



FULL LENGTH ARTICLE

Chemerin deficiency regulates adipogenesis is depot different through TIMP1

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KEYWORDS

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Abstract Adipocytes and immune cells are vital for the development of adipose tissue. Adipokines secreted by adipocytes regulate adipogenesis and body metabolism. Chemerin is one of the adipokines. However, the function and mechanism of chemerin in adipose tissue are not fully illuminated. Compared with wild type (WT) mice, *Rarres2*^{-/-} mice gained weight and significantly increased fat distribution in subcutaneous adipose tissue (SAT), rather than visceral adipose tissue (VAT) on high fat diet (HFD). PPAR γ and C/EBP α , the master regulators of adipogenesis, were up-regulated in SAT and down-regulated in VAT in *Rarres2*^{-/-} mice comparing with WT mice. In spite of chemerin deficiency or not, the ratio of adipocyte-progenitors to total cells and the differentiation capacity of adipocyte-progenitors were similar in SAT and VAT, but macrophage infiltration in VAT was more severe than in SAT in *Rarres2*^{-/-} mice. Furthermore, CD45⁺ immune cells supernatant from *Rarres2*^{-/-} SAT promoted the differentiation of adipocyte-progenitors and 3T3-L1 cells. Adipokine array assay of CD45⁺ immune cells supernatant revealed that metalloproteinase inhibitor 1 (TIMP1), an inhibitor of adipogenesis, was reduced in *Rarres2*^{-/-} SAT, but increased in *Rarres2*^{-/-} VAT. As we specifically knocked down chemerin in SAT, TIMP1 was down-regulated and adipogenesis was promoted with reducing infiltration of macrophages. The present study demonstrates that the effects of chemerin on adipose tissue is depot different, and specific knock down chemerin in SAT promote adipogenesis and improve glucose tolerance test (GTT) and insulin tolerance

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test (ITT). This suggests a potential therapeutic target for chemerin in the treatment of obesity related metabolic disorder.

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Introduction

Obesity is accompanied by expansion of adipose tissue and is highly involved in the development of metabolic diseases, such as cardiovascular disease and type 2 diabetes mellitus,¹ which threaten human health.^{2–5} Adipose tissue is not only the largest reservoir depot of triglycerides, but also an endocrine organ, which regulates energy and metabolic homeostasis by secreting various adipokines.^{6,7} The adipokines could affect the function of adipose tissue and other organs by autocrine and paracrine.⁷

Generally, white adipose tissue is divided into two broad categories according to adipose tissue distribution: subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT).⁸ Clinical and basic research evidences support many difference between SAT and VAT, such as the release of adipokines and the infiltration of immune cells.⁹ In obesity, expansion of VAT is tightly associated with metabolic diseases, such as insulin resistance.^{10,11} However, expansion of SAT potentially counteracts the strong trends toward the development of insulin resistance.^{12,13}

Chemerin, the gene name is retinoic acid response factor 2 (*Rarres2*) or Tazarotene-induced gene 2 (*TIG2*),¹⁴ was identified as a novel adipokine in 2007,^{15,16} has different effects on different tissues. It has been established that chemerin promotes 3T3-L1 cell differentiation, while down-regulated chemerin impairs 3T3-L1 adipogenesis and adipocyte-progenitors biological functions.^{16,17} Besides, chemerin altered the fate of myoblast cells from myogenesis to adipogenesis in C2C12 myoblasts.¹⁸ Chemerin appears as a potent chemoattractant protein of immune cells in obesity,^{19,20} especially for macrophages. Chemerin in circulation is positively with BMI,²¹ exacerbates insulin resistance in human skeletal muscle cells and glucose intolerance in diabetic (db/db) mice model.^{22–24} However, the exacerbation of glucose intolerance is a frequent finding in *Rarres2*^{−/−} mice fed with HFD.²⁵ These controversial data suggest chemerin is not just a byproduct during obesity and is essential for the development of adipose tissue. Meanwhile, it has been reported that SNPs of the chemerin are associated with increased visceral fat mass in lean subjects, which indicated an influence of chemerin on regional fat distribution.²⁶ However, the exact function and mechanism of chemerin in adipose tissue and adipogenesis have not been fully illustrated *in vivo*.

Here, we found that *Rarres2*^{−/−} mice had more deposition of fat in SAT rather than VAT, and chemerin deficiency promotes adipogenesis in SAT by reducing TIMP1, but contrary results were observed in VAT. Furthermore, specific down-regulation of chemerin in SAT promotes adipogenesis and ameliorates glucose intolerance and insulin resistance. These data indicate the essential role of chemerin in

modulating the development of adipose tissue is depot different.

Materials and methods

Mouse studies

Rarres2^{−/−} mice were kindly provided by Dr. Rui He from the Department of immunology, Fudan University. Targeting strategy for the disruption of the all coding regions of *Rarres2* (exon1, 2, 3) with a neomycin-resistant cassette.²⁷ Six to eight-week male mice were maintained under 12 h light and 12 h dark cycles with unlimited access to normal diet (ND) or HFD (60% kcal fat from Research Diet) and water. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. All applicable institutional and/or national guidelines for the care and use of animals were followed. All studies were approved by the Animal Care and Use Committee of the Chongqing Medical University and followed the National Institute of Health guidelines on the care and use of animals.

