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A Long Noncoding RNA, *lncRNA-Amhr2*, Plays a Role in *Amhr2* Gene Activation in Mouse Ovarian Granulosa Cells

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Précis: A 500-bp promoter was insufficient to mouse *Amhr2* gene activation, and a novel long noncoding RNA, *lncRNA-Amhr2*, contributes to the activation by enhancing promoter activity in granulosa cells.

Abbreviations: AMH, anti-Müllerian hormone; Amhr2, Anti-Müllerian hormone type II receptor; ANOVA, analysis of variance; BCA, bicinchoninic acid; CNS, conserved noncoding sequence; DMEM, Dulbecco Modified Eagle medium; Dox, doxycycline; EGFP, enhanced green fluorescent protein; ER, estrogen receptor; FBS, fetal bovine serum; lncRNA, long noncoding RNA; PCR, polymerase chain reaction; POP, prolyl oligopeptidase; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription-polymerase chain reaction; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; S.D., standard deviation; TSS, transcriptional start site; TTS, transcriptional termination site

Abstract

Anti-Müllerian hormone (AMH) is critical to the regression of Müllerian ducts during mammalian male differentiation and targets ovarian granulosa cells and testicular Sertoli and Leydig cells of adults. Specific effects of AMH are exerted via its receptor, AMH type II receptor (Amhr2), but the mechanism by which the *Amhr2* gene is specifically activated is not fully understood. To see whether a proximal promoter was sufficient to *Amhr2* gene activation, we generated transgenic mice that bore the enhanced green fluorescent protein (EGFP) gene driven by a 500-bp mouse *Amhr2* gene promoter. None of the established 10 lines, however, showed appropriate EGFP expression, indicating that the 500-bp promoter was insufficient to *Amhr2* gene activation. As a novel regulatory element, we found a long noncoding RNA, *lncRNA-Amhr2*, transcribed from upstream of the *Amhr2* gene in ovarian granulosa cells and testicular Sertoli cells. In primary granulosa cells, knock down of *lncRNA-Amhr2* resulted in a decrease of *Amhr2* mRNA level, and transient reporter gene assay showed that *lncRNA-Amhr2* activation increased *Amhr2* promoter activity. The activity was correlated with *lncRNA-Amhr2* transcription in stably transfected OV3121 cells that were derived from mouse granulosa cells. Moreover, by the Tet-on system, the induction of *lncRNA-Amhr2* transcription dramatically increased *Amhr2* promoter activity in OV3121 cells. These results indicate that *lncRNA-Amhr2* plays a role in *Amhr2* gene activation in ovarian granulosa cells by enhancing promoter activity, providing a novel insight into the *Amhr2* gene regulation underlying the AMH signaling in the female reproductive system.

Introduction

Anti-Müllerian hormone (AMH) is a glycoprotein which induces the regression of Müllerian ducts during male differentiation in embryos (1, 2) and controls the folliculogenesis and Leydig cell proliferation in the adult ovary and testis (3, 4). Its actions are mediated by type I and type II receptors that form a heterodimer, leading to activation of the Smad signaling pathway (5). Since the type I receptor is an activin-like kinase which is expressed in various tissues (5), it is the AMH type II receptor (Amhr2) that determines the specificity of AMH actions. In mammals, the *Amhr2* gene is highly expressed in embryonic and postnatal gonads, especially in ovarian granulosa cells and testicular Sertoli cells (6-9), although Leydig cells also express it (10, 11). Such specific activation of the *Amhr2* gene is essential to sex differentiation and adult reproductive functions (12).

In the adult ovary, AMH is secreted from granulosa cells of preantral follicles, and exhibits inhibitory effects on the entry of primordial follicles into the growing phase and on the sensitivity of antral follicles to follicle stimulating hormone (4, 13). When the AMH signaling is disturbed, the ovulation ceases at a younger age than usual (14). Moreover, in human, AMH is tightly associated with some ovarian dysfunction such as polycystic ovarian syndrome and premature ovarian insufficiency (4, 13), and the defect is caused by genetic mutations in the gene encoding AMH or its receptor (4) or by the aberrant regulation of *AMH* and *AMHR2* without genetic mutations (15). These findings, combined with the fact that the *AMH* gene is simply controlled by its promoter (16-18), and that Amhr2 is a major determinant of AMH actions, clearly underscore the importance of the regulatory mechanism of the *Amhr2* gene expression. Nevertheless, how the *Amhr2* gene is controlled is not fully understood.

The regulatory mechanism of the *AMHR2* gene was first reported in human. The binding of a transcription factor, SF-1, to the *AMHR2* proximal promoter and a synergistic effect of the β-catenin signaling pathway were shown to be important for gene activation (19, 20). A recent study showed that bone morphogenetic protein 15 could also enhance *AMHR2* promoter activity in granulosa cells (21).

In a rat Leydig cell line, a 1.6-kb *Amhr2* promoter exhibited high activity, and two SF-1 binding sites in the proximal region were critical (22). In mouse, a transcription factor, WT-1, binds to the *Amhr2* proximal promoter and plays a crucial role in gene activation during development (23). The chicken *AMHR2* gene was recently cloned, and found to be positively and negatively regulated by the *DMRT1* gene and estrogen, respectively (24). While all of these studies focused on the proximal promoter, it is not known whether the promoter is really sufficient to specific activation of the *Amhr2* gene and whether any other regulatory elements exist.

It has become clear that many genes require regulatory elements other than proximal promoters, such as distal enhancers and long noncoding RNAs (lncRNAs), to be fully and specifically activated (25-27). Especially, lncRNAs have recently received much attention due to their variability and abundance (28). lncRNAs are defined as RNAs that are longer than 200 nucleotides and function as RNA molecules without being translated (28). In many cases, lncRNA transcripts are important for their functions, but at some loci, noncoding transcription *per se* is effective independently of the resulting transcripts (29, 30). An increasing number of studies have shown that lncRNAs are key regulators in many biological fields, such as immunology, development, and neurobiology, by functioning through gene regulation at various levels (i.e. transcription, RNA stability, RNA degradation, and translation) (31-35). In the field of endocrinology, lncRNAs are known to participate in the hormonal control (36-41) and regulate the genes encoding hormones and hormone receptors (30, 42-45). Therefore, more lncRNAs are presumed to be associated with hormonal control or regulation of hormone/hormone receptor gene expression.

In this study, we assessed whether a proximal promoter was sufficient to *Amhr2* gene activation by generating transgenic mice, and found that a 500-bp promoter was insufficient to specific gene activation. We then identified a novel lncRNA, *lncRNA-Amhr2*, which was transcribed in correlation with *Amhr2*. By knock down and a series of experiments using reporter genes, *lncRNA-Amhr2* was found to enhance *Amhr2* promoter activity in ovarian granulosa cells. The results provide a novel insight into the *Amhr2* gene regulation.

Materials and Methods

Animals

C57/B6 and BDF1 (C57/B6 × DBA2 F1) mice were maintained at 25°C with a photoperiod of 14 hours light and 10 hours dark. Eight tissues (brain, heart, liver, kidney, ovary, spleen, testis, and uterus) were collected from male and/or female mice. Food and water were freely accessed. Experimental procedures used in this study were approved by the Institutional Animal Use and Care Committee at Hokkaido University.

Plasmid constructs

0.5k-EGFP: A 500-bp sequence of the mouse *Amhr2* promoter was amplified by polymerase chain reaction (PCR) with mouse genome DNA and KOD polymerase (Toyobo, Osaka, Japan) using a primer pair of 5'-CTACACAGAGAAACCCTGTC-3' and 5'-CCTCAGCCAAGGCTTCCTA-3'. The product was subcloned into a pBluescript II vector (Stratagene, La Jolla, CA, USA) at the *Eco*RV site, and the resulting plasmid was checked by DNA sequencing method. This plasmid was digested with *Hin*dIII and *Eco*RI, and a 0.5-kb fragment was blunted and inserted into a pEGFP-1 vector (Clontech, Palo Alto, CA, USA) at the blunted *Bam*HI site.

sh-lncAmhr2: A double strand DNA was generated by annealing 5'-GATCCGCAGAAAGCATGTCCATATGAGTGTGCTGTCCTCATATGGACATGCTTTCTGCTTTTTTA-3' and 5'-AGCTTAAAAAAGCAGAAAGCATGTCCATATGAGGACAGCACACTCATATGGACATGCTTTCTGCG-3' oligonucleotides. The DNA was inserted into a pBAsi-mU6 Neo DNA vector (Takara, Kusatsu, Japan) at *Bam*HI and *Hin*dIII sites. The construct was checked by DNA sequencing method.

0.5k-Luc: A 500-bp *Amhr2* promoter sequence was obtained as above and subcloned into a

pGL3-Basic vector (Promega, Madison, WI, USA) at the blunted *Hin*dIII site.

6.1k-Luc: 0.5k-Luc was digested with *Bgl*II, and a 607-bp fragment, which was obtained by digestion of a B6N mouse BAC clone, B6Ng01-240N22, (RIKEN Bioresource Center) with *Bgl*II, was inserted. The resulting vector was named 1.0k-Luc. A 6.0-kb fragment was obtained by digestion of B6Ng01-240N22 with *Eco*RI and subcloned into a pBluescript II vector at the *Eco*RI site. The plasmid was digested with *Eco*RI and *Nhe*I, and a 5126-bp fragment was inserted into 1.0k-Luc at the *Eco*RI-*Nhe*I site.

6.1k-toER-Luc: A 1.8-kb cDNA sequence of torazame estrogen receptor (ER) was subcloned into a pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA) (46). The plasmid was digested with *Eco*RI, and the 1.8-kb ER fragment was purified, blunted, and inserted into 6.1k-Luc which was digested with *Afl*II and *Tth*111I and blunted.

6.1k-ΔlncPr-Luc: 6.1k-Luc was digested with *Eco*RV and *Tth*111I, blunted, and self-ligated.

6.1k-POPpr-Luc: A 914-bp mouse prolyl oligopeptidase (POP) gene promoter was subcloned into a pEGFP-1 vector (Clontech) (47, 48). The plasmid was digested with *Bam*HI and *Eco*RI, and the POP promoter was purified, blunted, and inserted into 6.1k-Luc which was digested with *Eco*RV and *Tth*111I and blunted.

RevPOPpr-Luc and POPpr-Luc: The blunted 914-bp POP promoter prepared as above was inserted into pGL3-Basic which was digested with *Sma*I.

RevPOPpr-0.5k-Luc and POPpr-0.5k-Luc: The blunted 914-bp POP promoter prepared as above was inserted into 0.5k-Luc which was digested with *Sma*I.

6.1k-POPpr-toER-Luc: The blunted 914-bp POP promoter prepared as above was inserted into pcDNA3.1 containing toER cDNA which was digested with *Bam*HI and blunted. The resulting plasmid was digested with *Not*I and *Kpn*I, and a 2.7-kb fragment was purified, blunted, and inserted into 6.1k-Luc which was digested with *Eco*RV and *Afl*II and blunted.

6.1k-EGFP: A 1130-bp *Amhr2* promoter was amplified by PCR with mouse genome DNA and KOD FX Neo (Toyobo) using a primer pair of 5’-CTCTCCATCCCAAGTTGGTT-3’ and

5’-CCTCAGCCAAGGCTTCCTA-3’, and digested with *Kpn*I. The resulting 0.8-kb fragment was subcloned into a pEGFP-1 vector (Clontech) at the *Kpn*I-*Sma*I site, and the plasmid was named 0.8k-EGFP. 6.1k-Luc was digested with *Kpn*I, and a 5.3-kb fragment was purified and inserted into 0.8k-EGFP at the *Kpn*I site.

