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ORIGINAL ARTICLE

IGFBP-2 inhibits adipogenesis and lipogenesis in human visceral, but not subcutaneous, adipocytes

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BACKGROUND/OBJECTIVE: IGF-binding protein (IGFBP)-2 is the principal IGFBP produced by white adipocytes during adipogenesis, and circulating levels are reduced in obesity. Overexpression of IGFBP-2 in transgenic mice prevents obesity, but depot-specific effects of IGFBP-2 on adipo/lipogenesis are unknown. The present study aimed to investigate whether IGFBP-2 affects adipo/lipogenesis in a depot-specific manner and explore potential mechanisms.

METHODS: Following adipocyte characterisation, IGFBP-2 levels were measured from human subcutaneous and visceral preadipocytes, and IGFBP-2 dose–responses were then undertaken with exogenous IGFBP-2 in an *in vitro* IGF-I-free system to examine adipo/lipogenesis. Following this, both types of adipocytes were transfected with human siRNA IGFBP-2 to assess auto-/para-/intra-crine effects, with and without additional add-back IGFBP-2. To elucidate the potential mechanisms, visceral preadipocytes were treated with either wild-type or Heparin Binding Domain (HBD)-mutant IGFBP-2 (which is unable to bind to cell-surface components), and experiments were also undertaken using Echistatin (an integrin receptor blocker). Outcomes included gene expression profiles, protein levels and phosphorylation and lipid staining.

RESULTS: Human visceral adipocytes produced significantly more IGFBP-2 than subcutaneous adipocytes. Subsequent dose–responses to IGFBP-2 demonstrated significant reductions in adipo/lipogenesis in visceral, but not subcutaneous, adipocytes in response to increasing IGFBP-2. Silencing IGFBP-2 resulted in exaggerated adipo/lipogenesis in visceral, but not subcutaneous, adipocytes, an effect completely inhibited by add-back IGFBP-2. These effects occurred in the absence of changes in IGF-I levels. HBD-mutant IGFBP-2 had reduced effects compared with wild-type IGFBP-2. Wild-type IGFBP-2 increased phosphorylation of focal adhesion kinase (FAK) and decreased phosphatase and tensin homolog (PTEN) levels, suggestive of integrin-mediated signalling. Blockade of this signalling, using Echistatin, completely negated the effects of IGFBP-2 on visceral adipo/lipogenesis.

CONCLUSION: IGFBP-2 inhibits both adipogenesis and lipogenesis in visceral, but not subcutaneous, adipocytes. This depot-specific impairment appears to be independent of IGF-I and involves cell-surface association of IGFBP-2 and activation of integrin signalling pathways.

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INTRODUCTION

Obesity continues to be a major public health concern worldwide.[1] Excessive accumulation of fat is due to an imbalance in energy intake and energy expenditure.[2] Positive energy balance leads to adipose tissue expansion, which is caused by adipocyte hypertrophy (enlargement of adipocyte volume due to increased lipogenesis) and adipose tissue hyperplasia (enhancement of adipocyte cell number by proliferation and differentiation of preadipocytes).[3,4] Adipocytes are the primary cellular component of adipose tissue and are the main storage site of energy in the form of triglycerides.[5] Release of fatty acids from adipocytes can lead to an elevation of triglyceride content in the circulation and tissues, such as the liver and skeletal muscle, which may contribute to the pathogenesis of obesity-associated metabolic disorders.[6] Adipocytes also secrete a number of hormones, termed adipokines, which can affect peripheral insulin sensitivity.[7]

The distribution of body fat is more critical than total body fat content in the development of comorbidities in obesity.[8] Individuals with abdominal or central obesity carry a greater risk of developing metabolic and cardiovascular diseases than those with subcutaneous or peripheral obesity.[9] The detrimental metabolic effect of visceral adipocytes is associated with differences in gene expression, adipokine secretion and insulin action.[9] As adipocytes expand they become dysfunctional, because larger adipocytes appear to be insulin-resistant and hyperlipolytic.[3] Visceral adipose tissue contains a greater number of large adipocytes than other fat depots, and this metabolically more active site predicts morbidity/mortality more strongly than subcutaneous adipose tissue.[10]

Adipocyte differentiation is tightly regulated by a number of adipogenic transcription factors, including nuclear receptor peroxisome proliferator-activated receptor-gamma (PPARγ) and CCAAT/enhancer binding protein-alpha (C/EBPα), both of which have important roles in the complex coordination of the expression of numerous genes during adipogenesis.[11] These transcription factors control the expression of adipocyte-specific

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genes, such as adiponectin, sterol regulatory element binding protein-1c (SREBP-1c) and fatty acid synthase (FAS), which cause morphological changes and affect lipid accumulation within the cells.[12,13]

Insulin-like growth factor-I (IGF-I) has been shown to have an important role in adipocyte proliferation and differentiation.[14] The bioavailability of IGFs is modulated by six homologous IGF-binding proteins (IGFBPs), which bind IGFs but not insulin.[15] Different tissues secrete different IGFBPs, with IGFBP-2 being the predominant IGFBP produced by white preadipocytes during adipogenesis.[16] IGFBP-2 is the primary circulating IGFBP in infancy,[17] and low IGFBP-2 levels may be associated with increased adiposity.[18,19] Circulating levels of IGFBP-2 are reduced in obese adults[20] and children,[21,22] an effect which is more pronounced with visceral adiposity than subcutaneous adiposity.[23] Mice with transgenic overexpression of IGFBP-2 do not develop either obesity or insulin resistance, even when fed a high-fat diet, and *in vitro* data from murine 3T3-L1 adipocytes suggest that IGFBP-2 is capable of preventing adipocyte differentiation.[24] Effects in human adipocytes are not known.

Beside the ability to bind IGFs via the IGF-binding domain and modulate the activity of IGFs, IGFBP-2 also binds to the components of the cell surface (for example, proteoglycans) independent of IGFs, primarily via two heparin-binding domains (HBDs).[25,26] This is also involved in the binding of IGFBP-2 to the receptor-type protein tyrosine phosphatase-β (RPTP-β) in osteoblasts.[27] In addition, IGFBP-2 contains a Gly-Arg-Asp (RGD) integrin-binding motif,[25,28] which has been shown to bind integrins, including the α5β1 receptor.[29] Both of these functional domains of IGFBP-2 demonstrate its potential ability to mediate IGF-independent activities. Recent studies have also demonstrated that the HBD moieties of IGFBP-2 inhibits preadipocyte differentiation in male mice.[30] However, the depot-specific effects of IGFBP-2 in human adipocytes are yet to be explored. Given the functional and molecular differences between subcutaneous and visceral adipocytes, and that IGFBP-2 levels are reduced in visceral adiposity, our hypothesis was that IGFBP-2 would have differing effects on fat development in the two adipose depots. Therefore the present study was undertaken to investigate whether IGFBP-2 differentially affects adipogenesis and lipogenesis in human subcutaneous and visceral preadipocytes and investigate potential IGF-I independent mechanisms.

MATERIALS AND METHODS

Growth and differentiation of human subcutaneous and visceral preadipocytes

Human subcutaneous and visceral preadipocytes, derived from healthy non-obese and disease-free adults (Lonza, Walkersville, MD, USA) were cultured with Preadipocyte Growth Medium containing Preadipocyte Basal Medium-2 (PT-8202, Lonza) supplemented with 10% fetal bovine serum (PT-9000 H, Lonza), 1% L-Glutamine (PT-9001 H, Lonza) and 0.1% Gentamicin Sulfate Amphotericin-B (PT-4504 H, Lonza). At 90% confluency, growth medium was replaced with Differentiation Medium (Preadipocyte Growth Medium supplemented with dexamethasone, 3-isobutyl-1-methylxantine, indomethacin and insulin (PT-9502, Lonza). Human subcutaneous and visceral preadipocytes were differentiated over 7–10 days. Treatments were performed in preadipocytes or in serum-starved (24 h) differentiated adipocytes.

Characterisation of subcutaneous and visceral adipocytes

To characterise adipocyte differentiation as well as IGFBP-2 mRNA expression and IGFBP-2 protein levels in conditioned medium from subcutaneous and visceral preadipocytes, human subcutaneous and visceral preadipocytes were differentiated for 10 days with 1-ml samples of conditioned medium collected daily from cell cultures harvested on days 1, 2, 4, 6, 8 and 10 of differentiation. For gene expression analysis, harvested cultures were extracted with Trizol Reagent (Invitrogen, Auckland, New Zealand), while for protein analysis, cell monolayer was scraped in the presence of ice-cold phosphate-buffered saline (PBS). Samples were stored at − 80 °C.

IGFBP-2 treatments

To investigate the effects of IGFBP-2 on adipogenesis and lipogenesis, human subcutaneous and visceral adipocytes were treated with IGFBP-2. Human recombinant IGFBP-2 was purified as we previously described.[25] To examine the effects of IGFBP-2 on lipogenesis, on day 7 of differentiation, subcutaneous and visceral adipocytes were treated with IGFBP-2 at 0, 25, 50 or 100 ng ml^{-1} in serum-free medium for 24 h. Conditioned media and cells were harvested and stored as above. Subcutaneous and visceral preadipocytes were treated with IGFBP-2 (100 ng ml^{-1}) on day 0, 4 and 7 of differentiation. All treatments were terminated at day 8, when lipid staining was performed (as described below). To determine the effects of IGFBP-2 on adipogenesis, subcutaneous and visceral preadipocytes were treated with IGFBP-2 (100 ng ml^{-1}) on day 0 of culture. Cells were harvested for gene expression or lipid staining on days 4, 7 and 10 of differentiation.

