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Gender difference in development of steatohepatitis in *p62/Sqstm1 and Nrf2* double-knockout mice

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Abstract: Gender and menopause influence the severity and development manner of nonalcoholic steatohepatitis (NASH). Male *p62/Sqstm1* and nuclear factor E2-related factor-2 (*p62 and Nrf2*) double-knockout (DKO) mice exhibit severe steatohepatitis caused by hyperphagia-induced obesity, overload of lipopolysaccharide (LPS) into the liver, and potentiation of the inflammatory response in Kupffer cells. However, the pathogenetic phenotype of steatohepatitis in female DKO mice remains unknown. Phenotypic changes of steatohepatitis in DKO mice were compared in terms of gender differences. Compared with DKO male mice, DKO female mice exhibited later onset of steatohepatitis with obesity after 30 weeks of age, as well as milder severity of hepatic inflammation and fibrosis. Serum estradiol was higher in female than male mice, with levels increasing up to 30 weeks of age before decreasing until 50 weeks of age (corresponding to the post-menopausal period). Fecal and serum LPS were lower in female mice than male mice, and inflammatory signaling in the liver was attenuated in female compared with male mice. Correlating with LPS levels, the composition of intestinal microbiota in female mice was different from male mice. Gender differences were observed for the development of steatohepatitis in DKO mice. Low-grade inflammatory hit in the liver under *in vivo* conditions of high estradiol may be attributable to the milder pathological features of steatohepatitis in female mice.
Key words: estradiol, lipopolysaccharide, nonalcoholic steatohepatitis, *Nrf2*, *p62/Sqstm1*

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Introduction

Nonalcoholic steatohepatitis (NASH) is a progressive liver disease characterized by steatosis, inflammation, and fibrosis, leading to liver cirrhosis and hepatocellular carcinoma [4]. NASH is a hepatic phenotype of metabolic syndrome and an increasingly common health problem worldwide [28]. Pathogenesis of NASH includes multiple factors such as obesity, insulin resistance, intestinal microbiota-derived lipopolysaccharides (LPS), oxidative stress, lipotoxicity, and hepatocyte death [24]. Recently, several studies reported that gender influenced the clinical course of NASH in humans: men have a higher risk of severe fibrosis compared with women before the menopause, while after the menopause, women show the same severity of liver fibrosis as men [31]. Moreover, time from menopause is directly associated with an increased likelihood of severe fibrosis [14]. These results indicate that sex hormones, especially estrogen, may have protective effects against the progression of steatohepatitis. 17β-estradiol is the most abundant biologically active form of estrogen. Canonical estrogen signaling is mediated through nuclear hormone estrogen receptors 1 and 2, resulting in transcriptional target gene activation. Estradiol promotes proliferation of hepatocytes and regulates hepatocarcinogenesis through reduction of inflammatory cytokines [5, 19]. However, the molecular mechanisms corresponding to these pathogenic differences between men and women via estradiol are poorly understood.

p62/SQSTM1 (p62) is a cytoplasmic endosome-associated protein that acts as a scaffold for atypical protein kinase C [12], and plays an important role in selective autophagy of ubiquitin-binding protein [17]. *p62*-deficient (knockout) mice (*p62*-KO) develop mature-onset obesity with a standard diet that causes hyperphagia due to leptin resistance in the brain [10].

Nuclear factor E2-related factor-2 (NRF2) is a transcriptional factor and master regulator of the cellular adaptive response to oxidative stress [16]. *Nrf2*-deficient (knockout) mice (*Nrf2*-KO) exhibit a severe deficiency in the gene regulatory program of the antioxidant response, resulting in high susceptibility to oxidative stress-related disease [13]. In previous studies, *Nrf2*-KO mice developed more severe steatohepatitis induced by a methionine- and choline-deficient diet [21] or atherogenic plus high-fat diet [20] compared with wild-type (WT) mice. Moreover, activation of NRF2 prevented LPS-induced upregulation of proinflammatory cytokines including interleukin (IL)-6 and IL-1β [15].

Experimental animal models of NASH induced by diet (such as methionine- and choline-deficient or atherogenic plus high-fat diets) or gene deficiency show an unnatural disease course in terms of body weight and symptoms. Therefore, similarities of the models might deviate from clinical features of human NASH. Male *p62 and Nrf2* double-knockout mice (DKO) show steatohepatitis with obesity, insulin resistance, and adipokine imbalance, which mimics the pathogenesis of human NASH. Moreover, steatohepatitis of DKO male mice was associated with activation of innate immunity by excessive LPS flux from the intestine and a hyperinflammatory response in Kupffer cells [1]. Previous studies reported that a change of intestinal microbiota composition (dysbiosis) is associated with LPS production and progression of NASH [9]. Microbiota from patients with NASH have a higher proportion of the *Porphyromonadaceae* family (gram-negative bacteria) and a lower proportion of the *Lachnospiraceae* and *Ruminococcaceae* families (gram-positive bacteria) compared with healthy subjects [29, 32]. However, the association mechanisms in the pathogenesis of NASH between microbiota composition and sex hormone, especially estradiol are unknown.

In this study, our results showed that a low-grade inflammatory hit in the liver under *in vivo* conditions of high estradiol levels may be attributable to the mild pathological features of steatohepatitis. Further, we demonstrated phenotypic differences in the developmental manner of steatohepatitis between DKO male and female mice, which resembles the gender difference in clinical features of human NASH.

Materials and Methods

Animals

p62-KO and *Nrf2*-KO mice were generated and genotyped as previously described [10, 13]. *p62 and Nrf2* DKO mice were produced by crossing these mutant mice and re-genotyping [1]. Wild-type C57BL/6J mice were obtained from Charles River Laboratories Japan (Kanagawa, Japan). All mice were kept under specific pathogen-free conditions in an environmentally controlled clean room at the Laboratory Animal Resource Center, University of Tsukuba. They were housed in an ambient temperature of 20–23°C with a daily 12 h light/dark cycle. The mice had free access to drinking water and standard chow (MF chow: 5.1% fat, 23.1% protein, 360 kcal/100 g; Oriental Yeast, Tokyo, Japan). All experiments were performed under protocols approved by the Institutional Animal Care and Use Committees of the University of Tsukuba.