Glucose tolerance tests and insulin tolerance tests

For the glucose tolerance test (GTT), mice were intraperitoneally (i.p.) injected with 2 mg/g glucose after an overnight fast, whereas insulin tolerance test (ITT), 0.75 mU/g insulin was i.p. injected after 4 h fast. The blood glucose concentration in tail blood were detected using a glucometer at baseline and preassigned times after injection.

H&E staining and cell size quantitation

Standard H&E staining were performed on 5-μm paraffin sections of SAT and VAT. Cell diameter was measured in the H&E-stained sections of 6–8 individual samples in each group using Image J.

Isolation of SVF

Stromal vascular fraction (SVF) from the adipose tissue were isolated as described before.²⁸ Briefly, adipose tissue were harvested and cut into small pieces and treated with enzymatic digestion (collagenase VIII; Sigma), filtered in 100 μm mesh filter and collected the SVF after centrifugation, resuspended with an ammonium chloride lysis buffer to remove red blood cells.

Flow cytometry analyses

The stromal vascular fraction cells were stained with mAbs specific for CD45, CD31 and Sca1 (eBioscience) and analyzed on a BD Accuri C6 flow cytometer and the data analysis was performed by using FlowJo.

Cell culture

For adipocyte-progenitors differentiation, adipose tissue was dissected and digested as described above,²⁹ plated in 6-well CellBind plates, adherent cells were grown to confluence. And differentiation as previously described. For CD45⁺ cells conditional medium, SVF from the adipose tissue were digested, filtered, washed as described above, isolated as per the manufacturer's MACS protocol (Miltenyi) using positive selection for CD45. Briefly, cell suspensions were incubated with Fc block followed by CD45-conjugated magnetic beads, washed and passed over a lineage selection column. Retained cells were eluted, counted, and cultured in medium (DMEM supplemented with 10% heat-inactivated FBS, penicillin, streptomycin) for 24 h. The medium was centrifuged and collected the supernatant. Medium from 60 mm plates with no cells was used as control medium. Supernatant was filtered and used at a 1:1 dilution with fresh medium for 48 h stimulation during the first two days of adipocyte-progenitor differentiation.

Oil red O staining

In vitro differentiated cells were fixed for 20 min in 4% paraformaldehyde and stained with oil red O for 120 min. The stained fat droplets in the cells were visualized by light microscopy and photographed. To quantify the lipid content, the stained cells were treated with 100% isopropyl alcohol to extract the oil red O and measured at 520 nm by spectrophotometer.³⁰

ELISA

TIMP1 concentrations in supernatants of CD45⁺ immune cells were analyzed by Mouse TIMP1 DuoSet Elisa kits from R&D system according to the manufacturer's protocol.

RNA isolation and quantitative real time-PCR (qRT-PCR)

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA). Reverse transcribed involved cDNA synthesis kit (Thermo Fisher). Quantitative PCR (qPCR) involved Power SYBR green PCR master mix (Applied Biosystems, Carlsbad, CA) and a Q3 instrument (Applied Biosystems), with 18s mRNA as an endogenous control. Primer sequences for qPCR were from PrimerBank (<http://pga.mgh.harvard.edu/primerbank/>).

Western blotting and adipokine array assay

Cells and tissues were homogenized into lysis buffer containing 2% sodium dodecyl sulfate (SDS) and 60 mM Tris·HCl

(pH6.8). Lysates were quantitated and equal amounts of protein were loaded to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Antibodies used are C/EBP α and HSP90 (Santa Cruz Biotechnology), PPAR γ and Pref-1 (Cell Signaling Technology).

For the adipokine array, cells supernatants were analyzed by Mouse Adipokine Array kit (R&D), and quantified by ImageJ.

Energy expenditure

Energy expenditure was performed using CLAMS chamber (Columbus Instruments). Briefly, mice were housed in a chamber with free access to food and water for 72 h. Parameters of oxygen consumption (mL/kg·h), carbon dioxide production (mL/kg·h) and RER (VCO₂/VO₂) were calculated for each mouse divided by its body weight.

Generation and administration of recombinant adenovirus

The adenoviral expression vector pAd/BLOCK-iT encoding short hairpin RNA (shRNA) of *Rarres2* was constructed, with shLacZ as control. The sequences are as follows: sh*Rarres2*: CCGGCATCTATGATGATGA; shLacZ: AATTTAACGCCAGT-CAGGCT. Amplified adenovirus was purified using adenovirus purification kits (Sartorius, Göttingen, Germany) and injected twice a week in SAT in 8-week-old mice for 8 weeks challenged with HFD.

Statistical analysis

Data are presented as mean $\pm$ SEM. Statistical significance was measured by Student's unpaired *t* tests (2-tailed). A *P* value less than 0.05 was considered significant, for all data, **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

Results

Chemerin deficiency mice gained weight, and increased fat distribution in SAT rather than VAT

In order to clarify the roles of chemerin in adipogenesis and metabolism *in vivo*, we employed *Rarres2*^{-/-} mice to explore the function of chemerin. *Rarres2*^{-/-} mice displayed increased body weight compared with WT on HFD, but not on ND (Fig. 1A). Notably, its apparent that *Rarres2*^{-/-} mice displayed increased fat distribution of SAT rather than VAT (Fig. 1B and C). Specifically, the cell size of adipocytes elevated in both SAT and VAT (Fig. 1D).

