JY-1, an oocyte-specific gene, regulates granulosa cell function and early embryonic development in cattle

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Oocyte-specific gene products play a key role in regulation of fertility in mammals. Here, we describe the discovery, molecular characterization, and function of JY-1, a bovine oocyte-expressed gene shown to regulate both function of ovarian granulosa cells and early embryogenesis in cattle and characteristics of JY-1 loci in other species. The JY-1 gene encodes for a secreted protein with multiple mRNA transcripts containing an identical ORF but differing lengths of 3′ UTR. JY-1 mRNA and protein are oocyte-specific and detectable throughout folliculogenesis. Recombinant JY-1 protein regulates function of follicle-stimulating hormone-treated ovarian granulosa cells, resulting in enhanced progesterone synthesis accompanied by reduced cell numbers and estradiol production. JY-1 mRNA of maternal origin is also present in early bovine embryos, temporally regulated during the window from meiotic maturation through embryonic genome activation, and is required for blastocyst development. The JY-1 gene has three exons and is located on bovine chromosome 29. JY-1-like sequences are present on syntenic chromosomes of other vertebrate species, but lack exons 1 and 2, including the protein-coding region, suggestive of species specificity in evolution and function of this oocyte-specific gene.

The oocyte is a key regulator of multiple aspects of female fertility, including ovarian follicular development and early embryogenesis (1). The advent of oocyte genomics and EST sequencing projects have led to a dramatic increase in our understanding about the identities and functions of oocyte-specific genes in female reproduction (2, 3). However, inherent species-specific differences exist in the ovulation quota, follicular waves, duration of the ovarian cycle, and number of embryonic cell cycles required for embryonic genome activation (4) between the traditional animal model (polyovulatory mouse) versus monoovulatory species such as cattle and primates, including humans. Numerous examples suggest that oocyte-specific genes identified in the mouse may not have identical functions in other species. For instance, Belclare and Cambridge ewes with naturally occurring heterozygous mutations in the GDF9 gene have an increased ovulation rate and litter size (5), whereas homozygous BMP15 mutant mice are subfertile with defects in ovulation and fertilization (9). Thus, comparative genomics approaches coupled to functional studies in nontraditional model systems are needed to address dissimilarities in transcriptome composition between model organisms and provide information on existence of genes or gene families that may play important regulatory roles in fertility in nonmurine models, including the human. With this goal in mind, we previously constructed a bovine oocyte cDNA library and sequenced a number of ESTs (2). A highly abundant transcript (designated as JY-1) was identified and selected for further analysis, because it is entirely novel despite 7.95 million human, 4.74 million mouse, and 1.31 million bovine EST sequences in GenBank and because its expression is ovary specific. We thus hypothesized that JY-1 encodes for an oocyte-specific gene with important functions during folliculogenesis and early embryonic development. Here, we report the characterization and intraovarian localization of JY-1 mRNA and protein during folliculogenesis, evidence for a regulatory role for JY-1 in regulation of granulosa cell function and early embryonic development, and pronounced differences in characteristics of JY-1 loci in the genome of cattle versus other species examined (human, mouse, rat, chimpanzee, and dog).

Results

Tissue Distribution and Characterization of JY-1 mRNA Transcripts. Screening of RNA from various tissues by RT-PCR detected JY-1 mRNA only in fetal ovaries collected at days 180 and 210 of gestation but not in any other tissues examined [supporting information (SI) Fig. 6A] supporting tissue-specific expression of JY-1 mRNA. Northern analysis revealed three predominant JY-1 transcripts in RNA isolated from fetal ovaries (Fig. 1A). Further analysis of adult germinal vesicle (GV) oocytes (GVOs) by Northern blotting confirmed the presence of three major JY-1 transcripts of different lengths (∼1.8 kb, 1.2 kb, and 700 bp) (Fig. 1B). Because all 14 JY-1 inserts sequenced from the oocyte library were small (the longest is ∼455 bp in length) and could be partial cDNAs or represent the smaller predominant transcript detected by Northern analysis, a fetal ovary cDNA library was screened and two additional clones containing larger inserts were obtained. One clone contained an insert of ∼1.5 kb, and the other clone had an insert of ∼1.0 kb in length (GenBank accession nos. EF642496 and EF642497). Sequence analysis of these two larger JY-1 cDNAs and two original smaller cDNAs (455 and 355 bp) from the oocyte library revealed that the four cDNA represent four different transcripts of the JY-1 gene (SI Fig. 6B). Experiments using 5′RACE did not reveal any additional 5′ sequence confirming that the sequence observed at the 5′ end of all JY-1 transcripts is indeed complete (data not shown). Thus, the minor differences in the length of JY-1 transcripts observed in fetal ovary versus adult GVOs is most likely attributed to polyadenylation status of the mRNA transcripts. An identical ORF of 255 bp encoding for a predicted protein of 84 aa was identified in all four transcripts derived from the oocyte and fetal ovary libraries (SI Fig. 6B). Sequences

