Reproductive Biology 24 (2024) 100915

Contents lists available at ScienceDirect

Reproductive Biology

journal homepage: www.journals.elsevier.com/reproductive-biology

Original article

Characterization of bovine long non-coding RNAs, *OOSNCR1, OOSNCR2* and *OOSNCR3,* and their roles in oocyte maturation and early embryonic development

Jaelyn Z. Current, Heather L. Chaney, Mingxiang Zhang, Emily M. Dugan, Gianna L. Chimino, Jianbo Yao *

Laboratory of Animal Biotechnology and Genomics, Division of Animal and Nutritional Sciences, West Virginia University, Morgantown, WV 26506, United States

A R T I C L E I N F O

Keywords:
Long non-coding RNA
Oocyte maturation
Early embryonic development
SiRNA-mediated knockdown

A B S T R A C T

In mammals, early embryogenesis relies heavily on the regulation of maternal transcripts including protein-coding and non-coding RNAs stored in oocytes. In this study, the expression of three bovine oocyte expressed long non-coding RNAs (lncRNAs), *OOSNCR1, OOSNCR2,* and *OOSNCR3,* was characterized in somatic tissues, the ovarian follicle, and throughout early embryonic development. Moreover, the functional requirement of each transcript during oocyte maturation and early embryonic development was investigated using a siRNA-mediated knockdown approach. Tissue distribution analysis revealed that *OOSNCR1, OOSNCR2* and *OOSNCR3* are predominantly expressed in fetal ovaries. Follicular cell expression analysis revealed that these lncRNAs are highly expressed in the oocytes, with minor expression detected in the cumulus cells (CCs) and mural granulosa cells (mGCs). The expression for all three genes was highest during oocyte maturation, decreased at fertilization, and ceased altogether by the 16-cell stage. Knockdown of *OOSNCR1*, *OOSNCR2* and *OOSNCR3* in immature oocytes was achieved by microinjection of the cumulus-enclosed germinal vesicle (GV) oocytes with siRNAs targeting these lncRNAs. Knockdown of *OOSNCR1*, *OOSNCR2* and *OOSNCR3* did not affect cumulus expansion, but oocyte survival at 12 h post-insemination was significantly reduced. In addition, knockdown of *OOSNCR1*, *OOSNCR2* and *OOSNCR3* in immature oocytes resulted in a decreased rate of blastocyst development, and reduced expression of genes associated with oocyte competency such as nucleoplasmin 2 (*NPM2*), growth differentiation factor 9 (*GDF9*), bone morphogenetic protein 15 (*BMP15*), and *JY-1* in MII oocytes. The data herein suggest a functional requirement of *OOSNCR1, OOSNCR2,* and *OOSNCR3* during bovine oocyte maturation and early embryogenesis.

1. Introduction

Over the past decade, advancements in technology and the advent of deep sequencing have provided genome-wide transcriptional studies indicating a large portion of the genome is transcribed, much more than the 1–3 % that encodes proteins [10]. In humans, only 1.2 % of the genome represents protein-coding exons, whereas 24 % and 75 % are attributed to intronic and intergenic non-coding RNAs (ncRNAs) [29]. Further, ncRNAs are characterized by their specific expression patterns during critical developmental stages of certain tissues, and they play multiple roles in gene expression regulation [29]. What once was thought of as “genetic noise” from leaky transcriptional machinery has more recently come to the foreground of modern research in molecular biology due to its broad versatility in regulating gene expression.

Long non-coding RNAs (lncRNAs) are a class of RNAs that are greater than 200 nucleotides in length, usually 5’ capped, 3’ polyadenylated, spliced similarly to mRNAs, and to date have not been found to encode a protein [11]. With interest growing in the non-coding transcriptome, lncRNAs have been reported to play critical roles in various biological processes, including chromatin modification control of transcription [1,17,19,22,34,39], and regulation of gene expression at post-transcriptional [12,35] and post-translational levels [21]. With RNA sequencing technology more readily available, many studies have published large data sets identifying lncRNAs present in various reproductive tissues, including the ovary [27], oviduct [33], pregnant and nonpregnant uteri [38], and reproductive tissues inflicted with various

* Corresponding author.
E-mail address: jianbo.yao@mail.wvu.edu (J. Yao).

https://doi.org/10.1016/j.repbio.2024.100915
Received 31 October 2023; Received in revised form 11 June 2024; Accepted 16 June 2024
Available online 26 June 2024

Table 1
List of RT-qPCR primers and siRNA sequences used in the study.

RT-qPCR primers			
Gene	GenBank Acc #	Direction	Sequence (5'−3')
RPL19	NM_001040516.1	Forward	GAAATCGCCAATGCCAACTC
		Reverse	GAGCCTTGTCTGCCTTCA
GFP	MH777595	Forward	CAACAGCCACAACGTCTATATCATG
		Reverse	ATGTTGTGGCGGATCTTGAAG
OOSNCR1	PP158206	Forward	CCAACAGCTCATCTGTCAATT
		Reverse	GTTTCCTTGTGGCCATCTTTG
OOSNCR2	PP158207	Forward	GCAGAGAGAATCAGGCAGATG
		Reverse	GTATGATCTCGGAGTTCCAAC
OOSNCR3	PP158208	Forward	CTCTCATTCCAAACAGCATCC
		Reverse	CACACGGGCTTCAGTAGTTGC
USF1	NM_001001161.2	Forward	TGGCCGGACTTAGCACTCA
		Reverse	TCCGAGGAACTGGTCCTTCTT
JY-1	NM_001110098.1	Forward	TTGGAACTTCCATGGACGACC
		Reverse	ATTTGCTGGTGATCCCAAGAG
BMP15	NM_001031752.1	Forward	CCAAGAGGTAGTGAGGTTCGT
		Reverse	AGGGAGAGGTTTGGTCTTCTG
NPM2	NM_001168706.1	Forward	CGTTCTGGGGTTGGAGTTTTC
		Reverse	CGTCATCCTCCTCTTCCACTT
GDF9	NM_174681.2	Forward	GTAAAACCGTGAGTGTCCTGG
		Reverse	GCTCCTCCTTACACAACACAC
siRNA sequences			
siRNA	Position	Direction	Sequence (5'−3')
OOSNCR1_#1	701-726	Sense	AUACAUGAAACAGUUGGCUACUUGT
		Antisense	ACAAGUAGCCAACUGUUUCAUGUAUCU
OOSNCR1_#2	1027-1052	Sense	AAUCAGAGAAUUGUGCAAGAACUGA
		Antisense	UCAGUUCUUGCACAAUUCUCUGAUUGG
OOSNCR2_#1	80-105	Sense	AAAGACAGUCAUGAGCCAAAGGUTT
		Antisense	AAACCUUUGGCUCAUGACUGUCUUUGU
OOSNCR2_#2	186-211	Sense	GCAGUAUUAAGAUCUUGAUGACACC
		Antisense	GGUGUCAUCAAGAUCUUAAUACUGCUU
OOSNCR3_#1	82-107	Sense	GCUCUGUCCUCUCAUUCCAAACAGC
		Antisense	GCUGUUUGGAAUGAGAGGACAGAGCUG
OOSNCR3_#2	114-139	Sense	AAGCUUCCCUGAACUAUGAUUCACT
		Antisense	AGUGAAUCAUAGUUCAGGGAAGCUUCC

gynecological cancers [42].

Multiple studies have identified lncRNAs in ovarian follicular cells, including mural granulosa cells (mGCs) [16,24,40], cumulus cells (CCs) [5,41] and oocytes [37]. According to pathway analyses and bioinformatic predictions, many of these studies predicted lncRNAs to be associated with various cellular processes, including follicular development, oocyte maturation, cell cycle regulation, ovulation, estrogen production, cell proliferation, and apoptosis. Despite the minimal number of functional studies during oocyte maturation [18], lncRNAs have been identified and functionally characterized as critical regulators of zygotic genome activation (ZGA) in humans [2], mice [15], pigs [44], goats [9] and rabbits [31].

In mammals, the zygotes are transcriptionally silent, with all initial developmental events controlled by stored maternal RNAs and proteins encoded by genes known as maternal-effect genes [43]. Maternal-effect genes are transcribed during oogenesis and enable the activation of the embryonic genome. These oocyte-specific transcripts produce products that are expressed, stored, and secreted throughout oogenesis that regulates the follicular microenvironment to promote oocyte competence and successful fertilization and drive early embryonic development [8]. To date, bovine oocyte-specific lncRNAs and their corresponding mechanisms required for and utilized by the oocyte and early embryo are poorly understood, and our understanding of the contribution of such transcripts to oocyte maturation and early embryogenesis in cattle is limited. Thus, the objectives of this study were to characterize the expression of three novel oocyte-expressed lncRNAs, *OOSNCR1, OOSNCR2* and *OOSNCR3,* and elucidate their functional roles during oocyte maturation and early embryonic development in cattle.

2. Materials and methods

2.1. Bovine sample procurement

Bovine tissue samples, including liver, kidney, lung, thymus, spleen, adrenal, cortex, rumen, jejunum, vagina, caruncle, skeletal muscle, cardiac muscle, smooth muscle, fetal and adult ovaries, were collected at a local abattoir (Enterprise, WV) and a commercial facility (Souderton, PA). All tissue samples were excised in 1x1x1mm cubes from freshly slaughtered animals (n = 4), immediately snap-frozen, and then stored in liquid nitrogen. When a fetus was present in slaughtered female animals, sex was determined, and fetal age was predicted using the crown-rump length [30]. Whole fetal ovaries were immediately snap-frozen upon collection. All samples were stored at − 80 °C until further analysis.