6.1k-toER-EGFP: 6.1k-toER-Luc was digested with *Kpn*I, and a 5.3-kb fragment was purified and inserted into 0.8k-EGFP at the *Kpn*I site.

6.1k-POPpr-EGFP: 6.1k-POPpr-Luc was digested with *Kpn*I, and a 5.3-kb fragment was purified and inserted into 0.8k-EGFP at the *Kpn*I site.

6.1k-TO-Luc: A 459-bp hCMV*1 promoter sequence, which was responsive to doxycycline (Dox), was obtained by digestion of the pPBhCMV*1-cHA-pA plasmid (49) with *Sac*II and *Acc*I. The hCMV*1 promoter was blunted and inserted into 6.1k-Luc at the blunted *Tth*111I site.

Transgenic mouse generation

Transgenic mice were generated as previously described (48). The 0.5k-EGFP plasmid was digested with *Xho*I and *Afl*II and purified to exclude the vector sequence, and injected into fertilized eggs. 12 founders were obtained by dot blot analysis of tail DNAs with an EGFP fragment (772 bp) as a probe. The probe was prepared by digestion of pEGFP-1 vector with *Eco*RI and *Not*I. All founders were mated with wild type mice and 10 lines were established. Copy numbers of the established lines were determined by Southern blot analysis with genome DNAs digested with *Dra*I and *Pvu*II using an *Eco*RI-*Not*I EGFP fragment as a probe. A 422-bp probe, which was amplified from the *Amhr2* gene body by genome PCR using primers of 5’-ACATCTTCTCCAGGCTGGCA-3’ and 5’-CCAAGTGGATGGGATGTTG-3’, was used as a control of two copies. The procedure of Southern blot was previously described (50).

Reverse transcription-PCR (RT-PCR) and quantitative RT-PCR (qRT-PCR)

RNA isolation, DNase treatment, and reverse transcription followed by PCR or real time PCR

were performed as previously described (50-53). Reverse transcription was performed with a specific primer for *lncRNA-Amhr2* in Figure 2A and with the oligo(dT) primer in the other experiments. In real time PCR, detection of *lncRNA-Amhr2* was done with KOD SYBR qPCR Mix (Toyobo), and for the other transcripts, Power SYBR Green Master Mix (Life Technologies, Foster City, CA, USA) was used. Primer sequences for these experiments are listed in Table 1.

Cell sorting

The tunica albuginea was removed from testes of adult #326 mice and placed in Dulbecco Modified Eagle medium (DMEM) containing 0.5 mg/ml collagenase. The seminiferous tubules were cut into small pieces and incubated at 33˚C in water bath for 30 min with occasional agitation. The cells were dispersed by gentle pipetting, and filtrated through a cell strainer tube (falcon 352235) to remove seminiferous tubules. The cells were separated by EGFP intensity, and the EGFP-positive cells were sorted by JSAN cell sorter (Bay Bioscience, Kobe, Japan). OV3121 cells transfected with 6.1k-EGFP were also sorted into EGFP-positive and negative population.

Isolation and culture of primary Sertoli and granulosa cells

Primary Sertoli cells were isolated from 8-12 days old male mice and cultured on collagen-coated dishes as previously described (54). Briefly, testes were decapsulated and treated with 0.1% collagenase at 32˚C twice for 15-20 min, and the sedimented tubules were washed with phosphate buffered saline containing 1 mM EDTA three times. The tubules were cut into small pieces, and germ cells and Sertoli cells were dispersed by pipetting them. The dispersed cells were washed with DMEM/F12 supplemented with 10% fetal bovine serum (FBS) three times, and spread onto 24-well dishes that were coated with collagen. After cultured for 1 day at 32˚C, the cells were treated with 10 mM Tris-HCl (pH 7.4) for 3 min to eliminate germ cells, and the attached Sertoli cells were cultured for several days.

Primary granulosa cells were isolated from immature female mice that were administrated with

hormones and cultured in fibronectin-coated dishes as previously described (51). Briefly, 3-4 weeks old female mice were administrated with pregnant mare serum gonadotropin and human chorionic gonadotropin to induce follicular growth. Five hours after the injection of human chorionic gonadotropin, ovaries were collected, and follicles were punctured by needles in DMEM/F12 with 10% FBS. The cells were washed with the medium three times and spread onto 24-well dishes that were coated with fibronectin. They were cultured at 37˚C for several days.

Fractionation into cytoplasmic and nuclear fractions

Fractionation of primary granulosa cells into cytoplasmic and nuclear fractions followed by RT-PCR analysis was performed as previously described (52).

Rapid amplification of cDNA ends (RACE)

5'RACE and 3' RACE were performed with total RNAs from ovarian granulosa cells and the testis as previously described (52, 53). The first PCR product was diluted to 1:1000 and used for the second PCR. Ten subclones for each RACE product were checked by DNA sequencing method. Primers used for RACE analyses are listed in Table 2.

Knock down of *lncRNA-Amhr2*

sh-lncAmhr2 or pBAsi-mU6 Neo DNA plasmids were transfected into primary granulosa cells using GeneJuice transfection reagent (Merck, Darmstadt, Germany) according to the manufacturer's instruction. Two days later, 1 mg/ml G418 was added to the medium and the cells were cultured for another 4 days. Then, total RNAs were purified with ISOGEN II (Nippongene, Tokyo, Japan), and RNA levels were assessed by qRT-PCR.

Reporter gene assay by transient transfection

Constructs containing the luciferase gene were transfected into primary granulosa or Sertoli cells

with a pRL-CMV vector (Promega) using GeneJuice (Merck) or Genefectine (Genetrone Biotech, Seoul, South Korea) transfection reagents. Two days later, the cells were lysed and firefly luciferase activity was measured as previously described (51). Transfection efficiency was normalized by measuring *Renilla* luciferase activity that was derived from pRL-CMV.

Culture and establishment of OV3121 cells stably transfected with pEGFP plasmids

OV3121 cells derived from mouse granulosa cells were obtained from Japanese Collection of Research Bioresources cell bank (National Institutes of Biomedical Innovation, Health and Nutrition), and cultured in DMEM supplemented with 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 292 μg/ml L-glutamine. 0.5k-EGFP was linearized by digestion with *Xho*I, and 6.1k-EGFP, 6.1k-toER-EGFP, and 6.1k-POPpr-EGFP were digested with *Mlu*I. The linearized constructs were transfected into OV3121 cells using GeneJuice transfection reagent, and two days later, selection was started by adding 0.5 mg/ml G418 to the medium. Because the pEGFP-1 vector contained the neomycin-resistant gene, transfection of these constructs generated neomycin-resistant cells. The cells were cultured for another 10-14 days, and resulting survived cells were used for experiments.

Establishment of stably transfected OV3121 cells for Tet-on system and Dox treatment

The 6.1k-TO-Luc plasmid was linearized by digestion with *Mlu*I, and transfected into OV3121 cells with pPBCAGrtTA-IN and pPyCAG-PBase plasmids (49) using GeneJuice transfection reagent. pPBCAGrtTA-IN was a PiggyBac-based construct, and by transposase activity from pPyCAG-PBase, the rtTA gene would be efficiently integrated into the genome of OV3121 cells. Two days after transfection, 0.5 mg/ml G418 was added to the medium, and the cells were cultured for another 12 days. For Dox treatment, the cells were spread on 35-mm dishes, and 1 μg/ml Dox was added to the medium on the next day. Five and twenty-four hours after the addition of Dox, the cells were collected with ISOGEN II for qRT-PCR and with Passive Lysis Buffer (Promega) for luciferase assay. qRT-PCR was done as above, and luciferase activity was measured with LARII (Promega) using Lumat LB9507

(Berthold, Bad Wildbad, Germany). Protein concentration of the cell lysate was measured with a Pierce Bicinchoninic Acid (BCA) Protein Assay Reagent kit (Thermo Scientific, Waltham, MA) according to the manufacturer's instruction. Luciferase activity was normalized to the concentration.

Statistical analysis

The data were presented as means ± standard deviation (S.D.). Statistical comparison was made by Student *t*-test or one-way analysis of variance (ANOVA) followed by Tukey-Kramer test. $P < 0.05$ was considered statistically significant.

Results

A 500-bp promoter is insufficient to *Amhr2* gene activation

We obtained a 500-bp proximal promoter sequence by genomic PCR, based on the transcriptional start site (TSS) determined in rat and aligned to the mouse *Amhr2* locus (22, 23). This sequence contained the binding sites for SF1 and WT1 that were reported to be critical to *Amhr2/AMHR2* gene activation (23). We successfully established 10 transgenic lines bearing the EGFP gene driven by the 500-bp promoter, and the copy number was determined to be 1-25 (Table 3). We first checked whether green fluorescence was observed in the ovary and testis for all the lines, but most lines showed no fluorescence and only the testis from line #326 was green (Table 3, Figure 1A). To check the EGFP mRNA expression in adult tissues, we picked representative three lines, #321, #326, and #348, and performed RT-PCR with eight adult tissues. We detected no EGFP signals in any tissues of line #321 and faint bands in brain, heart, and spleen of line #348, while in line #326, a specific signal was observed in the testis (Figure 1B). These suggested that the transgene was integrated into an active chromatin region in the testis of #326. Thus, we checked embryos of #326, but did not observe any green fluorescence in gonads or urogenital regions at E12.5-15.5 (Table 3).

We next pursued the possibility that EGFP was specifically activated in somatic cells in the testis of #326. To determine which types of cells expressed EGFP, we dispersed the #326 testis, collected somatic and germ cells together, and sorted them by green fluorescence using a cell sorter. We investigated the expression of marker gene for each type of testicular cells: *Hsd3b* for Leydig cells, *Ddx4/Vasa* for germ cells, and *Sgp2* for Sertoli cells (55). qRT-PCR showed that EGFP-positive cells included both somatic cells and germ cells and were enriched in germ cells, which were inconsistent with the *Amhr2* expression enriched in somatic cells (Figure 1C). Thus, the data demonstrated that in #326, the 500-bp promoter did not reproduce the *Amhr2* expression pattern.

Collectively, the proximal promoter was insufficient to *Amhr2* gene activation, which suggested the presence of other regulatory elements.

Identification and characterization of *lncRNA-Amhr2*

In the process of searching for novel regulatory elements, we found noncoding transcription occurred in an upstream region of the *Amhr2* gene, and named the transcript *lncRNA-Amhr2*. RT-PCR using 8 tissues showed that *lncRNA-Amhr2* was specifically detected in the ovary and testis as with the *Amhr2* gene (Figure 2A). To check whether the cell type expressing *lncRNA-Amhr2* was coincided with *Amhr2*, we isolated and cultured primary granulosa and Sertoli cells and performed RT-PCR. The purity of these cell fractions was confirmed by qRT-PCR for marker genes in the testis and ovary (Supplemental Figure 1). The lncRNA signal was detected in both types of cells (Figure 2B), showing co-expression of *lncRNA-Amhr2* and *Amhr2*. We then examined the subcellular localization of *lncRNA-Amhr2* by RT-PCR with nuclear and cytoplasmic fractions from granulosa cells, and the signal was observed in the nucleus but not in the cytoplasm (Figure 2C). These data suggested the role of *lncRNA-Amhr2* in *Amhr2* gene activation, and we decided to determine the full length of this lncRNA.

