

## Transcriptional activation of the mouse *Scd2* gene by interdependent enhancers and long noncoding RNAs in ovarian granulosa cells

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**Abstract.** Specific gene expression in granulosa cells is key for the function of ovary, but the molecular mechanism of transcriptional activation is not well studied. Here we investigated the regulatory mechanism of the mouse stearoyl-CoA desaturase 2 (*Scd2*) gene encoding an enzyme for lipid metabolism. Northern blot and *in situ* hybridization indicated that the mouse *Scd2* mRNA was highly expressed in ovarian granulosa cells. We found four conserved noncoding sequences (CNSs) and two long noncoding RNAs (lncRNAs) transcribed from regions upstream of the *Scd2* gene as candidates of regulatory elements/factors. These lncRNAs were predominantly transcribed in the opposite direction to *Scd2* and localized in nuclei and showed the correlation with *Scd2* expression, raising the possibility of their transcriptional regulatory roles. Indeed, knockdown of both lncRNAs, *lncRNA-sc1* and *lncRNA-sc2*, significantly decreased the *Scd2* mRNA level in primary granulosa cells. Then, we investigated the histone modification pattern at this locus by a chromatin immunoprecipitation assay, and two CNSs, CNS1 and CNS2, were found to be marked with high levels of histone H3K9/K27 acetylation in primary granulosa cells. By a reporter gene assay, both CNS1 and CNS2 interdependently exhibited enhancer activity for the *Scd2* promoter in primary granulosa cells. These data suggest that the mouse *Scd2* gene is activated by two lncRNAs and interdependent enhancers in ovarian granulosa cells, which provides a new insight into transcriptional activation in granulosa cells.

**Key words:** Granulosa cell, Histone acetylation, Interdependent enhancer, Long noncoding RNA, Ovary, Stearoyl-CoA desaturase

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Tissue or cell characteristics are determined by specific patterns of gene expression, which is strictly controlled by mechanisms involving cis-regulatory elements (e.g. promoters, enhancers, and dual promoter–enhancers) and trans-regulatory factors (e.g. transcription factors) [1–4]. The cis-regulatory elements are epigenetically controlled in ways that several types of histone modifications loosen the chromatin encompassing the element, which allows transcription factors to bind and activate gene transcription [5]. In addition, long noncoding RNAs (lncRNAs) often bind to the chromatin at cis-regulatory elements and function in transcriptional regulation [6–8]. How these elements/factors coordinate to regulate gene transcription is different depending on tissues or cell types or different between genes in one cell type. Thus, a detailed understanding of transcriptional activation of each gene in each tissue or cell type is

of great importance, but only a small number of studies reported the mechanism in some tissues such as granulosa cells in the ovary.

The ovary is an organ to generate oocytes, and granulosa cells surround an oocyte in a follicle. Various genes are specifically expressed in granulosa cells and play essential roles in the progression of meiosis, oocyte maturation, follicular development, and ovulation [9–11], indicating the importance of gene regulation in this cell type. Nonetheless, the mechanism of transcriptional activation, especially the involvement of enhancers and lncRNAs, is not well understood in granulosa cells. We previously reported mechanisms of two genes that are functionally unrelated but highly expressed in mouse granulosa cells. The prolyl oligopeptidase (*Prep*) gene is ubiquitously expressed at different levels and the highest expression is observed in granulosa cells [12]. This robust transcription partly attributes to a potential enhancer overlapping with a CpG island and lncRNA transcribed downstream of the *Prep* gene [12, 13]. The anti-Müllerian hormone type II receptor (*Amhr2*) gene is specifically expressed in ovarian granulosa cells and testicular Sertoli and Leydig cells. An lncRNA, *lncRNA-Amhr2*, is transcribed upstream of the *Amhr2* gene and participates in its activation by enhancing promoter activity in granulosa cells [14]. These suggest that transcriptional activation in granulosa cells requires enhancers and/or lncRNAs,

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irrespective of the tissue specificity and function of genes.

To gain more insights into the gene activation in granulosa cells, here we focused on stearoyl-CoA desaturase 2 (*Scd2*) gene. The *Scd2* gene was identified as a gene which was upregulated during adipocyte differentiation [15]. It encodes an enzyme that catalyzes the desaturation of fatty acid and is required for lipid synthesis during skin and liver development in embryo [16]. In the ovary, saturated free fatty acids have negative effects on oocyte maturation and follicular development, and the SCD enzymes alleviate the toxicity by metabolizing them [17, 18]. Because the *Scd2* gene is expressed at high levels in rat granulosa cells, it is considered to play a major role in weakening the toxicity of saturated free fatty acids during oogenesis [19]. The transcriptional regulation was reported in mouse adipocytes and brain; SREBP1a and EGR2 bind to the *Scd2* promoter and enhance its activity [20, 21]. However, the regulatory mechanism in granulosa cells is unclear.

In this study, we attempted to identify cis-regulatory elements and lncRNAs that were responsible for *Scd2* gene activation in granulosa cells and found interdependent enhancers and two lncRNAs that possibly functioned in activation of this gene.

## Materials and Methods

### Animals

Mice (C57B/6 or BDF1) were kept at 25°C with a photoperiod 14 h light and 10 h dark, and 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.

### Northern blot analysis

Northern blot was performed as previously described [12]. Briefly, total RNAs were isolated from each tissue using ISOGEN (Nippongene, Tokyo, Japan) according to the manufacturer's instruction. Twenty micrograms of the RNA were electrophoresed on an agarose gel containing formaldehyde and transferred to a nylon membrane. The membrane was hybridized with radio-labelled probes and detected by autoradiography.

Probes were prepared as follows. *Scd2* and *Actb* sequences were amplified by reverse transcription-polymerase chain reaction (RT-PCR) with total RNA from the testis using primers shown in Table 1. The products were subcloned into a pBluescript II KS+ vector (Stratagene, La Jolla, CA, USA) and checked by DNA sequencing. The fragments were cut out from the vector by digestion with *SmaI/SalI* for *Scd2* and *EcoRI/HindIII* for *Actb* and purified with a GeneClean kit. Due to high sequence homology, the *Actb* probe also hybridized with *Actg1* and *Actg2* mRNAs.

