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RESEARCH ARTICLE

Role of gut microbiota in sex- and diet-dependent metabolic disorders that lead to early mortality of androgen receptor-deficient male mice

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Harada N, Hanada K, Minami Y, Kitakaze T, Ogata Y, Tokumoto H, Sato T, Kato S, Inui H, Yamaji R. Role of gut microbiota in sex- and diet-dependent metabolic disorders that lead to early mortality of androgen receptor-deficient male mice. *Am J Physiol Endocrinol Metab* 318: E525–E537, 2020. First published February 4, 2020; doi:10.1152/ajpendo.00461.2019.—The gut microbiota is involved in metabolic disorders induced by androgen deficiency after sexual maturation in males (late-onset hypogonadism). However, its role in the energy metabolism of congenital androgen deficiency (e.g., androgen-insensitive syndrome) remains elusive. Here, we examined the link between the gut microbiota and metabolic disease symptoms in androgen receptor knockout (ARKO) mouse by administering high-fat diet (HFD) and/or antibiotics. HFD-fed male, but not standard diet-fed male or HFD-fed female, ARKO mice exhibited increased feed efficiency, obesity with increased visceral adipocyte mass and hypertrophy, hepatic steatosis, glucose intolerance, insulin resistance, and loss of thigh muscle. In contrast, subcutaneous fat mass accumulated in ARKO mice irrespective of the diet and sex. Notably, all HFD-dependent metabolic disorders observed in ARKO males were abolished after antibiotics administration. The ratios of fecal weight-to-food weight and cecum weight-to-body weight were specifically reduced by ARKO in HFD-fed males. 16S rRNA sequencing of fecal microbiota from HFD-fed male mice revealed differences in microbiota composition between control and ARKO mice. Several genera or species (e.g., *Turicibacter* and *Lactobacillus reuteri*, respectively) were enriched in ARKO mice, and antibiotics treatment spoiled the changes. Furthermore, the life span of HFD-fed ARKO males was shorter than that of control mice, indicating that androgen deficiency causes metabolic dysfunctions leading to early death. These findings also suggest that AR signaling plays a role in the prevention of metabolic dysfunctions, presumably by influencing the gut microbiome, and improve our understanding of health consequences in subjects with hypogonadism and androgen insensitivity.

androgen-insensitive syndrome; longevity; metabolic syndrome; testosterone; type 2 diabetes

INTRODUCTION

Obesity has become a global public health problem (33). Abdominal visceral, but not subcutaneous, adiposity is a key characteristic of metabolic syndrome and an important risk factor for the development of type 2 diabetes mellitus (T2DM) (12, 33). The increasing prevalence of obesity, especially in mesenteric adipose deposits, and T2DM is also associated with nonalcoholic fatty liver disease (32, 50). These metabolic disorders are all associated with cardiovascular risk and early death (1, 12, 50). Accumulating evidence indicates that the gut microbiota is associated with energy metabolism and related diseases (28, 31, 34, 36, 41, 46, 53).

Androgens are a group of male sexual hormones involved in the development of sex-specific morphology and behavior (24, 35). Testosterone is a major circulating androgen secreted by the testis. Meta-analysis of prospective cohort studies (18) revealed that men with low testosterone levels are at an increased risk of abdominal obesity (5), metabolic syndrome (9), T2DM (8, 13), and cardiovascular and all-cause mortality (2, 10). Meta-analyses also indicate that androgen deprivation therapy, combined with chemical or physical castration, of subjects with prostate cancer leads to obesity (23), metabolic syndrome (4), diabetes (4, 54), and cardiovascular morbidity and mortality (3, 59). Intervention in the form of a testosterone replacement therapy improves these outcomes in subjects with low testosterone levels (18). Testosterone levels decrease with age (30) and are inversely associated with the age-related risk of metabolic disorders in men. In addition to late-onset hypogonadism after sex maturation in men, disorders of sex differentiation are suggested to be associated with increased risks of metabolic diseases as well (11, 17, 56). Androgen action is mediated by binding to the androgen receptor (AR), a member of the nuclear receptor superfamily of ligand-activated transcription factors. Recently, GPRC6A (a G protein-coupled receptor for L-amino acids), OXER1 (a G protein-coupled receptor for oxoeicosanoids), and ZIP9 (a zinc transporter) have also been found to mediate the action of androgens (29, 45, 51).

Despite the accumulating clinical evidence for the role of androgens in energy metabolism, the mechanisms by which a decline of testosterone levels induces metabolic disorders remain poorly understood. We have recently shown that castration after sexual maturation causes metabolic disorders in male

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mice fed high-fat diet (HFD) and that these disorders are not induced in mice with orally administered antibiotics (19, 20). These observations suggested that changes in the gut microbiota might act as a trigger of metabolic disorders upon androgen deprivation. Gut microbiota profiles are affected by sex hormones and sexual maturation (38, 44, 57). Disorders of sex differentiation cause ambiguity in genitalia and defects in testosterone biosynthesis and sexual maturation (55). However, the role of gut microbiota in the energy metabolism of subjects with disorders of sex differentiation remains unclear. In the present study, we examined the effects of gut microbiota on energy metabolism in AR knockout (ARKO) mice, a model for androgen insensitive syndrome (55), by administering antibiotics. Furthermore, we analyzed the sex-associated differences in metabolic symptoms caused by androgen deficiency in the ARKO model. We found that metabolic disorders are induced in HFD-fed ARKO males, and the gut microbiota prolife in HFD-fed ARKO males is different from that in HFD-fed control males. These findings are consistent with the metabolic disorders observed in subjects with disorders of sex differentiation.

MATERIALS AND METHODS

Animals. The *Ar* gene is located on the X chromosome, and ARKO male mice are infertile. Consequently, *Ar* null female mice cannot be obtained by breeding. Conditional ARKO male and female mice were therefore generated by using Cre-loxP system. $Ar^{flox/flox}$ female mice (Ar^{Tm1Ska}, C57BL/6 background) (47, 48) were crossed with male mice (C57BL/6 background) expressing Cre recombinase under the control of a ubiquitous hybrid CAG promoter (consisting of the cytomegalovirus immediate-early enhancer, the chicken beta-actin transcription start site, and a rabbit beta-globin intron with a 3′-splice acceptor site) (CAG-*cre*) (40) to generate conditional global ARKO male mice (CAG-*cre*; $Ar^{\Delta/y}$) and littermate controls ($Ar^{flox/y}$). ARKO female mice (CAG-*cre*; $Ar^{\Delta/\Delta}$) were generated by crossing $Ar^{flox/y}$ males and AR hetero-knockout females (CAG-*cre*; $Ar^{+/\Delta}$) and compared with littermate controls ($Ar^{+/flox}$). Genomic DNA was isolated from the ear and was used for genotyping to evaluate the presence of the *cre* and *Sry* genes and the truncation of the *Ar* gene. PCR was performed by using the following primers: *cre*, sense: 5′-GTTTCACTGGTTATGCGGCGG-3′ and antisense: 5′-TTCCAGGGCGCGCGAGTTGATAG-3′; *Sry*, sense: 5′-TTGTCTAGAGAGGCATGGAGGGCCATGTCAA-3′ and antisense: 5′-CCACTCCTCTGTGACACTTTAGCCCTCCGA-3′; *Il2*, sense: 5′-CTAGGCCACAGAATTGAAAGATCT-3′ and antisense: 5′- GTAGGTGGAAATTCTAGCATCATCC-3′; *Fabpi*, sense: 5′-CCTCCGGAGAGCAGCGATTAAAAGTGTCAG-3′ and antisense: 5′-TAGAGCTTTGCCACATCACAGGTCATTCAG-3′; and *Ar*, sense 1: 5′-AAGTGAATGGTCTTGGC-3′, antisense: 5′-TTACAGGTCTGGTGCAAGCC-3′, and sense 2: 5′-TGTTTTCACTGTCACTGCAGC-3′. Genes *Il2* and *Fabpi* were used as the internal controls in PCR reactions to analyze the *cre* and *Sry* status, respectively.

Mice were maintained under conventional conditions with controlled temperature (23 ± 3°C) and lighting (12:12-h light-dark cycle, lighting period starting at 0800). For the intraperitoneal glucose tolerance test (IPGTT) and insulin tolerance test (ITT), mice were fasted for 6 h, and then 2 g/kg glucose or 1 U bovine insulin were injected intraperitoneally, respectively. The blood was collected from the tail vein, and glucose levels were measured by using OneTouch Ultra (Johnson & Johnson, Milpitas, CA). The feces were collected from 17-wk-old animals for 1 wk, and freeze-dried fecal weight was measured. The digestion efficiency in 17-wk-old animals was calculated by dividing the dried fecal weight by consumed food weight. Body temperature was measured with a rectal thermometer (KN-91, Natsume Seisakusho, Tokyo, Japan) between 1400 and 1500 in 17-wk-old animals. Mice were euthanized at 20-wk-old under anesthesia after 4 h of fasting, and the plasma and organs were obtained for further analysis. All animal experiments were approved by the Animal Care and Use Committee of Osaka Prefecture University and were performed in compliance with its guidelines.

