Obesity, especially visceral obesity, is associated with insulin resistance and metabolic syndrome. We previously identified the cell surface proteoglycan glypican-4 as differentially expressed in subcutaneous versus visceral white fat depots. Here we show that glypican-4 is released from cells and adipose tissue explants of mice, and that circulating glypican-4 levels correlate with BMI and insulin sensitivity in humans. Furthermore, glypican-4 interacts with the insulin receptor, enhances insulin receptor signaling, and enhances adipocyte differentiation. Conversely, depletion of glypican-4 results in reduced activation of the insulin receptor and prevents adipocyte differentiation in vitro by inhibiting insulin-mediated C/EBPβ phosphorylation. These functions of glypican-4 are independent of its glycosphingolipid-lysinositol membrane anchor, as a nonmembrane–bound mutant of glypican-4 phenocopies the effects of native glypican-4 overexpression. In summary, glypican-4 is a novel circulating adipocyte sensitizing adipose-derived factor that, unlike other insulin sensitizers, acts directly on the insulin receptor to enhance signaling.

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Obesity is the main cause of insulin resistance in humans and, in many individuals, the first step in the development of type 2 diabetes and metabolic syndrome. The adverse metabolic effects of increasing fat mass depend heavily on its anatomical distribution, with visceral white adipose tissue (WAT) driving the development of insulin resistance and associated metabolic diseases (1). In contrast, increased subcutaneous WAT is not associated with insulin resistance, and in some circumstances has even been shown to have protective effects (1,2).

Expansion of adipose tissue is achieved by increased lipid storage in existing adipocytes and de novo differentiation of preadipocytes. Various autocrine, paracrine, and endocrine factors control adipocyte differentiation (3). Among them, insulin is important in regulation of differentiation and lipid accumulation in vitro and in vivo (4). WAT is also an important endocrine organ, secreting various cytokines and hormones (adipokines) regulating whole body metabolism and insulin sensitivity (5–7).

We previously identified a set of developmentally regulated genes that are differentially expressed in subcutaneous and visceral adipose tissue of mice and men (8). Among these, the patterning gene glypican-4 (Gpc4) is not only differentially expressed in these depots, but its expression in human WAT is also highly correlated with BMI and adipose distribution as measured by waist-to-hip ratio (WHR). Gpc4 belongs to a six-member family of glycosylphosphatidylinositol (GPI)-anchored heparan sulfate proteoglycans. Lacking transmembrane and intracellular domains, glypican family members are thus recently described as coreceptors for a variety of growth factors including Wnt, bone morphogenetic proteins (BMPs), fibroblast growth factors, and Hedgehog (9–11). Little is known about the signaling functions of Gpc4. Mammalian Gpc4 has been reported to bind to fibroblast growth factor 2 via its heparan sulfate chains in neuronal cells and to function as a low-affinity receptor for endostatin (12,13). The role of Gpc4 in adipocytes and its relationship to metabolic regulation remain unknown.

In this study, we demonstrate that Gpc4 is important for adipocyte differentiation by interacting with and regulating insulin receptor activation and its downstream signaling. This interaction is preserved in a soluble nonmembrane–anchored mutant of Gpc4. Furthermore, we provide evidence that Gpc4 is released from adipose tissue, and that serum Gpc4 is a marker for BMI and insulin sensitivity in mice and human. Thus, Gpc4 can serve as a novel adipokine being released from adipose tissue with the ability to enhance insulin sensitivity.

RESEARCH DESIGN AND METHODS
Human subjects. Paired samples of visceral and subcutaneous adipose tissue were obtained from 160 subjects as previously described (8). All subjects gave written informed consent before taking part in the study.

Mice. All protocols were approved by the Institutional Animal Care and Use Committee of the Joslin Diabetes Center and in accordance with National Institutes of Health (NIH) guidelines. Mice (Jackson Laboratory) were maintained on a 12-h light/dark cycle and fed a chow diet (95:520; PharmaServ) or high-fat diet (HFD) (OpenSource Diet D12492, Research Diet).

