Emerging Roles for the Transforming Growth Factor-β Superfamily in Regulating Adiposity and Energy Expenditure

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Members of the TGF-β superfamily regulate many aspects of development, including adipogenesis. Studies in cells and animal models have characterized the effects of superfamily signaling on adipocyte development, adiposity, and energy expenditure. Although bone morphogenetic protein (BMP) 4 is generally considered a protein that promotes the differentiation of white adipocytes, BMP7 has emerged as a selective regulator of brown adipogenesis. Conversely, TGF-β and activin A inhibit adipocyte development, a process augmented in TGF-β-treated cells by Smads 6 and 7, negative regulators of canonical TGF-β signaling. Other superfamily members have mixed effects on adipogenesis depending on cell culture conditions, the timing of expression, and the cell type, and many of these effects occur by altering the expression or activities of proteins that control the adipogenic cascade, including members of the CCAAT/enhancer binding protein family and peroxisome proliferator-activated receptor-γ. BMP7, growth differentiation factor (GDF) 8, and GDF3 are versatile in their mechanisms of action, and altering their normal expression characteristics has significant effects on adiposity *in vivo*. In addition to their roles in adipogenesis, activins and BMP7 regulate energy expenditure by affecting the expression of genes that contribute to mitochondrial biogenesis and function. GDF8 signals through its own receptors during adipogenesis while antagonizing BMP7, an example of a ligand from one major branch of the superfamily regulating the other. With such intricate relationships that ultimately affect adiposity, TGF-β superfamily signaling holds considerable promise as a target for treating human obesity and its comorbidities. **(*Endocrine Reviews* 32: 387–403, 2011)**

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I. Introduction

Overweight, obesity, and their impact on global health have been widely recognized as major medical problems. In the United States alone, estimates from the 1990s revealed that more than 300,000 deaths resulted from this condition, and greater than 10% of health care expenditures were devoted to its treatment and related disorders (1–5). Direct cost estimates for medical spending due to overweight and obesity are up to $92.6 billion an-

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in U.S.A.
Copyright © 2011 by The Endocrine Society
doi: 10.1210/er.2010-0018 Received August 10, 2010. Accepted November 23, 2010.
First Published Online December 20, 2010

Abbreviations: ActRIIB, Activin type IIb receptor; BAT, brown adipose tissue; BMP, bone morphogenetic protein; C/EBP, CCAAT/enhancer binding protein; CTGF, connective tissue growth factor; GDF, growth differentiation factor; HFD, high-fat diet; MSC, mesenchymal stem cell; PPAR, peroxisome proliferator-activated receptor; *Pref-1*, preadipocyte factor-1; R-Smad, regulatory Smad; UCP1, uncoupling protein 1.

nually in 2002 dollars, 9.1% of U.S. health expenditures (6). Trends for obesity prevalence since the 1970s are alarming, more than doubling from 1975 to 2004, and recent data suggest that the problem continued to worsen in 2009, with a projected 17.6% of the gross domestic product devoted to health spending (7, 8). The search for biological processes that affect adiposity has revealed contributions from the neurohormonal axis, ligand-receptor signaling pathways, and regulators of energy homeostasis, cell differentiation, and classical metabolic pathways (9–12), yet almost all current antiobesity drugs function in the brain as appetite suppressants. However, efficacy of these drugs is limited, and they can have a variety of undesirable side effects (13–15). This review focuses on recent discoveries in the TGF-β field that relate to the control of adipocyte differentiation and function, as well as effects on adiposity in animal models, and it highlights the clinical relevance in this regard in humans. These studies have broadened our awareness of TGF-β superfamily signaling as an important contributor to the regulation of lean *vs.* fat body mass and as a possible target for the treatment of human obesity.

II. TGF-β Signal Transduction

TGF-β superfamily signaling is well known as a key regulator of many biological processes. The 33 members of the mammalian superfamily use many of the same intermediates to exert a range of biological effects on target cells and tissues. In the canonical pathway, TGF-β ligands bind to their receptors and ultimately activate Smad proteins, which participate in transcriptional regulation (Fig. 1). The process is regulated on several levels to function

