Chapter

Novel Aspects of Follistatin/Transforming Growth Factor-β (TGF-β) Signaling in Adipose Tissue Metabolism: Implications in Metabolic Health

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Abstract

Obesity is a major risk factor for several metabolic disorders including insulin resistance, diabetes, and cardiovascular diseases. Chronic imbalance of calorie intake and expenditure results in storage of excess unused energy resulting in obesity and related metabolic dysfunctions. While most obesity therapies are focused on reducing the calorie intake and exercise, recent studies suggest that targeting cellular energy expenditure could be a fascinating alternative approach. Brown adipose tissue (BAT) not only has a remarkable calorie burning capacity, but it could also promote triglyceride clearance and glucose disposal. Induction of brown adipose mass and activity in relevant tissues are linked to relieve symptoms of various metabolic disorders such as diabetes, insulin resistance, and cardiovascular diseases. Follistatin (Fst), an extracellular protein that binds and antagonizes several members of the transforming growth factor beta (TGF-β)/myostatin (Mst) superfamily, promotes brown adipose characteristics in both white and brown adipose tissues by targeting distinct molecular pathways. Inhibition of Mst, on the other hand, leads to significant upregulation of adipose browning in white adipose tissues. This chapter will summarize most recent developments in targeting adipose tissue and their functional characteristics to explore therapeutic potential of Fst and TGF-β/Mst signaling to modulate adipose tissue metabolic functions to combat obesity and related metabolic syndromes.

Keywords: adipocyte, follistatin, myostatin, transforming growth factor beta, adipose browning, uncoupling protein 1, thermogenesis, insulin sensitivity

1. Introduction

Obesity is a global health problem that results from chronic imbalance between energy intake and its expenditure. Obesity is a major risk factor for several metabolic diseases including diabetes, dyslipidemia, insulin resistance, cardiovascular diseases, nonalcoholic fatty liver, and even some form of cancer. According to most recent global estimates, by year 2030 roughly 2.16 billion individuals will be obese

as defined by body mass index (BMI) of 30 or higher [1]. The economic impact of obesity and related metabolic complications has been estimated between 4 and 8% of gross domestic product which is comparable to 2018 defense budget ($643 billion) and Medicare ($588 billion) in the United States [2]. Thus the toll of obesity imposes massive and rapidly growing economic cost beyond human suffering. This economic burden of obesity, therefore, significantly impacts low-income and otherwise disadvantaged population. Staying physically active and maintaining a healthy diet are well accepted and proven strategies to prevent weight gain; however, an alarming increase of global obesity urgently requires the development of novel and highly effective anti-obesity therapies. According to the laws of thermodynamics, any treatment for obesity must require reduced energy intake, increased energy expenditure, or both. Recent data suggest that targeting cellular bioenergetics may provide attractive therapeutic avenues for the treatment and prevention of obesity. White adipose tissue (WAT) and brown adipose tissue (BAT) are two distinct adipose tissue types present in mammals. While WAT with larger unilocular lipid droplets store excess energy in the form of triglycerides, BAT consisting of multilocular smaller lipid droplets enriched with mitochondria that express uncoupling protein 1 (UCP1) has specialized capacity to dissipate excess energy via activating non-shivering thermogenesis. Pockets of UCP1-positive adipocytes have also been found within WAT depots which are called beige or brite (brown within white) adipocytes. These beige adipocytes show some morphological and functional similarities to classical brown adipocytes present with the BAT. Several molecular signaling pathways are reported to play significant roles in the development and differentiation of these white, beige, and brown adipose cells. Transforming growth factor beta (TGF-β) controls the development, growth, and cellular functions of diverse cell types by transmitting signals via dual serine/threonine kinase receptors and transcription factors called Smads, especially Smad3. TGF-β expression levels are significantly elevated in adipose tissues from obese mice [3], and blocking of TGF-β/Smad3 signaling results in protection from obesity and diabetes. These metabolic benefits are associated with increased appearance of brown-like adipocytes within the WAT [4]. Inactivation of myostatin (Mst) also called growth and differentiation factor 8 (GDF8), a key member of the TGF-β superfamily in both differentiating mouse embryonic fibroblast (MEF) primary cultures from wild type (WT) and Mst knockout (Mst KO) embryos, as well as in white adipose tissues of Mst KO mouse models, displays beige adipocyte phenotype and upregulation of key beige markers compared to the wild type [5]. Blockade of activin receptor IIB (ActRIIB) that integrates the actions of Mst and TGF-β-related ligands has been demonstrated to activate functional brown adipogenesis and thermogenesis [6]. Inhibition of Smad3 signaling, which has been identified as canonical pathway for Mst, induced WAT browning [7]. It therefore suggests that antagonizing TGF-β/Smad3/Mst signaling pathway would lead to significant favorable metabolic alterations by promoting adipose browning. Since follistatin (Fst) is a well-known inhibitor of TGF-β signaling pathway in a variety of cell lines [8–10], and a key antagonist of Mst, Braga et al. [11] hypothesized that Fst may promote browning of white adipocytes, and using differentiating MEF primary cultures from WT and Fst KO embryos provided the first evidence that Fst is a novel inducer of brown adipose characteristics. Subsequent studies using Fst-transgenic (Fst-Tg) mice overexpressing Fst under the control of skeletal muscle-specific myosin light chain promoter, the authors demonstrated that Fst targets distinct pathways to promote brown adipose characteristics in both BAT and WAT [11]. Combined together, these findings support the idea that targeting TGF-β/Smad3/Mst signaling either via direct genetic or pharmacological inhibition of this pathways or via directly upregulating Fst could be attractive therapeutic options for the treatment of obesity and related metabolic diseases.