2.2. Follicular cell collection and in vitro embryo production

Adult ovaries were harvested and stored at room temperature in 0.9 % NaCl solution until aspiration. Theca (TC) and mGCs were isolated from visible follicles using a previously established method (Murdoch et al., 1981). The cumulus-oocyte complexes (COCs) were aspirated from 2–12 mm follicles using an 18-gauge needle. After three washes of Boviplus oocyte washing medium (Minitube International, Tiefenbach, Germany) with BSA, heparin, and pen-strep per manufacturer's instructions, visibly healthy COCs were selected for IVM. Healthy COCs were defined as COCs with at least two consecutive layers of symmetrical CCs and a uniform cytoplasm. COCs were either collected at the germinal vesicle (GV) stage or pooled into groups of 50 and cultured in 500 μL of bovine IVM media (IVF Bioscience, Falmouth, United Kingdom) under embryo grade mineral oil for 22 h at 38.8 °C in 5 % CO_2 in humidified air.

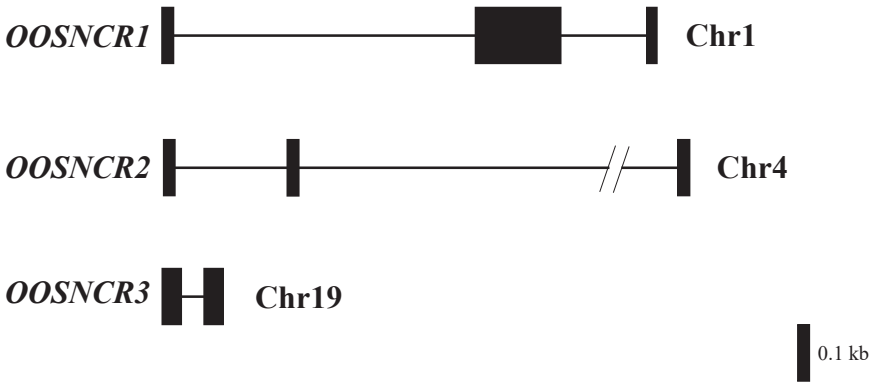

Fig. 1. Schematic diagrams of *OOSNCR1, OOSNCR2* and *OOSNCR3* genes. *OOSNCR1* is 1935 bps in length and contains 3 exons and 2 introns spanning about 10 kb on bovine chromosome 1. *OOSNCR2* is 292 bp in length and contains 3 exons and 2 introns spanning approximately 67 kb on chromosome 4. *OOSNCR3* is 384 bp and the gene is located on chromosome 19 containing 2 exons and 1 intron.

For GV oocyte and CC samples, CCs were removed using 0.1 % hyaluronidase solution (Sigma-Aldrich, St. Louis, MO) and vortexed for 5 min. Denuded GV oocytes were collected in pools of 20 with minimal volume and stored at − 80 °C. The remaining CCs were centrifuged into a pellet with the excess liquid removed, snap-frozen, and stored, as previously mentioned. Following IVM, the presence of metaphase II (MII) oocytes was confirmed based on the visual criteria of CC expansion and first polar body extrusion. Fully matured COCs were either collected, denuded, and stored in the same manner previously mentioned as MII oocytes and expanded CCs or selected to generate embryos.

For in vitro production of embryos, the COCs were washed 3 consecutive times each in 50 μL drops of bovine IVF medium (IVF Bioscience) and transferred to 4-well plates containing the same medium. Donated optixcell-extended bovine semen (Select Sires, Plain City, Ohio) was washed twice with bovine semen preparation medium (IVF Bioscience) per manufacturer's instructions. COCs in pools of 50 were co-cultured with sperm at a concentration of 2.0×10^6 sperm/mL for 12 h at 38.8 °C in 5 % CO_2 in humidified air. The presumptive zygotes were denuded and moved into 500 uL of bovine IVC (IVF Bioscience) with an oil overlay and incubated at 38.8 °C in 5 % CO_2 and 5 % O_2 until day 8 post-insemination. Embryos at two-cell, four-cell, eight-cell, and 16-cell stage were collected at 33-, 44-, 52-, and 72-hours post-insemination (hpi), and morula and blastocysts were collected at 5- and 8-days post-insemination.

2.3. Quantification of gene expression using quantitative real-time PCR (RT-qPCR)

Total RNA was isolated from tissues using TRIzol reagent (Ambion, Austin, TX) according to the manufacturer's instructions following homogenization with a MiniBeadBeater-16 (BioSpec Products, Bartlesville, OK). The RNA was treated with Turbo DNase I (Ambion), and RNA concentration and quality were measured using a Nanodrop Spectrophotometer, examining the absorbances at 260 nm and 280 nm. Total RNA from oocytes and embryos was isolated using the RNAqueous™ Micro Kit (Invitrogen, Waltham, MA) after each sample was spiked with 250 fg of green fluorescent protein (GFP) synthetic RNA (polyadenylated). cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems; Waltham, MA) per the manufacturer's instructions. Gene expression was quantified using Power-Up SYBR Green Master Mix (Applied Biosystems) and a CFX96 Real-Time System machine (Bio-Rad Laboratories, Hercules, CA). The RT-qPCR conditions were as follows: denaturing at 95 °C for 3 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. After the PCR cycling, a melting curve analysis was performed by a gradual temperature increase from 60 °C to 95 °C in increments of 0.5 °C for 10 s to confirm the specificity of the amplicon. A single sharp peak on a melting curve was observed for all genes analyzed confirming the specificity of the PCR reactions (Fig. S1). Standard curves for each target gene and the endogenous control gene were constructed using 10-fold serial dilutions of a bovine fetal ovary cDNA. Relative lncRNA or mRNA abundance was calculated via the standard curve method using *RPL19* as an endogenous control or *GFP* as an exogenous control (for oocytes and embryos) for normalization. The RT-qPCR primers were designed manually so that they span the adjacent exons and amplify products of less than 150 bp (Table 1).

2.4. siRNA-mediated knockdown of lncRNAs at the germinal vesicle stage

Microinjection of *OOSNCR1, OOSNCR2,* and *OOSNCR3* siRNA into GV oocytes was conducted using previously described methodologies with modifications [7]. Briefly, cumulus-enclosed GV-stage oocytes were microinjected with a cocktail of two siRNA species for each lncRNA. Two siRNA species (siRNA-1, siRNA-2) for each lncRNA were designed using the custom dicer-substrate siRNA (DsiRNA) design tool (Integrated DNA Technologies, Coralville, IA) targeting *OOSNCR1* at positions 701–726 and 1027–1052, *OOSNCR2* at positions 80–105 and 186–211, and *OOSNCR3* at positions 82–107 and 114–139, respectively (Table 1). Immature COCs were collected and randomly assigned to receive either each siRNA cocktail (50 μM), the negative control siRNA (50 μM, universal control species 1; Ambion Inc.) or remain as non-injected controls. All COCs injections were performed in M2 medium (Medium 199 containing HEPES supplemented with 2 % FBS) with approximately 15 pL of siRNA injected into each COC. To validate lncRNA knockdown, denuded MII oocytes injected with the siRNA cocktail for *OOSNCR1*, *OOSNCR2*, or *OOSNCR3* were collected in pools of 5 (n = 7), and expression of each lncRNA was quantified via RT-qPCR. Using ImageJ software, the effects of treatment on cumulus expansion were determined by tracing the area of individual COCs with each replicate (n = 5) having at least 20 COCs, and the percent survival of injected COCs reaching 12 hpi was determined via visual inspection (n = 3; pools of at least 20 COCs). The relative expression of select genes linked to bovine oocyte competency (*NPM2, GDF9, BMP15 JY-1,* and *USF-1)* in MII oocytes following siRNA-mediated knockdown of the lncRNAs at the GV stage was determined via RT-qPCR. Moreover, siRNA-injected COCs or uninjected controls were subjected to IVF, and the percent development reaching the blastocysts stage was determined on day 8 (n = 3).