Diets and antibiotics. Mice were fed with ad libitum access to standard chow diet (SD; CE2, CLEA Japan, Tokyo, Japan) and water. After being weaned at 4 wk of age, mice were individually housed and

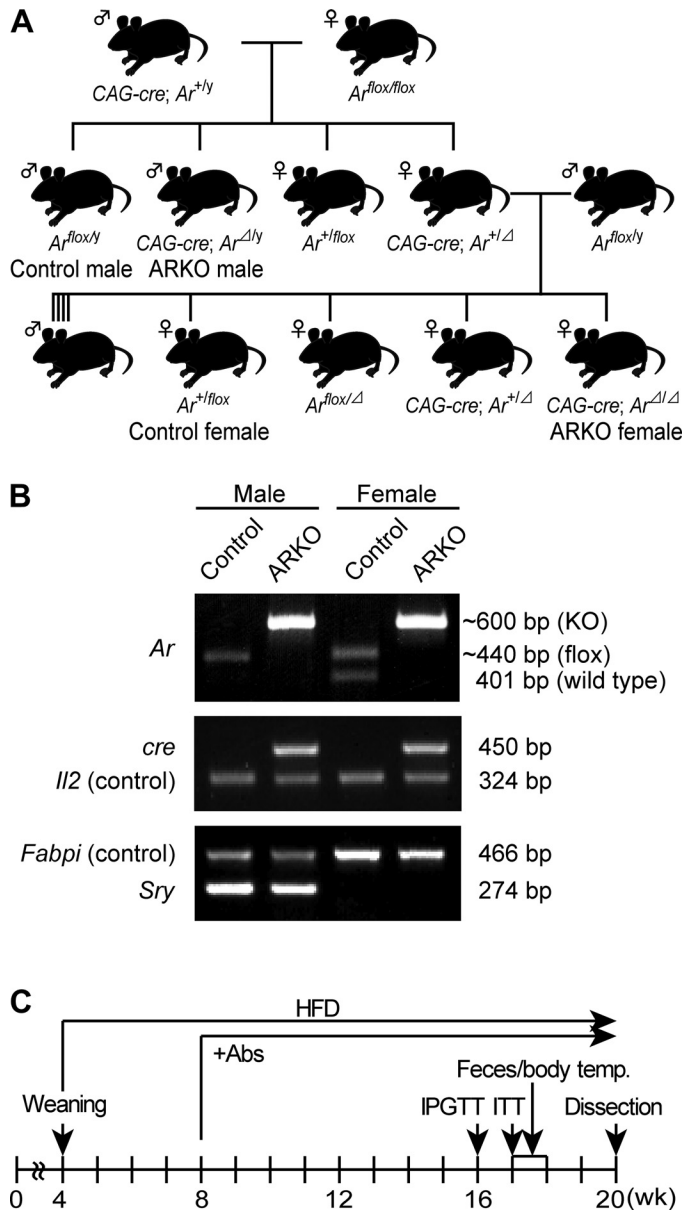

Fig. 1. Schema of generation of androgen receptor knockout (ARKO) and littermate control mice and their genotyping patterns and breeding. *A*: the *Ar* gene is located on the X chromosome. $Ar^{flox/flox}$ female mice were crossed with male CAG-*cre* mice to generate conditional global ARKO male mice (CAG-*cre*; $Ar^{\Delta/y}$) and littermate controls ($Ar^{flox/y}$). ARKO female mice (CAG-*cre*; $Ar^{\Delta/\Delta}$) and littermate controls ($Ar^{+/flox}$) were generated by crossing $Ar^{flox/y}$ males and AR hetero-knockout females (CAG-*cre*; $Ar^{+/\Delta}$). *B*: genotypic evaluation of *cre*, *Sry*, and truncation of *Ar*. *Il2* and *Fabpi* were used as the internal controls in PCR reactions to analyze *cre* and *Sry* status, respectively. *C*: experimental scheme of this study. Abs; antibiotics; HFD, high-fat diet; IPGTT, intraperitoneal glucose tolerance test; ITT, insulin tolerance test.

fed SD or HFD [Quick Fat, containing 25% (wt/wt) sucrose, CLEA Japan]. For the longevity analysis, the mice were housed in groups of control or ARKO mice. The percentage energy sources of the SD (3.44 kcal/g) were as follows: protein, 29%; carbohydrate, 58%; and fat, 13%. Those of the HFD (4.12 kcal/g) were as follows: protein, 24%; carbohydrate, 46%; and fat, 30%. Antibiotics (1 g/L ampicillin sodium salt and 1 g/L neomycin) were dissolved in drinking water. Feed efficiency was calculated each week based on body weight gain, divided by calorie intake, using the cumulated value from 4 wk of age.

Tissue staining. Paraffin sections (4 μm thick) were prepared as described elsewhere (22) and stained with hematoxylin and eosin. Tissue sections were observed under a light microscope (BZ9000, Keyence, Osaka, Japan). Densitometry measurements were performed by using ImageJ software version 1.48 [Research Resource Identifier (RRID):SCR_003070, National Institutes of Health, Bethesda, MD].

Determination of insulin, triglyceride, fatty acid, cholesterol, and adiponectin levels. Triglyceride, free fatty acid, and cholesterol levels in the liver or feces were determined by using the triglyceride E-Test (Wako, Osaka, Japan), NEFA C-Test (Wako), and cholesterol E-Test

