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High-fat-diet–induced modulations of leptin signaling and gastric microbiota drive precancerous lesions in the stomach

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ABSTRACT

Objectives: Obesity is a risk factor for malignancy in various tissues, and has been associated with gut microbiota alterations. However, the link between obesity-associated microbiota and gastric pathogenesis has not been clarified. We demonstrated that high-fat-diet (HFD) feeding causes intestinal metaplasia, which are precancerous lesions of the stomach, with augmented gastric leptin signaling. The aim of this study was to investigate the precise role of leptin signaling in the altered microbiota composition and pathogenesis in the stomach during diet-induced obesity.

Methods: Male C57 BL/6 J, leptin receptor (Lepr)-mutated *db/db*, and gastrointestinal epithelium-specific Lepr conditional knockout (T3 b-Lepr cKO) mice were fed a HFD or control diet. Gastrointestinal microbiota was analyzed by 16 S rRNA gene sequences and quantitative polymerase chain reaction. Transplantation of gastric microbiota of HFD-fed mice was performed to evaluate metaplasia onset in recipient mice.

Results: One week of HFD caused severe microbial dysbiosis in the stomach. The microbiota changes were accompanied by increased gastric leptin, leading to the consequent development of intestinal metaplasia. Transplantation of gastric microbiota from HFD-fed mice induced intestinal metaplasia in recipient mice; however, only a limited effect on pathogenesis was noted. HFD-fed *db/db* mice did not show a decrease in microbial abundance. Moreover, T3 b-Lepr cKO mice failed spontaneous obesity, and suppressed decreased abundance of gastric microbiota and occurrence of intestinal metaplasia during HFD feeding similar to *db/db* mice.

Conclusions: Gastric leptin signaling modulates the gastric microbiota community and regulates the pathogenesis in the gastric mucosa.

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Introduction

Gastric cancer (GC) is the second leading cause of cancer-related deaths worldwide [1]. Chronic *Helicobacter pylori* (*H. pylori*) infection is considered a major risk factor for GC, and approximately half of the world's population is infected with *H. pylori*. However, only a fraction of *H. pylori*-infected individuals ultimately develop GC [2,3]. Obesity augments the risk for gastric cardia adenocarcinoma [4]. A prevalence of *H. pylori* infection was not significantly higher in obese patients, but obesity increased the development of both chronic gastritis and GC [5]. Thus, obesity could potentially accelerate the intermediary steps in the formation of precancerous lesions and the consequent development of GCs. Accordingly, to prevent tumorigenesis in such high-risk individuals, the identification of signaling molecules and factors associated with obesity-induced gastric carcinogenesis is crucial.

Intestinal microbiota have been shown to play an important role in the development of obesity [6,7] and the progression of cancers [8]. High-fat diet (HFD)–induced metabolic diseases, including obesity, are closely associated with changes in the composition and number of gut microbiota (dysbiosis) [9,10], which suggests that nutrition can shape intestinal microbiota. In contrast, very few reports exist on the association between pathogenesis and microbiota in the stomach during diet-induced obesity because the gastric environment is considered unfavorable for bacterial growth, except for *H. pylori*. However, recent evidence points to the presence of core microbiota in the stomach and revealed gastric dysbiosis in patients with gastritis and GC [11,12].

Leptin is an adipocyte-derived hormone that acts centrally to suppress food intake and increase energy expenditure through its receptor in the hypothalamus [13]. Peripherally, leptin promotes angiogenesis, survival, and the proliferation of various cells [14]. The stomach also constitutively produces leptin and expresses Lepr [15,16]. Leptin signaling is mediated through the Janus kinase 2 and signal transducer and activator of transcription 3 (STAT3),

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phosphoinositide 3-kinase, and extracellular signal-regulated kinase 1/2 pathway and is blocked by the suppressor of cytokine signaling 3 (SOCS3).

Abundant leptin production is correlated with the progression of GC [17,18]. We demonstrated that SOCS3 deletion in the gastrointestinal epithelium of a murine model caused gastric tumors owing to enhanced gastric leptin signaling [19]. We further discovered that wild-type (WT) mice with diet-induced obesity exhibited enhanced leptin signaling in the stomach. These mice exhibited intestinal metaplasia, which are precancerous lesions of the stomach with increased expression of ectopic intestinal molecules, such as Muc2 (intestinal-type mucus protein), Cdx2 (transcription factor involved in the development of intestinal epithelial cells) [20], and the disruption of organelle homeostasis, which ultimately enhance cancer-stem-like properties [21]. These observations were abrogated in leptin-deficient *ob/ob* mice and leptin receptor-mutated *db/db* mice.

Based on these findings, in this study, we investigated the mechanism by which dietary fat modulates the microbial community composition to develop intestinal metaplasia mediated by gastric leptin signaling. These results can help to provide insight into the mechanisms linking HFD-induced leptin signaling followed by dysbiosis of the gastric microbiota and the development of intestinal metaplasia leading to obesity-related GC.

Methods

Animals and diets

Male C57 BL/6 J WT and *Lepr* mutated *db/db* mice (both purchased from SLIC Japan, Shizuoka, Japan) were housed under specific pathogen-free conditions and used in experiments at 7 wk of age. To further investigate the in vivo effect of leptin signaling on intestinal metaplasia, we adopted a gastrointestinal epithelial cell-specific *Lepr* knockout approach. Gastrointestinal epithelial cell-specific *Lepr* knockout mice were generated by crossing *T3 b-Cre* transgenic mice (i.e., gastrointestinal epithelial cell-specific expression of *Cre* under control of *T3 b* promoter) [19] and B6.129 P2-*Lepr^{flm1} Rck/J* (*Lepr^{fllox/fllox}*) mice, bearing homozygous floxed exon 1 *Lepr* alleles (The Jackson Laboratory, stock #008327). The weanlings were tail-snipped and genotyped using a sets of exon 1 primers, p1 (forward) 5'-GTC ACC TAG GTT AAT GTA TTC-3' and p2 (reverse) 5'-TCT AGC CCT CCA GCA CTG GAC-3' (The Jackson Laboratory stock #008327 protocol), and *Cre* primers as previously described [19].

The mice were provided with either a control diet (CD; 10% of calories from fat, D12450 J) or HFD (60% of calories from fat, D12492; Research Diets Inc., New Brunswick, NJ) and had access to water ad libitum. The mice were euthanized by isoflurane inhalation, and the stomach, large intestine (LI), and hypothalamus were removed. The stomach and LI were dissected longitudinally and the contents collected. The Animal Ethics Committee of the Prefectural University of Hiroshima approved the animal care and experiments.