The direction of the noncoding transcription was found to be opposite to *Amhr2* by RT-PCR with specific primers for reverse transcription (data not shown), so we attempted to determine the full

length in that direction. RACE analyses were performed with total RNAs from primary granulosa cells and the testis, and we identified tissue-specific TSSs by 5'RACE. In granulosa cells, two nucleotides were identified as TSSs by examining 10 subclones (Figure 3A and 3B). In the testis, we obtained two bands after the second PCR, and each contained one TSS (Figure 3A and 3C). By 3'RACE, we identified 10 transcriptional termination sites (TTSs) within a GA-rich region, and a guanine was determined to be the major one because it was TTS in more subclones than any other sites (Figure 3D, bold red font). Collectively, we discovered a novel lncRNA, namely *lncRNA-Amhr2*, which was co-expressed with *Amhr2*, and identified two variants in ovarian granulosa cells and two in the testis (Figure 3A). The length of the variants was 1655-2099 nucleotides, and the longest one in the testis contained one intron, while the other variants were a single exon gene. Although many lncRNAs were not evolutionarily conserved (33), *lncRNA-Amhr2* contained conserved noncoding sequences (CNSs), when we compared the mouse *Amhr2* locus with the human genome using the mVista program (http://genome.lbl.gov/vista/index.shtml) (Figure 3A). Nucleotide sequence data for these four *lncRNA-Amhr2* variants were deposited to DDBJ/EMBL/GenBank databases (LC274966, LC274967, LC274968, and LC274969).

Knock down of *lncRNA-Amhr2* decrease *Amhr2* expression in primary granulosa cells

The co-expression of *lncRNA-Amhr2* and *Amhr2* prompted us to explore the possibility of a novel mechanism for *Amhr2* gene activation by *lncRNA-Amhr2*. Thus, we attempted to knock down *lncRNA-Amhr2* in primary granulosa and Sertoli cells. However, the knock down did not work in Sertoli cells, and we could only obtain the result with granulosa cells. By transfection with the sh-lncAmhr2 construct and selection with G418 in primary granulosa cells, the *lncRNA-Amhr2* level was decreased by approximately 60% compared to control (Figure 4A). The endogenous *Amhr2* expression was significantly decreased to approximately 50% of control, while *Nr5a1/Sf1* and *Actb* mRNA levels were not changed by this knock down (Figure 4B-D). This indicated that *lncRNA-Amhr2* specifically contributed to *Amhr2* gene activation in granulosa cells.

lncRNA-Amhr2 activation increases _Amhr2_ promoter activity in primary granulosa cells

To see whether *lncRNA-Amhr2* activated the *Amhr2* gene by enhancing its promoter activity, we generated five constructs (Figure 5A). We obtained a 6.1-kb upstream sequence of the *Amhr2* gene and connected to the luciferase gene (6.1k-Luc). Then, we replaced most of the *lncRNA-Amhr2* sequence with torazame ER cDNA which was similar in length (6.1k-toER-Luc) (46), and the *lncRNA-Amhr2* promoter sequence with the POP promoter (6.1k-POPpr-Luc). The replacement with torazame ER would result in transcription of a sequence unrelated to *lncRNA-Amhr2*, and the POP promoter was known to show strong promoter activity in various types of cells and expected to drive *lncRNA-Amhr2* transcription at a higher level than other constructs (47, 48, 51). As controls, we also prepared the constructs that contained the 0.5-kb *Amhr2* promoter (0.5k-Luc) and lacked a *lncRNA-Amhr2* promoter sequence (6.1k-ΔlncPr-Luc).

We transiently transfected primary granulosa cells with these constructs as well as a control vector bearing no promoter (pGL3-Basic), and measured the luciferase activity. 0.5k-Luc showed significantly higher activity than 6.1k-Luc (Figure 5A), which was consistent with previous studies reporting that a shorter sequence often possessed higher promoter activity in transient assay (56-59). The replacement of lncRNA with torazame ER and the deletion of lncRNA promoter slightly reduced the activity but the difference was not statistically significant (Figure 5A, 6.1k-Luc vs 6.1k-toER-Luc and 6.1k-ΔlncPr-Luc). In contrast, the replacement of lncRNA promoter with POP promoter caused a significant increase in *Amhr2* promoter activity (Figure 5A, 6.1k-Luc vs 6.1k-POPpr-Luc). Notably, the POP sequence in the opposite direction exhibited neither promoter activity (Figure 5B) nor effects on the 0.5-kb *Amhr2* promoter (Figure 5C). These excluded the possibility that the reporter gene expression was increased by bidirectional promoter activity or enhancer activity of the POP promoter. In addition, we replaced both lncRNA sequence and lncRNA promoter with torazame ER and POP promoter (6.1k-POPpr-toER-Luc), and found that the activation of torazame ER transcription did not significantly increase *Amhr2* promoter activity (Figure 5D). These indicated that *lncRNA-Amhr2*

activation increased *Amhr2* promoter activity in granulosa cells.

In Sertoli cells, we observed significantly higher activity in 0.5k-Luc, similarly to granulosa cells (Figure 6). However, no significant difference was detected among the other constructs, even if 6.1k-POPpr-Luc showed higher activity (Figure 6). Therefore, we conducted further experiments only with granulosa cells.

lncRNA-Amhr2 transcription is correlated with _Amhr2_ promoter activity in stably transfected OV3121 cells

In the primary culture system, we could only perform transient transfection. We next investigated whether the *lncRNA-Amhr2* transcription also contributed to activation of the *Amhr2* promoter when the construct was integrated into genome DNA. For this purpose, we used OV3121 cells that were derived from mouse granulosa cell tumor and expected to maintain some properties as granulosa cells (60). Here we used the EGFP gene as a reporter and prepared four constructs: 0.5k-EGFP (the construct used for generating transgenic mice), 6.1k-EGFP, 6.1k-toER-EGFP, and 6.1k-POPpr-EGFP (Figure 7A and B). We transfected the constructs into OV3121 cells, established stable cells by the selection with G418, and checked expression of *lncRNA-Amhr2* and EGFP mRNA by qRT-PCR. *lncRNA-Amhr2* transcripts were detected at a significantly higher level in the cells with 6.1k-POPpr-EGFP than those with 6.1k-EGFP (Figure 7A). This showed that the POP promoter actually drove the *lncRNA-Amhr2* transcription more strongly than the original promoter. The other two constructs resulted in no *lncRNA-Amhr2* transcripts as expected (Figure 7A). The EGFP mRNA level was correlated with the *lncRNA-Amhr2* transcription. The cells with 6.1k-POPpr-EGFP showed a significantly higher level of EGFP expression than those with the other three constructs (Figure 7B). 6.1k-EGFP showed slightly higher EGFP expression than 0.5k-EGFP and 6.1k-toER-EGFP (Figure 7B), even in cells showing heterogeneous EGFP expression as observed by a fluorescent microscope (Figure 7C).

To further confirm the relationship between lncRNA and EGFP, we concentrated EGFP-positive

cells using a cell sorter, and performed RT-PCR. As a result, *lncRNA-Amhr2* transcripts were detected in EGFP-positive cells at a much higher level than in EGFP-negative cells (Figure 7D). This clearly indicated that the *lncRNA-Amhr2* transcription was well correlated with *Amhr2* promoter activity when the construct was integrated into genome DNA, and supported that *lncRNA-Amhr2* contributed to *Amhr2* activation.

Induction of *lncRNA-Amhr2* transcription increases *Amhr2* promoter activity

We finally attempted to clarify whether the *lncRNA-Amhr2* transcription could enhance *Amhr2* promoter activity, using the Tet-on system. We inserted the hCMV*1 promoter that was responsive to Dox into a restriction site at approximately 100-bp downstream to TSSs of *lncRNA-Amhr2* ovary variants in 6.1k-Luc (Figure 8A). This construct was expected to induce the transcription of most sequence of *lncRNA-Amhr2* in response to Dox. We transfected OV3121 cells with this construct and selected the cells that were integrated with the transgene into genome DNA. We added Dox to the selected cells, and 5 hours and 24 hours later, the *lncRNA-Amhr2* transcription was examined. By qRT-PCR, the *lncRNA-Amhr2* level was about 30-folds higher in the cells treated with Dox than in the control cells at both time points (Figure 8B). This indicated that the induction of *lncRNA-Amhr2* transcription was successful, so we checked the luciferase gene expression by qRT-PCR and luciferase activity assay. The luciferase mRNA level was 5.4-folds and 4.3-folds higher at 5 hours and 24 hours after the addition of Dox than in the control cells (Figure 8C), and luciferase activity was 6.9-folds and 12.5-folds higher with Dox than the control (Figure 8D). These data indicated that the induction of *lncRNA-Amhr2* transcription enhanced *Amhr2* promoter activity and increased the luciferase gene expression. Therefore, we concluded that *lncRNA-Amhr2* controlled the *Amhr2* gene through enhancing its promoter activity in ovarian granulosa cells.

Discussion

We first assessed *in vivo* activity of an *Amhr2* proximal promoter by generating transgenic mice. The EGFP fluorescence was not observed and the RT-PCR signal was not detected in the ovary and testis of transgenic lines except for the testis of #326 (Figure 1 and Table 3). This suggests that the transgene was integrated into genome regions where the chromatin was tightly packed in the nine lines except for #326, and that the 500-bp promoter does not contain any elements to open the chromatin. In addition, the majority of green cells from the #326 testis were found to be germ cells rather than somatic cells, and embryonic gonads were not green in this line. This further suggests that the transgene does not contain any elements to restrict the expression of a linked reporter gene to testicular somatic cells even if it is inserted into a genomic region that was specifically activated in the testis. We could still obtain some green Sertoli and Leydig cells and the promoter might be partly responsible for this activation, but obviously, the 500-bp sequence could not specify the expression to somatic cells in the testis. Collectively, our present data clearly show that the 500-bp promoter does not reproduce the *Amhr2* expression *in vivo*, in other words, the proximal promoter is insufficient to *Amhr2* gene activation. Given that reporter genes were expressed in mostly identical patterns to *Amhr2* when integrated into exon 5 of the native *Amhr2* gene (61, 62), other regulatory elements could be present at the locus, and we found *lncRNA-Amhr2* as an element responsible for *Amhr2* activation at least in granulosa cells.

Here we provided the following evidence supporting the regulation of *Amhr2* by *lncRNA-Amhr2*. First, knock down of *lncRNA-Amhr2* led to a decrease in the *Amhr2* mRNA level in primary granulosa cells (Figure 4). This, together with the data showing that the activation of torazame ER transcription did not increase *Amhr2* promoter activity (Figure 5), also revealed that it was the *lncRNA-Amhr2* transcript but not noncoding transcription *per se* that was required for gene activation. Second, transient reporter gene assay showed that *lncRNA-Amhr2* activation by POP promoter significantly increased *Amhr2* promoter activity in primary granulosa cells (Figure 5). Third, higher *lncRNA-Amhr2* transcription increased *Amhr2* promoter activity even when the transgene was integrated into the

genome DNA of granulosa cell-derived OV3121 cells (Figure 7). Fourth, in those stable OV3121 cells, the EGFP reporter gene was activated only in the cell population expressing *lncRNA-Amhr2* at high levels. Last, the induction of *lncRNA-Amhr2* transcription by the Tet-on system dramatically increased activity of the linked *Amhr2* promoter in stably transfected OV3121 cells (Figure 8). All of these results indicate that *lncRNA-Amhr2* plays an important role in *Amhr2* gene activation in ovarian granulosa cells, and therefore, this lncRNA is a novel regulatory element of the mouse *Amhr2* gene.

