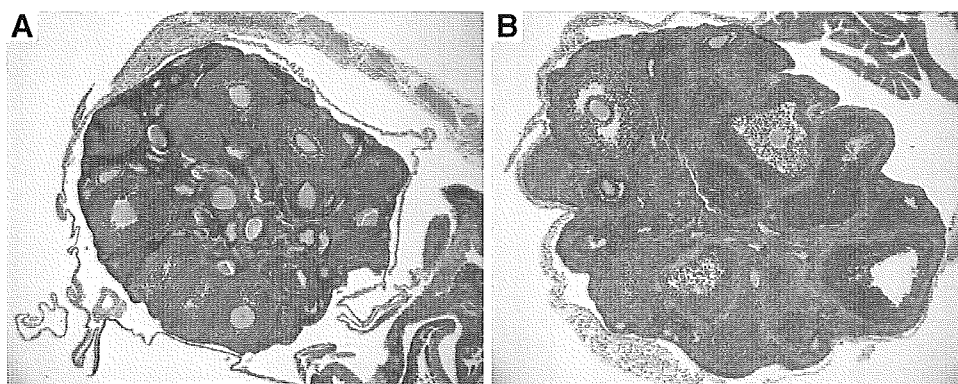


FIG. 5. Effects of PMSG and hCG treatments on follicular maturation in *klotho* mouse ovary. Five-week-old *klotho* mice were injected with 5 IU PMSG. Forty-eight hours after PMSG injection, animals were injected with 5 IU hCG. Animals were processed for histology 24 h after hCG treatment. A, Control *klotho* ovaries injected with saline instead of PMSG and hCG had no antral follicle. B, *klotho* ovaries 24 h after hCG administration had mature antral follicles and ovulating follicles.



(4.3 pups/litter) of which were agouti and heterozygous for the *klotho* locus (*kl/+*) (Table 1). These results indicate that *klotho* homozygous ovaries could give rise to offspring when

transplanted to wild-type mice. Second, we examined the effects of treatment with gonadotropins. Administration of PMSG to 5-wk-old female *klotho* mice induced antral forma-

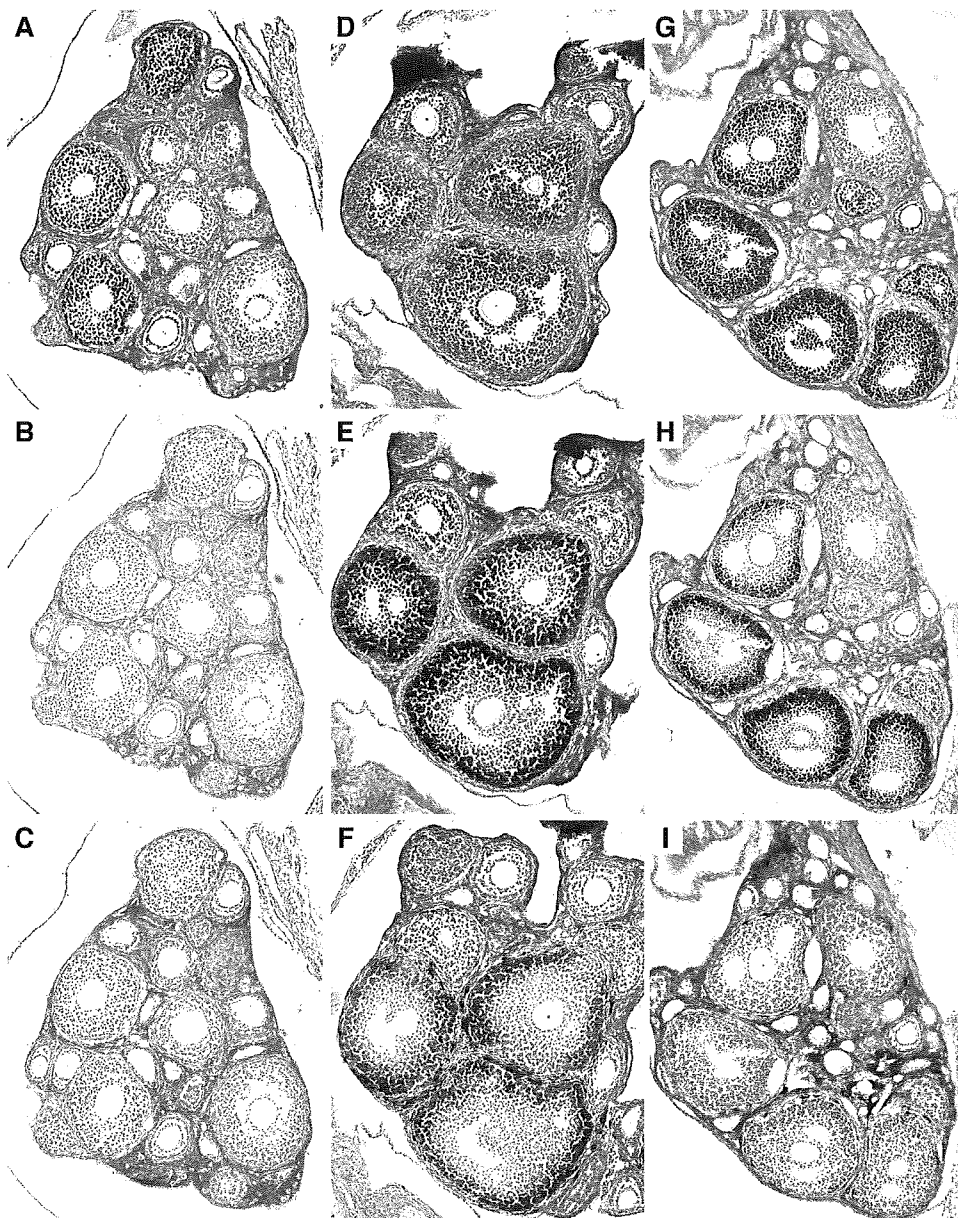


FIG. 6. Administration of FSH or GnRH induced expression of LHR and P450arom in *klotho* mouse ovary. Four- to 5-wk-old *klotho* mice were treated with vehicle (A–C), FSH (D–F), or GnRH (G–I). Expression of FSHR, P450arom, or LHR was examined by *in situ* hybridization analysis after these treatments. Sections of ovaries were hybridized with antisense probes for FSHR (A, D, and G), P450arom (B, E, and H), or LHR (C, F, and I). Either FSH or GnRH treatment induced both LHR and P450arom in *klotho* mouse ovaries (E, F, H, and I). There was no induction in the case of vehicle-treated *klotho* mice (B and C).

tion of ovarian follicles (data not shown). Subsequent administration of hCG after PMSG treatment induced continuation of follicular maturation and small number of ovulation in *klotho* ovaries (Fig. 5B), whereas maturation of ovarian follicles was never observed in *klotho* mice injected with saline (Fig. 5A). Functionally, PMSG/hCG treatment appeared to stimulate growth and maturation of the uterus, with a considerable increase in uterine size and development of endometrium (data not shown). These results indicate that female gonads in *klotho* mice are potentially functional and suggest that the severe atrophy of reproductive organs may be due to a deficiency of serum factors.

