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—Original Article—

Knockdown of Maternal Homeobox Transcription Factor SEBOX Gene Impaired Early Embryonic Development in Porcine Parthenotes

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Abstract. A number of germ cell-specific transcription factors essential for ovarian formation and folliculogenesis have been identified and studied. However, the role of these factors during early embryonic development has been poorly explored. In the present study, we investigated the role of SEBOX, a maternal homeobox transcription factor, during early embryonic development in porcine parthenotes. mRNA for *SEBOX* is preferentially expressed in oocytes, and expression persists until embryonic genome activation (EGA). Knockdown of SEBOX by siRNA disrupted early embryonic development, but not oocyte maturation. Many maternal genes essential for early embryonic development were upregulated in SEBOX-depleted embryos. Moreover, some pluripotency-associated genes, including *SOX2* and *NANOG*, were upregulated when SEBOX was knocked down. Therefore, our data demonstrate that SEBOX is required for early embryonic development in pigs and appears to regulate the degradation of maternal transcripts and the expression of pluripotency genes.
Key words: Homeobox, Maternal Factor, Porcine oocyte, SEBOX, Transcription factor

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In most organisms, including mammals, early embryonic development relies primarily on maternal factors that are encoded by maternal effect genes that accumulate during oogenesis [1, 2]. These maternal factors play crucial roles in successful embryo development before, and also after, embryonic genome activation (EGA) [3]. Although several maternal factors, including Mater, Stella, Zar1, Npm2 and Brg1, have been reported in the mouse [4–8], there is still limited data regarding the maternal factors involved in early embryo development in mammals.

Homeobox genes are a large family of genes that direct the formation of many body structures during early embryonic development. The homeobox genes share a highly conserved DNA-binding domain, known as a homeodomain [9], that recognizes and binds to specific DNA sequences in the regulatory regions of genes. Therefore, most proteins containing a homeodomain act as transcription factors that control the expression of other genes.

Recently, several homeodomain-containing maternal factors were shown to play crucial roles during oocyte development. For example, a deficiency in either newborn ovary homeobox (Nobox) or LIM-homeobox protein 8 (Lhx8) affects the expression of numerous germ cell-specific genes essential for germ cell development and, consequently, accelerates postnatal oocyte loss, causing female infertility [10–12]. More recently, skin-embryo-brain-oocyte homeobox (*Sebox*), a paired-like homeobox gene, was found to be essential for early oogenesis by regulating the expression of genes that are required during germ cell development in the mouse [13]. Despite extensive studies on the function of homeobox transcription factors during oogenesis, the role of these factors during early embryonic development is poorly understood. It is now becoming evident that these transcription factors are crucial for EGA and thereby regulate early embryo development. For example, knockdown of NOBOX in bovine zygotes impaired early embryo development and altered the expression profile of EGA-related genes at the 8-cell stage, as well as pluripotency genes at the blastocyst stage [14]. In addition, downregulation of Sebox in the mouse zygote blocked embryonic development [15].

Although these data clearly indicate that maternal transcription factors are involved in early embryonic development, as well as oogenesis, the molecular mechanics underlying the functions of these factors in early embryonic development have not been well defined. Therefore, in this study, we investigated the role of the maternal transcription factor SEBOX during early embryonic development in porcine parthenotes. We characterized the expression profile of *SEBOX* and its downstream target genes to understand the mechanisms that regulate early embryonic gene expression. Our data demonstrate that SEBOX is an essential maternal transcription factor that regulates both the degradation of mRNAs encoding many maternal factors and the expression of pluripotency genes.

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Materials and Methods

Isolation of denuded oocytes, cumulus cells and mural granulosa cells

Prepubertal porcine ovaries were collected from a local slaughterhouse and transported to the laboratory at 25 C in Dulbecco's phosphate-buffered saline (DPBS) supplemented with 75 μg/l penicillin G and 50 μg/l streptomycin sulfate. Cumulus-oocyte complexes (COCs) were aspirated from follicles 2–8 mm in diameter with an 18-gauge needle and a disposable 10 ml syringe. Clumps of mural granulosa cells were picked from the aspirate. Cumulus cells were obtained by repeated pipetting of COCs through a fine-bore pipette. Denuded oocytes were exposed to a 0.1% trypsin solution in DPBS to ensure complete removal of the cumulus cells. Samples were lysed in lysis buffer and stored frozen at –80 C.

