SMAD4-induced knockdown of the antisense long noncoding RNA BRE-AS contributes to granulosa cell apoptosis

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Antisense long noncoding RNAs (AS-lncRNAs), a sub-class of lncRNAs, are transcribed in the opposite direction from their overlapping protein-coding genes and are implicated in various physiological and pathological processes. However, their role in female reproduction remains largely unknown. Here, we report that BRE-AS, an AS-lncRNA transcript from intron 10 of the protein-coding gene BRE, is involved in granulosa cell (GC) apoptosis. Based on our previous RNA sequencing data, we identified 28 AS-lncRNAs as important in the initiation of porcine follicular atresia, with BRE-AS showing the most significant upregulation in early atretic follicles. In this study, gain- and loss-of-function assays demonstrated that BRE-AS induces early apoptosis in GCs. Mechanistically, BRE-AS acts in cis to suppress the expression of BRE, an anti-apoptotic factor, via direct interaction with the pre-mRNA transcript of the latter, inducing increased GC apoptosis. Notably, we also found that BRE-AS was upregulated in SMAD4-silenced GCs. SMAD4 was identified as a transcriptional repressor of BRE-AS because it inhibits BRE-AS expression and BRE-AS-mediated GC apoptosis. In conclusion, we not only identified a novel AS-lncRNA related to the early apoptosis of GCs and initiation of follicular atresia but also described a novel regulatory pathway, SMAD4/BRE-AS/BRE, coordinating GC function and female fertility.

INTRODUCTION

Long noncoding RNAs (lncRNAs) are defined as RNA transcripts of more than 200 nucleotides with no protein-coding potential, which function in multiple cellular processes, including cellular proliferation, development, differentiation, apoptosis, and autophagy.1–4 lncRNAs are classified as intronic, intergenic, sense, bidirectional, and antisense on the basis of their genomic location and relationship with the protein-coding genes on the chromosome.5 Antisense lncRNAs (AS-lncRNAs) are transcribed from the antisense strand of well-defined transcriptional units, mainly protein-coding genes.6,7 Owing to their double-stranded RNA (dsRNA) formation, AS-lncRNAs are well known for regulating the sense transcript of protein-coding genes facilitated by multiple mechanisms, including interfering with splicing and translation,8–10 affecting RNA stability,11,12 and inducing RNA editing.1

Emerging evidence suggests that AS-lncRNAs are involved in various biological processes, such as X chromosome inactivation,14,15 muscle atrophy,9 stem cell pluripotency,16 neural signal transduction,17 immune response,8 circadian clock function,18 and even tumorigenesis.19–20 Recently, a few reports have demonstrated that AS-lncRNAs are involved in female reproduction.21,22 For example, Inc-Amhr2, an AS-lncRNA transcript from the 5′ regulatory region of the Amhr2 gene, which is essential for various reproductive functions, has been shown to induce Amhr2 transcription in mouse granulosa cells (GCs) by enhancing the Amhr2 promoter activity.21 In human cumulus cells, HAS2-AS1 inhibition results in decreased GC migration and HAS2 expression, a marker of the cumulus expansion and ovulation.22 In addition, dysregulation of several AS-lncRNAs (e.g., CHL1-AS1, CHL1-AS2, and human zinc finger antisense 1 [ZFAS1]) causes ovarian dysfunction and diseases such as ovarian endometriosis or polycystic ovary syndrome.23,24

Mammalian folliculogenesis is an intricate process that begins with the establishment of a primordial follicle pool in fetal life,25 with only two final outcomes: either mature and ovulation or atresia and degeneration.26–28 The latter is the ultimate fate of most follicles. Follicular atresia has been shown to be triggered by GC apoptosis26,27 and is also affected by oocyte apoptosis.29 Increasing evidence demonstrates that both follicular atresia and GC apoptosis are regulated by numerous factors, such as gonadotropins (e.g., follicle-stimulating hormone [FSH]),30 steroid hormones (e.g., estrogen),31 cytokines (e.g., transforming growth factor beta 1 [TGF-β1]),32 and noncoding RNAs, including microRNAs (miRNAs), lncRNAs, and circular RNAs (circRNAs).3,34,35 However, the role of AS-lncRNAs in follicular atresia and GC apoptosis remains largely unknown. In this study, we aimed to identify AS-lncRNAs related to the initiation of follicular atresia. Furthermore, we investigated the mechanism underlying the initiation of follicular atresia induced by BRE-AS, the most significantly upregulated AS-lncRNA in early atretic follicles, and sought to understand the

Received 24 November 2020; accepted 7 May 2021; https://doi.org/10.1016/j.omtn.2021.05.006.

