Associations among Sebox and Other MEGs and Its Effects on Early Embryogenesis

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Abstract

In a previous report, we identified Sebox as a new candidate maternal effect gene that is essential for embryonic development and primarily impacts the two-cell (2C) stage. The present study was conducted to determine the mechanism of action for Sebox in this capacity, as shown by changes in the expression levels of other known MEG mRNAs after Sebox RNA interference (RNAi) in oocytes. Sebox-knockdown metaphase II (MII) oocytes displayed normal morphology, but among the 23 MEGs monitored, 8 genes were upregulated, and 15 genes were unchanged. We hypothesized that the perturbed gene expression of these MEGs may cause the arrest of embryo development at the 2C stage and examined the expression of several marker genes for the degradation of maternal factors and zygotic genome activation. We found that some maternal mRNAs, c-mos, Gbx2, and Gdf9, were not fully degraded in Sebox-knockdown 2C embryos, and that several zygotic genome activation markers, Mt1a, Rpl23, Ube2a and Wee1, were not fully expressed in conjunction with diminished embryonic transcriptional activity. In addition, Sebox may be involved in the formation of the subcortical maternal complex through its regulation of the upstream regulator, Figla. Therefore, we concluded that Sebox is important in preparing oocytes for embryonic development by orchestrating the expression of other important MEGs.

Introduction

During fertilization, oocytes resume their meiotic division upon penetration by sperm. Thereafter, the initial cleavage of the zygote early in embryogenesis proceeds without differentiation and growth of the zygote until successful implantation in the mother’s uterus occurs. The particular events that occur during the journey from the oviduct to the uterus rely on factors that are encoded by maternal effect genes (MEGs), which accumulate over the course of oogenesis [1]. A milestone in early embryogenesis that is essential for further embryonic development is the maternal-to-zygotic transition (MZT) [2]. This is the point at which oocyte-specific maternal factors selectively disappear and male or female zygotic genomes are selectively activated. Zygotic genome activation (ZGA) in mice occurs at the two-cell (2C) to four-cell (4C) embryonic transition [3], whereas in bovine, ovine, and human species, this transition occurs at the
Thus, MZT abnormalities may culminate in embryonic arrest or lead to deficiencies in factors that are required for further developmental stages.

Growing oocytes synthesize and accumulate RNAs and proteins that contribute to the normal early embryonic development. Using annealing control primer PCR (described elsewhere), we previously detected differential gene expression levels in the germinal vesicle (GV) and metaphase II (MII) stages of oocyte maturation [5]. We also previously identified that Sebox expression was greater in GV than in MII oocytes and that Sebox plays a role as an MEG that is essential for embryonic development, functioning primarily at the 2C stage; however, the precise molecular mechanisms of Sebox as an MEG have yet to be clarified [6].

Recently, other sources have substantiated the importance of Sebox in early oogenesis [7]. Sebox is a mouse paired-like homeobox gene that encodes a transcription factor with a 60 amino acid single homeodomain motif (Fig. 1). In 2000, Cinquanta and colleagues reported the Sebox expression in skin, brain, oocytes, and 2-cell stage embryos [8]. Homeobox genes are a large class of transcriptional regulators that are essential for regulating cell differentiation and the formation of body structures during early embryonic development. Homeobox genes share a highly conserved DNA-binding domain of 60 amino acids, named the homeodomain, which binds to a specific DNA sequence and regulates expression of genes. Therefore, proteins that include a homeodomain play an essential role in both intracellular interactions and control of the expression of target genes.

MEGs were first described in Drosophila [9], but the concept of mammalian MEGs was first reported in 2000 [10], with the subsequent discovery of approximately 30 MEGs. MEGs are generally grouped by function during embryonic development as follows: 1) degradation of maternal factors, 2) chromatin remodeling, 3) transcriptional activity, 4) DNA methylation, 5) subcortical maternal complex (SCMC), and 6) pre-implantation development [11]. Therefore, due to their major role in embryogenesis, mutations of MEGs not only place embryonic development in jeopardy but may also compromise oocyte maturation and meiotic division. The present study was conducted to explore the role(s) of Sebox in early embryogenesis, assessing the influence of the loss-of-function of Sebox on the expression levels of other MEGs in oocytes and on early embryogenesis, particularly the degradation of maternal factors and the transcriptional activity of zygotes during MZT.

**Materials and Methods**

**Research animals**

ICR mice (female and male), exclusively provided by Koatech (Pyeoungtack, Korea), were mated to produce embryos in the breeding facility at the CHA Research Institute of CHA University. All procedures described herein were reviewed and approved by the Institutional
Animal Care and Use Committee of CHA University and were performed in accordance with Guiding Principles for the Care and Use of Laboratory Animals.