2.5. Statistical analysis

All analyses were conducted in JMP Pro version 15.1.0 (JMP 1998–2023). All RT-qPCR data were log-transformed, and embryo data

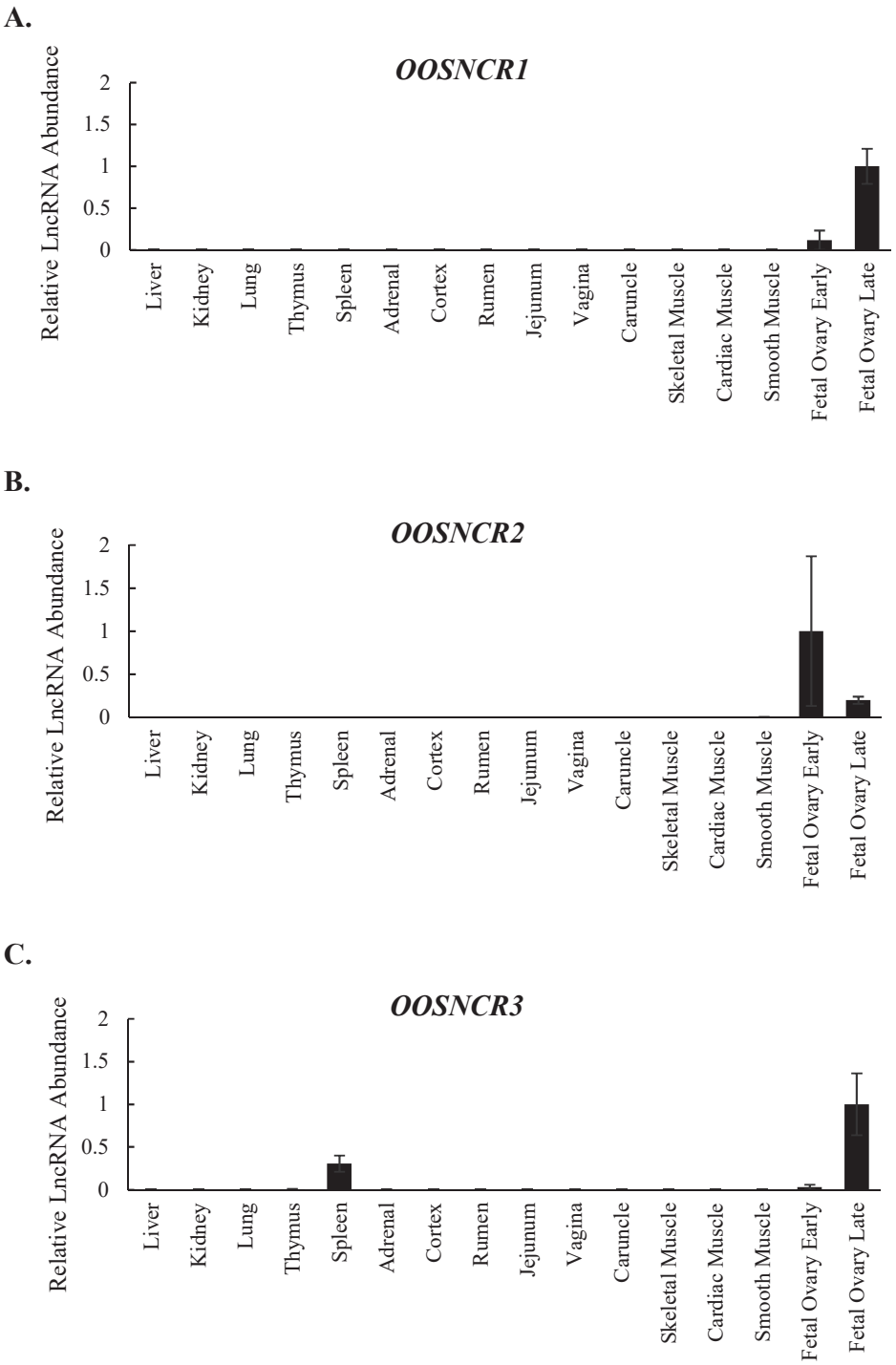

Fig. 2. Tissue expression profiles of *OOSNCR1, OOSNCR2* and *OOSNCR3* transcripts. Relative abundance of *OOSNCR1* (A)*, OOSNCR2* (B) and *OOSNCR3* transcripts (C) in bovine tissues (n = 4) including liver, kidney, lung, thymus, spleen, adrenal, cortex, rumen, jejunum, vagina, caruncle, skeletal muscle, cardiac muscle, smooth muscle, and fetal ovaries was determined by RT-qPCR analysis. *RPL19* was used as an endogenous control for data normalization. *OOSNCR1* and *OOSNCR2* were detected exclusively in early fetal ovaries (gestation day 90–105) and late fetal ovaries (gestation day 160–280). In contrast, *OOSNCR3* was detected in the fetal ovaries as well as in the spleen.

presented as percentages were arcsine transformed. Differences in gene expression and embryonic development were determined using a Student's *t*-test or One-way ANOVA. Following ANOVA, individual mean comparisons were performed using either Tukey's HSD or Dunnett's test. For all experiments, differences were considered statistically significant when $P < 0.05$ with a tendency range of $0.1 \geq P > 0.05$.

3. Results

3.1. Structural characterization of OOSNCR1, OOSNCR2 and OOSNCR3 genes

In a previous study, we identified lncRNAs that are specifically or predominantly expressed in bovine oocytes [37]. Three highly abundant

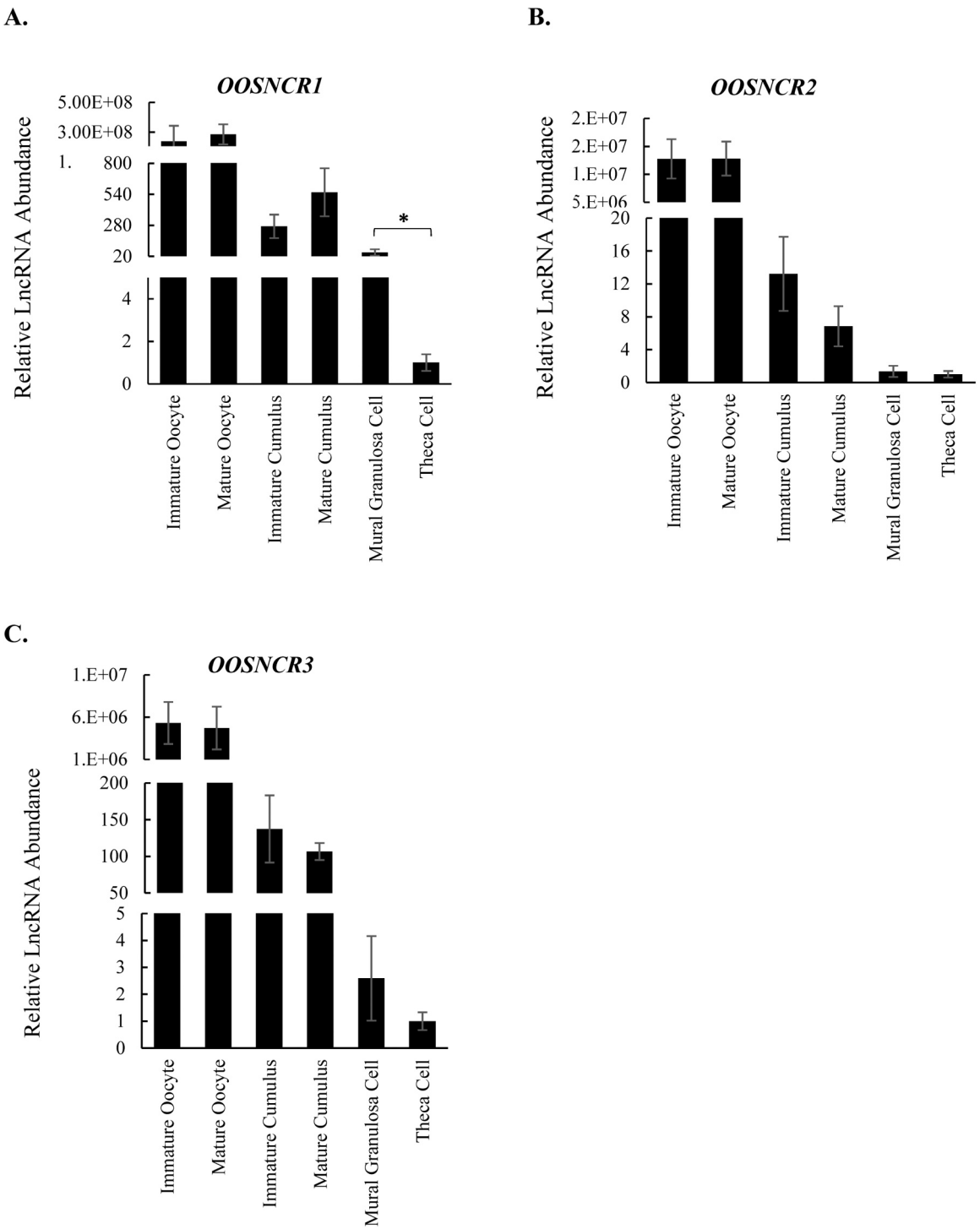

Fig. 3. Expression of *OOSNCR1, OOSNCR2* and *OOSNCR3* transcripts in follicular cells. Relative abundance of *OOSNCR1* (A), *OOSNCR2* (B) and *OOSNCR3* (C) in bovine follicular cells was determined by RT-qPCR analysis, and the differences in expression of the lncRNAs between cell types were determined by a Student's t-test. Theca and mural granulosa cells were isolated from individual follicles (n = 7), whereas cumulus cells were denuded from immature and mature oocytes in pools of 20 (n = 7). *RPL19* was used as an endogenous control for data normalization. *OOSNCR1, OOSNCR2,* and *OOSNCR3* were all detected at the highest level in the oocytes regardless of maturation status ($P = 0.32$). All three lncRNAs were more abundant in the CCs than the mGCs and TCs.