In contrast to granulosa cells, the knock down experiment did not work in Sertoli cells, similarly to some nuclear lncRNAs that were not efficiently knocked down with short hairpin RNAs (63, 64). Additionally, the usage of POP promoter for *lncRNA-Amhr2* transcription did not result in a significant increase of *Amhr2* promoter activity in primary Sertoli cells. However, these do not necessarily mean that *lncRNA-Amhr2* has nothing to do with *Amhr2* regulation in this cell lineage. We observed still higher *Amhr2* promoter activity in the cells with 6.1k-POPpr-Luc than 6.1k-Luc, 6.1k-toER-Luc, and 6.1k-ΔlncPr-Luc (Figure 6), suggesting a positive role of *lncRNA-Amhr2* in Sertoli cells. Alternatively, it may be possible that an intron-containing variant (Testis variant 2 in Figure 3A) activates the *Amhr2* gene by a different mechanism in Sertoli cells. Further analyses will be required for revealing the function of *lncRNA-Amhr2* in Sertoli cells.

lncRNAs control their target genes on the same chromosome in *cis* or on another chromosome in *trans*. Our present data strongly suggest that *lncRNA-Amhr2* acts on the *Amhr2* gene only in *cis*, which is consistent with many lncRNAs controlling linked protein-coding genes (26). In transgenic mice, the exogenous *Amhr2* promoter was not activated in the ovary of all 10 lines, despite the presence of endogenous *lncRNA-Amhr2* transcripts in granulosa cells. If *lncRNA-Amhr2* acted in *trans*, we could have observed the EGFP activation in their ovaries. In addition, our preliminary data showed that *Amhr2* expression was not influenced by overexpression of *lncRNA-Amhr2* in some types of cultured cells (our unpublished data). Thus, *lncRNA-Amhr2* is likely to act only in *cis*. *Cis*-acting lncRNAs generally need to interact with the chromatin region of their target gene loci (33, 65), and the endogenous *lncRNA-Amhr2* might not be able to reach genome regions integrated with the transgenes.

In general, lncRNAs are categorized by their localization and functional mechanisms, and *lncRNA-Amhr2* belongs to those localized in the nucleus and involved in gene activation in *cis*. There are some categories for such lncRNAs: enhancer RNAs, activating RNAs, and promoter associated noncoding RNAs. Enhancer RNAs are transcribed bidirectionally from enhancers and mediate the chromatin looping to allow the enhancers to physically contact with their target promoters (66, 67). Activating RNAs bind to mediators and promote the target gene transcription (26, 68), and promoter associated noncoding RNAs are located just upstream to their target gene promoters to control epigenetic modifications (69). *lncRNA-Amhr2* is similar to activating RNAs, in that it is transcribed from a neighboring region of its target, activates the target gene in *cis*, and the transcript is required. Thus, *lncRNA-Amhr2* may activate the *Amhr2* gene by a similar mechanism to activating RNAs.

Our present study revealed that a novel lncRNA, *lncRNA-Amhr2*, contributes to *Amhr2* gene activation in mouse ovarian granulosa cells. AMH is an important hormone for the regulation of folliculogenesis in the ovary, and the Amhr2 protein is a pivotal molecule to mediate the signaling. Given that the down- and up-regulation of this receptor gene results in abnormal folliculogenesis (14, 70, 71), *lncRNA-Amhr2* is highly likely to be a key molecule controlling the AMH signaling probably via interacting to the *Amhr2* promoter in *cis* and the consequent up-regulation of the *Amhr2* gene. Notably, one of the transcription factors for *Amhr2* activation, WT-1, binds to the proximal promoter of the *Amhr2* gene, and has RNA-binding activity in embryonic stem cells (72, 73). In combination, current results support the view that *lncRNA-Amhr2* interacts with transcription factors binding to the *Amhr2* promoter to activate this gene. Further investigation of the molecular mechanism of the *Amhr2* activation and the physiological functions in the regulation of endocrine system by *lncRNA-Amhr2* is underway.

In conclusion, we demonstrated that a proximal promoter was insufficient to *Amhr2* gene activation *in vivo* and identified a novel lncRNA, *lncRNA-Amhr2*, which plays a role in *Amhr2* gene activation in ovarian granulosa cells. The study reveals a novel mechanism of *Amhr2* gene activation, and provides a new insight into the regulation of AMH signaling.

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Figure legends

Figure 1. A 500-bp *Amhr2* promoter is insufficient to specific gene activation in granulosa and Sertoli cells. Transgenic mice were generated with the 0.5k-EGFP construct in which the EGFP gene was driven by a 500-bp *Amhr2* promoter. (A) Fluorescent microscopic images of testes from a wild type mouse (WT) and a #326 mouse. (B) RT-PCR analysis of the EGFP reporter gene in three transgenic mouse lines. Eight tissues were collected from the indicated transgenic mouse lines, #326, #321, and #348, and total RNAs were purified from them. cDNAs were synthesized with (RT+) or without reverse transcriptase (RT-) using the oligo(dT) primer, and PCR reactions were performed with primer pairs for the indicated genes. The cycle numbers were 30 for *Amhr2* and EGFP and 24 for *Gapdh*. Testis-specific amplification was observed in the #326 line. (C) qRT-PCR analysis of the EGFP gene in cell fractions before (Total) and after sorting (EGFP+). Testicular cells were isolated from testes of #326 mice, and green cells were collected by a cell sorter. Total RNAs were purified from the cells before and after the sorting, and cDNAs were synthesized using the olig(dT) primer. Quantitative PCR was performed to detect marker genes for Leydig cells (*Hsd3b*), germ cells (*Ddx4/Vasa*), and Sertoli cells (*Sgp2*) using the 7300 real-time PCR system (Applied Biosystems). The PCR was also performed for a housekeeping gene, *Gapdh*, and the data were normalized to the *Gapdh* level. The value in the cells before sorting (Total) was set to 1.0, and relative levels of the marker genes after sorting are shown. The EGFP-positive fraction was enriched in germ cells. The data are presented as mean ± S.D.

743 from four independent experiments with two sets of sorted cells, and the statistical significance was
744 analyzed by Student *t*-test. *$P < 0.05$, **$P < 0.01$.
745
746 **Figure 2.** Expression of *lncRNA-Amhr2*. (A) Tissue-specificity of *lncRNA-Amhr2* was investigated by
747 RT-PCR analysis. Eight tissues were isolated from wild type mice, and RT-PCR was performed by
748 using a specific primer for reverse transcription of *lncRNA-Amhr2* and oligo(dT) for *Gapdh*. We used a
749 primer pair that could amplify all four variants of *lncRNA-Amhr2*. The signal was specifically
750 observed in the ovary and testis. *Gapdh* was amplified as an internal control. The cycle numbers were
751 40 for *lncRNA-Amhr2* and 27 for *Gapdh*. (B) RT-PCR with primary cells. Sertoli cells were isolated
752 from testes of 8-12 days old male mice, and cultured on collagen-coated dishes for several days.
753 Primary granulosa cells were isolated from ovaries of immature female mice after administration with
754 hormones, and cultured on fibronectin-coated dishes for several days. Total RNAs were purified from
755 these cells, and RT-PCR analysis was performed as in Figure 1B. *Amhr2* was detected as a positive
756 control. The cycle number was 35. (C) Subcellular distribution of *lncRNA-Amhr2* was investigated by
757 RT-PCR. Primary granulosa cells were cultured and fractionated into cytoplasmic and nuclear
758 fractions. Total RNA was purified from each fraction (Cytoplasmic and Nuclear) as well as granulosa
759 cells without fractionation (Total), and RT-PCR was performed as above. A primer pair to amplify a
760 part of exon 6 of the *Gapdh* gene was used as a control. The *lncRNA-Amhr2* signal was exclusively
761 detected in the nuclear fraction. The cycle numbers were 35 for *lncRNA-Amhr2* and 30 for *Gapdh*.
762
763 **Figure 3.** Cloning of *lncRNA-Amhr2* and comparison with human genome. (A) Schematic drawing of
764 the mouse *Amhr2* gene locus. At the top, the *Amhr2* gene is depicted by boxes representing exons, and
765 translated and untranslated regions are indicated as black and white boxes. Below the gene structure, a
766 7-kb upstream region was enlarged. The 7-kb upstream sequence of the mouse *Amhr2* gene was
767 compared with the corresponding 7-kb sequence at the human *AMHR2* locus, using mVista program
768 (http://genome.lbl.gov/vista/index.shtml). This program detected conserved sequences presented by

pink or white painting. Y-axis indicates the percentage of identical nucleotides between the two species. At the bottom, positions of four *lncRNA-Amhr2* variants are shown with their length. Two variants were isolated from the testis and the other two was from ovarian granulosa cells. All variants have poly(A) tails. Testis variant 2 contains two exons. Some of the CNSs, marked with pink, overlapped with the region transcribed into *lncRNA-Amhr2*. (B) TSSs of *lncRNA-Amhr2* ovary variants. A specific band was obtained by 5'RACE, and ten subclones were checked. Two TSSs were identified as indicated by bold red fonts and bent arrows with the numbers of subclones. (C) TSSs of *lncRNA-Amhr2* testis variants. By 5'RACE, two specific bands were obtained and ten subclones were sequenced for each band. All of the ten subclones contained the same nucleotide as a TSS in both cases, and the TSSs are indicated by bold red fonts and bent arrows. (D) TTSs of *lncRNA-Amhr2*. By 3'RACE, a specific band was obtained in both the ovary and testis, and ten subclones were sequenced for each tissue. The TTS position of each subclone from ovary (lower, green fonts) and testis (upper, blue fonts) was indicated by bent arrows with numbers of subclones. A major TTS is shown by a bold red font.

Figure 4. Knock down of *lncRNA-Amhr2* in primary granulosa cells. Primary granulosa cells were cultured as in Figure 2B and transfected with a vector containing shRNA for *lncRNA-Amhr2* (Knock down) or a control vector (Control). The cells were selected by adding G418 to the culture medium for several days. Total RNAs were purified from the cells, and qRT-PCR was performed for expression of *lncRNA-Amhr2* (A), *Amhr2* (B), *Nr5a1/Sf1* (C), and *Actb* (D). The expression level was normalized to *Gapdh*, and the value in the control sample was set to 1.0. *lncRNA-Amhr2* was successfully knocked down, and *Amhr2* expression was significantly decreased. The data are presented as mean ± S.D. from four independent experiments, and the statistical significance was analyzed by Student *t*-test. $*P < 0.05$, $**P < 0.01$.

Figure 5. *lncRNA-Amhr2* activation increase *Amhr2* promoter activity in primary granulosa cells by

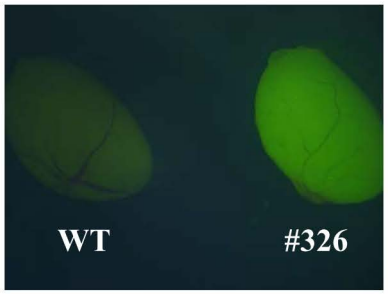
795 transient reporter gene assay. (A) Reporter gene assay in primary granulosa cells. Primary granulosa
796 cells were cultured and transfected with constructs indicated at left. Two days after the transfection,
797 cell lysates were collected and luciferase activity was measured. Relative luciferase activity is shown
798 in the graph at right. The data are presented as mean ± S.D. from three or five independent
799 experiments. Luciferase activity was significantly higher in 6.1k-POPpr-Luc than other constructs
800 containing the 6.1-kb sequence. (B) Unidirectional activity of the POP promoter. The POP promoter
801 was directly connected to the luciferase gene in both directions, and the constructs were transfected
802 into primary granulosa cells. Luciferase activity was measured and shown as above. The data are
803 presented as mean ± S.D. from four independent experiments. The POP promoter in the forward
804 direction exhibited much higher activity. (C) The effect of the POP promoter on *Amhr2* promoter
805 activity. The POP promoter was connected to upstream of the 0.5-kb *Amhr2* promoter in 0.5k-Luc, and
806 luciferase activity was assessed in primary granulosa cells as above. The data are presented as mean ±
807 S.D. from four independent experiments. The POP promoter in the reverse direction had no significant
808 effect on *Amhr2* promoter activity. (D) The effect of the torazame ER sequence driven by the POP
809 promoter on *Amhr2* promoter activity. *lncRNA-Amhr2* and its promoter sequence were replaced with
810 torazame ER and the POP promoter. Luciferase assay was performed in primary granulosa cells as
811 above. The data are presented as mean ± S.D. from four independent experiments. Torazame ER
812 transcription by the POP promoter did not significantly increase *Amhr2* promoter activity. For all the
813 data, the statistical significance was analyzed by one way ANOVA followed by Tukey-Kramer test.
814 $^{**}P < 0.01$.
815
816 **Figure 6.** Reporter gene assay in primary Sertoli cells. Primary Sertoli cells were cultured, and
817 transfection and luciferase activity assay were done as in Figure 5A. The data are presented as mean ±
818 S.D. from five or six independent experiments. The usage of POP promoter had no effect on *Amhr2*
819 promoter. The statistical significance was analyzed by one way ANOVA followed by Tukey-Kramer
820 test. $^{**}P < 0.01$.

