GHS-R suppression in adipose tissues protects against obesity and insulin resistance by regulating adipose angiogenesis and fibrosis

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Abstract

Background/Objectives: Ghrelin is an orexigenic hormone that increases food intake, adiposity, and insulin resistance through its receptor Growth Hormone Secretagogue Receptor (GHS-R). We previously showed that ghrelin/GHS-R signaling has important roles in regulation of energy homeostasis, and global deletion of GHS-R reduces obesity and improves insulin sensitivity by increasing thermogenesis. However, it is unknown whether GHS-R regulates thermogenic activation in adipose tissues directly.

Methods: We generated a novel adipose tissue-specific GHS-R deletion mouse model and characterized the mice under regular diet (RD) and high-fat diet (HFD) feeding. Body composition was measured by EchoMRI. Metabolic profiling was determined by indirect calorimetry. Response to environmental stress was assessed using a TH-8 temperature monitoring system. Insulin sensitivity was evaluated by glucose and insulin tolerance tests. Tissue histology was analyzed by hematoxylin/eosin and immunofluorescent staining. Expression of genes involved in thermogenesis, angiogenesis and fibrosis in adipose tissues were analyzed by real-time PCR.

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Results: Under RD feeding, adipose tissue-specific GHS-R deletion had little or no impact on metabolic parameters. However, under HFD feeding, adipose tissue-specific GHS-R deletion attenuated diet-induced obesity and insulin resistance, showing elevated physical activity and heat production. In addition, adipose tissue-specific GHS-R deletion increased expression of master adipose transcription regulator of peroxisome proliferator-activated receptor (PPAR) γ1 and adipokines of adiponectin and fibroblast growth factor (FGF) 21; and differentially modulated angiogenesis and fibrosis evident in both gene expression and histological analysis.

Conclusions: These results show that GHS-R has cell-autonomous effects in adipocytes, and suppression of GHS-R in adipose tissues protects against diet-induced obesity and insulin resistance by modulating adipose angiogenesis and fibrosis. These findings suggest adipose GHS-R may constitute a novel therapeutic target for treatment of obesity and metabolic syndrome.

Introduction

Obesity is considered the most prominent risk factor for insulin resistance, type 2 diabetes, and cardiovascular disease [1, 2]. Obesity is the consequence of sustained positive energy balance. Adipose tissue plays a critical role in energy- and glucose-homeostasis, working not only to store excess energy in white adipose tissue (WAT), but also to burn fat to dissipate energy in brown adipose tissue (BAT) [3-7]. Adipose tissue is considered to be one of the key organs involved in regulation of systemic insulin sensitivity, by producing adipokines such as leptin, adiponectin and fibroblast growth factors (FGFs) [8, 9].

Adipose tissue is a metabolically responsive endocrine organ that secretes a myriad of adipokines in response to the central and local signaling. Adipose tissue is extremely dynamic, undergoing constant remodeling that changes number and/or size of adipocytes in response to various metabolic stimuli [10, 11]. However, under the prolonged fat accumulation, adipose angiogenesis cannot maintain the remodeling process of rapid adipose tissue expansion. This unmatched challenge in adipose tissue expansion leads to hypoxia, inflammation, and metabolic stress in adipose tissue, which promotes adipose dysfunction and alters adipokine secretion. The adaptive adipose tissue remodeling in obesity is considered as a major contributing factor for pathophysiology of obesity and metabolic syndrome [11]. Angiogenesis is critical for adipose tissue remodeling in homeostatic and diseased conditions [10, 12]. Therefore, a better understanding of adipose tissue remodeling in obesity will facilitate the discovery of novel therapeutics for diet-induced obesity (DIO) and obesity-associated metabolic dysfunctions.

Ghrelin, an endogenous ligand for Growth Hormone Secretagogue Receptor (GHS-R), is the only known circulating orexigenic hormone [13, 14]. Ghrelin stimulates appetite and promotes adiposity; ghrelin signaling has important implications in the pathogenesis of obesity, insulin resistance, and type 2 diabetes [13, 15, 16]. GHS-R is selectively expressed in tissues; it is highly expressed in the hypothalamus of the brain, and its expression in peripheral tissues such as adipose tissue and muscle is much lower [17-19]. Accumulating evidence suggests that ghrelin/GHS-R signaling has important roles in regulating adiposity, energy balance and glucose homeostasis [20, 21]. Our previous studies show that GHS-R suppression prevents DIO by increasing thermogenesis in global and pan-neuronal GHS-R-

deleted mice [4, 18, 22]. Our recent study shows that adipocyte protein 2 (aP2)-Cre-mediated GHS-R knockdown mice exhibit thermogenic phenotype [23]. However, since GHS-R was ectopically deleted in non-adipose tissues such as hypothalamus in the aP2-Cre GHS-R deficient model, the direct effect of GHS-R in adipocytes still remains to be determined.

To investigate whether GHS-R has a cell-autonomous effect in adipocytes and assess the role of GHS-R in adipose tissue remodeling, we generated an adipocyte-specific GHS-R knockout mouse model by breeding the *Ghsr*$^{f/f}$ line with the adiponectin-Cre line in the current study. We investigated the role of adipose tissue-specific GHS-R in regulation of diet-induced adiposity and insulin resistance, and specifically investigated whether GHS-R signaling plays a key role in adipose tissue remodeling events such as angiogenesis and fibrosis.

Materials and Methods

Animals.

Adipose tissue-specific GHS-R-deleted mice were generated by breeding *adiponectin*-Cre mice (*adipoq*-Cre) with our *Ghsr*$^{f/f}$ mice based on a Cre-Lox system [18]. Mice were housed with 12-hour light/dark cycles (6 A.M. to 6 P.M.) and allowed free access to water and food. All diets used in this study were purchased from Harlan Teklad with the following composition in calorie distrubution: 6.5% fat, 60% carbohydrates, and 19.1% protein for the regular diet (RD) (2920X); 42% fat, 42.7% carbohydrates, and 15.2% protein for the high-fat diet (HFD) (TD 88137). Male mice were used in all studies and the mice were randomly assigned to RD or HFD. Besides the designed variables of genotype and diet, all other factors were tightly controlled to minimize variability. The age of mice used for each experiment was identical, and we tried to use littermate pairs as much as possible. In addition, we also paired mice with similar body weight and body fat as much as possible to enhance the rigor of the data set. All animal procedures were approved by the Institutional Animal Care and Use Committee at Baylor College of Medicine.

