Functional genomics studies of oocyte competence: evidence that reduced transcript abundance for follistatin is associated with poor developmental competence of bovine oocytes

Osman V Patel^{1,4}, Anilkumar Bettgowda^{1,4}, James J Ireland^{2,3,4}, Paul M Coussens^{3,4}, Patrick Lonergan⁶ and George W Smith^{1,3,4,5}

¹Laboratory of Mammalian Reproductive Biology and Genomics, ²Molecular Reproductive Endocrinology Laboratory, ³Center for Animal Functional Genomics, Departments of ⁴Animal Science and ⁵Physiology, Michigan State University, East Lansing, Michigan 48824, USA and ⁶Department of Animal Science and Centre for Integrative Biology, University College Dublin, Dublin, Ireland

Correspondence should be addressed to G W Smith; Email: smithge7@msu.edu

Abstract

Poor oocyte competence contributes to infertility in humans and livestock species. The molecular characteristics of such oocytes are generally unknown. Objectives of the present studies were to identify differences in RNA transcript abundance in oocytes and early embryos associated with reduced oocyte competence and development to the blastocyst stage. Microarray experiments were conducted using RNA isolated from germinal vesicle stage oocytes collected from adult versus prepubertal animals (model of poor oocyte competence). A total of 193 genes displaying greater mRNA abundance in adult oocytes and 223 genes displaying greater mRNA abundance in prepubertal oocytes were detected. Subsequent gene ontology analysis of microarray data revealed significant overrepresentation of transcripts encoding for genes in hormone secretion classification within adult oocytes and such genes were selected for further analysis. Real-time PCR experiments revealed greater abundance of mRNA for β A and β B subunits of inhibin/activin and follistatin, but not the α subunit in germinal vesicle stage oocytes collected from adult versus prepubertal animals. Cumulus cell follistatin and β B subunit mRNA abundance were similar in samples collected from prepubertal versus adult animals. A positive association between time of first cleavage (oocyte competence) and follistatin mRNA abundance was noted. Follistatin, β B, and α subunit mRNAs were temporally regulated during early bovine embryogenesis and peaked at the 16-cell stage. Collectively, results demonstrate a positive association of follistatin mRNA abundance with oocyte competence in two distinct models and dynamic regulation of follistatin, β B, and α subunit mRNAs in early embryos after initiation of transcription from the embryonic genome.

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Introduction

Oocyte competence, defined as the ability of an oocyte to be fertilized and develop to the blastocyst stage is progressively acquired during the period of oocyte growth accompanying follicular development (Eppig *et al.* 2002, Matzuk *et al.* 2002). Pituitary gonadotropins and bidirectional local communication between the oocyte and adjacent somatic (cumulus) cells are critical for both nuclear and cytoplasmic maturation (acquisition of ability to complete meiosis, ensure monospermic fertilization, and undergo preimplantation development; Eppig 1991, Eppig *et al.* 2002, Gosden 2002, Matzuk *et al.* 2002). Competencies acquired by the nuclear and the cytoplasmic compartments during final stages of

maturation support the notion that oocyte quality depends on a multiplicity of factors, many of which can be assessed only at the molecular level. However, the molecular characteristics (at the level of mRNA transcript profiles) of competent oocytes are not known.

To date, morphological parameters, such as shape, homogeneity of cytoplasm, presence of polar body, and compactness of cumulus cells have routinely been utilized as markers of oocyte competence, and in the final analysis select for and generate embryos of increased developmental potential (Armstrong 2001, Lonergan *et al.* 2001, 2003a, Coticchio *et al.* 2004, Krisher 2004). However, it is well known that morphological criteria alone are insufficient to distinguish competent oocytes that have the ability to bring about

a full-term pregnancy (Lonergan *et al.* 2003a, Coticchio *et al.* 2004, Krisher 2004). For these reasons, investigation of the molecular characteristics of oocytes of poor developmental competence is critical to form a foundation for the development of future classification criteria for the selection of oocytes with superior developmental capacity.

Two well-defined bovine models for investigation of oocyte developmental competence are the prepubertal calf model and the time of first cleavage. Antral follicles with fully grown oocytes are present in calves at or prior to birth, but overall success of pregnancies from *in vivo* and *in vitro* produced embryos derived from oocytes of prepubertal animals is low (Seidel *et al.* 1971, Revel *et al.* 1995, Gandolfi *et al.* 1998, Khatir *et al.* 1998a, 1998b, Armstrong 2001, Palma *et al.* 2001). Oocytes collected from prepubertal animals display reduced activity of maturation promoting factors, mitogen-activated protein kinase and cyclin B, altered protein synthesis, aberrant energy metabolism, less Ca^{2+} influx at fertilization, and an overall reduced embryo survival post-fertilization (Levesque & Sirard 1994, Revel *et al.* 1995, Gandolfi *et al.* 1998, Khatir *et al.* 1998a, 1998b, Steeves & Gardner 1999, Armstrong 2001, Palma *et al.* 2001, Salamone *et al.* 2001). Despite evidence of biochemical defects in prepubertal oocytes, comparatively less is known about the molecular characteristics of such oocytes, manifested at the level of differences in mRNA transcript profiles between oocytes collected from prepubertal versus adult animals.