2. Developmental origin, transcriptional regulators, and molecular signature of beige and brown adipocytes

Although white and brown adipocytes share many common features such as PPAR-γ-driven transcriptional control of adipogenesis, their gene expression profiles are distinct, and they do not share a direct common progenitor. Recent genetic studies using fat-mapping experiments have shown that while brown fat in the interscapular region and skeletal muscle share some common features and are derived from Myf5 expressing (Myf5+) cells (previously assumed to be exclusively present in committed skeletal muscle precursors), these Myf5+ precursor cells were absent in white and beige cells [12, 13]. Studies from several other laboratories using global gene expression as well as mitochondrial proteomics signature confirmed that BAT is highly related to the skeletal muscle and not WAT [14, 15]. The divergence of brown adipocyte precursor and skeletal muscle was investigated using Pax7, another myogenic marker in pulse-chase experiments, and it was reported that this divergence occurred between embryonic day 9.5 and 11.5 in mice [16]. UCP1-expressing beige adipocytes present in epididymal WAT (Epi WAT) are thought to be derived through the proliferation and differentiation of platelet-derived growth factor receptor α (PDGRF α), CD44, and SC1 precursor cells [17]. Beige or brown-like cells in the inguinal WAT, on the other hand, are suggested to be derived from Myf5-negative (Myf5-) precursor cells [12]. However, this view has recently been challenged by various groups on the basis of linage analysis studies that suggest that subsets of white adipocytes are derived from both Myf5+ and Myf5- precursors and respond to beta-3 adrenergic receptor (β3-AR) signaling suggesting that these beige adipocytes may have multiple origins [18–20]. A subset of UCP1-positive beige adipocytes is also recently reported to arise from Myh11, selectively expressed in smooth muscle cells [21]. It is also possible that beige cells can either originate from mesodermal stem cells or trans-differentiation of mature white adipocytes [22]. Beige cells may also originate from Ebf2+ precursors located in the subcutaneous adipose tissue (SAT) population characterized by specific markers Cd137 and Tmeme26 [23]. Furthermore, it is also possible that thermogenic adipocytes may arise from endothelial cells and capillaries where retinoic acid (RA) could induce adipose browning by activating VEGF signaling pathways [24]. RA is known to trigger angiogenesis and facilitate de novo generation of *Pdgrfα* expressing adipocyte precursors mediated via VEGFA/VEGFR2 signaling [25]. These findings, therefore, collectively suggest that beige adipocytes which are composed of heterogeneous cell populations may have distinct cellular origins.

The acquisition of morphological and molecular features of brown and beige fat is under the control of PPARγ-coactivator 1α (PGC-1α) [26]. PGC-1α is induced early in brown fat differentiation and is preferentially expressed in mature brown adipocytes. PGC1-1α ectopic expression is sufficient to promote various aspects of differentiation toward the brown fat lineage. PGC-1α is also rapidly and highly induced by cold exposure and turns on several key components of the adaptive thermogenic program including fatty acid oxidation, mitochondrial biogenesis, and increased oxygen consumption [27]. The expression levels of 140kD zinc figure containing transcription factor called PR domain containing 16 (PRDM16) are very high in BAT compared to the visceral WAT and appear to play a major role in brown adipose/skeletal muscle fate determination [28]. Ectopic expression of PRDM16 in cultured mesenchymal cells including white preadipocytes induced a complete brown fat differentiation program and activation of key thermogenic (*Ucp1*, *Pgc-1α*, *cidea*, and *elov3*) genes and coactivates the transcriptional activity

of PGC-1α/PGC-1β, as well as PPARα and PPARγ [12, 28]. Coincident with these changes, PRDM16 expression also led to suppression of several white fat and muscle-selective markers [29]. On the other hand, genetic ablation of PRDM16 in brown fat leads to significant increase in white adipose and muscle-specific genes [28]. These findings, therefore, suggest that PRDM16 acts as a critical cell fate regulator of brown fat, and careful analysis of its embryonic expression pattern will be extremely valuable to dissect out the putative brown fat-skeletal muscle precursors. Signaling molecules that control the timing and specificity of PRDM16 expression during development are unknown. Certain growth factors like bone morphogenetic proteins (BMPs), members of the TGF-β superfamily of secreted factors, are reported to influence both brown and white adipocyte differentiation [30–32]. While BMP2 and BMP4 are reported to promote white adipose cell differentiation, BMP7 is reported to selectively induce brown adipogenesis in committed precursor cells [32, 33]. BMP7 exposure to fibroblast cultures results in induction of full brown fat differentiation program, including induction of PRDM16 and UCP1 expression [30]. Importantly, significantly reduced amounts of BAT mass were observed in BMP7-deficient mice. However, it is not clear whether BMP7 plays any role in the regulation of PRDM16.

Under basal conditions both beige and brown adipocytes share some of the same key markers including UCP1 and PRDM16; however, data from clonal cell lines suggest that beige and brown adipose cells express related but distinctly different gene expression profiles [23]. Beige cells are highly enriched in Tmem26, Tbx1, and CD137 expression [23]. Comprehensive gene expression analysis of adipose tissues isolated from interscapular BAT and inguinal fat revealed several other beige-selective genes including *Ear2*, *Sp100*, *Klh113*, and *Slc27a* [23]. Molecular profiling and histological analysis of human BAT identified additional beige-selective markers HoxC8, HoxC9, Cited1, and Shox2 [34, 35]. On the other hand, classical brown adipocytes selectively express epithelial V-like antigen (Eva 1), Zic1, Lhx1, and Epsti [23, 36–38]. Using adipose tissues isolated from white and interscapular BAT from 129SVE mice, Wu et al. identified additional genes including *Hspb7*, *Ebf3*, *Pdk4*, *Fbxo31*, and *Oplah* that were enriched in BAT [23]. Using a combination of in silico, in vitro, as well as in vivo approaches, Ussar et al. reported the identification of three new cell surface markers of adipose tissues [39]. In this study, amino acid transporter Asc1 was identified as a white adipocyte-specific cell surface protein with very low to undetectable levels in brown adipocytes, whereas amino acid transporter PAT2 and the purinergic receptor P2RX5 are cell surface markers expressed in classical brown and beige adipocytes.

