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A long noncoding RNA transcribed from conserved noncoding sequences contributes to the mouse prolyl oligopeptidase gene activation

Shin Matsubara[1,*], Misuzu Kurihara[1], and Atsushi P. Kimura[1,2,†]

[1]Graduate School of Life Science, and [2]Department of Biological Sciences, Faculty of Science, Hokkaido University, Sapporo 060-0810, Japan

Running title: The POP gene activation by a long noncoding RNA

*Present address: Suntory Foundation for Life Sciences, Bioorganic Research Institute, Osaka 618-8503, Japan

†To whom correspondence should be addressed: Atsushi P. Kimura, Department of Biological Sciences, Faculty of Science, Hokkaido University, Sapporo 060-0810, Japan. Phone and Fax: 81-11-706-4452. E-mail: akimura@sci.hokudai.ac.jp

Note: Nucleotide sequence data for two types (2.2 kb and 2.8 kb) of *lncPrep+96kb* reported in this paper are available in the DDBJ/EMBL/GenBank databases under the accession numbers AB849012 and AB849013, respectively.

Abbreviations: Aip, aryl-hydrocarbon receptor-interacting protein; cDNA, complementary deoxyribonucleic acid; CGI, CpG island; ChIP, chromatin immunoprecipitation; CNS, conserved noncoding sequence; cRNA, complementary RNA; DIG, digoxigenin; eRNA, enhancer RNA; GFP, green fluorescent protein; Gapdh, glyceraldehyde 3-phosphate dehydrogenase; GSP, gene specific primer; hCG, human chorionic gonadotropin; lincRNA, long intergenic noncoding RNA; lncRNA, long noncoding RNA; nt, nucleotides; pancRNA, promoter-associated noncoding RNA; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; POP, prolyl oligopeptidase; PMSG, pregnant mare serum gonadotropin; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction; RACE, rapid amplification of cDNA ends; SD, standard deviation; shRNA, short-hairpin RNA; TSS, transcriptional start site

Summary

Prolyl oligopeptidase (POP) is a multifunctional protease which is involved in many physiological events, but its gene regulatory mechanism is poorly understood. To identify novel regulatory elements of the POP gene, we compared the genomic sequences at the mouse and human POP loci and found six conserved noncoding sequences (CNSs) at adjacent intergenic regions. From these CNSs, four long noncoding RNAs (lncRNAs) were transcribed and the expression pattern of one (*lncPrep+96kb*) was correlated with that of POP. *lncPrep+96kb* was transcribed as two forms due to the different transcriptional start sites and was localized at the nucleus and cytoplasm, although more was present at the nucleus. When we knocked down *lncPrep+96kb* in the primary ovarian granulosa cell and a hepatic cell line, the POP expression was decreased in both cells. In contrast, overexpression of *lncPrep+96kb* increased the POP expression only in the granulosa cell. Because *lncPrep+96kb* was upregulated with the same timing as POP in the hormone-treated ovary, this lncRNA could play a role in the POP gene activation in the granulosa cell. Moreover, a downstream region of the human POP gene was also transcribed. We propose a novel mechanism for the POP gene activation.

Keywords: prolyl oligopeptidase/ long noncoding RNA/ conserved noncoding sequence/ granulosa cell/ skeletal muscle

Introduction

Prolyl oligopeptidase (POP, E.C.3.4.21.26, also known as prolyl endopeptidase and post-proline cleaving enzyme; gene symbol *Prep*) is a serine endopeptidase which specifically digests small peptides up to 30 amino acids at the carboxyl side of an internal proline residue (1). POP is present in a wide range of species from bacteria to mammals and is involved in many important physiological events via its protease activity or by interacting with other proteins (2-5). In mammals, POP is expressed at different levels in different tissues (6-10), which probably guarantees its appropriate functions. Therefore, the POP gene expression must be strictly regulated in each tissue, but its regulatory mechanism is poorly understood.

The POP gene regulation has been investigated mainly by our group using the mouse as a model organism. Because the ovarian granulosa cell and the placenta expressed higher levels of POP mRNA than other tissues in mouse (8,11), we have focused on the activation mechanisms in these two tissues. First, we cloned a 914-bp promoter sequence and found that it has a strong transcriptional activity *in vitro* regardless of cell type (11,12). We then assessed the *in vivo* activity of this promoter and showed that it contained most elements to establish the appropriate POP localization in the placenta (13). In contrast, the promoter was not sufficient to appropriately activate the POP gene in the granulosa cell (13), and consistent with this, we identified a CpG island (CGI) in the gene body as a potential enhancer in the granulosa cell (11). However, this enhancer activity seemed too low to reproduce the endogenous level of POP mRNA, which suggested that other regulatory elements are required for the POP gene activation in the granulosa cell.

To identify gene regulatory elements, the comparison of noncoding genomic sequences between species is an effective approach. The genomic DNA sequence conserved beyond species, so-called ‘conserved noncoding sequence (CNS),’ has been often proved to be important for the gene regulation (14-16). In addition, more recent studies have revealed that the intergenic region which contains the entire or a part of CNS could be transcribed as a long noncoding RNA (lncRNA) which plays some roles in the regulation of adjacent genes (17,18).

The lncRNA is generally defined as an RNA which is greater than 200 nucleotides (nt) in length and is not translated into any proteins or peptides (19). Many lncRNAs are capped in 5’ end and polyadenylated in

3’ end of the transcript (20,21), but some like enhancer RNAs (eRNAs) does not have the poly(A) tail (22). Increasing number of lncRNAs has been recently reported to be essential in various biological processes such as the inflammatory response (23), cell differentiation and tissue development (24-29), the nuclear paraspeckle construction (30-32), and X chromosome inactivation (33). lncRNAs have also been found to play critical roles in some diseases (34-36). Importantly, many functions of lncRNAs were performed through their gene regulatory roles including the enhancer and silencer activity, the mRNA stability control, and the repression of microRNA activity (19,37).

In this study, we found that four lncRNAs were transcribed from CNSs located at the mouse POP gene locus, and the expression pattern of one lncRNA (*lncPrep+96kb*) was correlated with POP. When we knocked down and overexpressed *lncPrep+96kb* in the primary ovarian granulosa cell, the POP expression was decreased and increased, respectively. Moreover, the correlation between the POP and *lncPrep+96kb* expression was observed in the hormone primed ovary, and we also detected the noncoding transcription from 3’ flanking regions of the human POP gene. The results indicate that *lncPrep+96kb* contributes to the POP gene activation in the mouse ovarian granulosa cell.

Materials and Methods

Animals and tissue preparation

C57/BL6 and BDF1 (C57/BL6 × DBA2 F1) mice were purchased from CLEA Japan Inc. (Tokyo, Japan) and kept under the standard conditions (25 °C, 14-h light/10-h dark, and enough food and water). Tissues (liver, ovary, and testis) were collected from adult mice. Superovulation was induced using 3- and 4-week-old female mice as previously described (11) and the ovaries were collected at the indicated time points. The experimental procedures used in this study were approved by the Institutional Animal Use and Care Committee at Hokkaido University.

RNA extraction, DNase I treatment, and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from tissues or cultured cells using ISOGEN II (Nippon gene, Tokyo, Japan) according to the manufacturer's instructions. The RNA was further purified by the phenol/chloroform-isoamylalcohol extraction and ethanol precipitation. Total RNAs from the human skeletal muscle, liver, and testis were purchased from Clontech Laboratories, Inc (Mountain View, USA). The RNAs were treated with TURBO DNase I (Ambion, Austin, USA) for 30 min at 37 °C, and the reverse transcriptase reaction was performed with oligo$(dT)_{20}$ and Superscript III reverse transcriptase (Invitrogen, Carlsbad, USA). PCR reactions were done with the primers listed in Table I. Primer sequences for the human β-actin gene were described in a previous study by other researchers (38).

To determine the transcriptional direction of *lncPrep+96kb*, we conducted reverse transcriptase reaction using strand specific primers. To detect the sense-directed transcript, we used 5'-ACCGGAGCCTAGCAAATAG-3' for reverse transcription and amplified with primers 5'-GGAGCAGCTGAGATAGAAGC-3' and 5'-GGCCACCCATTTCTACTTAC-3'. For the antisense-directed transcript, 5'-GGAGCAGCTGAGATAGAAGC-3' was used for the reverse transcription and 5'-CTCAGCCCACACCTTTTGT-3' and 5'-ACCGGAGCCTAGCAAATAG-3' were for the following PCR.

5' and 3' rapid amplification of cDNA ends (5' RACE and 3' RACE)

For 5' RACE, cDNA was generated by using the total RNA from 3-week-old ovaries and a gene specific primer (GSP), 5'-GGCCACCCATTTCTACTTAC-3', for reverse transcription. Following the addition of oligodeoxycytidine by terminal deoxynucleotidyl transferase (Takara, Ohtsu, Japan), the first PCR was performed with the abridged anchor primer, GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG, and GSP1, 5'-GACTAACAGGCTAGCTTGGT-3'. The second nested PCR was performed using GSP2, 5'-GCTTGGTCATTTACCCAACC-3', and abridged universal amplification primer, GGCCACGCGTCGACTAGTAC.

For 3' RACE, the reverse transcriptase reaction was performed using oligo$(dT)_{20}$ connected to an adaptor sequence, CTGATCTAGAGGTACCGGATCC. The first and second PCRs were performed using GSP3, 5'-CTCAGCCCACACCTTTTGT-3', and GSP4, 5'-ATGCTACCAATCCCCAAAAC-3', respectively,

with the adaptor primer.

All the amplified products were subcloned into a pBluescript vector (Stratagene, La Jolla, USA) by the TA-cloning method, and twelve subclones for each sample were sequenced.

Cell culture

The mouse hepatic cell line, Hepa1-6, and the primary granulosa cell were obtained and maintained as previously described (11).

Preparation of subcellular fractions

The Hepa1-6 cell and the granulosa cell cultured in 35-mm dishes were washed with phosphate-buffered saline (PBS) and collected by the treatment with 0.25% trypsin/1 mM EDTA. After being washed with PBS, the cells were lysed in NP-40 lysis buffer (10 mM Tris-HCl, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP-40, pH 7.5) on ice for 10 min. Then the lysates were centrifuged at 1500 rpm for 5 min at 4°C and the resulting supernatants were collected as the ‘cytoplasm’ subfraction. The precipitations were washed with NP-40 lysis buffer several times, and the resulting pellets were used as the ‘nucleus’ subfraction. The total RNA of each subfraction was purified as described above. The successful fractionation was confirmed by amplifying the signals from mature and immature mRNA of the *glyceraldehyde 3-phosphate dehydrogenase (Gapdh)* gene which were present only in the cytoplasmic and nuclear subfractions, respectively. To detect the mature and immature mRNA of the *Gapdh* gene, forward primers at exon 5 and intron 5 and a common reverse primer at exon 6 were used. The primer sequences are listed in Table I.