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mechanism underlying high BRE-AS expression in early atretic follicles.

RESULTS

Differentially expressed AS-lncRNAs during porcine follicular atresia

We previously identified 94 differentially expressed lncRNAs (DEls) in follicles from healthy and early atretic samples using RNA sequencing (RNA-seq) (unpublished). Based on genomic location, we classified these DEls into long intergenic noncoding RNAs (lincRNAs) (46, 48.94%), bidirectional (9, 9.57%), sense (5, 5.32%), AS-lncRNAs (28, 29.79%), and lncRNAs that cannot be located within the genome (6, 6.38%) (Figure 1A). This shows that other than lincRNAs, AS-lncRNAs are the most abundant DELs in ovarian follicles during follicular atresia. In total, 28 differentially expressed AS-lncRNAs (DEAS-lncRNAs) (Figure 1B) were then sub-classified into three classical models: head-to-head (14, 50.0%), tail-to-tail (7, 25.0%), and overlap (7, 25.0%) (Figure 1C; Table S1). To further understand the potential function of these DEAS-lncRNAs, we performed a Gene Ontology (GO) enrichment analysis on the cis-target mRNAs of these DEAS-lncRNAs. Seventeen significant GO terms belonging to three GO categories, namely, biological process (BP) (11), cell component (CC) (5), and molecular function (MF) (1), were identified (Figure 1D; Table S2). GO terms in the BP category mainly included the anterior/posterior pattern specification (GO: 0009952), embryonic skeletal system morphogenesis (GO: 0048704), and skeletal system development (GO: 0001501). Those in the MF category mainly included sequence-specific DNA binding (GO: 0043565), transcription factor activity (GO: 0003700), and poly(A) RNA binding (GO: 0044822). The GO term in the CC category was transcription factor complexes alone (GO: 0005667). These data suggest that these DEAS-lncRNAs are related to the initiation of follicular atresia, possibly through the regulation of several important GO pathways.

BRE-AS is related to porcine follicular atresia

LOC102157709 is the most upregulated DEAS-lncRNA in early atretic follicles compared with that in healthy follicles (Figure 2A), indicating that it is strongly related to the initiation of follicular atresia. Therefore, LOC102157709 was further investigated. Rapid amplification of cDNA ends (RACE) assay showed that the full length of porcine LOC102157709 RNA was 468 bp (GenBank: MZ090592) (Figures 2B and 2C). Genomic location analysis showed that LOC102157709 is embedded within intron 10 of the porcine brain- and reproductive-organ-expressed protein (BRE) gene, and transcribed from the antisense sequence of this gene (Figure 2D), allowing us to refer to this as BRE-AS. The BRE-AS gene consists of two exons and one intron (Figure 2D), and the online tool coding potential assessment tool (CPAT) further
confirmed that BRE-AS is an AS-lncRNA with no protein-coding potential (Figure 2E). BRE-AS tissue expression patterns showed that it is specifically highly expressed in the ovary, lung, and intestine (Figure 2F). To further confirm the relationship between BRE-AS and follicular atresia in vivo, correlation analysis between follicular BRE-AS levels and the ratio of progesterone and 17ß-estradiol concentration (P4/E2) and GC density, both biomarkers of follicular atresia in follicular fluid, was performed. The results showed that follicular BRE-AS levels were positively correlated with the P4/E2 ratio and GC density in follicular fluid (Figures 2G and 2H), indicating that BRE-AS is related to follicular atresia in vivo. Follicular atresia is well known to be triggered by GC apoptosis. 28 We also showed that follicular BRE-AS levels were significantly associated with mRNA abundance of multiple pro-apoptotic genes, such as BAX, Caspase-3, and Caspase-8 (Figure 2I; Figure S1). These data suggest that BRE-AS is related to ovarian functions, especially follicular atresia.