Constructs. Gpc4 cDNA clones were obtained from Open Biosystems. An HA-tag was inserted after the signal peptide for native Gpc4, and the cDNA was cloned into the pCDH-puro lentiviral vector (Systems Biosciences). Gpc4ΔS208TAG531::HHHHHH (ΔGpc4) was created by site-directed mutagenesis (Stratagene) using the primers fwd:CGAGAAAGCTGACCACCATCACCATCA-CCATGGTGCCCATGCAG rev:CTGCATGGGCACCATGGTGATGGTGATGGTG-GTCAGCTTTCTCG. A 6xHis-tag was inserted at the N-terminus after the signal peptide and cloned into the pCDH-puro vector. All constructs were sequence verified. Short hairpin RNA (shRNA) lentiviral vectors (pLKO.1) were obtained from Open Biosystems. shGpc4 shRNA was targeted against the sequence GCCACCTGTTTTAAGCAATGT. A scrambled shRNA (shScr) targeting the sequence AGTTAAGTGGCCTGCT served as control.

Oligonucleotide pull-down assays. Pull downs were performed as previously described (14).

Cell culture. 3T3-L1 cells were cultured in Dulbecco’s modified Eagle’s medium 4.5 g/L glucose, 10% FBS and 2.5 μg/mL puromycin. Differentiation was induced with 170 mmol/L insulin, 500 μmol/L isobutylmethylxanthine, 400 ng/mL dexamethasone with or without 1 μmol/L troglitazone. Oil Red O staining was performed as previously described (14). Lentiviruses were produced in 293FT cells using the packaging plasmids psPAX2 and pMD2.G.
Quantitative real-time PCR. cDNA synthesis and quantitative real-time PCR (qPCR) were performed as previously described (1). Relative expression levels were calculated by the ΔΔCt method using TBP as reference. The primers used are described in references 8 and 14.

Western blots. Cells were lysed in 150 mmol/L NaCl, 50 mmol/L Tris- HCl (pH 7.4), 1 mmol/L EDTA, and 1% Triton X-100 with protease and phosphatase inhibitors (Sigma). The following antibodies were used: HRP-Actin (Santa Cruz Biotechnology), tyrosine (4G10), IRS-1 (BD Biosciences), pC/EBP (Invitrogen), insulin receptor substrate 1 (IRS-1) (BD Biosciences), pTyrosine (4G10), pIRS-1Y896 (Biosource), pIRSY612 (Invi

RESULTS

Gpc4 expression in fat of rodents at the mRNA and protein level. We have previously shown that in mice Gpc4 mRNA expression is twofold higher in perigonadal than in subcutaneous fat (8). To better understand Gpc4 physiology, we raised a peptide antibody against murine Gpc4 and used this to assess Gpc4 protein levels in tissues and serum of mice. As expected, Western blots of extracts from 3T3-L1 preadipocytes that are run under nonreducing conditions for native Gpc4 revealed a broad smear from ~100 to >170 kDa, representing the 63-kDa core protein with the attached heparan sulfate chains of varying lengths (Supplementary Fig. 1). As previously described, the core protein of Gpc4 undergoes furin-mediated cleavage, creating two disulfide-linked subunits of Gpc4 (11). Thus, when these same extracts were run under reducing conditions, we detected the proteolytically cleaved N-terminal α-subunit of Gpc4 as a sharp band at 37 kDa, allowing more precise quantitation (Supplementary Fig. 1).

Using this assay, we found that the difference in expression of Gpc4 between the murine fat depots was even more marked at the protein than at the mRNA level, and that perigonadal fat had ~fivefold higher Gpc4 levels than subcutaneous and brown adipose tissue (Fig. 1B). As in humans, Gpc4 expression in perigonadal fat of mice showed a bell-shaped relationship with level of obesity with upregulation of Gpc4 expression in mice with mild obesity due to an HFD, and lower levels in the very obese db/db mice. In subcutaneous fat, Gpc4 expression was also increased in mice fed an HFD and increased even further in db/db mice in this depot. This regulation by obesity state was specific to WAT with no change in Gpc4 in brown adipocyte tissue (BAT) in either the HFD or db/db mice (Fig. 1C).