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Abbreviations: FSH, follicle-stimulating hormone; GV, germinal vesicle; GVO, GV oocyte; JY-1, recombinant JY-1; MRI, metaphase I; IVF, in vitro fertilized; Chrom, chromosome.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. EF642496 and EF642497).

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elements (AUUUUAAAA and UAUUUUAAUA) were also noted in the 3′UTR of the two longest transcripts (SI Fig. 6C). The AU-rich putative cytoplasmic polyadenylation elements (AUUUUAAAAA and UAUUUUAAUA) were also noted in the 3′ UTR of the two longest transcripts (SI Fig. 6B).

**Characterization of JY-1 Protein.** The Signal IP3 program (10) predicted a signal peptide of 21 aa, indicating that JY-1 protein is likely to be secreted from the oocyte. The predicted molecular weight of JY-1 is ~9,000 Mᵣ, but the NetOGlyc-3.1 program (11) predicted two O-linked glycosylation sites in the deduced JY-1 amino acid sequence, suggesting probable glycosylation of the JY-1 protein. Polyclonal antiserum raised against recombinant JY-1 (rJY-1) protein (mature form without the signal peptide) was used in Western blot analysis to detect JY-1 protein.

**Immunoreactive JY-1 protein of ~11,000 Mᵣ and additional higher Mᵣ bands were detected in extracts of adult GVOs (Fig. 1C).** The polyclonal antiserum also detected the rJY-1 protein (6,700 Mᵣ, mature form lacking the signal peptide) that was used to generate the antiserum (Fig. 1C). Preincubation of JY-1 antiserum with excess antigen (rJY-1) blocked binding of the antibody specifically to the 11,000 Mᵣ protein and rJY-1 protein, but not to the higher Mᵣ bands (Fig. 1D), which represent nonspecific cross-reactivity. Immunoreactive JY-1 protein was detected in adult GVOs but not in any other cell/tissue samples examined (Fig. 1E). The 11,000 Mᵣ JY-1 protein also was not detected in GVOs when blots were incubated with preimmune rabbit serum (Fig. 1F). Publicly available databases were searched with the predicted amino acid sequence of JY-1 to identify functional domains and predict the structure of the JY-1 protein, and no significant orthologs of the JY-1 protein were found. A putative secondary structure for JY-1 protein was predicted by using the PSIPRED program (12), but we have not identified any motifs that are indicative of functional domains by using the conserved domain database (CDD) (13). Similarly, Pfam A and B (14) and a PSI-BLAST search of the Protein Data Bank at the National Center for Biotechnology Information (15) designed to designate sequences to protein families based on homology and identify 3D structures for homology modeling were unsuccessful. Thus, we conclude JY-1 is a member of a novel protein family.

