MicroRNA-181b Improves Glucose Homeostasis and Insulin Sensitivity by Regulating Endothelial Function in White Adipose Tissue

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Abstract

Rationale—The pathogenesis of insulin resistance involves dysregulated gene expression and function in multiple cell types including endothelial cells (ECs). Posttranscriptional mechanisms such as microRNA-mediated regulation of gene expression could affect insulin action by modulating EC function.

Objective—To determine whether microRNA-181b (miR-181b) affects the pathogenesis of insulin resistance by regulating EC function in white adipose tissue during obesity.

Methods and Results—MiR-181b expression was reduced in adipose tissue ECs of obese mice, and rescue of miR-181b expression improved glucose homeostasis and insulin sensitivity. Systemic intravenous delivery of miR-181b robustly accumulated in adipose tissue ECs, enhanced insulin-mediated Akt phosphorylation at Ser473, and reduced endothelial dysfunction, an effect

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signaling inhibits the expression of adhesion molecules involved in leukocyte rolling and adhesion to the vascular luminal wall.[18] Insulin resistance also leads to EC dysfunction through increased circulating free fatty acids and hyperglycemia.[19, 20] Indeed, ECs from visceral adipose tissue of obese mice or human subjects exhibit a marked inflammatory state with increased expression of chemokines, cytokines, and adhesion molecules.[17, 21] Several studies have shown insulin signaling in ECs protect endothelial function and attenuates the progression of atherosclerosis.[22–25] However, it remains unknown whether enhancing endothelial insulin signaling in adipose tissue may improve systemic insulin resistance.

Akt phosphorylation regulates many fundamental biological processes such as the insulin signaling cascade. Dysregulation of Akt phosphorylation often is involved in heart disease and diabetes.[26, 27] Full Akt activity depends on the phosphorylation of residues Thr308 and Ser473, which can be repressed by the lipid phosphatase – PTEN and the protein phosphatase – PH domain leucine-rich repeat phosphatases isoform 2 (PHLPP2). PHLPP2 inactivates Akt signaling by specifically dephosphorylating Ser473 but not Thr308 of Akt.[28, 29] It remains unknown whether altering the expression of PHLPP2 in ECs regulates Akt signaling, downstream substrates, insulin sensitivity, and glucose homeostasis.

MicroRNAs (miRNAs) are evolutionarily conserved small noncoding RNAs, which post-transcriptionally regulate gene expression by promoting mRNA degradation and/or inhibiting translation. It has been reported that miRNAs are differentially expressed in adipose tissue between lean and obese mice,[30, 31] as well as lean and obese human subjects.[32] We have shown that microRNA-181b (miR-181b) ameliorates NF-κB-mediated EC activation and vascular inflammation in mouse models of endotoxemia and atherosclerosis.[33, 34] However, it remains unknown whether: 1) miR-181b expression is dysregulated in ECs of adipose tissue, and 2) increasing miR-181b expression in adipose tissue will ameliorate obesity-associated insulin resistance and inflammatory responses.

In this study, we examined the expression of miR-181b in adipose tissue ECs of obese mice and the role of miR-181b and PHLPP2 in modulating glucose homeostasis and insulin sensitivity. Our findings reveal that miR-181b improves insulin signaling and reduces inflammation and EC dysfunction in white adipose tissue by targeting endothelial PHLPP2 without altering hepatic steatosis or lipid profiles.

METHODS

Methods are provided in the Online Supplement.

RESULTS

MiR-181b is reduced by diabetic stimuli in endothelial cells

Differential expression of miRNAs has been observed in adipose tissue from lean and obese mice as well as humans, although miRNA expression in adipose tissue ECs has not been previously explored.[30–32] To examine the expression of miR-181b, an anti-inflammatory microRNA that we previously identified in the macrovasculature[33, 34], ECs or adipocytes were isolated as described[21, 35–37] from eWAT of C57BL/6 mice fed a 60% HFD for 0, 3, 7,

or 14 days followed by qPCR analysis (Figure 1A). We found miR-181b is the most dominantly expressed among miR-181 family members in adipose tissue ECs as demonstrated that its expression is 3.6-fold higher than that of miR-181a. MiR-181b expression was reduced by 33% and 36% after HFD for 7 and 14 days, respectively, while the expression of miR-181a and miR-181c was reduced by 53% and 67% after HFD for 14 days (Figure 1A). In contrast, the expression of miR-181 family members was not significantly changed in adipocytes after HFD for the indicated days (Figure 1A), and miR-181b expression was not reduced in liver or skeletal muscle ECs (Online Figure I A). However, miR-181b expression was reduced by 24%, 32%, 31%, and 33% in ECs in vitro after treatment with glucose, PMA, TNF-α, or palmitate (Figure 1B). In addition, we examined miR-181b expression in ECs of eWAT over the period of 12-weeks of HFD. MiR-181b expression was decreased by 42%, 84% and 66% in ECs isolated from eWAT of mice fed a HFD for 2, 6, and 12 weeks, respectively (Online Figure I B). Although the ratios of phosphorylated Akt / total Akt were not changed under basal conditions (Online Figure I C), insulin responsiveness was blunted as indicated by reduced phosphorylation of Akt in ECs isolated from eWAT of mice fed on HFD at 2 weeks (Online Figure I D). The expression of VCAM-1, ICAM-1, and E-selectin were increased in ECs from eWAT of mice fed a HFD for 2 weeks compared to chow-fed mice (Online Figure I E). These data demonstrate that the reduction of miR-181b in ECs is an early event during the development of insulin resistance, which was associated with ongoing inflammatory responses.

MiR-181b improves glucose tolerance and insulin sensitivity in a mouse model of diet-induced diabetes

Our prior observations indicate that miR-181b serves as an anti-inflammatory regulator in the macrovasculature. Based upon the reduced expression of miR-181b in the microvasculature of white adipose tissue, we hypothesize that rescue of miR-181b expression may delay the progression of inflammation and insulin resistance, and improve insulin sensitivity. To examine the effect of miR-181b systemic delivery on glucose homeostasis and insulin sensitivity, C57BL/6 mice were fed a 60% HFD for 12 weeks. After 6 weeks HFD, mice were treated with miR-181b mimics (181b-m) or miRNA negative control (NS-m) for 6 weeks (twice a week, i.v. 1nmol/injection) (Figure 2A). Insulin Tolerance Tests (ITT) and Glucose Tolerance Tests (GTT) were performed at week 5 and 6, respectively, after miR-181b treatment (Figure 2A). The body weights were significantly increased in HFD-fed mice, which were independent of miRNA treatments (Figure 2B). However, miR-181b treatment markedly improved glucose tolerance (Figure 2C and 2D) and insulin sensitivity (Figure 2E and 2F) compared with NS-m treatment. MiR-181b reduced the area under curves for ITT and GTT by 57% and 38%, respectively, compared to control mice (Figure 2D and 2F). These beneficial effects occurred independent of any changes in lipid profiles, fat mass, or plasma levels of insulin and free fatty acids (Online Table I). Taken together, these data demonstrate that miR-181b delivery is able to improve glucose homeostasis and insulin sensitivity.