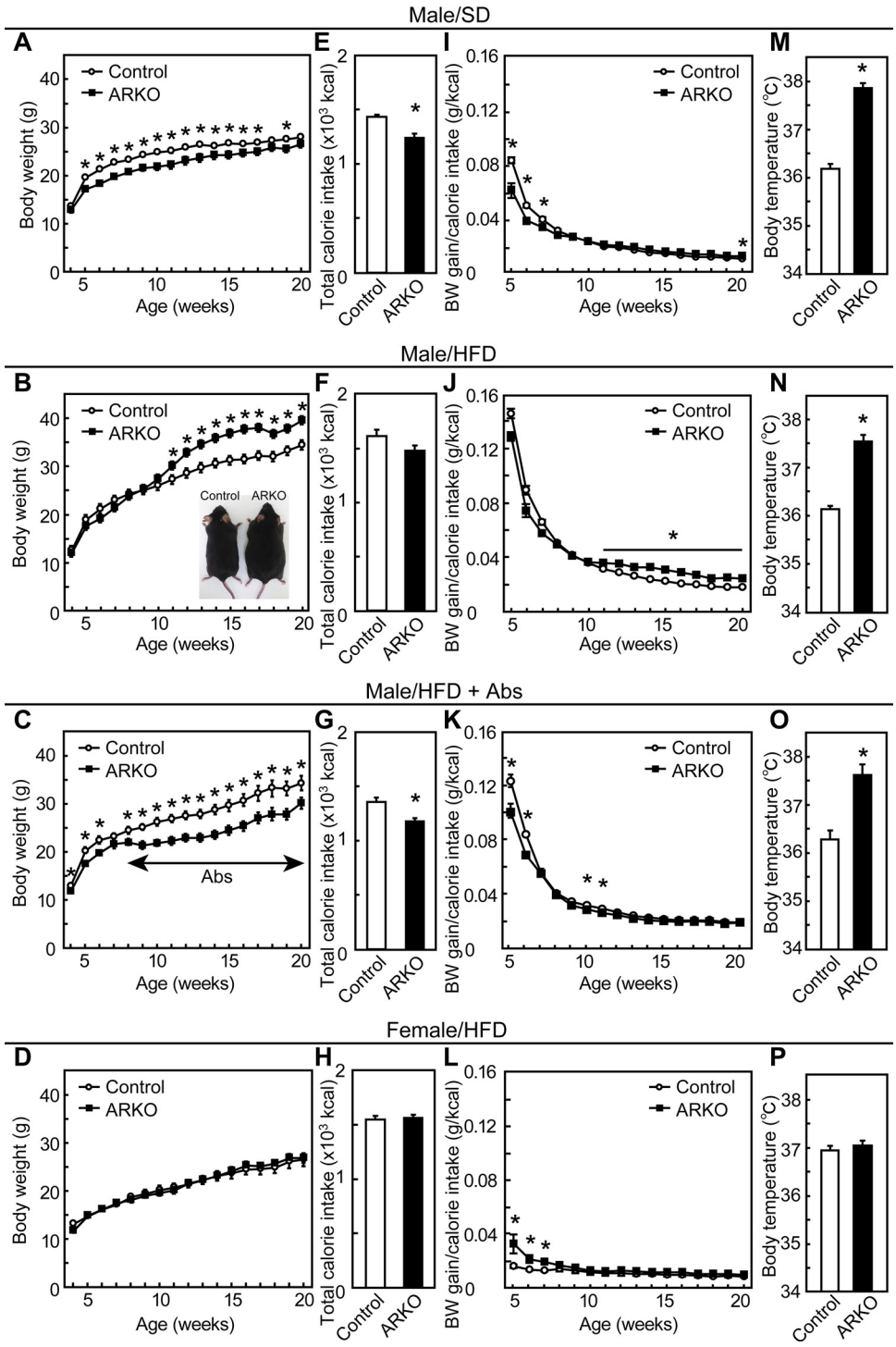

Fig. 2. Effect of androgen receptor knockout (ARKO) on the body weight, total calorie intake, cumulative feed efficiency, and body temperature in mouse. *A–P*: data are shown for males fed standard diet (SD) (*A*, *E*, *I*, and *M*); males fed high-fat diet (HFD) (*B*, *F*, *J*, and *N*); males fed HFD with antibiotics (Abs) (*C*, *G*, *K*, and *O*); and females fed HFD (*D*, *H*, *L*, and *P*). *A–D*: body weight. *E–H*: total calorie intake in 4- to 20-wk-old animals. *I–L*: calculated cumulative feed efficiency for 4-wk-old and older animals; BW, body weight. *M–P*: rectal body temperature. Data are expressed as the means ± SE. *Statistically significant differences [$P < 0.05$; $n = 10–19$ (*A–C*); $n = 5–7$ (*D*); $n = 5–15$ (*E–P*)].

(Wako), respectively, as described previously (20). To determine the adiponectin levels, plasma samples were resolved by SDS-PAGE, followed by Western blotting with polyclonal anti-adiponectin antibody (1/3,000, RRID: AB_367259, GTX23455, GeneTex, Irvine, CA). After reaction with anti-rabbit secondary antibody conjugated with horseradish peroxidase (1/10,000, RRID: AB_11125142, Bio-Rad, Hercules, CA), immunoreactive bands were visualized by using LAS4000 (GE Healthcare, Piscataway, NJ) as described previously (21). Band intensity was determined using ImageJ software version 1.48.

Analysis of the gut microbiota by 16S rRNA sequencing and real-time PCR. Genetic DNA of the gut microbiota was extracted from the feces as follows. Fresh feces (~100 mg) were suspended in 1.4 mL PBS by vortex mixing. After being incubated for 1 min, the supernatant (300 μL) was centrifuged at 20,000 *g* for 3 min. The

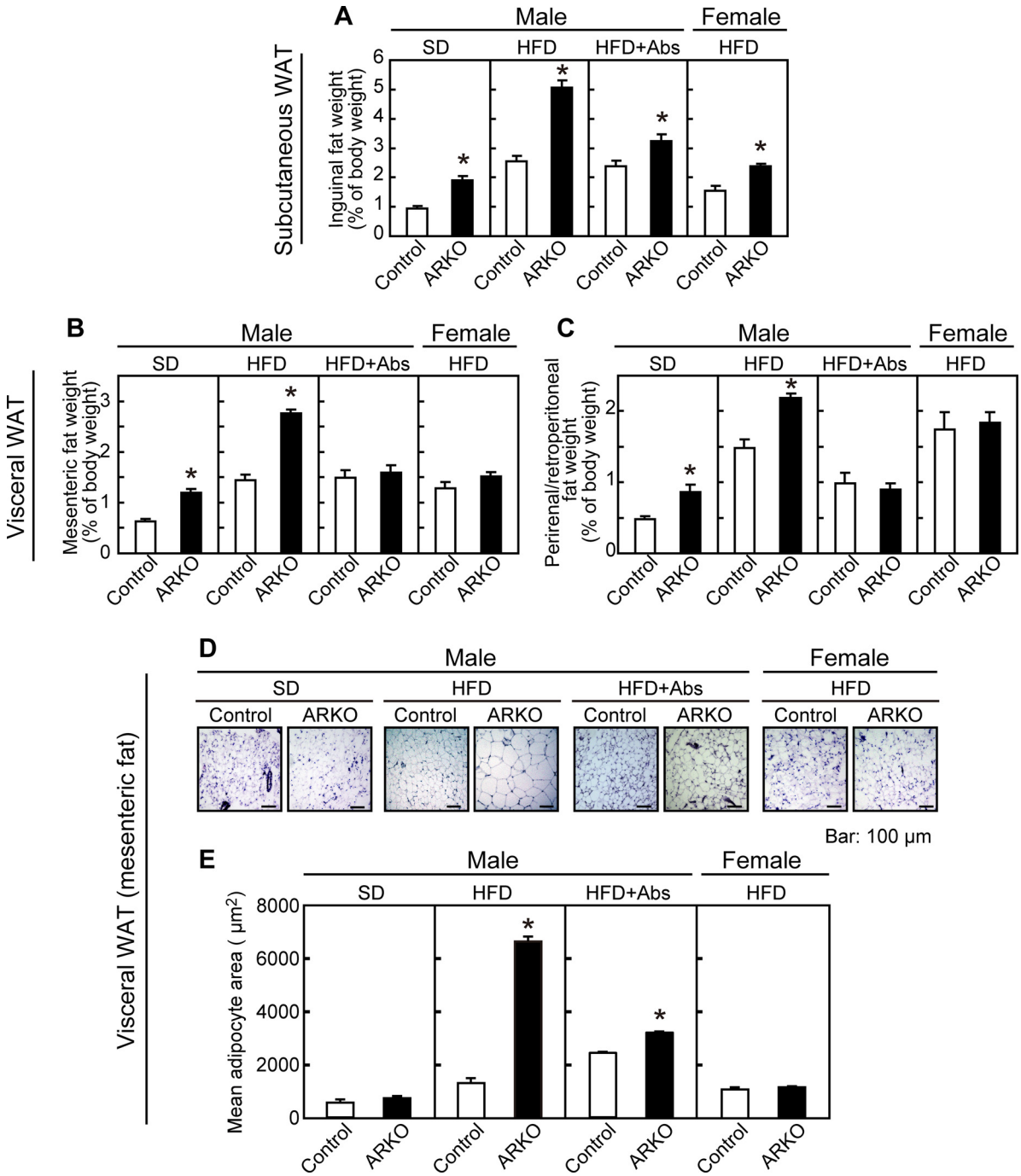

Fig. 3. Characterization of the visceral mesenteric adipose tissue in control and androgen receptor knockout (ARKO) mice. *A–C*: subcutaneous inguinal (*A*), visceral mesenteric (*B*), and visceral perirenal/retroperitoneal white adipose tissue (WAT) weights (*C*). *D*: representative images of mesenteric adipose tissue. Scale bar = 100 μm. *E*: mean adipocyte area. Data are expressed as means ± SE. *Statistically significant differences [$P < 0.05$; $n = 5$ (*A–E*), male standard diet (SD) control; $n = 6$ (*A–E*), male SD ARKO; $n = 19$ (*A–C*) or 5 (*D* and *E*), male high-fat diet (HFD) control; $n = 16$ (*A–C*) or 5 (*D* and *E*), male HFD ARKO; $n = 10$ (*A–C*) or 7 (*D* and *E*), male HFD + antibiotics (Abs) control; $n = 15$ (*A–C*) or 10 (*D* and *E*), male HFD + Abs ARKO; $n = 5$ (*A–E*), female HFD control; $n = 7$ (*A–C*) or 6 (*D* and *E*), female HFD ARKO].