BRE-AS induces early apoptosis of GCs

To investigate the role of BRE-AS in ovarian functions, especially follicular atresia, we overexpressed and knocked down BRE-AS in porcine GCs by transfection with a vector pcDNA3.1-BRE-AS or BRE-AS-specific small interfering RNA (BRE-AS-siRNA) (Figures 3A and 3B). Flow cytometry showed that BRE-AS overexpression strongly increased the GC apoptosis rate, especially the early apoptosis rate (Figure 3C). Furthermore, mRNA transcript ratios for anti-apoptotic BCL2 and pro-apoptotic BAX (BCL2/BAX) were decreased in BRE-AS-overexpressing GCs (Figure 3D). Conversely, BRE-AS knockdown decreased the GC apoptosis rate, especially the early apoptosis rate (Figure 3E), and increased the BCL2/BAX ratio in GCs (Figure 3F). These results suggest that BRE-AS induces the early apoptosis of GCs, thereby initiating follicular atresia.

BRE-AS modulates BRE expression by binding to its pre-mRNA

AS-lncRNAs play a critical biological role via their regulation of and interaction with their counterpart sense genes. 36 Therefore, we assessed whether BRE-AS affects BRE expression in GCs. As expected, BRE mRNA and protein levels were decreased in BRE-AS-overexpressing GCs (Figures 3A and 3B). Flow cytometry showed that BRE-AS overexpression strongly increased the GC apoptosis rate, especially the early apoptosis rate (Figure 3C). Furthermore, mRNA transcript ratios for anti-apoptotic BCL2 and pro-apoptotic BAX (BCL2/BAX) were decreased in BRE-AS-overexpressing GCs (Figure 3D). Conversely, BRE-AS knockdown decreased the GC apoptosis rate, especially the early apoptosis rate (Figure 3E), and increased the BCL2/BAX ratio in GCs (Figure 3F). These results suggest that BRE-AS induces the early apoptosis of GCs, thereby initiating follicular atresia.
cytoplasm, and co-RNA-fluorescence in situ hybridization (co-RNA-FISH) showed that BRE-AS and BRE pre-mRNA hybridized within the same nuclear foci (Figure 4F). Furthermore, when RNAs isolated from GCs were treated with RNase I, an RNase that specifically degrades single-stranded RNA (ssRNA), the overlapping region in both transcripts was found to be protected from degradation, indicating that BRE-AS and BRE pre-mRNA form dsRNA in the nuclei of GCs (Figure 4G). In addition, BRE-AS suppressed the pre-mRNA expression of BRE in GCs (Figure 4H). These data suggest that BRE-AS binds to BRE pre-mRNA to control BRE expression in GCs.

**BRE-AS induces GC apoptosis via downregulation of BRE expression**

To assess the role of BRE in GC apoptosis, we overexpressed or silenced BRE in GCs by transfecting with pcDNA3.1-BRE-AS vector or BRE-AS-siRNA, respectively (Figure S2). BRE overexpression decreased the apoptosis rate of GCs (Figure 5A) and increased the BCL2/BAX ratio (Figure 5B), whereas BRE inhibition had the opposite effect (Figures 5C and 5D), indicating that BRE attenuates GC apoptosis, which is contrary to the effect of BRE-AS. Additionally, BRE overexpression relieved the GC apoptosis induced by BRE-AS (Figure 5E), whereas BRE silencing inhibited the reduction in apoptosis caused by BRE-AS knockdown in GCs (Figure 5F). Taken together, these results suggest that BRE-AS induces GC apoptosis via the downregulation of BRE expression.

