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Identification of Novel Bovine Cumulus Cell Molecular Markers Predictive of Oocyte Competence: Functional and Diagnostic Implications[1]

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ABSTRACT

The present study was undertaken to discover molecular markers in bovine cumulus cells predictive of oocyte competence and to elucidate their functional significance. Differences in RNA transcript abundance in cumulus cells harvested from oocytes of adult versus prepubertal animals (a model of poor oocyte quality) were identified by microarray analysis. Four genes of interest encoding for the lysosomal cysteine proteinases cathepsins B, S, K, and Z and displaying greater transcript abundance in cumulus cells surrounding oocytes harvested from prepubertal animals were chosen for further investigation. Greater mRNA abundance for such genes in cumulus cells of prepubertal oocytes was confirmed by real-time RT-PCR. Elevated transcript abundance for cathepsins B, S, and Z also was observed in cumulus cells surrounding adult metaphase II oocytes that developed to the blastocyst stage at a low percentage following parthenogenetic activation versus those that developed at a high percentage. Functional significance of cumulus cell cathepsin expression to oocyte competence was confirmed by treatment of cumulus-oocyte complexes during in vitro oocyte maturation with a cell-permeable cysteine proteinase (cathepsin) inhibitor. Inhibitor treatment decreased apoptotic nuclei in the cumulus layer and enhanced development of parthenogenetically activated and in vitro-fertilized adult oocytes to the blastocyst stage. Stimulatory effects of inhibitor treatment during meiotic maturation on subsequent embryonic development were not observed when oocytes were matured in the absence of cumulus cells. The present results support a functional role for cumulus cell cathepsins in compromised oocyte competence and suggest that cumulus cell cathepsin mRNA abundance may be predictive of oocyte quality.

apoptosis, assisted reproductive technology, cathepsins, cumulus cells, embryo, oocyte quality

INTRODUCTION

The efficiency of assisted reproductive technologies (ART) is hampered by a lack of objective molecular markers predictive of oocyte quality. In ART, subjective morphological parameters are used as a primary criterion to select oocytes used for in vitro fertilization and intracytoplasmic sperm injection procedures and to assess healthy embryos with high implantation potential [1, 2]. Such criteria, however, do not truly predict the health of an embryo, because even normal-appearing embryos have abnormal chromosomes [3] and birth rates following embryo transfer are far from optimal. Therefore, to increase the live-birth rate per embryo transfer and reduce the cost of ART procedures, more than one embryo normally is transferred into the patient [2]. Transfer of multiple embryos, however, leads to obstetric complications, such as multifetal pregnancy resulting in twinning/triplets with increased rates of fetal morbidity and mortality [2, 4]. Although single-embryo transfers are practiced in some clinics [4, 5], the lack of both reliable markers and a valid definition of a high-quality oocyte/embryo has impeded selection of the optimal oocyte/embryo necessary for application of single-embryo transfers at a high efficiency. Therefore, identification of objective molecular markers predictive of an oocyte's capacity to develop into a healthy embryo has enormous implications for ART in humans as well as in embryo transfer programs with farm animal species.

The surrounding cumulus cell population of granulosa cells, herein referred to as cumulus cells, represents an attractive potential target for identification of objective molecular markers predictive of oocyte competence. The cumulus cells immediately surround the oocyte, and bidirectional communication between the oocyte and surrounding cumulus cells is critical to the development and function of both cell types [6]. For example, oocyte-secreted factors affect cumulus cell proliferation and expansion, differentiation, steriodogenesis, and gene expression in rodents [6, 7]. In exchange, cumulus cells provide substrates that are used by the oocytes for the energy metabolism necessary to promote oocyte meiotic maturation [7–9]. Similar evidence in the bovine model system indicates the oocyte is an important regulator of cumulus cell phenotype and survival, with marked effects on cell proliferation, cell death, and steroidogenesis [10, 11]. In turn, cumulus cells stimulate glutathione synthesis in bovine oocytes [12] and prevent damage induced by oxidative stress during in vitro maturation [13]. Given the bilateral communication that occurs between the cumulus cells and oocyte, it is plausible that changes in oocyte developmental competence likely affect cumulus cell phenotype and/or gene expression.

The cow is a relevant animal model for studies of oocyte competence in women, because ovarian physiology and many aspects of embryo development and ART are similar between

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these two single-ovulating species [14, 15]. A well-characterized bovine experimental model of poor oocyte competence is the prepubertal calf model. Although antral follicles with fully grown oocytes are present in prepubertal calves at or before birth, pregnancy rates from in vivo- or in vitro-produced embryos derived from oocytes of prepubertal animals are low [16–19]. This is attributed to the biochemical defects and/or changes in gene expression in prepubertal calf oocytes when compared to adult oocytes [20–24]. Recently, we reported the oocyte RNA transcript profiles associated with reduced developmental competence of prepubertal calf oocytes [25]. Complementary characterization of the molecular signatures (RNA transcript profiles) unique to cumulus cells surrounding oocytes harvested from the prepubertal calf model is predicted to unveil sets of genes or gene families for which the mRNA abundance in cumulus cells is associated with oocyte competence and that have functional significance. Thus, the objectives of the present study were to identify differences in the abundance of specific RNA transcripts in cumulus cells of bovine oocytes derived from adult versus prepubertal animals using cDNA microarray procedures and to investigate further the diagnostic utility and functional relationship of differences in mRNA abundance for select genes of interest (identified from microarray studies) with oocyte competence and early embryonic development in the bovine model system. The results of the present study support a functional role for cumulus cell cathepsin expression in compromised oocyte competence and suggest that cumulus cell cathepsin mRNA abundance (*CTSB*, *CTSS*, and *CTSZ*) may be predictive of oocyte quality.

MATERIALS AND METHODS

Materials

All materials were obtained from Sigma-Aldrich unless stated otherwise.

Cumulus Cell Recovery from Adult and Prepubertal Oocytes

Ovaries from adult and prepubertal (age, 24 wk) animals were collected at local abattoirs. The cumulus-oocyte complexes (COCs) were aspirated from all visible, 3- to 7-mm follicles, then selected (those with more than four compact layers of cumulus cells and homogenous cytoplasm) and washed three or four times in Hepes-buffered hamster embryo culture (HH) medium, after which the cumulus cells were completely separated by hyaluronidase (0.1%) digestion and repeated pipetting as described elsewhere [26]. The separated cumulus cells (four pools of cumulus cells with 18–20 oocytes each from adult and prepubertal animals) were snap-frozen in 100 μl of lysis solution (RNAqueous Micro Kit; Ambion, Inc.) and stored at −80°C until RNA isolation and subsequent microarray analysis. For real-time RT-PCR analysis, a separate set of samples consisting of five pools of cumulus cells derived from 13 to 14 oocytes each (from adult and prepubertal animals) were collected and processed as described previously.

