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Effect of α-naphthyl isothiocyanate on 2-amino-3-methylimidazo[4,5-*b*]pyridine (PhIP)-induced mammary carcinogenesis in rats

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The modifying effects of α-naphthyl isothiocyanate (ANIT) on 2-amino-3-methylimidazo[4,5-*b*]pyridine (PhIP)-induced mammary carcinogenesis were investigated in female Sprague-Dawley (SD) rats, and the hepatic activities of the phase II detoxifying enzymes glutathione *S*-transferase (GST) and quinone reductase (QR) were also assayed. Ninety-eight rats were divided into 4 groups. Starting at 6 weeks of age, rats were fed the high-fat diet without ANIT (Groups 1 and 4) or the experimental diet (high-fat diet mixed with 400 ppm ANIT, Groups 2 and 3). At 7 weeks of age, Groups 1 and 2 were given PhIP in corn oil (85 mg/kg body weight, 8 times for 11 days) by intragastric intubation. One week after the last PhIP injection, 5 rats in each group were sacrificed to assay GST and QR activities, and the experimental diets for Groups 2 and 3 were switched to the high-fat diet without ANIT until termination of the experiment. Group 4 served as the vehicle control. All rats were sacrificed at 24 weeks after the start of the experiment. At termination of the experiment, mammary tumours were detected in Groups 1 (PhIP alone) and 2 (PhIP + ANIT) and were shown histologically to be adenocarcinomas; their incidences (multiplicities) were 56.3% (1.66 ± 2.31/rat) in Group 1 and 6.7% (0.07 ± 0.25/rat) in Group 2 ($p < 0.001$). Mean sizes of the tumours were 10.6 ± 5.3 mm in Group 1 and 6.5 mm in Group 2. No mammary tumours were observed in rats of Groups 3 and 4. In addition, ANIT treatment significantly increased the activities of GST and QR in the livers of rats in Groups 2 and 3 as compared to Groups 1 and 4. These results imply that the isothiocyanate compound ANIT shows potent inhibitory effects on mammary carcinogenesis induced by PhIP in female SD rats when administered during the initiation stage.
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Key words: PhIP; α-naphthyl isothiocyanate; mammary carcinogenesis; chemoprevention; rat

Among a number of environmental factors, dietary habits have been regarded as the most important determinant for cancer development in humans.[1–3] Epidemiological data suggest that individuals eating fried or broiled meat have a significantly elevated risk of intestinal cancer.[4,5] A number of carcinogenic heterocyclic amines (HCAs) have been detected in cooked foods. These HCAs show potent mutagenicity in the Ames test and their carcinogenic potential has been demonstrated in various organs, including the forestomach, liver, small intestine, large intestine, mammary gland, Zymbal's gland, clitoral glands, haematopoietic systems, blood vessels and skin of rodents.[6,7] For example, 2-amino-3-methylimidazo[4,5-*b*]pyridine (PhIP) has attracted considerable attention for potent mutagenicity and carcinogenic potential in the mammary gland and colon of rats.[8,9] In most previous studies of their carcinogenesis, the long-term administration of HCAs in the diet has been needed for the development of tumours.[10] In rat mammary carcinogenesis induced by PhIP, mammary cancers are induced at a rate of 47% by administration of 0.04% PhIP in the diet for 52 weeks.[8] Ghoshal *et al.*[11] found mammary tumours in 53% of Sprague-Dawley (SD) rats fed a high-fat diet 25 weeks after treatment with PhIP in 10 doses of 75 mg/kg body weight for 12 days. It was also reported that mammary tumours developed in 77% of CD rats 41 weeks after treatment with PhIP at 50 μmol/rat/week for 8 weeks on a high-fat diet.[12] In these studies, a high-fat diet was given throughout the experiment. These results indicate that the development of PhIP-induced mammary tumours is enhanced and induced earlier by feeding with a high-fat diet. On the other hand, a high-fat diet did not affect PhIP-induced mammary carcinogenesis in SD × F344 F_1 hybrid rats.[13]

α-Naphthyl isothiocyanate (ANIT), present as the glucosinolate precursor, is a constituent of cruciferous vegetables.[14] ANIT has been reported to induce cholestasis, bile duct proliferation and focal necrosis of hepatocytes, without the development of liver cancer, and to inhibit hepatocarcinogenesis in rats.[15–17] The mechanisms of its chemopreventive effect have been discussed;[18] one possible mechanism is the regulation of phase I and phase II enzymes. ANIT has been shown to decrease hepatic cytochrome P-450 content,[19,20] and hepatic mixed-function oxidase activities,[21] and increase microsomal epoxide hydrolase and cytosolic DT-diaphorase activities.[19] The promutagenic PhIP found in cooked foods is converted to the active form mainly by cytochromes P450 1A1, 1A2 and 1B1 in the liver and partially in the target organs.[21–27] Glutathione *S*-transferase (GST) has been reported to inhibit DNA binding of *N*-acetyl-PhIP *in vitro*.[28]

These findings suggested that the administration of P450 inhibitor in the initiation phase may reduce the carcinogenicity of PhIP. ANIT was used in early studies of cancer chemoprevention, although it is toxic to the liver and is a cholestatic agent.[15–17] Possibly as a result of this, it is now used rarely in chemoprevention studies and has not been scheduled for trials in humans. Toxicity is a serious problem for trials of cancer chemopreventive agents. However, if lower doses of such compounds do not exert clear toxicity in any organs, including the liver, these compounds should not be omitted from basic research on cancer chemoprevention.

In our study, we examined the effects of a low dose (400 ppm in diet) of ANIT on PhIP-induced mammary carcinogenesis in SD rats. In addition, the activities of phase II detoxifying GST and quinone reductase (QR) in the liver were measured to clarify whether these enzymes are involved in its modification of PhIP-induced mammary carcinogenesis.

Material and methods

Animals, diet, water and carcinogen

Weanling female SD rats were purchased from Japan SLC, Co. (Hamamatsu, Japan). PhIP was supplied by Dr. Wakabayashi

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TABLE I – PERCENT COMPOSITION OF EXPERIMENTAL SEMIPURIFIED HIGH FAT DIETS CONTAINING CORN OIL

Diet ingredients	Percents of ingredients by weight
Casein, vitamin-free	23.50
DL-Methionine	0.35
Corn starch	32.90
Dextrose	8.30
Alphacel	5.90
Corn oil	**23.52**
Mineral, AIN	4.11
Vitamin, AIN (revised)	1.18
Choline bitartrate	0.24

AIN: American Institute of Nutrition.

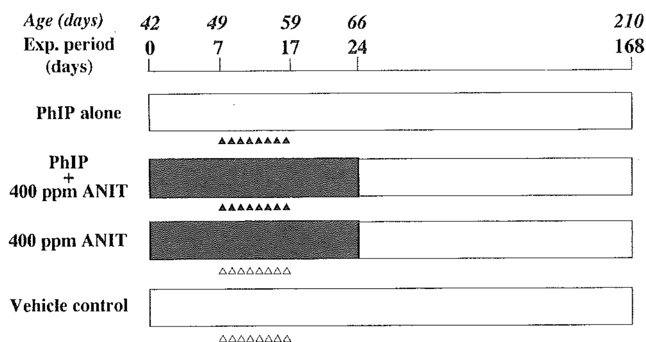

FIGURE 1 – Experimental protocol. Unshaded, high-fat diet; shaded, high-fat diet with 400 ppm α-naphthyl isothiocyanate (ANIT). Closed triangle, PhIP 85 mg/kg B.W. in corn oil by gavage Open triangle, Corn oil by gavage.

(National Cancer Center Research Institute, Tokyo, Japan). ANIT was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). All diet ingredients were obtained from CLEA Japan Inc. (Tokyo, Japan) and experimental and high-fat diets were prepared weekly in our laboratory and stored in a cold room (<4°C). The composition of the high-fat diet is shown in Table I. All animals were housed in wire cages (3 rats/cage). Animals had free access to water and diets under controlled environmental conditions of humidity (50 ± 10%), lighting (12 hr light/dark cycle) and temperature (23 ± 2°C).

Animal treatment and pathological examination

A total of 98 rats, 5 weeks of age, were divided into 4 groups: Group 1, 37 rats given PhIP alone; Group 2, 35 rats given PhIP and 400 ppm ANIT in the initiation phase; Group 3, 13 rats given ANIT alone and Group 4, 13 rats as vehicle controls. All animals were given the high-fat diet throughout the experiment as the basal diet (Fig. 1).

Rats in Groups 2 and 3 were given diet containing 400 ppm ANIT from the start of the experiment and animals in the other groups were kept on the basal diet. Animals in Groups 1 and 2 were given 8 doses of PhIP (85 mg/kg body weight, gastric intubation) in 0.1 ml of corn oil for 11 days, starting 7 days after the commencement of the experiment. Vehicle control rats received an equal volume of corn oil. For rats in Groups 2 and 3, the experimental diet was changed to the basal diet 1 week after final treatment with the carcinogen. Five rats in each group were sacrificed to assay GST and QR activities at 1 week after the last PhIP injection. All other animals were sacrificed 24 weeks after the start of the experiment to assess the modifying effect of ANIT on mammary carcinogenesis induced by PhIP. At the end of the experiment, complete autopsies on these animals were performed after sacrifice by ether anaesthesia. At autopsy, the location, number

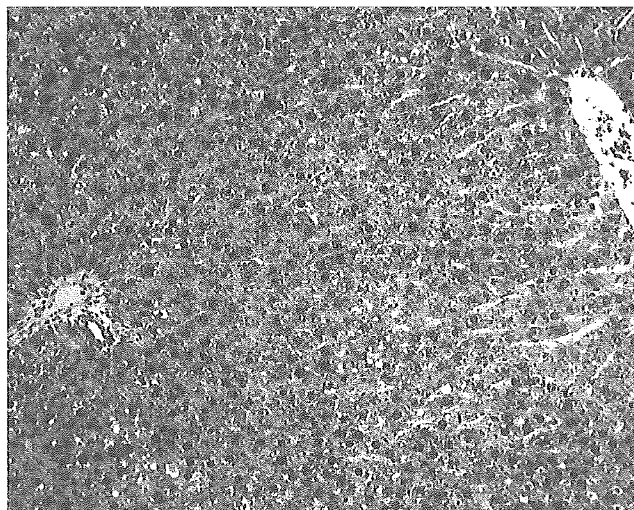

FIGURE 2 – Histology of liver of a rat from Group 3 (ANIT alone) reveals no abnormalities. Hematoxylin and eosin stain, ×10 (original magnification).

and size of mammary tumours were recorded. Tissues were fixed in 10% buffered formalin, embedded in paraffin blocks and processed for routine histological observation with haematoxylin and eosin staining. Pathological diagnosis of mammary tumours was performed according to the criteria outlined by Young and Hallowes.[29]

GST and QR assays

Aliquots of minced liver were processed to obtain the cytosolic fraction. The activities of GST and QR were determined using 1-chloro-2,4-dinitrobenzene (CDNB) or 1,2-dichloro-4-nitrobenzene (DCNB) for GST and NADH/menadione for QR as substrates, respectively, as described previously.[30–32] All spectrographic assays were based on absorption at 340 nm and all samples were examined in triplicate. One unit of enzyme activity was defined as the amount of enzyme catalysing the conversion of 1 μmol of substrate to product per min at 25°C. Cytosolic protein concentrations were determined by the Bradford method[33] using bovine serum albumin as the standard.

