FABP4 Attenuates PPARγ and Adipogenesis, and is Inversely Correlated With PPARγ in Adipose Tissues

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ABSTRACT

Fatty Acid Binding Protein 4 (FABP4, aP2) is a cytoplasmic fatty acid chaperone, expressed primarily in adipocytes and myeloid cells and implicated in the development of insulin resistance and atherosclerosis. Here we demonstrate that FABP4 triggers the ubiquitination and subsequent proteasomal degradation of PPARγ, a master regulator of adipogenesis and insulin responsiveness. Importantly, FABP4-null mouse pre-adipocytes as well as macrophages exhibited increased expression of PPARγ, and complementation of FABP4 in the macrophages reversed the increase in FABP4 expression. The FABP4-null pre-adipocytes exhibited a remarkably enhanced adipogenesis compared to WT cells, indicating that FABP4 regulates adipogenesis by down-regulating PPARγ. We found that FABP4 level was higher and PPARγ level was lower in human visceral fat and mouse epididymal fat compared with their subcutaneous fat. Furthermore, FABP4 was higher in adipose tissues of obese diabetic individuals compared to healthy ones. Suppression of PPARγ by FABP4 in visceral fat may explain the reported role of FABP4 in the development of obesity-related morbidities, including insulin resistance, diabetes, and atherosclerosis.
Adiposity is closely correlated with important physiological parameters such as blood pressure, systemic insulin sensitivity, dyslipidemia and serum triglyceride levels (1; 2), rendering obesity to an independent risk factor for myocardial infarction, stroke, type 2 diabetes mellitus, and certain cancers (3). Among adipose tissues, visceral fat is more closely correlated with obesity-associated pathology than overall adiposity (4-8).

The nuclear receptor PPARγ is a master regulator of adipose cell differentiation, playing a critical role in systemic lipid and glucose metabolism (9). PPARγ is activated by natural or synthetic agonists such as the anti-diabetic thiazolidinedione (TZD) (10). Activated PPARγ is a master regulator of adipogenesis, acting as a transcription factor of genes expressed in mature adipocytes, including FABP4, CD36, lipoprotein lipase (LPL) and adiponectin, all of which containing peroxisome proliferator response elements (PPREs) (11). PPARγ is also expressed in myeloid cells, and its activation promotes an anti-inflammatory phenotype (11). Disruption of PPARγ specifically in myeloid cells also predisposes mice to the development of diet-induced obesity, insulin resistance, and glucose intolerance (12), whereas activation of PPARγ within macrophages promotes lipid efflux, thereby stabilizing atherosclerotic lesions (13).

A major target gene of PPARγ is the lipid transporter FABP4, also known as aP2 (14). PPARγ induces FABP4 almost exclusively in adipocytes and macrophages. FABP4 acts as a fatty acids chaperone, which couples intracellular lipids to biological targets and signaling pathways (15; 16). FABP4 has been implicated in several aspects of the metabolic syndrome in mice, including insulin resistance and atherosclerosis (17-22). In humans, a T87C polymorphism in the FABP4 promoter leads to reduced transcriptional activity, resulting in lower serum triglycerides and a reduced risk of atherosclerosis and type 2 diabetes (23). In addition, the level of circulating human FABP4 was proposed as an independent prognostic marker for future development of diabetes (24; 25). Furthermore, PPARγ is considered a tumor suppressor gene (11), whereas FABP4 has a role in tumorigenesis (26; 27).

Understanding the mechanisms by which obesity alters adipose tissue biology is of major importance because the fat tissue acts as an endocrine organ that regulates systemic metabolism (28). Visceral adipose tissues (VAT) and subcutaneous adipose tissues (SAT) are functionally distinct and many clinical studies led to the conclusion that VAT rather
than SAT is the key factor inducing the metabolic syndrome, also serving as a source of inflammatory and stress-promoting factors (8; 29-31).

To date, the mechanisms by which FABP4 promotes insulin resistance and inflammation are not fully understood. The fact that FABP4 is induced by PPARγ but exerts metabolic and immunologic activities opposite to those of PPARγ led us to hypothesize that FABP4 might be involved in regulating PPARγ activity. Here we show that FABP4 negatively regulates PPARγ levels in macrophages and in adipocytes, thereby attenuating adipocyte differentiation; we elucidate a mechanism involving proteasomal PPARγ degradation and further demonstrate elevated FABP4/PPARγ ratio in intra-abdominal fat compared with SAT in mice and VAT compared with SAT in humans.

