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## Changes in brain ribonuclease (*BRB*) mRNA in granulosa cells (GC) of dominant versus subordinate ovarian follicles of cattle and the regulation of *BRB* gene expression in bovine GC

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### Abstract

Brain ribonuclease (BRB) is a member of the ribonuclease A superfamily that is constitutively expressed in a range of tissues, and is the functional homolog of human ribonuclease 1. This study was designed to characterize *BRB* gene expression in granulosa cells (GC) during development of bovine dominant ovarian follicles, and to determine the hormonal regulation of *BRB* in GC. Estrous cycles of Holstein cows ( $n = 18$ ) were synchronized and cows were ovariectomized on either day 3 to 4 or day 5 to 6 post-ovulation during dominant follicle growth and selection. Ovaries were collected, follicular fluid (FFL) was aspirated, and GC were collected for RNA isolation and quantitative PCR. Follicles were categorized as small (1 to 5 mm; pooled per ovary), medium (5 to 8 mm; individually collected) or large (8.1 to 17 mm; individually collected) based on surface diameter. Estradiol ( $E_2$ ) and progesterone ( $P_4$ ) levels were measured by RIA in FFL. Abundance of *BRB* mRNA in GC was 8.6- to 11.8-fold greater ( $P < 0.05$ ) in small ( $n = 31$ ), medium ( $n = 66$ ) and large ( $n = 33$ ) subordinate  $E_2$ -inactive (FFL  $E_2 < P_4$ ) follicles than in large ( $n = 16$ ) dominant  $E_2$ -active (FFL  $E_2 > P_4$ ) follicles. In the largest 4 follicles, GC *BRB* mRNA abundance was negatively correlated ( $P < 0.01$ ) with FFL  $E_2$  ( $r = -0.65$ ) and  $E_2/P_4$  ratio ( $r = -0.46$ ). In Exp. 2, GC from large (8 to 22 mm diameter) and small (1 to 5 mm diameter) follicles were treated with IGF1 (0 or 30 ng/mL), and/or tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) (0 or 30 ng/mL); IGF1 increased ( $P < 0.05$ ) *BRB* mRNA abundance and TNF $\alpha$  decreased ( $P < 0.001$ ) the IGF1-induced *BRB* mRNA abundance in large-follicle GC. In Exp. 3 to 6,  $E_2$ , FSH, fibroblast growth factor 9 (FGF9), cortisol, wingless 3A (WNT3A), or Sonic hedgehog (SHH) did not affect ( $P > 0.10$ ) abundance of *BRB* mRNA in GC; thyroxine and LH increased ( $P < 0.05$ ) whereas prostaglandin  $E_2$  (PGE $_2$ ) decreased ( $P < 0.05$ ) *BRB* mRNA abundance in small-follicle GC. Treatment of small-follicle GC with recombinant human RNase1 increased ( $P < 0.05$ ) GC numbers and estradiol production. In conclusion, *BRB* is a hormonally and developmentally regulated gene in bovine GC and may regulate estradiol production during follicular growth in cattle.

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## Keywords

Brain ribonuclease (BRB); granulosa cells; insulin-like growth factor 1 (IGF1); tumor necrosis factor  $\alpha$  (TNF $\alpha$ ); follicular growth

## 1. Introduction

Brain ribonuclease (BRB) was first isolated from bovine brain [1–3] and later found to have widespread tissue expression in cattle [3, 4]. Brain ribonuclease is a member of the ribonuclease A superfamily of 10 to 28 kDa proteins [5–8] and was recently identified as the functional homolog of human ribonuclease-1 (RNase1) [9]. Ribonuclease A superfamily proteins are multifaceted and exhibit immuno-modulatory effects [9], antitumoral activity [10] and pro-apoptotic activity [11]. Pancreatic RNase A is the better described RNase A superfamily member in cattle; this RNase is thought to function to breakdown the large amounts of RNA that accumulate in the ruminant gut [12, 13]. Another well-known RNase A superfamily member, seminal ribonuclease, is produced by bovine seminal vesicles, shares 80% identity with bovine pancreatic RNase A and has cytotoxic and immunosuppressive activity needed to protect spermatozoa from the female immune system [9, 14]. The ribonuclease A superfamily member, RNase5 (also called angiogenin), has been linked to morphological changes in the bovine ovary [15] and angiogenesis [16], but whether RNase1 homologs such as BRB change during follicular development or regulate follicular atresia is unknown. A previous study found that angiogenin (*ANG*) was the greatest up-regulated (by 20-fold) gene in granulosa cells of cystic vs. normal dominant follicles [17]. However, Affymetrix has revised the identity of their original *ANG* transcript on the microarray to be *BRB*. Therefore, we hypothesized that *BRB* expression in GC increases during normal follicular development in cattle. Other genes that were significantly different between cystic and normal follicles included the top 3 reproduction-related down-regulated genes: Indian hedgehog (*IHH*), fibroblast growth factor 9 (*FGF9*), secreted frizzled protein 4 (*SFRP4*), and the only other reproduction-related gene in the top 10 up-regulated genes was prostaglandin E2 receptor 4 (*PTGER4*) [17]. We further hypothesized that one or more of these intraovarian factors may regulate *BRB* mRNA in GC. Therefore, we evaluated the effects of IHH, FGF9, wingless 3A (WNT3A; a ligand for SFRP4) and PGE2 on *BRB* mRNA abundance in GC in the present study.

Hormones have been associated with production of other RNase A superfamily members such as RNase1 in non-ovarian tissues. For example, tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) decreases RNase1 production by human umbilical vein endothelial cells [18]. Although TNF $\alpha$  inhibits basal and FSH-induced steroidogenesis in granulosa cells (GC) and is thought to play a role in the regulation of ovarian function [19, 20], the effect of TNF $\alpha$  on ovarian BRB production is unknown. Similarly, IGF1 is a major ovarian tropic hormone [21, 22], but whether IGF1 alters production of BRB in ovarian cells is unknown. Other hormones such as thyroxine (T4) have been implicated in regulating both reproduction [23] and angiogenesis [24]. Investigation of the hormonal control of *BRB* mRNA expression in large- and small-follicle GC may reveal possible regulatory mechanisms in the ovarian ribonuclease A superfamily system. The objectives of this study were to characterize *BRB*

mRNA in GC during development of dominant follicles in cattle, and to evaluate the effect of ovarian trophic hormones LH, FSH, estradiol (E<sub>2</sub>) and IGF1, and other hormones and factors on ovarian *BRB* mRNA gene expression in cultured bovine GC.