The relationship between the kinetics of early embryonic cleavage and subsequent development has been well established in cattle and humans (McKiernan & Bavister 1994, Plante *et al.* 1994, Sakkas *et al.* 1998, Shoukir *et al.* 1998, Brevini *et al.* 2002, Rizos *et al.* 2002, Lonergan *et al.* 2003b, Gutierrez-Adan *et al.* 2004). Such relationships suggest that whether or not an embryo will reach the blastocyst stage is determined in part by events that occur prior to the two-cell stage. While limited transcription does occur at or before the two-cell stage (Memili & First 1999), the two-cell stage embryo is absolutely dependent on transcripts and proteins sequestered from the oocyte for its progressive development until the maternal-zygotic transition when the maternal RNA pool is replaced via significant transcription from the embryonic genome. The maternal-zygotic transition occurs at the 8–16 cell stage in cattle (Telford *et al.* 1990, Memili & First 1998, 2000). The differences in timing of first cleavage are plausibly reflective of inherent differences in transcriptome composition (maternally derived transcripts) between early and late cleaving embryos at the two-cell stage. However, there are no measurable objective molecular markers described to date that can accurately predict the developmental potential of an oocyte prior to cleavage. Investigation of the molecular constituents of oocytes in parallel from two well-established models of reduced

developmental competence may reveal common sets of genes or gene families whose mRNA abundance is associated with oocyte competence. Thus, the objectives of the present studies were to use cDNA microarray analysis to identify differences in abundance of specific RNA transcripts in bovine oocytes from adult versus prepubertal animals associated with reduced oocyte competence and to further investigate the relationship of mRNA abundance for select genes of interest (identified from microarray studies) with oocyte competence and early embryonic development.

Materials and Methods

Materials

All materials were obtained from Sigma Aldrich unless stated otherwise.

Adult and prepubertal oocyte recovery

Ovaries from adult and prepubertal (24 weeks of age) animals were collected at local abattoirs and transported to the laboratory in sterile 0.25 M NaCl. Transport time to the laboratory for ovaries collected from adult versus prepubertal animals was approximately 1.5 and 0.5 h respectively. Upon return to the laboratory, ovaries from adult and prepubertal animals were washed in sterile 0.25 M NaCl, and cumulus–oocyte complexes (COCs) were aspirated from all visible 3–7 mm follicles, selected (those with more than four compact layers of cumulus cells and homogeneous cytoplasm), washed three to four times in Hepes-buffered hamster embryo culture medium (HECM; 114 mM NaCl, 3.2 mM KCl, 2 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.5 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 100 $\mu\text{l}/\text{ml}$ MEM (minimum essential medium) non-essential ($10\times$) amino acids, 17 mM sodium lactate, 0.1 mM sodium pyruvate, 2 mM NaHCO_3 , 1 mM Hepes, 0.183 mM penicillin-G, 3 mg/ml BSA; pH 7.3–7.4; 275 ± 10 mOsm/kg) and cumulus cells were completely removed by hyaluronidase (0.1%) digestion and repeated pipetting as described previously (Bettegowda *et al.* 2006). The denuded germinal vesicle stage (GV) oocytes (five pools of 18–20 oocytes from adult and prepubertal animals; cumulus removal confirmed by visual inspection of individual oocytes) and their associated cumulus cells (five pools from same oocytes as mentioned previously) were separately snap-frozen in 100 μl lysis solution (RNAqueous Micro Kit, Ambion Inc., Austin, TX, USA) and stored at -80°C until RNA isolation (oocytes only) and subsequent microarray analysis. For real-time PCR analysis, a separate set of samples consisting of five pools of 13–14 oocytes each and matching samples of their associated cumulus cells (from adult and prepubertal animals) were collected and processed as described previously.

In vitro oocyte maturation

Germinal vesicle stage COCs (from adult ovaries; collected as described previously) were matured *in vitro* as described previously (Bettegowda *et al.* 2006). Briefly, COCs were matured in TCM (tissue culture medium) 199 (supplemented with 0.2 mM sodium pyruvate, 5 mg/ml gentamicin sulfate, 6.5 mM L-glutamine, 156 nM bovine LH (Sioux Biochemical, Sioux Center, IA, USA), 15.6 nM bovine follicle-stimulating hormone (FSH; Sioux Biochemical), 3.67 nM 17 β -estradiol, and 10% (v/v) defined (fetal bovine serum) FBS (Hyclone, Logan, UT, USA)) for 24 h in groups of 50 in four-well dishes containing 400 μ l maturation medium at 38.5 °C, 5% CO₂ in air with maximum humidity. Oocytes with expanded cumulus were subjected to *in vitro* fertilization as described later.