In situ hybridization for cultured cells

A probe for *lncPrep+96kb* was obtained by RT-PCR with the mouse ovarian total RNA using a primer pair of 5’-GGAGCAGCTGAGATAGAAGC-3’ and 5’-GGCCACCCATTTCTACTTAC-3’. Sense and antisense complementary RNA (cRNA) probes were synthesized by *in vitro* transcription using digoxigenin (DIG)-UTP (Roche Molecular Biochemicals, Mannheim, Germany) and T3 or T7 RNA polymerase (Promega Corporation, Madison, USA).

The Hepa1-6 cell (4×10^5) and the granulosa cell (from two superovulation-induced ovaries) were cultured on the cover glass in 35-mm dishes. The samples were fixed with 3.7% paraformaldehyde in PBS, permeabilized with 0.5% Triton-X in PBS, and washed with PBS. The cover glasses were then treated with prehybridization buffer (2×SSC, 20% formamide, 0.2% bovine serum albumin, 1 mg/ml of yeast transfer RNA) at 37°C for 15 min. Then the cover glasses were incubated at 37°C for 16-18 h in the same buffer containing 100-200 ng/ml DIG-labeled cRNA probes. The hybridized cover glasses were washed in a series of 2×SSC, 20% formamide, 2×SSC, and 1×SSC twice each for 5 min at 42°C. The blocking, antibody reaction, and signal development were performed as previously described (11).

Plasmid construction

For the knockdown experiment, a double-stranded DNA which contained a specific sequence against *lncPrep+96kb* or the green fluorescent protein (GFP) gene was generated by annealing the following synthetic oligonucleotides: the top strand 5’-GATCCGGTTCTCAATATCAATTAACCGTGTGCTGTCCGGTTAATTGATATTGAGAACCTTTTTTA-3’ and the bottom strand 5’-AGCTTAAAAAAGGTTCTCAATATCAATTAACCGGACAGCACACGGTTAATTGATATTGAGAACCG-3’ for *lncPrep+96kb* (specific sequences against *lncPrep+96kb* are underlined); the top strand 5’-GATCCGCATCAAGGTGAACTTCAAGTGTGCTGTCCTTGAAGTTCACCTTGATGCTTTTTTA-3’ and the bottom strand 5’-AGCTTAAAAAAGCATCAAGGTGAACTTCAAGGACAGCACACTTGAAGTTCACCTTGATGCG-3’ for GFP (specific sequences against GFP are underlined). The DNA fragment was inserted into pBAsi-mU6 Neo vector (Takara) at the *Bam*HI-*Hin*dIII site. After confirming the sequence, the resulting plasmids were named sh-lncPrep+96kb and sh-GFP, respectively.

For the overexpression experiment, full-length sequences of *lncPrep+96kb* (2.2 kb and 2.8 kb) were amplified by RT-PCR using cDNA from the 4-week-old ovary with KOD FX Neo (Toyobo, Osaka, Japan). The primer sequences for 2.2 kb and 2.8 kb variants are listed in Table I. The amplified products were subcloned into an *Eco*RV site of pBluescript (Stratagene) and the sequence was confirmed. The resulting

constructs were designated as pBS-2.2 kb and pBS-2.8 kb. We digested pBS-2.2 kb and pBS-2.8 kb with *Eco*RI and *Xho*I and inserted the lncRNA fragments into a pcDNA3.1 (+) vector (Invitrogen) at the *Eco*RI-*Xho*I site. These constructs were designated as pcDNA-2.2 kb and pcDNA-2.8 kb.

Knockdown and overexpression of lncPrep+96kb

The constructs for the knockdown and overexpression experiments (sh-lncPrep+96kb, sh-GFP, pcDNA-2.2kb, and pcDNA-2.8kb) were transfected into the Hepa1-6 or granulosa cell by using GeneJuice Transfection Reagent (Novagen, Medison, USA) according to the directions. Twenty-four hours after the transfection, the cells were selected by the treatment with 1-1.5 mg/ml of G418 (Nacalai Tesque, Kyoto, Japan) for 3-5 days. The medium containing G418 was changed every day. After the selection, the cells were dissolved in ISOGEN II (Nippon gene), and total RNAs were purified. These RNAs were used for RT-PCR or quantitative RT-PCR (qRT-PCR).

qRT-PCR analysis

cDNA from each tissue or cultured cell was synthesized as described above. qPCRs were performed as previously described (11). The primer sequences are listed in Table I.

Establishment of the Hepa1-6 cell line stably transfected with sh-lncPrep+96kb

The shRNA construct, sh-lncPrep+96kb, was transfected into the Hepa1-6 cell as described above. For the stable knockdown of the *lncPrep+96kb* expression, transfected cells were selected with 1-1.5 mg/ml of G418 for more than 20 days. The cells were passaged for several times in the medium containing G418. The medium containing G418 was changed every day. After the selection, the knockdown efficiency was evaluated by qRT-PCR and the results were shown in Supplementary Fig. 1.

Chromatin immunoprecipitation (ChIP) assay

The histone H3K9K27 acetylation pattern was investigated by the ChIP analysis according to the method we described previously (11).

Statistical analysis

Results were expressed as the average ± standard deviation (SD) of at least three independent experiments and analyzed by Student's t test using JSTAT statistical software (version 12.6 for Windows, Masato Sato, Japan). Differences were considered statistically significant at $P<0.05$.

Results

Search for CNSs and detection of lncRNAs at the mouse POP locus

To identify novel regulatory regions, we compared 132 kb of the mouse genomic sequence, which contained 92 kb of the POP gene and 20 kb each of the 5' and 3' adjacent region, with the corresponding human sequence. This was based on the concept that the primary sequences for important regulatory elements are generally well conserved beyond species (39). By this analysis, we found many CNSs within, upstream of, and downstream of the POP gene (Fig. 1A). Because recent studies have revealed that a large part of the intergenic region was transcribed as lncRNAs that were involved in the regulation of neighboring gene expression (20,21), this time we focused on two and four CNSs upstream and downstream, respectively, of the POP gene. We checked whether these CNSs were transcribed in the three mouse tissues, the ovary, testis, and liver (Fig. 1B). In these three tissues, POP mRNA was expressed at a higher level in the ovary than in the testis and liver (11). If any lncRNA was expressed in a similar pattern to POP, it could be involved in the POP gene regulation.

Our RT-PCR analysis demonstrated that at least five out of six CNSs were transcribed in the ovary, and two of them (at the position of +96kb) were expressed as a single transcript (Fig. 1B, specific bands marked by arrowheads). One CNS was completely overlapped with a repeat sequence and we could not design a specific primer set for it. We detected some nonspecific bands with lower molecular sizes, possibly from cDNA or contaminated genomic DNA, in some cases (Fig. 1B, marked by white circles). The lncRNAs derived from the positions of -20 kb and -18 kb were expressed at high levels in the ovary and testis, but not

in the liver (Fig. 1B), which meant that the expression of these lncRNAs was not correlated to POP. In contrast, the expression of lncRNAs from +96 kb (*lncPrep+96kb*) and +106 kb showed a correlation with POP. They were expressed at higher levels in the ovary than in the other two tissues. Especially, *lncPrep+96kb* contained two CNSs, was the largest in size among the four lncRNAs (Fig. 1A), and was detected by 30 cycles of PCR in contrast to 40 cycles for the other lncRNAs (Fig. 1B). Therefore, we hypothesized that *lncPrep+96kb* had some roles in the POP gene regulation and further analyzed this lncRNA by the following experiments.

Characterization of lncPrep+96kb

We first determined the transcriptional direction of *lncPrep+96kb* by RT-PCR using strand specific primers for reverse transcription. We obtained a specific band from sense-strand of *lncPrep+96kb* in the ovary, testis, and liver (Fig. 2A), which indicated that *lncPrep+96kb* was transcribed in the same direction as the POP gene. Consistent with figure 1B, the intensity of the signal was strongest in the ovary. To test the possibility that *lncPrep+96kb* could be a part of an exon of the POP gene, we conducted RT-PCR by using a sense primer designed in exon 15 of POP and an antisense primer in *lncPrep+96kb*, but no specific product was amplified (data not shown).

We next determined the 5’ and 3’ ends of *lncPrep+96kb* by the RACE analyses. By 3’RACE, we detected a single band, from which twelve subclones were sequenced and four different positions were found to be the 3’ end (Fig. 2B). Among them, we determined an adenine to be the major 3’ end of *lncPrep+96kb* as indicated in figure 2B, because more subclones contained this nucleotide as the end. In the 5’RACE experiment, two specific products were amplified and we examined the sequences of twelve subclones from each. For a longer product (2.8 kb), eleven subclones contained the same transcriptional start site (TSS) which should be the major TSS of this transcript (Fig. 2B). On the other hands, a shorter product (2.2 kb) included three different TSSs and we identified an adenine as the major TSS because eight subclones contained this position as the TSS (Fig. 2B). Taken together, *lncPrep+96kb* was assumed to be present as two major transcripts and their full-lengths were 2162 nt and 2820 nt long (Fig. 2B). To confirm that these two forms of *lncPrep+96kb* were really present as single transcripts, we performed RT-PCR with the ovary

RNA using primers designed at the major 5' and 3' ends and successfully amplified the entire sequences of both (data not shown).

Because we amplified both forms of *lncPrep+96kb* after reverse transcription with the oligo(dT) primer, and because poly(A) sequences longer than the oligo(dT) length were observed in some subclones by 3'RACE, *lncPrep+96kb* was presumably polyadenylated. To confirm the full length of this lncRNA and to see if there were any splice variants of it, we conducted the Northern blot analysis, but we did not detect any signals (data not shown). This was probably because the expression level of *lncPrep+96kb* was below the detection limit of our Northern analysis even though it was transcribed at a higher level than other lncRNAs at the mouse POP locus. However, as long as we performed the RACE analysis with the ovary total RNA, we did not obtain any splice variants, which suggested that no major splice variants for *lncPrep+96kb* were present in this tissue.

