METABOLIC, ENDOCRINE, AND GENITOURINARY PATHOBIOLOGY

A Disintegrin and Metalloproteinase with Thrombospondin Motifs 18 Deficiency Leads to Visceral Adiposity and Associated Metabolic Syndrome in Mice

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Visceral adiposity is of greater risk than obesity in s.c. adipose tissue for diabetes and cardiovascular disease. Its pathogenesis remains unclear, but it is associated with extracellular matrix (ECM) remodeling. A disintegrin and metalloproteinase with thrombospondin motifs (ADAMTSs) are a family of secreted zinc-dependent metalloproteinases that play crucial roles in development and various diseases because of their ECM remodeling activity. ADAMTS18 is an orphan ADAMTS whose function and substrate remain unclear. Herein, we showed that *Adamts18* mRNA was abundantly expressed in visceral (gonadal) white adipose tissue (vWAT) during the early stage of development after birth. *Adamts18* knockout (KO) mice showed increased body fat percentage and larger adipocyte size in vWAT relative to wild-type littermates. These findings may be partly attributed to ECM remodeling, especially increased expression of laminin 1 and adipokine thrombospondin 1 in vWAT. Attenuated extracellular signal-regulated kinase 1 and 2 activity, along with increased expression of adipocyte-specific transcription factors peroxisome proliferator-activated receptor- γ , CCAAT/enhancer binding protein β , and marker gene *Fabp4*, was detected in vWAT of *Adamts18* KO mice. Furthermore, *Adamts18* KO mice showed early metabolic syndrome, including hyperlipidemia, blood glucose metabolic disorder, and hypertension. ADAMTS18 deficiency promotes atherosclerosis in apolipoprotein E-deficient mice. These results indicate a novel function of ADAMTS18 in vWAT development and associated metabolic disorders. (*Am J Pathol* 2018, 188: 461–473; <https://doi.org/10.1016/j.ajpath.2017.10.020>)

Adipose tissue consists of adipocytes, stromal vascular fraction (SVF) cells, including preadipocytes, endothelial cells, fibroblasts, and macrophages, and extracellular matrix (ECM), such as collagen (Col) and laminin.^{1–3} ECM provides a critical microenvironment for adipocyte development and the phenotypic fate of mesenchymal stem cells.^{4,5} Adipocyte-ECM cross talk dysfunction may lead to lipid metabolism disorders, including abdominal (central) obesity, high serum triglycerides (TGs), abnormal glucose (GLU) regulation, and elevated blood pressure.^{6,7} Thus, identifying key molecules that orchestrate adipose tissue ECM is crucial for understanding the molecular basis of obesity and associated metabolic diseases.

A disintegrin and metalloproteinase with thrombospondin motifs (ADAMTSs) are a family of 19 secreted zinc metalloendopeptidases, which are known to cleave a wide range of substrates in ECM and have been implicated in development, tissue morphogenesis and remodeling, inflammation, tumorigenesis, and vascular biology.^{8–10} The functions of ADAMTSs include N-terminal procollagen

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R.Z. and M.C. contributed equally to this work.

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Table 1 Primary Antibodies Used in this Study

No.	Product name	Application	Source
1	p38 MAPK antibody	WB	CST (Boston, MA; 9212)
2	Phospho-p38 MAPK (Thr180/Tyr182) antibody	WB	CST (9211)
3	p44/42 MAPK (ERK1/2) antibody	WB	CST (9102)
4	Phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) antibody	WB	CST (9101)
5	Anti-PPAR γ	WB	Abcam (Cambridge, UK; ab191407)
6	Anti-C/EBP β	WB	Abcam (ab32358)
7	Anti-aP2	WB	Abcam (ab108311)
8	Anti-GAPDH	WB	Abways (Shanghai, China; AB0037)

aP2, adipocyte protein 2; C/EBP β , CCAAT/enhancer binding protein β ; ERK1/2, extracellular signal-regulated kinase 1 and 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MAPK, mitogen-activated protein kinase; PPAR γ , peroxisome proliferator-activated receptor- γ ; WB, Western blotting.

processing (ADAMTSs 2, 3, and 14)^{11–14}; spermatogenesis (ADAMTS2)¹⁵; follicular rupture and ovulation (ADAMTS1)¹⁶; angiogenesis inhibition (ADAMTSs 1, 8, and 9)^{17,18}; degradation of cartilage oligomeric matrix protein (ADAMTSs 7 and 12)¹⁹; cleavage of the matrix proteoglycan aggrecan, versican, and brevican (ADAMTSs 1, 4, 5, 8, 9, and 15)^{20–23}; and cleavage of ultralarge-molecular-weight von Willebrand factor (ADAMTS13).²⁴ Recent reports have shown that some ADAMTSs play crucial roles in adipogenesis and metabolic disorders. It was found that ADAMTS1 was decreased in the adipose tissue of obese mice, and ADAMTS1 inhibition increased adipose tissue mass via the ECM-dependent focal adhesion kinase/extracellular signal-regulated kinases 1 and 2 (ERK1/2) signaling pathway.²⁵ In humans, ADAMTS1 level is inversely related to body mass index.²⁵ In addition, a population-based cohort study showed that ADAMTS13 activity may be an independent risk factor for incident prediabetes and type 2 diabetes.²⁶ Furthermore, ADAMTS5 promoted murine adipogenesis and the development of visceral (gonadal) white adipose tissue (vWAT) and associated angiogenesis.²⁷ Moreover, loss of ADAMTS5 enhances brown adipose mass and promotes browning of white adipose tissue via cAMP response element-binding protein signaling in mice.²⁸