Computed tomography (CT) analysis

To analyze body fat and skeletal muscle composition, mice were anesthetized with intraperitoneal injections of 10% nembutal and scanned using a Latheta (LCT-200 M) experimental animal CT system (Hitachi Aloka Medical, Tokyo, Japan). Continuous 5 mm slice images between the liver and tail root were used for quantitative assessments using Latheta software. Visceral and subcutaneous fat were distinguished and evaluated quantitatively.

Biochemical analysis

Serum concentrations of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured by standard method in Oriental Yeast (Tokyo, Japan). Serum concentration of estradiol was measured by ASKA Pharma Medical Co., Ltd. (Kanagawa, Japan) using liquid chromatography with tandem mass spectrometry (LS-MS/MS). To adjust the sexual cycle of mice, blood samples were collected for four consecutive days and combined into one. Serum glucose was measured using a glucose CII test kit (Wako, Tokyo, Japan). Serum insulin was measured using a mouse insulin enzyme-linked immunosorbent assay (ELISA) kit (Morinaga, Kanagawa, Japan). Serum leptin was measured using a mouse/rat leptin ELISA kit (Morinaga, Kanagawa, Japan). Concentrations of LPS in plasma and feces were detected using a Pyrochrome limulus amebocyte lysate assay (Associated of Cape Cod Inc., East Falmouth, MA, USA). Levels of LPS were expressed as endotoxin units (EU).

Histological analysis

Liver tissue was fixed in 4% paraformaldehyde, embedded in paraffin, and stained with hematoxylin–eosin and Sirius Red solution. To determine the histopathological severity of steatohepatitis, the steatosis, activity, and fibrosis (SAF) score was assessed for the grade of steatosis (0–3), activity (0–4), and stage of fibrosis (0–4) [2, 3].

Real-time quantitative polymerase chain reaction (qPCR)

Steady-state mRNA levels in the liver were determined by real-time quantitative PCR. Total RNA was extracted from liver specimens, followed by cDNA synthesis. qPCR was performed with Fast SYBR Green Master Mix (Thermo Fisher Scientific, Waltham, MA, USA). Primers used for this study have been described previously [1]. Data were normalized to amounts of glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) present in each sample and then averaged.

Flow cytometric analysis

Following liver perfusion, primary cells were isolated from mice at 8 weeks of age. Kupffer cells were stained with allophycocyanin-conjugated anti-F4/80 antibody (Ab) (Thermo Fisher Scientific), PerCP/Cy 5.5 anti-mouse CD206 Ab (BioLegend San Diego, CA, USA) and PE anti-mouse CD11b Ab (BioLegend). Flow cytometry was performed using a Gallion flow cytometer (Beckman Coulter, Brea, CA, USA).

Fecal bacterial analysis

Feces samples were collected from mice, snap-frozen and stored at −80°C. DNA extraction and analysis of fecal microbiota using a 16S rDNA library and terminal restriction fragment length polymorphisms were measured by FASMAC Co., Ltd. (Kanagawa, Japan).

Statistics

Statistical analysis was conducted using IBM SPSS Statistics 22.0 (IBM, Armonk, NY, USA). Values are given as mean ± SEM. When two groups were compared, an unpaired *t*-test was used for data analysis. A P value of <0.05 was defined as statistically significant.

Results

Female DKO mice exhibit a later onset of hyperphagia and obesity

The body weights of WT male, WT female, DKO male, and DKO female mice fed a normal chow diet were monitored from 8 to 48 weeks of age. All mice had similar body weights at birth. After 16 weeks of age, DKO male mice gained weight much faster than WT male mice. DKO female mice started to gain weight dramatically at around 32 weeks of age and their body weights were similar to those of DKO male mice at 44 weeks of age. The period at which their obesity began was different between DKO male and DKO female mice, yet they exhibited obesity compared with WT male and WT female mice (46.4 ± 0.9 g for DKO male, 48.7 ± 1.3 g for DKO female, 41.3 ± 1.1 g for WT male, and 24.4 ± 1.0 g for WT female mice at 48 weeks of age; Fig. 1A). Food intake of DKO male mice was greater than DKO female mice until 40 weeks of age. Meanwhile food intake of DKO female mice increased at around 32 weeks of age, parallel with the body weight changes, and was equal to DKO male mice at 44 weeks of age (Fig. 1B). Using CT analysis, visceral fat to body weight ratio increased in DKO female mice from 30 weeks of age compared with DKO male mice, and the increase was greater at 50 weeks of age (Fig. 1C). Contrarily, skeletal muscle mass to body weight ratio decreased in DKO