Next, we assessed glucose homeostasis and oxygen consumption rates in mice. Basal oxygen consumption rates reduced while respiratory exchange ratio (RER) increased in *Rarres2*^{-/-} mice (Fig. 1E and F). It indicated the increasing utilization of carbohydrate compared with WT mice. Meanwhile, *Rarres2*^{-/-} mice showed deteriorated glucose tolerance and insulin resistance (Fig. 1G).

As our data show, increased SAT fat accumulation and decreased VAT fat accumulation in *Rarres2*^{-/-} mice. We

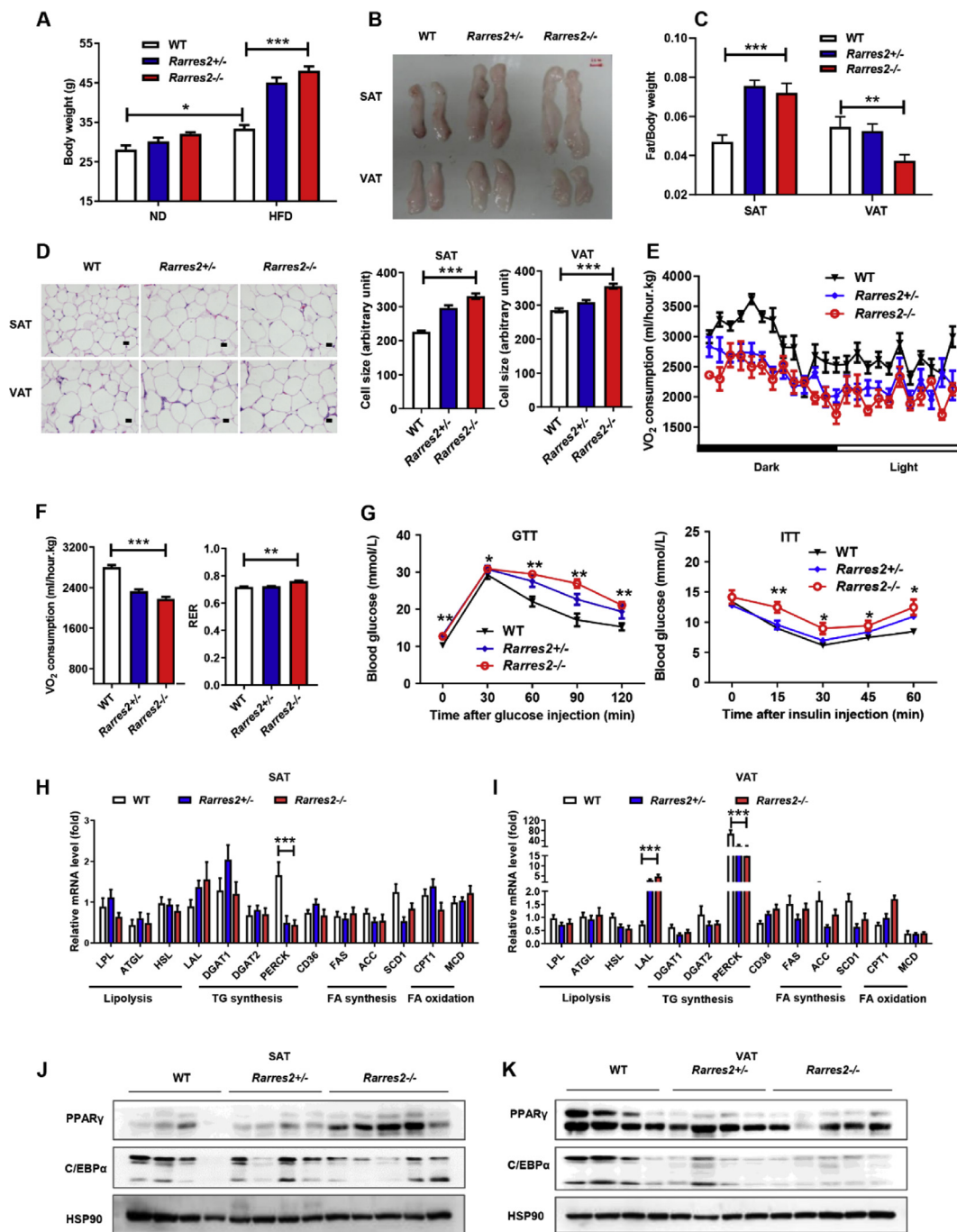


Figure 1 Chemerin deficiency regulates adipogenesis differently in SAT and VAT. (A) Body weight of WT, *Rarres2* heterozygous (*Rarres2*^{+/-}) and *Rarres2* knockout (*Rarres2*^{-/-}) mice, fed with ND or HFD. (B) Comparison of SAT and VAT. (C) Fat index (ratio of fat weight to whole body weight) of indicated mice. (D) H&E and quantification of cell size. (E-F) Whole-body oxygen consumption rate (E) and the average values of RER (F). (G) GTT and ITT analysis. (H-I) mRNA expression levels of lipolysis and lipogenic genes. (J-K) Western blot analysis of PPAR γ and C/EBP α in SAT (J) and VAT (K). All the mice were male and fed with HFD for 12 weeks if not indicated otherwise, $n = 8-12$ /group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

proposed that the effect of chemerin deficiency on different adipose depot is different, then we screened for the expression of lipogenic genes by RT-qPCR. Surprisingly, we found that the genes of triglyceride (TG) and fat acid (FA) synthesis were down-regulated while genes of lipolysis were no significant different in SAT of *Rarres2*^{-/-} mice compared with WT mice (Fig. 1H), data in VAT also

show same trend (Fig. 1I). However, the protein levels of PPAR γ and C/EBP α (key transcript factor of adipogenesis³¹) were significantly increased in SAT (Fig. 1J) while decreased in VAT (Fig. 1K) in *Rarres2*^{-/-} mice compared with WT mice. These data indicate that chemerin deficiency regulate adipogenesis differently in different adipose tissues.