Body composition, indirect calorimetry and functional tests.

Body composition was assessed by Echo MRI and metabolic profiling was assessed by Columbus Instruments Comprehensive Lab Animal Monitoring System (CLAMS), as we have previously described [18, 21]. Glucose tolerance test (GTT) and insulin tolerance test (ITT) were also performed as previously described [18, 21].

Cold stress test.

Core body temperature was used to assess the thermoregulation under 4°C cold stress. The rectal temperature was obtained using a TH-8 temperature monitoring system (Physitemp, Clifton, NJ) as previously described [18]. Briefly, mice were individually caged and placed in a 4°C cold room with free access to food and water. The rectal temperature was monitored hourly for 4 h.

Quantitative real-time PCR.

All procedures of RNA preparation and real-time quantitative PCR were performed as previously described [18, 24]. GHS-R 1a primers were designed to span an intron in order to distinguish from the expression of truncated GHS-R 1b: sense primer 5′-GGACCAGAACCACAAACAGACA-3′, anti-sense primer 5′-CAGCAGAGGATGAAAGCAAACA-3′. The rest of the primer information is listed in Supplemental Table 1.

Immunofluorescence staining.

Adipose tissue was immediately collected after terminations and fixed in 10% PBS-buffered formalin for 24 h. Following paraffin embedding, the tissue sections were stained with a primary antibody against Endomucin (1:200, R&D systems. Minneapolis, MN) or Mac-2 (1 mg/ml, 1:1,000; Tebu-Bio, France) followed by donkey α-goat Alexa Fluor 647-conjugated secondary antibodies (1.5 μg/mL, Jackson Immuno-Research Laboratories, West Grove, PA). Nuclei were stained with DAPI (1 μg/mL, Vector Laboratories, Burlingame, CA). Images were acquired with Carl Zeiss upright Apotome Axio Imager Z1/ZEN2 Core Imaging software.

Whole-mount immunofluorescence staining and confocal microscopy imaging.

The whole-mount immunofluorescence staining for adipose tissue has been described previously [25]. Briefly, after the mice were sacrificed, adipose tissues were excised and fixed with fresh 1% PBS-buffered paraformaldehyde for 30 min at room temperature. After washing with PBS for 3 times of 10 min each, the tissues were subdivided into 0.5 cm^3 sized pieces followed by blocking with 5% BSA-PBS for 30 min at room temperature. Then, the tissues were incubated with a α-Endomucin antibody (1:200, R&D systems. Minneapolis, MN) overnight at 4°C followed by staining with a donkey α–goat Alexa Fluor 647-conjugated secondary antibody (1:1000, Jackson Immuno-Research Laboratories). Images were acquired with the confocal Leica TCS SP5 microscope and the quantification was performed using the LAS AF software.

Statistical analysis.

Two-way ANOVA with repeated measures, or one-way ANOVA, was used for statistical analysis. At least n=5 was used for each genotype/treatment, and all gene expression samples were run in triplicates. Data are represented as mean ± SEM, and statistical significance is set to a minimum of $P < 0.05$.

Results

Generation of adipose tissue-specific GHS-R knockout mice

To investigate the role of GHS-R in adipose tissue, we generated an adipose tissue-specific GHS-R-deleted mouse model by breeding *adiponectin*-Cre mice (*adipoq*-Cre) with *Ghsr^f/f^* mice as we previously described [18]. As a result, GHS-R expression in epididymal WAT (epiWAT) and BAT of *adipoq*-Cre;*Ghsr^f/f^* mice were reduced by 34% and 42%, respectively; but were not reduced in hypothalamus, inguinal WAT (iWAT), and muscles (Fig. 1A). This

indicates that GHS-R deletion in *adipoq*-Cre;*Ghsr*$^{f/f}$ mice was adipose tissue-specific, specificlaly targeting epiWAT and BAT.

Adipose tissue-specific GHS-R deletion attenuates diet-induced obesity by increasing energy expenditure

We monitored body weight change and body composition under both RD and HFD feeding conditions, and the HFD feeding started from 6 weeks of age. Under RD feeding, adipose tissue-specific GHS-R deletion had no effect on body weight, fat mass or lean mass (Fig. S1A, S1B). In contrast, under HFD feeding, the *Adipoq*-Cre;*Ghsr*$^{f/f}$ mice had a lower body weight compared to *Ghsr*$^{f/f}$ mice starting at 16 weeks of age (Fig. 1B). Interestingly, *Adipoq*-Cre;*Ghsr*$^{f/f}$ mice exhibited a lower body fat mass starting at 10 weeks of age, 4 weeks post-HFD feeding (Fig. 1C). Consistently, the *Adipoq*-Cre;*Ghsr*$^{f/f}$ mice exhibited reduced sizes in epiWAT and BAT when compared to the *Ghsr*$^{f/f}$ mice (Fig. 1D).

To determine whether adipose tissue-specific GHS-R deletion affects other metabolic profiles, we conducted indirect calorimetry analysis. Under RD feeding, the total food intake was not different, whereas physical activity was reduced by nearly 25% in *Adipoq*-Cre;*Ghsr*$^{f/f}$ mice as compared to *Ghsr*$^{f/f}$ mice (Figs. S2A, S2B). Under RD feeding conditions, the energy expenditure during the day and the resting metabolic rate (RMR) of *Adipoq*-Cre;*Ghsr*$^{f/f}$ mice was increased compared to *Ghsr*$^{f/f}$ mice (Figs. S2C, S2D), while the respiratory exchange ratio (RER) was similar between the two genotypes (Fig. S2E). The opposing effects of decreased physical activity and increased RMR may explain the unremarkable metabolic changes under RD conditions. Similar to RD feeding, the total food intake of HFD-fed mice was comparable in both genotypes (Fig. 2A). Under HFD feeding conditions, *Adipoq*-Cre;*Ghsr*$^{f/f}$ mice exhibited significantly increased physical activity and energy expenditure during the dark/light cycle (Figs. 2B, 2C). To note that the RMR was significantly reduced when normalized to lean mass under HFD condition (Fig. 2D), which was opposite to that observed under RD feeding. Similar to RD, the RER scores were not altered under HFD feeding (Fig. 2E). Taken together, these results suggest that the leaner phenotype of HFD-fed *Adipoq*-Cre;*Ghsr*$^{f/f}$ mice was in part due to the enhanced energy expenditure resulted from increased physical activity and energy expenditure.