MiR-181b reduces inflammation in epididymal white adipose tissue

We have shown that miR-181b ameliorates NF-κB-mediated EC activation and vascular inflammation in mouse models of endotoxemia and atherosclerosis.[33, 34] This prompted us

to examine the effect of miR-181b delivery on inflammation and EC dysfunction in obese mice. First, paraffin sections of eWAT or liver were stained for the macrophage marker Mac2. Macrophage content indicated by Mac2 staining was significantly increased in eWAT of mice after 12-week of HFD. However, macrophage accumulation in eWAT of miR-181b-treated mice was reduced by 60% compared to NS ctrl-treated mice (Figure 3A). The macrophage content in liver was not reduced by miR-181b delivery. Second, macrophage M1 and M2 markers were examined by qPCR. MiR-181b delivery reduced the expression of the macrophage M1 markers *Tnfa*, *Il-1b*, and *Il12* by 48%, 51%, 39%, respectively, and increased the expression of M2 markers *Mrc2*, *Mgl2*, *Fizz1*, *Ym1 by* 43%, 33%, 60%, and 21%, respectively, in eWAT; while it exerted minimal effects on M1 and M2 markers of macrophage in liver (Figure 3B). Third, we observed that miR-181b delivery reduced ICAM-1 and VCAM-1 expression in eWAT from obese mice (Figure 3C), suggesting that EC dysfunction in eWAT in obese mice was ameliorated by miR-181b delivery. These effects of miR-181b treatment are associated with a 1.5-fold overexpression of miR-181b in eWAT and ~15-fold overexpression in ECs of eWAT (Online Figure II A). Systemic delivery of miR-181b could lead to more pronounced exogenous miR-181b expression in specific cell types within eWAT such as adipocytes or adipose tissue ECs. To test this, the expression of miR-181b in eWAT or ECs isolated from eWAT was examined after three consecutive injections of miRNA negative control (NS-m) or miR-181b. Three daily injections of miR-181b result in 105-fold expression of miR-181b in adipose tissue ECs, and 29-fold expression in eWAT (Online Figure II B). These data suggest that systemic delivery of miR-181b leads to predominant accumulation of exogenous miR-181b in adipose tissue ECs within eWAT, and reduces EC activation, macrophage accumulation, and inflammatory phenotype in white adipose tissue.

MiR-181b does not directly inhibit the cell-intrinsic capacity of monocytes/macrophages to migrate, proliferate, or be activated

Macrophage infiltration, proliferation, and activation are all involved in the pathogenesis of obesity-induced insulin resistance.[38, 39] Reduced macrophage content in eWAT could result from the direct effect of miR-181b on migratory and proliferative ability of monocytes/ macrophages or by directly reducing EC activation and dysfunction. To explore any direct effects of miR-181b on monocyte migration, adoptive transfer of monocytes was conducted as previously described.[40] Monocytes were isolated from HFD-fed mice treated with miR-181b or miRNA negative control (NS-m), labeled with PKH26, and injected into HFD-fed obese mice. Stromal vascular fractions were isolated from eWAT of the recipient mice two days later, and subjected to FACS analysis.[41] As shown in Figure 4A, miR-181b overexpression (25-fold, data not shown) in monocytes does not significantly inhibit their migration into eWAT revealed by the percentage of PKH26-positive cells among CD11b and F4/80 double-positive cells [NS-m: (22.1 ± 5.1) %; 181b-m: (19.2 ± 4.0) %]. To examine the effect of miR-181b on macrophage proliferation, staining for Ki67 (cell division marker), F4/80 (macrophage marker), and DAPI (nuclear marker) was performed on paraffin sections of eWAT from mice. The percentages of proliferating macrophages are (13.9 ± 1.9) % and (12.5 ± 1.1) % in NS-m or miR-181b treated mice respectively, suggesting systemic delivery of miR-181b did not affect macrophage proliferation in eWAT (Figure 4B). We previously showed that miR-181b does not inhibit NF-κB activation in

macrophages and NF-κB target gene expression in PBMCs.[33] Consistently, miR-181b did not affect TNF-α, IL-1β, and COX-2 gene expression in PBMCs isolated from insulin-resistant mice (Figure 4C). Collectively, these data indicate that miR-181b does not directly regulate cell-intrinsic monocytes/macrophage function including migration, proliferation, and activation.

MiR-181b expression promotes glucose uptake in adipocytes in a paracrine manner

Since miR-181b does not directly regulate cell-intrinsic functions of monocytes/macrophages, the protective effects of miR-181b delivery on insulin signaling in eWAT may result from the direct effects of miR-181b on adipocytes or ECs. To assess this, glucose uptake experiments were performed in differentiated, mature 3T3-L1 adipocytes. Consistent with our hypothesis, miR-181b overexpression in 3T3-L1 adipocytes did not promote glucose uptake (Figure 5A). ECs of inflamed adipose tissue may have adverse effects on insulin signaling in adipocytes, possibly via paracrine effects.[17] Since miR-181b attenuates endothelial inflammation in adipose tissues (Figure 3C), we reasoned that miR-181b improves insulin signaling in a paracrine manner by exerting protective effects in ECs. Indeed, the conditioned medium (Supe) from ECs overexpressing miR-181b markedly improved glucose uptake in adipocytes (Figure 5B). These data suggest that miR-181b overexpression in ECs may improve glucose uptake via paracrine mechanisms with adipocytes.

MiR-181b delivery improves insulin signaling by increasing Akt phosphorylation in epididymal white adipose tissue

Because Akt phosphorylation is a central event in insulin signaling cascade, we examined the expression of phospho-Akt (Ser473 and Thr308) in eWAT, skeletal muscle, and liver. HFD mice were treated with miR-181b or NS ctrl mimics in an analogous manner as Figure 2 and stimulated in the presence or absence of insulin for an additional 10 min prior to tissue harvest. In insulin-stimulated miR-181b-treated mice, phospho-Akt (Ser473) was significantly increased 2.2-fold in eWAT, whereas there were no significant differences in skeletal muscle or liver (Figure 6A). Phospho-Akt (Thr-308) was not changed by miR-181b delivery compared with controls in all the tissues examined. Moreover, the effect of miR-181b delivery on upstream insulin signaling was examined in eWAT. Since inflammatory stimuli can blunt insulin action by affecting the phosphorylation of mediators in upstream insulin signaling such as insulin receptor beta (InR-β) and insulin receptor substrate 1 (IRS1),[42–45] their phosphorylation status was examined by immunoprecipatation assays from eWAT lysates (Figure 6B). MiR-181b delivery did not affect the expression of phospho-InR-β (Tyr1162) and phospho-IRS1 (Ser307) in eWAT, suggesting upstream insulin signaling was not changed by miR-181b. Both FoxOs and eNOS are important molecules mediating insulin signaling events downstream of phospho-Akt in ECs.[46–48] We found phospho-FoxO1 (Ser 256) is increased up to 2.0-fold in eWAT of miR-181b treated mice compared with controls in response to insulin (Figure 6B). Consistently, miR-181b overexpression increased Akt phosphorylation at Ser473 and reduced nuclear accumulation of FoxO1 at 10 min after insulin stimulation in ECs (Figure 6C). Similarly, miR-181b increased phospho-eNOS (Ser1176) by 1.7-fold (Figure 6D) and nitric oxide (NO) activity by 1.5-fold (Online Figure III) in eWAT compared to controls after insulin stimulation. In a

separate experiment, obese mice were treated with miR-181b using the same dosing regimen as outlined in Figure 2, and ECs were isolated from eWATs to examine the effects of miR-181b delivery on insulin signaling and inflammation. Consistent with the effects of miR-181b on insulin signaling and inflammation in eWATs, we found miR-181b increased the phosphorylation of Akt by ~1.6-fold (Figure 6E) and reduced ICAM-1 expression by 43% in ECs isolated from eWATs (Online Figure IV). These data indicate that miR-181b delivery reduced insulin resistance and inflammation in ECs within eWATs. We also examined the expression of genes involved in thermogenesis and hepatic glucose production. The mRNA expression of UCP-1, PRDM16, and PGC-1α genes were not changed in brown fat and eWAT by miR-181b, suggesting that the thermogenic program is not affected by miR-181b delivery (Online Figure V A). To exclude any effects of miR-181b on hepatic glucose production, we examined miR-181b delivery on the expression of key metabolic enzymes involved in maintaining hepatic glucose homeostasis in liver from mice described in Figure 2. MiR-181b had no effect on mRNA expression for GS, GK, PK, GP, FBP1, G6P and PEPCK (Online Figure V B). Finally, miR-181b delivery had no effect on regulating lipid homeostasis in liver revealed by Oil Red O staining (Online Figure V C). These data suggest that systemic delivery of miR-181b improves glucose homeostasis and insulin sensitivity in a tissue-specific manner by promoting Akt phosphorylation and sensitizing insulin action in eWAT independent of any effects on insulin signaling in the liver and skeletal muscle, or lipid accumulation in the liver.

The reduction of endogenous miR-181b by inflammatory stimuli such as TNF-α could affect insulin signaling in ECs. Indeed, TNF-α treatment reduced insulin-induced Akt phosphorylation in ECs (Online Figure VI A) as previously described.[49] MiR-181b inhibition reduced insulin-induced Akt phosphorylation, which was potentiated in the presence of TNF-α treatment (Online Figure VI A). Furthermore, miR-181b overexpression rescued the reduction of insulin-induced Akt phosphorylation by TNF-α treatment (Online Figure VI B). The data imply that the early reduction of endogenous miR-181b in ECs of eWAT is likely involved in the pathogenesis of endothelial insulin resistance in eWATs during the development of obesity.

