



# TGF- $\beta$ /Smad3 signaling regulates brown adipocyte induction in white adipose tissue

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Recent identification of active brown fat reserves in adult humans has re-stimulated interest in the role of brown adipocytes in energy homeostasis. In addition, there is accumulating evidence to support the concept of an alteration in energy balance through acquisition of brown fat features in traditional white fat depots. We recently described an important role played by the TGF- $\beta$ /Smad3 signaling pathway in modulating the appearance of brown adipocytes in traditional white fat, and its implications to thermogenesis, mitochondrial energetics, energy expenditure, and protection from diabetes and obesity. Here we review the data supporting this phenomenon and put into perspective the promise of conversion of white fat to a brown fat state as a potential therapeutic option for obesity and diabetes.

**Keywords:** TGF-beta, Smad3, obesity, brown fat, white fat

The rise in the worldwide incidence of obesity and, by a tight correlation, diabetes is proof that we are failing as a populace to obey the energy balance equation. Obesity is principally characterized by accumulation of fat in adipose tissue (Gesta et al., 2007; Park et al., 2008). In conditions of energy excess, the white adipose tissue accumulates fat in the form of triglycerides, whilst brown adipose tissue has the potential stimulate energy expenditure by dissipation of fat to produce heat and maintain body temperature. White fat comprises of large unilocular lipid-containing adipocytes with few mitochondria. In contrast, brown fat comprises of small multilocular cells with abundant mitochondria. Brown adipocytes are uniquely characterized by the expression of uncoupling protein-1 (UCP1). Copious amount of brown fat exists in rodents and human infants and it was considered to be non-existent in adult humans. Recent findings that metabolically active brown fat exists in humans (Nedergaard et al., 2007; Cypess et al., 2009; van Marken Lichtenbelt et al., 2009; Virtanen et al., 2009) have stimulated interest concerning the therapeutic potential of augmenting brown fat to combat metabolic diseases (Enerback, 2010; Nedergaard and Cannon, 2010). Further, it appears that brown fat shares its developmental origin with muscle, and not white fat as it was long presumed (Atit et al., 2006; Timmons et al., 2007). Seale et al. (2008, 2009) provided the formal proof that brown fat is related to skeletal muscle and further showed that the transcription factor PRDM16 determines the fate of Myf5<sup>+</sup>-precursor cells toward brown fat lineage.

## **BROWN ADIPOCYTE INDUCTION IN WHITE ADIPOSE TISSUE**

Brown adipocytes are also found interspersed within the white adipose tissue, in response to chemical or hormonal stimulation, environmental changes, cold exposure, and defined genetic manipulation (Langin, 2009; Lefterova and Lazar, 2009; Frontini

and Cinti, 2010). The most well studied models whereby brown adipocytes appear in white fat are upon cold exposure or after stimulation of the beta(3)-adrenoceptor pathways. Cold exposure of mice results in expression of the brown adipocyte marker, UCP1, in inguinal white adipose tissue (Loncar, 1991) and in mesenteric, epididymal, retroperitoneal, inguinal, and periovarian adipose depots upon exposure to cold or to treatment with a beta-adrenoceptor agonist (Cousin et al., 1992). In agreement, beta 3-adrenoceptor knockout mice show suppressed occurrence of brown adipocytes in white fat upon cold exposure (Jimenez et al., 2003). In contrast, transgenic mice overexpressing the beta 1-adrenergic receptor in adipose tissue exhibit abundant appearance of brown fat cells in subcutaneous white adipose tissue and are resistant to obesity (Soloveva et al., 1997). Chronic treatment with the beta3-adrenoceptor agonist, CL 316,243, (Bloom et al., 1992), promotes thermogenesis, and the appearance of multilocular adipocytes in white fat while protecting from high-fat diet-induced obesity (Himms-Hagen et al., 1994). Infusion of CL 316,243 reduced abdominal fat, increased resting metabolic rate, and abundant multilocular brown adipocytes expressing uncoupling protein (UCP) appeared in retroperitoneal white fat (Ghorbani et al., 1997). Similarly, appearance of brown adipocytes in white adipose tissue during CL 316,243-treatment correlated with reversal of obesity and diabetes in Zucker fa/fa rats (Ghorbani and Himms-Hagen, 1997). Also, beta3-adrenergic receptors mediate CL 316,243 agonist-induced effects on energy expenditure, insulin secretion, and food intake (Grujic et al., 1997).