Statistical analysis

Differences in the incidence or multiplicity of mammary tumours between groups were analysed by Fisher's exact probability test, χ^2-test, Student's t-test or alternate Welch t-test.

Results

Table I shows body, liver and relative liver weights of rats in each group at the termination of the experiment. No significant differences were found in body or liver weights between Groups 1 (PhIP alone) and 2 (PhIP + ANIT). Body weight of Group 3 (ANIT alone) was significantly lower than that of Group 4 (vehicle control) ($p < 0.005$). Relative liver weight of rats in Group 3 (ANIT alone) was significantly greater than that in Group 4 (vehicle control) ($p < 0.005$). However, no clinical signs of toxicity of ANIT were noted during the study. Histological examination did not provide evidence of histological alterations, including cholestasis, bile duct proliferation or cell necrosis, in the livers of rats given ANIT alone (Fig. 2).

At the end of the experiment, mammary tumours were found in rats in Groups 1 and 2 but no tumours were found in animals of Groups 3 or 4 rats, as summarised in Table II. All of the mammary tumours were diagnosed as invasive or intraductal adenocarcino-

TABLE II – FINAL BODY AND LIVER WEIGHTS

Group	Treatment	Number of rats	Body weight (g)	Liver weight (g)	Relative liver weight (%)
1	PhIP alone	32	278 ± 20[1,2]	11.0 ± 2.4	3.94 ± 0.76
2	PhIP + ANIT	30	277 ± 19	10.3 ± 1.5	3.72 ± 0.52
3	ANIT alone	8	266 ± 19[2]	10.5 ± 1.1	3.94 ± 0.25[2]
4	No treatment	8	304 ± 20	10.4 ± 1.2	3.41 ± 0.30

[1]Mean ± SD.–[2]Significantly different from Group 4 by Student's *t*-test (b: $p < 0.005$).

TABLE III – INCIDENCE AND MULTIPLICITY OF MAMMARY CARCINOMA

Group	Treatment	Number of rats	Incidence (%)			Multiplicity (number/rat)		
			Total	Inv.d.Ca.[1]	Int.d.Ca.[2]	Total	Inv.d.Ca.	Int.d.Ca.
1	PhIP	32	18 (56.3)	17 (53.1)	8 (25.0)	1.66 ± 2.31[3]	1.19 ± 1.64	0.47 ± 1.29
2	PhIP + ANIT	30	2 (6.7)[4]	2 (6.7)[4]	0[5]	0.07 ± 0.25[6]	0.07 ± 0.25[6]	0
3	ANIT alone	8	0	0	0	0	0	0
4	Vehicle control	8	0	0	0	0	0	0

[1]Invasive ductal carcinoma.–[2]Intraductal carcinoma.–[3]a: Mean ± SD.–[4,5]Significantly different from Group 1 (PhIP alone) by Fisher's exact probability test (b: $p < 0.001$, c: $p < 0.005$).–[6]d: Significantly different from Group 1 (PhIP alone) by Student's *t*-test or alternate Welch *t*-test ($p < 0.001$).

TABLE IV – RESULTS OF LIVER GST AND QR ACTIVITIES

Group	Treatment	Number of rats	GST		QR
			CDNB (mU/mg)	DCNB (mU/mg)	NADH (mU/mg)
1	PhIP alone	5	415.6 ± 76.1[1,2]	8.41 ± 0.17[3]	126.7 ± 28.6[4]
2	PhIP + ANIT	5	491.0 ± 145.0	21.29 ± 0.44[5]	222.6 ± 2.2[6]
3	ANIT alone	5	322.4 ± 72.6	17.63 ± 0.39[3]	234.4 ± 94.5[7]
4	No treatment	5	269.0 ± 87.0	4.82 ± 0.87	61.4 ± 7.7

[1]Mean ± SD.–[2,3,4,7]Significantly different from Group 4 (No treatment) by Student's *t*-test or alternate Welch *t*-test (b: $p < 0.05$, c:$p < 0.001$, d: $p < 0.005$, g: $p < 0.02$).–[5,6]Significantly different from Group 1 (PhIP alone) by Student's *t*-test or alternate Welch *t*-test (e: $p < 0.001$, f: $p < 0.002$).

mas. The incidence of mammary adenocarcinoma in Group 2 (6.7%, $p < 0.001$) was significantly lower than that in Group 1 (56.3%). The average number of neoplasms in Group 2 (0.07 ± 0.25, $p < 0.001$) was also significantly lower than that in Group 1 (1.66 ± 2.31). Mean size of mammary tumours in Group 2 (6.5 mm) was smaller than that in Group 1 (10.6 ± 5.3 mm). No neoplastic lesions other than mammary tumours were observed in rats in any group.

Data regarding liver GST and QR activities as determined by short-term bioassay are summarised in Table III. GST activities toward CDNB in Group 1 was slightly but significantly ($p < 0.05$) higher than that in Group 4, whereas that in Group 2 was not significantly different from that in Group 1. GST activities toward DCNB in Groups 1 and 3 were significantly higher than that in Group 4 ($p < 0.001$). Combined treatment with PhIP and ANIT (Group 2) significantly increased GST-PCNB as compared to Group 1 treated with PhIP alone ($p < 0.001$) and showed a summation effect on induction of this activity. Liver QR activities in Groups 1 and 3 were significantly greater than that in Group 4 ($p < 0.005$). Combined treatment with PhIP and ANIT significantly increased QR activity ($p < 0.002$) but did not show a summation effect.

Discussion

In our study, a high incidence of mammary tumours was observed in rats given PhIP and a high-fat diet. It has been reported that long-term administration of HCAs is needed for the development of tumours in rodents.[8,11,12] We observed a higher incidence of mammary tumours as compared to the results reported previously by Ghoshal *et al.*[11] and El-Bayoumy *et al.*[12] Ghoshal *et al.*[11] reported that daily doses of 100 mg/kg of PhIP were toxic to the animals after the 7th dose and that weight loss was seen in surviving animals. In this experiment, 8 doses of 85 mg/kg PhIP were administered to rats for 11 days and did not cause body weight loss.

Treatment with ANIT alone in our study reduced the body weight as compared to that in vehicle-only controls. ANIT is known to induce hepatotoxicity and cholestasis in rats.[15–17] The dose (400 ppm in diet) of ANIT in our study was too low to induce liver toxicity or cholestasis and was about half the dose required to induce hepatotoxicity in rats. In fact, histological examination revealed neither hepatotoxicity nor cholestasis in rats given AINT. The experimental schedule (initiation feeding with ANIT) may be related to the lack of liver toxicity seen in our study.

Several chemopreventive agents for PhIP-induced mammary carcinogenesis, including synthetic or natural compounds with antioxidative properties, have been reported.[34–41] Their cancer chemopreventive effects were mostly observed when given during the entire experimental period or in the post-initiation phase of PhIP-induced mammary tumorigenesis, although diallyl disulfide and aspirin inhibited PhIP-induced mammary carcinogenesis when administered during the initiation phase.[34]

A number of mechanisms underlying chemoprevention by xenobiotics in PhIP-induced mammary tumorigenesis have been proposed. PhIP is oxidised to an *N*-hydroxy derivative [PhIP (NHOH)] in the liver by cytochrome P450 enzymes, and esterified by acetyltransferases or sulfotransferases to the ultimate carcinogen.[42–45] Adduct formation is thought to be crucial for PhIP-induced carcinogenesis and PhIP-DNA adducts have been detected in human tissues.[46] Certain isothiocyanates (*e.g.*, sulphoraphene, phenyl isothiocyanate and benzyl isothiocyanate (BITC)) have been reported to prevent chemically induced cancer development in laboratory animals.[47–55] Isothiocyanates exert their cancer chemopreventive action by modulating the activities of phase I and phase II drug metabolism enzymes.[47–53,55] ANIT can decrease hepatic cytochrome P-450 content.[16–18] PhIP is known to exert its carcinogenic

activity after metabolic activation by CYP1A2 or CYP1B1 mainly in the liver.[21–27] ANIT has been reported to inhibit carcinogenesis in the lung, liver, forestomach, intestine and mammary gland when given before or during carcinogen treatment.[15–18] In our study, ANIT elevated the activities of phase II enzymes, GST and QR in the liver. ANIT has been reported to increase the activity of GST,[20] which can inhibit DNA binding of *N*-acetyl-PhIP *in vitro*.[28] Thus, increased activities of GST and QR, particularly GST, in the liver may be one of the major causes of its cancer-suppressing effect observed in the present study. In our previous experiment, BITC failed to inhibit PhIP-induced mammary carcinogenesis in rats given 100 ppm of PhIP,[56] a higher dose than that used in the present study. The difference between the results of our previous and present studies may be due to the differences in enzyme induction capability of both compounds, BITC and ANIT; BITC shows weaker induction of enzymes involving oxidative metabolism and metabolic conversion than ANIT.[57]

In conclusion, ANIT significantly inhibited breast cancer development induced by PhIP, presumably through the induction of both GST and QR in the liver. Accordingly, ANIT may be a candidate chemopreventive agent for breast cancer. In relation to fat and PhIP, further studies of the effects on levels of CYP proteins, metabolic activation of PhIP by CYP isoforms and another typical phase II enzyme, UDP-glucuronyltrasferase, are necessary to confirm the suppression by ANIT in PhIP-induced mammary carcinogenesis, as UDP-glucuronyltransferase has also been reported to detoxify another heterocyclic amine, 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ).[58]

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Concurrent suppression of hyperlipidemia and intestinal polyp formation by NO-1886, increasing lipoprotein lipase activity in Min mice

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We have previously reported a hyperlipidemic state in two strains of *Apc*-deficient mice, Min and *Apc*^{1309}, associated with low expression levels of lipoprotein lipase (LPL) in the liver and small intestine, and enforced induction of LPL mRNA by peroxisome proliferator-activated receptor (PPAR)α and PPARγ agonists clearly suppressed hyperlipidemia and intestinal polyp formation in these mice. Meanwhile, a compound, NO-1886, has been shown to increase LPL mRNA and protein levels but not to possess PPARα and PPARγ agonistic activity. In this study, therefore, the effects of NO-1886 on hyperlipidemia and intestinal polyp formation were investigated in Min mice. Administration of 400 and 800 ppm NO-1886 in the diet for 13 weeks from 7 weeks of age caused a reduction of serum triglycerides to 39% and 31% of the untreated value, respectively, and the values for very low-density lipoprotein cholesterol, low-density lipoprotein cholesterol, and high-density lipoprotein cholesterol were improved almost to the wild-type level with a corresponding elevation of the LPL mRNA. Moreover, total numbers of intestinal polyps in the groups receiving NO-1886 at 400 and 800 ppm were decreased to 48% and 42% of the control value, respectively. We also found that NO-1886 suppressed cyclooxygenase-2 transcriptional promoter activity in a reporter gene assay and reduced cyclooxygenase-2 mRNA levels in the small intestine of Min mice. These results indicate that suppression of serum lipid levels by increasing LPL activity may contribute to a reduction of intestinal polyp formation with *Apc*-deficiency, and NO-1886 and its derivatives could be useful as chemopreventive agents for colon cancer.

