



CRISPR/Cas9-mediated *Angptl8* knockout suppresses plasma triglyceride concentrations and adiposity in rats^S

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Abstract Angiopoietin-like protein (ANGPTL)8 is a liver- and adipocyte-derived protein that controls plasma triglyceride (TG) levels. Most animal studies have used mouse models. Here, we generated an *Angptl8* KO rat model using a clustered regulatory interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) (CRISPR/Cas9) system to clarify the roles of ANGPTL8 in glucose and lipid metabolism. Compared with WT rats, *Angptl8* KO rats had lower body weight and fat content, associated with impaired lipogenesis in adipocytes; no differences existed between the groups in food intake or rectal temperature. Plasma TG levels in both the fasted and refeed states were significantly lower in KO than in WT rats, and an oral fat tolerance test showed decreased plasma TG excursion in *Angptl8* KO rats. Higher levels of lipase activity in the heart and greater expression of genes related to β -oxidation in heart and skeletal muscle were observed in *Angptl8* KO rats. However, there were no significant differences between KO and WT rats in glucose metabolism or the histology of pancreatic β -cells on both standard and high-fat diets. **In conclusion,** we demonstrated that *Angptl8* KO in rats resulted in lower body weight and plasma TG levels without affecting glucose metabolism. ANGPTL8 might be an important therapeutic target for obesity and dyslipidemia.—Izumi, R., T. Kusakabe, M. Noguchi, H. Iwakura, T. Tanaka, T. Miyazawa, D. Aotani, K. Hosoda, K. Kangawa, and K. Nakao. **CRISPR/Cas9-mediated *Angptl8* knockout suppresses plasma triglyceride concentrations and adiposity in rats.** *J. Lipid Res.* 2018. 59: 1575–1585.

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The metabolic syndrome is characterized by abdominal obesity with impaired glucose tolerance, dyslipidemia, and/or hypertension. Patients with the syndrome are at high risk of developing atherosclerotic CVD (1, 2). The number of patients with this syndrome has increased dramatically all over the world, posing a major public health issue (3, 4). There is an urgent need to develop novel therapeutic approaches to alleviate the burden of these diseases.

Angiopoietin-like protein (ANGPTL)8 has received increasing attention as a key regulator of glucose and lipid metabolism. ANGPTL8 is called an atypical ANGPTL in that it possesses an N-terminal coiled-coil domain but lacks a C-terminal fibrinogen-related domain (5). It is highly expressed in the liver and both white and brown adipose tissues and is secreted into the blood (6–8). ANGPTL8 expression is reduced by fasting and induced by refeeding (6, 7). It reportedly inhibits the activity of LPL by interacting with ANGPTL3 (5, 9), which has high homology with ANGPTL8. Therefore, the ANGPTL8 gene has also been

Abbreviations: ACOX, acyl-CoA oxidase; ANGPTL, angiopoietin-like protein; Cas9, clustered regulatory interspaced short palindromic repeat-associated protein 9; CM, chylomicron; CPT, carnitine palmitoyltransferase; CRISPR, clustered regulatory interspaced short palindromic repeat; FTT, fat tolerance test; gRNA, guide RNA; GTT, glucose tolerance test; Het, heterozygous; iAUC, incremental area under the curve; ITT, insulin tolerance test; SCD, stearoyl-CoA desaturase; TG, triglyceride.

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called “refeeding induced in fat and liver” (6) or “lipasin” (7). Mice lacking ANGPTL8 have been shown to have low plasma triglyceride (TG) levels (5, 6, 10, 11). Overexpression of ANGPTL8 in mice led to increased plasma TG levels (5, 7). ANGPTL8 has therefore been shown, mainly in mouse models, to regulate lipid metabolism.

In a subsequently retracted 2013 article, ANGPTL8 was renamed “betatrophin” to indicate that it regulated the replication of β -cells in response to insulin resistance (8). The authors reported that betatrophin/ANGPTL8 overexpression in mice induced a 17-fold increase in β -cell proliferation. However, subsequent studies in mice contested that claim, with findings that *Angptl8* KO mice exhibited normal glucose homeostasis, β -cell area, and compensatory β -cell expansion even if fed a high-fat diet (11, 12) or if given an insulin receptor antagonist (12). In addition, ANGPTL8 overexpression in multiple cohorts of mice of various ages and genetic strains had no effect on β -cell proliferation (13). The original article was finally retracted by the authors when they were unable to replicate their own findings in a larger number of mice (14). However, in a rat model, overexpression of ANGPTL8 by delivery of human ANGPTL8 gene plasmids to the pancreas, liver, and skeletal muscle reportedly increased β -cell proliferation (15). It may be, therefore, that there are species-specific effects accounting for the differences in the mouse and rat models.

Regardless of whether ANGPTL8 affects β -cells in certain species, the protein remains of great interest, and many investigators have tried to understand its role in metabolic disorders in humans, such as insulin resistance, obesity, dyslipidemia, and type 2 diabetes (16–19). As noted above, most studies of the function of ANGPTL8 have been performed in mouse models, but if there are indeed interspecies differences, it is important to use other animal models to yield a better understanding of the role of ANGPTL8 in metabolic homeostasis. Rats have long been used in biomedical research, including in the development of drugs, but they have fallen out of favor with the advent of gene-based research, as manipulating the rat genome has proven more difficult than working with the mouse genome (20). However, the development of the clustered regulatory interspaced short palindromic repeat (CRISPR) system using CRISPR-associated protein 9 (Cas9) has enabled editing of the rat genome (20, 21). In this study, we generated an *Angptl8* KO rat using the CRISPR/Cas9 system to investigate the protein’s roles in glucose and lipid metabolism.

MATERIALS AND METHODS

Animals

Angptl8 KO rats were generated using the CRISPR/Cas9 system as described previously (20–22). In brief, we designed two guide RNAs (gRNAs) targeting exons 2 and 3 in the ANGPTL8 gene (NM_001271710) (Fig. 1A), based on software tools predicting unique target sites throughout the rat genome. A mixture of transcribed Cas9 and gRNAs was microinjected into F344/Stm rat zygotes [National BioResource Project rat number: 0140] provided by the National BioResource Project for the Rat in Japan (www.anim.med.kyoto-u.ac.jp/nbr). We obtained two

lines of rats heterozygous (Het) for *Angptl8* (lines #1 and #2). Male and female Het rats were intercrossed to obtain homozygous *Angptl8* KO rats. Rats were genotyped by PCR with the following primers, 5'-CATCTGTTGAGCAGGCAGAA-3' (sense) and 5'-GTTTCAGTGGTGGCTTCCTTC-3' (antisense) for line #1 and 5'-GGGTGAGCAAAGCTGACCTA-3' (sense) and 5'-GAGTA-AACCCACCAGGCTCA-3' (antisense) for line #2. The PCR conditions were 35 cycles of 94°C for 30 s, 60°C for 60 s, and 72°C for 45 s. The amplification products were size-fractionated on 4% or 1.5% (w/v) agarose gels and visualized by UV illumination after staining with ethidium bromide (supplemental Fig. S1).

The rats were kept at a constant temperature of 23°C $\pm$ 1°C under a 14 h light and 10 h dark cycle (lights on 0700, lights off 2100) with free access to water and a standard diet (3.73 kcal/g, with 12% of calories from fat, F-2; Funabashi Farm, Chiba, Japan) unless otherwise stated. All animal care and experiments were conducted in accordance with the Guidelines for Animal Experiments of Kyoto University and were approved by the Animal Research Committee of Kyoto University.