Isolation of oocytes and embryos

Three-week-old female ICR mice were injected with 5 IU pregnant mare’s serum gonadotropin (PMSG; Sigma-Aldrich, St. Louis, MO, USA) and sacrificed 46 h later. Cumulus-enclosed oocyte complexes were then recovered from the ovaries by puncturing preovulatory follicles with 27-gauge needles. M2 medium (Sigma-Aldrich) containing 0.2 mM 3-isobutyl-1-methylxanthine (IBMX; Sigma-Aldrich) was used to inhibit germinal vesicle breakdown (GVBD). Cumulus cells were mechanically retrieved from oocytes by repeated extraction through a fine-bore pipette. Isolated murine oocytes were snap frozen and stored at -70°C prior to RNA isolation. Other female mice were superovulated and mated, and pronuclear embryos (PNs) were obtained 18–20 h after hCG injection.

Messenger RNA isolation

mRNA was isolated from oocytes and embryos at differing developmental stages using the Dynabeads mRNA DIRECT kit (Dynal Asa, Oslo, Norway) according to the manufacturer’s instructions. In short, oocytes were resuspended in 300 μl lysis/binding buffer (100 mM Tris-HCl [pH 7.5], 500 mM LiCl, 10 mM EDTA, 1% LiDS, and 5 mM dithiothreitol [DTT]) for 5 min at room temperature. After vortexing, 20 μl prewashed Dynabeads oligo dT25 was mixed with the lysate and annealed by rotating 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 14 μl Tris-HCl (10 mM Tris-HCl, pH 7.5) at 73°C for 2 min.

Reverse-transcriptase polymerase chain reaction (RT-PCR)

Purified mRNA and 0.5 μg oligo (dT) primer were mixed and incubated at 70°C for 10 min, and cDNA was synthesized. Single oocyte- and single embryo-equivalent cDNAs were used as templates for PCR analysis. Primer sequences for the genes encoding Sebox, Figla and H1foo and PCR conditions are listed in Table 1. Thereafter, PCR products were separated by 1.5% agarose gel electrophoresis and analyzed using the Gel Doc EZ Imager (Bio-Rad). Relative gene expression levels were normalized to those of H1foo. All experiments were repeated three times.

