Characterization of the Wnt Inhibitors Secreted Frizzled-Related Proteins (SFRPs) in Human Adipose Tissue

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Context: Wnt signaling regulates adipogenesis and adipocyte function. Secreted frizzled-related proteins (SFRPs) are a family of secreted proteins (SFRP1-5) that bind and inhibit Wnts. Several members, including SFRP5, have recently been implicated in adipocyte dysfunction in obesity.

Objective: Our objective was to characterize the expression, secretion, and function of the SFRP family in human white adipose tissue (WAT) and fat cells.

Design: *SFRP1-5* mRNA expression was measured in human sc and visceral WAT from lean and obese individuals and correlated to insulin sensitivity. SFRP secretion from WAT explants was assessed by ELISA. Gene expression of *SFRPs* in cultured adipocytes during and after differentiation was determined. Functional analyses were done by gene silencing or incubations with recombinant SFRPs.

Results: *SFRP1-4*, but not *SFRP5*, mRNA levels were altered in obesity. However, although *SFRP1* was down-regulated and correlated positively with insulin sensitivity, *SFRP2-4* were up-regulated, particularly in visceral WAT, and associated with insulin resistance. Only SFRP1, SFRP2, and SFRP4 were secreted from WAT, thereby constituting adipokines. Individual knockdowns of SFRP1, SFRP2, or SFRP4 during adipogenesis did not affect terminal differentiation. Incubations with SFRP1 reduced the secretion of the proinflammatory cytokines IL-6 and monocyte chemotactic protein-1 (MCP1) and increased the release of adiponectin.

Conclusions: SFRP1, SFRP2, and SFRP4 are adipokines, the expression of which correlates with insulin sensitivity. For SFRP1, this may be related to effects on the secretion of IL-6, MCP1, and adiponectin. In contrast to recent murine findings implicating SFRP5 in metabolic dysfunction, this SFRP is neither regulated by obesity nor actively secreted from human WAT. **(*J Clin Endocrinol Metab* 98: E503–E508, 2013)**

Obesity-related disturbances in white adipose tissue (WAT) are linked to the development of insulin resistance, and numerous factors affecting adipocyte function have been identified in recent years (1). For instance, WAT secretes >300 polypeptides termed adipokines, and the production of several is markedly altered in obesity and has been implicated in promoting a pernicious metabolic profile characterized by a chronic low-grade inflammation and reduced insulin sensitivity (2).

The Wingless-type mouse mammary tumor virus integration site (Wnt) family of secreted proteins has established roles in differentiation and cell function in several tissues including WAT (3). Several Wnts attenuate adipocyte differentiation by reducing the expression of proadipogenic transcription factors including peroxisome proliferator-activated receptor γ and members of the CAAT/enhancer-binding protein (C/EBP) family (3); transgenic overexpression of specific Wnts in adipocytes results in

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Abbreviations: BMI, body mass index; C/EBP, CAAT/enhancer-binding protein; DIO, diet-induced obesity; hADSC, human adipose-derived stem cell; $HOMA_{IR}$, homeostasis model assessment for insulin resistance; MCP1, monocyte chemotactic protein-1; SFRP, secreted frizzled-related protein; vWAT, visceral WAT; WAT, white adipose tissue.

reduced body fat and resistance to diet-induced obesity (DIO) (4).

Wnt signaling is modulated at several levels. A number of secreted proteins, including secreted frizzled-related proteins (SFRPs), bind Wnts, thereby inhibiting their action (5). In mammals, the SFRP family consists of 5 members termed SFRP1-5 (91%–98% amino acid identity between human and murine sequences) of which SFRP1, -2, and -4 stimulate adipogenesis in both human and murine in vitro models (5–7). SFRP1 expression is altered in both obese murine and human WAT (6), and SFRP1$^{-/-}$ mice display reduced fat mass (3). SFRP5 has recently been studied in different obese mouse models, but its role remains unclear because it has been shown to be both up-regulated (8) and down-regulated (9) in WAT and SFRP5$^{-/-}$ mice are reported to be either resistant (8) or sensitive (9) to DIO. However, a comprehensive investigation in human WAT has been lacking. Thus, we performed a functional mapping of SFRPs in human adipose tissue and adipocytes focusing on their expression, secretion, and possible function.