In vitro maturation and parthenogenetic activation

The COCs were washed three times with Hepes-buffered Tyrode's medium containing 0.1% (w/v) PVP (Hepes-TL-PVA). Each group of 50 COCs was matured in 500 μl of tissue culture medium (TCM)-199 (with Earle's salts; Gibco, Grand Island, NY, USA) supplemented with 0.57 mM cysteine (Sigma, St. Louis, MO, USA), 10 ng/ml EGF (Sigma), 10 IU/ml PMSG (Sigma) and 10 IU/ml hCG (Sigma) under paraffin oil at 39 C for 44 h. Following maturation, cumulus cells were removed by pipetting in the presence of 1 mg/ml hyaluronidase for 2–3 min. Oocytes were activated for parthenogenesis in 0.3 M mannitol (Sigma) supplemented with 1.0 mM Ca^{2+}, 0.1 mM $MgCl_2$ and 0.5 mM Hepes with two 110 kV/cm DC pulses of 50 μs in duration separated by 100 ms. After 3 h of culture in porcine zygote medium 3 (PZM3) containing 7.5 μg/ml cytochalasin B (Sigma), embryos were washed several times in PZM3 containing 0.4% (w/v) BSA and cultured in the same medium at 38.5 C in an atmosphere of 5% CO_2 and 95% air.

siRNA injection and embryo culture

Knockdown of endogenous SEBOX in porcine oocyte and embryos was performed via microinjection of *SEBOX* small interfering RNA (siRNA) at the GV stage and 6–8 h post activation, respectively. The siRNAs were designed by and purchased from a local company (Bioneer, Daejon, Korea, Table 2). The siRNA in medium was added to an injection pipette with a tip diameter of less than 1 μm using a micro-loader (5242 956.003, Eppendorf, Hamburg, Germany). The siRNA injections were performed using an inverted microscope (Nikon TE2000U) equipped with a micromanipulation system (Narishige, Tokyo, Japan). Fifty oocytes/embryos per group were transferred to 10 μl drops of manipulation medium (TCM-199 supplemented with 0.6 mM $NaCO_3$, 3 mM Hepes, 30 mM NaCl, and 0.1% BSA). The embryos were held in place using a holding pipette, and the plasma membrane was penetrated by the injection pipette with constant siRNA medium flow until obvious swelling was observed. To assess injection damage, oocytes/embryos were injected with elution buffer alone as a sham control. Oocytes after injection were cultured in maturation medium, while embryos were cultured in PZM3 droplets until collection. Germinal vesicle (GV) and metaphase II (MII) oocytes were collected before parthenogenetic activation, and embryo at the 1-cell (1C), 2-cell (2C), 4-cell (4C), 8-cell (8C), morula (MO), and blastocyst (BL) stages were collected at 6, 24, 48, 120, 124, and 168 h after parthenogenetic activation, respectively.

Quantitative polymerase chain reaction (qPCR)

The numbers of embryos used for qPCR at the GV, MII, 1C, 2C, 4C, MO, and BL stages were 20, 20, 20, 20, 15, 10, and 10, respectively. Extraction of mRNA and cDNA synthesis were performed with a Dynabeads mRNA Direct Kit (61012, Invitrogen) and SuperScript III First-Strand Synthesis Kit (18080-051, Invitrogen) according to the manufacturer's instructions. qPCR was conducted using a DyNAmo HS SYBR Green qPCR Kit (F-410L, Thermo Scientific, Vantaa, Finland) according to the manufacturer's instructions on a CFX96 Touch Real-time PCR Detection System (Bio-Rad). The PCR protocol was as follows: 95 C for 15 min, 40 cycles of 95 C for 20 sec, primer annealing temperature for 20 sec, 72 C for 30 sec, and finally 95 C for 10 sec. Gene-specific primers were designed with Primer Premier 6 (PREMIER Biosoft, Palo Alto, CA, USA) and the specificity for the target genes was confirmed using primer BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). *GAPDH* was used as an internal reference (Table 1). To visualize qPCR products, samples were separated by electrophoresis in 2% agarose gels.

Statistical analysis

Statistical analysis of qPCR and preimplantation developmental data was evaluated using a one-way analysis of variance (ANOVA) and a log-liner model with the IBM SPSS Statistics 19 software (IBM, Armonk, NY, USA). Data are presented as the mean ± SEM derived from at least three independent experiments. A value of $P<0.05$ was considered statistically significant.