pellet was resuspended in 150 μL of lysis buffer [100 mM Tris·HCl, pH 7.5, 4 M guanidine thiocyanate, 5% (wt/vol) *N*-lauroylsarcosine sodium salt, 10 mM EDTA, and 1% (vol/vol) 2-mercaptoethanol], the fecal bacteria were disrupted using zirconia beads (ϕ5 bead and 0.5 g of ϕ0.2-mm beads, TORAY, Osaka, Japan), and a multibeads shocker [MB755U(S), Yasui Kikai, Osaka, Japan] for 1 min at 2,500 rpm. Then, 150 μL of 1% (wt/vol) SDS were added and the lysate was incubated for 10 min at 70°C. After phenol-chloroform extraction, the genomic DNA was purified using DNA fragments extraction Kit (RBC Bioscience, New Taipei City, Taiwan). The V3–V4 region of 16S rRNA gene was PCR-amplified using PrimeSTAR GXL DNA polymerase (Takara Bio, Shiga, Japan) and specific primers (sense, 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′; and antisense, 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′). After purification using AMPure XP Beads (Beckman Coulter, Brea, CA), the amplicons were modified by attaching Illumina sequencing adaptors (Illumina, San Diego, CA) and dual-index barcode sequences by using PCR (Index PCR) with Kapa HiFi DNA polymerase (Kapa Biosystems, Cape Town, South Africa), followed by purification using AMPure XP Beads. The purified PCR products were subjected to MiSeq Reagentkit v2500 Cycle (Illumina) processing and analyzed using MiSeq (Illumina). Data were analyzed using the Quantitative insights into Microbial Ecology (QIIME, RRID: SCR_008249) pipeline. Clean Fastq data were aligned into operational taxonomic units (OTUs) at 97% similarity and profiled against a 16S rRNA database (Green genes, ver. gg_13_8). Principal coordinate analysis (PCoA) was performed to plot the variation of Bray-Curtis distances between samples using R software (RRID: SCR_001905).

Real-time PCR was performed using TB Green Premix Ex *Taq* II (Takara Bio) and specific primers [*Turicibacter* spp.: sense, 5′-CAGACGGGGACAACGATTGGA-3′ and antisense, 5′- TACGCATCGTCGCCTTGGTA-3′ (49); *Lactobacillus reuteri*: sense, 5′-GAACGCAYTGGCCCAA-3′ and antisense, 5′-TCCATTGTGGCCGATCAGT-3′ (39); and all bacteria: sense, 5′-GTGSTGCAYGGYTGTCGTCA-3′ and antisense, 5′-ACGTCRTCCMCACCTTCCTC-3′ (37)] with the extracted genomic DNA from the fecal microbiota as a template. The relative expression of target genes was calculated using the Ct value fitted to a standard curve that was obtained from a series of diluted cDNA and normalized by that of all bacteria.

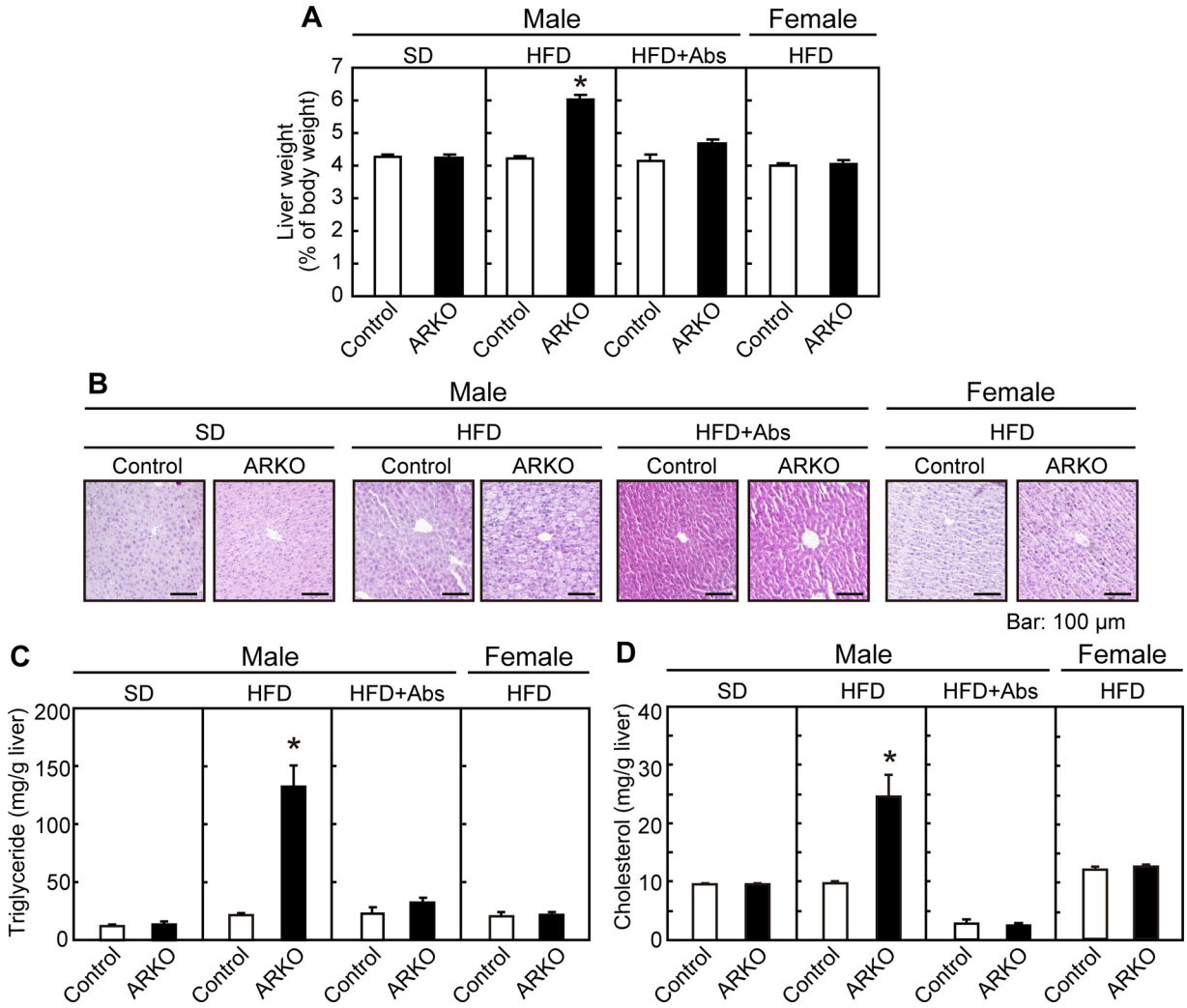

Fig. 4. Effect of androgen receptor knockout (ARKO) on lipid profiles in the mouse liver. *A*: relative liver weight in %body weight. *B*: representative images of liver sections. Scale bar = 100 μm. *C* and *D*: triglyceride (*C*) and (*D*) cholesterol levels in the liver. Data are expressed as means ± SE. *Statistically significant differences [$P < 0.05$; $n = 5$ (*A–D*), male standard diet (SD) control; $n = 6$ (*A–D*), male SD ARKO; $n = 19$ (*A*) or 8 (*B–D*), male high-fat diet (HFD) control; $n = 16$ (*A*) or 5 (*B–D*), male HFD ARKO; $n = 10$ (*A–D*), male HFD + antibiotics (Abs) control; $n = 15$ (*A–D*), male HFD + Abs ARKO; $n = 5$ (*A–D*), female HFD control; $n = 7$ (*A–D*), female HFD ARKO].

Table 1. *Plasma lipid levels and fecal lipid excretion in mice*

	Male/SD		Male/HFD		Male/HFD + Abs		Female/HFD	
	Control	ARKO	Control	ARKO	Control	ARKO	Control	ARKO
Plasma								
Triglyceride, mg/dL	49.0 ± 11.6	79.0 ± 11.7	25.5 ± 6.8	49.0 ± 5.3	53.3 ± 3.7	54.3 ± 3.6	65.0 ± 6.0	77.6 ± 18.2
Cholesterol, mg/dL	70.3 ± 14.0	64.2 ± 12.7	104.4 ± 8.6	127.8 ± 7.2	101.0 ± 7.0	105.8 ± 5.9	76.6 ± 13.5	72.3 ± 10.7
Free fatty acid, mEq/L	2.59 ± 0.09	3.19 ± 0.29	2.62 ± 0.15	2.55 ± 0.13	2.02 ± 0.12	2.42 ± 0.12*	2.44 ± 0.28	3.27 ± 0.26
Fecal excretion								
Triglyceride, mg/day	1.56 ± 0.15	1.20 ± 0.16	1.74 ± 0.14	1.48 ± 0.22	0.82 ± 0.10	0.64 ± 0.09	0.66 ± 0.07	0.77 ± 0.07
Cholesterol, mg/day	4.05 ± 0.31	3.06 ± 0.20*	2.41 ± 0.15	1.40 ± 0.14*	0.70 ± 0.09	0.62 ± 0.06	1.08 ± 0.06	1.74 ± 0.13*

Data are expressed as means ± SE. Plasma and fecal samples were collected from 20-wk-old and 17-wk-old mice, respectively. (n = 5–15). ARKO, androgen receptor knockout; HFD, high-fat diet; Abs, antibiotics; SD, standard diet. *$P < 0.05$, statistical differences were determined between control mice and ARKO mice.