Quantitative real time RT-PCR

Quantitative real time RT-PCR analysis of embryonic MEG mRNA relied on the iCycler iQ Detection System (Bio-Rad Laboratories Inc, Hercules, CA, USA). iQ SYBR Green Supermix PCR reagents (Bio-Rad) were used to monitor amplification, and the results were analyzed using the iCycler iQ proprietary 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). The primer sequences used for the genes analyzed are listed in Table 1. Templates were amplified through 40 cycles of denaturation (40 sec, 95°C), annealing (40 sec, 60°C), and extension (40 sec, 72°C). Upon completion of PCR, fluorescence was monitored continuously as the samples were slowly heated from 60°C to 95°C at 0.5°C intervals. The melting curves were used to identify any nonspecific amplification products. The expression levels of each mRNA species in oocytes and embryos were normalized to those of H1foo and
| Gene  | Accession numbers | Primer sequence* | Annealing temperature | Product size |
|-------|-------------------|------------------|-----------------------|--------------|
| Sebox-A | NM_008759         | F-AAAGCCAGGAGCCCTAAACT | 60°C                 | 334 bp       |
|        |                   | R-TTGAAGTGGTGCTGACATTGG |                      |              |
| Sebox-B | NM_008759         | F-GGAACATCAAGCCATCTCTT | 60°C                 | 293 bp       |
|        |                   | R-GGCCAGAGGCAAAGACTAAAA |                      |              |
| GFP    | KF111246.1        | F-CTGAAAAGTTACCTGCAACAC | 60°C                 | 334 bp       |
|        |                   | R-CGCCATGATATAGACGTTG |                      |              |
| H1foo  | NM_138311         | F-AAAGGAAGATGGCAGACATGG | 60°C                 | 137 bp       |
|        |                   | R-TCTTTGACGTTCTGCCCACTTA |                      |              |
| Actb   | NM_007393.3       | F-GGGTGTGTAGTGGAAGAATGGG | 60°C                 | 489 bp       |
|        |                   | R-GCTTGGTGTGAAGCTGTAG |                      |              |
| Gapdh  | BC092294          | F-ACCACAGTCCATGCGATCACC | 60°C                 | 451 bp       |
|        |                   | R-TCCACCACTCCTGACGTA |                      |              |
| Ago2   | NM_153178.4       | F-AGAAACTAGACGCAGCTGAAG | 60°C                 | 115 bp       |
|        |                   | R-AAAGTACATGGTGCGCAGTG |                      |              |
| Atg5   | NM_053069.5       | F-GCCATCTACAGAAGCTG | 60°C                 | 149 bp       |
|        |                   | R-TTGTTGCTATCCGCGTAATC |                      |              |
| Bmp15  | NM_009757.4       | F-TACAAGGTAGCTTCCACCA | 60°C                 | 135 bp       |
|        |                   | R-ATGGCATGGTTGCTGTA |                      |              |
| Bnc1   | NM_007562.2       | F-ACCACCTCGAGTTGACGAC | 60°C                 | 118 bp       |
|        |                   | R-TGCCATCAGCTGTGCTCATA |                      |              |
| Brf1   | NM_001174078.1    | F-CGCGCAGGATAGTGGAAGGA | 60°C                 | 119 bp       |
|        |                   | R-CACCAGCTGATCTCCACTGTT |                      |              |
| Btg1   | NM_007569         | F-CGACAGCTGACAGCTTTTCC | 60°C                 | 238 bp       |
|        |                   | R-GGTGAGCACCTTCGAGG |                      |              |
| Cdc2   | NM_007659.3       | F-AGACCTCAACAAGACCCCTTTC | 60°C                 | 262 bp       |
|        |                   | R-CAGGAAGAGAGCGAAGCTGTA |                      |              |
| c-mos  | NM_020021         | F-TGGCGTTCCTCAGTTTCTTTTCCC | 60°C                 | 273 bp       |
|        |                   | R-CTTTAATACACGAGCCAAAC |                      |              |
| Dicer1 | NM_148948.2       | F-AGTCTCTTGGTGCCGATTT | 60°C                 | 148 bp       |
|        |                   | R-GTTCCCATCTGAGCAATTC |                      |              |
| Dnmt3a | NM_007872.4       | F-CCCTCTCTCTGGCTTCTGGA | 60°C                 | 117 bp       |
|        |                   | R-TGCAGCAGACACTTCTTTGG |                      |              |
| Dnmt3l | NM_019448.3       | F-TGTCATGACTGAGGATGCCA | 60°C                 | 103 bp       |
|        |                   | R-ACCCGTGACTGATCTGGTA |                      |              |
| Eif1a  | NM_010120.5       | F-ATGCAGAGAATAGGACG | 60°C                 | 196 bp       |
|        |                   | R-AGGCGTGGAGAATGGCATGTT |                      |              |
| Figla  | NM_012013.1       | F-TGTCTTGGAGAAGCGAAGG | 60°C                 | 117 bp       |
|        |                   | R-TGGGTAGCATTTCACAGAG |                      |              |
| Filia  | NM_025890.3       | F-ATGGAGAGCAGCATCCACA | 60°C                 | 148 bp       |
|        |                   | R-TGAGCCGATAGCTGAGACA |                      |              |
| Floped | NM_026480.3       | F-ATCCTTTGGACAAACCAGTG | 60°C                 | 144 bp       |
|        |                   | R-TAGGTTGAGAGGGCAGA |                      |              |
| Gbx2   | NM_010262.3       | F-ATCTGGCTGGAGAATGAGTC | 60°C                 | 363 bp       |
|        |                   | R-TGCTACTGTAACAGG |                      |              |
| Gdf9   | NM_008110.2       | F-TTGGCAATCTCTTTCCACTC | 60°C                 | 106 bp       |
|        |                   | R-GGGAGATCCTTCCACCCATA |                      |              |

(Continued)
Table 1. (Continued)

