sporadic BAT activation for limited periods during the course of the day could conceivably have a significant cumulative impact on energy balance and adiposity over several months or years.

Can Human Brown Adipose Tissue Alter Glucose Homeostasis and Insulin Sensitivity?

Brown adipose tissue has been touted as therapeutic tissue which may protect against hyperglycemia in insulin-resistant individuals. In murine models of extreme cold exposure, BAT is responsible for the majority of whole-body glucose disposal (30), while transplantation of BAT into the abdominal visceral adipose tissue improves glucose tolerance and insulin sensitivity (31). In humans, the involvement of BAT in systemic glucose metabolism is evident from ^{18}F-FDG uptake images from PET/CT scans (**Figure 2**) performed for diagnostic purposes (32). Moreover, evidence from retrospective medical record review studies indicates an inverse relationship between BAT activity, diabetes, and glycemia (10, 19, 33, 34). Further, prospective studies have shown that acute cold exposure increases glucose uptake in BAT, where BAT glucose uptake rate per unit of tissue was higher than that of muscle (25, 35, 36), underscoring the oxidative potential of BAT. Specifically, Orava et al. reported that cold exposure resulted in a 12-fold increase in glucose disposal in BAT only, but not in other tissues (35). Similar results have been reported by Ouellet et al. (25). These data suggest that upon

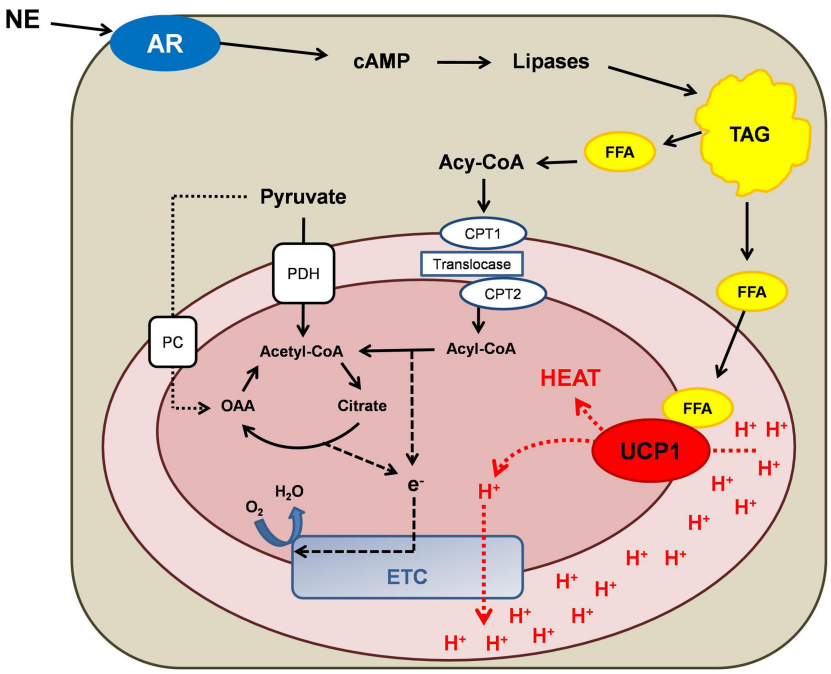


FIGURE 1 | Schematic overview of mitochondrial energy transduction within a brown adipocyte mitochondrion. Norepinephrine (NE) activation of adrenergic receptors (AR) causes an increase in cytosolic cyclic adenosine monophosphate (cAMP) levels. The subsequent activation of lipases results in the lipolysis of triacylglycerol (TAG), which increases intracellular free fatty acid (FFA) concentrations. FFAs fulfill two principal metabolic fates: (i) FFAs bind to and activate uncoupling protein 1 (UCP1), thus switching on UCP1-mediated proton (H^+) conductance. (ii) Activated FFAs (acyl-CoA) can be transported into the mitochondrion via the carnitine palmitoyl transferase (CPT) system and be oxidized to acetyl-CoA, thereby potentiating anaplerosis and providing reducing equivalents for the electron transport chain (ETC). Pyruvate also participates in mitochondrial anaplerosis and the production of reducing equivalent by being decarboxylated to acetyl-CoA by pyruvate dehydrogenase (PDH) or being carboxylated by pyruvate carboxylase (PC), forming oxaloacetate (OAA).

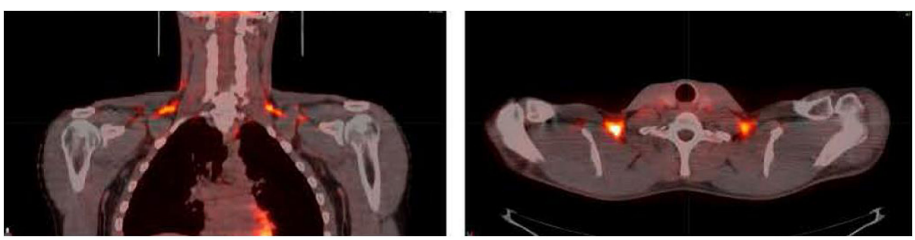


FIGURE 2 | Coronal (left) and transverse (right) 2-deoxy-2-[18F]fluoro-D-glucose (^{18}F-FDG) positron emission tomography (PET)-computed tomography (CT) images from a volunteer during cold exposure. The intense orange color in the supraclavicular area corresponds to brown adipose tissue. Image from Ref. (24). Reprinted with permission.

activation, BAT clears plasma glucose. However, Ouellet et al. demonstrated that BAT minimally contributed to whole-body plasma glucose utilization, which might be due to the short duration of cold exposure (3 h at ~18°C) and the presence of mild shivering during cold exposure (25).

The opposing results of the aforementioned studies question the physiological significance of human BAT in systemic glucose metabolism. In an attempt to address this, we studied glucose metabolism in individuals with BAT (BAT+) or without BAT (BAT−) under cold exposure and thermoneutral conditions by infusing isotopically labeled glucose in the fasted state and during a hyperinsulinemic-euglycemic clamp. We found that cold exposure increased whole-body glucose disposal in the fasted and insulin-stimulated state only in BAT+ individuals (24), suggesting that BAT can indeed play a significant role in systemic glucose metabolism in humans. Theoretically, if BAT remained chronically active, it could dispose ~23 g of glucose in 24 h [equating to approximately 8.4 kg of glucose (33,600 kcal) in a year]. Similarly, Lee et al. recently reported that increased BAT activity via cold acclimation improved post-prandial glycemia (29). Collectively, these studies support the notion that upon activation, BAT can indeed play a significant role in glucose homeostasis. Therefore,

BAT may represent a novel therapeutic target in the management of hyperglycemia.

Does Human Brown Adipose Tissue Play a Role in Systemic Lipid Metabolism?

Free fatty acids (FFAs) constitute the primary substrate for BAT (37). Moreover, FFAs bind to and activate UCP1, allowing proton transfer from the intermembrane space to the mitochondrial matrix resulting in heat production (9). Upon activation, BAT initially oxidizes intracellular lipid stores to produce heat (9). In contrast, prolonged BAT stimulation leads to increased uptake of FFA derived from circulating FFA and lipolysis of circulating lipoproteins, presumably when local lipid stores become depleted (9). BAT activation has been recently reported to increase the systemic clearance of TG-rich lipoproteins in rodents, lending credence to the notion that BAT may protect against hyperlipidemia (30, 38). Moreover, rodent BAT has been shown to release factors that increase vascular permeability in BAT, allowing TG-rich lipoproteins to enter the interstitial space (30).

The role of BAT in human lipid metabolism remains largely unexplored. Results from some (11, 35), but not all (23), cold exposure studies report a reduction in the respiratory gas exchange ratio during cold exposure, indicating increased fat oxidation in humans during BAT activation. Ouellet et al. (25) studied the role of BAT in lipid metabolism in more detail. Using PET/CT and a fatty acid tracer, ^{18}F-fluoro-thiaheptadecanoic acid (^{18}FTHA), they showed that 3 h of cold exposure increased BAT FFA uptake but that this accounted for <1% of the total FFA turnover (25). The conclusion of this study was that thermogenic BAT relies predominantly on the limited intracellular substrates (i.e., triglycerides stored inside BAT) to fuel mitochondrial thermogenesis (25), which questions the physiological significance of human BAT in whole-body lipid metabolism. However, the relatively short duration of the study might have been insufficient to deplete the intracellular lipid stores underestimating the contribution of BAT in the systemic lipid metabolism.

We recently studied BAT+ and BAT− individuals under mild cold exposure and thermoneutral conditions while infusing stable isotopes of glycerol and palmitate in order to directly trace lipid turnover in the fasted state and during a hyperinsulinemic-euglycemic clamp. We found that during cold exposure BAT+ individuals had greater whole-body lipolysis and FFA oxidation compared with BAT− participants, indicating that increased lipid mobilization from WAT provides FFA to sustain thermogenesis in BAT (39). Moreover, BAT+ individuals were protected from the cold-induced adipose tissue insulin resistance noted in the BAT− group (40). Our data suggest that BAT may indeed play an important role in the regulation of systemic lipid metabolism. The discord between our preliminary findings and those of others (25) may be related to the duration of cold exposure. For example, we studied individuals after ~5 h of cold exposure, while Ouellet et al. performed a 3 h cold exposure study. Whether there is a time-dependent depletion of BAT lipid stores, at which point BAT switches to using systemic FFAs as a fuel source, remains unknown.

Can Human WAT Really *Brown*?

More than 30 years ago, Young et al. demonstrated the appearance of brown adipocytes in the parametrial fat pad of mice following acclimation to severe cold exposure (41). This was confirmed when Cousin et al. demonstrated that severe cold exposure or treatment with a β3 adrenoreceptor agonist induced brown adipocytes within WAT (42). More recently, Shabalina et al. demonstrated that acclimation to cold (~5°) results in the browning of inguinal WAT. Importantly, these researchers showed the presence of UCP1 protein while demonstrating its function (via GDP-sensitive respiration) in inguinal WAT of cold-acclimated mice (43), thus demonstrating that these mitochondria had functional UCP1. Interestingly, per milligram of tissue, inguinal WAT from cold-acclimated mice developed a thermogenic capacity ~50% of that of intrascapular BAT (43). This striking plasticity of murine subcutaneous WAT to develop a thermogenic phenotype has potential implications for humans, given that most individuals have only 100 g or less of BAT, but many kilograms of subcutaneous WAT. If WAT in humans were able to exhibit the same plasticity as in mice, this would have huge implications for energy expenditure and substrate (glucose, lipids, and potentially amino acid) metabolism.

Unlike the inguinal subcutaneous WAT depot of mice, there is no evidence to suggest that human WAT has a comparable ability to alter its thermogenic capacity in response to an environmental stress. Indeed, chronic (10 days), albeit mild cold exposure, which activates BAT, does not alter the phenotype of WAT in humans (44). This suggests that a more profound and/or chronic adrenergic stress is likely needed to induce browning in humans. Indeed, this is perhaps reasonable considering that classic rodent models of browning typically acclimate rodents to extremely cold temperatures (~5°C) for several days, if not weeks (41–43). Considering that a thermoneutral temperature for a mouse is somewhere close to 30°C, it would be reasonable to theorize that chronic exposure to a temperature 25°C below thermoneutrality would be required to induce browning of WAT in humans.

Since cold exposure protocols used in rodents cannot be replicated in humans, researchers have looked to pathologies associated with chronic adrenergic stress. Over 30 years ago, it was reported that patients with pheochromocytoma, a tumor of the adrenal medulla which results in a chronic elevation in catecholamine secretion, had BAT surrounding their kidneys (45). More recently, Frontini et al. (46) reported the existence of multilocular adipocytes that stained positive for UCP1 within the omental adipose tissue of pheochromocytoma patients. This evidence suggests that human WAT may contain brown adipocytes, but unfortunately only histological and genomic measurements were made on these WAT samples. Whether these omental adipocytes, which showed morphological similarities to that of brown adipocyte and immune-reactivity to

UCP1, had functionally thermogenic mitochondria was not addressed.

Burn trauma represents a unique injury model as it is accompanied by prolonged adrenergic stress and a hypermetabolic state. To determine if subcutaneous WAT can acquire a brown fat phenotype, we collected biopsy samples of WAT from severely burned individuals. We found that when prospectively following patients after burn injury, UCP1 mRNA and UCP1 protein were induced in subcutaneous WAT at ~2 weeks post-injury (47). Moreover, this was accompanied by a similar time-dependent increase in mitochondria respiratory capacity and whole-body metabolic rate (47). Thus, it appears that human subcutaneous WAT has the ability to brown. In the context of burn trauma, this makes physiological sense, since following the destruction of their skin barrier burn victims have difficulty in conserving heat and maintaining core temperature.

To the best of our knowledge, our recent data provide the first evidence of browning of WAT in humans (47). However, we should note that while we did indeed see induction of UCP1, morphological adaptations, and increased mitochondrial respiratory in permeabilized WAT samples in a time-dependent manner after burn, the increase in thermogenic capacity (~threefold) was rather modest when compared with those seen in rodent WAT after chronic cold exposure. For example, Shabalina et al. (43) showed that the mitochondrial respiratory capacity of mitochondria isolated from the BAT of mice kept at 30°C was ~10-fold greater than that of mitochondria isolated from inguinal WAT. However, when acclimated to 5°C, the respiratory capacity of BAT mitochondria was only ~1.7-fold greater than that of inguinal WAT mitochondria. Indeed, BAT mitochondrial respiratory capacity was only marginally greater in cold-acclimated mice compared with thermoneutral mice. In contrast, WAT mitochondrial respiratory capacity was ~500% greater in inguinal WAT of cold-acclimated mice compared with thermoneutral animals. This remarkable plasticity, where murine inguinal WAT can adopt a thermogenic capacity akin to that of BAT, does not appear to be conserved in other mammals such as humans. Indeed, while we saw a threefold increase in subcutaneous WAT respiratory capacity after burn injury, respiration per milligram of tissue was still ~20-fold lower than what we have measured in human intrascapular BAT (47).

The Therapeutic Potential of UCP1-Positive Mitochondria: Should we Activate BAT or Brown WAT?

Much emphasis has been placed on manipulating UCP1 in order to increase thermogenesis and thus energy expenditure, which over time would reduce adiposity and consequently correct metabolic abnormalities associated with obesity. Broadly speaking, manipulation of UCP1 can be classified into two distinct ways: (i) acute activation of existing UCP1 and (ii) induction of UCP1. Induction of UCP1 can take several forms, i.e., the expansion of existing BAT depots, producing more UCP1 per gram of BAT, or inducing UCP1 within WAT (browning). However, the significance of either activating UCP1 within classical BAT depots or browning WAT in humans in terms of energy expenditure remains unclear. Using available data on organ-specific metabolic rates, and our own data on (a) the metabolic rate of human BAT following acute cold exposure and (b) human WAT that has undergone browning, we present theoretical calculations to estimate the potential impact of UCP1 on energy expenditure in humans (**Tables 1** and **2**).

Since UCP1 is inactive in the presence of inhibitory purine nucleotides such as ATP (50), BAT makes a negligible contribution to whole-body REE given that ~100 g of BAT represents ~0.1% of total body mass of a 70 kg individual. Similarly, in an individual with ~20% total fat mass, WAT accounts for a small (~4%) portion of whole-body REE (48) (see **Table 1**). Following acute (5 h) acclimation to mild cold (5 h at ~18°), our data (24) suggest that on average there is a 7.5% increase in REE. This acute intervention transforms BAT from being a quiescent tissue to one that makes a comparable contribution to whole-body REE as the heart or the kidneys (**Table 1**). In a 70 kg individual, this activation of BAT increases REE by 127 kcal (**Table 1**), which if maintained for 30 days would combust 0.5 kg of adipose tissue.

Burn trauma results in a significant increase in REE (51), which persists for months if not years post injury (52). While many ATP-consuming processes increase after burn, increased ATP turnover only explains 50–60% of burn-induced hypermetabolism (53). This means that thermogenesis represents a significant portion of hypermetabolism in burn victims, which makes sense since these patients have a compromised skin barrier and inability to thermoregulate. In order to estimate the

TABLE 1 | Impact of acute non-shivering cold exposure and severe burns on whole-body energy expenditure.

	Normal[a] (kcal/day)	Cold exposure (kcal/day)	Severe burns[d] (kcal/day)
BAT	1 (0.1)	127 (7.0)[c]	191 (6.3)[e]
WAT	68 (4.0)[b]	68 (3.7)	205 (6.7)[f]
Muscle	368 (21.7)	368 (20.2)	748 (24.5)
Liver	362 (21.3)	362 (19.8)	684 (22.4)
Heart	146 (8.6)	146 (8.0)	368 (12)
Kidney	137 (8.1)	137 (7.5)	244 (8.0)
Brain	338 (19.9)	338 (18.5)	338 (11.1)
Other	277 (16.3)	277 (15.2)	277 (9.1)
Whole body	1697	1823	3053

Values for each organ/tissue are presented as kilocalories/day with the percent contribution to whole-body energy expenditure in brackets.

[a]Values for thermoneutral conditions are taken from Rolfe and Brown (7).

[b]Values for white adipose tissue (WAT) are taken from Gallagher et al. (48).

[c]Values for cold exposure (5 h at ~18°) were derived from Chondronikola et al. (24), assuming a 7.5% increase in resting energy expenditure following acute cold exposure, which was attributable to brown adipose tissue (BAT) activation.

[d]Values for burn victims are taken from Wilmore and Aulick (49) for a patient with full-thickness burns encompassing ≥50% of their total body surface area, which results in an 80% increase in resting energy expenditure.

[e]As chronic cold exposure results in a 50% increase in BAT volume [van der Lans et al. (44)], BAT values derived from Chondronikola et al. (24) were multiplied by a factor of 1.5.

[f]WAT values for healthy individuals derived from Gallagher et al. (48) were multiplied by a factor of 3 to account for the increase in leak respiratory capacity of WAT seen in burn victims (47).

TABLE 2 | The contribution of various tissues to the increase in whole-body metabolic rate accompanying acute non-shivering cold exposure and severe burns.

	Cold exposure		Severe burns	
	Δ kcal/day	% of increase	Δ kcal/day	% of increase
BAT	126	100	190	14
WAT	0	–	136	10
Muscle	0	–	379	28
Liver	0	–	322	24
Heart	0	–	222	16
Kidney	0	–	107	8
Brain	0	–	0	0
Other	0	–	0	0
Whole body	126	100	1356	100

*Values for acute cold exposure and severe burn injury reported in **Table 1** are presented as the change (Δ) from healthy values and also as a percentage (%) of the increase in whole-body energy expenditure.*

potential contribution of BAT used burn injury as a model of severe adrenergic stress which is accompanied by hypermetabolism. We made calculations of whole-body and tissue-specific energy expenditure based on a patient with a burn covering 50% of their total body surface area, which resulted in an 80% increase in REE (49).

While the impact of burn trauma on BAT is largely unknown, we theorized that like chronic intermittent cold exposure, prolonged adrenergic stress following severe burn trauma would expand BAT depots. Assuming an ~50% increase in BAT volume as seen with 10 days of mild cold exposure (44), BAT would account for 191 kcal of total REE (**Table 1**). Here, while BAT makes a greater contribution to REE in absolute terms, since burn injury results in a large increase in REE, the relative contribution of BAT to whole-body REE is slightly lower (6.3%) when compared to cold exposure (7%) (**Table 1**). Perhaps more strikingly, when considering WATs contribution to REE in burn victims, the threefold increase in thermogenic capacity we found in subcutaneous WAT of burn victims would increase WATs contribution to whole-body REE to ~7% (**Table 1**). Thus, WAT and BAT may make similar absolute and relative contributions to whole-body REE in burn survivors. Again though, we should point out that these calculations are based on an individual with around 100 g of BAT and 15 kg of WAT, underscoring the profound difference in thermogenic potential per gram of tissue between BAT and WAT.

While the chronic adrenergic stress accompanying severe burn trauma provides a unique model where human WAT undergoes browning, it also offers significant insight into the physiological impact of achieving UCP1 activation and recruitment. In **Table 2**, we present the values reported in **Table 1** as the change from normal and also as a percentage in which each tissue contributes to the increase in whole-body REE. While the 127 kcal increase in REE following acute cold exposure is attributable to BAT, several organs contribute to the massive (1230 kcal) increase in REE following burn severe trauma. For example, while burn trauma may result in large absolute increases in BAT and WAT metabolic rates, their relative contributions to total REE are less impressive. Indeed, skeletal muscle (28%), the liver (24%), and the heart (16%) contribute most to burn-induced hypermetabolism, followed by BAT (14%) and WAT (10%) (**Table 2**).