**RESEARCH DESIGN AND METHODS**

**Cells and Reagents.** Human THP-1 (TIB-202) cells, human HEK 293T cells, HeLa (CCL-2.1) cells, mouse NIH-3T3 fibroblasts (CRL-1658) and mouse 3T3-L1 pre-adipocytes (CL-173) were from the ATCC. Immortalized WT and FABP4-null pre-adipocytes, as well as immortalized WT, FABP4-null and FABP4-complemented macrophage cell lines were generated from WT and FABP4-null mice as previously described (22; 32). 3T3-L1 cells and pre-adipocytes from FABP4 KO and WT mice were grown in DMEM containing 10% Bovine Serum (BS). THP-1 cells and immortalized macrophage cell lines were grown in RPMI 1640 medium containing 10% FBS, 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES and 1 mM sodium pyruvate. All other cell lines were grown in DMEM with 10% FBS (GibcoBRL), 100 IU/ml penicillin and 0.1 mg/ml streptomycin. The FABP4 inhibitor BMS309403 and the proteasome inhibitor MG262 were from Calbiochem. Rosiglitazone was from Cayman Chemical. JetPEI™ transfection reagent was from Polyplus-transfection. siRNA oligonucleotides were from Dharmacon. Cell Line Nucleofector kit V was used for siRNA transfections of THP-1 cells (Amaxa GmbH, Cologne, Germany). All other reagents were from Sigma.

**Differentiation of THP-1 Cells and Pre-Adipocytes.** THP-1 cells (1x10⁶/ml) were induced to differentiate by PMA (100 nM, 24 h). For experiments in which BMS309403 was included, the medium was supplemented with 10% lipoprotein deficient serum
(LPDS) instead of 10%FBS. 3T3-L1 pre-adipocytes were differentiated as described (33). Briefly, cells were sub-cultured at <70% confluence. To induce differentiation, confluent cell cultures were placed in medium containing 0.5 mM 1-Methyl-3-Isobutylxanthine (IBMX), 1 µM dexamethasone, and 5 µg/ml insulin in 10% FBS/DMEM for 48 h. Next, the medium was changed, and 5 µg/ml insulin was added for another 48 h. The medium was then replaced with 10% FBS/DMEM, and was renewed every 2 days until the cells became fully differentiated and lipid droplets were apparent.

**Oil Red O Staining.** Cells were washed, fixed with formaldehyde (4%, 20 min), washed 3X with PBS, stained with Oil red O (5% in isopropanol, freshly diluted 2:3 with water, 30 min.) and washed.

**Transient Transfection Assays.** Cells at 50%-70% confluence were transfected using the jetPEI™ reagent and the indicated plasmids, according to the manufacturer's instructions. Wherever needed, an empty vector was added to maintain a constant amount of 5 µg DNA/well. pCS6, (empty vector), pPPARγ and pFABP4 were from Open Biosystems. The following vectors were kindly provided by the indicated persons: pp53, Y. Shaul; pPPRE-luc; the lab of the late M. Liscovitch; pHA-Ub, C. Kahana. THP1 cells were transiently transfected using the Amaxa nucleofection technology (Amaxa GmbH, Cologne, Germany) before differentiation.

**Preparation of Adipose Tissue Lysates.** FABP4-null mice were generated as described (22). Biopsies from epididymal and subcutaneous depots of 12 week old mice were removed, frozen in liquid nitrogen, and stored at –80°. Fat tissues were homogenized in lysis buffer (10 mM Na₂HPO₄, pH 7.5, 5 mM EDTA, 100 mM NaCl, 1% Triton, 0.5% sodium deoxycholate, 0.1% SDS) containing protease inhibitors (1:1000, Sigma) and 1 mM PMSF. The Institutional Ethics Committee approved in advance all protocols of the study.

Paired human visceral and abdominal subcutaneous adipose tissue biopsies (VAT and SAT, respectively) were obtained during elective abdominal surgery for gastric banding, weight reduction surgery, or exploratory laparotomy (with negative findings) as described (34). The Institutional Ethics Committee approved in advance all protocols of the study, and all participants gave a written informed consent after all objectives and procedures were explained. Biopsies were delivered on ice, rinsed in saline, frozen in
liquid nitrogen, powdered and then homogenized in lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.5, 1% by volume Nonidet P-40, 0.25% sodium deoxycholate, 10 mM sodium β-glycerophosphate, 5 mM sodium pyrophosphate, 1 mM EGTA, 1 mM sodium vanadate, and 1 mM NaF), supplemented with protease inhibitor cocktail (1:1000; Sigma).