## 2. Materials and methods

### 2.1. Hormones and reagents

The hormones and reagents used in cell culture were: ovine FSH (NIDDK-oFSH-20; activity: 175 X NIH-FSH-S1 U/mg) and ovine LH (NIDDK-oLH-26; activity: 1.0 X NIH-LH-S1 U/mg) from the National Hormone & Pituitary Program (Torrance, CA, USA); carrier-free recombinant human ANG, WNT3A, FGF9, Sonic hedgehog (SHH) and IGF1, recombinant bovine TNF $\alpha$ , and recombinant mouse IHH (amino terminal peptide C28II) from R&D Systems (Minneapolis, MN); recombinant human RNase 1 RNase1) from Novoprotein Scientific, Inc. (Summit, NJ); testosterone from Steraloids (Wilton NH); and cortisol, T4 and E<sub>2</sub> from Sigma-Aldrich Corp. (St. Louis, MO, USA); and fetal calf serum (FCS) from EquiTech-Bio, Inc. (Kerrville, TX). Medium used for GC isolation and culture was Dulbecco's modified Eagle's medium and Ham's F-12 (1:1) containing gentamicin (0.12 mM), glutamine (2.0 mM), and sodium bicarbonate (38.5 mM; Sigma-Aldrich Corp.).

### 2.2. Animals and in vivo Experimental Design- Exp. 1

This experiment was performed to determine if abundance of *BRB* mRNA in GC changes during folliculogenesis in cattle and compare GC *BRB* mRNA abundance in dominant and subordinate follicles. The animal experimentation described in this report was approved by the Oklahoma State University Institutional Animal Care and Use Committee.

Non-lactating Holstein cows (n = 18) were used for this experiment. These cows were identified to be culled for non-reproductive reasons from the Oklahoma State University herd, and were housed on pasture and group-fed a total mixed ration consisting of alfalfa hay, whole cottonseed, and concentrate ad libitum. Estrous cycles were synchronized using 2 injections (im) of PGF2 $\alpha$  (Lutalyse®, 25 mg) with an interval of 11 d. From the first injection of prostaglandin F2 $\alpha$  to the occurrence of ovulation after the second injection, follicle development was monitored daily via ultrasonography using an Aloka 500V with a 7.5 MHz probe. Following ovulation, cows continued to be monitored with daily ultrasonography and were assigned to be ovariectomized either at days 3 to 4 (early growing phase of the first dominant follicle; n = 9 cows) or days 5 to 6 (late growing phase of the first dominant follicle; n = 9 cows). From the 18 cows used in the synchronization program, 2 failed (one from day 3 and one from day 6 groups) to ovulate and were excluded from this experiment. Both ovaries from each cow were removed via lateral incision through the left paralumbar fossa area after local anesthesia (2% lidocaine; 60 to 80 mL sc and im). After each ovariectomy, ovaries were identified as right and left, put on ice, and transported to the laboratory where diameters of all follicles  $\geq$  5 mm (surface diameter) in diameter were recorded, and ovarian tissue and follicular fluid (FFL) collected as previously described [25–27].

For GC sample collection, follicles were categorized by surface diameter as small (1 to 5 mm), medium (5.1 to 8 mm) or large (8.1 to 17 mm) follicles. The FFL from medium and

large follicles was aspirated individually and centrifuged to obtain GC, and FFL from small follicles was pooled within each ovary and then centrifuged to obtain GC as previously described [25, 28]. After centrifugation, FFL was aspirated and stored in another tube at  $-20^{\circ}\text{C}$  for measurement of  $\text{E}_2$  and progesterone ( $\text{P}_4$ ) via RIA. After aspiration of FFL, each medium and large follicle was bisected *in situ*, the inner wall was scraped, rinsed with cell culture medium to remove any remaining GC, and these GC were combined with GC collected from FFL as previously described [25, 29]. GC collected from small follicles were kept separate for each ovary. GC were lysed in 0.5 mL of TRIzol® reagent solution (Life Technologies, Inc., Grand Island, NY) and stored frozen at  $-80^{\circ}\text{C}$  until RNA extraction (see description below).

### 2.3. Cell culture and in vitro Experimental Design – Exp. 2 to 6

Ovaries from non-pregnant beef cows and heifers were collected from a local slaughterhouse, and based on surface diameter, GC were collected from small (1 to 5 mm) and large (8 to 22 mm) follicles as previously described [28–31]. Cells were re-suspended in medium containing collagenase and DNase (Sigma Chemical Co.) at 1.25 mg/mL and 0.5 mg/mL, respectively, to prevent cell clumping prior to plating.

Viable cells ( $2.0 \times 10^5$  in 20 to 80  $\mu\text{L}$  of medium) were plated on 24-well Falcon multiwell plates (Becton Dickinson, Lincoln Park, NJ, USA) in 1.0 mL of medium containing 10% FCS (v/v). Cells were cultured at  $38.5^{\circ}\text{C}$  in 10% FCS (v/v) for the first 48 h with a medium change at 24 h. Cells were then washed twice with serum-free medium and the various treatments (see below) were applied in serum-free medium for 24 h or 48 h after which medium was aspirated and TRIzol was added to the wells for collection of cellular RNA (see below).

*Exp. 2* was designed to test the effect of IGF1 and  $\text{TNF}\alpha$  on *BRB* mRNA in small- and large-follicle GC. Cells were cultured for 48 h in 10% FCS and then washed twice with serum free medium (0.5 mL) and treatments applied for 24 h. Treatments were as follows: Control (no additions), IGF1 (30 ng/mL),  $\text{TNF}\alpha$  (30 ng/mL), and  $\text{TNF}\alpha$  plus IGF1. Doses of IGF1 and  $\text{TNF}\alpha$  were selected based on previous studies showing that these doses significantly alter steroidogenesis [19, 32, 33]. After 24 h of treatment, cells were lysed in 0.5 mL of TRIzol for RNA extraction (see below).

