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Bovine oocytes and early embryos express Staufen and ELAVL RNA-binding proteins.

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For Submission to Zygote:

Bovine Oocytes and Early Embryos Express Staufen and ELAVL RNA Binding Proteins

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Running Title: RNA binding proteins in cow embryos

ABSTRACT:

RNA binding proteins (RBP) influence RNA editing, localization, stability and translation and may contribute to oocyte developmental competence by regulating the stability and turnover of oogenetic mRNAs. The expression of Staufen 1 and 2 and ELAVL1, ELAVL2 RNA binding proteins during cow early development was characterized. Cumulus-oocyte complexes were collected from slaughterhouse ovaries, matured, inseminated and subjected to embryo culture *in vitro*. Oocyte or preimplantation embryo pools were processed for RT-PCR and whole-mount immunofluorescence analysis of mRNA expression and protein distribution. STAU1 and STAU2, and ELAVL1 mRNAs and proteins were detected throughout cow preimplantation development from the germinal vesicle (GV) oocyte to the blastocyst stage. ELAVL2 mRNAs were detectable from the GV to the morula stage whereas ELAVL2 protein was in all stages examined and localized to both cytoplasm and nuclei. The findings provide a foundation for investigating the role of RBPs during mammalian oocyte maturation and early embryogenesis.

Key words: oogenesis, blastocyst, *in vitro* fertilization, gene expression, transcripts

INTRODUCTION:

Oocyte cytoplasmic maturation involves the accumulation of mRNAs, proteins, substrates and nutrients that are required to achieve the oocyte developmental competence that fosters embryonic development (Calder et al., 2003; Calder et al., 2001). RNA binding proteins (RBP) influence RNA editing, localization, stability and translation (Saunders and Barber, 2003). Therefore RBPs may contribute to oocyte competence by regulating the stability and turnover of oogenetic mRNAs. In addition, variations in oocyte competence may reflect differences in RBP levels and thus turnover of their target mRNAs during early development.

As a prelude to investigating these possibilities it is imperative that the expression of RBPs during mammalian oocyte maturation and early embryogenesis be first determined. Staufen is a Drosophila RBP that assists in establishing the anterior-posterior embryonic axis by regulating target mRNA localization in the Drosophila oocyte (St Johnston et al., 1991). There are two mammalian staufen genes, STAU1 (Marion et al., 1999) and STAU2 (Duchaine et al., 2000). ELAVL1 (HuR, HuA, elrA) is a widely expressed RBP (Fan and Steitz, 1998), which is present in the Xenopus embryo (Good, 1995) and can shuttle between the nucleus and cytoplasm (Atasoy et al., 1998). It binds to A-U rich sequences (AREs) to regulate mRNA half-life (Atasoy et al., 1998; Fan and Steitz, 1998). ELAVL2 (HuB, elrB, Hel-N1/2) is also an ARE binding RBP (Jain et al., 1997), which is predominantly neuronal but is also expressed in the ovary, testis, oocyte and Xenopus embryo (Good, 1995). ELAVL2 affects mRNA degradation, and may increase protein translation (Jain et al., 1997). The present study was conducted to characterize the expression of Staufen and ELAVL mRNAs and proteins during oocyte maturation and preimplantation development in the cow.

MATERIALS AND METHODS:

Production of Bovine Embryos In Vitro

Cumulus-oocyte complexes were isolated, placed into oocyte maturation and were fertilized *in vitro* and cultured up to the blastocyst stage as outlined in (Madan et al., 2005). Bovine ovaries were transported from a slaughterhouse in sterile saline at 32-37^{o}C for oocyte collection using standard protocols. Cumulus-oocyte complexes (COCs) from 3-6 mm follicles were aspirated into the follicular aspiration medium consisting of Dulbecos's phosphate buffered saline (Gibco BRL; Invitrogen, Burlington, ON), 0.3% bovine serum albumin (Sigma-Aldrich Canada Ltd, Oakville, ON) and 50 μg/ml Gentamycin (Sigma-Aldrich Canada Ltd.) using an 18G needle attached to vacuum suction apparatus. COCs that contained an oocyte with an evenly granulated cytoplasm and surrounded by more than three layers of cumulus cells were selected for maturation *in vitro*. For maturation *in vitro* COCs were cultured in oocyte maturation medium composed of modified synthetic oviductal fluid (SOF) medium with 0.8% BSA, modified Eagle medium (MEM) non essential amino acids (Gibco), MEM essential amino acids (Gibco),1mM glutamine, 0.5% μg/ml FSH and 1μg//ml 17β-estradiol. 50 COCs were placed in each well of a 4-well culture plate and incubated in a humidified atmosphere for 24h at 38.5°C and 5% CO_2 in air atmosphere.

For fertilization *in vitro,* 50 mature COCs were added to 330 μl drops containing modified Tyrode lactate medium (TLH) supplemented with 0.6% BSA fatty acid free (Sigma-Aldrich), 0.2 mM pyruvic acid, 10 μg/ml heparin and 50 μg/ml gentamycin under mineral oil. Frozen semen was thawed and processed through a standard "swim-up" method (Madan et al., 2005). Sperm concentration was adjusted to and added to each insemination culture drop at 1 x 10^6 spermatozoa/ml. COCs and spermatozoa were co-incubated in a humidified 5% CO_2 in air atmosphere at 38.5°C and for 15-18 h.

Following fertilization, zygotes were denuded of investing cumulus cells by vortexing, washing three times in culture media consisting of SOF1 plus 0.8% BSA, MEM non essential amino acids, 1mM glutamine and 1.5 mM glucose and 10 μM EDTA. Subsequently, 20-30 zygotes were placed into 50 μl culture drops under mineral oil and cultured at 38.5°C, 5% CO_2, and humidified air with reduced oxygen atmosphere (7%). Following 3 days of culture, SOF1 medium was replaced with SOF2 medium, which contained 0.8%, BSA, MEM non essential amino acids, MEM essential amino acids, 1mM glutamine and 1.5 mM glucose. Pools of 20 embryos were harvested at timed stages of development [2-, 4-, 8-cells, morulae, and blastocysts] for RNA extraction or application of immunofluorescence localization methods.

Primer Design

Primer sets were designed to recognize and amplify conserved nucleotide sequences encoding human and murine Staufen or ELAVL cDNAs. cDNA sequences and/or homologue(s) were identified using BLAST (Basic Local Alignment Search Tool) computer program (NCBI, Bethesda, MD). Primers were designed using the 'Primer3' computer program (Whitehead Institute, Cambridge, MA) and the corresponding oligonucleotides (Table 1), were synthesized (Invitrogen, Burlington, ON).

RNA extraction, reverse transcription and PCR

Total RNA was extracted from bovine embryos (pools of 20 embryos/stage at 1-, 2-, 4-, 8-cell, morula and blastocyst stages) as described (Madan et al., 2005). The total RNA extracts were digested with deoxyribonuclease (DNAse)-1 to eliminate possible contamination from genomic DNA. The RT reactions were conducted using oligo-dT primers (Gibco BRL) as previously described (Barcroft et al., 1998; Calder et al., 2005; Calder et al., 2003; Calder et al., 2001; Natale et al., 2004; Offenberg et al.,

2000). Samples were incubated for 90 min at 42°C in a 20 µl volume of 50mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.5 mM dNTPs, and 200 units of Superscript II (Gibco BRL) followed by heating the samples to 95°C for 5 min reaction termination.

PCR was conducted as described (Calder et al., 2005; Calder et al., 2003; Calder et al., 2001). Briefly, two embryo equivalents for each stage of development under investigation were used per PCR reaction, which was repeated a minimum of three times from pools of three different developmental series of embryos. PCR products were resolved on 2.0% agarose gels containing 0.5 µg/ml ethidium bromide (Invitrogen, Burlington, ON). To confirm the specificity of each PCR product, representative amplicons were extracted from the gels and purified using a QIAquick Gel Extraction Kit (Qiagen, Mississauga, ON) and submitted for nucleotide sequencing (DNA Sequencing Facility, Robarts Research Institute, London, ON, Canada). The nucleotide sequences were compared to sequences available in GenBank to confirm the specificity of each PCR product.