**Immunoblot Analysis.** Cell pellets were re-suspended on ice for 20 min. in a buffer consisting of 100 mM KCl, 0.5 mM EDTA, 20 mM HEPES, pH 7.6, 0.4% Nonidet P-40, 20% glycerol, protease inhibitors (Sigma) and 1 mM PMSF. The clarified lysate was stored at -80°C. Protein concentration was determined by BCA protein assay reagent kit (Pierce, Rockford, IL) using bovine serum albumin as a standard. Samples were subjected to 15% or 6%-18% gradient SDS-PAGE and immunoblotted with anti Actin (MP Biomedicals), anti FABP4, anti PPARγ, anti LPL (Santa Cruz Biotechnology), anti HA (Covance), and anti ubiquitinated proteins (clone FK2, Biomol). Mouse monoclonal anti p53 was a kind gift of C. Prives. Detection was performed using HRP-conjugated secondary antibodies (Jackson ImmunoResearch Labs), followed by Supersignal chemiluminescence detection system (Pierce). Quantitative densitometry was performed with EZ-Quant software. Protein levels were normalized to values obtained for β-actin.

**Immunoprecipitation Assays.** Cells were washed with ice-cold PBS and lysed with PBS containing 1% Triton X-100, 0.5% sodium deoxycholate, 10 mM sodium pyrophosphate, 2 mM sodium vanadate, 100 mM sodium fluoride, protease inhibitors mixture (Sigma), and 1 mM PMSF. Samples (500 µg protein) were pre-cleared by adding 60 µl protein G-Sepharose (GE Healthcare, Uppsala, Sweden, 1 h, 4°C). Anti-PPARγ antibody (1:100 in lysis buffer) was added for 17 h at 4°C, protein G-Sepharose was then added for another 1 h. The beads were then washed 4x with 1% Nonidet P-40 in PBS, extracted with Sample buffer and analyzed by SDS-PAGE and immunoblotting, as described above.

**Reporter Gene Assay.** NIH-3T3 cells (10-17x10³ cells/well) in 96 well plates were transfected using the jetPEI™ reagent with the following plasmids: 3X PPRE-TK-luciferase reporter (67 ng), pPPARγ (67 ng), pFABP4 (67 ng) and 10 ng pRL-TK (Renilla luciferase reporter vector, Promega). After 24 h, the medium was replaced by DMEM/10% FBS plus 10 µM rosiglitazone or vehicle (DMSO) for 48 h. Cell lysates were then analyzed for luciferase activity and normalized to Renilla activity using the
Dual-Luciferase® Reporter Assay Kit (Promega), according to the manufacturer’s instructions.

**Quantitative Real-Time PCR (qPCR).** Total RNA (1 µg), isolated using PerfectPure RNA Cultured Cell Kit (5 Prime) was reverse transcribed using random hexamers and High Capacity RNA-to-cDNA kit (Applied Biosystems). The reaction mixtures were diluted 15-fold, and 4.5 µl was then used as the template for real-time PCR. The following assays were performed: mFABP4 - Mn00445880_m1, mPPARγ - Mn00440945_ml, and mTBP – Mm00446973_m1 as a loading control. These probes were designed using Roche ProbeFinder Software (Roche Diagnostics). Quantitative RT-PCR was performed using a Roche LightCycler® 480 Real-Time PCR System and TaqMan Universal PCR Master Mix (Applied Biosystems). The amplification program was: initial denaturation at 95°C for 15 min, followed by 45 cycles of 95°C for 15 sec, 60°C for 1 min and 40°C for 30 sec. Gene expression was normalized to TBP mRNA was calculated using LightCycler Software (Roche). The results are means of fold change± SEM of triplicates.

**Inhibition of FABP4 Gene Expression by RNAi.** THP-1 cells were transfected using the Cell Line Nucleofector Kit V (Amaxa) with either siFABP4 siGENOME SMARTpool, 140 nM, or Non-Targeting siRNA Pool, 140 nM, Dharmacon, using the Amaxa nucleofection technology, program V-01. After 24 h, the cells were induced to differentiate to macrophages and the levels of FABP4 and PPARγ mRNA were determined by qPCR.

**Statistical Analysis.** ANOVA and Student’s t test were performed for statistical analysis, as appropriate. Statistical significance was designated at P<0.05. Values are expressed as mean±SEM.

**RESULTS**

**Inhibition of FABP4 enhances PPARγ level and activity.** THP-1 cells were induced to differentiate into macrophage-like cells and then incubated with either vehicle or the FABP4 inhibitor BMS309403. Inhibition of FABP4 did not affect the level of PPARγ mRNA (Fig. 1A) but elevated the basal level of PPARγ protein by 2.6±0.8 fold (P<0.005, n=3), as determined by immunoblotting and densitometry (Fig. 1B, C). To further study
the effect of FABP4 on PPARγ, we knocked down FABP4 mRNA in THP-1 cells by FABP4 targeting siRNA or non-targeting control siRNA. After 24 h, the cells were differentiated to macrophages and after an additional 48 h they were analyzed for the protein and the mRNA levels of both FABP4 and PPARγ. FABP4 siRNA effectively inhibited the expression of FABP4 mRNA and protein (Fig. 1C, D) and had only a minor effect on the expression of PPARγ mRNA (Fig. 1C). In contrast, FABP4 knockdown increased the protein level of PPARγ by 170±20% (n=3, P<0.01, Fig. 1D). These results suggested that PPARγ is negatively regulated by FABP4 predominantly at the post-transcriptional level.