Studies from microRNA (miRNA) signature analysis between beige and brown adipogenesis have provided significant differences in their molecular signature. MiRNA-193b-365 cluster is expressed in brown fat tissues and initially thought to be involved in the regulation of brown fat differentiation by inhibiting Runx1t1, which inhibits BAT differentiation [40]. However, subsequent in vivo studies show a normal BAT function in the absence of miRNA-193b-365 [41]. Inhibition of miRNA-182 and miRNA-203 in brown adipocytes led to downregulation of several genes involved in oxidative phosphorylation and electron transport [42]. Inhibition of miRNA-106b-93 led to induced expression of several adipogenic markers [43]. Similarly, positive (miRNA-196b) and negative (miRNA-26) regulation of beige adipogenesis have been identified [44, 45]. miRNAs that positively (miRNA-30 family) and negatively (miRNA-27 and miRNA-34a) regulate both brown and beige adipocytes are also identified [46–48]. Thus, there appear to be clear differences between BAT and beige miRNA gene signature in mouse and human tissues and cells.

3. Role of transforming growth factor-β (TGF-β) superfamily in adipose browning and metabolic health

The TGF-β superfamily consists of more than 33 members including TGFβ1, TGFβ2, and TGFβ3, bone morphogenetic proteins (BMPs), growth differentiation factors (GDFs), and activins that play important roles in growth, development, and function of diverse cell types including adipocytes [49, 50]. These evolutionary highly conserved superfamily members transmit their signals via dual serine/threonine kinase receptors and transcription factors called Smads. TGF-β superfamily members control various aspects of adipocyte biology. Adipose tissues from obese mice were reported to express elevated levels of TGF-β [3]. The binding of TGF-β family to their membrane receptors could be somewhat promiscuous and may allow 7 type I and 5 type II receptors to transduce signaling from these TGF-β superfamily members. Multiple cell types including adipose progenitors, preadipocytes, and adipocytes along with various immune cells are known to express protein belonging to TGF-β superfamily and their antagonists [51]. The role of TGF-β/Smad3 signaling in regulating beige adipocyte phenotype and metabolic characteristics were elegantly demonstrated by Yadav et al. [4]. They observed significant positive correlation between TGF-β1 levels and adiposity in both rodents and human subjects. Using $Smad3^{-/-}$ mice, they provided interesting link between Smad3 loss and protection against diet-induced obesity and related metabolic syndromes. These changes in metabolic parameters were associated with induction of white to brown phenotype and significantly increased mitochondrial biogenesis. In the same study, examination of a total of 184 nondiabetic human subjects from diverse ethnic groups, the authors identified direct relationship between circulating TGF-β1 levels and BMI, fat mass, and VO_2 consumption. Furthermore, anti-TGF-β antibody in $Lep^{ob/ob}$ and diet-induced obesity mouse models resulted in significantly reduced body weight, improved glucose and insulin tolerance, as well as significantly reduced fasting glucose and insulin levels. These metabolic improvements were associated with elevated expression of BAT/mitochondria-specific proteins in white adipose tissues. Such links between TGF-β signaling and mitochondrial energy metabolism pathway have also been reported by several other laboratories [52, 53]. Extracellular matrix protein microfibril-associated glycoprotein (MAGP) was found to be significantly altered in obese humans, and inactivation of MAGP1 gene ($Mfap2^{-/-}$) resulted in adipocyte hypertrophy and predisposition to metabolic diseases. $Mfap2^{-/-}$ mice had significantly lower expression of UCP1 expression in BAT and display reduced subcutaneous adipose browning and defective adaptation to cold exposure [53]. Treatment of these $Mfap2^{-/-}$ mice with neutralizing concentrations of anti-TGF-β antibody led to decreased adiposity and improved body temperature. Administration of a novel activin receptor type II B (ActRIIB) decoy receptor containing the extracellular domain of ActRIIB fused to human Fc (ActRIIB-Fc) resulted in suppression of diet-induced obesity and associated metabolic functions in mice [54]. In the same study, significantly increased adipose browning in epididymal white fat displaying robustly increased expression of UCP1 and PGC 1-α was observed following ActRIIB-Fc treatment. Furthermore, protection from diet-induced obesity in ActRIIB-Fc-treated mice was demonstrated to result from increased energy expenditure and not decreased caloric intake. Combined together, these interesting findings suggest novel insights into the role of TGF-β signaling in suppressing adipose browning program within white fat tissues in both mouse models and human subjects suggesting that efficient blockade of TGF-β activity could serve as an effective treatment strategy for obesity and diabetes.

Myostatin (Mst) is a key member of the TGF-β superfamily which is known to play a major role in the regulation of skeletal muscle growth. However, recent studies have clearly indicated that the effect of Mst extends beyond its role in skeletal