| Gene  | Accession numbers | Primer sequence* | Annealing temperature | Product size |
|-------|-------------------|------------------|-----------------------|--------------|
| Hr6a  | NM_019668.3       | F-CCAATAGTCCACACGC  | 60°C                  | 100 bp       |
|       |                   | R-CTCGGAGGCCCCGCTTTGTTTCTA |                      |              |
| Hsf1  | NM_008296.2       | F-CAACAGCTGCGTCTAGTTGCG | 60°C                  | 136 bp       |
|       |                   | R-CTCGGACACGAAAAAGGCAAAGAAG |                      |              |
| Hsp70.1| NM_010478.23     | F-ACAGCTGATCCTGCTCTGACCT | 60°C                  | 185 bp       |
|       |                   | R-TGGCTGTAGTCTCTTGGTTG |                      |              |
| Klf4  | NM_010637         | F-CAAGGACACGACCCACAC | 60°C                  | 227 bp       |
|       |                   | R-GAAAAGGGCCCAGTGAACCTT |                      |              |
| Kpna1 | NM_008465.5       | F-TCCAAGCAGTCTAGCTAGTCGCA | 60°C                  | 250 bp       |
|       |                   | R-CTGTCCCATATTTCCAGCTG |                      |              |
| Mater | NM_001039143.1    | F-CCCTTGGAATGCTCTGAGTA | 60°C                  | 112 bp       |
|       |                   | R-GTGGTGGAAGGCGTGGAGA |                      |              |
| Mt1a  | NM_001039368.1    | F-AAGAATCTCCTGAGGGGAGC | 60°C                  | 186 bp       |
|       |                   | R-TTCTAGCATCTGATGGGAT |                      |              |
| Muerv-1| Y12713            | F-TTGCTCTCTGTCCTCATAAC | 60°C                  | 132 bp       |
|       |                   | R-AAAATGACCAGGGGGGAACTC |                      |              |
| Nobox | NM_130869.3       | F-TTCTCCATCTCCTGACCTC | 60°C                  | 123 bp       |
|       |                   | R-TTCTGACTGAAGGCCAAAAGG |                      |              |
| Npm2  | NM_181345.3       | F-GAAAGCCAAAAGGAGGAGTGTA | 60°C                  | 146 bp       |
|       |                   | R-GGCGAAAGTTACTGAGAGGA |                      |              |
| Oct4  | NM_013633.3       | F-CGCGAAGGAGAAAGCCAGTTA | 60°C                  | 112 bp       |
|       |                   | R-CTGATTGGCMGATGAGGAGTGTA |                      |              |
| Omt2b | NM_205822.2       | F-AGCAGACAGAAGGCGACATT | 60°C                  | 215 bp       |
|       |                   | R-AGCAATAGTTCGGGACCTCAA |                      |              |
| Padi6 | NM_153106.2       | F-TGGAGAGGGAGAAAGCATGA | 60°C                  | 129 bp       |
|       |                   | R-TTCTGACTCAGGAGAGAGGTA |                      |              |
| Rpl23 | NM_022891.3       | F-CATGGTGATGCGACAGTTA | 60°C                  | 136 bp       |
|       |                   | R-GACCCTCGATCTCCTCAA |                      |              |
| Stella| NM_139218.1       | F-TGTTGCGTGCTGAAAGAC | 60°C                  | 151 bp       |
|       |                   | R-CATCGTCCGATCCAACTCAA |                      |              |
| Tcl1  | NM_009337.3       | F-GAAGCTATGTTCAGGTCCTC | 60°C                  | 150 bp       |
|       |                   | R-TTCAAGGACATCTGGTCCTCAA |                      |              |
| Tif1alpha| NM_145076.3     | F-ACCCAAATGCCGACTCTGAAACC | 60°C                  | 148 bp       |
|       |                   | R-CCAGCTTGGAGCATTCTGAAA |                      |              |
| Tie6  | NM_053254.2       | F-AACCTAAAGGCCCTAACA | 60°C                  | 134 bp       |
|       |                   | R-TGGAACAGATGCTCCAGT |                      |              |
| Ube2a | NM_019668.3       | F-TAAGGTGTTGGAATGCGGTCA | 60°C                  | 272 bp       |
|       |                   | R-TGTTCTGCTGACTATTGCGGA |                      |              |
| U2afb-p-rs| NC_000077.6     | F-TAAGCTGCAACCTGGAACCTC | 60°C                  | 109 bp       |
|       |                   | R-CCCTGCACTCAGCTCTGAAA |                      |              |
| Uchl1 | NM_011670.2       | F-GCCAGCTAAGGAAACTCTCAG | 60°C                  | 150 bp       |
|       |                   | R-CAGGGTTGCTTGGTTGTTGGA |                      |              |
| Wee1  | NM_009516.3       | F-AGCCATCTACCGAAGAAAGCAGA | 60°C                  | 375 bp       |
|       |                   | R-ATCTGGAAAGAGTGCCGCTT |                      |              |
| Zar1  | NM_174877.3       | F-GTCTGGCCAGGTGGTGAGA | 60°C                  | 143 bp       |
|       |                   | R-CACACAAGTCTTGCGAGTG |                      |              |
The relative expression levels of the target genes were evaluated using the comparative CT method [12, 13], and all analytic procedures were repeated at least three times.