Body weight, food intake, rectal temperature

Body weight was measured every week between 1500 and 1700 hours. Food intake was measured for 7 days and rectal temperature was determined with a digital thermometer (BAT7001H; Physitemp, Clifton, NJ).

Percent body fat and adipocyte size

Epididymal fat for calculation of percent body fat was dissected and weighed and immediately fixed in 10% neutral buffered formalin. For histology, 5 μ m-thick sections were stained with hematoxylin and eosin. Adipocyte size in the epididymal fat was evaluated on micrographs taken with a fluorescent microscope and analyzed (BioRevo BZ-9000; KEYENCE, Osaka, Japan), assessing the cross-sectional adipocyte area in 10 random fields (magnification $\times$ 200).

Adipocyte differentiation

For culture and differentiation of preadipocytes from subcutaneous fat, we applied a modification of a procedure described previously (23). Briefly, after isolation of subcutaneous inguinal fat from 15-week-old rats, the fat tissues were minced and digested with 0.2 mg/ml collagenase type IV at 37°C for 30–60 min. The suspension was passed through a 250 μ m pore mesh, and the effluent was centrifuged at 300 g for 5 min. After decantation, 20 ml of DMEM (Thermo Fisher Scientific, Waltham, MA) were added, and the suspension was passed through a 40 μ m pore mesh again to remove undigested clumps and debris. The effluent was centrifuged at 300 g for 5 min and the pellet was washed twice in 5 ml of DMEM. The resulting isolated cells were plated in a 24-well tissue culture dish and cultured in DMEM supplemented with FBS (Thermo Fisher Scientific). After growth to confluence, DMEM with 0.17 μ M insulin, 0.5 mM isobutyl-1-methylxanthine, 0.25 μ M dexamethasone, and 1 μ M pioglitazone was used to induce differentiation for 3 days. The medium was replaced with DMEM with 0.17 μ M insulin for two additional days. This procedure was performed twice, after which the differentiated adipocytes were analyzed.

Biochemical analysis

After an overnight fast and then again 2 h after refeeding, blood was collected from 12-week-old rats, and the plasma was isolated by centrifugation (1,150 g) for 10 min. Glucose levels were determined by the glucose oxidase method using a reflectance glucometer (LIFE CHECK; GUNZE, Kyoto, Japan). Plasma TG, total cholesterol, and NEFA were measured using enzymatic kits (Triglyceride E-test, Cholesterol E-test, and NEFA C-test; Wako

Pure Chemical Industries, Osaka, Japan). Plasma insulin levels were measured using an enzyme-linked immunosorbent assay kit (rat insulin kit; Morinaga Institute of Biological Science, Yokohama, Japan). Plasma TG and cholesterol levels in chylomicron (CM), VLDL, LDL, and HDL fractions were determined using high-performance liquid chromatography (LipoSEARCH; Skylight Biotech, Akita, Japan) as previously described (24). The upper limit of the size of CMs that pass through the column is about 500 nm.

Glucose, insulin, and fat tolerance tests

A glucose tolerance test (GTT) was performed by injecting rats intraperitoneally with 2.0 mg/g glucose after an overnight fast. Blood samples were obtained from the tail vein before and 15, 30, 60, and 120 min after the glucose load for measurement of blood glucose and plasma insulin levels. For an insulin tolerance test (ITT), rats were injected intraperitoneally with 1.0 U/kg human regular insulin (Humulin R; Eli Lilly Japan, Kobe, Japan) after an overnight fast, and blood glucose levels were measured before and 30, 60, 120, and 180 min after the insulin load. For a fat tolerance test (FTT), rats were given 10 μ l/g corn oil orally after an overnight fast, and blood samples were obtained before and 1, 2, 3, and 4 h after the fat load to measure plasma TG levels. Incremental area under the curve (iAUC) relative to the fasting value for TG during the FTT was calculated.

TG content in tissues

Rats aged between 30 and 48 weeks were euthanized between 1500 and 1700 hours, and individual organs were dissected to obtain tissue samples. For measurement of TG content in tissues, we applied a modification of a procedure described previously (25). Briefly, TGs in heart, skeletal muscle, and liver were extracted with 2-propanol (Nacalai Tesque, Kyoto, Japan) and heptane (Nacalai Tesque). After evaporation of the solvent, ethanol was added to solubilize the lipid and the TG content was measured using an enzymatic kit.

Lipase activity

To assess the plasma lipase activities, postheparin plasma was collected from each ad libitum-fed rat genotype 10 min after a bolus injection of heparin (0.2 U/g) into the tail vein. Heparin-releasable LPL activity was measured using the LPL/HTGL activity assay kit (Immuno-Biological Laboratories Co., Ltd., Gunma, Japan) according to the manufacturer's instructions. Total lipase activity in heart and skeletal muscle was determined using a LPL activity fluorometric assay kit (BioVision, Milpitas, CA) according to the manufacturer's instructions.

Total RNA extraction and quantitative real-time PCR

Total RNA was extracted from the heart, skeletal muscle, and liver with TRIzol (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Total RNA (1 μ g) was reverse-transcribed with an iScriptTM cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). Quantitative real-time PCR was performed with the StepOnePlus real-time PCR system using TaqMan (Applied Biosystems, Foster City, CA). Relative amounts of mRNAs were normalized with ribosomal 18 S RNA. The sets of primers and probes used are listed in supplemental Table S1.

Immunostaining

Pancreases were isolated and immediately fixed in 10% neutral buffered formalin. After proper fixation, the organs were routinely dehydrated through a graded series of ethanol concentrations, embedded in paraffin, sectioned, stained, and examined microscopically. Shortly after deparaffinization and hydrophilization, endogenous peroxidase activity was blocked with 3% hydrogen

peroxidase. For immunohistochemical staining for insulin, sections were incubated with guinea pig polyclonal anti-porcine insulin antibody (Dako, Carpinteria, CA). Immunolocalization was demonstrated using the EnVision+ system (anti-rabbit HRP-labeled polymer) (Dako) and DAB+ substrate kit (Dako).

Statistics

Data are expressed as mean $\pm$ SEM. Comparisons were made using a *t*-test or one-way factorial ANOVA followed by the Tukey-Kramer test, where appropriate. Statistical analysis of changes in body weight and the results of the GTT, ITT, and FTT were performed using two-way factorial ANOVA with repeated measurement followed by the Tukey test. A *P* value <0.05 was considered statistically significant.

RESULTS

Angptl8 KO rats were generated using the CRISPR/Cas9 system

Thirty-one pups (21 males and 10 females) were born. PCR amplification of the targeted loci showed that two male rats (lines #1 and #2) had *Angptl8* deletions. Further sequencing confirmed that the two rats had different frameshift mutations in exon 2 (Fig. 1A). Line #1 carried a 7 bp deletion and line #2 a 980 bp deletion in the ANGPTL8 gene, resulting in a premature termination codon. The expression of ANGPTL8 in the liver, white adipose tissue, and brown adipose tissue was significantly lower in *Angptl8* KO rats compared with WT rats (Fig. 1B). Rats Het for *Angptl8* had gene expression levels intermediate between those of the WT and *Angptl8* KO rats (Fig. 1B). Line #1 was chosen to establish a colony of *Angptl8* KO rats (designated as KO), as the phenotypes of lines #1 and #2 were identical.