Colon cancer is one of the most frequent cancers in developed countries, and many epidemiological studies have suggested a correlation with obesity and hyperlipidemia (1, 2). Recently, we reported an age-dependent hyperlipidemic state in *Apc*-deficient Min and *Apc*^{1309} mice, animal models of human familial adenomatous polyposis (3, 4). The mRNA levels for lipoprotein lipase (LPL), which catalyzes hydrolysis of triglycerides, were shown to be down-regulated in the livers and small intestines of these *Apc*-deficient animals compared with their wild-type counterparts. We also demonstrated that treatment with a peroxisome proliferator-activated receptor α (PPARα) agonist, bezafibrate, and a PPARγ agonist, pioglitazone, concomitantly suppressed hyperlipidemia and intestinal polyp formation in the mice with induction of LPL mRNA (3, 4). Thus, LPL expression levels may correlate with intestinal polyp development in *Apc*-deficient mice.

LPL is the major enzyme responsible for the hydrolysis of triglyceride-rich lipoproteins such as chylomicrons and very low-density lipoprotein (VLDL). LPL mRNA is expressed ubiquitously in the body but especially in adipose tissue and skeletal muscle, where it is synthesized then transferred to the surface of endothelial cells, to become bound to membrane-anchored heparan sulfate proteoglycans (5, 6). There are reports on association of hyperlipidemia with lowered or lack of LPL (7, 8). However, there have been no reports directly addressing links between LPL and colon carcinogenesis.

There has been great interest in development of an LPL-selective inducer for effective control of hypertriglyceridemia and low levels of high-density lipoprotein cholesterol (HDL-C) in serum. Several diethyl benzyl phosphonate derivatives were examined and one example, NO-1886, has been reported to increase LPL mRNA and protein levels, resulting in a reduction of plasma triglycerides and an increase in HDL-C levels in rats (9). NO-1886 also improves obesity in rats through induction of fatty acid oxidation-related enzymes, such as long-chain acyl-CoA dehydrogenase and acetyl-CoA acyltransferase (10). Moreover, NO-1886 reduces high-cholesterol diet-induced atherosclerotic lesions in rat coronary arteries (9).

It is well known that PPARα and PPARγ agonists improve hypertriglyceridemia and hypercholesterolemia through induction of lipid metabolism-related genes such as LPL (11). Moreover, these agonists have been documented to show antiproliferative and proapoptotic effects in various types of cancer cells, including colon cancer cells (12). Using a reporter gene assay, NO-1886 was revealed not to possess PPARα and PPARγ agonistic activity, unlike bezafibrate and pioglitazone (10). Thus, the LPL selective activator, NO-1886, may be a very essential agent for determining the relationship between hyperlipidemia due to LPL depression and colon carcinogenesis. In this study, we therefore examined the effects of 400 and 800 ppm NO-1886 in the diet on both hyperlipidemia and intestinal polyp formation in Min mice and demonstrated concomitant suppression of both. Taking account of the fact that NO-1886 also suppressed cyclooxygenase-2 (COX-2) mRNA expression, possible mechanisms of its action in *Apc*-deficient mice and the usage of NO-1886 or its derivatives as possible candidates of colon cancer prevention are also discussed.

Materials and Methods

Animals and Chemicals. Female C57BL/6-*Apc*$^{Min/+}$ mice (Min mice) were purchased from The Jackson Laboratory at 5 weeks of age and genotyped by the method reported in ref. 13. We used female animals for experimental convenience because there are no significant differences in the numbers of intestinal polyps and serum lipid levels between males and females (3, 4, 14). Heterozygotes of the Min strain and wild-type (C57BL/6J) mice were acclimated to laboratory conditions for 2 weeks. Three to five mice were housed per plastic cage with sterilized softwood chips as bedding in a barrier-sustained animal room air-conditioned at 24 ± 2°C and 55% humidity on a 12-h light/dark cycle. The LPL selective inducer NO-1886, 4-[(4-bromo-2-cyanophenyl)carbamoyl]benzylphosphonate, was chemically synthesized at Otsuka Pharmaceutical Factory (9). Its structure is shown in Fig. 1. NO-1886 was well mixed at concentrations of

Abbreviations: COX, cyclooxygenase; HDL, high-density lipoprotein; HDL-C, HDL cholesterol; iNOS, inducible NO; LDL, low-density lipoprotein; LDL-C, LDL cholesterol; LPL, lipoprotein lipase; PPAR, peroxisome proliferator-activated receptor; VLDL, very low-density lipoprotein; VLDL-C, VLDL cholesterol.

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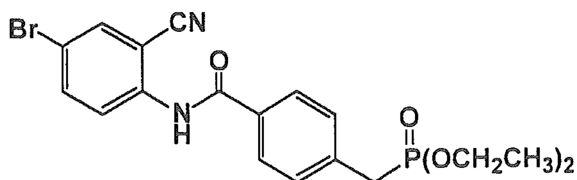

Fig. 1. Structure of NO-1886.

400 and 800 ppm with AIN-76A powdered basal diet (CLEA Japan, Tokyo). The chemical was confirmed to be stable under the experimental conditions used in this study.

Animal Experiments. To investigate the effects of NO-1886 on both hyperlipidemia and intestinal polyp formation, 10–13 female Min mice at 7 weeks of age were given 0 (control), 400, or 800 ppm NO-1886 in the diet for 13 weeks. Food and water were available ad libitum. The animals were observed daily for clinical signs and mortality. Body weights and food consumption were measured weekly. At the kill time points, animals were anesthetized with ether, and blood samples were collected from the abdominal aorta. Serum levels of triglycerides and total cholesterol were measured as reported in ref. 3. In addition, the levels of the major lipoprotein classes for cholesterol, VLDL, low-density lipoprotein (LDL), and high-density lipoprotein (HDL), were measured by HPLC (15). The experiments were conducted according to the "Guidelines for Animal Experiments in National Cancer Center" of the Committee for Ethics of Animal Experimentation of the National Cancer Center.

The intestinal tract was removed, filled with 10% buffered formalin, and separated into the small intestine, cecum, and colon. The small intestine was divided into the proximal segment (≈4 cm in length), and then the proximal (middle) and distal halves of the remainder. All segments were opened longitudinally and fixed flat between sheets of filter paper in 10% buffered formalin. The numbers and sizes of polyps and their distributions in the intestine were assessed with a stereoscopic microscope (3).

RT-PCR Analysis. Tissue samples from the normal parts of liver and small intestine of mice (n = 3 each) were rapidly deep-frozen in liquid nitrogen and stored at −80°C. Cells of a human colon adenocarcinoma cell line, DLD-1, were purchased from the Health Science Research Resources Bank (Osaka) and cultured according to the supplier's instructions. Total RNA was isolated from tissues by using Isogen (Nippon Gene, Tokyo), treated with DNase (Invitrogen) and applied at 3-μg aliquots in a final volume of 20 μl for synthesis of cDNA by using an Omniscript RT Kit (Qiagen, Hilden, Germany) and an oligo(dT) primer. The mixture was incubated for 10 min at 32°C, then 50 min at 42°C, and immediately cooled on ice. As an internal control to confirm the integrity of the isolated mRNA, β-actin was used (3). PCR was performed with specific primers for mouse LPL (5′-primer-GGATCCGTGGCCGCAGCAGACGCAGGA-AGA, 3′-primer-GAATTCCATCCAGTTGATGAATCTG-GCCAC) (16), COX-1 (5′-GTCATCAAGGAGTCCCGAG, 3′-CCAGTTTCTTCAGTGAGGC), COX-2 (5′-CACACTCTA-TCACTGGCACC, 3′-CTCTCTGCTCTGGTCAATGG), inducible nitric oxide (iNOS) (5′-CTTGGAGCGAGTTGTG-GATTG, 3′-CAGGAAGTAGGTGAGGGC), PPARγ (5′-TGAGACCAACAGCCTGACG, 3′-GATGTCAAAGGA-ATGCGAGTGG), PPARα (5′-TCTTACCTGTGAACACGA-CCTG, 3′-AGCAGTGGAAGAATCGGACC). PCR amplification of 1 μl of cDNA was carried out in a final volume of 10 μl with an PTC-200 DNA Engine (MJ Research, Waltham, MA), by using a HotStarTaq (Qiagen). Cycling conditions were as follows: 94°C for 20 sec, annealing temperature (60–64°C) for 30 sec, 72°C for 80 sec, and 25–35 cycles after an initial step of 95°C for 15 min. A final elongation step of 72°C for 10 min completed the PCR. The products were then electrophoresed in 2% agarose gels.