Results

Expression of porcine SEBOX mRNA

To determine the expression pattern of porcine *SEBOX*, we performed an RT-PCR analysis with normalized cDNAs derived from various porcine tissues (Fig. 1A). Expression was observed predominantly in the ovary and liver. Low level expression was also observed in the testis and lung. Moreover, RT-PCR analysis of oocytes, granulosa cells and cumulus cells revealed that porcine *SEBOX* is expressed exclusively in oocytes and not in other follicular somatic cells (Fig. 1B).

Next, we investigated the expression of porcine *SEBOX* during oocyte and early embryo development (Fig. 1C). Porcine *SEBOX* was highly expressed in GV and MII oocytes, as well as in 1-cell to 4-cell embryos, with the highest expression at the 2-cell stage. *SEBOX* mRNA expression was dramatically reduced in the 8-cell and morula stages, and was barely detectable by the blastocyst stage in porcine embryos. Considering that EGA occurs at the 4-cell stage in porcine embryos [16], these results suggest that porcine *SEBOX* is a maternally expressed gene.

The functions of porcine SEBOX during meiotic maturation

To investigate the functions of SEBOX during porcine meiotic maturation, siRNAs specifically targeting porcine *SEBOX* mRNA were designed and microinjected into GV oocytes. Knockdown of *SEBOX* mRNA was confirmed by quantitative RT-PCR (Fig. 2A). Interestingly, oocytes depleted of SEBOX developed to the MII stage with normal

Table 1. Primers used in the current study

Genes	GenBank accession no.	Primer sequence (5'→ 3')	Annealing temperature (C)	Product size
GAPDH	AF017079	F:GGGCATGAACCATGAGAAGT R: AGCAGGGATGATGTTCTGG	60	230
SEBOX	XM_003358176.1	F: CCTATCCCGACATTGGCACC R: ATTCTCTTGGCTCTGCGGTT	60	100
ZAR1	NM_001129956.1	F: CCATAAACCCTGGTCCTACTGA R: TCAAATACAGCCTTTCGTGAAG	55	118
GDF9	NM_001001909.1	F: CTACAACACTGTCCGGCTCTT R: CACCAGGCTGCACTCACATT	60	208
BMP15	NM_001005155	F: CCCTCGGGTACTACACTATG R: GGCTGGGCAATCATATCCT	60	192
MOS	NM_001113219.1	F: CGGCAACGTCACCTTACA R: CCAGGGAATACTTGAGACACTT	57	119
H1FOO	NM_001205063.1	F: AGTGGATGTCTTTCGGCTCA R: CGTCTTCCTGGACTGGATTT	60	248
POU5F1	NM_001113060.1	F: GTTCAGCCAAACGACCATCT R: TCTCGATACTTGTCCGCTTTC	58	182
NANOG	NM_001129971.1	F: GGGGTGGTTAGCTCCTGTTC R: CGCTGGAAATGGATGCTTCG	60	241
SOX2	NM_001123197.1	F: CCCGTGGTTACCTCTTCTTCC R: TACCGTTGATGGCCGTGCC	60	175
CDX2	XM_003130908.2	F: CAGGACAGGGCCTTGTTTAG R: CAGGTTGGCTCTGGCATTT	58	88
FSHR	NM_214386.1	F: CCAAGAACTTCCGCAGGGAT R: TGGAGGGCAGTGACCATTTC	60	134

Table 2. Porcine *SEBOX* siRNAs used in the current study

No.	Sequence (5'→ 3')
1	F: CUCUUCAGAGCGAGCUACUTT R: AGUAGCUCGCUCUGAAGAGTT
2	F: CAGCCUCCAUUCUCCAACATT R: UGUUGGAUAAUGGAGGCUGTT
3	F: CUCUCUUCAGAGCGAGCUATT R: UAGCUCGCUCUGAAGAGAGTT

polar body extrusion, and no morphological defects were observed (Fig. 2C). However, early embryonic development was significantly impaired when these oocytes were parthenogenetically activated (Fig. 2B). Collectively, these results suggest that SEBOX may play a role in embryonic development rather than in oocyte maturation.

The roles of porcine SEBOX during early embryo development

To further investigate the role of SEBOX during early embryo development, SEBOX was knocked down at the pronuclear stage of porcine embryos (6–8 h after parthenogenetic activation). Efficient knockdown of *SEBOX* mRNA in embryos was confirmed by PCR (Fig. 3A). Consistent with our data showing that parthenogenetically activated oocytes with SEBOX knocked downed of SEBOX during meiotic maturation exhibited impaired embryonic development, SEBOX knockdown in the zygote significantly disrupted embryonic development, further confirming an essential role for SEBOX during early embryonic development (Fig. 3B, C).