Statistical analysis. Data were analyzed by *t*-test or one- or two-way ANOVA with Tukey-Kramer's post hoc testing or log-rank test using JMP statistical software version 8.0.1 (SAS Institute, Cary, NC). Data are shown as means ± SE, and differences at $P < 0.05$ were considered statistically significant.

RESULTS

ARKO affects body weight, calorie intake, feed efficiency, and body temperature in mouse. ARKO mice and control littermate mice were generated by using the Cre-loxP system as described in the MATERIALS AND METHODS and illustrated in the schema shown in Fig. 1*A*. The genotyping patterns of *cre, Sry* (located on the Y-chromosome), and *Ar* from mice and the study experimental design are presented in Fig. 1, *B* and *C*, respectively. To examine whether the diet and sex affect energy metabolism in ARKO mouse, the body weight of ARKO mice was determined until the animals became 20 wk old. The body weight of ARKO SD-fed males was lower than that of control males throughout almost the entire observation

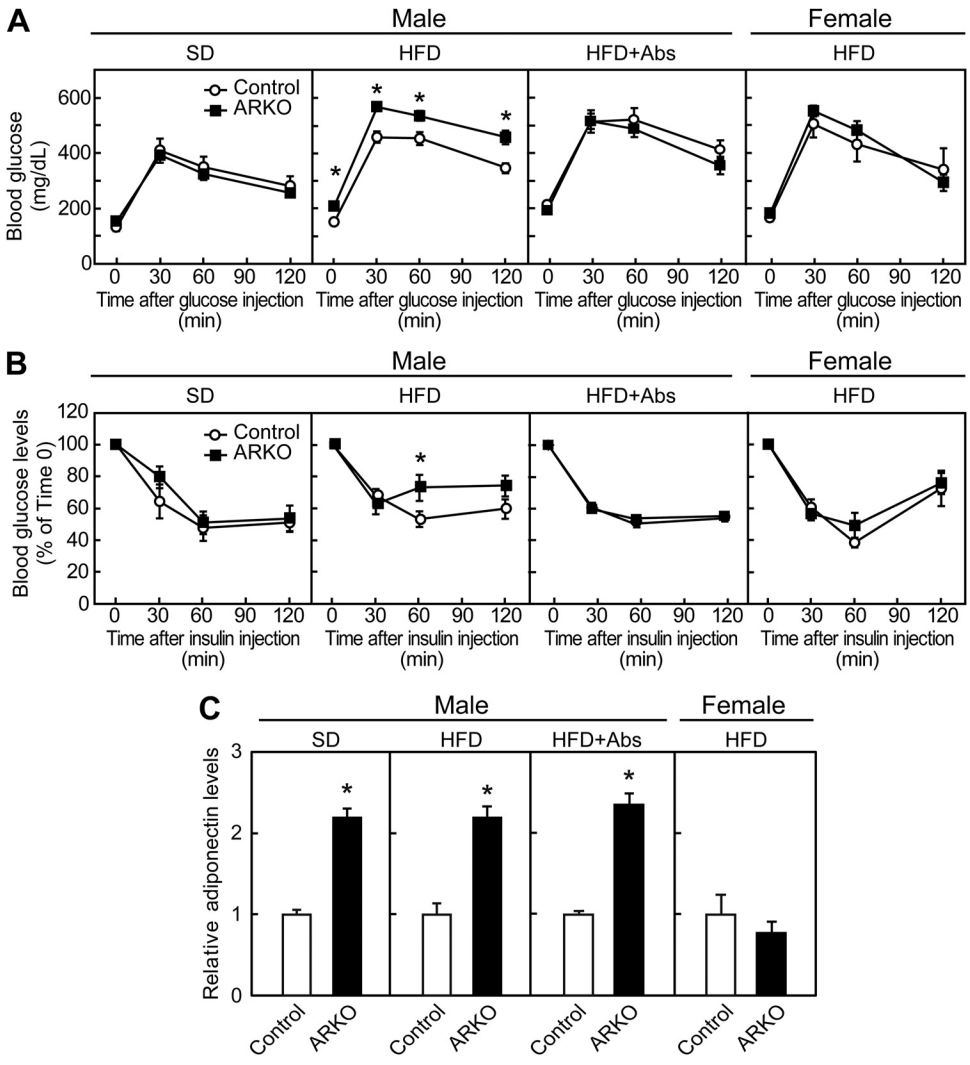

Fig. 5. Effect of androgen receptor knockout (ARKO) on glucose and insulin tolerance in mouse. *A*: intraperitoneal glucose tolerance test (IPGTT) in 17-wk-old animals. *B*: insulin tolerance test (ITT) at 18-wk-old animals. *C*: adiponectin levels in 20-wk-old animals. Data are expressed as means ± SE. *Statistically significant differences [$P < 0.05$; IPGTT and ITT: n = 5, male standard diet (SD) control; n = 6, male SD ARKO; n = 12, male high-fat diet (HFD) control; n = 11, male HFD ARKO; n = 10, male HFD + antibiotics (Abs) control; n = 15, male HFD + Abs ARKO; n = 6, female HFD control; n = 8, female HFD ARKO, adiponectin: n = 5–8].

period (Fig. 2*A*). By contrast, when mice were fed HFD, the body weight of ARKO males significantly exceeded that of the control males in mice older than 11 wk (Fig. 2*B*). Notably, the HFD-dependent overshoot of body weight in ARKO males was abolished when the mice were administered an antibiotic cocktail from 8 wk old (Fig. 2*C*). Meanwhile, in females, ARKO did not affect body weight even when the mice were fed HFD (Fig. 2*D*). The decrease of body weight in SD-fed ARKO males likely reflected reduced food intake (Fig. 2*E*) because the cumulated feed efficiency (i.e., body weight gain/calorie intake) was similar between the SD-fed groups, although some differences were observed during the initial and final periods (Fig. 2*I*). By contrast, in HFD-fed males, cumulative feed efficiency was significantly higher in the ARKO group than in the control group in animals that were older than 11 wk, without increased food intake (Fig. 2, *F* and *J*). Such HFD-dependent augmentation of feed efficiency, after considering food intake, in ARKO males was abolished by administration of antibiotics (Fig. 2, *G* and *K*). In females fed HFD, ARKO did not affect the total calorie intake and feed efficiency in mature animals (Fig. 2, *H* and *L*). In general, body temperature has a negative impact on feed efficiency because of increased energy usage for heat production. Body temperature was specifically increased in ARKO males, but not in ARKO females, in a diet- and antibiotic-independent manner (Fig. 2, *M–P*). The change in energy expenditure by heat production was inappropriate for the specific body weight gain in HFD-fed ARKO males. Collectively, these results indicated that the body weight gain accompanying the increase in feed efficiency occurred in an HFD-dependent and male-specific manner. These effects were abrogated by the administration of antibiotics, suggesting the involvement of gut microbiota.

ARKO potentiates lipid accumulation in adipocytes and hepatocytes. We next characterized the visceral and subcutaneous white adipose tissue (WAT) in the animals. ARKO increased subcutaneous inguinal WAT irrespective of sex, diet, or antibiotic treatment (Fig. 3*A*). By contrast, visceral mesenteric or perirenal/retroperitoneal WAT increased in ARKO male mice irrespective of the diet but not in antibiotics-treated male and HFD-fed female mice (Fig. 3, *B* and *C*). We then analyzed adipocyte size in the

