SEBOX is essential for early embryogenesis at the two-cell stage in the mouse

Subtitle: SEBOX in the mouse early embryo development

Summary: We showed that SEBOX is essential for early embryo development at the two-cell stage by using RNA interference.

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ABSTRACT

Previously, we found high levels of skin-embryo-brain-oocyte homeobox (Sebox) gene expression in germinal vesicle (GV) stage oocytes. The objective of the present study was to determine the role played by SEBOX in oocyte maturation and early embryogenesis by using RNA interference (RNAi). Microinjection of Sebox double stranded RNA (dsRNA) into GV oocytes resulted in a marked decrease in Sebox mRNA and protein expression. However, Sebox RNAi neither affects oocyte maturation rate nor morphological characteristics, including the spindle and chromosomal organization of metaphase II (MII) oocytes. In addition, Sebox RNAi had no discernible effect on the activities of M-phase promoting factor (MPF) or mitogen-activated protein kinase (MAPK). In contrast, microinjection of Sebox dsRNA into pronuclear (PN) stage embryos resulted in holding embryo development at the 2-cell (84.9%) and 4/8-cell (15.1%) stages. We concluded Sebox as a new addition to maternal-effect genes that produced and stored in oocytes and function in preimplantation embryo development.

oocyte maturation, preimplantation embryogenesis, RNA interference, Sebox
INTRODUCTION
During the period between the luteinizing hormone (LH) surge and ovulation, the oocyte nucleus and cytoplasm undergo marked changes, known as oocyte maturation. Oocyte maturation is a complex process involving the progression of nuclear maturation (meiotic division), cytoplasmic maturation (cytoplasmic reprogramming events) and epigenetic maturation [1-3]. Resumption of meiotic maturation starts with the disappearance of the nuclear membrane, germinal vesicle breakdown (GVBD), followed by restructuring chromatin and microtubules. Afterwards, oocytes undergo condensation of chromosomes, metaphase I (MI) spindle formation and separation of homologous chromosomes, with extrusion of the first polar body eventually stopping the cell cycle again at MII [4, 5]. With cytoplasmic maturation occurring throughout the oocyte growth and the meiosis, oocytes prepare the fertilization and embryo development [6]. The process of epigenetic maturation includes heritable chromatin dynamics that influence gene expression without changing the DNA sequence [7, 8].

Development of a mammalian embryo starts with fertilization, the union of a sperm and an oocyte. A fertilized oocyte, also termed zygote, undergoes several mitotic divisions to form the blastocyst before implantation. Until embryonic genome activation occurs at specific embryonic stages, the early embryonic development is controlled by maternal RNAs and proteins [9]. Therefore, identification of the genes expressed in the oocyte, will provide valuable resource to study oocyte maturation, fertilization, and early embryo development.

Previously, we identified a list of genes that differentially expressed between GV and MII during oocyte maturation by using annealing control primer-PCR [10]. Among the list, we found significantly high level of Sebox expression in GV oocytes relative to MII oocytes. There has been only one publication regarding the cloning and characterization of the Sebox gene [11]. Sebox is a mouse paired-like homeobox gene that encodes a protein with a 60 amino acid homeodomain motif that binds DNA and regulates gene expression [11]. Development is governed by molecular processes mediated by homeobox genes, which encode transcription factors. Hence, the homeodomain plays an important role in intracellular interactions and in the control of target gene transcription [12, 13]. The SEBOX homeodomain exhibits substantial similarity to mouse PHOX2 (65% amino acid identity) [14] and if it is a transcription factor, it may represent a key regulator in preimplantation embryo development. However, the function of Sebox is not yet known. Therefore, the objective of this study was to examine the roles played by Sebox in the mouse oocyte maturation and the preimplantation embryo development.
MATERIALS AND METHODS

Animals
All ICR mice were obtained from Koatech (Pyeoungtack, Korea) and mated to male mice of the same strain to produce embryos in the breeding facility at the CHA Stem Cell Institute of Pochon CHA University. All procedures described within were reviewed and approved by the University of Science Institutional Animal Care and Use Committee, and were performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals.

Total RNA isolation
The various organs from 7-wk-old male ICR mice were collected in Dulbecco’s PBS (GIBCO-BRL, Grand Island, NY) and homogenized on ice in 1 ml Trizol (Invitrogen, Carlsbad, CA) reagent and then incubated for 5 min at room temperature. 0.2 ml chloroform was added to supernatant and mixed with vortexing and incubated for 15 min at room temperature. Tube was centrifuged at 12,000 g for 15 min at 4°C. After centrifugation, supernatant was transferred to a new tube containing 0.5 ml of isopropanol and precipitated. The pellet was washed with 75% ethanol, dried, dissolved in DEPC treated water, and stored at -70°C.