RNA Extraction

Total RNA was extracted from each of the cumulus cell samples (used in microarray studies) collected from adult and prepubertal animals using the RNAqueous Micro Kit according to the manufacturer's instructions. The RNA was eluted twice using a 10-μl volume of prewarmed (75°C) elution solution according to manufacturer's instructions. Residual genomic DNA in all extracted samples was removed by DNase I (Ambion) digestion. The RNA from each pool of cumulus samples was divided into two 10-μl aliquots. One aliquot of extracted total RNA was used for cDNA microarray analysis. The total RNA for remaining cumulus cell samples (collected for real-time RT-PCR analysis) from germinal vesicle-stage oocytes of adult and prepubertal animals and in vitro-matured oocytes of adult animals was extracted with the same kit but with slight modifications. Before RNA extraction, each sample was spiked with 250 fg of green fluorescent protein (GFP) synthetic RNA as an exogenous control for RNA recovery and efficiency of cDNA synthesis [26]. Similarly, total RNA was eluted in 20 μl of eluent, but only 10 μl were used for each cDNA synthesis reaction.

Total RNA Amplification and cDNA Microarray Analysis

Total RNA (10 μl) from pools of cumulus cells (n = 4) collected from adult and prepubertal animals for microarray experiments was amplified using the RiboAmp Kit (Arcturus) according to previously validated procedures [27]. The quality and quantity of the amplified RNA generated were estimated using a ultraviolet spectrophotometer (Beckman Instruments, Fullerton, CA) and the Bioanalyzer 2100 RNA 6000 nanochip (Agilent Technologies). Only those amplification reactions yielding amplified RNA of consistent size range and quality across samples were used in subsequent microarray experiments. Two color microarray experiments were conducted as described previously [27] using a bovine cDNA array (Gene Expression Omnibus platform GPL325) containing expressed sequence tags representing approximately 15 200 unique genes [28]. A total of 15 μg of amplified RNA from cumulus samples harvested from adult and prepubertal animals were used for cDNA synthesis and labeling [27]. Hybridizations were performed on duplicate slides for each pair of samples (prepubertal vs. adult) and incorporated a dye swap (n = 8 slides)

In Vitro Oocyte Maturation

Germinal vesicle-stage COCs from adult animals (those with more than four compact layers of cumulus cells and homogenous cytoplasm) were in vitro matured as described previously [26]. Briefly, COCs were matured in Tissue Culture Medium-199 supplemented with 0.2 mM sodium pyruvate, 5 mg/ml of gentamicin sulfate, 6.5 mM L-glutamine, 156 mM bovine luteinizing hormone (Sioux Biochemical), 15.6 nM bovine follicle-stimulating hormone (Sioux Biochemical), 3.67 nM 17β-estradiol, and 10% fetal bovine serum (FBS; Hyclone) for 24 h in groups of 50 in four-well dishes containing 400 μl of maturation medium at 38.5°C and 5% CO_2 in air with maximum humidity.

Recovery of Cumulus Cells from In Vitro-Matured Oocytes, Parthenogenetic Activation, and Culture of Parthenogenetic Embryos

Germinal vesicle-stage COCs from adult animals (those with more than four compact layers of cumulus cells and homogenous cytoplasm) were in vitro matured for 24 h as described above. Groups of five in vitro-matured oocytes with expanded cumulus cells were randomly selected under a stereomicroscope, and cumulus cells were removed by repeated pipetting in a drop of HH medium, snap-frozen in 100 μl of lysis solution (Ambion), and stored at −80°C until RNA isolation and subsequent real-time RT-PCR analysis. After denuding and collecting cumulus cells, in vitro-matured oocytes were activated in 5 μM ionomycin (Calbiochem) in HH medium for 4 min, followed by 4 h of incubation in 2 mM 6-dimethylaminopurine in potassium simplex optimization medium (KSOM) supplemented with 3 mg/ml of BSA. After incubation, the oocytes were cultured in 30-μl drops of KSOM supplemented with 3 mg/ml of BSA under mineral oil. On Day 3, the cleavage rate was recorded, and both the uncleaved presumptive zygotes and cleaved embryos were transferred to drops of fresh KSOM supplemented with 3 mg/ml of BSA and 10% FBS until Day 7, when rates of blastocyst development were recorded. The matching cumulus cells corresponding to the groups of oocytes with an 80% cleavage rate (four of five oocytes cleaved) but a 0% blastocyst rate (low developmental competence group) and those with an 80–100% cleavage rate (four or five of five oocytes cleaved) but a 60–80% blastocyst rate (high developmental competence group) were used for RNA isolation and subsequent real-time RT-PCR analysis. As a control for parthenogenetic activation, the overall cleavage rate and the cumulative Day 7 blastocyst rate (from 10 groups of five oocytes each) were recorded for each experiment. Only cumulus cells (n = 4 samples each from the high and low developmental competence groups) collected from controlled experiments (n = 4 replicates) with overall rates of development to the blastocyst stage of greater than 20% (on Day 7) were used in the real-time RT-PCR analysis.

Quantitative Real-Time RT-PCR

Total RNA (10 μl) from each sample for real-time RT-PCR analysis was used for reverse transcription (RT) using oligo-$dT_{(15)}$ primers as described elsewhere [26]. After termination of cDNA synthesis, each RT reaction was then diluted with nuclease-free water (Ambion) to a final volume of 100 μl. The quantification of all gene transcripts (*CTSB*, *CTSK*, *CTSS*, *CTSZ*, *GFP*, and *18S* rRNA) was done by real-time quantitative RT-PCR using SYBR Green PCR Master Mix (Applied Biosystems). Primers were designed using the Primer Express program (Applied Biosystems) and derived from bovine sequences

TABLE 1. Sequences of primers (5′–3′) used for real-time RT-PCR.