*Exp. 3* was designed to test the effect of FSH,  $\text{E}_2$ , FGF9, and/or IHH on *BRB* mRNA abundance in small- and large-follicle GC. Cells were cultured for 48 h as described in Exp. 1. All 6 treatments contained IGF1 and either: Control (no additions), FSH (30 ng/mL),  $\text{E}_2$  (300 ng/mL), FSH plus  $\text{E}_2$ , FSH plus FGF9 (10 ng/mL), or FSH plus IHH (1  $\mu\text{g}/\text{mL}$ ); FSH,  $\text{E}_2$ , FGF9 and IHH were tested because of their known effects within the ovary including effects on steroidogenesis [28, 29, 33, 34] and recent implication in cystic follicle development [17]. Doses of  $\text{E}_2$ , FSH, FGF9 and IHH were selected based on previous studies showing that these doses significantly alter steroidogenesis [28, 29, 34–36]. After 24 h of treatment, cells were lysed in 0.5 mL of TRIzol for RNA extraction (see below).

*Exp. 4* was designed to test the effect of ANG, cortisol, PGE2, SHH, and WNT3A on *BRB* mRNA abundance in small-follicle bovine GC. Cortisol and PGE2 were tested because of

their effects on GC function [30, 37] and their reported effects on *RNase5* mRNA in non-ovarian tissues [38, 39], and SHH, WNT3A and ANG were tested because of their implication in ovarian IGF1 stimulation [40] and follicle development [15, 34, 41]. Cells were cultured as described above with treatments applied for 24 h as follows (all treatments included 30 ng/mL of IGF1): Control, cortisol (300 ng/mL), PGE2 (300 ng/mL), SHH (500 ng/mL), WNT3A (300 ng/mL), and ANG (300 ng/mL). Doses of cortisol, IGF1, SHH, PGE2, and WNT3A were selected based on previous studies showing that these doses significantly alter GC function [15, 30, 34, 41]. The concentration of ANG used was selected based on studies indicating that average concentrations of ANG in bovine plasma [42, 43] and human FFL [44, 45] range between 3 and 300 ng/mL. After 24 h of treatment, medium was aspirated and cells were lysed in 0.5 mL of TRIzol for RNA extraction (see below).

*Exp. 5* was designed to test the effect of PGE2 on *BRB* mRNA in large-follicle GC because of its effects in *Exp. 4*. Cells were cultured as described for *Exp. 2* and *4*. Both treatments contained IGF1 (30 ng/mL) and either: Control (no additions) or PGE2 (300 ng/mL). Medium was changed after 24 h. After 24 h of treatment, cells were lysed in 0.5 mL of TRIzol for RNA extraction (see below).

*Exp. 6* was designed to test the effect of LH and T4 and their combination on *BRB* mRNA in small-follicle GC. Cells were cultured as described for *Exp. 2* except that 4 treatments were applied in a 2 x 2 factorial arrangement for 48 h. All 4 treatments contained IGF1 (30 ng/mL) plus FSH (30 ng/mL) and either: LH (0 or 30 ng/mL), T4 (0 or 100 ng/mL), or both LH and T4. Medium was changed after 24 h. Doses of IGF1, FSH, LH and T4 were selected based on previous studies showing these doses affect steroidogenesis [23, 33]. After 48 h of treatment, cells were lysed in 0.5 mL of TRIzol for RNA extraction (see below).

*Exp. 7* was designed to evaluate the effect of RNase1, a homolog of BRB, on steroidogenesis and cell proliferation of small-follicle GC. GC were cultured as described for *Exp. 2* with the following treatments applied for 40 h in serum-free medium (containing 30 ng/mL of IGF1 and FSH, and 500 ng/mL of testosterone as an estrogen precursor) after a 6 h transfection with either control (plus lipofectomine medium) or RNase1 (300 ng/mL plus lipofectomine medium). Cells were treated with 300 ng/mL of recombinant human RNase1 per well using Lipofectamine 2000 in Opti-MEM-I (Invitrogen Corp.) to promote the entry of RNase1 into granulosa cells because the action of RNase1 is thought to be intracellular and RNase1 does not have membrane receptors [46]. Small-follicle GC were selected for this experiment rather than large-follicle GC because in vitro, small-follicle GC have less *BRB* mRNA and thus, presumably less endogenous BRB protein than in GC cultured from large follicles.

#### 2.4. RNA extraction and quantification

Total RNA was extracted using TRIzol reagent protocol (Life Technologies), and RNA was quantitated by spectrophotometry at 260 nm using a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE) as previously described [31, 47]. Quantification of *BRB* gene expression was conducted by fluorescent real-time PCR using an ABI Prism® 7500 sequence detection system as previously described [47]. The sequences for bovine *BRB*

(accession no. NM\_173891.2) primers and probe were: forward: CTGCTACCAGAGCAAATCTACC, reverse: CTAGTCTTGTAGGCACAGTTGG, and probe: TGCCGCGAGACAGGCAGCTCTAAGTA. The internal standard was *18S* rRNA. A target gene dual labeled probe (FAM-TAMRA) and an *18S* probe (VIC) for TaqMan were obtained from Applied Biosystems. Data analysis was done using the comparative threshold cycle (Ct) method as previously described [31, 47]. Fold changes in *BRB* mRNA abundance were calculated as being equal to  $2^{-Ct}$ .

## 2.5. Radioimmunoassays (RIA)

The P<sub>4</sub> and E<sub>2</sub> RIA were performed as previously described [23, 25]. All samples for each experiment were run in a single assay for each of the steroid RIA. The intra-assay coefficient of variation averaged 11.6 % for the P<sub>4</sub> RIA and 10.6% for the E<sub>2</sub> RIA.

## 2.6. Statistical analysis

The analysis of Exp. 1 data aimed to determine if *BRB* mRNA abundance changes in GC during different periods of follicular development and to detect its relationship with steroidogenesis. Data were analyzed via factorial ANOVA with MIXED procedures of SAS for Windows (version 9.2, SAS Institute Inc., Cary, NC) and are presented as means ( $\pm$  SEM) of measurements. Main factors in this split unit model were days post-ovulation (main unit factor; early, days 3 to 4, and late, days 5 to 6, growing phase of the first dominant follicle), follicle group (split unit factor; n = 4) based on follicle size (small, medium, or large) and their follicle estrogenic status (E<sub>2</sub> active: E<sub>2</sub> > P<sub>4</sub> concentrations or E<sub>2</sub> inactive: E<sub>2</sub> < P<sub>4</sub> concentrations), and their interactions. Random effect of cow nested in day was used as the error term for day effect, and follicle group by cow nested in day was used as the error term for follicle group effect and the follicle group by day interaction. To correct for heterogeneity of variance, abundance of *BRB* mRNA and E<sub>2</sub> concentrations in FFL were analyzed after transformation natural log (x + 1). Mean differences were determined by Fisher's protected least significant differences test [48] only if significant main effects in the ANOVA were detected. To evaluate the relationships among variables measured, Pearson correlation coefficients were generated using CORR procedure of SAS (SAS Institute, Cary, NC). Significance was declared at  $P < 0.05$ .