muscle. Genetic deletion of Mst displays favorable changes in several metabolic parameters including decreased fat deposition, enhanced fatty acid oxidation, improved insulin sensitivity, and increased resistance to diet-induced obesity besides increased skeletal muscle mass [55, 56]. Since Mst is expressed at very low levels in adipose tissues [57], it remains unclear how depletion of Mst can suppress fat accumulation. Earlier studies by Kim et al. show that treatment of mouse primary brown preadipocytes with recombinant Mst led to significant inhibition of brown adipogenic differentiation and reduced expression of markers *Ucp1*, *Prdm16*, and *Pgc-1α* [58]. A comparison of key thermogenic markers obtained from epididymal (Epi) and subcutaneous (SC) white adipose tissues shows significantly increased expression of UCP1 and PRDM16 in Mst KO mice compared to the WT littermates [54]. Using differentiating primary cultures isolated from WT and Mst KO mouse embryonic fibroblasts (MEFs) in the same study, Braga et al. further confirmed upregulation of key thermogenic markers in Mst KO mice compared to the WT mice [5]. Furthermore, recombinant Mst protein treatment of the differentiating MEFs significantly downregulated several key thermogenic markers including UCP1, PRDM16, PGC-1α/PGC-1β, and BMP7. Also, protein expression of adiponectin and phosphorylated AMP-activated protein kinase (pAMPK), which control the expression of genes involved in energy metabolism in coordination with NAD+-dependent sirtuin 1 (SirT1), were upregulated in Mst KO MEFs compared to the WT group [59, 60]. In another study, Chio et al. reported significantly increased energy expenditure and leptin sensitivity in Mst-deficient mice that could explain low fat mass in these mice compared to the WT group [61]. Shan et al. demonstrated that inhibition of Mst signaling in WAT SVF cells failed to induce browning of white adipocytes in vitro, suggesting that loss or inhibition of Mst signaling in preadipocytes does not account for adipose browning in white adipocyte tissues in Mst KO mice [62]. In order to test the possible non-cell autonomous effects of Mst, the authors thoroughly analyzed various muscle-derived circulating factors that could account for the browning phenotype. They reported that skeletal muscle-derived Fndc5 (irisin) plays a central role in mediating white adipose browning in Mst KO mice via activation of AMPK-PGC1α-Fndc5 pathway, suggesting the involvement of muscle-adipose cross talk during the process [63, 64]. Fndc5/irisin was initially identified as a PGC-1α-dependent myokine that is responsible for adipose browning both in vitro and in vivo and protects diet-induced obesity in obesity [65]. Possible involvement of Fndc5 in mediating adipose browning in Mst loss-of-function models has emerged from other laboratories. Dong et al. also confirmed possible intermediate role for Fndc5/irisin-mediated adipose browning in Mst KO mice. In another study, Mst signaling was shown to regulate Fndc5 expression and adipose browning via upregulation of miRNA-34a. Several laboratories have demonstrated that the absence of Mst in both in vitro and in vivo models improves insulin sensitivity [58, 66]. Several other laboratories provided additional evidence to support the view that Mst loss-of-function results in significant metabolic improvements resulting from adipose browning. Increased insulin sensitivity and WAT browning were reported in Meishan pigs with Mst functional deletion [67]. Several browning-related genes including UCP1, PGC-1α, PRDM16, Cidea, CD137, and Tmem26 were significantly upregulated in these Mst-deficient pigs. Protein expression levels of insulin receptor (IR) and insulin receptor substrate (IRS) were significantly induced in the skeletal muscle of these Mst-deficient pigs. Interestingly, serum irisin levels and skeletal muscle protein expression of irisin precursor protein FNDC5 were significantly higher in Mst-null pigs than wild-type pigs. These authors also demonstrated that inhibition of irisin expression was unable to block the activation of insulin signaling pathway, thus, implying that irisin may not be required for activation of insulin signaling in Mst-deficient skeletal muscle [67]. Genetic

disruption of Mst ($Mst^{-/-}$) in $LDLR^{-/-}$ ($Mst^{-/-}/LDLR^{-/-}$) mice was shown to reduce the development of proatherogenic dyslipidemia, improve insulin-mediated glucose disposal, and protect against hepatic steatosis [68]. Furthermore, Guo et al. demonstrated that administration of adeno-associated virus 9 (AAV9)-mediated Mst-pro-peptide in adult $LDLR^{-/-}$ mice reduced diet-induced hepatosteatosis and progression of atherosclerosis [69]. In both these reports, the beneficial metabolic effects were claimed to result from enlarged muscle mass following inactivation of functional Mst in $LDLR^{-/-}$ mice. Several recent reports have provided strong evidence suggesting that brown fat activation could reduce hypercholesterolemia and protect from atherosclerosis development [70–72]. Therefore, it is possible that observed beneficial effects of Mst inactivation in $LDLR^{-/-}$ background could be mediated at least in part via adipose browning.

More recently, Mst expression has been linked to mediate BAT-muscle cross talk [73]. Induction of Mst following loss of interferon regulatory factor 4 (IRF4) in BAT leads to significantly reduced exercise capacity, ribosomal protein synthesis, and mitochondrial function [73]. On the other hand, reduced serum levels of Mst resulting from IRF4 overexpression significantly increased exercise capacity in muscle. IRF4 expression was found to be induced in brown adipocytes following cold exposure and β3-adrenergic receptor (AR) agonist [74]. IRF4 expression was reported to be sufficient to induce thermogenic program in BAT, and loss of IRF4 in brown fat leads to significantly reduced energy expenditure. Also, IRF4 was shown to physically and functionally interact with PGC-1α to upregulate transcription of *Ucp1* gene and drive mitochondrial biogenesis and thermogenic program in BAT. In light of these exciting reports establishing IRF4 as a novel inhibitor of Mst, it is not surprising that IRF4 could antagonize the bioactivity of secreted Mst present in the blood to promote overall thermogenic program.

4. Follistatin regulation of white and brown adipose characteristics

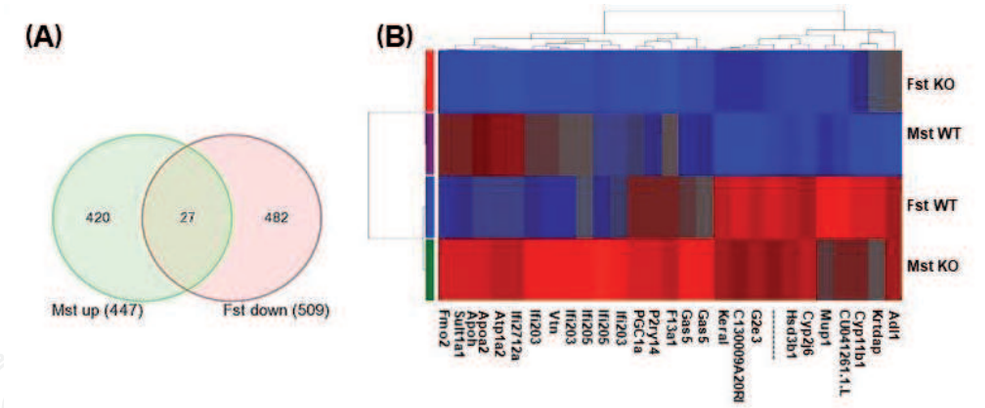
Follistatin (Fst) is a soluble secreted glycoprotein that is known to bind and neutralize the activity of several members of the TGF-β superfamily including activins and Mst in a variety of cell lines [9–10, 77]. Several genetic studies have convincingly demonstrated an essential role of Fst in the regulation of muscle mass. Elegant initial studies led by Lee and McPherron demonstrated that inhibition of Mst either by genetic manipulation or overexpressing Fst resulted in significantly increased muscle mass in mice [75]. The direct role for Fst in the regulation of muscle mass was also verified by several laboratories [8, 9, 76]. Fst was identified as a downstream target of testosterone during its pro-myogenic action in both in vitro and in vivo studies [8, 9]. Testosterone treatment of mouse mesenchymal multipotent C3H 10T1/C3H 10T2 cells led to upregulation of Fst and altered the expression of several key members of TGF-β superfamily [8]. Testosterone-induced upregulation of key myogenic markers MyoD and myosin heavy chain II proteins in C3H 10T1/C3H 10 T2 cells was abolished in cells simultaneously treated with anti-Fst antibody, suggesting an essential role of Fst during testosterone regulation of myogenic differentiation. The essential role of Fst was also established in in vivo studies using castrated male mice, where Fst gene expression level significantly reduced in the levator ani (LA) muscle compared to the sham-operated male mice, but testosterone supplementation in castrated mice upregulated Fst mRNA expression in LA muscle to the baseline levels [8]. Subsequent studies by Braga et al. reported that primary culture of muscle satellite cells express Fst and respond to testosterone treatment. Fst blocked TGF-β-induced inhibition of MHC II expression and induction of Smad2/Smad3 phosphorylation in satellite cells [9]. These