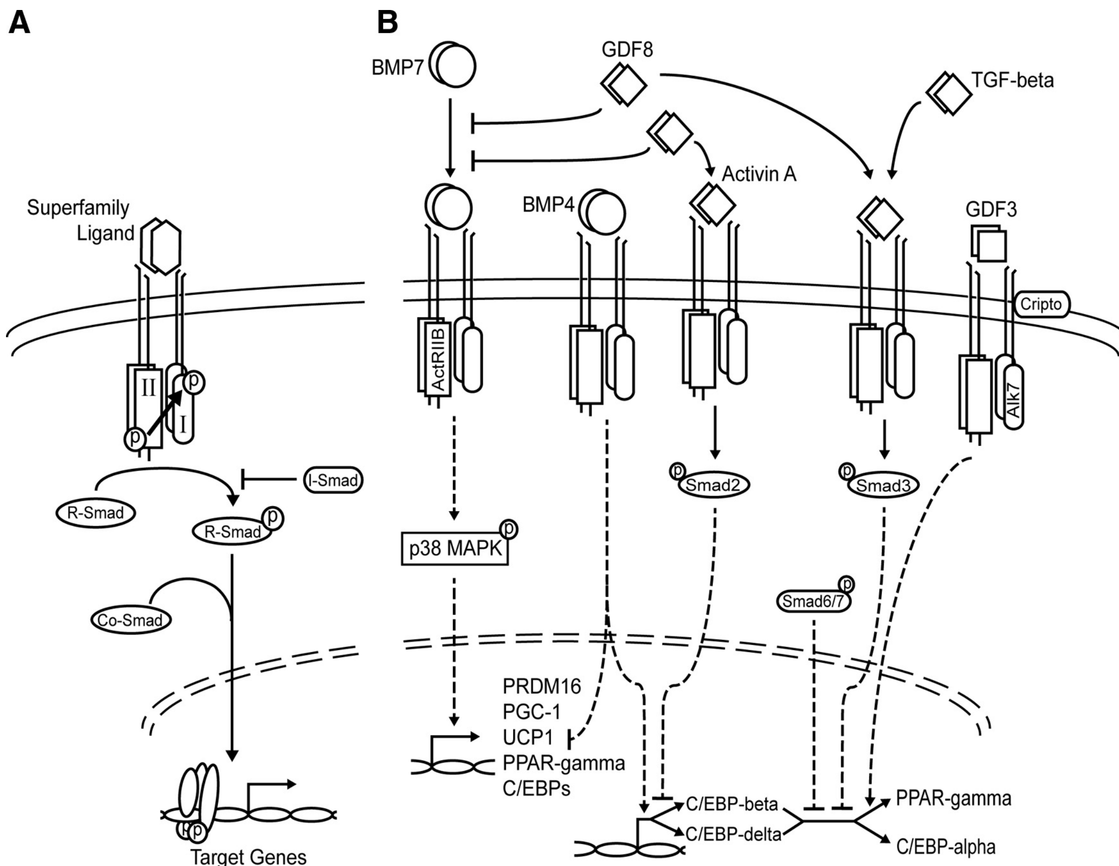

FIG. 1. TGF-β superfamily signaling. A, A superfamily dimer associates with type I and type II receptors, followed by receptor autophosphorylation (*diagonal arrow*). Subsequently, a R-Smad (Smads 1/5/8 for BMP signaling and Smads 2/3 for TGF-β/activin/GDF8 signaling) is phosphorylated and associates with Smad4 (Co-Smad, common to all Smad complexes). Receptor signaling induces an equilibrium shift of Smad complexes to the nucleus, resulting in direct effects on the expression of up to hundreds of target genes. Inhibitory Smads (I-Smads, Smads 6/7) block the association of R-Smads with Co-Smads or target ligand/receptor complexes for degradation, thereby antagonizing downstream signaling. B, Many superfamily members contribute to adipogenesis or mature adipocyte function. BMP7 is antagonized by activin A and GDF8—the latter competes with BMP7 for ActRIIB. p38 MAPK is activated by non-Smad-mediated (noncanonical) BMP7 signaling, and participates in brown adipogenesis, inducing the transcription of several genes important for mitochondrial biogenesis and function. BMP4 signaling is antagonistic to this effect by decreasing *Ucp1* expression while simultaneously supporting white adipocyte differentiation. In mature adipocytes, GDF3 signaling is likely mediated by Alk7, ActRIIB, and the EGF-CFC co-receptor, Cripto, and enhances the expression of PPAR-γ. The inhibitory role of GDF8 and TGF-β during white adipogenesis is mediated by Smad3 and is complemented by the actions of Smads 6 and 7. The *dashed lines* represent signaling events in which not all intermediates are shown.

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effectively in a variety of physiological networks. Recent excellent reviews and texts detail canonical and noncanonical TGF-β superfamily signaling and highlight the diversity of its downstream effects (16–19).

A. Ligands

TGF-β superfamily ligands include activins, inhibins, bone morphogenetic proteins (BMPs), Nodal, growth differentiation factors (GDFs), leftys, the TGF-β isoforms, and anti-Müllerian hormone (20). The ligands are synthesized as large, prepropeptides consisting of an amino-terminal signal peptide, a propeptide that is ultimately cleaved, and a mature peptide. Homo- or heterodimerization of mature peptides usually results in the production of tightly regulated signaling molecules that are released from the cell. Some enter the circulation and can function in an endocrine, autocrine, or paracrine fashion (17, 21). Dimerization occurs by the formation of intermolecular disulfide bonds through the interaction of the fourth of seven conserved cysteine residues found in almost all of the mature ligands (22–24). Five members, however, are missing this residue: BMP15, GDF3, GDF9, Lefty1, and Lefty2.

Functionally, the ligands are commonly divided into two main branches based on the receptors with which they interact and the Smad proteins that mediate their signals. In general, the TGF-β/activin/Nodal branch initiates transcription through activation of Smads 2/3, whereas the BMP branch activates Smads 1/5/8. Specific receptor interactions have not been definitively determined for many of the remaining superfamily ligands. Although useful, this classification does not entirely capture the complexity of combinatorial signaling in which ligands can signal through more than one receptor combination, sometimes activating different Smads in different cellular contexts.

B. Receptors

The receptors are transmembrane serine/threonine kinase glycoproteins that are divided into two groups: type I receptors that contain a unique GS domain preceding the kinase domain, and type II receptors (25). Although both branches of TGF-β family signaling require type I and type II receptors to initiate signal transduction, the order in which they do so differs. Ligands that use the branch that signals through Smads 2/3 (TGF-β/activin/Nodal) must first bind type II receptors, which subsequently recruit and activate the appropriate type I receptor by phosphorylation. This ternary complex can then activate either Smad2 or Smad3. Some BMPs, in contrast, can initially bind to either type I or type II receptors, but the formation of the ternary complex is still essential for the activation of the appropriate downstream regulatory Smads [R-Smads, referring to Smads 1/2/3/5/8 (16)].

An important characteristic of TGF-β superfamily ligands is their ability to bind more than one receptor combination, with each receptor having different binding domains (26, 27). This promiscuity is similarly present with the association of type II and type I receptors, allowing for the five type II and seven type I receptors to mediate the signaling of a relatively large group of ligands.

C. Signaling pathways

Once the appropriate R-Smad is phosphorylated by an activated type I receptor, it associates with the common Smad, Smad4 (Co-Smad). This complex shifts its equilibrium from the cytoplasm to favor the nucleus to drive or inhibit the transcription of downstream target genes (28) (Fig. 1). Accumulation of the R-Smad-Smad4 complex within the nucleus is regulated in part by TAZ, an essential nuclear transporter (29). After transcription, signaling is terminated by nuclear phosphatases, including PPM1a, that dephosphorylate R-Smads and subsequently result in the dissociation of heteromeric transcriptional complexes, allowing the Smads to be recycled to the cytoplasm (30).

Regulation of superfamily signaling occurs at several levels. Extracellular proteins (follistatin, follistatin-like family members, and others) have the ability to either compete for receptors or bind directly to TGF-β ligands, and a variety of intracellular regulatory proteins have also been described (31–35). Inhibitory Smads within the cell also antagonize superfamily signaling. Smad6, for example, inhibits BMP signaling (36), whereas Smad7 can inhibit both branches by interacting with intracellular domains of type I receptors (37).

In addition to the Smads, other noncanonical signaling pathways can be activated by TGF-β superfamily signaling. Activation of type I and II receptors has been associated with the activation of the Wnt signaling pathway (38), Erk (39), JNK (40), and p38 MAPK (41), indicating that superfamily signaling may have direct effects on growth and energy metabolism.

III. Roles of Superfamily Signaling in Adipogenesis and Adipocyte Function

A. Adipocyte differentiation

There are generally two types of adipose tissue, which have shared and distinct functional characteristics. Depending on energy balance and immediate physiological needs, white adipose tissue (WAT) specializes in energy storage and mobilization. The production of leptin and other adipokines by a variety of cell types within WAT also helps to regulate food intake, energy substrate metabolism, and metabolic rate, three important aspects of energy balance (reviewed in Ref. 42). Brown adipose tissue

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(BAT), in contrast, stores fewer lipids and contains a high density of mitochondria. The mitochondria in BAT contain high levels of uncoupling protein 1 (UCP1), a mitochondrial membrane protein that allows for the dissipation of the proton gradient as heat. As such, the primary functions of brown adipocytes are basal and adaptive thermogenesis as well as energy expenditure.

Adipocyte development *in vitro* is characterized by a multistep process whereby multipotent mesenchymal stem cells (MSCs) are progressively determined, then committed to the adipocyte lineage, and ultimately differentiate into mature adipocytes (reviewed in Ref. 43). Undifferentiated MSCs have the capacity to develop into several mesodermal cell types: osteoblasts, chondrocytes, myoblasts, and adipocytes (44–46). For the development of terminally differentiated adipocytes, the MSCs must first progress to the committed preadipocyte stage, a process that can be strongly influenced by BMP signaling (47–49), the hypomethylation of presumably lineage-specific gene(s) (45), and cell shape (50). The committed preadipocyte population proliferates to confluence, resulting in growth arrest and subsequent mitotic clonal expansion, followed by a second growth arrest stage and terminal adipocyte differentiation.