Bacterial 16 S rRNA gene amplicon analysis and quantitative polymerase chain reaction

DNA extraction from the gastrointestinal contents was performed using glass beads and phenol as described previously [22]. The mixture of glass beads and phenol was vortexed vigorously using a MicroSmash MS-100 R system (Tomy Digital Biology, Tokyo, Japan) at 5000 rpm for 30 s. Amplification and sequencing of the V1-V2 region of the bacterial 16 S rRNA gene were performed using the 27 Fmod2 primer and reverse 338 Rm primers, which contain index and Illumina adapter sequences, as described previously [23]. The MiSeq library was constructed by mixing an equal amount of DNA for every sample, and analyzed using MiSeq Reagent Kit v2 (Illumina, San Diego, CA). The resultant sequence data were analyzed by QIIME 2 [24]. A total of 1,738,049 raw reads across 95 samples, with an average 11 000 reads per sample, were processed with the QIIME 2 software package (version 2018.8).

Quantitative polymerase chain reaction (qPCR) analysis was carried out using the KOD SYBR qPCR Mix (TOYOBO, Osaka, Japan) with group- and subgroup-specific primer sets (Table S1), and standard DNA (provided by Yakult Central Institute, Japan). The products were detected on the AriaMx Real-time PCR system (version 1.61, Agilent Technologies, Foster City, CA).

Transplantation of gastric microbiota

For the depletion of gastrointestinal commensal microbiota, recipient mice were treated with an antibiotic mixture containing ampicillin (1 g/L), vancomycin

(500 mg/L), neomycin sulfate (1 g/L), and metronidazole (1 g/L), which was provided in the drinking water for 1 wk before transplantation [25]. Gastric microbiota for transplantation was prepared as reported previously [26] with a slight modification. In brief, the mice were fed the CD or HFD for 1, 3, and 7 mo.

After sacrifice, the gastric contents collected from five mice were pooled, mixed with a 9-fold volume of sterile saline, and homogenized immediately. The homogenate was left to stand on ice for 15 min and then centrifuged for 5 min at 6000 × g at 4°C. The supernatant was discarded, and the pellet was resuspended in sterile saline to form the bacterial suspension. Bacterial suspensions (100 μL) were gavaged into the recipient mice, which were subsequently maintained under normal conditions with a laboratory chow diet for 3 mo. For experiments involving the depletion of transplanted microbiota after transplantation, the mice were further administered the aforementioned antibiotic mixture for 1 wk and maintained under normal conditions with a laboratory chow diet for 3 mo.

Immunohistochemical analysis

Paraffin-embedded sections of 10% formalin-fixed tissues were stained either with hematoxylin and eosin or periodic-acid Schiff and Alcian blue. Detection of leptin, *P-Lepr*, *Cdx2*, and *Muc2* was performed in accordance with a previously published method [19]. In brief, the antigen retrieval sections using a Retrieval A kit (BD Biosciences, San Jose, CA) were incubated overnight with the primary antibodies (Abs), rabbit anti-leptin, anti-Muc2, and mouse anti-Cdx2 Abs (Santa Cruz Biotechnology Inc., Santa Cruz, CA) at 4°C.

Subsequently, the slides were stained with a biotinylated anti-rabbit or anti-mouse immunoglobulin G secondary Ab, along with streptavidin-labeled peroxidase using a Histofine SAB-PO or SAB-AP kit (Nichirei Biosciences Inc., Tokyo, Japan). Slides were developed using 3,3'-diaminobenzidine solution (ImmPact) or an alkaline phosphatase substrate kit (ImmPact Vector Red, Vector Laboratories, Burlingame, CA) in accordance with the manufacturer's protocol, followed by hematoxylin counterstaining. Slides were observed using a ZEISS Axio Imager 2 (Carl-Zeiss, Jena, Germany). A quantitative analysis of the immunohistochemistry-stained sections was performed using ImageJ software v1.52 (National Institutes of Health, Bethesda, MD).

Recombinant leptin treatment

To detect leptin-induced STAT3 activation in the gastric mucosa and hypothalamus, 5-week-old cKO and control mice were administered 1 mg/kg of recombinant leptin (Wako, Osaka, Japan) intraperitoneally, as described previously [27]. Lysates of the stomach and hypothalamus were prepared for a western blot analysis.

Western blot analysis

Lysates were prepared from the gastric tissues and hypothalamus for analysis with western blotting in accordance with a previously published method [19]. The Abs used in western blotting were rabbit antiphosphorylated STAT3 Ab, and mouse anti-STAT3 Ab were purchased from Cell Signaling Technology (Danvers, MA).

Statistical analysis

Significant variations in the frequency of genera across groups were extracted and analyzed with nonparametric *t* tests. Continuous data are presented as the means ± standard deviation and were analyzed by *t* test or analysis of variance, followed by the Holm–Sidak post hoc test for multiple comparisons, using Prism software, version 6 (GraphPad San Diego, CA). A *P*-value < 0.05 was considered statistically significant.

Results

Gastrointestinal microbiome dysbiosis in intestinal metaplasia by high-fat diet feeding

In line with our previous report [20], we confirmed that the gastric mucosa exhibited hyperplasia at 1 wk, reduction of parietal cells at 3 wk, complete loss of zymogenic and parietal cells, and glandular metaplasia at 12 wk, and no normal gastric gland at 20 wk (Fig. S1). In this time-course, we examined the abundance of microbiota in the stomach and LI in HFD-fed mice using next-generation sequencing of the 16 S rRNA gene. The microbiota of the stomach was dominantly populated by four phyla: Firmicutes (76.7%), Bacteroidetes (10.5%), Actinobacteria (8.5%), and Proteobacteria (2.1%; Fig. 1A).

One week of HFD feeding caused Firmicutes to increase, and by 20 wk, this phylum accounted for 95% of all bacteria.