For in vitro experiments, data are presented as the least squares means ( $\pm$  SEM) of measurements from 3 or more individual pools of large- and small-follicle GC used as experimental replicates. Each replicate experiment was conducted on cells collected from at least 3 animals. Each of the large-follicle GC pools was obtained from 5 to 10 follicles. Small-follicle GC were obtained from 6 to 20 ovaries within each experimental replicate. For RNA experiments (Exp. 2 to 6), treatments were applied to 4 different wells on 24-well plates, and duplicate samples for each pool and treatment were obtained by combining RNA from 2 wells. For Exp. 7, each treatment was applied to 3 different wells on 24-well plates, and medium and cells were collected from individual wells. Pool (i.e., experimental replicate) and its interaction with treatments were included in each ANOVA. Specific differences in relative fold mRNA abundance, steroid production or cell numbers among treatments were determined via ANOVA using GLM procedure of SAS (Statistical Analysis



System, Cary, NC) and Fisher's protected least significant difference procedure if significant main effects were observed [48]. Significance was declared at  $P < 0.05$ .

### 3. Results

#### 3.1. Exp. 1: E<sub>2</sub> and P<sub>4</sub> in FFL and BRB mRNA relative abundance in GC

**3.1.1. Size, E<sub>2</sub> and P<sub>4</sub> concentrations in FFL**—Follicle estrogenic status (i.e., size and E<sub>2</sub>-active or E<sub>2</sub>-inactive;  $P < 0.001$ ) but not day ( $P > 0.10$ ) or their interaction ( $P > 0.10$ ) influenced follicle size. Diameter of the large dominant E<sub>2</sub>-active follicles averaged  $12.0 \pm 0.4$  mm on days 3 to 4, and  $13.8 \pm 0.6$  mm on days 5 to 6. Diameter of large subordinate E<sub>2</sub>-inactive and medium subordinate E<sub>2</sub>-inactive follicles averaged  $9.81 \pm 0.36$  and  $6.36 \pm 0.23$  mm, respectively, on days 3 to 4, and  $9.14 \pm 0.35$  and  $6.38 \pm 0.27$  mm, respectively, on days 5 to 6. Because FFL was pooled among small follicles for each ovary, no individual diameters were recorded.

Concentrations of E<sub>2</sub> were influenced ( $P < 0.001$ ) by follicle status (i.e., size and E<sub>2</sub>-active or E<sub>2</sub>-inactive) but not day or their interaction ( $P > 0.10$ ). Concentrations of E<sub>2</sub> averaged  $186.5 \pm 29.5$ ,  $8.45 \pm 3.7$ ,  $2.3 \pm 0.8$ , and  $2.0 \pm 0.2$  ng/mL in large dominant E<sub>2</sub>-active, large subordinate E<sub>2</sub>-inactive, medium E<sub>2</sub>-inactive, and small E<sub>2</sub>-inactive follicles, respectively. In an analysis of the 4 largest follicles with 'rank' and 'day' as main effects, rank was significant but day and their interaction was not. Averaged across days, F1 follicles had greater ( $P < 0.001$ ) concentration of E<sub>2</sub> than F2, F3 and F4 follicles (169, 57, 6.3, and  $0.5 \pm 18$  ng/mL, respectively).

Concentrations of P<sub>4</sub> were not influenced ( $P > 0.10$ ) by day, follicle status (i.e., size and E<sub>2</sub>-active or E<sub>2</sub>-inactive) or their interaction. Concentrations of P<sub>4</sub> averaged  $61 \pm 7$ ,  $160 \pm 36$ ,  $236 \pm 42$ , and  $162 \pm 26$  ng/mL in large dominant E<sub>2</sub>-active, large subordinate E<sub>2</sub>-inactive, medium E<sub>2</sub>-inactive, and small E<sub>2</sub>-inactive follicles, respectively. In an analysis of the 4 largest follicles with 'rank' and 'day' as main effects, rank, day and their interaction were not significant. Concentrations of P<sub>4</sub> in F1, F2, F3 and F4 follicles averaged across days were 127, 136, 214, and  $139 \pm 49$  ng/mL, respectively.

**3.1.2. BRB mRNA relative abundance in GC**—Follicle status but not day or their interaction affected abundance of BRB mRNA such that BRB mRNA abundance was 8.6- to 11.8-fold greater ( $P < 0.01$ ) in subordinate large, medium and small E<sub>2</sub>-inactive (E<sub>2</sub>/P<sub>4</sub> ratio  $< 1$ ) follicles than in large dominant E<sub>2</sub>-active (E<sub>2</sub>/P<sub>4</sub> ratio  $> 1$ ) follicles on both days 3 and 6 (Fig. 1). In an analysis of the 4 largest follicles with 'rank' and 'day' as main effects, rank was significant but day or their interaction was not. Averaged across days, F1 follicles had 57% to 69% lower ( $P < 0.05$ ) BRB mRNA abundance than F2, F3 and F4 follicles (data not shown). In the largest 4 follicles ( $n = 64$ ) among cows, BRB mRNA abundance in GC was negatively correlated with FFL E<sub>2</sub> ( $r = -0.65$ ,  $P < 0.01$ ), E<sub>2</sub>/P<sub>4</sub> ratio ( $r = -0.46$ ,  $P < 0.01$ ), and diameter ( $r = -0.30$ ,  $P < 0.05$ ) whereas BRB mRNA abundance in GC was positively correlated with FFL P<sub>4</sub> ( $r = 0.29$ ,  $P < 0.05$ ). In small-follicles ( $n = 31$ ), BRB mRNA was not significantly correlated with either FFL E<sub>2</sub> ( $r = 0.04$ ) or P<sub>4</sub> ( $r = 0.13$ ).

### 3.2. Exp. 2: Effect of IGF1 and TNF $\alpha$ on small- and large-follicle GC BRB mRNA abundance

In small-follicle GC treated with IGF1, TNF $\alpha$  decreased ( $P < 0.05$ ) the abundance of *BRB* mRNA by 66%, but TNF $\alpha$  alone did not ( $P > 0.10$ ) alter *BRB* mRNA abundance (Fig. 2). In large-follicle GC, IGF1 increased ( $P < 0.0001$ ) *BRB* mRNA abundance by 3.2-fold, and this increase was completely blocked with the addition of TNF $\alpha$  (Fig. 2). Furthermore, TNF $\alpha$  decreased ( $P < 0.05$ ) *BRB* mRNA abundance by 68% compared to controls (Fig. 2).

