Gene silencing in adipose tissue macrophages regulates whole-body metabolism in obese mice

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Adipose tissue (AT) inflammation and infiltration by macrophages is associated with insulin resistance and type 2 diabetes in obese humans, offering a potential target for therapeutics. However, whether AT macrophages (ATMs) directly contribute to systemic glucose intolerance has not been determined. The reason is the lack of methods to ablate inflammatory genes expressed in macrophages specifically localized within AT depots, leaving macrophages in other tissues unaffected. Here we report that i.p. administration of siRNA encapsulated by glucan shells in obese mice selectively silences genes in epididymal ATMs, whereas macrophages within lung, spleen, kidney, heart, skeletal muscle, subcutaneous (SubQ) adipose, and liver are not targeted. Such administration of GeRPs to silence the inflammatory cytokines TNF-α or osteopontin in epididymal ATMs of obese mice caused significant improvement in glucose tolerance. These data are consistent with the hypothesis that cytokines produced by ATMs can exacerbate whole-body glucose intolerance.

obesity | RNAi | immune cells

The rapidly rising prevalence of obesity and type 2 diabetes mellitus (T2D) over the past several decades has highlighted a pressing need to develop new therapeutics for these metabolic diseases (1). In obese human subjects, the inability to appropriately expand subcutaneous (SubQ) adipose tissue (AT) leads to ectopic lipid deposition in liver and muscle and may be an underlying cause of insulin resistance (2–4). It is well appreciated that infiltration and activation of macrophages in the visceral AT correlate with a chronic inflammatory state (5–7). These macrophages secrete cytokines and other factors that could impair the ability of adipocytes to secrete beneficial adipokines or store lipid, leading to lipid deposition in nonadipose tissues and insulin resistance (5–7). Consistent with this concept, macrophage numbers in visceral AT correlate with systemic insulin resistance even in obese human subjects that are matched for body mass index (8, 9). In light of this evidence, it has been suggested that therapeutics that can attenuate visceral AT inflammation may alleviate the diabetic state (10, 11).

Despite abundant literature consistent with the paradigm that AT inflammation contributes to systemic insulin resistance and glucose intolerance, no direct data actually address this issue. The limitation has been a lack of available methodology to ablate inflammatory genes expressed in macrophages specifically localized within AT depots while leaving these immune cells in other tissues unaffected. This is particularly important because immune cells also infiltrate the liver, the major contributor to whole-body glucose homeostasis in mice under most conditions. Even studies on conditional gene knockouts in macrophages or bone marrow transplants are nonspecific in that they modulate immune cells throughout the body, not only in the AT (12). Therefore, a technique to decrease cytokine expression specifically in AT macrophages (ATMs) is required to define whether visceral ATM inflammation actually contributes to systemic glucose intolerance.

Here we report an siRNA delivery technology developed by our laboratory to silence inflammatory cytokine expression specifically in adipose macrophages in obese mice without accessing macrophages in other tissues, including liver, heart, or pancreas. The siRNA is encapsulated in micrometer-sized glucan shells (glucan-encapsulated siRNA particles, GeRPs) extracted from *Saccharomyces cerevisiae* (13) and composed mainly of β-1,3-D-glucan, a ligand of the dectin-1 receptor and other receptors that are expressed by macrophages (14). We show here that in contrast to lean mice, in which i.p.-injected GeRPs are phagocytosed by macrophages throughout the body (13, 15), i.p. injection of GeRPs into obese mice results in their accumulation and silencing of genes mostly in macrophages found in the visceral epididymal AT depots. Under the same conditions, we could not detect GeRPs in these immune cells within other tissues. Using this approach, we attenuated the expression of two inflammatory cytokines thought to play a role in the development of insulin resistance in rodents and humans—TNF-α (16, 17) and osteopontin (OPN) (18, 19)—and found an improved whole-body glucose tolerance. These results reveal i.p. administration of GeRPs as a unique tool to study the specific role of visceral ATMs in the development of insulin resistance induced by obesity.

Results

Biodistribution of GeRPs in Obese Mice. To study the localization of i.p.-injected GeRPs in obese mice, we used FITC-labeled GeRPs loaded with nontargeting scrambled (SCR) siRNA. Five-wk-old genetically obese (ob/ob) mice were administered GeRPs by i.p. injection daily for 5 d and various tissues, including liver, lung, spleen, pancreas, heart, and kidney and SubQ, mesenteric, perirenal, and epididymal ATs were analyzed by microscopy (Fig. 1 *A–J*). FITC-GeRPs were only observed in cells within the epididymal AT (Fig. 1*J*, enlarged images in Fig. S1*A*), and not in the other organs (Fig. 1 *A–F*) or other adipose depots (Fig. 1 *G–I*). Similar results were obtained when mice were treated with GeRPs for 10 d. To confirm these data, ob/ob mice were treated with GeRPs labeled with a ^{125}I probe (Fig. 1*K*). Consistent with the microscopy analysis, the epididymal AT exhibited the highest radiolabel per gram of tissue compared with all other tissues (Fig. 1*K*). These data document that GeRPs injected i.p. are mostly found in cells within the epididymal AT in obese ob/ob mice.