Adipose tissue-specific GHS-R deletion improves glucose tolerance and insulin sensitivity, but has no effect on thermogenesis

To determine whether adipose tissue-specific GHS-R regulates glucose homeostasis and insulin sensitivity, we performed insulin and glucose tolerance tests (ITT and GTT, respectively). Under RD feeding, *Adipoq*-Cre;*Ghsr*$^{f/f}$ mice showed comparable GTT and ITT profiles (Figs. S3A, S3B). In contrast, under HFD feeding, *Adipoq*-Cre;*Ghsr*$^{f/f}$ mice showed improved glucose tolerance and insulin sensitivity in GTT and ITT, respectively (Figs. 2F, 2G).

In our previous studies, global and brain-specific GHS-R deletions in mice induced a robust increase in thermogenesis [18, 21, 22]. Thus, we further assessed the thermogenic effect in *Adipoq*-Cre;*Ghsr*$^{f/f}$ mice under cold stress. The results revealed that the core body temperature of HFD-fed *Adipoq*-Cre;*Ghsr*$^{f/f}$ mice was slightly higher (p=0.07) within 4 h of

cold challenge (Fig. 3A). The expression of most known thermogenic marker genes in BAT and iWAT were not changed, except for β3 adrenergic receptor (β3-AR) in BAT (Figs. 3B, 3C). Collectively, these data suggest that the targeted GHS-R deletion in adipose tissues has little effect on thermogenesis. The increase in energy expenditure is likely attributable to increased physical activity.

Adipocyte GHS-R deficiency activates key transcription factors and adipokines in adipose tissue

To examine if the reduced adiposity in HFD-fed *Adipoq*-Cre;*Ghsr$^{f/f}$* mice was associated with decreased lipid accumulation in epiWAT, we assessed the expression of lipogenic genes. Surprisingly, the expression of adipocyte protein 2 (aP2), fatty acid synthase (FAS), perilipin, and preadipocyte factor 1 (pref-1) were not affected (Fig. 4A). Similarly, we found no difference in these lipogenic genes in epiWAT of RD-fed *Adipoq*-Cre;*Ghsr$^{f/f}$* mice (Fig. S4). Interestingly, we found adipose transcription factors peroxisome proliferator-activated receptor gamma (PPARγ) 1 mRNA expression robustly increased in epiWAT of HFD-fed *Adipoq*-Cre;*Ghsr$^{f/f}$* mice, whereas *PPARγ2* was not changed (Fig. 4B). This is noteworthy, because *PPARγ1* and *PPARγ2* are key transcription factors that regulate the differentiation and functions of adipocytes [26, 27]. Adipokines of FGF21, adiponectin, and leptin have been shown to have important roles in adipose metabolism; increased expression of FGF21 and adiponectin is known to be associated with the lean and insulin-sensitive phenotype [28, 29]. We next examined adipokines of FGF21, adiponectin, and leptin. FGF21 and adiponectin expression in epiWAT of HFD-fed *Adipoq*-Cre;*Ghsr$^{f/f}$* mice increased 5.9-fold and 1.6-fold, respectively, while leptin was not changed (Fig. 4C). These results suggest that GHS-R regulates adipocyte differentiation and adipokine expression. The increased healthy adipokines of FGF21 and adiponectin are in support of the metabolic improvements observed in the *Adipoq*-Cre;*Ghsr$^{f/f}$* mice .

Adipocyte GHS-R deficiency increases local angiogenesis and decreases inflammation and fibrosis in adipose tissue

Growing evidence indicates that adipose dysfunction in obesity is closely associated with adipose tissue remodeling [12, 30-32]. It has been shown that hypoxia, inflammation and inadequate angiogenic remodeling can elicit fibrosis [30, 32, 33]. To investigate the functional mechanisms that mediate the effects of GHS-R in adipose tissues, we assessed the genes associated with hypoxia, angiogenesis, inflammation and fibrosis. The epiWAT of *Adipoq*-Cre;*Ghsr$^{f/f}$* mice exhibited increased expression of hypoxia-inducible factor 2α (HIF2α), but there was no significant difference in HIF1α and HIF1β (Fig. 5A). The expression of angiogenic genes, such as vascular endothelial growth factor A (VEGF-A) and its receptor VEGF-R2, were increased by 44% and 56%, respectively, and hepatocyte growth factor (HGF) was increased by 57% (Fig. 5B). The expression of fibrotic genes Col1α and Col2α in epiWAT of HFD-fed *Adipoq*-Cre;*Ghsr$^{f/f}$* mice were decreased by 32% and 35%, respectively, while transforming growth factor beta (TGF-β) and its receptor (TGF-βR) were similar between the *Ghsr$^{f/f}$* and *Adipoq*-Cre;*Ghsr$^{f/f}$* mice (Fig. 5C). These results suggest that GHS-R may regulate hypoxia, angiogenesis and fibrosis in WAT.

To assess the adipose tissue composition, we performed histological analysis in epiWAT paraffin sections by conducting H&E staining and studied expression of signature markers for inflammation and vasculature. The H&E staining of adipose section revealed smaller sizes of adipocytes and reduced level of the macrophage marker Mac2 in epiWAT of HFD-fed *Adipoq*-Cre;*Ghsr*$^{f/f}$ mice (Fig. 5D), which is consistent with improved insulin sensitivity and reduced macrophage infiltration.

Adipose tissue expansion triggers the expansion of blood capillaries; adipose tissue endothelial cells promote pre-adipocyte proliferation [12, 30-32]. Indeed, we observed increased endothelial-specific marker endomucin staining in both paraffin sections and whole mount epiWAT of HFD-fed *Adipoq*-Cre;*Ghsr*$^{f/f}$ mice (Fig. 5D), which indicates increased microvasculature. Overall, the gene expression and histology data demonstrate that GHS-R in adipose tissue has profound effects on angiogenesis, inflammation, and fibrosis, suggesting that inhibition of GHS-R in adipocytes leads to a healthy metabolic microenvironment with increased angiogenesis and decreased inflammation and fibrosis, supporting the systemically improved insulin sensitivity.