Reporter Gene Assay for COX-2 Promoter-Dependent Transcriptional Activity. Stable transfectants containing pB2-Gal-BSD and pCOX-2/B2-Gal-BSD in the genome DNA of DLD-1 cells were prepared as described in ref. 17. Cells were seeded at a density of 2×10^4 cells per well in 96-well plates and precultured for 24 h. They were then cotreated with type α TGF (TGFα) (100 ng/ml) and/or NO-1886 (2.5, 5, and 10 μM), and the total β-gal activities of cells in each well were determined by colorimetric assay with *o*-nitrophenyl-β-D-gal as described in ref. 17. The background β-gal activity of DLD-1 cells was determined with a control nontreated culture of DLD-1/B2-Gal-BSD cells, and the value was set as 0. The basal β-gal activity of nontreated DLD-1/COX2-B2-Gal-BSD was set as 100%. The percentage of β-gal activity with each treatment was then calculated by using data from triplicate wells. The values for β-gal activity were normalized for total protein amount. All assays were carried out in triplicate, and the experiment was repeated at least twice.

Statistical Analysis. The results were expressed as mean ± SE values, and statistical analysis was performed with Student's t test. Differences were considered to be statistically significant with $P < 0.05$.

Results

Improvement of Serum Lipid Levels in Min Mice by NO-1886. Consistent with our previous reports (3, 4), a hyperlipidemic state was observed in the Min mice fed the basal diet at 20 weeks of age. Namely, serum levels of triglycerides at 20 weeks of age were dramatically increased to almost 30 times the wild-type value (Fig. 2*A*). Total cholesterol levels in Min mice were also increased 1.7-fold (Fig. 2*B*). Moreover, VLDL cholesterol

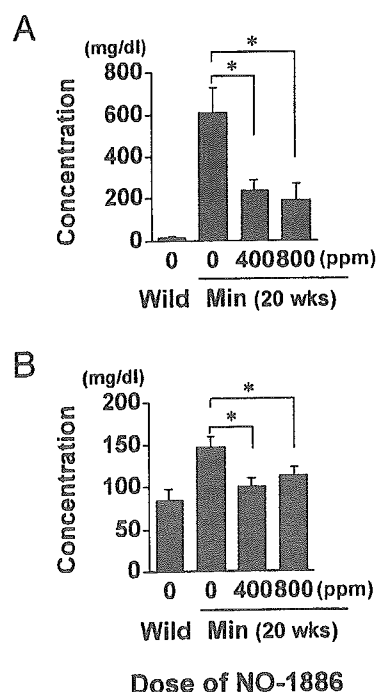

Fig. 2. Suppression of serum lipid levels in Min mice by NO-1886. Values for serum levels of triglyceride (*A*) and total cholesterol (*B*) in female Min mice given diet containing NO-1886 at doses of 0 (n = 7), 400 (n = 8), and 800 ppm (n = 10) for 13 weeks and wild-type mice (n = 6) are shown. Data are means; bars are SE. *, $P < 0.05$; **, $P < 0.01$.

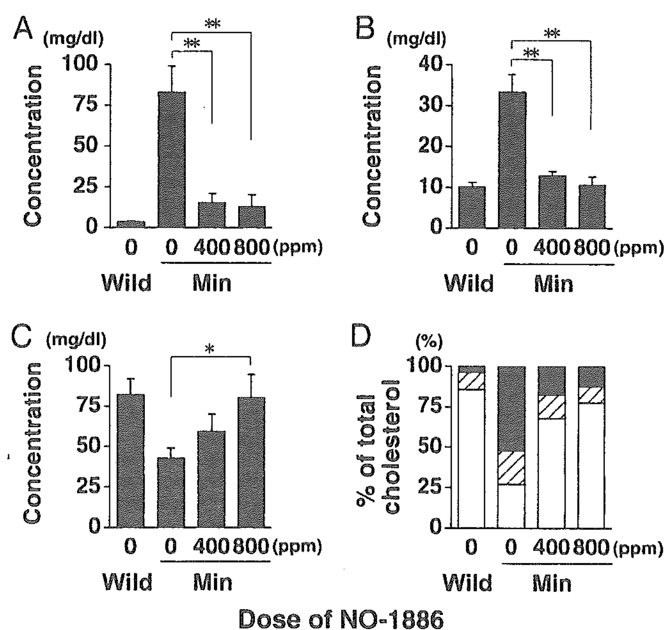

Fig. 3. Serum levels of cholesterol lipoproteins in female Min mice treated with NO-1886. Shown are lipoprotein classes in female Min mice given diets containing NO-1886 at doses of 0, 400, and 800 ppm for 13 weeks and wild-type mice. (*A*) VLDL cholesterol. (*B*) LDL cholesterol. (*C*) HDL cholesterol. (*D*) The proportions of cholesterol lipoproteins. Open box, HDL cholesterol; crosshatched box, LDL cholesterol; filled box, VLDL cholesterol). Data are means; bars are SE (n = 5). *, $P < 0.05$; **, $P < 0.01$.

(VLDL-C) levels in the basal diet group of Min mice were 24-fold higher than in their wild-type counterparts (Fig. 3*A*). LDL cholesterol (LDL-C) levels were increased 3.3-fold, whereas HDL-C was decreased to 50% of the wild-type value (Fig. 3 *B* and *C*). The proportions of HDL-C, LDL-C, and VLDL-C in the total cholesterol in Min mice (27%, 21%, and 52%, respectively) were almost opposite to those in wild-type mice (86%, 10%, and 4%, respectively) (Fig. 3*D*).

Administration of 400 and 800 ppm NO-1886 did not affect body weights or clinical signs of Min mice throughout the experimental period. Amounts of daily food intake were not different among groups, and daily intakes of NO-1886 in the 400- and 800-ppm groups of Min mice were 1.2–1.5 mg per mouse per day and 2.8–3.1 mg per mouse per day, respectively. In addition, there were no changes observed in any organ weights that could be attributable to toxicity. Administration of 400 and 800 ppm NO-1886 clearly decreased serum levels of triglycerides to 39% and 31% of the untreated control value, respectively (Fig. 2*A*). The levels of total cholesterol were also decreased to 69% and 77% of the untreated control value (Fig. 2*B*). Furthermore, levels of both triglyceride-rich lipoproteins, VLDL-C and LDL-C, were dramatically suppressed by NO-1886 treatment. The levels of VLDL-C in the groups treated with NO-1886 at 400 and 800 ppm were reduced to 19% and 15% of the untreated control value, and the levels of LDL-C were to 39% and 32% of the untreated control value, respectively (Fig. 3 *A* and *B*). In contrast, HDL-C levels were increased to the wild-type value at 800 ppm (Fig. 3*C*). Overall, administration of NO-1886 improved the balance of HDL-C, LDL-C, and VLDL-C in the total cholesterol of Min mice (Fig. 3*D*).

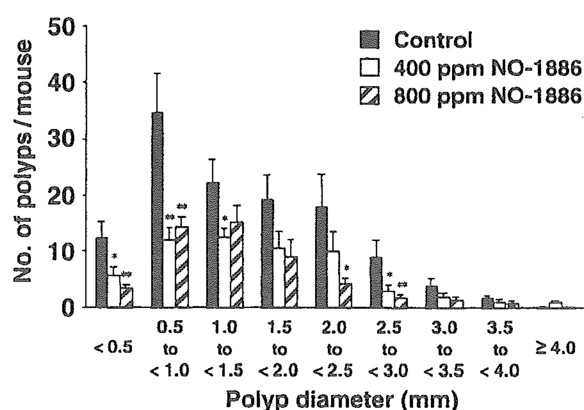

Fig. 4. Effects of NO-1886 on the size distribution of intestinal polyps in Min mice. Min mice were fed a basal diet (filled box) or a diet containing 400 ppm (open box) or 800 ppm (hatched box) NO-1886 for 13 weeks. The number of polyps per mouse in each size class is given as a mean value; bars are SE. *, $P < 0.05$; **, $P < 0.01$.

Suppression of Intestinal Polyp Formation in Min Mice by NO-1886. Table 1 summarizes data for the number and distribution of intestinal polyps in the basal diet and NO-1886-treated groups. Almost all polyps developed in the small intestine, with only a few in the colon (Table 1). The total number of polyps were significantly decreased by administration of 400- and 800-ppm NO-1886 to 48% and 42% of the untreated control value, respectively, with reduction in the proximal, middle, and distal parts by 63%, 57%, and 45% with 400 ppm, and by 74%, 63%, and 49% with 800 ppm. Treatment with NO-1886 also significantly decreased the numbers of colon polyps.

Fig. 4 shows the size distributions of intestinal polyps in the basal diet and NO-1886-treated groups. The main polyp sizes observed in the basal diet groups were 0.5–3.0 mm in diameter. Administration of NO-1886 reduced the numbers of polyps of all sizes.

Down-Regulation of COX-2 Transcriptional Activity by NO-1886. Expression of enzymes associated with inflammation has been reported to be increased in colon carcinogenesis (18). To cast light on mechanisms of the effect of NO-1886 on colon carcinogenesis, we investigated expression levels of mRNAs for COX-1, COX-2, and iNOS in DLD-1 human colon cancer cells by RT-PCR. As shown in Fig. 5*A*, the TGFα-stimulated mRNA levels for COX-2 were reduced to nonstimulated mRNA levels by NO-1886 in DLD-1 cells. On the other hand,

Table 1. Suppression of intestinal polyp development in Min mice by NO-1886

		Small intestine				
Dose, ppm	No. of mice	Proximal	Middle	Distal	Colon	Total
0	7	23.1 ± 4.2	37.1 ± 11.1	60.4 ± 12.2	1.0 ± 0.2	121.7 ± 26.0
400	8	8.5 ± 1.4 (37)*	16.1 ± 3.8 (43)	33.0 ± 6.5 (55)	0.4 ± 0.2 (38)†	58.0 ± 10.8 (48)†
800	10	5.9 ± 1.0 (26)*	13.8 ± 2.6 (37)†	30.5 ± 5.0 (51)†	0.3 ± 0.2 (30)†	50.5 ± 7.8 (42)*

Data are means ± SE of the number of polyps per mouse. Numbers in parentheses are percentages of the control value.
*Significantly different from the basal diet group at $P < 0.01$.
†Significantly different from the basal diet group at $P < 0.05$.