PPARγ is induced during the differentiation of pre-adipocytes into adipocytes, and in turn induces a set of differentiation-dependent genes, including FABP4, lipoprotein lipase and adiponectin (9; 35). Despite the fact that FABP4 is a robust marker of mature adipocytes, addition of its inhibitor BMS309403 to the adipogenic cocktail increased PPARγ protein expression by 3.9±0.2 fold (n=3, P<0.05), as well as expression of the PPARγ gene products FABP4, lipoprotein lipase and adiponectin (Fig. 1E). These findings suggested that FABP4 negatively regulates PPARγ-mediated genes activation during adipogenesis.

We then studied the interrelationships of FABP4 and PPARγ in vivo, comparing wild type mice with FABP4-null mice (17). We found that white adipose tissue of FABP4-null mice had a 2.7 fold higher level of PPARγ compared with that of WT mice (Figs. 2A, B). Furthermore, immunoblotting of extracts from immortalized WT and FABP4-null mouse macrophages, as well as immortalized FABP4-null macrophages, in which FABP4 expression was complemented, demonstrated that FABP4 deficiency led to higher expression of PPARγ, and that complementation of FABP4 expression reduced the expression level of PPARγ (Fig. 2C). In contrast with the protein level, FABP4 had no significant effect on the level of PPARγ mRNA in these cells (Fig. 2D). These results suggested that FABP4 regulates PPARγ at the protein level.

FABP4 attenuates adipogenesis. We then studied the role of FABP4 in adipogenesis. Upon induction of differentiation, we found that the FABP4-null adipocytes accumulated a larger number and a larger volume of lipid droplets compared with those accumulated in WT adipocytes (Figs. 3A, B). The enhanced adipogenesis in the FABP4-null adipocytes was apparent when the differentiation was performed both in the absence and
in the presence of rosiglitazone, a pro-adipogenic TZD. Furthermore, FABP4-null adipocytes expressed higher levels of PPARγ compared with their WT counterparts (Fig. 3C, D). PPARγ activity was also higher in the FABP4-null adipocytes, as evaluated by the level of adiponectin, both in the absence and presence of rosiglitazone (Fig. 3C, lower panel). We therefore propose that FABP4 attenuates the formation of fat cells by down-regulating the master regulator of adipogenesis, PPARγ. We noticed that the FABP4 null adipocytes exhibited stronger Oil red O staining compared with rosiglitazone-treated WT adipocytes (Fig. 3B), suggesting that FABP4 inhibition might be more effective in promoting insulin sensitivity than this antidiabetic drug.

**Over-expression of FABP4 reduces PPARγ protein expression and activity.** To further show the impact of FABP4 on PPARγ levels, we over-expressed PPARγ in NIH-3T3 cells, alone or in combination with FABP4. Immunoblotting showed that co-expression of FABP4 with PPARγ reduced the level of PPARγ by 70±2% (n=3, P<0.05, Fig. 4A). A similar result was obtained upon transfection the cells with an R126Q mutant of FABP4, which lacks the ability to bind fatty acids (data not shown). This result suggested that the fatty acid binding capacity of FABP4 is not required for its effect on PPARγ levels. The reduced expression of PPARγ upon its co-expression with FABP4 took place only at the protein level as no significant change in the expression of *PPARγ* mRNA was observed (Fig. 4B).

We next tested whether the FABP4-mediated reduction in PPARγ expression also affected its transactivation potential. NIH-3T3 cells were transfected with pPPRE-luc, a reporter gene containing three PPREs upstream of a luciferase gene, and either a control vector (pCS6) or the FABP4 expression vector pFABP4. The cells were then incubated either with the PPARγ ligand rosiglitazone or with a vehicle. Over-expression of FABP4 significantly reduced the basal as well as the rosiglitazone-induced transcriptional activity of PPARγ (Fig. 4C). This result indicates that FABP4 interferes with PPARγ activation both by its endogenous ligands and by its pharmacological ligands.

**FABP4 accelerates the proteasomal degradation of PPARγ.** Because *PPARγ* mRNA levels were not affected by *FABP4* silencing (Fig. 1A), we studied the possible regulation of PPARγ by FABP4 at the protein level. To this end, we followed the rate of PPARγ decay in the presence of cycloheximide, an inhibitor of protein synthesis. Human 293T
cells were transfected with pPPARγ, and either control vector (pCS6) or pFABP4. Cycloheximide was then added; whole cell extracts were prepared at various times and immunoblotted with a PPARγ antibody. Co-expression of FABP4 with PPARγ reduced the half-life of PPARγ at all time points (Figs. 5A, B). Thus, it is likely that FABP4 accelerates the rate of PPARγ degradation.