**Oocyte-Specific Localization of JY-1 mRNA and Protein within Ovarian Follicles.** Intraovarian expression of JY-1 mRNA and protein was restricted exclusively to oocytes. In situ hybridization localized JY-1 mRNA specifically to oocytes of preantral and antral follicles (SI Fig. 7). No significant hybridization to somatic ovarian cell types (granulosa, theca, and stroma) was noted. JY-1 protein was localized to oocytes of growing follicles at the primordial (single layer, with <10 flattened granulosa cells), primary (single layer with cuboidal granulosa cells) through antral follicle stages (Fig. 2) in fetal ovaries collected at day 230 of gestation. Immunoreactivity was not detected when tissue sections were incubated with preimmune rabbit IgG or the JY-1 antibody was preabsorbed with immunogen peptide (SI Fig. 8).
Effect of JY-1 Protein on Cell Number and Production of Estradiol and Progesterone by Cultured Granulosa Cells. The rJY-1 protein was used to test the ability of JY-1 to regulate bovine granulosa cell proliferation and steroidogenesis. Addition of rJY-1 to cultured granulosa cells inhibited the follicle-stimulating hormone (FSH)-induced increase in granulosa cell numbers at the 0.5 ng/ml dose (P < 0.05), and the response was maximal at 1 and 10 ng/ml doses (P < 0.05; Fig. 3A). Addition of rJY-1 at 0.1 ng/ml had no effect on granulosa cell numbers (Fig. 3A). However, in vitro production of estradiol was inhibited 2-fold in FSH-supplemented granulosa cells (P < 0.05) treated with the 0.1 ng/ml rJY-1 dose where significant effects on granulosa cell numbers were not observed (Fig. 3B). Further, the inhibitory effect on estradiol production was maximal at 0.5 ng/ml rJY-1, and supplementation with 1 and 10 ng/ml rJY-1 did not inhibit estradiol production. In contrast, addition of rJY-1 increased production of progesterone in a dose-dependent manner (P < 0.01), and the response was maximal at 1 and 10 ng/ml rJY-1 (Fig. 3C). Even though total cell numbers decreased by ~50% in response to treatment with 1 and 10 ng/ml rJY-1, progesterone production was doubled compared with cells cultured without rJY-1 (Fig. 3A and C). No effects of rJY-1 on granulosa cell numbers or estradiol and progesterone production were observed for granulosa cells cultured in the absence of FSH (data not shown).

Quantification of JY-1 mRNA During the Oocyte-to-Embryo Transition and Effect of JY-1 Knockdown on Early Embryonic Development. Given the observed oocyte-specific localization of JY-1 mRNA and protein, we hypothesized that JY-1 mRNA is regulated during meiotic maturation and early embryonic development. Temporal changes in abundance of polyadenylated versus total JY-1 transcripts during early development were characterized by quantitative real-time PCR. Abundance of polyadenylated JY-1 transcripts (cDNAs synthesized from oligo(dT) primers) decreased during meiotic maturation (P < 0.0001) increased (P < 0.05) at the pronuclear and four-cell stages relative to the metaphase II (MII) stage, and then decreased to nearly undetectable levels after the 16-cell stage of embryo development (Fig. 4A and SI Fig. 9A). In contrast, the amount of total JY-1 transcripts (cDNAs synthesized from random hexamers) gradually decreased from GV through 16-cell stages to nearly undetectable levels thereafter (SI Fig. 9B and C). The difference in abundance of polyadenylated versus total transcripts is probably the result of JY-1 mRNA deadenylation. Further, results of embryo culture experiments in the presence of the transcription inhibitor α-amanitin suggest that the JY-1 gene is not transcribed during the first and second embryonic cell cycles (SI Fig. 10), thus the JY-1 mRNA detected in early bovine embryos is maternal/oocyte derived.