reports provide conclusive evidence that Fst plays an important role in promoting myogenic differentiation and increasing muscle mass. In spite of several reports demonstrating as essential role of Fst in regulating muscle mass and its function, its role in lipid metabolism and energy balance was largely unknown. Fst-deficient mice die within hours after birth and have several defects including reduced size of diaphragm muscle [76]. These severe musculoskeletal defects were suggested to account for the neonatal death of these Fst-deficient pups. Since maintenance of body temperature through thermogenesis during early hours of neonatal life is extremely important, and both skeletal muscle and thermogenic brown fat share Myf5+ precursor cells, it is logical to test whether Fst could play a role in regulating the thermogenic program along with its established role in muscle development. Based on this logic and several published reports that Fst can bind and antagonize the biological actions of TGF-β/Mst signaling [8, 9], which are known inhibitors of thermogenic program, Braga et al. hypothesized that Fst may promote adipose browning and favorably alter energy metabolism [11]. Initial quantitative analysis of Fst gene expression in a mouse tissue panel consisting of several metabolic tissues demonstrated that Fst expression was highest in BAT along with skeletal muscle and was also expressed at a substantial level in inguinal WAT and the liver [11]. The expression levels in other tissues including the heart, intestine, and testis were significantly lower. This finding for the first time suggested a possible novel role of Fst in BAT and WAT and led to a series of subsequent in vitro and in vivo experimental approaches to delineate the precise role of Fst in adipose tissues of both origins. Using immortalized mouse brown preadipocytes, the authors clearly demonstrated that Fst protein expression was significantly induced in differentiated BAT cells displaying characteristic multilocular lipid droplets compared to the undifferentiated cells [11]. As expected, levels of key brown adipose markers such as UCP1 and PRDM16 were also significantly induced after differentiation of BAT cells. Furthermore, Fst gene expression in BAT was dramatically induced following cold exposure of the mice, suggesting that Fst is a novel cold-inducible gene and could play important role in regulating key metabolic functions. Since Fst KO mice are not viable, Braga et al. utilized primary cultures of differentiating mouse embryonic fibroblast (MEF) cultures isolated from Fst KO and WT embryos to test whether Fst loss-of-function results in defective thermogenic program [11]. Significant impairment in adipogenic differentiation and upregulation of BAT-specific markers were noted in Fst-deficient MEF cultures compared to the WT. Exogenous recombinant Fst protein treatment was able to rescue the thermogenic genes and proteins in Fst-deficient MEF cultures and further induced the expression of several BAT-specific genes in differentiated mouse BAT cells. Affymetrix global gene expression profiling clearly demonstrated lipid metabolism as the most significantly altered pathway and identified several genes involved in lipid metabolism and energy production such as *Adn*, *Thrsp*, *Hp*, *Acsl1*, *Fabp4*, *Pparg,* and *Cd36* were significantly downregulated in Fst KO MEFs compared to the WT. Significantly lower basal mitochondrial respiration in Fst KO MEFs compared to the WT cultures was rescued by exogenous recombinant Fst, suggesting that Fst increases cellular respiration [11].

In subsequent experiments, Singh et al. explored the in vivo actions of Fst overexpression on both white and brown adipose tissues using Fst-transgenic (Fst-Tg) mice to determine whether Fst promotes adipose browning and brown adipose mass and function in these mice and identify possible molecular targets of Fst in these adipose tissues. Fst-Tg mice express Fst under a muscle-specific promoter [75] in which the circulating Fst levels are 1.5-fold higher along with ~70% increased interscapular BAT mass compared to the WT mice [77]. BAT signature genes and several key proteins involved in mitochondrial biogenesis, fatty acid oxidation (FAO),

were significantly upregulated in iBAT as well as in Epi and SC adipose tissues of Fst-Tg mice compared to the WT mice [77]. The BAT marker UCP1 and beige-specific markers CD137 were significantly higher in both WAT depots of Fst-Tg mice compared to WT, with relatively larger differences observed in SC adipose depots. Several other markers involved in mitochondrial biogenesis and FAO were also found to be induced in both adipose depots from Fst-Tg mice compared to the WT mice. These observed differences in adipose browning capacity between the two WAT depots were found to be consistent with previous reports [78].