Isolation of oocytes and embryos
For isolation of GV oocytes from preovulatory follicles, 4-wk-old female ICR mice were injected with 5 IU of eCG and then sacrificed 46 h later. Cumulus-enclosed oocyte complexes (COCs) were recovered from ovaries by puncturing the preovulatory follicles with 27-guage needles. M2 medium (Sigma, St. Louis, MO) containing 0.2 mM 3-isobutyl-1-methyl-xanthine (IBMX, Sigma) was used to inhibit GVBD. Cumulus cells (CC) were removed from oocytes mechanically by repeated pipetting through a fine-bore pipette. Mural granulosa cells (GC) were recovered from the preovulatory follicles. Isolated oocytes, CC and GC were snap-frozen and stored at -70°C prior to RNA isolation.

To obtain MII oocytes, we injected female mice with 5 IU of eCG, followed by 5 IU hCG after 46 h. Super-ovulated MII oocytes were obtained from the oviduct 16 h after hCG injection. CCs surrounding MII oocytes were removed by treating COCs with hyaluronidase (300 U/ml, Sigma). Female mice were super-ovulated and mated, and embryos were obtained at specific time points post hCG injection as follows: pronucleus 1-cell embryo (PN) at 18-20 h, 2-cell embryos (2C) at 44-46 h, 4-cell embryos (4C) at 56-58 h, 8-cell embryos (8C) at 68-70 h, morula (MO) stage at 80-85 h, and blastocyst (BL) stage at 96-98 h.

Messenger RNA isolation
Messenger RNA was isolated from oocytes and embryos at different developmental stages using the Dynabeads mRNA DIRECT kit (Dynal Asa, Oslo, Norway), according to the manufacturer’s
instructions. To evaluate recovery, 0.1 pg rabbit α-globin mRNA (Sigma) or 0.1 ng Green Fluorescent Protein (GFP) synthetic RNA were added to each oocyte or embryo prior to mRNA extraction [15]. Briefly, oocytes were resuspended in 300 μl lysis/binding buffer (100 mM Tris-HCl [pH 7.5], 500 mM LiCl, 10 mM EDTA, 1% LiDS, 5 mM DTT) for 5 min at room temperature. After vortexing, 20 μl prewashed Dynabeads oligo dT25 were mixed with lysate and annealed by rotating for 5 min at room temperature. The beads were separated with a Dynal MPC-S magnetic particle concentrator and poly (A)^+ RNAs were eluted by incubation in 10 μl of Tris-HCl (10 mM Tris-HCl, pH 7.5) at 65°C for 2 min.

Reverse transcriptase polymerase chain reaction (RT-PCR)

Complementary DNA was synthesized from mRNA or total RNA using 0.5 μg oligo dT primer, according to the SuperScript Preamplification System protocol (Gibco). PCR reactions (20 μl) were carried out in 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl2, 0.2 mM dNTPs, 25 pM of each primer, 2.5 U of Taq DNA polymerase (Promega, Madison, WI). Single oocyte- and single embryo-equivalent cDNAs were used as templates for PCR analysis. PCR reaction conditions and primer sequences for the genes encoding Sebox, Gdf9, Fshr and GFP are listed in Table 1. The location of amplified products within mouse Sebox is indicated in Fig. 1. Sebox-A primer was used for dsRNA preparation while Sebox-B was used for analyzing RNAi-mediated Sebox-specific knockdown by RT-PCR.

PCR reaction products were then separated by 1.5% agarose gel electrophoresis, followed by quantitation using an Image Analyzer (Viber Lourmat). Relative gene expression levels were normalized to that of Gapdh. All experiments were repeated in triplicate.

Quantitative real-time RT-PCR

To measure the amount of Sebox mRNA in a single embryo, quantitative real-time RT-PCR analysis was performed using the iCycler (Bio-Rad, Hercules, CA). The iQ SYBR Green Supermix PCR reagents (Bio-Rad) were used for monitoring amplification and results were evaluated with the iCycler iQ real-time detection system software. The reaction mixture contained cDNA, 20 pmol forward and reverse primers and SYBR Green Supermix 2 (100 mM KCl, 40 mM Tris-HCl [pH 8.4], 0.4 mM each dNTP, 50 U/ml iTaq DNA polymerase, 6 mM MgCl2, SYBR Green I, 20 nM fluorescein and stabilizers). Template was amplified with 40 cycles of denaturation at 95°C for 40 s, annealing at 60°C for 40 s, and extension at 72°C for 40 s. Upon completion of PCR, fluorescence was monitored continuously while slowly heating the samples from 60 to 95°C at 0.5°C intervals. The melting curves were used to identify any nonspecific amplification products. Quantitation of gene amplification was performed by determining the cycle threshold (C_T), based on the fluorescence detected within the geometric region of the semi-log amplification plot. Expression of each mRNA species was normalized to that of rabbit-α-globin mRNA. Relative quantitation of target gene expression was evaluated using the comparative C_T method [16] and experiments were repeated at least three times using different sets of embryos.
Anti-SEBOX antibody production and purification

Rabbit anti-sera were prepared against SEBOX peptides. Polyclonal antibodies were produced against synthetic peptides containing the internal sequence (amino acid residues 67-80: NH2-QNRRAKRIKDRKPG-COOH) of the SEBOX protein. The peptides were coupled to the immunogenic carrier protein keyhole limpet hemocyanin (KLH) via an additional C-terminal cysteine, using the N-γ-maleimidobutyryloxy-succinimide ester (GMBS) conjugation method. Immunization with conjugated peptides and sampling of rabbit anti-sera were performed by a commercial facility (LabFrontier, Suwon, Korea). Crude serum was applied to a peptide-linked affinity resin and anti-Sebox IgG was eluted with 100 mM glycine (pH 2.5), followed by neutralization with 1 M Tris-HCl (pH 8.0).