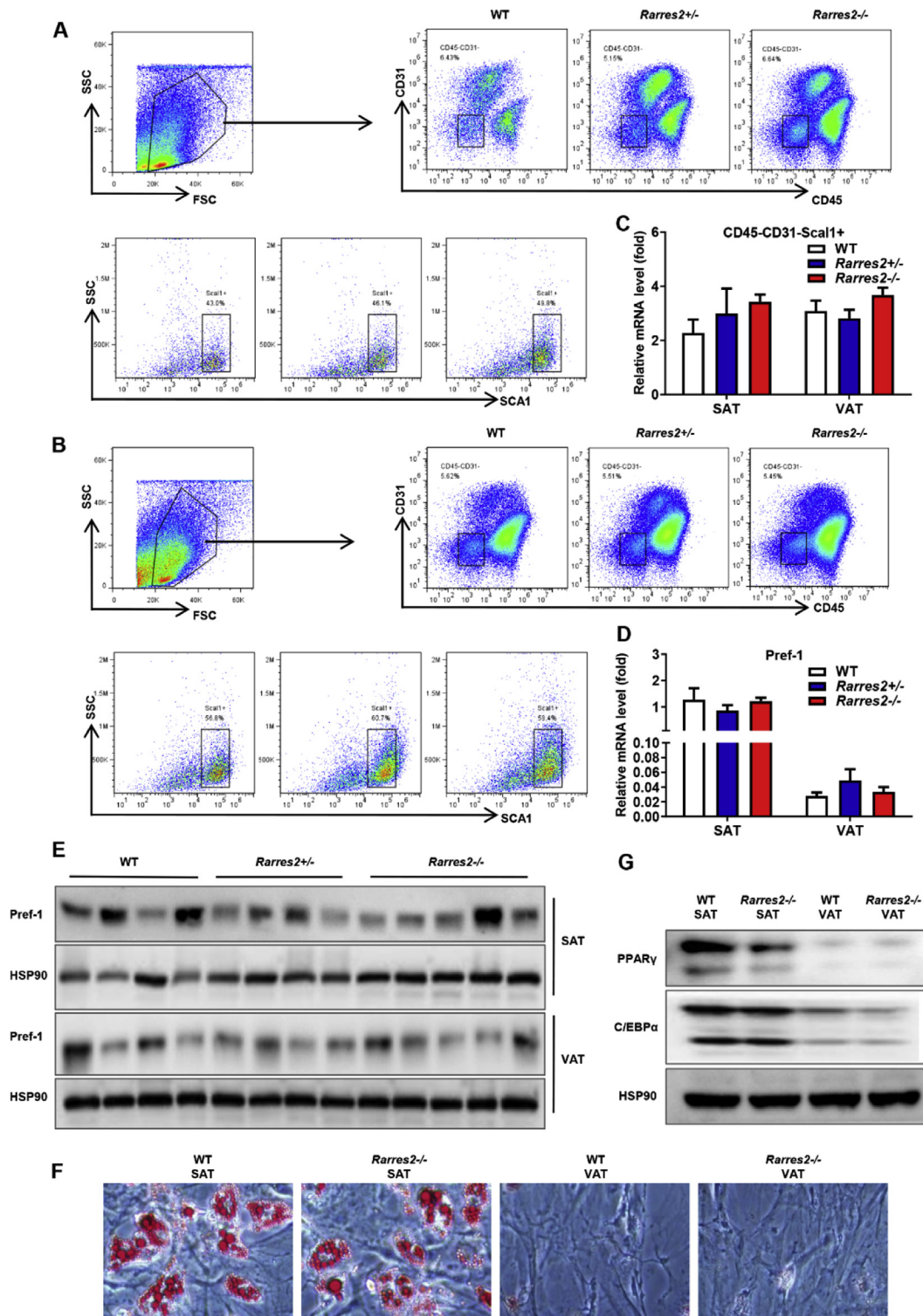


Figure 2 Chemerin had no effect on adipocyte-progenitors of SAT and VAT. (A-B) Representative flow cytometric analysis of adipocyte-progenitors in SAT (A) and VAT (B). (C) Percentage of adipocyte-progenitors measured by flow cytometry. (D-E) mRNA expression levels and Western blot analysis of Pref-1 in SAT and VAT. Oil red O staining (F), Western blot analysis of PPAR γ and C/EBP α (G). All the mice were male and fed with HFD for 12 weeks if not indicated otherwise, $n = 6-8$ /group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Chemerin deficiency has no effect on the proportion and differentiation capacity of adipocyte-progenitors

The process of adipogenesis is determined by several factors, such as the number, differentiation capability and microenvironment of adipocyte-progenitors. As CD31 is a marker of endothelial cells,^{32,33} CD45 is a marker of immune cells and SCA1 is stem cells marker,³⁴ previous reports took CD31⁺CD45⁺SCA1⁺ cells as adipocyte-progenitors.³⁵ We tested the proportion of adipocyte-progenitors by flow cytometry, found no significant difference in the proportion of adipocyte-progenitors to total cells in SAT and VAT in WT mice and *Rarres2*^{-/-} mice (Fig. 2A–C). Meanwhile, the mRNA and protein expression of Pref-1 (marker of adipocyte-progenitors) were also similar in SAT and VAT (Fig. 2D, E). To explore the differentiation capability of adipocyte-progenitors in different adipose depot, we isolated adipocyte-progenitors using MACS magnetic beads and

induced into adipocytes. As shown in Fig. 3F, G, there are no different on the fat accumulation and the expression of PPAR γ and C/EBP α in SAT and VAT. These data suggested that chemerin deficiency did not affect the adipogenesis of adipocyte-progenitors directly.