Angptl8 KO rats were resistant to diet-induced obesity

Body weights of Het and *Angptl8* KO rats were lower than those of WT rats throughout the study (Fig. 2A). However, food intake and rectal temperature measured at the age of 25 weeks to assess energy balance did not differ among the three groups (Fig. 2B, C). There was also no significant difference in weight between Het and *Angptl8* KO rats. As indicated by epididymal fat weight, the percent body fat of *Angptl8* KO rats was significantly lower than that of WT and Het rats (Fig. 2D). In addition, the adipocytes in epididymal fat were significantly smaller in *Angptl8* KO rats than in WT rats (Fig. 2E).

To confirm the influence of *Angptl8* KO on body weight, we measured weights when the rats were on a high-fat diet (5.24 kcal/g, with 60% of calories from fat, D12492; Research Diets Inc., New Brunswick, NJ), which increases the expression of ANGPTL8 in liver and adipose tissue (7, 26, 27). Rats were fed a high-fat diet from the age of 8 weeks, and they all gained weight gradually, but from the age of 13 weeks on, *Angptl8* KO rats gained significantly less weight than WT rats (Fig. 2F). There were, however, no differences in food intake or body temperature between *Angptl8* KO and WT rats (food intake, 35.7 $\pm$ 2.2 kcal/day vs. 36.5 $\pm$ 2.5 kcal/day; rectal temperature, 35.3°C $\pm$ 0.1°C vs. 35.4°C $\pm$ 0.3°C, measured at 25 weeks). Weight changes of Het rats

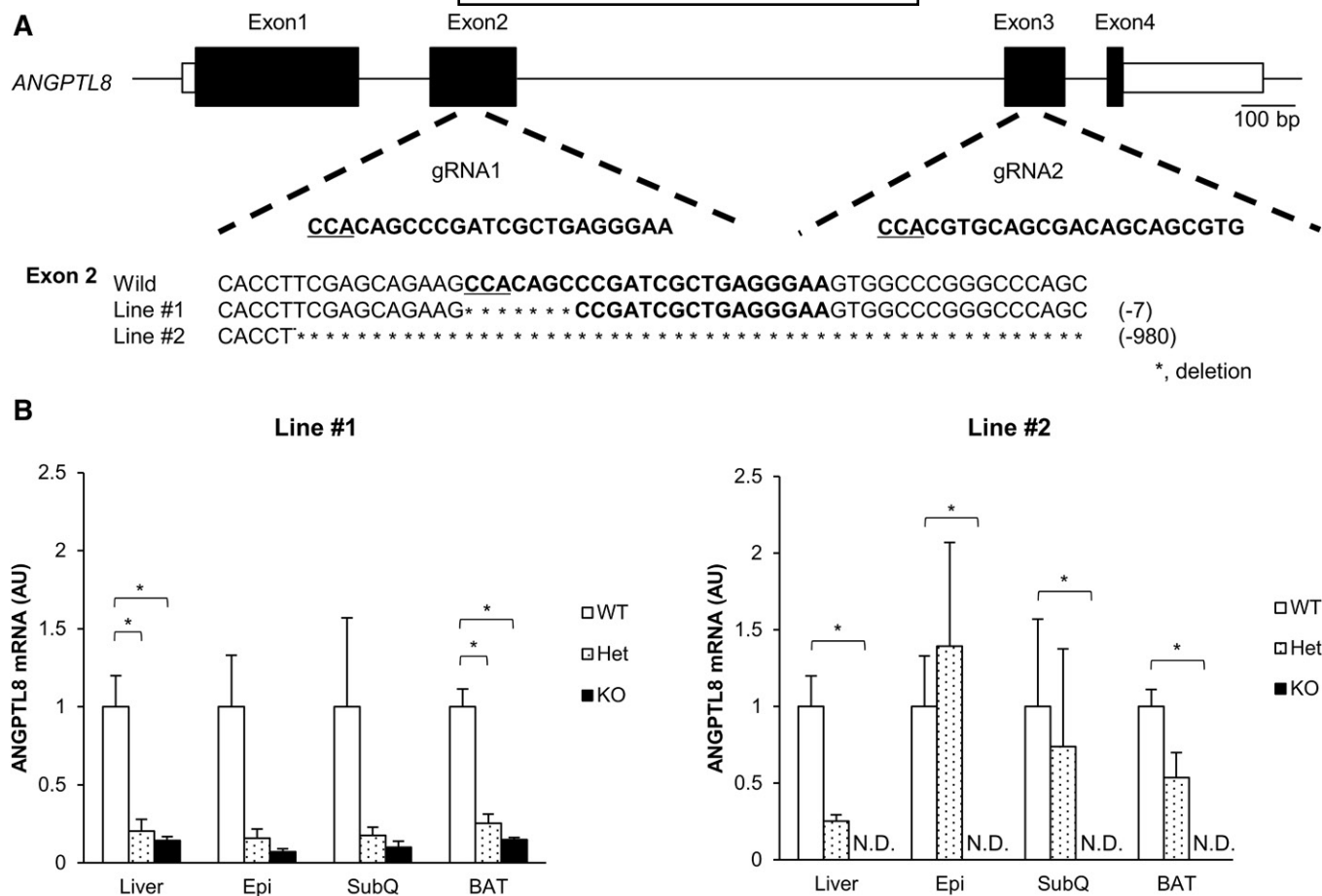


Fig. 1. Generation of *Angptl8* KO rats using the CRISPR/Cas9 system. **A:** Schematic diagram of gRNAs targeting at *Angptl8* loci. Proto-spacer adjacent motif sequences are underlined. **B:** Expression of ANGPTL8 mRNA in liver, epididymal fat (Epi), inguinal fat (SubQ), and brown adipose tissue (BAT) from ad libitum-fed WT, Het, and KO rats (n = 3–8 per group, age 35–48 weeks). Data were analyzed by one-way factorial ANOVA followed by the Tukey-Kramer test. **P* < 0.05.

were intermediate between those of the WT and *Angptl8* KO rats, indicating a dose-dependent effect of ANGPTL8 on body weight.

Angptl8 KO impaired adipogenesis

To investigate the influence of *Angptl8* KO on adipocytes themselves, we induced adipocyte differentiation to evaluate adipogenesis of preadipocytes from the stromal vascular fraction of the inguinal fat pads. Microscopic inspection of the culture dishes revealed lower numbers of Oil Red O-stained cells in KO cultures (**Fig. 3A**). In addition, we assessed the expression of adipocyte marker genes using real-time PCR and found no differences between WT and *Angptl8* KO cultures in the expression of PPAR- γ 2, the master regulator for the development of adipocytes. However, SREBP-1c and stearoyl-CoA desaturase (SCD)-1 expression was significantly lower in *Angptl8* KO cultures, and leptin expression appeared to be lower, although not significantly so (*P* = 0.058) (**Fig. 3B**).

Angptl8 KO rats had reduced plasma TG levels

To evaluate the influence of *Angptl8* KO on lipid metabolism in rats, we measured plasma lipid levels of each rat

genotype fed a standard diet. Plasma TG levels in both the fasted and refed states were more than 50% lower in *Angptl8* KO rats compared with WT rats, and Het rats had intermediate plasma TG levels (**Fig. 4A**). There were no significant differences in total cholesterol levels in the fasted and refed states among WT, Het, and *Angptl8* KO rats (**Fig. 4B**). Although NEFA levels in the refed state were not determined, they did not differ in the fasted state among WT, Het, and *Angptl8* KO rats (**Fig. 4C**). Plasma TG levels in the fed state were significantly lower in the CM, VLDL, and HDL fractions in *Angptl8* KO compared with WT rats (**Fig. 4D**).