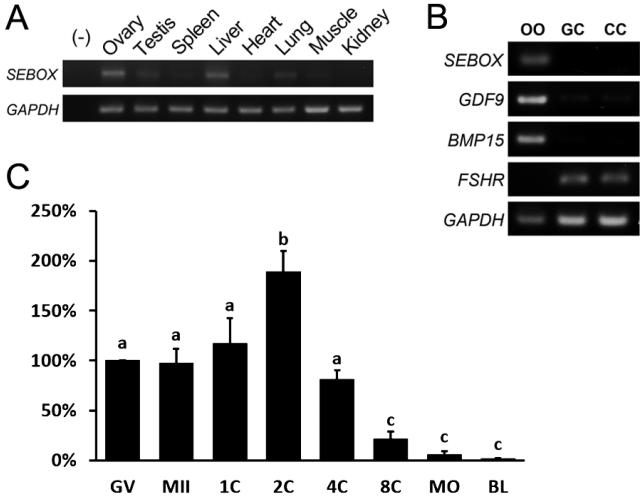

Fig. 1. *SEBOX* mRNA expression in porcine tissues, oocytes and parthenogenetic embryos. (A) Semiquantitative RT-PCR analysis was performed on various porcine tissues to evaluate the relative expression of *SEBOX*. (B) *SEBOX* expression in porcine denuded oocytes (OO), granulosa cells (GC) and cumulus cells (CC). *GDF9* and *BMP15* were used as markers for oocytes, while *FSHR* was used as a marker for GC and CC. (C) *SEBOX* expression in porcine oocytes and different preimplantation development stages of parthenogenetically-activated embryos. GV, germinal vesicle; MII, meiosis II; 1C, 1 cell; 2C, 2 cells; 4C, 4 cells; 8C, 8 cells; MO, morula; BL, blastocyst. Expression was normalized to the expression level in GV oocytes. The data are expressed as the mean ± SEM. Different letters indicate significant differences ($P<0.05$). *GAPDH* was used as an internal standard in all experiments.