We make the point above to underscore the fact that while the chronic stress response to burns may augment BAT and WAT thermogenesis, the systemic milieu which brings these adaptations in adipose tissue energy expenditure likely impacts many other organs. While our data on burn victims (47) provide novel evidence that human WAT can indeed undergo browning under very severe and extended adrenergic stress, this extreme pathophysiological state has an impact on nearly every organ system in the body. Thus, with all the available evidence to date, we suggest that the therapeutic potential of UCP1-positive mitochondria in combating obesity and its metabolic complications will likely be realized in humans by the acute activation of existing BAT and/or the expansion of BAT depots. If WAT browning holds any therapeutic potential in the context of obesity, the development of safe tissue-specific agents to induce browning is needed before this potential can be realized.

Summary

Adipocytes with an abundance of UCP1-positive mitochondria are unique in that gram for gram they have a respiratory capacity akin to that of muscle or liver, yet only minimal ability to produce ATP. The physiological role ascribed to this adaptation in mitochondrial energy transduction is heat production, facilitating thermoregulation in mammals. In the context of the global obesity epidemic, there is now much interest in the environmental and/or pharmacological exploitation of this thermoregulatory mechanism, since increased metabolic rate will combat obesity and the deleterious metabolic phenotype it incurs. In humans, a growing body of evidence suggests that acute activation of BAT can indeed have a meaningful impact on REE and intermediary glucose and lipid metabolism. However, whether brown adipocytes can be recruited in WAT and whether this alters REE or macronutrient metabolism remains unclear. Few human models mimic the extremity of confining rodents to near-freezing temperatures for several weeks, and in our study of severely burned patients, while prolonged stress does indeed brown WAT, several other organs are also significantly affected. Thus, to avoid the fate of the mitochondrial uncoupler dinitrophenol, future strategies aimed at augmenting UCP1 content and function in humans must strive to be tissue specific.

Acknowledgments

This work was supported by grants from the National Institutes of Health (P50-GM60338, R01-GM05668, T32 GM008256, H133P110012, and a Clinical and Translational Science Award UL1TR000071) as well as grants from the Shriners Hospitals for Children (85310 and 84090), the John Sealy Memorial Endowment Fund for Biomedical Research (66992), ADA (1-14-TS-35), and the Sealy Center on Aging at UTMB. CP was supported, in part, by a National Institute of Disability and Rehabilitation Research Training Grant (H133P110012). MC is supported by the Onassis Foundation.

References

1. Centers for Disease Control and Prevention/National Center for Health Statistics/Office of Analysis and Epidemiology. *Health Unites States, 2013* (2014). Available from: http://www.cdc.gov/nchs/data/hus/hus13.pdf[[x00023]]064
2. Ng M, Fleming T, Robinson M, Thomson B, Graetz N, Margono C, et al. Global, regional, and national prevalence of overweight and obesity in children and adults during 1980-2013: a systematic analysis for the Global Burden of Disease Study 2013. *Lancet* (2014) **384**(9945):766–81. doi:10.1016/S0140-6736(14)60460-8
3. Centers for Disease Control and Prevention. *National Diabetes Statistics Report* (2014). Available from: http://www.cdc.gov/diabetes/pubs/statsreport14/national-diabetes-report-web.pdf
4. Lim SS, Vos T, Flaxman AD, Danaei G, Shibuya K, Adair-Rohani H, et al. A comparative risk assessment of burden of disease and injury attributable to 67 risk factors and risk factor clusters in 21 regions, 1990–2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet* (2012) **380**(9859):2224–60. doi:10.1016/S0140-6736(12)61766-8
5. Pou KM, Massaro JM, Hoffmann U, Vasan RS, Maurovich-Horvat P, Larson MG, et al. Visceral and subcutaneous adipose tissue volumes are cross-sectionally related to markers of inflammation and oxidative stress: the Framingham Heart Study. *Circulation* (2007) **116**(11):1234–41. doi:10.1161/CIRCULATIONAHA.107.710509
6. Gastaldelli A, Cusi K, Pettiti M, Hardies J, Miyazaki Y, Berria R, et al. Relationship between hepatic/visceral fat and hepatic insulin resistance in nondiabetic and type 2 diabetic subjects. *Gastroenterology* (2007) **133**(2):496–506. doi:10.1053/j.gastro.2007.04.068
7. Rolfe D, Brown G. Cellular energy utilization and molecular origin of standard metabolic rate in mammals. *Physiol Rev* (1997) **77**:731–58.
8. van Marken Lichtenbelt WD, Schrauwen P. Implications of nonshivering thermogenesis for energy balance regulation in humans. *Am J Physiol Regul Integr Comp Physiol* (2011) **301**(2):R285–96. doi:10.1152/ajpregu.00652.2010
9. Cannon B, Nedergaard J. Brown adipose tissue: function and physiological significance. *Physiol Rev* (2004) **84**(1):277–359. doi:10.1152/physrev.00015.2003
10. Cypess AM, Lehman S, Williams G, Tal I, Rodman D, Goldfine AB, et al. Identification and importance of brown adipose tissue in adult humans. *N Engl J Med* (2009) **360**(15):1509–17. doi:10.1056/NEJMoa0810780
11. van Marken Lichtenbelt WD, Vanhommerig JW, Smulders NM, Drossaerts JM, Kemerink GJ, Bouvy ND, et al. Cold-activated brown adipose tissue in healthy men. *N Engl J Med* (2009) **360**(15):1500–8. doi:10.1056/NEJMoa0808718
12. Virtanen KA, Lidell ME, Orava J, Heglind M, Westergren R, Niemi T, et al. Functional brown adipose tissue in healthy adults. *N Engl J Med* (2009) **360**(15):1518–25. doi:10.1056/NEJMoa0808949
13. Nicholls D, Bernson V, Heaton G. The identification of the component in the inner membrane of brown adipose tissue mitochondria responsible for regulating energy dissipation. *Experientia Suppl* (1978) **32**:89–93. doi:10.1007/978-3-0348-5559-4_9
14. Fedorenko A, Lishko P, Kirichok Y. Mechanism of fatty-acid-dependent UCP1 uncoupling in brown fat mitochondria. *Cell* (2012) **151**:400–13. doi:10.1016/j.cell.2012.09.010
15. Feldmann HM, Golozoubova V, Cannon B, Nedergaard J. UCP1 ablation induces obesity and abolishes diet-induced thermogenesis in mice exempt from thermal stress by living at thermoneutrality. *Cell Metab* (2009) **9**(2):203–9. doi:10.1016/j.cmet.2008.12.014
16. Liu X, Rossmeisl M, McClaine J, Riachi M, Harper ME, Kozak LP. Paradoxical resistance to diet-induced obesity in UCP1-deficient mice. *J Clin Invest* (2003) **111**(3):399–407. doi:10.1172/JCI200315737
17. Inokuma K, Okamatsu-Ogura Y, Omachi A, Matsushita Y, Kimura K, Yamashita H, et al. Indispensable role of mitochondrial UCP1 for antiobesity effect of beta3-adrenergic stimulation. *Am J Physiol Endocrinol Metab* (2006) **290**(5):E1014–21. doi:10.1152/ajpendo.00105.2005
18. Lee P, Greenfield JR, Ho KK, Fulham MJ. A critical appraisal of the prevalence and metabolic significance of brown adipose tissue in adult humans. *Am J Physiol Endocrinol Metab* (2010) **299**(4):E601–6. doi:10.1152/ajpendo.00298.2010
19. Ouellet V, Routhier-Labadie A, Bellemare W, Lakhal-Chaieb L, Turcotte E, Carpentier AC, et al. Outdoor temperature, age, sex, body mass index, and diabetic status determine the prevalence, mass, and glucose-uptake activity of 18F-FDG-detected BAT in humans. *J Clin Endocrinol Metab* (2011) **96**(1):192–9. doi:10.1210/jc.2010-0989
20. Yilmaz Y, Ones T, Purnak T, Ozguven S, Kurt R, Atug O, et al. Association between the presence of brown adipose tissue and non-alcoholic fatty liver disease in adult humans. *Aliment Pharmacol Ther* (2011) **34**(3):318–23. doi:10.1111/j.1365-2036.2011.04723.x
21. Saito M, Okamatsu-Ogura Y, Matsushita M, Watanabe K, Yoneshiro T, Nio-Kobayashi J, et al. High incidence of metabolically active brown adipose tissue in healthy adult humans: effects of cold exposure and adiposity. *Diabetes* (2009) **58**(7):1526–31. doi:10.2337/db09-0530
22. Vijgen GH, Bouvy ND, Teule GJ, Brans B, Hoeks J, Schrauwen P, et al. Increase in brown adipose tissue activity after weight loss in morbidly obese subjects. *J Clin Endocrinol Metab* (2012) **97**(7):E1229–33. doi:10.1210/jc.2012-1289
23. Yoneshiro TAS, Matsushita M, Kameya T, Nakada K, Kawai Y, Saito M. Brown adipose tissue, whole-body energy expenditure, and thermogenesis in healthy adult men. *Obesity* (2011) **19**(1):13–6. doi:10.1038/oby.2010.105
24. Chondronikola M, Volpi E, Borsheim E, Porter C, Annamalai P, Enerback S, et al. Brown adipose tissue improves whole-body glucose homeostasis and insulin sensitivity in humans. *Diabetes* (2014) **63**(12):4089–99. doi:10.2337/db14-0746
25. Ouellet V, Labbe SM, Blondin DP, Phoenix S, Guerin B, Haman F, et al. Brown adipose tissue oxidative metabolism contributes to energy expenditure during acute cold exposure in humans. *J Clin Invest* (2012) **122**(2):545–52. doi:10.1172/JCI60433
26. Muzik O, Mangner TJ, Leonard WR, Kumar A, Janisse J, Granneman JG. 15O PET measurement of blood flow and oxygen consumption in cold-activated human brown fat. *J Nucl Med* (2013) **54**(4):523–31. doi:10.2967/jnumed.112.111336
27. Muzik O, Mangner TJ, Granneman JG. Assessment of oxidative metabolism in brown fat using PET imaging. *Front Endocrinol* (2012) **3**:15. doi:10.3389/fendo.2012.00015
28. Yoneshiro T, Aita S, Matsushita M, Kayahara T, Kameya T, Kawai Y, et al. Recruited brown adipose tissue as an antiobesity agent in humans. *J Clin Invest* (2013) **123**(8):3404–8. doi:10.1172/JCI67803
29. Lee P, Smith S, Linderman J, Courville AB, Brychta RJ, Dieckmann W, et al. Temperature-acclimated brown adipose tissue modulates insulin sensitivity in humans. *Diabetes* (2014) **63**(11):3686–98. doi:10.2337/db14-0513
30. Bartelt A, Bruns OT, Reimer R, Hohenberg H, Ittrich H, Peldschus K, et al. Brown adipose tissue activity controls triglyceride clearance. *Nat Med* (2011) **17**(2):200–5. doi:10.1038/nm.2297
31. Stanford KI, Middelbeek RJ, Townsend KL, An D, Nygaard EB, Hitchcox KM, et al. Brown adipose tissue regulates glucose homeostasis and insulin sensitivity. *J Clin Invest* (2013) **123**(1):215–23. doi:10.1172/JCI62308
32. Hany TF, Gharehpapagh E, Kamel EM, Buck A, Himms-Hagen J, von Schulthess GK. Brown adipose tissue: a factor to consider in symmetrical tracer uptake in the neck and upper chest region. *Eur J Nucl Med Mol Imaging* (2002) **29**(10):1393–8. doi:10.1007/s00259-002-0902-6
33. Jacene HA, Cohade CC, Zhang Z, Wahl RL. The relationship between patients' serum glucose levels and metabolically active brown adipose tissue detected by PET/CT. *Mol Imaging Biol* (2011) **13**(6):1278–83. doi:10.1007/s11307-010-0379-9
34. Matsushita M, Yoneshiro T, Aita S, Kameya T, Sugie H, Saito M. Impact of brown adipose tissue on body fatness and glucose metabolism in healthy humans. *Int J Obes (Lond)* (2013) **38**(6): 812–7. doi:10.1038/ijo.2013.206
35. Orava J, Nuutila P, Lidell ME, Oikonen V, Noponen T, Viljanen T, et al. Different metabolic responses of human brown adipose tissue to activation by cold and insulin. *Cell Metab* (2011) **14**(2):272–9. doi:10.1016/j.cmet.2011.06.012
36. Orava J, Nuutila P, Noponen T, Parkkola R, Viljanen T, Enerback S, et al. Blunted metabolic responses to cold and insulin stimulation in brown adipose tissue of obese humans. *Obesity (Silver Spring)* (2013) **21**(11): 2279–87. doi:10.1002/oby.20456
37. Ma SW, Foster DO. Uptake of glucose and release of fatty acids and glycerol by rat brown adipose tissue in vivo. *Can J Physiol Pharmacol* (1986) **64**(5):609–14. doi:10.1139/y86-101
38. Berbee JF, Boon MR, Khedoe PP, Bartelt A, Schlein C, Worthmann A, et al. Brown fat activation reduces hypercholesterolaemia and protects from atherosclerosis development. *Nat Commun* (2015) **6**:6356. doi:10.1038/ncomms7356

39. Chondronikola M, Porter C, Hurren N, Chao T, Yfanti C, Labbé S, et al. The functional role of brown adipose tissue in whole-body lipid metabolism in humans (1160.3). *FASEB J* (2014) **28**(1 Suppl).
40. Chondronikola M, Chao T, Porter C, Labbé S, Hurren N, Yfanti C, et al. Brown adipose tissue activation affects white adipose tissue insulin sensitivity in humans. *75th Scientific Sessions American Diabetes Association*. Boston, MA (2015).
41. Young P, Arch J, Ashwell M. Brown adipose tissue in the parametrial fat pad of the mouse. *FEBS Lett* (1984) **167**:10–4. doi:10.1016/0014-5793(84)80822-4
42. Cousin B, Cinti S, Morroni M, Raimbault S, Ricquier D, Pénicaud L, et al. Occurrence of brown adipocytes in rat white adipose tissue: molecular and morphological characterization. *J Cell Sci* (1992) **103**:931–42.
43. Shabalina I, Petrovic N, de Jong J, Kalinovich A, Cannon B, Nedergaard J. UCP1 in brite/beige adipose tissue mitochondria is functionally thermogenic. *Cell Rep* (2013) **5**:1196–203. doi:10.1016/j.celrep.2013.10.044
44. van der Lans A, Hoeks J, Brans B, Vijgen G, Visser M, Vosselman M, et al. Cold acclimation recruits human brown fat and increases nonshivering thermogenesis. *J Clin Invest* (2013) **123**:3395–403. doi:10.1172/JCI68993
45. Ricquier D, Nechad M, Mory G. Ultrastructural and biochemical characterization of human brown adipose tissue in pheochromocytoma. *J Clin Endocrinol Metab* (1982) **54**:803–7.
46. Frontini A, Vitali A, Perugini J, Murano I, Romiti C, Ricquier D, et al. White-to-brown transdifferentiation of omental adipocytes in patients affected by pheochromocytoma. *Biochim Biophys Acta* (2013) **1831**:950–9. doi:10.1016/j.bbalip.2013.02.005
47. Sidossis L, Porter C, Saraf M, Borsheim E, Radhakrishnan R, Chao T, et al. Browning of subcutaneous white adipose tissue in humans after severe adrenergic stress. *Cell Metab* (2015) **22**:219–27. doi:10.1016/j.cmet.2015.06.022
48. Gallagher D, Belmonte D, Deurenberg P, Wang Z, Krasnow N, Pi-Sunyer F, et al. Organ-tissue mass measurement allows modeling of REE and metabolically active tissue mass. *Am J Physiol* (1998) **275**:249–58.
49. Wilmore D, Aulick L. Systemic responses to injury and the healing wound. *JPEN J Parenter Enteral Nutr* (1980) **4**:147–51. doi:10.1177/014860718000400 2147
50. Shabalina I, Ost M, Petrovic N, Vrbacky M, Nedergaard J, Cannon B. Uncoupling protein-1 is not leaky. *Biochim Biophys Acta* (2010) **1797**:773–84. doi:10.1016/j.bbabio.2010.04.007
51. Wilmore D, Long J, Mason AJ, Skreen R, Pruitt BJ. Catecholamines: mediator of the hypermetabolic response to thermal injury. *Ann Surg* (1974) **180**:653–69. doi:10.1097/00000658-197410000-00031
52. Jeschke MG, Gauglitz GG, Kulp GA, Finnerty CC, Williams FN, Kraft R, et al. Long-term persistance of the pathophysiologic response to severe burn injury. *PLoS One* (2011) **6**:e21245. doi:10.1371/journal.pone.0021245
53. Yu YM, Tompkins RG, Ryan CM, Young VR. The metabolic basis of the increase of the increase in energy expenditure in severely burned patients. *JPEN J Parenter Enteral Nutr* (1999) **23**:160–8. doi:10.1177/0148607199023003160

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 09 November 2015
doi: 10.3389/fendo.2015.00169

Biodegradable Polymeric Microsphere-Based Drug Delivery for Inductive Browning of Fat

Chunhui Jiang[1,2†], Liangju Kuang[1,2†], Madeline P. Merkel[2,3], Feng Yue[4], Mario Alberto Cano-Vega[1,2], Naagarajan Narayanan[1,2], Shihuan Kuang[4,5] and Meng Deng[1,2,6,7]**

[1] Department of Agricultural and Biological Engineering, Purdue University, West Lafayette, IN, USA, [2] Bindley Bioscience Center, Purdue University, West Lafayette, IN, USA, [3] College of Pharmacy, Purdue University, West Lafayette, IN, USA, [4] Department of Animal Sciences, Purdue University, West Lafayette, IN, USA, [5] Center for Cancer Research, Purdue University, West Lafayette, IN, USA, [6] School of Materials Engineering, Purdue University, West Lafayette, IN, USA, [7] Weldon School of Biomedical Engineering, Purdue University, West Lafayette, IN, USA

Brown and beige adipocytes are potent therapeutic agents to increase energy expenditure and reduce risks of obesity and its affiliated metabolic symptoms. One strategy to increase beige adipocyte content is through inhibition of the evolutionarily conserved Notch signaling pathway. However, systemic delivery of Notch inhibitors is associated with off-target effects and multiple dosages of application further faces technical and translational challenges. Here, we report the development of a biodegradable polymeric microsphere-based drug delivery system for sustained, local release of a Notch inhibitor, DBZ. The microsphere-based delivery system was fabricated and optimized using an emulsion/solvent evaporation technique to encapsulate DBZ into poly(lactide-*co*-glycolide) (PLGA), a commonly used biodegradable polymer for controlled drug release. Release studies revealed the ability of PLGA microspheres to release DBZ in a sustained manner. Co-culture of white adipocytes with and without DBZ-loaded PLGA microspheres demonstrated that the released DBZ retained its bioactivity, and effectively inhibited Notch and promoted browning of white adipocytes. Injection of these DBZ-loaded PLGA microspheres into mouse inguinal white adipose tissue depots resulted in browning *in vivo*. Our results provide the encouraging proof-of-principle evidence for the application of biodegradable polymers as a controlled release platform for delivery of browning factors, and pave the way for development of new translational therapeutic strategies for treatment of obesity.

Keywords: Notch inhibition, browning, PLGA, microspheres, drug delivery, obesity

OPEN ACCESS

Edited by:
Jun Wu,
University of Michigan, USA

Reviewed by:
Sean Bong Lee,
Tulane University, USA
Wei Zhang,
Johns Hopkins University School of Medicine, USA

****Correspondence:***
Shihuan Kuang
skuang@purdue.edu;
Meng Deng
deng65@purdue.edu

†Chunhui Jiang and Liangju Kuang have contributed equally to this work.