To determine the extra-ovarian factor(s) lacking in *klotho* mice, which are necessary to gonadal maturation, we examined the response of *klotho* mouse gonads to these hormones. Administration of purified FSH and GnRH had effects on the maturation of gonads in *klotho* mice. The ovaries of FSH-treated *klotho* mice developed follicles that expressed P450arom and LHR (Fig. 6, E and F), whereas vehicle-treated *klotho* mouse ovaries scarcely expressed those (Fig. 6, B and C). To examine whether the Klotho protein is necessary for gonadotropin production or secretion, we further examined the effects of GnRH on *klotho* mice. This treatment also induced the expression of these two genes (Fig. 6, H and I). P450 aromatase was induced almost as much as seen in wild-type mice (data not shown). Induction of LHR was detected only in theca cells after treatment with GnRH (Fig. 6I). FSHR expression was observed in vehicle-, FSH-, and GnRH-treated *klotho* mouse ovaries (Fig. 6, A, D, and G). To confirm this effect, we carried out the thorough treatment of GnRH for *klotho* mice. This long-term GnRH treatment resulted in further maturation of gonads (Fig. 7). The ovarian

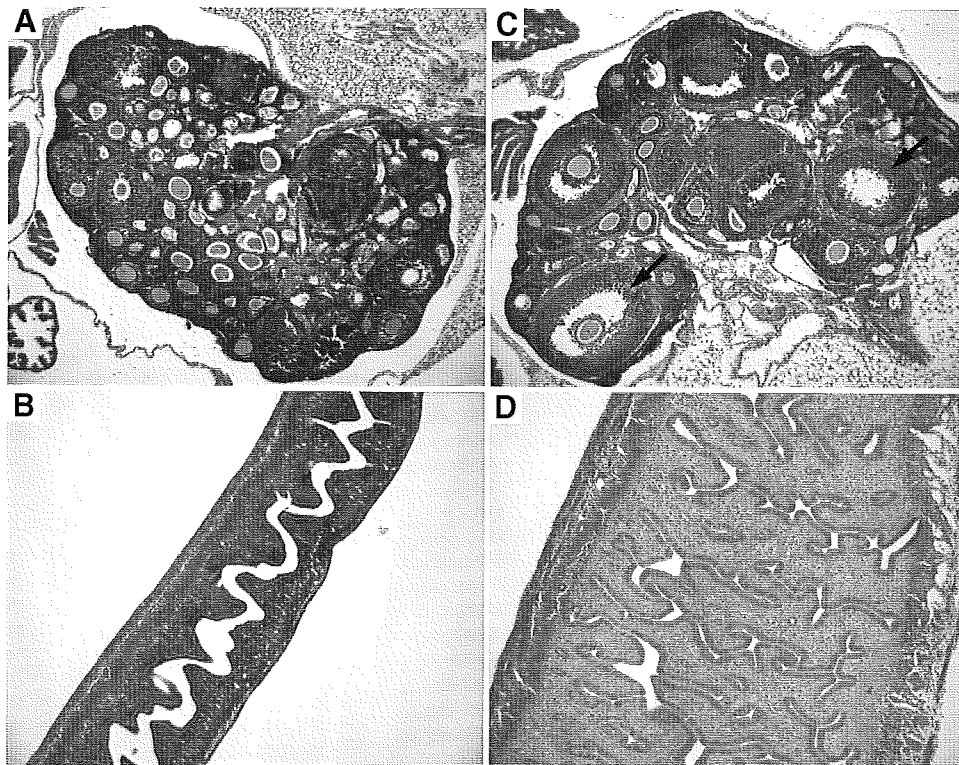
follicles matured to the preovulatory stage (Fig. 7C). Growth of uteri with marked increase in endometrial glands was observed (Fig. 7D). Effects of this long-term GnRH treatment were confirmed by *in situ* hybridization analysis. LHR were induced not only in theca cells but also mural granulosa cells of *klotho* mice (data not shown).

To reveal what is affected by the *klotho* deficiency in the pituitary gland, microscopic analysis was performed. Sections of female pituitary gland were immunologically stained for FSH or LH (Fig. 8). The number of FSH positive cells varied between animals in case of wild-type animals. FSH signals were also detected in the mutant animals; however, the number of positive cells was generally less in the mutants. Signals for LH were hardly observed in the *klotho* mice, whereas those were clearly detected in the wild-type mice. Because it is known that both LH and FSH are produced and secreted by identical cells, these suggest that the production of pituitary hormones is decreased in the mutant animals, although the hormone producing cells exist in the mutant pituitary. Further analysis for LH was performed by the immunoelectromicroscopy. The number of immunogold particles in secretory granules was clearly less in *klotho* mice compared with wild-type animals (Fig. 8, C and F). These also suggest that the LH production is decreased in the mutant mice, whereas the LH-producing cells exist in the mutant pituitary.

Discussion

To date, some cases of single-gene mutations resulting in sterility have been reported (29). In this study, we examined the cause of female sterility in *klotho* mice and inferred the

FIG. 7. Long-term GnRH stimulation induced further maturation of gonadal organs in *klotho* mice. *klotho* mice (4–5 wk old) were injected with 50 ng GnRH (C and D) or vehicle (A and B) every 2 h for 5 d. Development of follicles to preovulatory stages (arrows) and growth of endometrial gland in uterus were observed in GnRH-treated *klotho* mice (C and D). In vehicle-administered control animals, no histological changes were observed (A and B).