### In situ hybridization

Four-week-old female mice were administered with pregnant mare serum gonadotropin, and 48 h later, with human chorionic gonadotropin. Ovaries were collected 6 h after the injection of human chorionic gonadotropin, embedded in TissueTek, and frozen. Ten-micrometer sections were prepared, and *in situ* hybridization was performed as previously described [12]. We labeled the same *Scd2* sequence as used in Northern blot with digoxigenin by *in vitro* transcription in both directions and used as sense and antisense probes.

### Cell culture

Primary granulosa cells were obtained and cultured as previously described [12, 14]. Briefly, ovaries were collected from immature female mice administered with hormones as above 5 h after human chorionic gonadotropin injection, and preantral follicles were punctured with a 26G needle. The granulosa cells were spread onto 24-well plates or 35-mm dishes that were coated with fibronectin, and cultured in Dulbecco modified Eagles medium/F12 containing 10% fetal bovine serum.

Hepa1-6 cells (RCB1638; Riken Bioresource Center, Tsukuba, Japan) derived from mouse hepatic tumor were cultured in Dulbecco modified Eagles medium containing 10% fetal bovine serum.

### RT-PCR

Total RNAs from cells and tissues were purified as above. Nuclear and cytoplasmic RNAs were isolated by a method using NP-40 lysis buffer (10 mM Tris-HCl, 10 mM NaCl, 3 mM MgCl<sub>2</sub>, 0.5% NP-40, pH 7.5) for cell lysis as previously described [13]. RNA isolation, DNase I treatment, RT-PCR, and quantitative RT-PCR (qRT-PCR) were performed as previously described [12, 13, 22, 23]. The transcriptional direction was determined by qRT-PCR using gene-specific primers for reverse transcription. Primers are listed in Table 1.

### Knockdown of lncRNA-sc1 by shRNA

Short hairpin RNA (shRNA) constructs were generated by annealing oligonucleotides (Table 1) and inserting them into a pBasi-mU6 Neo DNA vector (Takara, Kusatsu, Japan) that was digested with *BamHI* and *HindIII*. The constructs were transfected into primary granulosa cells using GeneJuice transfection reagent (Merck, Darmstadt, Germany) according to the manufacturer's instruction. Twenty-four hours later, 1 mg/ml G418 was added to the medium and cultured for 4 days, changing the medium every day. Total RNAs were isolated using ISOGEN II (Nippongene) according to the manufacturer's protocol.

### Knockdown of lncRNA-sc2 by antisense oligonucleotide

Because shRNAs did not reduce the *lncRNA-sc2* expression, this lncRNA was knocked down by the 2'4'-bridged nucleic acid (2',4'-BNA) gapmer type of antisense oligonucleotide (ASO) with fully phosphorothioated linkage, which was generated by Hokkaido System Science (Sapporo, Japan). The ASO sequences were 5'-ATGTGATCGCGCTTCT-3' for the control (complementary to EGFP sequence) and 5'-GGATATCAGTCGTTCC-3' for the knockdown, and two nucleotides at each end were 2',4'-BNA. Each ASO was transfected into primary granulosa cells using LipoTrust Ex Oligo (Hokkaido System Science) according to the manufacturer's instruction. Two days later, the cells were collected with ISOGEN II (Nippongene) and total RNAs were purified.

### Chromatin immunoprecipitation (ChIP) assay

ChIP assay for histone acetylation was performed as previously described [12]. The antibody against acetylated histone H3 lysine 9 and lysine 27 (H3K9/K27ac) was kindly gifted by Dr Hiroshi Kimura at Tokyo Institute of Technology [24]. Primer sequences for quantitative PCR (qPCR) are listed in Table 1.

**Table 1.** Oligo DNAs used in this study

Designation	Forward	Reverse
[Probe for Northern blot]		
<i>Scd2</i>	GGTGGCAACTGAGGAACTTCG	TTGTGGGCACGACCATCATC
<i>Actb</i>	ACATCCGTAAGACCTCTATG	TAAAACGCAGCTCAGTAACAGT
[RT-PCR]		
<i>lncRNA-sc2</i>	TGTGGAGCCTCTGTATTCCTCG	TCCACCTGCTGCTGATACAAGT
<i>lncRNA-sc1</i>	AGCTGCAAGATGCCATTCT	CCATCCCTTGAGCTCACAAC
-6kb	CTTTCTGCTCAGAAACATTTG	GTTTTTGCTTGCAATCCAA
CNS1	GGAGATACAGTAATGGGGTGACC	TTGCCCATTCACACTGGTCTGG
CNS3	CACGTGCACATACAAAGATGGA	CCCCTTGGTCTGGATTATCC
<i>Scd2</i>	GACATTAATACCCCACTGCCA	GCTCCCCAGTGGTGAGAACTCT
<i>Gapdh</i>	CATGACCACAGTCCATGCCATC	TAGCCCAAGATGCCCTTCAGTG
[Strand-specific RT]		
RT for <i>lncRNA-sc2</i>	CAAAAGCTCTGCAAGCTGACA	TGGCAGGTCTTGTAAGAGAAATC
RT for <i>lncRNA-sc1</i>	AACCAGGCAAGACTAGAC	CCATCCCTTGAGCTCACAAC
[qRT-PCR]		
<i>lncRNA-sc1</i>	AGCTGCAAGATGCCATTCT	AGGCCAGCCTGGTCTACATCAT
<i>lncRNA-sc2</i>	TGACACAGGCTGGAGTTATC	TCCACCTGCTGCTGATACAAGT
<i>Scd2</i>	ACCCTTTGTCTGCTGAGGTCTGAAG	GATTGTGGTGGTGGCTGAGTAAGC
<i>Prep</i>	GGAATCGATGCTGCTGATTA	CCATCCAGCTTTATGCCTTT
<i>Gapdh</i>	CATGGCCTCCGTGTTCTTA	CCTGCTTACCACCTTCTTGA
[shRNA construct]		
shlncRNAsc1	GATCCGAACAAAGTCCACTAGAAACCGTGTGCT GTCCGGTTTCTAGTGGACTTTGTTCTTTTTTA	AGCTTAAAAAAGAACAAGTCCACTAGAAACCG GACAGCACACGGTTTCTAGTGGACTTTGTTCCG
[ChIP]		
<i>Aip</i> promoter	GGGCTTCAGCACAGAATCCA	TGAAAAATCCTGAGAGCCTCATT
<i>lncRNA-sc2</i>	CAAAAGCTCTGCAAGCTGACA	GGGATCTCCTCAGCGAGTCTT
<i>lncRNA-sc1</i>	AGCTGCAAGATGCCATTCT	GGCCCATCACAGGCTGTTTA
CNS1	GTGAGCCAGGCTGCAAT	GCATTCTCTGAGACCAAACCA
<i>Scd2</i> promoter	GTGAGAAAGCCAGGAGCAGAAA	TCGTCTGCTGCTCTCATTG
CNS2	GTGATGCAGGGCTCTGGTTAC	TCCCAACTCCCGTACCTCCTA
CNS3	CACGTGCACATACAAAGATGGA	CCCCTTGGTCTGGATTATCC
[Reporter construct]		
<i>Scd2</i> promoter	ATGGTCTCCACCTTTTCTTCC	TGCAGACACCAACACCGCATT
CNS2	TCCTGTAGGAGGTACGGGAGTT	CTGACACCCTCCCCCTATTGTC
CNS1 mutation	GCCATTTGCTGAGGACACCTGGCCTGG CATCTCTTCTTG	CAAGAAGAGAGATGCCAGGCCAGGTGT CCTCAGCAAATGGC