The non-cysteinylated peptide was coupled to Cyanogen bromide (CNBr)-activated Spheres 4B (GE Healthcare Life Science, Piscataway, NJ). CNBr-activated Sepharose 4B was rehydrated and washed using 1 mM HCl at room temperature. The coupling buffer (0.1 M NaHCO3 [pH 8.3], 0.5 M NaCl) was added to the gel for 4 h followed by addition of non-cysteinylated peptide in 1 ml of coupling buffer. Excess ligand was then washed away with five gel volumes of coupling buffer and the remaining active groups were blocked with 0.1 M Tris-HCl [pH 8.0] for 2 h. The gel was then washed with three cycles of alternating buffer: 0.1 M acetate buffer [pH 4.0] containing 0.5 M NaCl followed by 0.1 M Tris-HCl [pH 8.0] containing 0.5 M NaCl, and 10 ml PBS. The anti-SEBOX antibody (LabFrontier) was added and eluted with 0.1M Glycine [pH 2.8] and stored in 3 M Tris-HCl [pH 8.8] at -20°C.

Western blot analysis

Western blot was performed using an affinity-purified rabbit polyclonal anti-SEBOX antibody (LabFrontier). Protein extract (20 μg/lane) was subjected to 12 % SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences, Piscataway, NJ). The membrane was blocked for 1 h in Tris-buffered saline-Tween (TBST; 0.2 M NaCl, 0.1% Tween-20 and 10 mM Tris [pH 7.4]), containing 5% non-fat dry milk. The blocked membranes were then incubated with affinity-purified rabbit polyclonal anti-Sebox antibody (1:200; LabFrontier) or mouse monoclonal anti-α-tubulin antibody (1:500; sc-8035, Santa Cruz Biotechnology, Santa Cruz, CA) in TBST. After incubation, membranes were incubated with horseradish-peroxidase-conjugated anti-rabbit IgG (1:2000; sc-2030, Santa Cruz) or anti-mouse IgG (1:2000; A-2554, Sigma) in TBST for 1 h at room temperature. After each step, the membrane was washed several times with TBST and then bound antibody was detected using an enhanced chemiluminescence detection system (Santa Cruz) according to the manufacturer’s instruction.

Oocyte dot blot

Oocytes lysate were made by adding 2 μl of lysis buffer (150 mM NaCl, 50 mM Tris-HCl [pH 7.4], 1%
Triton X-100, 0.1% SDS, 5 mM EDTA and freshly-prepared 1 mM phenylmethylsulfonylfluoride) and then loaded onto a Hybond-P PVDF membrane (Amersham Biosciences). The next process of dot blotting is the same as that of Western blot.

Expressed protein levels were quantified by measuring intensity of the area for each dot using Bio 1D software (Vilber Lourmat, France). These values were then normalized by that of the α-tubulin dot, and expressed as a percentage in comparison to that of control oocytes.

**Preparation of Sebox dsRNA**

Sebox-A primers were used to amplify a region of Sebox cDNA (Table 1, Fig. 1), which was then cloned into pGEM-T Easy (Promega) and linearized with Spe I. Insert orientation was confirmed by PCR amplification using the T7 primer with each Sebox-A primer. Single stranded (ss) RNA for each orientation was synthesized using the MEGAscript RNAi Kit (Ambion, Austin, TX) and T7 RNA polymerase. Complementary RNAs were mixed and incubated at 75°C for 5 min, then cooled to room temperature. Formation of dsRNA was confirmed by 1% agarose gel electrophoresis, in which mobility of dsRNA was compared to that of the ssRNA. For microinjection, RNAs were diluted to a final concentration of 2 μg/μl.

**Microinjection and in vitro culture**

GV oocytes and PN stage embryos were microinjected with Sebox dsRNA in M2 medium containing 0.2 mM IBMX or M2 medium alone, respectively. An injection pipette containing dsRNA solution was inserted into the cytoplasm of an oocyte and 10 μl dsRNA were microinjected using a constant flow system (Transjector; Eppendorf, Hamburg, Germany). In order to assess injection damage, oocytes and embryos were injected with elution buffer alone, and used as sham controls. To determine the rate of maturation in vitro, oocytes were cultured in M16 medium for 16h or in M16 containing 0.2 mM IBMX for 8 h followed by culture in the plain M16 for 16 h in 5% CO₂ at 37°C.

