Targeted disruption of G protein-coupled bile acid receptor 1 (*Gpbar1/M-Bar*) in mice

Takaharu Maruyama, Kenichi Tanaka, Jun Suzuki, Hiroyuki Miyoshi, Naomoto Harada, Takao Nakamura, Yasuhisa Miyamoto, Akio Kanatani and **Yoshitaka Tamai**

Tsukuba Research Institute, Banyu Pharmaceutical Co., Ltd, Okubo 3, Tsukuba, Ibaraki 300-2611, Japan
(Requests for offprints should be addressed to K Tanaka; Email: kenichi_tanaka@merck.com)

Abstract

G protein-coupled bile acid receptor 1 (*Gpbar1/M-Bar*) is a novel G protein-coupled receptor for bile acid. Tissue distribution and cell-type specificity of Gpbar1 mRNA suggest a potential role for the receptor in the endocrine system; however, the precise physiological role of Gpbar1 still remains to be elucidated. To investigate the role of Gpbar1 *in vivo*, the *Gpbar1* gene was disrupted in mice. In homozygous mice, total bile acid pool size was significantly decreased by 21–25% compared with that of the wild-type mice, suggesting that Gpbar1 contributes to bile acid homeostasis. In order to assess the impact of Gpbar1 deficiency in bile acid homeostasis more precisely, *Gpbar1* homozygous mice were fed a high-fat diet for 2 months. As a result, female *Gpbar1* homozygous mice showed significant fat accumulation with body weight gain compared with that of the wild-type mice. These findings were also observed in heterozygous mice to the same extent. Although the precise mechanism for fat accumulation in female *Gpbar1* homozygous mice remains to be addressed, these data indicate that Gpbar1 is a potential new player in energy homeostasis. Thus, *Gpbar1*-deficient mice are useful in elucidating new physiological roles for Gpbar1.

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Introduction

Bile acids are synthesized from cholesterol in the liver and play pivotal roles not only in the solubilization of dietary fat, but also in the maintenance of cholesterol and bile acid homeostasis (Dietschy 1968, Russell & Setchell 1992). It is well known that bile acids regulate a number of biosynthetic enzymes and transporters through the activation of farnesoid X receptor (FXR), a bile acid nuclear receptor (Redinger 2003, Russell 2003). For instance, enzymes and transporters regulated by bile acids, such as cholesterol 7α-hydroxylase (CYP7A), Na^+-taurocholate cotransporting polypeptide (NTCP), and bile salt export pump (BSEP), are well known for their crucial roles in bile acid homeostasis (Grober *et al.* 1999, Chiang *et al.* 2000, Sinal *et al.* 2000, Tu *et al.* 2000, Ananthanarayanan *et al.* 2001).

Steroid hormones as well as bile acids modulate expressions of various genes by classical genomic actions through the stimulation of their nuclear receptors (Beato 1989, Aranda & Pascual 2001). However, there is substantial evidence that some steroid hormones stimulate second messengers by rapid non-genomic actions (Norman *et al.* 2004). It was reported that progestins inhibited cAMP formation in a cell line expressing membrane progestin receptor (mPR) and that the response was sensitive to the pertussis toxin, suggesting that mPR is coupled to the Gi/o protein (Zhu *et al.* 2003*a*,*b*). In addition, bile acid has been known to rapidly stimulate cAMP formation (Conley *et al.* 1976, Potter *et al.* 1991). Thus, the presence of a G protein-coupled receptor (GPCR) for bile acid was speculated. Recently, we and other investigators have successfully cloned a novel orphan GPCR, which did not show high homology to known GPCRs, and identified the endogenous ligand, bile acid (Maruyama *et al.* 2002, Kawamata *et al.* 2003). We have also revealed that the G protein-coupled bile acid receptor 1 (*Gpbar1/M-Bar/TGR5*) stimulated adenylate cyclase in response to bile acid without modulating the expression of FXR (Maruyama *et al.* 2002). Furthermore, Gpbar1 is endogenously expressed in enteroendocrine cell lines, such as NCI-H716, STC-1, and GLUTag, suggesting the potential role of Gpbar1 in the intestine (Maruyama *et al.* 2002). However, the precise role of Gpbar1 is yet to be determined.

In this study, we generated *Gpbar1*-deficient mice to elucidate the physiological role of Gpbar1 *in vivo*. First, we measured the total bile acid pool size and fecal excretion level in *Gpbar1*-deficient mice to investigate whether Gpbar1 would be involved in bile acid homeostasis. Then, we evaluated the impact of a high fat (HF) diet on *Gpbar1*-deficient mice to address the potential roles of Gpbar1.

Materials and Methods

Generation of Gpbar1*-deficient mice*

Mouse genomic *Gpbar1* clones were obtained by screening a 129/Sv mouse genomic λ phage library (Stratagene, La Jolla,

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CA, USA) using the cDNA probe of mouse *Gpbar1/M-Bar* (GenBank Accession no. AB086170). Most of the exon 2 region of the *Gpbar1* gene was replaced with a PGK-neo cassette. The targeting vector was linearized at a unique SalI site and introduced by electroporation into mouse embryonic stem (ES) cells, RW4. Neomycin-resistant ES clones were picked up and seven candidate clones were obtained by PCR screening. These PCR-positive clones were analyzed by genomic Southern blot analysis using probes A and B. Successful germline transmission was confirmed by genomic Southern blot analysis using probe B. *Gpbar1*-deficient mice were backcrossed to C57BL/6N mice for four generations before analysis.