lncRNAs (based on FPKM values) were selected for this study. They were previously named CUFF.2160.1, CUFF.34876.2 and CUFF.18208.1, and renamed here as *OOSNCR1* (Oocyte-Specific Non-Coding RNA), *OOSNCR2* and *OOSNCR3*, respectively. They have been deposited in the GenBank database under the accession numbers PP158206, PP158207 and PP158208, respectively. *OOSNCR1* is 1935 bp long, much longer than the average length of oocyte lncRNAs (782 bp). In contrast, *OOSNCR2* and *OOSNCR3* are short, 292 bp and 384 bp, respectively. Consistent with being a non-coding RNA, *OOSNCR1* does not contain open reading frames (ORFs) larger than 250 bp, and neither *OOSNCR2* nor *OOSNCR3* contains any significant ORFs. BLAST search revealed that all three lncRNAs do not match any lncRNAs in the NONCODE database (v5.0) and are intergenic. The schematic gene structure of these lncRNAs are shown in Fig. 1. The *OOSNCR1* gene contains three

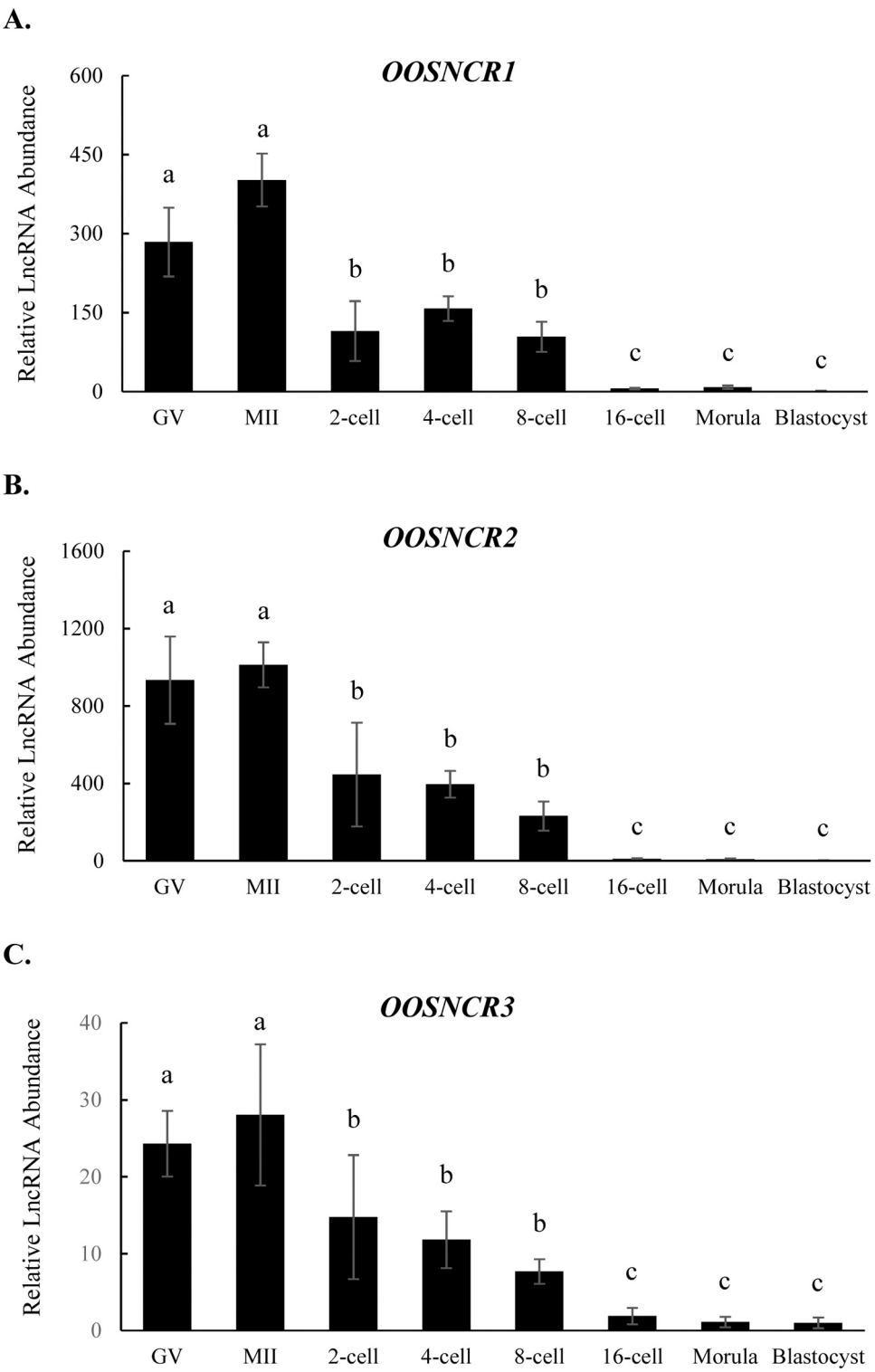

Fig. 4. Expression of *OOSNCR1, OOSNCR2* and *OOSNCR3* throughout oocyte maturation and early embryonic development. Relative abundance of *OOSNCR1* (A), *OOSNCR2* (B) and *OOSNCR3* (C) transcripts in pools of 20 denuded oocytes (GV and MII) and early embryos at the 2-cell, 4-cell, 8-cell, 16-cell, morula, and blastocysts stage was examined using RT-qPCR (n = 4 pools of oocytes or embryos). Data were normalized relative to abundance of GFP as an exogenous control. All three lncRNAs exhibited similar patterns of expression, being highest in immature and mature oocytes relative to the blastocysts ($P < 0.001$). Expression of all three lncRNAs decreased following fertilization and remained steady until the 8-cell stage ($P = 0.99$). Following the 8-cell stage, all three lncRNAs ceased expression by the blastocyst stage ($P < 0.05$).

exons and two introns spanning about 10 kb on bovine chromosome 1 (chr1:153866552–153875412). *OOSNCR2* is about 67 kb in length on chromosome 4 (chr4:40898664–40966028) and contains three exons and two introns, with intron 2 being about 64 kb. Further, *OOSNCR3* gene is about 550 bp in length on chromosome 19 (chr19:26557762–26558311) and contains two exons and one intron.

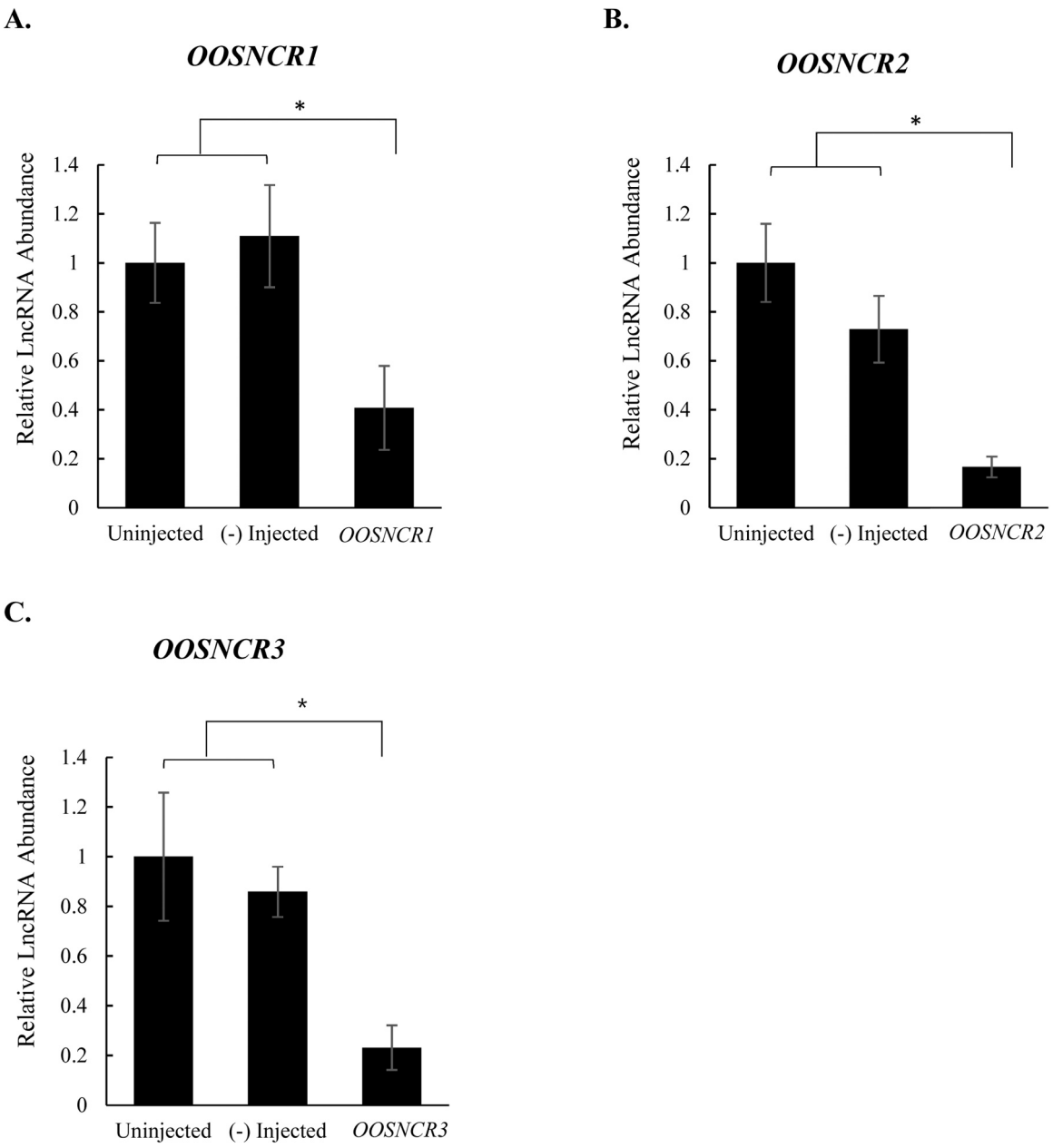

Fig. 5. Validation of siRNA-mediated knockdown of *OOSNCR1, OOSNCR2* and *OOSNCR3* in mature oocytes. Relative abundance of *OOSNCR1* (A), *OOSNCR2* (B) and *OOSNCR3* (C) in pools of 5 denuded mature oocytes (n = 7) was analyzed by RT-qPCR to validate gene knockdown. COCs were injected with a siRNA universal negative control, a cocktail of 2 siRNA species targeting each lncRNA, or remained as uninjected controls. LncRNA knockdown during oocyte maturation was achieved for all three lncRNAs ($P < 0.01$).