**SMAD4 is an inhibitor of BRE-AS in GCs**

Notably, our previous RNA-seq data showed that BRE-AS is also a DEL in SMAD4-silenced GCs (Figure 6A). Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) revealed that BRE-AS is upregulated in SMAD4-silenced GCs (Figure 6B), which is consistent with our RNA-seq data. Furthermore, the overexpression of SMAD4 by transfection with pcDNA3.1-SMAD4 reduced BRE-AS expression in GCs (Figure 6C). In addition, the silencing of SMAD4 increased BRE expression (Figures 6D and 6E), whereas the overexpression of
SMAD4 had the opposite effect in targeted cells (Figures 6F and 6G). However, this regulatory effect of SMAD4 on BRE expression was reversed by BRE-AS (Figures 6H and 6I), indicating that SMAD4 regulates the downstream target of BRE-AS via BRE-AS in GCs. These data suggest that SMAD4 is an inhibitor of BRE-AS in GCs.

SMAD4 is a transcriptional repressor of BRE-AS in GCs

SMAD4 is a well-known transcription factor.3,38 To elucidate the molecular mechanism by which SMAD4 inhibits BRE-AS in GCs, we first isolated a 1,100-bp sequence from the BRE-AS promoter region. Three SMAD4-binding elements (SBEs) were observed in this region (Figure 7A; Figure S3), and a DNA sequence from −161 nt to +148 nt of the promoter was identified as a putative core promoter region of BRE-AS in GCs using deletion constructs (Figure 7B). Furthermore, the activity of the BRE-AS core promoter was significantly decreased by SMAD4 overexpression (Figure 7C), whereas it was dramatically increased by SMAD4 knockdown (Figure 7D), revealing that SMAD4 can regulate the promoter activity of BRE-AS. Chromatin immunoprecipitation (ChIP) assay showed that SMAD4 directly binds to the SBE site in the core promoter of BRE-AS but not outside the promoter region (Figure 7E). Overall, these results and our previous data showed that SMAD4 is a transcriptional repressor of BRE-AS in GCs.

BRE-AS mediates SMAD4-induced inhibition of GC apoptosis

SMAD4 serves as an anti-apoptotic factor in porcine GCs.38 To determine whether SMAD4 controls GC apoptosis via BRE-AS, GCs were co-transfected with pcDNA3.1-SMAD4 and pcDNA3.1-BRE-AS or SMAD4-siRNA and BRE-AS-siRNA. As expected, the overexpression of SMAD4 reduced the GC apoptosis rate; however, this anti-apoptotic effect was reversed by BRE-AS overexpression (Figure 8A). In contrast, GC apoptosis was induced by SMAD4 knockdown; however, this was blocked by BRE-AS-siRNA (Figure 8B). Collectively, these results suggest that BRE-AS is a functional target of SMAD4 and mediates SMAD4-induced inhibition of GC apoptosis.

DISCUSSION

Atria is the ultimate fate of most ovarian follicles and limits the utilization efficiency of follicles and fertility potential in females. Follicular atresia is affected by many factors, such as follicular fluid factors,31,39,40 cell-death-related factors,26,29,41,42 and epigenetic mediators including miRNAs, lncRNAs, and circular RNAs.3,32,43 Here, we found that
AS-lncRNAs are related to the initiation of porcine follicular atresia and demonstrated that BRE-AS induces GC apoptosis, the primary cause of follicular atresia, by suppressing the protein-coding gene BRE, which encodes an anti-apoptotic factor. In addition, we showed that the anti-apoptotic factor SMAD4 is a transcriptional repressor of BRE-AS in GCs.

This study demonstrated for the first time that AS-lncRNAs participate in the regulation of follicular atresia and that BRE-AS, the most upregulated AS-lncRNA during follicular atresia, regulates the early apoptosis of GCs. Follicular atresia is triggered by GC apoptosis. Therefore, follicular-atresia-related factors including IncRNAs are usually involved in GC apoptosis. Noncoding RNA involved in the follicular atresia (NORFA), a lncRNA, was the first functional IncRNA linked to increased fecundity in Erhualian pigs, one of the most prolific pig breeds known worldwide, and is involved in follicular development and atresia via the inhibition of GC apoptosis. SMAD4-dependent noncoding RNA (SDNOR), an lncRNA, forms a regulatory axis with miR-29c to mediate crosstalk between the TGF-β and Wnt signaling pathways, inhibiting GC apoptosis and follicular atresia. Although there are no other reports describing the relationship between AS-lncRNAs and follicular atresia, the relationship between ZFAS1, an AS-lncRNA, and the regulation of GC apoptosis has recently been reported.