Materials and Methods

Adipose tissue samples

Abdominal scWAT or visceral WAT (vWAT) was obtained from healthy subjects undergoing surgery for nonmalignant diagnoses. All were examined after an overnight fast and classified as nonobese (herein defined as body mass index [BMI] < 27 kg/m^2) or obese (BMI ≥ 30 kg/m^2). Cohorts 1 to 3 (Supplemental Table 1, published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org) have been described previously (10–12). Written informed consent was obtained from all subjects. Studies were approved by the Regional Ethics board.

Cell culture

Human adipose-derived stem cells (hADSCs) were isolated from scWAT of a male donor (16 y old, BMI 24 kg/m^2). They were isolated, cultured, and differentiated to adipocytes using described protocols (13, 14).

Quantitative PCR

RNA isolation, cDNA synthesis, quantitative PCR, and data analysis were performed as described (11). Primers and probes are described in Supplemental Tables 2 and 3.

Small interfering RNA

Upon seeding, hADSCs (1 500 cells/cm^2) were reverse transfected using HiPerfect (Invitrogen, Life Technologies Corp, Carlsbad, California) and small interfering RNA (50 nM; Dharmacon, Thermo Fisher Scientific, Waltham, Massachusetts) (Supplemental Table 4). The cells were allowed to attach to the plate bottom for 24 hours before adipocyte differentiation was initiated.

ELISA and in vitro incubations

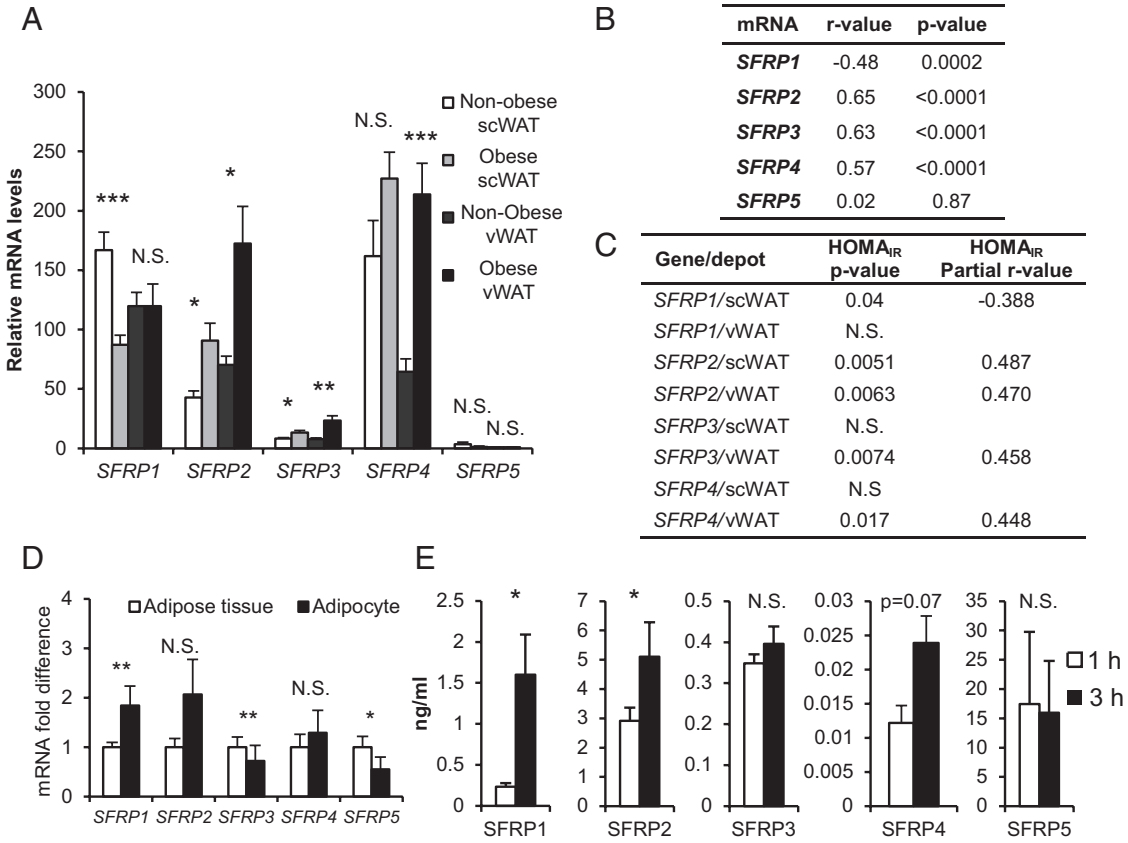
ELISA kits are detailed in Supplemental Table 4. Adipose tissue explants (BMI of donors detailed in Supplemental Table 1) were incubated in medium as described (15) and lactate dehydrogenase activity in conditioned media (a marker of necrosis) was determined using a kit from Roche (Basel, Switzerland; product 11644793001). Recombinant SFRPs (Supplemental Table 4) were added to the medium of hADSCs at day 12 of differentiation. Medium and RNA were collected after 48 hours. The amount of glycerol (lipolysis index) was assessed as described (16) and normalized to RNA concentration.