Gene	GenBank accession no.	Forward primer	Reverse primer
CTSB	BF868324	CGATGCCCGGGAACAGT	GAGCCAGGATCCCTGATC
CTSK	BF230198	CATATGAACTGGCCATGAACCA	TGAGTCCAGTCATCTTCTGAACCA
CTSS	BE482678	TCGTGGTTGGCTATGGTAACC	TGCAGGCCCCAGCTGTT
CTSZ	BE752253	GGGAGAAGATGATGGCAGAAAT	TCTTTTCGGTTGCCATTATGC
18S	BC102293	GTGGTGTTGAGGAAAGCAGACA	TGATCACACGTTCCACCTCATC
GFP		CAACAGCCACAACGTCTATATCATG	ATGTTGTGGCGGATCTTGAAG

found in GenBank (Table 1). The amplicon size for each of the genes studied ranged from 80 to 150 bp. A primer matrix was performed for each gene tested to determine the optimal primer concentrations. Each reaction mixture consisted of 2 μl of cDNA, 1.5 μl each of forward (5 μM) and reverse (5 μM) primers, 7.5 μl of nuclease-free water, and 12.5 μl of SYBR Green PCR Master Mix in a total reaction volume of 25 μl (96-well plates). Reactions were performed in duplicate for each sample in an ABI Prism 7000 Sequence Detection System (Applied Biosystems). For real-time RT-PCR experiments (for validation of microarray results), the amounts of the mRNAs of interest were normalized relative to the abundance of an endogenous control (*18S* rRNA) to account for differences in total RNA concentrations between samples. The mean sample threshold cycle (CT) and mean endogenous control CT for each sample were calculated from duplicate wells. The relative amounts of target gene expression for each sample were calculated using the formula $2^{-(\Delta\Delta CT)}$ as described elsewhere [29]. Copies of *GFP* RNA (external control) also were quantified to account for differences in RNA recovery and efficiency of reverse transcription using standard-curve technology for absolute quantification as described elsewhere [26, 30]. Similarly, quantification of gene transcripts (*CTSB*, *CTSK*, *CTSS*, *CTSZ*, *GFP*, and *18S* rRNA) from cumulus cell samples derived from high and low developmental competence groups was done by real-time RT-PCR using standard-curve technology for absolute quantification [30]. The identity of respective amplicons amplified from cumulus cell RNA was confirmed by melting-curve analysis [25] and comparison to amplicons amplified from respective standards. Partial cDNAs for *CTSB*, *CTSK*, *CTSS*, *CTSZ*, and *18S* rRNA were amplified from bovine granulosa cells, cloned into pCR2.1 TOPO vector (Invitrogen Life Technologies), and subjected to fluorescent dye primer sequencing to confirm identity. The resulting plasmids were used to construct standard curves. Copies of RNA for each individual gene of interest within each sample were normalized relative to copies of *18S* rRNA measured in each sample to account for differences in cell number or input RNA across samples.

Effect of Cysteine-Proteinase (Cathepsin) Inhibitor Treatment During Meiotic Maturation on Subsequent Embryonic Development

Germinal vesicle-stage COCs (those with more than four compact layers of cumulus cells and homogenous cytoplasm) from adult animals were in vitro matured as described above in groups of 50 in four-well dishes containing 400 μl of maturation medium for 24 h either in the absence (control) or presence of a cell-permeable, irreversible, and highly selective cysteine proteinase inhibitor, E-64 (inhibits CTSB) [31, 32], at concentrations of 1 and 10 μM. The E-64 was dissolved in embryo-tested water, and aliquots were stored at −20°C. The culture media containing 1 and 10 μM E-64 were prepared by adding 10 μl each of 1 mM and 100 μM E-64 stock solutions per milliliter of culture medium, respectively. Similarly, the control medium (without E-64) was prepared by adding 10 μl of embryo-tested water per milliliter of culture medium. The E-64 specifically inhibits cysteine proteinases and does not inhibit serine/aspartic proteinases [31]. In experiment 1, cumulus cells were separated from in vitro-matured oocytes, and oocytes were parthenogenetically activated as described above. The activated oocytes were cultured in four-well dishes containing 400 μl of KSOM supplemented with 3 mg/ml of BSA. After 72 h, embryos were cultured in fresh KSOM supplemented with 3 mg/ml of BSA and 10% FBS until Day 7, when the percentage of embryos reaching the blastocyst stage was recorded. Experiment 1 was repeated five times. In experiment 2, the oocytes that matured in the presence or absence of E-64 were subjected to in vitro fertilization and embryo culture as described previously [26]. The percentage of embryos developing to the blastocyst stage on Day 7 was recorded. The inner cell mass (ICM) and the trophectoderm (TE) cells of the Day 7 blastocysts obtained were counted by differential staining [33] as an indicator of blastocyst quality. Experiment 2 was repeated four times. In experiment 3, groups of 25 COCs or denuded oocytes were matured in the presence or absence of 10 μM E-64 and parthenogenetically activated, and embryos were cultured as described above until Day 7, when blastocyst rates were recorded. Experiment 3 was repeated four times.

Effect of Cysteine-Proteinase (Cathepsin) Inhibitor Treatment on Cumulus Cell Apoptosis

To determine the effects of cysteine proteinase inhibitor (E-64) treatment on DNA fragmentation indicative of apoptosis in cumulus cells, the TUNEL procedure was performed using the In Situ Cell Death Detection Kit (Fluorescein; Roche Diagnostics Corporation). Germinal vesicle-stage COCs were in vitro matured in the presence of 0 (control), 1, and 10 μM concentrations of E-64. The in vitro-matured COCs were washed once in PBS (10 mM potassium phosphate and 0.9% NaCl [pH 7.4]) supplemented with 1 mg/ml of polyvinylpyrrolidone (PBS-PVP). The COCs were fixed in 4% (w/v) paraformaldehyde in PBS (pH 7.4) for 1 h and then washed and stored in PBS-PVP at 4°C until TUNEL analysis. During TUNEL analysis, COCs were permeabilized in permeabilization solution (0.5% Triton X-100 and 0.1% sodium citrate) for 2 h at room temperature. Positive-control, in vitro-matured COCs were incubated in 200 μl of RQ1 RNase-free DNase (Promega) at 37°C for 1 h. The COCs were washed in PBS-PVP and incubated in 25 μl of TUNEL reaction mixture (prepared by the manufacturer) for 1 h at 37°C in the dark. Negative-control COCs were incubated in the absence of TUNEL reaction mixture. All COCs were then incubated in 500 μl of RNase A (50 μg/ml) for 1 h, followed by 30 min of incubation in 200 μl of propidium iodide (0.5 μg/ml) at room temperature. The COCs were washed three times in PBS-PVP, then placed on glass slides and mounted with ProLong antifade mounting medium (Invitrogen Life Technologies). Fluorescent images were acquired using a laser-scanning confocal microscope at the Michigan State University Center for Advanced Microscopy. Total and apoptotic cells in four fields per COC were counted, and data are expressed as the percentage of apoptotic cells. This experiment was repeated three times with 10 COCs per treatment group.