Specialty section:
This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *19 September 2015*
Accepted: *23 October 2015*
Published: *09 November 2015*

Citation:
Jiang C, Kuang L, Merkel MP, Yue F, Cano-Vega MA, Narayanan N, Kuang S and Deng M (2015) Biodegradable Polymeric Microsphere-Based Drug Delivery for Inductive Browning of Fat. Front. Endocrinol. 6:169. doi: 10.3389/fendo.2015.00169

INTRODUCTION

The obesity epidemic has posed a major concern for public health in modern society due to its association with a spectrum of metabolic diseases, including type 2 diabetes (T2D), heart diseases, hyperglycemia, and multiple cancers (1, 2). Obesity is morphologically characterized by the excessive lipid storage in the white adipose tissue (WAT) and featured as a complex disorder of energy imbalance wherein intake outpaces expenditure. Considerable efforts have been devoted to explore

the pathological mechanisms as well as the cellular and molecular therapeutic targets to combat obesity.

Currently, there are few medications available for obesity treatment (3). Mostly, those therapies are dedicated to decreasing energy intake by either suppressing appetite through stimulating the central nervous system or reducing nutrient digestion and absorption within the gastrointestinal tract (4). However, those medications only produce modest effects and are usually accompanied with unpleasant, potentially harmful side effects (5). Thus, alternative approaches to overcoming obesity are under extensive exploration. One such approach is through boosting energy expenditure. Recently, this strategy through stimulating thermogenesis has been emerging as an appealing alternative.

Brown adipose tissue (BAT) is the primary site of thermogenesis through uncoupling mitochondrial respiration and ATP synthesis via uncoupling protein 1 (Ucp1). While producing heat, BAT consumes not only free fatty acid but also large amounts of glucose (6, 7), thus providing benefits to metabolic health. Classical BAT is abundant in neonates but diminishes with age leading to limited quantities in adult humans. This compromises the therapeutic value of BAT in clinical intervention of obesity.

The recent discovery of inducible BAT (iBAT), also called beige adipose tissue, in WAT depots reignites the promise of obesity treatment through stimulating energy expenditure (8, 9). Beige adipocytes also possess robust thermogenic capacity, which has been confirmed recently by the transplantation of an engineered beige adipose tissue (10). More importantly, iBAT can be stimulated to burn energy in response to multiple pharmacological and genetic manipulations. Various factors have been identified to induce the transformation from white to beige adipose tissue (termed "browning") over recent years (11, 12).

We have recently reported that inhibition of the Notch signaling pathway leads to browning of WAT, and thus ameliorates obesity (13). Notch signaling is well known to play fundamental roles in organ development and cell fate determination, but its regulatory roles in energy homeostasis has previously been underappreciated. We showed that genetic deletion of Notch1 receptor or the nuclear mediator of Notch signaling (Rbpj) in mice improved their glucose tolerance and insulin sensitivity through inducing browning of WAT. Importantly, Notch inhibition through intraperitoneal injection of dibenzazepine (DBZ), a γ-secretase inhibitor, similarly improved insulin sensitivity and glucose homeostasis. However, it remains elusive whether this systemic administration of DBZ causes any uncontrolled drug distribution and potential off-target side effects as Notch has been recognized to play multifaceted roles in various types of cells (14). Therefore, it is imperative to develop an effective drug delivery system to locally and continuously exert effects of Notch inhibition specifically on WAT.

Polymer-based drug delivery systems provide a robust technology platform to enable sustained, spatio-temporally controlled drug release (15). Biodegradable polymers are of utmost interest, because they can be broken down and excreted or resorbed by the body without the necessity for removal interventions or surgical procedures (16, 17). Biodegradable polymer microspheres with predetermined degradation and drug release profiles have been a primary research focus for development of effective drug delivery systems for a variety of applications, including cancer, cardiovascular diseases, and vaccine development (18, 19). Among a variety of polymers that have been employed for drug delivery, poly(lactide-*co*-glycolide) (PLGA), copolymers of lactic acid and glycolic acid, have been particularly prominent due to biocompatibility, biodegradability, and convenient processability (20). PLGA microspheres constitute a versatile class of drug delivery vehicles from which the drug release kinetics can be controlled by modulating the microsphere size, molecular weight, and composition of PLGA (21, 22).

In this study, we aimed to develop a PLGA microsphere-based DBZ delivery system to promote browning of WAT for the treatment of obesity (**Figure 1**). PLGA with a 50:50 lactide to glycolide ratio was utilized since this specific polymer has a relatively fast degradation rate among the PLGA family, which is advantageous to induce browning. We formulated the DBZ-loaded PLGA microspheres using an emulsion/solvent evaporation technique and characterized the morphology and release profiles of those microspheres. Moreover, we demonstrated that the DBZ-loaded PLGA microspheres inhibited Notch, and consequently promoted browning both *in vitro* and *in vivo*. To the best of our knowledge, this is the first time that an effective bioengineered system was developed to deliver therapeutic agents to convert white adipocytes to beige adipocytes. This study not only contributes to our understanding of the underlying mechanism of Notch inhibition in the browning process but also paves the way for development of novel therapeutic strategies to counteract obesity and its associated metabolic syndrome in humans.

MATERIALS AND METHODS

Materials

PLGA (lactide:glycolide 50:50; Mw = 75,000) was obtained from lakeshore Biomaterials. Poly(vinyl alcohol) (PVA; 87–90% hydrolyzed, average Mw = 30,000–70,000), fluorescein isothiocyanate-labeled dextran (FITC-dextran) (Mw = 70,000), and DBZ were obtained from Sigma. Methylene chloride (DCM), dimethyl sulfoxide (DMSO), and acetonitrile were purchased from Fisher Scientific.

Animals

All the mice used in this study were wild-type lean mice kept under normal maintenance in a clean facility at Purdue University. All procedures regarding animal maintenance and experimental use were conducted following the regulation presented by Purdue University's Animal Care and Use Committee.

FITC-Dextran-Loaded Microsphere Formulation and Characterization

Fluorescein isothiocyanate-labeled dextran-loaded PLGA microsphere was prepared by a water-in-oil-in-water (WOW) double emulsion technique (23). Briefly, 0.4 g of PLGA was dissolved in 4 mL DCM. Into this organic phase (O), 330 μL of aqueous solution (W1) containing ~3.7 mg of FITC-dextran was emulsified using a vortex mixer operating at 1,000 rpm for 3 min to form the W1/O emulsion. This primary emulsion was injected into 400 mL of

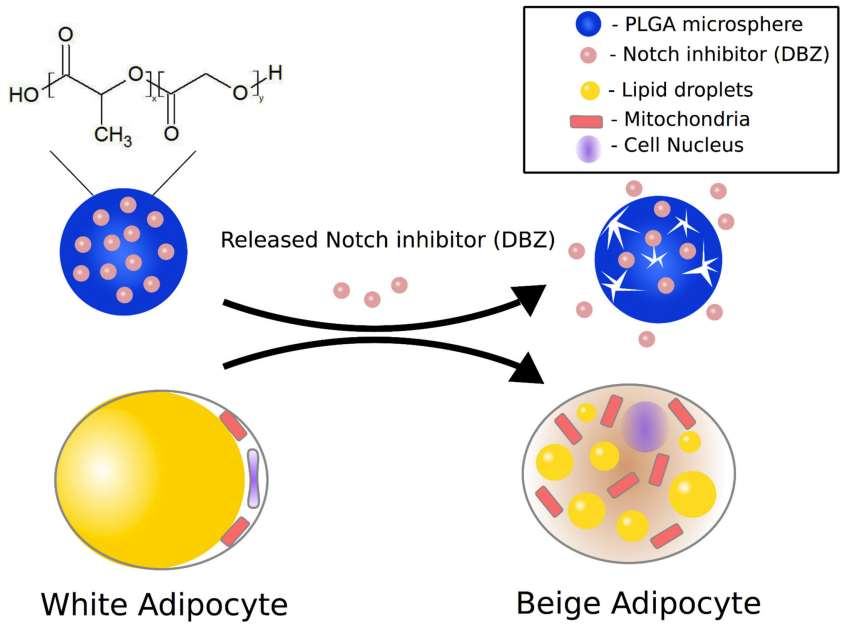


FIGURE 1 | Schematic illustration of DBZ-loaded PLGA microspheres enabling sustained release of DBZ to induce conversion of white adipocytes into beige adipocytes.

an aqueous phase containing 1% (w/v) PVA (W2). The resulting W1/O/W2 emulsion was stirred at 400 rpm for overnight with an overhead magnetic stirrer to allow solvent evaporation and microspheres hardening. The microspheres were then isolated by centrifugation, washed three times with distilled water, and were dried in a vacuum oven for 24 h. Final products were stored in a desiccator. FITC-dextran-loaded PLGA microspheres were imaged by fluorescence microscopy (EVOS FL).

DBZ-Loaded Microsphere Formulation and Characterization

PLGA microspheres loaded with DBZ were prepared by an oil-in-water (O/W) emulsion/solvent evaporation technique (24). The oil phase consisted of 540 mg of PLGA and 10.8 mg of DBZ dissolved in 2.7 mL DCM. This oil phase was injected into 400 mL of an aqueous phase containing 1% (w/v) PVA, which was stirred at 600 rpm to achieve an O/W emulsion system. The resulting emulsion was stirred overnight with an overhead magnetic stirrer to allow complete evaporation of the solvent and solidification of the droplets into microspheres. The microspheres were then isolated by centrifugation, washed three times with distilled water, and dried in a vacuum oven for 24 h. Final products were stored in a desiccator. The microsphere surface structure was investigated by scanning electron microscopy (SEM) (Nova Nano SEM). The microsphere diameter was measured using Image-J software, and about 130 microspheres were randomly selected for analysis.

The content of DBZ in microspheres was analyzed by a precipitation method. Five milligrams of DBZ-loaded PLGA microspheres were completely dissolved in chloroform. Once dissolved, the chloroform solution was added dropwise into 4 mL of methanol in a centrifuge tube to dissolve DBZ and precipitate PLGA. DBZ in the supernatant was collected and concentrated under nitrogen stream. Then, DBZ was dried in the vacuum oven for overnight and dissolved in 10 mL of 2:1 acetonitrile to Millipore water. The product was filtered for high-performance liquid chromatography (HPLC) analysis (Thermo HPLC). The samples were analyzed using a mobile phase of acetonitrile to 0.1% phosphoric acid (50:50) at a flow rate of 1 mL/min on a pentafluorophenylpropyl column and UV detection at 232 nm. Each sample was measured in duplicates. Actual drug loading and drug encapsulation efficiency were calculated using the following equations:

$$\text{Theoretical DBZ loading } \% = \frac{\text{DBZ (Total)}}{\text{DBZ(Total)} + \text{PLGA}} \times 100\% \quad (1.1)$$

$$\text{Actual DBZ loading } \% = \frac{\text{DBZ (Experiment)}}{\text{DBZ (Total)} + \text{PLGA}} \times 100\% \quad (1.2)$$

$$\text{Encapsulation efficiency } \% = \frac{\text{Actual DBZ loading}}{\text{Theoretical DBZ loading}} \times 100\% \quad (1.3)$$

In vitro FITC-Dextran Release from Microspheres

Fluorescein isothiocyanate-labeled dextran released from microspheres was measured by suspending ~20 mg microspheres in 5 mL PBS buffer at pH 7.4. The samples were placed

in a shaker maintained at 37°C and shaken at 200 rpm. At predetermined time intervals, the samples were removed from the shaker and centrifuged at 1,000 × *g* for 2 min. Two milliliters of medium was aliquoted for analysis and fresh medium of equal volume was added thereafter. The precipitated microsphere pellets were resuspended in the medium and placed back in the shaker. *In vitro* release of FITC-dextran was studied in triplicate. The FITC-dextran concentration in the aqueous phase was determined fluorometrically (excitation: 485 nm, emission: 520 nm, Synergy H1 microplate reader) using a standard calibration curve.

In vitro DBZ Release from Microspheres

The DBZ-loaded PLGA microspheres were characterized for drug release for 6 days in PBS at pH 7.4 and 37°C. DBZ release was measured using HPLC. In brief, 20 mg samples were placed in individual centrifuge tubes and filled with 2.5 mL of PBS. The samples were then placed in a shaker maintained at 37°C and shaken at 200 rpm. At specific time points, 1 mL medium was removed, saved for analysis, and replaced with same amount of fresh PBS. Time points were chosen such that perfect sink conditions were maintained. Each supernatant sample was extracted with 1 mL chloroform. The organic layer was then separated and allowed to evaporate. The dried DBZ was then reconstituted with 1 mL of a 40% solution of acetonitrile in water to provide a suitable solution for HPLC analysis (Thermo HPLC).

Isolation of Primary Preadipocytes

Primary preadipocytes were collected from limb subcutaneous WAT depots and minced into 2–5 mm^2 pieces. Then, these pieces were subject to 1.5 mg/mL collagenase digestion with agitation at 37°C for 1.5–2 h. The digestion was terminated with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). After that, the floating mature adipocytes were removed and the cell suspension was filtered through 100 μm mesh, followed by brief centrifugation at 450 × *g* for 5 min. The pellet was resuspended and seeded onto tissue culture plates.

Co-Culture of Primary Preadipocytes with Microspheres

Primary preadipocytes were cultured in growth medium containing DMEM, 20% FBS, and 1% penicillin/streptomycin at 37°C with 5% CO_2. The medium was changed every other day. Upon confluence, cells were subject to induction medium containing DMEM, 10% FBS, 2.85 μM insulin, 0.3 μM dexamethasone, and 0.63 mM 3-isobutyl-methylxanthine for 4 days, followed by differentiation medium containing DMEM, 200 nM insulin, and 10 nM triiodothyronine for four more days. Meanwhile, 5 mg polymer microspheres were added in permeable transwell inserts suspended in each well of 24-well plates while cells were induced for adipogenic differentiation. Other parallel treatment groups included cells in DBZ-containing medium (10 μM) and cells in DMSO vehicle control. To monitor adipogenic differentiation, the lipid droplets and nuclei of adipocytes during culture were counterstained with BODIPY and DAPI, respectively.

Quantitative Polymerase Chain Reaction

Total RNA was extracted from cell culture through Trizol. Random hexamer primers were utilized for the reverse transcription to synthesize cDNA. The quantitative polymerase chain reaction (qPCR) was performed with a Roche Light Cycler 96 machine (Roche). The 18S rRNA was applied as an internal control for normalization. For qPCR result analysis, the $2^{-\Delta\Delta ct}$ method was used to calculate the fold change.

Protein Extraction and Western Blots Analysis

Total protein was extracted from cells or tissue samples using RIPA buffer containing 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1% Non-idet P-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS). Protein concentrations were measured by using Pierce BCA protein assay reagent (Pierce Biotechnology, Rockford, IL, USA). Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore Corp., Billerica, MA, USA). Membranes were blocked in 5% milk for 1 h and incubated with primary antibodies at 4°C overnight. The PGC1-α (sc-13067) and GAPDH (sc-32233) antibodies were purchased from Santa Cruz Biotechnology, and both were diluted 1:1,000. The horseradish peroxidase (HRP)-conjugated secondary antibody (anti-rabbit IgG, 7074S, Cell Signaling) was diluted 1:5,000. Signals were detected with a ChemiDoc™ Touch Imaging System (Bio-Rad). The lanes were analyzed for densitometry quantification with Bio-Rad Image Lab V5.2.1.

In vivo Injection of PLGA Microspheres

Mice were first anesthetized by a ketamine–xylazine cocktail and then either FITC-dextran-loaded PLGA microspheres or DBZ-loaded PLGA microspheres (20 mg/30 g body weight) were injected into the inguinal WAT depot in one side of the body in a 1 mL solution of 0.5% Methocel E4M (wt/vol; Dow Chemical) and 0.1% Tween-80 (wt/vol; Sigma) in water. Similarly, PLGA microspheres were injected into the contralateral inguinal WAT depot. Adipose tissues were harvested after 24 h and 14 days post-injection for the study with FITC-dextran-loaded PLGA microspheres and DBZ-loaded PLGA microspheres, respectively. Oil Red O staining was performed to label the adipocytes for the study with FITC-dextran-loaded PLGA microspheres whereas hematoxylin and eosin (H&E) and immunohistochemistry staining was conducted for the study with DBZ-loaded PLGA microspheres.

H&E and Immunohistochemistry Staining

Adipose tissues were fixed in 10% formalin for 24 h at room temperature. Then, the tissues were embedded into paraffin and cut into 4 μm thick slices, deparaffinized, and rehydrated using xylene, ethanol, and water by standard methods. Immunohistochemistry was performed on a Dako Autostainer (Dako). Slides were incubated with 3% hydrogen peroxide and 2.5% normal horse serum (S-2012, Vector), followed by incubation with rabbit polyclonal anti-Ucp1 primary antibody diluted 1:200 in 2.5% normal horse

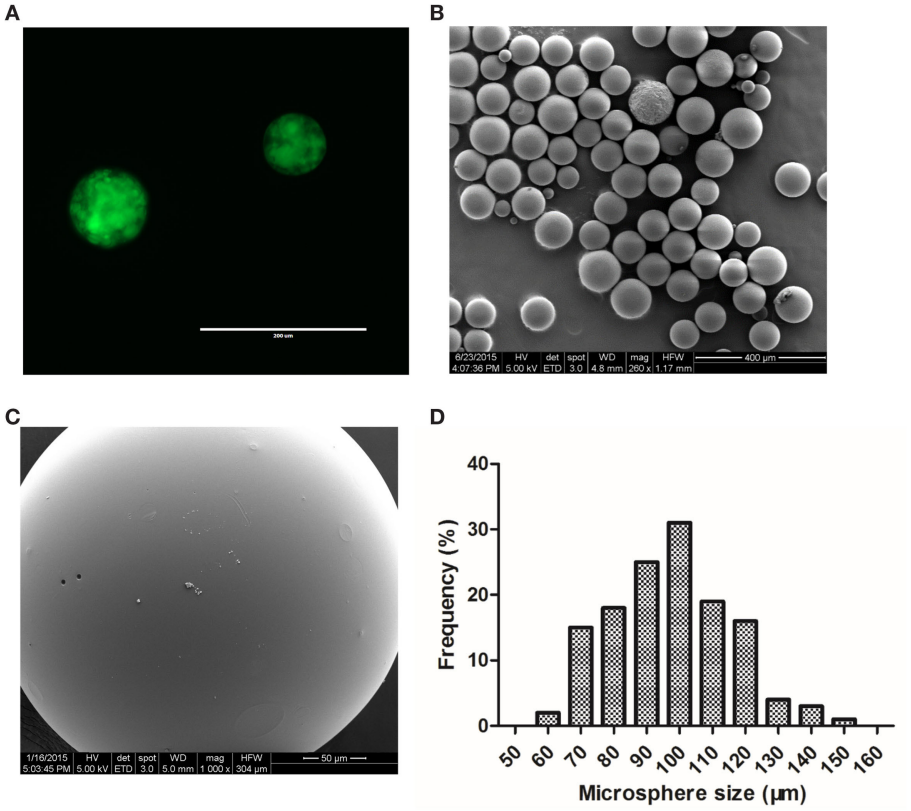


FIGURE 2 | Characterization of FITC-dextran and DBZ-loaded PLGA microspheres. **(A)** Fluorescent image of FITC-dextran-loaded microsphere. Scale bar, 200 μm. **(B)** SEM image of a population of DBZ-loaded PLGA microspheres. Scale bar, 400 μm. **(C)** Higher magnification SEM image of a single DBZ-loaded PLGA microsphere. Scale bar, 50 μm. **(D)** Size distribution of DBZ-loaded PLGA microspheres.

serum (S-2012, Vector) for 60 min. Signals were detected with an anti-rabbit IgG Polymer Detection Kit (MP-7401, Vector). Labeling was visualized with 3,3′-diaminobenzidine (DAB) as the chromogen (SK-4105, Vector). Slides were counterstained with Harris hematoxylin (EK Industries), and whole-slide digital images were collected with an Aperio Scan Scope slide scanner (Aperio).

Statistical Analysis

Quantitative data were reported as mean ± SEM *P*-values were calculated by a two-tailed Student's *t*-test. $P < 0.05$ was considered to be statistically significant.