**Preparation of Sebox and GFP dsRNA**

Sebox-A and GFP primers were used to amplify regions of Sebox and GFP cDNA, respectively, which were then cloned into pGEM-T Easy (Promega, Madison, WI, USA) and linearized with SpeI. A MEGAscript RNAi Kit (Ambion, Austin, TX, USA) and T7 RNA polymerase were used to synthesize single-stranded RNA (ssRNA) for each orientation. Complementary RNAs were mixed and incubated 5 min at 75°C and then cooled to room temperature. The formation of dsRNA was verified by 1% agarose gel electrophoresis, comparing the mobility of dsRNA with that of ssRNA. For microinjection, RNAs were diluted to a final concentration of 2 μg/μl. GFP RNAi was used as injection control.

**Microinjection and in vitro culture**

GV oocytes and PN embryos were microinjected with Sebox and GFP dsRNA in M2 medium containing 0.2 mM IBMX or in M2 medium alone, respectively. An injection pipette holding dsRNA solution was inserted into the cytoplasm of oocytes or embryos, and 10 pl dsRNA was microinjected with a constant-flow system (Femtojet; Eppendorf, Hamburg, Germany). To achieve the MII stage, oocytes were cultured in M16 containing 0.2 mM IBMX for 8 h, followed by culture in plain M16 for 16 h in 5% CO₂ at 37°C. Similarly, GFP and Sebox dsRNA-microinjected PN embryos were developed to the 2C stage in M16 medium containing 100 μM EDTA (Sigma-Aldrich).

**Transcriptional activity assay**

Newly synthesized RNAs, e.g., transcriptional activity, in embryos may be visualized by applying 5-ethynyl uridine (EU) to an in vitro embryonic transcriptional activity assay [12]. The Click-iT RNA Imaging Kit (Invitrogen, Carlsbad, CA, USA) was used for this purpose. After subjecting embryos to culture for 1 h in 2 mM EU-supplemented medium, embryos were washed three times for 10 min and fixed in 3.7% formaldehyde for 1 h. The preserved embryos were washed three times for 10 min and permeabilized by exposure to 0.2% Triton X-100 for 10 min. Finally, embryos were sequentially immersed in reaction buffer for 30 min, washed three times, and examined by confocal microscopy after the reaction buffer was eliminated with rinse buffer.

**Statistical analysis**

Statistical analysis of real time PCR data was carried out using student’s t-test. Data derived from at least three separate and independent experiments were expressed as the mean ± SEM.

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**Table 1. (Continued)**

| Gene | Accession numbers | Primer sequence* | Annealing temperature | Product size |
|------|-------------------|------------------|-----------------------|--------------|
| Zfp57 | NM_001013745.2 | F-CAGCCATCCAGGACACCAC 60°C | 144 bp |
| Zscan4 | NR_033707.1 | F-CAGATGCGAGTACACCAC 60°C | 514 bp |

*F = Forward, R = Reverse
doi:10.1371/journal.pone.0115050.t001
The p values were calculated based on a paired t-test of the triplicate delta CT values for each gene in the GFP RNAi group and Sebox RNAi group, and a value of p < 0.05 was considered statistically significant.

**Results**

**Expression levels of other MEGs impacted by Sebox RNAi**

We previously reported the expression of Sebox mRNA in GV oocytes [6]. Knockdown of Sebox mRNA and protein in GV oocytes did not affect the meiotic cell cycle of oocytes, so the oocytes without Sebox expression developed to MII but were arrested at the 2C stage of early embryonic development. Although Sebox-knockdown GV oocytes developed to normal MII in appearance, the expression levels of the 8 among 23 studied MEGs were up-regulated (Fig. 2). These results suggest that Sebox is an important regulatory transcription factor that may function in controlling the expression of other MEGs during preimplantational embryonic development. In particular, 1 gene pertaining to degradation of maternal factors (Dicer), 4 genes related to DNA methylation (Dnmt3l, Dnmt1, Stella, and Zfp57), and 2 genes pertaining to SCMC organization (Mater, and Padi6) were up-regulated after Sebox RNAi knockdown (Fig. 2). The expression of Uchl1 related to preimplantation development was also up-regulated.