Interestingly, genetic background modulates the relative degree of browning of white adipose tissue. CL 316,243 prevented the development of diet-induced obesity in A/J animals, but not in C57BL/6J animals. In agreement, CL 316,243-treated A/J mice, but not B/6J mice, showed abundant UCP1 expression in white

adipose depots (Collins et al., 1997). Also, significant stain-specific differences in UCP1 transcript levels were seen in various white fat depots derived from A/J and C57BL/6J strains of mice after stimulation of adrenergic signaling (Guerra et al., 1998). Further, cold exposure induced brown adipocytes in retroperitoneal fat of adult A/J mice but not in C57BL/6J mice. In contrast, induction of UCP1 in interscapular brown adipose tissue showed no such strain dependence (Xue et al., 2007).

### POSSIBLE MECHANISMS OF BROWNING IN WHITE FAT TRANSDIFFERENTIATION

Tissue plasticity which allows efficient conversion of white adipocyte to brown adipocyte and vice versa has been proposed as a potential mechanism (Frontini and Cinti, 2010). Thus, cold exposure conditions would promote white-to-brown conversion to fulfill the demand for thermogenesis, whereas, high-fat diet would promote conversion of brown-to-white fat to enable energy storage (Cinti, 2011). It is believed that multilocular fat cells that appear in white fat upon CL 316243-treatment derive from convertible unilocular adipocytes that become multilocular (Himms-Hagen et al., 2000) and that emergence of brown adipocytes in white fat upon cold exposure reflects beta(3)-adrenoceptor-mediated transdifferentiation (Barbatelli et al., 2010). The retinoblastoma (Rb) protein family acts as a molecular switch determining white versus brown adipocyte differentiation. Thus, inactivation of pRB results in the expression of UCP1 in the white adipose tissue and down-regulation of pRB expression is associated with white into brown adipocyte transdifferentiation in response to beta3-adrenergic receptor agonist treatment (Hansen et al., 2004). Further, mice lacking the Rb-family member, p107, possess white adipose tissue with multilocular adipocytes that express elevated levels of UCP1 and PGC-1 $\alpha$ , a transcriptional co-activator implicated in mitochondrial biogenesis and adaptive thermogenesis and pRb binds and represses PGC-1 $\alpha$  transcription (Scime et al., 2005).

### PRECURSOR POOL ORIGIN

Multilocular fat cells that appear in white fat upon CL 316243-treatment may derive from a precursor cell that gives rise to more typical brown adipocytes (Himms-Hagen et al., 2000). To date, such a precursor pool is unidentified. The evidence that the dermis, muscle, and brown fat all originate from the central dermomyotome (Atit et al., 2006) and that immature undifferentiated brown fat cells harbor muscle-specific transcripts and brown preadipocytes exhibit a myogenic signature (Timmons et al., 2007) stimulated a search for a common brown fat/muscle progenitor. Indeed, Seale et al. (2008, 2009) provided evidence that brown fat shares developmental ontogeny with skeletal muscle with the transcription factor PRDM16 promoting the differentiation of skeletal muscle-derived Myf5<sup>+</sup> precursor cells toward a classical brown adipocyte lineage. However, they did not detect Myf5<sup>+</sup> precursor cell derivatives in the examined white fat. These findings suggest that, either (i) Myf5<sup>+</sup> precursor cells do not promote the browning phenomenon in white fat, or (ii) a context dependent role for Myf5<sup>+</sup> precursor cells may yet exist with respect to induction of brown adipocytes in white fat, or (iii) other distinct precursor cell pools resident in white fat or other tissue/s may drive the

browning phenotype in white fat. Lineage tracing methods with the availability of unique gene expression markers for the three major types of adipose tissue depots – the brown (Zic1), the beige, or brite (Hoxc9), and the white (Tcf21; Walden et al., 2011) – would help identify the potential precursor pool that drives browning of white fat.