Role of Gpc4 in adipocyte differentiation and insulin signaling. To better understand the functional link between Gpc4 and adipogenesis, we created 3T3-L1 preadipocytes with stable knockdown of Gpc4 using lentivirally expressed shRNA (shGpc4). This resulted in a >95% depletion of Gpc4 mRNA (Fig. 2A) and a reduction of Gpc4 protein below the limits of detection when compared with control cells infected with scrambled shRNA (shScr) (Fig. 2B). The control 3T3-L1 cells differentiated efficiently into adipocytes within eight days after induction as visualized by Oil Red O (Fig. 2C). In contrast, Gpc4 knockdown cells failed to accumulate lipids. Furthermore, although stimulation by thiazolidinediones enhanced the differentiation of control cells, this had no significant effect on shGpc4 cells (Fig. 2C).

Failure to accumulate lipids was due to a blockade in differentiation. qPCR revealed that Gpc4 knockdown cells induced early adipogenic markers C/EBPβ and C/EBPδ at levels comparable with control. By contrast, treatment of knockdown cells with an induction cocktail did not induce the key downstream transcription factors for adipogenesis C/EBPβ and peroxisome proliferator–activated receptor γ (PPARγ), which were robustly increased in control cells (Fig. 2D) (17). Western blots from nuclear extracts 24 h after induction confirmed similar protein levels of C/EBPβ and C/EBPδ between control and knockdown cells (Fig. 2E); however, the important regulatory phosphorylation of
C/EBPβ Thr188 was reduced by 54% in Gpc4 knockdown cells compared with controls (Fig. 2E and F). Pull downs from nuclear lysates from these cells with oligonucleotides containing a C/EBP binding site revealed similar binding of C/EBPβ from control and shGpc4 cells; however, the bound C/EBPβ from Gpc4 knockdown cells showed greatly reduced Thr188 phosphorylation, indicating diminished activation of this key transcription factor (Fig. 2G). In addition to its role as activator of C/EBPα and PPARγ transcription, C/EBPβ is essential for clonal expansion in 3T3-L1 preadipocytes (18), and consistent with the diminished phosphorylation/activation of C/EBPβ, we also observed reduced mitotic clonal expansion in knockdown cells (Supplementary Fig. 2A).

Phosphorylation of C/EBPβ on Thr188 is mediated by MAPK and PI3-kinase signaling (19). Assessment of the phosphorylation/activation of ERK and Akt during the first 49 h of differentiation revealed a tendency for lower AktS473 phosphorylation, but no alterations of ERK phosphorylation (Supplementary Fig. 2B). Phosphorylation of IRS-1 on Y612 and Y896, sites required for insulin-mediated Akt and ERK activation, showed reduced phosphorylation, suggesting an effect of Gpc4 deletion on insulin signaling (Supplementary Fig. 2C).

Insulin stimulation of 3T3-L1 preadipocytes revealed 33% reduction in insulin receptor and reduced IGF-1 receptor (IGF1R) phosphorylation of Gpc4 knockdown cells compared with control (Fig. 3A and B). The reduced IR/IGF1R

**FIG. 1.** Gpc4 is differentially regulated in subcutaneous and visceral WAT upon weight gain. A: Gpc4 expression in subcutaneous (SCW) and visceral (Visc.) fat of 77 female and 83 male nondiabetic subjects, ranging from lean to obese, grouped by BMI. Visc. BMI 25–30 and visc. BMI >30 indicates subjects with a CT or MRI ratio between subcutaneous and visceral fat areas >0.4 in the given BMI range. B: Western blot for Gpc4 from 6-week-old C57BL/6 male mice. Actin is used as loading control. C: qPCR for Gpc4 from the indicated fat depots of C57BL/6 mice fed an HFD for 8 weeks, db/db and control mice. Control mice are C57BL/6 chow diet–fed mice and db/db mice combined (HFD, n = 4; db/db, n = 6; controls, n = 4–6). BAT, brown adipose tissue; PGF, perigonadal fat; SCF, subcutaneous flank fat. *P < 0.05; **P < 0.01; ***P < 0.001.
activation resulted in a reduction of IRS-1 phosphorylation and a 40–45% reduction in ERK activation (P < 0.01) and phosphorylation of Akt on Ser473 (P < 0.001) in Gpc4 knockdown cells (Fig. 3C and D). This was not caused by reduced insulin binding, as shGpc4 preadipocytes showed higher binding of the 125I insulin tracer, but lower affinity as judged by a rightward shift of the competition curve by unlabeled insulin (Supplementary Fig. 3A). Furthermore, AktS473 phosphorylation declined more rapidly in the Gpc4 knockdown cells during the 60 min time course (Fig. 3D), resulting in a ~50% reduction of AktS473 phosphorylation over the time course in Gpc4 knockdown cells as quantified by the area under the curve (Fig. 3E). This decreased AktS473 and ERK phosphorylation in Gpc4-depleted cells was observed in a wide range of insulin concentrations (Supplementary Fig. 3B). However, these changes were specific to insulin and not observed after stimulation with 10% FBS (Supplementary Fig. 3C).