Data Analysis

For microarray experiments, estimates of the false discovery rate (FDR) and differentially expressed genes were identified using the Significance Analysis of Microarrays program [34]. A minimum fold-change of ±1.7 ($P < 0.05$; FDR = 2%) was considered to be significant. Annotation of genes in the significant genes list was updated using the Goanna annotation tool at the Agbase resource [35]. Identification of biological themes (overrepresented genes) within lists of genes showing greater mRNA abundance in cumulus cells of prepubertal and adult oocytes was performed using Expression Analysis Systematic Explorer (EASE) [36], with an FDR of 10%. Microarray data can be accessed via Gene Expression Omnibus (accession no. GSE10809).

For real-time RT-PCR experiments, differences in mRNA abundance for *CTSB*, *CTSK*, *CTSS*, and *CTSZ* in cumulus cell samples from adult versus prepubertal oocytes and from oocytes of low versus high developmental competence were analyzed by ANOVA using the general linear models procedure of the SAS software (SAS Institute). For experiments evaluating the effects of cysteine proteinase (cathepsin) inhibitor (E-64) treatment during meiotic maturation on the percentage of cumulus cell apoptosis and subsequent rates of blastocyst development, data were analyzed by mixed linear models analysis procedures, followed by arcsin transformation of the percentage values. Differences in cumulus cell apoptosis and blastocyst rates across treatments were determined by using the Dunnett test.

RESULTS

Complementary DNA Microarray Analysis of RNA Transcript Profiles for Cumulus Cells Surrounding Adult and Prepubertal Oocytes

Microarray experiments revealed a significant number of genes encoding for transcripts of different abundance in cumulus cells surrounding germinal vesicle-stage oocytes harvested from adult versus prepubertal animals (see supple-

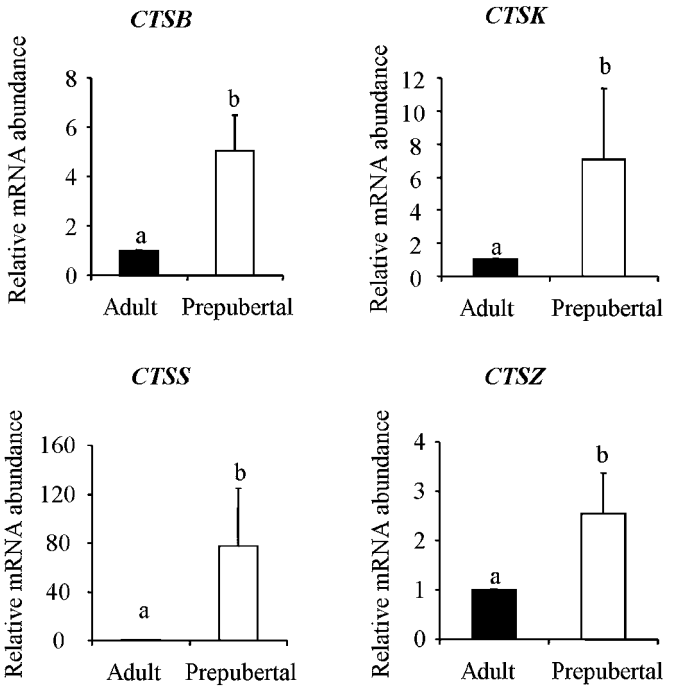

FIG. 1. Quantitative real-time RT-PCR analysis of *CTSB*, *CTSK*, *CTSS*, and *CTSZ* mRNA abundance in bovine cumulus cell samples harvested from germinal vesicle-stage COCs of adult (black bars) and prepubertal (white bars) animals (n = 5 each). Data were normalized relative to abundance of *18S* rRNA and are shown as the mean ± SEM. Time points within each panel without common letters differ significantly ($P < 0.05$).

mental data available at www.biolreprod.org). A total of 128 genes encoding for transcripts displaying greater mRNA abundance in cumulus cells surrounding oocytes collected from adult animals and 46 genes encoding for transcripts displaying greater mRNA abundance in cumulus cells surrounding compromised prepubertal oocytes were detected ($P < 0.05$; FDR = 2%). The most significant nonredundant and overrepresented gene categories (appearing more often in gene lists than would be predicted based on the distribution among all genes represented on the array) were determined using EASE analysis. The genes in the cysteine-type endopeptidase activity category were overrepresented in the cumulus cell samples harvested from oocytes of prepubertal animals. Given their documented biological activity, four of the genes listed belonging to the cathepsin family of lysosomal cysteine proteinases (*CTSB*, *CTSK*, *CTSS*, and *CTSZ*) were the focus of subsequent experiments to determine the functional and diagnostic relationship of such markers to oocyte competence.

Relative Abundance of CTSB, CTSK, CTSS, *and* CTSZ *Transcripts in Cumulus Cells of Adult and Prepubertal Oocytes*

Real-time RT-PCR analysis was performed on a set of cumulus cell samples separate from those used in the microarray experiments to evaluate the association of cumulus cell cathepsin mRNA abundance with oocyte competence. The results confirmed that higher amounts of mRNA for *CTSB*, *CTSK*, *CTSS*, and *CTSZ* are present in cumulus cells from oocytes collected from prepubertal versus adult animals (Fig. 1). The relative abundance of mRNAs for *CTSB*, *CTSK*, and *CTSZ* was approximately 2- to 6-fold higher in cumulus cells of oocytes from prepubertal versus adult animals ($P < 0.05$). The relative abundance of *CTSS* mRNA transcripts was approximately 80-fold higher in cumulus cells of oocytes collected from prepubertal versus adult animals ($P < 0.01$). The abundance of cathepsin S and cathepsin K mRNA was very low and approached undetectable levels in the cumulus cell samples from adult animals analyzed, which may account, in part, for the discrepancy in the magnitude of difference in relative expression between adult versus prepubertal cumulus cell samples observed with the two platforms (microarray vs. real-time RT-PCR). No difference in the abundance of *18S* rRNA was detected in cumulus samples from prepubertal versus adult animals (data not shown), further confirming that differences in the amounts of *CTSB*, *CTSK*, *CTSS*, and *CTSZ* mRNAs observed are not merely the result of differences in the abundance of total RNA or cell numbers in cumulus cell samples from adult versus prepubertal animals.

