Role of follistatin in promoting adipogenesis in women.

John N. Flanagan, Kristina Linder, Niklas Mejhert, Elisabeth Dungner, Kerstin Wahlen, Pauline Decaunes, Mikael Rydén, Peyman Björklund, Stefan Arver, Shalender Bhasin, et al.

Role of Follistatin in Promoting Adipogenesis in Women

John N. Flanagan, Kristina Linder, Niklas Mejhert, Elisabeth Dungner, Kerstin Wahlen, Pauline Decaunes, Mikael Rydén, Peyman Björklund, Stefan Arver, Shalender Bhasin, Anne Bouloumie, Peter Arner, and Ingrid Dahlman

Andrology Center (J.N.F., S.A.), and Department of Medicine (J.N.F., K.L., N.M., E.D., K.W., M.R., S.A., P.A., I.D.), Huddinge, Karolinska Institutet, S-141 86 Stockholm, Sweden; Institut National de la Santé et de la Recherche Médicale (P.D., A.B.), Unité 858 and Université Toulouse III Paul Sabatier, Institut de Médecine Moléculaire de Rangueil, 31432 Toulouse, France; Department of Surgical Sciences (P.B.), Uppsala University Hospital, SE-751 05 Uppsala, Sweden, and Yale Endocrine Neoplasia Laboratory, Yale University School of Medicin, New Haven, Connecticut 06520; and Section of Endocrinology, Diabetes, and Nutrition (S.B.), Boston University School of Medicine, Boston, Massachusetts 02218

Context: Follistatin is a glycoprotein that binds and neutralizes biological activities of TGFβ superfamily members including activin and myostatin. We previously identified by expression profiling that follistatin levels in white adipose tissue (WAT) were regulated by obesity.

Objective: The objective of the study was to elucidate the role of follistatin in human WAT and obesity.

Design: We measured secreted follistatin protein from WAT biopsies and fat cells *in vitro*. We also quantified follistatin mRNA expression in sc and visceral WAT and in WAT-fractionated cells and related it to obesity status, body region, and cellular origin. We investigated the effects of follistatin on adipocyte differentiation of progenitor cells *in vitro*.

Participants: Women (n = 66) with a wide variation in body mass index were recruited by advertisement and from a clinic for weight-reduction therapy.

Results: WAT secreted follistatin *in vitro*. Follistatin mRNA levels in sc but not visceral WAT were decreased in obesity and restored to nonobese levels after weight reduction. Follistatin mRNA levels were high in the stroma-vascular fraction of WAT and low in adipocytes. Recombinant follistatin treatment promoted adipogenic differentiation of progenitor cells and neutralized the inhibitory action of myostatin on differentiation *in vitro*. Moreover, activin and myostatin signaling receptors were detected in WAT and adipocytes.

Conclusion: Follistatin is a new adipokine important for adipogenesis. Down-regulated WAT expression of follistatin in obesity may counteract adiposity but could, by inhibiting adipogenesis, contribute to hypertrophic obesity (large fat cells) and insulin resistance. **(*J Clin Endocrinol Metab* 94: 3003–3009, 2009)**

Obesity is caused by an imbalance between food intake and energy expenditure. The central nervous system exercises a comprehensive control over food intake (1). The balance between energy expenditure and storage is primarily regulated by peripheral tissues such as liver, muscle, and white adipose tissue (WAT). Several pathways in WAT of importance for energy homeostasis are disturbed in obesity such as cell differentiation and lipid turnover (2). Moreover, WAT secretes a number of signal-

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in U.S.A.

doi: 10.1210/jc.2008-2005 Received September 11, 2008. Accepted May 19, 2009.
First Published Online May 26, 2009

Abbreviations: BMI, Body mass index; BMP, bone morphogenetic protein; CEBP, CCAAT enhancer-binding protein; DAPI, 4,6-diamidino-2 phenylindole; FABP, fatty acid binding protein; hMSC, human bone marrow-derived mesenchymal stem cell; PPAR, peroxisomal proliferator-activated receptor; Pref, preadipocyte factor; Smad, small mothers against decapentaplegic; TBS-T, Tris-buffered saline with Tween 20; TGFβR1, TGFβ type I receptor; WAT, white adipose tissue.

ing molecules, *i.e.* adipokines, which act locally or peripherally to control energy homeostasis and metabolism (3). Disturbances in adipokine secretion are associated with the metabolic complications of obesity such as insulin resistance. One potential new adipokine is follistatin, whose expression in human WAT is regulated by obesity in expression profile studies (4).

Follistatin is an extracellular glycoprotein that was originally identified as an inhibitor of pituitary FSH secretion (5). Follistatin functions as an antagonistic binding protein that neutralizes bioactivity of TGFβ superfamily members (6). Thus, follistatin prevents ligand-receptor interaction of activin, myostatin, and bone morphogenetic proteins (BMPs) (7–9). Activin and myostatin signal by binding to type II serine/threonine kinase receptor (activin type IIB) that partners with type I receptor (TGFβR1) to induce phosphorylation of small mothers against decapentaplegic (Smad)-2/3 and translocates to the nucleus to regulate transcription (10–12).

The physiological importance of follistatin potentially includes interfering with functions ascribed to activin, myostatin and BMPs. Both follistatin and activin are expressed in nearly all human tissues (13, 14). Activin overexpression results in cancer, cachexia, and liver necrosis (15). Activin is further implicated in ovarian follicular development, pituitary FSH secretion, insulin secretion, and bone metabolism (7, 16–18). Myostatin is expressed specifically in skeletal muscle and to a lesser extent in WAT (19, 20). Systemic overexpression induces muscle and fat loss (21). Myostatin-null animals have increased skeletal muscle mass and reduced adipogenesis, suggesting that myostatin regulates adiposity as well as muscularity by determining stem cell commitment (20). BMPs regulate differentiation of a broad set of cells (9).