In vitro fertilization and embryo culture

In vitro fertilization and embryo culture were performed as described previously (Bettegowda *et al.* 2006). Briefly, matured oocytes and sperm (10⁶ sperm/ml) were co-incubated for 20 h in groups of 50 in four-well dishes containing 400 μ l fertilization medium (114 mM NaCl, 25 mM NaHCO₃, 3.2 mM KCl, 0.34 mM NaH₂PO₄, 0.183 mM penicillin-G, 16.6 mM sodium lactate, 0.5 mM MgCl₂·6H₂O, 2.7 mM CaCl₂·2H₂O, 0.2 mM sodium pyruvate, 6 mg/ml BSA, and 1.5 U heparin) at 38.5 °C, 5% CO₂ in air with maximum humidity. To separate cumulus cells, the presumptive zygotes were vortexed for 2 min and washed thrice in Hepes-buffered HECM. Embryo culture was then performed in groups of 50 presumptive zygotes in four-well dishes containing 400 μ l KSOM medium (potassium simplex optimization medium; Specialty Media, Phillipsburg, NJ, USA) supplemented with 3 mg/ml BSA under mineral oil. Culture was carried out at 38.5 °C, 5% CO₂ in air with high humidity. Embryos at the 8–16-cell stage were separated 72 h after fertilization and cultured in fresh KSOM medium supplemented with 3 mg/ml BSA and 10% FBS until day 7. Pronucleus stage embryos (zygotes) were collected at 20 h post-insemination (hpi), two-cell embryos were collected at 33 hpi, four-cell embryos at 44 hpi, eight-cell embryos at 52 hpi, 16-cell embryos at 72 hpi, and morulae at 5 days and blastocysts at 7 days post-insemination. Embryos were processed for RNA isolation as described previously. As a control for the IVF procedure, a pool of embryos from each IVF run were cultured to the blastocyst stage to assess developmental competence of the fertilized eggs. Only embryos collected from controlled experiments with rates of development to blastocyst stage of >25% (on day 7) were used in the analysis. For each of the embryo stages, five pools of samples ($n=10$ embryos per pool) were collected from a total of 12 different IVF runs.

For collection of early and late cleaving embryos, pools of early cleaving two-cell embryos ($n=4$ pools of ten

embryos each) were collected at 30 h post-fertilization and processed as described previously. Pools of late cleaving embryos ($n=4$ pools of ten embryos each) were isolated from the same wells 6 h later and processed similarly. In each IVF run, a proportion of early and late cleaving two-cell embryos were cultured in separate wells (25 per well) as a control to assess the developmental competence of the fast and slow cleaving embryos. Percentage of development to the blastocyst stage (of oocytes subjected to fertilization) on day 7 was recorded.

RNA extraction

Total RNA was extracted from each pool of GV oocytes (used in microarray assays) collected from adult and prepubertal animals using the RNAqueous micro kit (Ambion) according to manufacturer's instructions. Total RNA from remaining samples was extracted with the same kit, but with slight modifications. Before RNA extraction, each sample was spiked with 250 fg green fluorescent protein (GFP) synthetic RNA as an exogenous control for RNA recovery and efficiency of cDNA synthesis (Bettegowda *et al.* 2006). Residual genomic DNA in all extracted samples was removed by DNase I digestion (Ambion). RNA was eluted twice from the silica-based microfilter cartridge using 10 μ l volume of prewarmed (75 °C) elution solution according to manufacturer's instructions. The RNA from each pool of oocytes collected from adult and prepubertal animals was divided into two 10 μ l aliquots. One aliquot of extracted total RNA was used for cDNA microarray analysis. Similarly, total RNA for real-time PCR analysis extracted from GV oocytes collected from prepubertal and adult animals, their matching cumulus cells, and *in vitro* produced early bovine embryos was eluted in 20 μ l eluent, but only 10 μ l was utilized for each cDNA synthesis reaction.

Total RNA amplification and cDNA microarray analysis

Total RNA (10 μ l) from the pools of oocytes ($n=5$) collected from adult and prepubertal animals for microarray experiments was amplified using the RiboAmp kit (Arcturus, Mountain View, CA, USA) using previously validated procedures (Patel *et al.* 2005). The quality and quantity of the amplified RNA generated were estimated using a u.v. spectrophotometer (Beckman Instruments, Fullerton, CA, USA), and the Bioanalyzer 2100 RNA 6000 nanochip (Agilent Technologies, Walbronn, Germany). Only those amplification reactions yielding amplified RNA of consistent size range and quantity across samples were utilized in subsequent microarray experiments.

Two color microarray experiments were conducted as described previously (Patel *et al.* 2005) using a bovine

cDNA array containing expressed sequence tags (ESTs) representing approximately 15 200 unique genes (Suchyta *et al.* 2003). A total of 15 µg amplified RNA from oocytes samples harvested from adult and prepubertal animals were used for cDNA synthesis and labeling (Patel *et al.* 2005). Hybridizations were performed on duplicate slides for each pair of samples (prepubertal versus adult) and incorporated a dye swap ($n=10$ slides total).

RT and quantitative real-time PCR

Total RNA (10 µl) from each sample for real-time PCR analysis was utilized for RT using oligo dT₍₁₅₎ primers as described elsewhere (Bettegowda *et al.* 2006). A 10 µl aliquot of RNA from early bovine embryos was also reverse transcribed as described previously using random hexamers (Bettegowda *et al.* 2006). 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 (follistatin, βB, and βA subunits of inhibin/activin, α subunit of inhibin, GFP, and 18S rRNA) was done by real-time quantitative RT-PCR using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The relative quantification method was utilized as described in detail elsewhere (Livak & Schmittgen 2001). Primers were designed using the Primer Express program (Applied Biosystems) and derived from bovine sequences found in GenBank (see 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 optimal primer concentrations. Each reaction mixture consisted of 2 µl cDNA, 1.5 µl each of forward (5 µM) and reverse primers (5 µM), 7.5 µl nuclease-free water, and 12.5 µl 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). Two non-template control samples were included on each plate for each primer set. Specificity of amplification with each primer set