Discussion

At present, the role of GHS-R signaling in adipose tissue is not fully understood due to the limitations of existing animal models. In order to unequivocally define the cell autonomous role of GHS-R in adipose tissue, we generated the adipose tissue-specific GHS-R knockout mouse model. We examined the GHS-R gene expression in multiple tissues including hypothalamus, epiWAT, BAT, iWAT, and muscle. The suppressed GHS-R expression in *Adipoq*-Cre*;Ghsr*$^{f/f}$ mice was confined to the epiWAT and BAT, not other central and peripheral tissues. We previously showed that GHS-R expression in iWAT is much lower than epiWAT [21]. Even though *adipoq*-Cre is capable of targeting both iWAT and epiWAT, the low expression of GHS-R in iWAT may obscure the difference. Our validation data indicate that the *adipoq*-Cre;*Ghsr*$^{f/f}$ mouse is a good model for studying the effects of GHS-R in epiWAT and BAT. Since epiWAT is the most important fat depot in regard to obesity and insulin resistance, this model would enable us to study the role of GHS-R in metabolic dysfunction.

In the current study, we demonstrated that adipose tissue-specific GHS-R deletion attenuates DIO and improves insulin sensitivity. We and others have shown that ghrelin exerts orexigenic effect to promote food intake and increase adiposity through stimulating orexigenic Agouti-Related Peptide (AgRP) neurons in the hypothalamus [22, 35]. However, homeostatic food intake is not affected in the global, pan-neuronal or AgRP-specific GHS-R knockout mice. Hence, in term of food intake, while neuronal GHS-R is required for food initiation and orexigenic effect of ghrelin, GHS-R has no effect on long-term total food intake. On the other hand, we showed that global, pan-neuronal and AgRP-specific GHS-R deletion protects against DIO by inducing thermogenesis to increase energy expenditure [18, 21, 22]. Considering the complex role of GHS-R in obesity and insulin resistance, it is possible that GHS-R exerts its effect differently through crosstalk with other systems, and these interactions maybe cell-type specific. GHS-R is a G protein-coupled receptor (GPCR) that can regulate feeding behavior through forming heterodimers with a variety of GPCRs in

its close proximity, such as melanocortin 3 (MC_3) receptor, dopamine receptors (D_1 and D_2), or serotonin 2C receptor ($5\text{-}HT_{2C}$), resulting in changes of signaling cascades involved in feeding behavior (intake of palatable food and motivation) [36-38]. In the current study, we showed a novel role for GHS-R on angiogenesis and fibrosis of WAT. It remains to be determined whether GHS-R dimerizes with other GPCRs to affect angiogenesis and fibrosis in adipocytes. Since GHS-R is an GPCR known to have high constitutive activity, it may also be important to determine whether the angiogenic and fibrotic effects of GHS-R is ghrelin-dependent or ghrelin-independent, perhaps by studying a diet-restriction model when endogenous ghrelin levels are elevated.

Energy homeostasis is determined by the balance between energy intake and energy expenditure. Energy expenditure is composed of RMR, physical activity and non-shivering thermogenesis [34]. Here we show that adipose tissue-specific GHS-R deletion increased energy expenditure by elevating physical activity and heat production without affecting energy intake. Remarkably, HFD-fed *Adipoq*-Cre*;Ghsr^{f/f}* mice exhibited lower body weight and adiposity compared to *Ghsr^{f/f}* mice, due to increased energy expenditure resulting from increased physical activity and heat production. Our previous studies showed increased thermogenic capacity in global, pan-neuronal-specific and AgRP-specific GHS-R knockout mice [4, 18, 21, 22]. Specifically, we showed GHS-R ablation shifts macrophage phenotype toward M2-like macrophages releasing anti-inflammatory cytokines and norepinephrine, thereby promoting lipid mobilization in epiWAT and thermogenesis in BAT in aging [24]. In addition, AgRP-specific deletion of GHS-R activates thermogenesis in both brown and subcutaneous fat by up-regulating the sympathetic outflow. Our previous aP2-Cre-mediated GHS-R knockdown mouse model also exhibits a thermogenic phenotype, as evident by improved thermogenesis in BAT and increased lipolysis and glucose uptake in epiWAT [23]. However, the thermogenic effect observed in of aP2-Cre-mediated GHS-R knockdown mice is not exclusively due to adipose tissue, because GHS-R is ectopically deleted in non-adipose tissues such as hypothalamus, peritoneal macrophages, and bone marrow. In contrast to global or neuronal GHS-R deletion, HFD-fed *Adipoq*-Cre;*Ghsr^{f/f}* mice did not exhibit significant thermogenic effects in either BAT nor iWAT, indicating that the thermogenic effect of GHS-R is primarily mediated by its central effect rather than its direct effect in adipose tissue. Therefore, the effect of GHS-R signaling on adipose tissue is unique and distinct from other non-adipose tissues. Taken together, these data suggest that GHS-R signaling likely regulates thermogenesis through by both centrally-mediated and adipose tissue mediated mechanisms. The current study specifically suggests that GHS-R autonomously remodels adipose tissues to regulate the angiogenic and fibrotic programs of WAT.

The potential functional mechanisms underlying the healthier phenotype of *Adipoq*-Cre;*Ghsr^{f/f}* mice were also investigated in epiWAT. Growing evidence suggests the presence of strong multi-directional correlations between aberrant adipose tissue remodeling, dysregulation of adipokine secretion, and metabolic stress in obese adipose tissue [39]. During the obesity progression, when angiogenesis can no longer keep up with tissue expansion, pathological adaptive processes such as hypoxia takes place and results in dysregulation of adipokines, fatty acid fluxes, cellular senescence, and necrotic adipocyte death; these pathological changes lead to inflammation and fibrosis that further exacerbate

insulin resistance [10, 12, 30]. Both inflammation and inadequate angiogenic remodeling can drive fibrosis, which in turn promote macrophage accumulation in adipose tissue impeding further angiogenesis [30, 32, 33]. Adipose tissue remodeling is a physiological process in response to alterations in nutritional status. In DIO, adipose tissue remodeling is accelerated, exhibiting adipose tissue expansion, hypoxia and metabolic dysfunction [10]. Angiogenesis is a crucial mechanism by which adipose tissue forms new blood vessels to meet the demand of adipose tissue expansion [39]. In our study, we found that the expression of HIF2α was elevated in epiWAT of HFD-fed *Adipoq*-Cre;*Ghsr$^{f/f}$* mice. HIF2α not only regulates a large set of transcription factors and co-regulators to modulate tissue hypoxic response; it also regulates genes involving in extracellular matrix remodeling and angiogenic signaling, including VEGF-A signaling [40]. VEGF-A functions through its two receptors, VEGF-R1 and VEGF-R2, which play major roles in angiogenesis [41]. Recent studies have shown that the administration of VEGF and HGF improves metabolism in humans and animals [42-44]. The metabolically regulatory role of VEGF-A has been documented [45]. Under HFD feeding, adipose tissue-specific VEGF knockout mice develop adipose hypoxia, inflammation, glucose intolerance and insulin resistance [45]. Conversely, overexpression of VEGF in mouse adipose tissue ameliorates the diet-induced metabolic dysregulation, acting as a protective mechanism [45]. Co-administration of HGF and VEGF-A have been shown to promote angiogenic responses and cell survival, and has been proposed as an effective therapeutic strategy to enhance angiogenesis [46]. Indeed, our study shows that GHS-R deletion promotes angiogenesis in epiWAT of HFD-fed *Adipoq*-Cre;*Ghsr$^{f/f}$* mice, consistent with the improved glucose homeostasis and insulin sensitivity. Thus, suppression of GHS-R signaling in WAT promotes a healthy angiogenic cascade in adipose tissue, leading to a protective effect against DIO.