*Scd2*, stearoyl-CoA desaturase 2; RT-PCR, reverse transcription-polymerase chain reaction; qRT-PCR, quantitative RT-PCR; CNS, conserved noncoding sequence.

**Reporter constructs**

**2.4kPr:** The transcriptional start site (TSS) of mouse *Scd2* gene was reported in the DBTSS database (<https://dbtss.hgc.jp/>). A 2377-bp *Scd2* promoter sequence was obtained by PCR with genome DNA and KOD FX (Toyobo, Osaka, Japan) using a primer pair shown in Table 1, and subcloned into a pBluescript II KS + vector. After we confirmed the sequence by DNA sequencing, the promoter was cut out by digestion with *EcoRI* and *HindIII*, blunted by T4 DNA polymerase (Takara), and inserted into pGL3-Basic (Promega, Madison, WI, USA) at the *SmaI* site.

**0.6kPr:** 2.4kPr was digested with *SacI* and self-ligated.

**1.3kPr:** 2.4kPr was digested with *MluI* and *BstXI*, blunted and self-ligated. The *BstXI* site was located in the 147-bp CNS1 sequence,

and the 1.3-kb promoter contained a 57-bp sequence of CNS1.

**1.3kPr-CNS2:** The CNS2 sequence was amplified by genomic PCR with the primer pair shown in Table 1, and inserted into 1.3kPr at the blunted *BamHI* site. The sequence was checked by DNA sequencing.

**2.4kPr-CNS2:** The CNS2 sequence was amplified as above and inserted into 2.4kPr at the blunted *BamHI* site. The sequence was checked by DNA sequencing.

**2.4kΔCNS1Pr:** The first PCR reactions were done with a sense primer of *Scd2* Promoter and an antisense primer of CNS1 mutation and with a sense primer of CNS1 mutation and an antisense primer of *Scd2* Promoter (Table 1). The products were purified, mixed and subjected to 2 cycles of PCR without additional primers, followed by 30 cycles of PCR with sense and antisense primers of *Scd2*

Promoter. The product was inserted into pGL3-Basic at the *Sma*I site and checked by DNA sequencing.

2.4kΔCNS1Pr-CNS2: The CNS2 sequence was amplified as above and inserted into 2.4kΔCNS1Pr at the blunted *Bam*HI site. The sequence was checked by DNA sequencing.

#### Reporter gene assay

Luciferase activity assay was performed as previously described [12]. Briefly, each construct was transfected into primary granulosa cells or Hepa1-6 cells using Lipofectamine 2000 (Thermo Fisher, Waltham, MA, USA) together with the pRL-CMV vector (Promega) according to the manufacturer's instruction. Two days later, luciferase activity was measured using a Dual-Luciferase Reporter Assay system (Promega).

#### Statistical analysis

The data are presented as means  $\pm$  standard deviation (SD). Statistical significance was assessed by Student *t* test or one-way analysis of variance (ANOVA) followed by Tukey-Kramer test. The *P* value less than 0.05 was considered statistically significant.

## Results

#### Mouse ovarian granulosa cells express *Scd2* mRNA at a high level

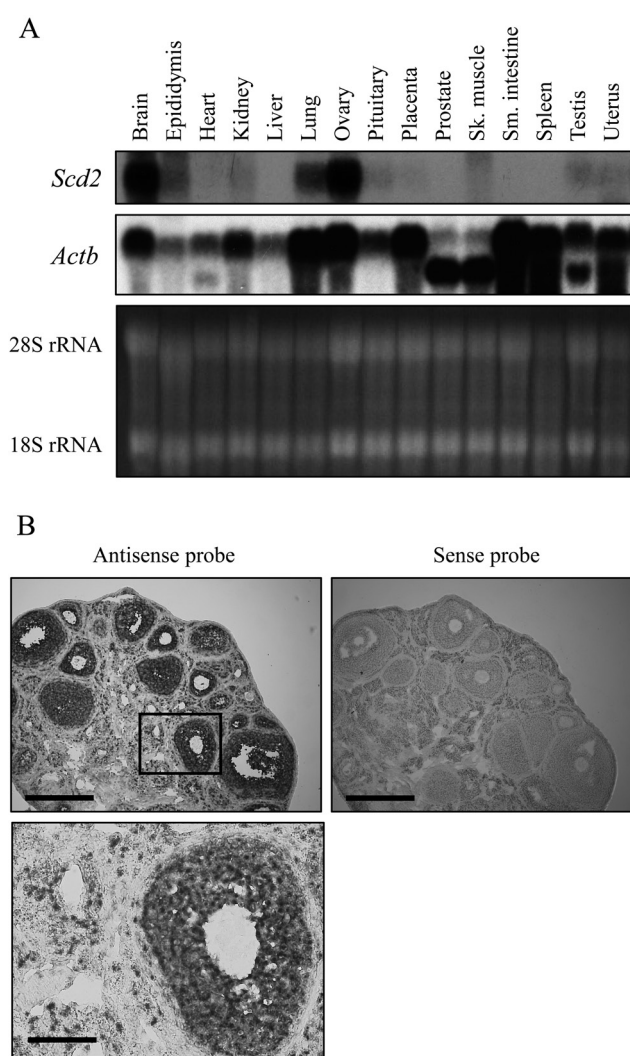
We first investigated the expression pattern of mouse *Scd2* mRNA to confirm the tissue specificity of this gene. Northern blot analysis, using 15 tissues from adult mice, showed strong signals in brain and ovary and detected fainter bands in several tissues (Fig. 1A), which was consistent with a previous result in rats [19]. Then, we checked the localization of *Scd2* mRNA in the ovary by *in situ* hybridization with superovulated mouse ovaries. The signal was observed specifically in granulosa cells with the antisense probe whereas the sense probe did not hybridize with any cells in the section (Fig. 1B). These data indicate that the mouse *Scd2* gene is highly expressed in ovarian granulosa cells.