in each assay was confirmed by melting curve analysis. The thermal cycler program consisted of 40 cycles of 95 °C for 15 s and 60 °C for 1 min. For real-time PCR experiments, amounts of mRNAs of interest were normalized relative to an exogenous control (GFP) to control for differences in RNA recovery and efficiency of RT (Bettegowda *et al.* 2006) and also separately normalized relative to abundance of an endogenous control (18S rRNA) to account for differences in RNA concentrations between samples. The mean sample threshold cycle (CT) and mean endogenous/exogenous control CT for each sample were calculated from duplicate wells. The mean CT of the control was then subtracted from the CT of samples to give the ΔCT. In each experiment, one of the experimental samples served as a control (calibrator). Subsequently, ΔCT of target sample was then subtracted from the calibrator ΔCT. The relative amounts of target gene expression for each sample were then calculated using the formula $2^{-(\Delta\Delta CT)}$ (Livak & Schmittgen 2001). Copies of 18S rRNA were also quantified using standard curve technology for absolute quantification (Whelan *et al.* 2003) as an index for differences in amounts of total RNA between samples of interest.

Data analysis

For microarray experiments, estimates of false discovery rate (FDR) and differentially expressed genes (FDR=5%) were identified using the significance analysis of microarrays program (Tusher *et al.* 2001). Differences in ratios of Cy3/Cy5 < 0.7 ($P<0.05$; FDR=5%) or Cy3/Cy5 > 1.3 ($P<0.05$; FDR=5%) were considered significant. Initial annotation and gene symbols for features on the array were obtained using the *Gene Links* toolkit (<http://cafg.msu.edu>).

Ontological classification and further annotation of genes encoding for transcripts showing differences in abundance in adult versus prepubertal oocytes were performed using the Database for Annotation, Visualization and Integrated Discovery (Dennis *et al.* 2003). Identification of biological themes (overrepresented genes) within lists of genes showing greater mRNA

Table 1 Sequence of primers used for real-time PCR.

Gene	GenBank Accession number	Sequence
Follistatin	BF774514	F: 5'-CAGAGCTGCAAGTCCAGTACCA-3' R: 5'-CATGTAGAGCTGCCTGGACAGA-3'
Inhibin α	BE721322	F: 5'-CCTCGGTGGAGGTTACTCTTTT-3' R: 5'-CGGGATCCCTTAGATGCAA-3'
Inhibin βA subunit	AW658434	F: 5'-GAGCAGTCGACAGACCTTTC-3' R: 5'-CCGGTGAGGATGGTCTTCAG-3'
Inhibin βB subunit	AW669304	F: 5'-GCATCTCCAGAATGCCTTCAC-3' R: 5'-GTGGAATGACTCTTATGCAATGG-3'
GFP	—	F: 5'-CAACAGCCACAACGCTATATCATG-3' R: 5'-ATGTTGTGGCGGATCTTGAAG-3'
18S rRNA	BC102293	F: 5'-GTGGTGTGAGGAAAGCAGACA-3' R: 5'-TGATCACACGTTCCACCTCATC-3'

abundance in prepubertal and adult oocytes was performed using EASE expression analysis systematic explorer; (Hosack *et al.* 2003) with a FDR of 10%. Non-redundant annotation classes with an EASE score of less than 5% are depicted.

For real-time PCR experiments, differences in abundance of specific transcripts of interest between adult and prepubertal oocytes and cumulus cell samples, early versus late cleaving embryos, and across stages of early embryonic development were analyzed by ANOVA using the General Linear Models procedure of SAS. Mean comparisons were performed when appropriate using Tukey's test.

Results

cDNA microarray analysis of RNA transcript profiles for oocytes collected from adult versus prepubertal animals

Microarray experiments revealed a significant number of genes encoding for transcripts of different abundance in GV oocytes harvested from adult versus prepubertal animals (see Supplementary data, Table 1). A total of 193 genes encoding for transcripts displaying greater mRNA abundance in adult oocytes and 223 genes encoding for transcripts displaying greater mRNA abundance in compromised prepubertal oocytes were detected ($P < 0.05$; FDR = 5%). Of the 193 bovine genes encoding for transcripts that were of greater abundance in adult versus prepubertal oocytes, 159 were annotated and 153 classified into ontology categories listed (Fig. 1). Of the 223 genes encoding for transcripts that were of greater abundance in prepubertal versus adult oocytes, 201 were annotated and 196 ontologically classified (Fig. 1). Non-redundant overrepresented gene categories (appearing more often in gene lists than would be predicted based on distribution among all genes represented on the array) in above gene lists are depicted in Table 2. Genes in the regulation of hormone secretion ontology category were found to overrepresented in the adult oocyte samples with lowest EASE score and given their documented biological activity, were selected for further analysis. Such genes encode for the β A and β B subunits of inhibin/activin and their binding protein, follistatin. A difference in hybridization intensity of 2.5, 2.4, and 1.7 for the β A subunit, follistatin, and the β B subunit respectively, corresponding to potentially higher mRNA abundance in adult versus prepubertal oocytes was observed in microarray studies (Supplementary data, Table 1).