Animal care

The mice were maintained on a 12 h light:12 h darkness cycle (0700–1900 h) and fed a standard rodent chow, CA-1 (CLEA, Tokyo, Japan) or high-fat (HF) diet (D12492, rodent diet with 60 kcal% fat; Research Diets, Inc., New Brunswick, NJ, USA) which were available *ad libitum*. Body weight was measured once a week. All animal procedures complied with the NIH guidelines and were approved by Banyu IACUC (Institutional Animal Care and Use Committee).

Bile acid analysis

In studies involving the measurement of the total bile acid pool size and fecal bile acid excretion, 13–14-week-old mice were housed individually in cages and food was available *ad libitum*. Total bile acid was extracted as previously described (Sinal *et al.* 2000). Briefly, for the measurement of the total bile acid pool size, the liver, gallbladder, and the entire small intestine were homogenized. Aliquots were extracted twice with ethanol under reflux. Subsequently, the extract was dried completely under a stream of nitrogen and resuspended in 50% ethanol. Feces were collected from each mouse over the 72-h period immediately prior to sacrifice, and then dried, weighed, and homogenized. The aliquots were extracted as mentioned previously. Total bile acid content was measured by an enzymatic method as previously described (Kitada *et al.* 2003).

Quantitative RT-PCR

Total RNA was prepared using ISOGEN (Nippon gene, Tokyo, Japan) and RNeasy kit (Qiagen) from each tissue of 6–10-week-old C57BL/6N mice, or 13–14-week-old Gpbar1 wild-type and homozygous mice. Random-primed cDNAs were synthesized by reverse-transcription (RT) and then subjected to quantitative PCR analysis using the PRISM 7900HT sequence detection system (Applied Biosystems, Foster City, CA, USA). Primers and probe sets for the detection of the expression of Gpbar1 (Mm00558112_s1), Cyp7a1 (Mm00484152_m1), Cyp7b1 (Mm00484157_m1), Cyp8b1 (Mm00501637_s1), Cyp27 (Mm00470430_m1), FXR (Mm00436419_m1), ileal bile acid transporter (IBAT) (Mm00488258_m1), short heterodimer partner (SHP) (Mm00442278_m1), BSEP (Mm00445168_m1), and ileal bile acid-binding protein (IBABP) (Mm00434316_m1) were purchased from Applied Biosystems. β-Actin was used for normalization of the gene expression level. The following primers and probe were used for the determination of β-actin: β-actin forward primer, 5′-AGGTCATCACTATTGGCAACGA-3′; β-actin reverse primer, 5′-CACAGGATTCCATACCCAAGAAG-3′; and β-actin probe, 5′-AGGTCATCACTATTGGCAACGA-3′.

Northern blot analysis

Aliquots (10 μg) of poly(A)RNA were isolated from the small intestine using a FastTrack 2.0 kit (Invitrogen). After

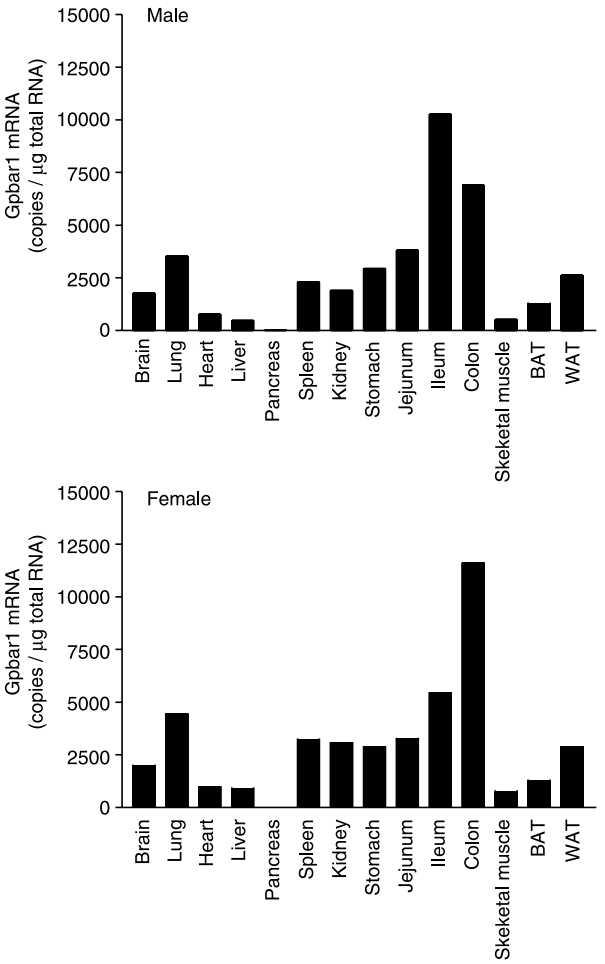

Figure 1 Tissue distribution of mouse Gpbar1 mRNA. Total RNA was subjected to reverse transcription and quantitative PCR using an ABI Prism 7900 sequence detector. Each column represents the mean value in duplicate. BAT, brown adipose tissue; WAT, white adipose tissue.

electrophoresis, the RNA was transferred to a Hybond-N^+ membrane (Amersham) and hybridized with ^{32}P-labeled probe for mouse Gpbar1 cDNA. The membrane was washed twice with 0·2×SSC containing 0·1% SDS at 65 °C and analyzed by FUJIX BAS2000 (Fuji film, Tokyo, Japan).