ADAMTS18 is an orphan ADAMTS whose function and substrate remain unclear. In C57Bl6/Ola mice, *Adamts18* deletion results in distinct developmental defects, including congenital disorders of the lens, lung, and female reproductive tract development.²⁹ In humans, *ADAMTS18* mutations have been linked to tumorigenesis,³⁰ eye diseases,^{31–33} reduced bone mineral density formation,³⁴ and white matter integrity of the brain.³⁵ We have previously reported that ADAMTS18 is associated with platelet lyses and cerebral infarction in a postischemic stroke model.³⁶ To further study the role of ADAMTS18 *in vivo*, we developed an *Adamts18* knockout (KO) mouse strain.³⁷ Our recent unpublished data (R.Z., N.Y., and W.Z.) showed that ADAMTS18 deficiency is associated with bone mass in mice. When measuring the bone mineral density by dual-energy X-ray absorptiometry,

we unexpectedly found that *Adamts18* KO mice exhibited significantly elevated body fat percentage compared with their wide-type (WT) littermates. This finding prompted a further search for a potential association between ADAMTS18 and adipogenesis. In this study, using *Adamts18* KO mice as an *in vivo* model, we investigated the role of ADAMTS18 in fat metabolism and associated disorders in mice.

Materials and Methods

Reagents

All reagents were from Sigma-Aldrich (St. Louis, MO) unless otherwise designed. Primary antibodies used in this study are listed in Table 1.

Animals

Adamts18 KO mice were generated as previously described.³⁷ Littermates of *Adamts18* KO and WT mice were obtained by mating between heterozygotes. Mice used in this study were on a mixed 129 Sv/C57BL6 background, were maintained under a 12-hour light/dark cycle, and were provided water and food ad libitum in a specific pathogen-free facility at East China Normal University (Shanghai, China). All procedures in animal experiments were approved by the Institutional Animal Care and Use Committee of East China Normal University. All methods were performed in accordance with the relevant guidelines and regulations.

Hematoxylin and Eosin Staining and Cell Size Quantification

Mice were sacrificed by cervical dislocation under narcosis. Adipose tissue samples were dissected and immediately fixed in 10% formaldehyde in phosphate-buffered saline, pH 7.4, dehydrated, embedded in paraffin, and divided into sections with a microtome. Standard hematoxylin and eosin staining was performed on paraffin sections (4 μ m thick) of

Table 2 Primers for Quantitative Real-Time RT-PCR

Gene	Primers	Length, bp
<i>Pparg</i>	F: 5'-AGCCCTTTGGTGACTTTATGGA-3' R: 5'-GCAGCAGGTTGTCTTGGATGT-3'	171
<i>Cebpb</i>	F: 5'-CGGGTTTCGGGACTTGATGC-3' R: 5'-TCAACAACCCCGCAGGAACAT-3'	133
<i>Fabp4</i>	F: 5'-GTGATGCCTTTGTGGGAACCT-3' R: 5'-TCCTGTCTGCTGCGGTGATT-3'	234
<i>Acsf4</i>	F: 5'-TCAAGAAGGGAAGCAAGGGTG-3' R: 5'-TTACAAAGAGGGGCGTCATAGC-3'	199
<i>Adamts16</i>	F: 5'-GCAACCCCAAGACACGACCT-3' R: 5'-ACACGCTCCAGTTTCCACA-3'	90
<i>Actb</i>	F: 5'-CCACCATGTACCCAGGCATT-3' R: 5'-AGGGTGTAACGCGAGCTCA-3'	253

F, forward; R, reverse.

adipose tissues. Single adipocyte size (μm^2 ; $\times 200$) was measured using ImageJ software version 1.50i (NIH, Bethesda, MD; <http://imagej.nih.gov/ij>).

Western Blotting

Adipose tissue samples were collected and lysed with radioimmunoprecipitation assay buffer (20 mmol/L tris, 2.5 mmol/L EDTA, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 40 mmol/L NaF, 10 mmol/L $\text{Na}_4\text{P}_2\text{O}_7$, and 1 mmol/L phenylmethylsulfonyl fluoride) supplemented with proteinase inhibitor cocktail (Calbiochem, San Diego, CA). Total protein concentration was determined using a Micro BCA Protein Assay kit (Pierce, Rockford, IL). Proteins were separated on 8% to 12% SDS-PAGE and immunoblotted with specific primary antibodies listed in Table 1, followed by species-appropriate horseradish peroxidase-coupled secondary antibody for 1 hour at room temperature. Proteins were visualized with an enhanced chemiluminescence kit (Millipore, Boston, MA), according to the manufacturer's instructions, and quantified using ImageJ version 1.50i.

RT-PCR

Total RNA was isolated from the vWAT, s.c. (inguinal) adipose tissue, and brown adipose tissue of mice using TRNzol-A⁺ Reagent (TIANGEN, Beijing, China). Total RNA was reverse transcribed to cDNA using a FastQuant RT kit (with gDNase) (TIANGEN), according to the manufacturer's instructions. An *Adamts18* cDNA fragment was amplified with primers specific to *Adamts18* exons 4 and 6. The primer sequences were as follows: exon 4 forward, 5'-TCCTCATCTCACCGC-TACCTCA-3'; and exon 6 reverse, 5'-GGTCCCATCTTT-GAACAGGCTA-3'. A β -actin cDNA fragment was amplified

as an internal control using the following primers: 5'-CCAC-CATGTACCCAGGCATT-3' (forward) and 5'-AGGGTG-TAAAACGCAGCTCA-3' (reverse).