Little is known about follistatin in adiposity. The circulating follistatin levels are high in human polycystic ovary syndrome but not affected by obesity (22, 23). Follistatin reverses the inhibitory effect of activin and myostatin on the differentiation of bovine preadipocytes (24, 25). In the present study, we investigated whether follistatin is secreted from human WAT *in vitro*. We also quantified follistatin mRNA levels in WAT and fractionated cells and related this to obesity status, body region, and cellular origin. Finally, we examined the effects of follistatin on human adipocyte differentiation *in vitro*.

Subjects and Methods

Participants

All subjects were recruited by advertisement or from the outpatient clinic for treatment of obesity at the local hospital for the purpose of studying genes regulating obesity. Obesity was defined as body mass index (BMI) 30 kg/m^2 or greater. All subjects were healthy and free of medication. Participants were investigated at 0800 h after an overnight fast. The study was approved by the ethics committee in Stockholm and Toulouse and written informed consent was obtained from all participants.

Subcutaneous and visceral WAT follistatin mRNA levels were quantified in cohort 1 comprising 13 nonobese (age 40 $\pm$ 13 yr, BMI 24 $\pm$ 2 kg/m^2) and 22 obese women (age 43 $\pm$ 9 yr, BMI 44 $\pm$ 4 kg/m^2). The nonobese subjects were operated for uncomplicated gallstone disease and the obese with antiobesity surgery. Subcutaneous adipose tissue from the surgical incision and omental adipose tissue were obtained at the beginning of surgery. These patients had been fasting overnight, and only saline was given as iv infusion until adipose tissue was removed. Subcutaneous WAT follistatin mRNA levels were further investigated in cohort 2 comprising 10 obese women (aged 39 $\pm$ 9 yr, BMI 40 $\pm$ 1 kg/m^2) investigated before and 2–4 yr after intense antiobesity therapy with antiobesity surgery or behavioral modification when they had reached a nonobese weight-stable state; these subjects have been described before (26). Cohorts 1 and 2 comprised third-generation Caucasian Swedish women living in Stockholm. Cohort 3 comprised women (n = 10) with a wide variation in BMI and was used to quantify follistatin mRNA levels in the isolated different cell fractions of sc WAT (27). Cohort 3 comprised Caucasian French women living in Toulouse. Activin and myostatin signaling receptors ACVRIIB and TGFβR1 were quantified in cohort 4 comprising 11 women (age 43 $\pm$ 14 yr, BMI 30 $\pm$ 8 kg/m^2) for whom mRNA from both sc WAT pieces and isolated adipocytes were available. Cohort 4 comprised third-generation Caucasian Swedish women living in Stockholm. Two women in cohort 4 were postmenopausal. Their ACVRIIB and TGFβR1 mRNA levels did not differ from those of the rest of the cohort. No other women in cohorts 1–4 had entered menopause.

Biopsies of the sc abdominal WAT (0.5–2 g) were obtained by needle aspiration under local anesthesia. WAT samples were brought to the laboratory in saline, and one part was used immediately for adipocyte experiments and another part was frozen in liquid nitrogen.

Isolation and differentiation of cells from WAT

From some WAT pieces we isolated the fat cells according to the collagenase procedure as previously described and determined mean fat cell volume and weight (28, 29). In some experiments we saved the nonadipose cells harvested from the stroma fraction of adipose tissue that was obtained after collagenase treatment. The stroma fraction was used for *in vitro* differentiation of preadipocytes under serum-free conditions exactly as described (29). Medium was replaced every fourth day. The different cell types of the stroma-vascular fraction were separated as described (27). The following cell types were identified: adipocytes, blood capillary endothelial cells (CD34+/CD31+), fraction containing cells with capacity to differentiate into fat cell, *i.e.* progenitor cells (CD34+/CD31−), macrophages (CD34−/CD14+), lymphatic and blood macrovessel endothelial cells (CD34−/CD14−/CD31+), and CD34−/CD14−/CD31− cells, mainly lymphocytes and mast cells (30). We did not use the CD105 antibody because it is not specific for human fat cell precursors.

Enzyme linked immunoassay

In vitro secretion of follistatin was examined in WAT pieces obtained in connection with breast surgery for cosmetic reasons or gastric by pass surgery for treating obesity (sc WAT, n = 4; omental WAT, n = 2). We also measured follistatin in medium from stroma cells undergoing *in vitro* differentiation to preadipocytes. WAT pieces (300 mg in 3 ml medium) or fat cells (300 μl packed cells in 3 ml medium) were incubated for different periods of time as described (31). Medium was saved at −70 C for later determination of follistatin levels by ELISA (catalog no. DFN00; R&D Systems, Abingdon, UK) according to the manufacturer's instructions.

Cell culture

Human bone marrow-derived mesenchymal stem cells (hMSC) from nonobese young male donors (BMI <25 kg/m^2, age 20–40 yr) were purchased from Lonza (Allendale, NJ). hMSCs were cultured and maintained in a formulated growth media MSCGM (Lonza) at 5% CO_2 and 37C. For adipogenesis experiments, cells were grown to 100% confluency and then induced with adipogenic medium that contains 3-isobutyl-*1*-methylxanthine (0.5 mM), dexamethasone (1 μM), and insulin (170 nM) as well as proprietary components provided by the vendor. Cells were treated with recombinant follistatin (500 ng/ml) (R&D Systems, Minneapolis, MN), recombinant myostatin (100 ng/ml; Wyeth, Cam-

bridge, MA) or carrier control (0.1% BSA in PBS) for 21 d. Medium was replaced every third day. Treated cells were analyzed for lipid content, differentiation, and RNA expression.