Blood chemistry

Plasma triglyceride (TG) and total cholesterol were measured using commercially available kits (Determiner L-TG II and L TC II (Kyowa medex, Tokyo, Japan)). Total plasma bile acid concentration was measured by an enzyme-colorimetric kit as previously described (Kitada *et al*. 2003).

Measurement of body composition

Whole body and lean body mass were measured by the Bruker minispec NMR analyzer (Bruker Optics, Woodlands, TX, USA), and male and female mice that were fed a HF diet were measured at 18 weeks of age.

Statistical analyses

All values are expressed as means ± S.E.M. Body weight changes were compared between groups using repeated measures ANOVA coupled to a *post hoc* Bonferroni test. Other data were analyzed by two-way ANOVA coupled to a *post hoc* Bonferroni test (StatView, SAS Institute, Cary, NC, USA).

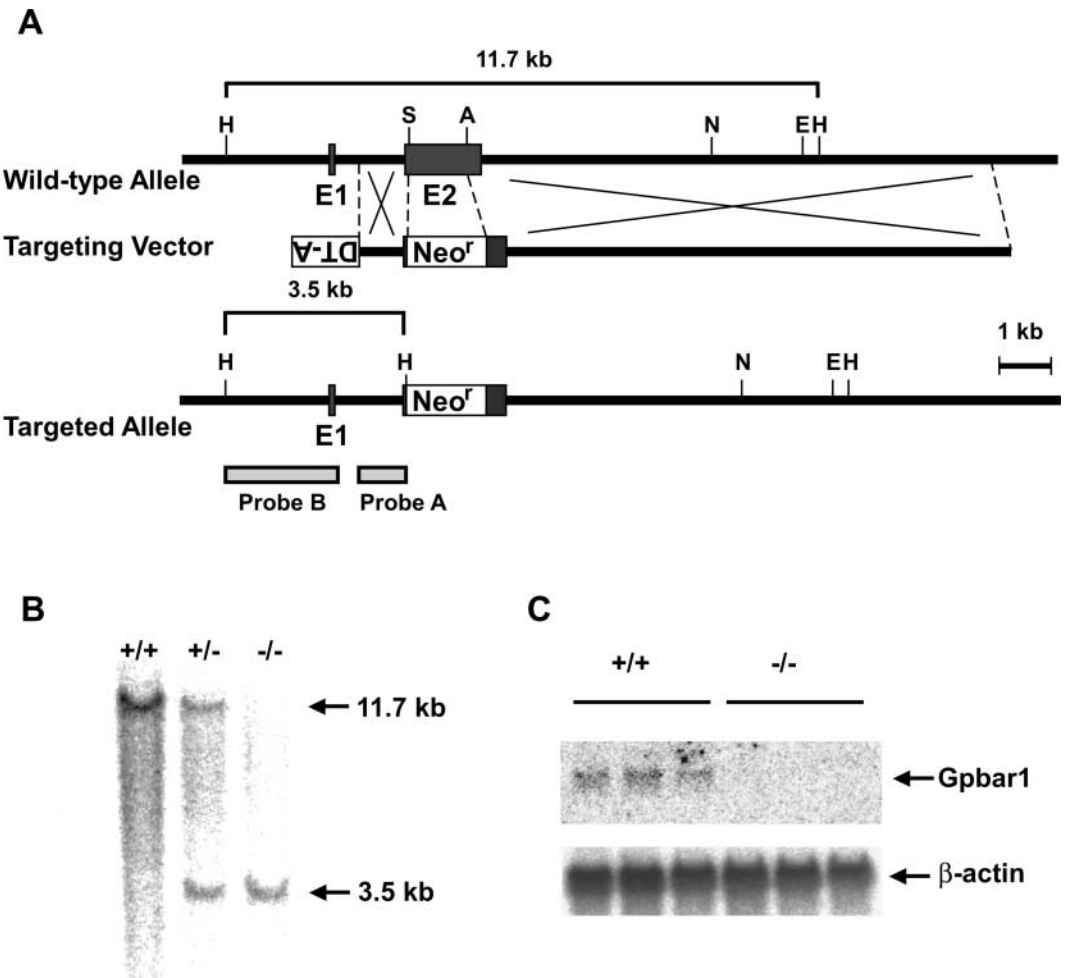

Figure 2 Targeted disruption of the mouse *Gpbar1* gene. (A) Targeting strategy for *Gpbar1*. A restriction enzyme map of the genomic locus and targeted allele is shown. The closed boxes represent exons (E1 and E2). Homologous recombination resulted in the replacement of exon 2, including ATG codon with the PGK-neo cassette. H, HindIII; S, SphI; A, ApaI; N, NsiI; E, EcoRI. (B) Genomic Southern blot analysis. HindIII-digested genomic DNA was blotted and hybridized with ^{32}P-labeled probe B. Wild-type band is 11·7 kb and the recombinant band is 3·5 kb in length. (C) Northern blot analysis of mouse Gpbar1 expression. Poly(A)RNA prepared from the small intestine in three wild-type (+/+) and three homozygous (−/−) mice was blotted and hybridized with ^{32}P-labeled probe of mouse Gpbar1 cDNA. Gpbar1 mRNA was 1·5 kb in length. β-Actin was used as a loading control.

Results

Tissue distribution of mouse Gpbar1 mRNA

The tissue distribution of mouse Gpbar1 mRNA was analyzed by quantitative RT-PCR (Fig. 1). High expression levels of Gpbar1 mRNA were detected in the ileum and colon of male mice, and in the colon of female mice. Medium expression levels were detected in the lung, spleen, kidney, stomach, jejunum, and gonadal white adipose tissue (WAT) of both male and female mice. Medium expression levels were also detected in the ileum of female mice. Among these tissues, Gpbar1 mRNA was substantially expressed in the intestine and/or colon, suggesting that Gpbar1 plays a certain role in bile acid homeostasis in these tissues.

Targeted disruption of the Gpbar1 *gene*