Quantitative Real-Time RT-PCR Analysis

Total RNA was isolated and transcribed, as described in *RT-PCR*. The quantitative real-time RT-PCR was performed using a StepOnePlus real-time PCR system (ThermoFisher, Carlsbad, CA) with SuperReal PreMix Plus (SYBR Green; TIANGEN), according to the manufacturer's instructions. Primers used in quantitative real-time RT-PCR are listed in Table 2. The relative quantity of target mRNA was analyzed using the $\Delta\Delta\text{Ct}$ method, with *Actb* as a reference gene. All reactions were performed as biological triplicates.

Isolation of Adipocytes and SVF Cells

Adipocytes and SVF cells were isolated from the vWAT of *Adamts18* KO mice and WT littermates at 3 to 4 weeks of age, as described.³⁸ Briefly, vWAT was harvested from mice, washed, and digested in 1.5 g/L collagenase type II solution containing 20 g/L bovine serum albumin and 1.2 g/L HEPES for 1 hour, with constant shaking at 37°C. Digestion was finished by adding the same volume of 0.01 mol/L phosphate-buffered saline, followed by a filtration using a 0.22- μm filter to remove undigested tissue. After centrifugation at $1000 \times g$ for 10 minutes, floating adipocytes were collected in a fresh tube. SVF cells from the precipitate were resuspended in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin.

Cell Culture and Induction of Adipogenic Differentiation

SVF cells were seeded equivalently in laminin-coated (10 $\mu\text{g}/\text{mL}$) or uncoated plates and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C with 5% CO_2 . Cells were seeded at a low density and allowed to grow until the following day, when the cell density reached 20% to 30% confluence. The cells were treated with induction medium [0.5 mmol/L isobutyl-methyl-xanthine, 1 $\mu\text{mol}/\text{L}$ dexamethasone, and 10 $\mu\text{g}/\text{mL}$ insulin (INS)] for 2 days to induce adipogenic differentiation. Then, cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 10 $\mu\text{g}/\text{mL}$ INS for another 2 days until they were prepared for oil red O staining.

Oil Red O Staining of Cultured Cells

Oil red O staining was performed according to the standard protocols.³⁹ Briefly, cells were washed with phosphate-buffered saline three times and fixed with 3.7% formaldehyde for 15 minutes. Oil red O (0.5% in isopropanol) was filtered through a 0.22- μm filter and incubated with cells for

Figure 1 Expression of *Adamts18* in mouse adipose tissues. **A:** Anatomical positions of visceral (gonadal) white adipose tissue (vWAT), s.c. WAT (sWAT), and brown adipose tissue (BAT) in mice with a C57BL/6/129 Sv background. WAT samples were harvested through making incisions through mouse abdominal skin, according to the sequence of numbers. **Left panels:** White arrows indicate the direction of incision. Black arrows point to the location of sWAT and vWAT. **Right panels:** BAT samples were harvested through making incisions through the mouse back skin, according to the sequence of numbers. White arrows indicate the direction of incision. The numbers represent the order in which the incisions were made. Black arrows point to the location of BAT. **B and C:** *Adamts18* mRNA expression profile in vWAT, sWAT, and BAT from both male and female wild-type (WT) mice at 2 and 6 weeks after birth, respectively. *Adamts18* mRNA expression levels in brain of WT and knockout (KO) mice were used as positive (+) and negative (-) control, respectively (**B**). Densitometric quantification normalized to β -actin was calculated to assess the expression change of *Adamts18* mRNA, and three animals per sex were pooled and analyzed (**C**). These experiments were repeated independently at least three times (**C**). Data are expressed as means $\pm$ SEM (**C**).

1 hour at room temperature. Cells were then washed with water, and the stained lipid droplets in adipocytes were visualized and imaged by a microscope. The positively stained adipocytes were quantified under the microscope.

Apoe^{-/-}/*Adamts18*^{-/-} Mouse Models for Atherosclerosis

Apoe knockout (*Apoe*^{-/-}) mice (C57/BL6; JunKe Co, Ltd, Nanjing, China) and *Adamts18*^{-/-} mice (C57/BL6/129 Sv) were crossbred to generate *Apoe*^{+/-}/*Adamts18*^{+/-} mice, which were used to obtain *Apoe*^{-/-}/*Adamts18*^{+/-} and *Apoe*^{-/-}/*Adamts18* knockout (*Adamts18*^{-/-}) littermates. Atherosclerosis was induced by feeding male mice at 8 weeks of age a high-fat diet containing 1.25% cholesterol (Research Diet; JunKe Co, Ltd) for 12 weeks. Blood was collected by heart puncture for measurement of serum lipids, including TG, low-density lipoprotein (LDL), high-density lipoprotein (HDL), and total cholesterol (TC), with kits. To assess lesions, heart-aorta complexes were excised, followed by thoracic-abdominal aortas (TAs) fixed with 10% formalin. Aortic sinuses and arches were embedded with OCT for frozen section preparation.