Relative abundance of inhibin/activin and follistatin transcripts in adult versus prepubertal oocytes and cumulus cells

Real-time PCR analysis using a set of samples distinct from those used in microarray experiments confirmed lower amounts of mRNA for above genes (follistatin, β B,

and β A subunits of inhibin/activin) in oocytes collected from prepubertal animals (Fig. 2). Differences in abundance of mRNA for the α subunit of inhibin were also examined to give a more complete picture of phenotypic differences in mRNA abundance for inhibin/activin subunits in oocytes collected from adult versus prepubertal animals, even though α subunit mRNA abundance was not significantly different in microarray studies (data not shown). The relative abundance of mRNAs for the β B and β A subunits of inhibin/activin and for follistatin was approximately three- to fivefold higher in oocytes from adult versus prepubertal animals ($P < 0.002$), but no difference in α subunit mRNA abundance was observed. No difference in abundance of 18S rRNA was detected in oocyte samples from adult versus prepubertal animals (data not shown), further confirming that differences in amounts of follistatin, β A, and β B subunit RNAs observed are not merely due to differences in abundance of total RNA in the two populations of oocytes. Differences in abundance of mRNA for follistatin and the β B subunit of inhibin/activin were specific to the oocyte compartment of COCs collected from adult versus prepubertal animals. There was no difference in mRNA abundance for the α and β B subunits of inhibin/activin and for follistatin in matching cumulus cells collected from oocytes harvested from adult versus prepubertal animals, but β A subunit mRNA was slightly higher ($P < 0.05$) in the cumulus cells surrounding oocytes collected from adult animals (Fig. 3).

Relative abundance of inhibin/activin and follistatin transcripts in early versus late cleaving two-cell bovine embryos

Given lower abundance of follistatin, β B, and β A subunit mRNAs observed in oocytes collected from prepubertal animals (poor oocyte competence) versus adult animals, we hypothesized that abundance of oocyte-derived transcripts for follistatin, β B, and β A subunits would be lower in late cleaving (reduced development to blastocyst stage) versus early cleaving two-cell stage bovine embryos. Early cleaving embryos had a greater blastocyst yield ($40 \pm 4\%$) than their late cleaving counterparts ($10 \pm 3\%$). Greater mRNA abundance for follistatin, but not for the β B and β A subunits of inhibin/activin, or the α subunit of inhibin was observed in early cleaving embryos compared with late cleaving embryos ($P < 0.05$; Fig. 4). No differences in abundance of 18S rRNA were detected in early versus late cleaving embryos (data not shown), indicating that differences in amounts of follistatin, β A, and β B subunit mRNAs observed were not due to differences in abundance of total RNA in the two populations of embryos.

Figure 1 Ontological classifications of genes encoding for transcripts of greater abundance in germinal vesicle stage oocytes collected from adult (top) and prepubertal animals (bottom). Of the 193 and 223 bovine genes encoding for transcripts displaying greater abundance in oocytes collected from adult and prepubertal animals, 159 and 201 were annotated and 153 and 196 were classified respectively into ontology categories listed for the two oocyte populations. Numbers in parentheses indicate percentage of total number of genes with transcripts of greater abundance for given population of oocytes that are represented in each ontology category.

Relative abundance of inhibin/activin and follistatin transcripts during early embryogenesis *in vitro*

Given observed association of follistatin transcript abundance with oocyte competence, we then investigated the temporal regulation of mRNA abundance for above genes during early embryonic development *in vitro*. Changes in relative abundance of polyadenylated

transcripts for follistatin, βA and βB subunits of inhibin/activin, and the α subunit of inhibin in embryos collected at the pronuclear, two-, four-, eight-, sixteen-cell, morula and blastocyst stages are illustrated in Fig. 5A. Follistatin, the βB subunit of inhibin/activin, and the α subunit of inhibin transcripts exhibited similar temporal changes in abundance, where mRNA

Table 2 EASE analysis of over-represented genes.

Sample	System	Gene category	Ease score	Gene symbol
Adult oocyte	GO biological processes	Regulation of hormone secretion	1.84×10^{-3}	FST, INHBA, INHBB
		Macromolecule biosynthesis	9.78×10^{-3}	ATIC, ATP5B, C20ORF155, EEF1A1, GCNT1, HSPB1, INHBA, INHBB, RPL10A, RPL9, RPS3A, SCD, TPI1
	GO molecular function	Protein binding	1.72×10^{-2}	CDK6, COTL1, CTNNBIP1, FLNA, FUS, INHBA, INHBB, JUP, PFN1, PRSS11, RIPK3, RTN3, SPARC, TPM1, TWSG1, VIM
Prepubertal oocyte	GO cellular component	Intracellular	2.08×10^{-2}	APLP2, B4GALT5, C10RF16, CCNB2, CTNND1, DIAPH1, DVL2, EIF3S4, FLJ10774, FRAP1, GBA, GNAI1, GTF2F1, HNRPF, INPP1, ISGF3G, KEAP1, KHSRP, KIF3C, MBNL3, MGAT1, MLL3, MSX1, MYH1, NAPA, NDUFV2, NFKBIA, ODC1, PEG10, POLS, PPARBP, PRC1, PRDM2, PRDX5, PRKAR1A, PSTPIP1, RASA1, REV1L, RTN3, SEC14L1, SPOP, STAR, TAP2, TOP1, TPM2, ZFYVE1

GO, gene ontology.

abundance for each gene remained low until the 16-cell stage when a significant ($P < 0.05$) increase was detected (12–40 fold). The increase in relative abundance of the three transcripts at the 16-cell stage was transient, as amounts of mRNA were significantly decreased at the morula and blastocyst stages (Fig. 5A). Similar results ($P < 0.05$) were obtained when abundance of total transcripts for follistatin, β A and β B subunits of inhibin/activin, and the α subunit of inhibin was determined using cDNA generated with random hexamers (Fig. 5B). Data depicted in Fig. 5A and B were normalized relative to abundance of 18S rRNA to account for differences in RNA content of embryos at various stages of development.