MiR-181b targets PHLPP2

MiR-181b could improve insulin signaling by directly regulating a target intrinsic to this pathway, or by suppressing target genes of other inter-related signaling pathways. Our previous studies demonstrated that miR-181b can repress importin-α3, a protein involved in the nuclear translocation of NF-κB (p65/p50), by binding to its 3′-UTR.[33, 34] MiR-181b could inhibit adipose tissue inflammation by reducing importin-α3 expression in ECs. We examined NF-κB p65 nuclear accumulation in adipose tissue ECs. Surprisingly, nuclear p65 expression was not significantly induced by HFD-feeding, and miR-181b delivery did not reduce p65 nuclear accumulation in adipose tissue ECs (Online Figure VII). A combined strategy using bioinformatics and microarray gene chip profiling was taken to identify potential miR-181b direct targets. Genes reduced by miR-181b overexpression identified by microarray gene chip analysis (GEO database accession no. GSE35030)[34] that are also predicted as direct targets of miR-181b by different algorithms will be of interest as potential miR-181b targets. Six algorithms predicted 1280 mouse and human orthologous

genes as miR-181b direct targets (Figure 7A). Among 4190 genes identified by microarray gene chip assay with more than 1.2-fold reduction by miR-181b overexpression, 306 genes were also found in the list of 1280 genes predicted by *in silico* algorithms[50] as miR-181b direct targets (Figure 7A). Gene ontology analysis of these 306 genes revealed that the targets of miR-181b encode regulators of biological processes such as protein amino acid phosphorylation, phosphorylation, intracellular signaling cascade, protein serine/threonine kinase activity, among others (Figure 7B). We focused on phosphatases since miR-181b likely promotes Akt phosphorylation at Serine 473 by reducing the expression of a phosphatase. Several serine/threonine phosphatases have been shown to regulate insulin signaling, and are involved in the pathogenesis of insulin resistance.[29, 51, 52] Among the combined list of 306 genes was Pleckstrin homology domain leucine-rich repeat protein phosphatase (PHLPP) isozyme2 (PHLPP2) which can directly de-phosphorylate and inactivate Akt.[28, 29] Indeed, we found miR-181b reduced PHLPP2 protein expression in ECs (Figure 7C). To verify that miR-181b directly targets PHLPP2, we performed Argonaute2 (AGO2) micro-ribonucleoprotein IP (miRNP-IP) studies to assess whether PHLPP2 mRNA is enriched in the RNA-induced silencing complex (RISC) following miR-181b overexpression. An approximately 4-fold enrichment of PHLPP2 mRNA was observed after AGO2 miRNP-IP in the presence of miR-181b, as compared with that with the miRNA-negative control (Figure 7D). Furthermore, miR-181b reduced the PHLPP2 3′-UTR activity by 54% (Figure 7E). To show the specificity of miR-181b on PHLPP2 3′UTR, the seed sequence in pcDNA3.1-miR-181b construct was mutated. MiR-181b mutant lost the ability to inhibit the activity of PHLPP2 3′UTR as shown in Figure 7E. Importantly, pharmacological inhibition[53] or siRNA knockdown of PHLPP2 promoted insulin-induced Akt phosphorylation in ECs (Figure 7F). To examine whether miR-181b targets PHLPP2 in vivo, PHLPP2 expression was examined in lysates of eWAT, skeletal muscle, and liver from obese mice treated with miR-181b (twice a week for 6 weeks). Systemic delivery of miR-181b reduced PHLPP2 protein expression in eWAT, but not in skeletal muscle or liver (Figure 7G and Online Figure VIII A). Because PHLPP2 expression is significantly lower in liver (nearly undetectable) than in eWAT and skeletal muscle, PHLPP2 may play a minimal role in regulating insulin signaling in liver (Online Figure VIII B). Moreover, miR-181b treatment (twice a week for 6 weeks) reduced PHLPP2 expression by 55% in ECs isolated from eWAT of obese mice (Figure 7H). These results identify PHLPP2 as a *bona fide* direct target of miR-181b. PHLPP2 expression was significantly increased in ECs of eWAT from mice fed a HFD for 2, 6, or 12 weeks (Online Figure VIII C), suggesting a counter-regulation of the miR-181b target PHLPP2 during the development of insulin resistance. In addition to PHLPP2, we examined the effects of miR-181b on other potential phosphatases including INPP5E, PPM1A, CTDSPL, and PPAR2B, which are predicted as miR-181b targets among 306 genes. MiR-181b overexpression reduced the expression of CTDSPL and PPAP2B by 41% and 56%, respectively, in HUVECs (Online Figure VIII D). In contrast, miR-181b overexpression had no effects on the expression of PPM1A and INPP5E. However, siRNA-mediated knockdown of CTDSPL or PPAP2B did not promote the phosphorylation of Akt at Serine 473 in response to insulin in HUVECs (Online Figure VIII E), suggesting CTDSPL and PPAR2B did not mediate miR-181b's effect on insulin-induced Akt phosphorylation. It has been reported that miR-181b reduces IGF-1R expression in tumor cells, and inhibits VEGF-induced PI3K-Akt signaling;[54] in contrast, in T cells it may

reduce PTEN expression, thereby promoting PI3K-Akt signaling.[55] Therefore, we examined whether miR-181b regulates Akt phosphorylation by reducing IGF-1R and PTEN expression in ECs. As shown in Online Figure VIII F, miR-181b overexpression did not reduce PTEN and IGF-1R expression in ECs, and also had no effect on PTEN expression in eWAT. These data suggest a cell-specific regulation of PTEN and IGF-1R expression by miR-181b. Collectively, these results identify PHLPP2 as a direct target of miR-181b that may mediate miR-181b's effects on the insulin signaling pathway.

PHLPP2 knockdown improves glucose tolerance and insulin sensitivity in diet-induced diabetic mice

To examine whether PHLPP2 knockdown will phenocopy miR-181b's effects to improve glucose homeostasis, insulin sensitivity, and reduce adipose tissue dysfunction in vivo, C57BL/6 obese mice were therapeutically treated with PHLPP2 siRNAs or negative control siRNAs for 6 weeks (twice a week, i.v. 1nmol/injection) (Figure 8A). PHLPP2 siRNAs injection did not affect the body weights of mice compared with control siRNAs (Figure 8B). However, GTT and ITT studies revealed that PHLPP2 knockdown significantly improved glucose tolerance (Figure 8C and 8D) and insulin sensitivity (Figure 8E and 8F) by 19% and 20%, respectively, compared to mice treated with control siRNAs controls (Figure 8D and 8F). The effect of PHLPP2 knockdown on ITT and GTT was associated with a ~1.5-fold increase of Akt phosphorylation at Ser473 in eWAT but not in liver (Figure 8G). Macrophage content indicated by Mac-2 staining was also reduced by 58% in eWAT of PHLPP2 siRNA treated mice (Figure 8H). In a separate experiment, three daily injections of PHLPP2 siRNA lead to a 72% and 12% reduction of PHLPP2 mRNA expression in adipose tissue ECs and adipocytes, respectively, suggesting that the majority of systemically delivered siRNAs accumulate in adipose tissue ECs (Figure 8I). Interestingly, PHLPP2 expression revealed by immunostaining was increased by 33% in ECs isolated from diabetic patients compared with control subjects (Figure 8J and Online Figure IX). PHLPP2 inhibition may represent a new therapeutic approach to reduce insulin resistance by improving vascular EC function within eWAT. These data suggest that knockdown of PHLPP2 improves glucose tolerance and insulin sensitivity, reduces adipose tissue endothelial inflammation, and inhibits macrophage accumulation.