BRE, also known as BARAM2 or BRCC45, is required for maintaining genomic integrity and plays a key role in DNA repair and anti-apoptotic responses. BRE is highly expressed in reproductive systems and is strongly involved in follicular development and atresia. A recent report showed that BRE mutants promote follicular atresia by inducing GC apoptosis via changes in the DNA damage repair pathways. In this study, we showed that BRE mediates BRE-AS-enhanced GC apoptosis, demonstrating that BRE is also an anti-apoptotic factor in porcine GCs. Notably, BRE has been identified as an anti-apoptotic factor in multiple cell types, including mouse hepatocytes, human hepatocellular carcinoma cells, lung cancer cells, and esophageal squamous cell carcinoma cells. Taken together, our findings describe the underlying regulatory mechanism facilitating BRE-AS-induced apoptosis in porcine GCs and further verify that BRE is an anti-apoptotic factor in the ovarian GCs of mammals.

AS-lncRNAs are a type of natural antisense transcripts (NATs), which are strongly associated with their counterpart sense genes. Increasing evidence suggests that AS-lncRNAs perform their biological functions primarily through the regulation of their counterpart sense genes by forming an RNA-RNA duplex or RNA-DNA triplex with their counterpart sense genes. In this study, we showed that BRE-AS suppresses BRE expression in porcine GCs by forming an RNA-RNA duplex with its pre-mRNA transcript. This dsRNA model has been shown to be involved in RNA splicing, RNA editing, and RNA silencing. For example, SAF, an antisense lncRNA of the apoptotic gene Fas, interacts with the pre-mRNA of the Fas receptor and recruits splicing factor 45 (SFP45) to facilitate the deletion of exon 6. This variant of FasΔEx6 produces soluble sFas protein and blocks FasL-induced apoptosis. The dsRNA structure can also trigger RNA editing of the pre-mRNA sequence of the sense transcript. Prostate cancer antigen 3 (PCA3), an antisense intronic lncRNA of PRUNE2, binds to intron 6 of PRUNE2 pre-mRNA and induces adenosine-to-inosine RNA editing mediated by ADAR2, which facilitates endogenous PRUNE2 silencing and tumorigeneresis. In addition, the nuclear-enriched antisense transcript GLS-AS is involved in pancreatic cancer metabolism and is known to suppress GLS expression at the post-transcriptional level via the production of a dsRNA construct including the AS-lncRNA sequence and the GLS pre-mRNA transcript. This dsRNA structure triggers ADAR/Dicer-dependent RNA interference.

SMAD4 is the only Co-SMAD protein in the TGF-β signaling pathway and plays an important role in GC function by acting as an anti-apoptotic protein. Many anti-apoptotic mechanisms of SMAD4 in GCs have been investigated, and the regulation of noncoding RNAs (e.g., miRNAs and IncRNAs) is one of the important mechanisms. SMAD4 controls GC apoptosis directly via miRNAs through two mechanisms: (1) influencing miRNA biosynthesis and (2) regulating single miRNA expression as a transcription factor. However, little is known about the interactions between SMAD4 and IncRNAs in the regulation of GC apoptosis. In this study, we showed that SMAD4 functions as a transcription factor to inhibit GC apoptosis by direct binding with the BRE-AS promoter region. We demonstrated that SMAD4 binds to the SBE motif in the promoter region of IncRNA SDNOR to promote SDNOR expression and control SDNOR-mediated apoptosis in porcine GCs. In addition, SMAD4 has been shown to be a transcriptional regulator of several IncRNAs in other cell types, such as IncRNA PCAAT7 in prostate cancer cells, IncRNA 9884 in renal inflammation cells, LINP1 in lung cancer cells, and LIN-LET in bladder cancer cells. Our findings provide new evidence for SMAD4 regulation of GC apoptosis by IncRNAs, especially AS-lncRNAs.