Figure 7. *lncRNA-Amhr2* transcription is correlated with *Amhr2* promoter activity in stably transfected OV3121 cells. (A) qRT-PCR analysis of *lncRNA-Amhr2* in stably transfected OV3121 cells. OV3121 cells were transfected with the indicated constructs, and the cells were selected with G418. Total RNAs were purified from the cells, and qRT-PCR was performed. Relative *lncRNA-Amhr2* levels were calculated by normalization to the *Gapdh* level and shown as a graph. The value of the cells with the 6.1k-EGFP construct was set to 1.0. The data are presented as mean ± S.D. from four independent qRT-PCR reactions by two transfection experiments. The *lncRNA-Amhr2* transcription was successfully enhanced by a POP promoter. Student *t*-test was performed to analyze the statistical significance. $^{**}P < 0.01$. (B) qRT-PCR analysis of the EGFP reporter gene in stably transfected OV3121 cells. Stably transfected OV3121 cells were established as above, and EGFP mRNA levels were examined and presented as above. The data are presented as mean ± S.D. from four independent qRT-PCR reactions by two transfection experiments. EGFP expression was the highest when *lncRNA-Amhr2* was driven by a POP promoter. The statistical significance was analyzed by one way ANOVA followed by Tukey-Kramer test. $^{**}P < 0.01$, $^{*}P < 0.05$. (C) The photograph of OV3121 cells stably transfected with the 6.1k-EGFP construct. Bright field (left) and fluorescent images (right) are shown. Some cells were green, but others were not. (D) RT-PCR analysis of the EGFP gene in stably transfected OV3121 cells sorted by a cell sorter. OV3121 cells that were stably transfected with 6.1k-EGFP were sorted into green cells (EGFP+) and non-fluorescent cells (EGFP-) by a cell sorter. Total RNA was purified from each fraction, and RT-PCR was performed to detect *lncRNA-Amhr2* transcription. The EGFP expression was correlated with *lncRNA-Amhr2* transcription. The cycle numbers were 30 for *lncRNA-Amhr2* and 25 for *Gapdh*.

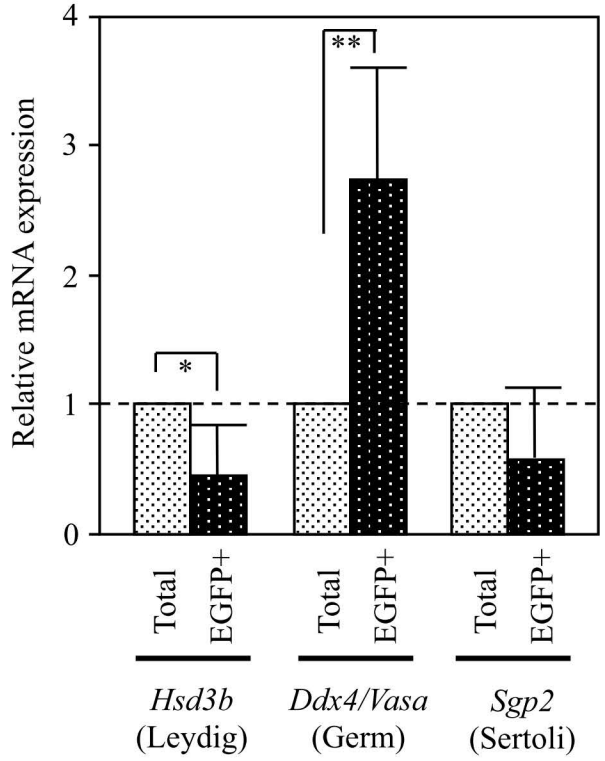
Figure 8. Induction of *lncRNA-Amhr2* transcription increased *Amhr2* promoter activity in the Tet-on system. (A) A schematic drawing of the 6.1k-TO-Luc construct for establishing the Tet-on inducible system. A 6.1-kb upstream region of the *Amhr2* gene was connected to the luciferase gene, and the

hCMV*1 promoter was inserted to induce the transcription of most sequence of *lncRNA-Amhr2*. (B-D) OV3121 cells were transfected with the construct and selected with G418. The established stable cells were treated with (Dox+) or without Dox (Dox-) for 5 or 24 hours, and total RNAs and protein extracts were collected. Using the total RNAs, qRT-PCR was performed to measure *lncRNA-Amhr2* (B) and luciferase mRNA levels (C). *Gapdh* was used as an internal control. The protein extracts were used for luciferase activity assay and BCA assay. The luciferase enzymatic activity was normalized to the value by BCA assay, and relative activity is shown as a graph (D). *lncRNA-Amhr2* transcription was successfully induced by Dox, and luciferase expression was dramatically increased. The data are presented as mean ± S.D. from three (B, C) or six independent experiments (D). Student *t*-test was performed to analyze the statistical significance. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

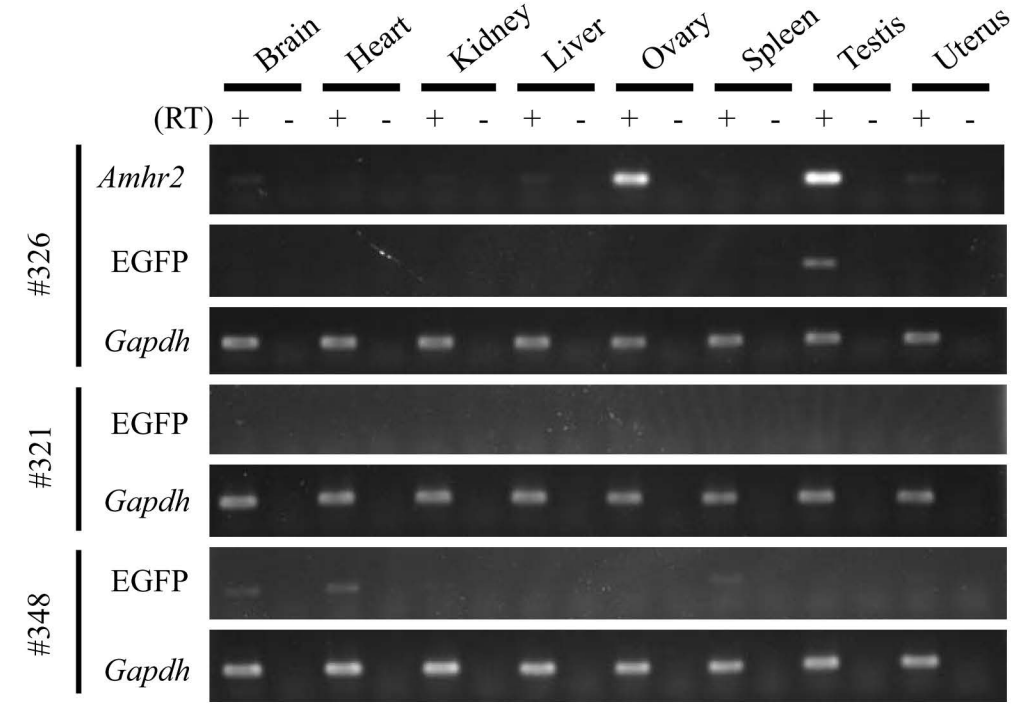
(A)


WT
#326

(C)


Relative mRNA expression
4
3
2
1
0
**
*
Total
EGFP+
Total
EGFP+
Total
EGFP+
Hsd3b
(Leydig)
Ddx4/Vasa
(Germ)
Sgp2
(Sertoli)

(B)


Brain
Heart
Kidney
Liver
Ovary
Spleen
Testis
Uterus
(RT) + - + - + - + - + - + - + - + -
#326
Amhr2
EGFP
Gapdh
#321
EGFP
Gapdh
#348
EGFP
Gapdh