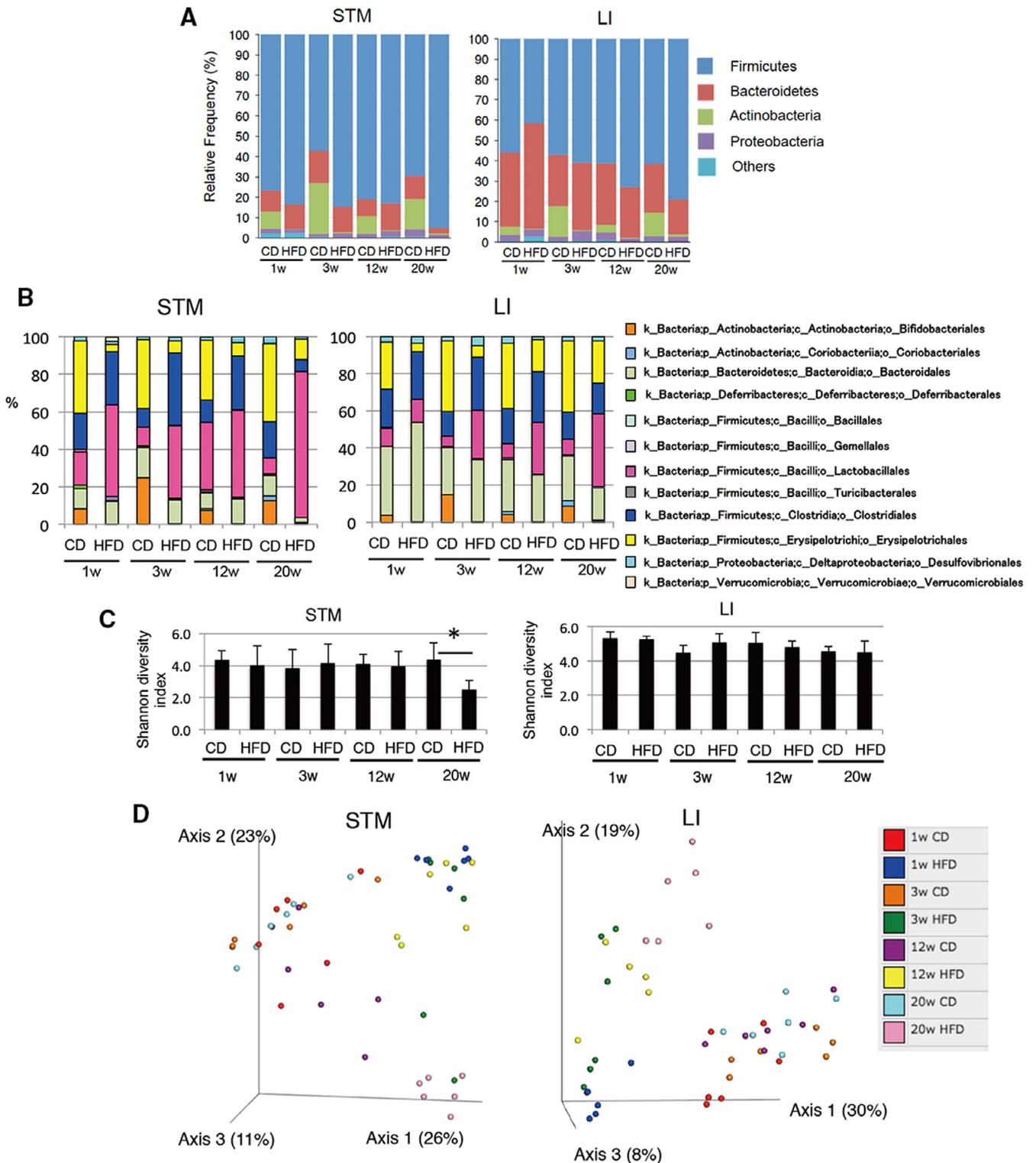


Fig. 1. Impact of a high-fat diet on gastrointestinal microbiota composition. Male C57 BL/6J mice were fed a control diet ($n = 6$) or high-fat diet ($n = 6$) for 1, 3, 12 and 20 wk. Microbial DNA in the stomach and large intestine of the mice was analyzed for relative bacterial 16S rRNA gene abundance. (A) Microbial composition and abundances of major phyla; (B) distribution of microbial orders in the stomach and large intestine. Data are presented as a stacked bar chart ($n = 6$ per group); (C) Shannon diversity index. The values represent the means \pm standard deviation ($n = 6$). * $P < 0.05$; (D) principal coordinate analysis of unweighted UniFrac distance. Each point represents an individual mouse.

Actinobacteria was present at very low levels in HFD-fed mice, and the abundance of Bacteroidetes was critically diminished by 20 wk of HFD feeding. Proteobacteria was present at a very

low proportion, which was not modulated by the HFD. By contrast, in LI, Firmicutes was not affected, whereas Actinobacteria decreased in the LI of HFD-fed mice, representing similar to the

pattern of change in the gastric region. Consistently, the abundance of proteobacteria did not differ between the CD and HFD groups, and its abundance in the LI matched that in the gastric region.

In response to the HFD, the proportion of Lactobacillales had a dominantly larger presence, but Bifidobacteriales was less abundant in the gastric microbiota (Fig. 1B). In particular, a clear increase in the proportion of *Lactobacillus reuteri* was observed, although no other species of the genus were identified (Fig. S2). In *Bifidobacterium*, *Bifidobacterium pseudolongum* completely disappeared in the stomach of HFD-fed mice. At 1 wk, Clostridiales (SMB53, family Clostridiaceae) increased temporarily, but Erysipelotrichales (*Allobaculum*, family Erysipelotrichaceae) exhibited a temporary decrease (Figs. 1B and S2).

The LI microbiota in HFD-fed mice exhibited an alteration pattern similar to that observed in the stomach, although the changes in the LI were milder than those in the stomach (Figs. 1B and S2). In the stomach of HFD-fed mice, the microbial diversity within the CD- and HFD-fed groups was not altered until 12 wk but strongly decreased at 20 wk, as demonstrated by the Shannon index, but no significant difference was observed in the LI (Fig. 1C). A principal coordinate analysis of the unweighted UniFrac distances also clearly separated HFD- and CD-fed mice into distinct clusters (Fig. 1D).

Next, we examined alterations in the number and composition of 12 major kinds of gastrointestinal microbiota by using group-specific 16 S rRNA-targeted primers. Bacterial number in the stomach (10^8 microbial cells) was comparable with that in the LI (10^9 microbial cells). The composition of gastric bacteria was similar to that of the LI (Fig. 2). One week of HFD feeding caused a 10% to 20% reduction in the microbial number of both the stomach and LI. Throughout the HFD-feeding period, *Bifidobacterium* was less abundant in the stomach than in the LI. In the stomach of HFD-fed mice, *Lactobacillus* increased at 3 wk (CD 7%, HFD 94% of the total of 12 major kinds of bacteria [100%]) and sustained the occupation at 12 wk (CD 9%, HFD 94%) and at 20 wk (CD 4%, HFD 88%).

Clostridium coccoides and *Clostridium leptum*, which are dominant microbiota in the LI, increased occupancy compared with those observed in CD-fed mice at 1 wk (*C. coccoides*: CD 11%, HFD 68%; *C. leptum*: CD 1%, HFD 8%). Microbiota changes in the LI were also *Lactobacillus*-dominant, similar to the patterns observed in the stomach, although with lower occupancy of *Lactobacillus* in the LI than in the stomach (CD 4%, HFD 73% from 3 wk to 12 wk; CD 1%, HFD 34% at 20 wk). These results obtained by qPCR analysis supported and reflected the metagenomics results, collectively, indicating that an HFD causes more rapid and severe dysbiosis in both the stomach and LI and ultimately enables the development of intestinal metaplasia.