DISCUSSION

A number of miRNAs have been identified as regulators of obesity-induced insulin resistance. For example, miR-802, miR-143, and miR-103/7 all regulate glucose metabolism and insulin sensitivity in obesity.[56–58] It remains unknown whether miRNAs affect insulin sensitivity by regulating EC function within eWAT. In the present study, we have discovered that: (1) the expression of miR-181b is reduced in white adipose tissue ECs of HFD mice, which can be rescued by miR-181b systemic delivery; (2) miR-181b delivery improves glucose homeostasis and insulin sensitivity associated with reduced EC activation, macrophage infiltration, and inflammatory phenotype in eWAT; (3) miR-181b had no direct effects on regulating monocyte/macrophage activation, proliferation, or recruitment in vivo; (3) miR-181b targets PHLPP2, a phophatase that dephosphorylates Akt at Ser473 in ECs; and (4) siRNA-mediated knockdown of PHLPP2 phenocopies miR-181b's effect on glucose

homeostasis, insulin sensitivity, and eWAT macrophage accumulation. We demonstrated that enhanced insulin signaling in adipose ECs exerts beneficial effects and promotes glucose uptake in adipocytes in a paracrine manner, and importantly, improves systemic glucose homeostasis and insulin sensitivity without altering hepatic steatosis or lipid profiles.

Several studies have been performed by other groups to uncover the causal relationships among vascular endothelial function, inflammation, and metabolic insulin resistance. For example, vascular inflammation (increased phosphorylation of IκBα and ICAM-1 expression) and insulin resistance (reduced phosphorylation of Akt and eNOS) are detectable in aortas of mice fed a HFD for 1 week,[15] which precedes the onset of peripheral insulin resistance in liver, skeletal muscle, and adipose tissue. Another group showed systemic insulin resistance occurred in mice fed a HFD for three days revealed by GTT and hyperinsulinemic-euglycemic clamp studies.[59] However, the relationship between EC function and endothelial insulin resistance within eWAT remains unclear. We examined Akt phoshporylation in ECs from eWATs of chow- and HFD-fed mice for 2 weeks. Our data suggest that ECs within eWAT display an insulin-resistant and inflammatory phenotype in mice after 2-week HFD (Online Figure I D and E). The reduction of miR-181b in ECs of eWAT was detected as early as 1 week after HFD (Figure 1A), suggesting that the reduction of miR-181b in ECs and associated endothelial activation was an early event during the development of insulin resistance.

Endothelial eNOS-NO signaling is impaired in ECs of visceral fat in obese patients.[60] Activation of eNOS by insulin through Akt in ECs is important for limiting obesity-induced insulin resistance and inflammation.[61] The production of NO by activated eNOS inhibits NF-κB activity, decreases cytokine-induced endothelial activation, and shifts macrophage polarization towards an M2 anti-inflammatory phenotype.[62–64] MiR-181b delivery increased the phosphorylation of Akt and eNOS, increased NO activity (Online Figure III), and decreased FoxO activity and ICAM-1 expression in eWATs (Figure 6). In ECs of eWATs, miR-181b also increased Akt phosphorylation and decreased ICAM-1 expression (Figure 6E and Online Figure IV). In contrast, miR-181b delivery had no intrinsic effects on macrophage activation, infiltration, and proliferation (Figure 4). However, miR-181b delivery reduced macrophage accumulation and shifted M1 macrophages to an M2 anti-inflammatory phenotype in eWATs (Figure 3A). These data indicate that miR-181b improved insulin resistance in eWATs by primarily improving endothelial Akt-eNOS-NO signaling through targeting PHLPP2, an effect reducing endothelial ICAM-1 expression, EC-leukocyte interactions, and favorably shifting M1 to M2 macrophages thereby generating an anti-inflammatory milieu in eWAT. This anti-inflammatory milieu in eWATs generated by miR-181b may also promote glucose uptake in adipocytes through a paracrine manner (Figure 5).

Our results demonstrate that miR-181b delivery improves insulin signaling in epididymal fat but not in liver or skeletal muscle. There are at least two reasons that may account for this tissue-specific effect: 1) miRNAs may confer target and functional specificity in different cell types or tissues.[65, 66] MiR-181b may target PHLPP2 in eWAT but not liver and skeletal muscle (Figure 7G and Online Figure VIII A); 2) tissue- and cell-specific accumulation of

exogenous miR-181b. Although miR-181b delivery leads to its highest overexpression in liver, exogenous miR-181b is enriched higher in adipose ECs than in liver or skeletal muscle ECs (Online Figure II B). A fenestrated, discontinuous endothelium in liver may cause this differential accumulation of exogenous miR-181b. Furthermore, expression of the miR-181b target PHLPP2 is minimally expressed in liver compared to eWAT, a finding that may account for the lack of miR-181b's effects on insulin signaling in liver. Interestingly, improved insulin signaling in liver ECs potentiated hepatic insulin resistance,[67] suggesting potential phenotype functional differences of liver ECs vs eWAT ECs.

NF-κB mediated vascular inflammation participates in the pathogenesis of obesity-associated insulin resistance.[15, 68, 69] We previously demonstrated that miR-181b reduces acute and chronic vascular inflammation by targeting endothelial importin-α3 and NF-κB signaling.[33, 34] While we anticipated that miR-181b could improve insulin sensitivity by reducing NF-κB-mediated EC activation and inflammation; surprisingly, we found that nuclear accumulation of NF-κB p65 is not significantly induced in white adipose tissue ECs after 12 weeks HFD, and miR-181b delivery had no inhibitory effect (Online Figure VII), suggesting that miR-181b targets different gene(s) than importin-α3 in adipose tissue ECs. Indeed, using a combination of bioinformatics and gene microarray profiling studies, our studies revealed that miR-181b directly targets PHLPP2 in ECs. As miRNAs are known to regulate several targets, our study cannot rule out the possibility that miR-181b targets additional gene(s) that mediate its beneficial effects on insulin sensitivity and glucose homeostasis.

In our study, epididymal fat mass in miR-181b treated HFD mice was not reduced, an effect suggesting that miR-181b delivery does not reduce the capacity of white adipocytes/adipose tissue to store lipids in the context of over-nutrition. Consistent with this, the level of circulating triglycerides and liver lipid content were not significantly different between conditions. Furthermore, adipocytes incubated with conditioned medium from ECs overexpressing miR-181b exhibited markedly improved insulin resistance, an effect highlighting the potential importance of EC-adipocyte interactions in regulating the insulin resistant state. Our data also suggest that miR-181b is able to reduce adipose tissue EC inflammation, leukocyte accumulation, and improve systemic glucose homeostasis and insulin sensitivity without potentiating the development of hepatic steatosis.

In conclusion, our study in HFD mice demonstrates that miR-181b expression is reduced in response to HFD-induced obesity and that rescue of miR-181b in the microvasculature of eWAT is sufficient to improve glucose homeostasis and insulin sensitivity. MiR-181b delivery decreases inflammation in adipose tissue ECs in eWAT by targeting the phosphatase PHLPP2, an effect that increases phospho-AKT (Ser473) to improve insulin signaling. MiR-181b-mediated effects were selective for ECs of eWAT because miR-181b overexpression in adipocytes did not promote glucose uptake. These data indicate that strategies aimed at improving microvascular EC function in visceral fat in general and restoring miR-181b expression (or inhibition of PHLPP2 expression) in adipose tissue ECs in particular, may provide the basis for the rationale design of novel therapies for insulin resistance and its attendant cardiovascular complications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations and Acronyms

EC	endothelial cell
HFD	high-fat diet
MiRNA	microRNA
miR-181b	microRNA-181b
3′UTR	3′-untranslated region
PBMCs	peripheral blood mononuclear cells
NS-m	miRNA negative control mimics
181b-m	microRNA-181 mimics
HUVECs	human umbilical vein endothelial cells
GTT	glucose tolerance test
ITT	insulin tolerance test
Ewat	epididymal white adipose tissue
PHLPP2	PH domain and leucine rich repeat protein phosphatase 2

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Novelty and Significance

What Is Known?

- Obesity-associated insulin resistance is a major risk factor for cardiovascular disease.

- Endothelial dysfunction contributes significantly to the pathogenesis of obesity-associated insulin resistance.

- We previously showed that the microRNA-181b (miR-181b) inhibits NF-κB-mediated endothelial activation in response to acute (endotoxemia) and chronic (atherosclerosis) inflammation. MiR-181b reduces expression of importin-α3, a protein critical for NF-κB translocation from cytoplasm to nucleus. However, the role of miRNA-181b in obesity-associated insulin resistance has not been examined.