To determine the rate of embryo development in vitro, control or Sebox dsRNA-microinjected PN stage embryos were cultured in M16 medium containing 100 μM EDTA (Sigma) for 3 days.

**Non-invasive examination of spindle structure**

Spindle structure observation was performed using the LC Polscope optics and controller system, combined with a computerized image analysis system (Oosight™ Meta Imaging System, CRI Inc., MA).

**Immunofluorescence staining**

Denuded oocytes were placed in Dulbecco’s PBS containing 0.1% polyvinyl alcohol (PBS-PVA), 4%
paraformaldehyde and 0.2% Triton X-100, and then fixed for 40 min at room temperature. Fixed oocytes were washed 3 times in PBS-PVA for 10 min each, and stored overnight in 1% BSA-supplemented PBS-PVA (BSA-PBS-PVA). Oocytes were blocked with 3% BSA-PBS-PVA for 1 h and incubated with the mouse monoclonal anti-α-tubulin antibody (1:100 dilution, sc-8035; Santa Cruz Biotechnology) at 4°C overnight. After washing, oocytes were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (1:40; Sigma) for 1 h at room temperature, and DNA was counterstained with propidium iodide (Sigma).

**Dual kinase activity assay**

Oocytes were washed in 0.1% PBS-PVA and then each oocyte was placed in an Eppendorf tube with 1 µl of 0.1% PBS-PVA and 4 µl ice-cold extraction buffer (80 mM β-glycerophosphate, 25 mM HEPES [pH 7.2], 20 mM EGTA, 15 mM MgCl₂, 1 mM DTT, 1 mM APMSF, 0.1 mM Na₃VO₄, 1 µg/ml leupeptin [Sigma] and 1 µg/ml aprotinin [Sigma]). Samples were frozen at -80°C until assayed. After thawing, oocytes were centrifuged at 13,000 g for 3 min, followed by the addition of 5 µl kinase buffer and 5 µl substrates, and incubation for 20 min at 37°C. The kinase buffer comprised 75 mM HEPES (pH 7.2), 75 mM β-glycerophosphate, 75 mM MgCl₂, 6 mM DTT, 10 mM EGTA, 60 µM ATP, 15 µM cAMP-dependent protein kinase inhibitor peptide (Sigma) and 0.3 µCi/µl [γ⁻³²P]-ATP (250 µCi/25 µl; Amersham Pharmacia Biotech, Amersham, UK). The substrate solution for the MPF and MAPK double kinase assay contained 4.5 µl histone H1 (5 mg/ml, calf thymus) and 0.5 µl of myelin basic protein (MBP; 5 mg/ml, bovine brain). The reaction was terminated by the addition of 5 µl 4 X SDS sample buffer and boiling for 5 min. Samples were separated by 15% PAGE and then labeled MBP and histone H1 were analyzed by autoradiogram.

**Statistical analysis**

Statistical analysis of real-time PCR data was evaluated using a one-way analysis of variance (ANOVA) and a log linear model. Data were presented as means ±SEM, derived from at least three separate and independent experiments, and a value of p<0.05 was considered as statistically significant.

**RESULTS**

**Sebox mRNA expression**

Sebox mRNA expressed ubiquitously in adult mice tissues with higher expression in the brain and ovaries (Fig. 2A). Among developing ovaries, the highest expression of Sebox mRNA was observed in the postnatal 5-day-old ovaries, which contain many primary follicles with growing oocytes and proliferating cuboidal granulosa cells (Fig. 2B). Sebox was expressed at higher levels in GV than in MII (Fig. 3A and C), and in oocytes than in follicular cells (Fig. 3D). Using quantitative real-time PCR (Fig. 4A), high levels of Sebox transcript were detected in the GV to 2C stage embryos, but thereafter
expression decreased dramatically and no transcript was detected after 4C up to blastocyst stages (Fig. 4B).

Effects of Sebox RNAi in GV oocytes

Maturation rates
To determine the role played by Sebox in oocyte maturation, RNAi was used to silence Sebox expression in mouse GV oocytes. Following the microinjection of Sebox dsRNA into the cytoplasm of GV oocytes, morphological changes and in vitro maturation rates were scored. Completion of meiosis I is morphologically marked by extrusion of the first polar body [17]. Disappearance of the GV and appearance of the first polar body were used as indices to score the maturation status of the oocytes. The maturation rate of the GV oocytes injected with Sebox dsRNA (Sebox RNAi; 82.5%) was not different significantly from those of the control (87.3%) or buffer-injected (80.3%) groups (Table 2). In spite of the knockdown of Sebox mRNA, these oocytes developed to morphologically normal MII similar to the control groups (Fig. 5A). RNAi-mediated knockdown of Sebox in MII oocytes (~86%, Fig. 5, B and E) did not appear to affect the expression of functionally and sequentially unrelated genes such as Plat, Mos and Gapdh. These results suggest that Sebox RNAi caused sequence-specific knockdown of Sebox alone (Fig. 5B).