Results

SFRP1-5 mRNA expression was measured in scWAT and vWAT from cohort 1 (Figure 1A). Using TaqMan probes, all *SFRPs* were detectable, but *SFRP3* and *-5* were detected at comparably lower levels. In scWAT, *SFRP1* was decreased, whereas *SFRP2* and *-3* were increased in obesity. *SFRP4* displayed a tendency ($P = .084$) toward higher levels in obesity. In vWAT, *SFRP1* was not affected by obesity, whereas the differences in *SFRP2-4* mRNA were even more pronounced. *SFRP5* mRNA was not influenced by obesity in either depot. To test the results in another cohort (cohort 2), we correlated gene expression in scWAT from an independent set of 56 nonobese and obese subjects and found that *SFRP1* was negatively and *SFRP2-4* were positively correlated with BMI, whereas *SFRP5* was unaffected (Figure 1B).

The correlation between the mRNA expression of the obesity-regulated *SFRP1-4* and homeostasis model assessment for insulin resistance ($HOMA_{IR}$) was evaluated using BMI as a covariate (cohort 1, Figure 1C). In scWAT, *SFRP1* was negatively and *SFRP2* positively associated with insulin resistance. In vWAT, *SFRP2-4* correlated with $HOMA_{IR}$. Because we measured expression on intact tissue, we assessed the cellular source of *SFRP1-5* by analyzing the mRNA levels in paired samples of isolated adipocytes versus intact scWAT (cohort 3, Figure 1D). *SFRP1* was higher whereas *SFRP3* and *SFRP5* were lower in the adipocyte fraction. For *SFRP2* and *SFRP4*, the differences were not statistically significant. This suggests that *SFRPs* are expressed in both adipocytes and the stromal vascular fraction, although *SFRP3* and *SFRP5* are primarily expressed in the nonadipocyte fraction.

SFRP1-5 secretion was assessed in scWAT explants (Figure 1E). Whereas SFRP1 and SFRP2 levels displayed a significant time-dependent increase in conditioned media, SFRP4 showed a borderline significance ($P = .07$). SFRP3 and SFRP5 levels remained unaltered at the 2 time points, suggesting that neither of these factors is actively secreted by scWAT. Notably, the absolute levels of SFRP3 and



B

mRNA	r-value	p-value
SFRP1	-0.48	0.0002
SFRP2	0.65	<0.0001
SFRP3	0.63	<0.0001
SFRP4	0.57	<0.0001
SFRP5	0.02	0.87

C

Gene/depot	$HOMA_{IR}$ p-value	$HOMA_{IR}$ Partial r-value
SFRP1/scWAT	0.04	-0.388
SFRP1/vWAT	N.S.	
SFRP2/scWAT	0.0051	0.487
SFRP2/vWAT	0.0063	0.470
SFRP3/scWAT	N.S.	
SFRP3/vWAT	0.0074	0.458
SFRP4/scWAT	N.S	
SFRP4/vWAT	0.017	0.448