Relative Abundance of CTSB, CTSK, CTSS, *and* CTSZ *Transcripts in Matching Cumulus Cells of In Vitro-Matured Oocytes with High and Low Rates of Blastocyst Development*

Given the higher abundance of *CTSB*, *CTSK*, *CTSS*, and *CTSZ* mRNA in cumulus cells of prepubertal oocytes (a model for poor oocyte competence) versus adult animals, we hypothesized that the abundance of the above-mentioned transcripts also would be higher in cumulus cells of developmentally compromised oocytes derived from adult animals. To fulfill this objective, parthenogenesis was used as an experimental tool to measure the quality of an oocyte as assessed by its potential to develop into a blastocyst following activation and its relationship with cathepsin mRNA abundance (design outlined in Fig. 2A). The matching cumulus cells harvested from groups of five oocytes (parthenogenetically activated and cultured together) with high rates of cleavage and blastocyst development (high developmental competence) versus groups with high cleavage rates but no blastocyst development (low developmental competence) were used for real-time RT-PCR analysis. As hypothesized, the relative abundance of mRNAs for *CTSB*, *CTSS*, and *CTSZ* was approximately 1.5- to 6-fold higher in cumulus cells collected from oocytes with low developmental competence versus those collected from oocytes with high developmental competence (Fig. 2B) ($P < 0.05$). The relative abundance of mRNA for *CTSK* displayed a trend to be higher in cumulus cells collected from adult oocytes of low developmental competence versus those collected from adult oocytes of high developmental competence.

Effect of Cysteine Proteinase (Cathepsin) Inhibitor Treatment During Meiotic Maturation on Developmental Capacity of Bovine Oocytes

Given the association of *CTSB*, *CTSS*, and *CTSZ* (belonging to the family of cysteine proteinases) transcript abundance with oocyte competence, we presumed that the activity of this family of enzymes in the cumulus cells during meiotic maturation may be detrimental to oocyte competence and subsequent early embryonic development. Thus, we investigated the effects of an irreversible, cell-permeable, and highly selective cysteine proteinase inhibitor (E-64, which inhibits CTSB) on oocyte meiotic maturation and early embryonic development. Treatment of COCs with E-64 during in vitro oocyte maturation did not affect the percentage of oocytes reaching the metaphase II stage (data not shown). The COCs matured in the presence of 1 and 10 μM E-64, however, displayed a significant, 40–50% increase in the rates of

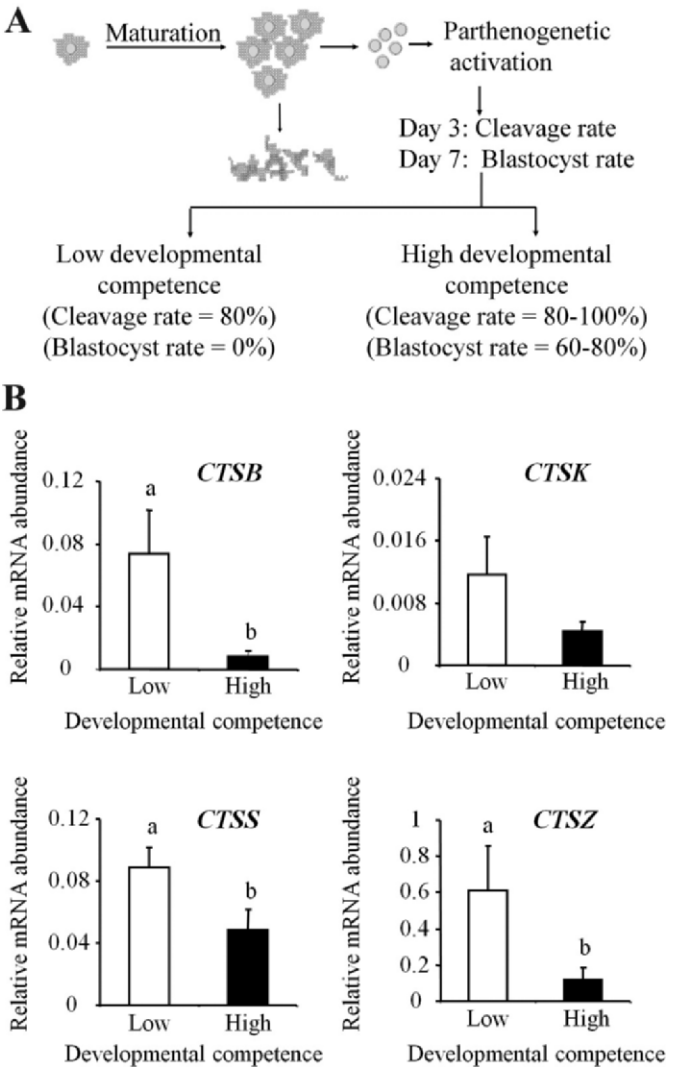

FIG. 2. Real-time RT-PCR analysis of the relationship between cumulus cell cathepsin mRNA abundance and competence of oocytes harvested from adult animals. **A**) Schematic illustration of the experimental design used in the present study. The matching bovine cumulus cells corresponding to the groups of oocytes with low (80% cleavage rate and 0% blastocyst rate; low developmental competence group) and high (80–100% cleavage rate and 60–80% blastocyst rate; high developmental competence group) rates of blastocyst development following parthenogenetic activation were used for real-time RT-PCR analysis. **B**) Results of quantitative real-time RT-PCR analysis of *CTSB*, *CTSK*, *CTSS*, and *CTSZ* mRNA abundance in matching cumulus cell samples harvested from in vitro-matured bovine oocytes of low (white bars) and high (black bars) developmental competence groups (n = 4 each). Data were normalized relative to abundance of *18S* rRNA and are shown as the mean ± SEM. Time points within each panel without common letters differ significantly ($P < 0.05$).