Fibrosis has also been shown to play a pathogenic role in adipose dysfunction, and can negatively affect insulin sensitivity and inflammation [33]. Hypoxia-induced tissue fibrosis is associated with the increased expression of Col1α and Col3α [47]. In this study, adipose tissue-specific deletion of GHS-R had no effect on TGF-β signaling (known as a central mediator for fibrogenesis), while Col1α and Col2α gene expression were significantly reduced, suggesting that GHS-R regulates selective adipose fibrosis signaling. PPARγ is a nuclear hormone receptor that is highly expressed in adipose tissue and considered a key regulator in adipose tissue homeostasis. PPARγ activation induces FGF21 production in adipose tissue [48]. In addition, thiazolidinediones, a synthetic PPARγ ligand, enhance the mRNA expression and secretion of adiponectin in cultured 3T3-L1 adipocytes [49, 50], indicating that PPARγ regulates FGF21 and adiponectin expression. Consistently, we observed increased gene expression of PPARγ, FGF21 and adiponectin in epiWAT of HFD-fed *Adipoq*-Cre;*Ghsr$^{f/f}$* mice, which suggests that GHS-R may modulate the adipose signaling cascade of PPARγ – FGF21/Adiponectin. FGF21 and adiponectin are key health-promoting adipokines that act on multiple tissues to coordinate glucose and lipid metabolism, enhancing insulin sensitivity and glucose homeostasis [51, 52]. Elevated FGF21 and adiponectin in GHS-R deficient adipose tissue may directly regulate adiposity, possibly controlling angiogenesis, inflammation and/or fibrosis, ultimately leading to a metabolically favorable phenotype of reduced adiposity and improved insulin sensitivity (Fig. 6).

In conclusion, we generated a novel mouse model with adipose tissue-specific GHS-R deletion. Our findings demonstrate that GHS-R has a cell-autonomous effect in adipocytes and adipose tissue-specific GHS-R deletion protects against DIO and improves insulin sensitivity. Our study further indicates that GHS-R signaling affects angiogenesis, inflammation and fibrosis in epiWAT under DIO, possibly by regulating adipose transcription factors and the expression of adipokines. Modulation of angiogenesis and treatment with angiostatic substances have been suggested to be a viable therapeutic strategy to improve adipose health. Adipose GHS-R may serve as a novel regulator of adipose angiogenesis, inflammation, and fibrosis, providing a valuable target for combating adipose dysfunction in obesity and insulin resistance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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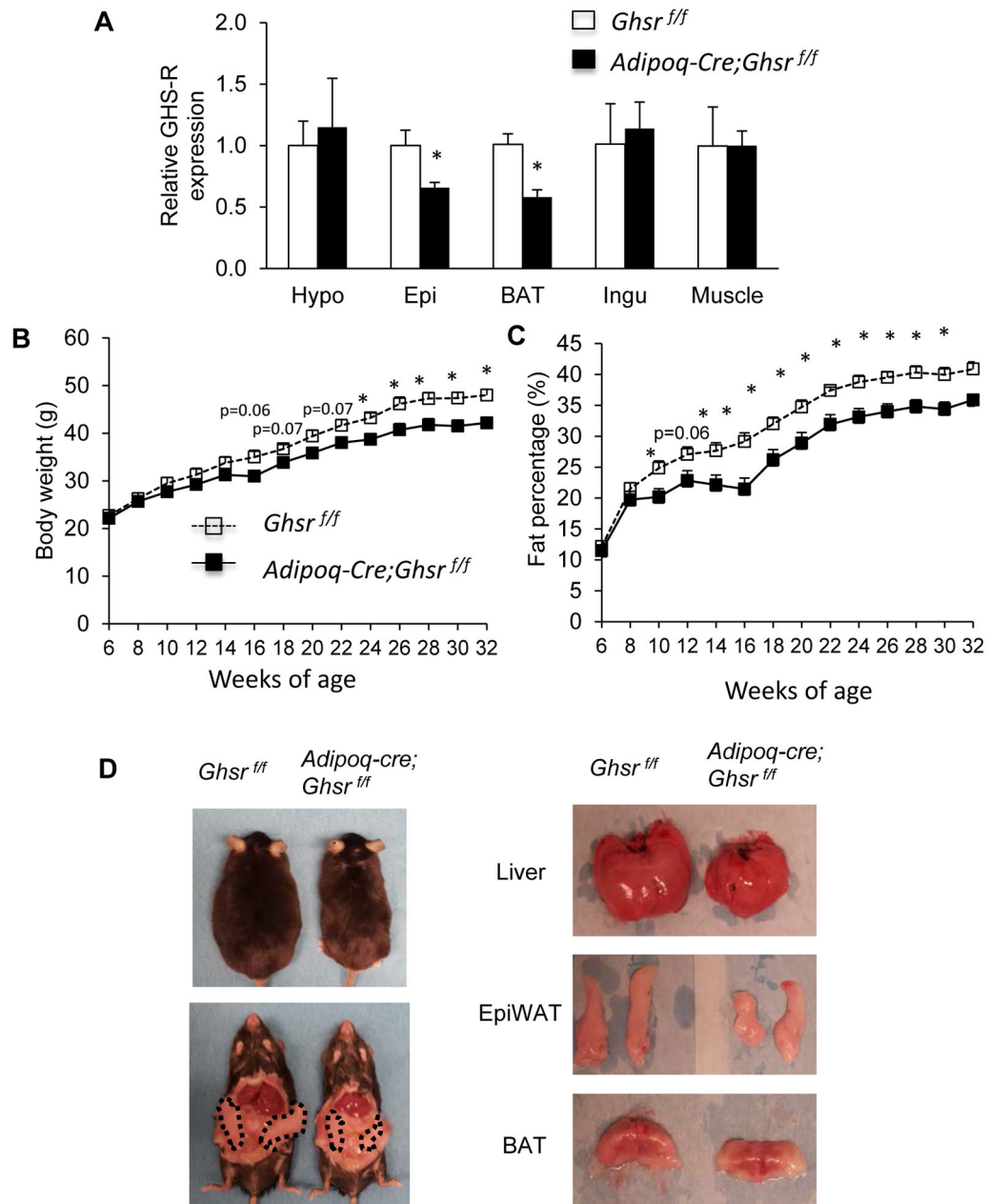52. Li H, Zhang J, Jia W: Fibroblast growth factor 21: a novel metabolic regulator from pharmacology to physiology. Frontiers of medicine 2013, 7:25–30. [PubMed: 23358894]