RESULTS

Formulation and Characterization of PLGA Microspheres

Using PLGA 50:50, drug-loaded PLGA microspheres were prepared and characterized for drug distribution and morphological characteristics. We first encapsulated FITC-dextran, a commonly used fluorescent probe, into PLGA microspheres to assess the distribution of FITC-dextran in microspheres by fluorescent microscopy. It was observed that green fluorescence was uniformly distributed throughout the microspheres (**Figure 2A**). Next, we formulated DBZ-loaded PLGA microspheres by optimizing the process parameters, such as PVA concentration and stirring speed, to obtain a narrow microsphere size range of 50–150 μm (**Figure 2B**). Higher magnification SEM micrograph revealed the smooth surface morphology of the microsphere (**Figure 2C**). The size distribution of the DBZ-loaded PLGA microspheres resembled a Gaussian distribution with more than 50% in the range of 90–110 μm (**Figure 2D**). The actual DBZ loading percentage was characterized to be ~1.4% (w/w), equivalent to ~68% DBZ encapsulation efficiency.

PLGA Microsphere System Enables FITC-Dextran and DBZ Release

To examine the cargo drug release capabilities from the PLGA microspheres, we first placed the FITC-dextran-loaded PLGA microspheres in the PBS at 37°C and release was quantified fluorometrically. The FITC-dextran-loaded PLGA microspheres showed a rapid release profile, which resulted in about 50% of total FITC-dextran released after 24 h (**Figure 3A**). This is presumably due to the hydrophilic nature of the FITC-dextran. We further characterized the release profile of DBZ from DBZ-loaded PLGA microspheres (**Figure 3B**). In general, the release

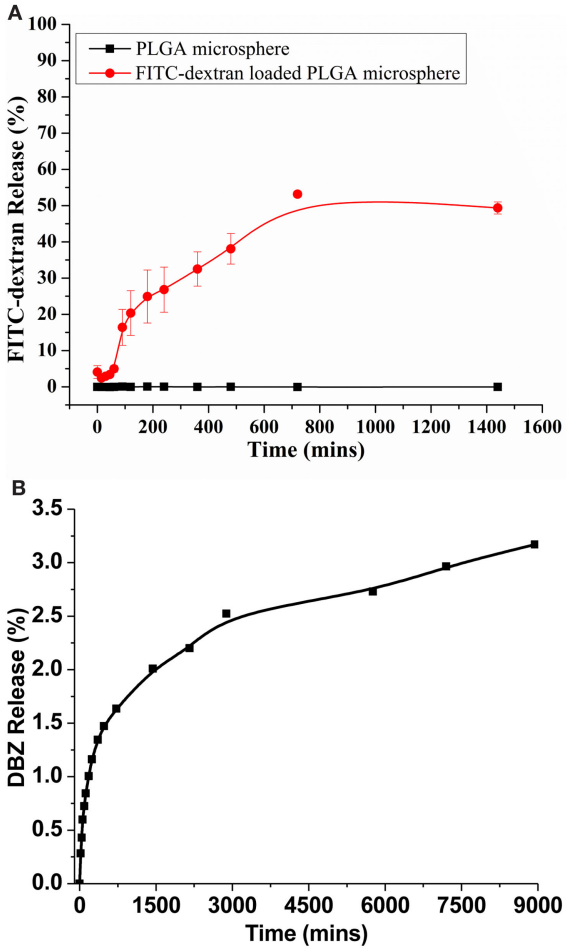


FIGURE 3 | *In vitro* release profiles of FITC-dextran and DBZ from PLGA microspheres. **(A)** *In vitro* release of FITC-dextran from microspheres in PBS at pH 7.4 and 37°C. **(B)** *In vitro* release of DBZ from microspheres in PBS at pH 7.4 and 37°C.

rate of DBZ from PLGA microspheres was much slower than that of FITC-dextran. Approximately, 2% DBZ was released from PLGA microspheres in the first 24 h. And the release rate became significantly decreased thereafter and around 3% DBZ (~8 μg) was released over 6 days, indicating that DBZ can be released from PLGA in a sustained manner.

DBZ-Loaded PLGA Microspheres Promote Browning in Adipocytes

As the initial attempt to test the bioactivity of DBZ released from PLGA microspheres, we co-cultured DBZ-loaded PLGA microspheres with primary adipocytes and examined the cellular responses (**Figure 4A**). Following induction of adipogenic differentiation for 8 days, we observed classical lipid droplets characterized by the BODIPY immunofluorescence (**Figure 4A**), which suggested that microspheres did not cause obvious adverse effects on cell culture. Then, we continued to analyze the mRNA levels of Notch target and thermogenic genes. As we expected, DBZ-loaded PLGA microspheres significantly inhibited Notch, demonstrated by the 40% mRNA reduction of Notch target genes, *Hes1* and *HeyL* (**Figure 4B**). Importantly, DBZ-loaded PLGA microspheres dramatically increased mRNA levels of browning markers, including *Ucp1*, *Cidea*, and *Ppargc1a* (**Figure 4C**), as well as mitochondria genes, including *Cox5b*, *Cox7a*, *Cpt1a*, and *Cpt2* (**Figure 4D**), suggesting that DBZ released from PLGA microspheres can inhibit the Notch pathway and consequently promote browning. In addition, western blot results shown in **Figure 4E** demonstrated that DBZ-loaded PLGA microspheres significantly elevated the protein level of Pgc1-α (encoded by *Ppargc1a*) by around 3.5-fold (**Figure 4F**), which plays key roles in mitochondrial biogenesis and oxidative metabolism. Collectively, all these data confirmed that DBZ remained bioactive to induce browning after being released from PLGA microspheres *in vitro*.

DBZ-Loaded PLGA Microspheres Stimulate Browning *In vivo*

To further test whether PLGA microspheres can locally deliver DBZ and promote browning *in vivo*, we sought to directly inject microspheres to the mouse inguinal WAT depots and assess the browning effect thereafter (**Figure 5A**). Initially, to ensure that microspheres could be precisely injected into the depots, we utilized the FITC-dextran-loaded microspheres to track microsphere placement. The microsphere injection sites were readily identified with green fluorescence via gross observation in the target inguinal adipose tissues (**Figure 5B**). Following 24 h of injection, FITC-dextran-loaded microspheres maintained round morphologies with expanded fluorescence, indicating the release of FITC-dextran from the microspheres over this time period (**Figures 5C,D**). This observation was also in agreement with our previous results regarding the release profile of FITC-dextran *in vitro*.

We next injected DBZ-loaded PLGA microspheres into the inguinal WAT depots using the same procedure. Fourteen days after injection, the inguinal WAT was collected and processed for paraffin embedding and sectioning. Through H&E staining, we observed that DBZ-loaded PLGA microspheres were dispersed within the WAT and maintained round morphologies in the size range of 50–150 μm. Importantly, these microspheres did not elicit any significant inflammatory responses (**Figure 6A**). Of note, DBZ-loaded PLGA microspheres drastically decreased the sizes of mature white adipocytes (**Figure 6B**), a hallmark indicator of browning effect. Multilocular Ucp1$^+$ beige adipocytes were evidently abundant with DBZ-loaded PLGA microspheres (**Figure 6B**). Also, this morphological feature of browning was further supported by western blot of tissue samples exhibiting increased protein levels of Pgc1-α and Ucp1 in the DBZ-loaded microsphere group compared to control (**Figure 6C**). All these data demonstrated that DBZ was successfully released from microspheres and retained its biological activity of Notch inhibition to promote browning *in vivo*.

DISCUSSION

There is accumulating evidence that significant metabolic differences that distinguish beige adipocytes from white adipocytes can potentially be successfully exploited to establish

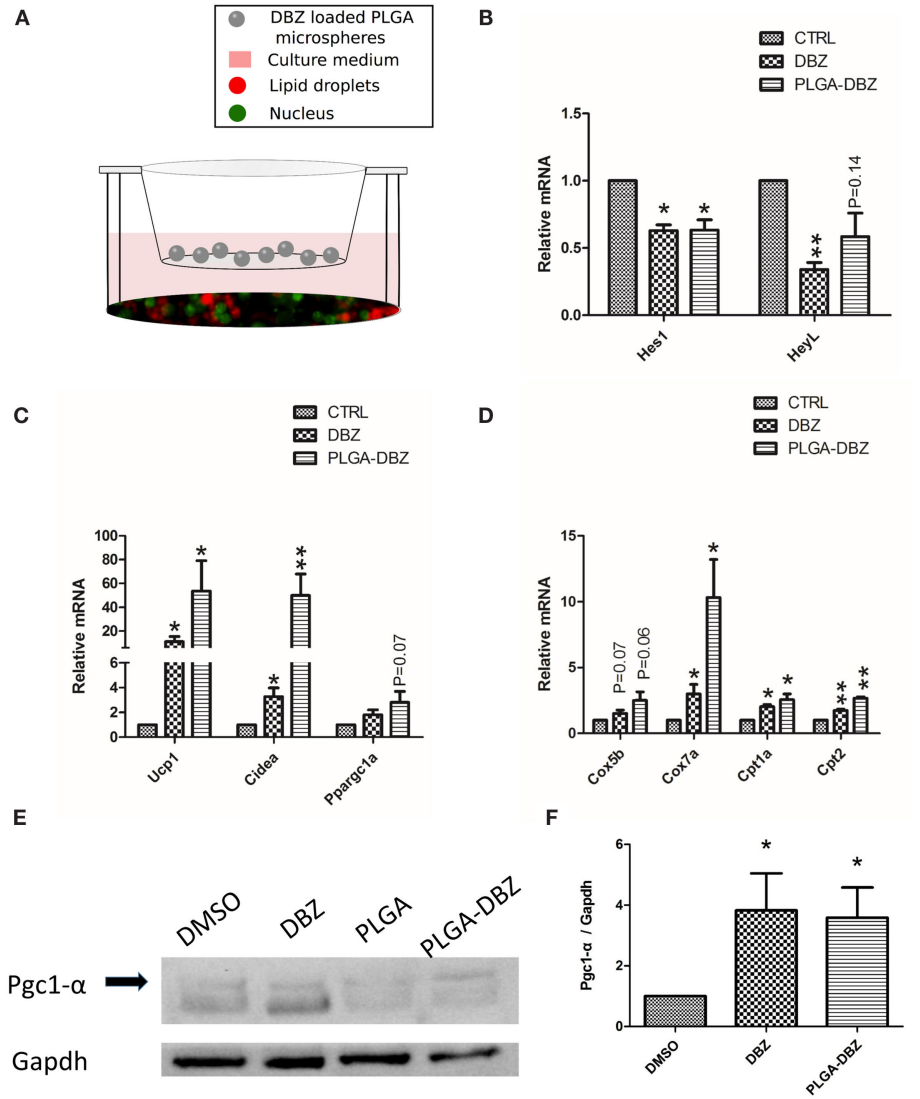


FIGURE 4 | DBZ-loaded PLGA microspheres inhibit Notch and promote browning *in vitro*. **(A)** Diagram of the co-culture system with primary white adipocytes seeded on well bottom and DBZ-loaded microspheres placed within a permeable insert. Mature adipocytes were labeled with BODIPY (red). Nuclei were counterstained with DAPI (green). Expression of **(B)** Notch target genes, **(C)** browning markers, and **(D)** mitochondria genes in white adipocytes co-cultured with DBZ-loaded PLGA microspheres. **(E)** Protein levels of Pgc1-α in white adipocytes co-cultured with DBZ-loaded PLGA microspheres. **(F)** Quantification of protein level for Pgc1-α relative to Gapdh. DBZ: cells in DBZ-containing medium (10 μM); CTRL: cells treated with DMSO vehicle control. $N = 3$. $^{*}P < 0.05$, $^{**}P < 0.01$.

new therapeutic strategies for treatment and/or prevention of obesity. Thus, identifying and targeting mechanisms underlying browning is crucial in the development of effective therapeutics to reduce adiposity. The Notch signaling pathway is important for cell–cell communication and cell fate determination during development and is required for adult tissue homeostasis. Notch target gene Hairy/enhancer-of-split 1 (Hes1) inhibits the transcriptions of PR domain-containing 16 (Prdm16), peroxisome proliferator-activated receptor gamma (Pparγ) coactivator 1 alpha (Ppargc1a) and Pparγ, all critical in the brown adipocyte biogenesis (13). This leads to reduced mitochondrion numbers and expression of Ucp1 (25–27). Notably, inhibition of Notch signaling through intraperitoneal injection of DBZ has been found to induce browning of white adipocytes, and consequently reduced obesity and improved glucose balance in obese mice (13). This strategy has been proposed to open up another novel avenue to treat obesity and its associated metabolic diseases. However, potential compliance issues associated with a requirement for multiple periodic drug injections, in combination with safety and efficacy concerns over widespread drug distribution in the body reduce the translational potential of this treatment. For instance, Notch inhibition has been proposed to affect multiple biological processes, such as angiogenesis (28, 29), bone formation (30, 31), and myogenesis (32, 33). Therefore, it would be beneficial if a carrier system were capable of delivering DBZ locally to the WAT in a sustained manner for treatment of obesity.

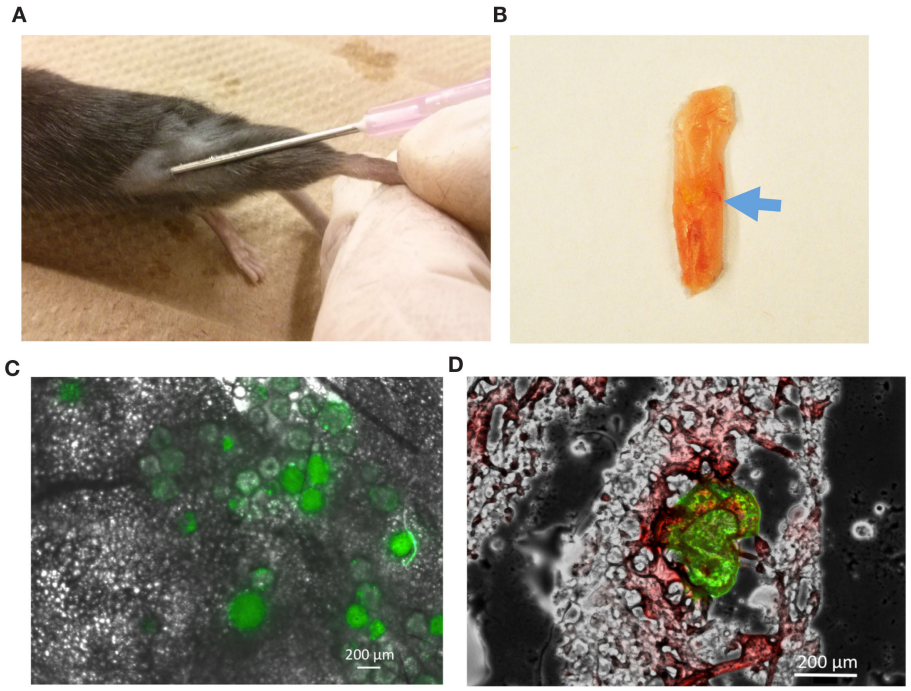


FIGURE 5 | Characterization of FITC-dextran-loaded microspheres following injection into mice. **(A)** Direct injection of microspheres into inguinal adipose depot of WT mice. **(B)** Injected microspheres (blue arrow) identified through gross observation of inguinal adipose tissues. **(C)** Fluorescent image illustrating the distribution of microspheres after 24 h of injection. **(D)** Representative image showing green fluorescence from FITC-dextran-loaded microspheres dispersed in white adipocytes labeled by Oil Red O staining. Green: FITC; Red: Oil Red O.

Polymer-based controlled drug delivery systems offer several unique advantages over conventional drug delivery including continuous drug release, decreased systemic side effects, and increased patient compliance (15). Polymer degradation is highly desirable in controlled drug delivery because it eliminates the need for the surgical removal of implants. PLGA is known to degrade by simple hydrolysis of the ester bonds into lactic and glycolic acids, which are ultimately metabolized to carbon dioxide and water (34). PLGA microspheres have been extensively investigated as carriers to deliver a variety of therapeutic agents (21, 22). The release of an encapsulated agent from PLGA microspheres is controlled by both diffusion and polymer degradation. The drug release kinetics can be tailored by varying the copolymer composition, molecular weight, and microsphere size (20–22). In particular, the degradation rate of PLGA depends on the copolymer composition. The molecular weight loss during hydrolysis is accelerated with an increase in glycolide content. This is attributed to greater absorption of water into the polymer matrix. For example, PLGA exhibited a degradation time in the order of PLGA 50:50 (1–2 months) < PLGA 75:25 (4–5 months) < PLGA 85:15 (5–6 months) (35). Polymers with higher molecular weight result in slower degradation rates (36). Furthermore, the size of PLGA microspheres is an important controlling factor on drug diffusion and polymer degradation. Berchane et al. demonstrated that the initial burst release rate of piroxicam from PLGA microspheres decreased with an increase in microsphere size from 13.9 to 81.2 μm, which is consistent with Fick's law of diffusion because an increase of diffusion pathways would reduce the drug release rate (37). It has been reported that large microspheres degrade in a heterogeneous manner wherein the degradation rate in the core is greater than that at the surface (38). In contrast, microspheres with diameter <300 μm undergo a homogeneous degradation whereby the core degradation rate is equivalent to the surface one (39). Small microspheres also degrade slower than large microspheres resulting from a decreased accumulation of acidic degradation products (40). *In vitro* release studies showed that the fabricated DBZ-loaded PLGA microspheres in the size range of 50–150 μm enabled sustained release of DBZ resulting in ~3% of encapsulated drug released over 6 days. This gradual release will be particularly beneficial to circumvent the need for multiple periodic injections of DBZ for induction of browning.

Co-culture of primary preadipocytes with DBZ-loaded PLGA microspheres demonstrated that released DBZ retained its bioactivity and effectively inhibited Notch signaling and upregulated expression of brown fat specific genes (**Figure 4**). For example, the expression of Notch downstream targets *Hes1* and *HeyL* in cultured white adipocytes with both DBZ and DBZ-loaded microspheres were reduced by more than 40%, indicating the bioactivity of DBZ released from polymers. Furthermore, DBZ-loaded microspheres resulted in an elevated expression of browning markers, including *Ucp1*, *Cidea*, and *Ppargc1a*, as well as mitochondria genes, including *Cox5b*, *Cox7a*, *Cpt1a*, and *Cpt2*. Notably, brown and beige adipocytes contain more mitochondria than white adipocytes, and possess ability to

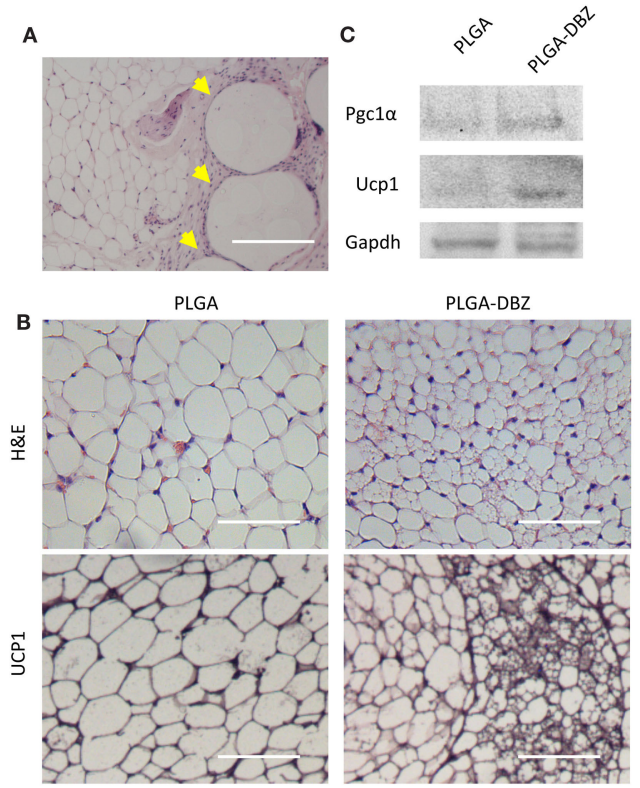


FIGURE 6 | DBZ-loaded PLGA microspheres induce browning *in vivo*. **(A)** H&E staining showing DBZ-loaded PLGA microspheres (yellow arrowhead) dispersed within the WAT depot. Scale bar, 200 μm. **(B)** H&E (top) and UCP1 (bottom) staining of inguinal WAT from mice at 14 days post-injection of DBZ-loaded PLGA microspheres. Scale bars, 100 μm. **(C)** Protein levels of Pgc1-α and Ucp1 of inguinal WAT from mice at 14 days post-injection of DBZ-loaded PLGA microspheres.

burn lipids (through β-oxidation) to generate heat (11). Pgc1-α (encoded by *Ppargc1a*) is also known to induce the expression of Ucp1 and other thermogenic components in adipocytes (26, 41). Our results are also in line with our earlier findings with the use of DBZ on browning of white adipocytes (13). The more pronounced increase in *Ucp1* and *Cidea* mRNA levels in the PLGA-DBZ-treated groups compared to the DBZ alone groups is presumably due to the continued release of DBZ from microspheres, which should have improved the biological activity of DBZ and thus highlights a major advantage of our drug delivery system for inductive browning.