What New Information Does This Article Contribute?

- MiR-181b expression in adipose tissue endothelial cells (ECs) is reduced early after high-fat diet in C57BL/6 mice, coincident with the onset of insulin resistance. Systemic intravenous delivery of miR-181b significantly improves glucose homeostasis and insulin sensitivity.

- MiR-181b reduces adipose EC inflammation, inhibits macrophage infiltration, and shifts M1 to anti-inflammatory M2 macrophages in epididymal white adipose tissue (eWAT). MiR-181b is identified as a new regulator of EC-adipocyte interactions in white adipose tissue, thereby controlling insulin resistance and inflammation.

- Mechanistically, miR-181b targets PHLPP2, a phosphatase that dephosphorylates Akt, leading to increased Akt phosphorylation at Serine 473 and improved EC insulin signaling. PhiosphoAkt in turn phosphorylate eNOS and increases nitric oxide activity in eWAT but not liver and skeletal muscle.

- Knockdown of PHLPP2 mimics miR-181b's effects including increased Akt phosphorylation in ECs and eWAT, reduced macrophage content in eWAT, and improved glucose homeostasis and insulin sensitivity.

Insulin resistance resulting from obesity commonly predisposes to the development of type 2 diabetes mellitus and cardiovascular disease. Chronic low-grade inflammation initiated in adipose tissue is a hallmark of obesity, which has been recognized as a key step in the development of insulin resistance. However, our understanding of adipose tissue EC dysfunction contributing to adipose tissue inflammation and insulin resistance remains inadequate. Results from this study will advance our understanding of miRNA-mediated regulation of adipose tissue EC function in the pathogenesis of adipose tissue insulin resistance and inflammation, which could provide novel and effective therapeutic strategies to manage a range of cardiovascular disease states that are exacerbated by insulin resistance.
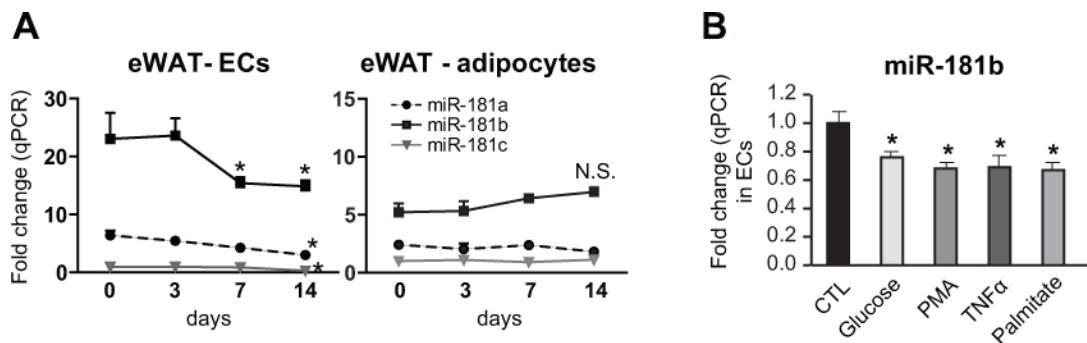
Figure 1. MiR-181b is reduced in response to inflammatory stimuli or hyperglycemia in endothelial cells

A, C57BL/6 mice were fed a high-fat diet as indicated (0, 3, 7, or 14 days). Endothelial cells (ECs) and adipocytes were isolated from eWAT for qPCR. The expression of miR-181 was normalized to small RNA U6 expression and compared to the expression of miR-181c in mice on chow that was subsequently set to a value of one, n=6 per group. **B**, ECs were cultured in the absence or presence of 30 mM D-glucose for 48 hours, 100 nM PMA for 24 hours, 10 ng/ml TNF-α or 100 μM palmitate for 4 hours and harvested for qPCR of miR-181b, n=3 per group. Data show Mean ± SEM; *, $P < 0.05$. N.S., non-significant.
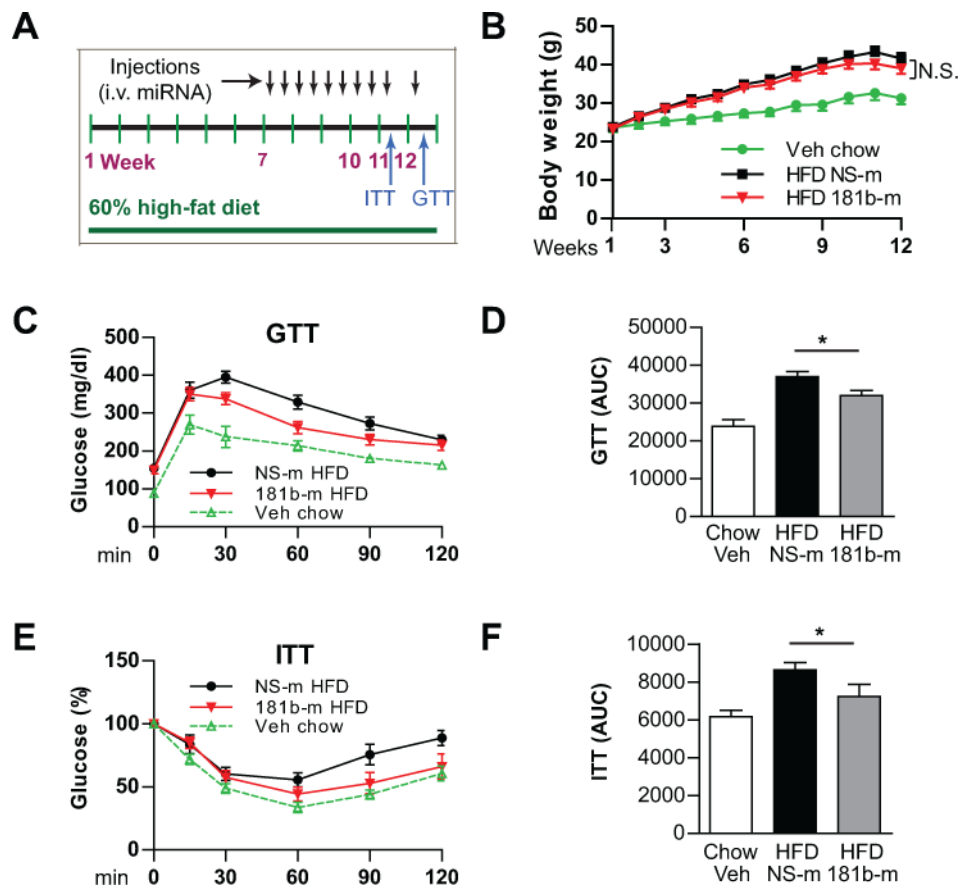
Figure 2. Systemic delivery of miR-181b improves glucose tolerance and insulin sensitivity in diet-induced obese mice