Supernatant of CD45⁺ immune cells from different depots has different effects on adipogenesis *in vitro*

In adipose tissue, there are many other cells excluding adipocytes. Among these cells, CD45⁺ immune cells contribute much to microenvironment. To confirm our hypothesis that chemerin deficiency may affect adipogenesis by regulating the microenvironment, we induced adipocyte-progenitors of WT with conditioned medium (supernatant of CD45⁺ immune cells isolated from WT-SAT, WT-VAT, *Rarres2*^{-/-}-SAT, *Rarres2*^{-/-}-VAT). In 3T3-L1 cells, lipid content and the

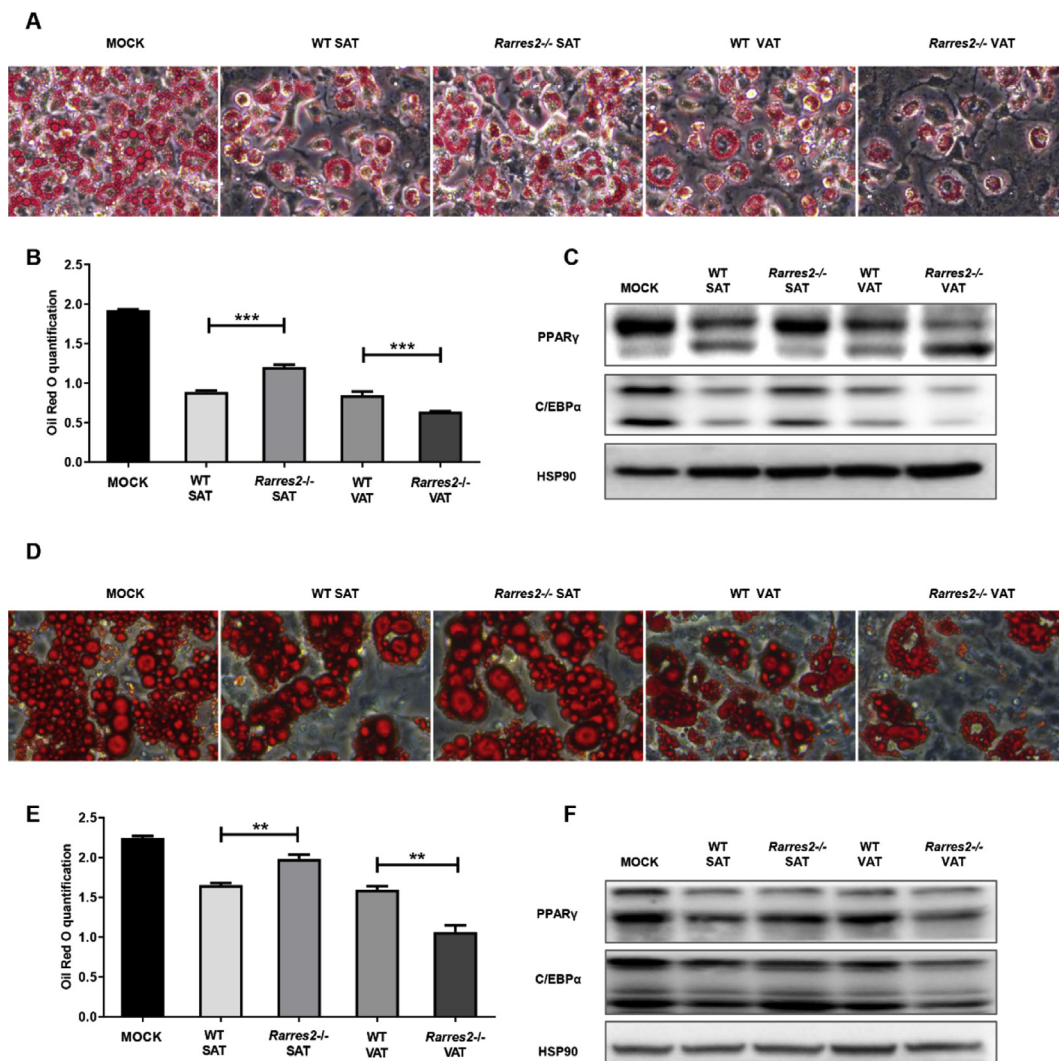


Figure 3 Supernatant of CD45⁺ cells from different depots have diverse effects on adipogenesis *in vitro*. 3T3-L1 (A–C) and adipocyte-progenitors sorted from 6-week-old mice (D–F) were induced by conditioned media (details in methods and materials). (A and C) Representative oil red O staining was photographed. (B and E) Oil red O staining in (A) and (C) were quantified. (C and F) Western blot analysis of PPAR γ and C/EBP α . All the supernatants of CD45⁺ cells in adipose tissue of WT or *Rarres2*^{-/-} male mice fed with HFD for 12 weeks. *n* = 6–8/group. ***P* < 0.01, ****P* < 0.001.

expression of key transcription factor (PPAR γ and C/EBP α) were increased with the conditioned medium from *Rarres2*^{-/-}-SAT and decreased from *Rarres2*^{-/-}-VAT (Fig. 3A–C). Consistent results observed in adipocyte-progenitors, which isolated from stromal vascular fraction of adipose tissue of WT mice (Fig. 3D–F). These data indicated that chemerin deficiency affect the microenvironment through CD45⁺ cells.