Small interfering RNA (siRNA) IGFBP-2

IGFBP-2 knockdown was achieved by transfecting differentiated subcutaneous and visceral adipocytes with human siRNA IGFBP-2 (SI00012502, Qiagen, Valencia, CA, USA) or with the non-silencing control AllStars Negative Control siRNA (Qiagen), in the presence of HiPerFect Transfection Reagent (Qiagen) as per the manufacturer's protocol. Adipocytes were transfected on day 7 of differentiation, IGFBP-2 was administered on day 8, and the cells were cultured for a further 24 h. Cells were harvested on day 9 for gene expression analysis or lipid staining.

HBD-mutant IGFBP-2

To explore which domain of IGFBP-2 is responsible for its effects on adipogenesis and lipogenesis in human visceral adipocytes, the HBD-mutant IGFBP-2 was utilised. Human recombinant HBD-mutant IGFBP-2 was purified as we previously described.[25] On day 0 of culture, visceral preadipocytes were treated with serum-free medium, PBS (vehicle), wild-type IGFBP-2 or HBD-mutant IGFBP-2 (100 ng ml^{-1}). Cultures were maintained until day 7 of differentiation, where cells were either harvested for gene expression or for protein analysis by cell scraping or lipid staining was performed.

Echistatin—integrin blocker

To determine whether IGFBP-2 regulates differentiation/adipogenesis in visceral preadipocytes through integrin signalling, on day 0 of culture, visceral preadipocytes were treated with 100 nM Echistatin (Disintegrin, Sigma-Aldrich, St Louis, MO, USA) or sterile water (vehicle) for 24 h prior to treatment with wild-type IGFBP-2 (100 ng ml^{-1}). Cultures were maintained until day 7 of differentiation, where cells were either harvested for gene expression or lipid staining was performed. To investigate whether the integrin-binding domain of IGFBP-2 is involved in the regulation of lipogenesis in differentiated visceral adipocytes, on day 7 of differentiation, visceral adipocytes were treated with serum-free medium or Echistatin at 100 nM for 24 h prior to treatment with or without IGFBP-2 (100 ng ml^{-1}) for a further 24 h. Cells were collected by cell scraping for protein analysis and stored at − 80 °C.

RNA analyses

Total RNA extraction and real-time quantitative-PCR (q-PCR). Total RNA was extracted using the Trizol method and processed as previously described.[31] Real-time q-PCR was performed using the LightCycler 480 II (Roche Diagnostics, Mannheim, Germany).[31] Quantitation was performed using the standard curve method, and gene expression levels were normalised to the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Human real-time q-PCR primers used are listed in Supplementary Table S1. These included primers for preadipocyte factor 1 (Pref-1), PPARγ, C/EBPα, adiponectin, phosphoenolpyruvate

carboxykinase (PEPCK), SREBP-1c, FAS and GAPDH (Sigma Chemical Co., St Louis, MO, USA).

Protein analyses

Cell lysates and western immunoblotting. Cells were lysed in RIPA buffer supplemented with Complete protease inhibitor cocktail (Roche, Mannheim, Germany) and PhosSToP Phosphatase Inhibitor cocktail (Roche, Penzberg, Germany) as previously described.[31] Protein concentration was quantified using the EZQ Protein Quantitation Kit (Invitrogen, Carlsbad, CA, USA). Protein samples (20 μg) were separated using gel electrophoresis and electrotransferred from gel to nitrocellulose membrane as previously described.[31] Membranes were blocked with 5% skim milk in Tris-buffered saline containing 0.05% Tween 20 for 1 h and probed overnight at 4 °C with antibodies raised against phosphorylation of focal adhesion kinase at site tyrosine 397 (pFAK^{y397}), FAK and phosphatase and tensin homolog (PTEN) (Cell Signalling Technology, Boston, MA, USA), Integrin α5 chain and Integrin β1 chain (BD Transduction Laboratories, San Jose, CA, USA), IGFBP-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:1000 dilution and GAPDH (Santa Cruz Biotechnology) at 1:10000 dilution. A secondary antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology) was applied at 1:2000 dilution for 1 h at room temperature. The SuperSignal West Pico Chemiluminescent Substrate system (Pierce, Thermo, Rockford, IL, USA) was used to detect immunoreactive bands, and results were analysed by densitometry using the ImageQuant TL software program (GE Healthcare, Sunnyvale, CA, USA).

Measurement of IGFBP-2 levels in conditioned media. The human IGFBP-2 DuoSet Enzyme-linked immunosorbent assay Development kit (DY674; R&D Systems, Inc., Minneapolis, MN, USA) was used to quantify human IGFBP-2 as per the manufacturer's instructions.

Measurement of Free IGF-I. Free IGF-I concentrations were measured using a human free IGF-I immunoassay kit (DFG100; R&D Systems, Inc.) as per the manufacturer's specifications.

Lipid staining. After treatments, adipocytes were washed with PBS, fixed in 4% formaldehyde solution, washed twice with PBS and incubated with a 1:200 dilution of high-content screening LipidTOX red neutral (Invitrogen, Eugene, OR, USA) in PBS for a minimum of 30 min before fluorescence microscopy imaging.

Statistical analysis

Data are presented as mean ± s.e.m. Results were analysed for statistically significant differences by one-way analysis of variance with Bonferroni's *post-hoc* test using SPSS (SPSS version 20.0; SPSS, Inc., Chicago, IL, USA). Values of $P < 0.05$ were considered significant.

RESULTS

Characterisation of subcutaneous and visceral preadipocytes

Degree of differentiation was assessed by gene expression patterns of preadipocyte marker pref-1, adipogenic transcription factors PPARγ and C/EBPα and an adipocyte-specific marker adiponectin. As expected, the expression of pref-1 in both human subcutaneous and visceral preadipocytes declined throughout differentiation and PPARγ increased from day 2 of differentiation, while C/EBPα increased from day 4 of differentiation and adiponectin increased from day 6 of differentiation (Figures 1a–d).[11,32,33] These data demonstrated effective adipocyte differentiation, alongside typical differentiated appearances (data not shown). Similar levels of pref-1 and PPARγ expression were found in subcutaneous and visceral adipocytes throughout differentiation. However, C/EBPα expression was lower on days 4–10, and adiponectin expression was higher on days 6–10 in visceral adipocytes when compared with subcutaneous adipocytes ($P < 0.001$).

Characterisation of IGFBP-2 gene expression, protein levels and production in subcutaneous and visceral preadipocytes

To examine whether subcutaneous and visceral adipocytes demonstrate depot-specific differences in IGFBP-2 gene and protein expression *in vitro*, IGFBP-2 gene expression and protein levels were quantified at days 0, 4, 7 and 10 of differentiation. IGFBP-2 gene expression increased by 1.6-fold (day 4; not significant), 3.4-fold (day 7; $P < 0.05$) and 4.7-fold (day 10; $P < 0.01$) in visceral adipocytes, while it increased by 1.9-fold (day 4; not significant), 2.8-fold (day 7; $P < 0.01$) and 3.4-fold (day 10; $P < 0.001$) in subcutaneous adipocytes (Figure 1e). There were no significant differences seen between visceral and subcutaneous IGFBP-2 gene expression. IGFBP-2 protein levels increased by 1.2-fold (day 4; not significant), 2.8-fold (day 7; $P < 0.001$) and 2.9-fold (day 10; $P < 0.001$) in subcutaneous adipocytes, while it increased by 1.4-fold (day 4; not significant), 3.6-fold (day 7; $P < 0.001$) and 3.9-fold (day 10; $P < 0.001$) in visceral adipocytes (Figure 1f). On days 7 and 10, IGFBP-2 protein levels were higher in visceral adipocytes than subcutaneous adipocytes ($P < 0.05$). IGFBP-2 concentrations were measured in conditioned media from paired differentiating subcutaneous and visceral adipocytes for 10 days. From day 4 of differentiation, visceral adipocytes produced higher (threefold to fourfold difference) amounts of IGFBP-2 than subcutaneous adipocytes despite identical plating densities ($P < 0.001$; Figures 1g and h).

IGFBP-2 downregulates gene expression of lipogenic markers in differentiated visceral adipocytes

To investigate the adipocyte depot-specific effects of IGFBP-2, day 7 differentiated human subcutaneous and visceral adipocytes were dosed with increasing concentrations of IGFBP-2. Given that visceral adipocytes appeared to produce up to 4.0 ng ml^{-1}ml of IGFBP-2 on day 10, treatments of 25, 50 and 100 ng ml^{-1} of IGFBP-2 were used to generate dose–response curves. In visceral adipocytes, IGFBP-2 treatment reduced the expression of PEPCK (a key marker of glyceroneogenesis), SREBP-1c and FAS (both markers of lipogenesis) in a dose-dependent manner (Figures 2a–c). The greatest reduction was seen at a dose of 100 ng ml^{-1}, in the order of 25% ($P < 0.001$). IGFBP-2 had no effect on PEPCK, SREBP-1c and FAS expression in subcutaneous adipocytes (Figures 2d–f).

IGFBP-2 inhibits adipocyte differentiation and lipogenesis in visceral, but not subcutaneous. adipocytes

The impact of IGFBP-2 on adipocyte differentiation was evaluated by treating subcutaneous and visceral preadipocytes with IGFBP-2 (at 100 ng ml^{-1}) throughout differentiation. In visceral adipocytes, IGFBP-2 inhibited preadipocyte differentiation into mature adipocytes as indicated by a reduction in PPARγ (36% on day 4; $P < 0.01$, 47% on day 7 and 73% on day 10; $P < 0.001$), C/EBPα (43% on day 4, 54% on day 7, 49% on day 10; $P < 0.001$) and adiponectin (day 4; $P < 0.05$, day 7; $P < 0.01$ and day 10; $P < 0.001$) expression compared with vehicle treatment (Figures 3A–C). In contrast, no differences were seen in PPARγ, C/EBPα or adiponectin expression in subcutaneous adipocytes on days 4, 7 or 10 of differentiation (Figures 3D–F). Consistent with this, IGFBP-2 reduced the number of lipid droplets per cell on day 4 (58%, $P < 0.01$), day 7 (62%, $P < 0.001$) and day 10 (72%, $P < 0.001$) of differentiation in visceral adipocytes, whereas it had no effect in differentiating subcutaneous adipocytes (Figure 3G).