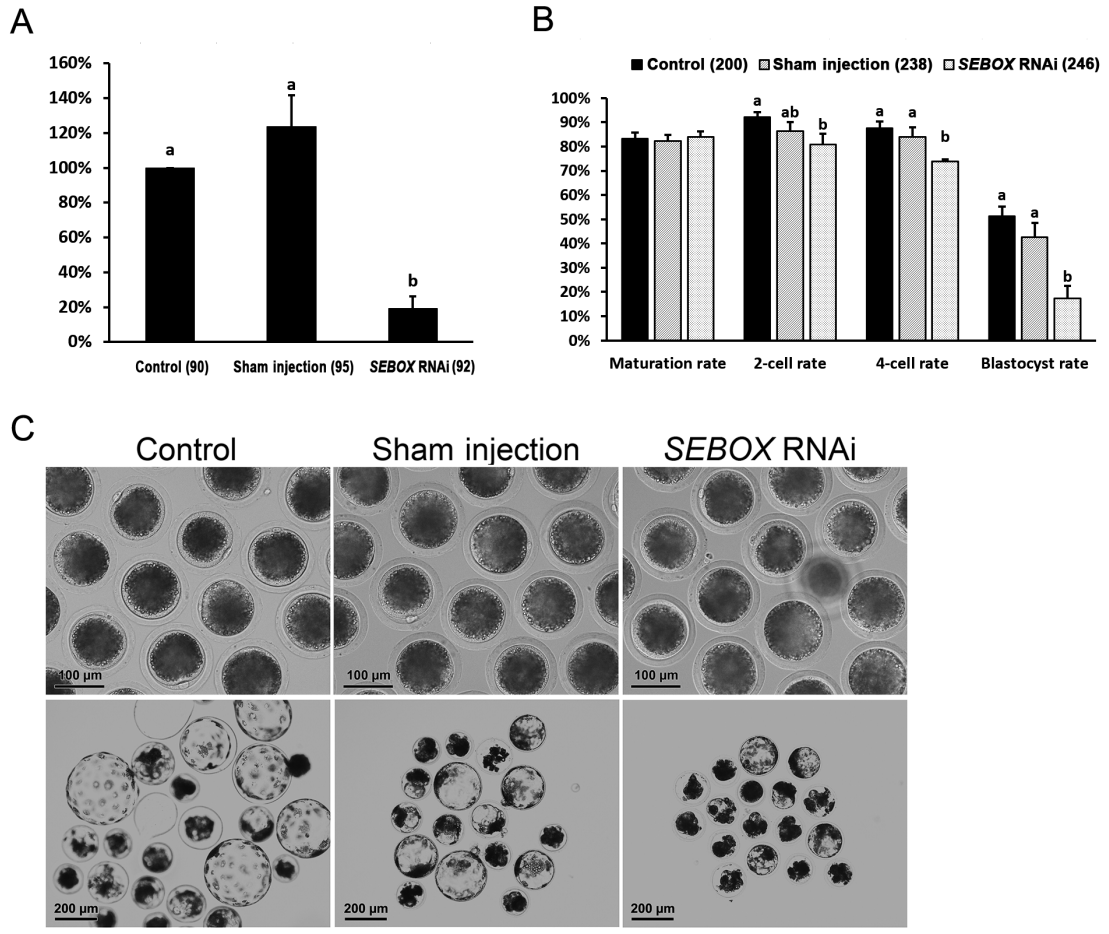

Fig. 2. The function of porcine SEBOX during meiotic maturation. DEPC water (sham-injected group) or *SEBOX* siRNA was injected into porcine oocytes at the GV stage. Non-injected oocytes were used as the control group. A: After 44 h of *in vitro* maturation, *SEBOX* mRNA levels of the control, sham-injected, and *SEBOX* RNAi groups were compared by qPCR. Expression was normalized to the expression level in the control group. *GAPDH* was used as an internal standard. The *SEBOX* mRNA level was greatly reduced in the RNAi group. B: Maturation and preimplantation developmental rates were compared among the control, sham-injected, and *SEBOX* RNAi groups. Cleavage, 4-cell and blastocyst rates were checked at 24 h, 48 h, and 7 days, respectively. C: Morphology of porcine MII oocytes and blastocysts in the control, sham-injected, and *SEBOX* RNAi groups. The numbers of oocytes used in this experiment are provided beside each group name, respectively. The data are expressed as the mean ± SEM. Different letters indicate significant differences ($P<0.05$).

SEBOX target genes during early embryo development

To dissect the molecular mechanisms underlying SEBOX function during embryonic development, we examined the expression levels of several maternally expressed genes that are essential for embryonic development. Surprisingly, all mRNA levels of maternally expressed genes examined (*GDF9*, *BMP15*, *ZAR1*, *MOS* and *H1FOO*) were higher than those in the control group (Fig. 4A). Furthermore, some genes involved in pluripotency, such as *NANOG* and *SOX2*, were also upregulated. By contrast, the expression of POU5F1 (also known as Oct4) and CDX2 was not affected by SEBOX knockdown (Fig. 4B). Given that no transcriptional activity is detected before the 4-cell stage in porcine embryos [16], it is likely that maternal mRNAs are not properly degraded in porcine embryos in which SEBOX has been knocked down.

Discussion

SEBOX is a maternally derived transcription factor that plays an important role in oogenesis, as well as in early embryo development [15, 17]. However, depletion of SEBOX in GV oocytes did not affect meiotic maturation of porcine oocytes, consistent with results from mouse oocytes [15], indicating that SEBOX is not required for transcription during meiotic maturation. Instead, SEBOX may be important in the activation of zygotic gene expression after fertilization. In this regard, it is not surprising that some transcripts are actively translated at the MII stage, although their transcript levels decrease during meiotic maturation. Indeed, genome-wide analysis of transcripts in mouse oocytes revealed that a number of transcripts dormant in the GV stage are actively recruited to polysomes to be translated at