Chemerin deficiency affects the infiltration of macrophage and TIMP1 inhibits adipogenesis

What is the key regulator in the supernatant? We sorted and cultured CD45⁺ immune cells by magnetic beads in SAT and VAT of *Rarres2*^{-/-} and WT mice, and detect the supernatant with adipokine array kit. Data revealed that expression

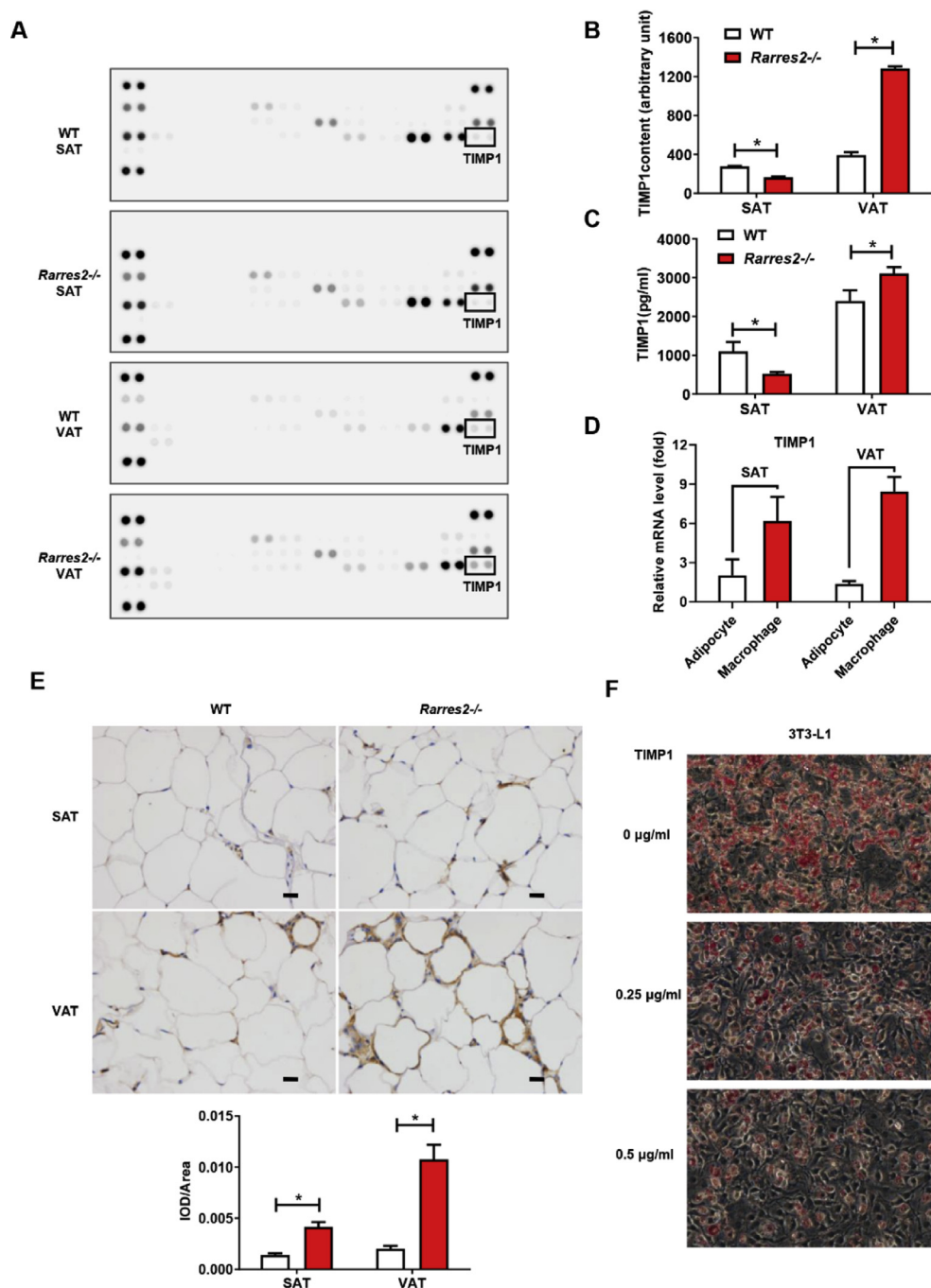


Figure 4 TIMP1 secreted by macrophage is involved in the regulation of adipogenesis. (A) Concentration of TIMP1 in supernatant of CD45⁺ cells in indicated depots of mice. (B) Concentration of TIMP1 were quantified. (C) Concentration of TIMP1 in supernatant of CD45⁺ cells in indicated depots of mice. (D) Oil red O stain of 3T3-L1 in different concentration of TIMP1. (E) mRNA expression of TIMP1 in adipocyte and macrophage. (F) Representative IHC staining of F4/80 in SAT and VAT from WT and *Rarres2*^{-/-} mice. All the mice were male and fed with HFD for 12 weeks if not indicated otherwise, $n = 6-8$ /group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

of metalloproteinase inhibitor 1 (TIMP1) was down-regulated in CD45⁺ immune cells conditioned medium from *Rarres2*^{-/-}-SAT and up-regulated in *Rarres2*^{-/-}-VAT (Fig. 4A and B). The expression of TIMP1 detected by ELISA was consistent with the data of adipokine array analysis (Fig. 4C).