To test the requirement of JY-1 during early embryonic development, we validated procedures for siRNA-mediated gene silencing in bovine embryos (SI Text). Multiple siRNA species were tested for efficacy and specificity of JY-1 mRNA knockdown via microinjection into MII oocytes followed by parthenogenetic activation. Parthenogenesis was used as a model to test the efficacy of JY-1 knockdown in embryos because it is easier to manipulate and allows for cumulus cell removal and genetic activation. Data were normalized relative to abundance of exogenous control (GFP) RNA and shown as mean ± SEM. (A) Effect of JY-1 siRNA microinjection on abundance of polyadenylated JY-1 mRNA in samples of two-cell embryos. Denuded MII oocytes were either microinjected with sham water or JY-1 siRNA mixture followed by parthenogenetic activation. Data were normalized relative to abundance of endogenous control (GFP) RNA and shown as mean ± SEM. (B) Effect of JY-1 siRNA microinjection on FSH-stimulated estradiol production in FSH-treated bovine granulosa cells. Note dose-dependent increase in progesterone in response to increasing concentrations of rJY-1 (P < 0.01). Concentrations of estradiol and progesterone were normalized to 30,000 cells. Data are depicted as mean ± SEM. Letters a and b indicate significant differences.
within the exons. (by two introns. The start (ATG) and stop (TAG) codons of the ORF are indicated
rat Chr 1 (syntenic Chrs to human Chr 11).
identified on human Chr 11, chimpanzee Chr 11, dog Chr 21, mouse Chr 7, and
1.5-kb bovine JY-1 cDNA.
JY-1
zebrafish, and
additional species. Genomic DNA databases at the National Center for Bio-
of the 3
(31.5%), and negative control siRNA-injected (33.7%) embryos
stage (7.4%) relative to uninjected (31.7%), sham-injected
control of the ORF, 5' UTR, and a portion of the 3' UTR. Note strong hybridization to a bovine genomic DNA fragment and weaker hybridization to sheep, pig, and human genomic DNA. (B) Gene structure of bovine JY-1. The JY-1 gene has three exons (E1, E2, E3) separated by two introns. The start (ATG) and stop (TAG) codons of the ORF are indicated within the exons. (C) Characterization of JY-1-like sequences in the genome of additional species. Genomic DNA databases at the National Center for Biotechnology Information for human, chimpanzee, dog, mouse, rat, chicken, zebrafish, and Drosophila were searched with the nucleotide sequence of the 1.5-kb bovine JY-1 cDNA. JY-1-like sequences corresponding to exon 3 were identified on human Chr 11, chimpanzee Chr 11, dog Chr 21, mouse Chr 7, and rat Chr 1 (syntenic Chrs to human Chr 11). JY-1-like sequences were not identified in genomic DNA databases for chicken, zebrafish, and Drosophila.

undetectable levels compared with uninjected control embryos (SI Fig. 13).

JY-1 siRNA mixture injection strikingly decreased the proportion of parthenogenetic embryos developing to the blastocyst stage (7.4%) relative to uninjected (31.7%), sham-injected (31.5%), and negative control siRNA-injected (33.7%) embryos (P < 0.05; Fig. 4C). Cleavage rates of embryos were not different between the groups. Similarly, JY-1 siRNA mixture injection into in vitro-fertilized (IVF) embryos did not affect the cleavage rates but dramatically reduced the proportion of IVF embryos developing to the blastocyst stage (4.2%) relative to uninjected (23.5%), sham-injected (24.1%), and negative control siRNA-injected (23.6%) embryos (P < 0.01; Fig. 4D). To further ensure the specificity of JY-1 siRNA in inhibiting embryonic development, experiments were repeated with the individual siRNAs injected separately. A reduction in the proportion of IVF embryos developing to the 8- to 16-cell stage (P < 0.0001; SI Fig. 14A) and proportion of embryos developing to the blastocyst stage (P < 0.0001; SI Fig. 14B) relative to uninjected and sham-injected controls was noted after injection of each siRNA individually.

Identification of JY-1-Like Sequences in Other Species. Southern blot analysis was used to investigate the presence of the JY-1 gene in the genome of cattle and other species. The 450-bp JY-1 cDNA strongly hybridized to an EcoRI genomic fragment in bovine genomic DNA, and weaker hybridization to sheep, pig, and human genomic DNA was also noted (Fig. 5A and SI Fig. 15). No significant hybridization to mouse, chicken, rainbow trout, and zebrafish genomic DNA was detected. The bovine JY-1 gene has three exons (25, 92, and 1,400 bp in length) separated by two introns (12.8 and 1.5 kb in length) (Fig. 5B and SI Fig. 16A) and is 16 kb in length and located on chromosome (Chr) 29 in the bovine genome. To identify putative cis elements that may confer tissue/cell-specific expression of JY-1, the 5' flanking sequence of the JY-1 gene was visually inspected. Five putative E-boxes [canonical sequence CANNTG; known to mediate oocyte-specific expression (16) in other species] were identified within 500 bp of the 5' flanking sequence of the bovine JY-1 gene (SI Fig. 16B).