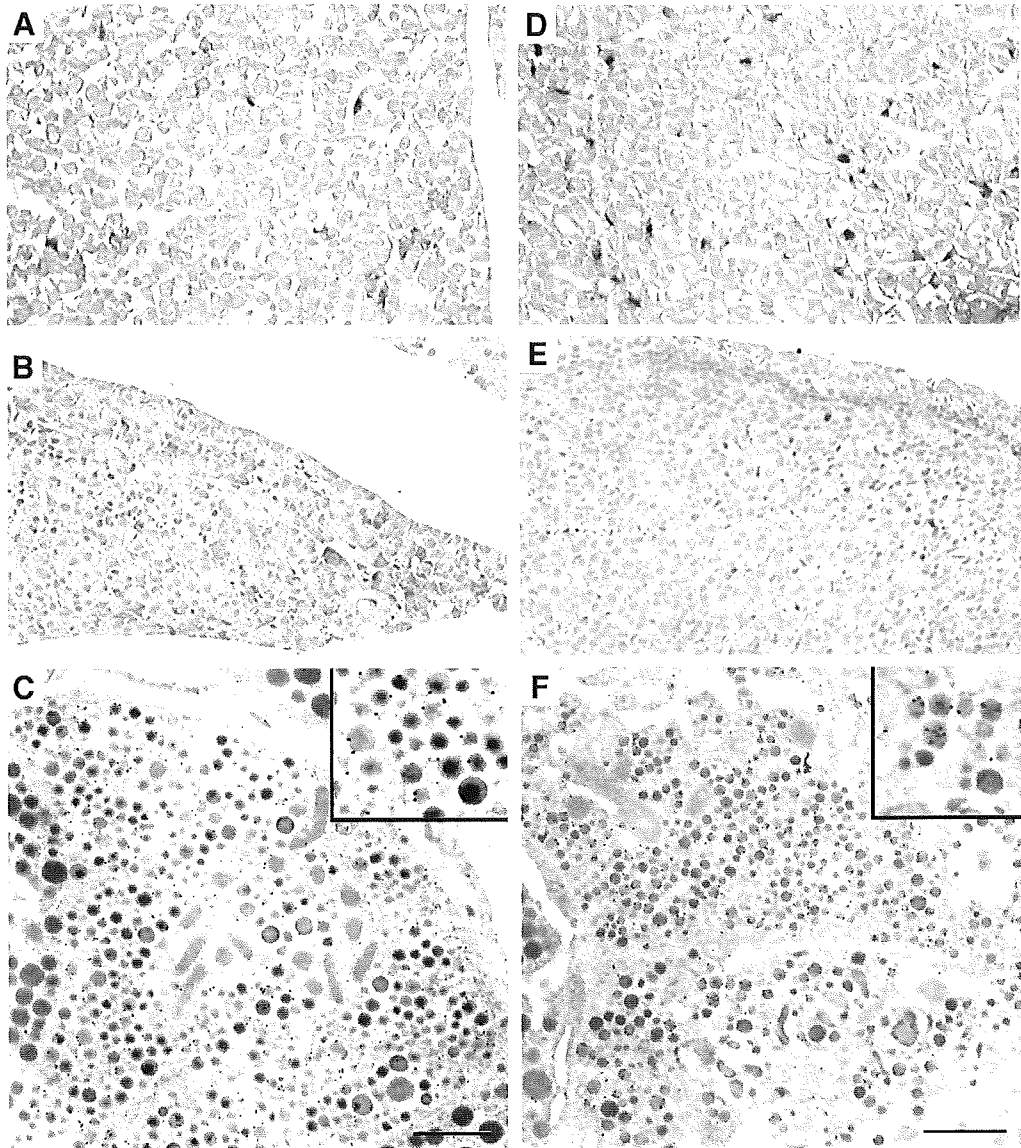


FIG. 8. Immunohistochemical analysis of gonadotropin-producing cells in the pituitary. Pituitary of wild-type (A–C) and *klotho* (D–F) mice was analyzed. FSH (A and D) and LH (B, C, E, and F) were detected on the section of pituitary. FSH signals were detected both in wild-type (A) and *klotho* (D) mice. LH-positive cells were rarely detected in the mutant (E), whereas these were clearly seen in the wild-type animals (B). LH-positive vesicles were detected by the analysis using immunoelectronmicroscopy (C and F). The amount of immunogold particles in secretory granules was less in the mutant (F) compared with the wild type (C).

mechanism by which Klotho protein exerts systemic effects. Although faint expression of *klotho* in the ovary and pituitary has been detected by RT-PCR, and atrophic phenotypes of these organs in *klotho* mice were reported (1), whether the absence of *klotho* in these regions is the primary cause has not been confirmed. In this study, we demonstrate that female gonads of *klotho* mice are out of estrus cycles, but they are potentially functional because transplanted ovaries could give rise to offspring (Table 1), and gonadotropin administration resulted in maturation of ovarian follicles (Figs. 5 and 6).

We have not observed either matured ovarian follicles or the vaginal opening of *klotho* female mice under normal conditions. At 12 d old, there was no apparent difference in gonadal organs between the *klotho* female and wild-type littermate (Fig. 1). At 8 wk old, there were apparent differ-

ences between genotypes (Figs. 2 and 3). Phenotypes in female reproductive system in *klotho* mice become apparent around the weaning stage the same as phenotypes observed in other organs.

Previous studies proposed the possibility that the soluble Klotho protein may exert hormonal effects on some organs including ovary to maintain their function (6, 7). In contrast to these studies, it was suggested that disrupted regulation of pituitary hormones induced the appearance of *klotho*-deficient phenotypes (1). We demonstrate here that sterility in *klotho* females is mainly caused by the absence of proper stimulus by gonadotropins. All of the following support this idea. First, few follicles were observed to develop beyond the preantral stage to antral stage in *klotho* mice (Fig. 2), confirmed by the nonexpression of both LHR and P450arom, the

maker genes of antral stage (Fig. 3). These ovarian phenotypes of *klotho* mice are very similar to those of hypophysectomized mice (25). The primary stimulus for antral transition is believed to be FSH (11, 30). Second, constant low levels of FSH and LH in sera were observed in *klotho* mice (Fig. 4). The production of FSH and LH was decreased in the pituitary of *klotho* mouse revealed by the immunohistochemistry (Fig. 8). Third, exogenous administration of purified FSH alone was sufficient to induce antral transition in *klotho* ovaries (Fig. 6). Nevertheless, the possibility that the Klotho protein might exert synergistic effects with gonadotropins on follicular maturation remains. Roles of ovarian intrinsic Klotho also remain to be elucidated. Several local factors are known to modulate or partially substitute the effects of gonadotropins on ovarian follicular maturation (31).

Higher serum level of progesterone in *klotho* mice was observed in this study (Fig. 4). The mechanism that leads to this elevation is not clear yet. Because LH levels were constantly low in the *klotho* mice, other factor(s) may be involved in this induction. Overactivation of adrenal gland might be a reason for this elevation (29). Slight elevation of serum ACTH was observed in the *klotho* mice (Toyama, R., unpublished data). This could be due to either the direct abnormality of hypothalamic-adrenal axis or feedback regulation, as observed in congenital adrenal hyperplasia (32).

Although the atrophy of gonadotrophs might be attributed to the deficiency of Klotho protein in the pituitary, the predominant cause of their dysfunction seems due to the deficiency of hypothalamic GnRH. Gonadotrophs of *klotho* mice sustained ability to develop ovarian follicles in response to exogenous GnRH injection. This result suggests potential reactivity of pituitary gonadotrophs and subsequent production of functional gonadotropins.

The mechanisms leading to the deficiency of GnRH stimulation in *klotho* mice remain to be elucidated. Loss of *klotho* gene expression in GnRH-producing cells might be the cause of dysregulation of GnRH. However, if any, deficiency of *klotho* in GnRH-producing cells should lead to dysregulation of only hypothalamic-pituitary-gonadal axis and its derived effects. We have not yet identified the *klotho*-expressing cells in hypothalamus because of its faint expression. Deficiency of *klotho* expression in choroid plexus might be the primary cause because *klotho* gene expression is abundant in choroid plexus in the brain (1). Absence of Klotho protein in this region might produce an imbalance in the cerebrospinal fluid such as dysregulation of mineral, sugar, protein, and Klotho concentrations. This imbalance would lead to failure of proper response by the endocrine system of the hypothalamus and pituitary gland. Decreased number of secretory granules in GH-producing cells of *klotho* mice was reported (1). Various stresses, such as abnormal mineral balance, itself could be the other candidate for the cause of sterility. Impaired up-regulation of serum phosphorus and calcium levels caused by impaired vitamin D metabolism in *klotho* mice was also reported (33).

It is possible that *klotho* mice cannot trigger the onset of puberty and stay in the prepubertal stage. Vaginal opening was not observed in *klotho* female mice (1). Abnormal circulation or absence of higher level of gonadotropins observed in this study might reflect the prepubertal absence of

GnRH pulse. A train of GnRH discharges is necessary to initiate the onset of puberty (34). Different immunoreactivity between two gonadotropins in the gonadotrophs might reflect the different requirement for the antral follicular development at the onset of puberty (35).

In this study, we revealed the potential reactivities, despite the atrophic phenotypes, of reproductive organs including the uterus, ovary, and gonadotrophs in female *klotho* mice. This is the first report demonstrating that some of the pathological phenotypes in *klotho* mice result from abnormal regulation of pituitary hormones. In other words, this suggests that Klotho might be involved in the regulatory control of hypothalamic-pituitary-axis and that the function of Klotho should be located upstream of gonadotropins.