To confirm the knockdown of SEBOX protein, we used purified anti-SEBOX antibody for Western analysis. A single, specific endogenous band of SEBOX at the size of ~21 kDa was detected in adult ovary and brain, but not in stomach (Fig. 5C). Dot blot analysis for oocytes using the same antibody showed a marked decrease (~53%) in SEBOX protein expression following Sebox RNAi (Fig. 5, D and E).

Sebox degradation rate and oocyte maturation
Sebox mRNA was highly expressed throughout the in vitro maturation (Fig. 6A). We found that Sebox mRNA gradually decreased after RNAi and almost completely disappear at around 8 h in culture (Fig. 6B). To verify the effect of complete knockdown of Sebox on the oocyte maturation, we carried out RNAi followed by 8 h of in vitro culture of oocytes in the IBMX medium and subsequent 16 h of main culture as shown in diagram Fig. 7A. Despite of the specific and marked decrease in Sebox mRNA expression in IBMX for 8 h (Fig. 7B), the consequence of Sebox RNAi was similar to that of control (76% vs. 89%) after 16 h of in vitro maturation (Fig 7C).

Spindle and chromosomal organization
After microinjection of Sebox RNAi, changes in the meiotic spindles of living oocytes were analyzed non-invasively using Polscope (Fig. 8A). Following RNAi treatment, the MII oocytes exhibited spindles of normal shape and localization, but the images were slightly lighter than those of control or buffer-
injected groups. Aceto-orcein staining did not show any differences in chromosomal configuration between oocytes in any of the three groups (Fig. 8B). Immunofluorescence staining with α-tubulin antibody confirmed that oocytes which received Sebox dsRNA underwent normal meiotic maturation, containing spindles and normally aligned chromosomes (Fig. 9).

**Dual kinase activity assay**

Mouse oocyte maturation is regulated by changes in MPF and MAPK activities [18-20]. These activities were measured by examining histone H1 and MBP phosphorylation. Similar to our previous findings, in which no discernible difference was found between uninjected control and Sebox dsRNA injected oocytes, no difference was detected in the activities of these two major regulator kinases (Fig. 10).

**Effects of Sebox RNAi in pronuclear stage embryo**

To evaluate the functional involvement of SEBOX in preimplantation embryo development, Sebox dsRNA was injected into PN embryos and embryo development was observed in vitro. After culturing for 72 h, the majority of control (82.8%) and buffer-injected (56.4%) zygotes had reached to the blastocyst stage, whereas the majority of zygotes injected with Sebox dsRNA remained at the 2C (84.9%) and 4C stages (15.1%; Table 3, Fig. 11). Microinjection of Sebox dsRNA into PN embryos also resulted in a markedly decreased expression of Sebox mRNA (~88%, Fig. 11, B and D) and protein (~47%, Fig. 11, C and D).

**DISCUSSION**

Using loss-of-function analysis, we found that SEBOX played no detectable role in regulating oocyte maturation process, but it involved in regulating embryo development. Microinjection or transfection of siRNA (small interfering RNA) enables selective inhibition of target transcript and protein expression, making it possible to examine function of a specific gene during oocyte maturation and preimplantation embryo development [21-26].

Long dsRNA (over 30 bp) can be used as a specific inhibitor of gene activity in the mouse oocytes and preimplantation embryos, since they do not exhibit a dsRNA-dependent protein kinase R (PKR) response that causes apoptosis in other mammalian cells [27]. In addition, long dsRNAs (~250-400 bp) may produce many intracellular siRNAs through the activity of endogenous Dicer [28]. Thus, we have been successful in obtaining gene-specific knockdown using long dsRNAs for various genes [23-24, unpublished data]. On the contrary, several different siRNAs should be randomly chosen throughout the entire gene sequence and experimentally tested to find the best out of them [29]. We had experienced that some synthetic siRNAs did not degrade targets and concluded that the microinjection of siRNAs one-by-one into the oocytes for testing its efficacy is not an appropriate tool.
The authors of the first report of SEBOX described that Sebox only expressed in the skin, brain by Northern blot, and in oocytes and embryos by in situ hybridization [11]. Approximately 100,000 copies of a DNA or RNA sequence are required for detection by blot hybridization. In contrast, PCR can amplify single copies of DNA or cDNA to readily detectable levels [30]. Therefore, we may detect Sebox mRNA ubiquitously in adult mice tissues by RT-PCR.

The spindle is a highly dynamic microtubular structure that orchestrates chromosome movements during oocyte meiotic maturation. The general chromosome-derived processes include chromosome condensation, spindle organization, kinetochore assembly and microtubule growth or responsiveness. In the present study, we observed that oocytes injected with Sebox dsRNA developed to the morphologically-normal MII. Additionally, we observed normal spindle organization and chromosomal configurations in those MII oocytes, despite the RNAi-mediated decrease in Sebox mRNA and protein expression. Therefore, we concluded that Sebox is not involved in regulation of the oocyte maturation processes.