Throughout adipocyte differentiation, temporally precise transcriptional events are required (reviewed in Ref. 43). Early differentiation is initiated when hormonal cues stimulate the production of CCAAT/enhancer binding proteins (C/EBP)-β and -δ (encoded by *Cebpb* and *Cebpd*, respectively) (51). The subsequent expression of peroxisome proliferator-activated receptor (PPAR)-γ (*Pparg*) and C/EBP-α (*Cebpa*) ends mitotic clonal expansion and is followed by a second growth arrest and terminal differentiation, characterized by the expression of FABP4/aP2 (hereafter, FABP4), leptin, and several other markers (52–55). The transition from mitotic clonal expansion to terminal adipocyte differentiation is marked by the activation of FoxO1 (56), which then activates the cell cycle inhibitor p21 (57) and results in the second growth arrest phase, allowing for terminal differentiation.

Much progress has been made toward understanding the developmental origins of brown and white adipocytes, although all aspects have not been resolved. It is clear that MSCs have the capacity to produce both cell types, and recent models suggest that white and brown adipocytes within WAT differentiate from a common $Myf5^{-}$ progenitor, whereas brown adipocytes within BAT arise from a distinct, myogenic/adipogenic $Myf5^{+}$ progenitor (58–60) (Fig. 2). Consistent with the common progenitor model in WAT is the white-to-brown phenotype conversion caused by transgenic overexpression of aP2-FoxC2 in mouse adipose tissues, with the corresponding increase in the expression of adipocyte differentiation markers, *Pparg* and *Cebpa*, as well as several markers characteristically expressed in brown adipocytes (61). However, the morphologies and numbers of adipocytes within WAT in response to cold exposure or pharmacological β3 adrenergic stimulation suggest that mature white and brown adipocytes may be capable of transdifferentiation (62–64). Considering the complexity of this developmental system, it is not surprising that many TGF-β ligands have mixed effects on adipogenesis under a variety of experimental conditions (Table 1).

B. Members with unidirectional effects on adipogenesis

1. BMP4 promotes MSC commitment to the adipocyte lineage

Although previous work confirms dual roles for many BMPs (BMP2, -4, -6, -7, and -9) in regulating adipogenesis and osteogenesis (65, 66), BMP4 is mostly recognized for its proadipogenic influence. In mouse embryonic stem cells, BMP4 can induce adipocyte formation in a dose-dependent manner in the absence of standard adipogenic “cocktails” (67, 68). Its role in directing commitment to the adipocyte lineage was also demonstrated by treating multipotent C3H10T1/2 cells with exogenous BMP4 (47). By treating these cells during the early proliferative stage, a high frequency of MSCs ultimately differentiate into adipocytes, and sc injection of these cells into athymic mice results in the development of adipose tissue (47). Not only does BMP4 support white adipogenesis, but it also functions in brown adipogenesis to decrease the expression of *UcpI*, a mitochondrial marker of brown adipocytes (49).

Additional evidence for BMP4’s role in directing commitment to the adipocyte lineage is provided by comparing gene expression profiles of the cell lines C3H10T1/2 and A33, the latter a committed preadipocyte line derived from C3H10T1/2 cells in which the timing of BMP4 peak expression is earlier than normal (48). During proliferation, C3H10T1/2 cells normally have very low *Bmp4* mRNA levels, which subsequently increase as the cells approach confluence. These cells differentiate poorly ($<$10%) under standard adipogenic conditions. In contrast, in A33 cells, *Bmp4* mRNA and protein levels, as well as the phosphorylation of downstream Smads 1/5/8, peak during early proliferation and markedly decrease as the cells reach confluence and growth arrest (69). In contrast to their parent cell line, A33 cells achieve approximately 90% differentiation under standard adipogenic conditions. Moreover, treatment of A33 cells with noggin, a BMP inhibitor (70), decreases cytoplasmic triglyceride accumulation and *Pparg* expression, indicating that the timing of BMP4 expression is important for commitment to the adipocyte lineage in C3H10T1/2 cells (48).

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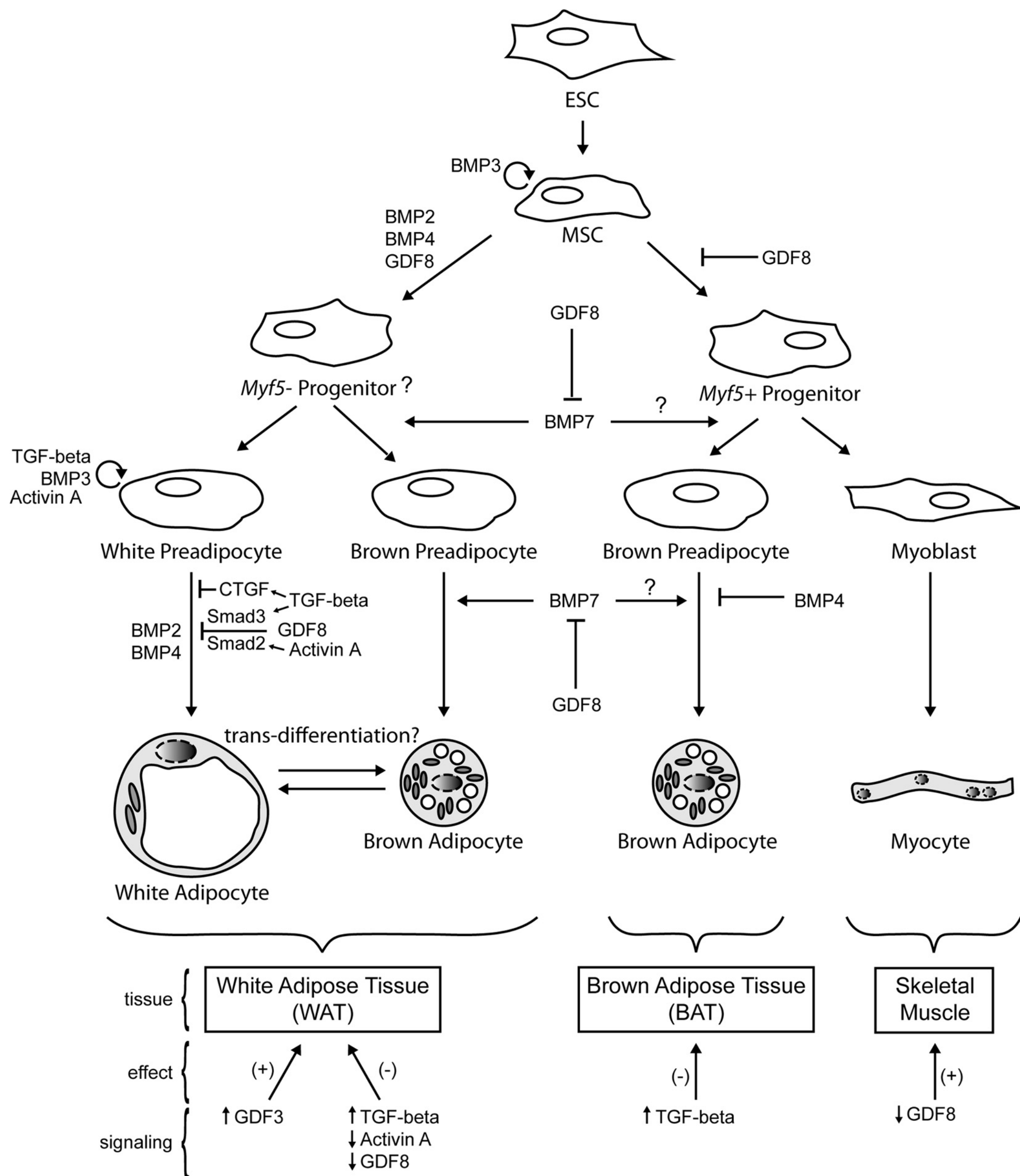