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Impaired negative feedback suppression of bile acid synthesis in mice lacking β Klotho

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We have generated a line of mutant mouse that lacks β Klotho, a protein that structurally resembles Klotho. The synthesis and excretion of bile acids were found to be dramatically elevated in these mutants, and the expression of 2 key bile acid synthase genes, *cholesterol 7 α -hydroxylase* (*Cyp7a1*) and *sterol 12 α -hydroxylase* (*Cyp8b1*), was strongly upregulated. Nuclear receptor pathways and the enterohepatic circulation, which regulates bile acid synthesis, seemed to be largely intact; however, bile acid-dependent induction of the small heterodimer partner (SHP) NR0B2, a common negative regulator of *Cyp7a1* and *Cyp8b1*, was significantly attenuated. The expression of *Cyp7a1* and *Cyp8b1* is known to be repressed by dietary bile acids via both SHP-dependent and -independent regulations. Interestingly, the suppression of *Cyp7a1* expression by dietary bile acids was impaired, whereas that of *Cyp8b1* expression was not substantially altered in β klotho^{-/-} mice. Therefore, β Klotho may stand as a novel contributor to *Cyp7a1*-selective regulation. Additionally, β Klotho-knockout mice exhibit resistance to gallstone formation, which suggests the potential future clinical relevance of the β Klotho system.

Introduction

We previously reported the identification of a mouse gene, *β klotho*, that encodes a protein that structurally resembles Klotho (1). Deficiency of the Klotho protein results in various human aging-like phenotypes in mouse, including calcium homeostasis disorders (2). Both Klotho and β Klotho are type I membrane proteins, which contain 2 family 1 glycosidase-like domains. They lack the characteristic glutamic acid residues essential for enzymatic activities and thus form a distinct subfamily within glycosidase family 1 (3, 4). *β klotho* is predominantly expressed in the liver, pancreas, and adipose tissues (1). With the exception of mRNA expression profiles, not much regarding the molecular function of β Klotho is known. We therefore generated *β klotho*^{-/-} mice. The gross appearance of knockout mice was normal upon initial examination. Interestingly and unexpectedly, however, bile acid metabolism was altered in these mice.

Bile acids are synthesized from cholesterol in the liver. They are vitally important for the intestinal absorption of lipids and lipid-soluble materials. In addition, bile acid biosynthesis is the sole pathway for the elimination of superfluous cholesterol. On the other hand, hydrophobic secondary bile acids generated by intestinal flora have toxic effects, leading to cholestasis and carcinogenesis (5, 6). Therefore, proper control of bile acid synthesis is crucial for animals. The rate-limiting step for the major bile acid synthetic pathway, which is catalyzed by cholesterol 7 α -hydroxylase (CYP7A1), is thus strictly regulated by numerous factors, with negative feedback regulated by bile acids representing the principal

one (7, 8). Nuclear receptors, kinases, and growth factor-mediated pathways have been demonstrated to be involved in this negative feedback regulation (6, 7, 9–16). After conjugation in hepatocytes, bile acids are secreted, reabsorbed from intestine, and delivered back to the liver. Transportation across the membrane is carried out by a number of solute carrier proteins and ABC transporters (17, 18). The intactness of this enterohepatic circulation is necessary for the appropriate regulation of bile acid metabolism.

Through the analysis of expression profiles of genes regulating bile acid synthesis, we propose potential roles for β Klotho in negative regulatory pathways.

Results

Generation of the *β klotho*^{-/-} mouse. The mouse *β klotho* locus consists of at least 8 exons. We have constructed a gene-targeting vector to delete most of the putative exon 1 that includes the first ATG codon via homologous recombination in ES cells (Figure 1A). Successful disruption of the *β klotho* locus was confirmed by genomic Southern blot analyses using a probe flanking the region upstream of the targeting vector (Figure 1, A and B). Elimination of the *β klotho* mRNA expression was confirmed by Northern blotting (Figure 1C) and further verified by RT-PCR using several primer pairs covering different regions of the remaining exons (data not shown). The absence of protein expression was also affirmed by Western blotting using a monoclonal antibody that recognizes the C-terminal portion of the second internal repeat (β KL2) of the β Klotho protein (Figure 1D). Although we could detect transcripts for enhanced GFP (EGFP) with the same tissue distribution as those for *β klotho*, we could not detect EGFP protein by Western blotting (data not shown).

β klotho^{-/-} mice were viable, fertile, and appeared grossly normal under standard conditions. The number of the homozygous mice obtained was less than would be expected according to Mendelian inheritance laws (297:624:193, *β klotho*^{+/+}/ *β klotho*^{+/-}/ *β klotho*^{-/-}). The body weights of homozygous animals were slightly

Nonstandard abbreviations used: CA, cholic acid; CDCA, chenodeoxycholic acid; CYP7A1, cholesterol 7 α -hydroxylase; CYP8B1, sterol 12 α -hydroxylase; EGFP, enhanced GFP; *Fgfr4*, fibroblast growth factor receptor 4; FXR, farnesoid X receptor; *Hmgcr*, 3-hydroxy-3-methylglutaryl coenzyme A reductase. LXR α , liver X receptor α ; SHP, small heterodimer partner.

Conflict of interest: The authors have declared that no conflict of interest exists.

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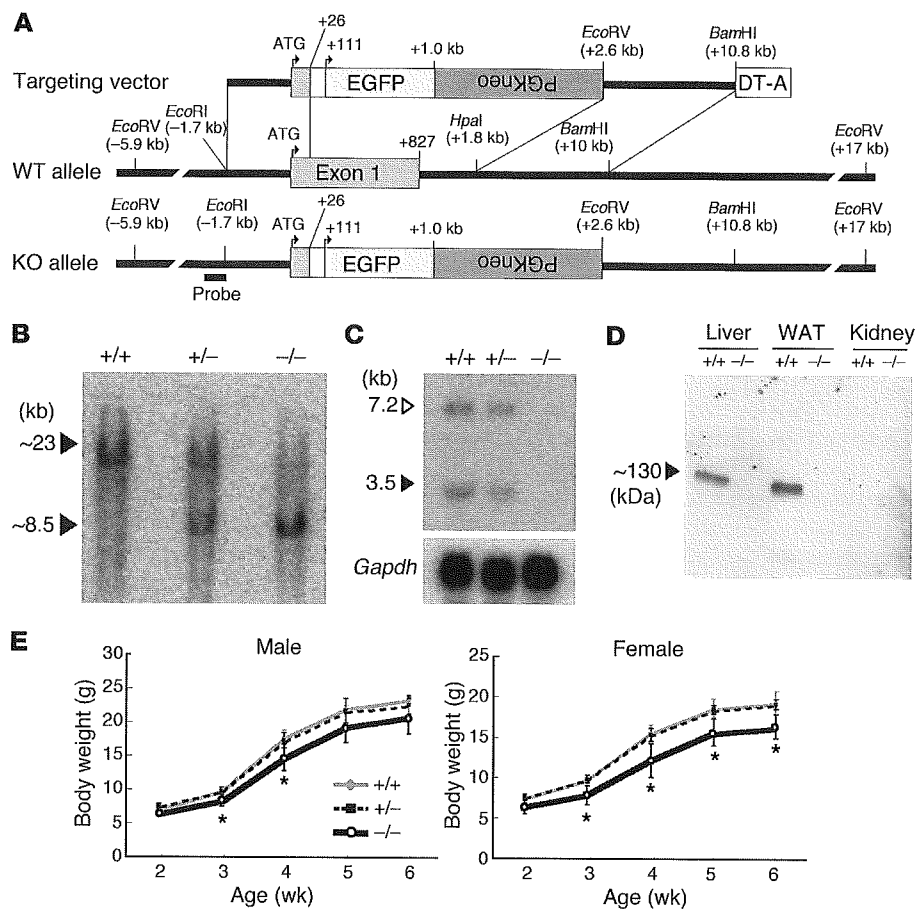