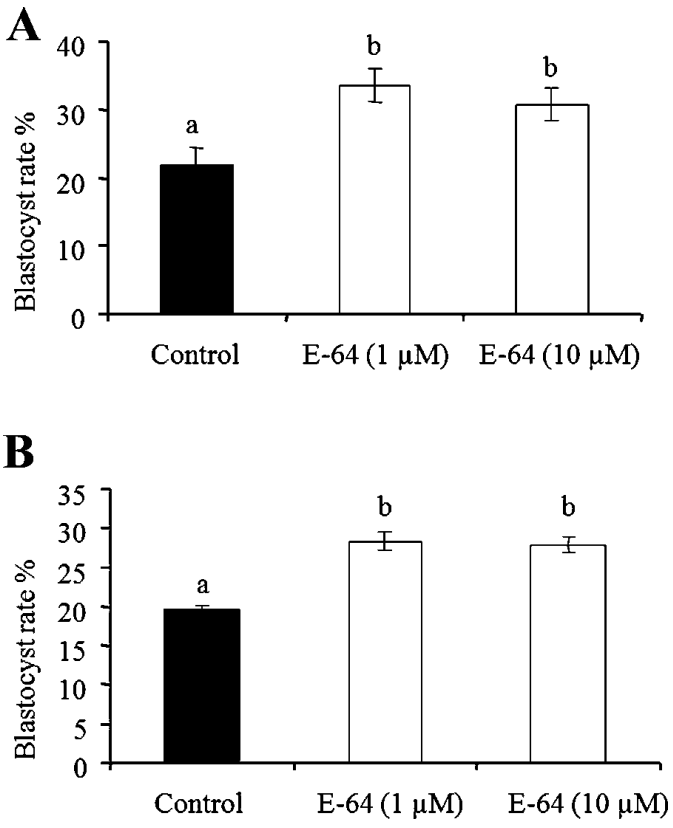

FIG. 3. Effects of cysteine proteinase (E-64) inhibitor treatment on developmental competence of bovine oocytes. Germinal vesicle-stage COCs were in vitro matured in the presence of 0 (control; black bars), 1, and 10 μM (both white bars) E-64, and effects of treatment on development to the blastocyst stage following parthenogenetic activation and in vitro fertilization were determined. **A**) Effect of E-64 treatments during meiotic maturation on parthenogenetic blastocyst development (assessed on Day 7 following activation). The rate of blastocyst development is expressed as the mean ± SEM (n = 5 replicates). Values with different letters across treatments are significantly different ($P < 0.05$). **B**) Effect of E-64 treatment during meiotic maturation on development of in vitro-fertilized embryos to the blastocyst stage (determined on Day 7 following insemination). The rate of blastocyst development is expressed as the mean ± SEM (n = 4 replicates). Values with different letters across treatments are significantly different ($P < 0.001$).

embryonic development to the blastocyst stage (Day 7) after parthenogenetic activation (Fig. 3A) ($P < 0.05$) or in vitro fertilization (Fig. 3B) ($P < 0.001$). The cleavage rate, however, was not affected by E-64 treatment (data not shown).

Differential staining was performed on blastocysts obtained following in vitro fertilization to determine if cysteine proteinase inhibitor treatment during meiotic maturation influenced subsequent blastocyst quality as assessed by total cell numbers and allocation to TE versus ICM. The ICM, TE, and total cell numbers, however, were not different across the treatment groups (Table 2).

To confirm that the effects of E-64 treatment during meiotic maturation on oocyte developmental competence are, in fact, mediated through the cumulus cells, the effects of E-64 treatment (during meiotic maturation) of COCs versus denuded oocytes devoid of cumulus cells on subsequent rates of blastocyst development following parthenogenetic activation were compared. Treatment of COCs with E-64 enhanced subsequent rates of blastocyst development following parthe-

TABLE 2. Effect of cathepsin inhibitor treatment during meiotic maturation on cell allocation in IVF embryos.[a]

Treatment	No. of blastocysts examined	ICM cells	TE cells	Total
Control	20	46.3 ± 3.4	67.9 ± 4.8	114.2 ± 4.5
E-64 (1 μM)	28	46.1 ± 3.4	75.4 ± 3.7	121.5 ± 4.2
E-64 (10 μM)	28	50.5 ± 2.8	69.5 ± 3.5	120.0 ± 3.8

[a] Cell numbers are represented as mean ± SEM.

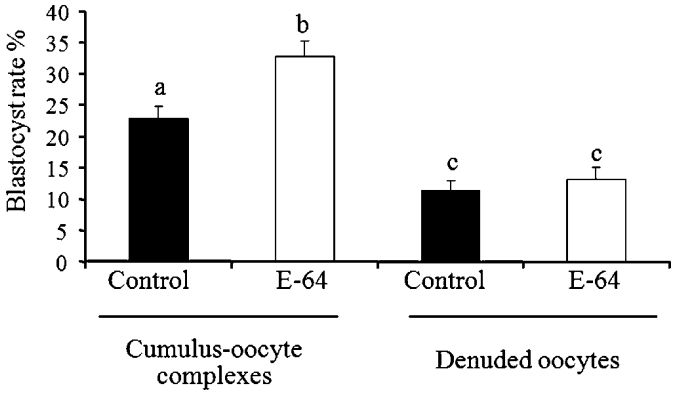

FIG. 4. Effects of cysteine proteinase (E-64) inhibitor treatment on developmental competence of bovine oocytes matured in the presence or absence of surrounding cumulus cells. Germinal vesicle-stage COCs or denuded oocytes were in vitro matured in the presence of 0 (control; black bars) or 10 μM (white bars) E-64, and effects of treatments on development of parthenogenetic embryos to the blastocyst stage were determined on Day 7 postactivation. The rate of blastocyst development is expressed as the mean ± SEM (n = 4 replicates). Values with different letters across treatments/groups are significantly different ($P < 0.01$).

nogenetic activation (Fig. 4) ($P < 0.01$). In contrast, blastocyst rates were lower for denuded oocytes following activation and were not influenced by E-64 treatment.

Effect of Cysteine Proteinase (Cathepsin) Inhibitor Treatment During Meiotic Maturation on Cumulus Cell Apoptosis

Given the association of cumulus cell cathepsin expression with oocyte competence, the observed stimulatory effects of cysteine proteinase inhibitor treatment during meiotic maturation on oocyte competence, and the reported stimulatory role of cathepsins in promoting apoptosis [37–39], we hypothesized that the stimulatory effects of inhibitor treatment may occur via the promotion of cumulus cell survival. Thus, the effects of E-64 treatment during meiotic maturation on cumulus cell apoptosis were examined. Figure 5 depicts representative fluorescent images of bovine COCs subjected to TUNEL labeling, with DNA fragmentation depicted by green fluorescence and total cells by red fluorescence. The COCs matured in the presence of 1 μM E-64 (Fig. 5, D–F) and 10 μM E-64 (Fig. 5, G–I) displayed a markedly reduced proportion of TUNEL-positive apoptotic cumulus cells (green color) relative to untreated controls (Fig. 5, A–C). Representative fluorescent images of DNase-treated, positive-control and negative-control COCs are displayed in Figure 5, J–L and M–O, respectively.

To quantify the effects of the cathepsin inhibitor treatment on cumulus cell apoptosis, experiments were repeated, and the percentage of apoptotic cells were counted on confocal images. The treatment with E-64 resulted in a dose-dependent decrease in cumulus cell apoptosis (Fig. 6). The treatment with 1 μM E-64 reduced cumulus cell apoptosis by approximately 60%, and the degree of apoptosis was further reduced to 14% of controls in response to treatment with 10 μM E-64 ($P < 0.0001$).

DISCUSSION

The cathepsin family of lysosomal cysteine proteinases, the catalytic activity of which is dependent on a cysteine residue in the active site, has been implicated in diverse biological functions and pathological disorders [37–43], but specific roles in ovarian/oocyte function for cathepsins are not well understood. Nevertheless, the results of the present study have established that a robust negative relationship exists between cumulus cell *CTSB*, *CTSS*, and *CTSZ* mRNA abundance and oocyte competence, and they suggest that cumulus cell cathepsin expression may be functionally coupled to, and potentially predictive of, oocyte competence.