JY-1-like sequences corresponding to exon 3 of the gene were also found in the human genomic and EST databases. JY-1-like sequence was identified on human Chr 11 (syntenic with bovine Chr 29) with the region of similarity corresponding to a portion (187 bp) of the protein-coding region and 850 bp in the 3' UTR of the 1.5-kb JY-1 cDNA (Fig. 5C). In the human EST database, a single EST derived from a human erythroid precursor cell (adult stem cell) cDNA library and lacking an ORF was identified (GenBank accession no. BU656412). The region of sequence similarity in the human EST is 187 bp and maps to human Chr 11 (11q14) with 100% identity and to the exact location where the sequence similar to bovine JY-1 is present (SI Fig. 17). JY-1-like sequences corresponding to exon 3 of the gene were also identified in the genome of additional vertebrate species. JY-1-like sequences were identified on chimpanzee Chr 11, dog Chr 21, mouse Chr 7, and rat Chr 1 (syntenic Chrs to human Chr 11 and bovine Chr 29; Fig. 5C).

Discussion

Results of the present studies demonstrate that the bovine JY-1 gene encodes for an oocyte-specific protein with important regulatory roles in granulosa cell function and early embryonic development and suggest that evolution of a functional JY-1 gene may be species-specific. Multiple oocyte-specific genes have been described in mice that directly regulate either folliculogenesis or early embryonic development (1). However, to our knowledge, establishment of a functional role for a single oocyte-specific gene (JY-1) in regulation of function of ovarian granulosa cells and early embryogenesis is unprecedented.

Identification of JY-1-like sequences corresponding to a small 3' portion of the ORF and/or the 3' UTR portion of the bovine JY-1 cDNA on syntenic Chrs to bovine Chr 29 in human, chimpanzee, mouse, rat, and dog (17–20) raises the possibility that the JY-1 locus is conserved in multiple vertebrate species. The sequence identity of the human EST (from erythroid precursor cells) with JY-1-like sequence on human Chr 11 (syntenic to bovine Chr 29) suggests that a mRNA transcript may be transcribed from the above locus. However, the syntenic loci do not encode for the complete JY-1 gene and lack sequences corresponding to exons 1 and 2 and thus a significant portion of the protein coding region. It appears unlikely based on extensive sequence analysis that the above loci in other species, including humans, encode for a protein of similar identity to bovine JY-1. Therefore, evolution of the oocyte-specific JY-1 protein is most likely species-specific. However, the presence of a functional ortholog performing similar roles as JY-1 in other mammalian species cannot be ruled out. Further, the significance of the conserved JY-1 3' UTR in multiple species is not known, but based on accumulating evidence for important regulatory roles of noncoding RNAs (21), a functional role for the observed JY-1-like sequence in the genome of other species cannot be discounted.

To suit the diverse reproductive functions in mammals, certain genes or gene families may have evolved by selection pressure during the course of evolution. For example, the trophoblast cell derived pregnancy recognition factor IFN-α produces IFN-α in mammalians in the Ruminantia suborder (e.g., cattle, sheep, goats), but not in unrelated species (22). Recent identification of the trophoblast kunitz domain protein (TKDP) gene family specifically expressed in ruminants (23) further supports the concept that certain genes in the reproductive system may have
evolved in a species-specific fashion and been selected for specialized functions. The evolution of the above genes in ruminants may be attributed to clear species-specific differences in the trophoblast and the type of placentaion mediating maternal-fetal communication (24). Species-specific attributes of oocyte function in general are not well understood, but bovine versus mouse oocytes do differ in their requirement for cumulus cell expansion and ability to promote glucose uptake by such cells (25–29). Studies in closely related species (e.g., sheep and goats) will be necessary to further determine the specificity in structure and function of the JY-1 gene.