Figure 1

Targeted disruption of the β klotho gene. (A) Strategy for disruption of mouse β klotho locus. Schematic diagrams of the targeting vector (top), wild-type allele (middle), and disrupted allele (bottom) are shown. Numbers correspond to the distance (bp) from the putative translation initiation site. The probe used for Southern blot analysis is shown at the bottom. (B) Southern blot analysis of EcoRV-digested genomic DNA. Blots were hybridized with the probe indicated in A. (C) Northern blot analysis of β klotho gene expression in the liver. The β klotho-specific probe hybridizes with 2 species of transcripts (1). (D) Western blot analysis of β klotho protein expression. Total proteins (15 μ g) extracted from indicated tissues were blotted on a PVDF membrane and incubated with a β klotho-specific monoclonal antibody. β klotho protein was detected only in β klotho-expressing tissues of wild-type mice and was absent in tissues of β klotho^{-/-} mice. WAT, white adipose tissue. (E) Growth curve comparison of β klotho^{+/+}, β klotho^{+/-}, and β klotho^{-/-} mice. Body weights were traced in mice from ages 2 to 6 weeks. Five to 12 mice for each group were examined. Error bars indicate SD. *P < 0.05.

but significantly reduced compared with those of wild-type and heterozygous littermates (Figure 1E). The decrease in body weight could not be attributed to the differences in the size of the liver or gonadal fat. The slight reduction in body weight was reproducibly seen in groups that were backcrossed 5 times into the C57BL/6 background (data not shown). Sections of various tissues including β klotho-expressing tissues stained with H&E were carefully examined; however, histological differences between β klotho^{-/-} and wild-type mice could not be found. Serum markers relevant to β klotho-expressing tissues such as serum triglyceride, fatty acids, cholesterol, phospholipid levels and enzyme activities derived from liver and pancreas were not significantly different between genotypes (data not shown).

Elevation of the synthesis and excretion of bile acids. β klotho-expressing tissues are all involved in lipid metabolism; therefore, we first speculated that this molecule might be involved in some aspects of lipid metabolism (1). However, serum markers indicative of lipid metabolism were not significantly altered in β klotho^{-/-} mice. We observed a slight reduction in serum cholesterol levels, although it did not reach statistical significance in mice of either sex (Figure 2A). This led us to focus on cholesterol and its metabolism in β klotho^{-/-} mice. Since β klotho is strongly expressed in the liver, where cholesterol is converted to bile acids (1), we examined the metabolism of these sterols in β klotho^{-/-} mice. We found that fecal bile acid excretion was dramatically elevated in the mutants compared with wild-type mice (Figure 2B). Daily food intake and total fecal amounts were similar in mice of both genotypes (Figure 2C), but the bile acid pool size

was slightly increased in β klotho^{-/-} mice, although the increase was statistically significant only in males (Figure 2D).

CYP7A1 is the most important enzyme for bile acid production, as it catalyzes the first and rate-limiting step of the classical bile acid synthetic pathway (8). We found that the expression of *Cyp7a1* was dramatically elevated in β klotho^{-/-} mice compared with that in the wild-type littermates (Figure 2E). More than 20 littermate pairs were examined, and the elevation was evident in all cases. It was also the case in mice that had been backcrossed 5 times into the C57BL/6 background (data not shown). Another enzyme, sterol 12 α -hydroxylase (CYP8B1), is also of importance, as it is indispensable for generation of the most abundant primary bile acid, cholic acid (CA) (8, 19). We examined the expression of *Cyp8b1* and found that it was also strongly upregulated in β klotho^{-/-} mice (Figure 2E).

It is known that interruption of the enterohepatic circulation elicits the elevation of bile acid synthesis (20, 21). For instance, cholestyramine, an inhibitor of intestinal bile acid reabsorption, dramatically increases bile acid synthesis and excretion (21). We examined the state of the circulation. The mRNA expression of *intestinal bile acid binding protein (I-babp)*, which is known to be induced in response to the absorption of bile acids (22), was considerably elevated in the small intestine of the β klotho^{-/-} mice (Figure 2F). This probably reflects the increased secretion of bile acids into the duodenum, and it is likely that bile acid absorption is largely intact in β klotho^{-/-} mice. Consistent genotype-dependent differences in the mRNA expression of hepatic canalicular transporters Na⁺-taurocholate-cotransporting protein 1 (Ntcp1;

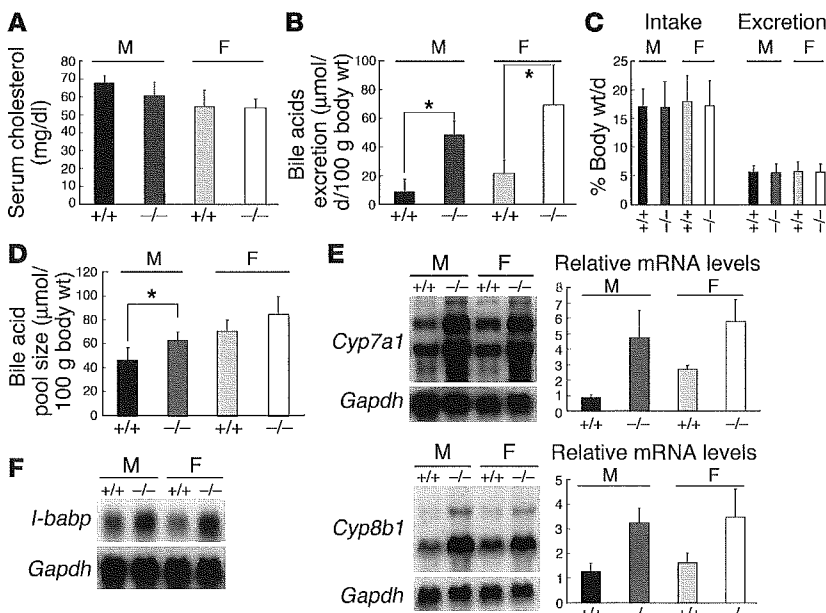


Figure 2 Altered bile acid metabolism in $\beta klotho^{-/-}$ mice. Graphs show the comparison of several parameters in wild-type and knockout mice. (A) Serum cholesterol levels were compared using 4–6 animals per group. (B) Stool samples were collected daily for 3 days from 4–6 mice per group. Fecal excretion of total bile acids was measured, and the averages of 12–18 samples per group are shown. * $P < 0.05$. (C) Daily food intake and fecal excretion of 4 individuals per each genotype were examined for 3 days. (D) Comparison of bile acid pool size. Four to 10 mice per group were examined. (E) Expression of bile acid synthase genes *Cyp7a1* and *Cyp8b1* in wild-type and $\beta klotho^{-/-}$ mice. Total RNA (30 μ g) from the livers of each genotype were analyzed. Representative results from 2 independent experiments are shown. Northern blots are shown in the left panel, and relative expression levels normalized for *Gapdh* levels are presented in the right panels. (F) Expression of *intestinal bile acid binding protein (I-babp)*. Total RNA (30 μ g) from the whole small intestine was analyzed. Results shown are from samples taken from age- and sex-matched littermates. All data in this figure were derived from 7-week-old mice fed standard diets. M, male; F, female. Error bars indicate SD.