#### Identification of conserved noncoding sequences at the *Scd2* gene locus

To identify cis-regulatory elements for *Scd2* gene activation, we searched for conserved noncoding sequences (CNSs) that are evolutionarily conserved between species and often overlap with transcriptional regulatory elements. We compared the genome sequence at the mouse *Scd2* locus with that at the human *SCD1* locus, given that only the *SCD1* gene is present in human in contrast to four paralogous *Scd* genes in rodents [25]. The mVista program (<http://genome.lbl.gov/vista/index.shtml>) identified four non-exonic sequences conserved in a 35-kb mouse genome encompassing 15-kb upstream and 7-kb downstream of *Scd2* gene. To identify cis-regulatory sequences outside the promoter, a conserved region just upstream of the *Scd2* gene was excluded. We designated the four conserved sequences as CNS1-CNS4 (Fig. 2).

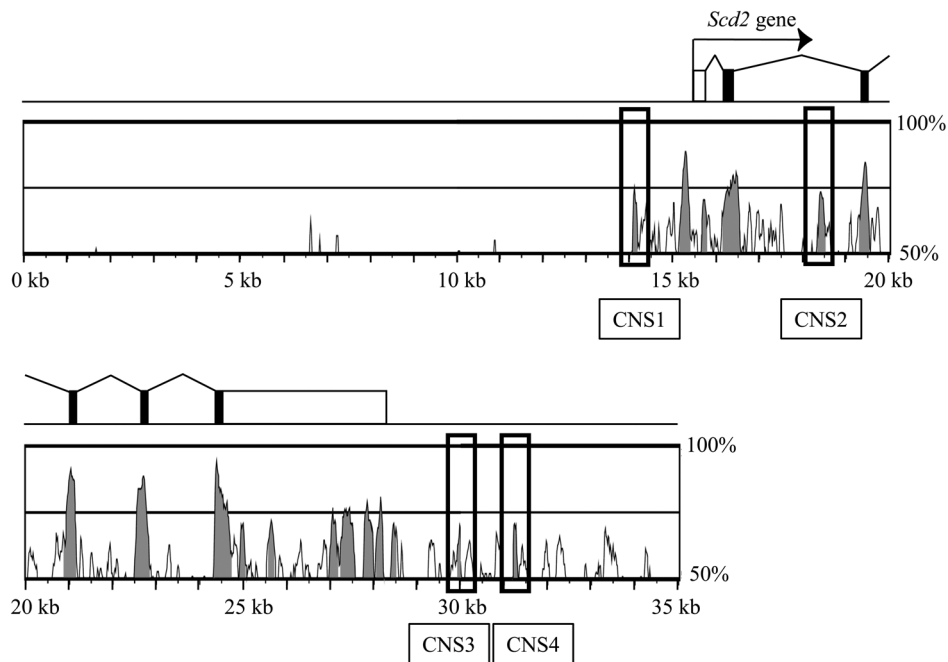
#### Noncoding transcription at the *Scd2* gene locus

We next examined whether any regions upstream or downstream of the mouse *Scd2* gene were transcribed by RT-PCR with total RNAs



**Fig. 1.** Mouse stearyl-CoA desaturase 2 (*Scd2*) mRNA expression. (A) Northern blot analysis of *Scd2* in various mouse tissues. Twenty micrograms of total RNAs from 15 tissues were electrophoresed on a denaturing agarose gel and stained with ethidium bromide (bottom). The gel was blotted to a nylon membrane and hybridized with a  $^{32}$ P-labeled *Scd2* or *Actb* probe. The signals were detected by autoradiography (top and middle). The probe for *Actb* cross-hybridized to *Actg1* and *Actg2* due to high sequence homology, and the upper band is the signal of *Actb* and *Actg1* and the lower band is that of *Actg2*. Intense *Scd2* signal was observed in brain and ovary. (B) *In situ* hybridization analysis of *Scd2* in the mouse ovary. Frozen sections (10  $\mu$ m) from an ovary of a superovulated mouse were hybridized with a digoxigenin-labeled antisense or sense cRNA probe. A region marked in a box in the upper panel is shown at higher magnification at the bottom. The scale bar represents 400  $\mu$ m in upper panels and 100  $\mu$ m in a lower panel.

from liver and primary granulosa cells. Based on the Long-RNA-seq database from ENCODE/Cold Spring Harbor Lab (GEO accession number: GSM900195 and GSM900183), a 15-kb upstream region of the *Scd2* gene was explored together with CNS1 and CNS3. CNS2 was not examined because it is so difficult to distinguish whether CNS2



**Fig. 2.** Conserved noncoding sequences (CNSs) at the stearoyl-CoA desaturase 2 (*Scd2*) locus. A 35-kb sequence of the mouse *Scd2* locus was compared with the corresponding region of the human *SCD1* locus. The mouse *Scd2* locus is depicted by rectangles indicating exons. White rectangles are 5' and 3' untranslated regions, and black shows the coding region. Below the gene structure, the sequence homology is shown. Highly conserved regions are painted with grey, and the grey regions not overlapping with *Scd2* promoter or exons are CNSs. Four CNSs were found at this locus and named CNS1-CNS4 as indicated.

is transcribed by itself or as a part of *Scd2* gene expression. CNS4 was not examined, either, because the entire region overlapped with repeat sequences. Intriguingly, noncoding transcripts were detected in both tissues for CNS1 and CNS3, while two upstream regions were exclusively or predominantly transcribed in granulosa cells (Fig. 3A). We named these transcripts *lncRNA-sc1* and *lncRNA-sc2* and further characterized them.