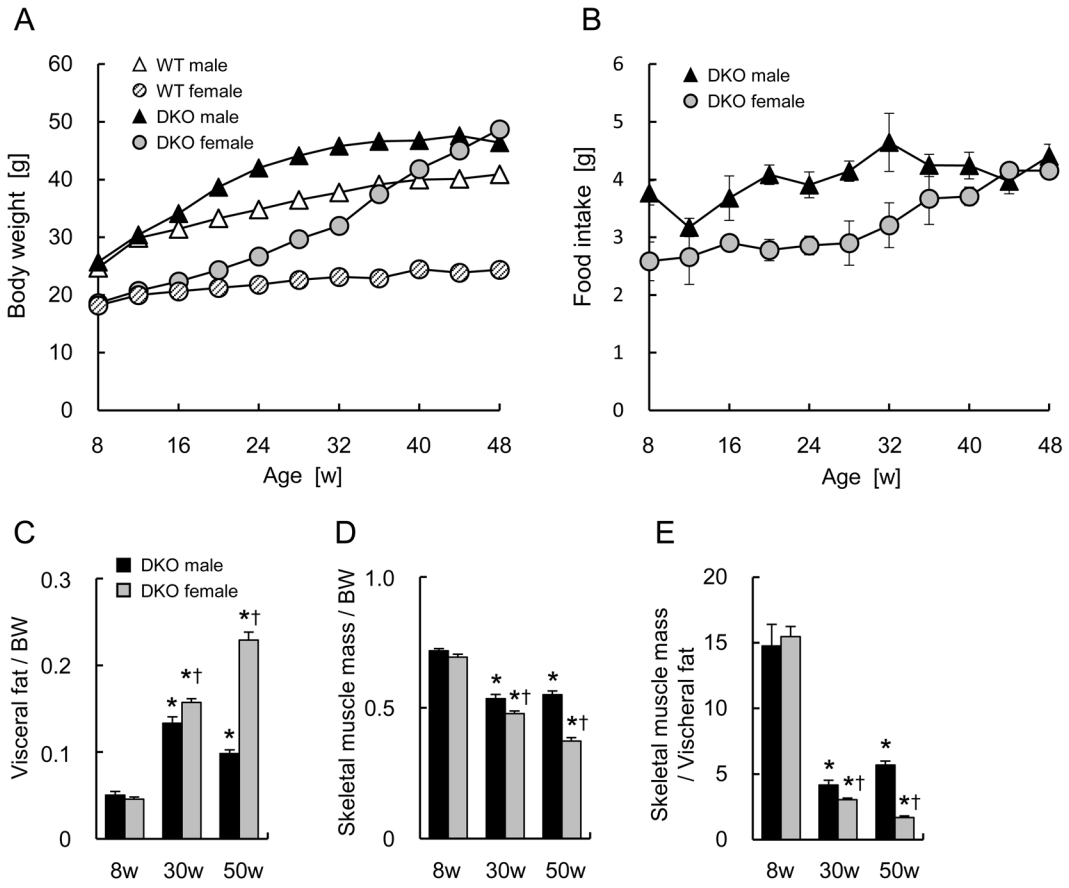

Fig. 1. Female *p62 and Nrf2* gene double-knockout (DKO) mice exhibit a later onset of obesity and hyperphagia compared with male DKO mice. (A) Time course of body weight changes in wild-type (WT) male, WT female, DKO male, and DKO female mice (n=17–20/group). (B) Time course of changes in food intake amount in DKO male and female mice (n=3–5/group). (C–E) Computed tomography-assisted analysis of body composition. Visceral fat (C), skeletal muscle mass (D), and skeletal muscle mass to visceral fat ratio in DKO male and female mice (n=4–9/group). Error bars represent SEM. **P*<0.05, 8 weeks of age (w) vs. 30 w, or 8 w vs. 50 w. †*P*<0.05, male vs. female.

female mice from 30 weeks of age compared with DKO male mice (Fig. 1D). Similarly, skeletal muscle mass to visceral fat ratio also decreased (Fig. 1E).

Serum estradiol levels in DKO mice

The comparison between male and female mice and the time course of changes in serum estradiol are shown in Figs. 2A and B. Levels of serum estradiol in DKO female mice are similar to those of WT female mice at 8 weeks of age, and greater than those in DKO male mice (Fig. 2A). Levels of serum estradiol in DKO female mice increased at 30 weeks of age compared with those at 8 weeks of age, but decreased potently by 50 weeks of age (Fig. 2B).

Female DKO mice exhibit a later onset of steatohepatitis with mild severity

The time course of changes in liver histology for DKO male and DKO female mice are shown in Figs. 3A and B. To determine the histopathological severity of steatohepatitis, SAF score was assessed (Fig. 3C). At 30 weeks of age, steatosis in histology and score of DKO female mice was milder compared with DKO male mice, but this reversed at 50 weeks of age (Figs. 3A and C, left panel). In the DKO male mice, the steatosis at 50 weeks of age tended to decrease compared with that at 30 weeks of age. These changes might show "burned-out NASH", which is accompanied by the loss of hepatic fat through advanced NASH-associated fibrosis. Hepatic inflammation and fibrosis in DKO female mice aggravated with age, but the severity was significantly suppressed compared with DKO male mice at 30 and 50 weeks of age (Figs. 3A and C, middle and right panels), despite similar body weights at 50 weeks of age (Fig. 1A).

Comparing blood biochemistry between DKO male and DKO female mice, DKO female mice had lower AST levels compared with DKO male mice at 30 weeks age (Fig. 4A), consistent with changes in liver histology (Fig. 3). Meanwhile, ALT levels were not different between DKO male and female mice at 30 weeks of age.

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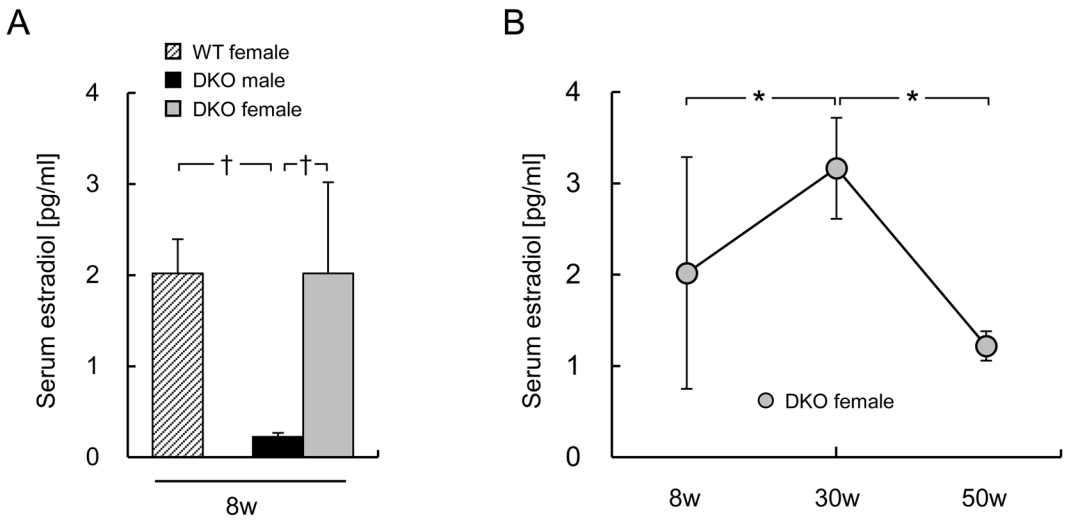

Fig. 2. Serum estradiol levels in *p62 and Nrf2* gene double-knockout (DKO) mice. (A) Comparison of serum estradiol levels in wild-type (WT) female, DKO male, and DKO female mice (n=5/group). (B) Time course of change in serum estradiol levels in DKO female mice (n=5/group). Error bars represent SEM. **P*<0.05, 8 weeks of age (w) vs. 30 w, or 30 w vs. 50 w. †*P*<0.05, male vs. female.