We then examined PPARγ levels in the presence of the proteasome inhibitor MG262. Human HeLa and mouse NIH-3T3 cells were transfected with pPPARγ and either pFABP4 or the control vector pCS6. MG262 abolished the FABP4-mediated reduction of PPARγ levels in both human and mouse cells (Figs. 5C, D). MG262 increased the level of PPARγ even in the absence of FABP4 (Fig. 5D), supporting an earlier report that the basal turnover of PPARγ is mediated by proteasomal degradation (36). These experiments suggest that FABP4 accelerates the proteasomal degradation of PPARγ.

**FABP4 enhances the ubiquitination of PPARγ.** Most proteasome substrates are ubiquitinated prior to their degradation. To investigate whether FABP4 affects the ubiquitination of PPARγ, we transiently expressed various combinations of pPPARγ, a plasmid encoding HA-tagged ubiquitin (pHA-Ub), and pFABP4 in 293T cells for 48 h, followed by addition of the proteasome inhibitor MG262 for 17 h to preserve short-lived ubiquitin conjugates. Immunoprecipitation with anti PPARγ antibody followed by immunoblotting with an anti HA tag antibody revealed that cells transfected with pPPARγ together with pHA-Ub expressed free PPARγ, as well as high molecular weight HA-positive bands (Fig. 6A). Upon co-expression of PPARγ and FABP4 the level of free PPARγ was reduced and expression of the ubiquitin-conjugated PPARγ practically disappeared (Fig. 6A). Over-expression of FABP4 robustly increased the conjugation of HA-ubiquitin to PPARγ, as determined by inhibiting its proteasomal degradation with MG262 (Fig. 6A, compare lanes 5 & 6).

We were also able to detect endogenous untagged ubiquitin conjugates of PPARγ using anti-ubiquitin antibody. Human 293T cells were co-transfected with pPPARγ and either pFABP4 or a control vector. In the absence of FABP4, mono-ubiquitinated PPARγ was the main conjugate as determined by immunoprecipitation with anti PPARγ antibody followed by immunoblotting with anti-ubiquitin antibody (Fig. 6B, lane 2). Upon co-expression of FABP4 and PPARγ, the level of the mono-ubiquitinated PPARγ was
reduced, whereas the level of the poly-ubiquitinated forms of PPARγ increased (Fig. 6B, compare lanes 2 & 3). As expected, MG262 considerably elevated the poly-ubiquitination of PPARγ and further reduced the level of the mono-ubiquitinated PPARγ (Fig. 6B, compare lanes 4 & 5 to 2 & 3). These experiments suggest that FABP4 triggers the proteasomal degradation of PPARγ by increasing its poly-ubiquitination. Unlike the case of PPARγ, co-expression of FABP4 with p53, a protein extensively characterized as a target of ubiquitin-dependent proteasomal degradation (37), had no effect on the protein level of p53, thereby demonstrating the specific effect of FABP4 on PPARγ degradation (Fig. 6C).

**FABP4 is increased and PPARγ is reduced in mouse epididymal fat compared with subcutaneous fat.** Because both FABP4 and visceral fat are implicated in obesity-related morbidities, we compared the expression of FABP4 and PPARγ in adipose tissues of mouse subcutaneous fat and epididymal fat, which resembles visceral fat in humans. In both WT and FABP4-null mouse strains, expression of FABP4 was higher in epididymal fat compared to subcutaneous fat, in correlation with reduced amounts of PPARγ (Figs. 7A, B). Similarly, PPARγ expression in adipose tissues of WT mice was reduced compared to its level in FABP4-null fat (Figs. 7A, B). These findings further confirm the role of FABP4 as a negative regulator of its master regulator PPARγ and demonstrate how these two genes are differentially expressed in the functionally distinct fat depots. Although the level of PPARγ in FABP4-null epididymal fat appears to be somewhat lower compared with that in subcutaneous fat, analysis of paired samples did not show a statistically significant difference between the levels of PPARγ in the mouse epididymal and subcutaneous fat (n=4 pairs, P=0.602).

**FABP4 is up-regulated in human visceral fat compared to subcutaneous fat.** We then measured the relative amount of FABP4 and PPARγ in paired samples of human VAT and SAT obtained from biopsies of lean (6), obese (11) and obese diabetic (9) individuals (Figs. 7C, D). VAT FABP4 of each individual was normalized to its SAT FABP4 level. No significant difference in the VAT/SAT ratio of FABP4 was observed upon comparing lean and healthy obese individuals. However, the VAT/SAT ratio of FABP4 was significantly higher in obese diabetic patients compared with lean or healthy obese individuals (Fig. 7D). Consistent with our previous results, PPARγ was negatively
correlated with FABP4, and was higher in the human SAT, compared with the human VAT (Fig. 7E).