GeRP Uptake in the Epididymal ATMs in Obese Mice. To study the profile of cells in the epididymal AT that internalize GeRPs, we performed fluorescence microscopy and flow cytometry analysis. The ob/ob mice were i.p. injected daily for 5 d with FITC-GeRPs. On day 6, epididymal AT sections were stained with an antibody against the macrophage marker F4/80 and analyzed by

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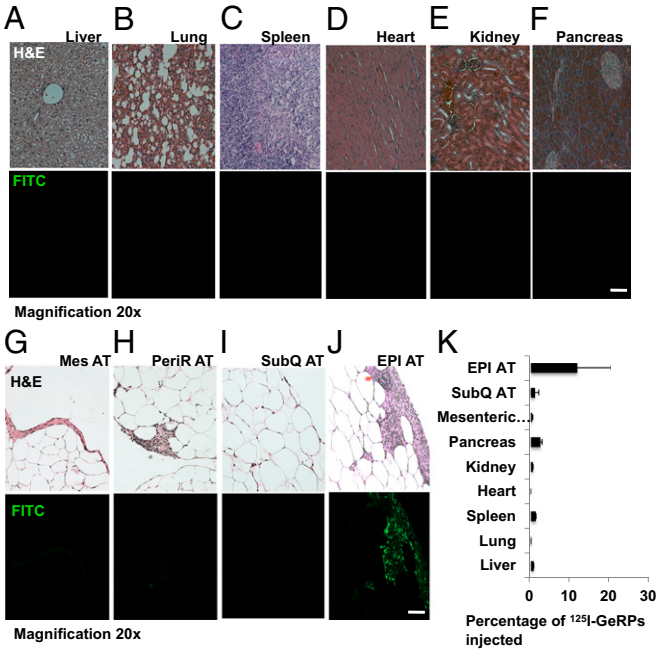

Fig. 1. GeRPs injected i.p. are localized in epididymal ATMs in obese mice. (*A–J*) Five-week-old ob/ob mice were i.p. injected once a day for 5 d with 5.6 mg/kg FITC-labeled GeRPs loaded with 2.1 mg/kg EP and 0.262 mg/kg SCR siRNA. On day 6, liver, lung, spleen, pancreas, heart, kidney, and (SubQ AT), mesenteric (Mes AT), perirenal (PeriR AT), and epididymal AT (EPI AT) were isolated. Tissues were fixed, sectioned, and then stained with H&E. Tissues were then analyzed by fluorescent microscopy. Images were obtained using a Zeiss Axiovert 200 inverted microscope. (Scale bar: 50 μm.) (*K*) Five-week-old ob/ob mice were i.p. injected with ^{125}I labeled GeRPs. Seven days later, liver, lung, spleen, pancreas, heart, kidney, and SubQ, mesenteric, and epididymal AT were isolated.

microscopy (Fig. 2*A*). Fig. 2*A* shows FITC-GeRPs in F4/80-positive cells in a region of the epididymal AT rich in macrophages termed crown-like structures (CLS). We next performed fluorescence microscopy on cells isolated from the stromal-vascular fraction (SVF), which contains all cells in the AT that do not float upon centrifugation, unlike lipid-laden adipocytes (Fig. 2*B*). SVF cells were isolated from the AT and stained with the F4/80 antibody (red) and the nuclear stain, DAPI (blue) (Fig. 2*B*). Images in Fig. 2*B*, *Left* show cells containing FITC-GeRPs only in GeRP-treated mice. Analyses at higher magnification (Fig. 2*B*, *Right*) show F4/80-positive cells containing multiple FITC-GeRPs (enlarged images in Fig. S1*B*). To further define the GeRP uptake profile in cells in the epididymal AT, SVF was also stained with antibodies against markers of multiple immune cell types (Fig. 2 C and *D*). Flow cytometry analysis confirmed the presence of FITC-GeRPs in F4/80-positive cells in the epididymal AT (Fig. 2*C*, *Upper Right*). Importantly, an FITC signal was detected in phagocytic cells, including F4/80-positive macrophages and F4/80 intermediate and negative monocytes and neutrophils (Fig. 2*C*, *Lower Right*). However, nonphagocytic cells, including eosinophils and T lymphocytes, showed no FITC signal, suggesting that these cells do not internalize GeRPs (Fig. 2*D*; for gating schemes, see Fig. S2). Similar results were obtained when mice were treated with GeRPs for 10 d. These results show that GeRPs injected i.p. are specifically delivered to phagocytes in the epididymal AT of obese ob/ob mice.

Gene Silencing in Primary Macrophages. We first measured the expression of TNF-α and OPN in epididymal and SubQ ATMs of 7-wk-old obese ob/ob mice and found strong increases of expression of these genes in epididymal ATMs of ob/ob mice compared with their lean WT littermates (Fig. 3 *A* and *B*). The increase in TNF-α and OPN expression in epididymal ATMs confirmed that the increase in macrophage content occurs mainly in the visceral AT in the obese state.

Primary peritoneal macrophages were used to screen for potent siRNAs against TNF-α or OPN, and two were chosen for each gene (Fig. 3 *C* and *D*). Both siRNA sequences against the target genes potently silenced the expression of TNF-α or OPN. Furthermore, secretion of both TNF-α and OPN was significantly silenced by the targeting siRNAs compared with SCR or untreated cells (Fig. 3 *E* and *F*). The data reveal that these genes could be silenced in primary macrophages in vitro both at the mRNA and protein levels.

Gene Silencing in Epididymal ATMs in Obese Mice. To test the ability of GeRPs to deliver functional siRNA and silence these genes in vivo, 5-wk-old ob/ob mice were injected daily for 10 d with GeRPs loaded with SCR or TNF-α siRNA, validated as shown in Fig. 3. The day after the last injection, TNF-α expression was measured in epididymal AT (Fig. 4*A*). TNF-α expression was significantly inhibited in the epididymal AT of ob/ob mice treated with