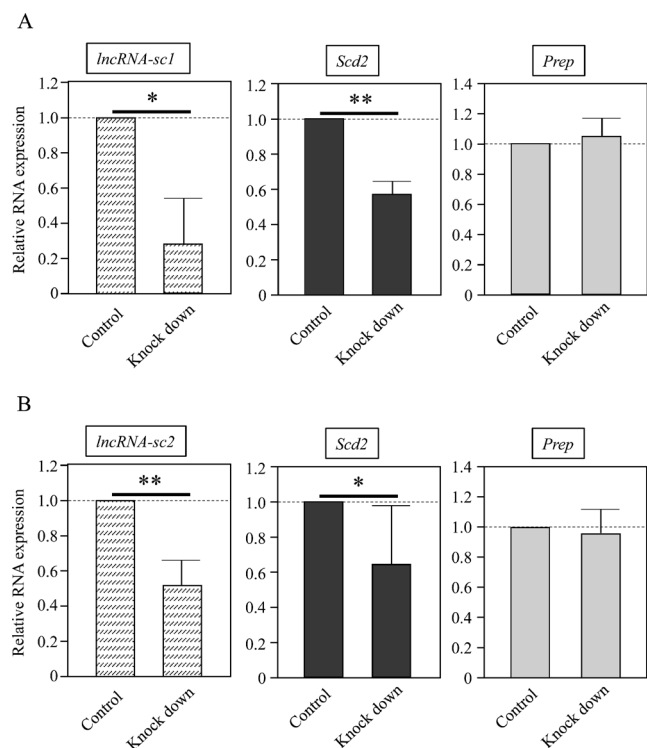
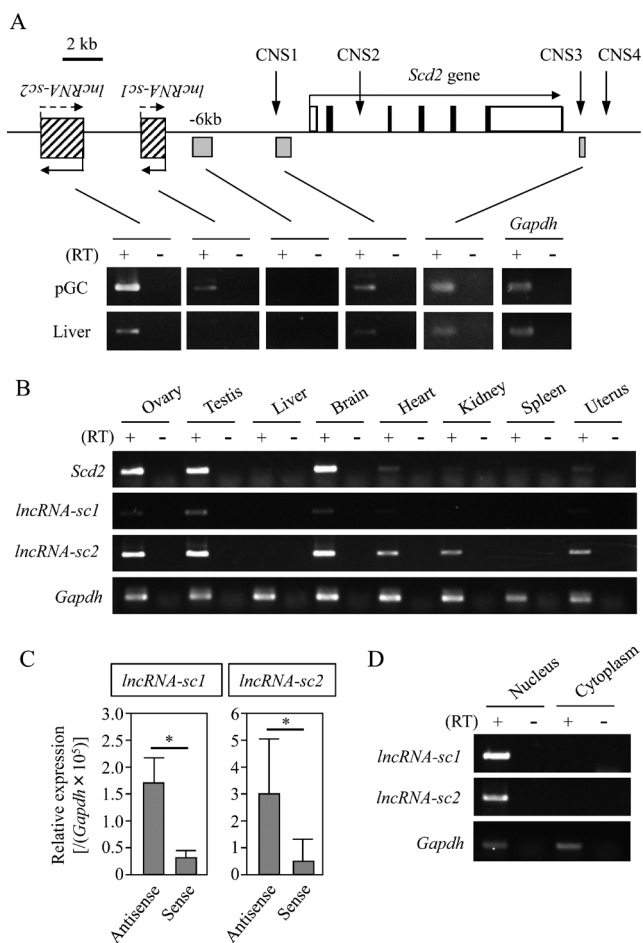
The tissue specificity of *lncRNA-sc1* and *lncRNA-sc2* was examined by RT-PCR with 8 mouse tissues, and the signals of both lncRNAs were detected in ovary, testis, and brain, similarly to *Scd2* mRNA (Fig. 3B). The transcriptional direction was determined by qRT-PCR using strand-specific primers for reverse transcription. The result indicated that both lncRNAs were transcribed in both directions, but more RNAs were in the antisense direction (Fig. 3C). The transcription level of antisense strand was 5.2-fold and 4.9-fold higher than that of sense for *lncRNA-sc1* and *lncRNA-sc2* respectively. The subcellular localization was also investigated by performing RT-PCR with total RNAs from nuclear and cytoplasmic fractions of granulosa cells. Both lncRNAs were present only in the nucleus, and no cytoplasmic signal was observed (Fig. 3D). Collectively, *lncRNA-sc1* and *lncRNA-sc2* transcription were correlated with *Scd2* expression, and both of them were localized in the nucleus of granulosa cells. This raised the possibility that these lncRNAs were involved in the *Scd2* gene activation in the ovary.

#### *Both lncRNA-sc1 and lncRNA-sc2 contributes to Scd2 gene activation*

To see whether the two lncRNAs were involved in the regulation of the *Scd2* gene, we attempted to knock them down. The knockdown with shRNA was only successful for *lncRNA-sc1*, and we used the 2',4'BNA gapmer type of ASO for *lncRNA-sc2*. By transfection with shRNA or ASO into primary granulosa cells, the level of *lncRNA-sc1* and *lncRNA-sc2* was decreased by 70% and 47%, respectively (Fig. 4). These knockdowns caused 45% and 36% significant reductions in the *Scd2* mRNA level compared to the control, while off-target knockdown of *Prep* mRNA was not observed by shRNA or ASO, suggesting the successful knockdown of specific lncRNAs (Fig. 4). These results raise the possibility that both *lncRNA-sc1* and *lncRNA-sc2* contribute to *Scd2* gene activation in mouse granulosa cells.