A, Schema of experimental procedures. C57BL/6J mice were fed a 60% high-fat diet (HFD) for 12 weeks. Six weeks after HFD, mice were treated with miR-181b (181b-m) or miRNA negative control (NS-m) for 6 weeks as indicated (i.v. 0.6 mg/kg). **B**, Body weights over time of mice on chow (injected with vehicle) or HFD (injected with NS-m or miR-181b). **C** and **E**, Blood glucose levels were measured on week 11 for the insulin tolerance test (ITT) and week 12 for the glucose tolerance test (GTT). Values were compared with basal glucose levels which were set as 100% for ITT. **D** and **F**, Area under the curve (AUC) of glucose and insulin tests was quantified. Mean ± SEM, n=7–9 mice per group; *, $P<0.05$.
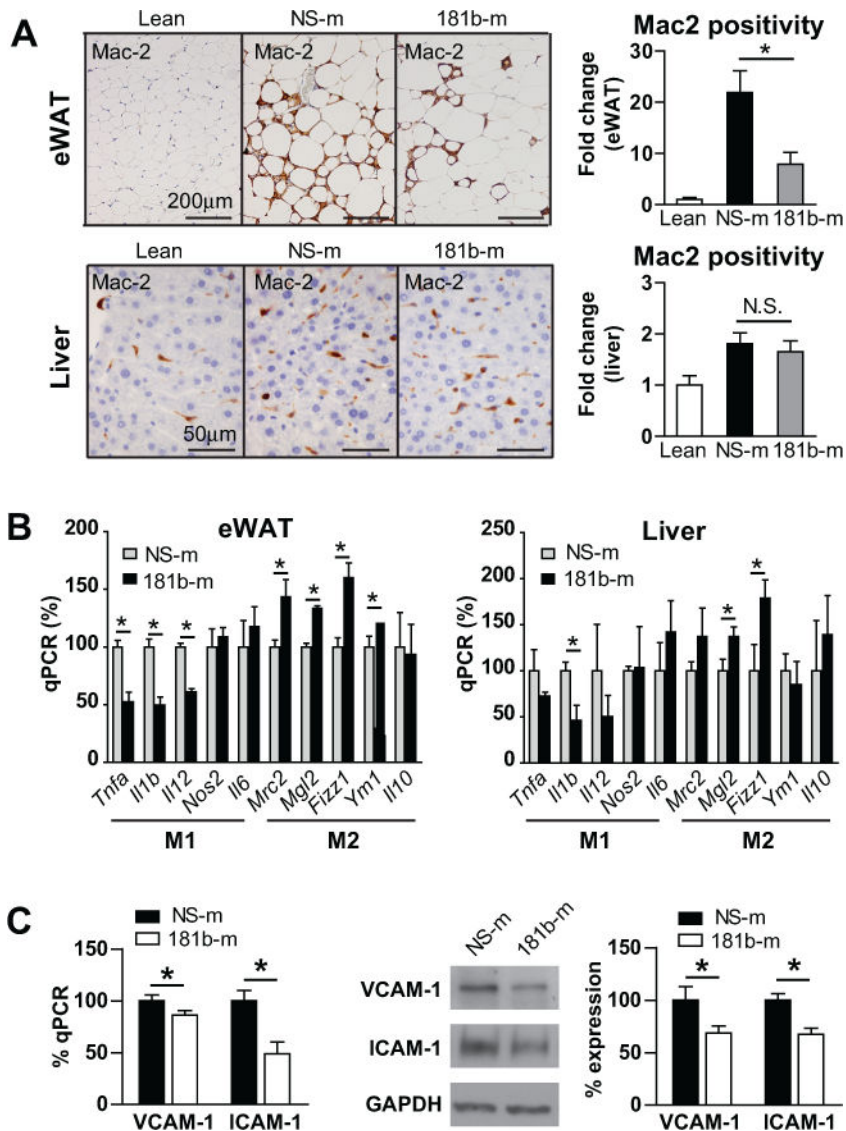
Figure 3. Systemic delivery of miR-181b reduces inflammation in epididymal fat
A, Paraffin sections of eWAT or liver were stained with Mac2, and the positive areas were quantified. **B**, qPCR of gene expression in epididymal white adipose tissue (eWAT) and liver; results were presented relative to those of miRNA negative control (NS-m) treated mice. **C**, qPCR and western blot analysis of VCAM-1 and ICAM-1 in eWAT. Mean ± SEM, n=6–9; *, $P < 0.05$.
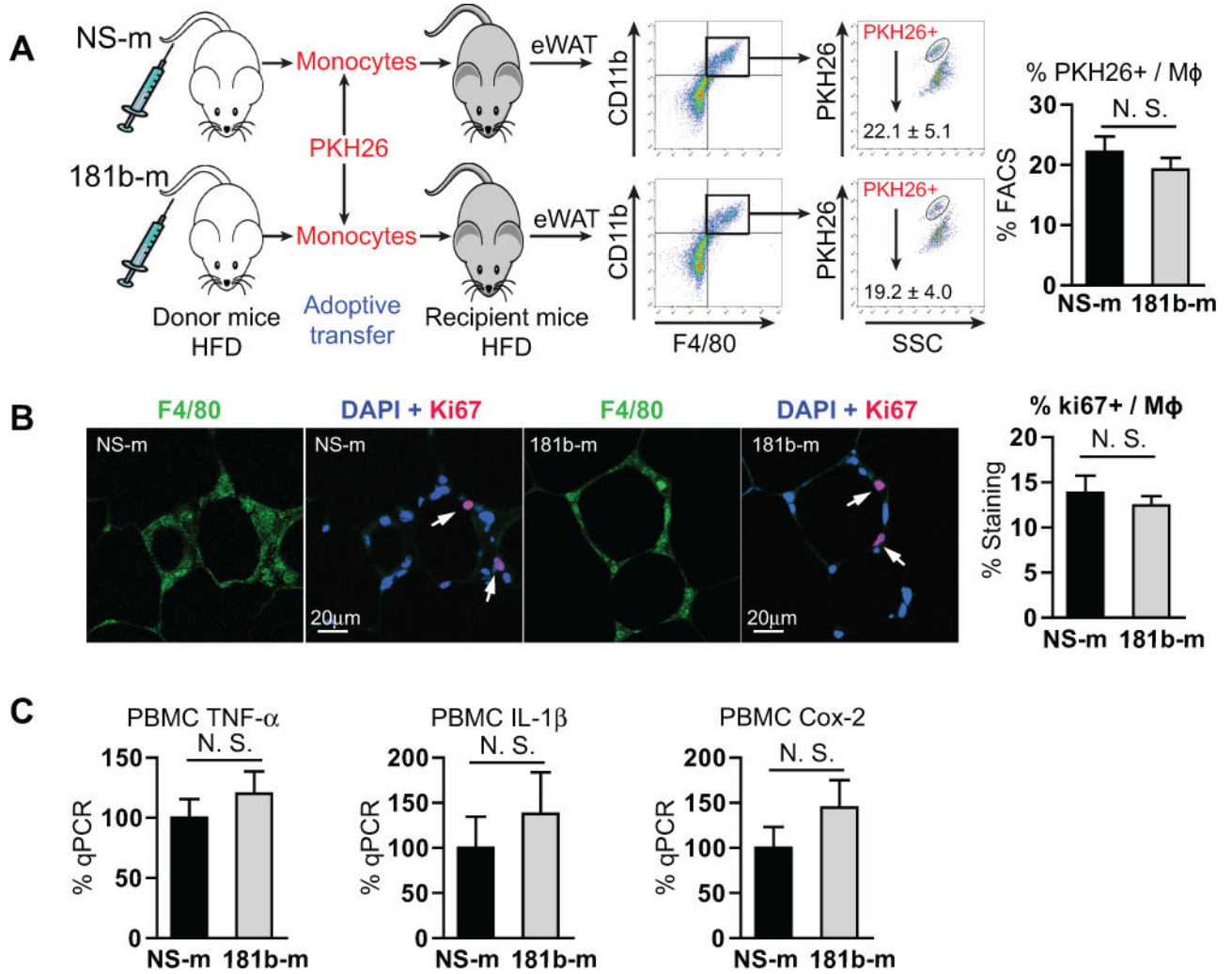
Figure 4. MiR-181b does not inhibit the proliferation, migration, and activation of monocytes/ macrophages

A, Paraffin sections of epididymal white adipose tissue (eWAT) were stained with F4/80, DAPI and Ki67. The percentages of Ki67 among F4/80 positive cells were calculated. Mean ± SEM, n=7–10 mice per group. **B**, Adoptive transfer of PKH26 labeled monocytes from obese mice overexpressing miRNA negative control (NS-m) or miR-181b, and FACS analysis of PKH26 positive cells among CD11b and F4/80 double positive cells in the eWAT of recipient obese mice. Mean ± SEM, n=4 mice per group. **C**, PBMCs were isolated from NS-m- or miR-181b-treated mice fed on a high-fat diet for qPCR analysis. Data show mean ± SEM, n=6 mice per group.
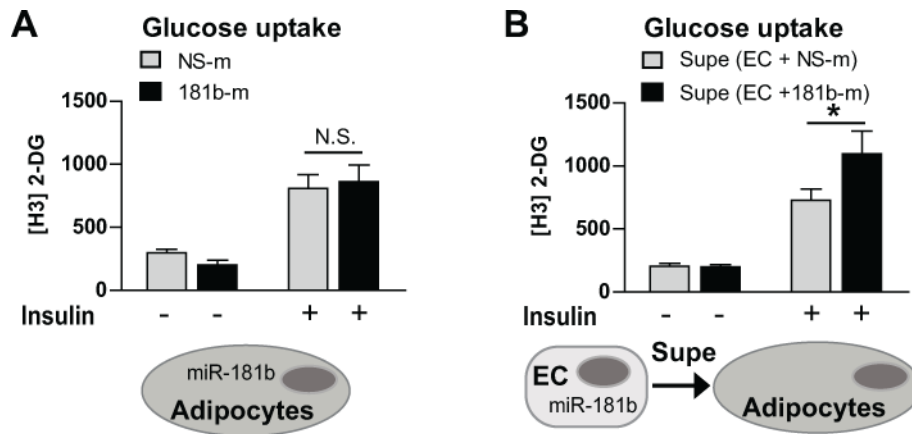
Figure 5. Glucose uptake in 3T3-L1 adipocytes is promoted in the presence of conditioned medium from endothelial cells overexpressing miR-181b