Oil Red O Staining of Artery

Oil red O staining was used to assess the size of the atherosclerotic lesion and its lipid content. Briefly, TAs were dissected, and *en face* oil red O staining of the artery plaque area was performed. Cross sections of aortic sinuses were fixed with 10% formalin, dehydrated with propylene glycol, and stained with 0.5% oil red O. For quantification, ImageJ version 1.50i was used to measure the lesion size of TAs and intimal areas of aortic sinuses. Lipid deposition was expressed as the percentage of oil red O-positive area in TA and the intima of sinuses.

Sandwich ELISA

For the sandwich enzyme-linked immunosorbent assay (ELISA) analysis, LDL (mouse LDL ELISA kit), HDL (mouse HDL ELISA kit), TG (mouse TG ELISA kit), TC (mouse TC ELISA kit), leptin (LEP; mouse LEP ELISA kit), INS (mouse INS ELISA kit), INS receptor (mouse INS receptor ELISA kit), GLU (mouse GLU ELISA kit), laminin (LN; mouse LN ELISA kit), thrombospondin 1 (THBS1; mouse THBS1 ELISA kit), Col IV (mouse Col type IV

Figure 2 Increased visceral (gonadal) white adipose tissue (vWAT) in *Adamts18* knockout (KO) mice. **A:** Cartoon depicting the method of isolating different fractions within the vWAT. **B and C:** RT-PCR analysis of expression of *Adamts18* mRNA in different fractions of vWAT of wild-type (WT) mice and *Adamts18* KO mice. β-actin was used as an internal reference. **D and E:** Body fat composition analysis. Body fat composition analysis by dual-energy X-ray absorptiometry in different genotype of male (**D**) and female (**E**) mice, aged 8 to 10 weeks. **F:** Representative hematoxylin and eosin staining of vWAT in different genotype of mice. **G and H:** Quantification of vWAT adipocyte size. Single adipocyte size was measured using ImageJ version 1.50i. Average adipocyte size of each mouse was calculated from approximately 270 adipocytes (three random fields of each section were selected, and three replicate sections from each mouse were analyzed). There were at least three individual sex-matched mice in each group (**C**, **G**, and **H**). Results are expressed as means ± SEM (**C**, **D**, **E**, **G**, and **H**). $n = 3$ per group (**B** and **C**); $n = 8$ per group (**D** and **E**, male mice); $n = 7$ per group (**D** and **E**, female mice). *** $P < 0.01$, **** $P < 0.001$ versus WT. Scale bars = 50 μm (**F**). Original magnification, ×200 (**F**). SVF, stromal vascular fraction.

ELISA kit), and Col VI (mouse Col type VI ELISA kit) were measured, according to the manufacturer's instructions of the ELISA kits (LYBD Bio-Technique Co, Ltd, Beijing, China).

IPGTT and IPIST

Adamts18 KO mice and WT littermates, at 8 to 10 weeks of age, were used for i.p. GLU tolerance test (IPGTT) and i.p. INS sensitivity test (IPIST). For IPGTT, mice were fasted overnight and then injected with GLU with 2 g/kg body weight. Blood GLU levels were measured by automatic GLU monitor from the tail blood before injection and 60 and 120 minutes after injection. For IPIST, mice were fasted 4 hours and injected with INS at 0.75 U/kg. Blood GLU levels were measured, as described earlier in this paragraph, immediately before injection and at 15, 30, 45, 60, and 120 minutes after injection.

Blood Pressure Determination

Mouse blood pressure was determined using an indirect tail cuff method with a Softron BP-98A indirect blood pressure meter (Softron Co Ltd, Tokyo, Japan). *Adamts18* KO mice and WT littermates, at 8 weeks of age, were used for this measurement. Mouse blood pressure was determined between

1:00 PM and 4:00 PM, and repeated five times for each mouse after a 5 days' training period for habituation. The mean of five values was taken as the final blood pressure level.

Statistical Analysis

All data are expressed as means ± SEM. Comparisons between two groups were made using unpaired *t*-tests. For IPGTT and IPIST study, two-way analyses of variance were used, followed by Bonferroni post hoc analysis when appropriate.

Results

Expression of *Adamts18* in Mouse Adipose Tissues

To determine whether ADAMTS18 is associated with adipogenesis, its expression profile was first examined in different types of fat and different stages of fat development (Figure 1, A and B). RT-PCR analysis demonstrated that *Adamts18* mRNA was abundantly expressed in vWAT at a postnatal age of 2 weeks in both male and female mice, whereas its expression decreased dramatically (approximately sixfold decrease) in vWAT of mice at 6 weeks after birth (Figure 1, B and C). *Adamts18* mRNA was almost

Figure 3 Altered extracellular matrix compositions in visceral (gonadal) white adipose tissue (vWAT) of *Adamts18* knockout (KO) mice. **A:** Sandwich enzyme-linked immunosorbent assay analysis of levels of thrombospondin 1 (THBS1), laminin 1 (LN1), type IV collagen (COL), and type VI COL in vWAT tissues of 8- to 10-week-old wild-type (WT) and *Adamts18* KO male mice. **B:** Expression levels of LN1 and THBS1 determined by Western blotting and quantification of relative expression level of the proteins. **C:** The representative images of LN-coated and uncoated (NLN) substrata on the spreading of stromal vascular fraction cells separated from the vWAT of 3-week-old mice. **D: Top row:** Postconfluent cells in both LN-coated and NLN dishes were induced to differentiate; adipocyte was assessed by oil red O staining at day 4. **Bottom row:** Quantification of relative positive stained cells by oil red O staining in both LN-coated and NLN dishes. Data are expressed as means $\pm$ SEM. $n = 8$ per group (**A**); $n = 4$ per group (**B**). * $P < 0.05$, ** $P < 0.01$ versus WT; $^{\dagger}P < 0.05$ versus uncoated. Scale bars = 100 μ m (**C** and **D**). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