Discussion

In the present study, differences in RNA transcript profiles associated with competence of oocytes harvested from prepubertal versus adult animals were examined using a functional genomics approach and novel information on oocyte transcripts potentially associated with developmental competence identified. Four distinct biological themes (based on EASE analysis) were delineated from gene lists resulting from microarray studies, which each form a foundation for future investigation. Based on EASE score and documented paracrine and autocrine reproductive roles of genes represented in the regulation of hormone secretion category, the dynamic regulation of mRNA for a subset of the transforming growth factor (TGF)- β superfamily (β A, β B, and α subunits of inhibin/activin) and their binding protein (follistatin) was further investigated in two well-established, albeit different models of oocyte competence in cattle.

To our knowledge, the present report is the first to document an association of follistatin mRNA abundance with oocyte competence and a confirmed association of mRNA abundance for a known gene with oocyte competence in two established model systems. Furthermore, a transient upregulation of follistatin and

inhibin/activin subunit mRNAs (α , β B) was observed at the 16-cell stage of early embryonic development, presumably resulting from new transcription from the embryonic genome. These results indicate that apart from the reported role of follistatin in regulating activin activity (Knight & Glister 2001, Phillips 2005), relative amounts of follistatin mRNA may be predictive of oocyte competence and suggest that follistatin may play a role in bovine early embryonic development.

A total of 193 and 223 transcripts showing greater abundance in oocytes collected from adult and prepubertal animals respectively were identified from microarray experiments. Given that >15 000 unique genes were represented on the bovine cDNA microarray utilized in the present studies (Suchyta *et al.* 2003), results suggest that differences in transcript abundance were not

Figure 2 Quantitative real-time RT-PCR analysis of α subunit of inhibin (α), β A (β A), and β B (β B) subunits of inhibin/activin and follistatin (FS) transcripts in oocyte samples collected from prepubertal (white bar) and adult (black bar) animals ($n=5$ each). Data were normalized relative to abundance of 18S rRNA (endogenous control) and are shown as mean $\pm$ S.E.M. Similar results were obtained when data were normalized relative to exogenous control RNA (GFP) to account specifically for differences in RNA recovery and efficiency of reverse transcription (data not shown). Time points within each panel without common superscripts differ, a, b = $P < 0.002$.

Figure 3 Quantitative real-time RT-PCR analysis of α subunit of inhibin (α), β A (BA), and β B (BB) subunits of inhibin/activin and follistatin (FS) transcripts in matching cumulus cell samples harvested from oocytes collected from prepubertal (white bar) and adult (black bar) animals ($n=5$ each). Data were normalized relative to abundance of endogenous control (18S rRNA) and are shown as mean $\pm$ S.E.M. Time points within each panel without common superscripts differ, a, b = $P < 0.05$.

solely due to differences in total RNA abundance between the two populations of oocytes, an observation that was also confirmed based on no observed difference in 18S rRNA abundance in adult versus prepubertal oocytes. Microarray approaches have been applied previously in mice to identify differences in RNA transcript profiles associated with oocyte aging (Hamanian *et al.* 2004) and development *in vitro* versus *in vivo* (Pan *et al.* 2005). In both cases, statistically significant differences in transcript abundance were observed for a relatively small percentage of genes ($<5\%$). Thus, the numbers of transcripts of greater abundance in adult and prepubertal oocytes detected in the present microarray

Figure 4 Quantitative real-time RT-PCR analysis of α subunit of inhibin (α), β A (BA), and β B (BB) subunits of inhibin/activin and follistatin (FS) transcripts in samples of early (white bar) and late (black bar) cleaving bovine two-cell embryos ($n=4$ each). Data were normalized relative to abundance of 18S rRNA (endogenous control) and are shown as mean $\pm$ S.E.M. Similar results were obtained when data were normalized relative to exogenous control RNA (GFP) to account specifically for differences in RNA recovery and efficiency of reverse transcription (data not shown). Time points within each panel without common superscripts differ, a, b = $P < 0.05$.

studies seems reasonable given the rigid analysis criteria employed. Future studies will be required to elucidate the association of additional transcripts identified in previously described microarray experiments with oocyte competence in other models and the potential functional and diagnostic significance of our results.