A, Adipocytes were transfected with miRNA negative control (NS-m) or miR-181b and glucose uptake was quantified. **B**, Adipocytes were cultured with conditioned medium from ECs overexpressing NS-m or miR-181b and glucose uptake was quantified. Mean ± SEM, n=4; *, $P < 0.05$. N.S., non-significant. Supe, supernatant.
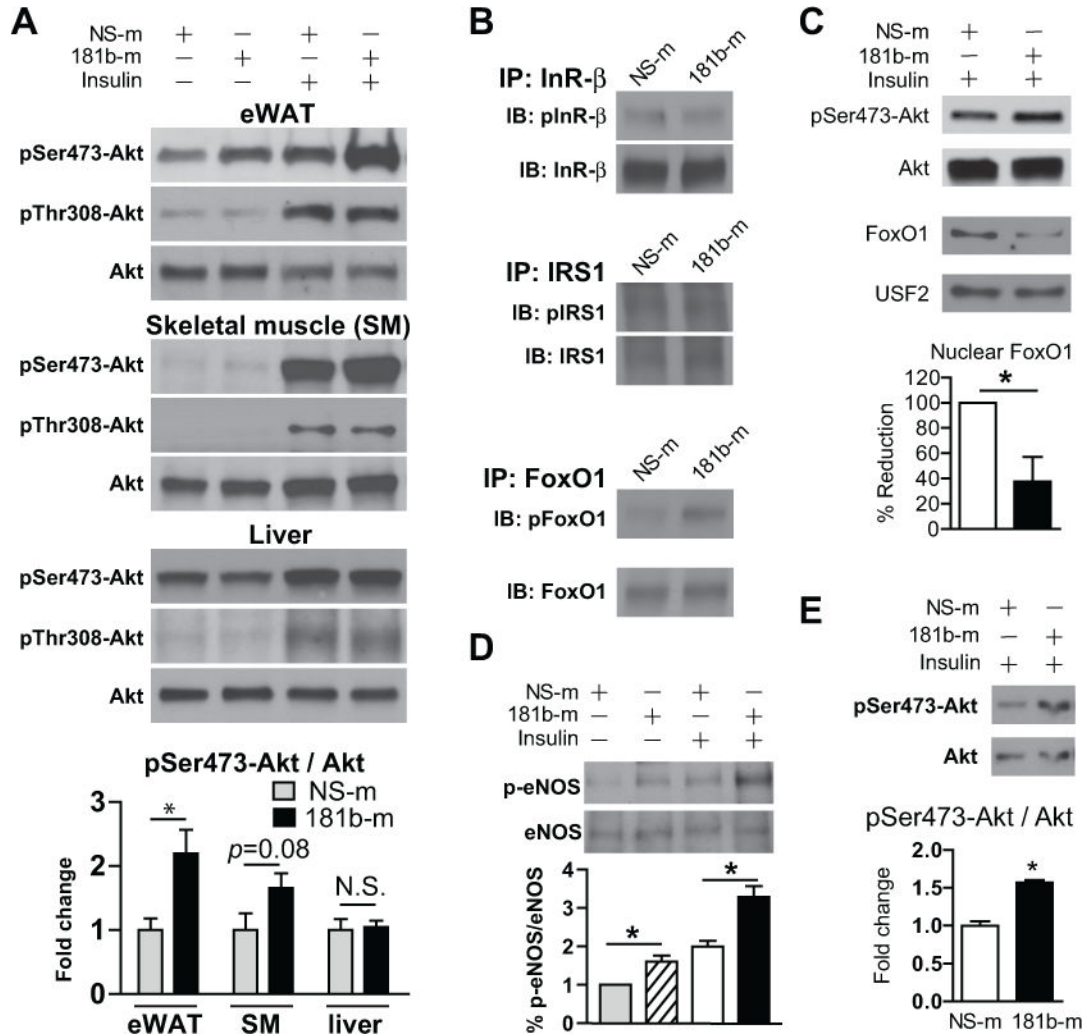
Figure 6. MiR-181b delivery improves Akt phosphorylation at Serine 473 in epididymal white adipose tissue

A, Western blot analysis of Akt phosphorylation at Serine 473 and Threonine 308, and quantifications of pSer473-Akt vs. total Akt in epididymal white adipose tissue (eWAT), skeletal muscle (SM), or liver from insulin-stimulated mice, n=6–9. **B**, Lysates of eWAT were used for immunoprecipitation and western blot analysis. **C**, MiRNA negative control (NS-m) or miR-181b-transfected HUVECs were treated with 100 nM insulin for 10 minutes. Western blot analysis of pSer473-Akt in total lysates, and total FoxO1 in nuclear fraction, n=3 independent experiments. **D**, Western blot analysis of eNOS phosphorylation at Serine 1176, and quantifications of pSer1176-eNOS vs. total eNOS in eWAT from insulin-stimulated mice, n=6–9. **E**, Western blot analysis of Akt phosphorylation at Serine 473 in ECs isolated from eWAT of obese mice treated with NS-m or miR-181b, n=6 mice / group. All values show Mean ± SEM; *, $P < 0.05$.
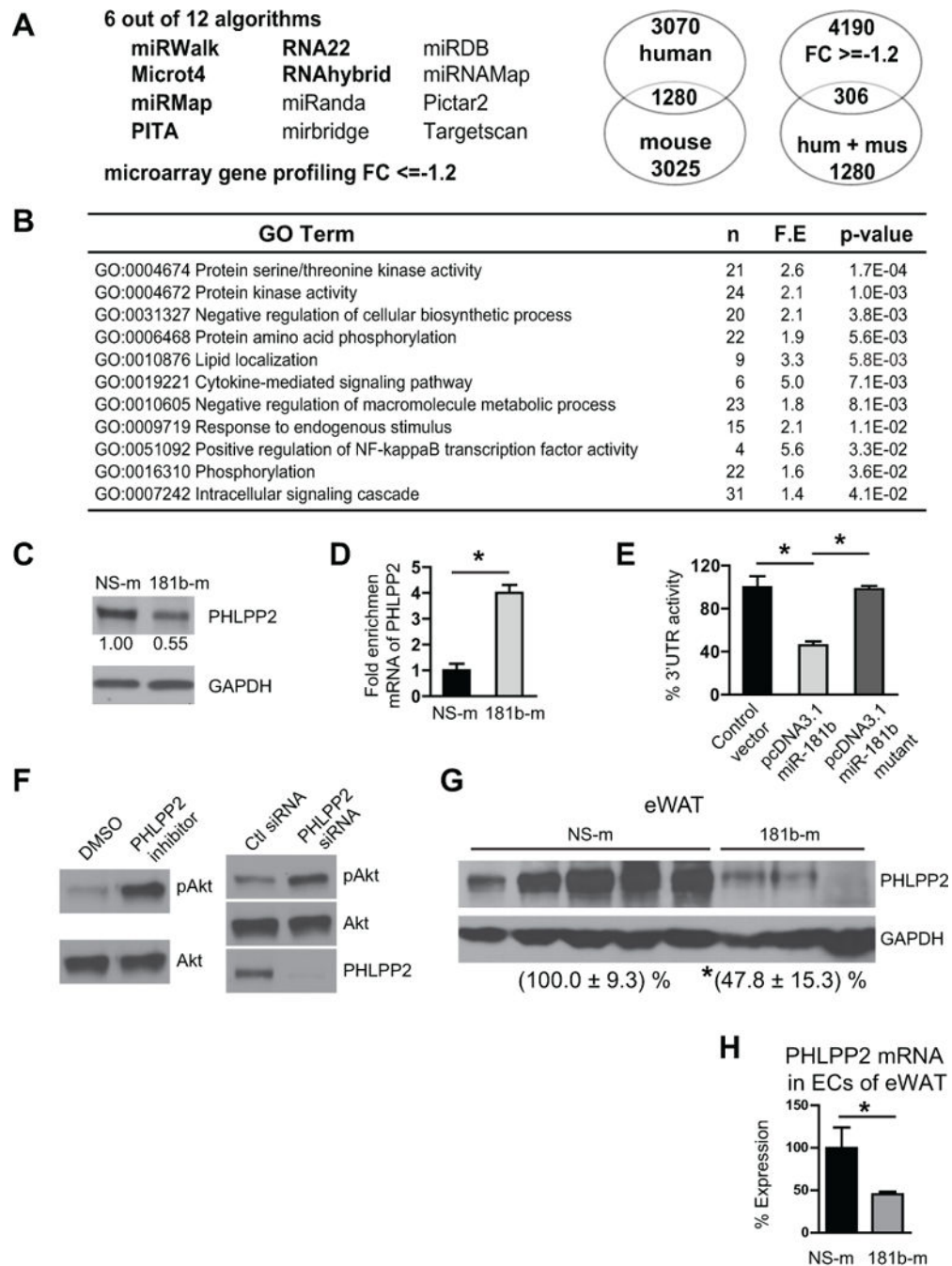
Figure 7. MiR-181b targets PHLPP2
A, Bioinformatic approach predicts miR-181b direct targets among genes reduced by
miR-181b overexpression in ECs identified by microarray gene chip profiling. FC, fold-
change. **B**, Gene ontology analysis of 306 genes identified in **A. C**, Western blot analysis of
endothelial cells (ECs) transfected with 10 nM miRNA negative control (NS-m) or 181b-m.
D, miRNP-IP analysis of enrichment of PHLPP2 mRNA in HUVECs transfected with NS-m
or 181b-m, n=2 independent experiments. **E**, Luciferase reporter assay of PHLPP2 3′UTR in
the presence of pcDNA3.1(+), pcDNA3.1(+)-miR-181b, and pcDNA3.1-miR-181b mutant