**Inadequate selective maternal mRNA degradation in Sebox knockdown 2C embryos**

To determine the exact effects of the up-regulated expression levels of several MEGs on the 2C arrest after Sebox RNAi knockdown, particularly on the degradation of some maternal factors, we measured the degradation of several well-known maternal factors. Changes in expression of the maternal mRNAs Bmp15, c-mos, Gbx2, Gdf9, Nobox, and Omt2b were evident in 2C stage

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Fig 2. Altered expression of 23 MEGs in Sebox-knockdown MII oocytes. Quantitative real time RT-PCR experiments were repeated at least three times, expressing data as the mean±SEM. Expression levels were calculated from the Ct values after normalization with H1foo. The statistical significance was assessed by a paired t-test with p values obtained by paired t-test within the delta Ct values. Asterisks, *, **, and *** represent statistical significance at p < 0.0001, p < 0.01, and p < 0.05, respectively. Control, GFP dsRNA-injected MII oocyte; Sebox RNAi, Sebox dsRNA-injected MII oocyte.

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embryos after Sebox RNAi knockdown (Fig. 3). Compared with control 2C stage embryos, the expression levels of c-mos, Gbx2, and Gdf9 were relatively high in arrested 2C embryos after Sebox RNAi knockdown. The expression levels of the other known maternal factors, such as Nobox and Omt2b, were not changed. These results strongly suggest that Sebox is fairly involved in the process of degrading maternal factors.

Incomplete expression of ZGA markers after Sebox RNAi knockdown
Embryos subjected to Sebox RNAi knockdown were blocked at the 2C stage of embryonic development. Normal embryonic development requires ZGA, which should be indicated by expression of typical ZGA markers [13,14]. Thus, we determined the expression levels of known ZGA markers by comparing the 2C controls and 2C Sebox-knockdown zygotes (Fig. 4A-C). The expression levels of Btg1, Klf4, Kpna1, Muerv-1 were significantly up-regulated, while the expression of Mt1a, Rpl23, Ube2a and Wee1 were down-regulated after Sebox RNAi knockdown. Expression of Cdc2, Eif1a, Hsp70.1, U2afbp-rs and Zscan-4 were not significantly changed. These results demonstrate that Sebox is partly, but not exclusively, involved in ZGA.

Diminished transcriptional activity in Sebox knockdown 2C embryos
Due to the imperfect pattern of expression of several ZGA markers described above, we decided to evaluate the transcriptional activity of the 2C embryos by measuring EU incorporation in embryos with or without Sebox RNAi knockdown. Sebox-knockdown embryos showed dramatically decreased EU incorporation compared with control and sham-control embryos, confirming that the halted maternal factor degradation and ZGA during the MZT period caused by Sebox RNAi knockdown resulted in decreased transcription in embryos arrested at the 2C stage (Fig. 5).

Expression of Figla in Sebox-knockdown MII oocytes
Because 2 out of the 5 genes involved in SCMC formation, i.e., Mater, and Padi6, were up-regulated in Sebox-knockdown MII oocytes, we evaluated changes in the known upstream regulators of SCMC. Figla, a germ-cell-specific, basic helix-loop-helix transcription factor, has been
reported to be a key regulatory molecule in coordinating the expression of the NALP family of genes [15]. Three members of the NALP gene family have oocyte-specific expression [16]. Among them, \textit{Nalp5} (also known as \textit{Mater}) is important in the formation of the SCMC complex [17]. Thus, we evaluated the expression levels of \textit{Figla} after \textit{Sebox} RNAi knockdown and found that expression levels increased 11.7-fold in \textit{Sebox}-knockdown Mll oocytes compared with the controls (Fig. 6). Consequently, we concluded that \textit{Sebox}, either directly and/or indirectly through \textit{Figla}, regulates the expression of SCMC component genes.

**Discussion**

During the MZT, gene expression is dramatically altered as a necessary step in embryonic development. By definition, MEGs are transcribed during oogenesis and are required for early developmental activities, such as establishing the overall polarity of the embryo. Some MEGs are expressed only in female gametes, whereas others are expressed after the embryonic genome is activated [18]. The timing of embryonic gene activation is species-specific [19]. In mice, embryonic gene activation occurs at the 2C stage, concurrently with the degradation of most
maternal mRNA transcripts [20]. Global expression profiles have identified distinctive patterns of maternal mRNA degradation and zygotic genome activation in mice, indicating remarkably dynamic reprogramming of gene expression at the 2C stage [21–23].

One major point of inquiry was whether developmental repercussion is found in Sebox-knockdown 2C embryos. In this study, Sebox-deficient MII oocytes displayed altered expression of several MEGs. First, the role of Sebox in degrading maternal factors was investigated. The degradation of maternal factors is initiated during oocyte maturation and proceeds after fertilization [24]. To support early embryogenesis, the degradation of previously existing factors is a crucial and selective process [25]. We measured the expression of known maternal mRNAs (Bmp15, c-mos, Gbx2, Gdf9, Nobox, and Omt2b), all of which should be degraded in normal 2C zygotes, and found incomplete elimination of c-mos, Gbx2, and Gdf9 after Sebox RNAi knockdown. Such abnormal clearance of maternal factors likely translates to latent defects in embryonic development.