To determine whether IGFBP-2 had a time-dependent effect on lipogenesis, it was administered from day 0, 4 or 7 of differentiation and terminated at day 8 when lipid staining was performed in subcutaneous and visceral preadipocytes. Treatment of IGFBP-2 on day 0 reduced lipid abundance by 70% ($P < 0.001$), on day 4 by 57% ($P < 0.001$) and on day 7 by 28% ($P < 0.05$) in

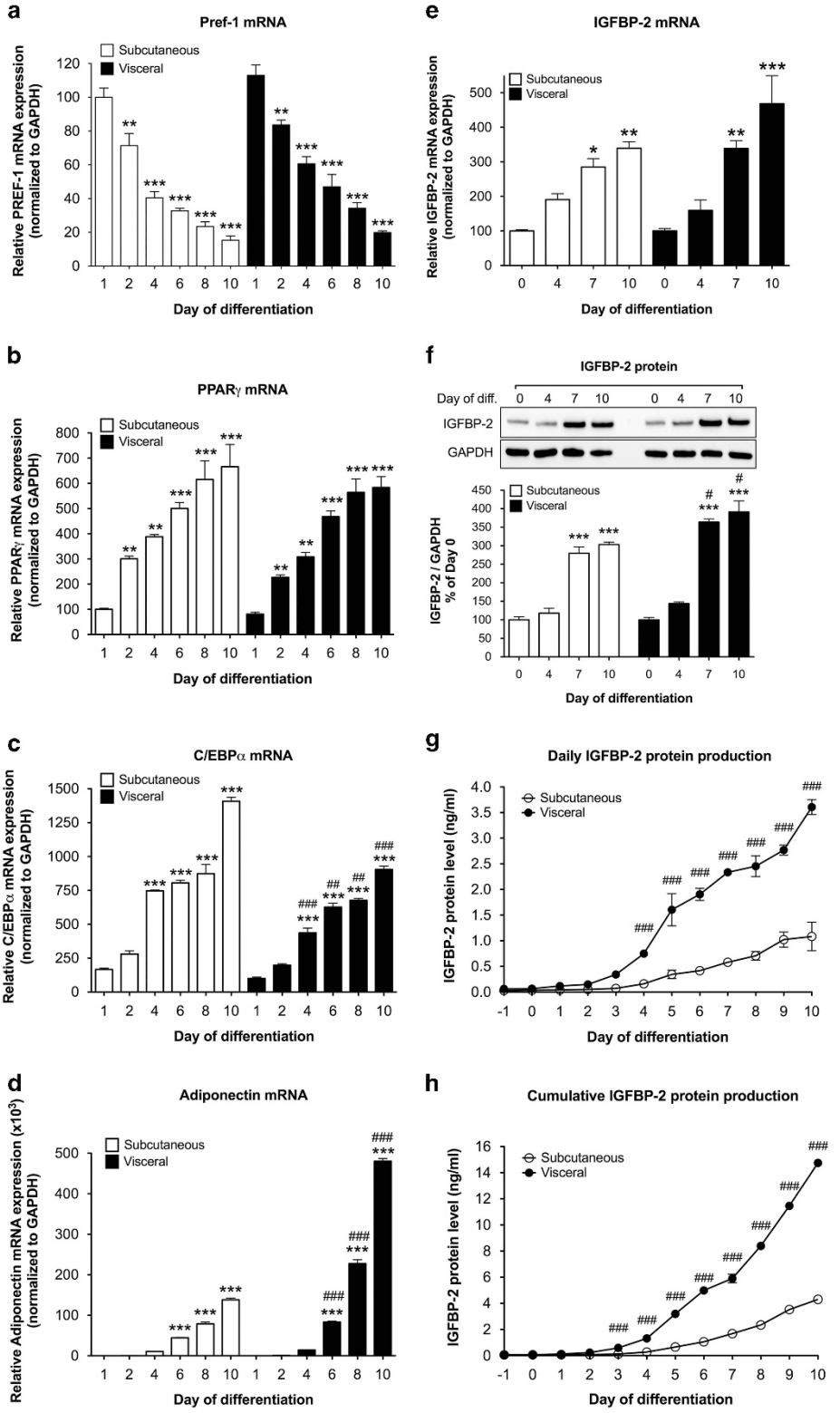


Figure 1. (**a**–**d**) Gene expression patterns of adipocyte-specific markers from differentiating human subcutaneous and visceral preadipocytes to adipocytes (day 1 to day 10). (**a**) Pref-1, (**b**) PPARγ, (**c**) C/EBPα and (**d**) adiponectin mRNA expression. Gene expression is corrected to the levels of GAPDH mRNA and is expressed relative to day 1 of adipocyte differentiation within adipose depot. (**e**, **f**) IGFBP-2 mRNA expression and protein levels in differentiating subcutaneous and visceral adipocytes (day 0, 4, 7 and 10). (**e**) IGFBP-2 mRNA expression is corrected to the levels of GAPDH mRNA and is expressed relative to day 0 of adipocyte differentiation within adipose depot. (**f**) IGFBP-2 protein level. Immunoblot quantification is normalised to GAPDH, and data are representative of three independent experiments. (**g**, **h**) Visceral adipocytes produce greater levels of IGFBP-2 protein than subcutaneous adipocytes. (**g**) Daily IGFBP-2 protein levels. (**h**) Cumulative IGFBP-2 protein levels. Quantification of IGFBP-2 protein in the conditioned media was by enzyme-linked immunosorbent assay. Gene expression and protein production data are representative of four independent experiments. All data are expressed as mean ± s.e.m. $^{*/\#}P < 0.05$, $^{**/\#\#}P < 0.01$, $^{***/\#\#\#}P < 0.001$, *Compared with control (first bar), #Compared with subcutaneous.

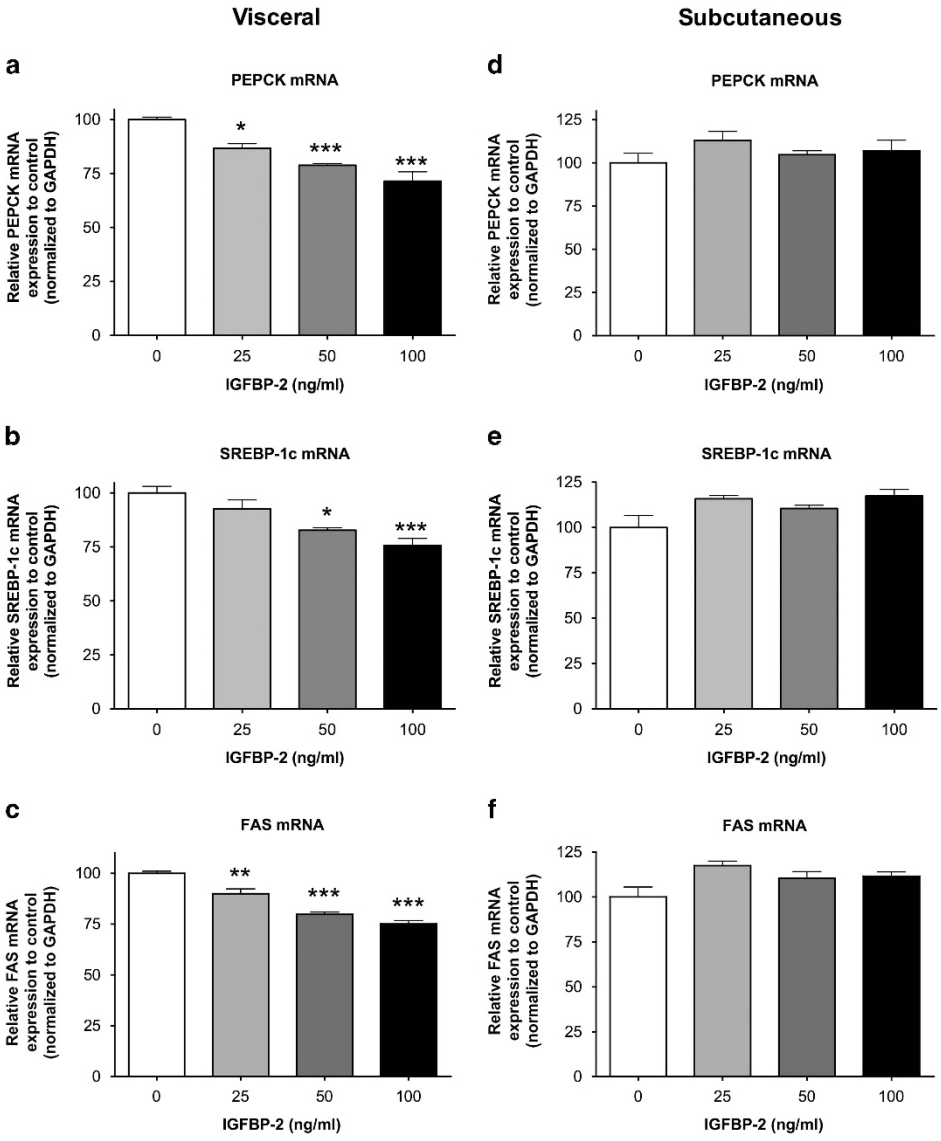


Figure 2. (**a**–**f**) Treatment of exogenous IGFBP-2 decreases gene expression of PEPCK (marker of gluceroneogenesis), SREBP-1c and FAS (markers of lipogenesis) in differentiated visceral adipocytes. Dose–response to IGFBP-2 (0–100 ng ml^{-1}) for 24 h in: visceral adipocytes (**a**) PEPCK, (**b**) SREBP-1c, and (**c**) FAS; and subcutaneous adipocyte (**d**) PEPCK (**e**) SREBP-1c, and (**f**) FAS mRNA expression. Gene expression is corrected to the levels of GAPDH mRNA and is expressed relative to vehicle (PBS)/control within adipose-depot. All data are expressed as mean ± s.e.m. Data are representative of four independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

visceral adipocytes, whereas there was no effect in subcutaneous adipocytes (Figure 3H).