Gpc4 interacts with the insulin receptor and enhances adipocyte differentiation independent of membrane anchorage. Gpc4 does not possess transmembrane or intracellular domains but is anchored to the cell membrane via a GPI anchor. Thus, Gpc4 itself cannot signal, but mediates its intracellular functions via interaction with other transmembrane proteins. Because depletion of Gpc4 resulted in reduced insulin/IGFIR activation (Fig. 3A and B), we tested for a possible interaction of Gpc4 with these receptors by performing coimmunoprecipitation experiments. This revealed commounoprecipitation of Gpc4 with the insulin receptor under basal growth conditions, which was lost upon insulin stimulation, indicating that Gpc4 interacts with the unoccupied insulin receptor, but dissociates upon insulin binding and receptor activation. Interestingly, interaction with the IGFIR showed a reciprocal pattern, as Gpc4 associated with the IGFIR after, but not prior to, insulin stimulation (Fig. 3F).

Table 1

| Gpc4-SCF | Gpc4-Visc |
|----------|----------|
| Gpc4-SCF/Visc | −0.446 ± 0.124 | −0.183 ± 0.051 |
| GIR | 0.301 ± 0.167 | 0.367 ± 0.104 |
| FGF | −2.99 ± 10.453 | −2.754 ± 6.691 |
| FPI | −0.294 ± 0.454 | 0.093 ± 0.291 |
| HOMA-IR | 8.277 ± 12.882 | −3.807 ± 8.254 |
| HbA1c | −14.145 ± 12.424 | 2.03 ± 7.989 |
| WHR | −67.304 ± 26.343 | 23.974 ± 17.128 |
| BMI | −0.821 ± 0.668 | 0.707 ± 0.426 |
| FFA | 3.226 ± 11.273 | 4.479 ± 7.211 |
| Cholesterol | −3.451 ± 5.653 | −1.742 ± 3.622 |
| HDL-C | 10.937 ± 9.864 | −0.797 ± 6.543 |
| LDLC | −5.614 ± 5.092 | −0.916 ± 3.273 |
| Sex | −10.416 ± 6.786 | 2.880 ± 4.374 |
| Age | 0.34 ± 0.201 | −0.44 ± 0.128 |

Shown are correlation coefficients ± SE. Values highlighted in boldface indicate significant correlations with a P value < 0.05. SCF, subcutaneous fat; Visc, visceral fat; FPG, fasting plasma glucose; FPI, fasting plasma insulin; FFA, free fatty acid.
Serum Gpc4 levels were ~1 ng/mL in the markedly obese ob/ob mice (Fig. 5C). Random-fed blood glucose and insulin measurements revealed that HFD-fed mice were still able to maintain normal glycemia and normal insulinemia, with much higher serum Gpc4 levels than controls, whereas ob/ob mice had elevated blood glucose levels despite hyperinsulinemia, which was accompanied with reduced serum Gpc4 levels (Supplementary Fig. 6B).