### 3.3 Exp. 3: Effect of IGF1, FSH, E<sub>2</sub>, FGF9, and IHH on small- and large-follicle GC BRB mRNA abundance

Treatment of small- and large-follicle GC with E<sub>2</sub> or FSH alone had no effect ( $P > 0.15$ ) on *BRB* mRNA abundance (Fig. 3). Also, E<sub>2</sub>, FGF9 and IHH had no effect ( $P > 0.10$ ) on *BRB* mRNA abundance in FSH-treated GC from small and large follicles (Fig. 3).

### 3.4. Exp. 4: Effect of ANG, cortisol, PGE2, SHH and WNT3A on small-follicle GC BRB mRNA abundance

PGE2 reduced ( $P < 0.05$ ) *BRB* mRNA abundance by 32% in IGF1-treated small-follicle GC, but there was no effect of ANG, cortisol, SHH or WNT3A ( $P > 0.50$ ) on *BRB* mRNA abundance after 24 h of treatment (Fig. 4).

### 3.5. Exp. 5: Effect of PGE2 on large-follicle GC BRB mRNA abundance

In contrast to Exp. 4, there was no effect of PGE2 ( $P > 0.50$ ) on *BRB* mRNA abundance in IGF1-treated large-follicle GC; relative *BRB* mRNA abundance averaged 10.1 and 8.77  $\pm$  1.5 for Control and PGE2-treated cultures, respectively.

### 3.6. Exp. 6: Effect of LH and T4 on small-follicle GC BRB mRNA abundance

Treatment with LH alone and T4 alone increased ( $P < 0.05$ ) *BRB* mRNA abundance by 2.1-fold and 1.9-fold, respectively in small-follicle GC treated with FSH plus IGF1 (Fig. 5). Combined treatments of LH and T4 also increased ( $P < 0.05$ ) *BRB* mRNA abundance above controls, but these levels did not differ ( $P > 0.10$ ) from levels found in GC treated with either LH alone or T4 alone (Fig. 5).

### 3.7. Exp. 7: Effect of RNase1 on small-follicle GC steroidogenesis and cell numbers

In small-follicle GC, RNase1 increased ( $P < 0.05$ ) cell numbers by 12% (Fig. 6A) and increased ( $P < 0.05$ ) E<sub>2</sub> production by 49% (Fig. 6B) compared to vehicle-treated controls. However, RNase1 did not ( $P > 0.10$ ) alter progesterone production (data not shown).

## 4. Discussion

The current study was conducted to determine if *BRB* mRNA abundance in GC changes during follicle development, and to determine if various hormones alter ovarian *BRB* mRNA gene expression in bovine GC in vitro. Results revealed that in GC: 1) *BRB* mRNA abundance was less in E<sub>2</sub>-active dominant follicles than E<sub>2</sub>-inactive subordinate follicles; 2) IGF1, LH and T4 increased *BRB* mRNA abundance, 3) TNF $\alpha$  and PGE2 inhibited IGF1-induced *BRB* mRNA abundance; 4) FSH, FGF9, E<sub>2</sub>, cortisol, and IHH/SHH had no effect on



IGF1-induced *BRB* mRNA abundance; and 5) exogenous RNase1 increased numbers of GC and increased GC E<sub>2</sub> production.

The abundance of *BRB* mRNA in GC was less in large E<sub>2</sub>-active dominant follicles at early and late growing phases of the first follicular wave than in small, medium and large E<sub>2</sub>-inactive subordinate follicles, indicating that BRB production likely decreases as the follicle becomes dominant and increased steroidogenesis is required for its further differentiation. Interestingly, treatment of small-follicle GC with RNase1, a homologue to BRB, increased E<sub>2</sub> production in the present study. Perhaps BRB stimulates E<sub>2</sub> production in small follicles but inhibits E<sub>2</sub> production in large follicles. The negative correlation between *BRB* mRNA and E<sub>2</sub> in large follicles but not small follicles supports this suggestion. If BRB is acting as a pro-apoptotic factor as suggested for some RNases [10], decreases in BRB would be needed during follicle growth and dominance. Another possibility is that if BRB has cytotoxic effects similar to bovine seminal RNase [6, 14] then BRB activity would have to be low in the oocyte-cumulus cell complexes when ovulated so the oocyte and sperm are allowed to survive during the fertilization process. However, our *in vitro* study indicated that RNase1 increases cell numbers, and thus would be considered anti-apoptotic, at least in small-follicle GC. Further research will be required to further clarify the functional role of BRB in follicular development of cattle.

Levels of E<sub>2</sub> in FFL and E<sub>2</sub>:P<sub>4</sub> ratio were negatively correlated with GC *BRB* mRNA abundance in the present study. The possibility that increased E<sub>2</sub> may directly inhibit *BRB* mRNA expression was discounted because *in vitro*, E<sub>2</sub> had no significant effect on bovine GC *BRB* mRNA abundance in Exp. 3. Similarly, based on results from Exp. 3, FSH is also not likely involved with changes in *BRB* expression in GC. Consistent with our findings that E<sub>2</sub> had no effect on *BRB* mRNA abundance, Koga et al. [49] found no differences in *RNase5* mRNA expression in human endometrial stromal cells treated with E<sub>2</sub> alone for 4 to 18 d. The absence of any change in FFL P<sub>4</sub> levels during significant changes in GC *BRB* mRNA abundance and its low positive correlation with FFL P<sub>4</sub> indicates that P<sub>4</sub> likely does not regulate *BRB* mRNA. Conversely, because treatment of GC with RNase1 had no effect on P<sub>4</sub> production in the present study, it is likely that BRB does not regulate progesterone production in bovine granulosa cells. Further research will be required to verify these suggestions.