DISCUSSION
The fatty acid chaperone FABP4 is induced by PPARγ, the master regulator of adipogenesis, yet these two regulators exert opposite effects on various metabolic parameters such as insulin resistance and inflammation (14). Here we demonstrate a previously unrecognized negative feedback loop, whereby FABP4 specifically triggers proteasomal degradation of PPARγ and consequently inhibits PPARγ-related functions, thereby providing a possible mechanism that explains their opposite effects. Our observations are consistent with an earlier finding that PPARγ activity is elevated in FABP4-null macrophages (20). FABP4 was reported to physically interact with PPARγ (38), hence, it is likely that such a physical interaction triggers the ubiquitination and the subsequent proteasomal degradation of PPARγ. This interaction took place through a site distinct from the fatty acid binding pocket of FABP4 (38) and therefore, the ability of the fatty acid binding inhibitor BMS309403 to block the effect of FABP4 on PPARγ might be the outcome of an indirect allosteric effect.

Our findings show that FABP4 expression is not just an outcome of adipogenesis. Rather, FABP4 negatively regulates PPARγ and pre-adipocyte differentiation. Our observation that FABP4-null adipocytes express higher PPARγ levels may explain their improved differentiation capacity. Expression array analysis of many cell types suggests that the changes in gene expression induced by TZDs take place mostly in adipocytes (39) as part of a de novo adipogenesis (40). Evidently, the influence of FABP4 on adipogenesis was far more extensive than that of rosiglitazone.

The higher level of FABP4 and the lower level of PPARγ in VAT compared with SAT suggests a causative link between VAT FABP4 and the metabolic syndrome and may explain some morphological and functional differences between the adipocyte subtypes comprising these two types of fat tissue. For instance, visceral pre-adipocytes proliferate and differentiate to mature adipocytes less readily than their subcutaneous counterparts. Visceral adipocytes are smaller and less responsive to TZD compared with subcutaneous
adipocytes (41), possibly due to the reduced PPARγ. Furthermore, the visceral adipose tissue secretes higher levels of pro-inflammatory cytokines (8; 42; 43).

Previous studies reported similar levels of FABP4 mRNA in human VAT and SAT (6; 44; 45). Our findings that FABP4 protein is higher in both human VAT and mouse intra-abdominal fat suggest that FABP4 may also be regulated post-transcriptionally. PPARγ mRNA levels were reported in paired samples of human VAT and SAT, but the data are rather inconsistent. In contrast, and in line with our observations, comparative data at the protein level consistently showed reduced levels and activity of PPARγ in human VAT (46-48), further supporting the notion of post-translational regulation of PPARγ by FABP4. Our finding of higher FABP4 levels in VAT relative to their levels in SAT in obese diabetic individuals may explain why obesity-associated pathology correlates better with visceral fat mass than with overall adiposity (4-8).

Our finding that FABP4 interferes with level and hence activity of PPARγ in adipocytes and macrophages may be critical to understanding the development of the metabolic syndrome and its related pathologies such as type 2 diabetes and atherosclerosis. We hypothesize that lipid accumulation elevates FABP4, thereby reducing PPARγ. In view of the impact of PPARγ on insulin sensitivity, our findings may explain the progression from insulin resistance to overt diabetic state when FABP4 levels increase, thereby compromising PPARγ levels and activities. FABP4 also promotes a pro-inflammatory state, further advancing the development of metabolic syndrome-associated pathologies.

A unique population of VAT-resident Treg cells was recently implicated in control of the inflammatory state of adipose tissue (49). PPARγ is a crucial regulator of this Treg cell population, necessary for complete restoration of insulin sensitivity by TZD in obese mice.

Pharmacologic activators of PPARγ such as TZDs significantly improve insulin sensitivity in type 2 diabetes and the net effect of PPARγ activation in murine macrophages is atheroprotective (13). However, TZDs trigger rapid degradation of PPARγ (36), possibly accounting for the recent reports implicating them in increased risk of cardiovascular death (50). We propose that similarly to TZD, FABP4 lowers PPARγ levels in macrophages, thereby triggering its adverse physiological effects (11). In this
respect, inhibition of FABP4, combined with activation of PPARγ by TZDs could attenuate the harmful effects of TZDs, while maintaining its beneficial activities.

AUTHOR CONTRIBUTIONS
T.G.S. designed and performed experiments and wrote the manuscript. A.R. provided samples, analyzed results and reviewed the manuscript. G.S.H. provided samples, analyzed results and reviewed the manuscript. M.R. designed experiments, analyzed results and wrote the manuscript.