As plasma TG levels of *Angptl8* KO rats were low in both the fasted and refed states, we performed oral FTTs to investigate plasma TG excursion. Plasma TG levels of *Angptl8* KO rats were significantly lower not only in the fasted state but also after a fat load compared with WT rats (**Fig. 4E**), and the iAUC for *Angptl8* KO rats was 51% lower than that for WT rats (205.3 ± 31.6 mg/dl/h vs. 419.8 ± 54.5 mg/dl/h, *P* < 0.05). Although CM production was not measured, this suggests that *Angptl8* KO rats had increased TG clearance. Finally, we determined postheparin LPL activity in each rat genotype. Postheparin plasma LPL activity

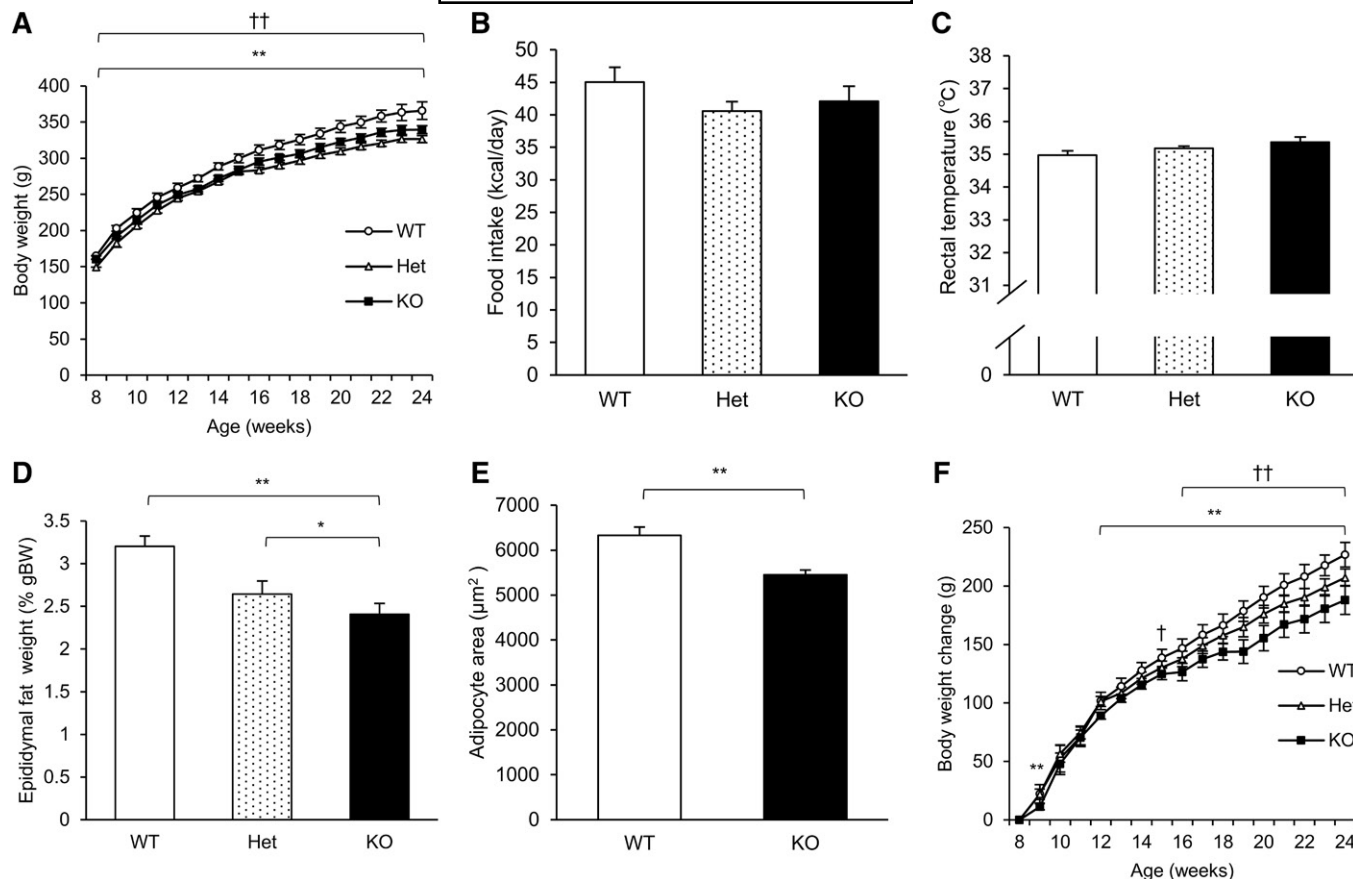


Fig. 2. Body weight and adipose tissue in *Angptl8* KO rats. A: Body weight monitored weekly in WT, Het, and KO rats fed a standard diet ($n = 5-14$ per group). Data were generated from four independent experiments. Data were analyzed by two-way factorial ANOVA with repeated measurement followed by the Tukey test. $**P < 0.01$ KO versus WT, $\dagger\dagger P < 0.01$ Het versus WT. Food intake (B) and rectal temperature (C) measured in WT, Het, and KO rats ($n = 4-14$ per group, age 25 weeks). Data were generated from four independent experiments. Data were analyzed by one-way factorial ANOVA followed by the Tukey-Kramer test. D: Epididymal fat weight of WT, Het, and KO rats ($n = 7-8$ per group, age 35–48 weeks). Data were analyzed by one-way factorial ANOVA followed by the Tukey-Kramer test. $*P < 0.05$, $**P < 0.01$. E: Adipocyte areas in epididymal fat of WT and KO rats ($n = 10$ per group, age 41 weeks). Data were analyzed by t -test. $**P < 0.01$. F: Body weight change monitored weekly in WT, Het, and KO rats fed a high-fat diet ($n = 4-9$ per group). Data were generated from three independent experiments. Data were analyzed by two-way factorial ANOVA with repeated measurement followed by the Tukey test. $**P < 0.01$ KO versus WT, $\dagger P < 0.05$ Het versus WT, $\dagger\dagger P < 0.01$ Het versus WT.

appeared to be higher by *Angptl8* KO in a dose-dependent manner, although not significantly so (Fig. 4F).

Angptl8 KO rats revealed accelerated β -oxidation in heart and skeletal muscle

Plasma TG levels of *Angptl8* KO rats were low in both the fasted and refed states even on a high-fat diet (Fig. 5A). Total lipase activity in heart tissue of *Angptl8* KO rats was higher than in WT rats, but not significantly so (25.4 ± 2.8 vs. 15.6 ± 4.3 , $P = 0.081$) (Fig. 5B). LPL expression levels in heart (1.00 ± 0.07 vs. 1.37 ± 0.21 , $P = 0.20$) and skeletal muscle (1.00 ± 0.15 vs. 1.02 ± 0.14 , $P = 0.91$) did not differ between WT and *Angptl8* KO rats (Fig. 5C). LPL catalyzes the hydrolytic cleavage of TGs into fatty acids for subsequent oxidation and/or storage. Therefore, we evaluated gene expressions related to β -oxidation and lipid synthesis in heart and skeletal muscle. The β -oxidation markers, carnitine palmitoyltransferase (CPT)-1b and acyl-CoA oxidase (ACOX), of *Angptl8* KO rats were significantly elevated in the heart (Fig. 5D), and a tendency toward this was observed in the skeletal muscle (Fig. 5E). On the other hand,

the lipid synthesis markers, SREBP-1c, SCD-1, and fatty acid synthase, did not differ between WT and *Angptl8* KO rats (supplemental Fig. S2A, B). Finally, we checked lipid accumulation in heart, skeletal muscle, and liver tissues and found that TG content in all those tissues did not differ between WT and *Angptl8* KO rats (Fig. 5F).