During oocyte maturation, MPF activity increases from GVBD until metaphase I and then drops sharply when the first polar body is extruded, and increase again as the oocytes progress into meiosis II [19]. The MAPK pathway is involved in regulation of microtubule organization during mammalian oocyte meiosis [18, 20]. MAPK is activated 2 h after GVBD and is inactivated around 6-8 h after fertilization [20]. Therefore, MPF and MAPK activities are high in a normal oocyte at MII stage. Using the dual kinase activity assay, we observed no change in the MPF and MAPK activities of MII oocytes injected with Sebox dsRNA. Therefore, we concluded that Sebox is not engaged in the activities of both MPF and MAPK during in vitro oocyte maturation.

During folliculogenesis and oogenesis, oocytes accumulate maternal-effect genes (MEGs) that are necessary to support early embryonic development. Maternal-effect genes produce and store mRNAs and proteins in the oocyte during oocytes growth and maturation play pivotal roles in early embryogenesis. In mice, eight (Nlrp5 (also known as Mater), Hsf1, Dnmt1 (also known as Dnmt1o), Pms2, Zar1, Npm2, Dppa3 (also known as Stella) and Zfp36l2) have been identified in the mouse [31-38] and three additional candidates (basonuclin, Smarca4 (also known as Brg1) and Oog1) have been proposed as MEGs [39-41]. Null mutations in mammalian MEGs do not affect follicular development, oocyte maturation, ovulation or fertilization, but they influence embryo development.

In the mouse, zygotic gene activation (ZGA) occurs at 1C to 2C stages. ZGA is crucial for the maternal-to-zygotic transition required for subsequent embryonic development and some MEGs may be involved in this process [10]. In embryos with disruption of Nlrp5 or Smarca4, development
becomes arrested at the 2C stage [31, 40]. In other mutants of MEGs, embryos are arrested at the 1C stage \((Npm2, Hsf1)\), at the PN stage \((Zar1)\), at later stages of preimplantation \((Dppa3, Pms2)\), and post-implantation \((Dnmt1)\) [32-37].

In this study, we observed that the microinjection of Sebox dsRNA at GV oocytes had no effect but at PN embryos resulted in developmental arrest of embryos at the 2C stage (84.9%). Therefore, we concluded that Sebox is essential for normal early embryogenesis in the mouse and proposed that Sebox is a new candidate for mammalian MEG. Further investigation is required to understand the mechanism underlying SEBOX function in regulation of embryo development.

ACKNOWLEDGMENTS

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REFERENCE

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FIGURE LEGENDS
Fig. 1. Schematic diagram of mouse Sebox (NM_008759) gene showing different sets of location of PCR-amplified products used for preparing Sebox dsRNA (Sebox A) and confirming knockdown of endogenous Sebox mRNA (Sebox B).
Fig. 2. Expression of Sebox mRNA. (A) Semi-quantitative RT-PCR analysis was performed on various mouse tissues to evaluate the relative expression of Sebox. M, muscle; H, heart; Lu, lung; St, stomach; B, brain; Sp, spleen; Li, liver; K, kidney; T, testis; U, uterus; 1d_O, neonatal 1-day-old ovary; 4w_O, 4-wk-old ovary. (B) Semiquantitative RT-PCR analysis for differential expression of Sebox mRNA according to the ovarian developmental stage. 1d, neonatal 1-day; 5d, 5-day-old; 2w, 2-wk-old; 3w, 3-wk-old; 4w, 4-wk-old ovaries. Gapdh was used as an internal control.

Fig. 3. Differential Sebox expression in oocytes and follicular cells. (A, B) Amplification profiles of Sebox (A) and rabbit α-globin (B) in oocytes. Each upper inner box represents the melting curves for amplified Sebox and rabbit α-globin confirming the right synthesis of the target without contamination with other products. (C) Expression of Sebox mRNA in oocyte was calculated from CT values from the real-time PCR data. Sebox levels were normalized with rabbit α-globin mRNA, and relative expression was calculated. Experiments were repeated at least three times and data were expressed as mean±SEM. Asterisks represent statistical significance at p<0.05. GV, germinal vesicle; MII, metaphase II. (D) RT-PCR analysis of Sebox mRNA in follicular components. OO, GV oocytes; CC, cumulus cells; GC, granulosa cells. Genes encoding Gdf9 and Fshr were used as markers for oocytes and granulosa cells, respectively. GFP was used as the external control.

Fig. 4. Quantitative real-time RT-PCR analysis of Sebox expression during embryo development. (A) Following reverse transcription of mRNAs isolated from oocytes and embryos at various stages of development, PCR amplification was performed using cDNAs equivalent to a single oocyte or single embryo. Upper inner box is melting curves for amplified Sebox. (B) Expression levels were calculated from CT values and normalized with rabbit α-globin mRNA, then expression ratio was determined relative to that of MII oocytes. Experiments were repeated at least three times and data were expressed as mean±SEM.