The actions of Fst in regulating WAT and BAT adipose characteristics were shown to be mediated via two distinct mechanisms. Fst increased phosphorylation of p38 mitogen-activated protein kinase (p38 MAPK) and extracellular signal-regulated kinase (ERK1/ERK2) in both WAT depots, while it increased Myf5 expression in iBAT of Fst-Tg mice [77]. The authors utilized in vitro studies to further confirm the obligatory and mechanistic basis for these distinctly different Fst targets. In differentiating 3T3-L1 cells, recombinant Fst treatment led to significant induction of UCP1 and beige-specific marker CD137. Pharmacological inhibition of p38 MAPK and ERK1/ERK2 phosphorylation by SB023580 (10 μM) and PD98059 (10 μM), respectively, either alone or in combination, led to significant blockade of Fst-induced (i) phosphorylation of both these proteins as expected and (ii) upregulation of UCP1 protein [77]. On the other hand, in BAT and differentiated mouse BAT cells, Fst increased Myf5 protein expression. Knockdown of Myf5 expression led to significant inhibition of recombinant Fst-mediated increase in UCP1 protein expression in differentiated mouse BAT cells. Additionally, Fst treatment was able to rescue Myf5 gene and protein expression in Fst KO MEFs, reinforcing that Myf5 is a critical mediator of Fst action in BAT [77]. Since BAT and skeletal muscle share Myf5-expressing progenitor cells [78, 79], the authors proposed that Fst promotes BAT activation and skeletal muscle growth by upregulating Myf5. Based on these novel findings, the authors proposed that Fst induces Myf5 expression in BAT and Myf5-positive progenitor cells to increase classical BAT activation, whereas it promotes phosphorylation of p38MAPK and ERK1/ERK2 in WAT to promote adipose browning. It is also possible that Fst could efficiently enhance the production of one or more of several myokines which are shown to induce white adipose browning including irisin (encoded by *Fndc5* gene), IL6, or FGF21 [80–82]. Both Fst and FGF21 were shown to be induced and secreted following exercise [81]. Also, secretion of irisin by skeletal muscle in response to exercise was reported to induce phosphorylation of p38 MAPK and ERK1/ERK2 leading to white adipose browning [80]. Upregulation of *Fndc5* gene expression was reported in skeletal muscle after treatment with both recombinant Fst protein and anti-Mst antibody [62], suggesting that Fst could target irisin/Mst-mediated pathway in muscle tissue to promote adipose browning mediated via muscle-adipose cross talk. Using similar MEF-based primary cultures obtained from WT and Fst KO and Mst KO, Braga et al. showed reciprocal regulation of BMP7 [5, 11], a key driver of brown adipogenesis and energy metabolism by Fst and Mst. These findings were confirmed by other laboratories in support of Fst-induced upregulation of BMP7 [83] and its downregulation by Mst [84]. Gene expression analysis of MEF primary cultures from WT and Fst KO versus WT and Mst KO shows several genes that were reciprocally regulated by Fst and Mst as identified by Affymetrix gene expression analysis and further validated by quantitative real-time PCR analysis (**Figure 1**). Analysis of basal oxygen consumption rate (OCR) in differentiated MEF cultures from these WT and Fst/Mst KO groups further suggests reciprocal effects of Fst and Mst on mitochondrial respiration (**Figure 2**). Combined together, these findings support the view that Fst may exert its pro-browning effects at least in part by inhibiting Mst signaling. Follistatin-like-3 (FSTL3) has been reported as another Mst binding protein that



(C) **27 common genes Mst up Fst down**

Gene Symbol	Fst KO vs. WT Fold-Change	Mst KO vs. WT Fold-Change
Apoh	-1.97088	1.53783
Apoa2	-2.81049	1.50383
Kera	-1.63685	1.76537
CU041261.1	-3.0673	1.7381
P2ry14	-1.54046	1.50554
Gas5	-1.87548	2.56952
Mup1	-3.96251	1.60283
Ifi27l2a	-1.53234	1.65737
Sult1a1	-1.62738	1.5307
Ppargc1a	-1.95601	1.73111
Krtdap	-1.70325	1.71661
G2e3	-1.61875	1.70135
Fmo2	-1.6254	1.56236
Gas5	-1.74474	2.64305
Adi1	-1.52644	5.60492
Cyp2j6	-1.56285	1.64017
Atp1a2	-4.17021	2.04007
C130009A20R1	-1.86572	1.90615
Hsd3b1	-1.693	1.52567
Ifi203	-1.62841	2.12467
F13a1	-2.49324	1.6117
Ifi203	-2.36551	2.41474
Ifi203	-2.48491	4.58089
Ifi205	-1.78756	5.27227
Vtn	-1.52667	2.15297
Cyp11b1	-1.62126	1.57936

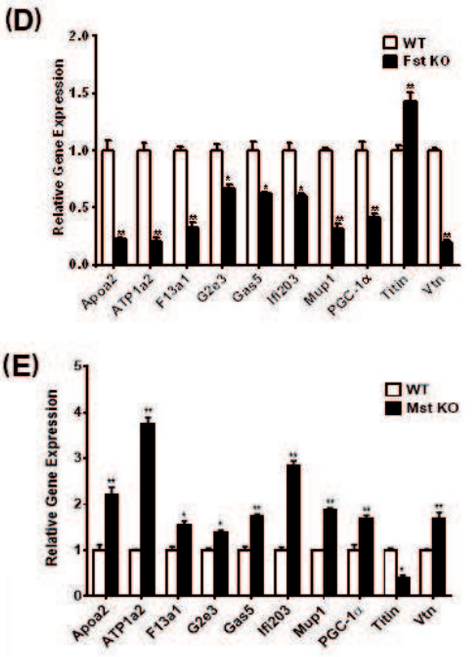


Figure 1.
*Reciprocal effects of Fst and Mst on several genes involved in lipid and energy metabolism. Primary cultures obtained from differentiating WT and Fst/Mst KO cells were analyzed by Affymetrix global gene profiling. (A) Van diagram showing 27 common genes that were reciprocally regulated by Fst and Mst. (B) Heat map showing differential expression of those 27 genes. (C) List of reciprocally regulated common genes. (D, E) Validation of Affymetrix data real-time PCR analysis. n = 3; *p ≤ 0.05; **p ≤ 0.01.*

could play an important role in regulating fat mass and glucose homeostasis [85]. FSTL3 knockout mice display distinct phenotype including decreased fat mass and improved insulin sensitivity. However, it is not known whether FSTL3 regulates BAT mass and thermogenic activity, suggesting that the role of Fst may be more complicated [86].