Glucose metabolism was not perturbed in ANGPTL8 KO rats

Fasting blood glucose and plasma insulin levels did not differ between WT and *Angptl8* KO rats fed a standard diet (Fig. 6A, B). To evaluate the influence of *Angptl8* KO on glucose tolerance and insulin sensitivity in rats, we performed GTTs and ITTs. In the GTTs, there were no significant differences in blood glucose or plasma insulin levels in the fasted state or after the glucose load between WT and *Angptl8* KO rats (Fig. 6C, D). The results of ITTs were similar, with no significant differences in blood glucose levels between WT and *Angptl8* KO rats (Fig. 6E), suggesting that *Angptl8* KO did not affect insulin sensitivity. Feeding a high-fat diet for 4 weeks induced insulin resistance and increased

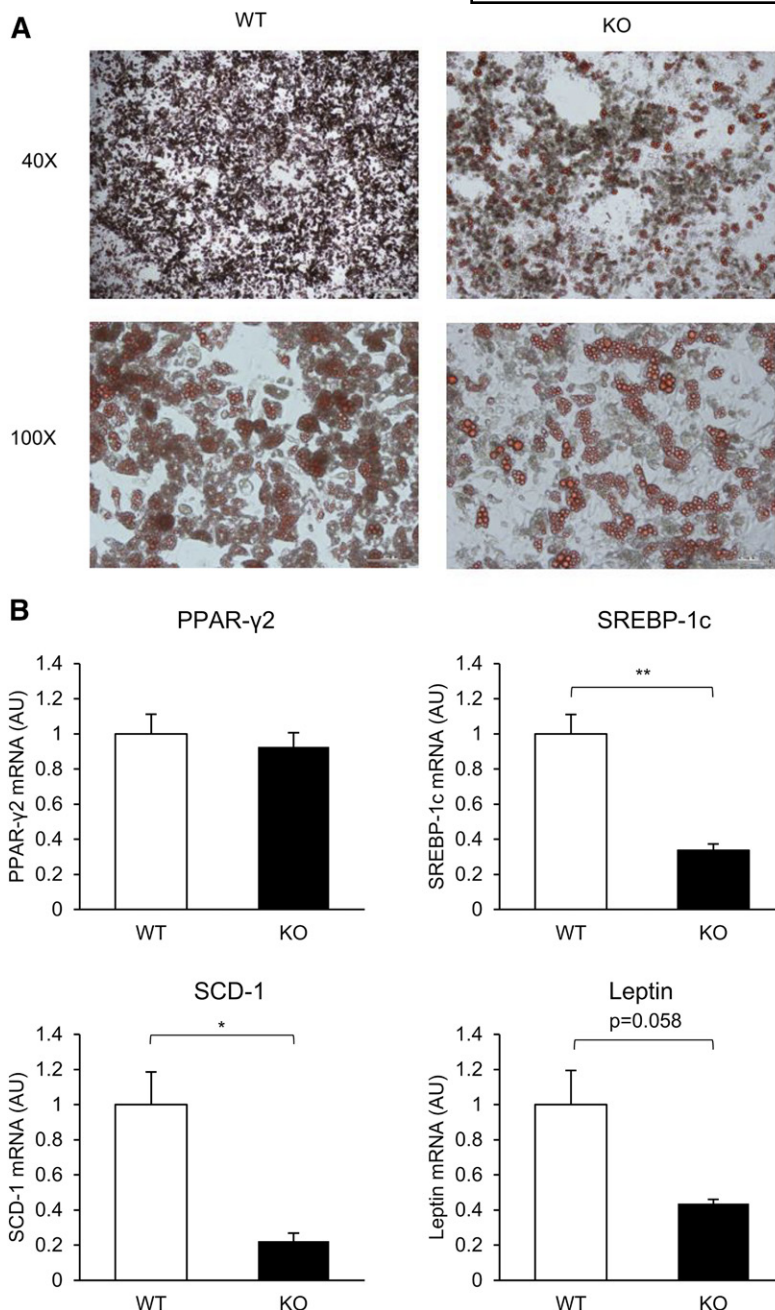


Fig. 3. Adipogenesis in *Angptl8* KO rats. A: Light microscopic images of differentiated adipocytes stained with Oil Red O derived from stromal vascular fraction cells of subcutaneous fat in WT and KO rats. B: Expression of adipocyte marker genes (n = 3–4 per group). Data were analyzed by *t*-test. **P* < 0.05, ***P* < 0.01.

plasma insulin levels in all rats, but there were again no differences in fasting blood glucose and plasma insulin levels between WT and *Angptl8* KO rats (Fig. 6A, B). Finally, we evaluated histologic changes in the pancreas. The increase in β -cell area of rats fed a high-fat diet for 34 weeks did not differ between WT and *Angptl8* KO rats (Fig. 6F).

DISCUSSION

To the best of our knowledge, this is the first report that CRISPR/Cas9-mediated *Angptl8* KO rats had reduced body weight and plasma TG levels without any changes in glucose metabolism. In addition, we demonstrated that deletion of *Angptl8* led to decreased fasting and postprandial plasma TG concentrations and increased expression of β -oxidation

genes in heart and muscle, without any extra lipid accumulation in heart, skeletal muscle, and liver. Rats are considered to be a better model than mice in terms of their physiologic and behavioral characteristics, which are in some respects more relevant to humans (20, 28, 29). As we have reported previously, for example, while Het *Pparg* KO mice have increased insulin sensitivity, Het *Pparg* KO rats show insulin resistance like patients with Het *Pparg* dominant-negative mutation (30). In this context, we have thus far generated KO rats for *Leptin* (31) and *Bscl2/Seipin* (32) besides *Pparg* (30) and investigated the roles of each gene. Therefore, we generated a rat model to analyze the functions of ANGPTL8. In this study, our data using *Angptl8* KO rats is similar to that found with *Angptl8* KO mice (11, 12), confirming physiologic roles of ANGPTL8 in energy, glucose, and lipid metabolism in two different species.