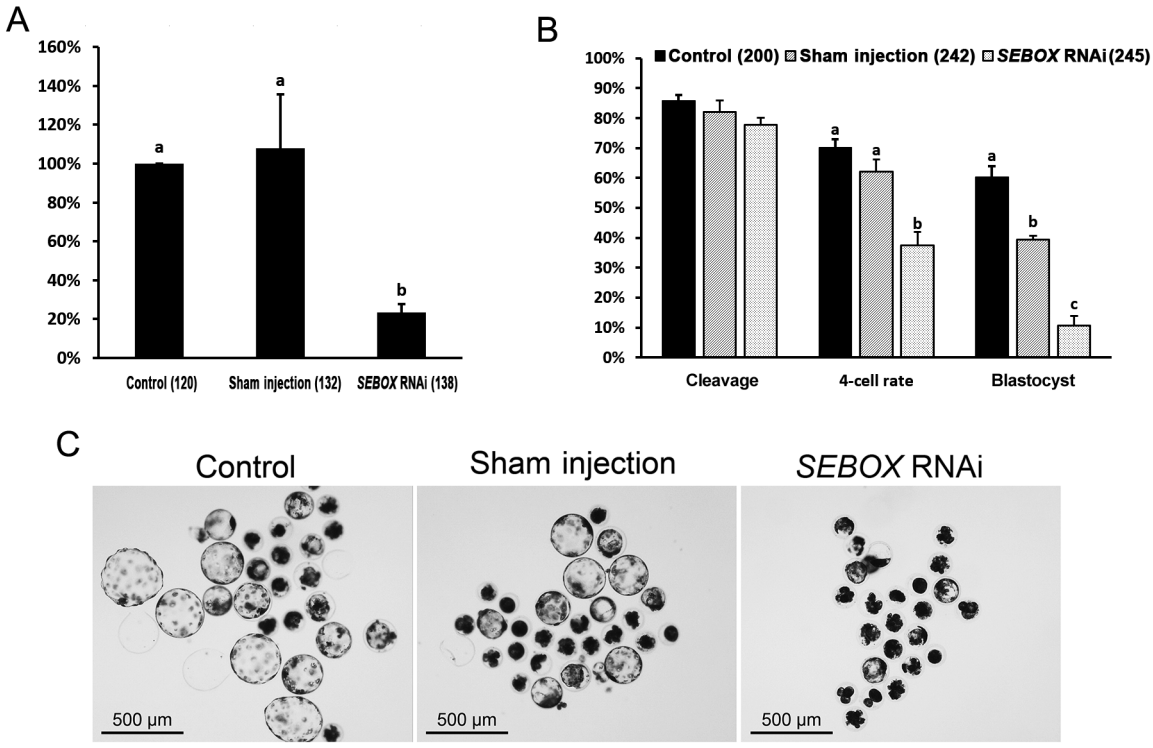

Fig. 3. The role of SEBOX during early development of porcine parthenogenetic embryos. DEPC water (sham-injected group) or *SEBOX* siRNA was injected into parthenogenetic embryos 6−8 h post activation (pronuclear stage). Non-injected embryos were used as controls. A: *SEBOX* mRNA levels were compared at the 2-cell stage. Expression was normalized to the level in the non-injected control group. *GAPDH* was used as an internal standard. *SEBOX* mRNA expression was suppressed in the RNAi group. B: Preimplantation developmental rates were compared among the control, sham-injected, and *SEBOX* RNAi groups. Cleavage, 4-cell and blastocyst rates were checked at 24 h, 48 h, and 7 days, respectively. C: Morphology of the parthenogenetic blastocysts in the control, sham-injected, and *SEBOX* RNAi groups. The numbers of embryos used in this experiment are provided beside each group name, respectively. The data are expressed as the mean ± SEM. Different letters indicate significant differences ($P<0.05$).

the MII stage [18]. These proteins are more likely to be required for embryonic development than for meiotic maturation. Therefore, it is of interest to determine whether SEBOX protein accumulates during meiotic maturation, even though *Sebox* transcript levels decrease by the MII stage in mouse oocytes [15].