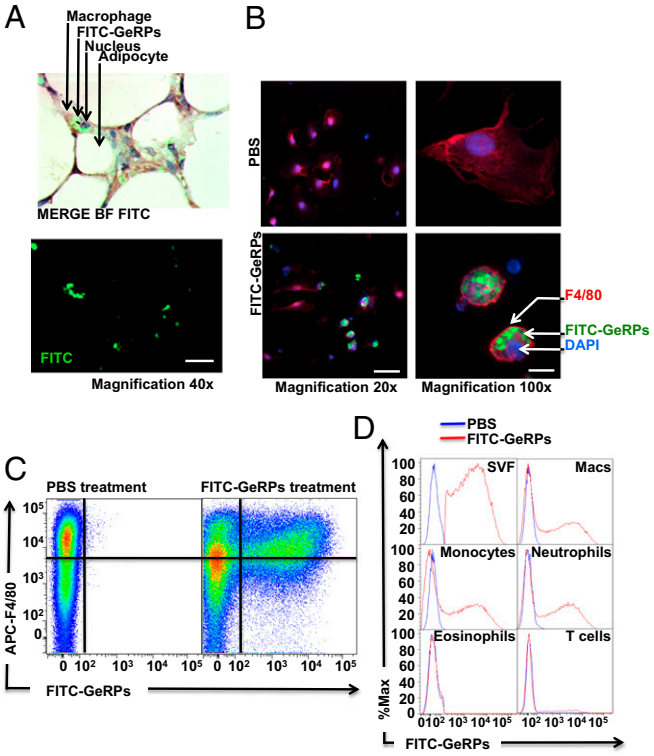

Fig. 2. GeRPs undergo phagocytosis by epididymal ATMs in obese mice. (*A*) Five-week-old ob/ob mice were i.p. injected once a day for 5 d with 5.6 mg/kg FITC-labeled GeRPs loaded with 2.1 mg/kg EP and 0.262 mg/kg SCR siRNA. On day 6, epididymal AT was isolated, fixed, sectioned, and stained with F4/80 antibody. Tissues were also counterstained with hematoxylin. Tissues were then analyzed by fluorescent microscopy. Spinning disk confocal microscopy showing crown-like structures composed of macrophages (dark brown) containing FITC-GeRPs (green). (Scale bar: 20 μm.) (*B*) Confocal microscopy showing F4/80 (red) and GeRPs (green) present in SVF cells 24 h after treatment. Nuclei were stained with DAPI (blue). (Scale bar: *Left*, 50 μm; *Right*, 10 μm.) (*C*) FACS analysis showing SVF cells isolated from mice treated with FITC-labeled GeRPs (FITC-GeRPs) and stained with F4/80 antibody. APC, allophycocyanin. (*D*) FITC level stain in epididymal AT total SVF cells, macrophages (F4/80+/CD11b+/Siglec-f-), monocytes (Ly6-C high and low), neutrophils (Gr1+), eosinophils (F4/80+/CD11b+/Siglec-f+), and T cells (CD3+) determined by flow cytometry.

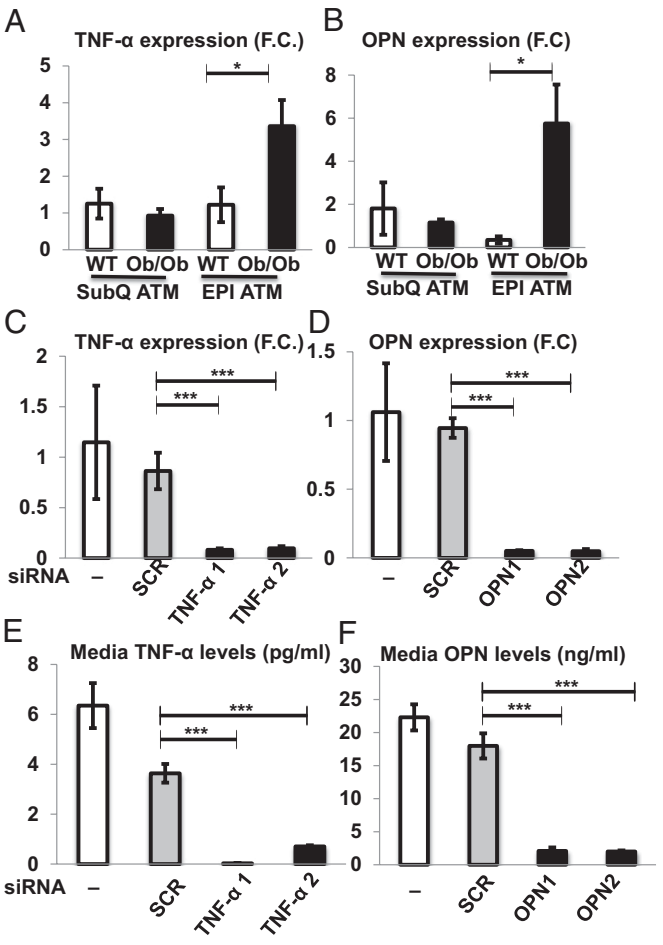

Fig. 3. Silencing genes in primary macrophages in vitro. (*A* and *B*) Expression of TNF-α and OPN measured by RT-PCR in isolated macrophages from epididymal and SubQ AT of 7-wk-old genetically obese ob/ob mice. Macrophages were isolated using CD11b antibody bound to magnetic beads. $n = 4$. Statistical significance was determined by *t* test. ***$P < 0.001$; *$P < 0.05$. Results are means expressed in fold change (F.C.) ± SEM. (*C* and *D*) 1×10^6 peritoneal macrophages were treated with particles made with a mixture of 160 pmoles siRNA and 3 nmol EP. Forty-eight hours after the treatment, mRNA levels were measured by RT-PCR. (*E*) TNF-α in media of peritoneal macrophages treated with 1 μg/mL LPS for 6 h measured by ELISA. (*F*) OPN protein basal levels in media measured by ELISA. $n = 3$ with three technical replicates for each experiment. Statistical significance was determined by ANOVA and Tukey post test. ***$P < 0.001$. Results are mean ± SEM.