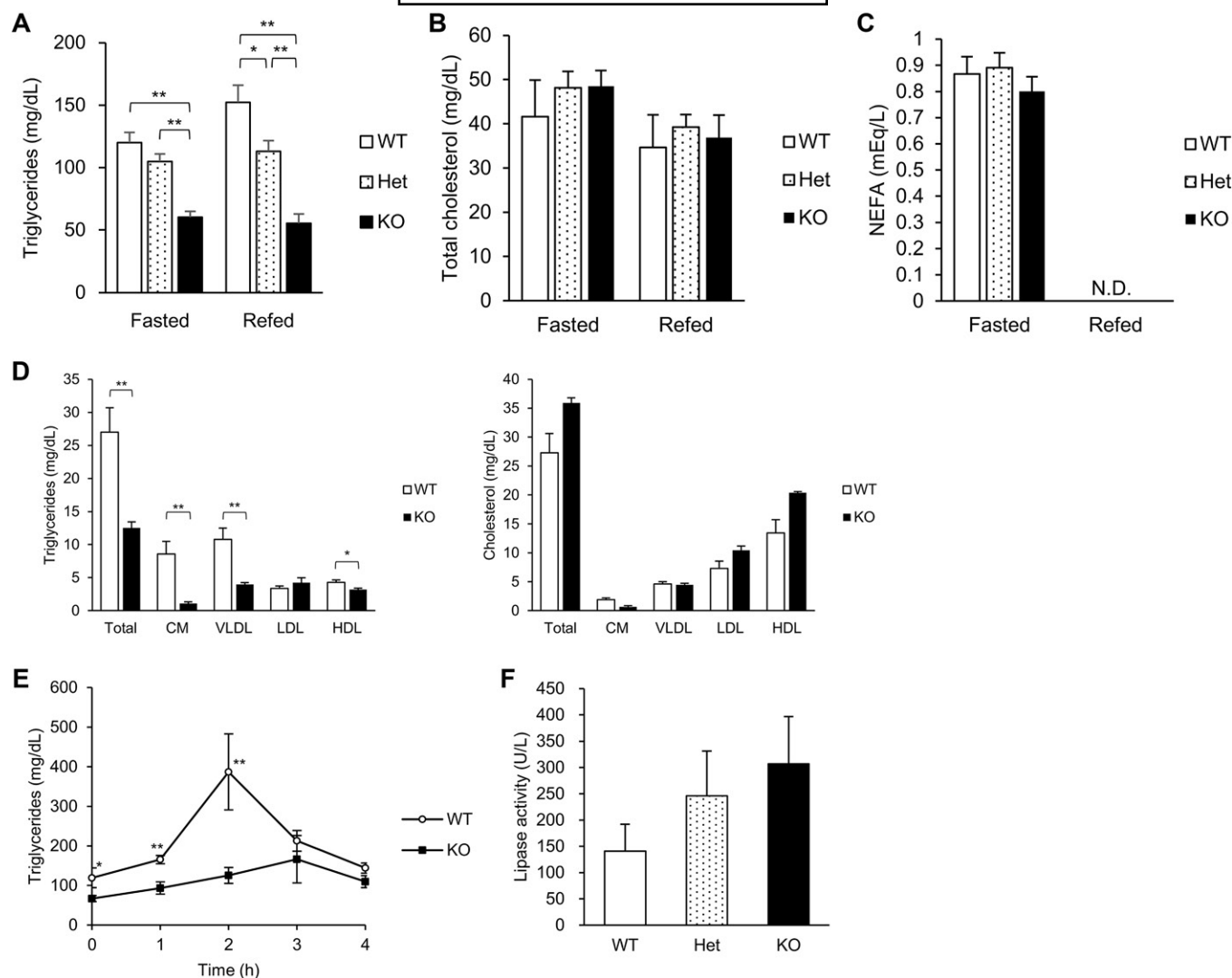


Fig. 4. Lipid metabolism in *Angptl8* KO rats. A–C: Plasma TG, total cholesterol, and NEFA levels in WT, Het, and KO rats fed a standard diet in the fasted and refed state ($n = 4$ –12 per group, age 12 weeks). Data were generated from four independent experiments. Data were analyzed by one-way factorial ANOVA followed by the Tukey-Kramer test. D: Plasma TG and cholesterol levels in lipoprotein fractions, including CMs, VLDL, LDL, and HDL in WT and KO rats measured by high-performance liquid chromatography ($n = 3$ –4 per group, age 12 weeks). Data were analyzed by *t*-test. E: Plasma TG levels during a FITT in WT and KO rats ($n = 3$ per group, age 20 weeks). Data were generated from two independent experiments. Data were analyzed by two-way factorial ANOVA with repeated measurement followed by the Tukey test. F: Postheparin plasma LPL activity of WT, Het, and KO rats fed with a standard diet ($n = 3$ –6 per group). Data were generated from two independent experiments. Data were analyzed by one-way factorial ANOVA followed by the Tukey-Kramer test. * $P < 0.05$, ** $P < 0.01$.

CRISPR/Cas9-mediated cleavage is achieved using the RNA-guided DNA nuclease, Cas9. Following generation of a double-stranded break, the DNA can be repaired by non-homologous end joining, which can generate KO mutations by the introduction of random deletions of a few to hundreds of base pairs (20). We obtained two lines of *Angptl8* KO rats with different deletion lengths, 7 bp and 980 bp, in the *ANGPTL8* gene (Fig. 1A). Decreased *ANGPTL8* transcript levels in KO rats were revealed both in lines #1 and #2 by real-time PCR (Fig. 1B). Unfortunately, because there are no good antibodies for rat *ANGPTL8*, we could not evaluate protein levels. Off-target mutations are a major concern with the CRISPR/Cas9 system (20–22). Therefore, we analyzed and carefully compared two lines of *Angptl8* KO rats to assess the adequacy of *Angptl8* KO and off-target mutations in our *Angptl8* KO rats. Both lines #1

and #2 exhibited similar phenotypes in terms of energy, glucose, and lipid metabolism (supplemental Fig. S3A–F), suggesting that the *ANGPTL8* gene was definitely knocked out and that off-target mutations were avoided in our *Angptl8* KO rats. Line #1 female *Angptl8* KO rats exhibited similar phenotypes to male *Angptl8* KO rats (supplemental Fig. S4A–F), indicating that the effect of *Angptl8* KO on energy, glucose, and lipid metabolism was similar regardless of the rats' sex.

Angptl8 KO suppressed weight gain in a dose-dependent manner, which was more evident when the rats were fed a high-fat diet (Fig. 2A, F). Suppression of weight gain in *Angptl8* KO rats was associated with reduced accumulation of adipose tissue (Fig. 2D, E), which is consistent with the phenotype of *Angptl8* KO mice (11). We speculate that there are two mechanisms underlying decreased adiposity. One of the mechanisms may be increased energy expenditure,