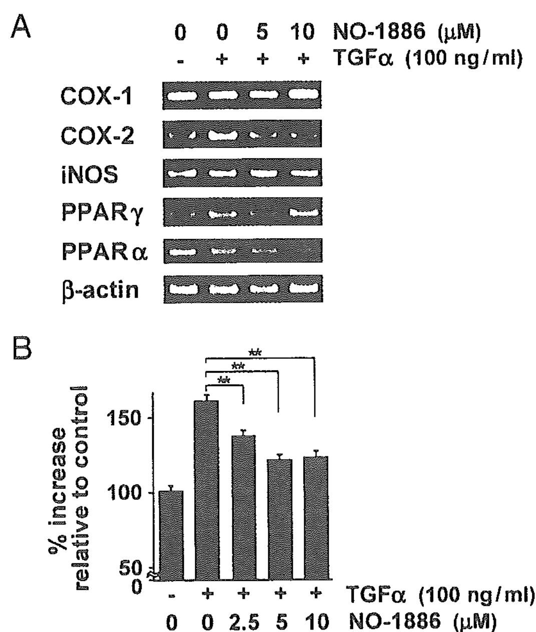

Fig. 5. Suppression of COX-2 mRNA level and COX-2 transcriptional activity in a human colon cancer cell line by NO-1886. (*A*) RT-PCR analysis of mRNA expression levels for COX-1, COX-2, iNOS, PPARγ, and PPARα in DLD-1 cells treated with the indicated dose of TGFα and/or NO-1886. (*B*) Reporter gene assay for COX-2 promoter-dependent transcriptional activity in DLD-1 cells. All assays were carried out in triplicate, and data are representative of at least two separate experiments. Data are means; bars are SD. **, $P < 0.01$.

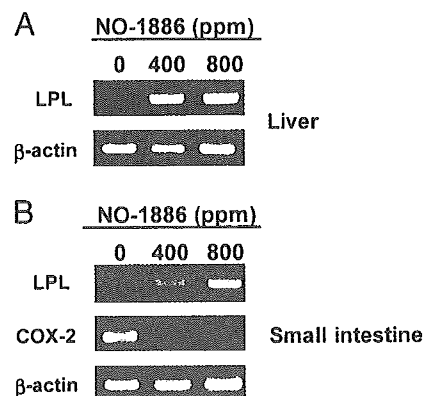

Fig. 6. Changes of mRNA levels for LPL and COX-2 in the liver and small intestine of female Min mice. (*A*) RT-PCR analysis of LPL mRNA expression in the livers of female Min mice given diets containing NO-1886 at doses of 0, 400, and 800 ppm for 13 weeks. (*B*) RT-PCR analysis of LPL and COX-2 mRNA expression in normal parts of the small intestine of female Min mice given diets containing NO-1886 at doses of 0, 400, and 800 ppm for 13 weeks. Data are representative of three mice of each group.

there was no obvious variation in the mRNA levels for COX-1 and iNOS (Fig. 5*A*). In addition, we confirmed that NO-1886 did not change the mRNA levels for PPARγ and PPARα in DLD-1 cells (Fig. 5*A*). Fig. 5*B* shows the results for β-gal reporter gene assay in DLD-1 cells. Treatment of cells with 100 ng/ml TGFα for 48 h increased COX-2 transcriptional levels to 1.6-fold of the control value, whereas NO-1886 at 5 and 10 μM suppressed TGFα-stimulated COX-2 transcriptional activity to 1.2-fold of the control value. No significant decrease of cell viability was observed after 48 h culture with NO-1886 at these concentrations.

Expression of LPL and COX-2 mRNAs in Liver and Small Intestine in Min Mice Assessed by RT-PCR. LPL mRNA levels in the liver and the small intestine in the Min mice at 20 weeks of age were very low but were markedly increased by the treatment with NO-1886 dose-dependently (Fig. 6). Consistent with the *in vitro* data in Fig. 5, administration of NO-1886 reduced mRNA levels of COX-2 in normal parts of small intestine of Min mice at 20 weeks of age (Fig. 6*B*).

Discussion

This study provided clear evidence that administration of the LPL selective inducer NO-1886, which increases LPL mRNA and protein levels, suppresses both hyperlipidemia and intestinal polyp formation in Min mice. Decrease in serum triglycerides, VLDL-C and LDL-C, and an increase in HDL-C were demonstrated, with elevation of LPL mRNA level and suppression of COX-2 expression. NO-1886 is shown not to have a potential of PPARα and PPARγ agonists (10). It is therefore speculated that LPL activity itself may play an important role in the intestinal polyp formation in *Apc*-deficient mice.

We earlier reported dramatically increased serum levels of triglycerides and markedly low levels of liver and small intestine LPL mRNA in Min mice compared with their wild-type counterparts (3, 4). This data provided concrete evidence that the expression levels of LPL, which catalyzes hydrolysis of triglycerides, correlate with hypertriglyceridemia in Min mice. At present, it still cannot be stated with certainty whether hyperlipidemia is a leading cause of intestinal polyp formation. Colon tumors induced by 1,2-dimethylhydrazine in rats, however, are not linked to serum lipid levels (19). As hyperlipidemia and polyp formation could be related to *Apc*-deficiency independently, we now address whether low LPL activity and high serum lipid levels could promote intestinal polyp formation in these mice.

It has been reported that inflammation-associated enzymes such as COX-2 and iNOS are overexpressed in colon carcinogenesis (18). Treatment with NO-1886 reduced COX-2 expression levels in a reporter gene assay as well as normal parts of the small intestine of Min mice. Immunohistochemically, expression of COX-2 is reported to be observed in normal parts of the small intestine in Min mice (20). Moreover, it is well known that expression of COX-2 is markedly elevated in colon cancers of humans and AOM-treated rats and in intestinal polyps of *Apc*-deficient mice (21–23), playing an important role in cancer cell proliferation and angiogenesis (24). Therefore, down-regulation of COX-2 by NO-1886 is clearly one possible mechanism underlying suppression of intestinal polyp development.

There is evidence that prostaglandin (PG)E_2, produced by COX-1 and COX-2, is a potent inhibitor of LPL expression in macrophages (25). PGE_2 levels are also known to be elevated in both human and rodent colon tumors (26, 27). These findings support the speculation that the role of LPL in intestinal polyp development may be associated with the arachidonic cascade.

In conclusion, this study indicates that the LPL selective inducer, NO-1886, has potential benefits for treatment of both hyperlipidemia and intestinal polyp development. LPL may be a good target for chemoprevention. Thus, NO-1886 and its derivatives are suggested to be promising candidate chemopreventive agents for colon cancer. It is very important to now clarify LPL functions and their significance for cancer development.

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Premature ovarian failure in androgen receptor-deficient mice

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Premature ovarian failure (POF) syndrome, an early decline of ovarian function in women, is frequently associated with X chromosome abnormalities ranging from various Xq deletions to complete loss of one of the X chromosomes. However, the genetic locus responsible for the POF remains unknown, and no candidate gene has been identified. Using the Cre/LoxP system, we have disrupted the mouse X chromosome androgen receptor (*Ar*) gene. Female $AR^{-/-}$ mice appeared normal but developed the POF phenotype with aberrant ovarian gene expression. Eight-week-old female $AR^{-/-}$ mice are fertile, but they have lower follicle numbers and impaired mammary development, and they produce only half of the normal number of pups per litter. Forty-week-old $AR^{-/-}$ mice are infertile because of complete loss of follicles. Genome-wide microarray analysis of mRNA from $AR^{-/-}$ ovaries revealed that a number of major regulators of folliculogenesis were under transcriptional control by AR. Our findings suggest that AR function is required for normal female reproduction, particularly folliculogenesis, and that AR is a potential therapeutic target in POF syndrome.

male hormone | nuclear receptor | female physiology | folliculogenesis | kit ligand

Premature ovarian failure (POF) is defined as an early decline of ovarian function after seemingly normal folliculogenesis (1). Genetic causes of POF have been frequently associated with X chromosome abnormalities (1, 2). Complete loss of one of the X chromosomes, as in Turner syndrome, and various Xq deletions are commonly identified as a cause of POF. However, responsible X-linked genes and their downstream targets have not been identified so far.

The androgen receptor (*Ar*) gene, which is the only sex hormone receptor gene on the X chromosome, is well known to be essential not only for the male reproductive system, but also for male physiology. In contrast, androgens are considered as male hormones; therefore, little is known about androgens' actions in female physiology, although AR expression in growing follicles has been described (3). However, because excessive androgen production in polycystic ovary syndrome causes infertility with abnormal menstrual cycles (4, 5), it is possible that AR-mediated androgen signaling also plays an important physiological role in the female reproductive system. Recently, using Cre/LoxP system, we generated an AR-null mutant mouse line (6) and demonstrated that inactivation of AR resulted in arrest of testicular development and spermatogenesis, impaired brain masculinization, high-turnover osteopenia, and late onset of obesity in males (7–9). At the same time, no overt physical or growth abnormalities were observed in female $AR^{-/-}$ mice. Therefore, to further examine potential role of AR in female physiology, we characterized female reproductive system in $AR^{-/-}$ females. Herein we show that female $AR^{-/-}$ mice develop the POF phenotype. At 3 weeks of age, $AR^{-/-}$ females had apparently normal ovaries with numbers of follicles similar to those in the wild-type females. However, thereafter the number of healthy follicles in the $AR^{-/-}$ ovary gradually declined, with a marked increase of atretic follicles, and by 40 weeks $AR^{-/-}$ mice became infertile, with no follicle detectable in the ovary. Reflecting this age-dependent progression in ovarian abnormality, several genes known to be involved in the oocyte–granulosa cell regulatory loop were identified by microarray analysis as AR downstream target genes. These findings clearly demonstrate that AR-mediated androgen signaling is indispensable for the maintenance of folliculogenesis and implicate impaired androgen signaling as a potential cause of the POF syndrome.

Materials and Methods

Generation of AR Knockout Mice. *AR* genomic clones were isolated from a TT2 embryonic stem cell genomic library by using human *AR* A/B domain cDNA as a probe (6). The targeting vector consisted of a 7.6-kb 5′ region containing exon 1, a 1.3-kb 3′ homologous region, a single loxP site, and a neo cassette with two loxP sites (10). Targeted clones (FB-18 and FC-61) were aggregated with single eight-cell embryos from CD-1 mice (11, 12). Floxed *AR* mice (C57BL/6) were then crossed with CMV-Cre transgenic mice (6). The two lines exhibited the same phenotypic abnormalities. The chromosomal sex of each pup was determined by genomic PCR amplification of the Y chromosome *Sry* gene (13).

Western Blot Analysis. To detect AR protein expression, ovarian cell lysates were separated by SDS/PAGE and transferred onto nitrocellulose membranes (14). Membranes were probed with polyclonal AR antibodies (N-20; Santa Cruz Biotechnology), and blots were visualized by using peroxidase-conjugated second antibody and an ECL detection kit (Amersham Pharmacia Biosciences).

Morphologic Classification of Growing Follicles. Sections were taken at intervals of 30 μm, and 6-μm paraffin-embedded sections were mounted on slides. Routine hematoxylin and eosin staining was performed for histologic examination by light microscopy. Follicle numbers in 12 sections per ovary were evaluated as primary follicles (oocyte surrounded by a single layer of cuboidal granulosa cells), preantral follicles (oocyte surrounded by two or

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Abbreviations: AR, androgen receptor; DHT, 5α-dihydrotestosterone; POF, premature ovarian failure.