3.2. Expression of OOSNCR1, OOSNCR2 and OOSNCR3 in tissues and follicular cells

To determine the tissue distributions of *OOSNCR1, OOSNCR2,* and *OOSNCR3*, a panel of tissues including liver, kidney, lung, thymus, spleen, adrenal, cortex, rumen, jejunum, vagina, caruncle, skeletal muscle, cardiac muscle, smooth muscle, and fetal ovaries was examined via RT-qPCR. As shown in Fig. 2, expression of *OOSNCR1* and *OOSNCR2* was detected exclusively in the fetal ovaries (Fig. 2A, B). In contrast, *OOSNCR3* expression was detected in both the spleen and fetal ovaries (Fig. 2C).

To examine the expression of *OOSNCR1, OOSNCR2,* and *OOSNCR3* in the ovary, follicular cells were isolated and analyzed using RT-qPCR. The results showed that the expression of *OOSNCR1, OOSNCR2,* and *OOSNCR3* was extremely high in oocytes relative to the follicular cells including CCs, mGCs and theca cells (Fig. 3). No significant difference in expression of these lncRNAs was observed between mature and immature oocytes. The maturation process also did not affect the expression of these lncRNAs in CCs, nor was there a difference detected in the expression of *OOSNCR2* and *OOSNCR3* in mGCs and theca cells. *OOSNCR1* expression was significantly higher in mGC when compared to theca cells ($P < 0.05$).

3.3. Expression of OOSNCR1, OOSNCR2 and OOSNCR3 during oocyte maturation and early embryonic development

The expression of three lncRNAs throughout oocyte maturation and early embryonic development was quantified using RT-qPCR. Pools of 20 denuded oocytes (GV and MII) and embryos at 2-cell, 4-cell, 8-cell, 16-cell stage, morula, and blastocysts were collected. All three

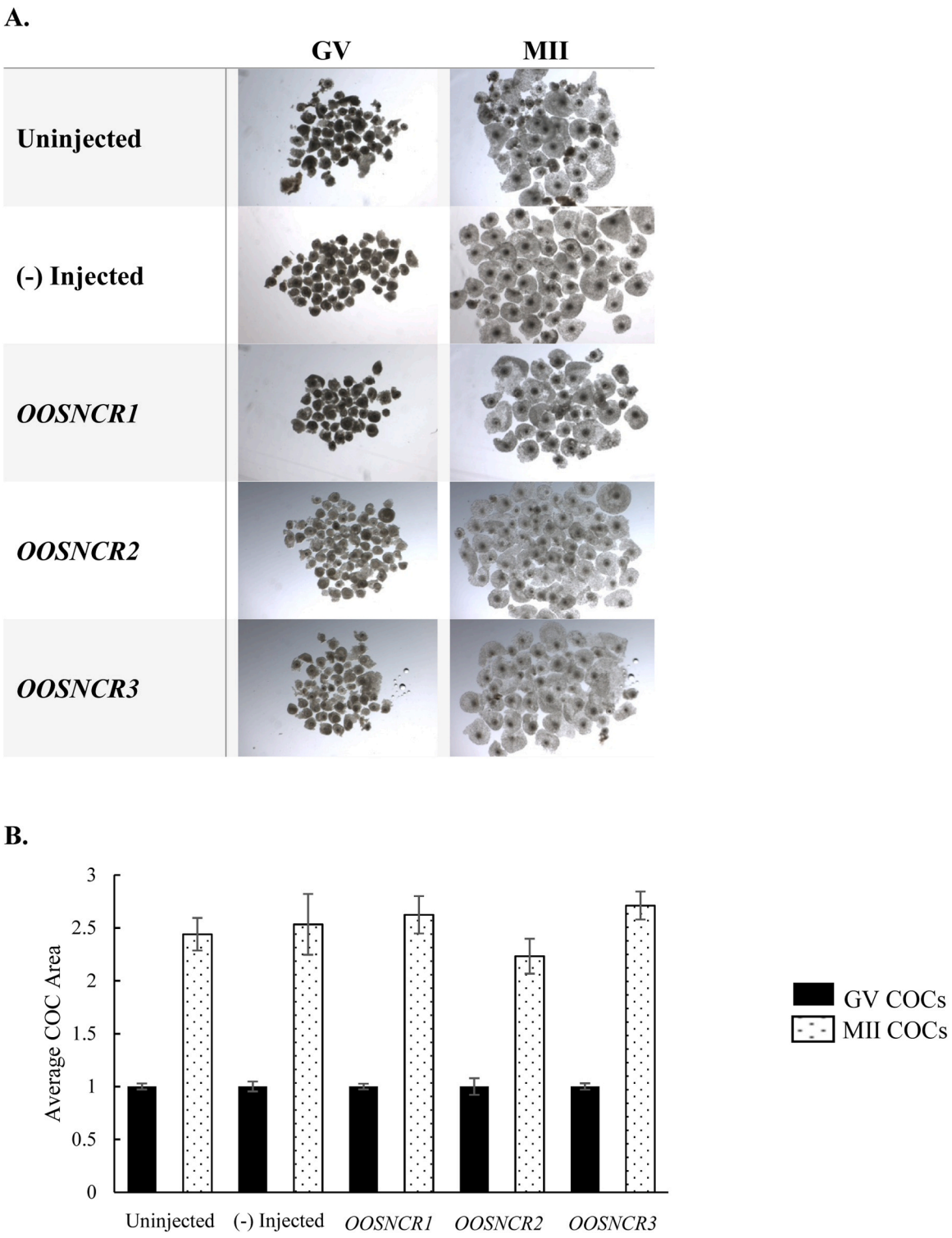

Fig. 6. Cumulus cell expansion during oocyte maturation following siRNA-mediated knockdown of *OOSNCR1, OOSNCR2* and *OOSNCR3.* (A) Representative image of COC expansion following treatment. (B) Effects of lncRNA knockdown on cumulus expansion for each lncRNA compared to the injected negative control and uninjected control. The area was measured for individual COCs, with each replicate (n = 5) having at least 20 COCs present. The average area was determined for each replicate. No difference in cumulus expansion following knockdown was detected in any treatment group ($P = 0.71$).

lncRNAs exhibited similar patterns of expression (Fig. 4). Expression of *OOSNCR1, OOSNCR2,* and *OOSNCR3* was highest in immature and mature oocytes compared to the blastocysts stage ($P < 0.001$). Following fertilization, expression of all three lncRNAs significantly decreased and remained steady until the 8-cell stage. Progressing past the 8-cell stage caused all three lncRNAs to cease expression by the blastocyst stage.

3.4. Effects of siRNA-mediated knockdown of lncRNAs at the germinal vesicle stage on oocyte maturation and subsequent embryonic development

The functional role of lncRNAs during oocyte maturation remains

Table 2
The percent survival of uninjected and siRNA injected COCs at 12 hpi.

Treatment	Alive at 12 hpi	Dead at 12 hpi	Survival at 12 hpi (%)	Average Survival (%) ± SEM
Uninjected	31	6	83.78	90.55 ± 4.87[a]
	29	4	87.88	
	17	0	100.00	
(-) Injected	8	2	80.00	59.49 ± 10.44[b]
	11	13	45.83	
	10	9	52.63	
OOSNCR1	10	30	25.00	21.42 ± 2.48[b]
	7	24	22.58	
	4	20	16.67	
OOSNCR2	10	16	38.46	35.68 ± 3.58[b]
	8	20	28.57	
	18	27	40.00	
OOSNCR3	9	7	56.25	48.33 ± 4.14[b]
	11	15	42.31	
	13	15	46.43	

[b] Different from the uninjected control; $P < 0.05$.

Table 3
Blastocyst rates following siRNA-mediated lncRNA knockdown in immature oocytes.