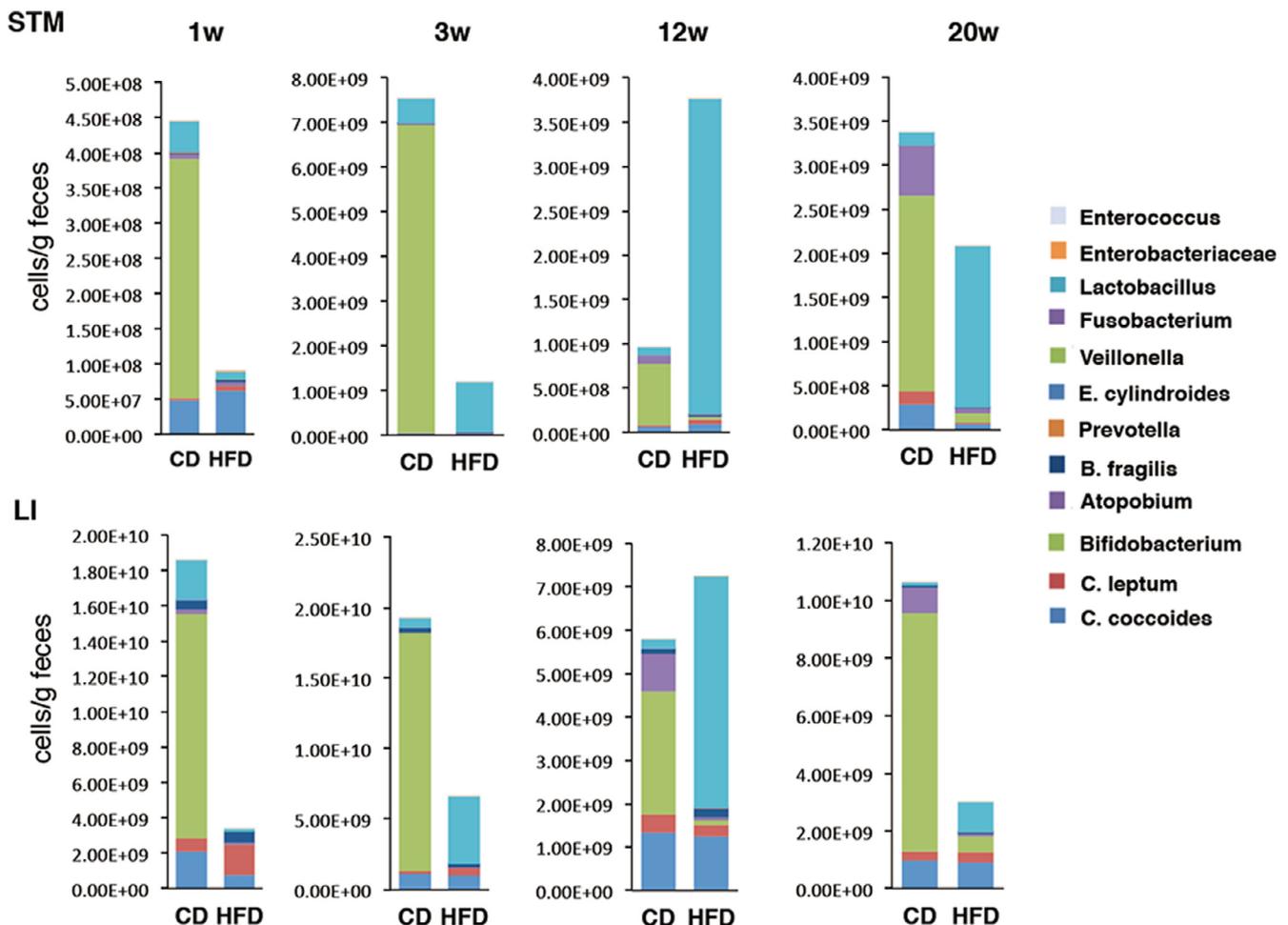


Fig. 2. High-fat diet remarkably decreases the microbial abundance in the gastrointestinal tract. Absolute numbers of the 12 major kinds of gastrointestinal bacteria, reflected by the 16 S rRNA gene abundance in the stomach and large intestine, quantified at the group-specific level using quantitative polymerase chain reaction. Data are represented as a stacked bar chart of $n = 6$ per group.

Limited involvement of gastric dysbiosis in induction of intestinal metaplasia

We transplanted gastric microbiota of HFD-fed mice into recipient mice to investigate whether gastric microbiota induces intestinal metaplasia. The transplantation of gastric contents of HFD-fed mice (3 mo) led to hyperplasia with atypical and irregular glandular cells, along with an increase in the frequency of Alcian blue-positive cells in the gastric cardia, reflecting an increase in acidic mucin, which is a marker of intestinal metaplasia in recipient mice (Fig. 3). Moreover, mice transplanted with gastric contents from HFD-fed mice (7 mo) further exhibited loss of parietal cells, which produce gastric acid, and the spread of Alcian blue-positive cells along the lamina muscularis mucosae. Mice transplanted with gastric microbiota from CD-fed mice showed normal mucosa.

The cardia in recipient mice showed abundant leptin and phosphorylated Lepr (Fig. 4A) and ectopic expression of Muc2 and Cdx2 (Fig. 4B). To confirm whether the gastric microbiota from HFD-fed mice directly affect the pathogenesis of intestinal metaplasia, we further treated recipient mice with antibiotic drugs after transplantation. The antibiotic treatment decreased the expression level of Muc2, Cdx2, leptin, and phosphorylated Lepr, but did not completely suppress their expression (Fig. 4). These results suggest that the HFD causes alteration of the gastric microbiota and pathogenesis of the intestinal metaplasia, but dysbiosis has a limited impact on augmented leptin expression during the early period.

We tried to dissect the relative contributions of microbiota and leptin/p-Lepr expression in triggering the rapid alterations observed by examining these effects under acute HFD conditions. One day of HFD feeding only slightly decreased the microbial numbers, with increased *Lactobacillus* and decreased *Bifidobacterium* abundance in the stomach. Moreover, the secretion of leptin was

significantly upregulated along with an increase of p-Lepr-positive cells in the stomach (Fig. S3). However, in the LI, HFD feeding caused a decrease in *Bifidobacterium* with no alteration of the total microbial number, and neither leptin nor p-Lepr was expressed. These results suggest that an HFD upregulates leptin production before the dynamic microbial changes in the stomach, and these alterations that occur very early during the feeding period could shape the subsequent microbial composition.

