BMP4 and BMP antagonists regulate human white and beige adipogenesis

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The limited expandability of subcutaneous adipose tissue, due to reduced ability to recruit and differentiate new adipocytes, prevents its buffering effect in obesity and is characterized by expanded adipocytes (hypertrophic obesity). Bone morphogenetic protein-4 (BMP4) plays a key role in regulating adipogenic precursor cell commitment and differentiation. We found BMP4 to be induced and secreted by differentiated (pre)adipocytes and BMP4 protein was increased in large adipose cells. However, the precursor cells exhibited a resistance to BMP4 due to increased secretion of the BMP inhibitor Gremlin-1 (GREM1). GREM1 is secreted by (pre)adipocytes and is an inhibitor of both BMP4 and BMP7. BMP4 alone, and/or silencing GREM1, increased transcriptional activation of peroxisome proliferator-activated receptor-γ (PPARγ) and promoted the preadipocytes to assume an oxidative beige/brown adipose phenotype including markers of increased mitochondria and PGC1α. Driving white adipose differentiation inhibited the beige/brown markers suggesting the presence of multipotent adipogenic precursor cells. However, silencing GREM1 and/or adding BMP4 during white adipogenic differentiation re-activated beige/brown markers suggesting that increased BMP4 preferentially regulates the beige/brown phenotype. Thus BMP4, secreted by white adipose cells, is an integral feedback regulator of both white and beige adipogenic commitment and differentiation and resistance to BMP4 by GREM1 characterizes hypertrophic obesity.

Obesity and its associated negative metabolic and health consequences are globally increasing at an epidemic rate. The subcutaneous adipose tissue (SAT) is the largest adipose depot of the body and it is also the major sink for excess fat storage. However, SAT has a limited expandability and, when exceeded, fat accumulates in different ectopic sites, including the liver and visceral adipose tissue, and this is the major driver of the metabolic consequences of obesity (1). Studies have also shown that individuals with T2D diabetes, compared to non-diabetic subjects, have increased amount of ectopic fat for the same amount of total body fat supporting a reduced ability to expand the subcutaneous depot (2). Consequently, ability to store excess fat in the subcutaneous depot has a protective effect against the obesity-associated complications; a concept supported by the ability of peroxisome proliferator-activated receptor-γ (PPARγ) ligands to reduce ectopic fat while subcutaneous body fat is increased (3). However, these ligands can only enhance the differentiation of already committed preadipocytes and cannot promote the commitment and recruitment of new adipose cells.

SAT contains a pool of preadipocytes and other precursor/stem cells that can differentiate into mature adipocytes (4). Regulation of adipogenesis is an important question since adipose cell expansion (hypertrophic adipocytes) is associated with a dysfunctional adipose tissue with local and systemic insulin resistance and both *in vivo* and *ex vivo* studies have shown that hypertrophic obesity is characterized by a reduced recruitment of new cells (4-9). Importantly, we have recently shown that adipose cell size in the abdominal SAT is considerably larger in individuals with a genetic predisposition (defined as being a first-degree relative) for T2D than in matched individuals lacking a known heredity for diabetes (5,8). This novel finding links hypertrophic obesity, ectopic fat accumulation and associated insulin resistance with genetic risk for T2D, a concept that has received strong recent support from large clinical studies (10,11).

The adipose tissue mesenchymal stem cells (MSC) serve as a reservoir and allow a continued renewal of precursor cells that can differentiate into adipocytes (12-14). The bone morphogenetic proteins (BMPs) are of particular interest since some members have been shown to recruit adipose precursor cells into the adipose lineage (6). BMP7 is reported to be a regulator of brown adipogenesis (15) while BMP2 and 4 are related to white adipogenesis (6,12,16). We recently demonstrated that human SAT preadipocytes induce BMP4 during differentiation and that BMP4 increased both commitment and differentiation of human precursor cells (6). In addition, recent studies with human precursor cells found BMP4 also to promote the induction of a beige phenotype (17).

Adipogenic commitment of early precursor cells by BMP4 is mediated by the dissociation of an intracellular complex consisting of the PPARγ transcriptional activator zinc finger protein-423 (ZNF423) (18) and the mesenchymal cell canonical WNT1 inducible signaling pathway protein-2 (WISP2), thereby allowing nuclear entry of ZNF423 and PPARγ induction (19). Thus, BMP4-signaling and its cross-talk with canonical WNT/WISP2 is an essential component of the early induction of adipogenesis. Consequently, inability to adequately increase BMP4 in precursor cells would decrease adipogenesis and, instead, promote enlargement of available cells, i.e., hypertrophic obesity similar to what was found when the commitment factor eBF1 was genetically deleted in mice (20).

The amount of BMP available for signaling is tightly regulated by the complex BMP receptor signaling pathways including a number of structurally distinct BMP antagonists that alter the ability of BMPs to bind to their receptors and regulate development of many different cell types (21,22). Very little is currently known about the endogenous BMP antagonists and their effects on BMP4 action in human adipogenesis and hypertrophic obesity.

Activin A, a secreted homodimer of inhibin-βA (INHBA) subunits (23,24), follistatin (25) and the pseudoreceptor BMP and activin membrane-bound inhibitor (BAMBI) (26) are all expressed in the adipose tissue. Follistatin is secreted by adipose tissue explants (27) and noggin is a well-established and secreted inhibitor which binds to the BMP receptors but its biological functions are mostly undetermined (6,28). Chordin and Chordin-like-1 (CHRDL1) are secreted proteins that bind BMP2, 4 and 7 (29,30) and CHRDL1 and has been shown to enhance the proliferation of human MSCs (31). Gremlin-1 (GREM1) is a potent extra- and intracellular inhibitor of BMP4 (32) and is involved in fibrosis and arthritis development (33).

Brown adipose tissue (BAT) is specialized for energy expenditure and maintaining body temperature. Until now, the physiological significance of BAT for whole-body metabolism in adult man has been unclear but cold exposure in men increased resting energy expenditure, glucose oxidation, and insulin sensitivity (34). An intermediate kind of brown adipocytes, the beige cells, has been demonstrated in SAT (35,36). Increasing the activation of beige cells in mice was associated with reduced weight gain and improved glucose tolerance (37).

In the present study, we examined the effects of BMP4 and several BMP inhibitors during adipogenic differentiation of human subcutaneous preadipocytes. Our results provide evidence for the concept that hypertrophic obesity is a condition of preadipocyte resistance to BMP4 as a consequence of increased secretion of GREM1. In addition, GREM1 regulates the ability of BMP4 to induce beige/brown adipogenesis making it an interesting target in obesity.

RESEARCH DESIGN AND METHODS

Human subjects

Genes/proteins were studied in isolated mature adipose cells and adipose tissue needle biopsies of the SAT from 33 individuals. The subjects were between 26 and 52 years, mean BMI 24.4±2.3 kg/m^2 (range 19.5-27.5) and adipose cell size 92.8±9.7 μm (range 71.5-118.4). Additional SAT was obtained from 24 individuals by needle biopsy (n=23) or bariatric surgery (n=1) for the adipogenic studies. The subjects were between 27 and 66 years of age, had a mean BMI of 27.5±7.1 kg/m^2 (range 19.3-54.8) and adipose cell size 95.9±17.0 μm (range 52.8-122.5 μm). All subjects had normal glucose levels and had no known chronic diseases. The Ethical Committee of the University of Gothenburg approved the study design and written informed consent was received from participants prior to inclusion in the study.