	Blastocyst rate (%) ± SEM
Uninjected	25 ± 4.1[a]
(-) Injected	21 ± 4.5[a]
OOSNCR1	3 ± 0.3[b]
OOSNCR2	2 ± 0.46[b]
OOSNCR3	6 ± 0.4[b]

[b] Different from the uninjected control; $P < 0.05$. Data are presented as mean ± SEM (n = 3).

unknown; therefore, we addressed the effects of *OOSNCR1, OOSNCR2,* and *OOSNCR3* knockdown on cumulus expansion, and the percent survival of COCs reaching 12 hpi. LncRNA knockdown was achieved for all three lncRNAs ($P < 0.01$; Fig. 5). Examination of CC expansion revealed no difference between uninjected and injected COCs ($P = 0.71$; Fig. 6). When examining COCs survival post-injection, the uninjected control COCs had a survival rate of 90.55 % ± 5.0 %, whereas injected COCs had a decreased survival rate ($P < 0.05$; Table 2). No difference was detected in the survival of injected COCs regardless of the siRNA cocktail.

To assess the effect of lncRNA knockdown during oocyte maturation on early embryonic development, uninjected and injected COCs were subjected to IVF. The percent development reaching the blastocysts stage was determined on day 8 (Table 3). Uninjected control and negative siRNA injected COCs resulted in a 25 % ± 4.1 % and 21 % ± 4.5 % blastocysts rate ($P = 0.796$), respectively. Knockdown of *OOSNCR1, OOSNCR2,* and *OOSNCR3* resulted in a decreased blastocyst rate of 3 % ± 0.3 %, 2 % ± 0.46 %, and 6 % ± 0.4 % ($P < 0.05$), respectively.

The relative abundance of select genes linked to bovine oocyte competency was examined in MII oocytes. Following the siRNA-mediated knockdown of all three lncRNAs at the GV stage, *NPM2* was downregulated ($P < 0.05$; Fig. 7A). Additionally, *GDF9* was downregulated following the knockdown of *OOSNCR1* and *OOSNCR3* ($P < 0.05$; Fig. 7B). Lastly, *OOSNCR1* knockdown caused a decrease of *NPM2, GDF9, BMP15,* and *JY-1* ($P < 0.05$; Fig. 7). *USF1* expression was not affected following the knockdown of any of these lncRNAs ($P = 0.645$; Fig. 7E).

4. Discussion

Results of the present study characterized the expression of three novel oocyte-expressed lncRNAs, *OOSNCR1*, *OOSNCR2* and *OOSNCR3,* in somatic tissues, the ovarian follicle, and throughout early embryonic development, and provided preliminary evidence suggesting a functional role of these lncRNAs during bovine oocyte maturation and early embryogenesis.

The three lncRNAs were selected from a group of lncRNAs predicted to be specific to bovine oocytes based on a previous study [37]. They were also selected based on high FPKM values indicating their abundance in bovine oocytes. Results of the present study confirmed that these lncRNAs are predominantly and abundantly expressed in immature and mature oocytes. Many protein-coding genes specifically or predominantly expressed in oocytes are known as maternal effect genes that are implicated in various aspects of early embryonic development [43]. Therefore, these lncRNAs may function as maternal effect genes playing important roles in maternal mRNA/protein degradation, epigenetic reprogramming, and initiation of embryonic genome activation.

The expression patterns of *OOSNCR1, OOSNCR2*, and *OOSNCR3* throughout oocyte maturation and early embryonic development were similar. They were expressed highest during oocyte maturation, followed by a significant decreased post-insemination where expression remained constant until expression ceased altogether by the 16-cell stage. In cattle, a low level of transcriptional activity (minor genome activation) occurs between the 1- and 4-cell stage embryo, with major genome activation happening at the 8-cell stage [20]. During genome activation, a shift from maternal to embryonic control is accompanied by widespread changes in chromatin structure and restructuring of the embryonic epigenome [14]. As lncRNAs are documented to regulate gene expression by modulating chromatin structure and function [32], future studies should aim to investigate changes in the chromatin landscape during EGA in response to lncRNA ablation.

Our results of siRNA-mediated gene knockdown of *OOSNCR1, OOSNCR2* and *OOSNCR3* in immature oocytes support the requirement of each of the lncRNAs for oocyte maturation. A recent study demonstrated that *Rose*, a mouse oocyte-specific lncRNA is important for successful oocyte development. Oocytes lacking *Rose* failed to extrude a polar body leading to abnormal MI, irregular polar body extrusion, and symmetrical division [18]. Further, oocytes lacking *Rose* that successfully extruded a polar body exhibited irregularities in their mitotic spindles and chromosome organization [18]. Our findings indicate that cumulus expansion was not affected by knockdown of these lncRNAs. However, COCs survival decreased following siRNA injection. Collectively, these data suggest an internal mechanism independent of CCs ultimately disrupting cytoplasmic maturation resulting in oocytes of lesser quality. Results of siRNA-mediated knockdown of the lncRNAs in GV oocytes also suggest a functional role of these lncRNAs in the development of embryos to the blastocyst stage. Interestingly, while our results showed that knockdown of the lncRNAs in immature oocytes resulted in a decreased rate of blastocyst development, a previous study by Caballero et al. demonstrated that knocking down of a bovine oocyte-expressed lncRNA in mature oocytes lead to an increased blastocyst development rate and larger blastocysts [4].

To evaluate the potential relationship between lncRNAs and oocyte competency, known genes linked to oocyte quality were quantified in the knockdown MII oocytes. Interestingly, *NPM2* was significantly decreased following the knockdown of each lncRNA. *NPM2* is an oocyte-specific nuclear protein essential for nuclear and nucleolar organization and early embryonic development. *NPM2* knockout female mice exhibited fertility defects, including impaired nucleolar organization and disrupted chromatin structure in oocytes and early embryos [3]. Further, *NPM2* has been identified as a histone chaperone in the oocyte and embryo [28], and the expression of bovine *NPM2* during early embryogenesis is potentially regulated by miR-181a [25]. Other oocyte-specific genes decreased following lncRNA knockdown included *GDF9*, *BMP15*, and *JY-1*. *GDF9* and *BMP15* genes are members of the TGFβ superfamily that encode proteins secreted by the oocytes into the surrounding follicular cells. The essential role of *GDF9* and *BMP15* genes in the ovarian follicle to mediate cell growth and differentiation of