Most of the exon 2 region, including the first ATG codon, was replaced with a PGK-neo cassette (Fig. 2A). We confirmed the disruption of *Gpbar1* by Southern and Northern blot analyses (Fig. 2B and C). *Gpbar1* heterozygous and homozygous mice were viable and fertile, appearing normal as compared with wild-type littermates under standard laboratory conditions. The intercrosses of heterozygous mice produced the wild-type, heterozygous, and homozygous mice in the predicted Mendelian ratios.

To investigate whether Gpbar1 plays a role in bile acid homeostasis, we measured the total bile acid pool size and fecal bile acid excretion levels of *Gpbar1* homozygous mice. As shown in Fig. 3A and C, the total bile acid pool size was significantly decreased by 25 and 21% in male and female homozygous mice, respectively, compared with that of the wild-type mice, suggesting that Gpbar1 contributes to bile acid homeostasis. In spite of the decrease in total bile acid pool size, there was no difference in fecal bile acid excretion levels between wild-type and homozygous mice (Fig. 3B and D).

The expression levels of enzymes for bile acid biosynthesis, Cyp7a1, Cyp7b1, Cyp8b1, and Cyp27, were determined by quantitative RT-PCR. The expression level of Cyp8b1, which is an important enzyme to determine cholic acid/chenodeoxycholic acid ratio (Bjorkhem & Eggertsen 2001, Li–Hawkins *et al.* 2002), was significantly increased by 81% in male homozygous mice compared with that of the wild-type mice, while the others remained unchanged in male homozygous mice (Fig. 4A). In female homozygous mice, the expression levels of these key enzymes were not significantly different from those of the wild-type mice (Fig. 4C). Next, we evaluated expression levels of FXR and FXR target genes (*SHP*, *BSEP*, and *IBABP*) in *Gpbar1* homozygous mice, because FXR is a well-known regulator of bile acid homeostasis (Sinal *et al.* 2000, Tu *et al.* 2000). The expression levels of FXR and SHP in male and female homozygous mice appeared similar to those of the wild-type mice (Fig. 4A–D). However, the expression levels of BSEP and IBABP in female homozygous mice were significantly increased by 32% and significantly decreased by 23%, respectively, compared with that of the wild-type mice (Fig. 4C and D). In contrast, the expression levels of BSEP and IBABP in male homozygous mice were not altered (Fig. 4A and B). We also determined the expression level of IBAT in homozygous mice to address the influence of Gpbar1