Fig. 5. Sebox RNAi in GV oocytes. Microinjection of Sebox dsRNA into the cytoplasm of GV oocytes resulted in normal MII formation, despite decreased expression of Sebox mRNA and protein. (A) Microphotographs of MII oocytes cultured in vitro for 16 h with and without Sebox RNAi. Magnification ×10. (B) Sebox RNAi resulted in specific suppression of Sebox mRNA expression. RT-PCR analysis was used to determine mRNA levels in a single oocyte. Unchanged expression of untargeted genes (Plat, Mos and Gapdh) suggesting Sebox-specific silencing. (C) Western blot analysis of SEBOX. The blot incubated by using affinity-purified rabbit polyclonal anti-SEBOX antibody. The sizes of the protein markers are indicated on the left side of the figure. α-tubulin was used as a loading control. O, ovary; B, brain; S, stomach. (D) Protein levels in oocytes were determined using dot blot analysis. Proteins were extracted from three MII oocytes for each dot. (E) Sebox mRNA and protein expression after Sebox RNAi in the GV oocytes. Graphical presentation of results shown in Fig. 5, B and D. The
experiment was performed three times, and the data are presented as the mean±SEM. Asterisks represent statistical significance at \( p<0.05 \). Light gray, Sebox mRNA; Dark gray, SEBOX protein. Control, uninjected; Buffer, buffer-injected (sham control); Sebox RNAi, Sebox dsRNA microinjected.

Fig. 6. Determination of critical time point for complete knockdown after Sebox RNAi. (A) Typical pattern of Sebox mRNA expression during normal \textit{in vitro} maturation. For the PCR reaction, single oocytes-equivalent cDNA was used as template for amplification. GV, GVBD, MI and MII were harvested 0 h, 2 h, 8 h and 16 h from \textit{in vitro} maturation, respectively. (B) Typical pattern of Sebox mRNA degradation during \textit{in vitro} maturation after Sebox RNAi. RT-PCR analysis was used to evaluate mRNA levels every 8 h after Sebox RNAi. The expression of H1foo represents an internal control.

Fig. 7. Effect of completely decreased Sebox mRNA on \textit{in vitro} oocyte maturation. (A) Diagram for experimental strategy. GV oocytes were microinjected with Sebox dsRNA, and oocytes were cultured in M16 supplemented with IBMX for 8 h and moved to the plain M16 for additional 16 h culture. (B) Sebox RNAi and culture in IBMX medium resulted in specific suppression of Sebox mRNA expression of the GV oocytes. Complete suppression of Sebox in GV oocytes before starting \textit{in vitro} maturation was confirmed by RT-PCR at 8 h with IBMX treatment. (C) Maturation rate of oocytes cultured in IBMX medium for 8 h followed by 16 h culture in plain M16 medium.

Fig. 8. Chromosomal configuration and spindle formation in oocytes following microinjection of Sebox RNAi. (A) Non-invasive analysis of spindle structure using Polscope. Micrographs show the same oocytes under bright field (left panel) and dark field (right panel). (B) Aceto-orcein staining of MII oocytes showed normal chromosomal configurations. Magnification ×20 (A) and ×40 (B).

Fig. 9. Immunofluorescent staining of \( \alpha \)-tubulin and DNA in MII oocytes. MII oocytes were fixed in 4% paraformaldehyde and then stained with \( \alpha \)-tubulin antibody (Green). DNA was counterstained with propidium iodide (Red). Magnification ×40.

Fig. 10. Dual kinase activity assay to assess the activities of MPF and MAPK. Amounts of phosphorylation of substrates Histone H1 and MBP reflect the activities of MPF and MAPK, respectively. One oocyte was loaded per lane. 1, uninjected oocyte; 2, buffer-injected oocyte (sham control); 3-5, Sebox dsRNA-injected oocytes.