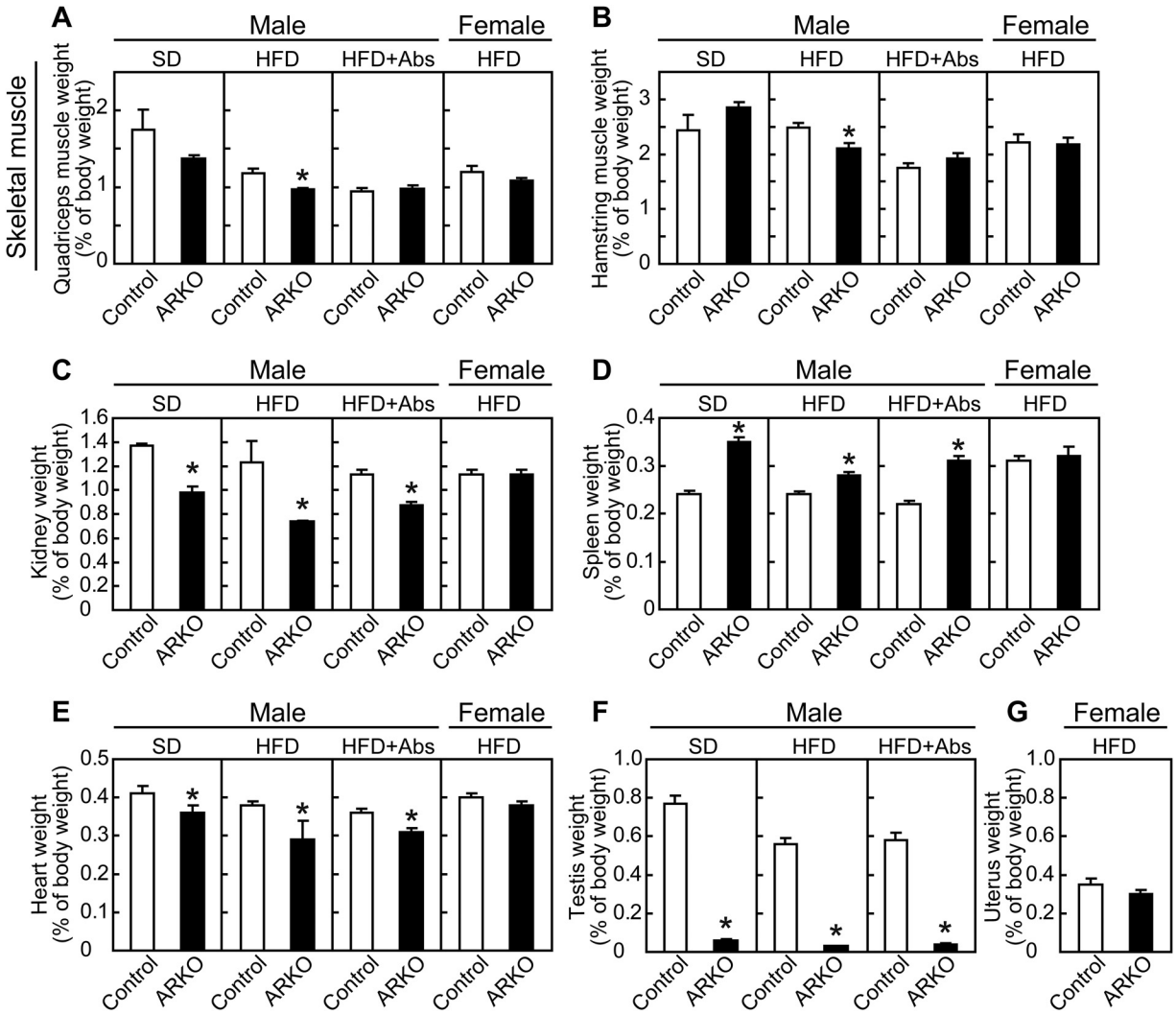

Fig. 6. Effect of androgen deficiency on relative weight of organs. *A–G*: quadriceps muscle (*A*), hamstring muscle (*B*), kidney (*C*), spleen (*D*), heart (*E*), testis (*F*), or uterus weight relative to body weight (*G*). Data are expressed as means ± SE. *Statistically significant differences [$P < 0.05$; $n = 5$, male standard diet (SD) control; $n = 6$, male SD androgen receptor knockout (ARKO); $n = 19$, male high-fat diet (HFD) control; $n = 16$, male HFD ARKO; $n = 10$, male HFD + antibiotics (Abs) control; $n = 15$, male HFD + Abs ARKO; $n = 5$, female HFD control; $n = 7$, female HFD ARKO].

visceral mesenteric fat by using a histological approach (Fig. 3*D*). ARKO significantly increased the mean adipocyte area in HFD-fed, but not SD-fed, male mice in a sex-dependent fashion (Fig. 3*E*). Although significant enlargement of the mean adipocyte area in ARKO was also apparent in HFD-fed mice administered antibiotics in drinking water, the magnitude of the effect was highly attenuated by antibiotic administration (4.1-fold increase in HFD-fed ARKO mice without antibiotics vs. 0.3-fold increase in HFD-fed ARKO mice with antibiotics). Collectively, ARKO-induced increase in WAT mass accompanying adipocyte hypertrophy was only observed in HFD-fed males without antibiotic treatment.

When male mice were fed HFD, the weight of liver in the ARKO group was markedly greater than that in the control group (Fig. 4*A*). The hepatic hypertrophy in HFD-fed ARKO males was not apparent in males fed SD and in females and was also attenuated by the antibiotic treatment. Histochemical analysis of the liver revealed ectopic lipid deposits in hepatocytes in HFD-fed ARKO male mice (Fig. 4*B*). The triglyceride and cholesterol levels in the liver were considerably higher in ARKO males than in control males when the mice were fed HFD (Fig. 4, *C* and *D*). Such sex- and diet-dependent fat accumulation in the liver was abolished by the administration of antibiotics. Despite abnormal fat deposits in WAT and hepatocytes, the plasma levels of triglycerides, cholesterol, and free fatty acids in HFD-fed ARKO males were not significantly affected compared with HFD-fed control males (Table 1).

ARKO leads to insulin resistance and glucose intolerance in HFD-fed male mouse. The impact of ARKO on glucose metabolism was next evaluated by glucose and insulin tolerance tests. As shown in Fig. 5*A*, blood glucose levels in ARKO males fed HFD were significantly higher than those in HFD-fed control males at all times points after the glucose challenge. Such glucose intolerance in ARKO mice was sex- and HFD-dependent and was eliminated by oral administration of antibiotics. Similarly, insulin sensitivity (i.e., the decrease of blood glucose levels after insulin injection) was lower in ARKO mice than that in the corresponding controls but only in male mice fed HFD and not receiving antibiotics (Fig. 5*B*). Plasma adiponectin levels were increased in ARKO males, but not in ARKO females, irrespective of the diet and antibiotic treatment and were thus not correlated with glucose tolerance and insulin resistance. Thigh muscle mass (i.e., quadriceps and hamstring mass) in ARKO mice was selectively decreased when male mice were fed HFD; antibiotic treatment abolished this effect (Fig. 6, *A* and *B*). Weights of the kidney, heart, and testis were also lower, and weight of the spleen was higher, in ARKO males than in control males; however, these changes were independent of diet and antibiotic treatment (Fig. 6, *C*–*F*). In ARKO females, muscle, kidney, heart, spleen, and uterus weights were unaffected (Fig. 6, *A*–*G*). These observations suggest that thigh muscle loss was involved in insulin resistance leading to glucose intolerance in HFD-fed ARKO males.

ARKO reduces fecal excretion in HFD-fed male mouse. When male mice were fed HFD, the ratio of feces-to-food intake was significantly decreased in ARKO mice compared with the controls, in 17-wk-old animals, but such difference was not observed in mice administered antibiotics (Fig. 7*A*). Similar to the ratio of feces-to-food intake, the ratio of cecum-to-body weight also decreased in ARKO mice, in an HFD- and sex-dependent manner, in 20-wk-old animals (Fig. 7*B*). Fecal excretion of triglycerides was not affected by ARKO under any of the experimental conditions tested (Table 1). Cholesterol excretion in the feces was decreased in ARKO males without antibiotics treatment but increased in ARKO females. These observations suggest that the gut environment is altered in HFD-fed ARKO male.