FIG. 2. Established contributions of TGF-β superfamily members to adipogenesis. MSCs have the capacity to differentiate into different cell types, including adipocytes and myocytes. Under normal conditions, WAT contains mostly white adipocytes and a relatively small number of brown adipocytes. The brown adipocytes in WAT (*left*) may differentiate from a Myf5$^-$ precursor, although the presence of this cell type within WAT (with the capacity to differentiate into either white or brown adipocytes) has not been definitively established. In addition, mature white adipocytes may have the ability to reversibly transdifferentiate into cells that have many of the characteristics of brown adipocytes. Although BMP3 has been shown to stimulate the proliferation of MSCs and preadipocytes, TGF-β and activin A enhance the proliferation of adipocyte precursors while also inhibiting their subsequent differentiation. BMP2 and BMP4 primarily support white adipocyte differentiation, whereas BMP7 has emerged as a factor that promotes brown adipogenesis, although the precise mechanisms in some contexts have not been resolved. The MSCs can alternatively give rise to a Myf5$^+$ progenitor that has the ability to differentiate into either myocytes or brown adipocytes (*right*). Brown adipocytes arising from Myf5$^+$ progenitors are predominantly located in brown adipose depots. Increased or decreased signaling by superfamily ligands (see "signaling" at the *bottom of the figure*), *in vivo*, can have stimulatory or inhibitory effects ("effect") on adipose and muscle mass ("tissue"). Proteins with positive effects on adipocyte differentiation or function are associated with *pointed arrows*, whereas those with negative/inhibitory effects are indicated by *blunt arrows*. *Question marks* denote processes or cell types for which there is supporting evidence, but that have not been unequivocally established. ESC, Embryonic stem cell.

2. BMP7 preferentially supports brown adipogenesis

BMP7 is involved in adipogenesis in a variety of cell types and animal models. In mouse bone marrow stromal cells, low concentrations of BMP7 stimulate adipocyte differentiation (71). BMP7 also initiates adipogenesis in high-density micromass cultures of adult human MSCs, a system that usually promotes chondrogenic, rather than adipogenic, differentiation (72). This proadipogenic effect includes increased expression of adipocyte markers, *FABP4* and *APM1*. BMP7 has been implicated in supporting brown adipogenesis while preventing osteogenesis by inhibiting the expression of runt-related transcription factor 2

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TABLE 1. Direct roles of TGF-β superfamily members in regulating adipogenesis and adiposity

Family member	Cell type/model	Effect on adipogenesis or adiposity	Ref.
BMP2	C3H10T1/2	Induces adipogenesis by increasing *Pparg* expression	96
	BMS2	Inhibits adipogenesis and induces *Alp* expression	87
	hMSC	Reduces leptin and lipid accumulation; increases ALP secretion	88
	Primary MEFs	Induces full adipogenic differentiation	163
	3T3-F442A	Represses adipocyte development; promotes osteoblast differentiation	90
	3T3-L1	Stimulates adipogenic differentiation in the presence of PPAR-γ agonists	95
	2T3	Induces adipogenesis by increasing *Pparg* expression via BMPR1A (ALK3)	92
BMP3	C3H10T1/2	Enhances proliferation via Smad3 stimulation	79
	3T3-L1	Enhances proliferation	79
BMP4	mESC	Promotes adipogenesis	68
	Chick ESC	Inhibits myogenesis in the presence of conditioned medium from MyoD-expressing cells	164
	C3H10T1/2	Induces adipocyte lineage commitment	47
	A33	Altered endogenous BMP4 expression confers preadipocyte phenotype	48
	Brown preadipocytes	Suppresses *UcpI* expression	49
BMP7	C3H10T1/2	Increases differentiation into brown adipocytes; up-regulates *Ucp1* expression	49
	BMS2	Stimulates adipocyte differentiation at low concentrations	71
	hMSC	Promotes adipogenic differentiation—increases *FABP4* and *APM1* expression	72
	3T3-L1	Stimulates Smads 1/5/8, but fails to induce differentiation	49
	Brown preadipocytes	Increases adipogenic markers, increases mitochondrial biogenesis, and suppresses adipogenic inhibitors	49
GDF3	P19	Inhibits BMP4 signaling	109
	3T3-L1	Increases *Pparg* expression in differentiated cells	104
	Human primary adipocytes	Increases *PPARG* expression	104
	Mouse models	GDF3 adenovirus enhances sensitivity to HFD	104
		KO: increases basal metabolic rate and confers protection against diet-induced obesity—selective effects on gene expression in white adipose	107, 113
GDF8/myostatin	C3H10T1/2	Promotes adipogenesis and inhibits myogenesis	97, 98
		Competitively inhibits BMP7 signaling	94
	hMSC	Suppresses transcriptional regulators and adipocyte markers	101
	3T3-L1	Inhibits preadipocyte differentiation	103
	Mouse models	Overexpression in adipose tissue: provides resistance to diet-induced obesity	98
		Overexpression in skeletal muscle of male mice: decreases skeletal muscle mass; increases adipose tissue mass	131
		Systemic overexpression: causes severe cachexia and loss of WAT	132
		KO: increases skeletal muscle mass, decreases adipose tissue mass	99, 100
Activins	hMADS	Activin A promotes proliferation and inhibits Smad2-mediated differentiation	84
	3T3-L1	Activin A inhibits differentiation—reduces *Pparg* and *Cebpa* expression	85
		Activin B inhibits lipolysis	146
	Mouse models	*Inhbb* insertion allele in the *Inhba* locus decreases WAT and increases mitochondrial biogenesis and function	117
TGF-β	hMSC	Prevents preadipocyte differentiation through cooperation with the Wnt signaling pathway	82
	NIH3T3	Represses the activity of C/EBP-β and -δ	80
	3T3-L1	Prevents differentiation to mature adipocytes	75
	3T3-F442A	Stimulates proliferation of preadipocytes	76, 77
		Decreases *Pparg* and *Cebpa* expression	76
	Mouse models	Overexpression decreases WAT and BAT	137
	Porcine adipose tissue	Decreases lipid accumulation	78

BMS2, Mouse marrow stromal cell line; hMSC, human marrow stromal cell line; MEFs, mouse embryo fibroblasts; KO, knockout; hMADS, human multipotent adipose-derived stem cells.