Next, we confirmed the presence of abnormal ZGA and found that Mt1a, Rpl23, Ube2a and Wee1 were down-regulated after Sebox RNAi knockdown but that Cdc2, Eif1a, Hsp70.1, U2afbp-rs, and Zscan4 were not. Furthermore, expression of 4 more genes, Btg1, Klf4, Kpna1, and Muerv-1 were even up-regulated after the loss of Sebox. This finding suggests that Sebox is certainly a significant regulator of ZGA, but it is not critical or exclusive because the expression levels of 5 out of 13 genes were not affected. SEBOX contains a homeodomain and may thus act as a transcription factor [8,26,27]. Indeed, a transcriptional activity assay confirmed a reduction of embryonic transcriptional activity after Sebox RNAi knockdown. Therefore, the regulation of ZGA by SEBOX and its control over the expression of other MEGs may occur at the transcriptional level. Further research on the interrelationship between SEBOX as a transcriptional factor and promoters of altered MEG expression levels is required.

An interesting outcome of this study was the finding that the expression levels of SCMC components were increased after Sebox RNAi knockdown. The SCMC encompasses many maternal proteins, of which FILIA, FLOPED, MATER, PADI6, and TLE6 are crucial for
progression beyond the first embryonic cell division [17]. Among these components, FLOPED, MATER, and TLE6 proteins show interactivity, whereas Filia and MATER bind directly in embryos [28]. According to previous findings, Figla is a key regulatory molecule of Nalp5, also known as Mater [16], and MATER has an important role in SCMC complex formation [17,28]. We confirmed a relationship between Sebox and Figla and found it noteworthy that Sebox depletion up-regulated Figla expression. These findings strongly suggest that Sebox, Figla, and SCMC components are linked. The specific interrelationships of MEGs have not been fully elucidated. Our results may provide a greater impetus to probe such relationships, exploring the direct/indirect interplay among SEBOX, FIGLA, and other MEGs, at both the transcriptional and post-translational levels.

Other publications have stressed the importance of the MZT in early embryonic development [29–31]. Arrest of α-amanitin-treated embryos at the 1C or 2C stage has been documented [32], and developmental block at the 2C stage has been attributed to delayed ZGA [33]. However, the specific molecular mechanism of the MZT in mice is still unclear. We believe that SEBOX is an important regulator of the MZT in addition to the genes that have been discovered to be active during the MZT [34].

Aside from their impact on embryonic development, a variety of functions have been ascribed to many MEGs in oocytes. Basonuclin-deficient oocytes containing cytoplasmic granules have been found to arrest at the 2C stage [35]; Ctf-deficient oocytes showed delayed GVBD and embryonic developmental arrest [36]; and Padi6 is thought to regulate microtubular and organelle dynamics during oocyte maturation and to contribute to the SCMC during early embryogenesis [37]. We previously reported that Gas6 contributes to the cytoplasmic maturation of oocytes and PN formation [38]. Additionally, in the present study, we report that even though Sebox-knockdown oocytes developed to the MII stage with normal morphology, Sebox knockdown may contribute to the incompetent cytoplasmic maturation of oocytes, which affects early embryo development.

In conclusion, our findings support an intimate association between Sebox and other MEGs, whereby Sebox is involved in regulating the elimination of maternal factors and promotion of embryonic gene expression required for normal developmental progression. These perturbed cytoplasmic expression levels that we observed for various genes in Sebox-deficient mouse oocytes signify impaired fertilization and embryonic development and thus merit further investigation.

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Author Contributions
Conceived and designed the experiments: MWP JJK KAL. Performed the experiments: MWP KHK EYK SYL. Analyzed the data: MWP KHK EYK SYL JJK KAL. Contributed reagents/materials/analysis tools: MWP KHK EYK SYL JJK KAL. Wrote the paper: MWP KHK SYL JJK KAL.