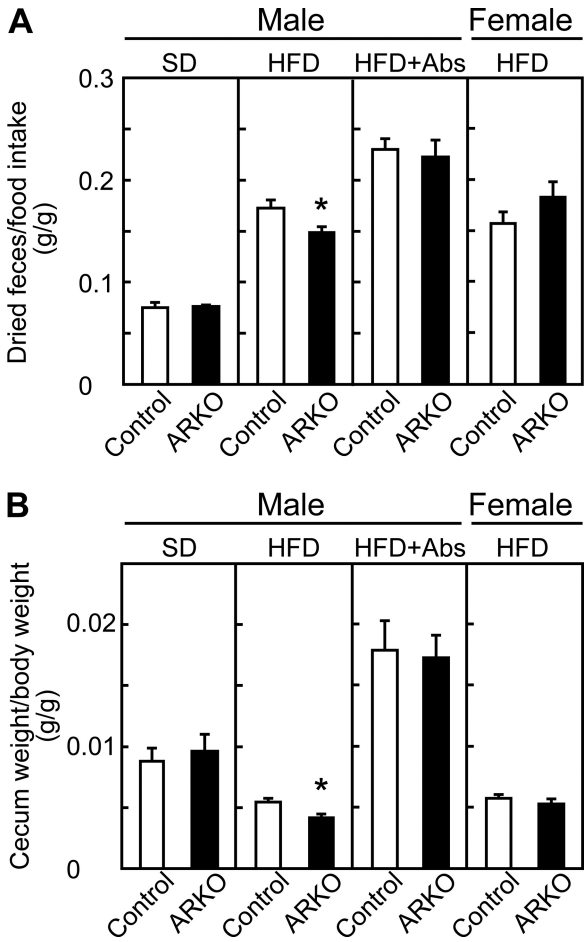

Fig. 7. Effect of androgen receptor knockout (ARKO) on the fecal and cecal weight in mouse. *A*: ratio of the weight of dried feces to the weight of food intake in 17-wk-old animals. *B*: ratio of the cecum wet weight to body weight in 20-wk-old animals. Data are expressed as means ± SE. *Statistically significant differences [$P < 0.05$; *A* and *B*, $n = 5$ and 12, respectively, male standard diet (SD) control; $n = 6$ and 12, respectively, male SD ARKO; $n = 11$ and 19, respectively, male high-fat diet (HFD) control; $n = 11$ and 16, respectively, male HFD ARKO; $n = 8$ and 10, respectively, male HFD + antibiotics (Abs) control; $n = 12$ and 15, respectively, male HFD + Abs ARKO; $n = 8$ and 5, respectively, female HFD control; $n = 8$ and 7, respectively, female HFD ARKO].

ARKO is associated with a different microbiota composition compared with controls in the gut of HFD-fed male mouse. The present experiments indicate that the gut microbiota may be involved in metabolic disorders in ARKO mice fed an HFD. To assess the effect of ARKO on the gut microbial community, the variable V3–V4 region of 16S rRNA gene from fecal samples 17-wk-old HFD-fed control and ARKO males at 17 wk of age was sequenced by using Illumina MiSeq. The relative abundance of taxa on genus level is shown in Fig. 8*A*. Phylogenic differences within samples were determined by

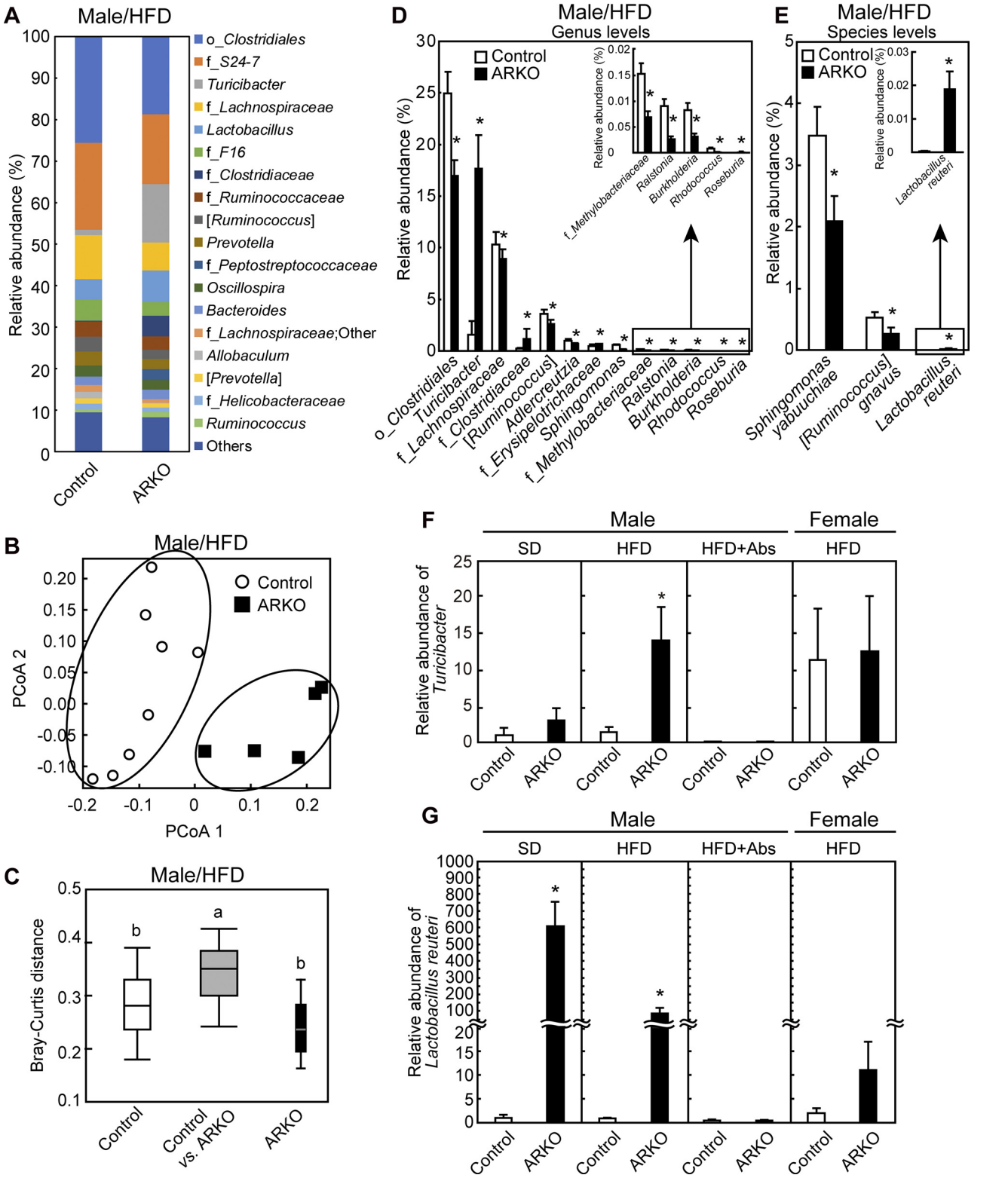

Fig. 8. 16S rRNA sequencing analysis of the microbiota from feces. *A*: microbial compositions at genus levels of high-fat diet (HFD)-fed males. *B*: principal coordinate analysis (PCoA) of HFD-fed males. *C*: the Bray-Curtis distance analysis within each group or between the two groups of HFD-fed males. *D* and *E*: taxonomic differences between the control and androgen receptor knockout (ARKO) mice at genus (*D*) or species (*E*) levels of HFD-fed males (*A–E*: $n = 8$, male HFD control; $n = 5$, male HFD ARKO). *F* and *G*: the abundance of *Turicibacter* and *Lactobacillus reuteri* was evaluated by real-time PCR [$n = 5$, male standard diet (SD) control; $n = 6$, male SD ARKO; $n = 19$, male HFD control; $n = 16$, male HFD ARKO; $n = 9$, male HFD + antibiotics (Abs) control; $n = 8$, male HFD + Abs ARKO; $n = 5$, female HFD control; $n = 7$, female HFD ARKO]. Data are expressed as means ± SE. Different letters or asterisks indicate statistically significant differences ($P < 0.05$).

using PCoA (Fig. 8*B*). As shown, microbiota compositions in the HFD-fed control and ARKO mice clustered separately. Furthermore, the Bray-Curtis distances were separated to a greater extent between the control and ARKO groups than those within each group (Fig. 8*C*). These observations indicate that the microbiota composition was different in the two groups. The abundances of the following were significantly different in the ARKO group in comparison with the control group: *Clostridiales* (decreased by ~30%), *Turicibacter* (increased by ~1,000%), *Lachnospiraceae* (decreased by ~10%), *Clostridiaceae* (increased by ~500%), *Ruminococcus* (decreased by ~30%), *Adlercreutzia* (decreased by ~30%), *Erysipelotrichaceae* (increased by ~50%), *Sphingomonas* (decreased by ~80%), *Methylobacteriaceae* (decreased by ~50%), *Ralstonia* (decreased by ~70%), *Burkholderia* (decreased by ~60%), *Rhodococcus* (decreased by ~90%), and *Roseburia* (not detected in the control group) (Fig. 8*D*). In addition, on the species level, the abundance of *Ruminococcus gnavus* (decreased by ~40% in the ARKO group in comparison with the control group), *Sphingomonas yabuuchiae* (decreased by ~60%), and *Lactobacillus reuteri* (increased by ~9,000%) was also influenced by ARKO (Fig. 8*E*). Based on their total abundance and rate of change, *Turicibacter* and *L. reuteri* are characteristic. We then examined sex-, diet-, and antibiotics-dependent status of *Turicibacter* and *L. reuteri* by real-time PCR. The increase of *Turicibacter* by ARKO was specific to HFD-fed male mice (Fig. 8*F*), whereas the increase of *L. reuteri* was observed in ARKO mice irrespective of diet (Fig. 8*G*). Treatment with antibiotics abolished these increases in HFD-fed ARKO male mice. Taken together, these observations indicate that alterations in the gut microbiota composition due to androgen deficiency are abrogated by antibiotics treatment.