Silencing IGFBP-2 increases lipogenesis and add-back IGFBP-2 restores inhibition of lipogenesis in differentiated visceral adipocytes

In these studies, we silenced the IGFBP-2 gene expression by 64% in day-7 differentiated visceral adipocytes and by 62% in subcutaneous adipocytes (Figure 4A). IGFBP-2 protein levels were reduced by 83% and 90% (Figure 4B), and IGFBP-2 production were reduced by 68% and 67% (Figure 4C), in visceral adipocytes and subcutaneous adipocytes, respectively. In visceral adipocytes, silencing of IGFBP-2 gene resulted in an increase in PEPCK (23%, $P < 0.05$), SREBP-1c (38%, $P < 0.001$) and FAS (26%, $P < 0.05$) expression. Adding back IGFBP-2 to already IGFBP-2-silenced adipocytes lowered SREBP-1c (24%, $P < 0.001$) and FAS (26%, $P < 0.01$) expression when compared with IGFBP-2-silenced adipocytes alone (Figures 4D–F). There were no differences seen with IGFBP-2 silencing or IGFBP-2 treatment in subcutaneous adipocytes (Figures 4G–I).

These findings were further supported by lipid staining studies where silencing IGFBP-2 increased lipid content (40% increase, $P < 0.01$) and treatment with IGFBP-2 decreased lipid content (65% reduction, $P < 0.01$). Adding back IGFBP-2 to already IGFBP-2-silenced visceral adipocytes decreased lipid staining compared with siRNA IGFBP-2 treatment alone (23% reduction, $P < 0.05$) (Figure 4J). Again, silencing IGFBP-2 or add-back IGFBP-2 dosing had no effect in subcutaneous adipocytes.

Given that no exogenous IGF-I was added to the system and to determine whether IGFBP-2 was inhibiting adipogenesis by modulating endogenous IGF-I activity, we quantified free IGF-I levels in the conditioned medium. Free IGF-I levels were unaltered (Figure 4K), suggesting that IGFBP-2 is capable of inhibiting adipogenesis via an IGF-I-independent-mediated pathway.

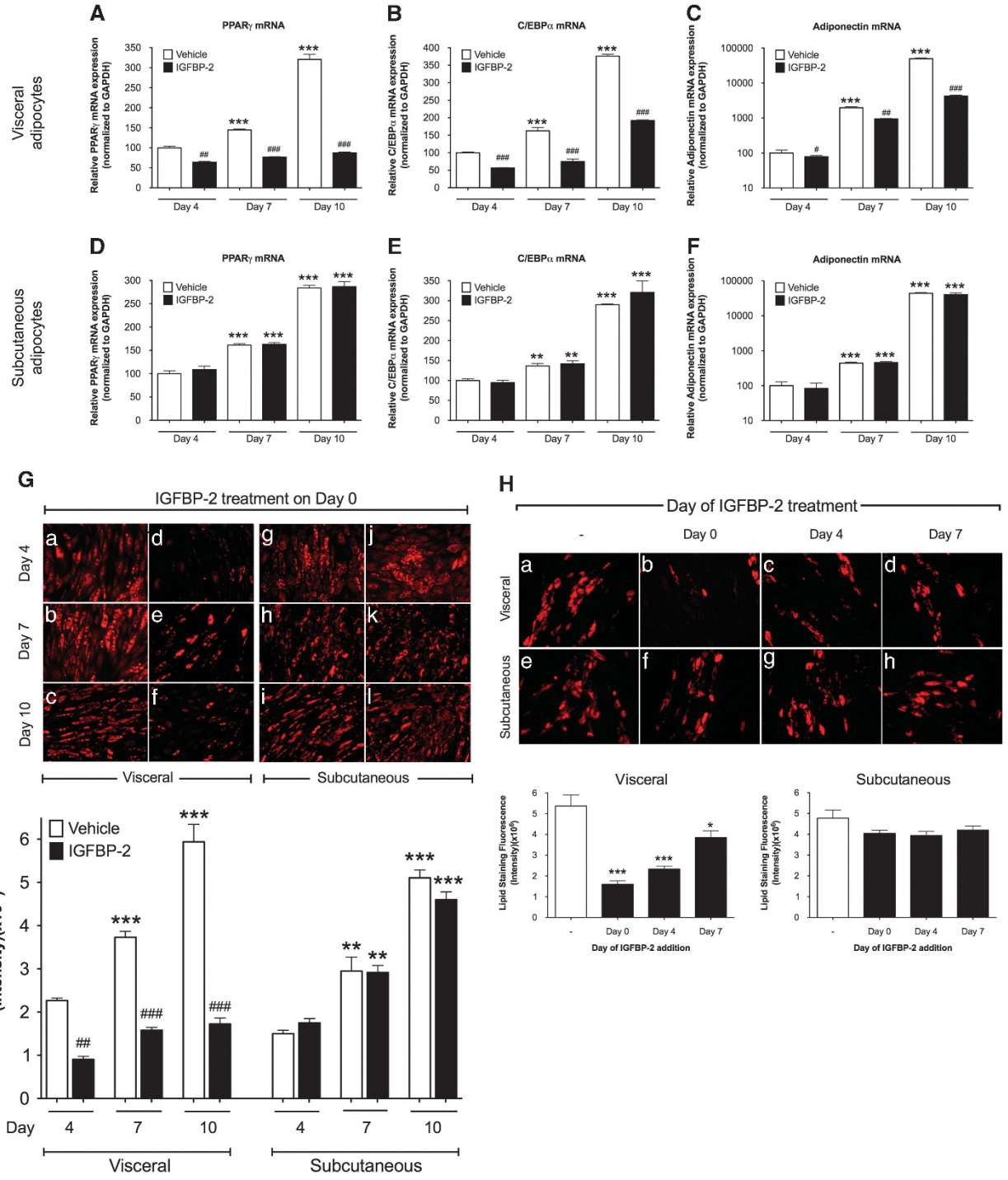


Figure 3. Treatment with exogenous IGFBP-2 downregulates adipocyte-specific markers in differentiating visceral adipocytes. IGFBP-2 (100 ng ml^{-1}) was administered on day 0 of differentiation in visceral and subcutaneous adipocytes, with gene expression quantified on day 4, 7 or 10 of differentiation. Visceral adipocytes: (**A**) PPARγ, (**B**) C/EBPα, and (**C**) adiponectin mRNA expression; subcutaneous adipocytes: (**D**) PPARγ, (**E**) C/EBPα, and (**F**) adiponectin mRNA expression. Note that the *y* axis for adiponectin mRNA expression is a log scale; reduction in adiponectin expression was 22% (day 4), 52% (day 7) and 91% (day 10). Gene expression is corrected to the levels of GAPDH mRNA and is expressed relative to day 4 of adipocyte differentiation with vehicle treatment within adipose depot. (**G**) Treatment with exogenous IGFBP-2 reduced lipid abundance in differentiating visceral adipocytes. IGFBP-2 (100 ng ml^{-1}) was administered on day 0 of differentiation in visceral and subcutaneous adipocytes, with lipid staining performed on day 4, 7 or 10 of differentiation. (**H**) Treatment of exogenous IGFBP-2 inhibits lipid abundance in visceral adipocytes. IGFBP-2 (100 ng ml^{-1}) was administered on day 0, 4 or 7 of differentiation in visceral and subcutaneous preadipocytes. Lipid staining was performed on day 8. Lipid staining using LipidTOX neutral red, magnification ×10. Lipid staining fluorescence intensity is expressed relative to control (no treatment): visceral (left), and subcutaneous (right). All data are expressed as mean ± s.e.m. Data are representative of four independent experiments. $^{*/\#}P < 0.05$, $^{**/\#\#}P < 0.01$, $^{***/\#\#\#}P < 0.001$, *Compared with first bar, #Compared with vehicle treatment.