One of the most important requirements for developing effective polymer-based drug delivery systems is tissue compatibility. PLGA polymers are considered a standard for drug delivery due to their recognized biocompatibility and approval by the Food and Drug Administration for a number of clinical applications (16, 17). The DBZ-loaded PLGA microspheres were well tolerated following *in vivo* injection into the WAT depots (**Figure 6A**). More importantly, we demonstrated that local DBZ-loaded microsphere injection successfully induced browning of WAT after 14 days (**Figures 6B,C**). The resultant browning further validates that Notch signaling plays a role in regulating the plasticity of white and beige adipocytes *in vivo* (13).

White adipose tissue is the primary site of long-term energy storage. In response to excess calorie intake, the size of the WAT expands through hyperplasia and hypertrophy of adipocytes. Our work pinpointed the potential of polymeric microspheres as a carrier platform to locally deliver DBZ to WAT in a sustained manner. Such a localized delivery system may eliminate the need for repeated injections as well as potential side effects that are accompanied with systemic administration of DBZ. Improved understanding of brown and beige adipocyte biology has led to recent discoveries of a variety of factors to promote WAT browning, such as fibroblast growth factors (42–44), bone morphogenetic proteins (BMPs) (45, 46), Irisin (47), T3 and T4 thyroid hormones (45, 46), natriuretic peptides (48), and β3-adrenergic pathway agonist (49–51). However, as with DBZ, there exist significant safety and efficacy concerns on systemic administration of these browning factors as uncontrolled tissue exposure may lead to potential off-target side effects and unpredictable kinetics. For instance, administration of FGF21 is associated with systemic side effects on bone loss (52). Therefore, development of effective browning-based therapeutics to address the ever-worsening obesity epidemic necessitates the integration of advances in controlled release technologies with discoveries in beige/brown fat cell biology.

CONCLUSION

DBZ-loaded PLGA microspheres with a size range of 50–150 μm were prepared as a DBZ delivery system using an emulsion/solvent evaporation technique. The DBZ-loaded PLGA microspheres supported release of DBZ in a sustained manner. The effectiveness of DBZ-loaded PLGA microspheres to induce browning was demonstrated by both *in vitro* and *in vivo* studies. This study for the first time demonstrates the feasibility of developing a bioengineered carrier system for controlled delivery of DBZ to achieve inductive browning using biodegradable polymeric microspheres. To facilitate the translation of our technology platform to have a positive impact in the therapeutic treatment, future efforts will be focused on the evaluation of this controlled delivery system with human cells and tissues.

AUTHORS CONTRIBUTION

MD, SK, CJ, and LK conceived and designed experiments. CJ, LK, MM, FY, MC-V, and NN performed experiments. CJ, LK, and MD analyzed the data. CJ, LK, MM, MD, and SK wrote the manuscript. MD had full access to all the data of the study and takes responsibility for the integrity of the data and accuracy of data analysis.

ACKNOWLEDGMENTS

We thank Donna Brooks for assistance with histology and immunohistochemistry. Funding support from Showalter Trust, Purdue Research Foundation, Transdisciplinary Obesity Prevention Research Sciences (TOPRS) Program, and Purdue Start-up Package is greatly appreciated.

REFERENCES

1. Lloyd-Jones D, Adams R, Carnethon M, De Simone G, Ferguson TB, Flegal K, et al. Heart disease and stroke statistics – 2009 update: a report from the American heart association statistics committee and stroke statistics subcommittee. *Circulation* (2009) **119**:e21–181. doi:10.1161/CIRCULATIONAHA.108.191261
2. Bornfeldt KE, Tabas I. Insulin resistance, hyperglycemia, and atherosclerosis. *Cell Metab* (2011) **14**:575–85. doi:10.1016/j.cmet.2011.07.015
3. Yanovski SZ, Yanovski JA. Long-term drug treatment for obesity a systematic and clinical review. *JAMA* (2014) **311**:74–86. doi:10.1001/jama.2014.2110
4. Chaudhri OB, Wynne K, Bloom SR. Can gut hormones control appetite and prevent obesity? *Diabetes Care* (2008) **31**(Suppl 2):S284–9. doi:10.2337/dc08-s269
5. Bray GA. Use and abuse of appetite-suppressant drugs in the treatment of obesity. *Ann Intern Med* (1993) **119**:707–13. doi:10.7326/0003-4819-119-7_Part_2-199310011-00016
6. Shibata H, Perusse F, Vallerand A, Bukowiecki LJ. Cold exposure reverses inhibitory effects of fasting on peripheral glucose uptake in rats. *Am J Physiol* (1989) **257**:R96–101.
7. Liu X, Perusse F, Bukowiecki LJ. Chronic norepinephrine infusion stimulates glucose uptake in white and brown adipose tissues. *Am J Physiol* (1994) **266**:R914–20.
8. Wu J, Bostrom P, Sparks LM, Ye L, Choi JH, Giang AH, et al. Beige adipocytes are a distinct type of thermogenic fat cell in mouse and human. *Cell* (2012) **150**:366–76. doi:10.1016/j.cell.2012.05.016
9. Wu J, Cohen P, Spiegelman BM. Adaptive thermogenesis in adipocytes: is beige the new brown? *Genes Dev* (2013) **27**:234–50. doi:10.1101/gad.211649.112
10. Tharp KM, Jha AK, Kraiczy J, Yesian A, Karateev G, Sinisi R, et al. Matrix assisted transplantation of functional beige adipose tissue. *Diabetes* (2015) **64**(11):3713–24. doi:10.2337/db15-0728
11. Harms M, Seale P. Brown and beige fat: development, function and therapeutic potential. *Nat Med* (2013) **19**:1252–63. doi:10.1038/nm.3361
12. Emont MP, Yu H, Wu J. Transcriptional control and hormonal response of thermogenic fat. *J Endocrinol* (2015) **225**:R35–47. doi:10.1530/JOE-15-0026
13. Bi P, Shan T, Liu W, Yue F, Yang X, Liang XR, et al. Inhibition of Notch signaling promotes browning of white adipose tissue and ameliorates obesity. *Nat Med* (2014) **20**:911–8. doi:10.1038/nm.3615
14. Bi P, Kuang S. Notch signaling as a novel regulator of metabolism. *Trends Endocrinol Metab* (2015) **26**:248–55. doi:10.1016/j.tem.2015.02.006
15. Uhrich KE, Cannizzaro SM, Langer RS, Shakesheff KM. Polymeric systems for controlled drug release. *Chem Rev* (1999) **99**:3181–98. doi:10.1021/cr940351u
16. Nair LS, Laurencin CT. Biodegradable polymers as biomaterials. *Prog Polym Sci* (2007) **32**:762–98. doi:10.1016/j.progpolymsci.2007.05.017
17. Kumbar SG, Laurencin CT, Deng M. *Natural and Synthetic Biomedical Polymers*. Burlington, San Diego: Elsevier (2014).
18. Varde NK, Pack DW. Microspheres for controlled release drug delivery. *Expert Opin Biol Ther* (2004) **4**:35–51. doi:10.1517/14712598.4.1.35
19. Brannon-Peppas L. Recent advances on the use of biodegradable microparticles and nanoparticles in controlled drug delivery. *Int J Pharm* (1995) **116**:1–9. doi:10.1016/0378-5173(94)00324-X
20. Makadia HK, Siegel SJ. Poly lactic-co-glycolic acid (PLGA) as biodegradable controlled drug delivery carrier. *Polymers (Basel)* (2011) **3**:1377–97. doi:10.3390/polym3031377
21. Berkland C, King M, Cox A, Kim K, Pack DW. Precise control of PLG microsphere size provides enhanced control of drug release rate. *J Control Release* (2002) **82**:137–47. doi:10.1016/S0168-3659(02)00136-0
22. Raman C, Berkland C, Kim K, Pack DW. Modeling small-molecule release from PLG microspheres: effects of polymer degradation and nonuniform drug distribution. *J Control Release* (2005) **103**:149–58. doi:10.1016/j.jconrel.2004.11.012
23. Mao S, Xu J, Cai C, Germershaus O, Schaper A, Kissel T. Effect of WOW process parameters on morphology and burst release of FITC-dextran loaded PLGA microspheres. *Int J Pharm* (2007) **334**:137–48. doi:10.1016/j.ijpharm.2006.10.036
24. Deng M, Kumbar SG, Wan YQ, Toti US, Allcock HR, Laurencin CT. Polyphosphazene polymers for tissue engineering: an analysis of material synthesis, characterization and applications. *Soft Matter* (2010) **6**:3119–32. doi:10.1039/b926402g
25. Austin S, St-Pierre J. PGC1alpha and mitochondrial metabolism – emerging concepts and relevance in ageing and neurodegenerative disorders. *J Cell Sci* (2012) **125**:4963–71. doi:10.1242/jcs.113662
26. Puigserver P, Wu Z, Park CW, Graves R, Wright M, Spiegelman BM. A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. *Cell* (1998) **92**:829–39. doi:10.1016/S0092-8674(00)81410-5
27. Cohen P, Levy JD, Zhang Y, Frontini A, Kolodin DP, Svensson KJ, et al. Ablation of PRDM16 and beige adipose causes metabolic dysfunction and a subcutaneous to visceral fat switch. *Cell* (2014) **156**:304–16. doi:10.1016/j.cell.2013.12.021
28. Benedito R, Roca C, Sorensen I, Adams S, Gossler A, Fruttiger M, et al. The notch ligands Dll4 and Jagged1 have opposing effects on angiogenesis. *Cell* (2009) **137**:1124–35. doi:10.1016/j.cell.2009.03.025
29. Noguera-Troise I, Daly C, Papadopoulos NJ, Coetzee S, Boland P, Gale NW, et al. Blockade of Dll4 inhibits tumour growth by promoting non-productive angiogenesis. *Nature* (2006) **444**:1032–7. doi:10.1038/nature05355
30. Chen L, Holmstrom K, Qiu W, Ditzel N, Shi K, Hokland L, et al. MicroRNA-34a inhibits osteoblast differentiation and in vivo bone formation of human stromal stem cells. *Stem Cells* (2014) **32**:902–12. doi:10.1002/stem.1615
31. Swarnkar G, Karuppaiah K, Mbalaviele G, Chen TH, Abu-Amer Y. Osteopetrosis in TAK1-deficient mice owing to defective NF-kappaB and NOTCH signaling. *Proc Natl Acad Sci U S A* (2015) **112**:154–9. doi:10.1073/pnas.1415213112
32. Jiang C, Wen Y, Kuroda K, Hannon K, Rudnicki MA, Kuang S. Notch signaling deficiency underlies age-dependent depletion of satellite cells in muscular dystrophy. *Dis Model Mech* (2014) **7**:997–1004. doi:10.1242/dmm.015917
33. Wen Y, Bi P, Liu W, Asakura A, Keller C, Kuang S. Constitutive Notch activation upregulates Pax7 and promotes the self-renewal of skeletal muscle satellite cells. *Mol Cell Biol* (2012) **32**:2300–11. doi:10.1128/MCB.06753-11
34. Deng M, Nair LS, Nukavarapu SP, Kumbar SG, Jiang T, Weikel AL, et al. In situ porous structures: a unique polymer erosion mechanism in biodegradable dipeptide-based polyphosphazene and polyester blends producing matrices for regenerative engineering. *Adv Funct Mater* (2010) **20**:2743–957. doi:10.1002/adfm.201090073
35. Middleton JC, Tipton AJ. Synthetic biodegradable polymers as orthopedic devices. *Biomaterials* (2000) **21**:2335–46. doi:10.1016/S0142-9612(00)00101-0
36. Kamei S, Inoue Y, Okada H, Yamada M, Ogawa Y, Toguchi H. New method for analysis of biodegradable polyesters by high-performance liquid chromatography after alkali hydrolysis. *Biomaterials* (1992) **13**:953–8. doi:10.1016/0142-9612(92)90120-D
37. Berchane NS, Carson KH, Rice-Ficht AC, Andrews MJ. Effect of mean diameter and polydispersity of PLG microspheres on drug release: experiment and theory. *Int J Pharm* (2007) **337**:118–26. doi:10.1016/j.ijpharm.2006.12.037
38. Grizzi I, Garreau H, Li S, Vert M. Hydrolytic degradation of devices based on poly(DL-lactic acid) size-dependence. *Biomaterials* (1995) **16**:305–11. doi:10.1016/0142-9612(95)93258-F
39. Spenlehauer G, Vert M, Benoit JP, Boddaert A. In vitro and in vivo degradation of poly(D,L lactide/glycolide) type microspheres made by solvent evaporation method. *Biomaterials* (1989) **10**:557–63. doi:10.1016/0142-9612(89)90063-X
40. Berkland C, Kim K, Pack DW. PLG microsphere size controls drug release rate through several competing factors. *Pharm Res* (2003) **20**:1055–62. doi:10.1023/A:1024466407849
41. Tiraby C, Tavernier G, Lefort C, Larrouy D, Bouillaud F, Ricquier D, et al. Acquirement of brown fat cell features by human white adipocytes. *J Biol Chem* (2003) **278**:33370–6. doi:10.1074/jbc.M305235200
42. Jonker JW, Suh JM, Atkins AR, Ahmadian M, Li P, Whyte J, et al. FGF1 axis is required for adaptive adipose remodelling and metabolic homeostasis. *Nature* (2012) **485**:391–4. doi:10.1038/nature10998
43. Fisher FM, Kleiner S, Douris N, Fox EC, Mepani RJ, Verdeguer F, et al. FGF21 regulates PGC-1alpha and browning of white adipose tissues in adaptive thermogenesis. *Genes Dev* (2012) **26**:271–81. doi:10.1101/gad.177857.111
44. Fu L, John LM, Adams SH, Yu XX, Tomlinson E, Renz M, et al. Fibroblast growth factor 19 increases metabolic rate and reverses dietary and leptin-deficient diabetes. *Endocrinology* (2004) **145**:2594–603. doi:10.1210/en.2003-1671

45. Whittle AJ, Carobbio S, Martins L, Slawik M, Hondares E, Vazquez MJ, et al. BMP8B increases brown adipose tissue thermogenesis through both central and peripheral actions. *Cell* (2012) **149**:871–85. doi:10.1016/j.cell.2012.02.066
46. Tseng YH, Kokkotou E, Schulz TJ, Huang TL, Winnay JN, Taniguchi CM, et al. New role of bone morphogenetic protein 7 in brown adipogenesis and energy expenditure. *Nature* (2008) **454**:1000–4. doi:10.1038/nature07221
47. Bostrom P, Wu J, Jedrychowski MP, Korde A, Ye L, Lo JC, et al. A PGC1-alpha-dependent myokine that drives brown-fat-like development of white fat and thermogenesis. *Nature* (2012) **481**:463–8. doi:10.1038/nature10777
48. Bordicchia M, Liu D, Amri EZ, Ailhaud G, Dessi-Fulgheri P, Zhang C, et al. Cardiac natriuretic peptides act via p38 MAPK to induce the brown fat thermogenic program in mouse and human adipocytes. *J Clin Invest* (2012) **122**:1022–36. doi:10.1172/JCI59701
49. Himms-Hagen J, Melnyk A, Zingaretti MC, Ceresi E, Barbatelli G, Cinti S. Multilocular fat cells in WAT of CL-316243-treated rats derive directly from white adipocytes. *Am J Physiol Cell Physiol* (2000) **279**:C670–81.
50. Nguyen KD, Qiu Y, Cui X, Goh YP, Mwangi J, David T, et al. Alternatively activated macrophages produce catecholamines to sustain adaptive thermogenesis. *Nature* (2011) **480**:104–8. doi:10.1038/nature10653
51. Collins S. Beta-adrenoceptor signaling networks in adipocytes for recruiting stored fat and energy expenditure. *Front Endocrinol* (2011) **2**:102. doi:10.3389/fendo.2011.00102
52. Wei W, Dutchak PA, Wang X, Ding X, Wang X, Bookout AL, et al. Fibroblast growth factor 21 promotes bone loss by potentiating the effects of peroxisome proliferator-activated receptor gamma. *Proc Natl Acad Sci U S A* (2012) **109**:3143–8. doi:10.1073/pnas.1200797109

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

MINI REVIEW
published: 20 October 2015
doi: 10.3389/fendo.2015.00164

Bioengineering beige adipose tissue therapeutics

Kevin M. Tharp[1,2] *and Andreas Stahl*[1]*

[1] Program in Metabolic Biology, Department of Nutritional Science and Toxicology, University of California Berkeley, Berkeley, CA, USA, [2] Department of Bioengineering, University of California Berkeley, Berkeley, CA, USA

Unlocking the therapeutic potential of brown/beige adipose tissue requires technological advancements that enable the controlled expansion of this uniquely thermogenic tissue. Transplantation of brown fat in small animal model systems has confirmed the expectation that brown fat expansion could possibly provide a novel therapeutic to combat obesity and related disorders. Expansion and/or stimulation of uncoupling protein-1 (UCP1)-positive adipose tissues have repeatedly demonstrated physiologically beneficial reductions in circulating glucose and lipids. The recent discovery that brown adipose tissue (BAT)-derived secreted factors positively alter whole body metabolism further expands potential benefits of brown or beige/brite adipose expansion. Unfortunately, there are no sources of transplantable BATs for human therapeutic purposes at this time. Recent developments in bioengineering, including novel hyaluronic acid-based hydrogels, have enabled non-immunogenic, functional tissue allografts that can be used to generate large quantities of UCP1-positive adipose tissue. These sophisticated tissue-engineering systems have provided the methodology to develop metabolically active brown or beige/brite adipose tissue implants with the potential to be used as a metabolic therapy. Unlike the pharmacological browning of white adipose depots, implantation of bioengineered UCP1-positive adipose tissues offers a spatially controlled therapeutic. Moving forward, new insights into the mechanisms by which extracellular cues govern stem-cell differentiation and progenitor cell recruitment may enable cell-free matrix implant approaches, which generate a niche sufficient to recruit white adipose tissue-derived stem cells and support their differentiation into functional beige/brite adipose tissues. This review summarizes clinically relevant discoveries in tissue-engineering and biology leading toward the recent development of biomaterial supported beige adipose tissue implants and their potential for the metabolic therapies.

Keywords: beige, adipose, brown fat, brown adipose, obesity, diabetes mellitus, bioengineering, hydrogel

OPEN ACCESS

Edited by:
Jun Wu,
University of Michigan, USA

Reviewed by:
Alexandros Vegiopoulos,
German Cancer Research Center (DKFZ), Germany
Meilian Liu,
University of New Mexico Health Sciences Center, USA

***Correspondence:**
Andreas Stahl
astahl@berkeley.edu

Specialty section:
This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *01 September 2015*
Accepted: *05 October 2015*
Published: *20 October 2015*

Citation:
Tharp KM and Stahl A (2015) Bioengineering beige adipose tissue therapeutics. Front. Endocrinol. 6:164. doi: 10.3389/fendo.2015.00164

INTRODUCTION

Proposing brown adipose tissue (BAT) expansion as a therapeutic treatment for obesity and obesity-related disorders has recently gained significant traction (1, 2). BAT, as well as beige adipocytes (3, 4), has high metabolic capacity due to high mitochondrial content and expression of uncoupling protein-1 (UCP1) (5), a long-chain fatty acid anion/proton-symporter (6), found in the inner mitochondrial membrane. UCP1 decouples the action of ATP-synthase and dissipates the proton gradient produced by the electron transport chain, thus generating heat. BAT mass inversely

correlates with body mass index (BMI), which supports the notion that BAT may regulate overall bodyweight and metabolic health (7). The benefits of expanded UCP1-expressing adipose may not be limited to their metabolic characteristics, as brown adipose has been shown to possess a potent secretome (8). Notably, implanted BAT has been shown to produce secretions of IGF-1, which was reported to enable the insulin-independent reversal of type-1 diabetes (9). BAT also appears to exert significant effects on the lipid metabolism in the liver (10) and bone mineral density (11, 12). Overall, the generation of functional beige or brown adipocytes for therapeutic purposes appears to hold significant merit for a number of possible treatments.