TNF-α-GeRPs compared with SCR-GeRPs (Fig. 4*A*). Mice were also treated for 5 d with GeRPs loaded with SCR siRNA or OPN siRNA. A significant 70% knockdown of OPN expression was observed in the epididymal AT of ob/ob mice treated with OPN-GeRPs compared with SCR-GeRPs (Fig. 4*B*). To confirm the specificity of the siRNA-mediated knockdown, the expression of several other macrophage and immune cell factors was measured, including CD11b, CD11c, F4/80, CD68, IL-1β, IL-6, IL-10, IL-4, CC-motif chemokine receptor-2 (CCR2), monocyte chemoattractant protein 1 (MCP-1), and adipocyte protein 2 (aP2). Expression of these markers was unchanged in mice treated with TNF-α-GeRPs or OPN-GeRPs compared with SCR-GeRPs (Fig. S3). This result confirmed the specificity of siRNA-mediated knockdowns and suggested that silencing TNF-α and OPN expression had no effect on global AT inflammation during the experiment. Consistent with the biodistribution studies showing GeRPs were present only in phagocytic cells of epididymal AT and not in liver, no depletion of the target gene products was observed in liver of treated mice (Fig. 4 *C* and *D*).

To further analyze the tissue specificity of GeRP-mediated knockdown, different adipose depots including SubQ, mesenteric, and perirenal depots were isolated from mice treated with TNF-α-GeRPs and SCR-GeRPs (Fig. 4 *E*–*G*). Although mice treated with TNF-α-GeRPs exhibited a significant TNF-α depletion in the epididymal AT (Fig. 4*A*), there was no knockdown in any of the other adipose depots tested (Fig. 4 *E*–*G*). These data corroborated biodistribution results showing GeRPs present only in the epididymal AT and not in other adipose depots. TNF-α protein levels and bioactivity in media of SVF isolated from epididymal AT of mice treated with TNF-α-GeRPs was also significantly reduced

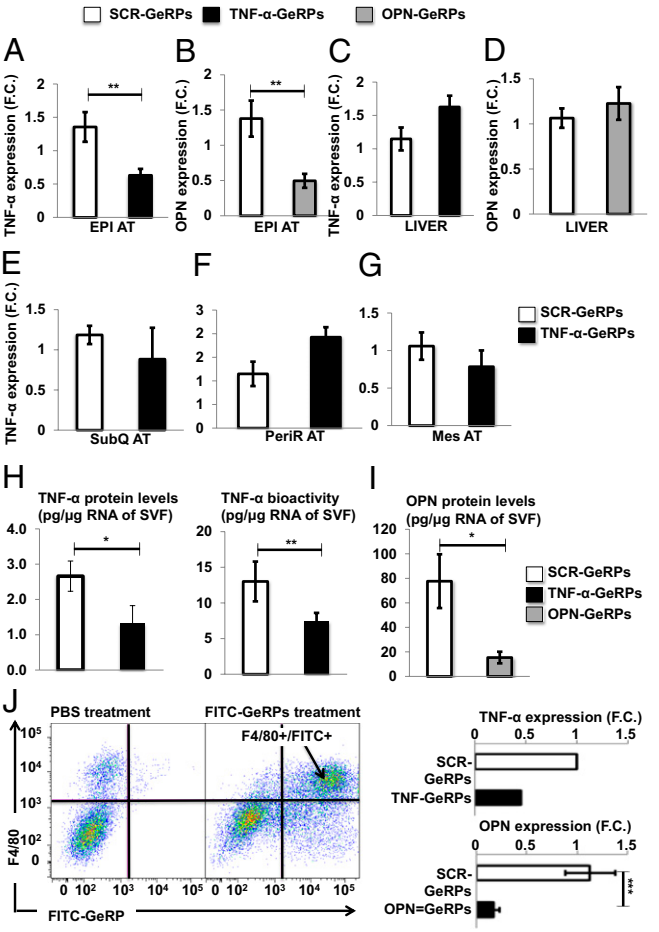

Fig. 4. Gene silencing in epididymal ATMs in obese mice without affecting macrophages in liver or other adipose depots. Expression of TNF-α in (*A*) epididymal AT and (*C*) liver from mice treated for 10 d with SCR- or TNF-α-GeRPs. $n = 23$–24. Expression of OPN in (*B*) epididymal AT and (*D*) liver from mice treated for 5 d with SCR- or OPN-GeRPs. $n = 28$. Statistical significance was determined by *t* test. ***$P < 0.001$; *$P < 0.05$. Results are means in F.C. ± SEM. TNF-α expression in (*E*) SubQ (*F*) perirenal, and (*G*) mesenteric AT from mice treated for 10 d with SCR- or TNF-α-GeRPs. $n = 5$. (*H*) TNF-α protein levels and bioactivity in LPS-treated epididymal SVF media of mice treated with SCR- or TNF-α-GeRPs. $n = 10$. (*I*) OPN protein levels in epididymal SVF media of mice treated with SCR- or OPN-GeRPs. $n = 10$. Statistical significance was determined by *t* test. **$P < 0.01$; *$P < 0.05$. (*J*) Representative dot-plot of F4/80-stained epididymal SVF from mice treated with PBS or FITC-labeled SCR-GeRPs for 5 or 10 d. F4/80+ cells containing GeRPs (FITC+) were sorted by FACS and mRNA levels were measured by RT-PCR in mice treated with SCR-, TNF-α-, or OPN-GeRPs. For TNF-α, $n = 5$ mice pooled together. For OPN, $n = 3$ groups of three mice pooled together. Statistical significance was determined by *t* test. ***$P < 0.001$.

compared with mice treated with SCR-GeRPs (Fig. 4*H*). Mice treated with OPN-GeRPs had a significant 81% decrease of OPN secretion in SVF isolated from epididymal AT compared with mice treated with SCR-GeRPs (Fig. 4*I*).

FACS was performed to analyze the knockdown of the target genes in ATMs containing FITC-labeled GeRPs (Fig. 4*J*). Mice were treated with FITC-labeled SCR-GeRPs, TNF-α-GeRPs, or OPN-GeRPs, and epididymal SVF was plated and stained with the F4/80 antibody. Flow cytometry analysis showed that 80% of epididymal ATMs contained FITC-GeRPs (Fig. S4*A*). TNF-α and OPN expression was significantly silenced in sorted F4/80+/FITC+ ATMs in mice treated with TNF-α-GeRPs or OPN-GeRPs, respectively, compared with SCR-GeRPs (Fig. 4*J*). The expression of TNF-α and OPN was unchanged in cells that did not contain GeRPs (FITC–, Fig. S4*B*). Taken together, these data reveal that i.p.-injected GeRPs can specifically silence genes in epididymal ATMs in vivo without affecting gene expression in macrophages in other major organs such as liver or other adipose depots.