### AUTOCRINE OR PARACRINE FACTORS

Autocrine or paracrine factors – classic examples being insulin and leptin – play critical roles in regulating not only the cells from which they derive, but also play overarching roles in regulating the biology of other cells that orchestrate glucose homeostasis. This is possible via these factors engaging their specific receptors in the cell type where the action is elicited. Several facts support a notion that a similar such secretagogue might regulate the appearance of brown adipocytes in white fat: (i) the white fat is a reservoir that synthesizes and secretes multiple cytokines and hormones such as leptin, (ii) classical brown fat might produce factors that promote brown adipocyte induction locally as well as at a distance in white fat, and (iii) considering the muscle-specific origin of brown adipocytes, it is logical to assume that a muscle-specific factor might serve as an inducer of brown adipocytes. Indeed, a muscle-derived hormone, irisin, has been identified that promotes induction of brown adipocytes in white fat (Bostrom et al., 2012). Irisin, whose receptor is presently unidentified, is induced in the muscle by PGC-1 $\alpha$  expression. It is induced with exercise and a rise in plasma irisin levels correlate with increased energy expenditure, enhanced glucose homeostasis, and protection from obesity. The observation that irisin has paracrine effects on white fat to stimulate induction of a brown fat program has utility in not only furthering the knowledge about browning of white fat but also has obvious therapeutic potential. It is plausible that other factors such as irisin, produced locally in white fat or acting in a paracrine manner, may stimulate the browning of white fat.

### Neural circuits

Neural circuits are also recognized as critical modifiers of the white fat to brown fat phenomenon. An enriched environment induced browning of white fat associated with expression of genes involved in brown adipogenesis, thermogenesis, and beta-adrenergic signaling pathways along with improved energy homeostasis and glucose tolerance (Cao et al., 2011). Hypothalamic overexpression of the brain-derived neurotrophic factor (BDNF) promoted a lean phenotype accompanied with acquisition of brown fat features in the white fat, whereas, inhibition of BDNF signaling reversed this phenotype (Cao et al., 2011). Also, hypothalamic neuropeptide Y (NPY) has been implicated in promoting brown fat features in white fat (Chao et al., 2011). Knockdown of NPY expression in the dorsomedial hypothalamus (DMH) reduced white fat depots, increased energy expenditure, and suppressed high-fat diet-induced obesity. Interestingly, these phenomena were accompanied by appearance of brown adipocytes in inguinal white adipose tissue (Chao et al., 2011).

### Signaling pathways

Increased FOXC2 expression in adipocytes results in a lean phenotype and protects from hypertriglyceridemia and diet-induced insulin resistance and obesity primarily by increasing the

beta-adrenergic–cAMP–protein kinase A (PKA) signaling pathway (Cederberg et al., 2001). PPAR $\alpha$  stimulated the expression of the PRDM16 gene in brown adipocytes and PPAR $\alpha$  activation in white adipocytes induced expression of brown fat markers (Hondares et al., 2011). C/EBP $\alpha$  and the corepressors CtBP1 and CtBP2 repress visceral white adipose genes and activate UCP1 transcription during PPAR $\gamma$  agonist-mediated induction of the brown phenotype in white adipocytes (Vernochet et al., 2009). Induction of UCP1 expression in white adipose tissue, but not in classic interscapular brown adipose tissue, is dependent on cyclooxygenase activity (Madsen et al., 2010). Cyclooxygenase (COX)-2, a rate-limiting enzyme in prostaglandin synthesis, is a downstream effector of beta-adrenergic signaling in white adipose tissue and is required for appearance of brown fat features in white fat. Prostaglandin induced differentiation of mesenchymal progenitors toward a brown adipocyte phenotype and overexpression of COX-2 in white fat induced brown adipogenesis in white fat, increased energy expenditure, and protected mice against high-fat diet-induced obesity (Vegiopoulos et al., 2010). Retinoic acid reduces body weight, increases body temperature and adiposity in rodent models and stimulates UCP1 expression in brown adipose tissue and skeletal muscle. Administration of all-trans retinoic acid results in reduced adiposity and adipocyte cell size with a rise in multilocular adipocytes expressing brown fat markers in white fat depots (Mercader et al., 2006). Wnt10b blocks beta3-agonist-induced brown adipose tissue differentiation and suppresses UCP1 expression through repression of PGC-1 $\alpha$ , while promoting the appearance of unilocular lipid droplets and expression of white adipocyte genes consistent with conversion of brown fat to white fat (Kang et al., 2005). AMP-kinase (AMPK) signaling is also implicated in the brown fat to white fat conversion. Adipose-specific ablation of an AMP-kinase substrate, desnutrin/ATGL, converts brown fat to a white fat-like tissue where the mice exhibit severely impaired thermogenesis, increased expression of white fat genes and decreased brown fat genes (Ahmadian et al., 2011).