The hormonal regulation of cumulus cell *CTSB*, *CTSS*, and *CTSZ* expression is not known. The mRNA and protein for *CTSB*, *CTSK*, and *CTSS*, however, are expressed in mouse ovarian follicles [44], and evidence indicates hormonal regulation during the estrous cycle, with elevated mRNA being observed during late proestrus and estrus [44]. Cathepsin L (*CTSL*), another member of the lysosomal cysteine proteinase family, also exhibits increased mRNA expression in the ovulating mouse and rat follicle in response to an ovulatory stimulus (injection of human chorionic gonadotropin) [45].

To improve the efficiency of ART, the assay of mRNA abundance for cumulus cell-expressed genes has been proposed as a valid approach to predict the physiological characteristics/developmental potential of resulting human embryos [46, 47]. Greater mRNA abundance has been reported for *HAS2*, *PTGS2*, and *GREM1* in cumulus cells from matching human oocytes that produce higher-grade embryos [46], and a positive association of increased cumulus cell *PTX3* mRNA abundance with successful in vitro fertilization and developmental/implantation potential of human embryos in vitro has been observed [47]. The functional significance of the above-described human cumulus cell markers to oocyte competence, however, is not known. In the present study, higher mRNA abundance for *CTSB*, *CTSS*, and *CTSZ* was detected in the cumulus cells harvested from groups of five adult oocytes of poor developmental competence (80% cleavage rate, 0% blastocyst development rate) versus those of higher developmental competence (80–100% cleavage rate, 60–80% blastocyst development rate). This finding, coupled with the demonstration that cathepsin inhibitor treatment of COCs during meiotic maturation improves blastocyst development, implies an important role for cumulus cell cathepsins not only as diagnostic markers but also in influencing the competency of oocytes to develop into blastocysts following fertilization. The relationship of cumulus cell expression of both the above-mentioned markers in the human and those identified in the bovine model system with pregnancy success will need to be determined to elucidate their true diagnostic utility.

The mechanisms by which changes in cumulus cell phenotype influence oocyte competence are not completely understood. Cumulus cells provide a network of gap junction transmembrane channels facilitating two-way communication for exchange of nutrients and paracrine factors between the oocyte and the cumulus cells [48–50]. Sufficient biochemical evidence from domestic animal species (e.g., cow and pig) indicate that the attachment of cumulus cells to the oocyte during meiotic maturation and fertilization is crucial for promoting subsequent embryo development [49, 51–57]. Furthermore, cumulus cells have a protective role in preventing oxidative stress-induced apoptosis and DNA damage [55] by enhancing the glutathione content in oocytes of swine [13] and, thus, functionally influence oocyte competence. Cumulus cells apposed to bovine oocytes undergo some degree of apoptotic DNA fragmentation during in vitro maturation [58], and considerable variation exists in the degree of cumulus cell apoptosis even among bovine oocytes of the highest morphological or quality grade [59]. The mechanisms that promote cumulus cell apoptosis are not clear, but perturbated stimulation of cumulus cells by oocyte-secreted antiapoptotic factors,

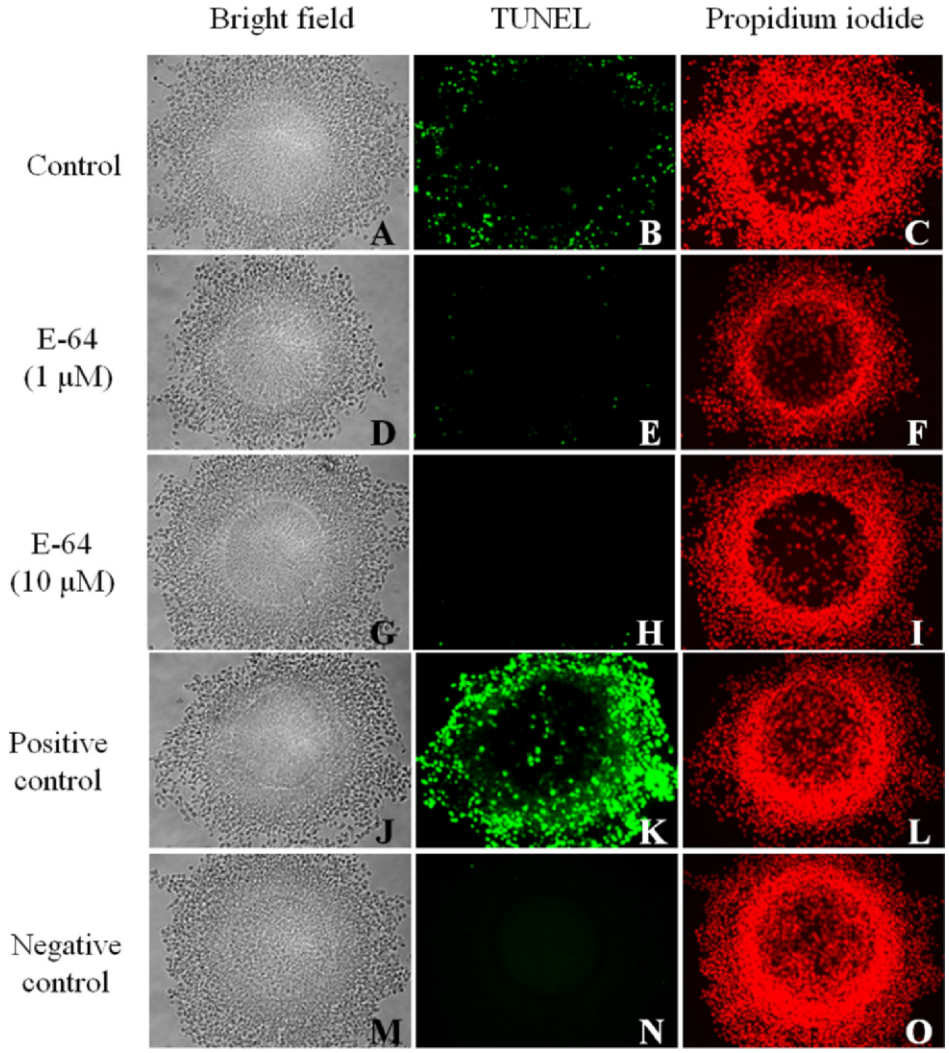

FIG. 5. Effect of cysteine proteinase (E-64) inhibitor treatment during in vitro meiotic maturation on apoptosis of bovine COCs. Bovine COCs were in vitro matured in the presence of 0, 1, and 10 μM E-64 and then subjected to TUNEL staining. **A**–**C**) Control COCs matured without E-64. **D**–**I**) Bovine COCs matured in the presence of 1 (**D**–**F**) and 10 (**G**–**I**) μM of E-64. **J**–**L**) Positive-control, mature COCs treated with DNase before TUNEL staining. **M**–**O**) Negative-control, mature COCs treated with DNase but incubated in the absence of TUNEL reagent used for detection of DNA fragmentation. **B**, **E**, **H**, **K**, and **N**) TUNEL staining and detection of DNA fragmentation (green). **C**, **F**, **I**, **L**, and **O**) Propidium iodide staining used for detection of total cells. **A**, **D**, **G**, **J**, and **M**) Corresponding bright-field images. Original magnification ×200.