Silencing Inflammatory Genes in Epididymal ATMs Affects Whole-Body Metabolism in ob/ob Mice. To test the effect of GeRP-mediated gene silencing in epididymal ATMs on whole-body metabolism, glucose tolerance tests were performed in ob/ob mice treated with TNF-α-GeRPs, OPN-GeRPs, or SCR-GeRPs (Fig. 5 *A* and *B*). Both TNF-α and OPN silencing in epididymal ATMs improved glucose tolerance in obese mice (Fig. 5 *A* and *B*). Area under the glucose tolerance test curves showed that mice treated with TNF-α-GeRPs or OPN-GeRPs were significantly more glucose tolerant compared with mice treated with PBS or SCR-GeRPs (Fig. 5 *C* and *D*). Importantly, this occurred without affecting TNF-α or OPN expression in other tissues or protein levels in serum, in which TNF-α levels were undetectable and OPN levels unchanged (Fig. 4 and Fig. S5*A*). The effect of TNF-α and OPN silencing on glucose tolerance was independent of an effect on weight gain during treatments (Fig. S5 *B* and *C*). Taken together, these results suggest that decreasing production of these cytokines specifically in epididymal ATMs has a beneficial effect on whole-body metabolism.

Experiments on ob/ob mice were also performed with GeRPs loaded with siRNA against genes in which expression in epididymal ATMs was unchanged or decreased with obesity. These included a complement component 3a protein receptor (C3aR) and a protein of the leukotriene production pathway, 5-lipoxygenase activating protein (FLAP) (Fig. S6*A*). Although inhibition of FLAP and whole-body knockout of C3aR prevent diet-induced insulin resistance (18, 19), we failed to observe effects of silencing these genes on glucose tolerance, despite significant gene knockdowns in epididymal ATMs (Fig. S6 *B* and *C*). This observation suggests that C3aR and FLAP may play a role in cells other than ATMs in regulating glucose metabolism.

TNF-α can induce the release of lipids from adipocytes into the circulation, a process known as lipolysis, causing ectopic lipid deposition in liver (20). Although silencing TNF-α in ATMs prevents the development of glucose intolerance in obese mice, it did not change circulating lipid levels [free fatty acid or triglycerides (TG)] (Fig. S7*A*). To analyze the degree of ectopic lipid deposition, microscopy was performed on H&E liver sections of mice treated with PBS, SCR-GeRPs, or TNF-α-GeRPs (Fig. S7*B*). The ob/ob mice treated with PBS had a high degree of liver steatosis, represented by enlarged lipid droplets in hepatocytes compared with their WT littermates (Fig. S7*B*). A similar degree of liver steatosis was observed in PBS-treated mice compared with mice treated with SCR-GeRPs or TNF-α-GeRPs (Fig. S7*B*). Consistently, liver TG content was similar in mice treated with PBS, SCR-GeRPs, or TNF-α-GeRPs (Fig. S7*C*). This suggests that the beneficial effect of silencing TNF-α in epididymal ATMs may be due to a process independent of lipolysis.

The role of OPN in the development of insulin resistance has been attributed to its role in attracting macrophages in the

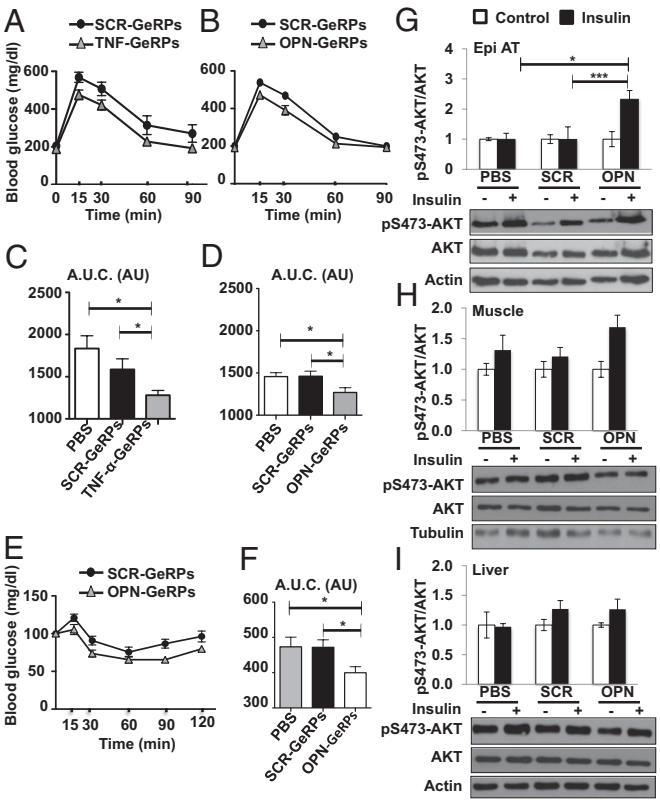

Fig. 5. Gene silencing in epididymal ATMs in obese mice regulates whole-body metabolism. Five-week-old ob/ob mice were treated as described in Fig. 4. Twenty-four hours after the last injection, glucose tolerance tests (GTT) were performed on mice that were fasted for 16 h. Mice were treated with SCR-GeRPs, (*A*) TNF-α-GeRPs, or (*B*) OPN-GeRPs. (*C* and *D*) Area under the curve (AUC) of corresponding GTT graphs. $n = 10–18$. Statistical significance was determined by ANOVA and Tukey posttest. $^{*}P < 0.05$. Results are mean ± SEM. (*E*) Insulin tolerance tests (ITT) were performed on day 6 in ob/ob mice treated with PBS or SCR- or OPN-GeRPs by injecting 1 U/kg of insulin. (*F*) AUC of corresponding ITT graph. $n = 15$. Statistical significance was determined by ANOVA and Tukey posttest. $^{*}P < 0.05$. Results are mean ± SEM. PBS or SCR- or OPN-GeRPs treated mice were also fasted for 4 h and then treated by i.p. injection with 1 U/kg of insulin (15 min). Western blotting and multiplexed ELISA were used to detect Akt and activated (pSer473) Akt in (*G*) epididymal AT (EPI AT), (*H*) gastrocnemius muscle, and (*I*) liver. $n = 5–6$. Statistical significance was determined by ANOVA and Tukey posttest. $^{***}P < 0.001$; $^{*}P < 0.05$. Results are mean ± SEM.