Slc10a1) and organic anion-transporting polypeptide 1 (Oatp1; Slc27a) and apical transporters bile acid-exporting pump (Bsep; Abcb11) and multidrug resistance-associated protein 2 (Mrp2; Abcc2) were not observed. Also, the expression of ileal apical sodium-dependent bile acid transporter (Asbt; Slc10a2) was not reduced in $\beta klotho^{-/-}$ mice (data not shown). Additionally, the bile acid pool size was increased in $\beta klotho^{-/-}$ mice, whereas it is reduced in enterohepatic circulation-deficient mice (20). These results led us to conclude that the enterohepatic circulation of bile acids is not significantly impaired in $\beta klotho^{-/-}$ mice.

Resistance to cholesterol gallstone formation. It has been reported that C57BL/6 mice develop cholesterol gallstones when cholesterol and bile acid-containing atherogenic (lithogenic) diets are administered (23). The formation is more severe in males than females and can be prevented by the overexpression of rat CYP7A1 in mice (23). We examined the response of $\beta klotho^{-/-}$ mice to long-term administration of the atherogenic diet, which contains 1% cholesterol and 0.5% CA. Consistent with previous reports, wild-type mice developed severe gallstones after being fed the atherogenic diet for 8 weeks (Figure 3A, left). In contrast, gallstone formation was not observed in $\beta klotho^{-/-}$ mice of either sex (Figure 3A, right). More than 14 mice for each genotype were examined, and no exceptions

were seen. We also observed that the size of the gallbladder was smaller in $\beta klotho^{-/-}$ mice compared with that in wild-type mice of both sexes (Figure 3A arrows).

When supersaturated in bile, cholesterol crystals form gallstones. Many factors are known to be involved in the initial steps of gallstone formation (24). However, the most important factor for gallstone formation is excessive cholesterol relative to bile acids and phospholipids (25, 26). Since rat CYP7A1-overexpressing mice exhibit resistance to gallstone formation (23), increased bile acid synthesis appears to be sufficient for its prevention. This is also supported by the fact that the long-term administration of ursodeoxycholic acid solubilizes gallstones in bile (27).

To further investigate the mechanisms underlying the gallstone resistance, we examined hepatic, fecal, and serum cholesterol levels. Under normal feeding conditions, hepatic, fecal, and serum cholesterol levels were not significantly different between genotypes (Figures 3, B and C, left, and Figure 2A). When mice were challenged with an atherogenic diet, these parameters were dramatically elevated in both genotypes; however, we could not find any genotype-dependent differences common to both sexes (Figures 3, B and C, right, and Figure 3D). In both standard and atherogenic diet-fed conditions, we examined the expression of hepatic free cholesterol transporters *Abcg5* and *Abcg8* (also called *sterolin-1* and *sterolin-2*), which are responsible for the secretion of free cholesterol into bile (28). The atherogenic diet dramatically elevated their expression; however, we could not find any overt genotype-dependent differences in either dietary condition (data not shown).

Together, the results suggest that the gross cholesterol macrodynamics in the liver are not significantly altered in $\beta klotho^{-/-}$ mice. These observations suggested that gallstone resistance in $\beta klotho^{-/-}$ mice resulted from the overproduction and secretion of bile acids.

Because hepatic and serum cholesterol levels were similarly elevated by the atherogenic diet in both genotypes, it was suggested that $\beta klotho$ is not a dominant factor for gross cholesterol metabolism in the liver. It might play a rather limited role that is specific to the suppression of bile acid synthesis. However, since loss of $\beta klotho$ did cause a slight decrease in serum cholesterol levels, it might be an important regulator for long-term maintenance of cholesterol homeostasis, at least under standard dietary conditions, despite its mild effects.

Gene expression profiles under basal and high bile acid dietary conditions. Gene expression profiles in the liver were examined in order to identify genes with distinct genotype-specific expression. The expression of more than 30 genes encoding nuclear receptors, transporters, P-450s and other enzymes, lipoprotein receptors, and apolipoproteins related to lipid, steroid, and xenobiotic metabolism were compared between wild-type and $\beta klotho^{-/-}$ genotypes by Northern blotting in sex-matched littermate pairs. In addition to the 2 bile acid synthase genes already described, we

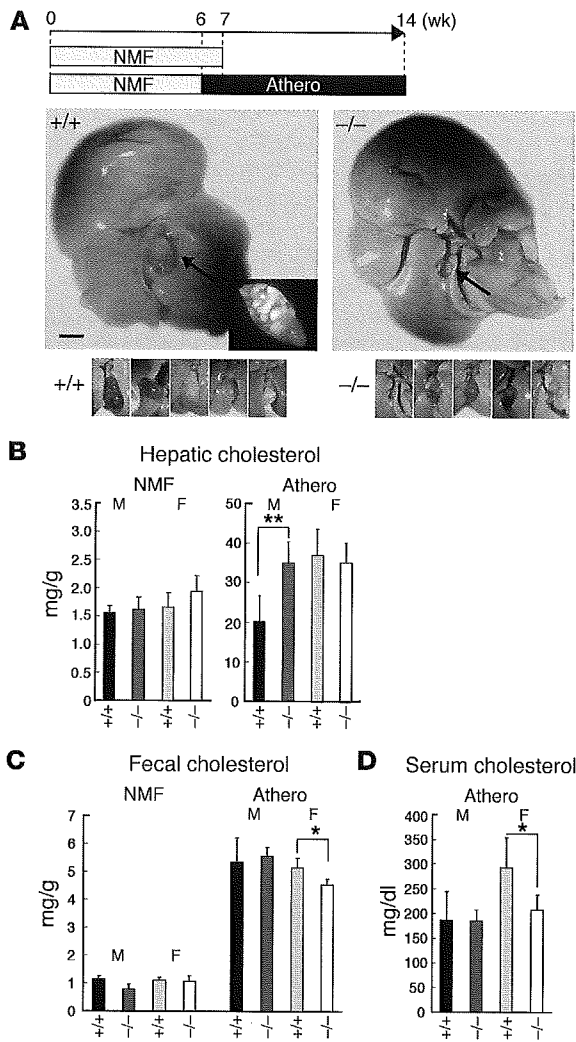


Figure 3

Resistance of β *klotho*^{-/-} mice to gallstone formation. (A) Cholesterol gallstones formed in wild-type mice (left) but not in β *klotho*^{-/-} mice on the atherogenic diet (Athero) (right). Diet administration regimens are shown at the top. Mice were sacrificed at the end of each regimen. Gallbladder is indicated (arrows). A magnified image of a wild-type gallbladder with severe gallstones is shown in the inset in the left panel. Examples of gallbladders from other individuals are shown below. Scale bars: 5 mm. (B) The average hepatic cholesterol levels of 7-week-old standard diet-fed mice (NMF; left panel) and that of 14-week-old mice on the atherogenic regimen (right panel). Four to 7 mice per group were examined. (C) Average fecal cholesterol concentrations. Stool samples were collected daily for 3 days from 7-week-old standard diet-fed mice (left) and 14-week-old mice on the atherogenic regimen (right). Three to 4 mice per group were examined. Results for stool samples from the same individuals were combined for the analyses. (D) Serum cholesterol levels of 14-week-old mice on the atherogenic regimen. Four to 7 mice per group were examined. Error bars indicate SD. **P* < 0.05; ***P* < 0.005.

diminished after dietary administration of bile acids, irrespective of genotype (Figure 4B). The expression levels were slightly higher in β *klotho*^{-/-} mice after the administration; however, a statistically significant difference between genotypes was not observed (Figure 4B). The expression levels of *Hmgr* under high bile acid conditions were not significantly different between genotypes (data not shown).