Digestion of adipose tissue biopsies and preadipocyte differentiation

Subcutaneous adipose tissue was digested with collagenase and cell size measured as previously described (6,7). The remaining stromal-vascular progenitor cells were isolated and differentiated as described (6). When indicated 100 ng/mL (3 nmol/L) BMP4 was added.

Quantitative real-time PCR (Q-PCR)

Details of real-time PCR assays were described previously (6). Gene-specific primers and probes were designed using Primer Express software or purchased as Assay-on-Demand (Life Technologies, Stockholm, Sweden).

Over expression of CHRDL1

To overexpress CHRDL1, cells were transfected with a myc-DDK-tagged ORF clone of CHRDL1 obtained from Origene (TrueORF Gold RC202635, BioNordika Sweden) using Lipofectamine 2000 (Life Technologies, Stockholm, Sweden) according to the manufacturers protocol. An empty vector was used as negative control and GFP expression to monitor transfection efficiency. Adipogenic differentiation was induced 24h after transfection.

Small interfering RNA (siRNA)

Human isolated preadipocytes were transfected with either CHRDL1 or noggin or GREM1 siRNA using RNAiMAX (Life Technologies, Stockholm, Sweden) according to the manufacturer's instructions. After 48h, medium was changed to adipocyte differentiation media with and without BMP4 (10-100 ng/mL) as stated. RNA was extracted with EZNA total RNA kit (VWR, Stockholm Sweden) after 6 days culture.

Immunohistochemistry and confocal imaging of BMP4 and mitochondria

Human adipose tissue stromal cells were grown on glass slides, fixed with 4% formaldehyde for 15 min and permeabilized in 0.1% Triton X-100 for 5 min. Cells were then blocked with 20% FBS for 30 min followed by incubation with anti-BMP4 antibody (MAB1049, Merck Millipore, Solna Sweden) and UCP1 (ab10983, Abcam, Cambridge, UK) for 3h. After washing in PBS and incubation with secondary antibody conjugated with Alexa-594 for 1h confocal images were collected by the Leica SP5 confocal system. Cells were also stained with Mito Tracker Red according to the manufacturer (Life Technologies).

Whole cell extracts and immunoblotting

Protein lysates and immunoblotting were performed as described (38) using the following antibodies; SMAD1/5/8 (sc-6031 Santa Cruz, AH Diagnostics, Solna Sweden), pSMAD1/5/8

(Cell Signaling 9511, BioNordika, Stockholm, Sweden), pSMAD3 (Cell Signaling 8769), AKT (Cell Signaling 9272), SMAD3 (sc-8332 Santa Cruz), BMP4 (ab93939) and BMP7 (sc-53917).

Detection of secreted GREM1 and BMP4

AlphaLISA® was used to measure secreted human Gremlin-1 (MedImmune, Gaithersburg, MD) and secreted BMP4 was detected with an ELISA from Abcam, ab99982, according to the manufacturer´s instructions.

Statistical analysis

The experimental data are presented as means ±SEM. Data analyzes were carried out with the PASWstatistics (SPSS Inc.) for Macintosh. For cells differentiated *in vitro,* Student's paired *t*-test was used for comparison of gene expression with basal samples within groups and one-way ANOVA (post hoc Tukey's test) was used for direct comparisons between groups, correcting for multiple variables where applicable. Differences were considered statistically significant at $P < 0.05$ level.

RESULTS

BMP4 is secreted by adipose cells and increased in hypertrophic obesity

To address the potential role of BMP4 in hypertrophic obesity, we first asked if BMP4 is expressed in mature adipose cells and, if so, if it is reduced in enlarged cells. However, *BMP4* transcript levels were high in isolated mature adipocytes and correlated positively with the cell size (Fig. 1A), albeit not with BMI over this limited range (19.5 – 27.5 kg/m^2) (Fig. 1B).

We have previously shown that BMP4 mRNA levels are increased during human preadipocyte differentiation (6) To ensure that BMP4 protein also is present in adipose cells we examined differentiated and undifferentiated preadipocytes with both immunofluorescence and Western blot. BMP4 protein was clearly induced following differentiation (Fig. 1C). Furthermore, intermediate- and low-molecular weight secreted BMP4 protein in adipose tissue biopsies was also positively correlated with cell size (Fig. 1E). To validate that BMP4 also was secreted from differentiated human adipocytes we collected cell culture medium from day 0 and days 0-3 and 9-12 from preadipocytes undergoing differentiation and measured BMP4 protein secretion with ELISA. BMP4 protein was also secreted by differentiated (pre)adipocytes (Fig. 1F).

BMP inhibitors are increased in hypertrophic obesity

A possible explanation for the unexpected finding of increased BMP4 in hypertrophic obesity could be that the endogenous BMP inhibitors also are increased, thereby inducing a cellular resistance to BMP4 and its pro-adipogenic effect. All measured BMP4 inhibitors were robustly expressed in isolated mature adipose cells as well as in undifferentiated preadipocytes and adipocytes differentiated *in vitro* (Supplementary Table 1). Furthermore, transcript levels of *GREM1 and CHRDL1* correlated positively with those of *BMP4* in both mature adipocytes and intact adipose tissue biopsies (Supplementary Table 2) supporting the

concept of a BMP4 resistance. In addition, *GREM1* and *CHRDL1* mRNA levels in the human adipose tissue biopsies correlated positively and significantly (P=0.02 and P=0.003, respectively) with adipose cell size of the donors while there was no significant correlation for *noggin* or *follistatin* (data not shown). These results show that both BMP4 and certain BMP4 inhibitors are increased in adipose tissue characterized by expanded adipose cells supporting the possibility that hypertrophic obesity is a condition of cellular BMP4 resistance.

Of note, *CHRDL1* is the most highly expressed BMP inhibitor in differentiated (pre)adipocytes and, surprisingly, the highest expression is seen in mature adipose cells (Supplementary Table 1). Human tissue expression pattern also shows *CHRDL1* to be most highly expressed in the subcutaneous adipose tissue (Supplementary Fig. 1A) and to be higher in obese than in lean individuals in this depot (Supplementary Fig. 1B).

Regulation of BMP4 and BMP4 Inhibitors during Adipogenesis

To characterize the potential role of the BMP4 inhibitors, we examined their expression following differentiation of subcutaneous adipogenic precursor cells. *BMP4* increased as expected and all but two of the inhibitors (*CHRDL1* and *noggin*) were reduced in differentiated cells (Fig. 2A). We also analyzed *BMP2* and *BMP7* following differentiation. However, *BMP2* transcript levels decreased (Fig. 2A) while *BMP7* was generally not expressed in human preadipocytes or differentiated adipose cells and we could not detect BMP7 protein in Western blots (data not shown). Thus, these were not further studied.

CHRDL1 increased gradually during differentiation while *noggin* showed a transient early increase followed by inhibition (Fig. 2B-C). The expression of these inhibitors was accentuated by the presence of BMP4, probably due to the enhanced differentiation induced by BMP4 (Fig. 2B-C). We characterized the time-course for these inhibitors in relation to *PPARγ* transcript levels and both the increase in *CHRDL1* and the decrease in *GREM1*

followed a similar time-course as *PPARγ* induction (Fig. 2D). The other BMP inhibitors (*CHRD*, *FST* and *BAMBI)* only showed minor differences in mRNA levels following preadipocyte differentiation suggesting that they played a less important role for the apparent BMP4 resistance (Supplementary Table 1).