Our study is the first to identify the relationship between AS-lncRNAs and the initiation of follicular atresia. We demonstrated that BRE-AS initiates follicular atresia by inducing early apoptosis in GCs. Furthermore, BRE-AS suppresses host BRE expression by forming an

Figure 5. BRE mediates BRE-AS induction of GC apoptosis
(A and B) GCs were transfected with pcDNA3.1-BRE, apoptosis rate was determined by FACS (A), BCL2 and BAX levels were detected by qRT-PCR, and BCL2/BAX ratio was calculated (B). (C and D) GCs were transfected with BRE-siRNA, apoptosis rate was determined by FACS (C), BCL2 and BAX levels were detected by qRT-PCR, and BCL2/BAX ratio was calculated (D). (E) GCs were co-transfected with pcDNA3.1-BRE-AS and pcDNA3.1-BRE, and apoptosis rate was determined by FACS. Each group has at least 3 replicates. Each group is represented by mean ± SEM; **p < 0.01.
RNA-RNA duplex with the BRE pre-mRNA transcript and facilitates GC apoptosis. In addition, we identified SMAD4 as a transcriptional repressor of BRE-AS and described a novel pathway for the regulation of follicular atresia and GC apoptosis, that is, the SMAD4/BRE-AS/BRE pathway (Figure 8C). Overall, our findings provide new insights into the epigenetic mechanisms underlying follicular atresia and female fertility. AS-lncRNA BRE-AS is expected to become a new target and non-hormone drug for molecular therapy of follicular atresia and other ovarian diseases.

MATERIALS AND METHODS

IncRNA locus classification

Data on DELs in healthy and early atresia follicle samples from porcine ovaries were obtained from our previous RNA-seq study (unpublished), and the DELs were classified into locus biotypes according to their genomic distribution patterns. InCAs were defined as either genic (≤5 kb) or intergenic (>5 kb) using a 5 kb cutoff for nearby protein-coding genes. Genic IncRNAs were then further categorized as bidirectional, sense, or antisense based on their transcriptional orientation and start site with respect to any nearby protein-coding locus (Figure S4).

Bioinformatics analysis

Differential expression analysis of AS-lncRNAs was performed using the DESeq2 package in R project (https://www.r-project.org/), and the cis roles of these DEAS-lncRNAs were determined using GO enrichment analysis, based on the coding genes up to 200 kb upstream or downstream of the DEAS-lncRNAs. This analysis was performed using the DAVID 6.8 database (https://david.ncifcrf.gov/tools.jsp), and a p-value < 0.05 was considered to indicate statistical significance. The
locations of the AS-lncRNAs and their nearby genes were annotated using the National Center for Biotechnology Information database (https://www.ncbi.nlm.nih.gov/). The CPAT website tool65 (http://lilab.research.bcm.edu/cpat/) was used to predict the protein-coding ability of AS-lncRNAs. The transcriptional factors in the promoter region of BRE-AS were identified using the JASPAR database (http://jaspar.genereg.net/).

Follicle collection and P4 or E2 detection
Follicles were collected as previously described.35 Briefly, antral follicles, 3–5 mm in diameter, were dissociated from the ovaries by surgical tweezers and scalpels. After tearing the follicular membrane with forceps, the follicular fluid was isolated by centrifugation at 1,000 rpm for 1 min. All animal experiments were approved by the Ethical Committee of Nanjing Agricultural University. The GC density was counted using a hemocytometer in follicular fluid diluted in phosphate-buffered saline (PBS) (1:10) under a light microscope. For P4 or E2 detection, after centrifugation for 3 min at 1,000 rpm, the supernatant of follicular fluid was diluted at 1:100 in PBS, and radioimmunoassay was performed to measure steroid hormone levels by using iodine [125I] P4 or E2 Radiolmmunoassay Kit (North Institute, Beijing, China) in Jinling Hospital (Nanjing, China) according to the manufacturer’s instructions.