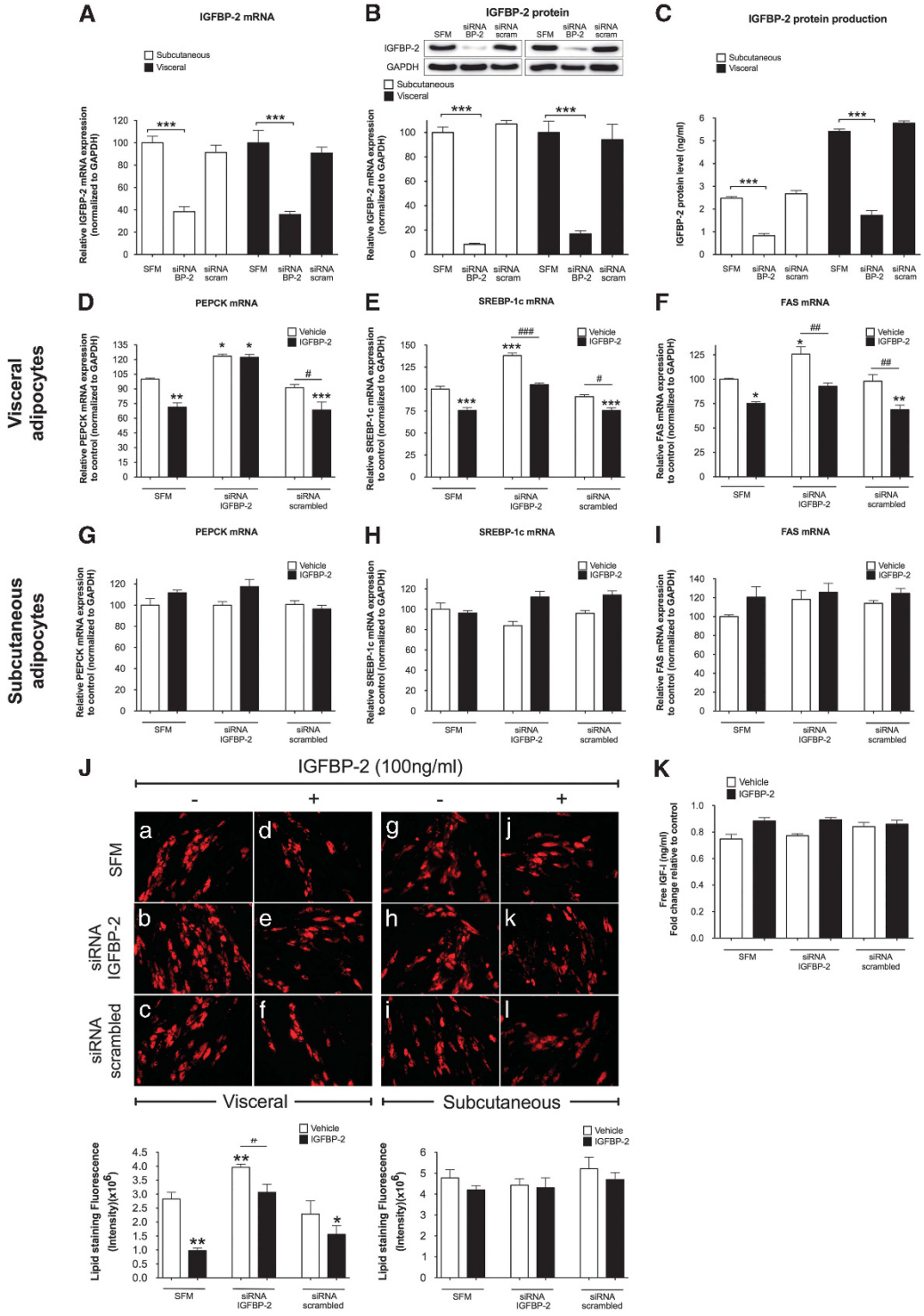


Figure 4. Silencing of IGFBP-2 in subcutaneous and visceral adipocyte: IGFBP-2 (**A**) mRNA, (**B**) protein level, and (**C**) protein production. Adipocytes were transfected with IGFBP-2 siRNA on day 7 of differentiation and IGFBP-2 was administered at day 8 of differentiation and cultured for a further 24 h. Gene expression and lipid staining (LipidTOX neutral red) was performed on day 9 of differentiation. (**D**–**I**) IGFBP-2 decreases expression of lipogenic genes in visceral adipocytes, whereas silencing of IGFBP-2 increases the gene expression of PEPCK, SREBP-1c and FAS expression. Subsequent treatment with exogenous IGFBP-2 decreases SREBP-1c and FAS expression in visceral adipocytes. Visceral adipocytes: (**D**) PEPCK, (**E**) SREBP-1c, and (**F**) FAS mRNA expression; subcutaneous adipocytes: (**G**) PEPCK (**H**) SREBP-1c, and (**I**) FAS mRNA expression. Gene expression is corrected to the levels of GAPDH mRNA and is expressed relative to vehicle (PBS)/control within adipose depot. (**J**) Silencing of IGFBP-2 increases lipid abundance in differentiated visceral adipocytes. Subsequent treatment of exogenous IGFBP-2 decreases lipid abundance in visceral adipocytes. Lipid staining, magnification × 10. Lipid staining fluorescence intensity is expressed relative to control (no treatment): visceral (left), and subcutaneous (right). (**K**) Free IGF-I levels were unaltered with IGFBP-2 silencing or treatment in differentiated visceral adipocytes. Quantification of free IGF-I levels in the conditioned medium by enzyme-linked immunosorbent assay. All data are expressed as mean ± s.e.m. Data are representative of four independent experiments. $^{*/\#}P < 0.05$, $^{**/\#\#}P < 0.01$, $^{***/\#\#\#}P < 0.001$, *Compared with serum-free media (SFM), #Compared with vehicle treatment.

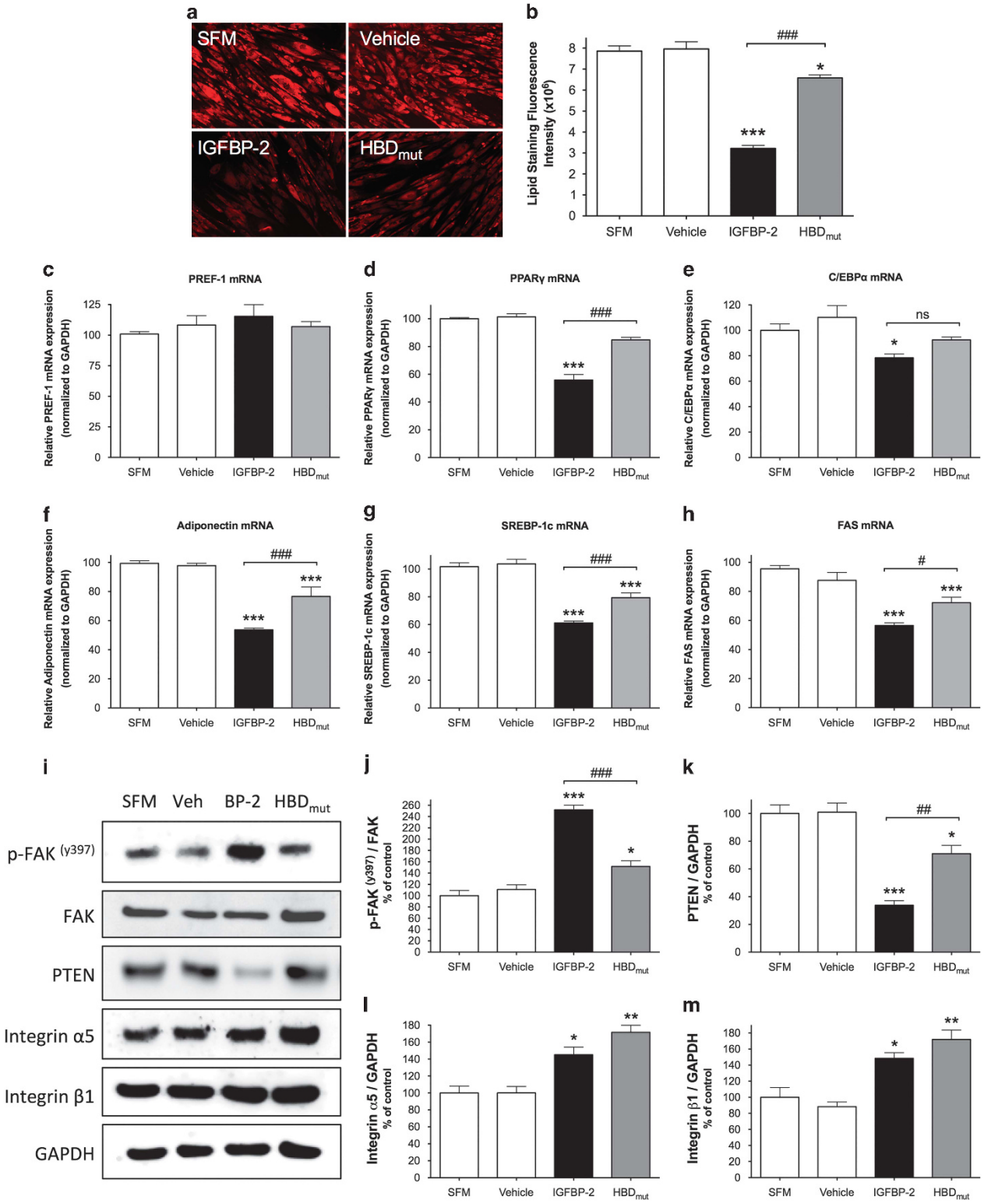
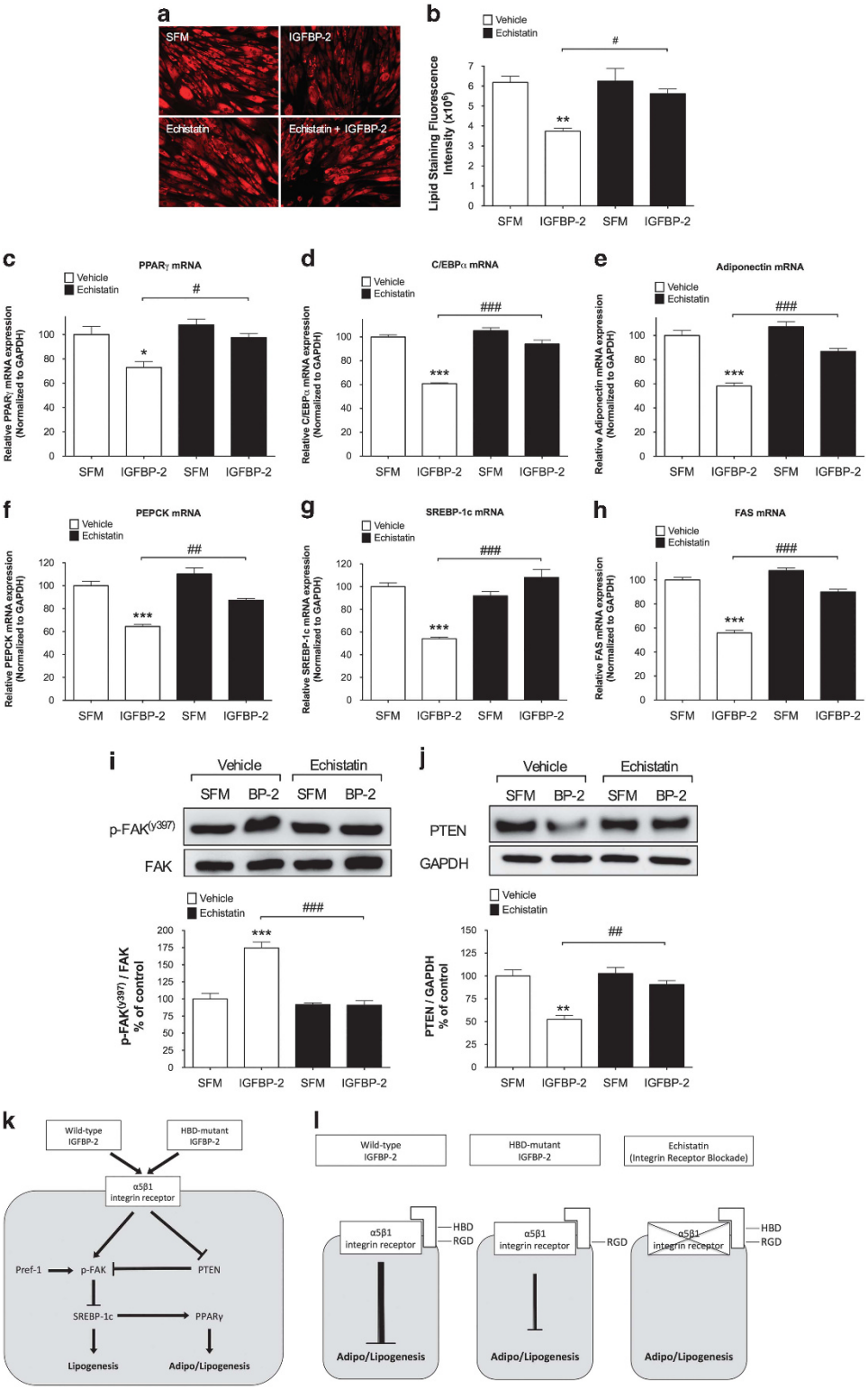