To determine whether Gpc4 was circulating in humans, we used a human Gpc4 ELISA assay to assess serum Gpc4 levels in the same cohort that had been used for expression analysis of Gpc4 mRNA in adipose. In males, serum Gpc4 levels paralleled the gene expression data from visceral fat (Fig. 5D), with the highest serum Gpc4 levels in individuals who were overweight with a visceral distribution and lower levels in both lean and viscerally obese subjects. By contrast, females showed a continuous increase in serum Gpc4 levels from lean to overweight and obese. When both male and female subjects were divided into the lowest and highest quartile of serum Gpc4 levels, those individuals with highest serum Gpc4 had significantly higher percentage body fat, higher BMI, larger WHR, and higher levels of free fatty acids and leptin, all markers of body fat content. Additionally, high serum Gpc4 was associated with increased markers of insulin resistance, including high homeostasis model assessment of insulin resistance (HOMA-IR), high fasting plasma insulin, and insulin resistance as assessed by decreased GIR (Fig. 5E and Supplementary Fig. 6C). We did not observe any association with fasting plasma glucose, cholesterol, HDL-C, LDL-C, or serum adiponectin, although in this group of nondiabetics, those with high serum Gpc4 did have significantly higher HbA1c values, although still within the normal range (Supplementary Fig. 6C). Multivariate analysis of 15 parameters including Gpc4 expression in subcutaneous and visceral fat confirmed a positive correlation.
of BMI and a negative correlation of GIR with serum Gpc4 levels (Table 2 and Supplementary Fig. 7A). When subjects were divided into subgroups of nonobese and obese subjects with either low serum Gpc4 ($\leq 5$ ng/mL) or high serum Gpc4 ($\geq 9$ ng/mL), nonobese subjects with high serum Gpc4 levels showed the same degree of insulin resistance, measured by fasting plasma insulin, GIR, and HOMA-IR, as obese subjects with either low

FIG. 3. Gpc4 regulates insulin receptor activation and downstream signaling. A: Western blots from insulin- and IGF1R $\beta$-subunit immunoprecipitations of confluent shScr and shGpc4 preadipocytes, blotted for insulin/IGF1R $\beta$ and pTyrosine before and after 5 min of 10 nmol/L insulin stimulation. B: Quantification of tyrosine phosphorylated insulin receptor in 3T3-L1 preadipocytes, normalized to total insulin receptor levels ($n = 6$). C: Western blots of confluent shScr and shGpc4 preadipocytes from total cell lysates before and after 5-min stimulation with 10 nmol/L insulin. D: Quantification of ERK and AktS473 phosphorylation at 0, 5, 10, 20, 40, and 60 min after insulin stimulation. pERK and pAktS473 were normalized to total ERK and Akt levels ($n = 8$). E: Area under the curve of AktS473 phosphorylation shown in D. F: Coimmunoprecipitation of Gpc4 with insulin and IGF1R $\beta$-subunit in 3T3-L1 cells. For all stimulation experiments, confluent undifferentiated preadipocytes were serum-starved for 3 h and stimulated with 10 nmol/L insulin. **$P < 0.01$; ***$P < 0.001$. (A high-quality color representation of this figure is available in the online issue.)
or high serum Gpc4 levels (Fig. 5F and Supplementary Fig. 7B). In an independent set of 30 age-, sex-, and BMI-matched obese insulin-sensitive and insulin-resistant patients (21), we observed ~2 times higher sGpc4 levels in insulin-resistant compared with insulin-sensitive patients (Fig. 5G).

DISCUSSION

Glypican-4 belongs to the family of GPI-anchored heparan sulfate proteoglycans, which includes six members in mammals (10). We previously found that Gpc4 is differentially expressed between fat depots and is highly regulated in obesity (8). We now show that Gpc4 regulates insulin...
FIG. 5. Gpc4 is released from adipocytes and correlates with markers of body fat and insulin resistance. A: Western blot for Gpc4 from conditioned serum-free Opti-MEMI of cultured isolated subcutaneous, perigonadal, and brown adipocytes and the corresponding SVF. Ponceau-S staining shows equal loading of proteins. Cells were isolated by collagenase digest and medium was conditioned for 12 h. B: Western blot of serum Gpc4. Glycoproteins from serum of 4-month-old C57BL/6 male and female mice were purified using anion exchange chromatography. Western blots from concentrated eluates were probed for Gpc4. C: Gpc4 ELISA from serum of C57BL/6 mice fed an HFD for 8 weeks, ob/ob and control mice. Control mice are C57BL/6 chow diet–fed mice and ob/+ mice combined (n = 6 per genotype). D: Gpc4 ELISA from serum of nondiabetic females (n = 77) and males (n = 83) grouped according to BMI and body fat distribution. E: Comparison of BMI, WHR, and GIR during a euglycemic hyperinsulinemic clamp and HOMA-IR of the lowest and highest quartile of serum Gpc4 levels of females and males (n = 19 and 20 per quartile, respectively). F: Comparison of GIR from nonobese (BMI <30) and obese (BMI >30) subjects divided into groups with low serum Gpc4 levels (≤5 ng/mL) and high serum Gpc4 levels (≥9 ng/mL). G: Serum Gpc4 levels in 30 obese age-, sex-, and BMI-matched insulin-sensitive and insulin-resistant subjects. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.
signaling via interaction with the insulin receptor. As a result, reducing levels of Gpc4 diminishes insulin signaling. In preadipocytes, this results in blunted activation of C/EBPβ and a block in adipocyte differentiation. We also demonstrate that Gpc4 is released from adipose tissue and that circulating Gpc4 in rodents and humans positively correlates with body fat content and insulin resistance.