Consistent with findings of the present study (where LH increased GC *BRB* mRNA in Exp. 6), Koga et al. [44] found that *RNase5* mRNA was upregulated by hCG in cultured human GC. Because RNase A family members exhibit pro-apoptotic activity [10], BRB may be involved in initiating atresia at the end of the first follicular wave in a monovulatory species such as the bovine. In addition to LH, IGF1 increased *BRB* mRNA abundance in large-follicle GC by several fold in the present study. IGF1 has been shown to be positively correlated with RNase5 levels in blood serum in humans [50] and further supports a role for IGF1 in regulating ribonucleases. Because dominant follicles have greater free IGF1 than subordinate follicles [22, 51] and greater LH receptors in GC [52, 53] but less *BRB* mRNA in GC, the hormonal regulation of decreased *BRB* mRNA in E<sub>2</sub>-active dominant follicles will require further elucidation.

In the present study, TNF $\alpha$  decreased IGF1-induced *BRB* mRNA abundance in small- and large-follicle GC implicating the immune system in regulating ovarian ribonucleases. Similarly, Gansler et al. [18] found that TNF $\alpha$  decreases RNase1 production by human umbilical vein endothelial cells. In contrast, Etoh et al. [54] found that TNF $\alpha$  induced *RNase5* mRNA expression in human colon cancer cells. Perhaps differences in the specific RNase, species and/or tissue exist in their response to TNF $\alpha$ , and will require further elucidation. The present finding that the inhibitory effect of TNF $\alpha$  and stimulatory effect of IGF1 on *BRB* mRNA abundance was more pronounced in large- than small-follicle GC indicates that *BRB* gene expression is under developmental and opposing control by TNF $\alpha$  and IGF1. Developmental differences in the steroidogenic response to TNF $\alpha$  in bovine GC [19, 55] has also been reported. In another physiological context, TNF $\alpha$  increases within the corpus luteum during luteal regression [20] and increases during infections [56–58] in cattle. Thus, our studies support the hypothesis that increased TNF $\alpha$  either during normal luteal regression or during acute-phase responses (i.e., disease) may impact ovarian follicular function via altered follicular BRB production. Because there were developmental differences in the *BRB* mRNA response to TNF $\alpha$ , changes in this response during follicular growth should be explored in further detail in cattle and other species.

In the present study, ANG, cortisol, FGF9, IHH/SHH, and WNT3A had no effect on *BRB* mRNA abundance in GC, whereas T4 increased and PGE2 decreased *BRB* mRNA expression in GC, a novel finding. It should be cautioned that the present study evaluated only a single dose of these intraovarian factors and thus, further work will be required to ascertain whether lower or higher doses of these factors affect *BRB* expression in GC. The inhibitory effect of PGE2 on *BRB* mRNA was observed in small-follicle GC but not in large-follicle GC. In cattle, FFL levels of PGE2 increase dramatically after the LH surge/GnRH treatment [59, 60], LH induces PGE2 production by GC [61], and PGE2 amplifies insulin-induced oxytocin release by GC [37]. Perhaps increased PGE2 released by the ovulatory follicle acts as a paracrine factor inhibiting small-follicle BRB production. The novel finding that T4 increased *BRB* mRNA further links the thyroid gland to ovarian function in cattle. In bovine theca cells, T4 stimulates LH-induced steroidogenesis [23] and is thought to play a role in the regulation of angiogenesis in several tissues [24] including the ovary [62]. The present study further expands the role of PGE2 and T4 to that of regulating production of BRB within the follicle.

## Conclusions

In conclusion, *BRB* mRNA expression in GC is less in E<sub>2</sub>-active dominant follicles than E<sub>2</sub>-inactive subordinate follicles and is positively regulated by IGF1, T4 and LH and negatively regulated by PGE2 and TNF $\alpha$ . Because *BRB* mRNA is decreased in dominant follicles and is increased in cystic and E<sub>2</sub>-inactive follicles, BRB could be preventing differentiation and/or ovulation in bovine follicles. Based on studies with RNase1, a BRB homologue, indicating that BRB may stimulate E<sub>2</sub> production and proliferation of small-follicle GC, we hypothesize that inhibition of BRB may be more important for successful ovulation rather than for differentiation. Additional research is needed to further elucidate the physiological functions of BRB and its interactions with IGF1, LH, T4, PGE2 and TNF $\alpha$  during growth and atresia of ovarian follicles in cattle.

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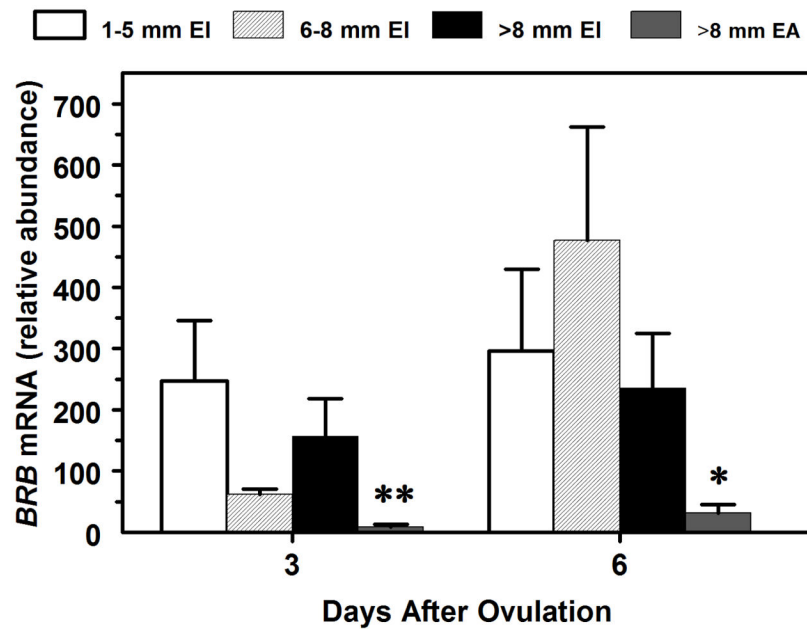
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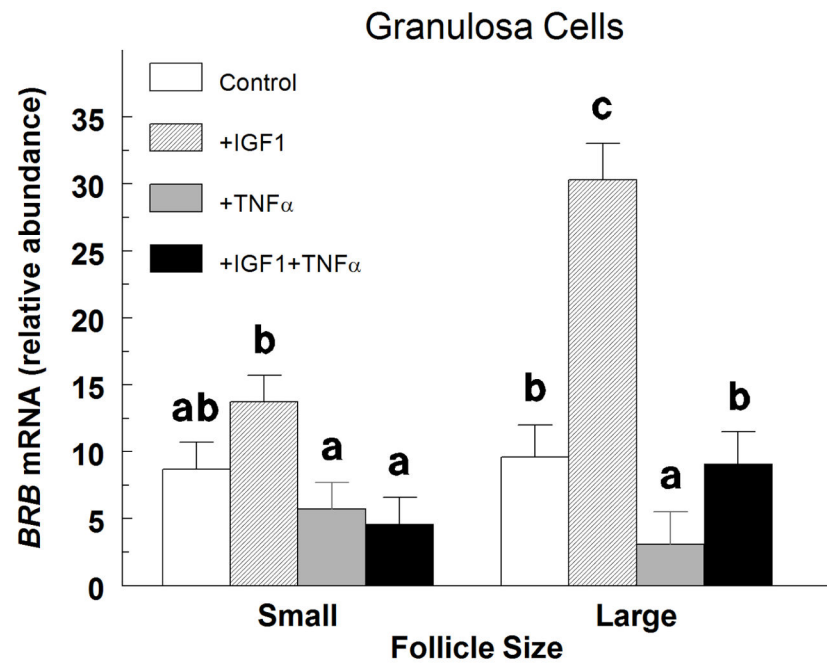