To determine the subcellular distribution, we fractionated the primary granulosa cell and the Hepa1-6 cell, which derived from the mouse liver, into the cytoplasmic and nuclear subfractions. We purified total RNAs from these fractions and assessed the *lncPrep+96kb* expression. RT-PCR to amplify mature (Fig. 3A, ex5-ex6) and immature (Fig. 3A, int5-ex6) mRNA of the *Gapdh* gene indicated that our fractionation was performed successfully. The *lncPrep+96kb* transcript was detected in both the cytoplasmic and nuclear subfractions of both cells, although more signals appeared to be present in the nucleus (Fig. 3A). In fact, when we quantified and normalized the *lncPrep+96kb* signal to *Gapdh* (ex6-ex6), the nucleus contained 2.3-fold higher level of *lncPrep+96kb* than the cytoplasm in the granulosa cell (Fig. 3A, graph). In the Hepa1-6 cell, the nuclear *lncPrep+96kb* level was 6.5-fold higher than the cytoplasmic RNA (Fig. 3A, graph). We repeated this analysis for confirmation, and the result showed that the nucleus contained 5.6- and 3.4-fold higher levels of *lncPrep+96kb* than the cytoplasm in the granulosa cell and the Hepa1-6 cell, respectively (data not shown). These indicated that *lncPrep+96kb* was localized at both the nucleus and the cytoplasm, but more was present in the nucleus.

To further confirm the subcellular localization of *lncPrep+96kb*, we performed the *in situ* hybridization analysis with the Hepa1-6 cell and the granulosa cell. This showed that the *lncPrep+96kb* transcript was localized in both the cytoplasm and nucleus of these cells (Fig. 3B). No signal was observed in the cells

hybridized with a sense probe (Fig. 3B). Although this analysis was not so quantitative, the signals seemed to be stronger in the nucleus than in the cytoplasm of both cell types. The nuclear localization suggested that *lncPrep+96kb* could perform its function without being translated, and indeed the Coding Potential Calculator (http://cpc.cbi.pku.edu.cn/) classified both forms of *lncPrep+96kb* as lncRNAs.

Knockdown and overexpression of lncPrep+96kb

In order to evaluate whether *lncPrep+96kb* is involved in the POP gene regulation, we conducted the knockdown experiment using a short-hairpin RNA (shRNA) against *lncPrep+96kb* (sh-lncPrep+96kb). The shRNA against the GFP gene (sh-GFP) was used as a negative control. Three different shRNAs for *lncPrep+96kb* were tested (data not shown) and the most effective one was used for the analysis. We transfected the shRNAs into the primary granulosa and Hepa1-6 cell, selected the cells with G418 to eliminate non-transfected cells, and assessed the POP expression level by qRT-PCR. Successful knockdown was confirmed by RT-PCR of *lncPrep+96kb* (Fig. 4A, electrophoretic image). By this knockdown, the POP mRNA level was decreased to approximately 60% of the control in both cells (Fig. 4A, graph), which indicated that *lncPrep+96kb* played a role in the POP gene activation in these cells.

It was very important to determine which of the nuclear and cytoplasmic *lncPrep+96kb* was involved in the POP gene activation. However, in our knockdown experiment, we transiently transfected the shRNA construct and selected the G418-resistent cell, which resulted in low yield of the transfected cell. This made the cell fractionation into nucleus and cytoplasm impossible. Therefore, we decided to generate the cell stably transfected with the shRNA construct. Because it was impossible to passage the primary granulosa cell, we used the Hepa1-6 cell for this experiment. We introduced sh-lncPrep+96kb into the Hepa1-6 cell, selected the transfected cells by the treatment with G418 for more than 20 days, and successfully established the stable cell line. In this cell, *lncPrep+96kb* and POP mRNA were downregulated to 24% and 37%, respectively, of the non-transfected Hepa1-6 cell (Supplementary Fig. 1). Then, we fractionated this cell into the nuclear and cytoplasmic subfractions and measured the *lncPrep+96kb* expression level by qRT-PCR. The result demonstrated that the nuclear lncRNA was decreased to 34% of the non-transfected cell, but the cytoplasmic lncRNA was not affected (Fig. 4B). This indicated that, at least in the Hepa1-6 cell, the nuclear

lncPrep+96kb activated the POP gene expression.

We next investigated an effect of overexpression of *lncPrep+96kb* on the POP gene expression. By the transfection with two types of vectors (pcDNA-2.2kb and pcDNA-2.8kb) and the selection with G418, we could successfully overexpressed the two types of *lncPrep+96kb* (Fig. 4C, electrophoretic image). The 2.2-kb transcript was detected by the overexpression of both pcDNA-2.2kb and pcDNA-2.8kb because the 2.8-kb transcript contained the entire 2.2-kb sequence. In the granulosa cell, overexpression of the *lncPrep+96kb* transcripts resulted in 2- to 2.5-fold increase of the POP mRNA level (Fig. 4C, graph). However, interestingly, the POP expression was unaltered in Hepa1-6 by the overexpression, which suggested that *lncPrep+96kb* activated the POP expression by different mechanisms between these two cell types. The data showed that the ectopic *lncPrep+96kb* expression could activate the POP gene in the granulosa cell.

Histone acetylation in the Hepa1-6 cell stably transfected with the sh-lncPrep+96kb construct

To gain mechanistic insight into how *lncPrep+96kb* activated the POP gene expression, we investigated the histone acetylation, a well-known mark for active chromatin. We have already found that the POP promoter was acetylated at a high level both in the primary granulosa cell and in the liver and a potential enhancer, CGI-2, was acetylated only in the granulosa cell (11). By using the Hepa1-6 cell line which was stably transfected with the sh-lncPrep+96kb construct, we performed the ChIP analysis to determine the histone H3K9K27 acetylation pattern at the mouse POP locus. As our previous study (11), we examined acetylation levels at five regions of the mouse POP locus: 5’ flanking, promoter, gene body, CGI-2, and 3’ flanking (Fig. 5A). The 3’ flanking region did not overlap with the region from which *lncPrep+96kb* was transcribed but was positioned just downstream of it. In the non-transfected Hepa1-6 cell, a high level of acetylation was observed only at the promoter, and CGI-2 was not acetylated (Fig. 5B, black bars). This pattern was similar to that in the liver which expressed POP mRNA at a lower level than the granulosa cell (11). Indeed, the Hepa1-6 cell expressed a lower level of POP mRNA than the granulosa cell (data not shown). In the Hepa1-6 cell which was stably transfected with the sh-lncPrep+96kb construct, the histone acetylation pattern was not different from that in the non-transfected cell (Fig. 5B). This indicated that

lncPrep+96kb was not involved in the regulation of histone acetylation at least in the Hepa1-6 cell.

Hormonal response of POP and lncPrep+96kb in the mouse ovary

Our current data demonstrated that *lncPrep+96kb* could activate the POP mRNA expression in the granulosa cell. We then assessed whether *lncPrep+96kb* had the physiological significance in the mouse ovary. POP is specifically expressed in the granulosa cell, which plays an important role in generating functional oocytes in response to gonadotropins, and POP is known to be regulated by the hormones in the rat and porcine ovary (7,40). Therefore, we investigated the hormonal response of POP and *lncPrep+96kb* in the mouse ovary. We primed pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) into 3- or 4-week-old mice at a 48-hr interval to induce the follicular development and ovulation. By this treatment, follicles were expected to grow mainly after the hCG injection, and the ovulation occurred around 12 hr later than that. Consistent with our previous study (7), the qRT-PCR analysis demonstrated that the POP mRNA expression gradually increased by the hCG injection and reached a peak at 6 hr (Fig. 6A). Interestingly, the *lncPrep+96kb* expression was also induced to the highest level at 6 hr after the hCG injection (Fig. 6B). This coincidence of the POP expression with *lncPrep+96kb* suggested that this lncRNA could be actually involved in the POP gene activation in the mouse ovary.

lncRNA expression from CNSs at the human POP locus

We finally assessed the possibility that lncRNAs could also be transcribed at the human POP locus, because *lncPrep+96kb* encompassed CNSs between mouse and human. Human POP mRNA was known to be expressed at a high level in the skeletal muscle (6,10), so we purchased total RNA from this tissue and performed RT-PCR. Two other tissues, the liver and the testis, were also used for comparison. As a result, the skeletal muscle and the testis expressed higher levels of human POP mRNA than the liver (Fig. 7). While the high expression in the skeletal muscle was consistent with previous studies (6,10), the high level of POP in the testis was unexpected. One study performed the Northern blot analysis with the human testis RNA and showed that this tissue did not express POP mRNA at such a high level (10). However, the RNA amount applied to each lane in that Northern analysis might be different as evidenced by the GAPDH levels. Based

on our current data, we claim that the human POP expression is high not only in the skeletal muscle but also in the testis.

We then examined whether the human genomic regions corresponding to the CNSs, which was included in *lncPrep+96kb*, were transcribed (Fig. 7A). Surprisingly, we failed to detect any specific transcripts from CNSs in the skeletal muscle and liver, but the signals were observed in the testis (Fig. 7B, CNS1, CNS2). This indicated that CNS1 and CNS2 were transcribed at the human POP locus, but their expression was not correlated to POP. To further examine whether there were any 3' region of the human POP gene transcribed in the skeletal muscle, we investigated a sequence upstream of the CNSs. As a result, this region was transcribed in the skeletal muscle and testis, although it could not be an ortholog of *lncPrep+96kb* (Fig. 7B, 3' proximal). These results indicated that, in human, some sequences downstream of the POP gene were transcribed probably as lncRNAs, but their expression patterns were not necessarily correlated to POP.