The current strategies to clinically deploy BAT fall into two main categories: pharmaceutical or genetic interventions to induce endogenous BAT/beige differentiation pathways, and the *ex vivo* generation of autologous cell/tissue transplants (13–17). Current gene-therapy approaches still have challenges to overcome before they are applied as anti-obesity therapeutics (18), but have proven to be significant instruments for investigating BAT biology (16, 19). Pharmacological activation of the pathways that drive a white adipose tissue (WAT) to beige/brite transition, a process known as "browning", offer little control over the location and temporal extent of the effects. Transplantation of autologous BAT in small animal models has shown clear metabolic enhancements (20–22) but approaches are unlikely to be suitable for human therapies since there are few sources of transplantable mature human BAT and immune-rejection would need to be overcome. To successfully harness the therapeutic potential of BAT, a readily available source of transplantable cells with the potential to robustly generate UCP1-positive (UCP1+) adipocytes must first be identified or created. Second, the basis to form a functional, non-immunogenic, highly localized, and metabolically active brown/beige tissue must be defined.

TISSUES FOR TISSUE-BASED THERAPIES

The use of small molecules or growth factors to induce the browning of WAT is a promising area of research, but systemic dosing has the potential to produce off target effects with undesirable consequences. One notable browning agent, rosiglitazone, was widely used to treat diabetes but has disconcerting side effects that included heart failure (23). Browning factors (24–39) generally target conserved signaling pathways, making specificity a concern. By using biomaterials to localize and control release kinetics of browning factors to desired anatomical locations, an acceptable level of specificity may be achieved (40–46). When increasing the number of brown adipocytes, other supporting cell types are required for producing a functional tissue. For BAT, this includes interactions between the nervous, vascular, and immune systems, with UCP1+ adipocytes. Efforts to understand how brown or beige fat responds to cold temperature have uncovered evidence that classical brown fat relies on the sympathetic nervous system (47–49) and immune system (50, 51) to initiate and maintain the thermogenic response. While immune (33, 34) and nervous systems (52) also play a major role in beige fat development and activation, beige adipocytes have the innate ability to sense temperature and independently respond by either differentiating into UCP1+ adipose and/or by inducing uncoupled respiration (53). Thus, classical brown, but not beige, tissue therapeutics will likely require sympathetic innervation for thermogenic function and persistence. The vascular system enables access to metabolic substrates, as well as the oxygen required for the metabolic activity of brown/beige fat (10). Additionally, vascular networks circulate activating or browning signals (25, 30, 35, 54–56). In addition to innervation and vascularization, tissue-based therapies facilitate important cell–cell and cell–extracellular matrix (ECM) interactions that provide function altering chemical and physical inputs (57–62).

Natural ECM is comprised of collagens, elastins, fibronectin, laminins, proteoglycans, and glycosaminoglycans (63). The ECM is a highly organized network of physical signals that dynamically interact with the cells it supports. The topology and composition of the ECM is heavily remodeled, especially during differentiation. Remodeling of the ECM is a balance of specific degradation by matrix metalloproteinases (MMPs), new matrix component deposition, unmasking of cryptic binding sites in response to cleavage or tension, crosslinking or bonding of ECM components, and inside-out signaling from adhesion receptors on cellular surfaces. Growth factor signaling is regulated through the ECM by controlling their capture or exclusion, rate of delivery to the cell surface receptor, and molecular presentation (64). The ECM establishes a biological framework that provides physical support to cells, but also regulates signaling through adhesion receptors and alters endocrine, paracrine, autocrine, and juxtacrine signals (62). To date, many groups have remade "synthetic" tissues by decellularizing the desired tissue, then reseeding the remaining native ECM with cell populations (65, 66). However, given the inaccessibility of mature BAT, this approach will be difficult to translate to artificial UCP1+ tissues.

Tissue architecture has been known to exert strong effects on cell behavior, and *in vitro* hydrogels, meant to mimic natural ECMs, appear to be a successful option to improve cell culture models (67). These 3-dimensional (3D) hydrogels, which are water swollen polymer networks, have been utilized to enhance hepatocyte (68) and pancreatic islet function (69). Not only does 3D-organization affect the function and viability of cells (70), but it mediates the differentiation of many cell types (71), especially adipocytes. Adipogenesis is highly dependent on cytoskeletal rearrangements where cell shape is altered to accommodate the intracellular accumulation and organization of lipids. This was discovered when preadipocytes could not differentiate when cultured on a surface of fibronectin (72); this phenotype was rescued by disrupting the cytoskeletal response to the supraphysiological abundance of fibronectin. Laminin also has been found to play a role in WAT expansion (73). Another adipose ECM component, Collagen VI, was found to be an essential microenvironmental signal for adipocytes to regulate the amount of TAG they accumulate (74). When Collagen VI is removed from the adipose ECM, adipocytes develop a hypertrophic state in which the adipocytes are capable of sequestering significantly more lipids than normal. Specific ECM degradation is also a critical feature of WAT development (75). These phenomenal findings support the notion that the adipose ECM is an integral signal for adipocyte behavior and overall condition and function of the organ. When working with *in vitro* models of white adipocytes, it is also evident that the prototypical unilocular lipid

droplets observed *in vivo* are not present unless cultured in 3D-matrices (76, 77).

Comparatively little is known about the ECMs of WAT, BAT, and the changes in adipose-matrix during browning. As this area of research is explored further, ECM-derived signals will hopefully be identified to build our understanding of adipocyte-ECM interactions and provide applicable systems to induce the formation of UCP1+ adipocytes. Biomaterials have utilized the incorporation of whole ECM components, such as laminin (78) or collagen (79), to improve their biocompatibility and effectiveness as cell scaffolds. Whether or not specific mixtures of ECM components can be combined with preadipocytes to form functional brown fat has yet to be determined, but this approach has already been applied to WAT (66, 80, 81). ECM molecules could theoretically be derived from the same tissues used to isolate the primary precursor cell populations for the generation of an adipose allograft (66) but thermogenic fat may have a distinct structural niche capable of engaging specific integrin (82) and syndecan (83) populations. Integrin expression is known to be highly dynamic during adipogenisis (84) and the beta-1 integrin has been extensively used to purify preadipocytes (85). Therefore, targeting integrin signaling appears to be a logical method to alter adipogenesis and it is likely that UCP1+ tissue function is dependent on specific 3D interactions and organization. This has now been demonstrated via integrin-ligands, in the form of matrix-derived peptide sequences and secreted molecules, on both UCP1-expression and lipid accumulation (86, 87).

MATERIALS TO BUILD TISSUES

Decellularized tissue-based tissue-engineering is not the only method to produce synthetic tissues (81, 88, 89). Polymeric biomaterials with bioactive modifications have been employed to generate many tissue types (61, 90–92). Tissue-engineering is a sophisticated endeavor that requires significant tuning, therefore modular approaches should be considered for the construction of functional synthetic tissues. Specifically, the physical properties, degradation kinetics, and biologically interactive components should be able to be altered independently. Bioengineered tissues can be generated through the selection of a core polymer, a crosslinking system, bioactive modifications, and the incorporated cell populations (**Figure 1**). The core material of the

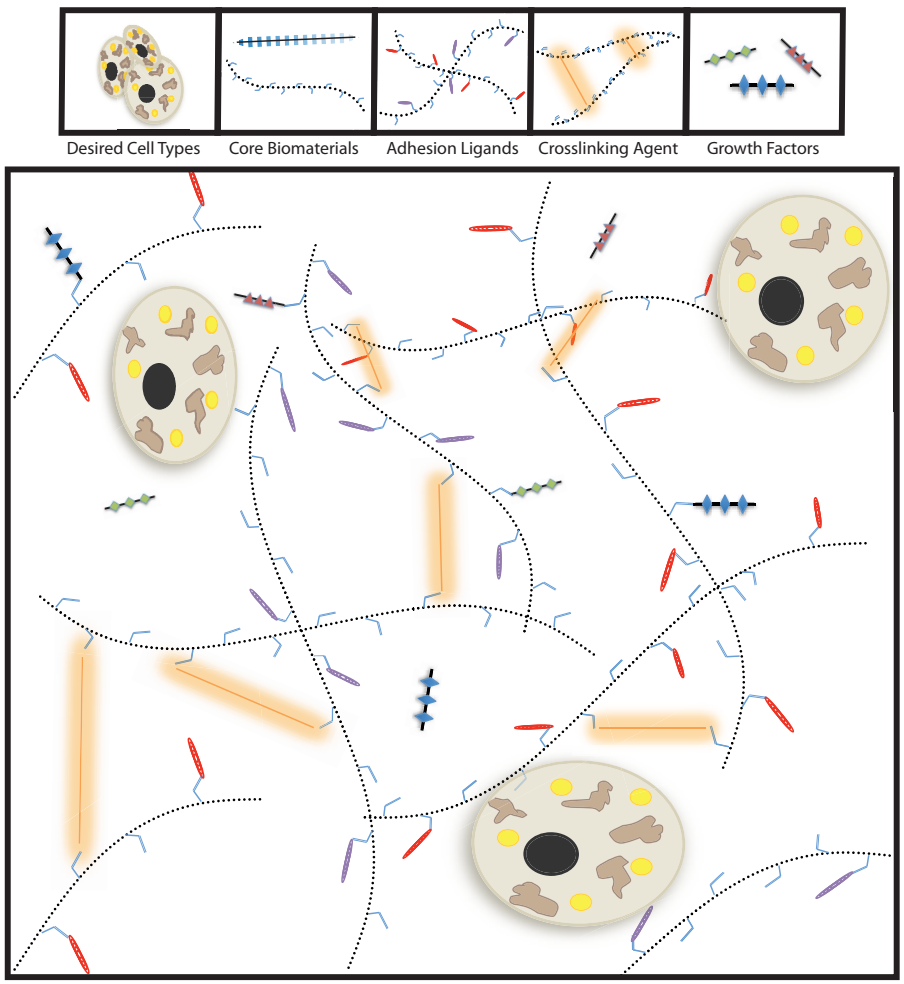


FIGURE 1 | Modular tissue-engineering. Here, we visually depict the five bioengineering variables discussed throughout this review. By tailoring the properties biochemical and physical properties of the biomaterial, synthetic tissues for therapeutic purposes can be successfully generated.

biomaterial determines the method of deployment (injectable-vs.-implantable), macrostructure, and the behavior of the cellular payload.

While many biocompatible materials have been proposed to engineer beige/brown-adipose tissue (93), HyA-based hydrogels show particular promise for the engineering of adipose tissue for therapeutic purposes (94, 95) and is the only material that has been successfully used to establish brown/beige fat implants *in vivo* (86). HyA is a naturally occurring glycosaminoglycan, consisting of β-1,4-D-glucuronic acid– β-1,3-*N*-acetyl-D-glucosamine, that is highly tractable for tissue-engineering for biomedical purposes (94, 95). Endogenous HyA is synthesized by hyaluronic acid synthase and extruded into the extracellular microenvironment, where it functions as an essential component of the native ECM and interacts with cell surface receptors such as CD44 or RHAMM (96). HyA is highly variable in length, spanning lengths up to 10 μM and ranging from 100 kDa to 8 mDa. Hyaluronic acid plays a pivotal role in ECM organization through its interactions with the other major components of the ECM (63).

HyA scaffolds enhance survival of autologous adipose stem-cell implants (97–100) and possibly promote adipose expansion (101). Not only are HyA-based hydrogels naturally occurring, biocompatible, modifiable, injectable, biodegradable, non-immunogenic, and anti-thrombogenic (94, 102), but also HyA has already been FDA approved for a number of clinical applications such as correction of facial lipoatrophy, wrinkle and scar removal, amelioration of osteoarthritic joint pain, dietary supplementation, ulcers, and cataract surgery. HyA has also showed clinical success for the temporary esthetic augmentation of lips, breasts, and buttocks (103), and achieved impressive results as a replacement of traditional dressings of epidermal burns and lesions (104). Importantly, utilizing HyA avoids fibrotic encapsulation of implants, a problem that has plagued the early literature of bioengineering (105). In general, avoiding encapsulation requires the use of non-immunogenic materials and cells, biodegradable materials, nearly anisotropic physical properties to the surrounding tissues, and possibly growth factors to induce recruitment of host-derived cells into the implant. HyA has been shown to have significant effects on tissue remodeling and cell signaling, and is naturally degraded by hyaluronidase or oxidizing agents (106).

CELLS FOR METABOLIC THERAPEUTICS

Generation of bioengineered-BAT will rely on an easily accessible and ample source of progenitor cells. A key discovery in this regard came from the observation that UCP1+ adipocytes can be generated by certain WAT depots (107). These distinct adipocytes are described as beige/brite, and they express unique surface markers (3). While genetic factors play a major role in the ability to generate beige adipocytes (108, 109), the expansion of beige adipose mass has been linked to improved metabolic health. Therefore, the isolation of adipose-derived multipotent stem cells (MSCs) from undesirable WAT depots and subsequent reintroduction as autologous BAT shows therapeutic promise. One of the most abundant sources of preadipocytes is WAT, and the implantation of WAT-derived stem cells is an FDA accepted procedure (110).

The most common source of WAT-derived stem cells is the stromal vascular fraction (SVF), which contains T cells, B cells, mast cells, adipose tissue macrophages, and MSCs such as preadipocytes and endothelial progenitor cells. This cellular fraction can be further purified to enrich preadipocytes by selecting for cell surface markers such as Pref-1+, Lin−, CD29+, CD34+, Sca-1+, CD24+, CD45−, Mac1−, PDGFRα+ (15, 17, 111). Additionally, preadipocyte sorting can be used to enrich for populations that are known to readily transdifferentiate into beige/brite fat such as CD137, TMEM26, ASC-1, PAT2, and P2RX5 (112). Alternatively, as stem-cell therapies become more accepted, induced pluripotent cells might be a suitable option since iPSC-derived brown adipocytes have also been generated and transplants of these cells show promising metabolic effects (16, 113).

While a purified population of preadipocytes or adipocytes may provide more conclusive insights for biological experiments, it may not be optimal for building a functional tissue. As previously mentioned, the immune system and vascular systems are essential for supporting the function and formation of beige adipose tissue and other SVF components could contribute to these tissue types. For example, macrophages and T cells have been shown to be an important part of beige/brown-adipose function and development (33, 51, 114) and interactions between the adipocytes and the vascular niche may also be important for browning (115–118) particularly through cell–cell interactions, cytokines, and growth factors such as Il-33 (35) or VEGF-A (119). Thus, the use of multiple purified cell population or utilization of non-purified-SVF, as recently demonstrated for the generation of bioengineered-BAT (86), may offer distinct advantages.

BIOMATERIAL OPTIMIZATION

Degradation and remodeling ability of the synthetic ECM is just as important as the initial structure itself. If the synthetic tissues are not biodegradable through mechanisms that cells naturally use for movement and reorganization, integrating with the host will be jeopardized. Specifically, to facilitate effective remodeling and reorganization of the tissue by the immune and vascular systems, biodegradable and biologically interactive biomaterials should be utilized. The simplest way to imbue a biomaterial with biodegradability is to use MMP-sensitive crosslinking agents. Most of the available core materials can be easily modified to accommodate the current MMP-sensitive crosslinkers, which are short peptides containing an MMP-specific cleavage site (61, 120–122). Most of these core materials will be modified to facilitate efficient crosslinking by spontaneous aqueous phase reactions, such as the Michael addition where a thiol and acrylate form a thio-ether bond (92, 123). These types of biodegradable crosslinkers have been shown to be essential for the recruitment of host cells for the successful *in vivo* integration of biomaterial implants (122, 124).

Additionally, the elastic modulus of biomaterials has been shown to be highly instructive for the differentiation of MSCs into adipocytes (125–127). This mechanotransductive control of differentiation can be accomplished without applying direct physical forces to cells. By presenting a cell with an adhesion-promoting environment, matrix-associated adhesions form and produce an

intrinsic mechanotransductive signal for the cell, as well as adjacent cells in the microenvironment. Therefore, a soft biomaterial optimized with specific adhesion-promoting ligands may be capable of inducing the same mechanotransductive signals as a much stiffer material. Numerous biological processes are affected by mechanical signals; notably, nuclear envelope plasticity and permeability (128, 129), splicing (130), and signal transduction (131). The prominent browning factor, BMP7, is known to alter cytoskeletal dynamics in adipocytes and other cell types, which supports the notion that physical cues may be important for bioengineered-BAT (32, 132–134). Interestingly, our group found that the storage modulus of WAT seems to differ from that of BAT (WAT ~ 3 kPa, BAT ~ 4 kPa). How important this difference in modulus is for brown fat development and function remains to be explored more systematically.

Bioactive modifications, such as integrin-binding domains conjugated to hydrogels, have become commonplace to enhance the bioactivity of biomaterials (61, 90, 91) and HyA-hydrogels augment integrin signaling (135). These materials provide some degree of ECM-mimicry without replicating the entire complexity of the native ECM. For example, alginate conjugated with RGD-containing ligands is supportive to cardiomyocytes and also promotes adipogenisis (136, 137). However, alginate's effects on adipogenisis may be due to the rounded morphology, shown to be strongly instructive to adipogenesis (72), cells undergo when encapsulated in alginate-based materials (138). Other peptide-modified hydrogels promote the formation of bone via collagen mimetic peptides (139). ADMSC-spheroids entrapped in Poly(ethylene glycol)-hydrogels have been proposed to form beige adipocytes *in vitro* (140) and have been modified to drive MSCs toward adipogenic or osteogenic fates (141). Interestingly, Vaicik et al. find UCP1-expression highest when the storage modulus of the hydrogel is BAT-equivalent.

Recent work by our laboratory to develop bioengineered-BAT with HyA-based biomaterials, differentiation promoting adhesion ligands, MMP-sensitive crosslinkers, and ADMSCs has produced a synthetic beige adipose tissue. The discovery of these bioactive modifications came from an effort to screen for ligands that preferentially promoted the attachment of UCP1+ adipocytes. Incidentally, it was discovered that some of these bioactive components, derived from laminin, directly enhanced UCP1-expression (86). The identified bioactive peptides were then conjugated to a HyA core material to promote browning. The assembly of the optimized hydrogel with adipocytes was termed beige adipose tissue-matrix-assisted cellular transplant (BAT-MACT), and was shown to successfully scaffold beige adipocytes for therapeutic purposes in animal models. What is particularly exciting about these HyA-hydrogels and MMP-sensitive crosslinkers is that they are injectable, making this approach as non-invasive as possible. Due to HyA's viscoelastic properties, it can be easily mixed with the desired cell populations and just before implantation, mixed with the crosslinker. This allows for a short period of time where the cell-laden hydrogel is still a liquid and can be injected to the desired anatomical location with subsequent crosslinking to solidify a functional organoid.

The BAT-MACT implant system (**Figure 2**) has shown that implanted beige fat can have a nearly immediate effect on glucose homeostasis that persists during the duration of the implant's lifespan. Implanted beige adipose, similar to brown adipose, responds to cold stimulus by increasing lipid uptake an oxidation. The expansion of beige fat attenuates weight gain on a high-fat diet and induces a thermo-responsive metabolic augmentation to the recipient (86). Being that adipocytes derived from WAT sources are capable of sensing temperature independent neuronal signaling, it is not surprising that an implant of beige fat behaves this way. However, implanted classical brown adipose may not necessarily function in this manner, as the organ relies heavily on the sympathetic neuronal stimulation to induce the thermogenic program.