Activation of p38MAPK pathway that promotes adipose browning by β3-adrenergic receptor (β3-AR) has been well documented [87, 88]. Since intraperitoneal injection of β3-AR agonist CL316,243 in Fst-Tg mice resulted in additive response to UCP1 levels in WAT and BAT compared to the WT mice, it is possible that β3-AR signaling could play an important role upstream of p38 MAPK pathway during Fst-induced adipose browning. More recently, elegant studies by Liu et al. demonstrated that inhibition of follicle-stimulating hormone (FSH) through a polyclonal antibody induced adipose browning and activated BAT and thermogenesis [89]. Since Fst was initially isolated from follicular fluid and found to inhibit secretion of FSH from anterior pituitary, this recent report further validates the identification of Fst as a novel inducer of adipose browning.

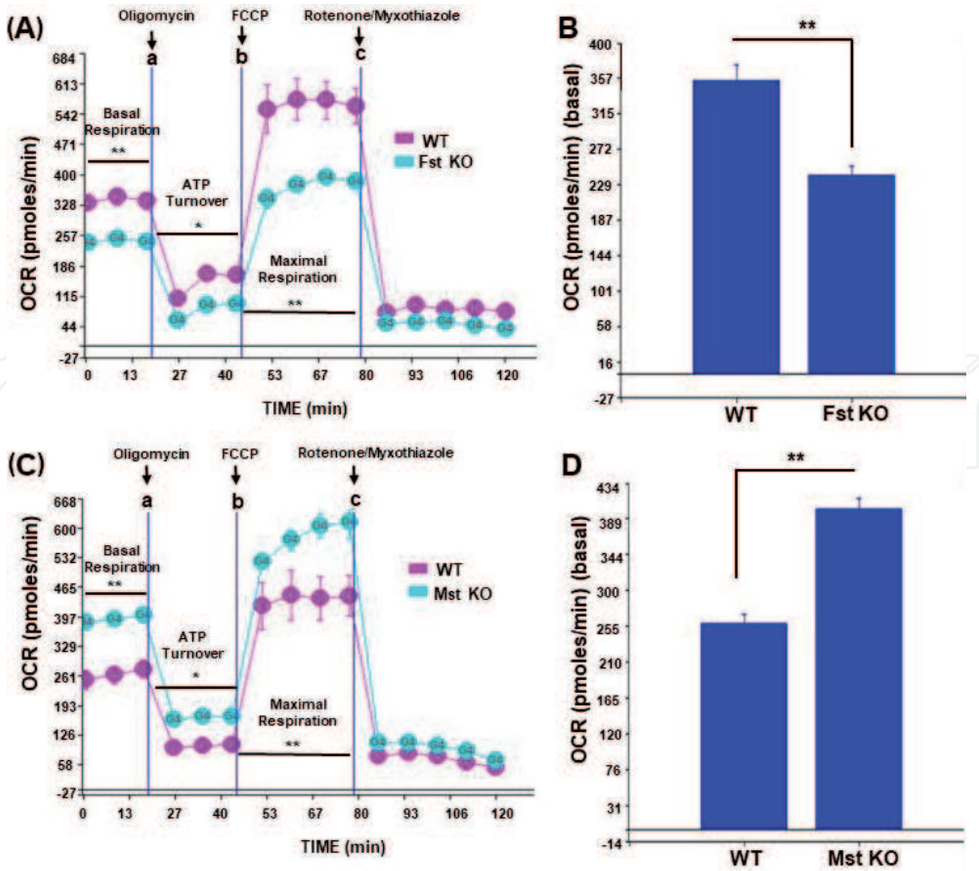


Figure 2.
*Reciprocal effects of Fst (A, B) and Mst (C, D) on basal oxygen consumption rate (OCR) as analyzed by the seahorse bioscience XF24 extracellular flux analyzer. Data are expressed as mean +/− SEM. *p ≤ 0.05; **p ≤ 0.01.*

5. Metabolic profiling of Fst overexpression and relevance to metabolic diseases

In order to get better understanding of observed adipose browning and its metabolic consequences in Fst overexpressing in mice (Fst-Tg) as well as in differentiated 3T3-L1 (3T3-L1 Fst) preadipocyte, Singh et al. initially performed quantitative analysis of abdominal fat volume by CT scan, glucose clearance, and serum lipid profiles [90]. Fst-Tg mice displayed significant decrease in abdominal fat mass, increased glucose clearance, and significantly lower triglyceride (TG) and free fatty acid (FFA) levels compared to the WT control mice. A comparison of the overall lipidomic profiles using gas chromatography time-of-flight technology (GC TOF) shows a general reduction in diglycerides (DG), triglycerides (TG), ceramide (D42:0), fatty acids (FA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), and lysophosphatidylethanolamines (LPE: 16.0) in Fst overexpressing 3T3-L1 (3T3-L1 Fst) cells compared to the basal 3T3-L1 cells after adipogenic differentiation (ref). On the other hand, a significant increase in several lysophosphatidylcholines (LPC) including LPC 16:0, LPC 18:0, and LPC 18:1 was observed in 3T3-L1 Fst cells in comparison to 3T3-L1 cells. The decreased levels of several of these lipid metabolites observed in 3T3-L1 Fst cells are known contributors toward the development of obesity and related metabolic diseases [91, 92]. Increased levels of several of these LPCs following Fst overexpression also suggest a beneficial role for Fst as these LPCs are reported to be significantly reduced in obesity and type 2 diabetes [93, 94]. Comprehensive analysis of metabolites obtained from epi and SC adipose tissue between WT and Fst-Tg mice provided significant differences between metabolites involved in energy and