Levels of fasting glucose in DKO female mice were significantly lower compared with DKO male mice at 8 weeks of age, however this difference disappeared at 30 weeks of age (Fig. 4B, left panel). Levels of fasting insulin were significantly inhibited in DKO female mice at 30 weeks of age compared with DKO male mice. The results in Fig. 4B indicate that DKO male mice had greater insulin resistance compared with DKO female mice at 30 weeks of age. Levels of serum leptin were lower in DKO female mice compared with DKO male mice at 8 weeks of age, but increased significantly fast, and had reversed at 30 weeks of age compared with DKO male mice (Fig. 4C).

Inflammatory signaling is attenuated in the liver of DKO female mice

Hepatic mRNA expression levels of inflammatory cytokines (*Tnf-α* and *Il-1β*) and a fibrosis-related gene (*Col1a1*) were examined in DKO mice by qPCR (Fig. 5A). mRNA levels of the inflammatory cytokines and fibrosis-related gene increased with age in DKO male mice, whereas they were significantly suppressed in DKO female mice (Fig. 5A). These results appear to correlate with the degree of inflammatory cell infiltration and fibrosis in liver tissue sections (Fig. 3). mRNA levels of toll-like receptor (*Tlr*)-4 in DKO male mice showed a tendency to increase at 50 weeks of age, whereas they were significantly suppressed in DKO female mice (Fig. 5B). mRNA levels of *Trl6* also increased at 30 weeks of age and these increases were inhibited in DKO female mice (Fig. 5B). mRNA levels of *Trl9* in DKO male mice increased at 30 and 50 weeks of age compared to those at 8 weeks of age. However, those increases were not observed in DKO female mice (Fig. 5B).

Macrophage polarization is a process by which macrophages express different functions in response to signals. Further, M1 macrophages are implicated in initiation and maintenance of inflammation. We reported that M1 phenotype Kupffer cells but not M2 phenotype Kupffer cells increased in the liver of DKO male mice at 8 weeks of age, the time point before the development of obesity and NASH, compared with WT mice [1]. To examine differences between the genders, Kupffer cell phonotype was determined using flow cytometry with CD11b as a marker for M1 macrophages and CD206 as a marker for M2 macrophages in the liver of DKO male and female mice at 8 weeks of age. A gated percentage of both M1 and M2 macrophages in DKO female mice decreased relatively but not significantly changed (Fig. 5C). These results indicated that the acceleration of inflammatory response in DKO mice is similar between male and female basically.

LPS polarizes macrophages towards a M1 phenotype and is the predominant cause of liver inflammation in steatohepatitis. Thus, levels of fecal and serum LPS were determined. Both fecal and serum LPS levels were similar between DKO male and DKO female mice at 8 weeks of age. Moreover, LPS levels increased significantly in both DKO male and DKO female mice at 30 weeks of age, but were suppressed significantly in DKO female mice compared with DKO male mice (Fig. 5D).