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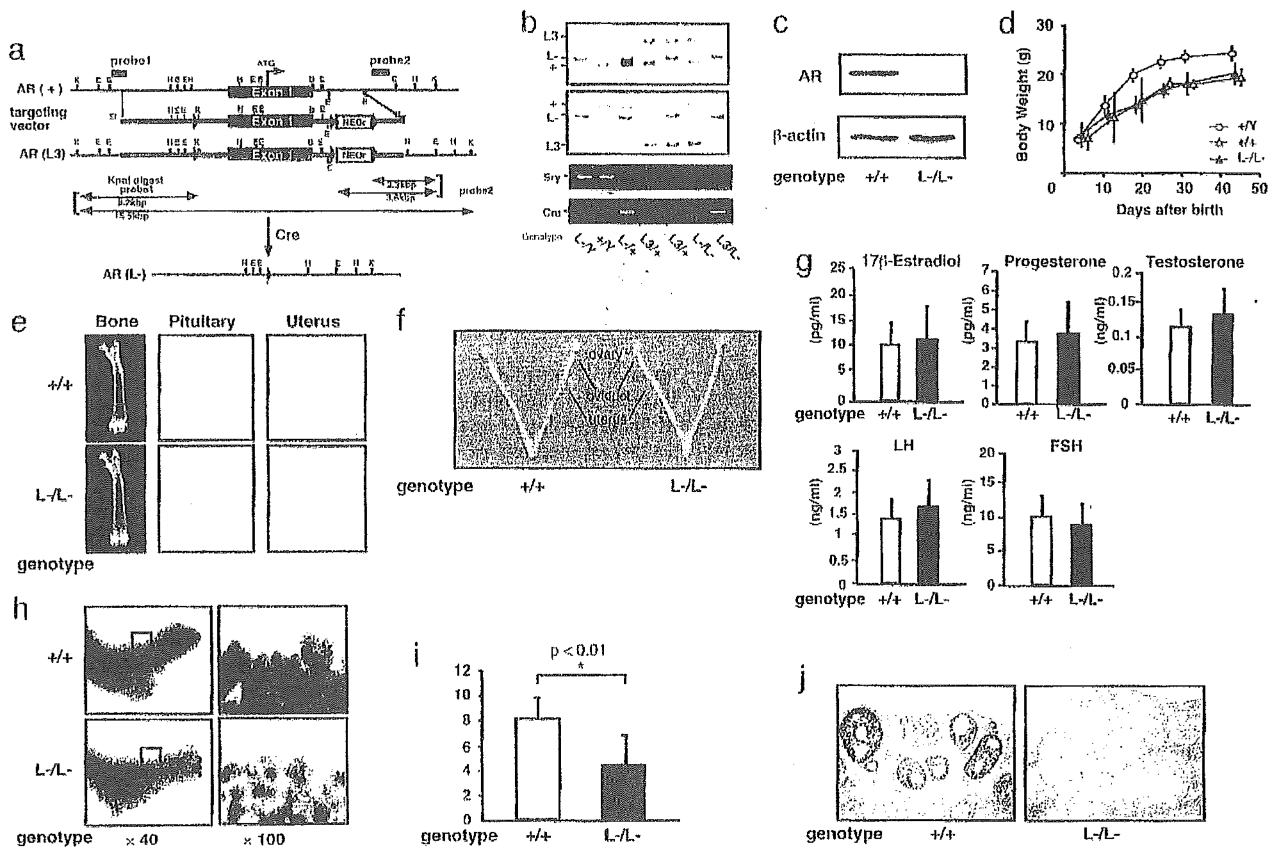

Fig. 1. Phenotypic characterization of AR knockout female mice. (*a*) Diagram of the wild-type *Ar* genomic locus (+), floxed *AR* L3 allele (L3), and *AR* allele (L-) obtained after Cre-mediated excision of exon 1. K, KpnI; E, EcoRI; H, HindIII; B, BamHI. LoxP sites are indicated by arrowheads. The targeting vector consisted of a 7.6-kb 5′ homologous region containing exon 1, a 1.3-kb 3′ homologous region, a single loxP site, and the neo cassette with two loxP sites. (*b*) Detection of the Y chromosome-specific *Sry* gene in $AR^{-/Y}$ mice by PCR. (*c*) Absence of AR protein in $AR^{-/-}$ mice ovaries by Western blot analysis using a specific C-terminal antibody. (*d*) Normal weight gain in $AR^{-/-}$ females. (*e*) Histology of pituitary, uterus, and bone tissues in $AR^{+/+}$ and $AR^{-/-}$ females at 8 weeks of age. (*f*) Female reproductive organs were macroscopically normal in $AR^{-/-}$ mice. (*g*) Serum hormone levels at the proestrus stage in $AR^{-/-}$ mice were not significantly altered. Serum 17β-estradiol, progesterone, testosterone, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) levels in $AR^{+/+}$ ($n = 13$) and $AR^{-/-}$ ($n = 10$) females at 8–10 weeks of age are shown. (*h*) Lobuloalveolar development is impaired in $AR^{-/-}$ mammary glands. Whole mount of inguinal mammary glands (*Left*) and its higher magnification (*Right*) were prepared on day 3 of lactation. (*i*) Average number of pups per litter is markedly reduced in $AR^{-/-}$ mice at 8 weeks of age. Data are shown as mean ± SEM and analyzed by using Student's *t* test. (*j*) AR immunocytochemistry in $AR^{+/+}$ and $AR^{-/-}$ ovaries. Sections were counterstained with eosin.

more layers of granulosa cells with no antrum), or antral follicles (antrum within the granulosa cell layers enclosing the oocyte). Follicles were determined to be atretic if they displayed two or more of the following criteria within a single cross section: more than two pyknotic nuclei, granulosa cells within the antral cavity, granulosa cells pulling away from the basement membrane, or uneven granulosa cell layers (15).

Immunohistochemistry. Sections were subjected to a microwave antigen retrieval technique by boiling in 10 mM citrate buffer (pH 6.0) in a microwave oven for 30 min (16). The cooled sections were incubated in 1% H_2O_2 for 30 min to quench endogenous peroxidase and then incubated with 1% Triton X-100 in PBS for 10 min. To block nonspecific antibody binding, sections were incubated in normal goat serum for 1 h at 4°C. Sections were then incubated with anti-AR (1:100) or anti-cleaved caspase-3 (1:100) in 3% BSA overnight at 4°C. Negative controls were incubated in 3% BSA without primary antibody. The ABC method was used to visualize signals according to the manufacturer's instructions. Sections were incubated in biotinylated goat anti-rabbit IgG (1:200 dilution) for 2 h at room temperature, washed with PBS, and incubated in avidin–biotin–horseradish peroxidase for 1 h. After thorough washing in PBS, sections were developed with 3,3′-diaminobendizine tetrahydrochloride substrate, slightly counterstained with eosin, dehydrated through an ethanol series and xylene, and mounted.

Estrus Cycles and Fertility Test. To determine the stage of the estrus cycle (proestrus, estrus, and diestrus), vaginal smears were taken every morning and stained with Giemsa solution. For evaluation of female fertility for 15 weeks, an 8- or 24-week-old wild-type or $AR^{-/-}$ female was mated with a wild-type fertile male, replaced every 2 weeks with the other fertile male. Cages were monitored daily and for an additional 23 days, and the presence of seminal plugs and number of litters were recorded.

RNA Extraction and Quantitative Competitive RT-PCR. Total ovarian RNA was extracted by using TRIzol (Invitrogen) (16). Oligo-dT-primed cDNA was synthesized from 1 μg of ovarian RNA by using SuperScript reverse transcriptase (Gibco BRL, Gaithersburg, MD) in a 20-μl reaction volume, 1 μl of which was then diluted serially (2- to 128-fold) and used to PCR-amplify an internal control gene, *cycA*, to allow concentration estimation.

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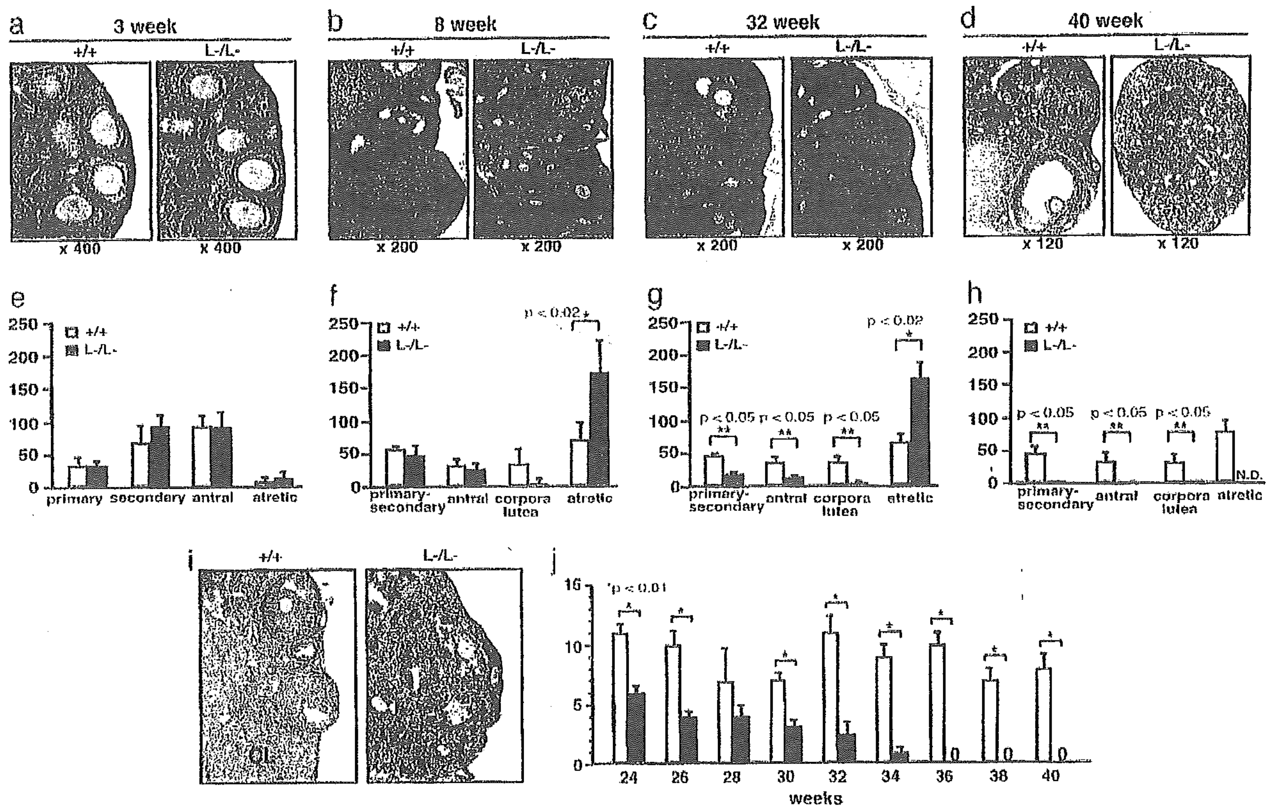