ARKO is associated with reduced life span of HFD-fed male mouse. Finally, the life span of HFD-fed control and ARKO male mice was determined using the Kaplan-Meier method (Fig. 9). According to the log-rank test analysis, the life span was significantly and substantially shortened in ARKO males compared with the control males ($P < 0.001$, mean lifetime in the control vs. ARKO males: 22.0 ± 0.9 mo vs. 17.8 ± 0.5 mo, respectively). These observations indicate that the metabolic disorders in HFD-fed ARKO mouse shorten the animal life span.

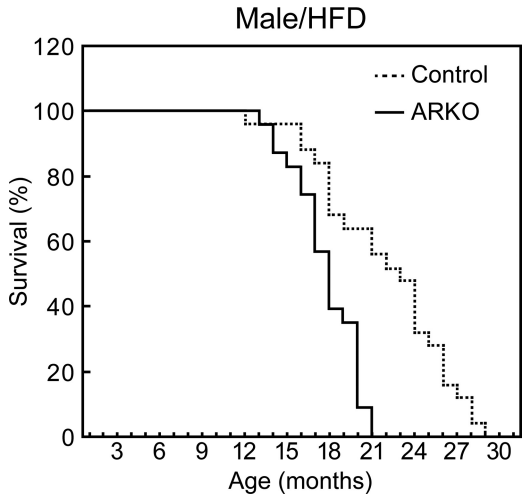

Fig. 9. Effect of androgen receptor knockout (ARKO) on longevity in high-fat diet (HFD)-fed mice. The life span of control and ARKO mice fed HFD was determined. Data are presented as the Kaplan-Meier plot, expressed as %surviving animals plotted against age in months ($n = 25$ for control, $n = 23$ for ARKO).

DISCUSSION

Although ARKO in male mice is known to cause metabolic disorders (7, 15, 16, 48), the involvement of gut microbiota, sex-associated differences, and effect on longevity are not known and this has been addressed. In the present study, we have demonstrated that ARKO caused metabolic disorders, such as increased feed efficiency, obesity with increased visceral adipocyte mass and hypertrophy, hepatic steatosis, glucose intolerance, insulin resistance, and loss of thigh muscle in male, but not female, mouse fed HFD. Among HFD-fed males, the life span of ARKO mice was significantly shorter than that of control mice. To the best of our knowledge, this is the first study to assess the effect of androgen signaling on longevity in laboratory animals. Of note, antibiotic administration apparently abolished metabolic disorders observed in HFD-fed ARKO males. In addition, the composition of gut microbiota in HFD-fed ARKO males was different from that in HFD-fed control males, and this difference was abrogated by antibiotics treatment. Collectively, these observations indicate that AR signaling plays a role in the prevention of metabolic dysfunctions, presumably by influencing the gut microbiome.

Clinical and epidemiological studies indicate that hypogonadism is associated with obesity, metabolic syndrome, and T2DM (reviewed in Ref. 18). We have previously shown that androgen deficiency after sexual maturation causes metabolic abnormalities in HFD-fed male mice in the castration model, while antibiotic treatment counters these effects (20). In the present study, ARKO mice was designed to assess the role of gut microbiota in subjects with disorders of sex differentiation in which metabolic disorders have been recently reported (11, 17, 56). In addition, although the castration model cannot be used to assess the role of sex in the observed effects of androgen signaling, the ARKO model can indeed be used to address this issue. The data obtained in the current study of the ARKO model indicated that the defects in AR signaling caused dysfunction in the energy metabolism in males but not in females. In female, although hyperandrogenism contributes to metabolic disorders observed in the polycystic ovary syndrome (58), defects in AR signaling only lead to subcutaneous fat accumulation. The castration model (20) and the ARKO model yielded similar results for male mice. Therefore, data from these two models indicate that defects in AR signaling play a critical role in the development of metabolic disorders caused by both late-onset hypogonadism and by disorders of sex differentiation.

In the current study, late-onset obesity accompanying visceral fat accumulation and glucose intolerance was observed in HFD-fed ARKO males. Considerable controversy surrounds the effect of ARKO on disorders in visceral fat accumulation and glucose metabolism (7, 15, 16, 18, 48). On the other hand, the observations related to energy metabolism disorders (i.e., enhanced feed efficiency, obesity, excessive visceral fat mass and hypertrophy, hepatic steatosis, glucose intolerance, insulin resistance, and sarcopenia) in the ARKO model presented

herein were considerably dependent on HFD and were virtually abolished by antibiotics. Future studies will elucidate the contribution of different tissues to insulin insensitivity in HFD-fed ARKO mice. These observations indicate the importance of gut microbiome in metabolic disorders in ARKO mouse. Unlike the visceral fat mass, subcutaneous fat mass was increased in association with ARKO in a sex- and antibiotic treatment-independent manner. The visceral fat mass might be more strongly affected by the gut microbiome than direct androgen signaling. This notion is supported by the fact that the gut microbiome influences visceral fat accumulation more strongly than subcutaneous fat accumulation (34). Taken together, it is reasonable to propose that the development of metabolic dysfunctions in ARKO highly depends on the experimental conditions affecting the gut microbiota.

The gut microbiome impacts feed efficiency (53), obesity (36, 53), hepatic steatosis (31), T2DM (46), insulin resistance (28), and sarcopenia (52). In the current study, when male mice were fed HFD, ARKO resulted in a decreased fecal weight-to-food weight ratio and cecum weight-to-body weight ratio and changes in the gut microbiota composition. Therefore, these two indexes may be useful for the evaluation of the difference in gut circumstance (e.g., gut microbiome status) in future studies. According to previous studies, *Lactobacillus* species levels are increased in HFD-fed obese castrated mouse compared with sham-operated HFD-fed controls (19, 20). Although the difference did not reach statistical significance, relative *Lactobacillus* abundance tended to increase in HFD-fed ARKO male mice (control: 4.6 ± 1.1% vs. ARKO: 8.9 ± 1.9%, $P = 0.06$) in the current study. *Lactobacillus* bacteria are both positively and negatively associated with obesity, depending on the species or strain (14, 42). *L. reuteri* is positively associated with obesity (14, 43), and its abundance was greatly increased in ARKO mice in the current study. The HFD used in the current study contains 25% (wt/wt) fructose, and fructose intake may be key for the association of abundance of *L. reuteri* and obesity (25). *Turicibacter* was abundant in the feces, with the abundance greatly increased in ARKO mice. The association of *Turicibacter* and obesity has been reported earlier (6), although it remains controversial. The observed increases in *Turicibacter* and *L. reuteri* in HFD-fed ARKO mice were abolished by antibiotics treatment. Collectively, the changes in composition of the gut microbiota and the microbiome may be linked to metabolic disorders induced by androgen deficiency (i.e., both late-onset hypogonadism and disorders of sex differentiation).

Hypogonadism in man is a risk factor for early death (2, 10, 18). That is because of the incidence of metabolic diseases, such as obesity (5, 23), metabolic syndrome (4, 9), T2DM (4, 8, 13, 54), and nonalcoholic fatty liver disease (27), in hypogonadal men. In the current study, metabolic dysfunctions associated with defective AR signaling (in ARKO) were observed in male, but not female, mice fed with HFD, leading to early death. In addition, the results shown using ARKO mice, an androgen-insensitive model, indicate the involvement of the gut microbiota in the observed metabolic dysfunctions and are similar to those of the castration model, a late-onset hypogonadism model (19, 20). Collectively, our findings support the notion that sex hormones influence the gut microbe composition in male and female mice, the disruption of which is involved in metabolic disorders (20, 26, 38, 44, 57). A limitation of this study is the difference in gut microbiota composition between mice and humans. Therefore, future clinical trials are needed to test this notion in humans.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

N.H., K.H., and Y.M. conceived and designed research; N.H., K.H., and Y.M. performed experiments; N.H., K.H., Y.M., T.K., Y.O., H.T., T.S., S.K., H.I., and R.Y. analyzed data; N.H., K.H., Y.M., T.K., Y.O., H.T., T.S., S.K., H.I., and R.Y. interpreted results of experiments; N.H., K.H., and Y.M. prepared figures; N.H. drafted manuscript; N.H. edited and revised manuscript; N.H., K.H., Y.M., T.K., Y.O., H.T., T.S., S.K., H.I., and R.Y. approved final version of manuscript.

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