FUTURE DIRECTIONS

The first iteration of BAT-MACTs had an *in vivo* lifespan of approximately 3–4 weeks (86), which allows for well-controlled spatio-temporal applications but may be too short for single-application-based metabolic interventions. Further effort is needed to understand what would promote the maintenance of such a metabolically active depot for longer periods of time and if a microenvironment capable of promoting beige adipose

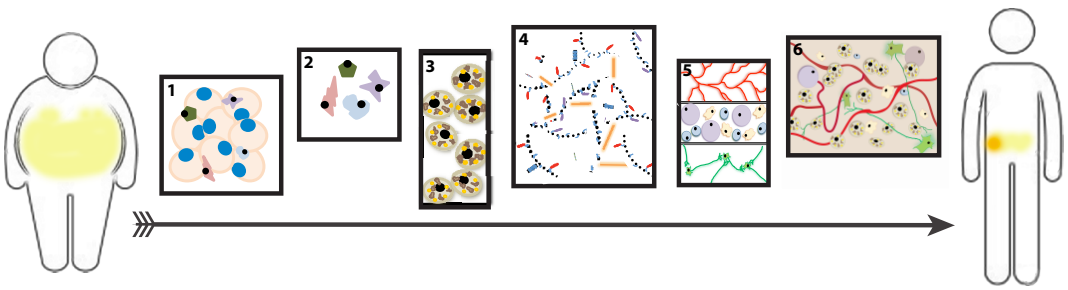


FIGURE 2 | Beige adipose tissue-matrix-assisted cellular transplant (BAT-MACT). The strategy used to expand beige adipose tissue for therapeutic purposes can be deconstructed into six stages: (1) Isolation of white adipose tissue (WAT) from patient. (2) Purification of stromal vascular fraction from the explanted WAT. (3) *In vitro* differentiation of SVF-derived preadipocytes toward the beige fat phenotype. (4) Suspension of beige adipocytes (SVF) into optimized hydrogel for implantation. (5) Post implantation, the hydrogel is biocompatible and degradable so that it is permissive to integrate the recipient's vascular, immune, and nervous systems. (6) The established beige adipose tissue functions as a metabolically active organ. If a sufficient increase of metabolic rate is achieved via BAT-MACT implantation, weight loss will occur.

self-renewal could be created (71). The inclusion of modifications that enhance beige adipogenesis or metabolic activity, such as sequestered growth factors, small molecules, or mixed materials will certainly be pursued. Incorporating metabolic activators of beige adipocytes into the BAT-MACT system may be an essential step toward promoting weight loss and metabolic improvement in the face of thermoneutrality. At this stage, by using immobilized synthetic physical cues, the biomaterial component has a significantly lower risk of off target effects relative to a similarly functioning implant generated with secreted factors temporarily sequestered within the matrix. At its extreme, the implanted matrix could be endowed with sufficient biochemical and biophysical clues for the recruitment and directed differentiation of stem cells to allow for cell-free implants that are sufficient to establish beige adipose tissue. Such an acellular implant would be attractive as it could avoid many cost and health concerns associated with cellular implants.

Overall, extension of tissue-engineering principles to brown fat biology holds the promise of furthering both our mechanistic understanding of the factors required for brown/beige fat function and development, the rapid testing of hypothesis regarding the physiological impact of brown/beige tissue expansion, and ultimately novel treatment options for metabolic disorders.

ACKNOWLEDGMENTS

This work was supported in part by NIH grants R01DK101293 and R01DK089202, and the American Diabetes Association basic science award 1-14-BS-191 awarded to AS. We thank the Dr. Kevin Healy and Dr. Amit Jha for their contributions, and Jordan Tharp for assistance with figures and syntax.

REFERENCES

1. Liu Y, Vaughan CH, Bartness TJ, Yoneshiro T, Aita S, Matsushita M, et al. Recruited brown adipose tissue as an antiobesity agent in humans. *J Neurosci* (2013) **123**:3404–8. doi:10.1172/JCI67803
2. Boss O, Farmer SR. Recruitment of brown adipose tissue as a therapy for obesity-associated diseases. *Front Endocrinol* (2012) **3**:14. doi:10.3389/fendo.2012.00014
3. Wu J, Bostrom P, Sparks LM, Ye L, Choi JH, Giang AH, et al. Beige adipocytes are a distinct type of thermogenic fat cell in mouse and human. *Cell* (2012) **150**:366–76. doi:10.1016/j.cell.2012.05.016
4. Rosen ED, Spiegelman BM. What we talk about when we talk about fat. *Cell* (2014) **156**:20–44. doi:10.1016/j.cell.2013.12.012
5. Nicholls DG, Locke RM. Thermogenic mechanisms in brown fat. *Physiol Rev* (1984) **64**:1–64.
6. Fedorenko A, Lishko PV, Kirichok Y. Mechanism of fatty-acid-dependent UCP1 uncoupling in brown fat mitochondria. *Cell* (2012) **151**:400–13. doi:10.1016/j.cell.2012.09.010
7. Celi FS. Brown adipose tissue – when it pays to be inefficient. *N Engl J Med* (2009) **360**:1553–6. doi:10.1056/NEJMe0900466
8. Wang GX, Zhao XY, Lin JD. The brown fat secretome: metabolic functions beyond thermogenesis. *Trends Endocrinol Metab* (2015) **26**:231–7. doi:10.1016/j.tem.2015.03.002
9. Gunawardana SC, Piston DW. Insulin-independent reversal of type 1 diabetes in nonobese diabetic mice with brown adipose tissue transplant. *Am J Physiol Endocrinol Metab* (2015) **308**:E1043–55. doi:10.1152/ajpendo.00570.2014
10. Wang GX, Zhao XY, Meng ZX, Kern M, Dietrich A, Chen Z, et al. The brown fat-enriched secreted factor Nrg4 preserves metabolic homeostasis through attenuation of hepatic lipogenesis. *Nat Med* (2014) **20**:1436–43. doi:10.1038/nm.3713
11. Bredella MA, Gill CM, Rosen CJ, Klibanski A, Torriani M. Positive effects of brown adipose tissue on femoral bone structure. *Bone* (2014) **58**:55–8. doi:10.1016/j.bone.2013.10.007
12. Nguyen AD, Lee NJ, Wee NK, Zhang L, Enriquez RF, Khor EC, et al. Uncoupling protein-1 is protective of bone mass under mild cold stress conditions. *Bone* (2015). doi:10.1016/j.bone.2015.05.037
13. Ohno H, Shinoda K, Spiegelman BM, Kajimura S. PPARgamma agonists induce a white-to-brown fat conversion through stabilization of PRDM16 protein. *Cell Metab* (2012) **15**:395–404. doi:10.1016/j.cmet.2012.01.019
14. Elabd C, Chiellini C, Carmona M, Galitzky J, Cochet O, Petersen R, et al. Human multipotent adipose-derived stem cells differentiate into functional brown adipocytes. *Stem Cells* (2009) **27**:2753–60. doi:10.1002/stem.200
15. Schulz TJ, Huang TL, Tran TT, Zhang H, Townsend KL, Shadrach JL, et al. Identification of inducible brown adipocyte progenitors residing in skeletal muscle and white fat. *Proc Natl Acad Sci U S A* (2011) **108**:143–8. doi:10.1073/pnas.1010929108
16. Ahfeldt T, Schinzel RT, Lee YK, Hendrickson D, Kaplan A, Lum DH, et al. Programming human pluripotent stem cells into white and brown adipocytes. *Nat Cell Biol* (2012) **14**:209–19. doi:10.1038/ncb2411
17. Rodeheffer MS, Birsoy K, Friedman JM. Identification of white adipocyte progenitor cells in vivo. *Cell* (2008) **135**:240–9. doi:10.1016/j.cell.2008.09.036
18. Edelstein ML, Abedi MR, Wixon J, Edelstein RM. Gene therapy clinical trials worldwide 1989-2004-an overview. *J Gene Med* (2004) **6**:597–602. doi:10.1002/jgm.619
19. Kajimura S, Seale P, Kubota K, Lunsford E, Frangioni JV, Gygi SP, et al. Initiation of myoblast to brown fat switch by a PRDM16-C/EBP-beta transcriptional complex. *Nature* (2009) **460**:1154–8. doi:10.1038/nature08262
20. Liu X, Wang S, You Y, Meng M, Zheng Z, Dong M, et al. Brown adipose tissue transplantation reverses obesity in ob/ob mice. *Endocrinology* (2015) **156**:2461–9. doi:10.1210/en.2014-1598
21. Gunawardana SC, Piston DW. Reversal of type 1 diabetes in mice by brown adipose tissue transplant. *Diabetes* (2012) **61**:674–82. doi:10.2337/db11-0510
22. Stanford KI, Middelbeek RJ, Townsend KL, An D, Nygaard EB, Hitchcox KM, et al. Brown adipose tissue regulates glucose homeostasis and insulin sensitivity. *J Clin Invest* (2013) **123**:215–23. doi:10.1172/JCI62308
23. Komajda M, McMurray JJ, Beck-Nielsen H, Gomis R, Hanefeld M, Pocock SJ, et al. Heart failure events with rosiglitazone in type 2 diabetes: data from the record clinical trial. *Eur Heart J* (2010) **31**:824–31. doi:10.1093/eurheartj/ehp604
24. Moisan A, Lee YK, Zhang JD, Hudak CS, Meyer CA, Prummer M, et al. White-to-brown metabolic conversion of human adipocytes by JAK inhibition. *Nat Cell Biol* (2015) **17**:57–67. doi:10.1038/ncb3075
25. Bostrom P, Wu J, Jedrychowski MP, Korde A, Ye L, Lo JC, et al. A PGC1-alpha-dependent myokine that drives brown-fat-like development of white fat and thermogenesis. *Nature* (2012) **481**:463–8. doi:10.1038/nature10777
26. Jedrychowski MP, Wrann CD, Paulo JA, Gerber KK, Szpyt J, Robinson MM, et al. Detection and quantitation of circulating human irisin by tandem mass spectrometry. *Cell Metab* (2015) **22**734–40. doi:10.1016/j.cmet.2015.08.001
27. Rao RR, Long JZ, White JP, Svensson KJ, Lou J, Lokurkar I, et al. Meteorin-like is a hormone that regulates immune-adipose interactions to increase beige fat thermogenesis. *Cell* (2014) **157**:1279–91. doi:10.1016/j.cell.2014.03.065
28. Sellayah D, Sikder D. Orexin restores aging-related brown adipose tissue dysfunction in male mice. *Endocrinology* (2014) **155**:485–501. doi:10.1210/en.2013-1629
29. Than A, He HL, Chua SH, Xu D, Sun L, Leow MK, et al. Apelin enhances brown adipogenesis and browning of white adipocytes. *J Biol Chem* (2015) **290**:14679–91. doi:10.1074/jbc.M115.643817
30. Fisher FM, Kleiner S, Douris N, Fox EC, Mepani RJ, Verdeguer F, et al. FGF21 regulates PGC-1alpha and browning of white adipose tissues in adaptive thermogenesis. *Genes Dev* (2012) **26**:271–81. doi:10.1101/gad.177857.111
31. Tseng YH, Kokkotou E, Schulz TJ, Huang TL, Winnay JN, Taniguchi CM, et al. New role of bone morphogenetic protein 7 in brown adipogenesis and energy expenditure. *Nature* (2008) **454**:1000–4. doi:10.1038/nature07221

32. McDonald ME, Li C, Bian H, Smith BD, Layne MD, Farmer SR. Myocardin-related transcription factor A regulates conversion of progenitors to beige adipocytes. *Cell* (2015) **160**:105–18. doi:10.1016/j.cell.2014.12.005
33. Qiu Y, Nguyen KD, Odegaard JI, Cui X, Tian X, Locksley RM, et al. Eosinophils and type 2 cytokine signaling in macrophages orchestrate development of functional beige fat. *Cell* (2014) **157**:1292–308. doi:10.1016/j.cell.2014.03.066
34. Lee MW, Odegaard JI, Mukundan L, Qiu Y, Molofsky AB, Nussbaum JC, et al. Activated type 2 innate lymphoid cells regulate beige fat biogenesis. *Cell* (2015) **160**:74–87. doi:10.1016/j.cell.2014.12.011
35. Brestoff JR, Kim BS, Saenz SA, Stine RR, Monticelli LA, Sonnenberg GF, et al. Group 2 innate lymphoid cells promote beiging of white adipose tissue and limit obesity. *Nature* (2015) **519**:242–6. doi:10.1038/nature14115
36. Daquinag AC, Tseng C, Salameh A, Zhang Y, Amaya-Manzanares F, Dadbin A, et al. Depletion of white adipocyte progenitors induces beige adipocyte differentiation and suppresses obesity development. *Cell Death Differ* (2015) **22**:351–63. doi:10.1038/cdd.2014.148
37. Himms-Hagen J, Cui J, Danforth E Jr, Taatjes DJ, Lang SS, Waters BL, et al. Effect of CL-316,243, a thermogenic beta 3-agonist, on energy balance and brown and white adipose tissues in rats. *Am J Physiol* (1994) **266**:R1371–82.
38. Bonet ML, Oliver P, Palou A. Pharmacological and nutritional agents promoting browning of white adipose tissue. *Biochim Biophys Acta* (2013) **1831**:969–85. doi:10.1016/j.bbalip.2012.12.002
39. Villarroya F, Vidal-Puig A. Beyond the sympathetic tone: the new brown fat activators. *Cell Metab* (2013) **17**:638–43. doi:10.1016/j.cmet.2013.02.020
40. Jha AK, Mathur A, Svedlund FL, Ye J, Yeghiazarians Y, Healy KE. Molecular weight and concentration of heparin in hyaluronic acid-based matrices modulates growth factor retention kinetics and stem cell fate. *J Control Release* (2015) **209**:308–16. doi:10.1016/j.jconrel.2015.04.034
41. Xu X, Jha AK, Duncan RL, Jia X. Heparin-decorated, hyaluronic acid-based hydrogel particles for the controlled release of bone morphogenetic protein 2. *Acta Biomater* (2011) **7**:3050–9. doi:10.1016/j.actbio.2011.04.018
42. Fan M, Ma Y, Zhang Z, Mao J, Tan H, Hu X. Biodegradable hyaluronic acid hydrogels to control release of dexamethasone through aqueous Diels-Alder chemistry for adipose tissue engineering. *Mater Sci Eng C Mater Biol Appl* (2015) **56**:311–7. doi:10.1016/j.msec.2015.04.004
43. Bhakta G, Rai B, Lim ZX, Hui JH, Stein GS, van Wijnen AJ, et al. Hyaluronic acid-based hydrogels functionalized with heparin that support controlled release of bioactive BMP-2. *Biomaterials* (2012) **33**:6113–22. doi:10.1016/j.biomaterials.2012.05.030
44. Yamamoto M, Takahashi Y, Tabata Y. Controlled release by biodegradable hydrogels enhances the ectopic bone formation of bone morphogenetic protein. *Biomaterials* (2003) **24**:4375–83. doi:10.1016/S0142-9612(03)00337-5
45. Ishihara M, Fujita M, Obara K, Hattori H, Nakamura S, Nambu M, et al. Controlled releases of FGF-2 and paclitaxel from chitosan hydrogels and their subsequent effects on wound repair, angiogenesis, and tumor growth. *Curr Drug Deliv* (2006) **3**:351–8. doi:10.2174/156720106778559047
46. Tae G, Kornfield JA, Hubbell JA. Sustained release of human growth hormone from in situ forming hydrogels using self-assembly of fluoroalkyl-ended poly(ethylene glycol). *Biomaterials* (2005) **26**:5259–66. doi:10.1016/j.biomaterials.2005.01.042
47. Schulz TJ, Huang P, Huang TL, Xue R, McDougall LE, Townsend KL, et al. Brown-fat paucity due to impaired BMP signalling induces compensatory browning of white fat. *Nature* (2013) **495**:379–83. doi:10.1038/nature11943
48. Collins S. beta-adrenoceptor signaling networks in adipocytes for recruiting stored fat and energy expenditure. *Front Endocrinol* (2011) **2**:102. doi:10.3389/fendo.2011.00102
49. Ryu V, Garretson JT, Liu Y, Vaughan CH, Bartness TJ, Brown adipose tissue has sympathetic-sensory feedback circuits. *J Neurosci* (2015) **35**:2181–90. doi:10.1523/JNEUROSCI.3306-14.2015
50. Nguyen KD, Qiu Y, Cui X, Goh YP, Mwangi J, David T, et al. Alternatively activated macrophages produce catecholamines to sustain adaptive thermogenesis. *Nature* (2011) **480**:104–8. doi:10.1038/nature10653
51. Wu D, Molofsky AB, Liang HE, Ricardo-Gonzalez RR, Jouihan HA, Bando JK, et al. Eosinophils sustain adipose alternatively activated macrophages associated with glucose homeostasis. *Science* (2011) **332**:243–7. doi:10.1126/science.1201475
52. Murano I, Barbatelli G, Giordano A, Cinti S. Noradrenergic parenchymal nerve fiber branching after cold acclimatisation correlates with brown adipocyte density in mouse adipose organ. *J Anat* (2009) **214**:171–8. doi:10.1111/j.1469-7580.2008.01001.x
53. Ye L, Wu J, Cohen P, Kazak L, Khandekar MJ, Jedrychowski MP, et al. Fat cells directly sense temperature to activate thermogenesis. *Proc Natl Acad Sci U S A* (2013) **110**:12480–5. doi:10.1073/pnas.1310261110
54. Watanabe M, Houten SM, Mataki C, Christoffolete MA, Kim BW, Sato H, et al. Bile acids induce energy expenditure by promoting intracellular thyroid hormone activation. *Nature* (2006) **439**:484–9. doi:10.1038/nature04330
55. Bordicchia M, Liu D, Amri EZ, Ailhaud G, Dessi-Fulgheri P, Zhang C, et al. Cardiac natriuretic peptides act via p38 MAPK to induce the brown fat thermogenic program in mouse and human adipocytes. *J Clin Invest* (2012) **122**:1022–36. doi:10.1172/JCI59701
56. Obregon MJ. Adipose tissues and thyroid hormones. *Front Physiol* (2014) **5**:479. doi:10.3389/fphys.2014.00479
57. Scadden DT. The stem-cell niche as an entity of action. *Nature* (2006) **441**:1075–9. doi:10.1038/nature04957
58. Jones DL, Wagers AJ. No place like home: anatomy and function of the stem cell niche. *Nat Rev Mol Cell Biol* (2008) **9**:11–21. doi:10.1038/nrm2319
59. Bissell MJ, Kenny PA, Radisky DC. Microenvironmental regulators of tissue structure and function also regulate tumor induction and progression: the role of extracellular matrix and its degrading enzymes. *Cold Spring Harb Symp Quant Biol* (2005) **70**:343–56. doi:10.1101/sqb.2005.70.013
60. Saha K, Pollock JF, Schaffer DV, Healy KE. Designing synthetic materials to control stem cell phenotype. *Curr Opin Chem Biol* (2007) **11**:381–7. doi:10.1016/j.cbpa.2007.05.030
61. Lutolf MP, Hubbell JA. Synthetic biomaterials as instructive extracellular microenvironments for morphogenesis in tissue engineering. *Nat Biotechnol* (2005) **23**:47–55. doi:10.1038/nbt1055
62. Bhat R, Bissell MJ. Of plasticity and specificity: dialectics of the microenvironment and macroenvironment and the organ phenotype. *Wiley Interdiscip Rev Dev Biol* (2014) **3**:147–63. doi:10.1002/wdev.130
63. Mouw JK, Ou G, Weaver VM. Extracellular matrix assembly: a multiscale deconstruction. *Nat Rev Mol Cell Biol* (2014) **15**:771–85. doi:10.1038/nrm3902
64. Wang Y, Botvinick EL, Zhao Y, Berns MW, Usami S, Tsien RY, et al. Visualizing the mechanical activation of src. *Nature* (2005) **434**:1040–5. doi:10.1038/nature03469
65. Badylak SF, Taylor D, Uygun K. Whole-organ tissue engineering: decellularization and recellularization of three-dimensional matrix scaffolds. *Annu Rev Biomed Eng* (2011) **13**:27–53. doi:10.1146/annurev-bioeng-071910-124743
66. Young DA, Ibrahim DO, Hu D, Christman KL. Injectable hydrogel scaffold from decellularized human lipoaspirate. *Acta Biomater* (2011) **7**:1040–9. doi:10.1016/j.actbio.2010.09.035
67. Tibbitt MW, Anseth KS. Hydrogels as extracellular matrix mimics for 3D cell culture. *Biotechnol Bioeng* (2009) **103**:655–63. doi:10.1002/bit.22361
68. Kim M, Lee JY, Jones CN, Revzin A, Tae G. Heparin-based hydrogel as a matrix for encapsulation and cultivation of primary hepatocytes. *Biomaterials* (2010) **31**:3596–603. doi:10.1016/j.biomaterials.2010.01.068
69. Weber LM, Anseth KS. Hydrogel encapsulation environments functionalized with extracellular matrix interactions increase islet insulin secretion. *Matrix Biol* (2008) **27**:667–73. doi:10.1016/j.matbio.2008.08.001
70. Friedland JC, Lakins JN, Kazanietz MG, Chernoff J, Boettiger D, Weaver VM. alpha6beta4 integrin activates Rac-dependent p21-activated kinase 1 to drive NF-kappaB-dependent resistance to apoptosis in 3D mammary acini. *J Cell Sci* (2007) **120**:3700–12. doi:10.1242/jcs.03484
71. Lee ST, Yun JI, Jo YS, Mochizuki M, van der Vlies AJ, Kontos S, et al. Engineering integrin signaling for promoting embryonic stem cell self-renewal in a precisely defined niche. *Biomaterials* (2010) **31**:1219–26. doi:10.1016/j.biomaterials.2009.10.054
72. Spiegelman BM, Ginty CA. Fibronectin modulation of cell shape and lipogenic gene expression in 3T3-adipocytes. *Cell* (1983) **35**:657–66. doi:10.1016/0092-8674(83)90098-3
73. Vaicik MK, Thyboll Kortesmaa J, Moverare-Skrtic S, Kortesmaa J, Soininen R, Bergstrom G, et al. Laminin alpha4 deficient mice exhibit decreased capacity for adipose tissue expansion and weight gain. *PLoS One* (2014) **9**:e109854. doi:10.1371/journal.pone.0109854
74. Khan T, Muise ES, Iyengar P, Wang ZV, Chandalia M, Abate N, et al. Metabolic dysregulation and adipose tissue fibrosis: role of collagen VI. *Mol Cell Biol* (2009) **29**:1575–91. doi:10.1128/MCB.01300-08