lipid metabolism of the groups. Several components of the Krebs cycle including citrate, succinylcarnitine, and fumarate were significantly downregulated in Fst-Tg Epi tissues compared to the WT tissues, whereas only reduced levels of succinate were found in SC adipose tissues form Fst-Tg mice compared to the WT mice. Also, several long-chain FAs, components of the carnitine metabolism, glycerolipids, ketone bodies, and lysolipids were selectively found to be lower in Fst-Tg Epi tissues than the WT group. Interestingly, levels of Epi cholesterol levels were also selectively reduced in Epi tissues only. Several key amino acids including tyrosine, components of tryptophan, branched-chain amino acid (BCAA), and urea cycle metabolism were also found to be dramatically reduced in Epi adipose tissues in Fst-Tg mice compared to the WT mice. A comparison of omega-3 polyunsaturated fatty acid (ω-3 PUFAs) levels between the groups shows highly significant increase selectively in SC adipose tissues of Fst-Tg mice. Interestingly, ω-3 PUFAs are reported to improve not only obesity-associated metabolic disorders including insulin resistance and dyslipidemia but also several aspects of energy and lipid metabolism and inflammation [95–97]. Significantly decreased levels of beta-hydroxybutyric acid (BHBA), the end product of FA beta-oxidation and key contributor to metabolic syndrome, were also observed in the Epi adipose tissues of Fst-Tg mice compared to the WT. Combined together, these comprehensive metabolomic profilings of Fst in vitro and in vivo overexpression show a clear pattern of favorable changes in several metabolites implicated in metabolic complications and provide future impetus to thoroughly investigate the novel therapeutic role of Fst.

6. Conclusions

Several lines of evidence support the view that brown and beige adipocytes play important roles in regulating various aspects of lipid and glucose metabolism. The browning process in WAT that entails a shift in WAT primary function from storing excess energy to the dissipation of energy has been linked to the prevention of progression and development of obesity and related metabolic abnormalities including insulin resistance, hyperlipidemia, and type 2 diabetes. Data generated from several laboratories collectively suggest that blocking of TGF-β/Smad3/Mst signaling efficiently increases brown adipose phenotypic and metabolic characteristics and possible downstream mediators during the process as summarized in **Figure 3**.

Accordingly, new strategies to identify and develop novel TGF-β/Mst inhibitors to increase BAT and beige adipose mass and activities are currently being explored with the hope that blockade of this signaling pathway could lead to the development of therapeutic avenues. Since Fst has been demonstrated to efficiently antagonize Mst and inhibit overall TGF-β signaling in several in vitro and in vivo models, the novel therapeutic potential of Fst for the treatment of obesity and related metabolic diseases needs to be thoroughly explored in preclinical studies. It is, therefore, necessary to identify the key molecular and cellular targets of Fst responsible for its regulation of overall thermogenic program. Although phosphorylation and activation of the p38MAPK/ERK1/ERK2 signaling by Fst in WAT have recently been linked to Fst-induced browning, the possible intermediate role of irisin during the process could not be ruled out. Similar to Fst, secretion of irisin by skeletal muscle is induced following exercise which could induce p38MAPK/ERK1/ERK2 phosphorylation and lead to browning of WAT [80]. Fibroblast growth factor 21 (FGF21), another exercise-induced secretory protein linked to adipose browning and which promotes brown adipose characteristics, has also been shown to be influenced by recombinant Fst (rFst) treatment in 3T3-L1 as well as in WAT of

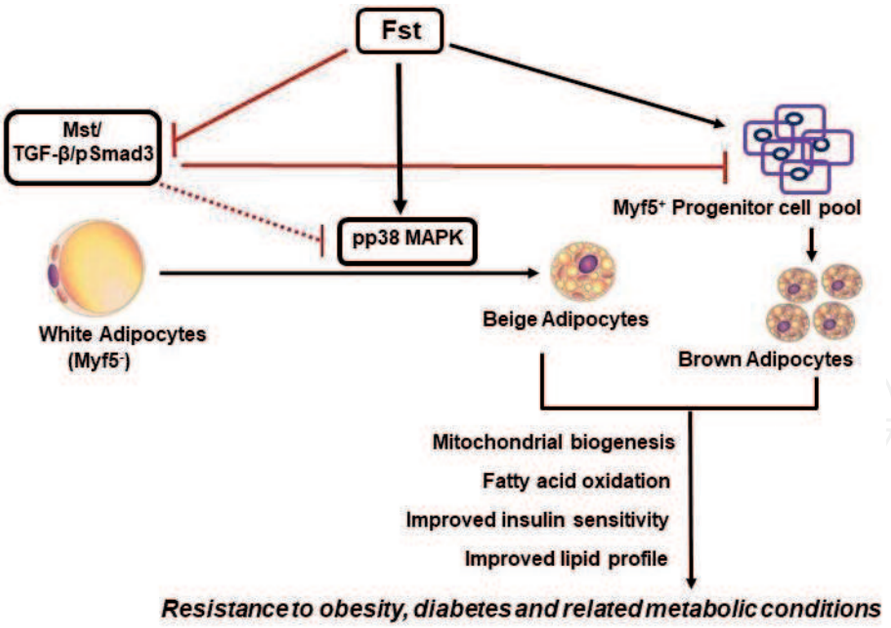


Figure 3.
Proposed hypothetical model for Fst and Mst/TGF-β regulation of adipose browning characteristics of white and brown adipose tissue and their metabolic implications.

Fst-transgenic (Fst-Tg) mice. Robust upregulation of FGF21/adiponectin/AMPK signaling pathway observed under both conditions suggests a possible mechanistic link between Fst and FGF21. Preclinical studies using an alternatively spliced cDNA of follistatin (FS344) delivered by adeno-associated virus (AAV) to muscle in both human patients with certain degenerative muscle disorders [98, 99] and nonhuman primates [100] show no apparent structural or functional aberrations in a variety of organs, suggesting the potential of Fst use in clinical trials, although these studies did not assess adipose tissue metabolic parameters. Therefore, novel antagonists of TGF-β/Mst signaling pathways, including Fst [101], hold a great promise for the treatment of not only muscle loss and dysfunction but also for obesity and related metabolic diseases.

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