While it is clear that many signals can promote the browning of white fat, the mechanistic details are poorly understood. Further, some of the prior observations utilized cell culture models like primary mouse embryonic fibroblasts differentiated fat cells that harbor both lineages of “brown” fat cells. Also, many of the mouse studies were not performed at thermo-neutral temperature, and instead at an adapted colder temperature of 22–24°C which complicates the role of these molecules in thermogenesis and energy expenditure. Further, whether the repressing of white fat phenotype of these molecules is executed via functional regulation of adaptive thermogenesis or determination of cell fate and it remains unclear how many of these signals regulate the Myf5<sup>+</sup> lineage brown fat.

### TGF- $\beta$ SIGNALING REGULATES APPEARANCE OF BROWN ADIPOCYTES IN WHITE ADIPOSE TISSUE

We recently elucidated the importance of TGF- $\beta$  signaling in the appearance of brown adipocytes within the white adipose tissue (Crunkhorn, 2011; Yadav et al., 2011). The TGF- $\beta$  superfamily member, BMP7, has been implicated in brown adipogenesis (Tseng et al., 2008). Whether, there exists cross-talk between TGF- $\beta$  and BMP signaling in mediating brown adipogenesis remains to be

determined. Similarly, whether BMP signals, or signals emanating from other TGF- $\beta$  superfamily members, play a role in promoting browning of white fat remains unclear. Moreover, it is also not known if there exists a region-specific importance of TGF- $\beta$  signaling as it pertains to brown adipocyte development within the different white fat depots, such as the mesenteric, epididymal, retroperitoneal, inguinal, and periovarian adipose depots.

BMP proteins promote differentiation to either white adipocytes or brown adipocytes (Schulz et al., 2011). BMP7 triggers commitment of mesenchymal progenitor cells to a brown adipocyte lineage, and implantation of these cells into nude mice results in development of adipose tissue containing mostly brown adipocytes (Tseng et al., 2008). Subpopulation of adipogenic progenitors (termed ScaPCs) residing in murine brown fat, white fat, and skeletal muscle were isolated and it was shown that muscle and white fat derived Sca-1(+) cells were able to differentiate into brown-like adipocytes upon stimulation with BMP7 (Schulz et al., 2011). Also, Bmp7 knockout embryos show a marked reduction of brown fat, whereas, adenoviral-mediated expression of BMP7 in mice results in a significant increase in brown fat mass and leads to an increase in energy expenditure and a reduction in weight gain (Tseng et al., 2008). However, the role of BMP7 in promoting browning of white fat remains unclear. A more recent study identified Zfp423, a BMP-Smad signaling effector, as a transcriptional regulator of both brown and white preadipocyte differentiation (Gupta et al., 2010), although whether Zfp423 plays a role in promoting brown fat features in white fat is unknown.

The molecular signature that we obtain in white fat derived from Smad3-deficient mice and mice treated with the TGF- $\beta$  neutralization antibody, 1D11, provides interesting insight into the development of brown adipocytes within the white fat milieu. Thus, white fat from *Smad3*<sup>-/-</sup> mice and mice treated with 1D11 antibodies, expresses a preponderance of genes that represent brown fat, mitochondrial, and skeletal muscle biology (Yadav et al., 2011). These findings are in contrast to studies where PPAR $\gamma$  agonist rosiglitazone promotes norepinephrine augmentable UCP1 gene expression in a subset of white adipocyte cells, without concurrent expression of brown fat/muscle-specific markers (Petrovic et al., 2010). Thus, chronic treatment with rosiglitazone promotes the expression of brown fat genes, mitochondriogenesis, and thermogenesis capacity in a significant subset of white fat cells. However, the cells, referred to as “brite” adipocytes, are devoid of transcripts for classic brown adipocytes markers (including PRDM16) or for myocyte-associated genes. We reconcile these observations as evidence of at least two separate pathways that promote browning in the white fat. Rosiglitazone might promote a pool of brown adipocyte precursors that does not share a muscle origin, while the TGF $\beta$ 1 effect could act on a pool of cells that might potentially represent a common progenitor for white, brown, and muscle cells. Smad3 cooccupies the genome with cell type specific master transcription factors, including Myod1 in myotubes, PU.1 in pro-B cells, and Oct4 in ESCs (Mullen et al., 2011). It is conceivable that in white fat cells, Smad2/3 interacts with factor/s that specify and maintain cell identity and cellular function. This would also provide plausible explanation for the increased expression of brown fat and skeletal muscle-specific genes in *Smad3*<sup>-/-</sup> white fat.