(*Runx2*) (49). In C3H/10T1/2 cells, pretreatment with BMP7 results in brown adipogenesis with lipid accrual and expression of *Ucp1* (49).

Although BMP7 increases phosphorylation of Smads 1/5/8 in 3T3-L1 cells and brown preadipocytes, enhanced phosphorylation of p38 MAPK and ATF2, as well as increased expression of several genes that participate in mitochondrial biogenesis and function, are only observed in brown preadipocytes (49). BMP7 also has a substantial effect on the adipogenic cascade, suppressing the expression of adipogenic inhibitors nectin, preadipocyte factor-1 (*Pref-1*), and *Wnt10a*, while up-regulating *Pparg*, *Cebpa*, *Fabp4*, and *Prdm16*, an early marker of brown adipocytes (59). Moreover, the impaired differentiation of insulin re-

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ceptor substrate-1-deficient brown preadipocytes can be overcome by treatment with BMP7, which positively affects the expression of insulin signaling components inhibiting the expression of *Pref-1* through the direct interaction of Smad1/4 complexes with the *Pref-1* promoter (73). Thus, BMP7 promotes brown adipogenesis in MSCs as well as committed brown preadipocytes. This provides a transcriptional environment that suppresses adipogenic inhibition and augments the expression of genes that promote the phenotypic characteristics and functional activity of mature brown adipocytes while potentially improving insulin sensitivity.

3. TGF-β is a negative regulator of adipogenesis

Within the context of adipogenesis, TGF-β1 has been the most extensively studied family member. In contrast to other ligands that have proadipogenic effects, TGF-β's influence is mostly inhibitory. Although an early study of rat brown adipocytes showed that TGF-β induces the expression of lipogenic enzymes (74), other studies show that it inhibits the early stages of 3T3-L1 differentiation into mature adipocytes, a property that is restricted to the first 35–40 h after onset of differentiation (75) because the availability of TGF-β type I and II receptors decreases after induction (76). TGF-β also increases proliferation of 3T3-F442A preadipocytes (76, 77), promoting an increase in the progenitor population while simultaneously inhibiting their differentiation (17). Experiments with primary cultures from sc porcine adipose tissue further show that TGF-β inhibits lipid accumulation (78). Similarly, BMP3 increases proliferation, but neither the commitment nor the differentiation of MSCs or committed 3T3-L1 preadipocytes (79).

In an attempt to identify the mechanism of TGF-β's action, studies have aimed to determine the relevant downstream targets of TGF-β signaling. Although overexpression of Smads 3, 6, or 7 all enhance TGF-β-induced proliferation of 3T3-F442A preadipocytes, only Smad3 overexpression exhibits a similar trend in the absence of TGF-β, indicating that the mechanism by which Smad3 mediates TGF-β activity is different than that of the inhibitory Smads 6 and 7 (76). Because Smads 6 and 7 normally block canonical TGF-β superfamily signaling, it is likely that they enhance the inhibitory effect of TGF-β on adipogenesis by reducing the signaling of other superfamily ligands (perhaps those of the Smads1/5/8 branch) with a net negative effect on adipogenesis.

The mechanism of Smad3 inhibition of adipogenesis was further elucidated through observations that TGF-β treatment of 3T3-F442A cells decreases expression of *Pparg* and *Cebpa* (76) by functionally repressing their upstream factors, *Cebpb* and *Cebpd* (80). Smads 3/4 physically interact with the two upstream proteins and disrupt their function, thereby preventing the subsequent transcription of downstream targets essential for adipogenesis (80).

Connective tissue growth factor (CTGF) is a downstream mediator of TGF-β1 signaling in many cell types, whose mRNA levels increase in response to TGF-β in 3T3-L1 preadipocytes (81). Similar to TGF-β, CTGF has antiadipogenic effects in these cells and in primary adipocytes, and it may also be an important mediator of cell fate decisions because it also promotes osteogenesis *in vitro*. The mechanism of inhibition is thought to include effects on the nuclear localization of C/EBP-β, with consequential reduction of *Cebpa* transcription (81). In human MSCs, Smad3-mediated inhibition by TGF-β also involves the up-regulation of genes from the Wnt signaling pathway (82), a cascade that also inhibits adipocyte differentiation (83). Thus, several processes working in concert appear to mediate TGF-β's inhibitory role in adipogenesis.

4. Activin A promotes proliferation but inhibits differentiation of human preadipocytes

Activin A is expressed in human preadipocytes, and its levels can be increased by secreted factors from macrophages isolated from WATs or decreased by dexamethasone (84). Similar to TGF-β, the roles of activin A in adipogenesis are mostly inhibitory, with positive effects on the proliferation of human adipocyte precursors, but negative effects on their subsequent differentiation (84). Upon induction of differentiation, activin A levels rapidly decline. This is supported in other cellular models in which activin A treatment of 3T3-L1 cells inhibited differentiation, reflected by reduced expression of *Pparg* and *Cebpa* (85). In contrast to TGF-β, however, the inhibition of differentiation by activin A is mediated by Smad2 and C/EBP-β, rather than Smad3 (84), suggesting that the inhibition of adipogenesis by TGF-β superfamily ligands exhibits selectivity for R-Smads, even within the same branch of signaling, similar to the recent observation of Smad3 selectivity in developing Sertoli cells (86). As such, it would be interesting to determine whether the inhibitory effects of activin A on adipogenesis are unique to Smad2 or whether Smad3 is also a contributing mediator.

C. Members with mixed effects on adipogenesis

1. BMP2 effects on adipogenesis depend on the cell type and culture conditions

Although some members of the TGF-β superfamily have clearly been implicated as pro- or antiadipogenic, others exhibit mixed effects. BMP2, for example, induces the differentiation of MSCs into osteoblasts, chondrocytes, and adipocytes (65). In murine and conditionally immortalized human bone marrow stromal cell lines, BMP2 not only inhibits adipocyte differentiation and mat-

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uration, but also promotes the expression of alkaline phosphatase (*Alp*), a marker for early osteoblast differentiation (87, 88). BMP2 also enhances osteoblast lineage commitment by increasing *Osf2/Cbfa1*, an osteoblast marker (89), while decreasing leptin (*LEP*) and lipid accumulation (88). As such, BMP2 directs these uncommitted precursor cells toward the osteoblast lineage. Similarly, BMP2 cooperates with retinoic acid in 3T3-F442A preadipocytes in a manner that promotes osteoblast differentiation while suppressing adipocyte development (90). Because BMP2 activates BMPR1A (ALK3) and BMPR1B (ALK6) (91), constitutive expression of either activated receptor in conjunction with BMPRII also inhibits adipocyte differentiation, similar to exogenous BMP2 treatment (90).

In contrast, a study in 2T3 cells concluded that although BMP2 has the ability to induce osteogenesis, it can also signal through ALK3 to stimulate adipocyte differentiation by increasing expression of *Pparg* (92). Consistent with this observation, microarray data from A33 preadipocytes show an approximate 2-fold increase in *Alk3* mRNA when compared with C3H10T1/2 cells, whereas *Alk6* was not detected in the cells (48). Although these observations are consistent with a model in which ALK3 promotes adipogenesis whereas ALK6 is coupled to osteogenesis, it seems that BMP-induced differentiation is cell type dependent. In C3H10T1/2 cells, for example, the constitutive expression of ALK3 or ALK6 induces adipocyte commitment in the absence of either BMP2 or BMP4, demonstrated by the accumulation of lipid and expression of *Fabp4* (93).