References
1. Latham KE (1999) Mechanisms and control of embryonic genome activation in mammalian embryos. Int Rev Cytol 193: 71–124. PMID: 10494621
2. Schultz RM (2002) The molecular foundations of the maternal to zygotic transition in the preimplantation embryo. Hum Reprod Update 8: 323–331. PMID: 12206467
19. Zernicka-Goetz M (1994) Activation of embryonic genes during preimplantation rat development. Mol

20. Schultz RM (1993) Regulation of zygotic gene activation in the mouse. Bioessays 15: 531

21. Li L, Zheng P, Dean J (2010) Maternal control of early mouse development. Development 137: 859–870. doi: 10.1242/dev.039487 PMID: 20179092

22. Wang QT, Piotrowska K, Ciemerych MA, Milenkovic L, Scott MP, et al. (2004) A genome-wide study of maternal gene expression reveals developmental signaling pathways in the preimplantation mouse embryo. Dev Biol 283: 40–57. PMID: 15975430

23. Kigami D, Minami N, Takayama H, Imai H (2003) MuERV-L is one of the earliest transcribed genes in the mouse. Cell 6: 133

24. Paynton BV, Rempel R, Bachvarova R (1988) Changes in state of adenylation and time course of degradation of maternal mRNAs during oocyte maturation and early embryonic development in the mouse. Dev Biol 129: 304–314. PMID: 2455285

25. Alizadeh Z, Kageyama S, Aoki F (2005) Degradation of maternal mRNA in mouse embryos: selective degradation of specific mRNAs after fertilization. Mol Reprod Dev 72: 281–290. PMID: 16094646

26. Lee HS, Kim EY, Lee KA (2011) Changes in gene expression associated with oocyte meiosis after Obox4 RNAi. Clin Exp Reprod Med 38: 68–74. doi: 10.5653/cerm.2011.38.2.68 PMID: 22384421

27. Park GT, Lee KA (2013) Nuclear localization of Obox4 is dependent on its homeobox domain. Clin Exp Reprod Med 40: 1–6. doi: 10.5653/cerm.2013.40.1.1 PMID: 23614109

28. Ohsugi M, Zheng P, Baibakov B, Li L, Dean J (2008) Maternally derived FIGLA-MATER complex localizes asymmetrically in cleavage-stage mouse embryos. Development 135: 259–269. PMID: 18057100
29. Tadros W, Lipshitz HD (2009) The maternal-to-zygotic transition: a play in two acts. Development 136: 3033–3042. doi: 10.1242/dev.033183 PMID: 19700615
30. Tsai TC, Lin W, Yang SH, Cheng WT, Cheng EH, et al. (2010) Granzyme G is expressed in the two-cell stage mouse embryo and is required for the maternal-zygotic transition. BMC Dev Biol 10: 88. doi: 10.1186/1471-213X-10-88 PMID: 20704734
31. Rother F, Shmidt T, Popova E, Krivokharchenko A, Hugel S, et al. (2011) Importin alpha7 is essential for zygotic genome activation and early mouse development. PLoS One 6: e18310. doi: 10.1371/journal.pone.0018310 PMID: 21479251
32. Golbus MS, Calarco PG, Epstein CJ (1973) The effects of inhibitors of RNA synthesis (alpha-amanitin and actinomycin D) on preimplantation mouse embryogenesis. J Exp Zool 186: 207–216. PMID: 4795793
33. Qiu JJ, Zhang WW, Wu ZL, Wang YH, Qian M, et al. (2003) Delay of ZGA initiation occurred in 2-cell blocked mouse embryos. Cell Res 13: 179–185. PMID: 12862318
34. Lykke-Andersen K, Gilchrist MJ, Grabarek JB, Das P, Miska E, et al. (2008) Maternal Argonaute 2 is essential for early mouse development at the maternal-zygotic transition. Mol Biol Cell 19: 4383–4392. doi: 10.1091/mbc.E08-02-0219 PMID: 18701707
35. Ma J, Zeng F, Schultz RM, Tseng H (2006) Basonuclin: a novel mammalian maternal-effect gene. Development 133: 2053–2062. PMID: 16624857
36. Wan LB, Pan H, Hannenhalli S, Cheng Y, Ma J, et al. (2008) Maternal depletion of CTCF reveals multiple functions during oocyte and preimplantation embryo development. Development 135: 2729–2738. doi: 10.1242/dev.024539 PMID: 18614575
37. Yurttas P, Vitale AM, Fitzhenry RJ, Cohen-Gould L, Wu W, et al. (2008) Role for PADI6 and the cytoplasmic lattices in ribosomal storage in oocytes and translational control in the early mouse embryo. Development 135: 2627–2636. doi: 10.1242/dev.016329 PMID: 18599511
38. Kim KH, Kim EY, Kim Y, Kim E, Lee HS, et al. (2011) Gas6 downregulation impaired cytoplasmic maturation and pronuclear formation independent to the MPF activity. PLoS One 6: e23304. doi: 10.1371/journal.pone.0023304 PMID: 21850267