The TGF- β (inhibin/activin) family members and their binding protein follistatin play key roles in folliculogenesis and an association with oocyte maturation is well established (Knight & Glister 2001, Phillips 2005). We acknowledge that results of the present studies merely establish an association of follistatin mRNA abundance with oocyte competence in the two model systems examined and do not prove a functional role for follistatin and/or inhibins/activins in oocyte competence. A number of previous studies have investigated the effect of supplementation with exogenous activin, inhibin, and follistatin *in vitro* on early embryonic development (Izadyar *et al.* 1996, Yoshioka & Kamomae 1996, Stock *et al.* 1997, Silva & Knight 1998, Yoshioka *et al.* 1998) and with mixed results. Stock *et al.* (1997) reported that supplementation of media with inhibin, activin, or the combination during *in vitro* bovine oocyte maturation significantly increased cleavage rates post-fertilization. Based on these results, they concluded that inhibin and activin may be important in the acquisition of oocyte competence. However, an antagonistic relationship between inhibin and activin is essential to the control of pituitary FSH release and to normal gonadal function (Knight & Glister 2003, Phillips 2005). Thus, it is interesting that both inhibin and activin were able to unvaryingly influence cleavage rates following supplementation during oocyte maturation (Stock *et al.* 1997). Other independent studies found no effect of inhibin and activin supplementation during *in vitro* oocyte maturation on subsequent development to the blastocyst stage following *in vitro* fertilization (Van Tol *et al.* 1994, Izadyar *et al.* 1996, Silva & Knight 1998). Similar findings of no substantial effect on post-fertilization cleavage rates were also reported following follistatin supplementation during *in vitro* oocyte maturation (Silva & Knight 1998). On the other hand, activin supplementation during *in vitro* embryo culture was reported to increase blastocyst yield, whereas an inhibitory effect of follistatin was noted (Yoshioka & Kamomae 1996, Yoshioka *et al.* 1998). It remains to be elucidated whether endogenous production of above growth factors compromised effects of exogenously supplemented growth factors and thus complicated interpretation of the physiological role of endogenous activin, inhibin, and/or follistatin. Secondly, it remains to be verified if time of administration coincided with the biologically relevant stages of development when oocyte-derived endogenous inhibin, activin, and follistatin potentially act. Hence, it will be important to delineate the stage-specific regulatory role of endogenous oocyte-derived inhibin, activin, and follistatin to

Figure 5 Quantitative real-time RT-PCR analysis of α subunit of inhibin (α), βA (βA), and βB (βB) subunits of inhibin/activin and follistatin (FS) transcripts in samples of *in vitro* derived bovine embryos collected at pronucleus (PN), two-cell (2C), four-cell (4C), eight-cell (8C), sixteen-cell (16C), morula (MO), and blastocyst (BT) stages ($n=5$ each). (A) Differences in abundance of polyadenylated transcripts for genes of interest (RNA reverse transcribed with oligo dT₁₅ primers) at specific stages of early embryonic development. (B) Differences in abundance of total transcripts for genes of interest (RNA reverse transcribed with random hexamers) at specific stages of early embryonic development. Data were normalized relative to abundance of 18S rRNA (endogenous control) and are shown as mean $\pm$ S.E.M. Similar results were obtained when data were normalized relative to exogenous control RNA (GFP) to account specifically for differences in RNA recovery and efficiency of reverse transcription (data not shown). Time points within each panel without common superscripts differ, $P < 0.001$.

facilitate interpretations of earlier studies employing exogenously supplemented treatments.

To our knowledge, no studies have addressed the relationship between endogenous follistatin production and early embryonic development in cattle. Matzuk (1995) utilizing activin- and follistatin-deficient mice proposed that low doses of follistatin may actually play an agonistic role in the activin response. Low levels of follistatin could thus increase the bioavailability of activin rather than neutralize its actions (Silva & Knight 1998). The latter is substantiated by the fact that administration of lower doses of follistatin to COCs increased their developmental potential and did not antagonize the effects of activin A (Silva & Knight 1998). Thus, a potential functional association of oocyte-derived follistatin with oocyte competence is supported by results of the present study, but additional experiments are necessary to determine cause–effect relationships.

The oocyte and its surrounding somatic cells are interdependent in regulation of growth and development of the oocyte and ovarian follicle (Eppig *et al.* 2002, Matzuk *et al.* 2002). Follistatin and inhibin/activin mRNAs and protein are detectable in the germ and somatic cell compartments of antral follicles (Izadyar *et al.* 1998, Silva *et al.* 2003, 2004). Moreover, there is ample evidence that germ cell-derived factors influence secretion of a plethora of substances by the surrounding somatic cells, including TGF- β family members (Eppig 1991, Eppig *et al.* 2002, Matzuk *et al.* 2002). Two recent studies have described cyclooxygenase-2, hyaluronic acid synthase-2, gremlin, and pentraxin-3 expression in the cumulus cells as potential markers of the quality of the enclosed oocyte (McKenzie *et al.* 2004, Zhang *et al.* 2005). However, neither of the latter studies elaborated whether the same described genes were correspondingly altered in the germ cells encompassed by these somatic

cells, because such experiments would require destruction of the oocyte. To our knowledge, this study is the first to correlate altered mRNA abundance between the germ and somatic cells in a known model of developmentally compromised oocytes. Abundance of mRNA for the βA subunit of inhibin/activin was higher both in the oocyte and the cumulus cell compartment of COCs collected from adult versus prepubertal animals. However, transcript abundance for follistatin and the α and βB subunits of inhibin/activin was similar in cumulus cells surrounding the same oocytes collected from prepubertal versus adult animals where differences in oocyte mRNA abundance for follistatin and the βB subunit were observed. This may suggest that although follistatin and activins/inhibins may be necessary for the synchronized development of germ and somatic cells (Knight & Glister 2001, Phillips 2005), their overall regulation in the two cell types is generally distinct.