epididymal AT (21, 22). To test this, macrophage content was analyzed in the epididymal AT of mice treated with OPN-GeRPs compared with SCR-GeRPs (Fig. S8*A*). Epididymal ATM content was unchanged in OPN-GeRP–treated mice compared with mice treated with SCR-GeRPs (Fig. S8*A*). This suggests that OPN silencing in epididymal ATMs prevents obesity-induced insulin resistance independently of macrophage recruitment. Analysis of circulating lipids showed that OPN silencing in epididymal AT had no effect on lipolysis (Fig. S8 *B* and *C*). To study the effect of OPN silencing in ATMs on systemic insulin sensitivity, we performed insulin tolerance tests in mice treated with PBS-, SCR-, or OPN-GeRPs (Fig. 5 *E* and *F*). Mice treated with OPN-GeRPs had a significant improvement in insulin response compared with mice treated with PBS or SCR-GeRPs (Fig. 5 *E* and *F*). Measurement of fasting insulin levels showed that mice treated with OPN-GeRPs were still hyperinsulinemic compared with mice treated with SCR-GeRPs (Fig. S8*D*). These results suggested that OPN silencing in ATMs increases systemic insulin sensitivity. Therefore, we also performed biochemical studies to investigate insulin-stimulated Akt activation in mice treated with

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PBS, SCR-GeRPs, or OPN-GeRPs (Fig. 5 *G–I*). Obesity suppressed insulin-stimulated Akt activation in liver, AT, and skeletal muscle of mice treated with PBS or SCR-GeRPs but not in AT of mice treated with OPN-GeRPs (Fig. 5 *G–I*). Together, these data demonstrate that silencing OPN in ATMs improves insulin sensitivity. Furthermore, OPN and TNF-α levels were specifically decreased in epididymal AT and not in other tissues or serum (Fig. 4 and Fig. S5*A*), suggesting that they may play a paracrine role in the AT.

Addressing whether OPN and TNF-α silencing may affect the expression of circulating factors, we did not detect any difference in serum adiponectin or fibroblast growth factor 21 (FGF21) levels in mice treated with PBS or SCR-GeRPs, TNF-α-GeRPs, or OPN-GeRPs (Fig. S9 *A–D*). FGF21 has been shown to regulate brown adipose tissue (BAT) mass and activity (23, 24). Consistent with a lack of change in serum FGF21, we failed to observe any difference in BAT morphology, weight, or expression of specific markers such as uncoupling protein 1 in treated mice (Fig. S9*E*).

Discussion

The major findings reported here include the development of a powerful RNAi-based method to selectively silence genes in epididymal ATMs in obese mice while leaving such cells unperturbed in other tissues (Figs. 1 and 2). This selective biodistribution profile of i.p.-injected GeRPs in obese mice is in keeping with the known high accumulation of macrophages in visceral AT compared with other adipose depots and metabolic tissues in obese rodents and humans (9, 25, 26). In previous studies using lean healthy animals, i.p.-injected GeRPs were internalized by macrophages that could be detected throughout the body (13, 15). In contrast, using obese insulin-resistant animals, we detected GeRPs mostly in the epididymal ATMs following i.p. injection. One explanation for the different patterns of GeRP distribution observed in lean versus obese mice could be the increase in AT chemoattractants in obesity. Adipocytes produce a wide range of such factors, such as MCP-1, which may recruit monocytes expressing its receptor CCR2 to adipose tissue in obese mice (27, 28).

Although multiple siRNA delivery systems have been recently described in the literature (29, 30), none has been used in an obese, insulin-resistant animal model. A previous study described delivery of lipidoid nanoparticles carrying siRNA to immune cells, including lymphocytes, in spleen, blood, and bone marrow following i.v. injection in lean mice (31). In contrast, we show here that GeRPs are only found in phagocytes and not in nonphagocytic cells, such as T cells or eosinophils, which could represent a major clinical advantage. This important feature of the GeRPs is conferred by their size (2–4 μm) and the β-1,3-D-glucan that is specifically recognized by receptors expressed by phagocytic cells (14).

Decreasing inflammation in obese rodents often results in improvement of insulin sensitivity (10, 11). However, blocking cytokines by injection of antibodies or antagonists has yielded mixed results in alleviating insulin resistance in humans (32–35). An explanation for the frequently observed absence of effects of these drugs on insulin resistance in obese subjects may be their low penetrance in AT in which the endogenous cytokines act in a paracrine fashion (36). However, no study has actually shown a direct role of macrophages in the AT in the regulation of systemic metabolism because experimental anti-inflammatory gene knockouts and other procedures are not restricted to AT, but in general act on immune cells within all tissues. Macrophages in particular are present in all tissues, and cytokines are highly expressed by various immune and nonimmune cells (37). Therefore, the physiological role of ATMs has been a key unanswered question in the field, and is particularly important considering the variable results obtained with cytokine blockers in human subjects (36, 38, 39).