To validate the potential importance of CHRDL1 and noggin in regulating human white adipogenesis we examined their expression after 9 days in preadipocytes undergoing poor or very good adipogenic differentiation. We also examined factors in the differentiation cocktail, which affected their expression. Table 1 shows that induction of *CHRDL1* was *positively* related to that of *PPARγ* as well as ability of the cells to undergo differentiation. This is consistent with an overall positive correlation between *CHRDL1* and *PPARγ* expression in fully differentiated (pre)adipose cells (Fig. 2E). Thus, CHRDL1 is a good marker of adipogenic differentiation consistent with the very high expression in mature adipose cells (Supplementary Table 1).

In contrast to *CHRDL1*, there was no clear difference in *noggin* expression. This finding, together with the lack of correlation with cell size/BMI, suggests that noggin is an unlikely contributor to the preadipocyte BMP4-resistance in hypertrophic obesity. In contrast, the ability to inhibit *GREM1* following differentiation was positively associated with *PPARγ* transcript activation suggesting that GREM1 could be an important endogenous regulator of BMP4 resistance and adipogenesis (Fig. 2F).

Effect of Silencing or Overexpressing CHRDL1

CHRDL1 was silenced by around 90% with siRNA (Fig. 3A) but this reduction did not increase, but markedly *reduced,* transcriptional activation of *PPARγ, adiponectin* and *FABP4* during differentiation (Fig. 3B). Furthermore, addition of BMP4 did not rescue the inhibitory effect of siCHRDL1 on adipogenic differentiation (Fig. 3B).

Interestingly, cells transfected with CHRDL1 siRNA showed increased *ACTA2* (*α-SMA*), a marker of the myofibroblast phenotype, as well as *INHBA* mRNA levels (Fig. 3C) suggesting that CHRDL1 may cross-talk with other signaling pathways, possibly TGFβ which is known to inhibit adipogenesis and to increase these markers of fibrosis (23,39-41). Thus, CHRDL1 is not an endogenous inhibitor of BMP4 in human preadipocytes and we also saw no inhibitory or positive effect on pSMAD1/5/8 activation by BMP4 following CHRDL1 overexpression in human preadipocytes (Fig. 3D-E).

Consistent with the concept that CHRDL1 cross-talks with other signaling pathways, we did not find that overexpressing CHRDL1 in preadipocytes directly enhanced differentiation (Fig. 3F) but, instead, it decreased *ACTA2*, *CTGF* and *INHBA* further supporting potential crosstalk with TGFβ (Fig. 3G). We also examined if silencing CHRDL1 altered initial upstream signaling of TGFβ, measured as pSMAD3 increase by TGFβ after 120 min, but saw no such direct upstream effect (data not shown).

GREM1 is Increased in Hypertrophic Obesity

GREM1 mRNA levels in whole SAT biopsies were positively correlated with adipose cell size of the donors (Fig. 4A) and ability to inhibit GREM1 during differentiation of preadipocytes was also markedly reduced in hypertrophic obesity and correlated with the cell size of the donors (Fig. 4B). This is consistent with the negative correlation between *GREM1* and *PPARγ* transcriptional activation seen in differentiated cells (Fig. 2F).

Since GREM1 is a secreted BMP inhibitor, we measured GREM1 in the culture medium of human preadipocytes undergoing differentiation. Secretion of GREM1 correlated positively with *GREM1* mRNA levels (Fig. 4C) and it remained essentially stable after day 6 of differentiation (Fig. 4D). This is in accordance with the rapid down-regulation of GREM1 during initiation of differentiation and the partial rebound effect seen at later time points (Fig.

4E). Furthermore, GREM1 secretion by differentiated preadipocytes correlated positively with BMI of the donors and also tended to correlate with cell size (Fig. 4F-G). We also verified that GREM1 protein is a bona fide inhibitor of BMP4 (as well as BMP7 - data not shown) signaling and pSMAD1/5/8 activation (Fig. 4H-I).

We also analyzed the expression of *GREM1* in different human tissues. It is highly expressed in the adipose tissue and has a higher expression in omental than in subcutaneous adipose tissue (Supplementary Fig. 2).

Effect of Silencing GREM1 on White and Beige Adipogenesis

We then examined the effect of silencing GREM1 (>90% inhibition, Fig. 3A) in undifferentiated preadipocytes and found *PPARγ* induction to be significantly ($P<0.05$) increased to a similar extent as in control cells incubated with BMP4 and the effect of pioglitazone was also significantly higher ($P<0.02$) (Fig. 5A). Similarly, the PPARγ transcriptional activator *ZNF423* (42) was significantly increased (Fig. 5B) and the effect of both BMP4 and pioglitazone were also higher in siGREM1 cells (Fig. 5B). These effects of silencing GREM1 indicate that it is an important regulator of the pro-adipogenic effect of endogenous BMP4 in human preadipocytes.

BMP4 has also recently been shown to enhance beige adipogenesis in human precursor cells (17). We also found addition of BMP4 to increase the beige adipose marker *TMEM26* in the preadipocytes ($P<0.05$) and this effect was markedly increased in siGREM1 cells while adding the PPARγ ligand pioglitazone alone had no effect on *TMEM26* under any of these conditions (Fig. 5C). Interestingly, and as also previously noted (17), the beige adipose cell marker *TBX1* was expressed at low levels but it increased significantly following addition of BMP4 (data not shown). Nevertheless, the expression of other markers of beige cells, *TMEM26* and *CD137* (*TNFRSF9*), were closely correlated in human preadipocytes (Fig. 5D).

The brown adipose cell marker, *ZIC1*, but not *PRDM16* (data not shown), was clearly increased in siGREM1 cells ($P<0.02$) and this was further enhanced by BMP4 (Fig. 5E). Unexpectedly, we also found *BMP8B*, a regulator of thermogenesis and BAT activation in mice (43), to be robustly expressed in human SAT as well as in the preadipocytes but only slightly increased by the addition of BMP4 and/or by silencing GREM1 (data not shown). However, *BMP8B* expression correlated closely with that of *TMEM26* suggesting that it is a marker of beige adipogenesis in human adipose tissue (Fig. 5F). The mRNA levels of *UCP1* in control human preadipocytes were low but the expression was increased and became measurable in most siGREM1 cells whether or not cAMP or BMP4 was present (data not shown). Importantly, silencing GREM1 in human preadipocytes increased the mitochondrial content as measured by the Mito Tracker Red and this was further increased by BMP4 (Fig. 5G-H). Furthermore, induction of UCP1 was also seen under these conditions (Fig 5G-H). *PGC1α* was also increased by both silencing GREM1 and/or adding BMP4 and further markedly increased by adding pioglitazone (Fig 5I).

Taken together, GREM1 is an attractive target for overcoming the BMP4 resistance in hypertrophic obesity since silencing GREM1 enhances endogenous BMP4 signaling and action, increases *ZNF423* and *PPARγ* induction as well as expression of markers of an oxidative beige/brown adipose cell phenotype.

White Adipogenic Differentiation Reduces Beige/brown Adipogenesis

We also examined the beige/brown adipose cell markers *CD137, TMEM26* and *ZIC1* following induction of white adipose cell differentiation of the preadipocytes. All beige markers were all dramatically reduced suggesting the presence of multipotent precursor cells, which could undergo either beige/brown or white differentiation depending on the ambient signals (Fig. 5J). Similarly, *BMP8B* was also reduced following induction of white

adipogenesis (Fig. 5J). However, silencing GREM1 before white adipogenic differentiation re-activated *TMEM26* (Fig. 5K) and *ZIC1* (data not shown) suggesting that GREM1 and/or the enhanced BMP4 signaling exerted a particularly prominent effect in promoting the beige/brown phenotype of human preadipocytes. Ongoing studies are aimed at clarifying detailed molecular mechanisms for this.