undetectable in s.c. (inguinal) adipose tissue and brown adipose tissue at both 2 and 6 weeks after birth (Figure 1, B and C). The expression of *Adamts18* mRNA was then examined in different fractions of the vWAT (Figure 2A). The results showed that *Adamts18* was mostly expressed in SVF cells in vWAT (Figure 2, B and C). Body composition analysis by dual-energy X-ray absorptiometry revealed significantly elevated body fat percentage in adult *Adamts18* KO mice, aged 8 to 10 weeks, relative to WT littermates ($P < 0.001$) (Figure 2, D and E). Histopathological examinations showed that the size of adipocytes and lipid

droplets in vWAT of *Adamts18* KO mice was obviously larger than that in WT controls (Figure 2, F–H), whereas no apparent morphologic differences were observed in s.c. (inguinal) adipose tissue and brown adipose tissue (data not shown). The expression of uncoupling protein-1 was also examined in vWAT. RT-PCR analysis showed that the expression of uncoupling protein-1 mRNA in vWAT of *Adamts18* KO mice was not significantly different from that in WT mice (Supplemental Figure S1). These results suggest that ADAMTS18 exerts an important influence on the early adipocyte directional differentiation of vWAT.

Figure 4 ADAMTS18 deficiency promotes visceral (gonadal) white adipose tissue (vWAT) differentiation by inhibition of the extracellular signal-regulated kinase 1 and 2 (ERK1/2) signaling pathway in mice. **A: Top panel:** Western blot analysis of the protein levels of total ERK1/2 (t-ERK1/2) and phosphorylated ERK1/2 (p-ERK1/2) in vWAT from age- and sex-matched mice with different genotypes. **Bottom panel:** Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control; the activity of ERK1/2 was expressed as the ratio of p-ERK1/2/t-ERK1/2. **B: Top panel:** Western blot analysis of the protein levels of peroxisome proliferator-activated receptor-γ (PPARγ), CCAAT/enhancer binding protein β (C/EBPβ), and adipocyte protein 2 (aP2) in vWAT from age- and sex-matched mice with different genotypes with the indicated antibodies. **Bottom panel:** Relative expression levels of the proteins are represented as protein/GAPDH. **C–E:** The relative mRNA expression of Pparγ (**C**), C/ebpβ (**D**), and aP2 (**E**) in mouse vWAT determined by quantitative real-time RT-PCR. The relative quantity of target gene was normalized to housekeeping gene β-actin using the $\Delta\Delta C_t$ method. Data are expressed as means $\pm$ SEM (**A–E**). $n = 4$ (**A–E**). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus WT (t -test). KO, knockout; WT, wild type.

ADAMTS18 Deficiency Alters ECM Composition in vWAT, which Promotes Adipocyte Differentiation

The ECM microenvironment plays important roles in adipocyte development and expansion and in the phenotypic fate of mesenchymal stem cells.^{4,5} To clarify whether enlarged vWAT in *Adamts18* KO mice is related to ECM remodeling, the key adipogenesis-related ECM proteins were examined. A sandwich ELISA showed that the protein levels of LN1 and THBS1 were significantly increased in vWAT tissue of *Adamts18* KO mice compared with those in WT controls; however, the protein levels of Col IV and VI showed no significant differences between *Adamts18* KO mice and WT controls (Figure 3A). Western blotting analysis further confirmed that both LN1 and THBS1 were increased in vWAT of *Adamts18* KO mice compared with those of WT controls (Figure 3B). *In vitro*, SVF cells separated from the vWAT of mice were respectively seeded in LN-coated and uncoated culture dishes and treated with a

standard adipocyte differentiation protocol. Markedly enhanced preadipocyte spreading (from both WT and *Adamts18* KO mice) was observed in LN-coated dishes relative to uncoated dishes (Figure 3C). After 6 days of differentiation induction, the number of cells stained by oil red O in LN-coated dishes (from both WT and *Adamts18* KO mice) was obviously more than that in LN-uncoated dishes (Figure 3D). Furthermore, oil red O-stained cells, originated from SVF cells of *Adamts18* KO mice, were obviously more than those from WT mice in LN-uncoated dishes (Figure 3D). These results suggest that enlarged vWAT in *Adamts18* KO mice may be attributed, in part, to ECM remodeling, especially increased LN expression.

ADAMTS18 Deficiency Promotes vWAT Differentiation through Inhibition of the ERK1/2 Pathway in Mice

It is well known that ECM components critically regulate adipocyte differentiation by affecting ERK1/2 pathways and