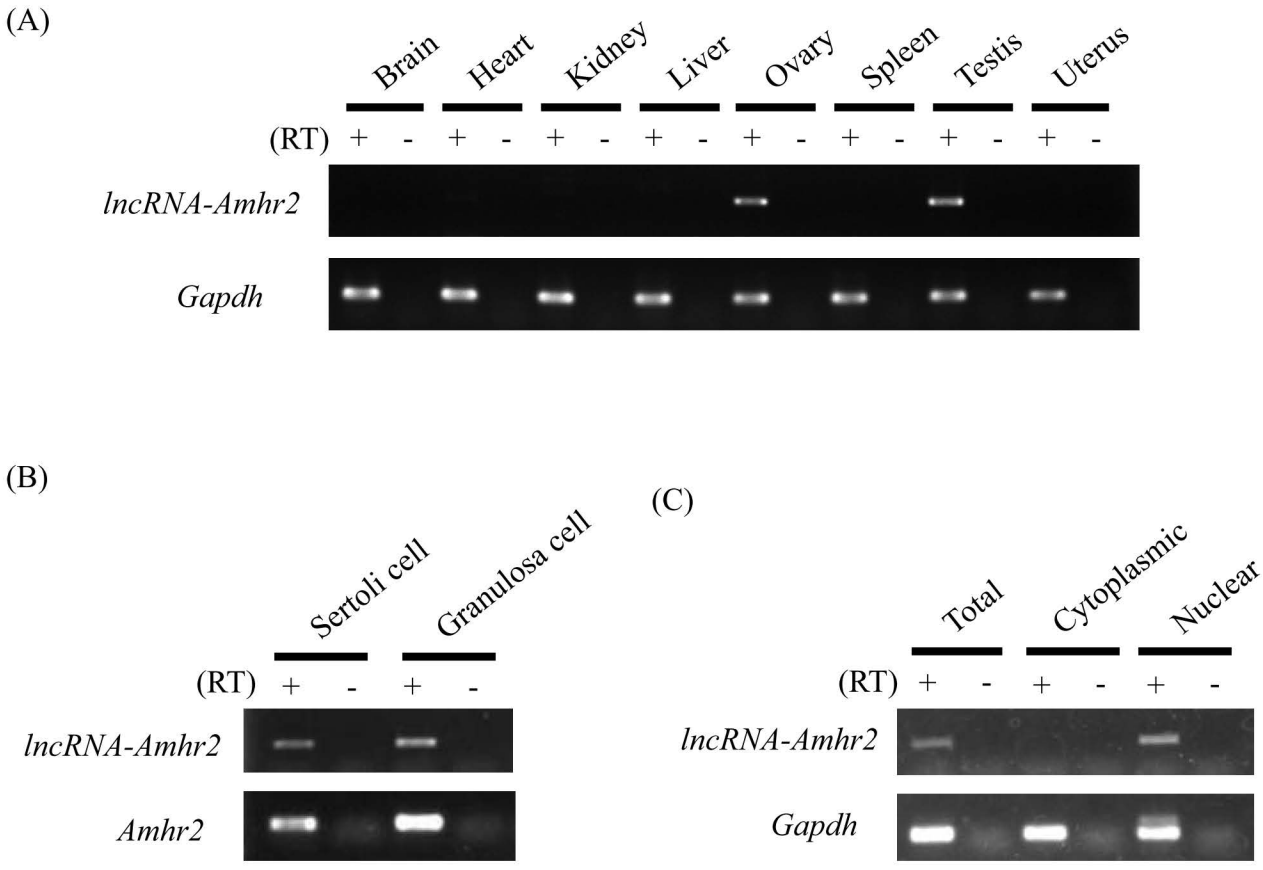
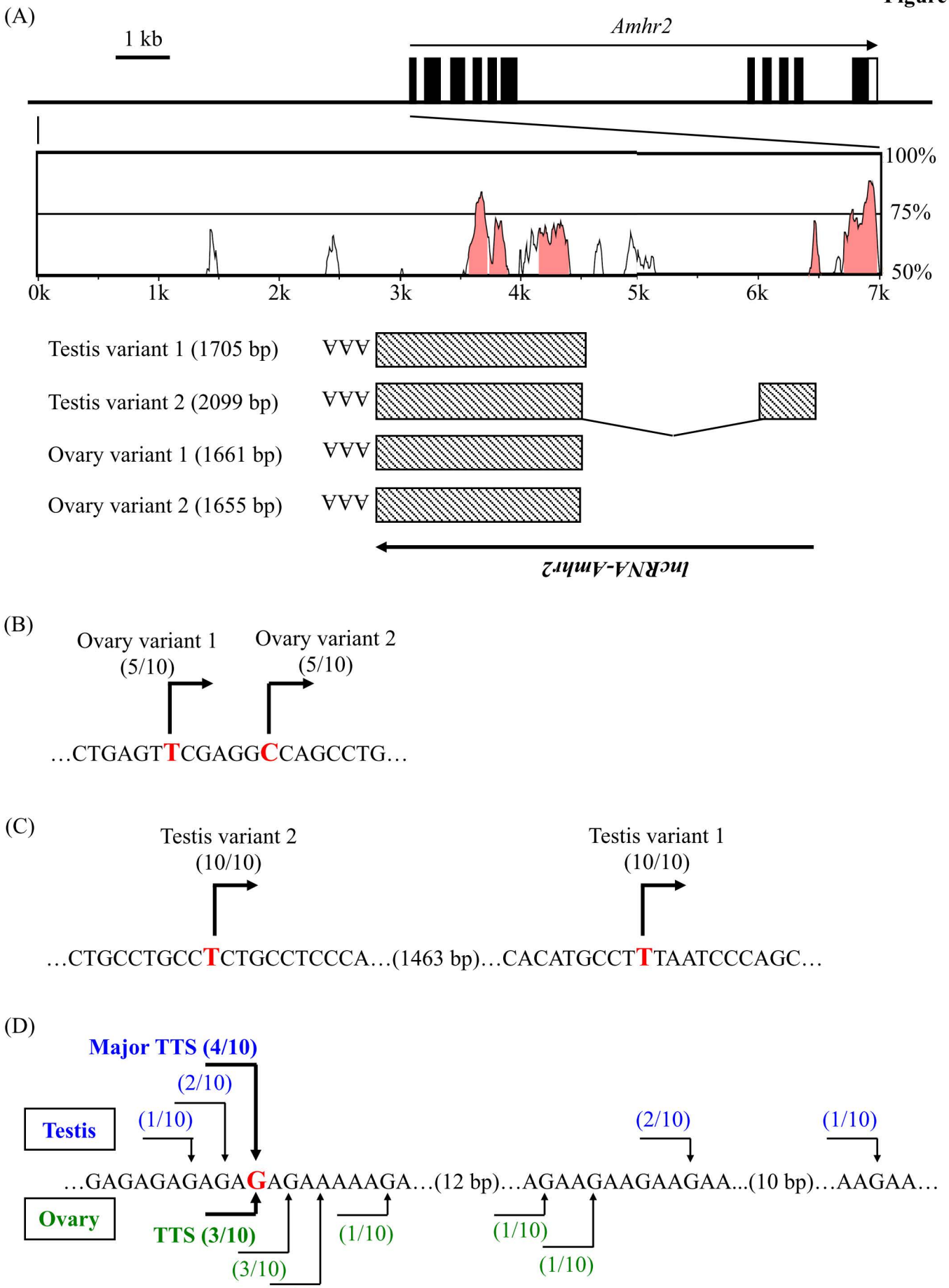

(A)
Brain
Heart
Kidney
Liver
Ovary
Spleen
Testis
Uterus
(RT) + - + - + - + - + - + - + - + -
lncRNA-Amhr2
Gapdh
(B)
Sertoli cell
Granulosa cell
(RT) + - + -
lncRNA-Amhr2
Amhr2
(C)
Total
Cytoplasmic
Nuclear
(RT) + - + - + -
lncRNA-Amhr2
Gapdh

Figure 3


(A)
1 kb
Amhr2
100%
75%
50%
0k
1k
2k
3k
4k
5k
6k
7k
Testis variant 1 (1705 bp)
Testis variant 2 (2099 bp)
Ovary variant 1 (1661 bp)
Ovary variant 2 (1655 bp)
lncRNA-Amhr2
(B)
Ovary variant 1
(5/10)
Ovary variant 2
(5/10)
…CTGAGTTCGAGGCCAGCCTG…
(C)
Testis variant 2
(10/10)
Testis variant 1
(10/10)
…CTGCCTGCCTCTGCCTCCCA…(1463 bp)…CACATGCCTTTAATCCCAGC…
(D)
Major TTS (4/10)
(2/10)
Testis
(1/10)
(2/10)
(1/10)
…GAGAGAGAGAGAGAAAAAGA…(12 bp)…AGAAGAAGAAGAA...(10 bp)…AAGAA…
Ovary
TTS (3/10)
(1/10)
(1/10)
(3/10)
(1/10)
(1/10)

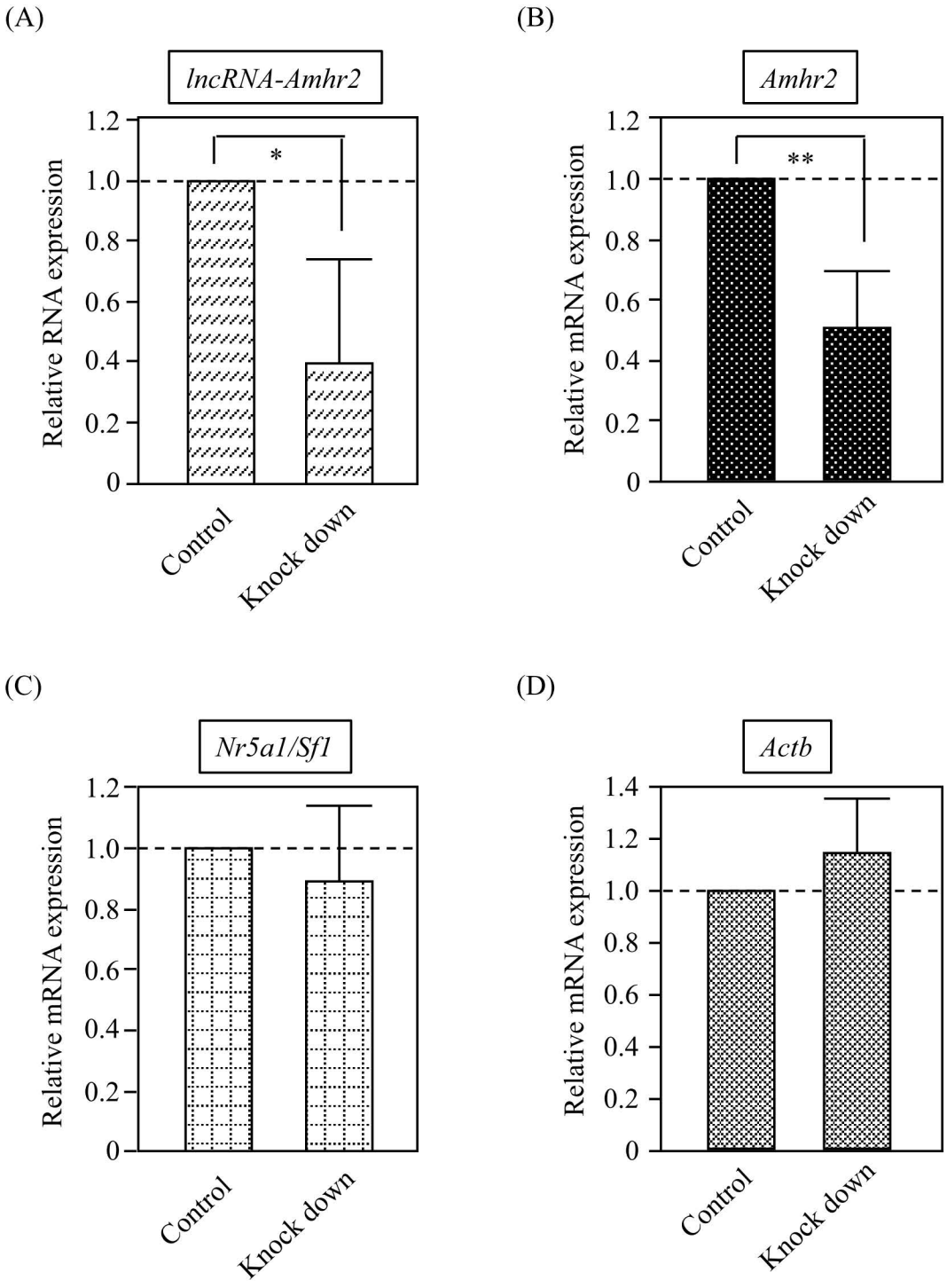

(A)
lncRNA-Amhr2
Relative RNA expression
1.2
1.0
0.8
0.6
0.4
0.2
0
*
Control
Knock down
(B)
Amhr2
Relative mRNA expression
1.2
1.0
0.8
0.6
0.4
0.2
0
**
Control
Knock down
(C)
Nr5a1/Sf1
Relative mRNA expression
1.2
1.0
0.8
0.6
0.4
0.2
0
Control
Knock down
(D)
Actb
Relative mRNA expression
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Control
Knock down