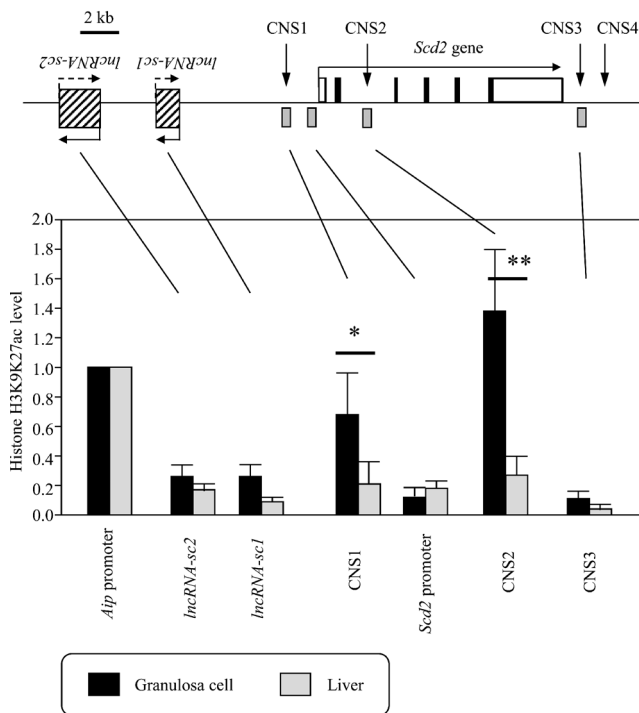
#### *Histone acetylation pattern at the Scd2 locus*

In contrast to *lncRNA-sc1* and *lncRNA-sc2*, the transcription at CNSs was not correlated with *Scd2*, but they might function as enhancers. Because the chromatin at active cis-regulatory elements are often acetylated, we next investigated histone H3K9/K27ac patterns at the *Scd2* locus. We performed the ChIP-qPCR analysis with nuclei from primary granulosa cells and liver and measured the histone acetylation level in each CNS, each lncRNA sequence, and the *Scd2* promoter. The acetylation level was normalized to that at the *Aip* promoter because this gene is recognized as one of the best control genes for expression analysis and is positively marked with H3K9/K27ac in both tissues [12, 26]. In the liver, H3K9/K27ac levels



**Fig. 4.** Knockdown of *lncRNA-sc1* and *lncRNA-sc2* in primary granulosa cells. (A) Primary granulosa cells were transfected with a vector containing shRNA for *lncRNA-sc1* (Knock down) or a control vector (Control). After selection with G418, total RNAs were purified and the expression of *lncRNA-sc1*, *Scd2*, and *Prep* was investigated by quantitative RT-PCR (qRT-PCR). The level was normalized to *Gapdh*, and the value in the control sample was set to 1.0. Successful knockdown of *lncRNA-sc1* significantly decreased stearoyl-CoA desaturase 2 (*Scd2*) expression. The data are presented as mean  $\pm$  SD from three independent experiments, and the statistical significance was analyzed by Student's *t* test. \*  $P < 0.05$ ; \*\*  $P < 0.01$ . (B) Primary granulosa cells were transfected with the 2',4'RNA gapmer type of ASO for *lncRNA-sc2* (Knock down) or EGFP (Control), and total RNAs were isolated two days later. The expression of *lncRNA-sc2*, *Scd2*, and *Prep* was investigated and presented as in (A). The knockdown of *lncRNA-sc2* significantly reduced *Scd2* expression. The data are presented as mean  $\pm$  SD from six independent experiments, and the statistical significance was analyzed by Student's *t* test. \*  $P < 0.05$ ; \*\*  $P < 0.01$ .

at all regions were lower than the *Aip* promoter, while in granulosa cells, CNS1 and CNS2 showed a comparable level to and a higher level than the *Aip* promoter, respectively (Fig. 5). The levels at CNS1 and CNS2 were significantly higher in granulosa cells than in the liver (Fig. 5). The two lncRNA sequences showed slightly higher H3K9/K27ac levels in granulosa cells than in the liver, but the difference was not statistically significant. These results raised the possibility that CNS1 and CNS2, rather than their transcripts, were cis-regulatory elements for *Scd2* gene activation in granulosa cells.



**Fig. 5.** Histone acetylation patterns at the mouse stearyl-CoA desaturase 2 (*Scd2*) locus. ChIP was performed with nuclei from primary granulosa cells and liver using anti-histone H3K9/K27ac antibody. DNAs were purified from the chromatin precipitated with the antibody (bound) and before immunoprecipitation (input), and subjected to qPCR for the indicated regions. The acetylation level was calculated as the bound to input ratio, and further normalized to the level at the housekeeping *Aip* gene promoter, which was set to 1.0. The levels in granulosa cells and liver are shown by black and light gray bars, respectively. The data are presented as mean  $\pm$  SD from four independent experiments with two sets of cell/tissue samples, and the statistical significance was analyzed by one-way ANOVA followed by Tukey-Kramer test. \*  $P < 0.05$ ; \*\*  $P < 0.01$ .

#### *CNS1 and CNS2 exhibit interdependent enhancer activity for the Scd2 promoter*

Finally, we assessed the enhancer activity of CNS1 and CNS2 by a transient reporter gene assay. We obtained a 2.4-kb *Scd2* promoter sequence, which contained CNS1, by PCR, and connected it to the luciferase gene. 0.6-kb and 1.3-kb promoter sequences that contained no and partial CNS1, respectively, were obtained by restriction digestion of the 2.4-kb promoter. The 2.4-kb promoter, in which the CNS1 sequence was deleted, was also generated, and then CNS2 was connected to each construct. These constructs were transfected into primary granulosa cells or mouse hepatic Hepa1-6 cells, and luciferase activity was measured two days later.