Since it is known that the expression of *Cyp7a1* and *Cyp8b1* is strongly regulated by the regulatory cascade that is mediated through farnesoid X receptor (FXR; NR1H4) and SHP, the expression of *Shp* was examined in both basal and high bile acid states (9, 30). The expression level of *Shp* was slightly lower in the mutant, but the difference was not statistically significant (Figure 4B). After the administration of bile acid-enriched diet, the expression levels of *Shp* were significantly elevated in both mutant and wild-type mice. However, although the expression was dramatically increased in the wild-type mice (2.48- and 2.46-fold for CA and CDCA, respectively), the increase was rather weak in the mutant (1.64- and 1.79-fold for CA and CDCA, respectively) (Figure 4B). The expression profiles of *Fxr* did not differ significantly between genotypes, and those of β *klotho* did not differ among the various dietary conditions (data not shown). Similar experiments were also performed with NMF (a standard diet) and NMF supplemented with 1% CA, although the animals were not age-matched in these experiments. Identical results were also obtained from these alternative experiments (data not shown).

Discussion

In order to define the molecular function of β Klotho, we generated a β *klotho*^{-/-} mutant mouse. β *klotho*^{-/-} mice appeared grossly normal, although a slight reduction in body weight compared with wild-type and heterozygous mice was noted (Figure 1E). Hepatic, fecal, and serum cholesterol levels were not significantly altered in β *klotho*^{-/-} mice. Unexpectedly, however, we found that bile acid synthesis in the liver and its fecal excretion were dramatically enhanced in these mutants (Figure 2). Additionally, β *klotho*^{-/-} mice exhibited resistance to the formation of cholesterol gallstones when they were fed an atherogenic diet (Figure 3A). These phenotypes are likely to have resulted from the impaired negative feedback suppression of the bile acid synthesis.

It is believed that one of the major negative feedback pathways for bile acid production is mediated by nuclear receptors,

observed distinct genotype-dependent difference in the expression of 3-hydroxy-3-methylglutaryl coenzyme A reductase (*Hmgr*), the rate-limiting enzyme for cholesterol biosynthesis (29). The expression of *Hmgr* was also strongly upregulated in β *klotho*^{-/-} mice, in both males and females (Figure 4A).

Since bile acid metabolism was significantly altered in β *klotho*^{-/-} mice, likely as a result of the upregulation of the bile acid synthase genes, we examined the effect of dietary bile acids on the expression of *Cyp7a1*, *Cyp8b1*, and regulatory factors for these genes. Mice were fed the basal diet from the lactation period with their mothers up to 6 weeks of age. After that, the same diet or bile acid-containing derivatives of the basal diet were administered for an additional week. Two kinds of primary bile acids, CA and chenodeoxycholic acid (CDCA), were used at the concentrations of 1% (Figure 4B, top).

In wild-type animals, the expression of *Cyp7a1* was detected when they were fed the basal diet and was completely diminished under administration of diets that contained bile acids. In β *klotho*^{-/-} mice, *Cyp7a1* expression was dramatically elevated compared with that of wild-type mice under the basal conditions. After dietary administration of bile acids, the expression levels were reduced; however, a significant portion of expression could not be eliminated (Figure 4B). In clear contrast, *Cyp8b1* expression was almost completely

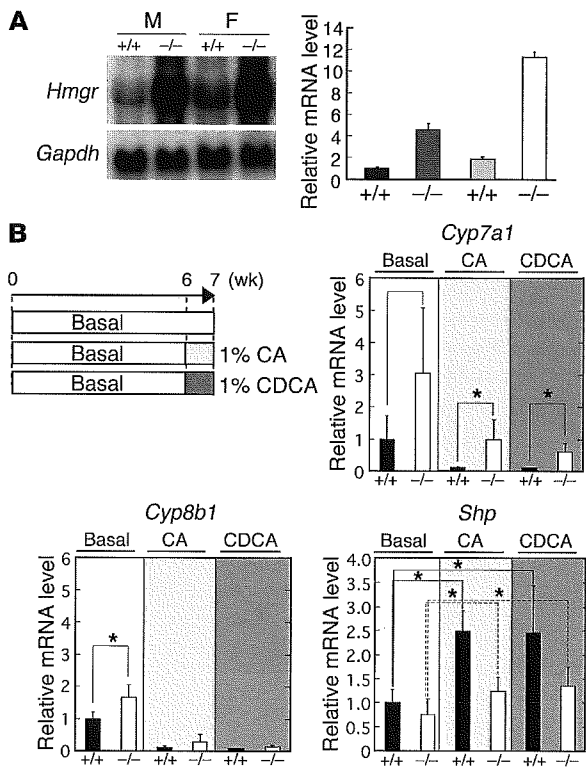


Figure 4 Gene expression profiles under various dietary conditions. (A) *Hmgr* mRNA expression in wild-type and β *klotho*^{-/-} mice. Total RNA (30 μ g) from the liver was analyzed. Representative examples of 2 independent trials are shown. Northern blots are shown at the left, and relative expression levels compensated for *Gapdh* are shown in the right panel. (B) Relative expression levels of selected genes. The expression levels of *Gapdh* were used as internal controls. Relative expression levels of each gene were further standardized to the mean expression levels of wild-type animals fed the basal diet. The dietary regimens used for the examination of the effect of dietary bile acids are shown at the top. Mice were sacrificed at the end of each regimen. Effects of dietary CA and CDCA on the expression of *Cyp7a1*, *Cyp8b1*, and *Shp* were examined. Total RNA (30 μ g) from the liver was subjected to Northern blot analysis. The mean values obtained from 6 independent animals for each group are shown. Error bars indicate SD. **P* < 0.05.

including FXR and SHP. The bile acid receptor FXR induces SHP expression to suppress bile acid synthase gene expressions (30–32). In β *klotho*^{-/-} mice, bile acid-mediated induction of *Shp* was significantly attenuated (Figure 4B). This limited blunting would be expected to contribute to the abnormal regulation of bile acid synthesis at least in a part. β Klotho should participate in some aspects of the FXR/SHP cascade. On the other hand, other FXR target genes, *I-babp* (Figure 2F) and *Fgf15* (data not shown), were appropriately upregulated in the intestine, responding to the increased bile acid influx. Therefore, FXR function seems to be only impaired in the induction of *Shp* in the liver.

Since bile acid-dependent suppression of bile acid synthase genes *Cyp7a1* and *Cyp8b1* is virtually intact in *Shp*^{-/-} mice (33, 34), the expression of these genes must be diminished by bile acids, even though FXR/SHP cascade is impaired. In β *klotho*^{-/-} mice, however, *Cyp8b1* expression was completely suppressed, whereas that of *Cyp7a1* was not. Therefore, β Klotho should be involved not only in

the FXR/SHP cascade but also in a *Cyp7a1*-specific regulation that is independent of SHP. SHP-independent divergent regulation of *Cyp7a1* and *Cyp8b1* was also demonstrated in *Shp*^{-/-} mice chronically treated with bile acids (35).

Pathways mediated via pregnane X receptor (NR1I2) and PPAR α (NR1C1) are possible candidates for SHP-independent redundant regulation. In addition to their authentic target genes, these nuclear receptors also suppresses the expression of *Cyp7a1* (6, 10, 11). We found that the expression levels of these receptors and their authentic target genes did not vary between genotypes (data not shown). Thus, these nuclear receptor-mediated pathways are likely to be largely functional in β *klotho*^{-/-} mice.