Figure 5

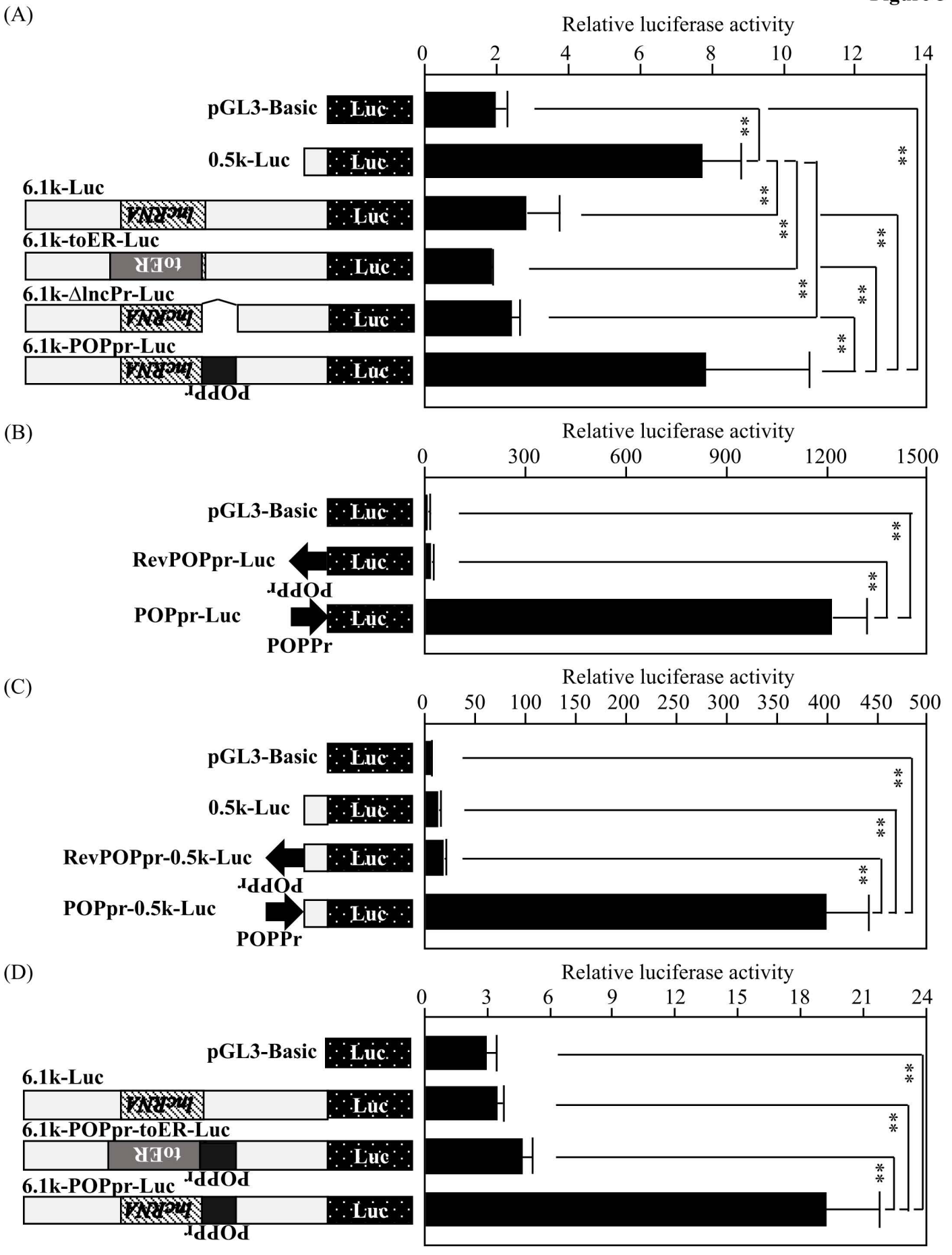
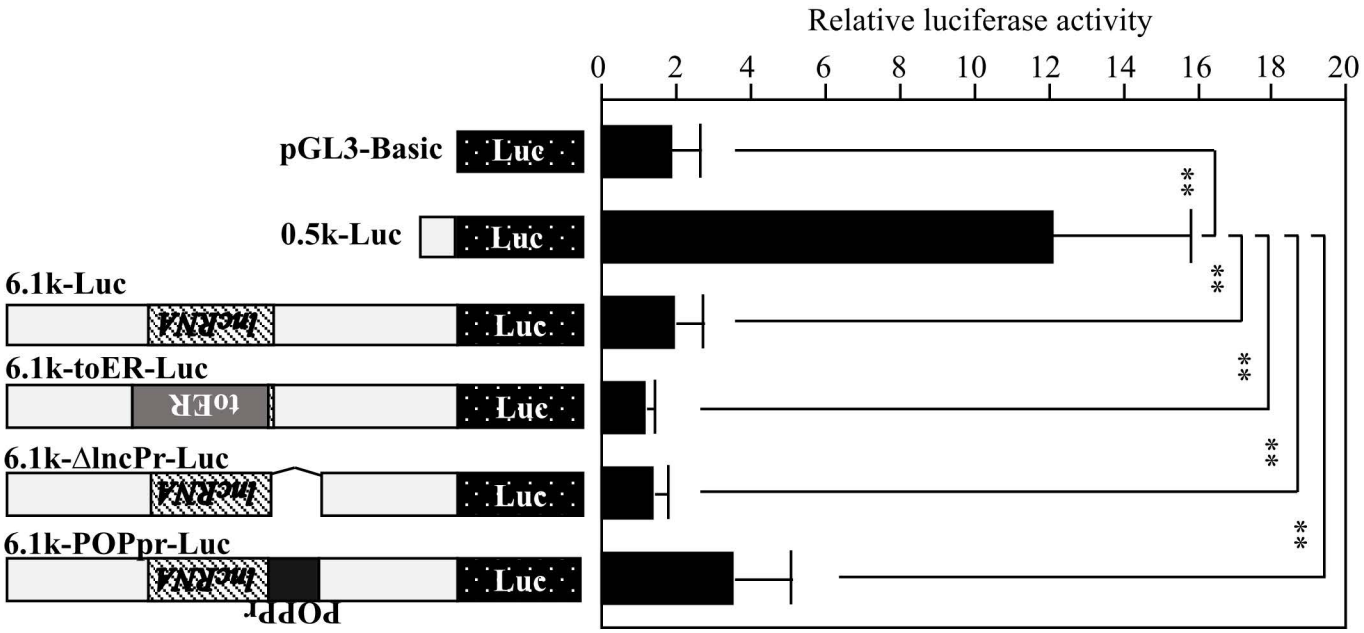
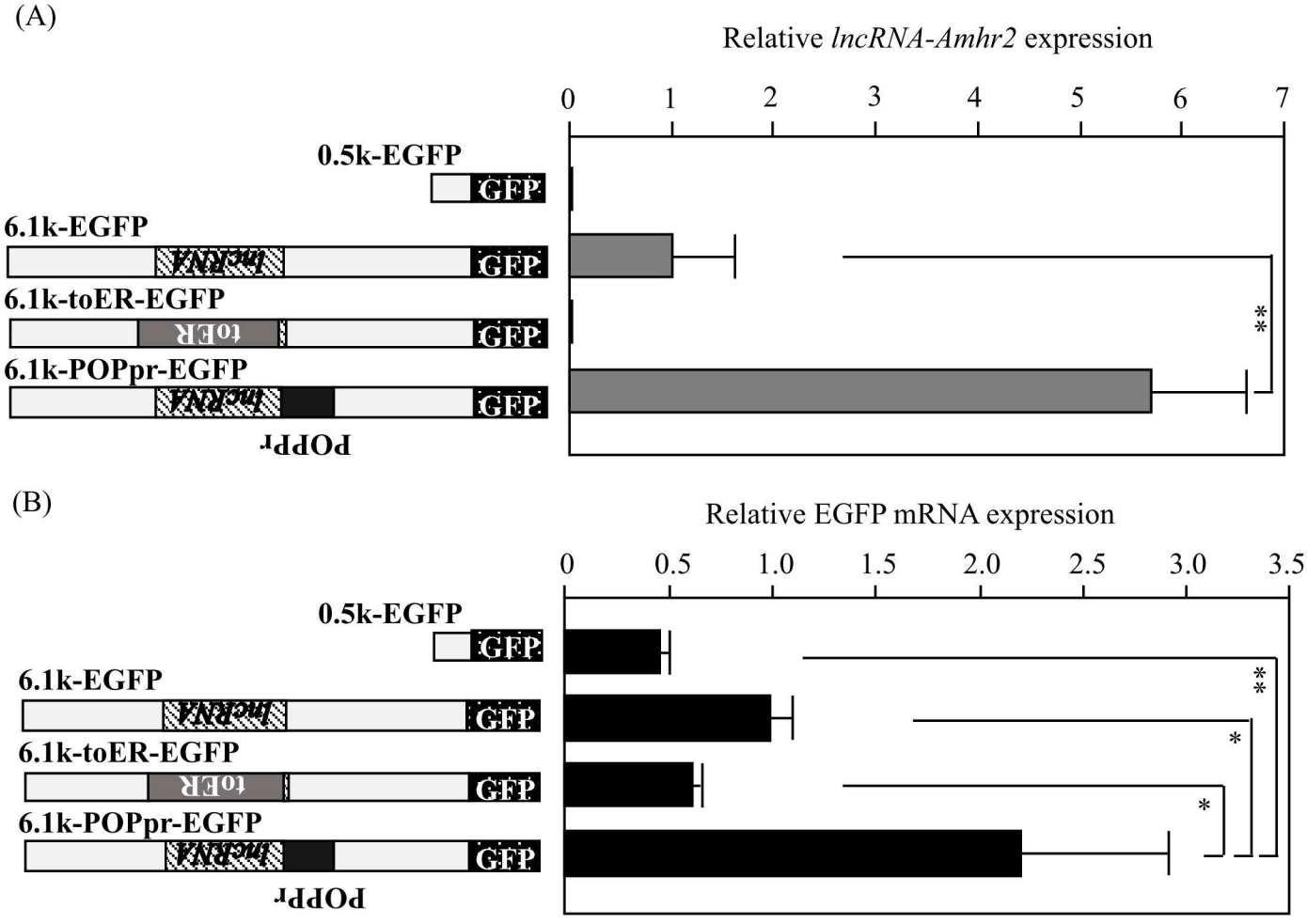
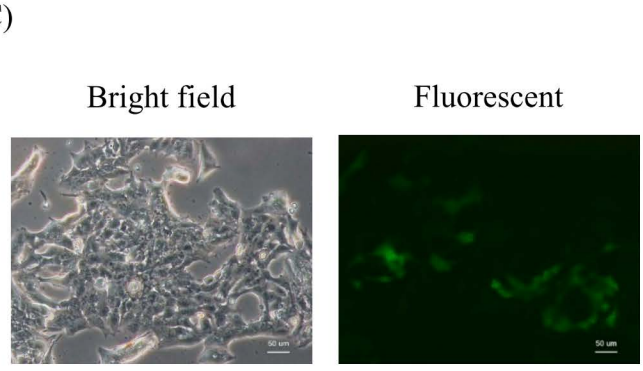
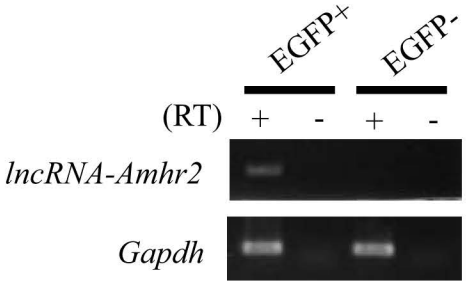

(A)
Relative luciferase activity
0
2
4
6
8
10
12
14
pGL3-Basic
Luc
0.5k-Luc
6.1k-Luc
lncRNA
6.1k-toER-Luc
toER
6.1k-ΔlncPr-Luc
6.1k-POPpr-Luc
POPPr
**
(B)
Relative luciferase activity
0
300
600
900
1200
1500
pGL3-Basic
RevPOPpr-Luc
POPpr-Luc
(C)
Relative luciferase activity
0
50
100
150
200
250
300
350
400
450
500
pGL3-Basic
0.5k-Luc
RevPOPpr-0.5k-Luc
POPpr-0.5k-Luc
(D)
Relative luciferase activity
0
3
6
9
12
15
18
21
24
pGL3-Basic
6.1k-Luc
6.1k-POPpr-toER-Luc
6.1k-POPpr-Luc

Figure 6


Relative luciferase activity
0
2
4
6
8
10
12
14
16
18
20
pGL3-Basic
Luc
0.5k-Luc
Luc
6.1k-Luc
lncRNA
Luc
6.1k-toER-Luc
toER
Luc
6.1k-ΔlncPr-Luc
lncRNA
Luc
6.1k-POPpr-Luc
lncRNA
Luc
POPpr
**
**
**
**
**


(A)
Relative lncRNA-Amhr2 expression
0
1
2
3
4
5
6
7
0.5k-EGFP
GFP
6.1k-EGFP
lncRNA
GFP
6.1k-toER-EGFP
toER
GFP
6.1k-POPpr-EGFP
lncRNA
GFP
POPpr
**
(B)
Relative EGFP mRNA expression
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
0.5k-EGFP
GFP
6.1k-EGFP
lncRNA
GFP
6.1k-toER-EGFP
toER
GFP
6.1k-POPpr-EGFP
lncRNA
GFP
POPpr
**
*
*