Figure 1. SFRP gene expression and protein secretion in human adipose tissue and adipocytes. A, Relative mRNA expression (measured by quantitative PCR) of *SFRPs* in scWAT and vWAT of obese and lean subjects from cohort 1 (n = 37). B, Relative mRNA expression (measured by microarray in scWAT) of individual *SFRPs* from subjects in cohort 2 (n = 56) correlated with BMI. *P* and *r* values for linear regression are shown. C, Multiple regression analysis was performed to correlate relative *SFRP* mRNA levels with $\log_{10}$ $HOMA_{IR}$ score using BMI as a covariate. D, *SFRP* mRNA expression was determined in paired samples of intact scWAT and isolated adipocytes (cohort 3, n = 14). Expression levels in adipocytes are expressed relative to those observed in tissue. E, Human adipose tissue explants (donors detailed in Supplemental Table 1) were incubated in suspension, and conditioned medium was taken at 1 and 3 hours after the start of incubation. SFRP levels were determined by ELISA. There was no sign of increased cell death in explant cultures as determined by lactate dehydrogenase activity assay (data not shown). The number of samples (n) was as follows: SFRP1, n = 6; SFRP2, n = 6; SFRP3, n = 7; SFRP4, n = 8; SFRP5, n = 6. For all determinations of mRNA levels, statistical comparisons were done using Student's *t* test, for adipocyte vs adipose tissue mRNA, comparisons were performed on $\log_{10}$ values. SFRP release from adipose explants was assessed using Wilcoxon signed rank test. Values in graphs are presented as mean + SD except for E, which shows variability as SEM. $*P < .05$; $**P < .01$; $***P < .001$; N.S., not significant.

SFRP4 were considerably lower than those of the other SFRPs.

SFRP gene expression was determined during in vitro differentiation of hADSCs. *SFRP1* and in particular *SFRP4* were expressed at higher levels in nondifferentiated cells with lower levels toward the end of the differentiation process (Figure 2A). In contrast, *SFRP2* and *SFRP3* (not shown) displayed no distinct alterations in mRNA levels, although the variations in the *SFRP2* measurements were high. *SFRP5* was not detectable in these cells (data not shown). Further studies were focused on SFRP1, SFRP2, and SFRP4 because these were the only SFRPs that were actively secreted from human WAT. Their impact on adipogenesis was assessed by knockdown before the start of the differentiation (Figure 2A) followed by mRNA expression analysis of adipogenesis-associated genes at different time points (Figure 2B). Pivotal adipogenic transcription factors including peroxisome proliferator-activated receptor γ (*PPARG*, Figure 2B) and C/EBPα (data not shown) were unaffected by SFRP down-regulation, whereas C/EBPδ (*CEBPD*, Figure 2B) levels were slightly increased throughout the differentiation process. However, this did not have an impact on adipocyte phenotype because the expression of several adipocyte marker genes including *FABP4* (Figure 2B), *PLIN1*, *ADIPOQ*, *PNPLA2*, and *LIPE* as well as cell morphology (data not shown) were unaltered at full differentiation, suggesting that, at least under the present conditions, SFRPs do not influence adipogenesis to any significant degree. To investigate whether recombinant SFRP1, SFRP2, and SFRP4 could influence adipocyte function, in vitro differentiated hADSCs were incubated with different concentrations of each factor for 48 hours (Figure 2C). Glycerol concentrations in conditioned media were unaltered, implying that