Suppression of dysbiosis by absence of leptin receptor signaling

Dysbiosis associated with the upregulation of Lepr signaling caused intestinal metaplasia in WT mice. Therefore, we used Lepr-mutated *db/db* mice to investigate if Lepr affects the gastrointestinal microbiota composition. The population of gastric microbiota in CD-fed *db/db* mice was two orders of magnitude lower than that in WT mice (Fig. 5). In contrast with WT mice, at 1 wk after feeding, the gastric microbiota in HFD-fed *db/db* mice inversely increased compared with that in CD-fed *db/db* mice, although the *Lactobacillus* occupancy increased more rapidly in HFD-fed *db/db* mice than in WT mice. At 3 wk, the gastric microbiota of CD-fed *db/db* mice reached a level similar to that of WT mice, whereas the *Lactobacillus* dominance was maintained. In contrast, neither diet- nor time-dependent dysbiosis was observed in the LI. These results suggest that a lack of leptin signaling influences the gastric microbiota more than the LI microbiota.

Deletion of Lepr in gastrointestinal epithelium does not induce dysbiosis or intestinal metaplasia

We generated gastrointestinal epithelial-specific Lepr cKO mice because Lepr is systemically mutated in *db/db* mice. To generate a gastrointestinal epithelium-specific deletion, Lepr^{flox/flox} mice

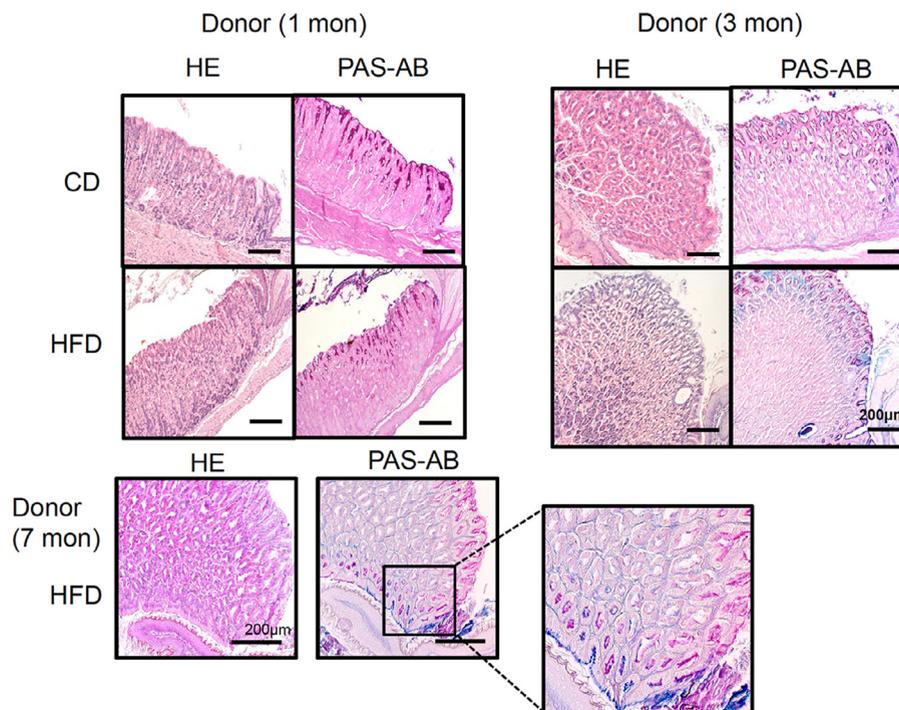


Fig. 3. Induction of acidic mucin in the gastric mucosa by transplantation of the gastric contents of high-fat diet-fed mice. The gastric microbiota of mice fed the high-fat diet for 1, 3, and 7 mo was transferred to recipient mice and maintained for 3 mo until sacrifice. Representative gastric sections stained for hematoxylin and eosin and periodic-acid Schiff-Alcian blue (Alcian blue) are shown. The magnified image shows the gastric cardia in recipient mice receiving the gastric contents of mice fed the high-fat diet for 7 mo. Strong Alcian blue-positive cells were observed.