DISCUSSION

Hypertrophic obesity is associated with a dysregulated adipose tissue, inflammation and local and systemic insulin resistance (4,5,8,9) and the degree of insulin resistance is positively correlated with adipose cell size (16) as well as future risk of developing T2D (44). Thus, understanding adipose precursor cell recruitment and differentiation in the large SAT can open new possibilities for preventing ectopic fat accumulation and the metabolic complications of obesity. Furthermore, recent animal data have shown that cells in SAT have the greatest plasticity in terms of inducing beige/brown adipose cells and that these cells play an important role in total body energy expenditure and weight gain (45). BMP4 regulates adipose precursor cell commitment into the white adipose lineage (6,13,46), is induced in human preadipocytes undergoing differentiation (16) and overexpression in the adipose tissue in mice leads to an increased beige/brown phenotype in SAT (46) and BMP4 can also activate beige adipose cell development in human precursor cells (17).

Since hypertrophic obesity is associated with an impaired subcutaneous adipogenesis (16), we postulated that this could be due to reduced BMP4 in the precursor cells. However, we found that cellular *BMP4* transcript and protein levels are increased in hypertrophic obesity and BMP4 is secreted by the adipose cells supporting a functional feedback regulation promoting the recruitment of new adipose cells when needed rather than just expanding existing cells. These results suggest that the precursor cells are resistant to secreted BMP4 possibly due to increased activity of the endogenous BMP inhibitors.

The time-course for the induction of the BMP inhibitors during preadipocyte differentiation showed that all but *noggin* and *CHRDL1* were markedly decreased. *CHRDL1* increased during differentiation and was a positive marker of *PPARγ* induction and adipogenesis and, in fact, it is not an inhibitor of BMP4 in human preadipocytes. Expressing CHRDL1 in the undifferentiated preadipocytes had no direct effect on *PPARγ* but reduced

several markers of fibrosis and TGFβ activation. Silencing CHRDL1 reduced *PPARγ* and increased *ACTA2* (*αSMA*), *CTGF* and *INHBA* as markers of a myofibroblast phenotype. These findings indicate that CHRDL1 cross-talks with, and inhibits, the pro-fibrotic TGFβ pathway which is consistent with the finding that genetic deletion of CHRDL1 in mice enhances renal fibrosis. (31). Interestingly, CHRDL1 has been found in the human adipose tissue secretome (47) indicating that it may be a circulating protein but nothing is known about potential endocrine effects.

Noggin is a well-established inhibitor of BMP4 and, as expected, antagonizing noggin enhances endogenous BMP4-stimulated preadipocyte differentiation to both white (6) and beige phenotype (data not shown). However, its temporal expression, lack of association with adipose cell size and precursor cells differentiation capacity suggests that it is not specifically related to the reduced adipogenesis and BMP4 resistance in hypertrophic obesity.

In contrast, GREM1 is a secreted and powerful inhibitor of both BMP4 and BMP7 signaling as well as of adipogenic differentiation. The time-course for *GREM1* is consistent with an important regulatory effect on adipogenesis. It is secreted by human (pre)adipocytes, secreted protein and mRNA levels were positively correlated and transcript levels were closely correlated with adipose cell size. Silencing GREM1 also increased the effect of BMP4 on *PPARγ* induction. Thus, GREM1 is an important regulator of white adipogenesis and the ability of BMP4 to induce commitment and differentiation.

Interestingly, GREM1 also regulates BMP4-induced beige/brown adipogenesis as well as the effect of BMP4 on markers of mitochondrial content and *PGC1α* induction. Unexpectedly, we also found BMP4 and GREM1 to be involved in the regulation of *BMP8B*. BMP8B activates thermogenesis in brown adipose tissue in mice but it is not expressed in white adipose tissue (43). However, *BMP8B* is robustly expressed and closely associated with TMEM26 suggesting that it is a marker of beige adipose cells in human SAT.

Induction of white adipogenesis markedly reduced *TMEM26, CD137, BMP8B and ZIC1* in the human cells suggesting the presence of multipotent precursor cells which could enter both white and beige/brown adipogenesis. This concept is similar to the proposal by Lee et al. (48) in murine SAT and lineage-tracing experiments have shown that beige adipose cells can revert to white cells (49). Importantly, we also found that silencing GREM1 in partly *differentiated* white adipocytes re-activated *TMEM26* and *ZIC1* suggesting a transdifferentiation potential and that GREM1/BMP4 strongly promotes a beige/brown phenotype of already committed and differentiating preadipocytes. Our findings are schematically illustrated in Fig. 6.

Taken together, our results show that BMP4 is an important endogenous regulator of human white and beige adipogenesis. However, the powerful BMP4/7 inhibitor GREM1, which is specifically increased in hypertrophic obesity, antagonizes these effects and is an interesting target in human obesity/T2D.

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Duality of Interest. J.G and C.R are employees of MedImmune LLC.

Author Contribution. U.S designed the experiments, did the data analysis and interpretation, and wrote the manuscript. B.G performed experiments and analyzed data and wrote the manuscript. A.H, S.H and J.M.H performed experiments. P.A.S contributed with data in human tissues. C.R and J.G contributed with discussion and reviewed the manuscript. U.S. is the guarantor of this work and, as such, takes full responsibility for the work as a whole, including the study design, access to data, and the decision to submit and publish the manuscript.

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Table 1

To identify factors in the differentiation cocktail that regulated *CHRDL1* and *Noggin* and their relation to preadipocyte differentiation potential, single additions or combinations thereof were added to DMEM/10% FBS. IBMX/pio/dexa/insulin represents full differentiation cocktail, shown in bold. DMEM/FBS is used as reference. Results from two individuals with different degrees of differentiation.

Degree of differentiation	≈ 5%			≈ 50%		
	CHRDL1	*Noggin*	*PPARγ2*	*CHRDL1*	*Noggin*	*PPARγ2*
DMEM	0.85	0.91	1.15	1.05	0.94	1.00
IBMX	0.58	0.89	1.35	0.88	0.77	1.11
Pio	0.70	1.08	1.21	1.17	0.76	2.75
IBMX/pio	0.99	0.69	10.2	8.15	0.68	573
IBMX/dexa	2.65	3.05	20.2	5.18	1.62	268
Cocktail	**6.16**	**1.93**	**274**	**45.2**	**1.28**	**3243**

Differentiation in % is related to % surface that was covered with lipid droplets. The table shows data from differentiation day 9 when the cells had started acquiring lipids. Pio = pioglitazone and dexa = dexamethasone.

Figure 1 - *BMP4* mRNA levels in mature adipose cells correlate with adipocyte cell size. Human adipocytes were isolated from subcutaneous adipose tissue biopsies after collagenase digestion and RNA extracted. (*A*) *BMP4* mRNA levels correlate significantly with adipose cell size ($P<0.01$, n=33), (*B*) but not with BMI over this limited range. (*C*) BMP4 protein is induced during differentiation of human preadipocytes. Preadipocytes from two individuals were isolated from human adipose tissue, differentiated or not into adipocytes and BMP4 protein detected with immunofluorescence. Double staining with anti-BMP4 and DAPI. (*D*) Human adipose tissue biopsies were analyzed by immunoblot for intermediate- and low-molecular weight BMP4 protein. Results from 7 different donors from the same immunoblot. AKT was used as loading control and rhBMP4 was included as control. (*E*) Ratio of total BMP4/AKT, $P=0.01$. (F) Secretion of BMP4 to the medium of preadipocytes undergoing differentiation from day 0 to days 0-3 and 9-12. Gene expressions were first normalized to 18S rRNA then normalized to one individual (=1). Spearman's rank correlation was used to analyze data which were not normally distributed.