Whole-mount indirect immunofluorescence

To localize Staufen and ELAVL proteins in bovine oocytes and preimplantation embryos we employed whole-mount immunofluorescence methods as outlined in (Calder et al., 2005; Calder et al., 2003; Calder et al., 2001; Madan et al., 2005). Embryos (oocytes, 2-, 4- 8-cell, morula and blastocyst stages) were washed in 1X phosphate buffer saline (PBS) and then fixed in 2% paraformaldehyde (PFA) in PBS for 20 min at room temperature. These fixed embryos were washed in 1X PBS and either processed immediately for immuno-labeling or stored at 4°C in PBS + 0.09% sodium azide for a maximum of 3 weeks. Fixed embryos were permeabilized and blocked in 1X PBS + 5% goat Serum + 0.01% Triton X-100 for 1 hour at room temperature. Embryos were washed in 1X PBS and incubated with primary antibody diluted 1:100 in 1X PBS +1% goat Serum + 0.005% Triton X-100 for one hour at

room temperature followed by additional washes totaling 1 hour at 37ºC. Primary antibodies were detected by exposure for one hour to FITC-conjugated secondary antibodies (Jackson Immuno Labs, MA, USA) diluted 1:200. Embryos were then treated with rhodamine-conjugated phalloidin (5µg/ml; 1:20) and DAPI (1mg/ml; 1:2000) for 30 minutes at 37ºC followed by 2 washes for 2 hours each at 37ºC. Embryos were mounted in Fluoro-Guard Antifade Mounting Reagent (BioRad, Mississauga, ON, Canada). Fluorescence patterns were examined using a Zeiss LSM 410 (laser scanning microscope) with an inverted Axiovert 100 microscope under 20-40X magnification. The images were then captured and stored as TIFF files by the Zeiss LSM software package. Rabbit polyclonal anti-human STAU1, ELAVL1 and ELAVL2 antibodies were obtained from (Chemicon, Temecula, CA).

RESULTS AND DISCUSSION:

STAU1 mRNA was detected from the germinal vesicle (GV) oocyte to the blastocyst stage (Fig. 1A). The primers used to amplify STAU1 revealed two mRNA isoforms expressed in oocytes and embryos, which differ due to a splicing event resulting in an insertion of 18bp (6aa) in RNA binding domain 3, similar to isoforms reported in mouse (Duchaine et al., 2000). STAU1 protein was detected in the cytoplasm of bovine oocytes and embryos at all stages examined (Fig.1C-K). Staufen was observed in the GV, pronuclei or nuclei up to the morula stage but this localization was rarely stronger than cytoplasmic fluorescence (Fig. 1 C-K). STAU2 mRNA was also detected in all cow preimplantation stages (Figure 1B). ELAVL1 mRNA was detected continuously from the GV oocyte to the blastocyst stage (Fig. 2A). ELAVL1 protein was cytoplasmic at all stages examined, but was also present in the GV and most nuclei at all stages, but was less evident at the blastocyst stage (Fig. 2 C-K). ELAVL2 mRNAs were detected from the GV oocyte to the 8-cell stage in all replicates (Fig. 2B). ELAVL2 mRNA was detected only in some replicates at the morula stage and not at all at the blastocyst stage

(Fig. 2B). Two ELAVL2 mRNAs were detected. The larger band corresponds to a 539bp product common to human and mouse sequences in Genbank (mouse, **NM_207685**). In the mouse, there is an ELAVL2 mRNA splice variant lacking 36nt, this isoform would amplify as 503bp (**NM_207686**). Another isoform lacks the bases AGG (R) before this splice junction, the 36 bp and has the addition of AGT (S) at the end of the splice junction, making it also 503bp **(AY035379)**. Yet another splice variant reported contains the 5' R, misses the 36nt, and contains the 3' S, making it 506bp **(AY035378)**. However, a human variant contains only 500bp and the 5' R, the 36nt and the 3' S are missing (**BC030692**). ELAVL2 immunofluorescence was cytoplasmic at all stages examined, but was also detectable in the GV, pronuclei and most nuclei, and was brighter in the nucleus than cytoplasm at particularly the 4- and 8-cell stages (Fig. 2 L-T). At the blastocyst stage, ELAVL2 also appeared at the borders of trophectoderm cells (Fig. 2S).