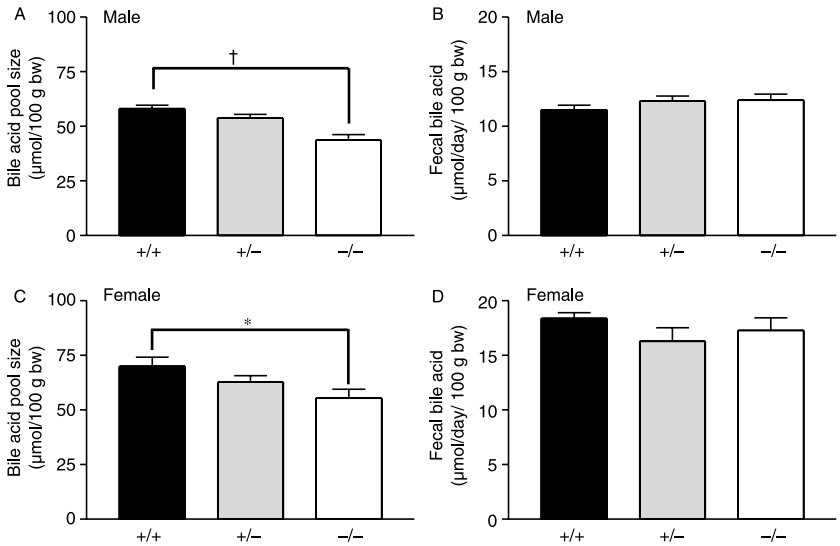

Figure 3 Total bile acid pool size and fecal bile acid excretion in *Gpbar1*-deficient mice. Total bile acid pool size (A) and (C) and fecal bile acid excretion (B) and (D) were determined by an enzymatic method as described in the Materials and Methods. All values are expressed as mean±S.E.M. of data from wild-type (+/+), heterozygous (+/−) and homozygous mice (−/−) respectively (n=7–16). *P<0·05, †P< 0·001, compared with the wild-type group. bw, body weight.

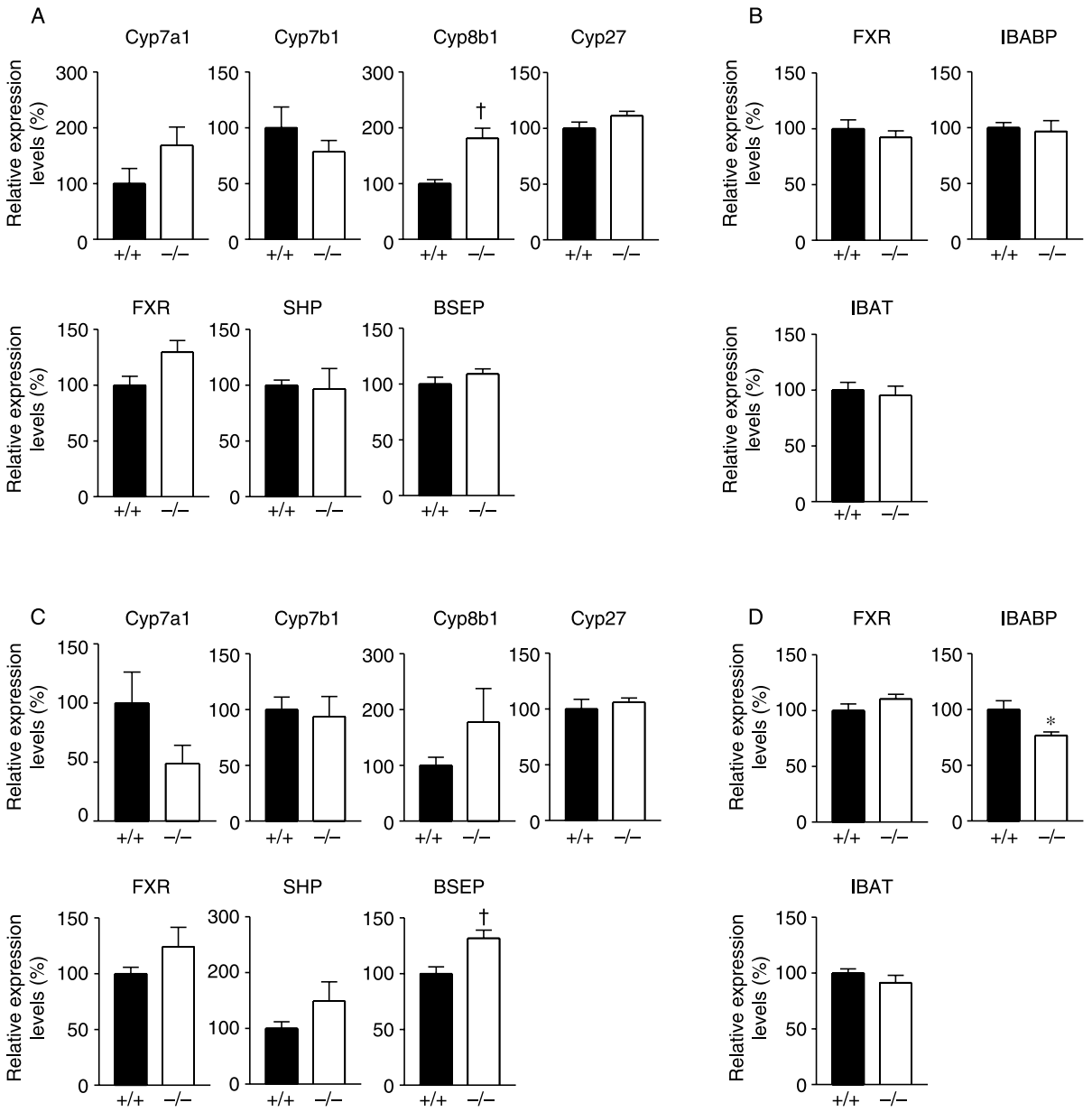

Figure 4 Reverse transcription PCR as determined by quantitative Taqman PCR. Total RNA was prepared from the liver and ileum of Gpbar1 wild-type and homozygous mice. The relative expression levels of Cyp7a1, Cyp7b1, Cyp8b1, Cyp27, FXR, SHP, and BSEP in male and female liver are shown in (A) and (C) respectively. The relative expression levels of FXR, IBABP, and IBAT in male and female ileum are shown in (B) and (D) respectively. The relative expression levels are shown compared with 100% expression level in the wild-type mouse after normalization to the β-actin expression level in each tissue. All values are expressed as mean ± S.E.M. of data from wild-type (+/+) and homozygous mice (−/−) respectively ($n=5$). $^{*}P<0{\cdot}05$, $^{\dagger}P<0{\cdot}01$, compared with the wild-type mice.