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FIGURE LEGENDS

FIG. 1. FABP4 inhibition increases PPARγ protein expression and activity in macrophage-like cells. A: Relative PPARγ mRNA levels, isolated from differentiated macrophage-like THP-1 cells incubated with or without the FABP4 inhibitor BMS309403 (BMS, 25 µM, 48 h) and measured by qPCR (n=6). B: THP-1 macrophage-like cells were similarly treated and whole cell lysates were immunoblotted with antibodies against PPARγ. The results shown are representative of three independent experiments. C: THP-1 macrophage-like cells were transfected with non-targeting control (open bars) or FABP4 siRNA (closed bars) and then induced to differentiate with PMA for 48 h. Shown are the relative levels of FABP4 mRNA and PPARγ mRNA, isolated from the cell extracts and measured by qPCR (n=6). D: Whole extracts from THP1 cells treated as in C and immunoblotted with antibodies against FABP4, PPARγ and β-actin. E: Immunoblots of the indicated proteins isolated from control 3T3-L1 pre-adipocytes (Un-diff.), 3T3-L1 cells induced to differentiate for 8 days (Diff.) or induced to differentiate in the presence of 25 µM BMS309403 (Diff./BMS). The results shown are representative of three independent experiments. The lanes were run on the same gel but were non-contiguous. Molecular mass markers (kDa) are indicated on the right sides of panels (B), (D) and (E). Data are presented as means±SEM. N.S. - not significant, *P < 0.05, ***P<0.001.

FIG. 2. PPARγ expression is higher in FABP4-null mice. A: Immunoblots of subcutaneous white adipose tissue lysates from FABP4-null (KO) and WT mice (showing 4 of 8 mice per group) with antibodies to FABP4, PPARγ and β-actin. B: Densitometric analysis of the immunoblots of PPARγ of the above adipose tissues. Data are presented as means±SEM. of the densitometric readings (n=8 mice per group; *P<0.05). C: Immunoblots of PPARγ and FABP4 in immortalized macrophage cell lines from WT and FABP4-null mice and the FABP4-null macrophage cell line in which FABP4 expression was complemented. Data are representative of three independent experiments. D: Relative expression level of
PPARγ mRNA in FABP4-null and FABP4-complemented immortalized mouse macrophage cell lines (n=3; N.S. - not significant, P=0.48).

FIG. 3. FABP4-null mouse pre-adipocytes (from embryonic fibroblasts) undergo enhanced adipogenesis and express higher levels of PPARγ. WT and FABP4-null (KO) cells were induced to differentiate into adipocytes in the absence or presence of rosiglitazone (rosi, 10 µM) for 9 days. A: Light microscopic images of undifferentiated WT pre-adipocytes, differentiated WT and FABP4-null adipocytes. Notice the difference in the number and size of the oil droplets. B: Oil red O staining of neutral lipids in WT and FABP4-null adipocytes following differentiation as above in the absence or presence of rosiglitazone (Rosi). C: Immunoblots of cell lysates derived from WT or FABP4-null adipocytes. Data shown in (B) & (C) are representative of three independent experiments, each one performed in triplicates. D: Densitometric analysis of the immunoblots of PPARγ, FABP4 and β-actin as shown in (C). Open bars: WT adipocytes; closed bars: FABP4-null adipocytes. Data are presented as means±SEM of the densitometric readings (n=4; *P<0.05; **P<0.01).

FIG. 4. Co-expression of PPARγ and FABP4 reduces PPARγ levels and activity in NIH-3T3 Cells. A: Immunoblots of extracts from NIH-3T3 cells transiently transfected with the indicated expression plasmids for 48 h. Wherever required, control vector pCS6 was added to obtain identical amounts of DNA vector. Immunoblots were probed with antibodies to FABP4, PPARγ and β-actin. B: Relative expression level of PPARγ mRNA in NIH-3T3 cells co-transfected with the indicated combinations of pControl, pFABP4 and pPPARγ. (n=3; N.S. - not significant, P=0.865). C: Normalized luciferase activity of extracts from NIH-3T3 cells transfected for 72 h with pPPRE-luc, pRL-TK Renilla luc and either pPPARγ+pCS6 (open bars) or pPPARγ+pFABP4 (closed bars). Vehicle (Control) or rosiglitazone (10 µM) were added 24 h after initiation of the transfection. Data are presented as means±SEM (n=3; ***P<0.001).
FIG. 5. FABP4 accelerates proteasomal degradation of PPARγ. A: Human 293T cells were transfected with pPPARγ and either pControl or pFABP4. After 48 h, the cells were treated with cycloheximide (350 µM) and harvested at the indicated times after cycloheximide addition. Immunoblots of cell extracts with antibodies directed against PPARγ, FABP4 and β-actin are shown. B: Densitometric analysis of the bands shown in A, relative to time 0. Data are presented as means±SEM of the densitometric readings (n=3, *P<0.05). C: HeLa cells were transfected with pPPARγ and either pCS6 (control vector) or pFABP4. After 48 h, the proteasome inhibitor MG262 (0.1 µM) was added for 17 h, cell extracts were then prepared and immunoblotted with antibodies to PPARγ, FABP4 and β-actin. D: Mouse NIH-3T3 cells were transfected, treated with MG262 and extracts were immunoblotted as in (C). Data shown are representative of three independent experiments.