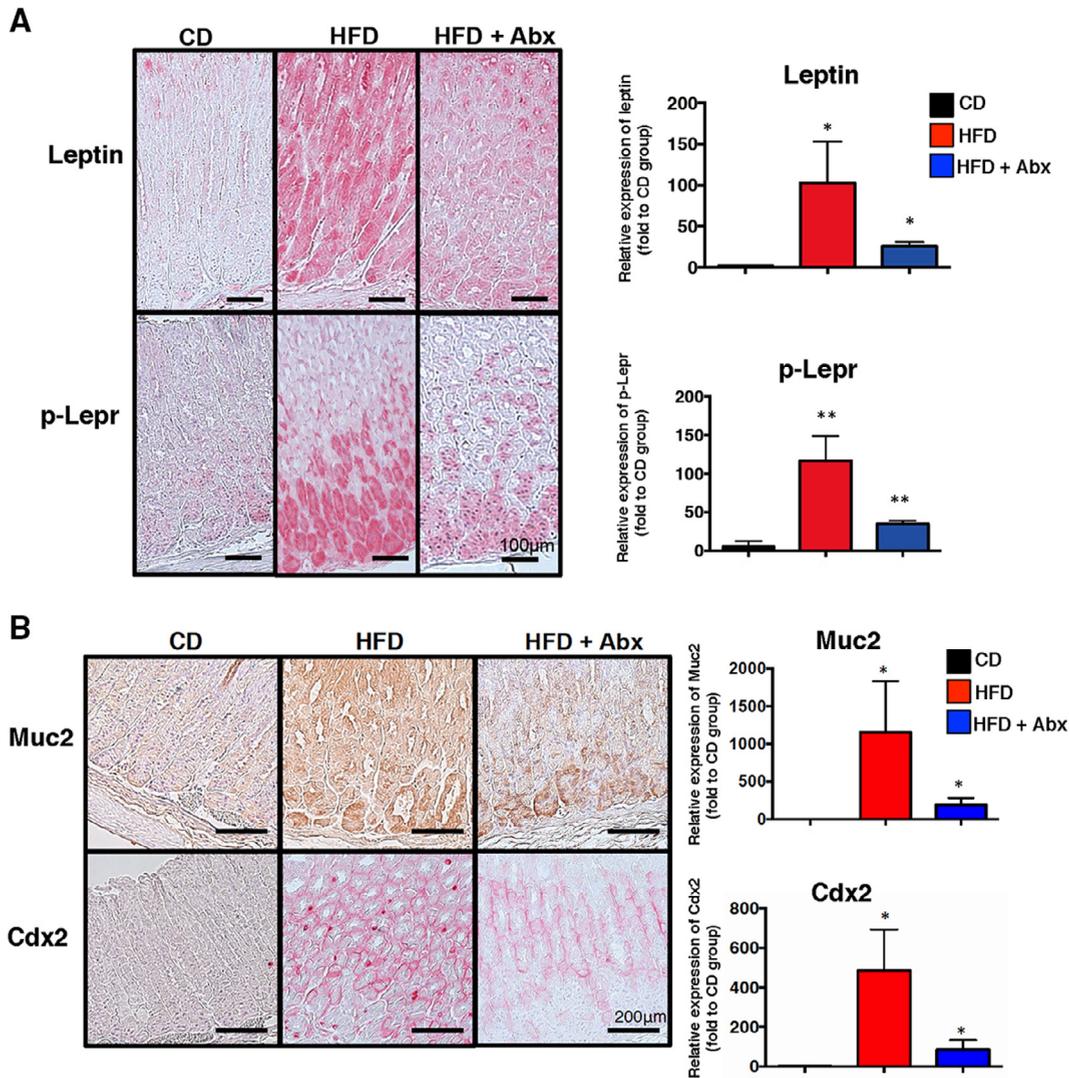


Fig. 4. Antibiotic drugs only partially suppress intestinal metaplasia in recipient mice transplanted with the gastric microbiota of high-fat diet-fed mice. Gastric contents of mice fed the control or high-fat diet for 7 mo were transferred to recipient mice (control or high-fat diet) with simultaneous antibiotic administration after gastric content of high-fat diet-fed mice and subsequently maintained for 3 mo. (A) Gastric sections stained for leptin and p-Lepr, and (B) Cdx2 and Muc2. Representative data are shown. Image J was used to calculate the staining intensity and calculate fold change in expression levels of treatment compared with the control-diet group. Data is shown as mean ± standard deviation of three independent experiments. *P* values were obtained using the Student's *t* test (two-tailed) by comparing with the control-diet group. Significant difference: * *P* < 0.05; ** *P* < 0.01.

were crossed with *T3 b-Cre* transgenic mice (Figs. 6A and 6B). In leptin-treated *T3 b-Lepr* cKO mice, STAT3 phosphorylation was observed in the hypothalamus but not in the gastric mucosa (Fig. 6C), which indicates that the Cre-mediated specific gastrointestinal epithelium-deletion of the *Lepr* gene of *T3 b-Lepr* cKO mice was efficient and functional. In contrast with *db/db* mice, *T3 b-Lepr* cKO mice did not spontaneously develop obesity (Fig. 6D). There was a comparable bacterial number in the stomach of *T3 b-Lepr* cKO with WT mice (Fig. 7). After 3 wk of HFD feeding, the gastric microbiota abundance in *T3 b-Lepr* cKO mice slightly decreased, but potently decreased in control mice. In the LI, HFD feeding caused a slight increase in bacterial number in cKO mice. Moreover, the gastric mucosa in HFD-fed *T3 b-Lepr* cKO mice was normal, with intact parietal cells and a mucosal structure, whereas in control mice, the HFD induced the disappearance of parietal cells and hyperplasia, and strongly stained Alcian blue-positive cells were observed, which is indicative of dispersed acidic mucin (Fig. 8). These results indicate that although the microbiota

is similar between *T3 b-Lepr* cKO and WT mice, HFD-induced dysbiosis in *T3 b-Lepr* cKO mice more closely resemble that of *db/db* mice.

Discussion

This work first provided the murine model that gastric *Lepr* signaling can shape the microbial community and cause intestinal metaplasia in diet-induced obesity. We found that HFD feeding induces dysbiosis more rapidly in the stomach than the LI and that dysbiotic gastric microbiota is associated with intestinal metaplasia onset, albeit with a limited contribution to pathogenicity. Mice that lack *Lepr* in the gastrointestinal tract are protected against dysbiosis and HFD-induced intestinal metaplasia. Dysbiosis has been considered to cause many gastrointestinal and systemic diseases, including cancer [28]. Thus, these results greatly contribute to improving the current understanding of the role of gastric leptin in gastric malignancies.

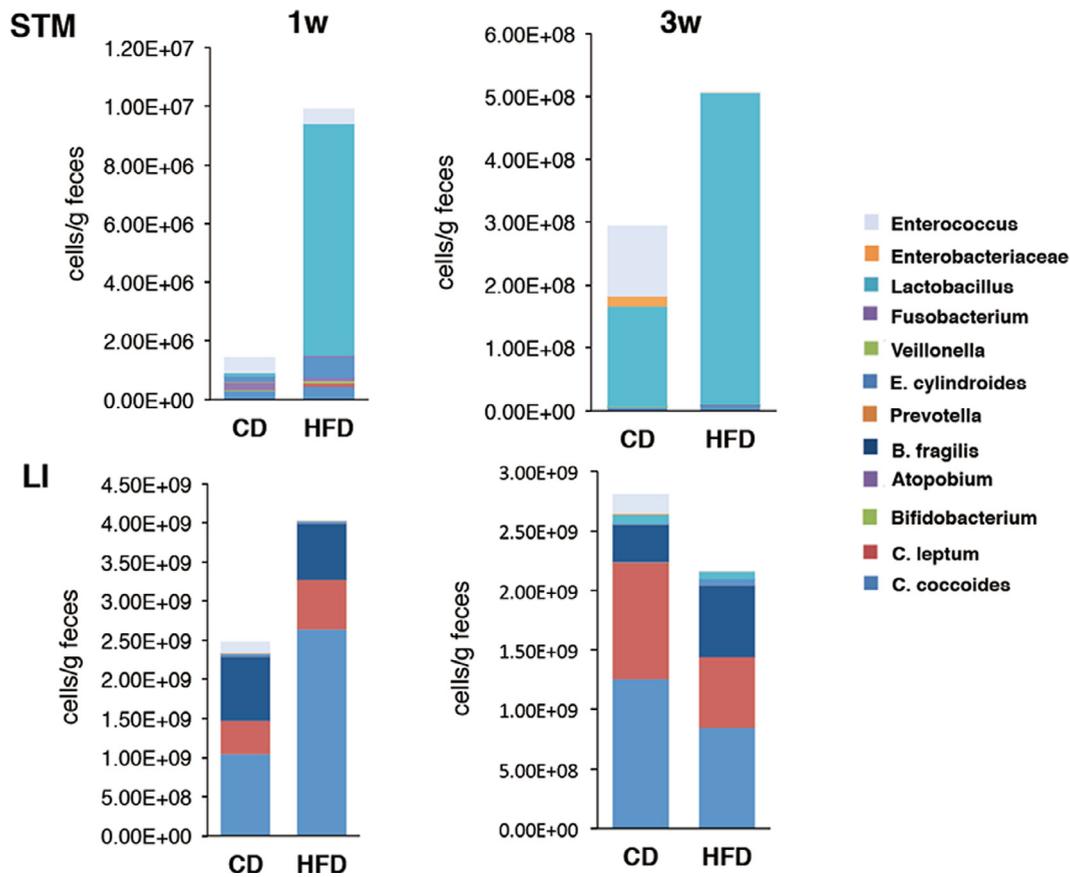


Fig. 5. Changes in gastrointestinal microbial number in high-fat diet-fed *db/db* mice. *db/db* mice were fed the control diet ($n = 5$) or high-fat diet ($n = 5$) for 1 and 3 wk, respectively. Absolute numbers of the 12 major kinds of gastrointestinal bacteria based on 16 S rRNA gene abundance in the stomach and large intestine were quantified at the group-specific level using quantitative polymerase chain reaction.