Percentage differentiated cells

The percentage of adipocyte differentiated hMSCs was determined by counting cells stained positive for lipid-specific dye oil-Red-O-positive cells and relate to nuclear 4,6-diamidino-2 phenylindole (DAPI) cells for total cell number. After 21 d of differentiation, cells were fixed in 10% formalin for 10 min, stained with 0.3% oil-Red-O solution for 10 min, washed three times with distilled water, mounted in VECTASHIELD mounting medium with DAPI (Vector Laboratories, Burlingame, CA), and photographed. The photomicrographs were used to calculate percentage of cells differentiated to adipocytes by determining the ratio of oil-Red-O-positive cells to the DAPI-stained cells for total cell number. Results for each condition represent cell number from five photomicrographs of randomly selected fields (× 400) from two independent experiments.

Determination of lipid content

Lipid content was assessed by AdipoRed assay reagent (Lonza). After 21 d of treatment, cells were rinsed twice with PBS and then incubated with AdipoRed assay reagent for 10 min. AdipoRed is a Nile Red fluorescent reagent that enables the quantification of intracellular lipid droplets. Intracellular triglyceride accumulation was measured in a 7000 Hitachi fluorometer (Indianapolis, IN) with excitation at 485 nm and emission at 572 nm.

RNA preparation and cDNA synthesis

Adipose tissue pieces (300 mg) or 200 μl isolated cells were kept at −70 C for subsequent RNA extraction using the RNeasy minikit (QIAGEN, Hilden, Germany). RNA samples were treated with ribonuclease-free deoxyribonuclease (QIAGEN). The RNA concentration was determined using spectrophotometer. High-quality RNA was confirmed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). One microgram of RNA was reverse transcribed to cDNA using the Omniscript reverse transcriptase kit (QIAGEN) and random hexamer primers. For hMSCs, total RNA was extracted using SV Total RNA isolation kit (Promega, Madison WI). cDNA was generated with 2 μg of total RNA using Superscript RNAase H− (Invitrogen, Carlsbad, CA) with random hexamer primers.

Quantitative real-time PCR

WAT follistatin and 18S mRNA were in cohorts 1 and 2 quantified using the SYBR Green-based technology. Primer pairs were designed to span exon-intron boundaries. The following primers were used for specific mRNA quantification: follistatin, 5′-AGTCCAGTACCAAGGCAGATGT-3′ and 5′-GGTCACACAGTAGGCATTATTGG-3′, and 18S, 5′-CACATGGCCTCCAAGGAGTAAG-3′ and 5′-CCAGCAGTGAGGGTCTCTCT-3′. Dissociation curve analyses and agarose gel electrophoresis were used to validate that a single amplicon was amplified. For SYBR Green assays, 5 ng of cDNA were mixed with gene-specific primers (final concentration 300 nM) and IQ SYBR green supermix (Bio-Rad Laboratories, Hercules, CA) and amplified according to the manufacturer's instructions.

For isolated cells, the SYBR Green based assay was not sensitive enough to reproducible measure follistatin, so in these experiments we used a Taqman assay (follistatin, Hs00246260_m1; Applied Biosystems, Foster City, CA). Other specific mRNA measurements on isolated or *in vitro* differentiated cells were also performed with Taqman assays [ACVRIIB, Hs00609603_m1; TGFβR1, Hs00610318_m1; fatty acid binding protein (FABP)-4, Hs00609791_m1; CCAAT enhancer-binding protein (CEBP)-α, Hs00269972_m1; preadipocyte factor (Pref)-1, Hs-00171584_m1, and peroxisomal proliferator-activated receptor (PPAR)-γ, Hs00234592_m1] with the above 18S SYBR Green-based assay as control. For Taqman assays, 20 ng of cDNA, gene-specific Taqman primer/probe, and Taqman universal master mix (4318157; Applied Biosystems) were mixed to a total volume of 25 μl and amplified according to the manufacturer's instructions. PCR was performed with an ABI Prism 7500 (Applied Biosystems) or iCycler IQ (Bio-Rad Laboratories). A direct comparative method was used for data analysis, *i.e.* $2^{(\text{Ct target gene calibrator} - \text{Ct target gene sample})}/2^{(\text{Ct 18S calibrator} - \text{Ct 18S sample})}$.