Expansion of visceral adipose tissue, i.e., central obesity, is associated with insulin resistance, whereas expansion of subcutaneous adipose tissue, i.e., peripheral obesity, is not (5,7). Defining the mechanisms underlying body fat distribution and this differential link to insulin resistance is important for understanding the development of comorbidities associated with obesity, including type 2 diabetes, stroke, hypertension, and cardiovascular disease (22). We find that expression of Gpc4 is not only differential between subcutaneous and visceral fat, but that Gpc4 expression in visceral adipose positively correlates with both BMI and, independently, with insulin resistance as measured by euglycemic hyperinsulinemic clamps. Of greater significance, Gpc4 is present in serum of mice and humans, and serum Gpc4 levels are positively correlated with body fat content and insulin resistance. In nonobese persons, serum Gpc4 increases progressively with BMI, especially in viscerally obese women and viscerally overweight men. Multivariate analysis revealed an independent negative correlation of serum Gpc4 with GIR, i.e., higher serum Gpc4 levels are associated with greater insulin resistance. Indeed, nonobese subjects (BMI <30) with high serum Gpc4 (>9 ng/mL) levels have the same degree of insulin resistance by euglycemic clamp, fasting insulin, and HOMA-IR as obese subjects, independent of serum Gpc4 levels. Furthermore, sGpc4 levels are doubled in insulin-resistant obese subjects compared with age-, sex-, and BMI-matched insulin-sensitive subjects. Thus, serum Gpc4 is not only a marker for BMI, but it is also an independent marker of insulin resistance.

This link between Gpc4 and changes in insulin sensitivity appears to involve two novel mechanisms. First, glypicans are released from the cell surface by an enzymatically regulated process mediated by GPI-lipases. [125,126] Gpc4 is shed by adipocytes and hepatocytes and is released from white and brown adipose tissue and other organs (14,23). The source of serum Gpc4 is not known. Gpc4 is expressed in kidney, pituitary, and WAT, indicating that other tissues could contribute to serum Gpc4. However, the strong association of serum Gpc4 levels with BMI in humans and the fact that Gpc4 can be released from cultured primary adipocytes make adipose tissue one likely source of serum Gpc4.

To date, no circulating factor has been shown to directly enhance the activation of the insulin receptor itself. Both the transmembrane glycoprotein plasma cell membrane glycoprotein-1 or ectonucleotide pyrophosphatase/phosphodiesterase and circulating alpha 2-HS glycoprotein are known to interact with the extracellular domains of the insulin receptor and to negatively affect insulin binding and activation of the insulin receptor (28,29). By contrast, we find that both membrane- and nonmembrane-bound Gpc4 can interact with the insulin receptor and enhance insulin signaling. This interaction occurs with the unoccupied insulin receptor, and stimulation by insulin disrupts the interaction of Gpc4 with the insulin receptor. Thus, overexpression of native Gpc4 or ΔGpc4 or addition of recombinant ΔGpc4 enhances insulin signaling in 3T3-L1 cells, whereas the depletion of Gpc4 results in reduced insulin receptor phosphorylation and downstream signaling.