## THERAPEUTIC RELEVANCE

Discovering approaches to either prevent fat storage or promote fat dissipation will have a major clinical impact (Tseng et al., 2010). Human white adipocytes have the potential to acquire brown fat cell features (Tiraby et al., 2003). Our findings support the notion that promoting brown adipocyte like features in white fat might favorably alter energy balance and protect from obesity and diabetes (Tiraby and Langin, 2003). Further, our anti-TGF- $\beta$  antibody studies strongly suggest that this strategy has translational potential in treatment of human obesity and diabetes. Similarly, the role of neuronal signaling regulators, NPY, and BDNF, have been elucidated in the promotion of browning of white fat (Cao et al., 2011; Chao et al., 2011) making these factors amenable for consideration as therapeutic targets. Importantly, the fact that simple changes in the environment promotes hypothalamic BDNF expression and leads to browning of white fat and increased energy dissipation (Cao et al., 2011) is of particular relevance with respect to behavioral modulation as a means to manage and treat obesity and diabetes. Interestingly, nicotine induces browning of white fat, along with associated histological and molecular characteristics, decreases food intake and protects from obesity (Yoshida et al., 1999) thus presenting a potential anti-obesity modality. Furthermore, evidence that prostaglandins, cyclooxygenase 2, and retinoic acid mediated pathways promote the accumulation of brown adipocyte like cells in traditional white fat depots (Madsen et al., 2010; Vegiopoulos et al., 2010; Mercader et al., 2006) argues for utility of these molecules and their downstream effectors as candidate therapeutic targets. Finally, considering its ability to induce browning of white fat and improving glucose homeostasis, the hormone irisin can now be added to list of therapeutic candidates for metabolic disease (Bostrom et al., 2012).

As elevated TGF- $\beta$  levels are common in many disease conditions, there is a push to develop TGF- $\beta$  antagonist therapies, including TGF- $\beta$  neutralization antibodies and small molecule TGF- $\beta$  receptor antagonists (Yingling et al., 2004). Indeed, such regimens are being clinically evaluated for diseases, such as cancer, fibrosis, scarring, diabetic nephropathy, where elevated TGF- $\beta$

levels are implicated (Massague et al., 2000; Rane et al., 2006; Massague, 2008). Findings that TGF- $\beta$ 1 levels are elevated with obesity, taken together with our illustration of beneficial effects of the anti-TGF $\beta$  neutralization in mouse models of obesity and diabetes, provide a rationale to consider anti-TGF- $\beta$  modalities for these diseases. The efficacy of 1D11 ( $\alpha$ -TGF- $\beta$ ) has been tested in pre-clinical disease models (Ling et al., 2003; Nam et al., 2008) and a closely related human version of this antibody, designated Fresolimumab, is currently under evaluation in human clinical studies of pulmonary fibrosis, renal disease, and cancer. However, the TGF- $\beta$  family proteins engage specific receptors in virtually every cell type (Roberts and Sporn, 1985). In addition to the canonical TGF- $\beta$ /Smad signaling node, cross-talk with other signaling networks is a common feature of TGF- $\beta$  signals (Derynck and Zhang, 2003) and inhibiting the TGF- $\beta$  pathway at the ligand-receptor level may damage essential signaling networks that cross-talk with TGF- $\beta$ .

Although we propose that modulation of TGF- $\beta$ /Smad3 signaling activates a brown adipocyte like phenotype in rodent white fat, its implication in browning of human white fat is unclear at this time. Further, understanding the molecular pathways of TGF- $\beta$ /Smad3 signaling as it pertains to appearance of brown adipocytes in white fat depots is essential to decipher mechanistic details. Does TGF- $\beta$ /Smad3 signaling affect transdifferentiation or precursor cell dynamics as it induces browning of white fat? Which white fat depots are the primary targets, and how do the TGF- $\beta$ /Smad3 signals harmonize with other molecules, hormones, and pathways that also regulate browning of white fat? Answers to these questions, while yielding greater mechanistic insight, will uncover novel targets for development of rational therapeutics to counter obesity and diabetes.

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