As chemerin is a chemokine, recent literatures showed chemerin effect macrophage infiltration. Besides, macrophage account for the majority of CD45⁺ cells in adipose tissue. Furthermore, as Mariana et al reported that TIMP1 is mainly expressed in macrophages but not adipocytes in adipose tissue,³⁶ and our data confirm this (Fig. 4D).

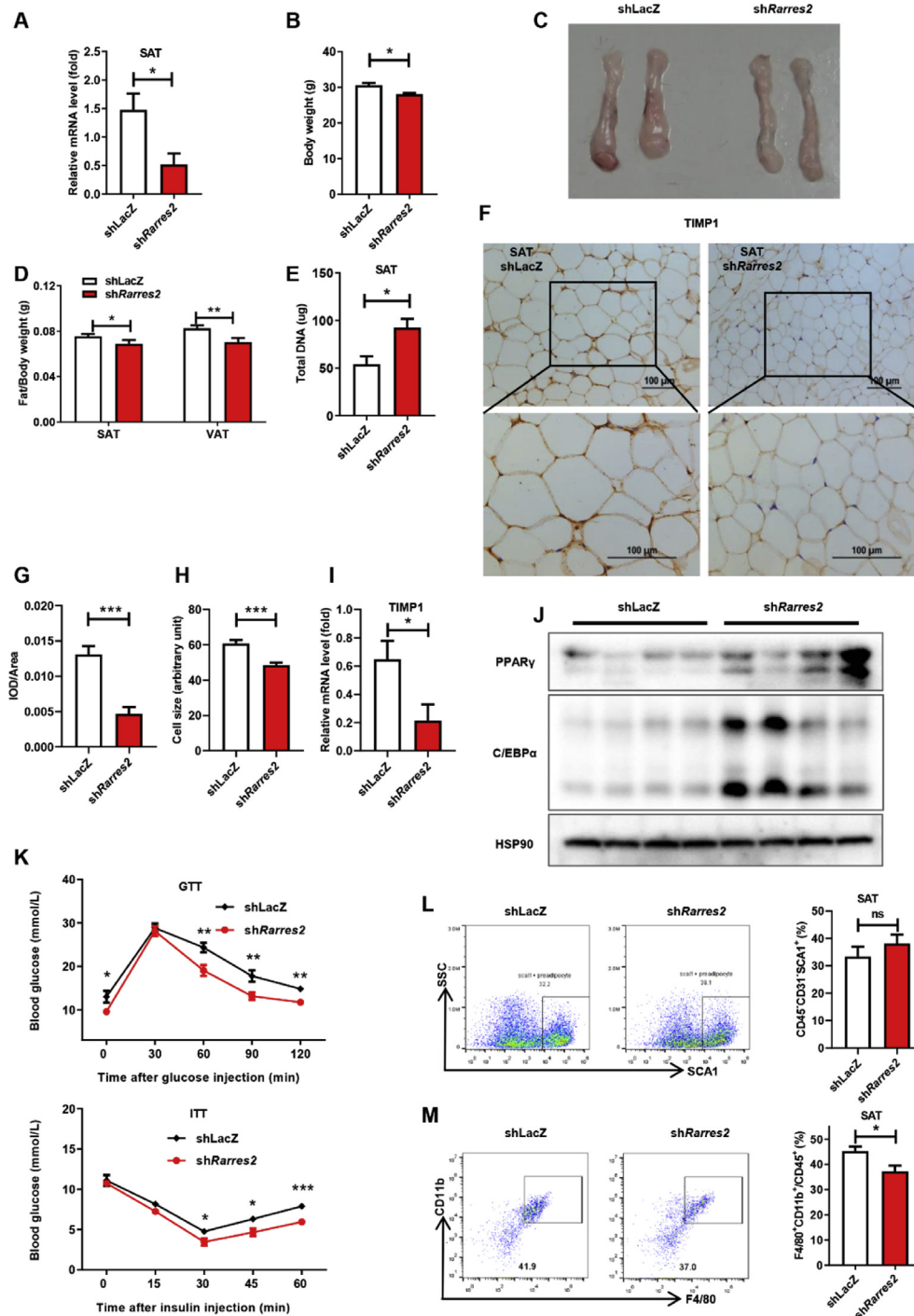


Figure 5 Knock down of chemerin specific in SAT promote adipogenesis and improve GTT and ITT. Male mice were fed with HFD and infected with shLacZ or shRarres2 twice weekly via subcutaneous injection adjacent to inguinal SAT for 8 weeks. (A) Gene expression levels of chemerin in SAT. (B) Body weight of mice. (C) Comparison of SAT. (D) Ratio of fat weight to body weight. (E) Total DNA of SAT. (F and H) Adipocyte cell size of SAT. (F and G) IHC of TIMP1 in SAT. (I) mRNA expression levels of TIMP1 in SAT. (J) Western blot analysis of PPARγ and C/EBPα. (K) GTT and ITT analysis. (L-M) Representative flow cytometric analysis of adipocyte-progenitors (L) and macrophage (M) in SAT of indicated mice. *n* = 6/group. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

Macrophage infiltration is more severe in VAT compared with SAT in *Rarres2*^{-/-} mice (Fig. 4E). We found that TIMP1 could inhibit the differentiation of 3T3-L1 (Fig. 4F). These data suggested that different levels of TIMP1 may have different effects on adipocyte-progenitors differentiation.