Western blot

Abdominal sc WAT follistatin protein was detected by Western blot from six women not selected based on age or BMI. Three hundred milligrams of sc WAT in 500 μl radioimmunoprecipitation assay buffer [50 mM Tris (pH 7.4); 150 mM NaCl; 0.25% nadeoxycholate; 1% Nonidet P-40; 1 mM phenylmethylsulfonyl fluoride; 1 mM Na_2VO_4; 1 mM NaF; 1 mM protein inhibitor cocktail; Roche Diagnostics, Mannheim, Germany] was homogenized using a microtome. The tissue lysates were centrifuged at 14,000 rpm for 30 min after which the infranatant was collected. Protein content was assayed spectrophotometrically using BCA protein assay reagent kit (Pierce, Rockford, IL) on 96-well microtiter plates with BSA (Sigma, St. Louis, MO) as a standard. One hundred milligrams of total cellular protein were loaded on polyacrylamide gels and separated by standard 12% SDS-PAGE. Gels were transferred to polyvinylidine fluoride membranes (Amersham Pharmacia Biotech, Little Chaffore, UK). The blot was blocked for 1 h at room temperature in Tris-buffered saline with 0.1% Tween 20 (TBS-T) and 5% nonfat dried milk. This was followed by an overnight incubation at 4 C with primary antibodies directed against follistatin (0.1 μg/ml) (AF669; R&D Systems) and β-actin (A2066; Sigma). The following day the membrane was rinsed in TBS-T and secondary α-goat antibodies (follistatin) or α-rabbit antibodies (β-actin) conjugated to horseradish peroxidase (Sigma) were added (1:10,000). After incubation, the membrane was rinsed with TBS-T. Antigen-antibody complexes were detected by chemiluminescence using LumiGLO reagent (Cell Signaling Technology, Boston, MA) and exposed to high-performance chemiluminescence film (Amersham Biosciences).

Statistical analysis

mRNA levels were $\log_{10}$ transformed before analysis. Unless otherwise indicated, unpaired or paired *t* test (two sided) was used to compare mRNA levels between two groups. Follistatin secretion from WAT was evaluated by one-way ANOVA with repeated measures. Values are mean ± SD.

Results

WAT mRNA levels of follistatin are reduced in obesity

Subcutaneous WAT mRNA levels of follistatin are inversely associated with obesity ($P < 0.001$) (Fig. 1A, *left panel*). Marked-long term weight loss restores follistatin levels ($P < 0.001$ (Fig. 1B). Follistatin mRNA levels are higher in sc compared with visceral fat (6.0 ± 1.8 *vs.* 2.6 ± 1.1 AU, *P* value 2×10^{-12}). Furthermore, visceral fat follistatin levels are not significantly affected by obesity (Fig. 1A, *right panel*). There was, however, no correlation between fat cell size and follistatin mRNA (figure not shown).

Source of follistatin mRNA in WAT

The stroma-vascular fraction is the major source of follistatin in WAT (Fig. 1D). Highest levels of follistatin were observed in the CD34+/CD31− cells, which contain adipocytes progenitor cells, and in the capillary endothelial cell fraction. Very low levels of follistatin mRNA were detected in isolated adipocytes. mRNA levels of follistatin in cells of the immune system, *i.e.* macro-

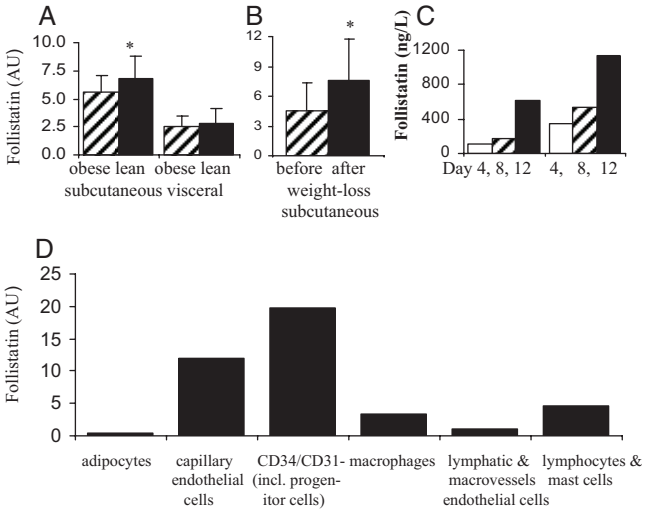

FIG. 1. Follistatin mRNA levels in WAT of obese (n = 22) and nonobese (n = 13) women (A) and in obese women before and after marked long term weight reduction (n = 10) (B). C, Follistatin protein levels in medium collected at d 4, 8, and 12 from stroma cells differentiated *in vitro* to preadipocytes (n = 2). D, Follistatin mRNA levels in the different cell fractions in WAT (n = 10) (see *Subjects and Methods* to see how the cells were isolated). AU = $2^{(Ct\ target\ gene\ calibrator - Ct\ target\ gene\ sample)}/2^{(Ct\ 18S\ calibrator - Ct\ 18S\ sample)}$, where the calibrator is a random sample. Values are mean ± SD. *, $P < 0.05$; ** $P \leq 0.01$.

phages and lymphocytes, were in between. Pref-1, described as a preadipocyte marker in rodents, was detected at low levels in the CD34+/CD31− cell fraction. In one third of these samples, Pref-1 was undetected. Pref-1 was undetected in the other cell fractions of the stroma vascular fraction and in isolated adipocytes. We validated our Pref-1 assay by measuring Pref-1 in two normal human adrenal glands. Pref-1 levels in the adrenal gland were about 1000 times higher than in the CD34+/CD31− cells (results not shown). At present we do not know which cell type(s) in the stroma that contains Pref-1.