Figure 5 ADAMTS18 deficiency promotes atherosclerosis in *ApoE*^{-/-} mice. **A and B:** Serum lipid levels in 8-week-old wild-type (WT) and *Adamts18* knockout (KO) males with a normal chow diet (**A**) or after a high-fat diet (HFD) for 12 weeks (**B**). **C:** Serum lipid levels in *ApoE*^{-/-} male mice with different genotypes of *Adamts18*. **D–F:** Oil red O staining of artery plaque area in the thoracic-abdominal aorta (TA) of WT and *Adamts18* KO mice (**D**) or in TA of *ApoE*^{-/-}/*Adamts18*^{+/+} and *ApoE*^{-/-}/*Adamts18*^{-/-} mice (**E**) or oil red O staining of lesion size in the aortic sinus of *ApoE*^{-/-}/*Adamts18*^{+/+} and *ApoE*^{-/-}/*Adamts18*^{-/-} mice (**F**) after an HFD for 12 weeks. **D–F: Top panels:** Representative images of oil red O staining. **Bottom panels:** For quantification, ImageJ version 1.50i was used to measure the plaque area or lesion size of TA of aortic sinuses. **Arrows in F** indicate the location of Oil red O staining of lesion size in the aortic sinus of *ApoE*^{-/-}/*Adamts18*^{+/+} and *ApoE*^{-/-}/*Adamts18*^{-/-} mice. Data are expressed as means ± SEM (**A–F**). *n* = 7 per group (**A**); *n* = 6 per group (**B** and **D**); *n* = 10 to 11 per group (**C**); *n* = 5 per group (**E** and **F**). **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 versus control. Scale bars = 200 μm (**F**). HDL, high-density lipoprotein; LDL, low-density lipoprotein; TC, total cholesterol; TG, triglyceride.

the downstream adipocyte-specific signaling pathway.⁴⁰ ADAMTS18 deficiency significantly decreased the level of phosphorylated ERK1/2 in vWAT (Figure 4A). Consistently, the protein levels of adipogenic transcription factors, including peroxisome proliferator-activated receptor-γ, CCAAT/enhancer binding protein β, and adipocyte-specific marker adipocyte protein 2, were significantly increased in vWAT of *Adamts18* KO mice relative to WT controls (Figure 4B). The expression of these adipogenic factors was also checked at the mRNA level by quantitative real-time RT-PCR, and similar results were shown (Figure 4, C–E). However, ADAMTS18 deficiency did not affect the protein level of total and phosphorylated p38 mitogen-activated protein kinase (data not shown). In addition, expression of acyl-CoA synthetase long-chain family member 4, which plays a crucial role in lipolysis, showed no significant difference between *Adamts18* KO mice and WT littermates (Supplemental Table S1).

ADAMTS18 Deficiency Causes Increased Serum Lipid Levels in Mice

Increased visceral fat volume is a major contributory factor in hyperlipidemia, because excessive TG is continually released

by fat cells into the bloodstream.^{1,2} To further investigate the relevance of ADAMTS18 and hyperlipidemia, the blood lipid level of mice was examined. Compared with WT controls, *Adamts18* KO mice fed a regular chow diet showed increased LDL (KO versus WT, 3.56 ± 0.28 versus 3.25 ± 0.22 mmol/L; *P* = 0.0408), decreased HDL (KO versus WT, 1.30 ± 0.09 versus 1.48 ± 0.15 mmol/L; *P* = 0.018), increased TG (KO versus WT, 1.31 ± 0.07 versus 0.84 ± 0.09 mmol/L; *P* < 0.001), and increased TC (KO versus WT, 4.79 ± 0.43 versus 4.1 ± 0.33 mmol/L; *P* = 0.0058; *n* = 7 per group) (Figure 5A).

ADAMTS18 Deficiency Promotes Atherosclerosis in *ApoE*^{-/-} Mice

Because increased serum lipid level is a major risk factor for atherosclerosis,^{6,7} the effect of ADAMTS18 was further evaluated on atherosclerosis. After a high-fat diet for 12 weeks, *Adamts18* KO mice displayed significantly increased LDL, TG, and TC and decreased HDL relative to WT littermates (Figure 5B). Similarly, *ApoE*^{-/-}/*Adamts18*^{-/-} mice showed increased LDL, TG, and TC and decreased HDL relative to *ApoE*^{-/-}/*Adamts18*^{+/+} littermates (Figure 5C). Oil red O staining showed that artery plaque area in TA of *Adamts18* KO

Figure 6 ADAMTS18 deficiency results in abnormal blood glucose regulation in mice. **A–C:** Levels of leptin (LEP; **A**), insulin (INS; **B**), and insulin receptor (ISR; **C**) in serum of 8- to 10-week-old male *Adamts18* knockout (KO) mice and wild-type (WT) littermates measured by sandwich enzyme-linked immunosorbent assay. **D:** Random blood glucose (RBG) and fasting blood glucose (FBG) measured by glucometer in 8- to 10-week-old male *Adamts18* KO mice and WT littermates. **E and F:** Glucose concentrations measured by glucometer in 8- to 10-week-old male *Adamts18* KO mice and WT littermates during an i.p. glucose tolerance test (IPGTT; **E**) and an insulin sensitivity test (IPIST; **F**). Two-way analyses of variance were used for IPGTT and IPIST, followed by the Bonferroni post hoc analysis. Data are expressed as means ± SEM (**A–F**). $n = 7$ per group (**A–C**); $n = 6$ per group (**D**); $n = 12$ (**E and F**, KO mice); $n = 11$ (**E and F**, WT mice). * $P < 0.05$, *** $P < 0.001$.

mice was significantly increased by approximately 1.42-fold in comparison to that of WT littermates (Figure 5D). Consistently, artery plaque area in TA of *Apoe*^{-/-}/*Adamts18*^{-/-} mice was significantly increased by approximately 1.85-fold in comparison to that of *Apoe*^{-/-}/*Adamts18*^{+/+} littermates (Figure 5E). Moreover, *Apoe*^{-/-}/*Adamts18*^{-/-} mice showed a significantly increased lesion size by approximately 1.72-fold in the aortic sinus compared with *Apoe*^{-/-}/*Adamts18*^{+/+} littermates (Figure 5F).