Figure 2 - Induction of *BMP4* and BMP4 inhibitors during differentiation of human preadipocytes. Isolated human stromal cells from subcutaneous adipose tissue were induced to differentiate. mRNA was extracted and analyzed with Q-PCR. (*A*) mRNA levels of BMP2 and 4 and the BMP4 inhibitors at day 14 of differentiation (n=24). The BMP4 inhibitors *CHRDL1* (*B*) and *noggin* (*C*) are rapidly induced during differentiation. (*B-C*) controls, grey bars and BMP4 stimulation, black bars (n=3). (*D*) Time course for changes in *CHRDL1*, *PPARγ* and *GREM1* mRNA levels during initiation of differentiation. *CHRDL1* (black bars), *PPARγ* (grey bars), *GREM1* (dark grey bars). Log scale was used due to large differences between genes. (*E*) There was a positive correlation between changes in *PPARγ* and *CHRDL1* mRNA, $P<0.01$ even when the individual with high *CHRDL1* expression was omitted

(*P*<0.01), while (*F*) suppression of *GREM1* correlated with the increase of *PPARγ mRNA levels* after differentiation, *P*<0.01 (n=38, differentiation day 14). Results were first normalized to 18S rRNA then normalized to expression levels in the control sample/DMEM (=1) at day 0. Results are means ±SEM. (*)*P* <0.1 **P* <0.05, ***P* <0.02, ****P* <0.002.

Figure 3 - Silencing the BMP4 inhibitor CHRDL1 in preadipocytes elicits negative effect on adipogenesis. Human stromal cells were induced to differentiate into adipocytes for 5 days with scrambled or specific siRNA. (*A*) *CHRDL1* and *GREM1* expression in differentiated cells was reduced with 87% respective 92% after silencing and *GREM1* expression in preadipocytes was reduced with 92%. (*B*) *PPARγ, adiponectin (APM1) and FABP4* mRNA levels were reduced by CHRDL1 siRNA. (*C*) Silencing CHRDL1 increases *ACTA2* and *INHBA* mRNA levels. (*D-E*) Phosphorylation of SMAD1/5/8 is not changed in cells transfected with CHRDL1. The cells were incubated for 2h with 40 ng/mL BMP4. Noggin, 100 ng/mL, was used as BMP4 inhibitor. Results are means ±SEM of 3 experiments. (*F*) Human preadipocytes were transfected with CHRDL1 or empty vector. Differentiation was initiated 48h after transfection and mRNA levels were analyzed at differentiation day 6. *CHRDL1* expression in cells transfected with an empty vector was ≈10-fold induced at differentiation day 6 while transfection with CHRDL1 further increased *CHRDL1* ≈20-fold, i.e., 150-200-fold. (*G*) CHRDL1 overexpression reduces *ACTA2, CTGF* and *INHBA* mRNA levels in preadipocytes. Results were first normalized to 18S rRNA then normalized to expression levels in the control sample (scrambled = 1) or vector. Results are means ±SEM of 4 experiments. (*)*P*<0.1, **P*<0.05, ***P*<0.02, ****P*<0.002.

Figure 4 - *GREM1* mRNA levels are positively correlated with cell size of the donors. (*A*) *GREM1* mRNA and cell size in whole adipose tissue, *P*<0.05, n=29. (*B*) *GREM1* mRNA and

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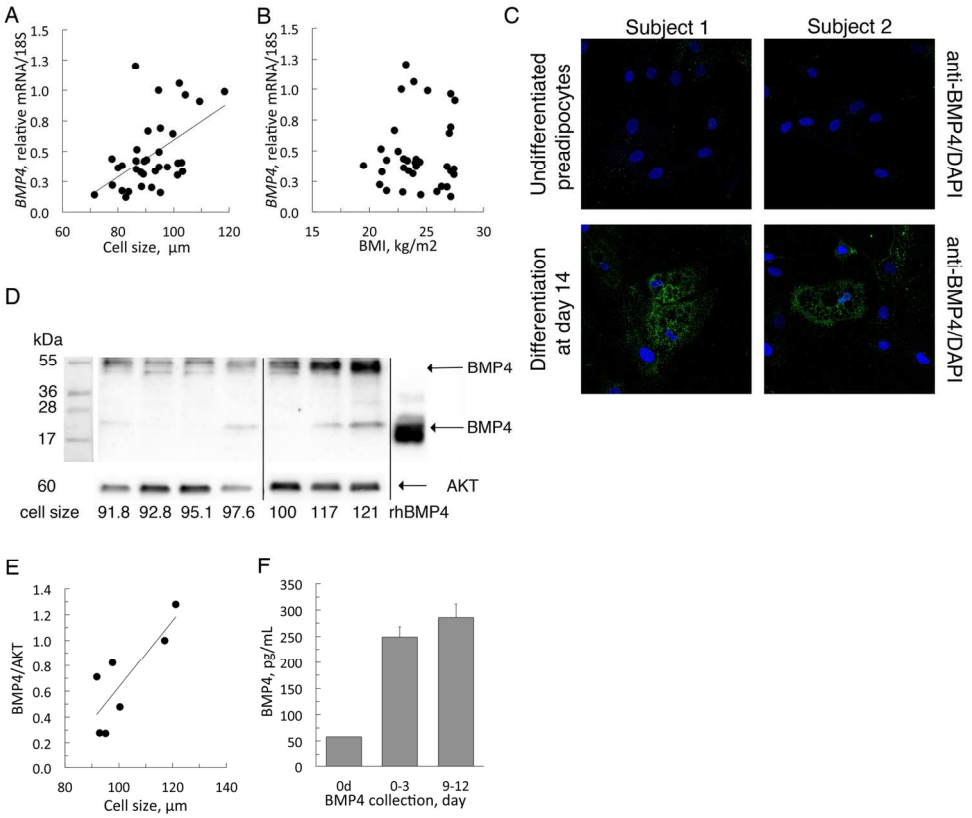
Figure 5 - Silencing GREM1 in preadipocytes induced a beige/brown adipose phenotype. (*A*) *PPARγ2* was increased by silencing GREM1 and further induced by 1 μM pioglitazone, (*B*) comparable to the PPARγ transcriptional activator *ZNF423*. (*C*) Similarly, *TMEM26* was significantly increased but pioglitazone (pio) had no direct effect. (*D*) *TMEM26* and *CD137* mRNA levels were correlated in undifferentiated preadipocytes, $P<0.001$, n=23. The correlation remained significant when the outlier was omitted, $P<0.05$. Results were first normalized to 18S rRNA and than to one subject =1. (*E*) The brown adipose marker *ZIC1* was induced in siGREM1 cells and further increased by BMP4. (*F*) *TMEM26* correlated also with the transcript level of *BMP8B*, $P<0.01$, n=14, in undifferentiated preadipocytes. Results were first normalized to 18S rRNA and than to one subject =1. (*G*) Silencing GREM1 increased the mitochondrial content in human preadipocytes. Mitochondria were stained with Mito Tracker Red. Silencing GREM1 also induced expression of UCP1, which was further enhanced by

BMP4. Double staining with DAPI. (*H*) Quantification of Mito Tracker Red (4 subjects) and UCP1 staining (3 subjects). (*I*) *PGC1α* was induced by silencing GREM1 and an additive effect was seen with pioglitazone and BMP4 in combination. (*J*) Inducing white adipogenesis reduced *TMEM26, CD137, BMP8B* and *ZIC1* mRNA levels. Results from 2-5 individuals. *BMP8B* was only analyzed day 0 and 9. (*K*) Silencing GREM1 before induction of adipogenesis increased *TMEM26* in partly differentiated cells. Results from 6 individuals at differentiation day 9. Results were first normalized to 18S rRNA then normalized to expression levels in the undifferentiated sample at 0h or (*A-C, E, H-J*) the control sample (scrambled = 1). Results are means ±SEM of 6-9 experiments. (*)$P<0.1$, *$P<0.05$, **$P<0.02$, ***$P<0.002$.