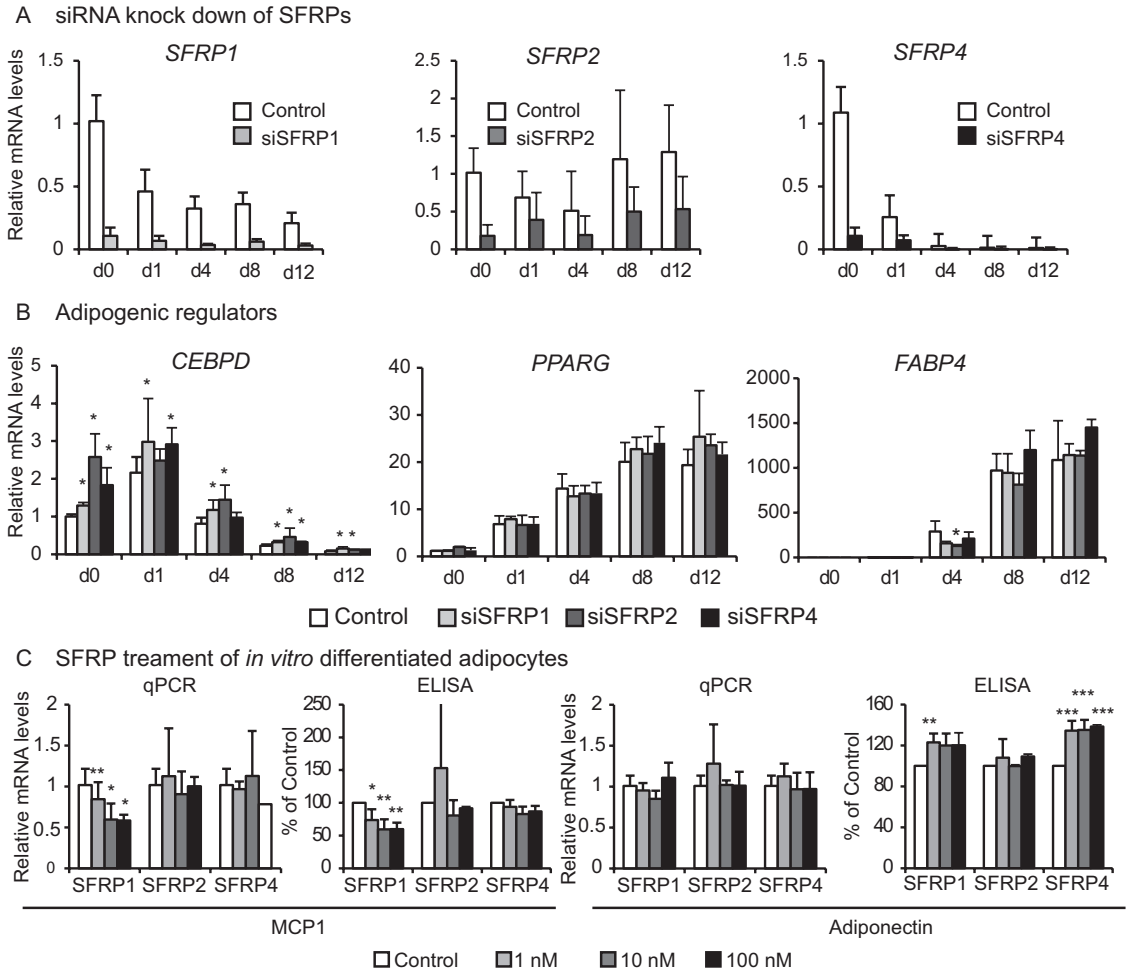


Figure 2. Effects of *SFRP* RNA interference (RNAi) and recombinant protein in human in vitro differentiated adipocytes. A, The mRNA expression of *SFRP1, -2*, and *-4* was determined in in vitro differentiated human adipocytes at the start of differentiation (day 0) and days 1, 4, 8, and 12 after induction of differentiation. Cells were transfected with small interfering RNA targeting *SFRP1, -2*, or *-4* 1 day before induction of differentiation. Quantitative RT-PCR measurements confirmed the silencing effect. For all 3 transcripts, gene knockdown by RNAi was efficient and selective with no off-target effects on other *SFRPs* (data not shown). For *SFRP1* and *-4*, the RNAi effect persisted for the entire 12-day follow-up, whereas for *SFRP2*, gene knockdown was somewhat less pronounced after day 1. B, Measurements of mRNA expression were also performed for adipogenic transcription factors and other adipocyte marker genes. C, Cells were incubated for 48 hours with the indicated concentrations of SFRPs. Treatment with SFRP1 attenuated the mRNA levels and secretion of MCP1 (left panels). Both SFRP1 and SFRP4 treatment increased adiponectin secretion without affecting *ADIPOQ* mRNA levels (right panels). Error bars represent SDs, and statistical significance was calculated using Student's *t* test on 3 independent experiments. $*P < .05$; $**P < .01$; $***P < .001$.

basal lipolysis was not influenced (data not shown). Adiponectin secretion was increased by both SFRP1 and SFRP4, whereas *ADIPOQ* (the adiponectin gene) mRNA expression was unaffected. SFRP1 reduced the levels of the proinflammatory factors monocyte chemotactic protein-1 (MCP1) (Figure 2C) and IL-6 (data not shown), which for the former were also observed at the mRNA level.