(C)
Bright field
Fluorescent


(D)
EGFP+
EGFP-
(RT)
+
-
+
-
lncRNA-Amhr2
Gapdh

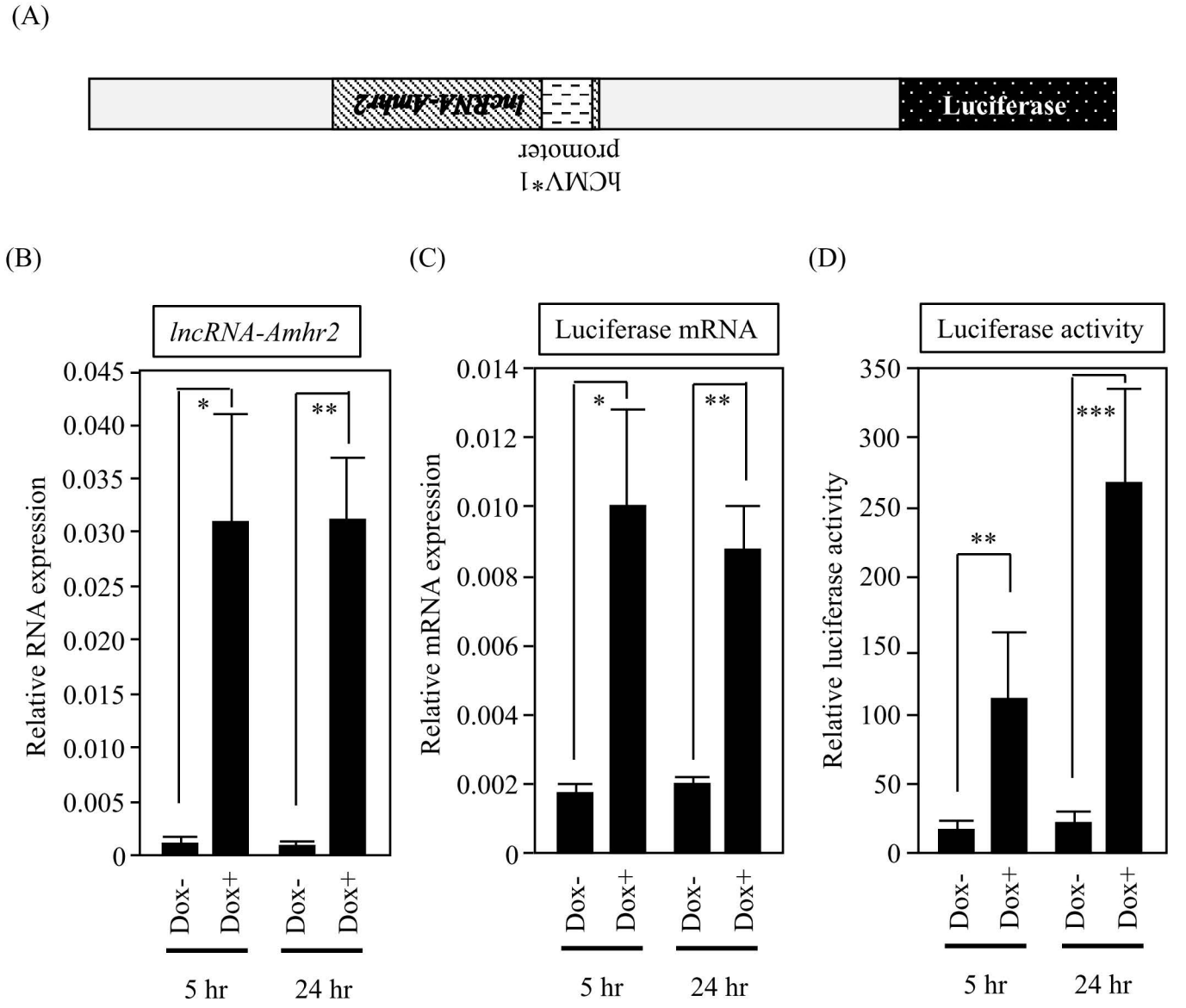

(A)
lncRNA-Amhr2
Luciferase
hCMV*1
promoter
(B)
lncRNA-Amhr2
Relative RNA expression
0.045
0.040
0.035
0.030
0.025
0.020
0.015
0.010
0.005
0
*
**
Dox-
Dox+
Dox-
Dox+
5 hr
24 hr
(C)
Luciferase mRNA
Relative mRNA expression
0.014
0.012
0.010
0.008
0.006
0.004
0.002
0
*
**
Dox-
Dox+
Dox-
Dox+
5 hr
24 hr
(D)
Luciferase activity
Relative luciferase activity
350
300
250
200
150
100
50
0
**
***
Dox-
Dox+
Dox-
Dox+
5 hr
24 hr

Table 1. Oligonucleotide primers used for RT-PCR and qRT-PCR

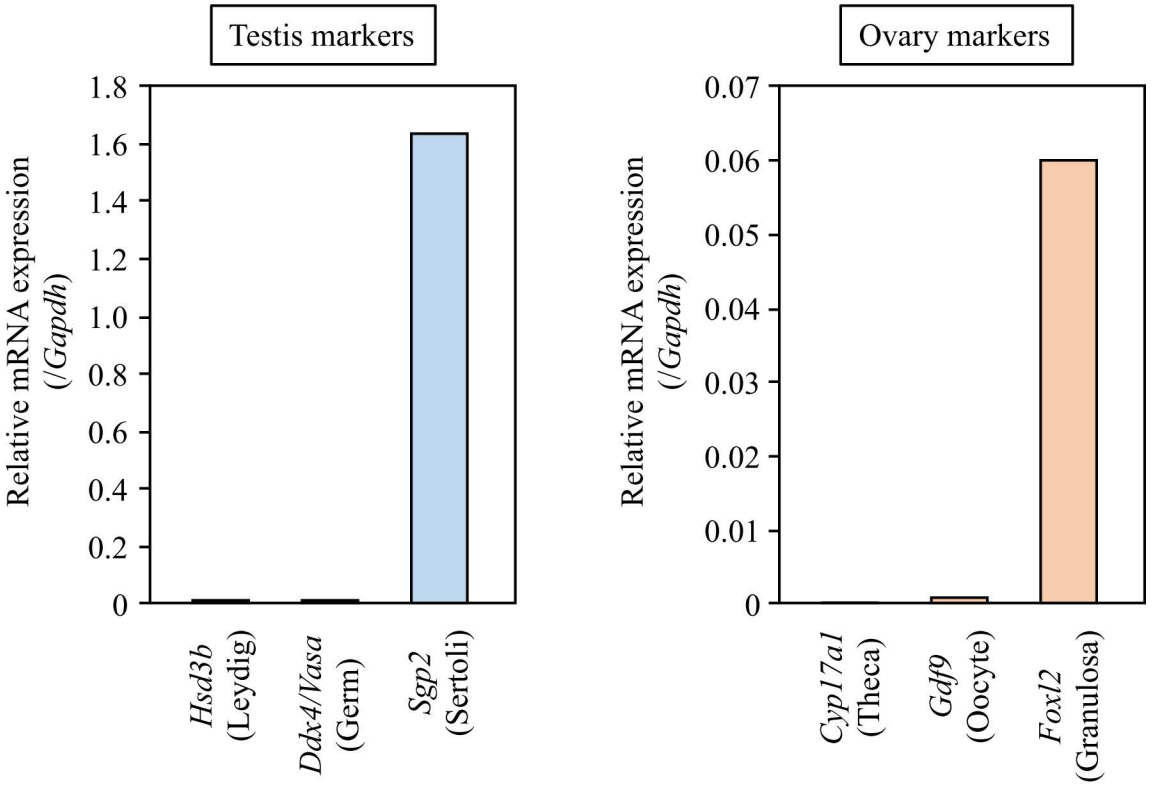
Designation	Forward	Reverse
RT-PCR followed by electrophoresis		
Amhr2	5’-GCTCCTGGAAGATTGCTGGG-3’	5’-ATGTCGGCCACACGCATGTC-3’
lncRNA-Amhr2	5’-ACAGGGCTGGAGACAGCAT-3’	5’-CCACCTCAGCCAAGTAACTG-3’
EGFP	5’-ACGTAAACGGCCACAAGTTC-3’	5’-TTGAAGTTCACCTTGATGCC-3’
Gapdh	5’-CATGACCACAGTCCATGCCATC-3’	5’-TAGCCCAAGATGCCCTTCAGTG-3’
RT-PCR with a specific primer for RT		
Primer for RT		5’-CCACCTCAGCCAAGTAACTG-3’
lncRNA-Amhr2	5’-AAAGACACTTGTCCCTGGCA-3’	5’-AAGCAGTCTTCCTGCCTCTT-3’
qRT-PCR		
Amhr2	5’-TGCTAGGCCTACAGCATGAC-3’	5’-TCGCTCGTGTACTGAGTCAA-3’
lncRNA-Amhr2	5’-GGCTTGTGTTGCCATAGAGA-3’	5’-AAGCAGTCTTCCTGCCTCTT-3’
Nr5a1/Sf1	5’-GCCACCCTACAGCTATCCAG-3’	5’-CTGGTCCTCCTCTGGCTCTA-3’
Actb	5’-GGTCATCACTATTGGCAACG-3’	5’-ACGGATGTCAACGTCACACT-3’
EGFP	5’-AGCAAAGACCCCAACGAGAA-3'	5’-GGCGGCGGTCACGAA-3'
Luciferase	5’-GGGACGAAGACGAACACTTC-3’	5’-GGTGTTGGAGCAAGATGGAT-3’
Gapdh	5’-CATGGCCTTCCGTGTTCCTA-3’	5’-CCTGCTTCACCACCTTCTTGA-3’

Table 2. Oligonucleotide primers used for RACE analyses

Designation	Forward	Reverse
5’RACE with ovarian granulosa cells		
Reverse transcription		5’-CCGTGCTTGGGTTCTGGGTT-3’
Gene specific primer for the first PCR		5’-ACTTATCTGATCTGGAGGCT-3’
Gene specific primer for the second PCR		5’-ATGCTGTCTCCAGCCCTGT-3’
5’RACE with the testis		
Reverse transcription		5’-ATGCTGTCTCCAGCCCTGT-3’
Gene specific primer for the first PCR		5’-AGAGACAGGAGCAGGAACCA-3’
Gene specific primer for the second PCR		5’-TGCCAGGGACAAGTGTCTTT-3’
3’RACE		
Gene specific primer for the first PCR	5’-CATCCTGGGCCTTGTCTCAA-3’	
Gene specific primer for the second PCR	5’-CAGTTACTTGGCTGAGGTGG-3’	

Table 3. Transgene expression and copy number of each transgenic line

Line	Green ovary	Green testis	Green gonad in embryo	Copy number
#318	No	No		1
#321	No	No		10
#326	No	Yes	No	3
#337	No	No		4
#338	No	No		2
#348	No	No		4
#356	No	No		2
#373	No	No		3
#376	No	No		4
#379	No	No		25



Supplemental Figure 1. qRT-PCR analysis of marker genes for the mouse ovary and testis in primary Sertoli and granulosa cells. Primary Sertoli cells were collected from 8-12 days old male mice, and primary granulosa cells were from immature female mice that were administrated with hormones. The cells were cultured for several days, and total RNAs were purified. cDNAs were synthesized with the oligo(dT) primer, and real time PCR was performed to detect the marker gene expression for each cell type. In the testis, *Hsd3b*, *Ddx4/Vasa*, and *Sgp2* were used as markers for Leydig cells, germ cells, and Sertoli cells, and in the ovary, *Cyp17a1*, *Gdf9*, and *Foxl2* were used for theca cells, oocytes, and granulosa cells.