ADAMTS18 Deficiency Causes Abnormal Blood GLU Metabolism in Mice

Visceral fat is an important endocrine organ.^{1,2} The results of sandwich ELISA demonstrated that *Adamts18* KO mice showed significantly increased serum LEP level relative to WT littermates (KO versus WT, 5.69 ± 0.78 versus 4.33 ± 0.77 $\mu\text{g/L}$; $P = 0.01$; $n = 7$ per group) (Figure 6A). Furthermore, *Adamts18* KO mice showed a significantly decreased serum INS level relative to WT littermates (KO versus WT, 10.2 ± 1.6 versus 12.5 ± 1.5 mU/L; $P = 0.026$; $n = 7$ per group) (Figure 6B). However, the concentration of INS receptor showed no significant difference between *Adamts18* KO and WT mice (KO versus WT, 4555.6 ± 621.8 versus 4533.2 ± 654.1 pg/mL; $P = 0.95$; $n = 7$ per group) (Figure 6C). There was no significant difference in fasting blood GLU and random blood GLU between *Adamts18* KO and WT mice (Figure 6D). IPGTT demonstrated that *Adamts18* KO mice showed higher blood GLU levels relative to WT controls at 60 and 120 minutes after i.p. GLU loading [GLU, KO versus WT, 60 minutes: 8.89 ± 0.95 versus

7.34 ± 0.83 mmol/L ($P < 0.001$); 120 minutes: 6.44 ± 0.95 versus 5.12 ± 0.57 mmol/L ($P < 0.001$)] (Figure 6E). IPIST showed no significant differences in blood GLU levels between *Adamts18* KO and WT mice at multiple time points after INS injection (Figure 6F), which suggests that there is no significant difference in INS sensitivity between *Adamts18* KO and WT mice.

ADAMTS18 Deficiency Causes Hypertension in Mice

A blood pressure assay showed that both male and female *Adamts18* KO mice showed higher blood pressure relative to WT littermates, and female mice showed this inclination more distinctively [male systolic pressure, KO versus WT, 124.17 ± 22.97 versus 110.33 ± 8.52 mmHg; female systolic pressure, KO versus WT, 120.67 ± 14.80 versus 95.83 ± 8.66 mmHg ($P < 0.01$); male diastolic pressure, KO versus WT, 84.17 ± 11.82 versus 70.5 ± 3.08 mmHg ($P < 0.05$); female diastolic pressure, KO versus WT, 85 ± 12.66 versus 69 ± 7.72 mmHg ($P < 0.05$); male mean pressure, KO versus WT, 88.17 ± 6.74 versus 77.67 ± 8.02 mmHg ($P < 0.05$); female mean blood pressure, KO versus WT, 88.17 ± 6.74 versus 77.67 ± 8.02 mmHg ($P < 0.05$)] (Figure 7, A and B). Histological analysis showed that the thickness of the blood vessel wall in the common carotid artery of *Adamts18* KO mice increased significantly (approximately 1.3-fold) relative to WT littermates (Figure 7C).

Discussion

ADAMTS18 is an orphan ADAMTS whose pathophysiological roles remain unclear. In this study, we used

Figure 7 ADAMTS18 deficiency induces high blood pressure in mice. An indirect tail-cuff method was used to record the systolic blood pressure, diastolic blood pressure, and mean pressure. Pulse pressure refers to the difference between the systolic blood pressure and diastolic blood pressure. **A** and **B**: Blood pressure monitoring in 8-week-old male *Adamts18* knockout (KO) mice and wild-type (WT) littermates (**A**) and female *Adamts18* KO mice and WT littermates (**B**). Each mouse was measured five times, and the mean of five values was taken as the final blood pressure level. **C**: Representative hematoxylin and eosin staining of common carotid artery (CCA) and the relative wall thickness of each group was analyzed. Data are expressed as means $\pm$ SEM (**A–C**). $n = 6$ (**A** and **B**); $n = 4$ (**C**). * $P < 0.05$, ** $P < 0.01$ versus WT. Scale bars = 200 μ m (**C**). A, adventitia; E, endothelium; M, media.

Adamts18 KO mice as an *in vivo* model and demonstrated, for the first time, that ADAMTS18 deficiency promotes vWAT differentiation and leads to associated metabolic syndrome, including hyperlipidemia, abnormal blood GLU regulation, and hypertension, in mice. This study adds to the growing body of evidence that ADAMTSs are associated with adipogenesis and metabolic disorders.^{25–28}

The ADAMTS family plays a pivotal role in organ development and various diseases because of its ECM remodeling activity.^{8–10} ECM composition and dynamics are of crucial importance to adipocyte function.³ ADAMTS18 deficiency increased LN1 and THBS1 in mouse vWAT

(Figure 3, A and B). LN is a major component of the basal lamina, and it plays an exclusive role in the morphologic aspects of preadipocyte development. It was reported that preadipocytes showed relatively higher affinity for LN1 than other ECM proteins, and this is conducive to preadipocyte adhesion, migration, and proliferation.⁴¹ Markedly enhanced preadipocyte spreading (Figure 3C) and an increased number of induced mature adipocytes were found in laminin-coated dishes relative to uncoated dishes (Figure 3D). This suggests that increased LN1 in vWAT of *Adamts18* KO mice is a key factor for promoting adipocyte differentiation. THBS1 is an adipokine, and it is highly expressed in obese