Results support an important role for JY-1 in early embryonic development in cattle. Our evidence indicates that JY-1 mRNA is dynamically regulated during the window from meiotic maturation through embryonic genome activation. Results of siRNA-mediated gene knockdown experiments in two different in vitro models of early embryogenesis support a requirement of JY-1 for development to the blastocyst stage in cattle. Results also suggest that the maternal JY-1 mRNA is translated during bovine early embryogenesis because siRNA-mediated mRNA knockdown prevented the accumulation of JY-1 protein in early embryos. Our results further suggest that JY-1 is required during the early embryonic stages before embryonic genome activation, because JY-1 siRNA injection reduced development of embryos to the 8- to 16-cell stages by ~40%, and only 25% of injected embryos reaching the 8-to 16-cell stage developed into blastocysts. Gene targeting approaches have demonstrated the role of oocyte-specific MATER, ZAR1, and NPM2 genes for early embryo development in mice (30). Embryonic genome activation occurs much later in domestic ruminants (e.g., 8- to 16-cell stages in cattle and sheep) compared with the mouse (at the two-cell stage), thus additional maternal effect genes may be required to promote early embryogenesis in such species. While MATER and ZAR1 expression in bovine oocytes/embryos has been reported (31, 32), experimental evidence is lacking to support the requirement of the above genes for bovine early embryogenesis. To our knowledge, JY-1 is the only known oocyte-specific maternal factor demonstrated to govern early embryonic development in nonmurine species.

Oocyte regulation of folliculogenesis and phenotype/function of ovarian somatic (cumulus and granulosa) cells has been well established (1, 33). Results of the present studies demonstrate pronounced effects of rJY-1 protein on the granulosa cell phenotype in a manner mimicking preovulatory events characteristic of the luteinization process. Biological actions of JY-1 on bovine granulosa cells do not mimic the reported effects of the oocyte-specific protein in cattle and demonstrate multiple distinct roles for an oocyte-specific gene related to both folliculogenesis and early embryonic development. Structure function studies and future investigation of the signaling pathways or mechanisms mediating JY-1 action will provide further insight into functional domains that mediate JY-1 activity and into fundamental mechanisms regulating folliculogenesis and early embryonic development.

Materials and Methods

Northern Blot Analysis. Northern blotting was performed as described (39). For details see SI Text.

Bovine Fetal Ovary cDNA Library Construction. See SI Text.

Western Blotting. Recombinant JY-1 protein (rJY-1, predicted mature protein without signal peptide) was expressed in BL21 Escherichia coli and purified commercially by C & P Biotech (Thornhill, ON, Canada) using the pET15b vector. Polyclonal antiserum was generated in rabbits against rJY-1 protein by Affinity Bioreagents (Golden, CO). Western blotting was performed with our established protocols (39). See SI Text.

In situ Hybridization. In situ hybridization was performed according to our established procedure (40). See SI Text.

Immunohistochemistry. Polyclonal antiserum was generated against a 20-aa synthetic peptide corresponding to a portion of the carboxyl terminus of the predicted amino acid sequence of bovine JY-1 (C55-A74). Peptide synthesis, conjugation to keyhole limpet hemocyanin, immunization, and immunoaffinity purification was conducted commercially by Bethyl Laboratories (Montgomery, TX). Immunocytochemical localization of JY-1 protein was performed according to our published procedures (n = 3 samples) (39). See SI Text.

Effect of rJY-1 on Granulosa Cell Function. Serum-free long-term granulosa cell culture was performed as described (41, 42). See SI Text.

Quantification of JY1 mRNA in Oocytes and Early Embryos. Oocyte recovery, in vitro maturation, in vitro fertilization, embryo culture, and quantification of mRNA by real-time PCR were performed as described (43). See SI Text.

Synthesis and Validation of siRNA Species by Microinjection. To determine the effects of JY-1 knockdown on blastocyst development, microinjection experiments were performed in two different in vitro models of embryo development: parthenogenes- is and IVF (n = 4–5 replicates per treatment) (see SI Text).

Genomic Southern Blot Analysis, Genomic Library Screening, and Bioinformatics Analysis. See SI Text.
Statistical Analysis. For real-time PCR experiments, differences in mRNA abundance were determined by one-way ANOVA using the GLM procedure of SAS. For microinjection experiments, rates of embryo development to 8- to 16-cell and blastocyst stages were analyzed after arcsin transformation by using the GLM procedure of SAS. Similarly, differences in progesterone, estradiol, and cell numbers were determined by the Mixed Linear Models procedure of SAS. Mean comparisons were performed with Tukey’s test. The dose–response relationship between rJY-1 and progesterone was determined by regression analysis. Differences of \( P < 0.05 \) were considered significant.

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