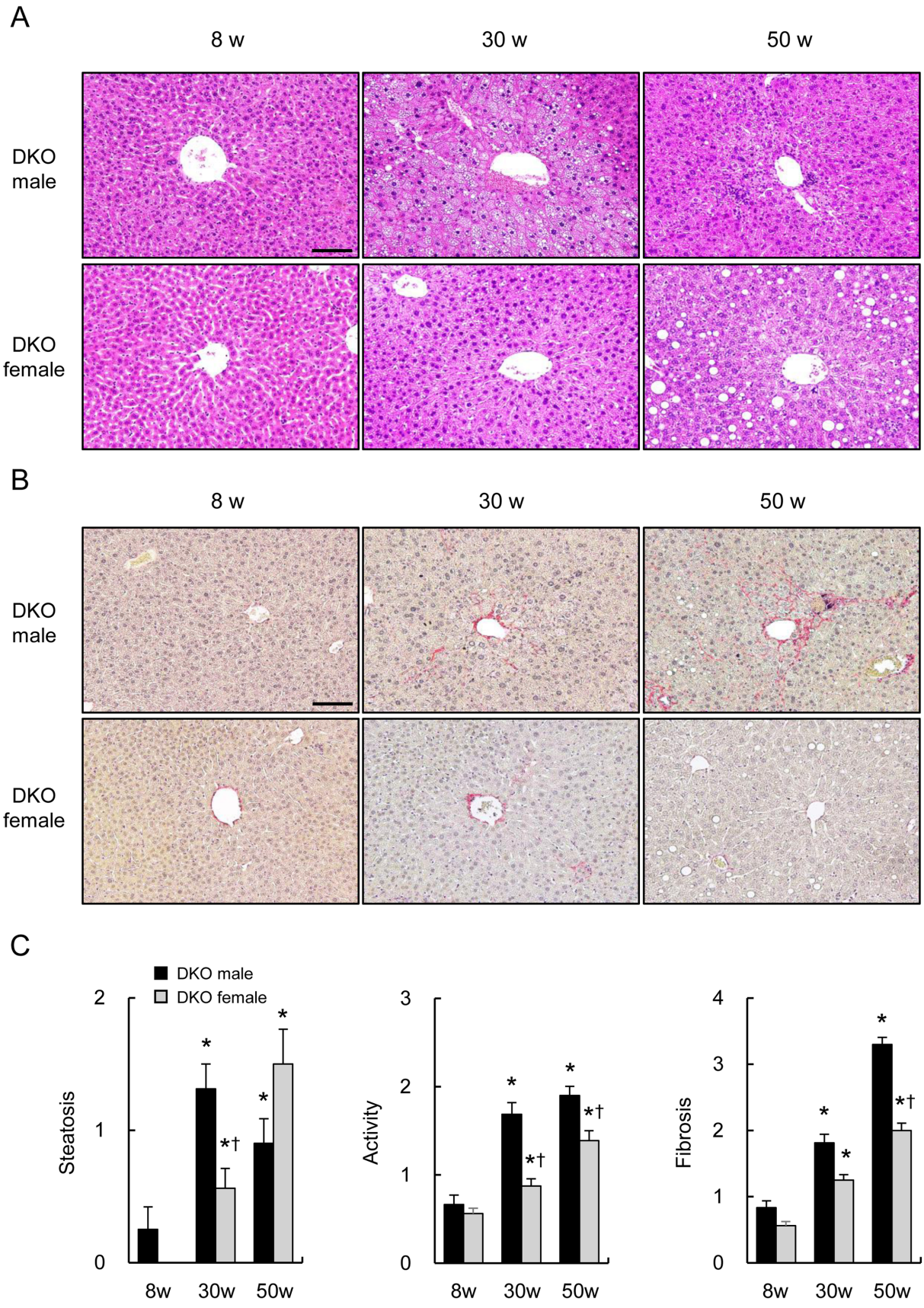

Fig. 3. Histopathology of steatohepatitis in *p62 and Nrf2* gene double-knockout (DKO) male and female mice. The severity of inflammation and fibrosis are milder in female mice than male mice. (A) Hematoxylin and eosin-stained sections (scale bar, 100 μm) of liver specimens from DKO male and female mice at 8, 30, and 50 weeks of age. (B) Sirius Red-stained sections (scale bar, 100 μm). (C–E) The steatosis, activity, and fibrosis (SAF) score (n=5–9/group). Error bars represent SEM. *P<0.05, 8 weeks of age (w) vs. 30 w, or 8 w vs. 50 w. $^{\dagger}P$<0.05, male vs. female.

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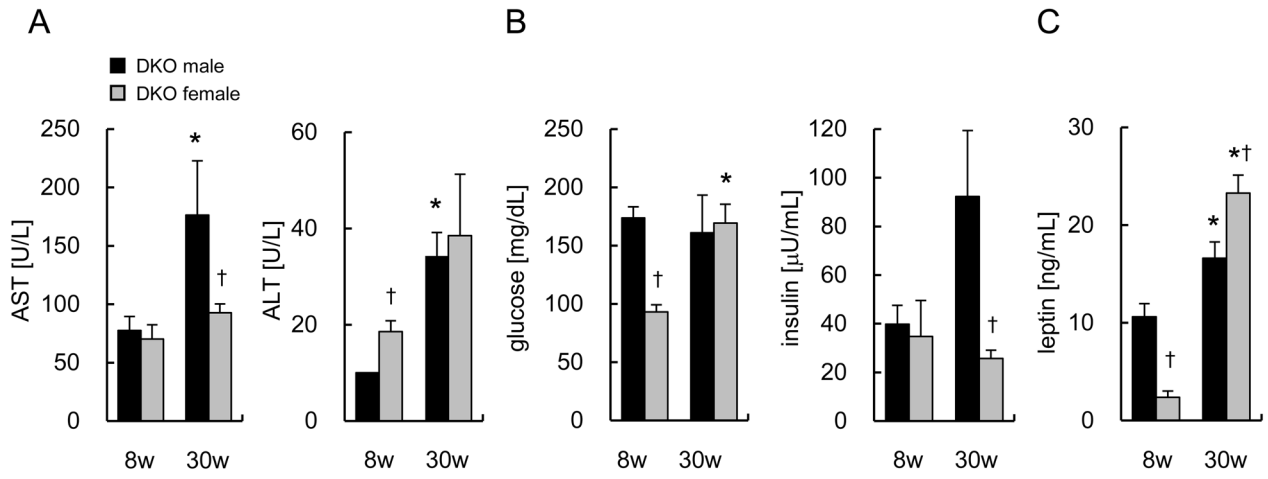

Fig. 4. Analysis of blood biochemistry (aspartate aminotransferase [AST], alanine aminotransferase [ALT], glucose, insulin, and leptin) in *p62 and Nrf2* gene double-knockout (DKO) male and female mice at 8 and 30 weeks of age (n=4–10/group). Glucose and insulin levels were measured in a fasting condition. Error bars represent SEM. **P*<0.05, 8 weeks of age (w) vs. 30 w. †*P*<0.05, male vs. female.

Intestinal microbiota composition in DKO mice

Fecal microbiota composition was determined in WT male, WT female, DKO male, and DKO female mice at 8 and 30 weeks of age. Gram-positive bacteria are predominant in intestinal microbiota composition in both WT and DKO mice at both 8 and 30 weeks of age (Fig. 6A). At 30 weeks of age, gram-negative bacteria were increased in WT female mice compared with WT male mice (Fig. 6A). Alternatively, the percentage of gram-negative bacteria in DKO male and female mice at 30 weeks of age was similar to WT female mice, and differences were not observed between DKO male and DKO female mice (Fig. 6A). The *Lachnospiraceae* and *Ruminococcaceae* families are gram-positive bacteria. *Ruminococcaceae* families were less abundant in DKO male mice at both 8 and 30 weeks of age, whereas these declines were not observed in DKO female mice and the abundant significantly increased in DKO female mice compared with DKO male mice (Fig. 6B). *Lachnospiraceae* families showed similar patterns to the *Ruminococcaceae* families, and the changes were also small (Fig. 6B, left two panels). The *Porphyromonadaceae* and *Paraprevotellaceae* families, which are gram-negative bacteria, were more abundant in both DKO male and DKO female mice compared with WT male and WT female mice at both 8 and 30 weeks of age (Fig. 6B, right two panels). Furthermore, a significant difference between DKO male and DKO female mice in abundant of the *Porphyromonadaceae* and *Paraprevotellaceae* families was not observed (Fig. 6B, right two panels).