Figure 5. HBD-mutant IGFBP-2 is less effective in inhibiting adipocyte differentiation and lipogenesis than wild-type IGFBP-2 in visceral adipocytes. Visceral preadipocytes were exposed to wild-type IGFBP-2 or HBD-mutant IGFBP-2 at day 0 of differentiation and on day 7; (**a**, **b**) Lipid staining (LipidTOX neutral red), magnification ×10, (**c**) Pref-1 (**d**) PPARγ, (**e**) C/EBPα, (**f**) adiponectin, (**g**) SREBP-1c and (**h**) FAS mRNA expression. Gene expression is corrected to the levels of GAPDH mRNA and is expressed relative to serum-free media (SFM)/control. Data are representative of four independent experiments. (**i**) Representative immunoblots of (**j**) p-FAK^{y397} (**k**) PTEN (**l**) Integrin α5 and (**m**) Integrin β1. Immunoblot quantification is normalised to total FAK or GAPDH. All data are expressed as mean ± s.e.m. Data are representative of three independent experiments. $^{*}P < 0.05$, $^{**/\#\#}P < 0.01$, $^{***/\#\#\#}P < 0.001$, *Compared with SFM (first bar), $^{\#}$HBD-mutant treatment compared with wild-type IGFBP-2.

The inhibitory effect of IGFBP-2 on adipogenesis and lipogenesis is partially mediated by the HBD

The findings thus far demonstrate that IGFBP-2 inhibits adipogenesis and lipogenesis in a depot-specific manner, affecting only visceral preadipocytes, and therefore further experiments were only performed in visceral preadipocytes. To elucidate which domain/s of IGFBP-2 is responsible for its effects on adipogenesis and lipogenesis in human visceral adipocytes, the HBD-mutant IGFBP-2 was utilised. Visceral preadipocytes were treated with serum-free medium or supplemented with either PBS (vehicle), IGFBP-2 (wild-type) or HBD-mutant IGFBP-2 on day 0 of culture. By day 7, lipid abundance was reduced with HBD-mutant IGFBP-2 treatment (20%, $P < 0.05$) but not to the same extent as seen with wild-type IGFBP-2 (60%, $P < 0.01$; Figures 5a and b). Wild-type



IGFBP-2 led to a significant reduction in PPARγ (43%, $P < 0.001$), C/EBPα (22%, $P < 0.05$), adiponectin (46%, $P < 0.001$), SREBP-1c (39%, $P < 0.001$) and FAS (43%, $P < 0.001$) expression, while HBD-mutant IGFBP-2 reduced only adiponectin (23%, $P < 0.001$), SREBP-1c (21%, $P < 0.001$) and FAS expression (28%, $P < 0.001$) on day 7 of differentiation. In contrast, there was a significant difference in PPARγ ($P < 0.001$), adiponectin ($P < 0.001$), SREBP-1c ($P < 0.001$) and FAS ($P < 0.05$) expression between wild-type IGFBP-2 compared with HBD-mutant IGFBP-2 treatments (Figures 5c–h). Wild-type IGFBP-2 increased *P*-FAK^{y397} by 2.5-fold ($P < 0.001$), while HBD-mutant IGFBP-2 only increased *P*-FAK^{y397} by 50% ($P < 0.05$)(Figures 5i and j). Furthermore, wild-type IGFBP-2 and HBD-mutant IGFBP-2 reduced PTEN levels by 66% ($P < 0.001$) and 30% ($P < 0.05$), respectively (Figures 5i and k). Wild-type IGFBP-2 increased integrin α5 by 45% ($P < 0.05$) and integrin β1 by 49% ($P < 0.05$), while HBD-mutant IGFBP-2 treatment increased both integrin α5 ($P < 0.001$) and integrin β1 ($P < 0.01$) by 70% (Figures 5i, l and m).

Inhibition of adipogenesis and lipogenesis are primarily mediated via the RGD domain of IGFBP-2

Although the HBD-mutant IGFBP-2 did not have the same inhibitory effect as wild-type IGFBP-2 on adipocyte differentiation and lipogenesis, it did possess modest anti-adipogenic and lipogenic properties. We therefore speculated that the effects of IGFBP-2 could also be via the RGD domain and signalling through integrin receptors. On day 0 of culture, visceral preadipocytes were incubated with Echistatin (disintegrin—an integrin inhibitor) for 24 h, prior to subsequent treatment with wild-type IGFBP-2. Prior Echistatin treatment negated IGFBP-2 inhibitory effects on lipid abundance ($P < 0.05$) (Figures 6a and b), PPARγ ($P < 0.05$), C/EBPα ($P < 0.001$), adiponectin ($P < 0.001$), PEPCK ($P < 0.01$), SREBP-1c ($P < 0.001$) and FAS ($P < 0.001$) mRNA expression (Figures 6c–h). In addition, IGFBP-2 treatment on day-7 differentiated visceral adipocytes led to increased FAK phosphorylation by 75% ($P < 0.01$) (Figure 6i) and decreased PTEN by 48% ($P < 0.01$) (Figure 6j). However, in the presence of Echistatin, IGFBP-2 treatment had no effect on phosphorylation of FAK and PTEN in differentiated visceral adipocytes.

DISCUSSION

Previous studies have indicated that IGFBP-2 inhibits 3T3-L1 cell differentiation *in vitro*,[24] but depot-specific effects in human adipocytes have not yet been assessed. We demonstrate a specific effect of IGFBP-2 in visceral adipocytes through downregulation of adipogenic transcription factor genes, which led to a coordinated cascade of reduction in the expression of adipocyte-specific markers. IGFBP-2 also exhibited anti-lipogenic effects in a time-dependent manner, with greatest inhibition of lipid droplets occurring in the earlier stages of adipocyte differentiation in visceral preadipocytes. Furthermore, silencing IGFBP-2 resulted in increased lipogenesis in visceral, but not subcutaneous, adipocytes. This depot-specific effect of IGFBP-2 appears to involve cell-surface associations and activation of integrin signalling.

Previous studies have shown that IGFBP-2 mRNA levels in adipose tissue are significantly lower in obese individuals, an effect which is more pronounced with visceral than subcutaneous adiposity.[23] In the present study, we demonstrate that visceral adipocytes produce greater levels of IGFBP-2 than subcutaneous adipocytes. Perhaps in a similar way, circulating adiponectin levels have been shown to be lower in obesity and yet high-molecular-weight adiponectin secretion has been shown to be higher from visceral (when compared with subcutaneous) adipocytes derived from normal-weight individuals.[34] We suggest that IGFBP-2 may behave in a similar way to adiponectin, where IGFBP-2 levels are reduced with obesity, and that this is an effect which is more pronounced with visceral adiposity than subcutaneous adiposity. Previous studies have shown that visceral and subcutaneous adipocytes respond differently to different stimuli, such as fatty acids,[35] and nutrients,[19,36] and differential effects in terms of adipokine secretion are clearly demonstrated.[37] Furthermore, we have recently demonstrated that intracerebroventricular leptin infusion led to an increase in IGFBP-2 mRNA levels in visceral and subcutaneous adipose tissue in sheep,[31] and leptin resistance may therefore contribute to different levels of IGFBP-2 secretion from the two fat compartments in obesity. Collectively, these findings indicate that IGFBP-2 levels are dysregulated in obesity, and, in particular, in those with visceral obesity. Furthermore, this depot-specific effect may impact on the development of obesity-related conditions, such as the metabolic syndrome.