Figure 8 Schematic diagram of the role of *Adamts18* in visceral (gonadal) white adipose tissue (vWAT) development and associated metabolic syndrome in mice. *Adamts18* was mostly expressed by stromal vascular fraction cells of vWAT. It exerts influence on the early adipocyte directional differentiation of vWAT through modulating extracellular matrix (ECM) remodeling. ADAMTS18 deficiency increases laminin 1 (LN1) and thrombospondin 1 (THBS1) expression, which inhibits extracellular signal-regulated kinase 1 and 2 (ERK1/2) activity and increases expression of adipogenic transcription factors peroxisome proliferator-activated receptor- γ (PPAR γ), CCAAT/enhancer binding protein β (C/EBP β), and adipocyte-specific marker adipocyte protein 2 (aP2). Accordingly, it contributes to enlarged vWAT. Enlarged vWAT increases the risks of hyperlipidemia, atherosclerosis, blood glucose metabolic disorder, and hypertension in mice.

individuals.⁴² Loss of THBS1 attenuated weight gain and fat accumulation in mice with a high-fat diet.⁴³ The data that demonstrated increased THBS1 expression in enlarged vWAT tissue of *Adamts18* KO mice are consistent with these reports. In addition, SVF cells separated from vWAT of *Adamts18* KO mice were more prone to be induced to mature adipocytes than those from vWAT of WT mice (Figure 3D). These results suggest that enlarged vWAT in *Adamts18* KO mice may be attributed, in part, to altered ECM composition, especially increased LN and THBS1 expression. ECM remodeling is a complex systematic process involving many molecules. Herein, the focus was on the regulation of LN, THBS1, and Col during adipogenesis. However, other molecules, like secreted protein acidic and rich in cysteine, which functions as an inhibitor of adipogenesis, might also serve as a target for ADAMTS18 during adipocyte differentiation and lipid metabolism. Altogether, we propose that ADAMTS18 regulates adipogenesis by influencing the cell microenvironment through modifying ECM components rather than regulating a single target or substrate.

Adhesion to ECM regulates a variety of cellular processes, including proliferation, survival, and differentiation.⁴⁴ Integrins are the main receptors that recognize fibronectin, laminin, and Col within the ECM.⁴⁵ Engagement and clustering of integrins leads to intracellular signaling transduction.⁴⁶ The ERK1/2 cascade is one of the most important pathways regulated by adhesion⁴⁷ and is crucial for adipogenesis.^{48,49} ERK1/2 needs to be activated during early adipogenesis for normal clonal expansion, whereas phosphorylated ERK1/2 stimulates the phosphorylation of peroxisome proliferator-activated receptor- γ , which leads to the inhibition of adipogenesis in the later stage of adipocyte differentiation. Data showed that ERK1/2 activity was inhibited (Figure 4A), whereas the expression of its downstream adipocyte-specific transcription factors (peroxisome proliferator-activated receptor- γ , CCAAT/enhancer binding protein β , and marker gene *Fabp4*) was increased, in vWAT of *Adamts18* KO mice (Figure 4, B–E). Therefore, these findings suggest that ADAMTS18 regulates adipogenesis by affecting ERK1/2 activation through ECM remodeling.

ADAMTS18 deficiency led to hyperlipidemia in mice (Figure 5A). Hyperlipidemia is a major risk factor for atherosclerosis.⁵⁰ *Apoe*^{-/-} mouse has been used as an experimental model of atherosclerosis because it spontaneously develops hypercholesterolemia and atherosclerosis in a reproducible manner, similar to what is observed in humans.⁵¹ ADAMTS18 deficiency synergistically aggravated atherosclerosis in *Apoe*^{-/-} mice (Figure 5, B–F). In this regard, ADAMTS18 might be a potential clinical biomarker for screening of atherosclerotic patients.

This study also demonstrates that ADAMTS18 deficiency causes hypertension (Figure 7, A and B) and increased common carotid artery thickness (Figure 7C) in mice. ADAMTS18 and ADAMTS16 form a phylogenetic clade.^{52,53} The close evolutionary relationship between

ADAMTS18 and ADAMTS16 suggests that these proteins may also be functionally similar. Indeed, *Adamts16* KO rats showed inherited hypotension because of increased Col in the aortic adventitia.⁵⁴ However, a significant difference in the expression of *Adamts16* mRNA between *Adamts18* KO mice and WT controls was not observed (Supplemental Table S2). We speculated that hypertension in *Adamts18* KO mice may be partly attributed to increased serum LEP level (Figure 6A). Several studies have shown that LEP may regulate blood pressure by exciting the sympathetic nervous system,⁵⁵ lowering endothelial vasodilation function,⁵⁶ and promoting the proliferation of vascular smooth muscle cells, thereby increasing peripheral vascular resistance.⁵⁷

Adipose tissue distribution is a strong predictor of the occurrence of the metabolic syndrome in the context of obesity.^{6,7} Obese individuals who preferentially expand visceral adipose tissue are at a greater risk for diabetes and cardiovascular disease than are equally obese individuals who store excess energy in s.c. adipose tissue.^{6,7} To our knowledge, this is the first study that recognizes ADAMTS18 as a novel and important regulator of vWAT development and related metabolic syndrome by affecting ECM remodeling (Figure 8). In the future, ADAMTS18 might also be a target for the diagnosis and treatment of visceral adiposity and associated metabolic risks.

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Supplemental Data

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