Discussion

The findings of this study demonstrated gender differences in the phenotypes of steatohepatitis between DKO male and female mice. Male mice develop steatohepatitis with a normal chow diet due to multiple parallel hits and show phenotypic similarities to human NASH [1]. In contrast, in female mice, the low-grade inflammatory hit in the liver under *in vivo* conditions of high estradiol levels may be attributable to the milder pathological features of steatohepatitis. Estradiol may counteract the multiple factors for NASH, especially hepatic inflammatory signaling (Fig. 7).

Several studies have reported that gender influences disease progression of NASH in humans. Prevalence of NASH increases dramatically around 50 years old in females [11], while males have a higher risk of more severe fibrosis compared with females before the menopause, while after the menopause, females have a similar severity of liver fibrosis compared with males [31]. Moreover, post-menopausal conditions are directly associated with an increased likelihood of severe fibrosis [14]. These data indicate that female hormones such as estrogen could have protective effects against disease progression of NASH.

In this study, levels of serum estradiol in female mice increased up to 30 weeks of age (i.e., mature age), and then decreased down to 50 weeks of age (i.e., middle age) (Fig. 2). The period of menopause in B6J female mice is 40–55 weeks of age because childbirth decreases or stops from 24 to 32 weeks of age in mice [7]. In humans, serum estradiol levels in women significantly decrease 2–3 years before the onset of menopause. More-

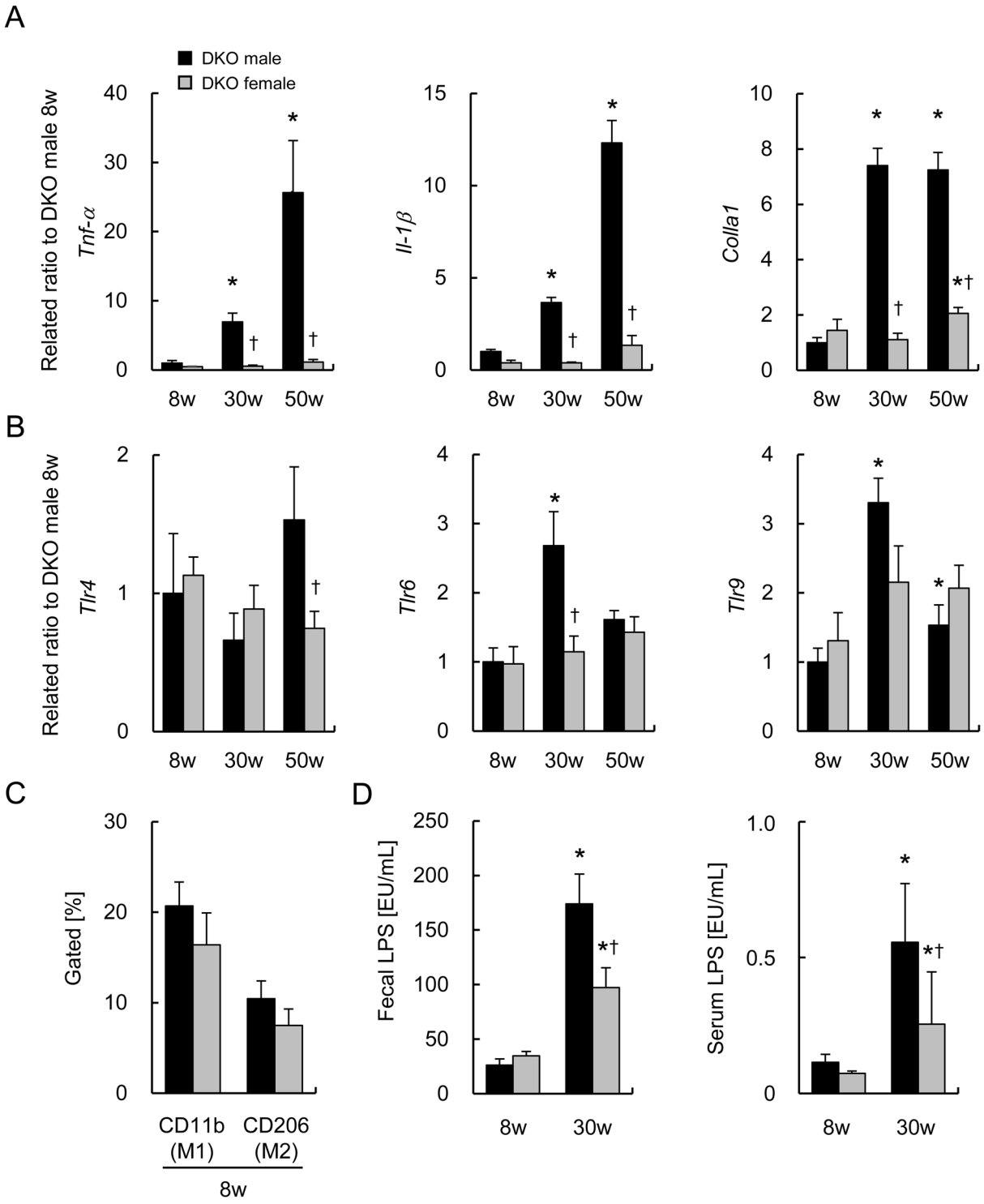

Fig. 5. Analysis of inflammatory signaling in the liver of *p62 and Nrf2* gene double-knockout (DKO) male and female mice at 8 and 30 weeks of age. (A) mRNA levels of inflammatory cytokines (*Tnf-α* and *Il-1β*), a fibrosis-related gene (*Colla1*), and (B) toll-like receptors (*Tlr*)-*4*, -*6*, and -*9* (n=5–9/group). (C) CD11b (M1) and CD206 (M2) expression in F4/80 positive cells (Kupffer cells) in the liver (n=3–5/group). Primary Kupffer cells were isolated from mice at 8 weeks of age. (D) Fecal and serum lipopolysaccharide (LPS) levels (n=3–5/group). Error bars represent SEM. **P*<0.05, 8 weeks of age (w) vs. 30 w, or 8 w vs. 50 w. †*P*<0.05, male vs. female.