WAT secretes follistatin *in vitro*

We demonstrated by ELISA that WAT secretes follistatin *in vitro* in a time-dependent manner (Fig. 2). In investigated sc (n = 4) and visceral (n = 2) WAT pieces no major difference in follistatin secretion between the two fat depots was detected. In the six experiments put together, there was a significant overtime effect on follistatin secretion ($P = 0.013$ by one way ANOVA repeated measure). *Post hoc* test (one sided paired *t* test) showed

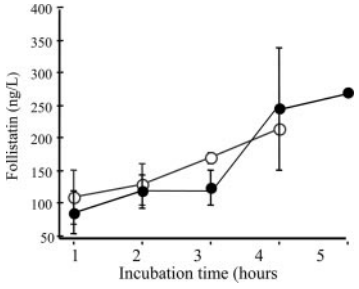

FIG. 2. Follistatin secretion from WAT *in vitro*. Pieces of WAT (300 mg) were incubated in medium (3 ml) and concentration of follistatin in medium determined with ELISA. *Filled dot*, sc WAT (n = 4); *unfilled dot*, visceral WAT (n = 2). One-way ANOVA with repeated measures for all six observations put together at 1–4 h gives *P* value of 0.013.

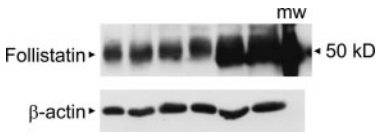

FIG. 3. Follistatin protein detected by Western blot in six women not selected for age or BMI. β-Actin was used to control for differences in overall protein between samples.

that the difference between 1 and 2 h and between 3 and 4 h were significant ($P = 0.011$ and 0.047, respectively). We did not have samples to relate follistatin secretion to obesity. Follistatin secretion was undetectable in isolated fat cell cultures after short-term cultures up to 4 h (values not shown).

We were able to detect increasing levels of follistatin in medium from differentiating stroma cells (Fig. 1C). Medium was saved from two experiments only and we were therefore not able to do any statistical analysis of these results. Only one isoform of follistatin with a size close to 50 kDa was detected by Western blot (Fig. 3).

Follistatin promotes adipogenesis *in vitro*

We examined the effects of recombinant human follistatin treatment on adipogenic differentiation of two human primary progenitor cell types, bone marrow-derived mesenchymal stem cells (hMSCs) and sc WAT-derived preadipocytes. After 21 d of follistatin treatment, adipogenic differentiated hMSCs had overall more visual lipid content (Fig. 4A, *top right panel*) compared with control cells (Fig. 4A, *top left panel*). To examine follistatin neutralizing activity of TGFβ family members, we cotreated hMSCs with follistatin and myostatin under adipogenic differentiation conditions. Myostatin treatment alone inhibited adipogenic differentiation of hMSCs (Fig. 4A, *bottom right panel*). Follistatin and myostatin cotreatment appeared to reverse myostatin inhibitory effects on adipogenesis and restoring the number of visual adipocytes (Fig. 4A, *bottom left panel*) to numbers comparable with hMSC-derived control cells (Fig. 4A, *top left panel*). Quantitatively, follistatin increased lipid content in adipogenic differentiated hMSCs by 50% above adipogenic control cells ($P < 0.01$) and reversed the inhibitory effect of myostatin on lipid accumulation from 70% ($P < 0.01$) to 135% ($P < 0.02$) compared with adipogenic control cells (Fig. 4B). Similar effects of follistatin treatment on lipid content were observed in differentiated human preadipocytes (Fig. 4C).

Subsequently we evaluated the impact of follistatin treatment on the percentage of adipogenic differentiated hMSCs. After 21 d of differentiation, cells were stained with lipid specific dye oil-Red-O to determine the percentage of differentiated cells. In adipogenic control cultures, 61 ± 2% of hMSCs were oil-Red-O-positive cells compared with 78 ± 3% in follistatin-treated cells. Myostatin treatment cultures had 48 ± 2% oil-Red-O-positive hMSCs, whereas cotreatment with follistatin increased the differentiated cells to 57 ± 2%.

Follistatin promotion of adipogenesis in hMSCs was further investigated by assessing mRNA levels of adipogenic markers. We examined two initiating transcription factors of adipogenesis, CEBPα and PPARγ, and one late marker of adipogenesis, FABP4. mRNA was measured after 21 d of chronic treatment of

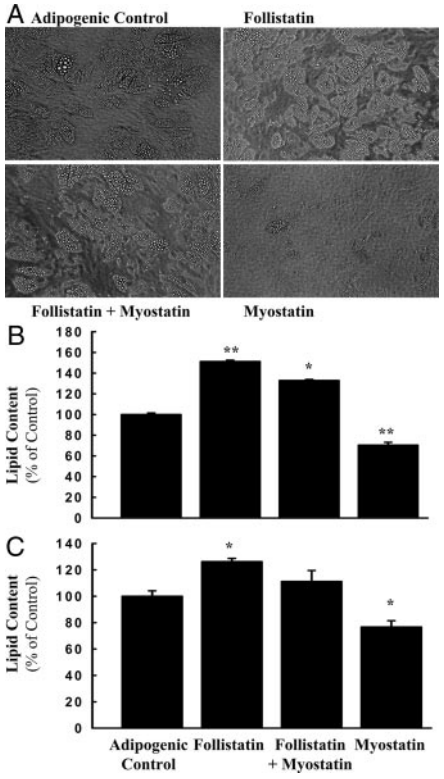

FIG. 4. Adipogenic differentiation of cultured hMSCs and preadipocytes treated for 21 d with Follistatin (500 ng/ml) alone, Myostatin (100 ng/ml) alone, both in combination, or 0.1% BSA-PBS control under adipogenic conditions. A, Images represent differentiated hMSCs. B and C, Quantitative determination of lipid content by AdipoRed reagent (Lonza) in differentiated hMSCs (B) and human preadipocytes (C) at 21 d of treatment (n = 6–8 determinations from two separate experiments). Values are mean ± SD. *, $P < 0.02$; **, $P < 0.01$.