### Highlights

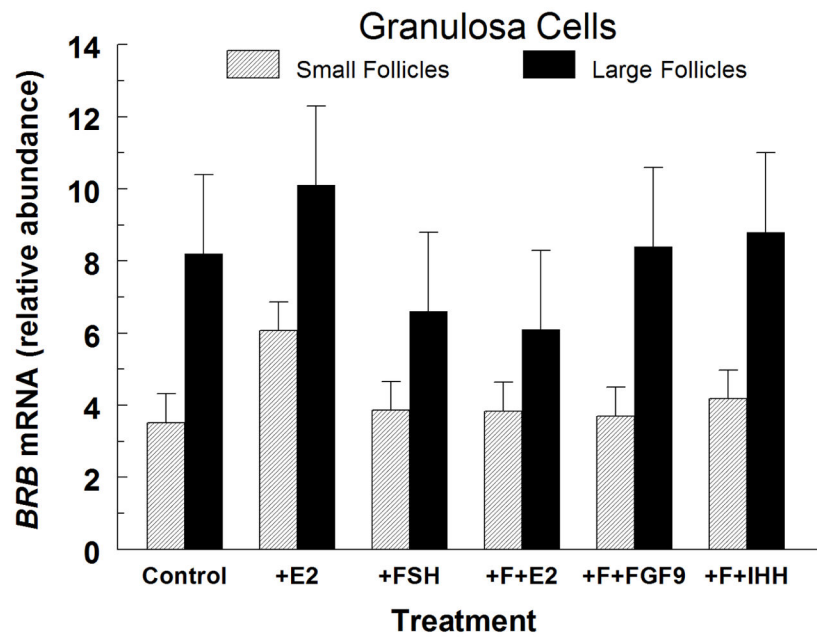
- Brain ribonuclease (BRB) mRNA is lower in dominant vs. subordinate follicles.
- We examine which hormones regulate BRB mRNA in granulosa cells.
- IGF1, LH and thyroxine increase whereas TNFalpha and PGE2 decrease BRB mRNA.
- RNase1, a BRB homologue, stimulates granulosa cell estradiol production.



**Fig. 1.** Effects of follicular size (Large are > 8 mm diameter; Medium are 5.1 to 8 mm (6–8 mm); Small are 1 to 5 mm in diameter) and E<sub>2</sub> status (EA = estrogen active; EI = estrogen inactive) (Panel A) and day post-ovulation (Panel B) on *BRB* mRNA by bovine GC. Values (n = 6 to 38 follicles per group) are normalized to constitutively expressed *18S* ribosomal RNA and are least squares means ± SEM. \*\*For day 3, mean differs ( $P < 0.01$ ) from all other means. \*For day 6, mean differs ( $P < 0.05$ ) from small- and medium-sized follicle means.

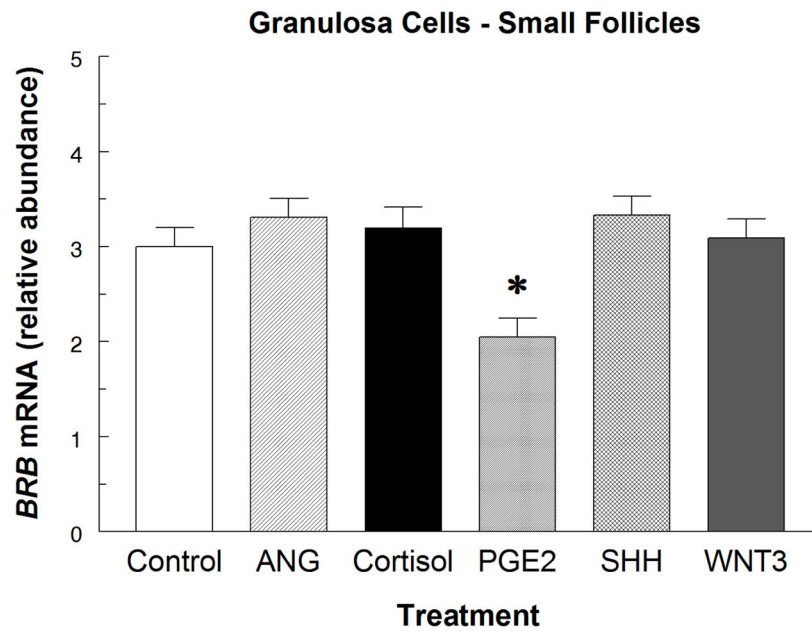


**Fig. 2.** Effect of insulin-like growth factor 1 (IGF1) and tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) on small- and large-follicle GC *BRB* mRNA abundance in Exp. 2. Cells were cultured for 48 h as described in Materials and Methods, and then treated for an additional 24 h with: Control (no additions), IGF1 (30 ng/mL), TNF $\alpha$  (30 ng/mL) or TNF $\alpha$  plus IGF1. Values are means  $\pm$  SEM of 3 separate experiments. <sup>abc</sup>Within follicle size group, means without a common letter differ ( $P < 0.05$ ).