deficiency on bile acid re-uptake. However, the expression level was not affected in male and female homozygous mice compared with that of the wild-type mice (Fig. 4B and D). Therefore, the different expression levels of Cyp8b1, BSEP, and IBABP between male and female homozygous mice were unlikely to be involved in the decreased bile acid pool size observed in both male and female *Gpbar1* homozygous mice, although changes in their enzymatic activities have been uncharted so far.

Under regular dietary conditions, we determined body weight of the *Gpbar1* homozygous and heterozygous mice. Male and female homozygous and heterozygous mice showed no difference in body weight as compared with that of the wild-type mice (Fig. 5). We measured concentrations of the

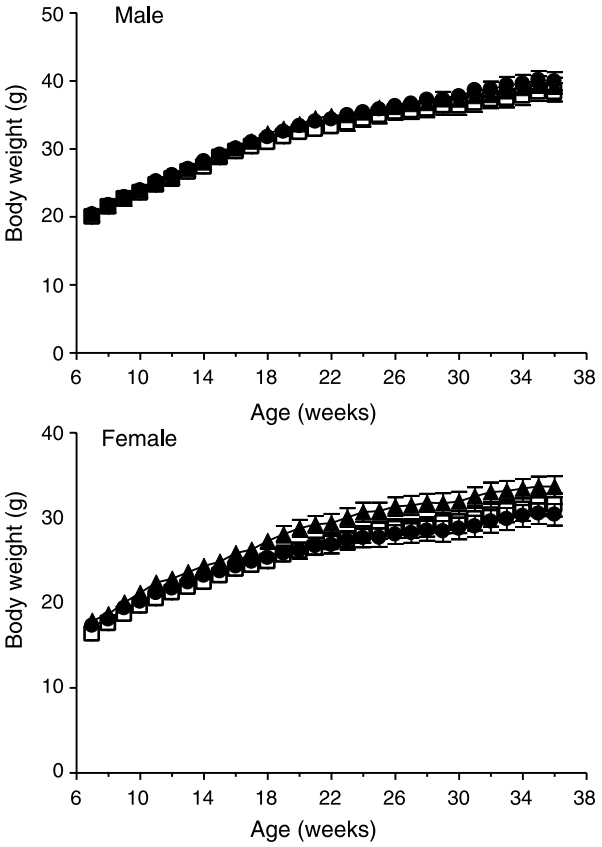

Figure 5 Growth curves of *Gpbar1* homozygous mice on regular chow diets. Wild-type, heterozygous, and homozygous mice are indicated by ●, ▲, and □ respectively. All values are expressed as mean±S.E.M. (n=15 or 16).

total bile acids and lipids in the plasma to investigate the effect of *Gpbar1* gene disruption. Plasma levels of total bile acids and triglyceride were not different between wild-type and homozygous mice. However, total plasma cholesterol levels significantly increased by 16% in male ($P<0{\cdot}05$) but not in female homozygous mice (data not shown).

Analysis of Gpbar1-deficient mice on a HF diet

Next, we investigated the response of *Gpbar1* homozygous mice in a HF diet in order to assess the impact of *Gpbar1* deficiency in bile acid homeostasis more precisely. The HF diet was given from 9 weeks of age for 2 months. From 12 weeks of age, the female homozygous mice had a significant change in body weight compared with that of the wild-type mice (Fig. 6D). Female heterozygous mice also gained body weight compared with that of the wild-type mice, although it was not statistically significant (Fig. 6D). The amount of food intake was independent from genotypes in both male and female mice (Fig. 6E and F). Male homozygous mice tended to gain more weight than the wild-type mice, although it was not statistically significant (Fig. 6A and C). At 18 weeks of age, the fat mass of female homozygous mice significantly increased without change in lean mass, indicating that the gain in body weight resulted from fat accumulation (Fig. 7C and D). Female heterozygous mice also showed increased fat mass without change in lean mass, although not significantly, compared with that of the wild-type mice, indicating that like female homozygous mice there was an increase in fat accumulation with body weight gain (Fig. 7C and D). Similar to the results of the growth curve, male homozygous mice tended to accumulate more fat than the wild-type mice; however, it was not statistically significant (Fig. 7A and B).