Fig. 11. Sebox RNAi in PN embryos. (A) Injection of Sebox dsRNA into PN embryos inhibited early embryogenesis. PN embryos were microinjected with Sebox dsRNA and cultured for 3 days \textit{in vitro} until the blastocyst stage. Magnification ×15. (B) Sebox RNAi resulted in specific suppression of Sebox mRNA expression. RT-PCR analysis was used to determine mRNA levels in a single 2C
embryo. Unchanged expression of untargeted genes (Plat, Mos and Gapdh) suggesting Sebox-specific silencing. (C) Reduced Sebox protein expression in 2C embryos following microinjection of Sebox dsRNA was measured by dot blot analysis. Proteins were extracted from three 2C embryos for each dot. (D) Sebox mRNA and protein expression after Sebox RNAi in the PN embryos. Graphical presentation of results shown in Fig. 11, B and C. Experiment was accomplished three times and the data are presented as the mean±SEM. Asterisks represent statistical significance at p<0.05. Light gray, Sebox mRNA; Dark gray, SEBOX protein.
Table 1. Primer sequences and RT-PCR conditions.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession numbers</th>
<th>Primer sequence *</th>
<th>Annealing temperature</th>
<th>Product size</th>
</tr>
</thead>
</table>
| **Sebox-A** | NM_008759 | For- AAAGCCAGGAGCCCTAAACT  
Rev- TTAGAAGTGTTCTACATTGG | 60°C | 334 bp |
| **Sebox-B** | NM_008759 | For- GGAACATCAAGCCATCCTCT  
Rev- GGCCAGAGCCAAGACTTTA | 60°C | 293 bp |
| **Gdf9** | NM_008110 | For- GGTTCATCTCTGATAGGCAG  
Rev- GGGGCTGAAGGAGGGAGG | 65°C | 446 bp |
| **Plat** | NM_008872 | For- CATGGGCAAAGAGTTACACAG  
Rev- CAGAGAAGAAATGGAGACG | 60°C | 631 bp |
| **Mos** | NM_020021 | For- TGGCTGTCTCTACACTTTC  
Rev- CTTTATACACCGAGCCAA | 60°C | 273 bp |
| **Fshr** | NM_013523 | For- TCCTTCTATGGGACTGACTT  
Rev- AGAGGCTCCCTGCAAACAT | 60°C | 165 bp |
| **H1foo** | NM_138311 | For- GCAGAACCAGAAAGAGGTCAGAA  
Rev- TGGAGGAGGTCTTTGGGAAGT | 60°C | 378 bp |
| **GFP** | EU056363 | For- ATGGTGAGCAAGGGCGAG  
Rev- CTTGTACAGCTCGTCCAT | 60°C | 717 bp |
| **Gapdh** | BC092294 | For- ACCACAGTCCATGCCCAC  
Rev- TCCACCACCCTGTGGCTGA | 60°C | 451 bp |

* For = Forward; Rev = Reverse

Sebox-A primers were used for RT-PCR and preparation of dsRNA. Sebox-B primers were used to confirm the knockdown of Sebox mRNA after RNAi.
Table 2. *In vitro* maturation of mouse oocytes after injection of *Sebox* dsRNA in GV oocytes.

<table>
<thead>
<tr>
<th></th>
<th>Number of oocytes (%)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Germinal vesicle (GV)</td>
<td>Metaphase I (MI)</td>
<td>Metaphase II (MII)</td>
</tr>
<tr>
<td>Control</td>
<td>252</td>
<td>0 (0)</td>
<td>32 (12.7)</td>
<td>220 (87.3)</td>
</tr>
<tr>
<td>Buffer</td>
<td>244</td>
<td>30 (12.3)</td>
<td>18 (7.4)</td>
<td>196 (80.3)</td>
</tr>
<tr>
<td><em>Sebox</em> RNAi</td>
<td>240</td>
<td>26 (10.8)</td>
<td>16 (6.7)</td>
<td>198 (82.5)</td>
</tr>
</tbody>
</table>
Table 3. *In vitro* development of mouse preimplantation embryos after injection of *Sebox* dsRNA into PN embryos.

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>2-cell</th>
<th>4/8-cell</th>
<th>Morula</th>
<th>Blastocyst</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>151</td>
<td>-</td>
<td>9 (5.9)</td>
<td>17 (11.3)</td>
<td>125 (82.8)</td>
</tr>
<tr>
<td>Buffer</td>
<td>165</td>
<td>9 (5.5)</td>
<td>39 (23.6)</td>
<td>24 (14.5)</td>
<td>93 (56.4)</td>
</tr>
<tr>
<td><em>Sebox RNAi</em></td>
<td>126</td>
<td>107 (84.9)*</td>
<td>19 (15.1)</td>
<td>-</td>
<td>-</td>
</tr>
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</table>

*Sebox RNAi* indicates a significant change compared to the control group.
Figure 1

Mouse Sebox: NM_008759
Figure 2

A

B
Figure 5

A

Control Buffer Sebox RNAi

B

<table>
<thead>
<tr>
<th>MII</th>
<th>Sebox</th>
<th>Control Buffer RNAi</th>
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<tbody>
<tr>
<td>Sebox</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plat</td>
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<tr>
<td>Mos</td>
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<tr>
<td>Gapdh</td>
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</table>

C

D

Control Buffer Sebox RNAi

E

Relative expression level (%)

Control Buffer Sebox RNAi
Figure 6

A

<table>
<thead>
<tr>
<th></th>
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<tbody>
<tr>
<td></td>
<td>GV</td>
<td>GVBD</td>
<td>MI</td>
<td>MII</td>
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<tr>
<td>0 h</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 h</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>8 h</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16 h</td>
<td></td>
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</table>

Sebox

H1foo

B

<table>
<thead>
<tr>
<th></th>
<th>Sebox RNAi</th>
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</tr>
<tr>
<td>4 h</td>
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<td>6 h</td>
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<tr>
<td>8 h</td>
<td></td>
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</tr>
</tbody>
</table>

Sebox

H1foo
Figure 10

MII oocyte

1  2  3  4  5

Histone H1 (MPF)

MBP (MAPK)