Figure. 1. Validation of *Adipoq*-Cre;*Ghsr^{f/f}* mouse model and adipose tissue-specific GHS-R deletion reduced body weight and fat mass under HFD-feeding.
(A) Relative GHS-R gene expression in hypothalamus (Hypo), epididymal white adipose tissue (Epi), brown adipose tissue (BAT), inguinal white adipose tissue (Ingu), and muscle. n=5-8. *, $p<0.05$, *Ghsr^{f/f}* vs. *Adipoq*-Cre;*Ghsr^{f/f}*. (B) Body weight and (C) Fat percentage at 6-32 weeks of age. (D) The representative images of mouse, liver, epiWAT, and BAT. n=6-8. *, $p<0.05$, *Ghsr^{f/f}* vs. *Adipoq*-Cre;*Ghsr^{f/f}*.
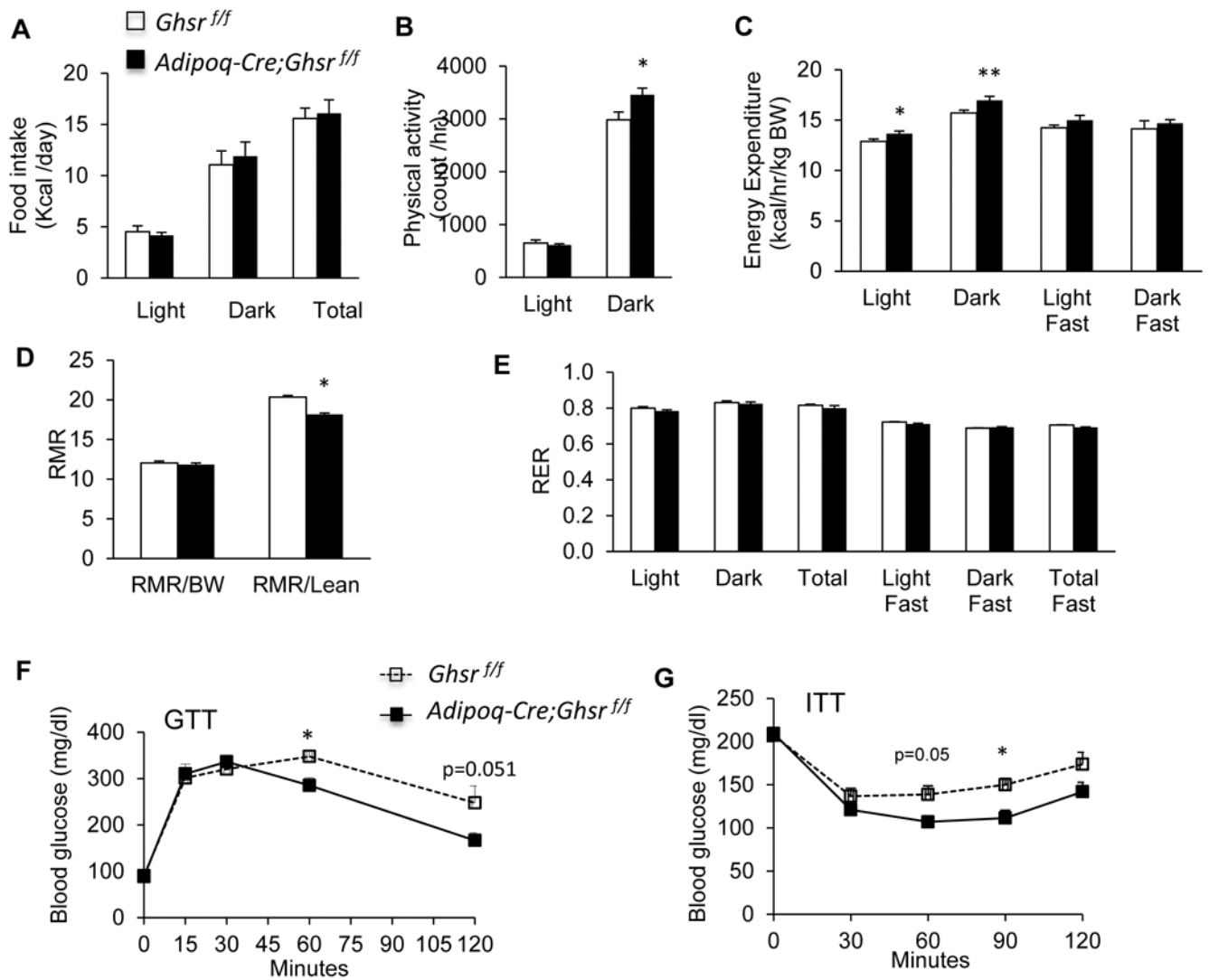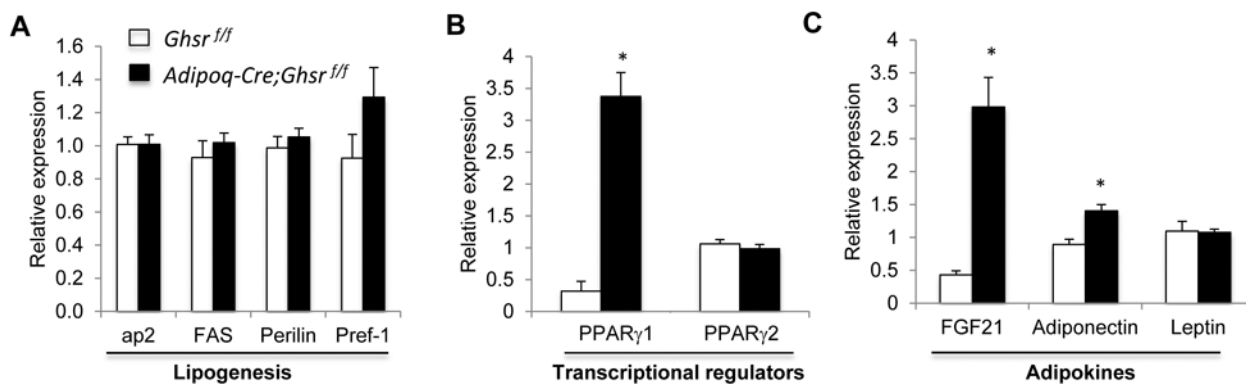
Figure. 2. HFD-fed adipose tissue-specific GHS-R deletion improved insulin sensitivity.
Metabolic profiles were performed using indirect calorimetry at 15-16 weeks of age; data
under fed conditions present an average of last 3 days, and mice were fasted for 24 h before
termination. (A) Food intake, (B) Physical activity, (C) Energy Expenditure (Heat
production), (D) Resting metabolic rate (RMR), and (E) Respiratory exchange ratio (RER).
n=5. *, $p<0.05$, $Ghsr^{f/f}$ vs. $Adipoq$-Cre;$Ghsr^{f/f}$. (F) Blood glucose levels during GTT at 17
weeks of age. (G) Blood glucose levels during ITT at 22 weeks of age. n=6-8. *, $p<0.05$,
$Ghsr^{f/f}$ vs. $Adipoq$-Cre;$Ghsr^{f/f}$.
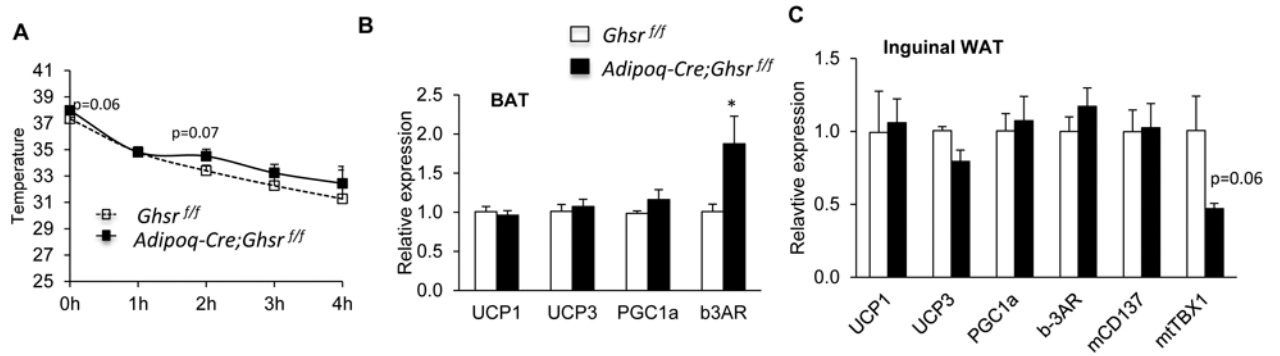
Figure. 3. Adipose tissue-specific GHS-R deletion had no effect on thermogenesis.
HFD-fed mice at 32 weeks of age were individually caged at 4°C and provided with free access to food and water. (A) Rectal temperature was recorded hourly for 4 h. (B) Expression of thermogenic genes in BAT. (C) Expression of thermogenic genes in iWAT. n=5-6. *, $p<0.05$, $Ghsr^{f/f}$ vs. Adipoq-Cre;$Ghsr^{f/f}$.