RNA isolation and qRT-PCR
Total RNA from porcine tissues, ovarian follicles (3–5 mm), or GCs were isolated using a High-Purity Total RNA Extraction Kit (Biotek, Beijing, China), and cDNAs were reverse-transcribed using a HiScript Q RT SuperMix kit (Vazyme Biotech, Nanjing, China) according to the manufacturer’s instructions. qRT-PCR was performed using an AceQ qPCR SYBR Green Master Mix (Vazyme Biotech, Nanjing, China) on an ABI-7500 Real-Time PCR System (Applied Biosystems, Beverly, MA, USA), and the relative gene expression was calculated using the standard ΔΔCt method. The specific primers used to quantify BRE, BRE-AS, BCL2, BAX, and GAPDH are listed in Table S3.

RACE
To determine the transcriptional initiation and termination sites in BRE-AS, 5’-RACE and 3’-RACE assays were performed using a SMARTer RACE cDNA Amplification Kit (TaKaRa, Dalian, China). The gene-specific primers used in these assays were 5’-TGG GGT
CTT GGG TCC ACC TGA GGT C-3' for the 5'-RACE assay and 5'-CGG CAG AGC CAG CCA GGA TTC TGA C-3' for the 3'-RACE assay.

Cell culture and transfection

GCs were isolated from 3- to 5-mm healthy follicles, grown in 12- or 6-well plates, and maintained in Dulbecco’s modified Eagle’s medium/F-12 (Gibco, Carlsbad, CA, USA) supplemented with 15% fetal bovine serum (FBS, Gibco, Carlsbad, CA, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco, Carlsbad, CA, USA). After being cultured at 37°C and 5% CO2 for 36 h, the GCs were washed twice with PBS and prepared for transfection. KGN cell lines were obtained from Procell (Procell, Wuhan, China), maintained in Roswell Park Memorial Institute 1640 medium supplemented with 10% FBS (Gibco, Carlsbad, CA, USA), penicillin (100 U/mL), and streptomycin (100 mg/mL), and grown at 37°C in a humidified atmosphere containing 5% CO2. Transfection was performed at 80% confluence using Lipofectamine 3000 Reagent (Invitrogen, Shanghai, China).

Plasmid and oligonucleotide construction

To construct the BRE and BRE-AS overexpression vectors, we cloned the full-length cDNA of BRE or BRE-AS into a pcDNA3.1 expression vector (GenePharma, Shanghai, China) using NHEI and XhoI enzymes; the pcDNA3.1-SMAD4 vector was constructed as previously described.37 To identify the core promoter region of BRE-AS, 309-, 541-, 805-, and 1,100-bp fragments from the BRE-AS promoter region were cloned and inserted into the pGL3-Basic vector (Promega, Madison, WI, USA) using KpnI and XhoI. BRE-AS-, BRE-, and SMAD4-specific siRNAs were synthesized by GenePharma (Shanghai, China). All the relevant oligonucleotide sequences are summarized in Table S4.

Apoptosis assay

Cellular apoptosis was evaluated using an Annexin V-FITC/PI Apoptosis Detection Kit (Vazyme, Nanjing, China). Briefly, GCs were double-stained using 5 μL of annexin V and 5 μL of propidium iodide, and the number of apoptotic cells was calculated using a BD FACScan flow cytometry system (Becton Dickinson, Franklin, NJ, USA) with an excitation wavelength of 488 nm and an emission wavelength of 605 nm.