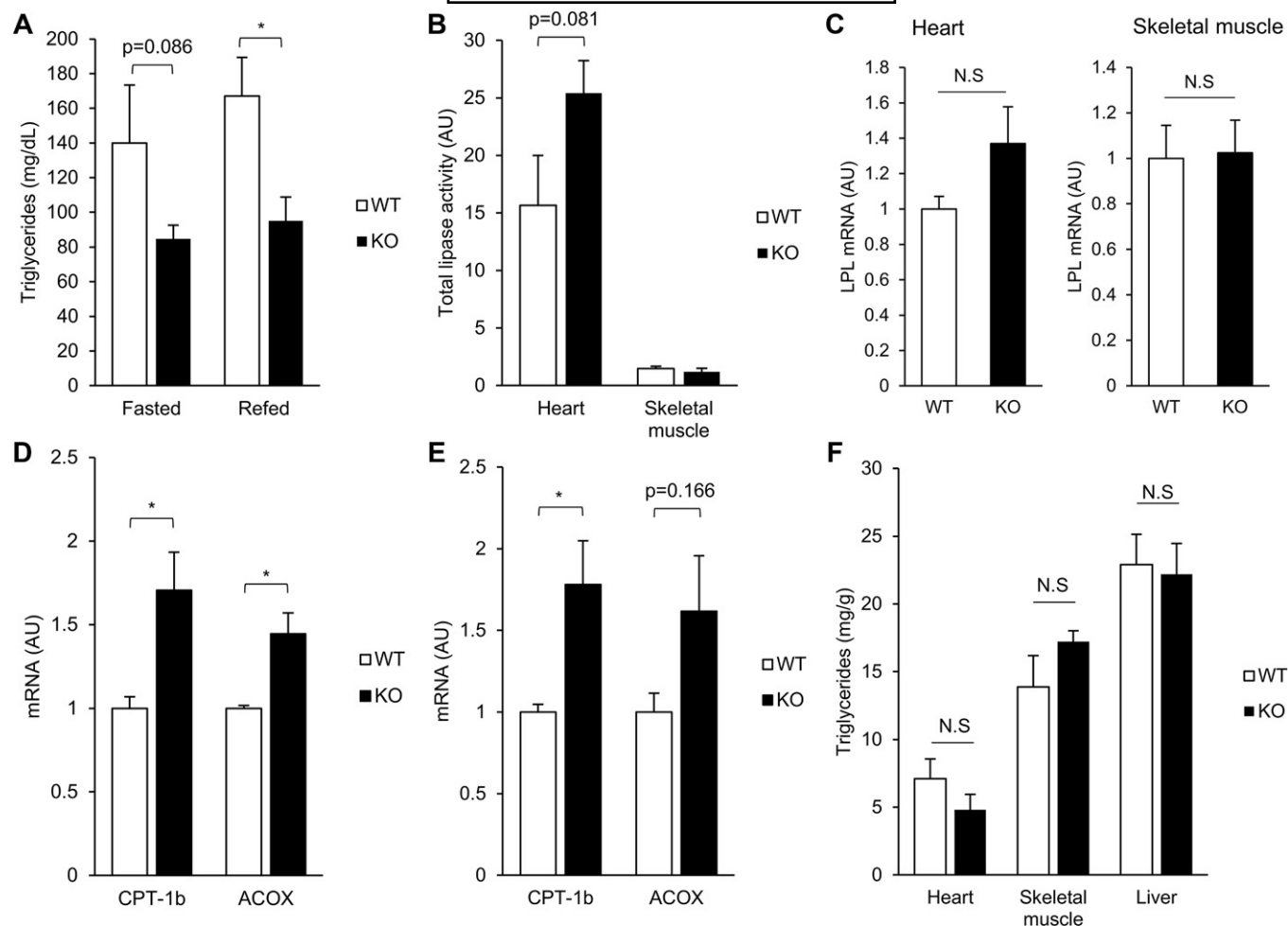


Fig. 5. Effects of *Angptl8* KO on lipase activity in oxidative tissues and ectopic fat accumulation. A: Plasma TG levels in WT and KO rats fed a high-fat diet in the fasted and refed state ($n = 4$ –12 per group, age 12 weeks). B: Total lipase activity in heart and skeletal muscle of WT and KO rats fed a high-fat diet ($n = 4$ –6 per group, age 30–42 weeks). C: Expression of LPL in heart and skeletal muscle of WT and KO rats fed a high-fat diet ($n = 4$ –6 per group, age 30–42 weeks). Expression of genes related to β -oxidation in heart (D) and skeletal muscle (E) of WT and KO rats fed a high-fat diet ($n = 4$ –6 per group, age 30–42 weeks). F: TG content in heart, skeletal muscle, and liver of WT and KO rats fed a high-fat diet ($n = 4$ –6 per group, age 30–42 weeks). Data were generated from three independent experiments. Data were analyzed by t -test. $*P < 0.05$.

because ANGPTL8 blockade with a monoclonal antibody reduced body fat content and weight secondary to increased energy expenditure in mice (33). Although we did not measure whole-body expenditure in the present study, there were no differences in rectal temperature or food intake (Fig. 2B, C). In addition, there were no differences in core body temperature or energy expenditure, evaluated by oxygen consumption and respiratory exchange ratio, between *Angptl8* KO mice and WT littermates (11). Taken together, the main cause of suppressed weight gain may be other than increased energy expenditure. The other mechanism may be due to the adipocytes themselves. The expression of ANGPTL8 is exclusive to mature adipocytes in the adipose tissue, and its level is markedly increased during adipogenesis (6). When we examined adipocyte differentiation using primary cultures, we found that lipid accumulation was impaired in *Angptl8* KO cultures (Fig. 3A). Knockdown of *Angptl8* in murine 3T3L1 cells with siRNA was reported to reduce lipid accumulation (6), which is compatible with our results in rats. Real-time PCR analyses showed that PPAR- γ 2 transcript levels were

similar, but lipogenic genes, such as SREBP-1c and SCD-1, were significantly lower in *Angptl8* KO compared with WT rats (Fig. 3B), suggesting that lipogenesis in adipocytes was impaired by *Angptl8* KO. The reason for suppression of weight gain in *Angptl8* KO rats remains to be elucidated.

Plasma TG levels in both the fasted and refed states were almost half in *Angptl8* KO rats compared with WT rats (Figs. 4A, 5B), and the iAUC during the FTT showed decreased plasma TG excursions in *Angptl8* KO rats (Fig. 4E). TG clearance is regulated by the balance of production and consumption of TG. Lipid synthesis and fatty acid oxidation in the liver, the main TG synthetic organ, were examined by gene expressions, but no differences were observed (supplemental Fig. S5). On the other hand, tissues such as the heart, skeletal muscle, and white adipose tissue rely on plasma TGs as an important source of fatty acids for oxidation or to be stored (34). Utilization of plasma TGs is dependent on LPL, which is attached to the capillary endothelium and catalyzes the hydrolytic cleavage of TGs into fatty acids. LPL is minutely regulated to adjust fatty acid uptake by multiple mechanisms at the transcriptional and