FIG. 6. FABP4 enhances ubiquitination of PPARγ. A: 293T cells were transfected with the indicated expression vectors. Wherever required, control vector pCS6 was added to obtain identical amounts of vector DNA. After 48 h, the cells were treated with vehicle or with MG262 (0.1 µM; 17 h), cell lysates were immunoprecipitated with an anti-PPARγ antibody and the immunoprecipitates were immunoblotted with antibodies directed against HA-ubiquitin (top panels) or PPARγ (bottom panels). The lanes were run on the same gel but were non-contiguous. B: 293T cells were transiently transfected with pPPARγ and either pFABP4 or an empty vector. After 48 h, the cells were treated with MG262 as above, cell lysates were immunoprecipitated with an anti-PPARγ antibody and the immunoprecipitates were immunoblotted with the anti-ubiquitin antibody. C: 293T cells were transiently transfected with pPPARγ or pp53 and either pFABP4 or an empty vector. After 48 h, cell lysates were immunoblotted with the anti-PPARγ or anti-p53 antibodies. Data shown in (A), (B) & (C) are representative of three independent experiments.

FIG. 7. FABP4 and PPARγ levels are inversely correlated in mouse and human adipose tissues. A: Immunoblots of PPARγ, FABP4 and β-actin from paired subcutaneous (S) and epididymal (E) fat of WT and FABP4-null mice. Data shown
are representative of four independent experiments. B: Densitometric analysis of the immunoblots of PPARγ and FABP4 from paired subcutaneous adipose tissue (open bars) and epididymal adipose tissue (closed bars) from WT mice. Data shown are means±SEM of the densitometric readings. (n=4). C: Immunoblots of PPARγ and FABP4 from paired human subcutaneous (S) and visceral fat (V). Shown are immunoblots of paired SAT and VAT lysates representing 28 individuals. D: FABP4 ratios in SAT and VAT as determined by densitometric analysis of the FABP4 immunoblots of adipose tissues from lean (6), obese (11) and obese diabetic (9) individuals. Data are means±SEM of the densitometric readings. E: PPARγ levels in SAT and VAT as determined by densitometric analysis of the PPARγ immunoblots (n=28). Densitometric values in (B), (D) & (E) were normalized to β-actin. All data are presented as means±SEM of the densitometric readings. ***P<0.001.
A

Un-differentiated – wild type

Differentiated – wild type

Differentiated – FABP4-null

B

Wild type

FABP4-null

C

PPARγ

FABP4

β Actin

Rosi:

WT

KO

Rosi:

WT

KO

Adiponectin

β Actin

Rosi:

WT

KO

D

PPARγ/β actin

Rosi:

- - + +

* **
**A**

<table>
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<tr>
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<th>Rosiglitazone</th>
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<td>pPPARγ:</td>
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**B**

- **Data 3**
  - pPPARγ + pControl
  - pPPARγ + pFABP4

- PPARγ mRNA (Rel. expression)
  - N.S.

**C**

- **Luciferase / Renilla**
  - Control
  - Rosiglitazone

**Note:**
- PPARγ: Peroxisome Proliferator-Activated Receptor γ
- FABP4: fatty acid-binding protein 4
- β-actin: beta-actin
## A

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<thead>
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## B

- **Graph**: PPARγ/β actin levels over time (h) for pCS6 and pFABP4.
- **Legend**: pCS6 (△), pFABP4 (■).
- **Time (h)**: 0, 2, 4, 6, 8, 10.
- **Y-axis**: PPARγ/β actin levels.

## C

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## D

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<td>β actin</td>
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**A**

Diabetes

PPARγ  
FABP4  
β actin

Wild type  |  FABP4-null
--- | ---
S | E | S | E

**B**

![Graph showing relative density of FABP4 and PPARγ](image)

***

**C**

![Graph showing relative density of PPARγ, FABP4, and β actin](image)

**D**

![Bar chart showing FABP4 ratio in VAT/SAT](image)

***

**E**

![Bar chart showing PPARγ/β actin ratio](image)