Discussion

In the present study, we found four intergenic transcripts from upstream and downstream of the mouse POP gene (Fig. 1). Although this time we focused on *lncPrep+96kb*, the other transcripts might also be lncRNAs involved in the POP gene regulation irrespective of their low levels of expression. Indeed, many lncRNAs, which were expressed at low levels, have been reported to participate in the regulation of various genes (41). It is interesting that expression patterns of these transcripts were different between tissues. Relatively high levels of lncRNAs were expressed from CNSs upstream and downstream of the POP gene in the ovary, but only from upstream in the testis, while these CNSs were transcriptionally inactive in the liver (Fig. 1B). If these lncRNAs were really involved in the POP gene regulation, the combination of these transcripts might affect its tissue specific expression.

lncPrep+96kb was localized in both the nucleus and cytoplasm although more was present in the nucleus (Fig. 3). Considering that its full length contained an open reading frame for a peptide of 62 amino acids, *lncPrep+96kb* might possibly be translated to a peptide. Indeed, recent ribosome profiling analyses

indicated that many lncRNAs were bound by ribosomes (42,43). However, other studies argued that a majority of lncRNAs did not encode proteins (44,45), suggesting the function of *lncPrep+96kb* as an RNA molecule. To see if *lncPrep+96kb* activated the mouse POP expression in the nucleus or in the cytoplasm, we generated the Hepa1-6 cell line in which *lncPrep+96kb* was stably knocked down. In this cell line, shRNA exclusively downregulated the nuclear type of *lncPrep+96kb* (Fig. 4B), which indicated that *lncPrep+96kb* activated the POP gene expression in the nucleus. This suggests that *lncPrep+96kb* activates the POP expression in the nucleus of the granulosa cell, too. Taken together with that more *lncPrep+96kb* was localized in the nucleus than in the cytoplasm of both the Hepa1-6 and granulosa cell (Fig. 3), *lncPrep+96kb* is likely to be a lncRNA which controls the POP gene transcription.

lncRNAs are now classified into many categories. Noncoding RNAs transcribed from an intron, a promoter, and an intergenic region are called as an intronic RNA, a promoter-associated noncoding RNA (pancRNA), and a long intergenic noncoding RNA (lincRNA), respectively (46,47). When a lincRNA has an enhancer activity to increase the transcription of other genes, it is called as an enhancer-like RNA or an activating RNA (48). An antisense RNA is transcribed in the opposite direction to a coding gene (49), and eRNAs are transcribed from enhancers (22,50). Noncoding transcription is also observed in locus control regions at the β-globin and human growth hormone gene loci (51-53), and some types of cells contain circular RNAs (54-57). Although not all of these RNAs were functionally investigated, many of them were found to be involved in the repression or activation of protein-coding genes. Among these RNAs, *lncPrep+96kb* was similar to enhancer-like RNAs of lincRNAs in that it was transcribed from an intergenic region, was likely to be polyadenylated at its 3’ end, and contributed to the activation of a protein-coding gene (POP) (48).

Several studies have reported how lncRNAs activated the expression of protein-coding genes in the nucleus. *HOTTIP* and *Mistral* were transcribed from intergenic regions at the human and mouse *HOXA/HoxA* gene cluster and activated the cluster genes by recruiting the histone methyltransferase, MLL complex, to the target genes (58,59). pancRNAs were transcribed just upstream of TSSs of protein-coding genes and changed their epigenetic states in rat cell lines (47). Some enhancer-like RNAs were reported to interact with mediators and change the chromatin conformation to activate the target genes (60). eRNAs have

been reported to establish the chromatin looping and generate the interaction of the enhancers with their target genes (61-63). In the current study, the *lncPrep+96kb* knockdown did not change the histone acetylation pattern in the Hepa1-6 cell (Fig. 5), which suggested that it might activate the POP gene, for example, by associating with mediators to establish the active chromatin domain. In this case, by knocking down *lncPrep+96kb*, the mediator complex might become unstable, which might result in the downregulation of the POP expression. Alternatively, *lncPrep+96kb* might be involved in the regulation of other epigenetic status such as histone H3K4 methylation or DNA methylation.

The lncRNA can regulate the gene expression by acting in *cis* and in *trans*. Generally, the lncRNA derived from the same chromosome as the target gene is considered to act in *cis*, whereas it from the different chromosome is in *trans*. For example, Kcnq1ot1, transcribed from the same chromosome as its target, Kncq1, induced a repressive histone mark in *cis* (64). In contrast, lincRNA-p21 mediated p53-dependent repression of several genes, which were located on different chromosomes, in *trans* (65). In case of *lncPrep+96kb*, its ectopic expression upregulated the endogenous POP expression in the granulosa cell in *trans* (Fig. 4), although it did not necessarily mean that *lncPrep+96kb* actually worked in *trans in vivo*. By contrast, in the Hepa1-6 cell, the overexpression did not increase the POP expression (Fig. 4). This might be because the mechanism by which *lncPrep+96kb* activated the POP gene expression was different between the two cells or because the exogenous *lncPrep+96kb* was overexpressed mainly in the cytoplasm of the Hepa1-6 cell. In any case, these data suggest that *lncPrep+96kb* works by different mechanisms between various types of cells and may have distinct functions in different tissues.

The ovary is controlled by gonadotropins to generate mature oocytes and many genes are known to be actually regulated by follicle stimulating hormone and luteinizing hormone (66). We previously reported that POP is regulated by gonadotropins in rat and porcine (7,40), and consistent with the results, here we showed that the injection of PMSG and hCG induced the POP expression (Fig. 6). Interestingly, the *lncPrep+96kb* level increased with the same timing as POP mRNA (Fig. 6). Considering that *lncPrep+96kb* could activate the POP expression in the granulosa cell, *lncPrep+96kb* might mediate the hormone signal to the POP gene. In other words, hCG might first increase the *lncPrep+96kb* level, and thereby the POP expression might be upregulated by the lncRNA. In this context, it is noteworthy that some lncRNAs were upregulated by

hormones (62,67-69). Especially, a very recent report showed that eRNAs are important for the induction of target genes by estrogen (62). A similar mechanism may work for the POP gene activation by *lncPrep+96kb* in the granulosa cell.

It is not conclusive whether *lncPrep+96kb* is involved in the basal level or induced level of the POP expression. However, it probably contributes to the basal level of the POP expression, because of two reasons. First, *lncPrep+96kb* was expressed in the ovary from immature mice which were not administrated with gonadotropins (Fig. 6B, PMSG 0 h). Second, in the Hepa1-6 cell, which was not exposed to any hormones, *lncPrep+96kb* was involved in the POP gene activation as shown by our knockdown experiments (Fig. 4). On the other hands, it is not clear whether this lncRNA is involved in the hormone induction. In this study, we indicated that *lncPrep+96kb* could activate the POP gene expression in the granulosa cell collected from hormone-primed immature mice, and that its expression after the PMSG and hCG injection was correlated to POP. These suggest that *lncPrep+96kb* may play a role in the POP gene induction by gonadotropins.

Taken together with our previous findings, we propose the following model for the mouse POP gene activation in the ovarian granulosa cell. A 0.9-kb promoter has a minimum activity in the granulosa cell (13), so the transcription needs to be enhanced. As we previously reported (11), CGI-2 located around exon 15 is a potential enhancer for the POP gene (Fig. 1A), and it may actually increase the POP transcription *in vivo*. *lncPrep+96kb* is transcribed from the 3' flanking sequence of the POP gene at a high level in the granulosa cell and probably enhances the POP gene transcription. These two elements (CGI-2 and *lncPrep+96kb*) may be able to act synergistically or independently to activate the POP gene. Alternatively, CGI-2 may possibly be related to the activation of *lncPrep+96kb*. However, because *lncPrep+96kb* and CGI-2 could both increase the POP expression only by a few folds, additional element(s) are probably needed for the ovarian POP gene activation.

Finally, we investigated noncoding transcription at the human POP locus to see if any lncRNAs were transcribed from the 3' region of the human POP gene. Unexpectedly, we could observe the transcription signals from CNS1 and CNS2 only in the testis, and another human tissue expressing a high level of POP mRNA, the skeletal muscle, did not express the CNSs. The data indicates that a human ortholog of

lncPrep+96kb probably exists, but its expression is not correlated to POP. This in turn suggests that noncoding transcription from CNS1 and CNS2 is not involved in the human POP gene activation. Instead, we found that an upstream region of the CNSs was transcribed at a higher level in both the testis and the skeletal muscle but not in the liver (Fig. 7). This indicated that the expression of this lncRNA was correlated to POP. Therefore, it may be possible that the lncRNA transcribed from the 3' proximal region plays a role in the human POP gene activation, although it is not an ortholog of *lncPrep+96kb*. In general, the primary structure of lncRNAs is not well conserved beyond species, but there are some lncRNAs that are functionally conserved (70,71). The transcript from the 3' proximal region of the human POP gene may be functionally similar to *lncPrep+96kb*. Further studies will be necessary to reveal the whole mechanism of the POP gene regulation.

In conclusion, we identified a novel lncRNA transcribed from CNSs downstream of the mouse POP gene. This lncRNA, namely *lncPrep+96kb*, contributed to the POP gene activation in the granulosa cell by our knockdown and overexpression experiments, and was induced with the same timing as POP by gonadotropins. In human, some lncRNAs were transcribed at the 3' flanking region of the POP gene, but their expression patterns were not necessarily correlated to POP. These data provide a novel insight into the mechanism for the POP gene activation.

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Conflict of interest

The authors certify that they have nothing to disclose.

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Figure legends

Fig. 1. CNSs and their noncoding transcription at the mouse POP locus. (A) The mouse POP locus was illustrated with exon numbers and CpG islands (CGI-1 and CGI-2) at the top. The graph shows the sequence homology to the human POP locus. Conserved sequences depicted with dark blue are exons and the other conserved regions are CNSs (pink). Four intergenic regions (light blue boxes) are enlarged below and the amplified regions are indicated by orange bars with their lengths. Note that the region at +96kb contains two CNSs. (B) RT-PCR analysis was performed using total RNAs from the mouse ovary, testis, and liver. The RNAs were treated with TURBO DNase I and subject to reverse transcription. *Gapdh* was used as an internal control. The numbers given in the parentheses indicate the cycle number of PCR. Specific bands are marked with arrowheads, and nonspecific bands were with white circles.