Figure. 4. Adipose tissue-specific GHS-R deletion did not affect lipogenesis but increased transcription factor PPARγ1, and adipokines of FGF21 and adiponectin in epiWAT.
(A) Expression of lipogenic genes. (B) Expression of master adipose transcription factors PPARγ1 and PPARγ2. (C) Expression of adipokines of FGF21, adiponectin and leptin. n=5. *, $p<0.05$, *Ghsr*[f/f] *vs. Adipoq*-Cre;*Ghsr*[f/f].
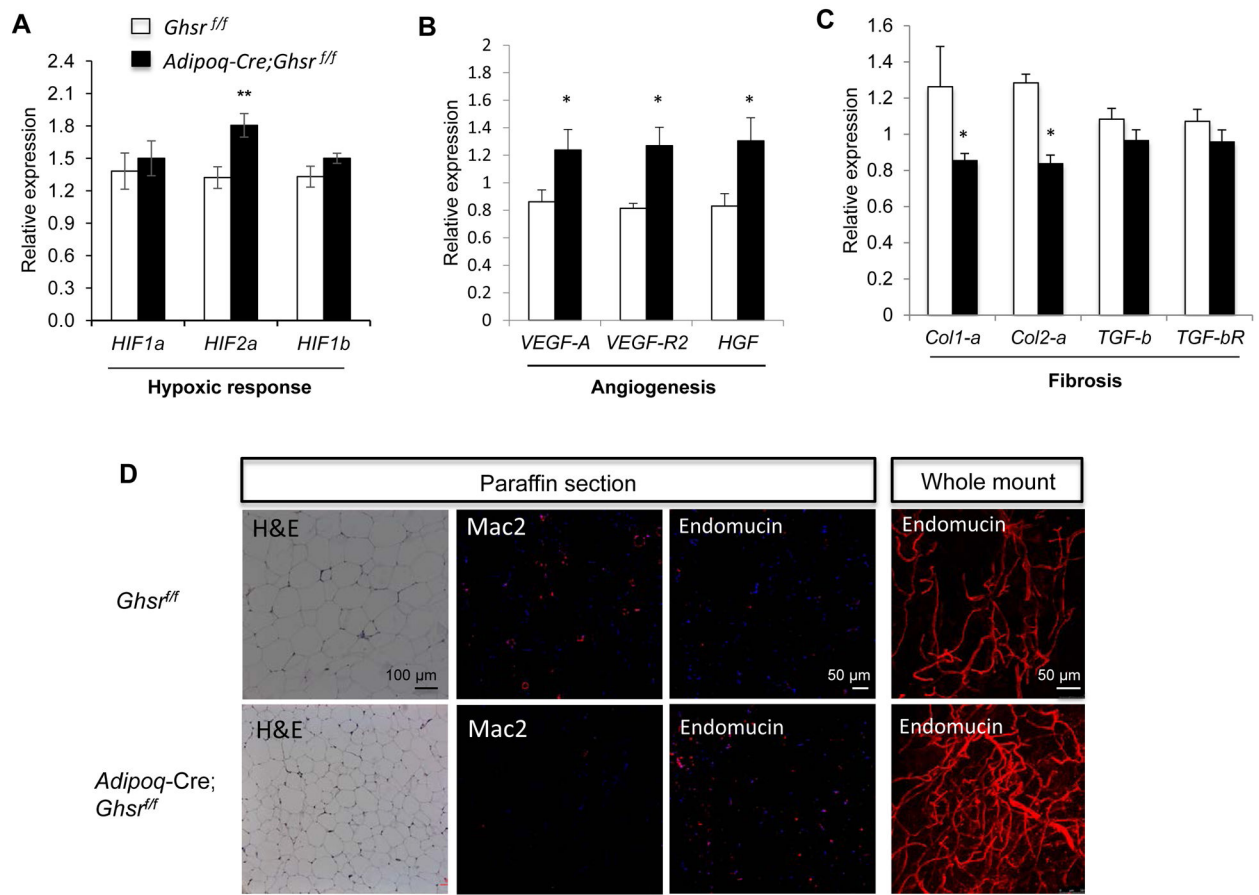
Figure. 5. Adipose tissue-specific GHS-R deletion modulated angiogenesis and fibrosis in epiWAT under HFD feeding.
(A) Expression of hypoxia-inducible transcription factors. (B) Expression of angiogenic genes. (C) Expression of fibrotic genes. (D) H&E staining, macrophages marker Mac2 (red), and vasculature marker endomucin (red) staining in paraffin sections; endomucin staining in tissue whole mount, showing decreased macrophage