such as BMP15 and BMP6, could be involved [11]. We cannot prove direct cause-and-effect relationships based on the higher expression of *CTSB*, *CTSS*, and *CTSZ* mRNA in the cumulus cells surrounding oocytes of poor developmental competence,

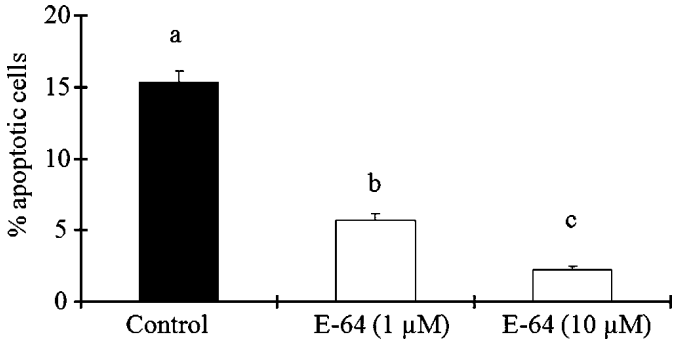

FIG. 6. Quantitative analysis of the effect of cysteine proteinase (E-64) inhibitor treatment during in vitro meiotic maturation on apoptosis of cumulus cells within bovine COCs. Bovine COCs were in vitro matured in the presence of 0, 1 and 10 μM E-64 and then subjected to TUNEL staining. Images were captured using confocal microscopy. Total and apoptotic cells in four fields per COC were counted, and data are expressed as the percentage of apoptotic cells (mean ± SEM). The experiment was repeated three times with 10 COCs per treatment group. Values with different letters across treatments are significantly different ($P < 0.0001$).

but coupled with stimulatory effects of cysteine proteinase (cathepsin) inhibitor treatment on oocyte competence (as determined by development of parthenogenetic and in vitro-fertilized embryos to the blastocyst stage) and the ability of such an inhibitor to reduce cumulus cell apoptosis, we can speculate that elevated cumulus cell CTSB, CTSS, and CTSZ may favor enhanced cumulus cell apoptosis and contribute to reduced oocyte competence. A relationship between maternal age and increased cumulus cell apoptosis [60] and negative ART outcomes (poor blastocyst development and quality as well as pregnancy rates) has been reported in women [61, 62].

Members of the cathepsin family of cysteine proteinases are implicated in proapoptotic pathways and originally were believed to directly activate caspases, a family of proteinases involved in programmed cell death [37–39, 63, 64]. Leptin treatment, which prevents cumulus cell apoptosis within cumulus cell-enclosed bovine oocytes, is known to have positive effects on fertilization rate and blastocyst development [65]. The above-described effects are mediated through the cumulus cells, because leptin treatment had no effect on rates of blastocyst development when provided to denuded oocytes (without surrounding cumulus cells) during culture [65]. Similarly, midkine, a heparin-binding growth differentiation factor, has been implicated in promoting oocyte developmental competence by mediating antiapoptotic actions through the cumulus cells [66, 67]. We are unaware, however, of any

evidence supporting a relationship between cumulus-oocyte expression of leptin or midkine and oocyte competence.

The cell-permeable cathepsin inhibitor used in the present study (E-64) inhibits many cysteine proteinases, and its specificity is not limited to cathepsins [31, 32]. Testing of specific inhibitors of individual cathepsins (cell permeable and impermeable), both individually and in combination, is of future interest to elucidate whether all the specific cathepsins of interest that have been identified are linked to poor competence of oocytes and to their site of action. Although the stimulatory effects of E-64 treatment during meiotic maturation on blastocyst development could be mediated by inhibition of enzymes outside the cathepsin family, our microarray experiments only revealed a negative association of mRNA abundance for the cysteine proteinases investigated here (*CTSB*, *CTSK*, *CTSS*, and *CTSZ*) with oocyte competence. Thus, inhibition of these enzymes during meiotic maturation likely has the greatest effect on blastocyst development. Furthermore, of the cathepsins that were observed to be differentially expressed in our microarray analysis, E-64 only inhibits CTSB, suggesting that the stimulatory effects of inhibitor treatment on blastocyst development may, potentially, be enhanced if the activity of additional cathepsins associated with oocyte competence (CTSS and CTSZ) also is pharmacologically inhibited.

The results of the present study indicate that the stimulatory effects of E-64 treatment during meiotic maturation on blastocyst development is unlikely to be mediated by inhibition of enzyme activity present in the oocyte. As observed for the cumulus cells, our previous microarray experiments comparing RNA transcript profiles for prepubertal oocytes (a model for poor oocyte competence) versus adult oocytes [25] did not reveal significant expression of *CTSB*, *CTSS*, and *CTSZ* mRNA. Furthermore, additional direct evidence indicates that stimulatory effects of E-64 on oocyte developmental competence are mediated through the cumulus cell layer. The treatment of denuded oocytes with E-64 during meiotic maturation did not enhance the ability of such oocytes to develop to the blastocyst stage following parthenogenetic activation. Attachment of cumulus cells to the oocyte is absolutely necessary during bovine meiotic maturation and fertilization to promote subsequent embryo development [52, 68]. At a minimum, the present results suggest that E-64 treatment cannot rescue the development competence of oocytes matured in the absence of a surrounding cumulus cell layer.

In conclusion, the results of the present study support a negative association and potential functional relationship of cumulus cell cathepsin mRNA (*CTSB*, *CTSS*, and *CTSZ*) expression with oocyte competence. The present results also suggest that transcript levels for *CTSB*, *CTSS*, and *CTSZ* in the cumulus cells may hold promise as an indicator/novel diagnostic approach to predict the capacity of bovine oocytes to support development to the blastocyst stage following fertilization, an early indicator of pregnancy potential. Additional studies will be required to determine the translational utility of the above-described findings to oocyte competence in other species, including humans, and their true diagnostic utility in predicting pregnancy success.

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