In both granulosa and hepatic cells, CNS2 showed enhancer activity only when CNS1 was present because luciferase activity was significantly higher in 2.4kPr-CNS2 than 2.4kPr while no difference was observed between 1.3kPr and 1.3kPr-CNS2 or between 2.4k $\Delta$ CNS1Pr and 2.4k $\Delta$ CNS1Pr-CNS2 (Fig. 6). However, the fold increase in luciferase activity of 2.4kPr-CNS2 to 2.4kPr was higher

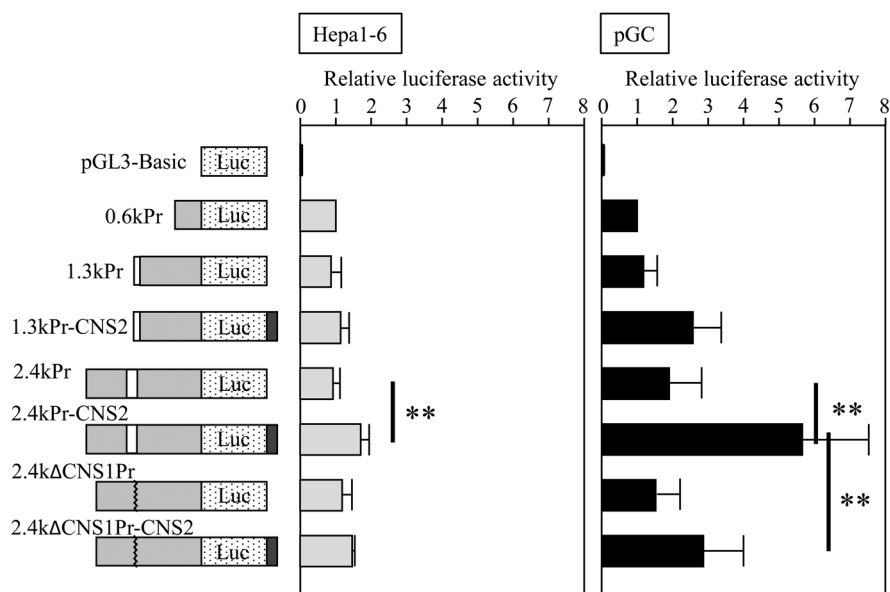
in granulosa cells (3.0) than in hepatic cells (1.8), suggesting the physiological significance of CNS2 in granulosa cells. By contrast, CNS1 showed enhancer activity only in granulosa cells, because a significant difference was detected between 2.4kPr-CNS2 and 2.4k $\Delta$ CNS1Pr-CNS2 in granulosa cells but not in hepatic cells (Fig. 6). The enhancer activity was also dependent on the presence of CNS2, since luciferase activity was not significantly different between 1.3kPr and 2.4kPr or between 2.4kPr and 2.4k $\Delta$ CNS1Pr in granulosa cells (Fig. 6). These raised the possibility that both CNS1 and CNS2 possessed enhancer activity for the *Scd2* promoter to function predominantly and specifically in granulosa cells and they were interdependent.

## Discussion

Here we report a possible mechanism of mouse *Scd2* gene activation in ovarian granulosa cells by two enhancers and two lncRNAs. The potential enhancers are CNSs identified by comparison of the mouse *Scd2* locus with the human *SCD1* locus, but these two genes show different tissue distribution: mouse *Scd2* in ovary and brain and human *SCD1* in liver and brain [27]. Transcriptional regulation of human *SCD1* gene has been well studied, and several transcription factors contribute to the gene activation by binding to a promoter region mostly within 1 kb from TSS [28]. On the other hand, sequences more than 1 kb distant from TSS are shown to have little effect on the transcription [29]. CNS1 is located further upstream of the 1-kb promoter region, so it is unlikely to be involved in human *SCD1* regulation, and the function of CNS2 has never been assessed in any species. These CNSs may have unknown functions in human.

It is interesting that CNS1 and CNS3 are transcribed in both granulosa cells and liver, even if the *Scd2* gene is not expressed in the adult liver. These noncoding transcripts may have some common functions to both tissues such as the construction of subnuclear or subcellular structures and the regulation of RNA stability [6, 30]. Alternatively, the transcripts may contribute to the repression of a neighboring gene in both tissues. Indeed, mouse genome contains four *Scd* genes consisting of a gene cluster, and *Scd3* and *Scd4* genes are expressed at undetectable levels in liver and ovary according to the Mouse ENCODE transcriptome data [31] and some literatures [32, 33]. In contrast to these noncoding RNAs from CNSs, *lncRNA-sc1* and *lncRNA-sc2* are transcribed from non-conserved sequences.

Both *lncRNA-sc1* and *lncRNA-sc2* transcripts are longer than 200 bases and have little coding potential, and therefore, they are highly likely to function as lncRNAs. They were detected by RT-PCR with the oligo(dT) primer, which suggests that they are polyadenylated. However, we cannot conclude it because the identification of 5'- and 3'-ends of these lncRNAs was not successful by the methods that were successful for other lncRNAs [13, 14, 22, 23, 34]. *lncRNA-sc1* and *lncRNA-sc2* may have multiple transcriptional start and termination sites, as is the case for some lncRNAs [35–37]. These lncRNAs were localized to nuclei of granulosa cells, which encouraged us to examine their contribution to transcriptional regulation. Using shRNA and ASO, we successfully knocked down these lncRNAs and observed significant decreases in *Scd2* expression. This and the correlation of both lncRNAs with *Scd2* in tissue distribution support that they are regulatory factors of *Scd2* gene activation in



**Fig. 6.** Reporter gene assay in mouse hepatic and primary granulosa cells. The indicated constructs were transfected into Hepa1-6 cells or primary granulosa cells (pGC), and luciferase activity was measured two days later. White and black rectangle indicated CNS1 and CNS2, respectively in the illustration of the constructs. The activity was normalized to that of 0.6kPr, which was set to 1.0. Light gray and black bars in the graph show the data in mouse hepatic Hepa1-6 cells and pGC, respectively. The data are presented as mean  $\pm$  SD from five to eleven independent experiments, and the statistical significance was analyzed by one-way ANOVA followed by Tukey-Kramer test. \*\*  $P < 0.01$ .

mouse granulosa cells. However, the mechanism by which these lncRNAs contribute to *Scd2* gene activation remains unknown, and they may directly interact with the *Scd2* gene or be involved in the regulation indirectly.

Besides lncRNAs, we found CNS1 and CNS2 to be potential enhancers for *Scd2* gene in granulosa cells. CNS2 significantly increased promoter activity in hepatic cells, but the fold increase was small and only a low level of histone acetylation was associated with CNS2 in the liver. This suggests that CNS2 does not physiologically function as an enhancer in the liver even if it showed significant activity by an *in vitro* reporter gene assay. In contrast, both CNS1 and CNS2 clearly increased *Scd2* promoter activity and the chromatin at these regions were highly acetylated at H3K9/K27 in granulosa cells. H3K9ac is widely distributed in active chromatin and actively transcribed regions [38], and H3K27ac is known to be an enhancer mark in some cell types [39, 40]. Therefore, the high levels of H3K9/K27ac at CNS1 and CNS2 regions raises the possibility that they are located at active chromatin in granulosa cells and function as granulosa cell-specific enhancers for *Scd2* gene.