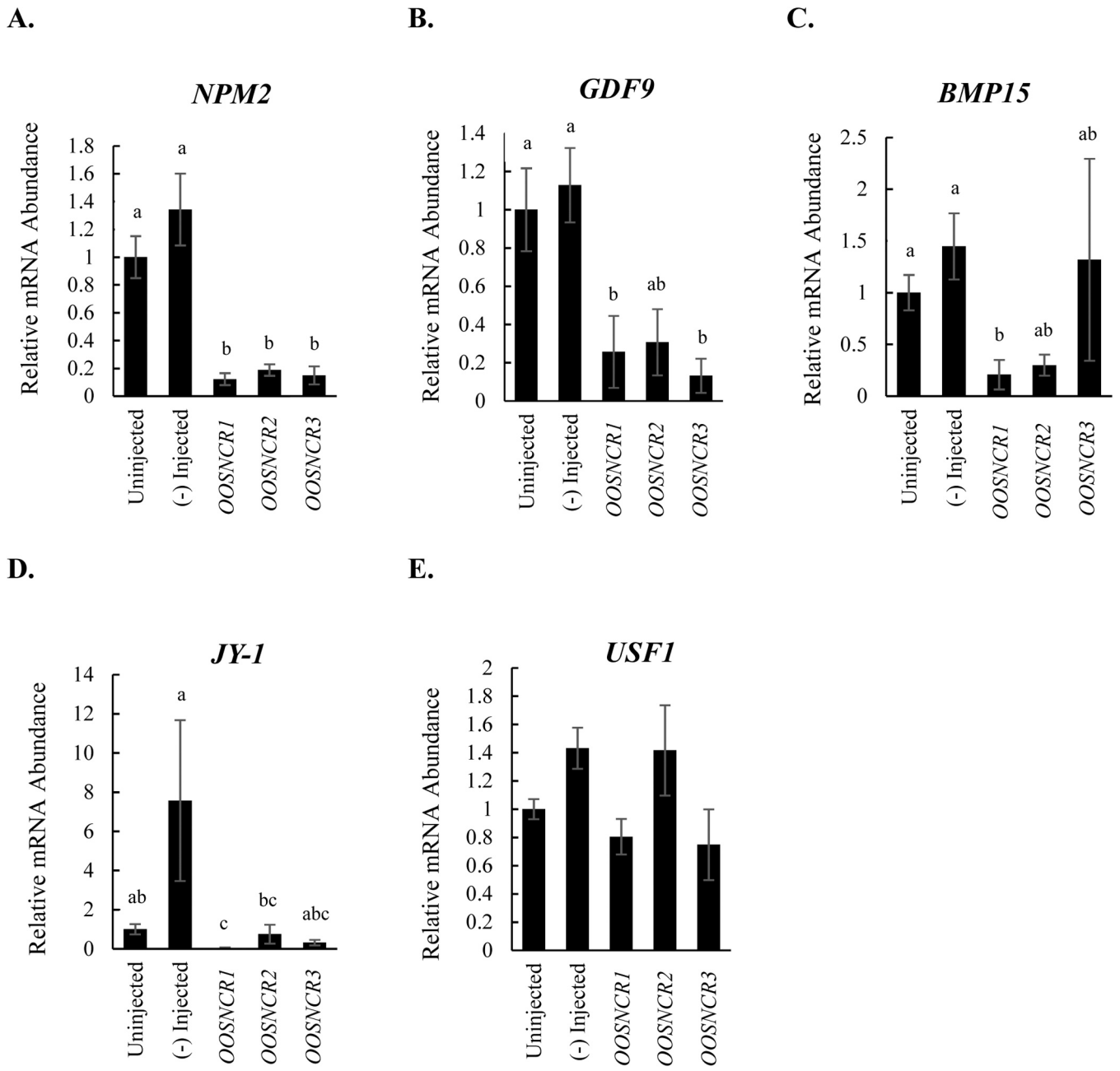

Fig. 7. Relative abundance of select genes linked to bovine oocyte competency following siRNA-mediated knockdown of *OOSNCR1, OOSNCR2* and *OOSNCR3*. Select genes including *NPM2, GDF9*, *BMP15, JY-1* and USF1were quantified using RT-qPCR in denuded MII oocytes (n = 7; pools of 5) following siRNA-mediated knockdown of *OOSNCR1, OOSNCR2,* and *OOSNCR3*. (A) *NPM2* expression was downregulated following the knockdown of all three lncRNAs ($P < 0.05$). (B) *GDF9* was downregulated following the knockdown of *OOSNCR1* and *OOSNCR3* ($P < 0.05$). (C) *BMP15* and (D) *JY-1* were downregulated following *OOSNCR1* knockdown ($P < 0.05$). (E) *USF1* expression was not affected by lncRNA knockdown ($P = 0.645$).

follicular cells has been well documented [26]. Expression of *GDF9* in oocytes during early folliculogenesis is regulated by an oocyte-specific transcription factor, NOBOX [6], and transcription of *BMP15* is likely controlled by LHX8, a transcription factor highly expressed in oocytes [36]. *JY-1* is an oocyte-specific maternal effect gene in cattle and knockdown of this gene in the cumulus-enclosed germinal vesicle oocytes resulted in impaired oocyte maturation, partial cumulus expansion, and a significant decrease in early embryos reaching the blastocyst stage [23]. The mechanisms of how these genes important for oocyte competence are regulated by these lncRNAs will require further investigation. Future studies on the subcellular location of these lncRNAs in oocyte would provide valuable information on how these lncRNAs function as the lncRNAs located in the nucleus are likely involved in epigenetic and transcriptional regulation of gene expression while the lncRNAs present in the cytoplasm are frequently involved in post-transcriptional regulation processes [13].

In summary, results of the present study demonstrated that *OOSNCR1, OOSNCR2*, and *OOSNCR3* are predominantly and abundantly expressed in bovine oocytes, and provided evidence suggesting that these lncRNAs are important for oocyte maturation and early embryonic development. The mechanisms of how these lncRNAs function during oocyte maturation and early embryonic development remain to be investigated.

Author contributions

JZC planned the study design, collected samples, performed experiments, analyzed data, interpreted data, and drafted the manuscript. HLC collected ovary samples and performed in vitro embryo production. MZ performed in vitro embryo production. EMD and GLC assisted with in vitro embryo production and gene expression data. JY aided in data analysis, interpretation, and manuscript preparation.

Declaration of Competing Interest

The authors declare no conflicts of interest.

Acknowledgments

This project was supported by the National Institute of Food and Agriculture, U.S. Department of Agriculture, award number 2020–38640-31520 through the Northeast Sustainable Agriculture Research and Education (SARE) program under sub-award number GNE19–196-33243, and the USDA National Institute of Food and Agriculture, Multistate Research Project 1014002. In addition, the authors would like to thank Dr. Ida Holaskova for her assistance in statistical analysis, Select Sires (Plain City, OH) for semen donations, and the West Virginia Department of Agriculture, Hyde's Meat Packing (Enterprise, WV), and JBS Beef Plant (Souderton, PA) for their generous donation of bovine ovaries for this work.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.repbio.2024.100915.