Discussion

To investigate the physiological role of Gpbar1 *in vivo*, we generated *Gpbar1*-deficient mice. Total bile acid pool size was significantly decreased in homozygous mice compared with that of the wild-type mice (Fig. 3A and C), suggesting that Gpbar1 plays a regulatory role in bile acid homeostasis. To reveal why bile acid pool size was decreased in homozygous mice, we assessed the expression levels of key players in bile acid homeostasis by quantitative RT-PCR analysis (Fig. 4). Although the expression levels of some key genes were significantly changed compared with that of the wild-type mice, significant gender differences were also observed. Therefore, the expression change is unlikely to contribute mainly to the decrease in bile acid pool size observed in both male and female *Gpbar1* homozygous mice. However, enzymatic activities of these key players remain to be addressed.

Female *Gpbar1* homozygous and even heterozygous mice showed more weight gain than the wild-type mice under the HF diet conditions (Fig. 6D). Body composition analysis revealed that the increased body weight was due to fat accumulation (Fig. 7C). Male homozygous mice showed a tendency to fat accumulation with body weight gain compared with that of the wild-type mice, although it was not statistically significant (Figs 6C and 7A). In this experimental model, the amounts of food intake in homozygous mice were similar to that of the wild-type mice (Fig. 6E and F). Furthermore, locomotor activity was not changed between homozygous and wild-type mice (data not shown). These data suggest that energy expenditure in homozygous mice was decreased compared with that of the wild-type mice. With respect to the energy expenditure, it was recently reported that bile acid induced energy expenditure through activating cAMP-dependent thyroid hormone-activating enzyme type 2 iodothyronine deiodinase (D2) (Watanabe *et al.* 2006). The efficacy is unlikely to be from the FXR pathway, but it is mediated by TGR5 (Gpbar1), which can increase the cAMP level stimulated by bile acid. The lack of Gpbar1-cAMP-D2 pathway may decrease energy expenditure and elicit adiposity in homozygous mice when fed a HF diet.

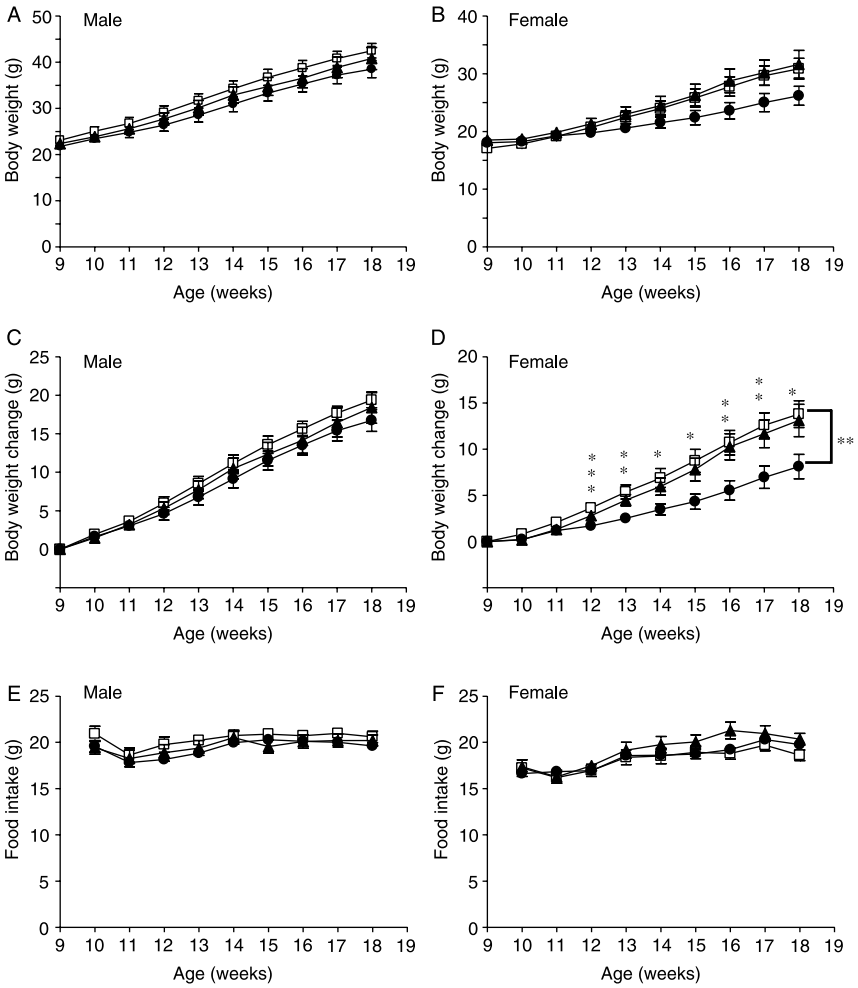

Figure 6 Growth curves (A) and (B), body weight change (C) and (D) and food intake (E) and (F) of *Gpbar1* homozygous male (A), (C) and (E) and female (B), (D) and (F) mice on HF diets. Wild-type, heterozygous, and homozygous mice are indicated by ●, ▲, and □ respectively ($n=10$). *$P<0{\cdot}05$, **$P<0{\cdot}01$, ***$P<0{\cdot}001$ compared between homozygous and wild-type group.