Extensive metagenomics and phylogenomic data of animal/human intestinal microbiota in the context of metabolic disorders and cancers are increasingly available [8]. Ley et al. [10] reported the occurrence of a high Firmicutes-to-Bacteroidetes ratio in gut fecal samples of obese animals, which was further confirmed in obese humans [6,29]. However, other studies [30,31], including the present study (Fig. 1A), did not reproduce this result, which highlights the controversy in understanding the connection between intestinal dysbiosis and general health. In the present study, the gastric and LI microbiota showed similar trends in the alteration of the Actinobacteria population, which suggests that gastrointestinal microbiota might act in a coordinated manner to affect the health of the host.

We found a considerable abundance of gastric microbiota in mice. The enriched *Lactobacillus* population in the gastric microbiota under the reduced gastric acid secretion conditions in chronic *H. pylori*-infected patients has been associated with GC, indicating an altered gastric milieu that leads to intestinal metaplasia and GC development [12]. HFD also induces intestinal metaplasia, as we reported previously [20,21]. In the present study, we found that *Lactobacillus* dominance along with loss of *Bifidobacterium* occurs in the stomach of HFD-fed mice, implying a potential association between microbial alterations and pathogenesis. *L. reuteri* has been reported to increase in abundance in the gut of obese patients [32,33]. *L. reuteri* also increased in the stomach and LI of HFD-fed mice (Fig. S2).

In addition, *B. pseudolongum*, which has been reported in lean mice [34], drastically decreased in both the stomach and LI of HFD-fed mice (Fig. S2). An abundance of SMB53, a bacterium reported in type 2 diabetes mellitus model mice [35] and coexpressed with *H. pylori* in intestinal metaplasia [11], transiently increased in both the stomach and LI at 1 wk, but decreased in the stomach and reached the same level in the LI at a later period. *Allobaculum*, a bacterium in healthy condition and reduced abundance in obese animals [36], showed decreased levels during HFD feeding in the stomach. The gastric microbial abundance closely resembled that of the LI. Furthermore, both the gastric and LI microbial abundance severely decreased in response to the HFD.

Nonetheless, apparent structural alterations and the pathogenesis were restricted to the stomach. He et al. also reported that an HFD induced changes in the gastric microbial occupation rate by 24 wk on a HFD; however, the association between microbial alterations and gastrointestinal pathogenesis has not been discussed previously [37]. Beyez et al. reported that in intestinal-specific PPAR δ cKO mice, stemness and tumorigenesis were observed in the intestine after long-term HFD feeding (9–14 mo) [38]. Our study suggests that drastic dysbiosis itself, irrespective of the species involved, has been associated with the onset of metaplastic lesions in the stomach. Taken together with the results from the present study, the stomach appears to be more susceptible to short-term HFD feeding, and a different mechanism for predisposition to neoplasia may be at play. Further studies are required to

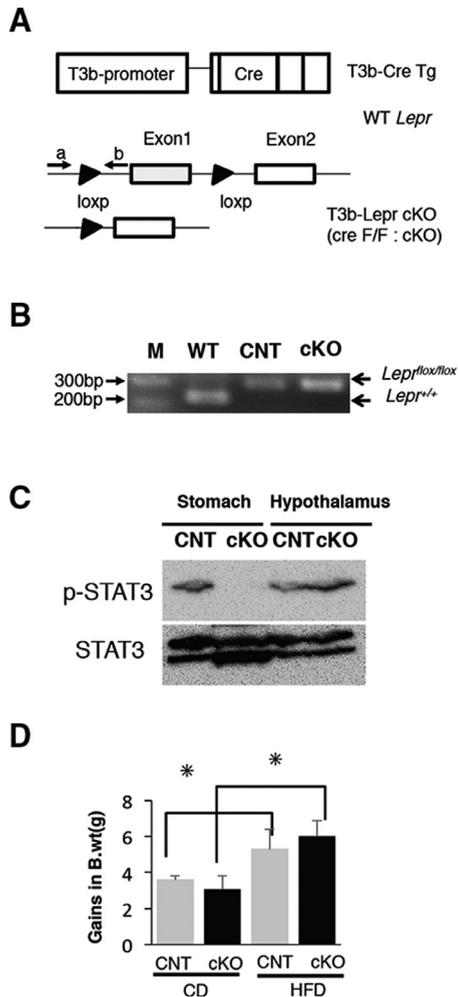


Fig. 6. Generation of T3 b-Lepr conditional knockout mice. (A) Schema of *Lepr* floxed and deleted loci. Exon 1 is flanked by two LoxP sites (arrowheads); (B) polymerase chain reaction genotyping of floxed alleles using the primer sets a and b against the tail genome; (C) Western blotting analysis for phosphorylated STAT3 in the gastric mucosa and hypothalamus of control and conditional knockout mice after administration of recombinant leptin for 45 min; (D) changes in body weight gain in T3 b-Lepr conditional knockout and control mice fed the control diet (n = 4) or high-fat diet (n = 5) for 3 wk. Values represent the means ± standard deviation. Significant difference: * P < 0.001.