Fig. 2. Cloning of *lncPrep+96kb*. (A) The transcriptional direction was determined by RT-PCR using strand specific primers for reverse transcription. The sense-directed transcript was detected in the ovary, testis, and liver. The cycle number of these PCR reactions was 30. (B) Schematic drawing of the 3' flanking region of the mouse POP gene is shown at the top. The full-length form of *lncPrep+96kb* was determined by 3' RACE

and 5’ RACE using the 3-week-old ovary. Two major TSSs (bent arrows) and one major transcriptional end site (a vertical line) are shown. Two full-length forms of *lncPrep+96kb* (2.2 kb and 2.8kb) are indicated by red lines. Since the cDNA was prepared by reverse transcription with the oligo(dT) primer and the poly(A) sequences were observed by 3’RACE, *lncPrep+96kb* was presumed to have a poly(A) tail. CNSs included in *lncPrep+96kb* are indicated with pink paintings. At the bottom, results of the RACE analyses are summarized. Sequences around the two TSSs and an end site are shown. For each RACE product, twelve subclones were sequenced and the positions of TSSs or transcriptional end sites are indicated with the numbers of subclones. The nucleotides which were identified as TSSs or the end site in more subclones were determined as a major TSS or end site (bold bent arrows or a bold vertical line). Putative poly(A) signal sequences are underlined.

Fig. 3. Subcellular distribution of *lncPrep+96kb*. (A) RT-PCR analysis using the Hepa1-6 cell, which was derived from the mouse liver, and the primary granulosa cell (pGC). Hepa1-6 and pGC were fractionated into cytoplasmic and nuclear subfractions, and the total RNA was purified from each subfraction. As a control, the total RNA was also isolated from the entire cell, and the data from this control is shown as ‘Total’. After the treatment with TURBO DNase I, RT-PCR was performed. The successful fractionation was confirmed by amplifying mature and immature RNAs of the *Gapdh* gene. The mature RNA was detected with a primer pair designed at exon 5 and exon 6 (ex5-ex6) while the immature RNA was with a primer pair at intron 5 and exon 6 (int5-ex6). The mature RNA should be present mainly in the cytoplasmic fraction and the immature RNA in the nucleus. The primers designed within a single exon (exon 6) should detect both immature and mature RNAs (ex6-ex6). The signal for *lncPrep+96kb* was detected in both cytoplasmic and nuclear subfractions. The intensity of each band was measured by using the ImageJ program, and the *lncPrep+96kb* level was calculated by normalizing to *Gapdh* (ex6-ex6). In both cells, the nucleus contained more *lncPrep+96kb*. The PCR reactions were performed with different cycle numbers for linear amplification. We reproduced this result twice, and a representative result is shown here. (B) *In situ* hybridization was performed using DIG-labeled sense (SS) and antisense (AS) cRNA probes for *lncPrep+96kb*. Hepa1-6 (right) and pGC (left) were cultured on cover slips and hybridized with the probes.

Signals (purple) were developed by using the NBT/BCIP substrate. Magnified images of the red boxes are shown below. The bars represent 100 μm (upper four images) and 50 μm (bottom two images). The nucleus is surrounded by white dashed lines.

Fig. 4. Knockdown and overexpression of *lncPrep+96kb*. (A) *lncPrep+96kb* was knocked down by using an shRNA (sh-lncPrep+96kb). An shRNA against the GFP gene (sh-GFP) was used as a negative control. The shRNA constructs were transfected into pGC (left) and Hepa1-6 (right). The expression of POP and *lncPrep+96kb* were analyzed by qRT-PCR (graphs) and RT-PCR (electrophoretic images), respectively. The RT-PCR for *lncPrep+96kb* was performed in the linear amplification and a representative image is shown. This result confirmed successful knockdown. The POP mRNA level was normalized with *aryl-hydrocarbon receptor-interacting protein (Aip)* and the value in the cell transfected with sh-GFP was set as 1.0. The data were presented as means ± SD. By the *lncPrep+96kb* knockdown, the POP expression decreased by about 40% in both cells. (B) *lncPrep+96kb* in the nucleus was downregulated by knockdown. A stable Hepa1-6 cell line was generated by transfection of the sh-lncPrep+96kb construct and the following selection with G418 for more than 20 days. The established cell line was fractionated into the nuclear and cytoplasmic subfractions and total RNAs were purified from them. The level of *lncPrep+96kb* in each subfraction was measured by qRT-PCR. Compared to the non-transfected Hepa1-6 cell, *lncPrep+96kb* was downregulated in the nucleus but not in the cytoplasm. (C) Two forms of *lncPrep+96kb* (2.2 kb and 2.8 kb) were subcloned into the pcDNA3.1 vector (pcDNA-2.2kb and pcDNA-2.8kb). The constructs were transfected into pGC (left) and Hepa1-6 (right) and the expression of POP and *lncPrep+96kb* were analyzed by qRT-PCR (graphs) and RT-PCR (representative electrophoretic images), respectively. Successful overexpression was confirmed by detecting each form of *lncPrep+96kb* by RT-PCR in the linear amplification (bottom). Because the entire sequence of the 2.2-kb form was included in the 2.8-kb transcript, the signal for the 2.2-kb form was also detected by the overexpression of pcDNA-2.8kb. The data are presented as in (A). The overexpression increased the POP expression by 2- to 2.5-folds only in the granulosa cell.

Fig. 5. Histone acetylation pattern at the mouse POP gene locus in the Hepa1-6 cell stably transfected

with sh-lncPrep+96kb. (A) A genomic structure of the mouse POP gene locus is illustrated. Exons are indicated by vertical lines or open boxes with the exon number. Positions of amplicons for ChIP are shown by black arrows. The region from which *lncPrep+96kb* is transcribed is shown by a black bar below the gene structure. (B) Histone acetylation pattern at the mouse POP locus. ChIP was conducted with chromatin isolated from the non-transfected Hepa1-6 cell and the cell stably transfected with the sh-lncPrep+96kb construct. The sheared chromatin was immunoprecipitated with a monoclonal antibody against acetylated histone H3K9K27 (H3K9K27ac) or normal mouse IgG. Purified DNA from before (Input) and after (bound) immunoprecipitation was subjected to real time PCR for the amplicons shown in (A) and the amplification efficiency was normalized by calculating the signal ratio between bound and input fraction. The value was further normalized by the constitutively active *Aip* gene promoter designated as 1.0. The black and white bars represent the H3K9K27ac levels of the non-transfected Hepa1-6 cell and the stably transfected cell, respectively. The graph was shown as the average ± SD (n=3-4). Because the immunoprecipitation with normal mouse IgG resulted in background levels of histone acetylation in all the regions examined, the data are not shown here.

Fig. 6. The expression of POP and *lncPrep+96kb* in the mouse ovary primed with gonadotropins. Three- and four-week-old female mice were injected with PMSG and hCG at a 48-h interval, and the ovaries were collected at the indicated time points. The expression of POP (A) and *lncPrep+96kb* (B) were determined by qRT-PCR. The expression levels were normalized with *Aip*. Both POP and *lncPrep+96kb* were induced by the hCG injection and their highest expression was observed at 6 h after that. The data are presented as mean ± SD. $^{**}P < 0.01$ compared with the 0 h.

Fig. 7. Expression of lncRNAs at the human POP locus. (A) The human POP locus at chromosome 6 is illustrated. The human POP gene consists of 15 exons each of which is indicated by a vertical line with the exon number. At the bottom, a 3’ region is enlarged, and CNSs are indicated with pink paintings. Horizontal lines are the amplified regions with their names. (B) RT-PCR analysis with human RNAs. Total RNAs from the human skeletal muscle, testis, and liver were treated with TURBO DNase I and reverse-transcribed with

the oligo(dT) primer. PCR was performed with the primer pairs for the three regions indicated in (A) as well as for POP and β-ACTIN mRNAs. An intense signal for POP was detected in the skeletal muscle and the testis. Among the three intergenic regions, two CNSs were transcribed only in the testis, and a 3’proximal sequence was amplified in the skeletal muscle and the testis.

Table I. Oligonucleotide primers used in this study.

Designation	Forward	Reverse
RT-PCR		
lncPrep-20kb	5’-CTCTACACTTTGGTGGGACT-3’	5’-ACCCCTTTCAGAGTCTCTTC-3’
lncPrep-18kb	5’-CCAGACAGGTCAAACTTCTT-3’	5’-CAAGACACCCATTCTAAAGC-3’
lncPrep+96kb	5’-GGAGCAGCTGAGATAGAAGC-3’	5’-ACCGGAGCCTAGCAAATAG-3’
lncPrep+106kb	5’-ATCTTCATTACGGATTCTGG-3’	5’-ATAACCCCATCAGTCACATT-3’
Gapdh	5’-CATGACCACAGTCCATGCCATC-3’	5’-TAGCCCAAGATGCCCTTCAGTG-3’
Subcellular distribution		
Gapdh at exon 5	5’-TTGTGATGGGTGTGAACCAC-3’	5’-TAGCCCAAGATGCCCTTCAGTG-3’
Gapdh at intron 5	5’-CCTTCTTTGTAGGTGTCCCT-3’	5’-TAGCCCAAGATGCCCTTCAGTG-3’
Full length cloning		
lncPrep+96kb-2.2kb	5’-AGCTTGTGTATTGCTCATAT-3’	5’-TTTGCTTTTTAATTTTTATTTG-3’
lncPrep+96kb-2.8kb	5’-AAACAGCATTGGTGTGAATG-3’	5’-TTTGCTTTTTAATTTTTATTTG-3’
qRT-PCR		
POP	5’-GGAATCGATGCTGCTGATTA-3’	5’-CCATCCAGCTTTATGCCTTT-3’
lncPrep+96kb	5’-TGACATGCTATGGGAAAAGC-3’	5’-TCCCTATCCTACCCCAAACA-3’
Aip	5’-GAGGACGGGATCCAAAAGC-3’	5’-CTGTGCAGCGTCCGAAAGT-3’
RT-PCR for human		
POP	5’-GATGATGGCCGCTATGTCTT-3’	5’- TTCTTCTGACCGCTGTACCC-3’
3’proximal	5’-ATACTGTTGGGGTGGTCAGG-3’	5’-CCAGCTCCTGGGGTTACTG-3’
CNS1	5’-GCAATGACCCTTTCTGGATG-3’	5’-CATTGAACGAACCCTCCTTG-3’
CNS2	5’-CTGATGTTGGTTCCCAGGAG-3’	5’-ATGTTCTTTGCTGGACACTG-3’