BMP2 also stimulates adipogenesis in C3H10T1/2 and 3T3-L1 cells (94). Although it was initially shown that BMP2 only stimulates adipogenesis in the presence of PPAR-γ agonists in 3T3-L1 cells (95), others have demonstrated that BMP2 alone is sufficient to increase adipogenesis in C3H10T1/2 cells by inducing *Pparg* expression (96). In this context, BMP2 simultaneously activates Smad1 and p38 MAPK signaling, which in turn induce and up-regulate *Pparg* expression (96). Although BMP2 induces the phosphorylation of Smads 1/5/8 as well as p38 MAPK, disruption of Smad activity through Smad4-directed RNA interference blocks commitment and differentiation of MSCs into adipocytes, whereas knockdown of p38 MAPK only partially disrupts the preadipocyte phenotype (93). Therefore, of the two pathways activated by BMP2 in C3H10T1/2 cells, BMP/Smad signaling has the greater influence on adipocyte lineage determination.

2. GDF8 affects early commitment in mice but prevents differentiation of human cell lines

GDF8 (myostatin) also promotes adipogenesis in C3H10T1/2 cells while inhibiting myogenesis (97, 98). These findings are consistent with observations in *Gdf8*-null mice, which have markedly increased skeletal muscle mass (99, 100). However, adipocytes from GDF8-treated MSCs are smaller than wild type, with a gene expression profile consistent with immature adipocytes (98).

In contrast, GDF8 inhibits differentiation of human MSCs where it has a dose-dependent negative effect on lipid accumulation, down-regulating the expression of *PPARG*, *CEBPA*, *LEP*, and *FABP4* (101). Because *CEBPB* expression is not affected, and considering that it is required for mitotic clonal expansion (102), GDF8's inhibition of adipogenesis is likely to occur after mitotic clonal expansion and during subsequent early events of differentiation. This inhibitory effect, similar to that exerted by TGF-β (80), is Smad3-dependent and also involves Smad3 interactions with transducers of canonical Wnt signaling (101).

These findings in human cells correlate with GDF8's inhibition of differentiation in 3T3-L1 cells (103), although the expression of *Lep* in GDF8-treated 3T3-L1 cultures is unchanged. This may indicate either that GDF8 plays a more significant role as a modulator of energy homeostasis in humans than in mice or that the leptin response differs between the cell types. Furthermore, the observation that GDF8 can induce adipogenesis in murine MSCs but not in committed 3T3-L1 cells suggests that GDF8's effects are sensitive to the stage of adipocyte development.

GDF8 has significant effects on the signaling of other superfamily members. Through a direct interaction with activin type IIb receptor (ActRIIB) in MSCs, GDF8 can compete with BMP7 to block adipogenesis and the subsequent expression of late differentiation markers (94). Moreover, because the BMP inhibitory effect of GDF8 seems to be selective for BMP7, GDF8 may also negatively regulate brown adipose development.

3. Growth differentiation factor 3 (GDF3)

GDF3 increases *Pparg* expression in fully differentiated 3T3-L1 cells and primary cultures of human adipocytes while having no effect on undifferentiated preadipocytes (104). GDF3 acts in an activin/Nodal-like signaling pathway using ALK4 or ALK7, ActRIIB, and the co-ligand/receptor Cripto (105–107), with ALK7 serving as a type I receptor in mature adipocytes (107, 108). *Alk7*-null mice phenocopy the protection from diet-induced obesity and the decrease in adipocyte size observed in *Gdf3*-null mouse models (see *Section IV*), implicating Alk7 as a type I receptor for GDF3 in adipocytes *in vivo* (107). Others have shown in other contexts that GDF3 inhibits BMP4 *in vitro*, suggesting that GDF3 might also function by inhibiting Smads 1/5/8-mediated BMP signaling (109, 110). Considering the dual roles of GDF8 in selectively inhib-

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iting BMPs, while concurrently activating the Smad 2/3 branch of TGF-β signaling, GDF3-mediated BMP inhibition may similarly prove to be an important mechanism in the control of adipocyte differentiation and function.

IV. TGF-β Superfamily Signaling in the Regulation of Adiposity

A. BMPs contribute to adipocyte differentiation and energy expenditure

Coupled to BMP7's role as a promoter of brown adipogenesis is its effects on energy expenditure. Tail vein injection of adenovirus expressing BMP7 increases BAT, without affecting the mass of WAT (49). Although BMP7 increases the expression of *Prdm16* and *Ucp1* in brown adipose, there are no changes in the expression of genes involved in energy metabolism in white adipose, muscle, or liver. The increase in BAT mass results in increased energy expenditure, higher basal body temperature, and decreased body weight—attributes that clearly link BMP7 signaling to energy balance. Furthermore, sc implantation of BMP7-treated MSCs into athymic mice results in ectopic brown adipose formation. Conversely, *Bmp7*-null mice fail to properly develop BAT (49).

Adipogenic effects of BMP2 are mediated by the transcriptional coactivator Schnurri-2 (Shn-2) (111). After BMP2-mediated Smad1 activation, Shn-2 is recruited into the nucleus and assembles at the *Pparg* promoter with the Smad1/4 complex and is required for efficient transcription of *Pparg* in cooperation with C/EBP-α. Consistent with this role, *Shn2*-null mice (with consequentially reduced BMP2 signaling) have reduced adiposity, and embryo fibroblasts derived from these mice differentiate poorly into adipocytes (111).

B. GDF3 affects susceptibility to diet-induced obesity

GDF3 is expressed in adipose tissue (112, 113) and is up-regulated selectively in white adipose by high-fat diet (HFD) conditions (113). Adenoviral transfer of GDF3 renders mice more susceptible to the adipogenic effects of HFD but has no effect under regular diet conditions (104). Conversely, *Gdf3*-null mice are protected from diet-induced obesity (107, 113), accumulating less white adipose than wild-type mice under HFD conditions, but displaying no significant difference in body weight with regular diet (113). The protection from obesity is not due to decreased food intake, malabsorption, or physical activity, but rather is due to a higher basal metabolic rate, which is further increased by HFD conditions (113). Corresponding to the higher metabolic rates of *Gdf3*-null mice, genes that are important for mitochondrial biogenesis and function are selectively up-regulated in WAT (113). The gene expression profile is more reminiscent of BAT, implicating GDF3 as a potential mediator between the two lineages, and suggesting one possible mechanism for GDF3's effects on adiposity. Because these effects are only observed under HFD conditions, GDF3 may also provide an important link between the nutrient environment and downstream processes that affect adiposity, including the metabolic rate.