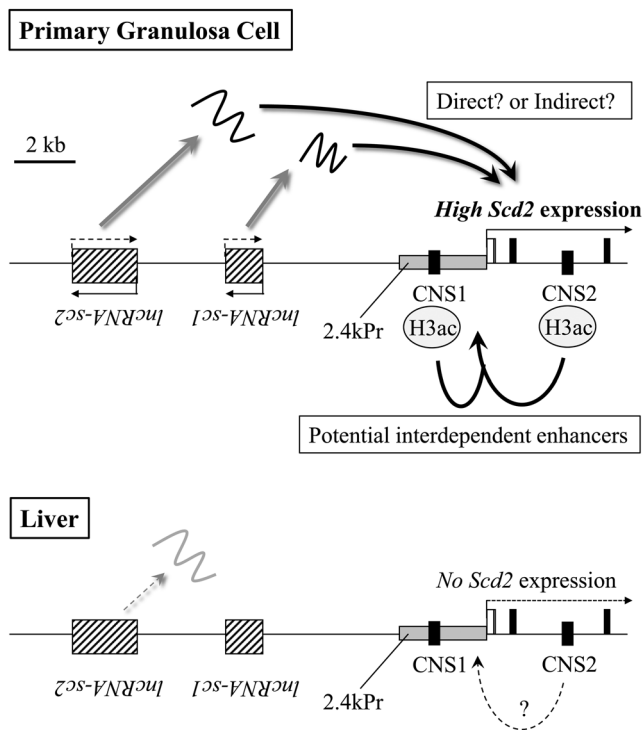
Notably, our data show that CNS1 and CNS2 enhance *Scd2* promoter activity in an interdependent manner. A pair of interdependent enhancers was shown by some studies demonstrating that they increase promoter activity of their target genes only when both are present [41]. The mechanism is largely unclear, but at the human CIITA locus, distal enhancers interact with each other via chromatin looping and cooperate together for gene activation [42]. In this case, a chromatin-remodeling enzyme BRG1 is necessary for the cooperation. It is reported that two transcription factors, SREBP1a and EGR2, bind to the *Scd2* promoter for the transcriptional activation in other

tissues [20, 21], and these factors are expressed in mouse granulosa cells [43, 44]. In addition, their binding motifs are found in both CNS1 and CNS2 by searching at TFBIND (<http://tfbind.hgc.jp/>). These raise the possibility that these or other transcription factors mediate the chromatin opening and facilitate the genomic interaction between CNSs and the *Scd2* promoter in granulosa cells, leading to enhancing the gene expression. More studies will be required for detailed mechanisms.

From these data, we propose a possible model of mouse *Scd2* gene activation in granulosa cells (Fig. 7). In this model, transcription of mouse *Scd2* gene in granulosa cells is activated by two enhancers and two lncRNAs. *lncRNA-sc1* and *lncRNA-sc2* that are not expressed or expressed at a low level in the liver are transcribed approximately 7 kb and 12 kb upstream of the *Scd2* gene, respectively, and contribute to the gene activation by a direct or indirect mechanism in granulosa cells. CNS1 located 1.2 kb upstream of the *Scd2* gene and CNS2 in intron 2 are acetylated at histone H3K9/K27, which may allow these CNSs to interact with each other and enhance *Scd2* gene transcription in granulosa cells. CNSs with low levels of histone acetylation in the liver may not be competent for enhancing or even transcribing the *Scd2* gene. Although the causal relationship between lncRNAs and CNSs is not clear, it may be possible that the noncoding transcription recruits a histone acetyltransferase for acetylation of CNSs, or vice versa.

Specific gene expression in granulosa cells plays central roles in control of oogenesis, and the regulatory mechanism of transcriptional activation has been studied for several genes such as *Cyp11a1*, *Cyp19a1*, *Star*, and *Adams-1* [45–48]. Most of them focused on their promoters and upstream enhancers and a few investigated the





**Fig. 7.** A proposed model of stearoyl-CoA desaturase 2 (*Scd2*) gene activation in mouse granulosa cells and liver. Two lncRNAs are transcribed at the upstream of *Scd2* gene, and both *lncRNA-sc1* and *lncRNA-sc2* play roles in *Scd2* gene activation in granulosa cells. In the liver, *lncRNA-sc2* may be expressed at a low level but *lncRNA-sc1* is not expressed. CNS1 and CNS2 are predominantly marked with acetylated histone H3 in granulosa cells and possibly function as granulosa cell-specific enhancers in an interdependent manner. In the liver, CNS2 showed low enhancer activity *in vitro*, which may not be physiologically significant. Two CNSs and two lncRNAs may thus function in the mouse *Scd2* gene activation in granulosa cells. Details are described in the text. The genome regions transcribed into lncRNAs are depicted with striped rectangles, and CNS1 and CNS2 are black rectangles marked with each name. The 2.4-kb *Scd2* promoter in our reporter gene assay is indicated as a grey rectangle pointed with '2.4kPr'.

significance of epigenetic regulation. As a result, it was found that the chromatin at their promoters and enhancers are highly acetylated at histone H3 and thereby transcription factors bind to the regulatory sequences to activate transcription. However, the knowledge of cis-regulatory elements and epigenetic regulation in granulosa cells is still limited and little is known about the function of lncRNAs. The current study, together with our previous data [12–14], extends our understanding of cis-elements and epigenetics and suggests the existence of another gene whose expression is regulated by lncRNAs for gene activation in granulosa cells. These predict a complex mechanism of gene regulation in granulosa cells.

In conclusion, we identified two potential enhancers and two lncRNAs (*lncRNA-sc1* and *lncRNA-sc2*) possibly involved in mouse *Scd2* gene activation in granulosa cells. The potential enhancers (CNS1 and CNS2) are marked with high levels of H3K9/K27ac and can function in an interdependent manner. This is an interesting model

of granulosa cell-specific gene activation and provides significant insights into the regulation of the function of granulosa cells.

**Conflict of interest:** The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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