Western blotting

Western blotting was performed as previously described.3 After being denatured at 99°C for 10 min, total proteins were separated using 10%–12% sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes (Merck Millipore), and probed with primary antibodies against BRE (diluted at 1:1,000, Abcam, Cambridge, MA, USA) or beta-tubulin (diluted at 1:2,000, Proteintech, China). After incubation with horseradish peroxidase-conjugated AffiniPure goat anti-rabbit immunoglobulin G (IgG) secondary antibodies (diluted at 1:5,000, Sangon Biotech, China), the antigen–antibody reactions were evaluated using an enhanced chemiluminescence reagent (Biosharp, Nanjing, China) and visualized using the Bio-Rad Quantity One 1-D system (Bio-Rad, Hercules, CA, USA).
Subcellular localization
GCs were incubated in 200 µL of cold cytoplasmic lysate buffer (0.15% NP-40, 10 µM Tris, and 150 µM NaCl) for 5 min, and then 500 µL of cold sucrose buffer (10 µM Tris, 150 µM NaCl, and 24% sucrose) was added. The samples were centrifuged at 10,000 × g for 3 min at 4°C. The supernatant containing the cytoplasmic components was collected and stored at −80°C. Further, the nuclei in the precipitate were resuspended in ddH2O without RNase and centrifuged at 10,000 × g for 3 min at 4°C. The supernatant was removed, and the precipitate containing the nuclear components was retained. We isolated RNA from the cytoplasmic and nuclear components as described above and then performed qRT-PCR to evaluate BRE-AS expression.

RNA FISH assay
GCs were fixed in 4% formaldehyde for 20 min at 20°C and then permeabilized using PBS containing 0.39% Triton X-100 for 5 min on ice. After washing in PBS and 2× saline sodium citrate buffer, the cy3- and FAM-labeled oligonucleotide probes against BRE-AS (5′-CCG CAC GCA TCT GGA CTG GAC AGC ACA ACT-3′) and BRE pre-mRNA (5′-AGC GCA AGA CCT CAG GTG GAC CCA AGA-3′) were incubated with the treated GCs and hybridized overnight at 37°C in a humidified chamber. The nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI), and BRE-AS and BRE pre-mRNA were detected using fluorescence microscopy at 543 nm and 647 nm wavelengths, respectively.

Ribonuclease protection assay (RPA)
RPA was used to identify any dsRNA in the total RNA isolated from GC samples treated with DNase I (Vazyme Biotech, Nanjing, China) and RNase I (Invitrogen, Shanghai, China), which removed all genomic DNA contamination and cleaved any ssRNA. We used a specific primer probe set designed against a non-overlapping and overlapping region of the transcript to detect degradation via PCR. The sequences for the primers used in these assays are described in Table S3.

Luciferase reporter assay
Luciferase activity was measured using Luciferase Assay Buffer II and Stop & GLO Stratech using a Modulus Assay System (Promega, Madison, WI, USA) according to the manufacturer’s instructions. The firefly and Renilla luciferase activity were then evaluated and normalized for each sample.

ChIP assay
The ChIP assay was performed using a Pierce Agarose ChIP Kit (Thermo Scientific, Waltham, MA, USA). Briefly, a total of 2 × 10^6 cells were fixed in 1% formaldehyde via gentle swirling at 20°C for 10 min, and the fixation reaction was stopped by the addition of glycine solution. Nucleic acid was digested into 300–600-bp fragments using 6 U of micrococcal nuclease incubated in a water bath at 37°C for 15 min. IP samples were incubated with 2 µg of SMAD4 antibody or normal rabbit IgG overnight at 4°C on a rocking platform, and precipitated DNA fragments containing SBE motifs were detected using PCR with the specific primers listed in Table S3.

Statistical analysis
Data are presented as mean ± standard error of mean, and IBM SPSS Statistics v.20.0 (SPSS) was used for all statistical analyses. Significance of data was evaluated using Student’s t test or one-way analysis of variance. *p < 0.05 and **p < 0.01 were considered to indicate statistical significance, and GraphPad Prism v.5.0 software was used to create all figures.

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.omtn.2021.05.006.

ACKNOWLEDGMENTS
This work was supported by the Qing Lan Project of Jiangsu Province (2020) and the National Natural Science Foundation of China (no. 31772568).

AUTHOR CONTRIBUTIONS
W.Y. and Q.L. designed the research; W.Y., X.D., and M.W. performed the experiments; W.Y., Y.W., and Z.P. analyzed the data; W.Y., J.Z., and Q.L. wrote the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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