Figure 6 - Schematic view of the regulation of commitment and differentiation of human subcutaneous adipose precursor cells by BMP4 and GREM1. BMP4 increases during differentiation of human preadipocytes and can exert autocrine/paracrine effects as a secreted molecule. This effect of BMP4 enhances commitment and differentiation of new preadipocytes and therefore strives to prevent adipose cell hypertrophy. The effect of BMP4 is regulated by the endogenous BMP inhibitors and, in human preadipocytes, GREM1 is a key regulator of the effect of BMP4 to promote both white and beige/brown differentiation.



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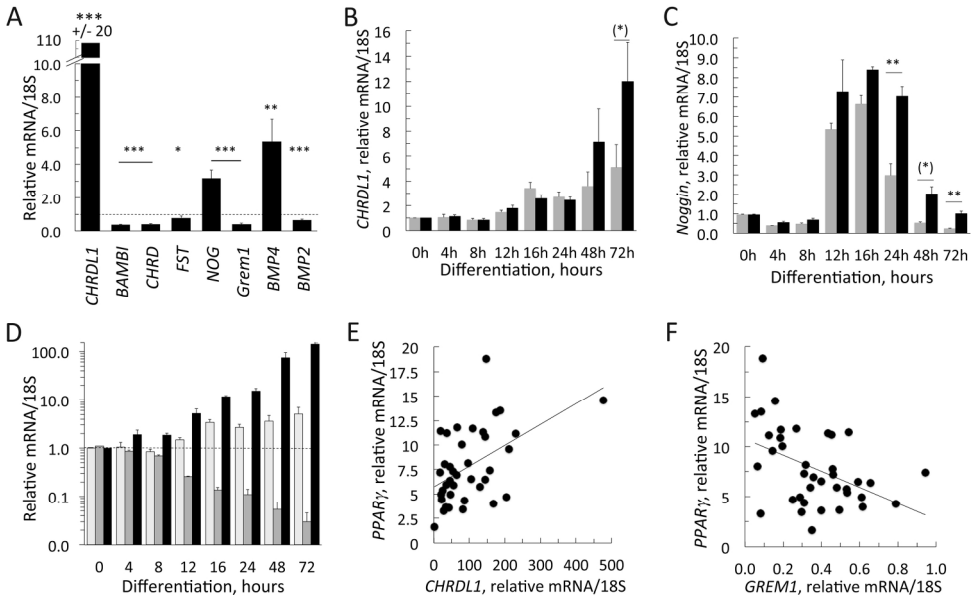

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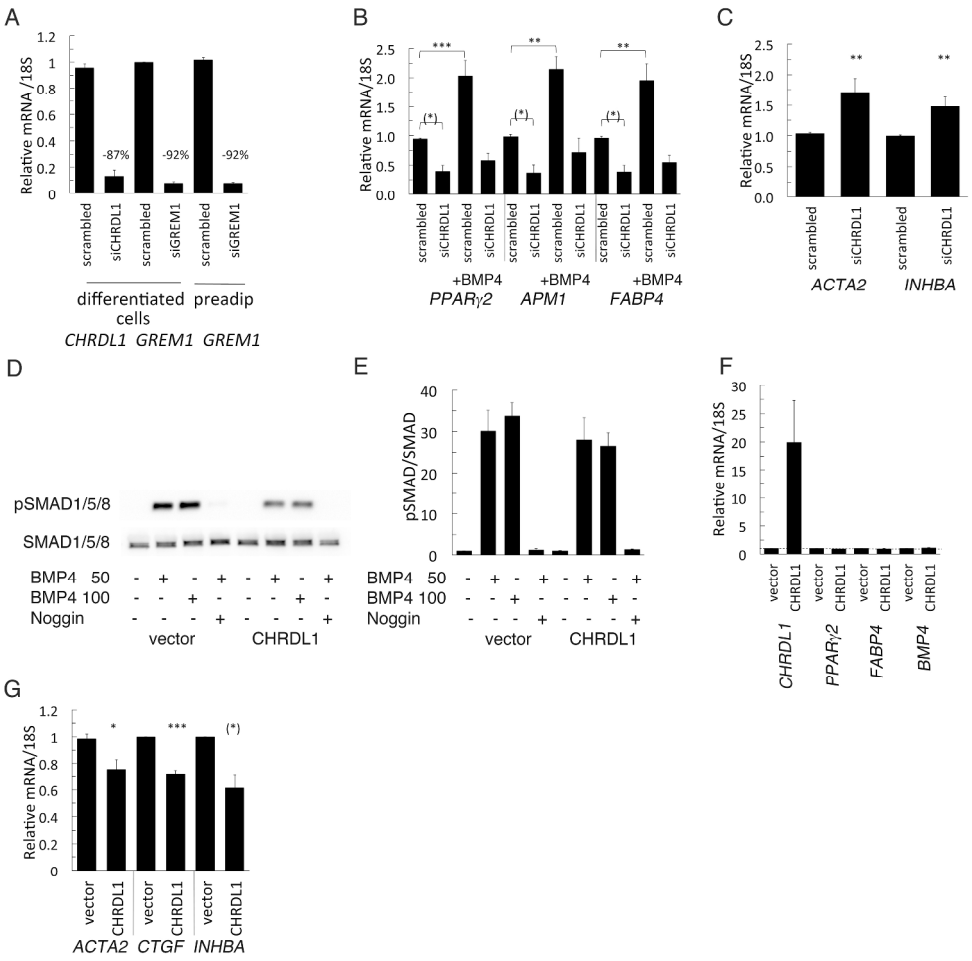