C. Activins influence mitochondrial biogenesis and function

The activin subgroup is comprised of four mammalian superfamily members, activins β A, B, C, and E. Activins A, B, or AB are comprised of two closely related β A or β B subunits (encoded by *Inhba* and *Inhbb* genes, respectively), that form homo- or heterodimers. Unlike *INHBA* which is down-regulated during the induction of adipocyte differentiation (84), *INHBB* levels are high in human adipocytes (114, 115). Mice were generated that contain an *Inhbb* insertion allele at the *Inhba* locus (116). Designated *Inhba*^{BK}, this allele allows activin B to assume the spatiotemporal expression of activin A. When compared with wild type, *Inhba*^{BK} mice have much less WAT, which contains small, immature adipocytes, most abundant in visceral depots (117). Moreover, the basal metabolic rates of *Inhba*^{BK} mice are markedly elevated, with corresponding up-regulation of genes involved in mitochondrial biogenesis and function in several tissues and an increase in the basal level of mitochondrial uncoupling (117). Nevertheless, HFD is sufficient to restore body weight to normal levels, whereas diet-induced obesity and glucose intolerance do not develop (117). Thus, activin signaling plays an important role in the regulation of mitochondrial energy expenditure, with consequential effects on adiposity in addition to its autocrine/paracrine effects on adipocyte differentiation and function.

D. GDF8 affects adiposity by more than one mechanism

GDF8 is a negative regulator of skeletal muscle growth because GDF8 deficiency causes increased muscle mass in several species (118–124). However, *Gdf8*-null mice also have reduced adiposity, with decreased adipocyte cell size and number despite normal body temperatures, normal food intake, and lower metabolic rates than wild-type controls (99, 100). These characteristics may indicate that the decrease in adipose tissue is a secondary consequence of muscle hypertrophy, a contention that is supported by other mouse models of increased muscle mass (125–127). Supporting an indirect mechanism for regulating adiposity, the inhibition of GDF8 signaling in skeletal muscle of transgenic male mice containing a muscle-specific dominant negative ActRIIB receptor resulted in a similar phenotype to GDF8 knockout mice, whereas inhibition of

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GDF8 signaling in only adipose tissue did not affect body composition (128). This indicates that the regulation of adiposity in GDF8 null models is likely to be secondary to the metabolic changes associated with increased skeletal muscle mass. Gene expression and proteomic profiles of skeletal muscle from *Gdf8*-null mice show changes in genes encoding glycolytic enzymes and mitochondrial proteins, reflecting a metabolic shift in favor of glycolysis (129). Thus, failure of fat accumulation in *Gdf8*-null mice may result from a shift in skeletal muscle glucose metabolism with global effects on energy storage. However, GDF8's roles in metabolism are likely to be multifaceted, as demonstrated by overexpression models.

Gdf8 overexpression in mouse adipose tissue not only decreases adipocyte size, but also confers resistance to diet-induced obesity, consistent with its antiadipogenic roles *in vitro* (94, 98, 130). In contrast, *Gdf8* overexpression in skeletal muscle results in larger epididymal fat pads (131), the reciprocal phenotype of muscle-specific inactivation. Systemic overexpression, however, achieved by im injection of GDF8-producing CHO cells, results in reduced skeletal muscle mass and a nearly complete loss of WAT (132). Similarly, sc injection of GDF8 significantly reduces adiposity, but does not affect muscle mass or total body weight, possibly due to differences in the levels of bioactive protein in the two models (132). Although the effects on skeletal muscle might have been predicted with CHO cell overexpression, the repressive effects on adipose cannot be easily reconciled with the "glycolytic shift" model detailed above and suggest that more than one mechanism mediates GDF8's effects on adiposity. Direct (GDF8 signaling) or indirect (BMP inhibition) effects on adipocyte differentiation or function are potential mechanisms that are consistent with the observations from systemic overexpression models.

E. Follistatin and follistatin-like expression correlate with obesity and adipogenesis

The properties of some activin and GDF8 binding proteins implicate them as possible contributors to the regulation of adiposity. Genetic (*ob/ob*) and diet-induced obesity mouse models exhibit an increase in sc and decrease in visceral adipose expression of follistatin-like 3 (*Fstl3*) relative to regular diet controls (133). Similarly, follistatin (*Fst*) mRNA displays adipose depot-selective expression characteristics in obese women; however, in contrast to *Fstl3*, *Fst* mRNA levels are diminished in sc but not in visceral adipose depots of these individuals. Follistatin is also a proadipogenic factor in human bone marrow-derived MSCs and sc WAT-derived preadipocytes (134). In contrast, expression of follistatin-like 1 (Fstl-1) protein, which occurs at high levels in undifferentiated 3T3-L1 cells and primary preadipocytes, declines rapidly during adipocyte differentiation (135).

Fstl3 knockout mice exhibit no significant effects on muscle mass, but instead display reduced adiposity that is selective for visceral fat depots, presumably due to alternative distribution of lipids to other fat depots and the liver because total body fat content is not different from controls (136). This may be due in part to the stimulatory effects of the mutation on pancreatic islet development and consequential effects on insulin production and nutrient metabolism.

F. TGF-β is increased in obesity, yet overexpression blocks adipogenesis

Despite its inhibitory effect on adipogenesis *in vitro*, conflicting reports characterize the effects of TGF-β (*Tgfb1*) *in vivo*. Consistent with *in vitro* studies, constitutively overexpressing an active human *TGFB1* transgene in several tissues, including WAT, BAT, and in circulation, results in a severe reduction in both white and brown adipose depots in mice (137). The expression of the transgene in *ob/ob* mice prevents the morbid obesity that typically develops (137). However, *Tgfb1* mRNA levels are increased in the adipose tissues of *ob/ob* and *db/db* mice (138), possibly reflecting physiologically increased TGF-β production corresponding to increased fat mass, as commonly seen with leptin and other adipokines (42, 139).

Because TGF-β expands the population of preadipocytes without promoting their subsequent differentiation (17), the high levels of TGF-β in obese animal models could also reflect an important early role in supporting the undifferentiated population in a calorie-rich environment, but also in restricting subsequent differentiation. Once the differentiation process is initiated and TGF-β receptor availability decreases (76), the "braking effect" of TGF-β on adipogenesis would be reduced and would allow for increased fat accumulation. The pharmacological levels of TGF-β in transgenic models, in contrast, could override the regulatory mechanisms of TGF-β signaling, thereby saturating all available receptors with consequential inhibitory effects on adipogenesis.

V. Clinical Associations

A. Activins, GDF8, and lean/fat body mass

Although *in vitro* studies suggest that activin A has greater biopotency than activin B (comprised of *Inhbb* dimers) in many contexts, it is also evident that activin B contributes in regulating development and body composition, especially in the absence of activin A (140–142). Activin B signals, in part, through interactions with the type I receptor, ALK7 (143, 144). Because activin B has been identified as the predominant activin in human (114)

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and mouse adipose (145), and considering that ALK7 is abundantly expressed in adipocytes (115), it has been hypothesized that activin B plays a role in human adipocyte function by signaling through ALK7. Consistent with this possibility, 3T3-L1 preadipocytes express ALK7 during late-stage adipocyte differentiation (108), and activin B treatment of fully differentiated 3T3-L1 cells significantly decreases the expression and activity of lipases involved in triglyceride breakdown, thereby establishing an inverse relationship between activin B signaling and lipolysis in adipocytes (146).