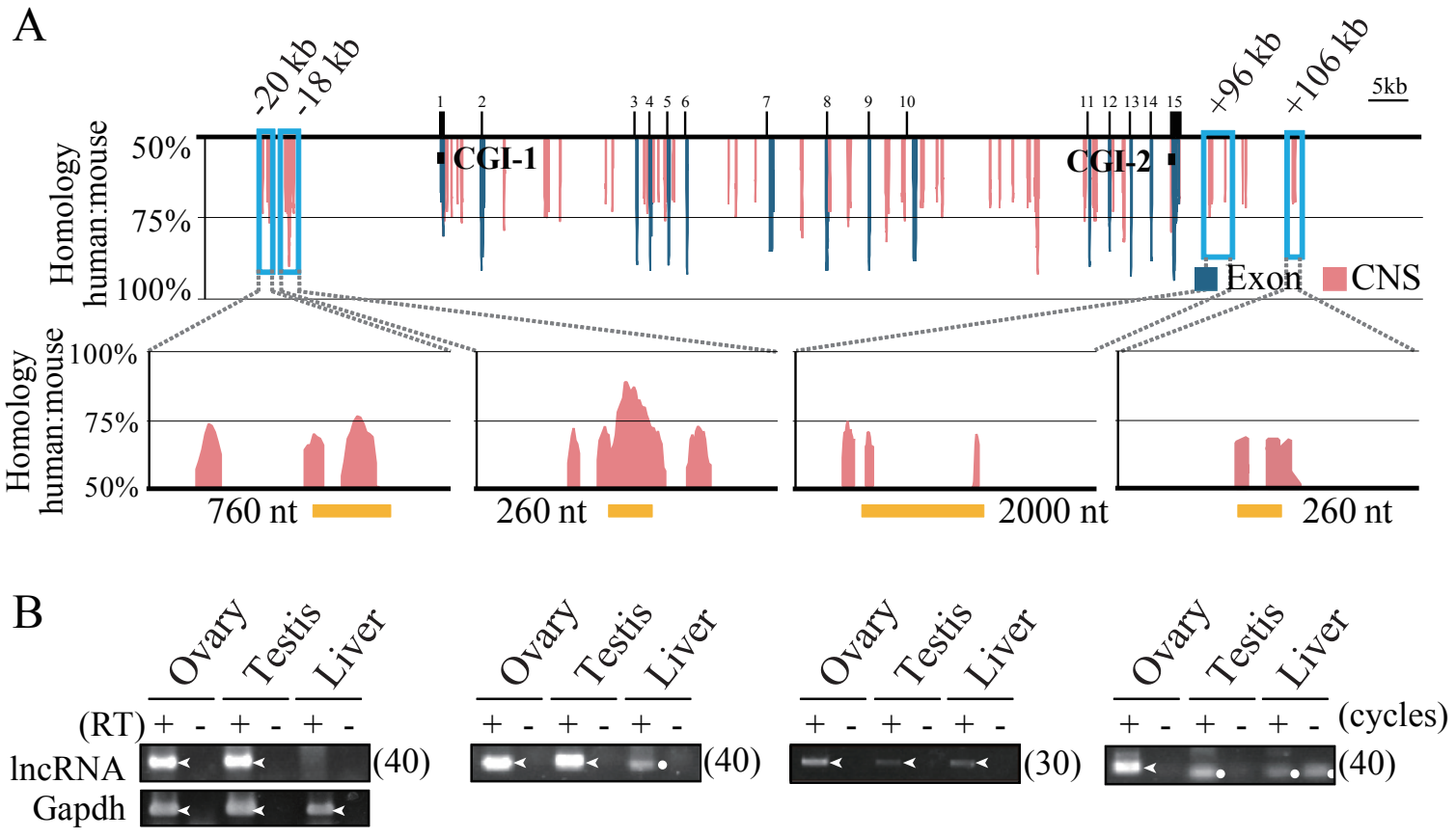

A
-20 kb
-18 kb
+96 kb
+106 kb
5kb
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
CGI-1
CGI-2
Homology human:mouse
50%
75%
100%
Exon
CNS
760 nt
260 nt
2000 nt
260 nt
B
Ovary
Testis
Liver
(RT) + - + - + -
lncRNA
Gapdh
(40)
(40)
(30)
(40)
(cycles)

Fig. 1

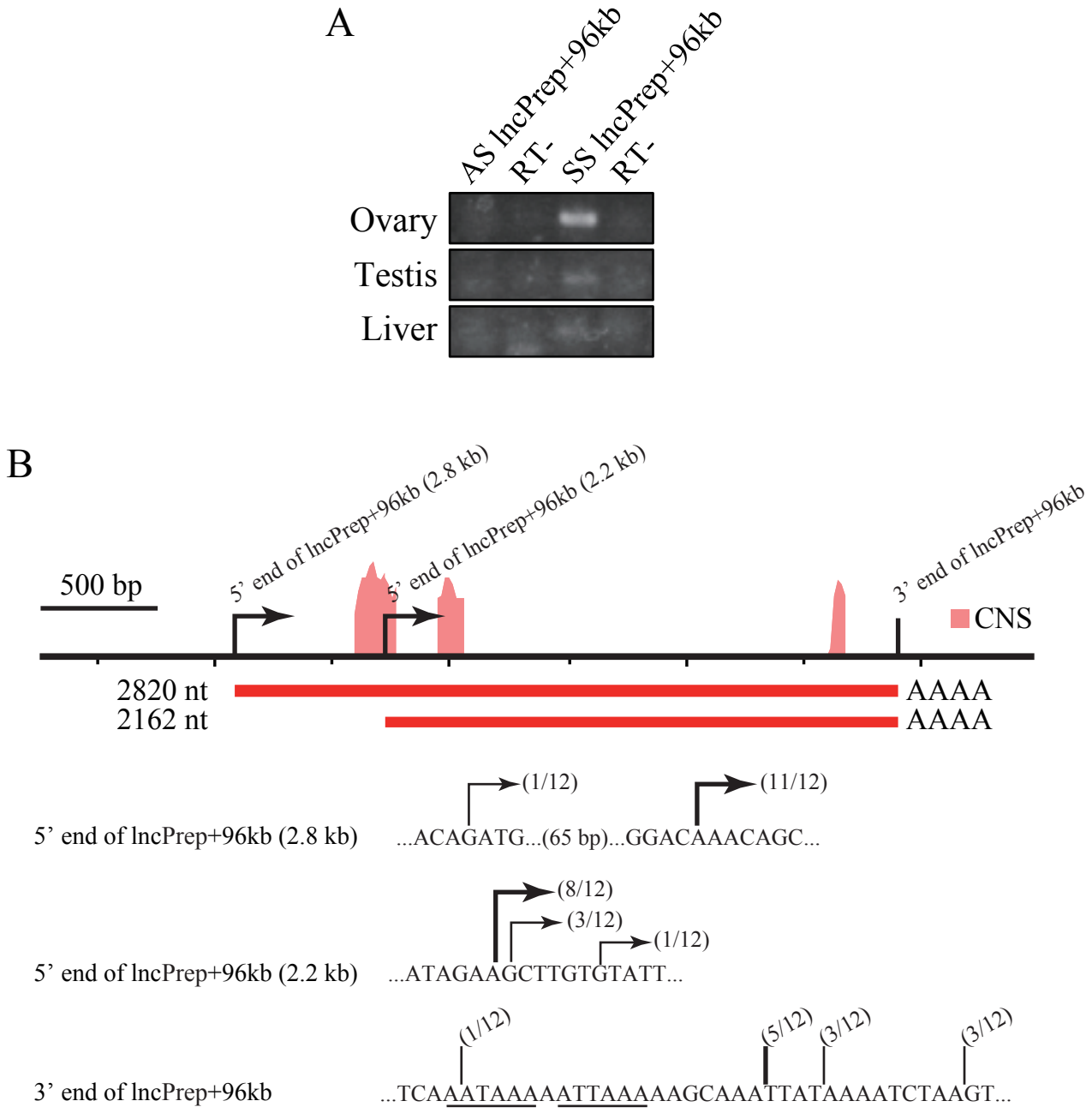

A
AS lncPrep+96kb
RT-
SS lncPrep+96kb
RT-
Ovary
Testis
Liver
B
500 bp
5' end of lncPrep+96kb (2.8 kb)
5' end of lncPrep+96kb (2.2 kb)
3' end of lncPrep+96kb
CNS
2820 nt
2162 nt
AAAA
AAAA
5' end of lncPrep+96kb (2.8 kb)
(1/12)
(11/12)
...ACAGATG...(65 bp)...GGACAAACAGC...
5' end of lncPrep+96kb (2.2 kb)
(8/12)
(3/12)
(1/12)
...ATAGAAGCTTGTGTATT...
3' end of lncPrep+96kb
(1/12)
(5/12)
(3/12)
(3/12)
...TCAAATAAAAATTAAAAAGCAAATTATAAAATCTAAGT...