The control of early development is dependent upon tight regulation of gene activity. At the time of EGA, transcription is under the control of maternally inherited factors. To prevent aberrant expression of genes normally transcribed at, or soon after EGA, the activity of these maternal transcription factors must be tightly regulated. In most cases, maternally-derived mRNAs are degraded as embryonic transcripts begin to be transcribed [19]. This prepares the embryo for later developmental events and is essential for embryo survival. However, knockdown of SEBOX led to the upregulation of many transcripts encoding maternal factors. Because there is no transcriptional activity before the 4-cell stage of porcine embryos [16], maternal mRNAs derived from oocytes may remain intact when SEBOX is knocked down. Although SEBOX has been identified as a transcription factor, the protein seems to have an additional role in stabilizing certain mRNAs. It is also possible that SEBOX regulates the expression of miRNAs that target maternal transcripts. Indeed, when Ago2, one of the major components of miRNA-mediated RNA silencing, is knocked down at the pronuclear stage, embryos arrest at the 2-cell stage, primarily as the result of a failure to degrade maternal mRNAs [20]. Moreover, miRNA-196a is highly expressed at the time of EGA in bovine embryos, and binds and thereby degrades maternal transcripts encoding NOBOX [21]. Moreover, we could not exclude the possibility that the length of poly(A) may be affected by SEBOX knockdown. In SEBOX-depleted embryos, the prolonged expression of maternal genes that are normally degraded or replaced by embryonic genes upon EGA may either interfere with the timing of activation of embryonic genes or may compete with embryonic genes that have been expressed. Disruption of the balance in expression between maternal and embryonic genes delays or inhibits the normal development of pig embryos. In the Xenopus, persistent expression of maternal c-mos, which is normally degraded soon after fertilization, delayed the onset of embryonic cell cycles [22]. In addition, some maternal factors may play a role in switching on embryonic gene expression, including genes involved in pluripotency. Thus, the excessive levels of maternal factors induced by SEBOX depletion may induce overexpression of pluripotency genes, such as *SOX2* and *NANOG*. *POU5F1* and *NANOG* are indeed downregulated when

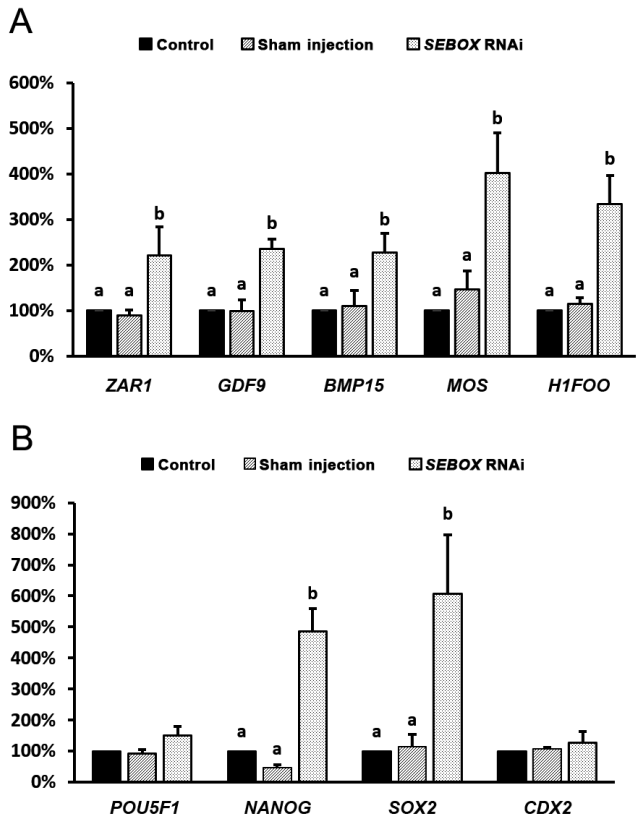

Fig. 4. Effect of SEBOX on expression of maternally expressed genes and pluripotent genes in porcine parthenogenetic embryos. DEPC water (sham-injected group) or *SEBOX* siRNA was injected into parthenogenetic embryos at the 1-cell stage. Non-injected embryos were used as controls. A: Expression levels of the maternally expressed genes *ZAR1*, *GDF9*, *BMP15*, *MOS*, and *H1FOO* were compared at the 4-cell stage. B: Expression levels of pluripotent-related genes were compared at the blastocyst stage. Expression was normalized to the level in the non-injected control group. *GAPDH* was used as an internal standard. The data are expressed as the mean ± SEM. Different letters indicate significant differences ($P<0.05$).

NOBOX is downregulated during early embryonic development as well as oogenesis [12, 14].

In conclusion, our data demonstrate that porcine SEBOX is an essential maternal factor that regulates early embryonic development. Maternal factors may activate certain genes in embryos during EGA. These genes may have a specific role within the embryo, perhaps as activators for transcription of genes essential for subsequent development. Considering the role of SEBOX in the induction of downstream transcription factors, SEBOX is a logical candidate to participate in EGA. It is important to identify genes involved in EGA to clarify the basic mechanisms controlling cell proliferation and differentiation during early embryo development. This knowledge could lead to improvements in embryo culture systems, transgenics and cloning, and would potentially provide a marker for viable embryos for transfer or freezing.

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