The specificity of GeRPs in targeting epididymal ATMs when administered i.p. permitted us to address this critical question (Figs. 4 and 5). The motivation for targeting TNF-α and OPN in epididymal ATMs in our studies was based on three major points: (*i*) both TNF-$\alpha^{-/-}$ and $OPN^{-/-}$ mice are protected from obesity-induced insulin resistance (17, 22); (*ii*) the expression of these genes is high in AT from obese mice (Fig. 3) (21, 22, 40, 41); and (*iii*) anti-TNF-α and anti-OPN neutralizing antibodies both reverse obesity-induced insulin resistance in rodents (21, 42). Based on these considerations, GeRPs were loaded with siRNAs to silence TNF-α or OPN in genetically obese ob/ob mice. Although previous work by our laboratory and others demonstrated that GeRPs can mediate potent gene silencing in peritoneal macrophages in lean mice (13, 15, 43), here we found specific gene knockdowns within epididymal ATMs in obese mice. Importantly, GeRP-mediated TNF-α or OPN silencing in epididymal ATMs improved the glucose tolerance of ob/ob mice (Fig. 5). Silencing TNF-α or OPN had no effect on the expression of CD11b, CD11c, F4/80, CD68, IL-1β, IL-6, IL-10, IL-4, CCR2, MCP-1, or aP2 in AT, suggesting that none of these inflammatory mediators is downstream of TNF-α or OPN in this context. Thus, the selective depletion of TNF or OPN in the absence of changes in these other factors was sufficient to improve glucose tolerance (Fig. S3). Importantly, OPN silencing in ATMs specifically increased insulin-stimulated Akt activation in AT, suggesting a paracrine role of macrophage OPN in improving insulin signaling (Fig. 5). Thus, this study shows a direct link between epididymal ATM-derived cytokines and insulin resistance.

The present study illustrates the crucial role of macrophage local environment and emphasizes the importance of studying macrophage function within a specific tissue. Although additional work is needed to define the molecular mechanisms by which these macrophage proteins regulate whole-body metabolism, the GeRP siRNA delivery system provides a unique tool for such studies.

Materials and Methods

All mice were purchased from Jackson Laboratory. Mice were housed on a 12-h light/dark schedule and had free access to water and food. All procedures involving animals were approved by the Institutional Animal Care and Use Committee at the University of Massachusetts Medical School.

Preparation of GeRPs. Glucan shells were prepared as previously described (15) (see *SI Materials and Methods* for the detailed protocol). To load siRNA in glucan shells, 3 nmol siRNA (Dharmacon) were incubated with 50 nmoles Endo-Porter (EP; Gene Tools) in 30 mM sodium acetate pH 4.8 for 15 min at room temperature in a final volume of 20 μL. The siRNA/EP solution was added to 1 mg (~10^9) of glucan shells and then vortexed and incubated for 1 h. The siRNA-loaded GeRPs were then resuspended in PBS and sonicated to ensure homogeneity of the GeRP preparation. GeRPs were kept at 4 °C.

GeRP Administration and Tissue Isolation. Five-week-old C57BL6 ob/ob male mice were i.p. injected once a day for 5 or 10 d with 5.6 mg/kg GeRPs loaded with 2.1 mg/kg EP and 0.262 mg/kg siRNA. Twenty-four hours after the last injection, mice were killed and tissues were isolated and used for RT-PCR and microscopy.

Metabolic Studies. Glucose and insulin tolerance tests were performed on ob/ob animals at 5 or 10 d after GeRP treatment. Glucose (1 g/kg) and insulin (1 IU/kg) were administrated by i.p. injection. Blood samples were withdrawn from the tail vein at the indicated time, and glycemia was determined using glucometers (Bayer and alpha-trak).