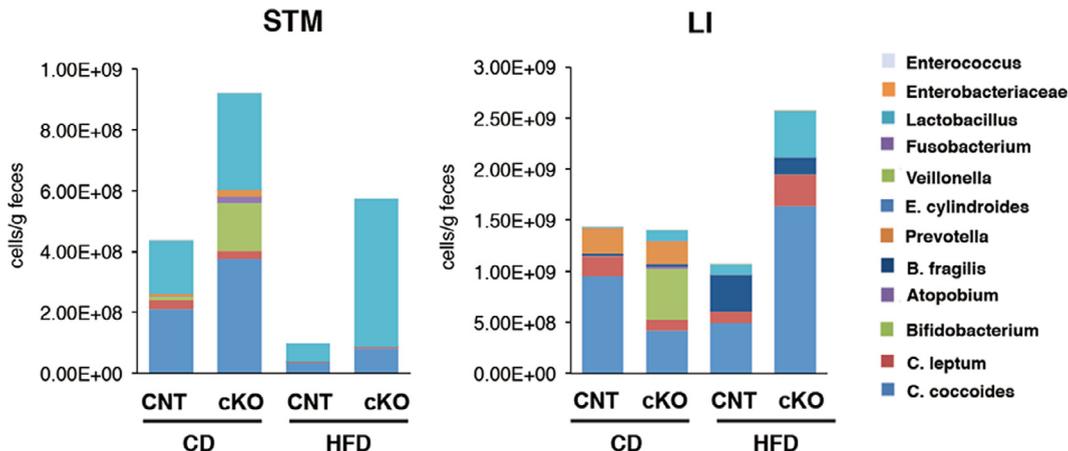


Fig. 7. Suppression of dysbiosis in high-fat diet-fed T3 b-Lepr conditional knockout mice. Abundance of 12 major kinds of gastrointestinal bacterial monitored by group-specific quantification of 16 S rRNA gene abundance in the stomach and large intestine using quantitative polymerase chain reaction. Control and T3 b-Lepr conditional knockout mice were fed the control or high-fat diet for 3 wk. Data are presented as a stacked bar chart (n = 4–5 per group).

clarify the significance of similar types of dysbiosis in different tissues of the same individuals.

Leptin has pleiotropic effects and is significantly associated with both gastrointestinal malignancies and tumorigenesis [17,39]. Our earlier work demonstrated that aberrant leptin signaling led to the development of gastric tumors in mice with gastrointestinal SOCS3 deletion [19]. Obesity is one of the known risk factors for the onset of GC; however, both *ob/ob* and *db/db* mice, which are extraordinarily obese and lack leptin signaling, do not spontaneously develop gastric tumors. Furthermore, an elevated expression level of markers of pluripotent stem cells were suppressed in *ob/ob* and *db/db* mice under HFD feeding [21]. Collectively, these results imply that obesity in conjunction with altered leptin signaling is necessary for the induction of tumorigenesis.

Leptin receptor signaling in the peripheral tissues remains uncertain. Unlike *db/db* mice, T3 b-Lepr cKO mice did not exhibit spontaneous obesity, which is similar to mice that lack intestinal Lepr [40]. This implies that gastrointestinal Lepr does not affect body weight. In the present study, HFD-induced metaplasia was suppressed in T3 b-Lepr cKO mice with no occurrence of dysbiosis (Fig. 7). In addition, the gastric and LI microbiota abundances in *db/db* mice were lower than those of WT mice throughout the experimental period, whereas T3 b-Lepr cKO and WT mice had a similar abundance of gastric and LI microbiota, regardless of the diet, which suggests that the total gastrointestinal microbial number could be dependent on body mass. Both *db/db* and T3 b-Lepr cKO mice had parietal cells in the stomach with gastric *Lactobacillus* dominance, irrespective of diet.

Although the link of gastric neoplasia with decreased parietal cells and enriched *Lactobacillus* population has been considered, our results imply that these factors are independent contributors in diet-induced obesity. Interestingly, neither *db/db* nor T3 b-Lepr cKO mice showed a decrease in gastric and LI microbial numbers under the HFD (Figs. 5 and 7), but the HFD induced a drastic microbial decrease in WT mice and control mice. Thus, a constant microbial number seems to be maintained with a lack of leptin signaling, albeit with *Lactobacillus* dominance. Conversely, leptin signaling accelerates dysbiosis. Moreover, with a HFD, the control and WT mice had high numbers of Alcian blue-positive cells, but the T3 b-Lepr cKO mice did not, which indicates that gastric Lepr signaling contributes to the development of precancerous lesions of the stomach. Thus, gastric Lepr-mediated signaling acts tumorigenesis and differs from central Lepr signaling, which is essential for body mass and energy expenditure.

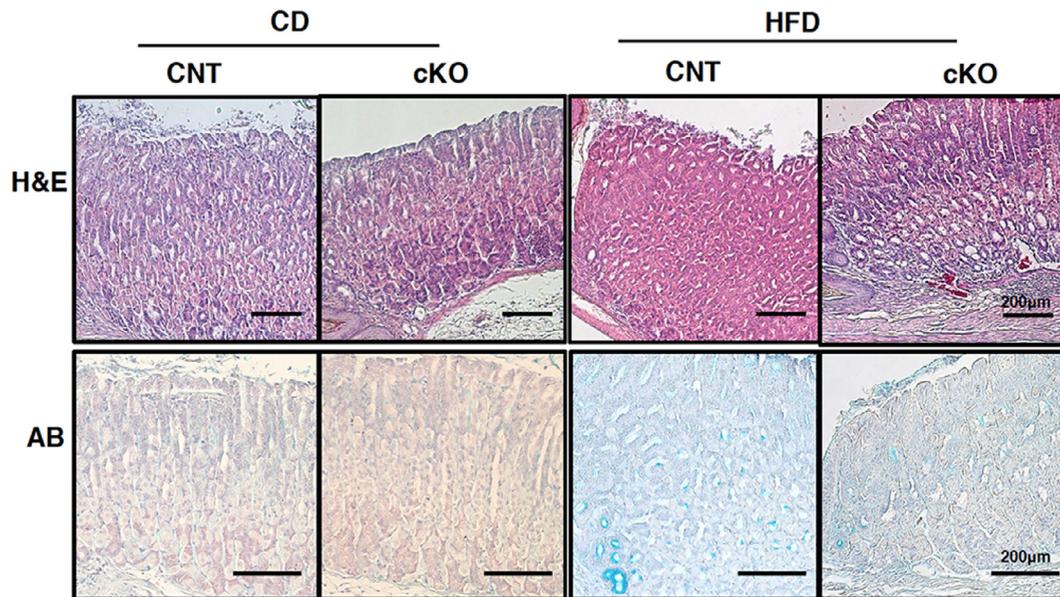


Fig 8. Suppression of high-fat diet-induced intestinal metaplasia in T3 b-LepR conditional knockout mice. Representative gastric sections stained for hematoxylin and eosin and Alcian blue in control and T3 b-LepR control knockout mice fed the control or high-fat diet for 8 wk.

Conclusions

We demonstrated the role of HFD-induced gastric Lepr signaling in intestinal metaplasia, which shapes the microbial community to collectively modulate pathologic progression in the stomach. Further studies are needed to clarify how gastric leptin affects cell development normally or neoplasia and determines the cell fate in the stomach. Our findings suggest the possibility of novel targets for the development of preventive or therapeutic strategies against GC, especially in the context of obesity.

Declaration of Competing Interest

None

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Supplementary materials

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