Discussion

We demonstrate, in 2 independent cohorts, that 4 of 5 SFRP family members are differentially expressed in human WAT comparing nonobese and obese subjects. In addition, SFRP1 and -2 and possibly SFRP4 are actively secreted from human WAT and can thus be regarded as adipokines. SFRP1 is the only SFRP being reduced in obese scWAT, and the mRNA expression is negatively correlated with insulin resistance, independently of BMI. Although the exact mechanisms remain to be elucidated, a causal link may be via effects on inflammation because recombinant SFRP1 reduced the secretion of the proinflammatory factors MCP1 and IL-6 and increased the release of adiponectin in vitro. These results are in agreement with data in nonadipose tissue, demonstrating that SFRP1 may exert an anti-inflammatory action by reducing cytokine release (IL-6 among them) in a mouse model of myocardial infarction (17). Interestingly, SFRP5 has recently been proposed to play a similar role in murine models (9).

However, because SFRP5 in human WAT is neither regulated by obesity nor actively secreted by WAT, it is tempting to speculate that SFRP1 may play the corresponding role in human WAT.

SFRP1 expression in scWAT has previously been reported to display an inverse U-shaped relationship with the highest mRNA levels observed in subjects with mild obesity (BMI 30–35 kg/m^2) (6). In contrast, in our cohorts comprising a total of 93 subjects with a very broad BMI range, *SFRP1* expression displayed a negative linear relationship with BMI over the entire weight range.

We found a slight decrease in *SFRP1* expression during adipogenesis, whereas Lagathu et al (6) observed an increase in SFRP1 mRNA expression in murine and human adipose stroma-vascular cultures and 3T3-L1 cells. The reasons for these discrepancies are probably related to the use of different cell systems and differentiation cocktails, and we cannot exclude that SFRPs may affect adipogenesis in human cells differentiated under less potent adipogenic conditions. Moreover, we cannot preclude potential redundancies in the SFRP family that would be observable only if 2 or more SFRPs were silenced concomitantly.

Ouchi et al (9) reported that SFRP5-deficient mice are sensitive to DIO and develop severe glucose intolerance and an inflamed insulin-resistant adipose tissue. They also reported lower SFRP5 expression in obese human subjects with significant macrophage infiltration. These data were to some degree supported by a study reporting that although circulating SFRP5 were similar in lean and obese subjects, levels increased after caloric restriction (18). In contrast, Mori et al (8) reported that SFRP5$^{-/-}$ mice are resistant to DIO and have smaller adipocytes with enhanced mitochondrial metabolism compared with wild-type mice. In line with these findings, *ob/ob* mice (19) as well as mice with DIO (20) display elevated expression of SFRP5 in WAT. Our findings do not support a role for SFRP5 in human WAT, because the mRNA levels in WAT are low and unaltered by obesity and SFRP5 is not actively secreted from human WAT. The observation that SFRP5 gene expression in WAT was low, whereas protein levels in conditioned media of WAT explants were relatively high, indicates that SFRP5 protein may predominantly be present within WAT blood vessels. This notion is corroborated by the fact that SFRP5 is clearly detectable in the circulation (18). Admittedly, although SFRP5 was not actively secreted from human WAT and its gene expression was not influenced by obesity, this does not exclude functional roles in human adipocytes that remain to be elucidated.

In conclusion, the expression of several SFRPs is perturbed in obesity and associates with markers of insulin resistance. Although their exact functional role remains to be defined, SFRP1 is a factor positively correlated with insulin sensitivity, possibly via effects on adipokine secretion. Finally, in contrast to recent findings in mice, *SFRP5* expression in human WAT does not appear to be influenced by obesity.

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