Fig. 2

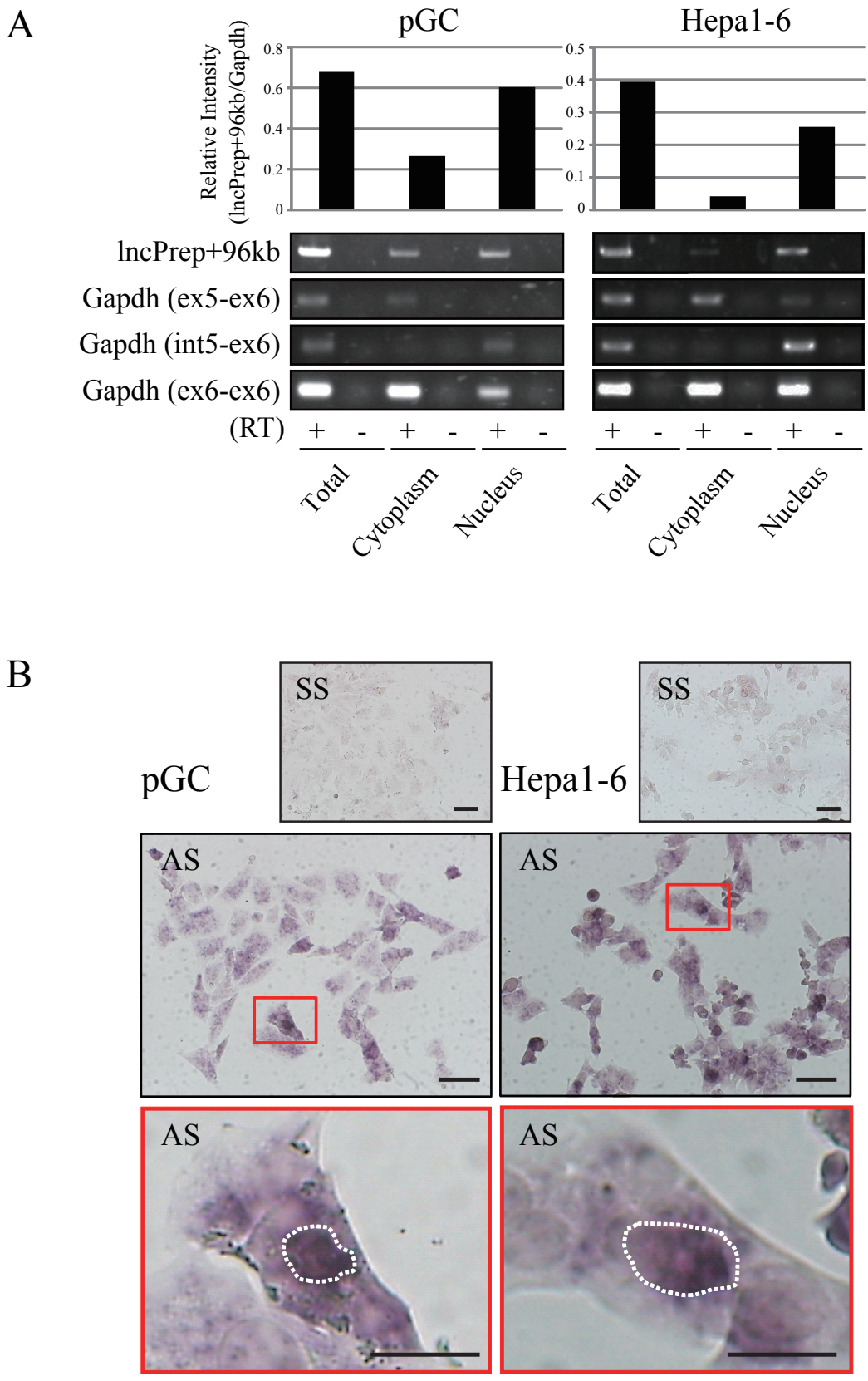

A
pGC
Hepa1-6
Relative Intensity
(lncPrep+96kb/Gapdh)
0.8
0.6
0.4
0.2
0
0.5
0.4
0.3
0.2
0.1
0
lncPrep+96kb
Gapdh (ex5-ex6)
Gapdh (int5-ex6)
Gapdh (ex6-ex6)
(RT)
+ - + - + -
+ - + - + -
Total
Cytoplasm
Nucleus
Total
Cytoplasm
Nucleus
B
pGC
SS
Hepa1-6
SS
AS
AS
AS
AS

Fig. 3

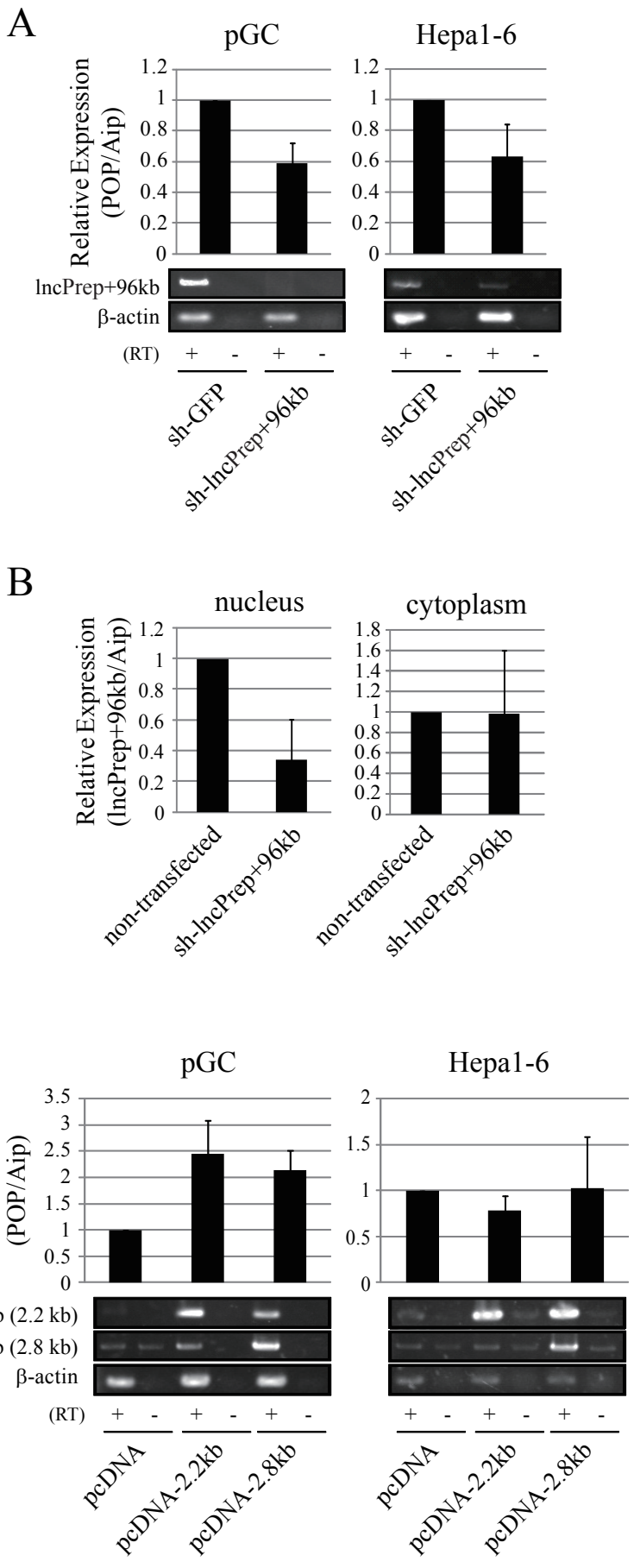

A
pGC
Hepa1-6
Relative Expression (POP/Aip)
1.2
1
0.8
0.6
0.4
0.2
0
lncPrep+96kb
β-actin
(RT) + - + - + - + -
sh-GFP
sh-lncPrep+96kb
B
nucleus
cytoplasm
Relative Expression (lncPrep+96kb/Aip)
1.8
1.6
1.4
1.2
non-transfected
sh-lncPrep+96kb
C
3.5
3
2.5
2
1.5
0.5
lncPrep+96kb (2.2 kb)
lncPrep+96kb (2.8 kb)
pcDNA
pcDNA-2.2kb
pcDNA-2.8kb

Fig. 4

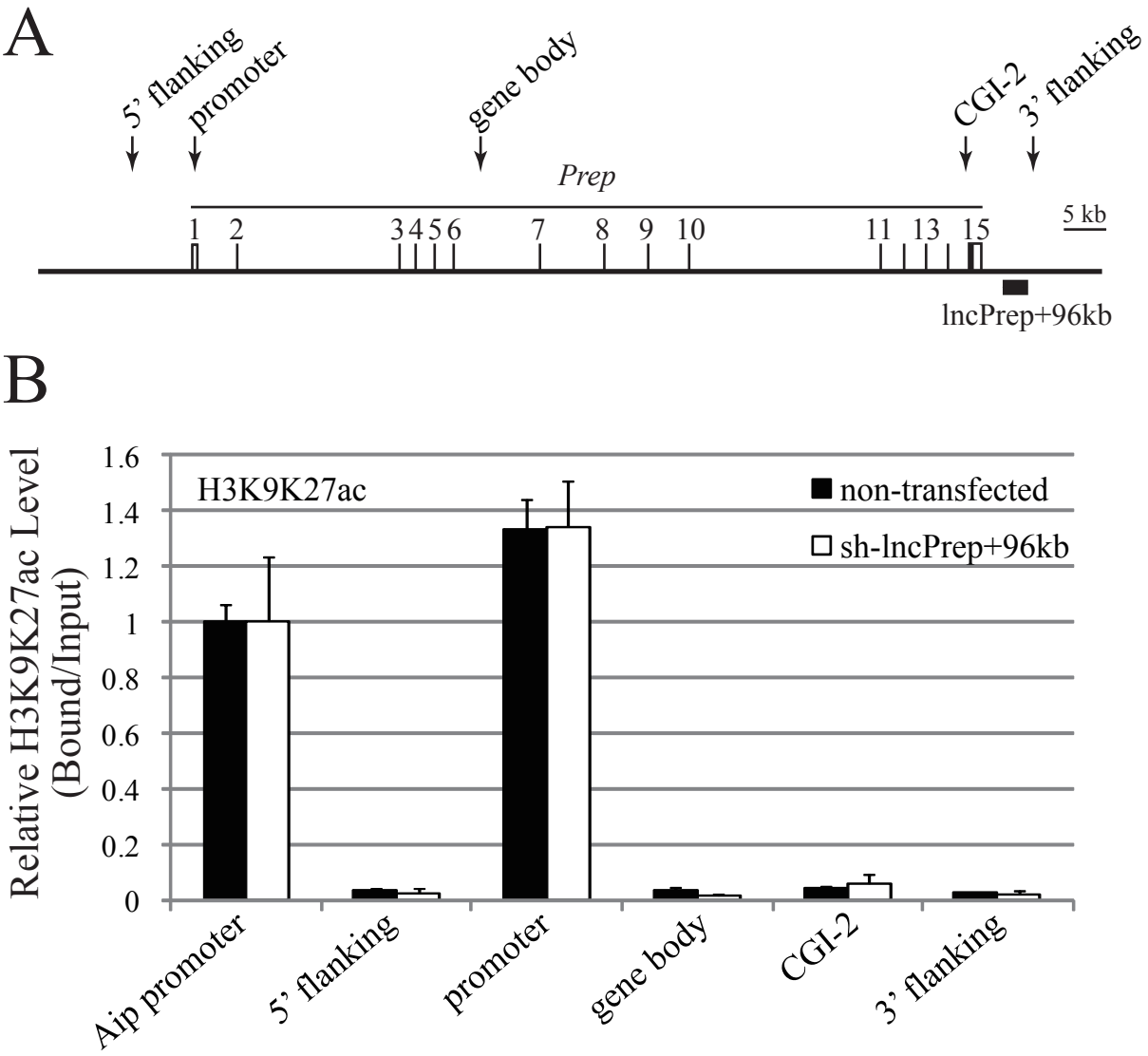
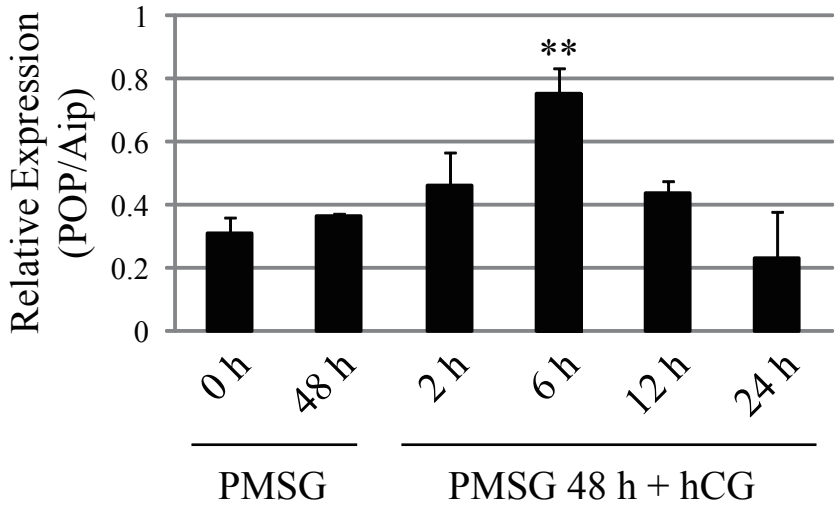