In humans, *INHBB* and *INHBA* mRNA levels positively correlate with obesity (84, 115) and with the amount of total and sc adipose tissue (114). Furthermore, *INHBB* transcript levels decrease during diet-induced weight loss and positively correlate with serum insulin and cholesterol levels, waist circumference, and body mass index (114). In contrast to these relationships between activin B and adiposity, however, ALK7 expression in adipose is lower in obese subjects than normal controls (115), suggesting that a complex relationship exists in regulating activin B and ALK7 expression, possibly indicating an autoregulatory mechanism for activin signaling in response to the nutritional environment.

Similar to the cachexia observed in mouse models (132), an increase in GDF8 in humans has been associated with loss of muscle mass, including disuse atrophy (147, 148), and AIDS wasting syndrome (149). Consistent with its role as a negative regulator of muscle growth, GDF8 levels decrease after acute aerobic exercise (150) and postatrophic resistance training (151). Conversely, cultured muscle cells obtained from obese women demonstrate increased secretion of biologically active GDF8 (152), and weight loss in morbidly obese patients results in a significant decrease in *GDF8* expression in skeletal muscle biopsies (153, 154).

Pharmacological disruption of signaling for GDF8, activin A, and possibly other superfamily ligands such as GDF11 through the use of soluble ActRIIb-Fc receptor reduces adipose tissue mass in lean and obese mice (155, 156) and prevents or reverses the cancer-cachexia syndrome in a variety of cancer models in mice (156, 157). Clinically, these observations suggest that GDF8 and activin signaling are very likely to be involved in the normal maintenance of lean/fat body mass that is disrupted in a variety of diseases, including HIV/AIDS, cancer, sepsis, severe burn injury, obesity, and others. As such, modulating GDF8 and activin signaling through activin receptors may prove to be an effective strategy to offset the effects of these conditions in humans.

B. TGF-β and body mass index

An early study of 25 obese women indicated that circulating TGF-β levels are decreased in obesity (158). However, others have shown that obesity in humans results in increased expression of TGF-β in adipose tissues (159), similar to animal models (138). The source of adipose-derived TGF-β is cells from the stromal vascular (nonadipocyte) fraction of white adipose (160). Circulating TGF-β levels also positively correlate with obesity, body mass index, and leptin levels in hypertensive patients (161).

C. BMP receptor 1a and obesity-related traits

BMPs and their receptors have also been associated with obesity. mRNA levels of *BMPR1A* (*ALK3*) are increased in visceral and sc adipose of overweight and obese adults and are positively correlated with several parameters used to gauge health, including percentage body fat as well as fasting plasma glucose and insulin levels. In the same study, three single nucleotide polymorphisms within *BMPR1A* were identified as obesity risk alleles, showed an association with increased BMI in two independent cohorts, and were also correlated with increased adipose *BMPR1A* mRNA levels (162).

VI. Perspectives and Future Directions

It is evident that the TGF-β superfamily ligands participate in an increasingly complex signaling network that is regulated at several levels and mediated by a variety of interactions between receptors, signaling intermediates, and downstream effectors. The net effect of signal transduction is highly dependent on spatiotemporal expression of these components. Another important consideration is that many of the ligands are present in the circulation, and so, endocrine contributions of the superfamily, particularly to the regulation of energy metabolism, should also be carefully considered. Recent studies have led to the emergence of new roles in regulating adipogenesis *in vitro* and adiposity *in vivo*, with substantial influence on energy expenditure.

BMPs have emerged as specific regulators of differentiation that promote adipocyte development from multipotent precursors. Although BMP2 and BMP4 promote the differentiation of white adipocytes, BMP7 directs brown adipogenesis in MSCs and committed brown preadipocytes. These proadipogenic events, however, can be modulated by other TGF-β family members, such as TGF-β, GDF8, and activins. It remains to be determined whether other members have similar effects.

GDF3 is an adipogenic factor that can inhibit BMP4 signaling. Considering that mice overexpressing GDF3 have increased sensitivity to the effects of HFD, whereas mice deficient in GDF3 exhibit protection from diet-induced obesity, GDF3 clearly has a net positive effect on adiposity. In both cases, these effects occur with HFD,

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suggesting that GDF3 is an important mediator between the nutritional environment and downstream effects on adiposity. Hypotheses to reconcile GDF3's seemingly antagonistic roles as a proadipogenic factor and a BMP4 inhibitor include spatiotemporal differences in expression of the two ligands during adipogenesis; selective effects on BMP inhibition, such as preferential inhibition of BMP7 or BMP2 rather than BMP4; or simultaneous effects on more than one superfamily signaling event, similar to GDF8's ability to inhibit BMP7 while also activating its own receptors. Alternatively, GDF3's main effects could be on mature adipocytes, in which the roles of TGF-β superfamily signaling are just being explored. Thus, understanding GDF3 effects at the cellular level will require a careful consideration of the milieu of factors that can potentially interact to influence adipogenesis and adipocyte function.

With the emergence of these relationships, more work is required to clarify the role of TGF-β ligands not only on recruitment and differentiation but also on the function of mature adipocytes. Clinically, TGF-β family members have been associated with a number of pathologies, including cachexia, diabetes, as well as obesity and its related comorbidities. Therefore, the information that will undoubtedly emerge about TGF-β superfamily signaling over the coming years will be essential to gain a more complete understanding of the ways in which superfamily signaling influences these human diseases.

Determining the mechanisms by which TGF-β superfamily signaling affects adipogenesis and mature adipocyte function will be further aided by a comprehensive assessment of gene expression during adipogenesis and in mature adipocytes that are treated with specific ligands. In addition to providing a comprehensive overview of the expression characteristics for superfamily signaling components, genome-wide expression profiling and other approaches are likely to reveal biological networks that link TGF-β superfamily signaling to adipocyte differentiation and function.

Current treatment of human obesity is restricted to behavioral modification, the central control of satiety, induced intestinal malabsorption, and a variety of surgical procedures, all with limited efficacy, undesirable side effects, and/or unknown long-term consequences. The effects of TGF-β superfamily signaling on lean and fat body mass suggest a possible new approach to the problem that focuses on the metabolic activities of fat and muscle cells with consequential, beneficial effects on energy expenditure. This approach will require a better understanding of the effects of exogenous activation or inhibition of specific signaling events *in vivo* to determine safety and efficacy. It will also require the development of specific inhibitors or mimetic agents to reproduce the effects observed in a variety of experimental models. Toward this aim, soluble ActRIIB receptor (ACE-031) is in phase I clinical trials in humans for the treatment of neuromuscular disorders and possibly other diseases, with a goal to increase lean body mass. Recent data in mouse models certainly suggest that such an approach has great potential to be clinically beneficial. The next decade of therapeutics targeting TGF-β superfamily signaling promises to be an exciting one.

Acknowledgments

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Work described herein from the Brown laboratory was supported by National Institutes of Health Grants DK073572, HD01156, and HD27823, and the Robert Wood Johnson Foundation, and in part by Research Grant 5Y-01-482 from the March of Dimes Birth Defects Foundation. C.W.B. was the recipient of a Burroughs Wellcome Fund Career Award in the Biomedical Sciences.

Disclosure Summary: The authors have nothing to disclose.

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