Insulin is an important regulator of adipocyte differentiation and function (4). In line with that, adipocyte differentiation is increased in Gpc4 or ΔGpc4 overexpressing cells and blocked in Gpc4 knockout cells. The latter is due to an inability to induce C/EBPα and PPARγ, the key transcription factors required for differentiation, secondary to reduced phosphorylation of C/EBPβ at the ERK/GSK3β consensus site Thr188. Phosphorylation of Thr188 is essential for DNA binding and transactivation of C/EBPα and PPARγ (19,30). Block of adipocyte differentiation at this stage of differentiation is also seen in IRS-1/IRS-2 double knockout cells (31), further indicating a link between insulin signaling and the adipocyte differentiation defect. Overexpression of the Akt and ERK inhibitor TRB3 also prevents activation of C/EBPβ and thereby inhibits adipocyte differentiation (14). However, it is possible that Gpc4 could affect additional signaling pathways, or that other factors within the insulin signaling pathway contribute to the differentiation defect, as insulin signaling induces a variety of transcription factors that might regulate adipocyte differentiation (32).

Taken together, our data show that Gpc4 is an insulin-sensitizing "adipokine" that directly interacts with the insulin receptor to regulate its activation and downstream signaling. The importance of Gpc4 in modulating insulin signaling is underlined by the inability of Gpc4 knockout cells to differentiate into adipocytes because of a lack of insulin signaling. In addition to its biological activity, serum levels of Gpc4 are correlated with insulin resistance. The role of Gpc4 as an insulin sensitizer and its higher serum levels in insulin-resistant individuals may seem counterintuitive at first. However, insulin itself shows a similar distribution, with lower levels in insulin-resistant subjects. Thus, serum Gpc4 is not only a marker for BMI, but it is also an independent marker of insulin resistance.

**Table 2**

Multivariate regression analysis of serum Gpc4 with clinical parameters and Gpc4 expression in WAT

| Parameter | Coefficient | SE | P value |
|-----------|-------------|----|---------|
| Serum Gpc4 | -0.004 ± 0.009 | | |
| Gpc4-SCF | -0.21 ± 0.014 | | |
| GIR | -0.46 ± 0.019 | | |
| FPG | -0.002 ± 1.164 | | |
| FPI | 0.0004788 ± 0.00515 | | |
| HOMA-IR | -0.59 ± 1.436 | | |
| Hba1c | 0.585 ± 1.389 | | |
| WHR | 3.023 ± 2.908 | | |
| BMI | 0.179 ± 0.075 | | |
| FFA | 0.855 ± 1.555 | | |
| Cholesterol | 0.217 ± 0.63 | | |
| HDL-C | 0.518 ± 1.103 | | |
| LDL-C | -0.996 ± 0.569 | | |
| Sex | 1.434 ± 0.762 | | |
| Age | 0.002 ± 0.022 | | |

Shown are correlation coefficients ± SE. Values highlighted in boldface indicate significant correlations with a P value < 0.05. SCF, subcutaneous fat; Visc, visceral fat; FPG, fasting plasma glucose; FPI, fasting plasma insulin; FFA, free fatty acid. [125,126] Gpc4-SCF, Gpc4-Visc, and Gpc4-GIR are independently, with insulin resistance as measured by visceral adipose tissue positively correlating with both BMI and, cutaneous and visceral fat, but that Gpc4 expression in WAT, and that Gpc4 expression in kidney, pituitary, and WAT, indicating that other tissues could contribute to serum Gpc4. However, the strong association of serum Gpc4 levels with BMI in humans and the fact that Gpc4 can be released from cultured primary adipocytes make adipose tissue one likely source of serum Gpc4.
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versus insulin-resistant individuals. Given that GPD1 is the most likely candidate to cleave Gpc4 and is itself an insulin-regulated gene, it is possible that increasing levels of insulin early in obesity lead to increased Gpc4 cleavage, which results in increased circulating Gpc4 levels. With disease progression, as in the *ob/ob* mouse, increased insulin resistance in GPD1-producing cells would result in a reduction of GPD1 activity and a drop in circulating Gpc4 levels, further decreasing insulin sensitivity and accelerating disease progression. Thus, our data suggest that increased circulating Gpc4 levels could be a novel regulatory mechanism by which fat acts to counteract insulin resistance, and maintaining high serum Gpc4 levels in severely insulin-resistant or diabetic subjects could lower insulin demands. Although further studies are required to dissect the various function of soluble versus membrane-bound Gpc4, glypican-4 forms a novel adipokine and a novel mechanism by which adipose tissue can modulate insulin signaling.

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S.U. wrote the manuscript and researched data. O.B. and M.B. researched data and reviewed and edited the manuscript. C.R.K. helped design experiments and edited the manuscript. C.R.K. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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