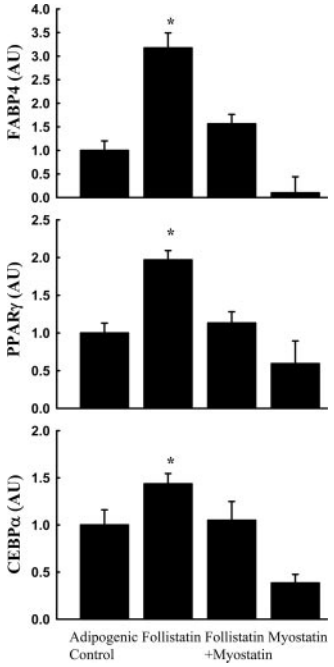

FIG. 5. mRNA levels of adipogenic markers FABP4, PPARγ, and CEBPα in hMSCs treated for 21 d with Follistatin (500 ng/ml) alone, Myostatin (100 ng/ml) alone, both in combination, or 0.1% BSA-PBS control under adipogenic conditions (n = 3 determinations from three independent experiments). AU = $2^{(Ct\ target\ gene\ calibrator - Ct\ target\ gene\ sample)}/2^{(Ct\ 18S\ calibrator - Ct\ 18S\ sample)}$, where the calibrator is an adipogenic control sample. Values are mean ± SD. *, $P < 0.02$.

hMSCs under adipogenic conditions with follistatin and myostatin alone or in combination or with carrier control. FABP4, CEBPα, and PPARγ mRNA expression levels were increased after chronic follistatin treatment compared with adipogenic control cells (Fig. 5, $P < 0.02$). Furthermore, we were able to rescue the inhibitory effect of myostatin on adipogenesis by coincubation with follistatin.

Expression of follistatin targets ACVRIIB and TGFβR1 in WAT

We next quantified mRNA of the activin/myostatin-receptors mediating the effects of follistatin, ACVRIIB, and TGFβR1. TGFβR1 was expressed at similar levels in sc isolated adipocytes and WAT pieces, 1.80 + 0.80 *vs.* 1.65 + 0.69 AU ($P = 0.64$). ACVRIIB was expressed at lower levels in sc isolated adipocytes *vs.* WAT pieces, 1.10 + 0.39 *vs.* 2.24 + 1.07 AU ($P = 0.0044$).

Discussion

We have established that follistatin is a novel protein secreted from human adipose tissue that promotes adipogenesis. sc and visceral WAT release follistatin *in vitro*. Only one isoform of follistatin, which most probably corresponds to the largest isoform FS315 was detected in WAT (32, 33). Only women were investigated in this study. We therefore cannot draw any conclusions on the role of follistatin in men.

Because follistatin is an extracellular protein, it can potentially function on all cell types within WAT that activin and myostatin signal upon. Importantly, we detected the expression of activin and myostatin receptor complex ACVRIIB and TGFβR1 in isolated adipocytes and WAT. This supports that all the necessary machinery for activin/myostatin/follistatin signaling are present within WAT. Furthermore, follistatin secretion was increased during adipocyte differentiation *in vitro*, implying a role in differentiation. Indeed, we found that recombinant follistatin treatment promotes adipogenic differentiation of progenitor cells *in vitro*, resulting in increased lipid content and CEBPα, PPARγ, and FABP4 mRNA expression.

Follistatin was able to reverse myostatin inhibition of adipogenesis in human progenitor cells by neutralizing the activity of this TGFβ family member. Others have similarly reported that follistatin reverse activin and myostatin inhibition of bovine adipogenesis (24, 25). Previous experiments have established that activin and myostatin inhibit adipogenesis by receptor-mediated phosphorylation of Smad2/Smad3 that directly suppresses CEBPα and PPARγ mRNA expression (11, 12). Furthermore, myostatin has been shown to inhibit adipogenesis through Smad-mediated impairment of the transactivation function of CEBPβ (12). Therefore, the interaction between myostatin and follistatin would release the Smad-mediated impairment of CEBPβ to allow adipogenesis to go forward.

Of note, a substantially higher concentration of follistatin is required to promote adipocyte differentiation *in vitro* than the amount of follistatin that was detected in supernatants from

incubated WAT biopsies. The main aim of the *in vitro* incubation is to show that WAT can secrete the protein of interest, whereas the interpretation of absolute amounts of secreted protein is uncertain because we do not have tools to measure the *in vivo* secretion and how it is related to the *in vitro* results.

In vitro-differentiated preadipocytes secreted follistatin, whereas no secretion was detected from isolated adipocytes. This discrepancy may be due to the much shorter incubation time in medium for the isolated adipocytes *vs.* preadipocyte cultures. The long incubation time for preadipocytes may induce secondary changes in adipokine secretion that are not seen in the isolated adipocyte cultures. It was also surprising that high levels of follistatin mRNA were observed in the stroma vascular fraction and in particular in the CD34+/CD31− cell fraction, which contains the adipocyte progenitor cells, despite that follistatin secretion increased during fat cell differentiation. However, whereas mRNA gives a measure at a specific time point, protein secretion reflects production during a period. Dynamic changes in mRNA expression may explain the discrepancies between mRNA and protein. As an alternative, posttranscriptional regulation may explain the difference.

In this report, we observed a decreased follistatin expression in obese subjects in sc WAT compared with nonobese controls. Furthermore, antiobesity intervention restored sc WAT follistatin levels to those of nonobese controls. Importantly, human WAT follistatin is probably acting locally because circulating follistatin levels are not affected by obesity status (23). Interestingly, the expression of a follistatin ligand, activin B, was shown to be reduced by diet-induced weight loss in sc WAT from obese subjects (34). These data suggest that obesity decreases the follistatin to activin ratio. Because follistatin *in vitro* stimulates adipogenesis a plausible explanation is that sc WAT follistatin is down-regulated in obesity to counteract adiposity. The role in visceral WAT is more uncertain.