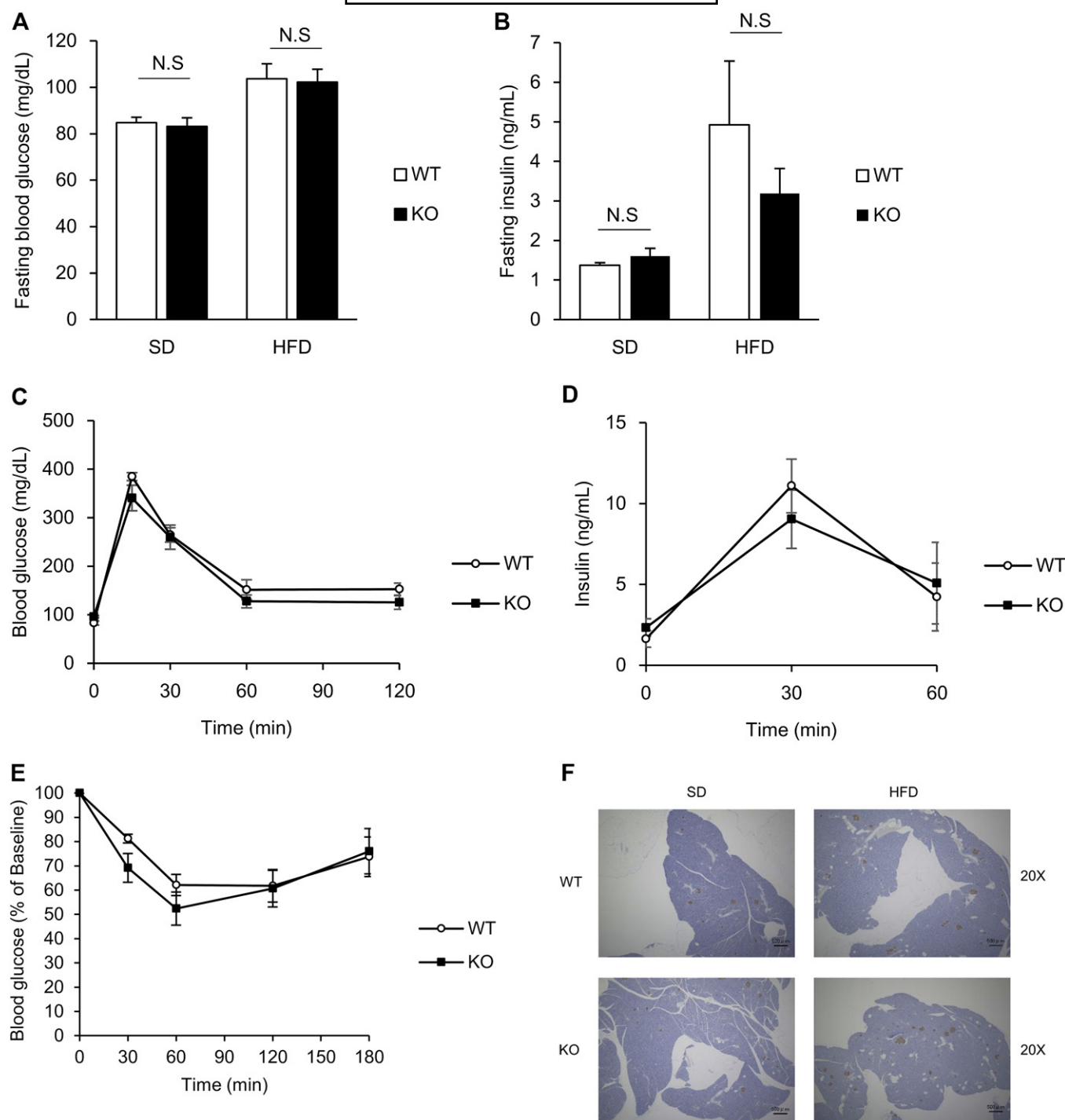


Fig. 6. Glucose metabolism in *Angptl8* KO rats. Fasting blood glucose (A) and fasting plasma insulin (B) levels of WT and KO rats fed a standard (SD) or a high-fat diet (HFD) ($n = 4-6$ per group, age 12 weeks). Data were generated from four independent experiments. Data were analyzed by *t*-test. Blood glucose levels (C) and plasma insulin levels (D) during a GITT in WT and KO rats ($n = 3-7$ per group, age 26 weeks). Data were generated from four independent experiments. Data were analyzed by two-way factorial ANOVA with repeated measurement followed by the Tukey test. E: Blood glucose levels during an ITT in WT and KO rats ($n = 3-7$ per group, age 27 weeks). Data were generated from four independent experiments. Data were analyzed by two-way factorial ANOVA with repeated measurement followed by the Tukey test. F: Representative images of the pancreas with staining for insulin in WT and KO rats (magnification $\times 20$, SD, age 37 weeks; HFD, age 42 weeks).

posttranslational levels to meet the requirements of the underlying tissue. In the fed state, LPL activity is increased in white adipose tissue and reduced in heart and skeletal muscle (34, 35), suggesting that LPL is triggered to deliver TGs to adipose tissue rather than to oxidative tissues in the fed state. In this study, the postheparin plasma LPL activity

appeared to be higher by *Angptl8* KO in a dose-dependent manner, although not significantly so (Fig. 4F). In addition, total lipase activity in the heart of fed *Angptl8* KO rats was slightly higher, though not significantly so, compared with that of WT rats (Fig. 5B). The transcriptional levels of LPL did not differ (Fig. 5C). LPL in the heart and the skeletal

muscle catalyzes lipolysis of plasma lipoproteins, reducing plasma TGs and providing free fatty acids as substrates for oxidation to produce energy (36, 37). Changes of LPL activity in heart and skeletal muscle, especially, can strongly affect plasma TG levels. For example, mice with cardiac muscle-specific overexpression of LPL reportedly have lower plasma TG levels (38). In addition, overexpression of CPT-1 in skeletal muscle reportedly increases fatty acid oxidation and decreases plasma TG levels in rats (39). In this study, expression levels of genes related to β -oxidation were significantly higher both in the heart and the skeletal muscle of fed *Angptl8* KO rats associated with increased LPL activity (Fig. 5D, E). These data suggest that greater LPL activity in *Angptl8* KO rats accelerated β -oxidation in oxidative tissues, leading to increased TG clearance and decreased plasma TG levels. However, the mechanism linking ANGPTL8 deficiency and decreased LPL activity with increased β -oxidation is unknown.

Glucose metabolism was unchanged in *Angptl8* KO rats (Fig. 6A–F), similar to findings in *Angptl8* KO mice (11, 12). Despite the findings of Chen et al. (15) that ANGPTL8 promoted β -cell proliferation in rats, we found that deletion of *Angptl8* did not affect β -cells or glucose metabolism in rats. According to the previous rat study, deletion of *Angptl8* was likely to exacerbate glucose metabolism. However, based on our data, suppression of ANGPTL8 for treating dyslipidemia in humans would not worsen the control of diabetes, which will provide new evidence for the translational research toward a therapy targeted to ANGPTL8 in humans. The discrepancy between studies of the same species may relate to differences in study design, the one using overexpression of ANGPTL8 and ours using deletion of ANGPTL8. Further investigation is needed to determine whether ANGPTL8 regulates β -cell replication in rats.

Increased plasma ANGPTL8 levels have been reported in several diseases, including obesity, dyslipidemia, and type 2 diabetes (16–19), disorders that are all risk factors for atherosclerosis leading to CVD (40). In this regard, inhibition of ANGPTL8 is anticipated as a potential therapy to prevent CVD events. In particular, despite lowering of plasma LDL-cholesterol levels with a statin, elevated TG levels may remain a residual risk for CVD (41, 42). The Japan Diabetes Complications Study, a prospective study to assess risk factors for coronary heart disease and stroke, showed that serum TG was a leading predictor of coronary heart disease (43). Therefore, decreasing ANGPTL8 levels, for example by a monoclonal antibody (33, 44), might well prevent CVD both by preventing weight gain and by substantially reducing plasma TG levels.

ANGPTL3 and ANGPTL4, having high homology with ANGPTL8, have also been reported as regulators of LPL activity and are expected to be potential targets to treat dyslipidemia (45). *Angptl3* KO mice showed increased postheparin plasma LPL activity and decreased plasma TG levels (46). A phase I study of antisense inhibitor of ANGPTL3 was conducted, reporting that decreased TG levels were observed in the treatment group (47). *Angptl4* KO mice were also reported to have increased postheparin plasma LPL activity (46), and treatment with monoclonal

antibody for mouse ANGPTL4 demonstrated that inhibition of ANGPTL4 led to decreased TG levels in mice (48). Similar to *Angptl8* KO rats, both *Angptl3* and *Angptl4* KO mice showed increased LPL activity in heart or skeletal muscle (49, 50). However, neither *Angptl3* nor *Angptl4* KO mice showed a significant change in body weight compared with WT mice (46). Although the reason for this discrepancy is not clear, decreased adipocyte expression of lipogenic genes, suggestive of impaired lipogenesis, could contribute to the lower body weight in *Angptl8* KO rats. With its unique effect on body weight, ANGPTL8 may be a more suitable target for treating dyslipidemia with obesity.

In conclusion, we generated for the first time *Angptl8* KO rats using the CRISPR/Cas9 system and demonstrated that *Angptl8* KO reduced body weight, adiposity, and plasma TG levels without affecting glucose metabolism. These data provide proof of concept for studying *Angptl8* KO in the rat model and indicate that ANGPTL8 may be a novel target in developing drugs to treat obesity and dyslipidemia. 

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