over, dietary energy, protein, carbohydrate, and fiber intake are significantly higher 3–4 years before the menopause and visceral adipose tissue increases around menopause [18]. Food intake, body weight, and visceral fat started to increase in female mice at 28–32 weeks of age. In contrast, skeletal muscle mass decreased around 30 weeks of age (Fig. 1). Estradiol has an effect similar to leptin, which reduces appetite and adiposity

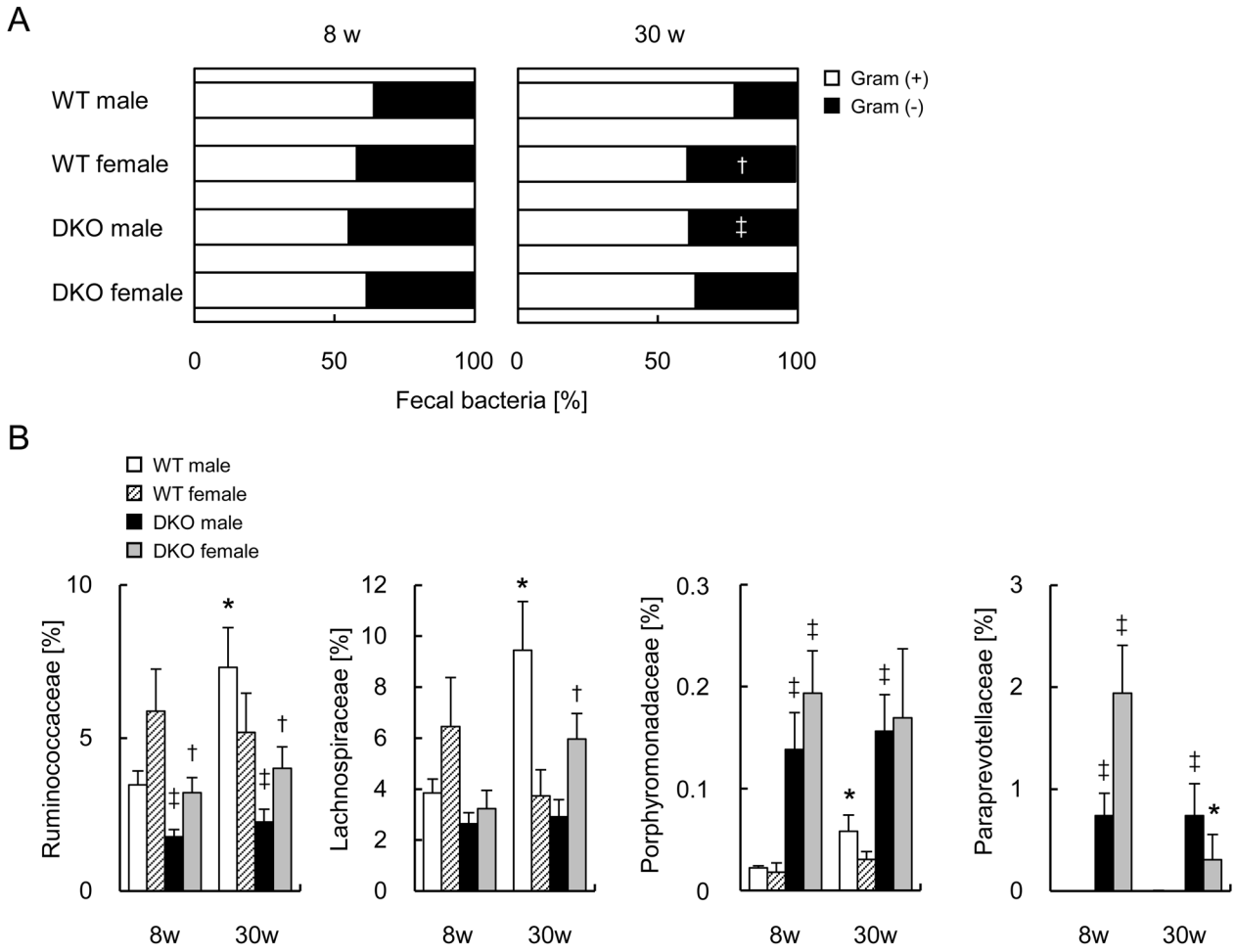

Fig. 6. Analysis of intestinal microbiota composition. (A) Relative abundance of gram-positive and -negative bacteria calculated as percentage relative to total bacteria in feces (n=5–8/group). (B) Relative abundance of families of bacteria in feces calculated as percentage relative to total bacteria (n=5–8/group). Error bars represent SEM. **P*<0.05, 8 weeks of age (w) vs. 30 w. †*P*<0.05, male vs. female. ‡*P*<0.05, wild-type (WT) vs. double-knockout (DKO) mice.

[8]. Loss of the effects of estradiol in the post-menopausal period may be associated with an increase of food intake and adiposity, and also development of obesity and hepatic steatosis.

While body weight was similar between male and female mice at 48 weeks of age (male: 46.4 ± 0.9 g, female: 48.7 ± 1.3 g; Fig. 1), the inflammatory conditions of steatohepatitis were milder in female mice than male mice in terms of histopathological grade (Fig. 3) and serum AST levels (Fig. 4A). Moreover, expression levels of *Tlr*, which mediates LPS-stimulated induction of proinflammatory cytokines in Kupffer cells (*Tnf-α* and *Il-1β*), were attenuated significantly in female mice (Figs. 5A and B). Estradiol shortens the LPS-induced proinflammatory phase and triggers resolution of the inflammatory signal in macrophages through regulation of the SOCS3 and STAT3 signaling pathway [27]. While subtypes of Kupffer cells (such as M1 and M2) were not different between male and female mice (Fig. 5C), the effect of estradiol might attenuate the inflammatory signal in Kupffer cells in the liver of female mice.