IGFBP-2 is able to bind IGF-I via its IGF-binding domain, and so we undertook our experiments in an IGF-I-free system. However, to also account for the possibility that the anti-adipogenic effects of IGFBP-2 may occur through reduced action of adipocyte-secreted IGF-I,[38] we quantified free IGF-I levels in media from the cultures of subcutaneous and visceral adipocytes with or without IGFBP-2 silencing and IGFBP-2 treatment. No differences were seen in free IGF-I levels between each treatment, suggesting that IGFBP-2 does not cause anti-adipogenic and anti-lipogenic effects in visceral adipocytes via an IGF-I-dependent mechanism. We did not conduct experiments with the addition of IGF-I, as a previous study has already demonstrated that the adipocyte medium (including our adipocyte medium) already contains insulin at concentrations adequate to activate the IGF-I receptor.[30] In the cited study, IGF-I addition did not lead to an additional effect on cell differentiation.[30] As IGFBP-2 or HBD-mutant IGFBP-2 cannot bind insulin, it can be deduced that IGFBP-2 or HBD-mutant

Figure 6. Wild-type IGFBP-2 inhibition of adipo/lipogenesis is abolished with prior treatment of Echistatin (disintegrin). Visceral preadipocytes were treated with Echistatin or vehicle for 24 h prior to wild-type IGFBP-2. On day 7: (**a**, **b**) Lipid staining (LipidTOX neutral red), magnification ×10, (**c**) PPARγ, (**d**) C/EBPα, (**e**) adiponectin (**f**) PEPCK, (**g**) SREBP-1c, and (**h**) FAS mRNA expression. Gene expression is corrected to the levels of GAPDH mRNA and is expressed relative to serum-free media (SFM)/control. Data are representative of four independent experiments. (**i**, **j**) IGFBP-2 increases FAK phosphorylation and PTEN via integrin signalling in visceral adipocytes. On day 7 of differentiation, visceral adipocytes were treated with Echistatin 24 h prior to IGFBP-2 treatment for another 24 h. Western immunoblotting of cell lysates: (**i**) p-FAK^{y397} and (**j**) PTEN. Immunoblot quantification is normalised to total FAK or GAPDH, respectively. All data are expressed as the mean ± s.e.m. Data are representative of three independent experiments. $^{**/\#\#}P < 0.01$, $^{***/\#\#\#}P < 0.001$, *Compared with first bar (SFM), #Echistatin compared with vehicle treatment. (**k**) A model by which IGFBP-2 regulates adipogenesis and lipogenesis in visceral adipocytes. Wild-type IGFBP-2 and HBD-mutant IGFBP-2 both contain the RGD sequences that bind α5β1 integrin receptors. It has been shown that α5β1 integrin receptor activation activates FAK phosphorylation and inhibits PTEN. PTEN has also been demonstrated to inhibit phosphorylation of FAK, while pref-1 induces FAK phosphorylation. The net outcome results in the activation of FAK signalling, which has been suggested to inhibit adipogenesis and lipogenesis mediated by master regulators SREBP-1c and PPARγ. (**l**) Overall summary of findings indicate that: wild-type IGFBP-2 inhibits adipo/lipogenesis via both the RGD and HBD motifs; HBD-mutant IGFBP-2 inhibits adipo/lipogenesis to a lesser degree via only the RGD motif; while when the integrin α5β1 is blocked by Echistatin, wild-type IGFBP-2 is unable to have any effect on adipo/lipogenesis demonstrating that IGFBP-2 inhibits adipo/lipogenesis via integrin signalling in visceral adipocytes.

IGFBP-2 does not inhibit adipogenesis by impeding IGF-I or insulin binding to their receptors.

A recent study demonstrated that both HBDs (HBD at the linker region (HBD1) and HBD at the C-terminal (HBD2)) of IGFBP-2 have some ability to inhibit murine preadipocyte differentiation *in vitro*, with HBD2 being more potent.[30] It has, however, been shown that, at neutral pH, the HBD2 motif of IGFBP-2 is not exposed,[39] consequently not allowing surface binding. In addition, it has also been demonstrated that the HBD1 of IGFBP-2 is able to bind to a receptor tyrosine phosphatase, RPTP-β, and inhibits its ability to dephosphorylate PTEN resulting in enhancement of osteoblast proliferation.[27] Therefore mutagenesis of this HBD region of IGFBP-2 would result in a loss of capability to bind RPTP-β. To dissect the molecular mechanisms underlying the anti-adipogenic and anti-lipogenic effects of IGFBP-2 in visceral preadipocytes, we utilised the HBD-mutant IGFBP-2 (mutation of the HBD region at the linker region (HBD1)) as previously described.[25] We demonstrate here that the HBD-mutant IGFBP-2 inhibits lipid abundance and reduces gene expression of adipocyte-specific markers to a lesser extent than wild-type IGFBP-2, suggesting that this HBD region of IGFBP-2 has a role in the mediation of adipogenesis in human visceral adipocytes.

Studies have indicated that peptides comprising the RGD sequence are able to bind to the α5β1 integrin, resulting in FAK phosphorylation and impeding murine preadipocyte differentiation.[40,41] Here we found that the HBD-mutant IGFBP-2 led to a minor enhancement in FAK phosphorylation and a minor reduction in PTEN protein, whereas wild-type IGFBP-2 produced robust integrin activation as determined by FAK activation and cognate PTEN inhibition. In addition, levels of the cell membrane integrin α5 and β1 chain were higher with HBD-mutant IGFBP-2 treatment than with wild-type IGFBP-2 treatment, indicating inability of the HBD-mutant IGFBP-2 to promote integrin internalisation, an event required for canonical integrin signalling. Levels of integrin α5 and β1 chain were also higher with wild-type IGFBP-2 treatment, and this may be due to the result of canonical integrin signalling, integrin internalisation and rapid recycling of internalised integrin back to the plasma membrane.[42,43] Further experiments using Echistatin, the most potent integrin blocker, abolished all of IGFBP-2's cellular effects and thus revealed that, in adipocytes, IGFBP-2 induces FAK phosphorylation and PTEN inhibition via canonical integrin signalling. Taken together, these findings suggest that IGFBP-2 induces FAK phosphorylation predominantly through the RGD domain. In accordance with this, previous work has shown that pref-1 cannot inhibit adipogenesis in the absence of integrins, and FAK phosphorylation was increased 2.5-fold when pref-1 was administered in 3T3-L1 adipocytes, indicating that integrins are necessary for pref-1 inhibition of adipogenesis.[44] Furthermore, there is evidence that adipocyte-specific FAK deletion is associated with an increase in SREBP-1c,[45] supporting the notion that FAK activation by IGFBP-2 may represent a potential mechanism for its inhibition of adipogenesis. Taken together, these results suggest that both the RGD and HBD motifs in IGFBP-2 are necessary for effective inhibition of integrin signalling and, in turn, inhibition of adipogenesis and lipogenesis. Whether the interaction between the HBD-mutant IGFBP-2 and integrin α5β1 is less stable than that of wild-type IGFBP-2 remains to be demonstrated.

To understand the inter-relationship between IGFBP-2 and the regulation of adipogenesis and lipogenesis, Figure 6k presents a model in which IGFBP-2 may mediate adipogenesis and lipogenesis in visceral adipocytes. Both wild-type IGFBP-2 and HBD-mutant IGFBP-2 contain the RGD sequence that allows binding with α5β1 integrin receptors. It has been shown that α5β1 integrin receptor activation leads to FAK phosphorylation and inhibits PTEN. PTEN inhibits phosphorylation of FAK, while pref-1 induces FAK phosphorylation. The net outcome is activation of FAK signalling, which is suggested to inhibit adipogenesis and lipogenesis through the master regulators SREBP-1c and PPARγ. An overall summary, shown in Figure 6l, indicates that: (1) wild-type IGFBP-2 inhibits adipogenesis and lipogenesis via both the RGD and HBD motifs; (2) HBD-mutant IGFBP-2 inhibits adipogenesis and lipogenesis to a lesser degree via the RGD motif only; and (3) when integrin α5β1 is blocked by Echistatin, wild-type IGFBP-2 is unable to affect adipogenesis and lipogenesis. Collectively, our findings demonstrate that IGFBP-2 inhibits adipogenesis and lipogenesis via integrin signalling in visceral adipocytes and that this is mainly mediated by the RGD domain. As IGFBP-2 levels are very low in preadipocytes, IGFBP-2 silencing experiments in preadipocytes were not examined, as it would not provide relevant insight by which IGFBP-2 regulates adipo/lipogenesis. However, future experiments are required to fully elucidate whether the effect of IGFBP-2 on integrin signalling is a cause or a consequence of the associated impairment of adipo/lipogenesis.