in HUVECs, n=3 independent experiments. **F**, Pharmacological inhibition or siRNA knockdown of PHLPP2 increases pSer473-Akt in ECs in response to 100 nM insulin at 10 minutes. **G**, Western blot analysis of PHLPP2 expression in eWAT, n= 3 – 5. **H**, PHLPP2 expression was detected in ECs isolated from epididymal white adipose tissue (eWAT) of obese mice treated with NS-m or miR-181b using the dosing regimen as outlined in Figure 2, n=6 mice per group. All values showmean ± SEM; *, $P < 0.05$.
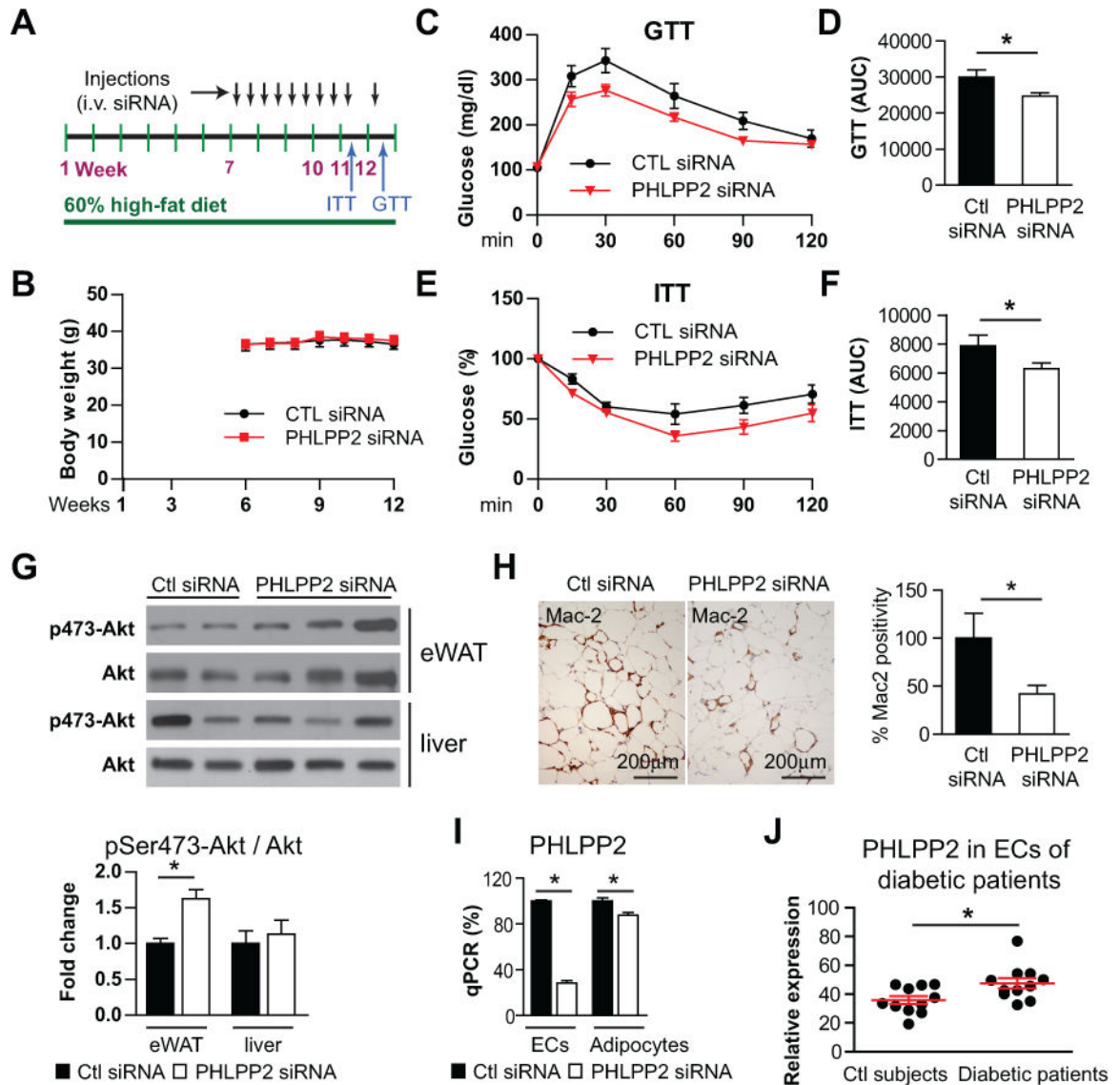
Figure 8. Systemic delivery of PHLPP2 siRNA improves glucose tolerance and insulin sensitivity in diet-induced obese mice

A, Schema of experimental procedure. C57BL/6J mice were fed a 60% high-fat diet (HFD) for 12 weeks. Six weeks after HFD, mice were treated with negative control siRNA or PHLPP2 siRNA for the subsequent 6 weeks as indicated (i.v. 0.6 mg/kg). **B**, Body weights over time of mice injected with control siRNAs or PHLPP2 siRNAs respectively. **C** and **E**, Blood glucose levels were measured on week 11 for the insulin tolerance test (ITT) and week 12 for the glucose tolerance test (GTT). Values were compared with basal glucose levels which were set as 100% for ITT. **D** and **F**, Area under the curves (AUC) for ITT and GTT were quantified. **G**, Western blot analysis of Akt and pSer473-Akt in epididymal white adipose tissue (eWAT) and liver. **H**, Paraffin sections of eWAT were stained with Mac2, and the positive areas were quantified. **B–H**, n=6–7. **I**, Mice were injected with negative control or PHLPP2 siRNAs three times on consecutive days, and the expression of PHLPP2 was examined in endothelial cells (ECs) or adipocytes isolated from eWAT, n=3 mice per

group. **J**, Quantification of PHLPP2 protein expression in ECs freshly isolated from control and diabetic subjects, n=11 subjects each group. All data show mean ± SEM; *, $P<0.05$.