Fig. 2. POF in $AR^{-/-}$ female mice. (a–d) Histology of $AR^{+/+}$ and $AR^{-/-}$ ovaries at 3 weeks, 8 weeks, 32 weeks, and 40 weeks of age. All sections were stained with hematoxylin and eosin. An asterisk marks the atretic follicle. CL, corpus luteum. (e–h) Relative follicle counts at 3 weeks (e), 8 weeks (f), 32 weeks (g), and 40 weeks (h) of age. Numbers represent total counts of every fifth section from serially sectioned ovaries ($n = 4$ animals per genotype). (i) Immunohistochemical study for activated, cleaved caspase-3 revealed increased positive cells (apoptotic cells) in $AR^{-/-}$ ovaries. Sections were counterstained with hematoxylin. An asterisk marks the caspase-3-positive cell. CL, corpus luteum. (j) Age-dependent reduction in the number of pups per litter in $AR^{-/-}$ female mice. A continuous breeding assay was started at 24 weeks of age ($n = 6$–10 animals per genotype). For all panels, data are shown as mean ± SEM and were analyzed by using Student's t test.

Primers were designed from cDNA sequences of *Kitl* (M57647; nucleotides 1099–1751), Gdf9 (NM008110; nucleotides 720–1532), *Bmp15* (NM009757; nucleotides 146–973), *Ers2* (NM010157; nucleotides 1139–1921), *Pgr* (NM008829; nucleotides 1587–2425), *Cyp11a1* (NM019779; nucleotides 761–1697), *Cyp17a1* (M64863; nucleotides 522–932), *Cyp19* (D00659; nucleotides 699–1049), *Fshr* (AF095642; nucleotides 625–1427), *Lhr* (M81310; nucleotides 592–1331), *Ptgs2* (AF338730; nucleotides 3–605), and *Ccnd2* (NM009829; nucleotides 150–1065) and chosen from different exons to avoid amplification from genomic DNA.

GeneChip Analysis. Ovaries were isolated and stabilized in RNA*later* RNA Stabilization Reagent (Ambion, Austin, TX) before RNA purification (17). Total RNA was purified by using an RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. First-strand cDNA was synthesized from 5 μg of RNA by using 200 units of SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA), 100 pmol T7-$(dT)_{24}$ primer [5′-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG-$(dT)_{24}$-3′], 1× first-strand buffer, and 0.5 mM dNTPs at 42°C for 1 h. Second-strand synthesis was performed by incubating first-strand cDNA with 10 units of *Escherichia coli* ligase (Invitrogen), 40 units of DNA polymerase I (Invitrogen), 2 units of RNase H (Invitrogen), 1× reaction buffer, and 0.2 mM dNTPs at 16°C for 2 h, followed by 10 units of T4 DNA polymerase (Invitrogen) and incubation for another 5 min at 16°C. Double-stranded cDNA was purified by using GeneChip Sample Cleanup Module (Affymetrix, Santa Clara, CA) according to the manufacturer's instructions and labeled by *in vitro* transcription by using a BioArray HighYield RNA transcript labeling kit (Enzo Diagnostics, Farmingdale, NY). Briefly, dsDNA was mixed with 1× HY reaction buffer, 1× biotin-labeled ribonucleotides (NTPs with Bio-UTP and Bio-CTP), 1× DTT, 1× RNase inhibitor mix, and 1× T7 RNA polymerase and incubated at 37°C for 4 h. Labeled cRNA was then purified by using GeneChip Sample Cleanup Module and fragmented in 1× fragmentation buffer at 94°C for 35 min. For hybridization to the GeneChip Mouse Expression Array 430A or 430B or Mouse Genome 430 2.0 Array (Affymetrix), 15 μg of fragmented cRNA probe was incubated with 50 pM control oligonucleotide B2, 1× eukaryotic hybridization control, 0.1 mg/ml herring sperm DNA, 0.5 mg/ml acetylated BSA, and 1× hybridization buffer in a 45°C rotisserie oven for 16 h. Washing and staining were performed by using a GeneChip Fluidic Station (Affymetrix) according to the manufacturer's protocol. Phycoerythrin-stained arrays were scanned as digital image files and analyzed with GENECHIP OPERATING SOFTWARE (Affymetrix) (17).

Luciferase Assay. The *Kitl* promoter region (−2866 to −1 bp) was inserted into the pGL3-basic vector (Promega) for assay using the Luciferase Assay System (Promega) (14, 16). Cells at 40–50% confluence were transfected with a reference pRL-CMV

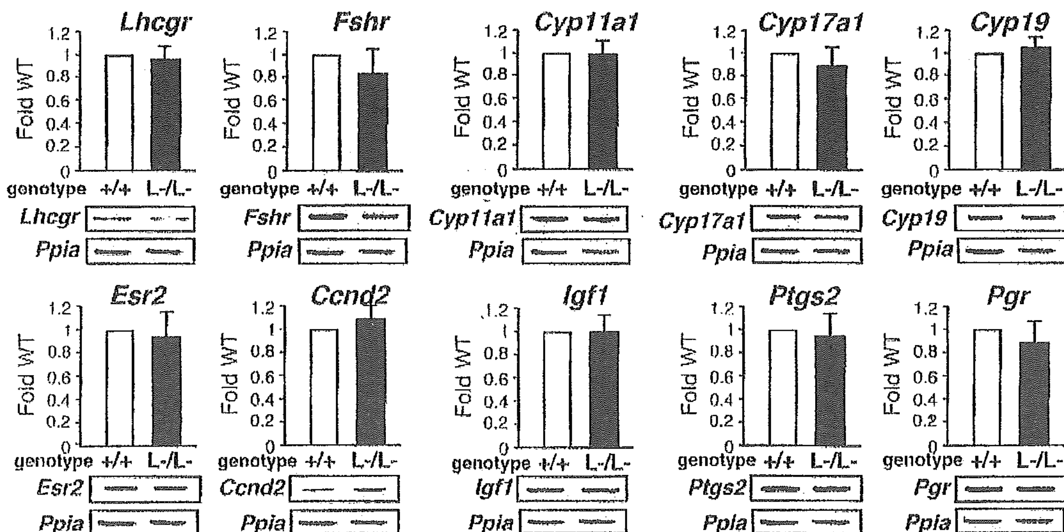

Fig. 3. No significant alterations in mRNA levels of several major regulators in folliculogenesis. Shown is semiquantitative RT-PCR of LH receptor (*Lhr*), FSH receptor (*Fshr*), p450 side chain cleavage enzyme (*Cyp11a1*), 17-α-hydroxylase (*Cyp17a1*), Aromatase (*Cyp19*), estrogen receptor-β (*Esr2*), cyclin D2 (*Ccnd2*), insulin-like growth factor 1 (*Igf1*), cyclooxygenase 2 (*Ptgs2*), or progesterone receptor (*Pgr*) gene expression in $AR^{+/+}$ and $AR^{-/-}$ ovaries. Results shown were representative (using one ovary per genotype in each experiment) of five independent experiments.

plasmid (Promega) using Lipofectamine reagent (GIBCO/BRL, Grand Island, NY) to normalize transfection. Results shown are representative of five independent experiments.

Results and Discussion

Subfertility of $AR^{-/-}$ Female Mice at 8 Weeks of Age. The *Ar* gene located on the X chromosome was disrupted in mice by using the Cre/Lox P system (6) (Fig. 1 *a–c*). Female $AR^{-/-}$ mice showed normal growth compared with the wild-type littermates (Fig. 1*d*), with no detectable bone loss (Fig. 1*e*) or obesity common for male $AR^{-/Y}$ mice (8, 9). Young (8-week-old) $AR^{-/-}$ females appeared indistinguishable from the wild-type littermates, displayed normal sexual behavior (7), and produced the first offspring of normal body size at the expected age. Macroscopic appearance of their reproductive organs, including uteri, oviducts, and ovaries, also appeared normal (Fig. 1*f*). Histological analysis showed no significant abnormality in the uterus or pituitary (Fig. 1*e*), whereas mammary ductal branching and elongation were substantially reduced, as revealed by whole-mount analysis (Fig. 1*h*). Serum levels of 17β-estradiol, progesterone, testosterone, luteinizing hormone, and follicle-stimulating hormone were also within normal range in 8-week-old mutant females at the proestrus stage (Fig. 1*g*), suggesting that the two-cell two-gonadotrophin system in female reproductive and endocrine organs (18) was intact in $AR^{-/-}$ mice at 8 weeks of age. The most obvious early sign of abnormal reproductive function in the $AR^{-/-}$ females was that their average numbers of pups per litter were only about half of those of the wild-type littermates, ($AR^{+/-}$, 8.3 ± 0.4 pups per litter; $AR^{-/-}$, 4.5 ± 0.5 pups per litter) (Fig. 1*i*).

$AR^{-/-}$ Female Mice Developed POF Phenotypes. Histological analysis of 8-week-old $AR^{-/-}$ ovaries clearly showed that numbers of atretic follicles were significantly increased, with decreased numbers of corpora lutea (Fig. 2 *b* and *f*). This finding suggests that the reduced pup numbers were due to impaired folliculogenesis in AR-deficient ovaries. Indeed, AR protein expression was readily detectable in the wild-type 8-week-old ovaries (Fig. 1*j*), with AR expressed at the highest levels in growing follicle granulosa cells at all developmental stages and at relatively low levels in corpora lutea. Thus, AR appears to play a regulatory role in granulosa cells during their maturation to the luteal phase.