A
5' flanking
promoter
gene body
CGI-2
3' flanking
Prep
1 2 3 4 5 6 7 8 9 10 11 13 15
5 kb
lncPrep+96kb
B
Relative H3K9K27ac Level (Bound/Input)
H3K9K27ac
non-transfected
sh-lncPrep+96kb
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Aip promoter
5' flanking
promoter
gene body
CGI-2
3' flanking

Fig. 5

A


Relative Expression
(POP/Aip)
1
0.8
0.6
0.4
0.2
0
**
0 h
48 h
2 h
6 h
12 h
24 h
PMSG
PMSG 48 h + hCG

B

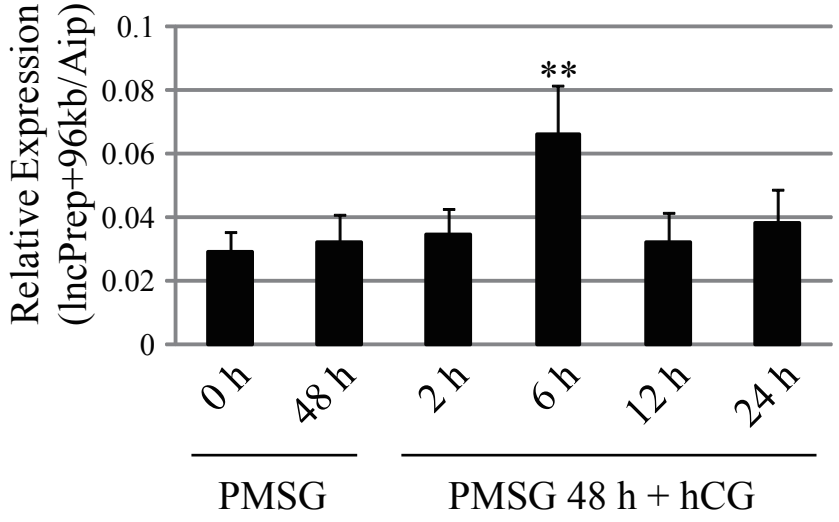
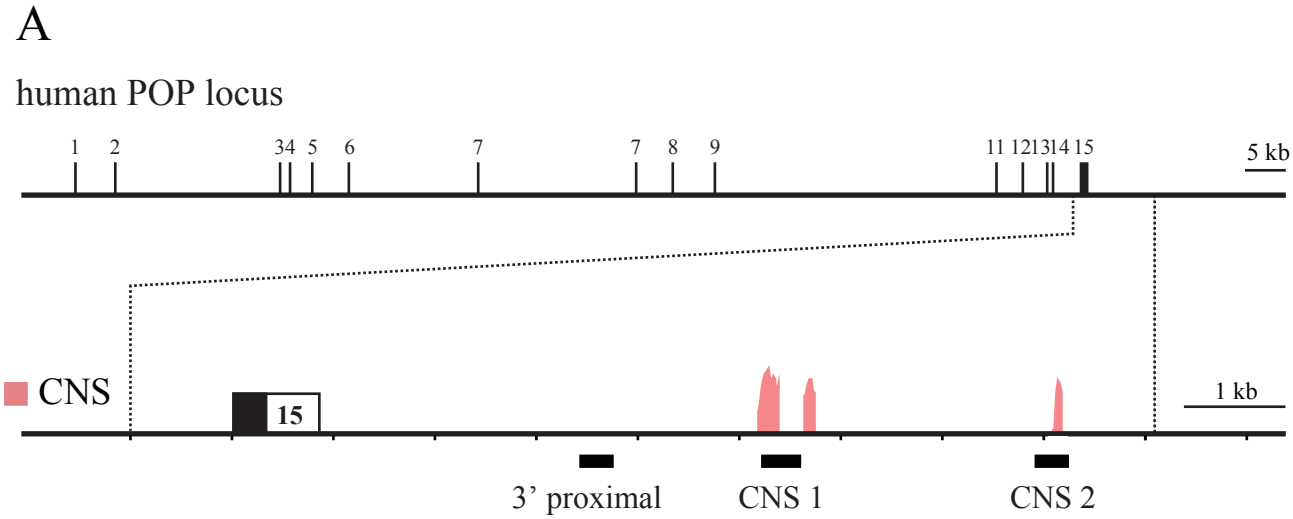

Relative Expression
(lncPrep+96kb/Aip)
0.1
0.08
0.06
0.04
0.02
0
**
0 h
48 h
2 h
6 h
12 h
24 h
PMSG
PMSG 48 h + hCG

Fig. 6


A
human POP locus
1 2 34 5 6 7 7 8 9 11 12 13 14 15
5 kb
CNS
15
1 kb
3' proximal
CNS 1
CNS 2

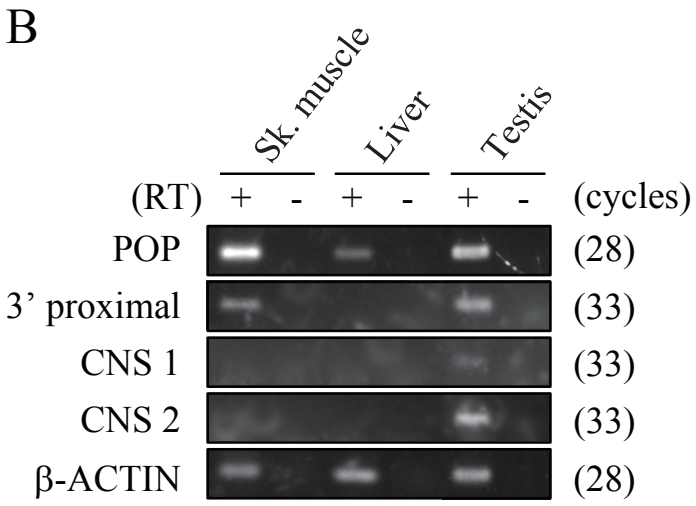
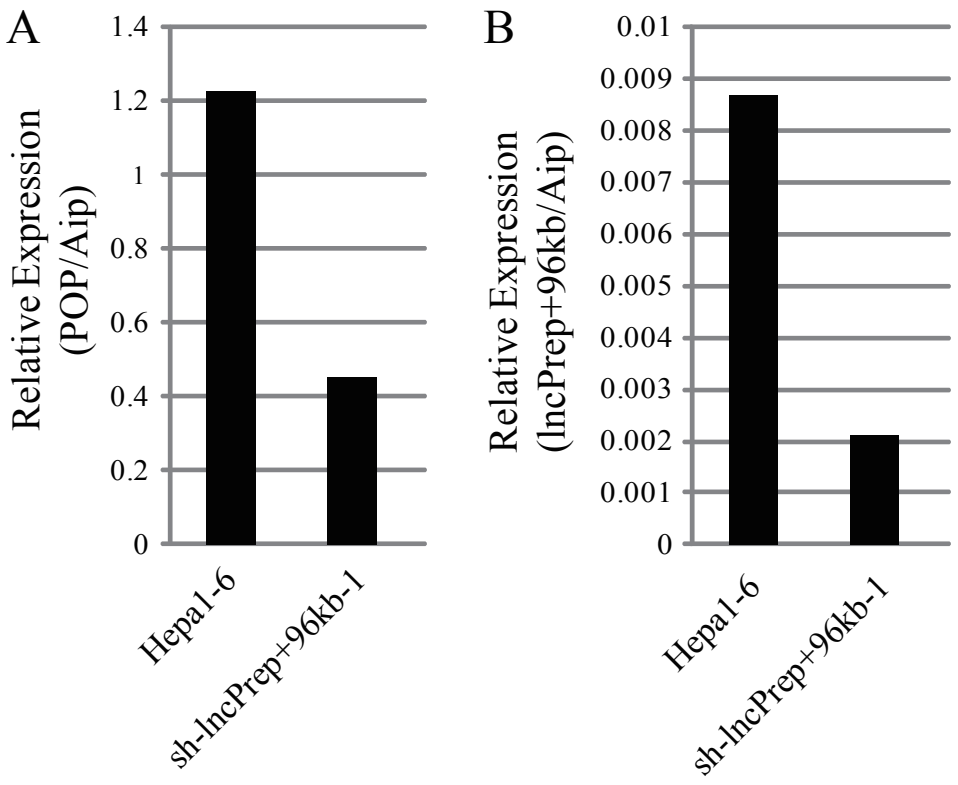

B
Sk. muscle
Liver
Testis
(RT) + - + - + -
(cycles)
POP (28)
3' proximal (33)
CNS 1 (33)
CNS 2 (33)
β-ACTIN (28)

Fig. 7



Supplementary Fig. 1. Stable knockdown of lncPrep+96kb in the Hepa1-6 cell. lncPrep+96kb was knocked down by using an shRNA (sh-lncPrep+96kb). The shRNA construct was transfected into the Hepa1-6 cell. After the selection with 1-1.5 mg/ml G418 for more than 20 days, the expressions of POP (A) and lncPrep+96kb (B) were analyzed by qRT-PCR. The expression levels were normalized with *Aip*. Successful knockdown was confirmed. By the lncPrep+96kb knockdown, the POP expression was decreased to 37%.

Supplementary Fig. 1