75. Chun TH, Hotary KB, Sabeh F, Saltiel AR, Allen ED, Weiss SJ. A pericellular collagenase directs the 3-dimensional development of white adipose tissue. *Cell* (2006) **125**:577–91. doi:10.1016/j.cell.2006.02.050
76. Gerlach JC, Lin YC, Brayfield CA, Minteer DM, Li H, Rubin JP, et al. Adipogenesis of human adipose-derived stem cells within three-dimensional hollow fiber-based bioreactors. *Tissue Eng Part C Methods* (2012) **18**:54–61. doi:10.1089/ten.TEC.2011.0216
77. Emont MP, Yu H, Jun H, Hong X, Maganti N, Stegemann JP, et al. Using a 3D culture system to differentiate visceral adipocytes in vitro. *Endocrinology* (2015): en20151567.
78. Francisco AT, Mancino RJ, Bowles RD, Brunger JM, Tainter DM, Chen YT, et al. Injectable laminin-functionalized hydrogel for nucleus pulposus regeneration. *Biomaterials* (2013) **34**:7381–8. doi:10.1016/j.biomaterials.2013.06.038
79. Branco da Cunha C, Klumpers DD, Li WA, Koshy ST, Weaver JC, Chaudhuri O, et al. Influence of the stiffness of three-dimensional alginate/collagen-I interpenetrating networks on fibroblast biology. *Biomaterials* (2014) **35**:8927–36. doi:10.1016/j.biomaterials.2014.06.047
80. Abberton KM, Bortolotto SK, Woods AA, Findlay M, Morrison WA, Thompson EW, et al. Myogel, a novel, basement membrane-rich, extracellular matrix derived from skeletal muscle, is highly adipogenic in vivo and in vitro. *Cells Tissues Organs* (2008) **188**:347–58. doi:10.1159/000121575
81. Flynn LE. The use of decellularized adipose tissue to provide an inductive microenvironment for the adipogenic differentiation of human adipose-derived stem cells. *Biomaterials* (2010) **31**:4715–24. doi:10.1016/j.biomaterials.2010.02.046
82. Xue R, Lynes MD, Dreyfuss JM, Shamsi F, Schulz TJ, Zhang H, et al. Clonal analyses and gene profiling identify genetic biomarkers of the thermogenic potential of human brown and white preadipocytes. *Nat Med* (2015) **21**:760–8. doi:10.1038/nm.3881
83. Kasza I, Suh Y, Wollny D, Clark RJ, Roopra A, Colman RJ, et al. Syndecan-1 is required to maintain intradermal fat and prevent cold stress. *PLoS Genet* (2014) **10**:e1004514. doi:10.1371/journal.pgen.1004514
84. Liu J, DeYoung SM, Zhang M, Zhang M, Cheng A, Saltiel AR. Changes in integrin expression during adipocyte differentiation. *Cell Metab* (2005) **2**:165–77. doi:10.1016/j.cmet.2005.08.006
85. Wu J, Cohen P, Spiegelman BM. Adaptive thermogenesis in adipocytes: is beige the new brown? *Genes Dev* (2013) **27**:234–50. doi:10.1101/gad.211649.112
86. Tharp KM, Jha AK, Kraiczy J, Yesian A, Karateev G, Sinisi R, et al. Matrix assisted transplantation of functional beige adipose tissue. *Diabetes* (2015). doi:10.2337/db15-0728
87. Khalifeh-Soltani A, McKleroy W, Sakuma S, Cheung YY, Tharp K, Qiu Y, et al. Mfge8 promotes obesity by mediating the uptake of dietary fats and serum fatty acids. *Nat Med* (2014) **20**:175–83. doi:10.1038/nm.3450
88. Tapias LF, Ott HC. Decellularized scaffolds as a platform for bioengineered organs. *Curr Opin Organ Transplant* (2014) **19**:145–52. doi:10.1097/MOT.0000000000000051
89. Silva FJ, Holt DJ, Vargas V, Yockman J, Boudina S, Atkinson D, et al. Metabolically active human brown adipose tissue derived stem cells. *Stem Cells* (2014) **32**:572–81. doi:10.1002/stem.1595
90. Griffith LG. Emerging design principles in biomaterials and scaffolds for tissue engineering. *Ann N Y Acad Sci* (2002) **961**:83–95. doi:10.1111/j.1749-6632.2002.tb03056.x
91. Rice JJ, Martino MM, De Laporte L, Tortelli F, Briquez PS, Hubbell JA. Engineering the regenerative microenvironment with biomaterials. *Adv Healthc Mater* (2013) **2**:57–71. doi:10.1002/adhm.201200197
92. Lutolf MP, Raeber GP, Zisch AH, Tirelli N, Hubbell JA. Cell-responsive synthetic hydrogels. *Adv Mater Deerfield* (2003) **15**:888–92. doi:10.1002/adma.200304621
93. Unser AM, Tian Y, Xie Y. Opportunities and challenges in three-dimensional brown adipogenesis of stem cells. *Biotechnol Adv* (2015) **33**:962–79. doi:10.1016/j.biotechadv.2015.07.005
94. Xu X, Jha AK, Harrington DA, Farach-Carson MC, Jia X. Hyaluronic acid-based hydrogels: from a natural polysaccharide to complex networks. *Soft Matter* (2012) **8**:3280–94. doi:10.1039/c2sm06463d
95. Burdick JA, Prestwich GD. Hyaluronic acid hydrogels for biomedical applications. *Adv Mater* (2011) **23**:H41–56. doi:10.1002/adma.201003963
96. Yang B, Yang BL, Savani RC, Turley EA. Identification of a common hyaluronan binding motif in the hyaluronan binding proteins RHAMM, CD44 and link protein. *EMBO J* (1994) **13**:286–96.
97. Stillaert FB, Di Bartolo C, Hunt JA, Rhodes NP, Tognana E, Monstrey S, et al. Human clinical experience with adipose precursor cells seeded on hyaluronic acid-based spongy scaffolds. *Biomaterials* (2008) **29**:3953–9. doi:10.1016/j.biomaterials.2008.06.005
98. Piccinno MS, Veronesi E, Loschi P, Pignatti M, Murgia A, Grisendi G, et al. Adipose stromal/stem cells assist fat transplantation reducing necrosis and increasing graft performance. *Apoptosis* (2013) **18**:1274–89. doi:10.1007/s10495-013-0878-7
99. Kim YM, Oh SH, Choi JS, Lee S, Ra JC, Lee JH, et al. Adipose-derived stem cell-containing hyaluronic acid/alginate hydrogel improves vocal fold wound healing. *Laryngoscope* (2014) **124**:E64–72. doi:10.1002/lary.24405
100. Moreno A, Martinez A, Olmedillas S, Bello S, de Miguel F. Hyaluronic acid effect on adipose-derived stem cells. Biological in vitro evaluation. *Rev Esp Cir Ortop Traumatol* (2015) **59**:215–21. doi:10.1016/j.recot.2014.10.004
101. Huang SH, Lin YN, Lee SS, Chai CY, Chang HW, Lin TM, et al. New adipose tissue formation by human adipose-derived stem cells with hyaluronic acid gel in immunodeficient mice. *Int J Med Sci* (2015) **12**:154–62. doi:10.7150/ijms.9964
102. Prestwich GD. Engineering a clinically-useful matrix for cell therapy. *Organogenesis* (2008) **4**:42–7. doi:10.4161/org.6152
103. De Meyere B, Mir-Mir S, Penas J, Camenisch CC, Heden P. Stabilized hyaluronic acid gel for volume restoration and contouring of the buttocks: 24-month efficacy and safety. *Aesthetic Plast Surg* (2014) **38**:404–12. doi:10.1007/s00266-013-0251-9
104. Voigt J, Driver VR. Hyaluronic acid derivatives and their healing effect on burns, epithelial surgical wounds, and chronic wounds: a systematic review and meta-analysis of randomized controlled trials. *Wound Repair Regen* (2012) **20**:317–31. doi:10.1111/j.1524-475X.2012.00777.x
105. Anderson JM, Rodriguez A, Chang DT. Foreign body reaction to biomaterials. *Semin Immunol* (2008) **20**:86–100. doi:10.1016/j.smim.2007.11.004
106. Burdick JA, Chung C, Jia X, Randolph MA, Langer R. Controlled degradation and mechanical behavior of photopolymerized hyaluronic acid networks. *Biomacromolecules* (2005) **6**:386–91. doi:10.1021/bm049508a
107. Cousin B, Cinti S, Morroni M, Raimbault S, Ricquier D, Penicaud L, et al. Occurrence of brown adipocytes in rat white adipose tissue: molecular and morphological characterization. *J Cell Sci* (1992) **103**(Pt 4):931–42.
108. Xue B, Rim JS, Hogan JC, Coulter AA, Koza RA, Kozak LP. Genetic variability affects the development of brown adipocytes in white fat but not in interscapular brown fat. *J Lipid Res* (2007) **48**:41–51. doi:10.1194/jlr.M600287-JLR200
109. Kozak LP. The genetics of brown adipocyte induction in white fat depots. *Front Endocrinol* (2011) **2**:64. doi:10.3389/fendo.2011.00064
110. Yoshimura K, Suga H, Eto H. Adipose-derived stem/progenitor cells: roles in adipose tissue remodeling and potential use for soft tissue augmentation. *Regen Med* (2009) **4**:265–73. doi:10.2217/17460751.4.2.265
111. Lee YH, Petkova AP, Mottillo EP, Granneman JG. In vivo identification of bipotential adipocyte progenitors recruited by beta3-adrenoceptor activation and high-fat feeding. *Cell Metab* (2012) **15**:480–91. doi:10.1016/j.cmet.2012.03.009
112. Ussar S, Lee KY, Dankel SN, Boucher J, Haering MF, Kleinridders A, et al. ASC-1, PAT2, and P2RX5 are cell surface markers for white, beige, and brown adipocytes. *Sci Transl Med* (2014) **6**:247ra103. doi:10.1126/scitranslmed.3008490
113. Nishio M, Yoneshiro T, Nakahara M, Suzuki S, Saeki K, Hasegawa M, et al. Production of functional classical brown adipocytes from human pluripotent stem cells using specific hemopoietin cocktail without gene transfer. *Cell Metab* (2012) **16**:394–406. doi:10.1016/j.cmet.2012.08.001
114. Medrikova D, Sijmonsma TP, Sowodniok K, Richards DM, Delacher M, Sticht C, et al. Brown adipose tissue harbors a distinct sub-population of regulatory T cells. *PLoS One* (2015) **10**:e0118534. doi:10.1371/journal.pone.0118534
115. Tang W, Zeve D, Suh JM, Bosnakovski D, Kyba M, Hammer RE, et al. White fat progenitor cells reside in the adipose vasculature. *Science* (2008) **322**:583–6. doi:10.1126/science.1156232
116. Nolan DJ, Ginsberg M, Israely E, Palikuqi B, Poulos MG, James D, et al. Molecular signatures of tissue-specific microvascular endothelial cell heterogeneity in organ maintenance and regeneration. *Dev Cell* (2013) **26**:204–19. doi:10.1016/j.devcel.2013.06.017
117. Butler JM, Kobayashi H, Rafii S. Instructive role of the vascular niche in promoting tumour growth and tissue repair by angiocrine factors. *Nat Rev Cancer* (2010) **10**:138–46. doi:10.1038/nrc2791

118. Bi P, Shan T, Liu W, Yue F, Yang X, Liang XR, et al. Inhibition of notch signaling promotes browning of white adipose tissue and ameliorates obesity. *Nat Med* (2014) **20**:911–8. doi:10.1038/nm.3615
119. Sun K, Wernstedt Asterholm I, Kusminski CM, Bueno AC, Wang ZV, Pollard JW, et al. Dichotomous effects of VEGF-A on adipose tissue dysfunction. *Proc Natl Acad Sci U S A* (2012) **109**:5874–9. doi:10.1073/pnas.1200447109
120. Schultz KM, Kyburz KA, Anseth KS. Measuring dynamic cell-material interactions and remodeling during 3D human mesenchymal stem cell migration in hydrogels. *Proc Natl Acad Sci U S A* (2015) **112**:E3757–64. doi:10.1073/pnas.1511304112
121. Wall ST, Yeh CC, Tu RY, Mann MJ, Healy KE. Biomimetic matrices for myocardial stabilization and stem cell transplantation. *J Biomed Mater Res A* (2010) **95**:1055–66. doi:10.1002/jbm.a.32904
122. Kim S, Chung EH, Gilbert M, Healy KE. Synthetic MMP-13 degradable ECMs based on poly(N-isopropylacrylamide-co-acrylic acid) semi-interpenetrating polymer networks. I. Degradation and cell migration. *J Biomed Mater Res A* (2005) **75**:73–88. doi:10.1002/jbm.a.30375
123. Mather BD, Viswanathan K, Miller KM, Long TE. Michael addition reactions in macromolecular design for emerging technologies. *Prog Polym Sci* (2006) **31**:487–531. doi:10.1016/j.progpolymsci.2006.03.001
124. Lutolf MP, Lauer-Fields JL, Schmoekel HG, Metters AT, Weber FE, Fields GB, et al. Synthetic matrix metalloproteinase-sensitive hydrogels for the conduction of tissue regeneration: engineering cell-invasion characteristics. *Proc Natl Acad Sci U S A* (2003) **100**:5413–8. doi:10.1073/pnas.0737381100
125. Engler AJ, Sen S, Sweeney HL, Discher DE. Matrix elasticity directs stem cell lineage specification. *Cell* (2006) **126**:677–89. doi:10.1016/j.cell.2006.06.044
126. Guvendiren M, Burdick JA. Stiffening hydrogels to probe short- and long-term cellular responses to dynamic mechanics. *Nat Commun* (2012) **3**:792. doi:10.1038/ncomms1792
127. Young DA, Choi YS, Engler AJ, Christman KL. Stimulation of adipogenesis of adult adipose-derived stem cells using substrates that mimic the stiffness of adipose tissue. *Biomaterials* (2013) **34**:8581–8. doi:10.1016/j.biomaterials.2013.07.103
128. Kumar A, Mazzanti M, Mistrik M, Kosar M, Beznoussenko GV, Mironov AA, et al. ATR mediates a checkpoint at the nuclear envelope in response to mechanical stress. *Cell* (2014) **158**:633–46. doi:10.1016/j.cell.2014.05.046
129. Shivashankar GV. Mechanosignaling to the cell nucleus and gene regulation. *Annu Rev Biophys* (2011) **40**:361–78. doi:10.1146/annurev-biophys-042910-155319
130. Jakkaraju S, Zhe X, Pan D, Choudhury R, Schuger L. TIPs are tension-responsive proteins involved in myogenic versus adipogenic differentiation. *Dev Cell* (2005) **9**:39–49. doi:10.1016/j.devcel.2005.04.015
131. Hughes-Fulford M. Signal transduction and mechanical stress. *Sci STKE* (2004) **2004**:RE12. doi:10.1126/stke.2492004re12
132. Vinall RL, Lo SH, Reddi AH. Regulation of articular chondrocyte phenotype by bone morphogenetic protein 7, interleukin 1, and cellular context is dependent on the cytoskeleton. *Exp Cell Res* (2002) **272**:32–44. doi:10.1006/excr.2001.5395
133. Fuchshofer R, Stephan DA, Russell P, Tamm ER. Gene expression profiling of TGFbeta2- and/or BMP7-treated trabecular meshwork cells: identification of Smad7 as a critical inhibitor of TGF-beta2 signaling. *Exp Eye Res* (2009) **88**:1020–32. doi:10.1016/j.exer.2009.01.002
134. Konstantinidis G, Moustakas A, Stournaras C. Regulation of myosin light chain function by BMP signaling controls actin cytoskeleton remodeling. *Cell Physiol Biochem* (2011) **28**:1031–44. doi:10.1159/000335790
135. Chopra A, Murray ME, Byfield FJ, Mendez MG, Halleluyan R, Restle DJ, et al. Augmentation of integrin-mediated mechanotransduction by hyaluronic acid. *Biomaterials* (2014) **35**:71–82. doi:10.1016/j.biomaterials.2013.09.066
136. Shachar M, Tsur-Gang O, Dvir T, Leor J, Cohen S. The effect of immobilized RGD peptide in alginate scaffolds on cardiac tissue engineering. *Acta Biomater* (2011) **7**:152–62. doi:10.1016/j.actbio.2010.07.034
137. Kang SW, Cha BH, Park H, Park KS, Lee KY, Lee SH. The effect of conjugating RGD into 3D alginate hydrogels on adipogenic differentiation of human adipose-derived stromal cells. *Macromol Biosci* (2011) **11**:673–9. doi:10.1002/mabi.201000479
138. Lee KY, Mooney DJ. Hydrogels for tissue engineering. *Chem Rev* (2001) **101**:1869–79. doi:10.1021/cr000108x
139. Mehta M, Madl CM, Lee S, Duda GN, Mooney DJ. The collagen I mimetic peptide DGEA enhances an osteogenic phenotype in mesenchymal stem cells when presented from cell-encapsulating hydrogels. *J Biomed Mater Res A* (2015) **103**:3516–25. doi:10.1002/jbm.a.35497
140. Vaicik MK, Morse M, Blagajcevic A, Rios J, Larson JC, Yang F, et al. Hydrogel-based engineering of beige adipose tissue. *J Mater Chem B* (2015) **3**:7903–11. doi:10.1039/C5TB00952A
141. Benoit DS, Schwartz MP, Durney AR, Anseth KS. Small functional groups for controlled differentiation of hydrogel-encapsulated human mesenchymal stem cells. *Nat Mater* (2008) **7**:816–23. doi:10.1038/nmat2269

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read, for greatest visibility

COLLABORATIVE PEER-REVIEW
Designed to be rigorous – yet also collaborative, fair and constructive

FAST PUBLICATION
Average 85 days from submission to publication (across all journals)

COPYRIGHT TO AUTHORS
No limit to article distribution and re-use

TRANSPARENT
Editors and reviewers acknowledged by name on published articles

SUPPORT
By our Swiss-based editorial team

IMPACT METRICS
Advanced metrics track your article's impact

GLOBAL SPREAD
5'100'000+ monthly article views and downloads

LOOP RESEARCH NETWORK
Our network increases readership for your article

Frontiers
EPFL Innovation Park, Building I • 1015 Lausanne • Switzerland
Tel +41 21 510 17 00 • Fax +41 21 510 17 01 • info@frontiersin.org
www.frontiersin.org

Find us on