In summary, we demonstrate depot-specific differences in the regulation adipogenesis and lipogenesis by IGFBP-2. Specifically, IGFBP-2 inhibits both adipogenesis and lipogenesis in visceral, but not subcutaneous, adipocytes, and this appears to involve cell-surface associations and activation of integrin signalling via the RGD domain and through enhancement of FAK phosphorylation. This may potentially represent a novel pathway for drug development to treat central obesity.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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REFERENCES

1 Swinburn BA, Sacks G, Hall KD, McPherson K, Finegood DT, Moodie ML *et al.* The global obesity pandemic: shaped by global drivers and local environments. *Lancet* 2011; **378**: 804–814.
2 Tataranni PA, Ravussin E. Effect of fat intake on energy balance. *Ann NY Acad Sci* 1997; **819**: 37–43.
3 Arner E, Westermark PO, Spalding KL, Britton T, Rydén M, Frisén J *et al.* Adipocyte turnover: relevance to human adipose tissue morphology. *Diabetes* 2010; **59**: 105–109.
4 Spalding KL, Arner E, Westermark PO, Bernard S, Buchholz BA, Bergmann O *et al.* Dynamics of fat cell turnover in humans. *Nature* 2008; **453**: 783–787.
5 Spiegelman BM, Flier JS. Adipogenesis and obesity: rounding out the big picture. *Cell* 1996; **87**: 377–389.
6 Attie AD, Scherer PE. Adipocyte metabolism and obesity. *J Lipid Res* 2009; **50**: S395–S399.
7 Guilherme A, Virbasius JV, Puri V, Czech MP. Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes. *Nat Rev Mol Cell Biol* 2008; **9**: 367–377.
8 Larsson B. Obesity, fat distribution and cardiovascular disease. *Int J Obes* 1991; **15**: 53–57.
9 Wajchenberg BL. Subcutaneous and visceral adipose tissue: their relation to the metabolic syndrome. *Endocr Rev* 2000; **21**: 697–738.
10 Ibrahim MM. Subcutaneous and visceral adipose tissue: Structural and functional differences. *Obes Rev* 2010; **11**: 11–18.
11 Brun RP, Kim JB, Hu E, Altiok S, Spiegelman BM. Adipocyte differentiation: a transcriptional regulatory cascade. *Curr Opin Cell Biol* 1996; **8**: 826–832.
12 Gregoire F, Smas C, Sul H. Understanding adipocyte differentiation. *Physiol Rev* 1998; **78**: 783–809.
13 Cristancho A, Lazar M. Forming functional fat: a growing understanding of adipocyte differentiation. *Nat Rev Mol Cell Biol* 2011; **28**: 722–734.

14 Smith PJ, Wise LS, Berkowitz R, Wan C, Rubin CS. Insulin-like growth factor-I is an essential regulator of the differentiation of 3T3-L1 adipocytes. *J Biol Chem* 1988; **263**: 9402–9408.
15 Russo VC, Gluckman PD, Feldman EL, Werther GA. The insulin-like growth factor system and its pleiotropic functions in brain. *Endocr Rev* 2005; **26**: 916–943.
16 Boney CM, Moats-Staats BM, Stiles AD, D'Ercole AJ. Expression of insulin-like growth factor-I (IGF-I) and IGF-binding proteins during adipogenesis. *Endocrinology* 1994; **135**: 1863–1868.
17 Bhala A, Harris M, Zirin S, Corcoran L, Cohen P. Insulin-like growth factor axis parameters in sick hospitalized neonates. *J Pediatr Endocrinol Metab* 1998; **11**: 451–459.
18 Sabin MA, Russo VC, Azar WJ, Yau SW, Kiess W, Werther GA. IGFBP-2 at the interface of growth and metabolism- implications for childhood obesity. *Pediatr Endocrinol Rev* 2011; **8**: 382–393.
19 Sabin MA, Yau SW, Russo VC, Clarke IJ, Dunshea FR, Chau J *et al.* Dietary mono-unsaturated fat in early life regulates IGFBP2: implications for fat mass accretion and insulin sensitivity. *Obesity* 2011; **19**: 2374–2381.
20 Nam S, Lee E, Kim K, Cha B, Song Y, Lim S. Effect of obesity on total and free insulin-like growth factor (IGF)-1, and their relationship to IGF-binding protein (BP)-1, IGFBP-2, IGFBP-3, insulin, and growth hormone. *Int J Obes Relat Metab Disord* 1997; **21**: 355–359.
21 Ballerini MG, Ropelato MG, Domene HM, Pennisi P, Heinrich JJ, Jasper HG. Differential impact of simple childhood obesity on the components of the growth hormone-insulin-like growth factor (IGF)-IGF binding proteins axis. *J Pediatr Endocrinol Metab* 2004; **17**: 749–757.
22 Claudio M, Benjamim F, Riccardo B, Massimiliano C, Francesco B, Luciano C. Adipocytes IGFBP-2 expression in prepubertal obese children. *Obesity* 2010; **18**: 2055–2057.
23 Li Z, Picard F. Modulation of IGFBP2 mRNA expression in white adipose tissue upon aging and obesity. *Horm Metab Res* 2010; 787–791.
24 Wheatcroft S, Kearney M, Shah A, Ezzat V, Miell J, Modo M. IGF-binding protein-2 protects against the development of obesity and insulin resistance. *Diabetes* 2007; **56**: 284–294.
25 Russo VC, Schütt BS, Andaloro E, Ymer SI, Hoeflich A, Ranke MB *et al.* Insulin-like growth factor binding protein-2 binding to extracellular matrix plays a critical role in neuroblastoma cell proliferation, migration, and invasion. *Endocrinology* 2005; **146**: 4445–4455.
26 Russo VC, Bach LA, Fosang AJ, Baker NL, Werther GA. Insulin-like growth factor binding protein-2 binds to cell surface proteoglycans in the rat brain olfactory bulb. *Endocrinology* 1997; **138**: 4858–4867.
27 Shen X, Xi G, Maile LA, Wai C, Rosen CJ, Clemmons DR. Insulin-like growth factor (IGF) binding protein 2 functions coordinately with receptor protein tyrosine phosphatase β and the IGF-I receptor to regulate IGF-I-stimulated signaling. *Mol Cell Biol* 2012; **32**: 4116–4130.
28 Schutt B, Langkamp M, Rauschnabel U, Ranke M, Elmlinger M. Integrin-mediated action of insulin-like growth factor binding protein-2 in tumor cells. *J Mol Endocrinol* 2004; **32**: 859–868.
29 Wheatcroft SB, Kearney MT. IGF-dependent and IGF-independent actions of IGF-binding protein-1 and -2: implications for metabolic homeostasis. *Trends Endocrinol Metab* 2009; **20**: 153–162.
30 Xi G, Solum MA, Wai C, Maile LA, Rosen CJ, Clemmons DR. The heparin-binding domains of IGFBP-2 mediate its inhibitory effect on preadipocyte differentiation and fat development in male mice. *Endocrinology* 2013; **154**: 4146–4157.
31 Yau SW, Henry BA, Russo VC, McConell GK, Clarke IJ, Werther GA *et al.* Leptin enhances insulin sensitivity by direct and sympathetic nervous system regulation of muscle IGFBP-2 expression: evidence from nonrodent models. *Endocrinology* 2014; **155**: 2133–2143.
32 Hu ED, Kim JB, Sarraf P, Spiegelman BM. Inhibition of adipogenesis through MAP kinase-mediated phosphorylation of PPAR gamma. *Science* 1996; **274**: 2100–2103.
33 Smas CM, Sul HS. Pref-1 a protein containing EGF-like repeats, inhibits adipocyte differentiation. *Cell* 1993; **73**: 725–734.
34 Kovacova Z, Tencerova M, Roussel B, Wedellova Z, Rossmeislova L, Langin D *et al.* The impact of obesity on secretion of adiponectin multimeric isoforms differs in visceral and subcutaneous adipose tissue. *Int J Obes* 2012; **36**: 1360–1365.
35 Sabin MA, Crowne EC, Stewart CE, Hunt LP, Turner SJ, Welsh GI *et al.* Depot-specific effects of fatty acids on lipid accumulation in children's adipocytes. *Biochem Biophys Res Commun* 2007; **361**: 356–361.
36 Einstein FH, Atzmon G, Yang XM, Ma XH, Rincon M, Rudin E *et al.* Differential responses of visceral and subcutaneous fat depots to nutrients. *Diabetes* 2005; **54**: 672–678.
37 Lihn AS, Bruun JM, He GS, Pedersen SB, Jensen PF, Richelsen B. Lower expression of adiponectin mRNA in visceral adipose tissue in lean and obese subjects. *Mol Cell Endocrinol* 2004; **219**: 9–15.
38 Holly JMP, Sabin MA, Perks CM, Shield JP. Adipogenesis and IGF-1. *Metab Syndr Relat Disord* 2006; **4**: 43–50.
39 Kuang Z, Yao S, Keizer DW, Wang CC, Bach LA, Forbes BE *et al.* Structure, dynamics and heparin binding of the C-terminal domain of insulin-like growth factor-binding protein-2 (IGFBP-2). *J Mol Biol* 2006; **364**: 690–704.
40 Lin YT, Tang CH, Chuang WJ, Wang SM, Huang TF, Fu WM. Inhibition of adipogenesis by RGD-dependent disintegrin. *Biochem Pharmacol* 2005; **70**: 1469–1478.
41 Liu J, DeYoung SM, Zhang M, Zhang M, Cheng A, Saltiel AR. Changes in integrin expression during adipocyte differentiation. *Cell Metab* 2005; **2**: 165–177.
42 Bretscher MS. Circulating integrins: alpha 5 beta 1, alpha 6 beta 4 and Mac-1, but not alpha 3 beta 1, alpha 4 beta 1 or LFA-1. *EMBO J* 1992; **11**: 405–410.
43 Caswell PT, Norman JC. Integrin trafficking and the control of cell migration. *Traffic* 2006; **7**: 14–21.
44 Wang Y, Zhao L, Smas C, Sul HS. Pref-1 interacts with fibronectin to inhibit adipocyte differentiation. *Mol Cell Biol* 2010; **30**: 3480–3492.
45 Luk CT, Shi SY, Cai EP, Sivasubramaniyam T, Schroer SA, Woo M. Adipocyte-specific FAK deletion in mice leads to insulin resistance but divergent adipose tissue remodelling under lean and obese conditions. *Can J Diabetes* 2013; **37**: 433–434.

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