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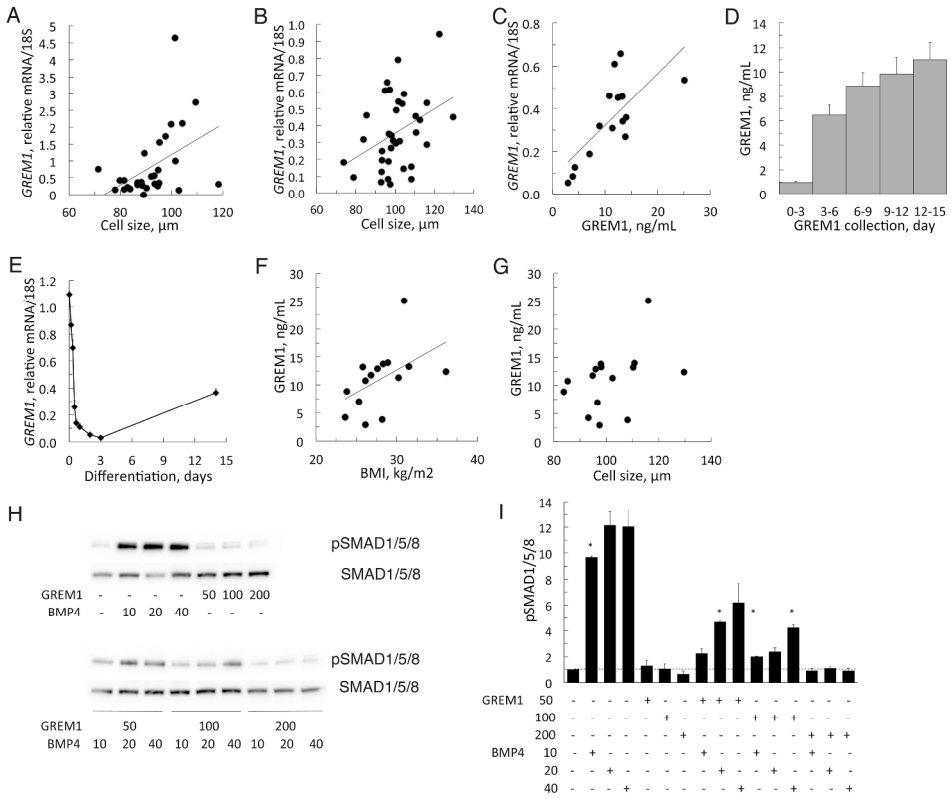

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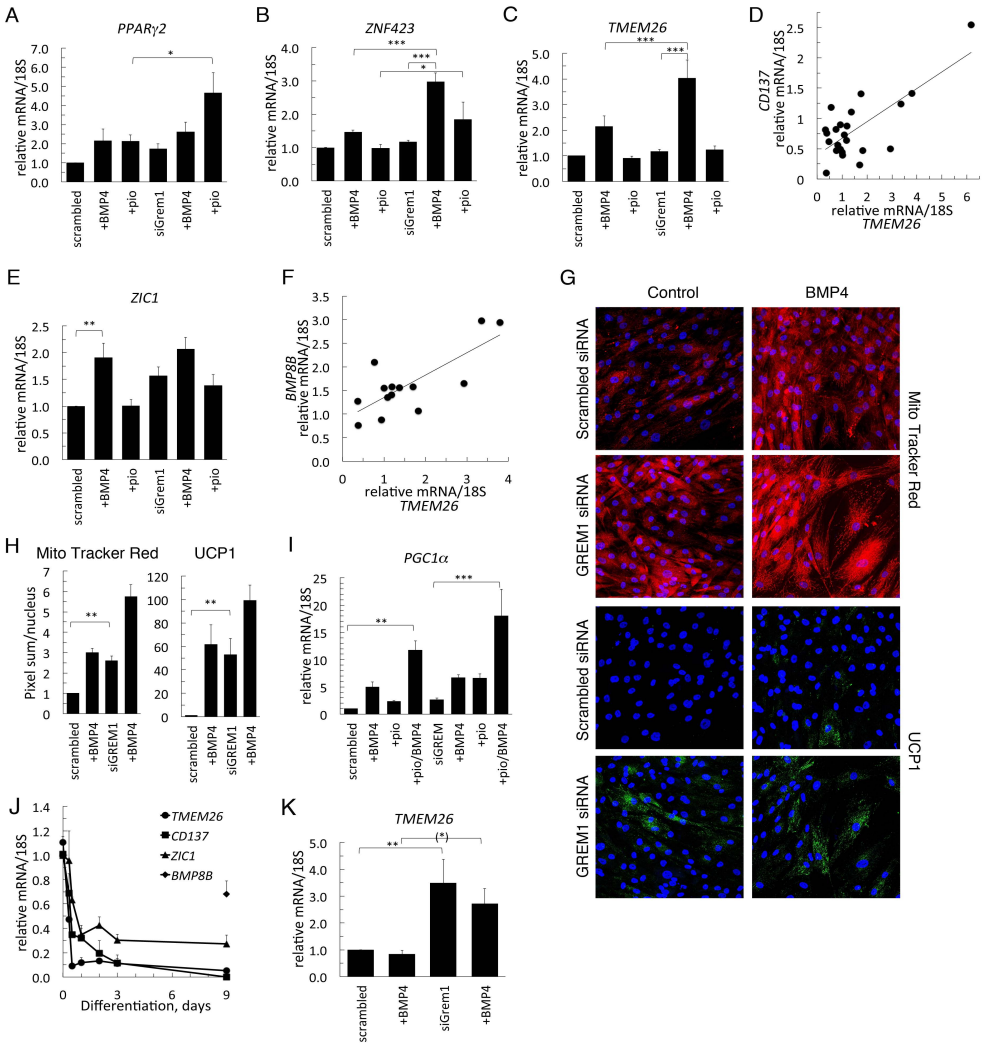

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(scrambled = 1). Results are means ±SEM of 6-9 experiments. (*)P<0.1, *P<0.05, **P<0.02, ***P<0.002.
192x210mm (300 x 300 DPI)

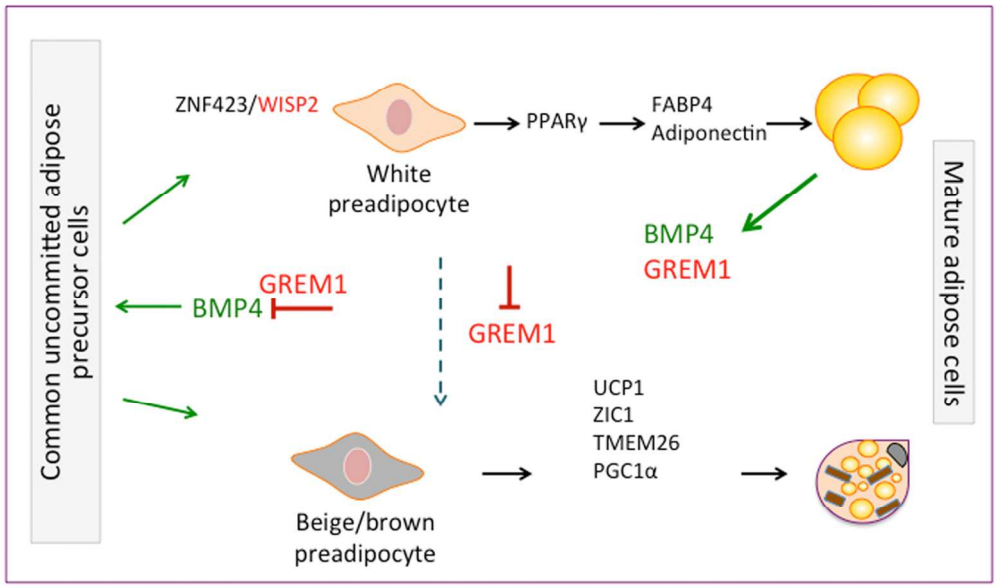


Figure 6 - Schematic view of the regulation of commitment and differentiation of human subcutaneous adipose precursor cells by BMP4 and GREM1. BMP4 increases during differentiation of human preadipocytes and can exert autocrine/paracrine effects as a secreted molecule. This effect of BMP4 enhances commitment and differentiation of new preadipocytes and therefore strives to prevent adipose cell hypertrophy. The effect of BMP4 is regulated by the endogenous BMP inhibitors and, in human preadipocytes, GREM1 is a key regulator of the effect of BMP4 to promote both white and beige/brown differentiation.
119x75mm (300 x 300 DPI)

SUPPLEMENTARY DATA

Supplementary Table 1. Ct-values for BMP antagonists in undifferentiated human preadipocytes, isolated mature adipocytes and *in vitro* differentiated human preadipocytes.