References

[1] Arab K, Karaulanov E, Musheev M, Trnka P, Schäfer A, Grummt I, Niehrs C. GADD45A binds R-loops and recruits TET1 to CpG island promoters. Nat Genet 2019;51:217–23. https://doi.org/10.1038/s41588-018-0306-6.
[2] Bouckenheimer J, Assou S, Riquier S, Hou C, Philippe N, Sansac C, Lavabre-Bertrand T, Commes T, Lemaître J-M, Boureux A, De Vos J. Long non-coding RNAs in human early embryonic development and their potential in ART. Hum Reprod Update 2016;23:19–40. https://doi.org/10.1093/humupd/dmw035.
[3] Burns KH, Viveiros MM, Ren Y, Wang P, DeMayo FJ, Frail DE, Eppig JJ, Matzuk MM. Roles of NPM2 in chromatin and nucleolar organization in oocytes and embryos. Science 2003;300:633–6. https://doi.org/10.1126/science.1081813.
[4] Caballero J, Gilbert I, Fournier E, Gagné D, Scantland S, Macaulay A, Robert C. Exploring the function of long non-coding RNA in the development of bovine early embryos. Reprod Fertil Dev 2014;27:40–52. https://doi.org/10.1071/RD14338.
[5] Caponnetto A, Battaglia R, Ferrara C, Vento ME, Borzì P, Paradiso M, Scollo P, Purrello M, Longobardi S, D'Hooghe T, Valerio D, Di Pietro C, Italian Society of Embryology, Reproduction, Research (SIERR). Down-regulation of long non-coding RNAs in reproductive aging and analysis of the lncRNA-miRNA-mRNA networks in human cumulus cells. J Assist Reprod Genet 2022;39:919–31. https://doi.org/10.1007/s10815-022-02446-8.
[6] Choi Y, Rajkovic A. Characterization of NOBOX DNA binding specificity and its regulation of Gdf9 and Pou5f1 promoters. J Biol Chem 2006;281(47):35747–56. https://doi.org/10.1074/jbc.M604008200.
[7] Datta TK, Rajput SK, Wee G, Lee K, Folger JK, Smith GW. Requirement of the transcription factor USF1 in bovine oocyte and early embryonic development. Reproduction 2015;149:203–12. https://doi.org/10.1530/REP-14-0445.
[8] De Sousa PA, Caveney A, Westhusin ME, Watson AJ. Temporal patterns of embryonic gene expression and their dependence on oogenetic factors. Theriogenology 1998;49:115–28. https://doi.org/10.1016/S0093-691X(97)00406-8.
[9] Deng M, Liu Z, Ren C, Zhang G, Pang J, Zhang Y, Wang F, Wan Y. Long noncoding RNAs exchange during zygotic genome activation in goat. Biol Reprod 2018;99:707–17. https://doi.org/10.1093/biolre/ioy118.
[10] Djebali S, Davis CA, Merkel A, Dobin A, Lassmann T, Mortazavi A, Tanzer A, Lagarde J, Lin W, Schlesinger F, Xue C, Marinov GK, Khatun J, Williams BA, Zaleski C, Rozowsky J, Röder M, Kokocinski F, Abdelhamid RF, Alioto T, Antoshechkin I, Baer MT, Bar NS, Batut P, Bell K, Bell I, Chakrabortty S, Chen X, Chrast J, Curado J, Derrien T, Drenkow J, Dumais E, Dumais J, Duttagupta R, Falconnet E, Fastuca M, Fejes-Toth K, Ferreira P, Foissac S, Fullwood MJ, Gao H, Gonzalez D, Gordon A, Gunawardena H, Howald C, Jha S, Johnson R, Kapranov P, King B, Kingswood C, Luo OJ, Park E, Persaud K, Preall JB, Ribeca P, Risk B, Robyr D, Sammeth M, Schaffer L, See L-H, Shahab A, Skancke J, Suzuki AM, Takahashi H, Tilgner H, Trout D, Walters N, Wang H, Wrobel J, Yu Y, Ruan X, Hayashizaki Y, Harrow J, Gerstein M, Hubbard T, Reymond A, Antonarakis SE, Hannon G, Giddings MC, Ruan Y, Wold B, Carninci P, Guigó R, Gingeras TR. Landscape of transcription in human cells. Nature 2012;489:101–8. https://doi.org/10.1038/nature11233.
[11] ENCODE Project Consortium. An integrated encyclopedia of DNA elements in the human genome. Nature 2012;489:57–74. https://doi.org/10.1038/nature11247.
[12] Engreitz JM, Sirokman K, McDonel P, Shishkin A, Surka C, Russell P, Grossman SR, Chow AY, Guttman M, Lander ES. RNA-RNA Interactions Enable Specific Targeting of Noncoding RNAs to Nascent Pre-mRNAs and Chromatin Sites. Cell 2014;159:188–99. https://doi.org/10.1016/j.cell.2014.08.018.
[13] Gao N, Li Y, Li J, Gao Z, Yang Z, Li Y, Liu H, Fan T. Long Non-Coding RNAs: The Regulatory Mechanisms, Research Strategies, and Future Directions in Cancers. Front Oncol 2020;10:598817. https://doi.org/10.3389/fonc.2020.598817.
[14] Halstead MM, Ma X, Zhou C, Schultz RM, Ross PJ. Chromatin remodeling in bovine embryos indicates species-specific regulation of genome activation. Nat Commun 2020;11:4654. https://doi.org/10.1038/s41467-020-18508-3.
[15] Hamazaki N, Uesaka M, Nakashima K, Agata K, Imamura T. Gene activation-associated long noncoding RNAs function in mouse preimplantation development. Development 2015;142:910–20. https://doi.org/10.1242/dev.116996.
[16] Hu K, He C, Ren H, Wang H, Liu K, Li L, Liao Y, Liang M. LncRNA Gm2044 promotes 17β-estradiol synthesis in mpGCs by acting as miR-138-5p sponge. Mol Reprod Dev 2019;86:1023–32. https://doi.org/10.1002/mrd.23179.
[17] Hung T, Wang Yulei, Lin MF, Koegel AK, Kotake Y, Grant GD, Horlings HM, Shah N, Umbricht C, Wang P, Wang Yu, Kong B, Langerød A, Børresen-Dale A-L, Kim SK, van de Vijver M, Sukumar S, Whitfield ML, Kellis M, Xiong Y, Wong DJ, Chang HY. Extensive and coordinated transcription of noncoding RNAs within cell-cycle promoters. Nat Genet 2011;43:621–9. https://doi.org/10.1038/ng.848.
[18] Iyyappan R, Aleshkina D, Zhu L, Jiang Z, Kinterova V, Susor A. Oocyte specific lncRNA variant Rose influences oocyte and embryo development. Noncoding RNA Res 2021;6:107–13. https://doi.org/10.1016/j.ncrna.2021.06.001.
[19] Jain AK, Xi Y, McCarthy R, Allton K, Akdemir KC, Patel LR, Aronow B, Lin C, Li W, Yang L, Barton MC. LncPRESS1 Is a p53-Regulated LncRNA that Safeguards Pluripotency by Disrupting SIRT6-Mediated De-acetylation of Histone H3K56. Mol Cell 2016;64:967–81. https://doi.org/10.1016/j.molcel.2016.10.039.
[20] Kaňka J, Kepková K, Němcová L. Gene expression during minor genome activation in preimplantation bovine development. Theriogenology 2009;72:572–83. https://doi.org/10.1016/j.theriogenology.2009.04.014.
[21] Karakas D, Ozpolat B. The Role of LncRNAs in Translation. Non-Coding RNA 2021;7:16. https://doi.org/10.3390/ncrna7010016.
[22] Kino T, Hurt DE, Ichijo T, Nader N, Chrousos GP. Noncoding RNA gas5 is a growth arrest- and starvation-associated repressor of the glucocorticoid receptor. ra8 Sci Signal 2010;3. https://doi.org/10.1126/scisignal.2000568.
[23] Lee K-B, Wee G, Zhang K, Folger JK, Knott JG, Smith GW. Functional role of the bovine oocyte-specific protein JY-1 in meiotic maturation, cumulus expansion, and subsequent embryonic development. Biol. Reprod 2014;90:69. https://doi.org/10.1095/biolreprod.113.115071.
[24] Li Y, Liu Y, Chen S, Chen X, Ye D, Zhou X, Zhe J, Zhang J. Down-regulation of long non-coding RNA MALAT1 inhibits granulosa cell proliferation in endometriosis by up-regulating P21 via activation of the ERK/MAPK pathway. Mol Hum Reprod 2019;25:17–29. https://doi.org/10.1093/molehr/gay045.
[25] Lingenfelter BM, Tripurani SK, Tejomurtula J, Smith GW, Yao J. Molecular cloning and expression of bovine nucleoplasmin 2 (NPM2): a maternal effect gene regulated by miR-181a. Reprod Biol Endocrinol 2011;9:40. https://doi.org/10.1186/1477-7827-9-40.
[26] Paulini F, Melo EO. The Role of Oocyte-Secreted Factors GDF9 and BMP15 in Follicular Development and Oogenesis. Reprod Domest Anim 2011;46:354–61. https://doi.org/10.1111/j.1439-0531.2010.01739.x.
[27] Peng Y, Chang L, Wang Y, Wang R, Hu L, Zhao Z, Geng L, Liu Z, Gong Y, Li J, Li X, Zhang C. Genome-wide differential expression of long noncoding RNAs and mRNAs in ovarian follicles of two different chicken breeds. Genomics 2019;111:1395–403. https://doi.org/10.1016/j.ygeno.2018.09.012.
[28] Platonova O, Akey IV, Head JF, Akey CW. Crystal Structure and Function of Human Nucleoplasmin (Npm2): A Histone Chaperone in Oocytes and Embryos. Biochemistry 2011;50:8078–89. https://doi.org/10.1021/bi2006652.
[29] Rao MRS. Long Non Coding RNA Biology. : Adv Exp Med Biol 2017;Vol. 1008:1–46.
[30] Rexroad CE, Casida LE, Tyler WJ. Crown-Rump Length of Fetuses in Purebred Holstein-Friesian Cows1,2. J Dairy Sci 1974;57:346–7. https://doi.org/10.3168/jds.S0022-0302(74)84891-5.
[31] Shi Y, Cai M, Du K, Bai X, Tang L, Jia X, Chen S, Wang J, Lai S. Dynamics of Known Long Non-Coding RNAs during the Maternal-to-Zygotic Transition in Rabbit. Animals 2021;11:3592. https://doi.org/10.3390/ani11123592.
[32] Statello L, Guo C-J, Chen L-L, Huarte M. Gene regulation by long non-coding RNAs and its biological functions. Nat Rev Mol Cell Biol 2021;22:96–118. https://doi.org/10.1038/s41580-020-00315-9.
[33] Sun Z, Zhang Z, Liu Y, Ren C, He X, Jiang Y, Ouyang Y, Hong Q, Chu M. Integrated Analysis of mRNAs and Long Non-Coding RNAs Expression of Oviduct That Provides Novel Insights into the Prolificacy Mechanism of Goat (Capra hircus). Genes 2022;13:1031. https://doi.org/10.3390/genes13061031.
[34] Taylor DH, Chu ET-J, Spektor R, Soloway PD. Long non-coding RNA regulation of reproduction and development. Mol Reprod Dev 2015;82:932–56. https://doi.org/10.1002/mrd.22581.
[35] Tripathi V, Ellis JD, Shen Z, Song DY, Pan Q, Watt AT, Freier SM, Bennett CF, Sharma A, Bubulya PA, Blencowe BJ, Prasanth SG, Prasanth KV. The nuclear-retained noncoding RNA MALAT1 regulates alternative splicing by modulating SR

splicing factor phosphorylation. Mol Cell 2010;39:925–38. https://doi.org/10.1016/j.molcel.2010.08.011.
[36] Wan Q, Wang Y, Wang H. Identification and Analysis of Regulatory Elements in Porcine Bone Morphogenetic Protein 15 Gene Promoter. Int J Mol Sci 2015;16(10):25759–72. https://doi.org/10.3390/ijms161025759.
[37] Wang J, Koganti PP, Yao J. Systematic identification of long intergenic non-coding RNAs expressed in bovine oocytes. Reprod Biol Endocrinol 2020;18:13. https://doi.org/10.1186/s12958-020-00573-4.
[38] Wang Y, Xue S, Liu X, Liu H, Hu T, Qiu X, Zhang J, Lei M. Analyses of Long Non-Coding RNA and mRNA profiling using RNA sequencing during the pre-implantation phases in pig endometrium. Sci Rep 2016;6:20238. https://doi.org/10.1038/srep20238.
[39] Xing Z, Lin A, Li C, Liang K, Wang S, Liu Y, Park PK, Qin L, Wei Y, Hawke DH, Hung M-C, Lin C, Yang L. lncRNA directs cooperative epigenetic regulation downstream of chemokine signals. Cell 2014;159:1110–25. https://doi.org/10.1016/j.cell.2014.10.013.
[40] Zhang F-L, Li N, Wang H, Ma J-M, Shen W, Li L. Zearalenone Exposure Induces the Apoptosis of Porcine Granulosa Cells and Changes Long Noncoding RNA Expression To Promote Antiapoptosis by Activating the JAK2–STAT3 Pathway. J Agric Food Chem 2019;67:12117–28. https://doi.org/10.1021/acs.jafc.9b05189.
[41] Zhao L, Pan Y, Wang M, Wang Junqian, Wang Y, Han X, Wang Jinglei, Zhang T, Zhao T, He H, Cui Y, Yu S. Integrated analysis of the expression profiles of the lncRNA-miRNA-mRNA ceRNA network in granulosa and cumulus cells from yak ovaries. BMC Genom 2022;23:633. https://doi.org/10.1186/s12864-022-08848-3.
[42] Zhao M, Qiu Y, Yang B, Sun L, Hei K, Du X, Li Y. Long Non-Coding RNAs Involved in Gynecological Cancer. Int J Gynecol Cancer 2014;24. https://doi.org/10.1097/IGC.0000000000000212. Available from: ⟨https://ijgc.bmj.com/content/24/7/1140⟩.
[43] Zhang K, Smith GW. Maternal control of early embryogenesis in mammals. Reprod Fertil Dev 2015;27(6):880. https://doi.org/10.1071/RD14441.
[44] Zhong L, Mu H, Wen B, Zhang W, Wei Q, Gao G, Han J, Cao S. Long non-coding RNAs involved in the regulatory network during porcine pre-implantation embryonic development and iPSC induction. Sci Rep 2018;8:6649. https://doi.org/10.1038/s41598-018-24863-5.