FGF-mediated regulation is also a potential candidate for SHP-independent regulation. Lack of *fibroblast growth factor receptor 4* (*Fgfr4*) elicits enhanced expression of *Cyp7a1* in mice (15), and exogenous expression of FGF19, a specific ligand for FGFR4, suppresses *Cyp7a1* expression (16). In human hepatocytes, FGF19 is induced by bile acids and suppresses *CYP7A1* expression regardless of SHP expression levels (16). In addition, *Shp* expression levels decreased in constitutively activated human FGFR4-transgenic mice along with the levels of bile acid pools but did not increase in response to elevated bile acid levels in FGFR4-deficient mice (36). These results suggest that FGFR4-mediated repression of *Cyp7a1* is likely to be independent of SHP expression levels (36). Interestingly, there are several common phenotypes between β *klotho*^{-/-} and *Fgfr4*^{-/-} mice. To our knowledge, simultaneous induction of these genes has not been found except in β *klotho*^{-/-} and *Fgfr4*^{-/-} mice. Additionally, β *klotho*^{-/-} and *Fgfr4*^{-/-} mice similarly exhibit a small gallbladder size (15).

Other candidates for SHP-independent pathways are those mediated via JNK. In rat hepatocytes, bile acids elicit JNK activation concurrent with suppression of *Cyp7a1* expression (13). Since JNK activation leads to the induction of SHP in these cells, JNK-mediated regulation of *Cyp7a1* probably involves SHP at least in a part (13). However, *Cyp7a1* expression was enhanced even in the *Shp*^{-/-} hepatocytes when treated with JNK inhibitor. Therefore, not all downstream targets of JNK signals are mediated through SHP (33). Notably, it has been demonstrated that FGFR4 is involved in JNK-signaling pathways, which suggests that these pathways are related to each other at least to some extent (15, 16, 36).

The expression of *Cyp7a1* is also under the strong feedforward regulation of cholesterol via liver X receptor α (LXR α ; NR1H3) (37). However, that is generally hypostatic to the negative feedback regulation (38). We were unable to precisely evaluate its effect; however, it is likely that LXR α -mediated regulation by cholesterol is largely intact in the β *klotho*^{-/-} mouse, as the expression of other LXR α target genes, *Abcg5* and *Abcg8* (39), seems to be normally induced by the elevation of hepatic cholesterol levels (data not shown). Further study is necessary in order to understand the molecular mechanisms underlying β Klotho's involvement in regulation of bile acid synthesis.

The underlying cause of the growth retardation in β *klotho*^{-/-} mice remains unclear. One might predict that it is also a result of the elevation in bile acid synthesis, although the expression of *Cyp7a1* at early stages should be examined.

The results in β *klotho*^{-/-} mice revealed the potential of the β Klotho system for future medical applications. An increasingly large percentage of the population suffers from cholesterol gallstone formation. Without exhibiting other obvious disease-related phenotypes, β *klotho*^{-/-} mice produce and excrete higher amounts



of bile acids and were resistant to cholesterol gallstone formation. Therefore, reagents that interfere with β Klotho activity might help to prevent gallstone formation. Also, such reagents may help in the development of novel approaches to facilitating the excretion of lipid-soluble toxins from the body with bile flow.

Methods

Targeting vector construction and generation of β klotho^{-/-} mouse. Genomic DNA clones spanning the β klotho locus were isolated from a mouse 129SVJ genomic library. The 5' arm of the targeting vector covered a 6.4-kb *EcoRI* fragment including part of the putative first ATG-containing exon. An 8.0-kb *HpaI*-*BamHI* fragment was used for the 3' arm. A promoter-less EGFP gene and a PGKneo cassette were inserted between these 2 arms with the EGFP gene in the forward direction flanked by loxP5171 (5' side) (40) and loxP sequences (3' side). The PGKneo cassette was in the reverse direction, flanked by loxP sequences. Vector DNA was linearized and introduced into TT2 ES cells via electroporation. G418-resistant clones were selected and screened by Southern blotting. Out of 439 clones, 3 were selected and injected into 8 cell stage Crlj:CD1 (ICR) embryos. Germline transmission occurred in 2 chimeras derived from a clone. Male chimeras were crossed with female C57BL/6 mice, and heterozygous offspring were intercrossed to obtain β klotho^{-/-} mice. Offspring from these heterozygous parents were used in this study; however, we confirmed that the same phenotypes were also observed in offspring of heterozygous parents backcrossed 5 times to C57BL/6.

Animals and diets. Mice were housed in plastic colony cages in a temperature-controlled room (24°C) with a 12-hour light/12-hour dark cycle under specific pathogen-free conditions, and all experimental procedures were approved by the Committee on Animal Research (Faculty of Medicine, Kyoto University). Standard feed was the NMF diet (Oriental Yeast Co.). The other diets, including the basal diet (no. 5755), 1% CA diet (no. 58794), 1% CDCA diet (no. 5T11), and atherogenic diet (no. 5806C-Q), were purchased from PMI Feeds Inc. In metabolic experiments, mice were individually housed in metabolic cages, and stool and urine samples were collected every 24 hours for 3 days. Food, water, stool, and urine were weighed daily.

Antibodies and protein analyses. A portion of the β klotho cDNA (corresponding to amino acids 814–967, C-terminal half of the β KL2 domain; ref. 1) was cloned into the *EcoRI* site of the pGEX-4T-1 expression vector (Amersham Biosciences) to produce a partial β klotho-GST fusion protein, which was introduced into *E. coli* (BL21). After separation by SDS-PAGE, the band corresponding to the partial β klotho-GST fusion protein was excised from the gel and injected into an SD rat purchased from Japan SLC Inc. The splenocytes of the immunized rat were used for the establishment of monoclonal anti- β klotho antibodies after hybridoma selection. Western blotting was performed with diluted anti- β klotho antibody (1:2,500) against 15 μ g of total protein.

Northern blot analysis. Total RNAs were extracted using the Total RNA Isolation System (Promega), and 30 μ g aliquots were subjected to the analysis. Blots were exposed to the imaging plate (Fujifilm) and analyzed by a Storm 860 scanner (Molecular Dynamics). Detection probes were obtained from cDNA fragments amplified by RT-PCR with gene-specific primer pairs. Each fragment was cloned into a pT7Blue T-Vector (Novagen), and sequences were confirmed. After excision from the vector by restriction enzymes, the fragments were labeled by α^{32} P-dCTP using a Megaprime DNA Labeling Kit (Amersham Biosciences) and subjected to the hybridization.

Plasma and tissue chemistry. Mice were anesthetized by diethylether, and whole blood was collected from hearts. Serum was separated from collected blood by centrifugation. Serum cholesterol concentrations were determined using Cholesterol E-Test Wako (Wako Pure Chemical Industries). The concentrations of other plasma constituents were determined by SRL Inc. Hepatic total lipids were extracted using Bligh and Dyer's method (41) and resuspended in 2% Triton X-100 by sonication. Following extraction, hepatic cholesterol levels were determined by using the Cholesterol E-Test Wako (Wako Pure Chemical Industries). Bile acid pool size was determined using Total Bile Acids Test Wako (Wako Pure Chemical Industries) following ethanol extraction from a mixed tissue sample including liver, gallbladder, and whole small intestine (15). Fecal total bile acids were also determined using the same kit after ethanol extraction (15). Fecal cholesterol was determined by Japan Food Research Laboratories using gas chromatography (GC-1700, Shimadzu; and DB-1 column, Agilent).

Statistical analysis. Unless otherwise noted, all values are expressed as the mean \pm SD. All data were analyzed by 2-tailed, unpaired Student's *t* test for significant differences between the mean values using Statcel (OMS Publishing Inc.) for Macintosh. *P* values less than 0.05 were considered to be statistically significant.

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