Gene	Undifferentiated preadipocytes mean deltaCt-value	Differentiated preadipocytes mean deltaCt-value	Isolated mature Adipocytes mean deltaCt-value
CHRDL1	19.8	13.7	10.9
CHRD	18.6	20.0	19.1
NOG	17.2	16.1	24.4
FST	14.2	14.7	20.2
BAMBI	13.2	14.7	16.3
GREM1	13.8	15.6	24.3

Analyses of mRNA were determined with quantitative real-time PCR (Q-PCR) and 18S was used as reference. A deltaCt-value >26 is considered as not expressed, between 22-26 as low expression and <15 is considered as high expression. Results are means of 34 individuals (isolated cells) and 24-38 individuals (*in vitro* differentiated cells) analyzed in duplicates.

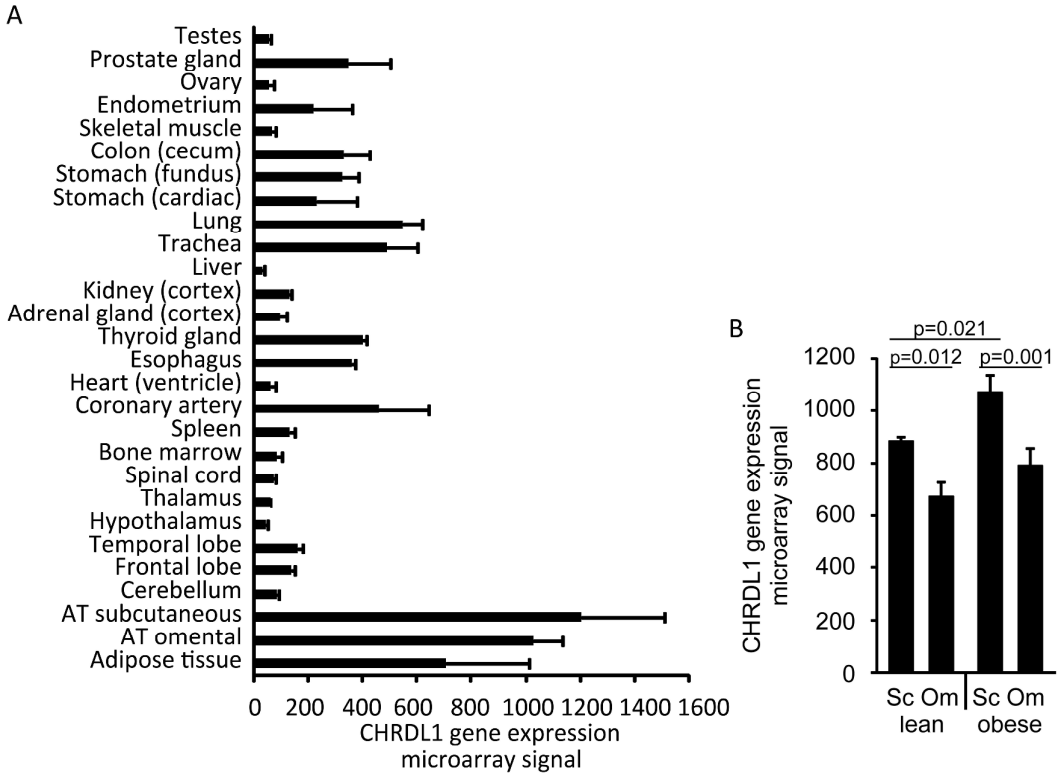
SUPPLEMENTARY DATA

Supplementary Table 2. Correlations between mRNA levels for BMP4 and BMP inhibitors in isolated adipocytes and adipose tissue biopsies.

Gene	Isolated adipocytes		Adipose tissue biopsies	
	R	*P*-value	R	*P*-value
CHRD	0.34	0.047	0.26	0.17
FST	0.04	0.82	-0.26	0.17
NOG	0.37	0.03	0.20	0.29
CHRDL1	0.69	<0.001	0.50	0.005
GREM1	0.38	0.044	0.38	0.02

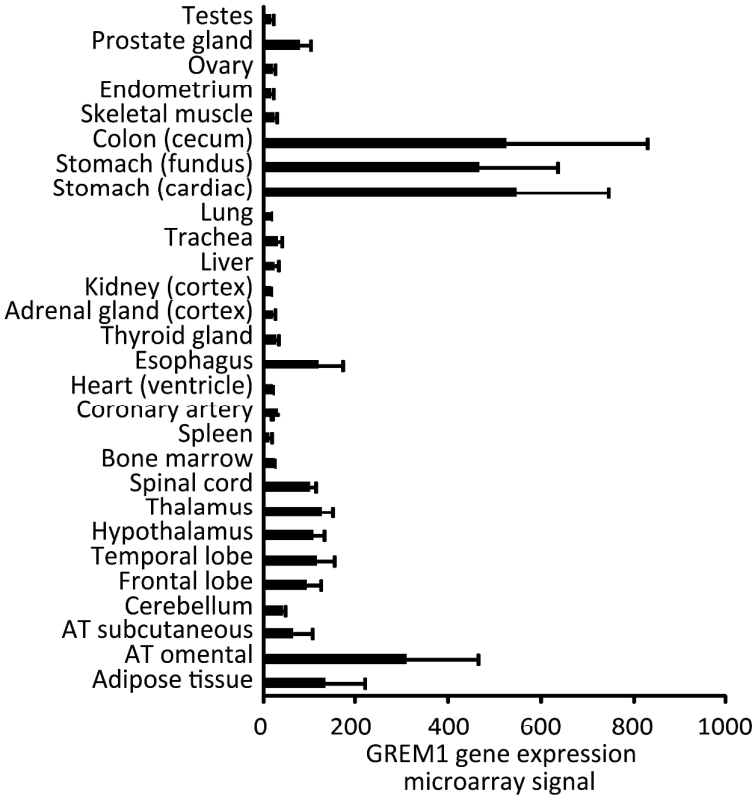
Results are means of 34 individuals analyzed in duplicates. mRNA levels were analyzed with Q-PCR and related to one individual (RQ=1). Spearman's rank correlation was used to analyze data, which were not normally distributed. A *P*-value <0.05 was considered as significant.

SUPPLEMENTARY DATA



Supplemental Figure 1. *CHRDL1* is highly expressed in human adipose tissues. (*A*) *CHRDL1* expression in human tissues analyzed by DNA microarrays. Data from the GSE3526 data set in the GEO database and is presented as mean expression +/- SEM. (*B*) *CHRDL1* gene expression in adipose tissue from lean vs. obese individuals. Paired abdominal subcutaneous (Sc) and omental (Om) adipose tissue. Surgical biopsies were obtained from ten women undergoing elective surgery (liposuction and elective gynecological procedures). These procedures were in accordance with guidelines of the South Birmingham Ethics Committee. The subjects were divided into two cohorts according to BMI (lean, BMI 23.0 ± 1.2 kg/m^2, n=5; obese, BMI 33.2 ± 3.1 kg/m^2, n=5). Gene expression was analyzed using the Human Genome U133 plus 2.0 DNA microarrays (Affymetrix). *CHRDL1* gene expression was analyzed using the 209763_at probe set.

SUPPLEMENTARY DATA



Supplemental Figure 2. Expression of *GREM1* in different human tissues. *GREM1* expression was analyzed by DNA microarrays. Data is from the GSE3526 data set in the GEO database and is presented as mean expression +/- SEM.