Alternatively, the expression of Gpbar1 in WAT raised the possibility that Gpbar1 is involved in the regulation of energy homeostasis. It is well known that lipolysis is induced by various hormones and cytokines in adipocytes (Carmen & Victor 2006). Furthermore, this lipolysis was also stimulated by forskolin, isobutyl-methylxanthine (IBMX) or dibutyryl-cAMP in adipocytes prepared from lean or obese Zucker rats (Fruhbeck *et al.* 2001), indicating that the lipolysis is regulated by intracellular cAMP levels. As Gpbar1 also stimulates cAMP formation, Gpbar1 may play a role in the regulation of the lipolysis in WAT; however, further investigation is required.

Moreover, the Gpbar1 expression was confirmed in enteroendocrine cell lines like STC-1, but not in epithelial cell lines (Maruyama *et al.* 2002). It was also reported that bile acid stimulated glucagon-like peptide-1 (GLP-1) secretion from STC-1 cells via Gpbar1/TGR5 (Katsuma *et al.* 2005). GLP-1 is secreted from the L-cells in the intestine after meals and indirectly decreases blood glucose levels both by stimulating insulin secretion in a glucose-dependent manner and inhibiting glucagon secretion (Tang-Christensen *et al.* 1996). The endocrine L-cells were detected throughout in the small and large intestine, with the majority of L-cells localized to the distal ileum and colon (Drucker 2002). It is noteworthy that the high expression level of Gpbar1 mRNA was observed in the colon (Fig. 1), where the primary bile acids such as cholic acid were converted to the secondary bile acids such as deoxycholic acid by micro-organisms (Hylemon *et al.* 1994). Such secondary bile acids were more potent than the primary bile acids for Gpbar1 activation (Maruyama *et al.* 2002, Kawamata *et al.* 2003). These observations strongly support the idea that Gpbar1 facilitates GLP-1 secretion in response to bile acid *in vivo*. Mice lacking dipeptidyl peptidase

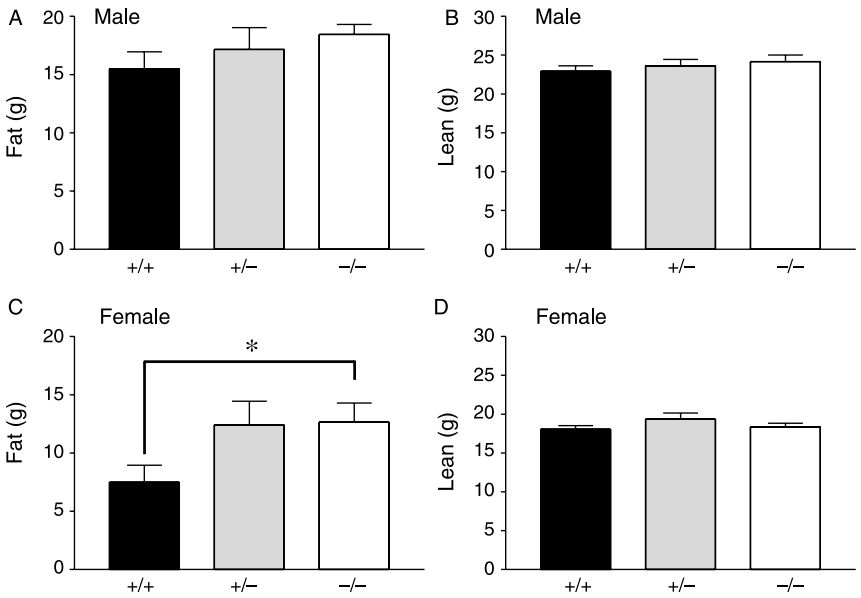

Figure 7 Body composition analysis of Gpbar1 homozygous mice on a HF diet. Fat mass (A) and (C) and lean mass (B) and (D). All values are expressed as mean ± S.E.M. of data from wild-type (+/+), heterozygous (+/−), and homozygous mice (−/−) (n=10). *P<0·05, compared with the wild-type group.

IV, which inactivates GLP-1, showed resistance to HF diet-induced obesity (Conarello *et al.* 2003). Further investigation is required; however, this observation raises the possibility that decreases in the GLP-1 signaling from the intestine result in the adiposity in *Gpbar1* homozygous mice. To investigate the correlation between bile acid and GLP-1 signaling, and to evaluate the therapeutic potential of Gpbar1 as a target for facilitation of GLP-1 secretion, the *Gpbar1* homozygous mouse may prove to be a useful tool.

In summary, targeted disruption of *Gpbar1* in mice showed a decrease in total bile acid pool size, suggesting that Gpbar1 contributes to bile acid homeostasis. In addition, female *Gpbar1* homozygous mice showed significant fat accumulation with body weight gain compared with that of the wild-type mice when fed a HF diet, demonstrating that Gpbar1 could be a key player in energy homeostasis as well. Although several mechanisms showing critical roles of Gpbar1 still remain to be addressed, Gpbar1 is an intriguing molecule to elucidate the physiological roles of bile acids. Thus, *Gpbar1*-deficient mice are useful tools for further investigations of bile acid physiology and pathophysiology.

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