LPS is an inflammatory mediator derived from intestinal microbiota and may play a central role in the cascade of hepatic inflammation and development of fibrosis upon the multiple parallel hits hypothesis [1, 24]. In this study, both fecal and serum LPS levels were decreased in female mice compared with male mice (Fig. 5C). Decreased LPS levels could result in developmental attenuation of inflammation and fibrosis progression in the liver.

LPS is produced by gram-negative bacteria [26]. However, the percentage of gram-negative bacteria did not differ significantly between male and female mice at 8 and 30 weeks of age (Fig. 6A). Cumulative data suggest that a change of intestinal microbiota composition (dysbiosis) is associated with LPS production and progression of NASH [9]. Microbiota from patients with NASH have a higher proportion of the *Porphyromonadaceae* family (gram-negative bacteria) and a lower proportion of the *Lachnospiraceae* and *Ruminococcaceae* families

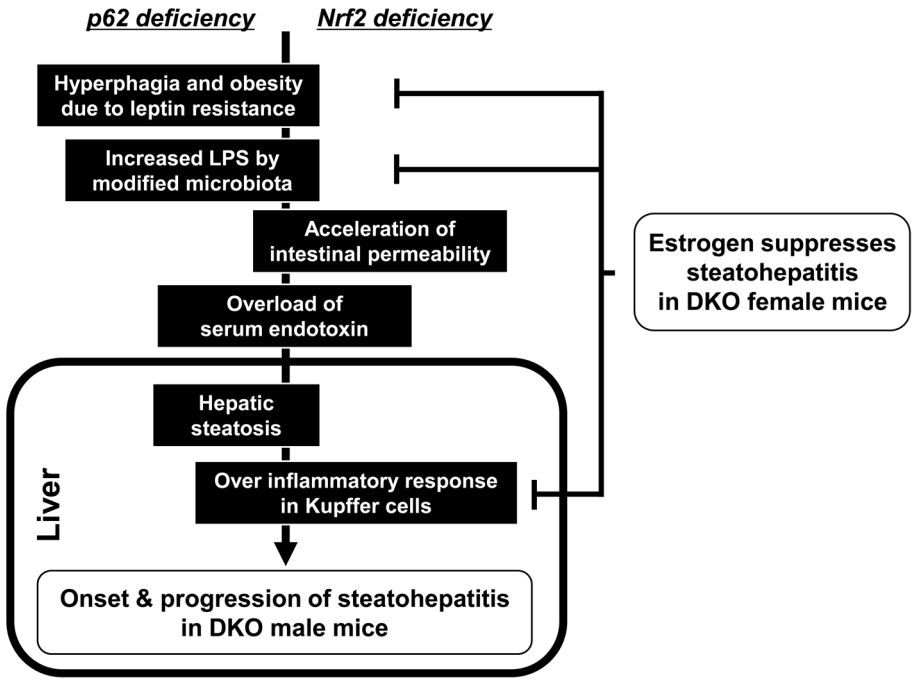

Fig. 7. Schematic hypothesis of steatohepatitis in double-knockout (DKO) mice. Deficiency of the *p62* gene in mice induces hyperphagia and obesity due to leptin resistance, which in turn leads to hepatic steatosis. Moreover, *p62* deficiency modifies microbiota composition and increases lipopolysaccharide (LPS) production in the intestine. Deficiency of *Nrf2* results in acceleration of intestinal permeability through downregulation of tight junction proteins in the intestine. These changes lead to overload flux of LPS into the liver. Furthermore, *Nrf2* deficiency results in an increased inflammatory response to LPS in Kupffer cells. The above multiple hits lead to the onset of steatohepatitis in DKO mice. Gender differences are observed for the development of steatohepatitis in DKO mice. In female mice, a low-grade inflammatory hit in the liver under *in vivo* conditions of high estradiol levels may be attributable to attenuation of hyperphagia-induced obesity, LPS production in modified microbiota, and inflammatory responses in the liver, all of which lead to the milder pathological features of steatohepatitis.

(gram-positive bacteria) compared with healthy subjects [29, 32]. In this study, flora analysis showed an increased proportion of *Porphyromonadaceae* and *Paraprevotellaceae* in DKO mice compared with WT mice, but they did not differ significantly between male and female mice. In contrast, *Ruminococcaceae* was decreased significantly in DKO male mice compared with WT male mice, and increased significantly in DKO female mice compared with DKO male mice (Fig. 6B). Recent studies have shown that gender or female hormones affect microbiota composition in both humans and mice [23, 30], and another study revealed that gonadectomy and hormone replacement changed gut microbiota composition dramatically [22]. These data indicate that sex hormone, especially estrogen could influence gut microbiota. Moreover, estrogen and microbiota may interact to influence the pathological conditions of inflammatory diseases, including NAFLD, through modification of LPS production in microbiota [6]. Taken together, the effect of estrogen in microbiota might result in attenuation of LPS production in intestinal microbiota.

Despite the theoretical beneficial effect of estrogen replacement therapy on human NASH, whether estrogen replacement therapy reduces fibrosis risk or progression of NASH among post-menopausal women remains uncertain and inconclusive [14]. In this study, estrogen replacement therapy was not performed and needs further analysis to demonstrate a direct effect of estrogen against steatohepatitis in DKO mice. Insulin resistance was milder in female mice than male mice (Fig. 4B). It is likely that a protective role of endogenous estrogen is important for glucose homeostasis and insulin resistance [25]. In the post-menopausal period, with decreased estrogen levels, insulin resistance is associated with inflammatory conditions involved in endoplasmic reticulum stress and oxidative stress in NASH [24].

In conclusion, gender differences are observed for development of steatohepatitis in DKO mice, which resembles the clinical features of human NASH. Low-grade inflammatory hit in the liver under *in vivo* conditions of high estradiol levels may be attributable to the milder pathological features of steatohepatitis in female mice. A therapeutic approach targeting inflammatory hits with estrogen or its analogues may be useful for potential management of steatohepatitis.

Conflict of Interest

The authors declare that they have no conflicts of interest.

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