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1. Chen L, Magliano DJ, Zimmet PZ (2011) [The worldwide epidemiology of type 2 diabetes mellitus—present and future perspectives.] *Nat Rev Endocrinol* 8(4):228–236.
2. Guilherme A, Virbasius JV, Puri V, Czech MP (2008) Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes. *Nat Rev Mol Cell Biol* 9(5):367–377.
3. Kusminski CM, Shetty S, Orci L, Unger RH, Scherer PE (2009) Diabetes and apoptosis: Lipotoxicity. *Apoptosis* 14(12):1484–1495.
4. Samuel VT, Petersen KF, Shulman GI (2010) Lipid-induced insulin resistance: Unravelling the mechanism. *Lancet* 375(9733):2267–2277.
5. Lumeng CN, Saltiel AR (2011) Inflammatory links between obesity and metabolic disease. *J Clin Invest* 121(6):2111–2117.
6. Olefsky JM, Glass CK (2010) Macrophages, inflammation, and insulin resistance. *Annu Rev Physiol* 72:219–246.
7. Weisberg SP, et al. (2003) Obesity is associated with macrophage accumulation in adipose tissue. *J Clin Invest* 112(12):1796–1808.
8. Blüher M (2010) The distinction of metabolically 'healthy' from 'unhealthy' obese individuals. *Curr Opin Lipidol* 21(1):38–43.
9. Hardy OT, et al. [Body mass index-independent inflammation in omental adipose tissue associated with insulin resistance in morbid obesity.] *Surg Obes Relat Dis* 7(1): 60–67.
10. Burke JR, Gregor KR, Tramposch KM (1995) Mechanism of inhibition of human nonpancreatic secreted phospholipase A2 by the anti-inflammatory agent BMS-181162. *J Biol Chem* 270(1):274–280.
11. Yuan M, et al. (2001) Reversal of obesity- and diet-induced insulin resistance with salicylates or targeted disruption of Ikkbeta. *Science* 293(5535):1673–1677.
12. Romeo GR, Lee J, Shoelson SE (2012) Metabolic syndrome, insulin resistance, and roles of inflammation—mechanisms and therapeutic targets. *Arterioscler Thromb Vasc Biol* 32(8):1771–1776.
13. Aouadi M, et al. (2009) Orally delivered siRNA targeting macrophage Map4k4 suppresses systemic inflammation. *Nature* 458(7242):1180–1184.
14. Herre J, Gordon S, Brown GD (2004) Dectin-1 and its role in the recognition of beta-glucans by macrophages. *Mol Immunol* 40(12):869–876.
15. Tesz GJ, et al. (2011) Glucan particles for selective delivery of siRNA to phagocytic cells in mice. *Biochem J* 436(2):351–362.
16. De Taeye BM, et al. (2007) Macrophage TNF-alpha contributes to insulin resistance and hepatic steatosis in diet-induced obesity. *Am J Physiol Endocrinol Metab* 293(3): E713–E725.
17. Hotamisligil GS (1999) Mechanisms of TNF-alpha-induced insulin resistance. *Exp Clin Endocrinol Diabetes* 107(2):119–125.
18. Horrillo R, et al. (2010) 5-lipoxygenase activating protein signals adipose tissue inflammation and lipid dysfunction in experimental obesity. *J Immunol* 184(7): 3978–3987.
19. Mamane Y, et al. (2009) The C3a anaphylatoxin receptor is a key mediator of insulin resistance and functions by modulating adipose tissue macrophage infiltration and activation. *Diabetes* 58(9):2006–2017.
20. Plomgaard P, Fischer CP, Ibfelt T, Pedersen BK, van Hall G (2008) Tumor necrosis factor-alpha modulates human in vivo lipolysis. *J Clin Endocrinol Metab* 93(2): 543–549.
21. Kiefer FW, et al. (2010) Neutralization of osteopontin inhibits obesity-induced inflammation and insulin resistance. *Diabetes* 59(4):935–946.
22. Nomiyama T, et al. (2007) Osteopontin mediates obesity-induced adipose tissue macrophage infiltration and insulin resistance in mice. *J Clin Invest* 117(10): 2877–2888.
23. Badman MK, Koester A, Flier JS, Kharitonenkov A, Maratos-Flier E (2009) Fibroblast growth factor 21-deficient mice demonstrate impaired adaptation to ketosis. *Endocrinology* 150(11):4931–4940.
24. Fisher FM, et al. (2012) FGF21 regulates PGC-1α and browning of white adipose tissues in adaptive thermogenesis. *Genes Dev* 26(3):271–281.
25. Alvehus M, Burén J, Sjöström M, Goedecke J, Olsson T (2010) The human visceral fat depot has a unique inflammatory profile. *Obesity (Silver Spring)* 18(5):879–883.
26. Fontana L, Eagon JC, Trujillo ME, Scherer PE, Klein S (2007) Visceral fat adipokine secretion is associated with systemic inflammation in obese humans. *Diabetes* 56(4): 1010–1013.
27. Chen A, et al. (2005) Diet induction of monocyte chemoattractant protein-1 and its impact on obesity. *Obes Res* 13(8):1311–1320.
28. Kamei N, et al. (2006) Overexpression of monocyte chemoattractant protein-1 in adipose tissues causes macrophage recruitment and insulin resistance. *J Biol Chem* 281(36):26602–26614.
29. Binkhathlan Z, Alshamsan A (2012) Emerging nanodelivery strategies of RNAi molecules for colon cancer therapy: preclinical developments. *Ther Deliv* 3(9):1117–1130.
30. Gavrilov K, Saltzman WM (2012) Therapeutic siRNA: Principles, challenges, and strategies. *Yale J Biol Med* 85(2):187–200.
31. Leuschner F, et al. (2011) Therapeutic siRNA silencing in inflammatory monocytes in mice. *Nat Biotechnol* 29(11):1005–1010.
32. Bernstein LE, Berry J, Kim S, Canavan B, Grinspoon SK (2006) Effects of etanercept in patients with the metabolic syndrome. *Arch Intern Med* 166(8):902–908.
33. Larsen CM, et al. (2007) Interleukin-1-receptor antagonist in type 2 diabetes mellitus. *N Engl J Med* 356(15):1517–1526.
34. Ofei F, Hurel S, Newkirk J, Sopwith M, Taylor R (1996) Effects of an engineered human anti-TNF-alpha antibody (CDP571) on insulin sensitivity and glycemic control in patients with NIDDM. *Diabetes* 45(7):881–885.
35. van Asseldonk EJ, et al. (2011) Treatment with Anakinra improves disposition index but not insulin sensitivity in nondiabetic subjects with the metabolic syndrome: A randomized, double-blind, placebo-controlled study. *J Clin Endocrinol Metab* 96(7): 2119–2126.
36. Di Rocco P, Manco M, Rosa G, Greco AV, Mingrone G (2004) Lowered tumor necrosis factor receptors, but not increased insulin sensitivity, with infliximab. *Obes Res* 12(4): 734–739.
37. Gordon S (2012) Innate immune functions of macrophages in different tissue environments. *J Innate Immun* 4(5-6):409–410.
38. Stanley TL, et al. (2011) TNF-alpha antagonism with etanercept decreases glucose and increases the proportion of high molecular weight adiponectin in obese subjects with features of the metabolic syndrome. *J Clin Endocrinol Metab* 96(1):E146–E150.
39. Solomon DH, et al. (2011) Association between disease-modifying antirheumatic drugs and diabetes risk in patients with rheumatoid arthritis and psoriasis. *JAMA* 305 (24):2525–2531.
40. Hotamisligil GS, Arner P, Caro JF, Atkinson RL, Spiegelman BM (1995) Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance. *J Clin Invest* 95(5):2409–2415.
41. Hotamisligil GS, Shargill NS, Spiegelman BM (1993) Adipose expression of tumor necrosis factor-alpha: Direct role in obesity-linked insulin resistance. *Science* 259 (5091):87–91.
42. Borst SE, Bagby GJ (2002) Neutralization of tumor necrosis factor reverses age-induced impairment of insulin responsiveness in skeletal muscle of Sprague-Dawley rats. *Metabolism* 51(8):1061–1064.
43. Huang W, et al. (2011) Coronin 2A mediates actin-dependent de-repression of inflammatory response genes. *Nature* 470(7334):414–418.