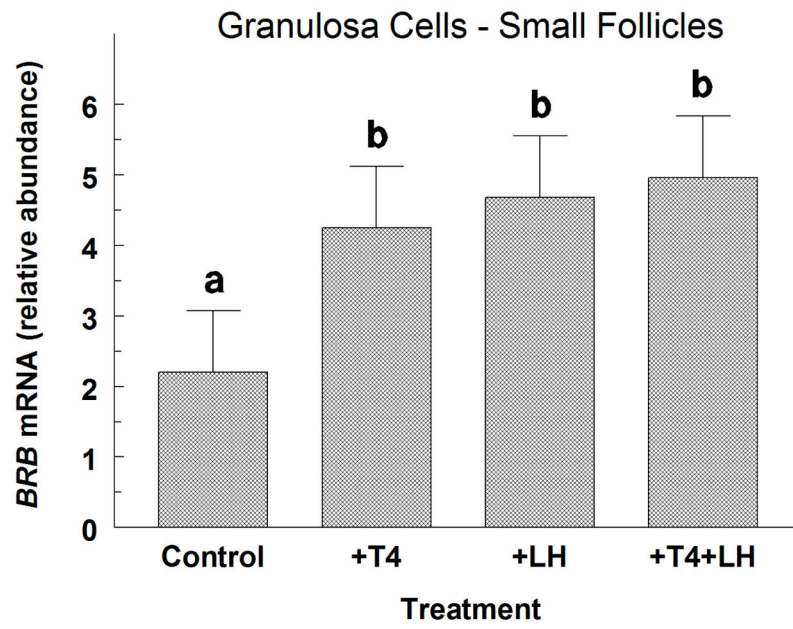


**Fig. 3.**

Effect of estradiol (300 ng/mL; E<sub>2</sub>), FSH (30 ng/mL), FSH plus E<sub>2</sub> (F+E<sub>2</sub>), FSH plus 30 ng/mL of FGF9 (F+FGF9), and FSH plus 1 μg/mL of IHH (F+IHH) on *BRB* mRNA abundance in IGF1-treated (Control) small-follicle GC of Exp. 3. Cells were cultured for 48 h as described in Materials and Methods, and then treated for 24 h with 100 ng/mL of IGF1 and the indicated hormones for 24 h. Values are means ± SEM of 3 separate experiments.

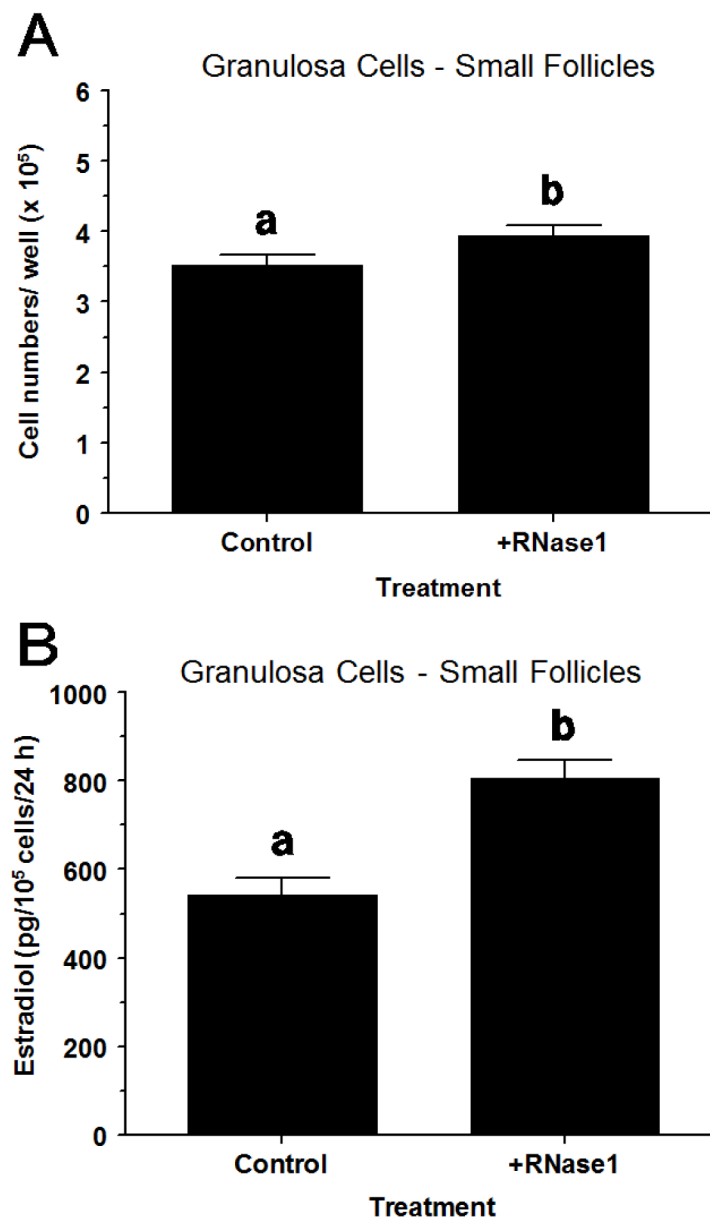


**Fig. 4.** Effect of angiogenin (300 ng/mL; ANG), cortisol (300 ng/mL), prostaglandin E2 (PGE2; 300 ng/mL), Sonic hedgehog (SHH; 500 ng/mL), and wntless-3A (WNT3; 300 ng/mL) on *BRB* mRNA abundance in IGF1-treated (Control) small-follicle GC of Exp. 4. Cells were cultured for 48 h as described in Materials and Methods, and then treated for 24 h with 30 ng/mL of IGF1 and the indicated hormones for 24 h. Values are means  $\pm$  SEM of 3 separate experiments. \*Asterisk indicates mean differs ( $P < 0.05$ ) from Control.



**Fig. 5.** Effect of thyroxine (T4; 100 ng/mL) and LH (30 ng/mL) on *BRB* mRNA abundance in FSH plus IGF1-treated (Control) small-follicle GC of Exp. 6. Cells were cultured for 48 h as described in Materials and Methods, and then treated for 48 h with 30 ng/mL of FSH and IGF1 and either T4 or LH. Values are means  $\pm$  SEM of 3 separate experiments. <sup>ab</sup>Means without a common letter differ ( $P < 0.05$ ).





**Fig. 6.** Effect of recombinant human RNase1 on proliferation and estradiol production in FSH plus IGF1-treated small-follicle GC of Exp. 7. Cells were cultured for 48 h as described in Materials and Methods, and then treated for 40 h with RNase1 (0 or 300 ng/mL), FSH (30 ng/mL) and IGF1 (30 ng/mL). Values are means  $\pm$  SEM of 3 separate experiments. <sup>ab</sup>Within a panel, means without a common letter differ ( $P < 0.05$ ).