To investigate this possibility, we examined the ovarian phenotype of female $AR^{-/-}$ mice at different ages. At 3 weeks, ovaries contain various stages of follicles, including primary, secondary, and antral follicles in wild-type animals (Fig. 2*a*) (19). In $AR^{-/-}$ ovaries at 3 weeks of age, the folliculogenesis appeared to be unaltered, with normal numbers and localization of primary and secondary follicles (Fig. 2 *a* and *e*). However, degenerated folliculogenesis became evident with further aging. Although follicles and corpora lutea at all developmental stages were still present, corpora lutea numbers were clearly reduced in 8-week-old $AR^{-/-}$ mutants (Fig. 2 *b* and *f*), similar to that observed in another mouse line (20). Expected apoptosis was seen in atretic follicles by activated caspase-3 immunohistochemistry assays (Fig. 2*i*). But, by 32 weeks of age, defects in folliculogenesis in $AR^{-/-}$ ovaries became profound, with fewer follicles observed and increased atretic follicles (Fig. 2 *c* and *g*), and >40% (5 of 12 mice) of the $AR^{-/-}$ females were already infertile. By 40 weeks, all $AR^{-/-}$ females became infertile, with no follicles remaining (Fig. 2 *d* and *h*); at the same age, $AR^{+/+}$ females were fertile and had normal follicle numbers. Consistent with progressive deficiency in folliculogenesis, the pup number per litter steadily decreased in aging $AR^{-/-}$ females (Fig. 2*i*). These data indicate that AR plays an important physiological role at the preluteal phase of folliculogenesis.

Alteration in Gene Expressions of Several Major Regulators Involved in the Oocyte–Granulosa Cell Regulatory Loop. To explore the molecular basis underlying the impaired folliculogenesis in $AR^{-/-}$ ovaries, we analyzed expression of several major known regulators and markers of folliculogenesis (21–23). Surprisingly, no significant alterations in mRNA levels of LH receptor (*Lhr*), FSH receptor (*Fshr*), p450 side chain cleavage enzyme (*Cyp11a1*), 17-α-hydroxylase (*Cyp17a1*), aromatase (*Cyp19*), estrogen receptor-β (*Esr2*), cyclin D2 (*Ccnd2*), or insulin-like growth factor 1 (*Igf1*) of 8-week-old $AR^{-/-}$ ovaries at the proestrus stage, and further cyclooxygenase 2 (*Ptgs2*) or progesterone receptor (*Pgr*) at the estrus stage, were detected by

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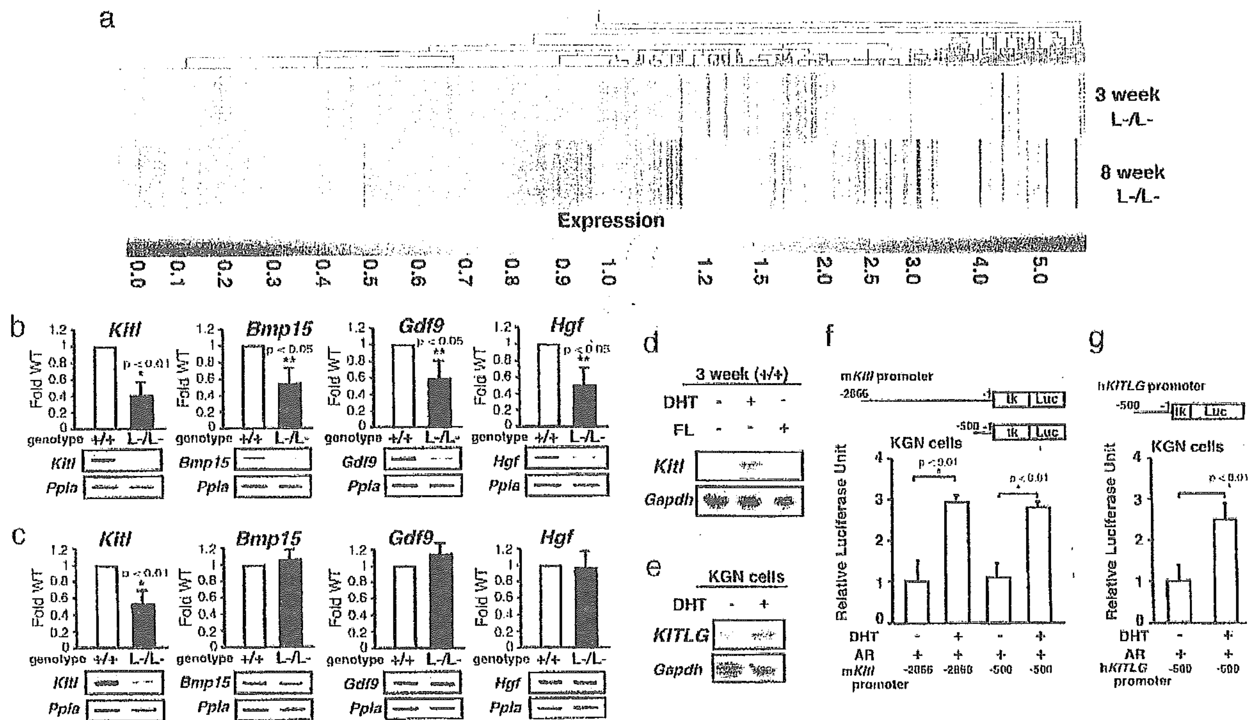

Fig. 4. Genome-wide microarray analysis and semiquantitative RT-PCR revealed that expression of the oocyte–granulosa cell regulator loop was down-regulated in $AR^{-/-}$ ovaries. (*a*) Microarray analysis of $AR^{-/-}$ compared with $AR^{+/+}$ ovaries at 3 and 8 weeks of age. Data obtained from microarray analysis as described in *Materials and Methods* were used to generate a cluster analysis. Each vertical line represents a single gene. The ratios of gene expression levels in $AR^{-/-}$ ovaries compared with wild type are presented. (*b* and *c*) Semiquantitative RT-PCR analysis of AR-regulated genes identified from the microarray study. Results shown are representative (using one ovary per genotype in each experiment) of five independent experiments. Data are shown as mean ± SEM and were analyzed by using Student's *t* test. (*d*) Comparison of *Kitl* gene expression by Northern blot analysis among placebo-, DHT-, and flutamide (FL)-treated $AR^{+/+}$ mouse ovaries. (*e*) Induction of *KITLG* gene expression by DHT treatment in KGN cells. (*f* and *g*) Androgen responsiveness in the mouse and human *kit ligand* promoters by a luciferase assay performed by using KGN cells. Data are shown as mean ± SEM and were analyzed by using Student's *t* test.

semiquantitative RT-PCR analysis (Fig. 3). Genome-wide microarray analysis (17) of RNA from 8-week-old $AR^{-/-}$ ovaries at the proestrus stage has been undertaken to identify AR-regulated genes. In comparison with $AR^{+/+}$ ovaries, expressions of 772 genes were down-regulated, whereas 351 genes were up-regulated in $AR^{-/-}$ ovaries (Fig. 4*a*; see also Tables 1 and 2, which are published as supporting information on the PNAS web site). Several genes known to be involved in the oocyte–granulosa cell regulatory loop (24) were identified as candidate AR target genes, including KIT ligand (*Kitl*) (25), morphogenetic protein 15 (*Bmp15*) (26), growth differentiation factor-9 (*Gdf9*) (27), and hepatocyte growth factor (*Hgf*) (28). Impaired folliculogenesis had been reported in mice deficient in each of these three regulators (26, 27, 29). To validate the microarray data, we performed semiquantitative RT-PCR analysis of 8-week-old $AR^{-/-}$ ovary RNA and confirmed that expression of these factors was down-regulated (Fig. 4*b*). To identify a regulator downstream of the AR signaling at an earlier stage of folliculogenesis, 3-week-old $AR^{-/-}$ ovaries that, as pointed out earlier, display no apparent phenotypic abnormality were examined. Fewer genes had altered expression levels (519 genes up-regulated; 326 genes down-regulated) (Fig. 4*a*; see also Tables 3 and 4, which are published as supporting information on the PNAS web site), and, of the four regulators tested by RT-PCR, only *Kitl* was found to be down-regulated at this age (Fig. 4*c*). Because *Kitl* is a granulosa cell-derived factor and stimulates oocyte growth and maturation (29–31), down-regulation of the *Kitl* expression in 3-week-old or even younger $AR^{-/-}$ ovaries may trigger impairment in folliculogenesis at a later age. To test for possible *Kitl* gene regulation by AR, 3-week-old wild-type females were treated with 5α-dihydrotestosterone (DHT). At 4 h after hormone injection, a clear induction of *Kitl* expression was observed in the ovaries, whereas a known antiandrogen flutamide attenuated the induction by DHT (Fig. 4*d*). The induction of endogenous human *kit ligand* (*KITLG*) gene by DHT was also observed in human granulosa-like tumor cells (KGN) in culture (Fig. 4*e*). Furthermore, androgen-induced transactivation of mouse and human *kit ligand* promoters (32) was observed by a luciferase reporter assay (33) in KGN (Fig. 4 *f* and *g*), 293T, and HeLa (data not shown) cells. However, no response to DHT was detected in the similar assay using promoters of the *Bmp15*, *Gdf9*, and *Hgf* genes (data not shown). Thus, we have shown that, in a regulatory cascade controlling folliculogenesis, *Kitl* represents a direct downstream target of androgen signaling.

As an upstream regulator, AR may also be indirectly involved in control of expression of other genes critical for folliculogenesis, because an age-dependent down-regulation of *Bmp15*, *Gdf9*, and *Hgf* gene expression was also observed in $AR^{-/-}$ ovaries. *Bmp15* and *Gdf9* are oocyte-derived factors that promote the development of surrounding granulosa cells in growing follicles (34, 35), whereas *Hgf* is secreted by theca cells and acts as a granulosa cell growth factor (36). Down-regulation of these factors, presumably due to decreased *Kitl* expression, may lead to impaired bidirectional communication between oocyte and granulosa cells (24) and, eventually, to early termination of folliculogenesis, as in POF syndrome.

Thus, we have identified AR as a novel regulator of follicu-

logenesis that apparently acts in the regulatory cascade upstream of the major factors controlling ovarian function, confirming the previous findings of the AR expression in granulose cells of growing follicles (3). Although not immediately relevant to the ovarian physiology, abnormal development of the mammary glands observed in our AR-deficient mice adds further strong evidence of an essential role of the AR not only in male, but also in female, reproductive function.

With increasing age of the first childbirth by women in the modern society, POF syndrome has become an important social and medical problem. Our findings suggest that POF syndrome may be caused by an impairment in androgen signaling and that X chromosomal mutations affecting the *AR* gene function may play a key role in hereditary POF. From clinical perspective, the present study provides evidence that AR can be a beneficial therapeutic target in treatment of POF syndrome patients.

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