As the vast majority of mammalian *in vitro* matured oocytes are meiotically competent, deficiencies in cytoplasmic maturation are proposed as a primary reason for their low developmental rates. Transcription and storage of maternal mRNAs occurs during follicular growth and slows as the oocyte reaches mature size (Fair et al., 1995). The embryo is dependent on stored maternal mRNAs until at least the maternal-zygotic transition (MZT), when transcription of embryonic genes begins in earnest with a major burst of embryonic transcription initiating at the 8-cell stage in the cow (Telford et al., 1990). Many maternal mRNAs become deadenylated during oocyte maturation and early cleavage (Brevini-Gandolfi et al., 1999). Over 200 RBP candidate genes have been identified in Drosophila (Lasko, 2000). Regulated mRNA stability occurs in mammalian cells in response to nutrient levels, hormones and environmental stresses such as hypoxia and heat stress (reviewed by (Guhaniyogi and Brewer, 2001). It has been suggested that variations in transcriptional activity largely govern changes in embryonic mRNA abundance associated with exposure to sub-optimal environments (Niemann and

Wrenzycki, 2000). However, oocyte and embryo competence could also be affected by environmental effects acting through RBPs to alter transcript stability or translation.

During oogenesis, oocytes acquire a depository of maternally-encoded transcripts that must be stored and remain dormant until their translation is required following oocyte activation and insemination. This delay between their production and translation is important as the oocyte becomes transcriptionally silent during the final stages of folliculogenesis and transcription is not renewed until post-insemination and early embryogenesis (Schultz, 2005). Yet few studies have investigated mRNA stability during preimplantation development. Instead, the majority of studies have focused upon understanding transcriptional regulation (Knijn et al., 2002; Knijn et al., 2005; Niemann and Wrenzycki, 2000; Rinaudo and Schultz, 2004; Wrenzycki et al., 1999; Wrenzycki et al., 2005). Our study provides the first indications that gene products encoding Staufen and ELAVl RBPs are expressed during bovine preimplantation development.

Preimplantation mammalian embryos attempt to respond positively to the pressures that sub-optimal culture environments place upon them (Ho et al., 1994; Ho et al., 1995; Niemann and Wrenzycki, 2000; Watson et al., 2000). The embryo, (at least partially), compensates for missing components or offsets the presence of deleterious components, by regulating its developmental program (Niemann and Wrenzycki, 2000). This capacity however must operate within a defined range of tolerances, (Bavister, 2000; Bolton, 1992; Gardner et al., 2002; Leese, 2002; Schultz and Williams, 2002) and current media, are still sub-optimal for all species. We are aware that current media are still sub-optimal since cultured embryos from all species display a reduced pregnancy rate following embryo transfer, and are also prone to metabolic and growth disorders that may find their origin in a culture induced “metabolic re-programming” during the preimplantation development period as compared to their *in vivo* derived counterparts (Adamiak et al., 2005; Barker, 2003; Boerjan et al., 2000; McEvoy et

al., 2001; McEvoy et al., 2000; Sinclair et al., 1999; Sinclair et al., 2003; van Wagtendonk-de Leeuw et al., 2000).