WAT mass can increase through a combination of hypertrophic increase in size of preexisting adipocytes and *de novo* hyperplasia of adipocytes by differentiation from residential progenitor cells in the stroma-vascular portion of WAT (35). Although follistatin expression and adipocyte size did not correlate, down-regulation of follistatin in obesity could aim to limit adiposity by inhibiting adipogenesis but, by failing to limit fat accumulation, indirectly contribute to hypertrophic obesity. Low follistatin levels may result in enlargement of existing adipocytes at the expense of new fat cell formation. However, we cannot rule out that changes in the degree of vascularization in WAT that accompanies the development of obesity might account for the changes in follistatin expression.

We were able to confirm our findings with two different native fat cell types. We did not use immortalized human fat cells because their functional properties, including adipokine production, compared with native human fat cells are not well established. We presently used three validated markers for human fat cell differentiation. Other markers, such as GATA-2 and GATA-3, have been validated only in rodent fat cells.

In summary, the present study demonstrated that follistatin is expressed and secreted in WAT. The expression of follistatin in sc WAT is decreased in obesity but can be recovered by antiobesity intervention. Follistatin promotes differentiation of progenitor cells *in vitro*. We therefore propose that follistatin is a novel adipokine with an important regulatory function in adipogenesis.

Acknowledgments

Address all correspondence and requests for reprints to: Ingrid Dahlman, Department of Medicine, Huddinge, Karolinska Institutet, S-141 86 Stockholm, Sweden. E-mail: ingrid.dahlman@ki.se.

This work was supported by the Swedish Research Council, European Union [HEPADIP (LSHM-CT-2005-018734) and ADAPT (HEALTH-F2-2008-201100)], NordForsk (SYSDIET-070014), Karolinska Institutet, Swedish Diabetes Association, Swedish Heart and Lung Association, Novo Nordisk Foundation, Magnus Bergvalls Stiftelse, and Åke Wibergs stiftelse.

Disclosure Summary: The authors have nothing to disclose.

References

1. **Spiegelman BM, Flier JS** 2001 Obesity and the regulation of energy balance. Cell 104:531–543
2. **Large V, Peroni O, Letexier D, Ray H, Beylot M** 2004 Metabolism of lipids in human white adipocyte. Diabetes Metab 30:294–309
3. **Arner P** 2005 Insulin resistance in type 2 diabetes—role of the adipokines. Curr Mol Med 5:333–339
4. **Dahlman I, Kaaman M, Olsson T, Tan GD, Bickerton AS, Wahlen K, Andersson J, Nordstrom EA, Blomqvist L, Sjogren A, Forsgren M, Attersand A, Arner P** 2005 A unique role of monocyte chemoattractant protein 1 among chemokines in adipose tissue of obese subjects. J Clin Endocrinol Metab 90:5834–5840
5. **Ueno N, Ling N, Ying SY, Esch F, Shimasaki S, Guillemin R** 1987 Isolation and partial characterization of follistatin: a single-chain Mr 35,000 monomeric protein that inhibits the release of follicle-stimulating hormone. Proc Natl Acad Sci USA 84:8282–8286
6. **Mather JP, Moore A, Li RH** 1997 Activins, inhibins, and follistatins: further thoughts on a growing family of regulators. Proc Soc Exp Biol Med 215:209–222
7. **Nakamura T, Takio K, Eto Y, Shibai H, Titani K, Sugino H** 1990 Activin-binding protein from rat ovary is follistatin. Science 247:836–838
8. **Lee SJ, McPherron AC** 2001 Regulation of myostatin activity and muscle growth. Proc Natl Acad Sci USA 98:9306–9311
9. **Bessa PC, Casal M, Reis RL** 2008 Bone morphogenetic proteins in tissue engineering: the road from the laboratory to the clinic, part I (basic concepts). J Tissue Eng Regen Med 2:1–13
10. **Shimizu A, Kato M, Nakao A, Imamura T, ten Dijke P, Heldin CH, Kawabata M, Shimada S, Miyazono K** 1998 Identification of receptors and Smad proteins involved in activin signalling in a human epidermal keratinocyte cell line. Genes Cells 3:125–134
11. **Rebbapragada A, Benchabane H, Wrana JL, Celeste AJ, Attisano L** 2003 Myostatin signals through a transforming growth factor β-like signaling pathway to block adipogenesis. Mol Cell Biol 23:7230–7242
12. **Guo W, Flanagan J, Jasuja R, Kirkland J, Jiang L, Bhasin S** 2008 The effects of myostatin on adipogenic differentiation of human bone marrow-derived mesenchymal stem cells are mediated through cross-communication between Smad3 and WNT/β-cantenin signaling pathways. J Biol Chem 283:9136–9145
13. **Tuuri T, Eramaa M, Hilden K, Ritvos O** 1994 The tissue distribution of activin βA- and βB-subunit and follistatin messenger ribonucleic acids suggests multiple sites of action for the activin-follistatin system during human development. J Clin Endocrinol Metab 78:1521–1524
14. **Tortoriello DV, Sidis Y, Holtzman DA, Holmes WE, Schneyer AL** 2001 Human follistatin-related protein: a structural homologue of follistatin with nuclear localization. Endocrinology 142:3426–3434
15. **Cipriano SC, Chen L, Kumar TR, Matzuk MM** 2000 Follistatin is a modulator of gonadal tumor progression and the activin-induced wasting syndrome in inhibin-deficient mice. Endocrinology 141:2319–2327
16. **Shibata H, Kanzaki M, Takeuchi T, Miyazaki J, Kojima I** 1996 Two distinct