Specific knockdown of chemerin in SAT promotes adipogenesis and improves GTT and ITT

Given that expand SAT could improve glucose tolerance,¹³ but our data shows *Rarres2*^{-/-} mice with expand SAT exhibiting worse glucose tolerance. Reports showed that chemerin plays an important role in glucose homeostasis by regulating multiple organs including β -cell.²⁵ To exclude the effects of chemerin deficiency on other tissues and verify the roles of chemerin on adipogenesis in SAT, we knocked down chemerin with adenovirus specific in SAT (Fig. 5A). Body weight and the ratio of SAT or VAT to body weight were decreased (Fig. 5B–D). The cell number (based on DNA content) of SAT increased as well as the reduced fat cells volume (Fig. 5D, F–H). Thus, down-regulation of chemerin in SAT increased number of adipocytes *in situ*, associating with the down-regulated the expression of TIMP1 both in protein and gene level (Fig. 5F, I). Interestingly, the expression of key transcription factors also had slightly but significantly increase (Fig. 5J). Moreover, GTT and ITT analysis showed improved glucose tolerance and insulin resistance (Fig. 5K), consistency with the *Rarres2*^{-/-} mice, down-regulated chemerin had no effect on the number of adipocyte-progenitors, but reduces the infiltration of macrophages in SAT (Fig. 5L and M).

Discussion

Adipose tissue is composed not only adipocytes, but also of immune cells, endothelial cells and adipocyte-progenitors. Immunity and metabolism are two fundamental systems of adipose tissue. The presence of CD45⁺ immune cells (macrophages, NK cells, dendritic cells, eosinophils, etc.) recognized as an indispensable component of adipose tissue involved in physiologic and pathologic remodeling.³⁷ In lean conditions, eosinophils, anti-inflammatory macrophages, and innate lymphoid cells, contribute to the maintenance of metabolic homeostasis. In obese conditions, NK cells secrete a vast array of active substances, such as interferon gamma (IFN- γ), which promote adipose inflammation,³⁸ pro-inflammatory macrophages are the most abundant immune cell type, which express not only inflammatory cytokines (IL-1 β , TNF α)³⁹ which aggravate inflammation and promote insulin resistance via serine phosphorylation of IRS.⁴⁰ In addition, macrophages secrete osteopontin, which attracts preadipocyte proliferation, promotes the proliferation and differentiation of preadipocytes.⁴¹

Chemerin was reported as an enhancer for 3T3-L1 cell differentiation,¹⁶ and positively with BMI in circulation.²¹ Our data shows that development of adipose tissue is not affected in *Rarres2*^{-/-} mice on ND. Rather, *Rarres2*^{-/-} mice gained more body weight, and SAT increased, VAT decreased on HFD. As our data showed no significant difference in the proportion of adipocyte-progenitors and the adipogenesis capability in WT and *Rarres2*^{-/-} mice (Fig. 2).

The infiltration of macrophage is significantly different between SAT and VAT, and infiltration in VAT is more severe than SAT (Fig. 4F). The literature showed that the low-grade chronic inflammation perpetuated by HFD promotes the development of obesity.⁴² This may be the reason for infiltration of macrophages both increased in SAT and VAT, but the adipogenesis was different. Furthermore, we found the expression of TIMP1 were significantly different between SAT and VAT, in adipose tissue, TIMP1 was mainly secreted from macrophages,³⁶ that is consistent with our data that high degree inflammation increasing TIMP1 expression.

Remodeling of extracellular matrix is essential for the differentiation of neonatal adipocytes from fibroblast-like adipocyte-progenitors during body fat formation *in vivo*.^{43,44} In this process, as reports revealed that MMP9 promotes angiogenesis^{45,46} and adipogenesis.⁴⁷ TIMP1 negatively regulated adipogenesis due to directly inhibit adipocyte differentiation⁴⁸ and abated adipogenesis by antagonizing the function of MMPs.⁴⁶

Considering that the increase in SAT improve glucose tolerance.¹³ We were puzzled by deteriorated glucose tolerance in *Rarres2*^{-/-} mice which has more SAT. Based on chemerin could affect multiple organs, the chemerin deficiency resulted in the impaired function of hepatic glucose production and islet β -cell.²⁵ In order to clarify the exact roles of chemerin in adipose tissue, we specifically knocked down chemerin in SAT to exclude the effect of chemerin on hepatocyte and islet β -cell, and these mice displayed improved adipogenesis, glucose tolerance and insulin resistance (Fig. 5) accompany with decreased macrophage infiltration and TIMP1.

Conclusion

The study demonstrated that the down-regulated chemerin inhibited the expression of TIMP1 by decreasing the infiltration of macrophage in SAT, we first revealed chemerin deficiency affect adipogenesis is different in SAT and VAT *in vivo*. The specific down-regulation of chemerin in SAT promotes adipogenesis and improves GTT and ITT. This suggests a potential therapeutic target for chemerin in the treatment of obesity related metabolic disorders.

Conflict of Interests

The authors declare no conflict of interest.

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