infiltration and increased microvasculature in epiWAT of HFD-fed *Adipoq*-Cre;*Ghsr^f/f* mice. n=5. *, *p*<0.05, *Ghsr^f/f* vs. *Adipoq*-Cre;*Ghsr^f/f*.
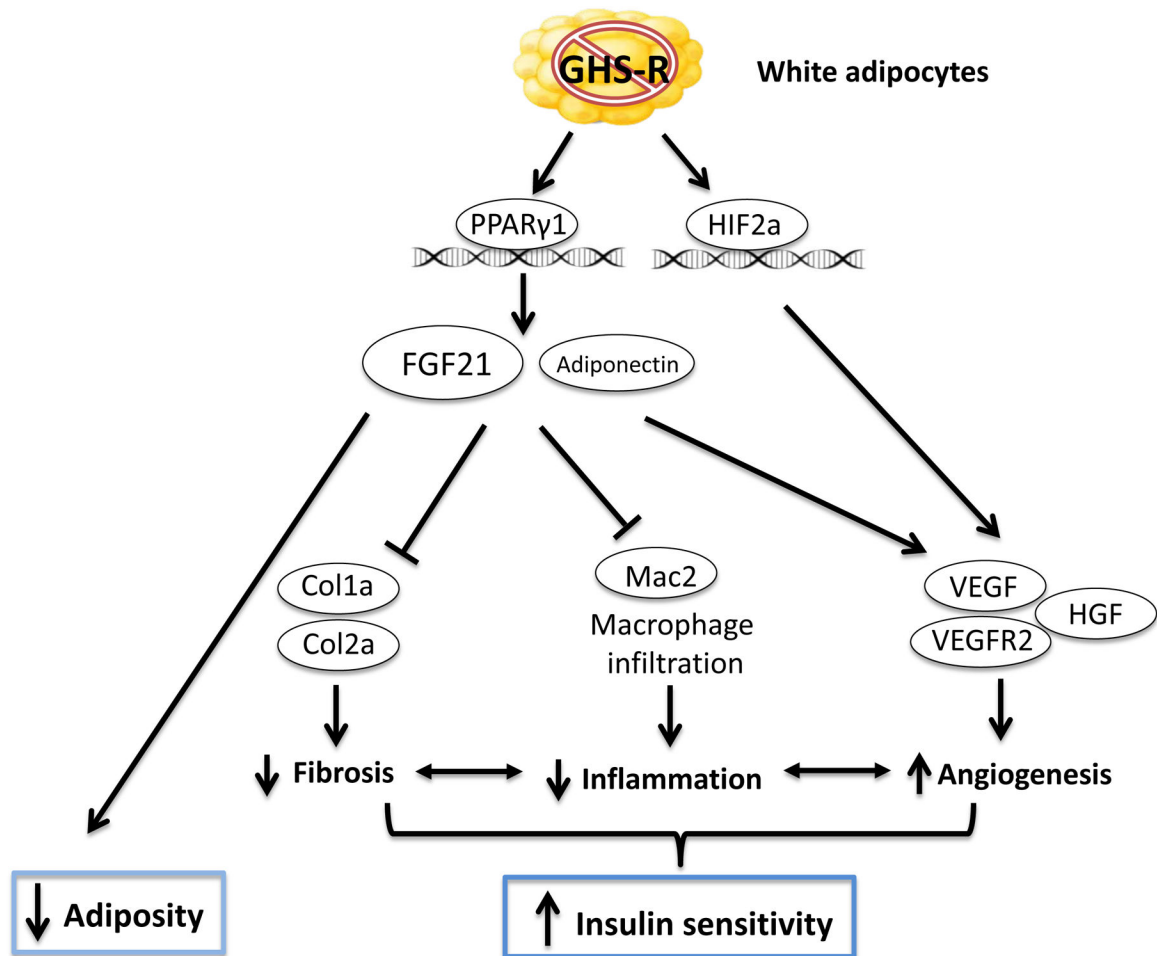
Figure. 6. Schematic diagram of the proposed actions of GHS-R in adipose tissue under obesity.
Under obesity conditions, GHS-R inhibition in adipocytes mitigates adiposity and improves insulin sensitivity by the following potential mechanisms: 1) Activating transcriptional regulator PPARγ1 to enhance healthy adipokines of FGF21 and adiponectin, which decreases macrophage infiltration to reduce inflammation and Col1α/Col2α expression to suppress fibrosis; 2) Stimulating hypoxia transcription regulator HIF2α to increase the expression of VEGF, VEGF-R2, and HGF to promote angiogenesis. Together, these functional signaling cascades lead to reduced adiposity and improved insulin sensitivity.