signaling pathways activated by activin A in glucose-responsive pancreatic β-cell lines. J Mol Endocrinol 16:249–258
17. **Florio P, Luisi S, Marchetti P, Lupi R, Cobellis L, Falaschi C, Sugino H, Navalesi R, Genazzani AR, Petraglia F** 2000 Activin A stimulates insulin secretion in cultured human pancreatic islets. J Endocrinol Invest 23:231–234
18. **Sakai R, Eto Y** 2001 Involvement of activin in the regulation of bone metabolism. Mol Cell Endocrinol 180:183–188
19. **Gonzalez-Cadavid NF, Taylor WE, Yarasheski K, Sinha-Hikim I, Ma K, Ezzat S, Shen R, Lalani R, Asa S, Mamita M, Nair G, Arver S, Bhasin S** 1998 Organization of the human myostatin gene and expression in healthy men and HIV-infected men with muscle wasting. Proc Natl Acad Sci USA 95:14938–14943
20. **McPherron AC, Lawler AM, Lee SJ** 1997 Regulation of skeletal muscle mass in mice by a new TGF-β superfamily member. Nature 387:83–90
21. **Zimmers TA, Davies MV, Koniaris LG, Haynes P, Esquela AF, Tomkinson KN, McPherron AC, Wolfman NM, Lee SJ** 2002 Induction of cachexia in mice by systemically administered myostatin. Science 296:1486–1488
22. **Norman RJ, Milner CR, Groome NP, Robertson DM** 2001 Circulating follistatin concentrations are higher and activin concentrations are lower in polycystic ovarian syndrome. Hum Reprod 16:668–672
23. **Eldar-Geva T, Spitz IM, Groome NP, Margalioth EJ, Homburg R** 2001 Follistatin and activin A serum concentrations in obese and non-obese patients with polycystic ovary syndrome. Hum Reprod 16:2552–2556
24. **Hirai S, Matsumoto H, Hino N, Kawachi H, Matsui T, Yano H** 2007 Myostatin inhibits differentiation of bovine preadipocyte. Domest Anim Endocrinol 32:1–14
25. **Hirai S, Matsumoto H, Moriya NH, Kawachi H, Yano H** 2007 Follistatin rescues the inhibitory effect of activin A on the differentiation of bovine preadipocyte. Domest Anim Endocrinol 33:269–280
26. **Lofgren P, Andersson I, Adolfsson B, Leijonhufvud BM, Hertel K, Hoffstedt J, Arner P** 2005 Long-term prospective and controlled studies demonstrate adipose tissue hypercellularity and relative leptin deficiency in the postobese state. J Clin Endocrinol Metab 90:6207–6213
27. **Curat CA, Miranville A, Sengenes C, Diehl M, Tonus C, Busse R, Bouloumie A** 2004 From blood monocytes to adipose tissue-resident macrophages: induction of diapedesis by human mature adipocytes. Diabetes 53:1285–1292
28. **Rodbell M, Krishna G** 1974 Preparation of isolated fat cells and fat cell "ghosts": methods for assaying adenylate cyclase activity and levels of cyclic AMP. Methods Enzymol 31:103–114
29. **van Harmelen V, Dicker A, Ryden M, Hauner H, Lonnqvist F, Naslund E, Arner P** 2002 Increased lipolysis and decreased leptin production by human omental as compared with subcutaneous preadipocytes. Diabetes 51:2029–2036
30. **Sengenes C, Lolmede K, Zakaroff-Girard A, Busse R, Bouloumie A** 2005 Preadipocytes in the human subcutaneous adipose tissue display distinct features from the adult mesenchymal and hematopoietic stem cells. J Cell Physiol 205:114–122
31. **Lonnqvist F, Nordfors L, Jansson M, Thorne A, Schalling M, Arner P** 1997 Leptin secretion from adipose tissue in women. Relationship to plasma levels and gene expression. J Clin Invest 99:2398–2404
32. **Shimasaki S, Koga M, Esch F, Cooksey K, Mercado M, Koba A, Ueno N, Ying SY, Ling N, Guillemin R** 1988 Primary structure of the human follistatin precursor and its genomic organization. Proc Natl Acad Sci USA 85:4218–4222
33. **Sidis Y, Mukherjee A, Keutmann H, Delbaere A, Sadatsuki A, Schneyer A** 2006 Biological activity of follistatin isoforms and follistatin-like-3 is dependent on differential cell surface binding and specificity for activin, myostatin, and bone morphogenetic proteins. Endocrinology 147:3586–3597
34. **Sjoholm K, Palming J, Lystig TC, Jennische E, Woodruff TK, Carlsson B, Carlsson LM** 2006 The expression of inhibin βB is high in human adipocytes, reduced by weight loss, and correlates to factors implicated in metabolic disease. Biochem Biophys Res Commun 344:1308–1314
35. **Spalding KL, Arner E, Westermark PO, Bernard S, Buchholz BA, Bergmann O, Blomqvist L, Hoffstedt J, Naslund E, Britton T, Concha H, Hassan M, Ryden M, Frisen J, Arner P** 2008 Dynamics of fat cell turnover in humans. Nature 453:783–787