Due to these concerns great effort has been focused on understanding how early embryos adjust their developmental program to compensate for exposure to sub-optimal culture environments. Approaches in this research area have included studies directly comparing the levels of "marker" gene expression, also variations in embryo metabolism between *in vitro* and *in vivo* derived preimplantation embryos and most recently epigenetic re-programming as signified by variation in DNA methylation patterns (Brevini-Gandolfi et al., 1999; De Sousa et al., 1998; Ecker et al., 2004; Knijn et al., 2005; Leese, 2002; Mann et al., 2004; Watson et al., 2000; Wrenzycki et al., 1999; Wrenzycki et al., 2005). The prevailing interpretation of the majority of these studies is that gene transcription is influenced by exposure to varying culture environments. Only a very few studies have begun to examine the influences of varying culture environments on mRNA deadenylation, and mRNA stability (Brevini-Gandolfi et al., 1999; Gandolfi and Gandolfi, 2001; Temeles and Schultz, 1997). In addition, there is a need to understand the role of RBPs in regulating preimplantation mRNA stability as these proteins represent reasonable targets for culture induced influences on embryonic mRNAs (Yang et al., 2005; Yang et al., 2006; Yang et al., 2005; Yu et al., 2003). This study therefore provides a foundation for investigating the roles of RBPs during early development and will allow for investigations regarding their putative roles in regulating preimplantation development and more importantly, eventually, their collective roles in regulating mRNA transcripts during early embryogenesis.

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Table 1. Primers used to detect RBP mRNAs in cow oocytes and embryos

		Sequence	Anneal Temp.	Size in bp	Accession #
STAU1	5’	ATTTCCAGTC/TCCACCTTTAC	53	318bp 300bp	BC082277 mouse NM_001037328 human
STAU1	3’	TGGTCACAAAGTTCTTCAT			BC082277 mouse NM_001037328 human
STAU2	5’	GAAGTTGCTACTGGAACAGG	52	342bp	XM_872380 cow
STAU2	3’	AGCTGAACTACTCGATGTGG			XM_872380 cow
ELAVL1	5’	AAGACCACATGGCG/CGAAGAC	59	474bp	NM_010485 mouse NM_001419 human
ELAVL1	3’	TTGCCTCTTCTGCC/TTCC/TGAC			NM_010485 mouse NM_001419 human
ELAVL2	5’	AGGTCACTGGCATATCAAGG	55	539bp 500-506bp	NM_207686 mouse NM_004432 human
ELAVL2	3’	TCCATTGAGGCTAC/GC/GTATCG			NM_207686 mouse NM_004432 human

Fig 1. Staufen mRNA and protein during cow preimplantation development. **A:** Staufen 1; **B**: Staufen 2; **C-K**: Staufen protein in cow oocytes and embryos. Green is FITC-labeled 2' antibody against rabbit anti-staufen. Red is rhodamine-phalloidin stain for F-actin. Blue is DAPI stained nuclei. GV-germinal vesicle stage; 24h, *in vitro* matured 24h; Mat-matured 24h *in vitro;* 1c-1 cell stage; 2c-2 cell stage; 4c-4 cell stage; 8c-8 cell stage; Mor-morula stage; Bl-blastocyst stage; Neg-negative control; MW, 100bp molecular weight standard; +, positive control tissue, br=brain. Bar is approximately 50µm. N=3 replicates, 25-39 embryos examined at each stage.

Fig.2. ELAVL mRNA and protein during cow preimplantation development. **A**: ELAVL1; **B**: ELAVL2; **C-K** ELAVL1 protein, N=3 replicates, 29-48 embryos examined at each stage; **L-T** ELAVL2 protein, N=3 replicates, 25-43 embryos examined at each stage. Green is FITC-labeled 2' antibody against rabbit anti-ELAVL1 or 2. Red is rhodamine-phalloidin stain for F-actin. Blue is DAPI stained nuclei. GV-germinal vesicle stage; 24h, *in vitro* matured 24h; Mat-matured 24h *in vitro*; 1c-1 cell stage; 2c-2 cell stage; 4c-4 cell stage; 8c-8 cell stage; Mor-morula stage; Bl-blastocyst stage; Neg-negative control; MW, 100bp molecular weight standard; +, positive control tissue, br=brain, gc=granulosa cells. Bar is approximately 50µm.