It is well known that transcription in bovine oocytes declines to very low levels upon attainment of full size (approximately 120 μ m) in a follicle of about 3 mm in diameter (Fair *et al.* 2002), and does not reinitiate at significant levels until the maternal-zygotic transition (Telford *et al.* 1990, Memili & First 1998, 2000). Therefore, prior to this stage, post-fertilization development is dependent on timely translation of maternally derived oocyte mRNAs. Since the maternal-zygotic transition and initiation of significant new transcription are characteristic of later stages (8–16 cell) of embryonic development in cattle (Telford *et al.* 1990, Memili & First 1998, 2000), potential markers of high-quality oocytes are also likely to be of greater abundance in early versus late cleaving two-cell stage bovine embryos. We thus explored the association of mRNAs for follistatin, the βA and the βB subunits of inhibin/activin, and the α subunit of inhibin with time of first cleavage in this model of

oocyte competence. After IVF, early cleaving embryos have enhanced competence to reach the blastocyst stages as compared with their later cleaving counterparts (McKiernan & Bavister 1994, Sakkas *et al.* 1998, Shoukir *et al.* 1998). In our study, the early cleaving control embryos attained higher rates of development to the blastocyst stage in comparison with late cleaving embryos, in agreement with earlier reports (Loneragan *et al.* 2003a). In addition, increased abundance of follistatin mRNA was evident in early versus late cleaving bovine embryos, similar to the relationship observed in oocytes collected from adult versus prepubertal animals. Although the relationship between the kinetics of early embryonic cleavage and subsequent development have been reported in several species (McKiernan & Bavister 1994, Sakkas *et al.* 1998, Loneragan *et al.* 2003b), the mechanisms influencing the kinetics of initial early cleavage divisions are not clear. However, all authors do agree that time of first cleavage is linked to the intrinsic quality of the oocyte (Plante *et al.* 1994, Brevini *et al.* 2002, Rizos *et al.* 2002, Loneragan *et al.* 2003b, Gutierrez-Adan *et al.* 2004). Furthermore, while follistatin is best known for its ability to bind activin with a high affinity, there is evidence of additional roles for follistatin independent of activin binding and important functional roles during development. For example, follistatin binds to bone morphogenetic proteins (BMPs) and the BMPs are also important for oocyte and embryonic development (Fainsod *et al.* 1997, Iemura *et al.* 1998). In addition, in *Xenopus*, follistatin is reported to act as an organizer factor during early embryogenesis (Iemura *et al.* 1998). Thus, further studies will be required to determine the functional significance of alterations in follistatin transcript abundance to oocyte competence and time of first cleavage and the mechanisms involved.

An association of mRNA abundance for several other genes with time of first cleavage has been established previously. Gutierrez-Adan *et al.* (2004) reported that mRNAs for stress-induced genes, such as sarcosine oxidase, mitochondrial Mn-superoxide dismutase, apoptosis regulator bax- α , interferon τ , and glucose-6-phosphate dehydrogenase are more abundant in slow developing embryos, but the authors previously noted that culture conditions significantly altered mRNA abundance for the same stress-associated genes (Loneragan *et al.* 2003b). Greater mRNA abundance for histone H3 and the preimplantation embryo development (Ped) genes has been observed in early versus late cleaving two-cell bovine embryos (Fair *et al.* 2004a, 2004b), as has greater mRNA abundance for isocitrate dehydrogenase, histone H2A, and YY1- and E4TF1-associated factor 1 (Dode *et al.* 2006). However, to our knowledge, an association of oocyte mRNA abundance for above genes with developmental competence has not been confirmed in additional experimental models. This study is the first to show that follistatin mRNA abundance in oocytes is regulated and associated with oocyte competence in two

well-described experimental model systems. Therefore, our results suggest that maternally derived follistatin may play a regulatory role in bovine early embryogenesis.

This study also demonstrated that follistatin, β B, β A, and α subunits of inhibin/activin mRNAs are dynamically regulated during early embryogenesis. During the early cleavage stages up until zygotic genome activation and initiation of new transcription, embryonic development is entirely dependent on maternal mRNAs and proteins synthesized and stored during oogenesis (Telford *et al.* 1990, Memili & First 1998, 2000). In the present study, mRNA abundance for follistatin, the β B subunit of inhibin/activin, and the α subunit of inhibin remained low until the 16-cell stage, where a significant increase in mRNA abundance for above genes was observed. This rapid increase in mRNA abundance at the 16-cell stage is likely due to new transcription of such genes from the embryonic genome and not due to post-transcriptional modification of existing RNA stores, since a similar temporal upregulation was observed when both total and polyadenylated transcripts for genes of interest were quantified.

Interestingly, Majerus *et al.* (2000) found that a significant proportion of calf oocyte-derived embryos arrested development before the nine-cell stage and those that developed to the blastocyst stage had a longer lag phase preceding the major onset of zygotic genome activation than oocytes derived from adult animals. Similar results were reported by Camargo *et al.* (2005). Thus, it is plausible that increased mRNA abundance for follistatin, the β B subunit of inhibin/activin, and the α subunit of inhibin at the 16-cell stage could be associated with important developmental events, including compaction of blastomeres and subsequent cavitation. However, the functional significance of observed changes in mRNA abundance remains to be determined.

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