Fig.1. Staufen mRNA and Protein During Cow Preimplantation Development

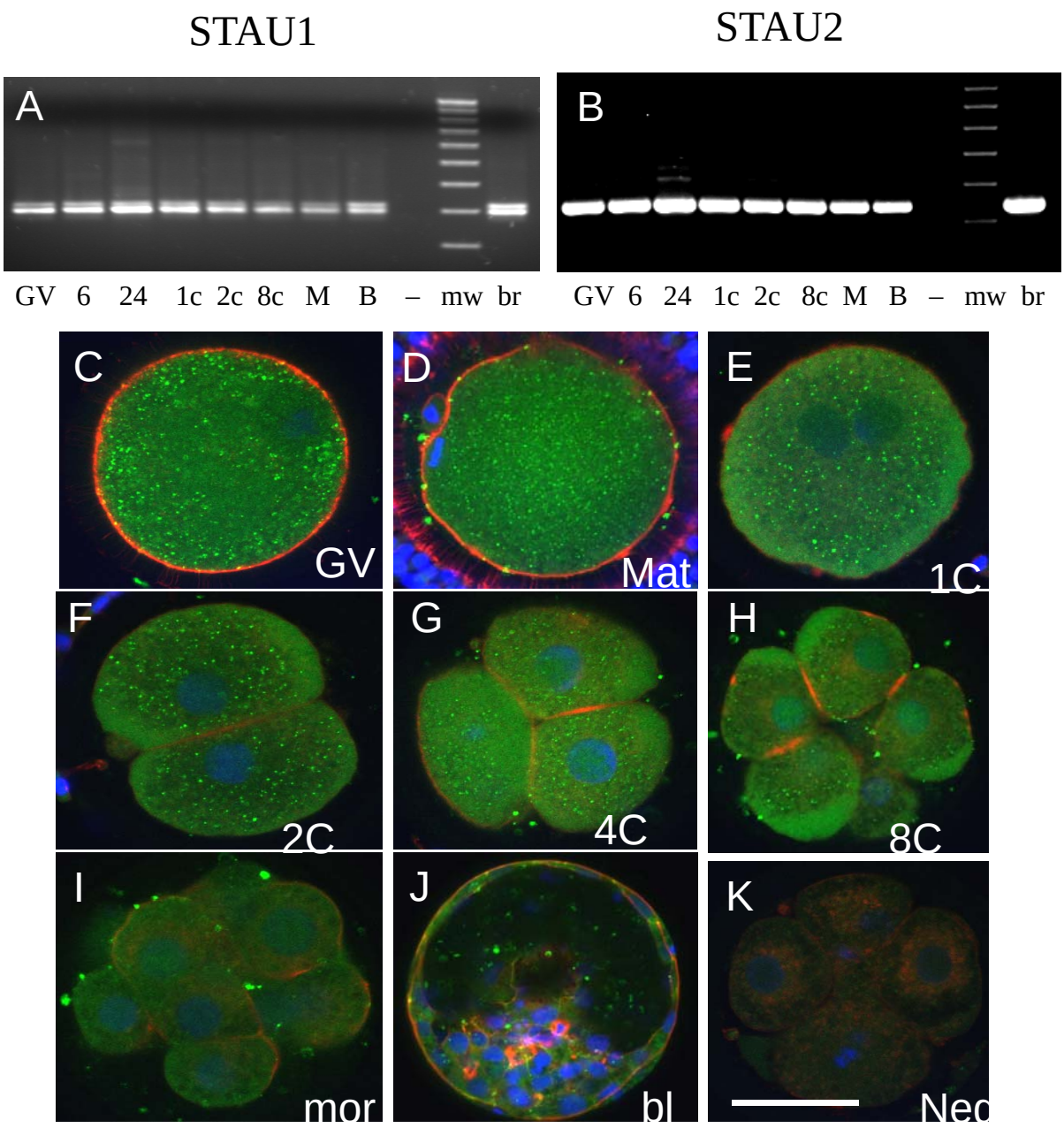

Fig. 2. ELAV mRNA and Protein During Cow Preimplantation Development