ABSTRACTS FOR POSTER PRESENTATION

Gene Expression

230 TRANSCRIPTOMICS ANALYSIS OF CHANGES IN GENE EXPRESSION IN BOVINE OVIDUCT EPITHELIAL CELLS DURING THE ESTROUS CYCLE

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The oviduct epithelium undergoes marked morphological and functional changes during the estrous cycle. It has been shown that a dramatic change in the frequencies of ciliated and non-ciliated cells occurs during the estrous cycle. At estrus the epithelium consists of secretory and ciliated cells and at diestrus mainly of ciliated cells. The oviduct provides the microenvironment for sperm capacitation, fertilization, and early cleavage-stage embryonic development. At the molecular level, only a few genes or proteins are known that change during the estrous cycle and which may be important for fertility, so as the bovine oviduct-specific glycoprotein, the major secretory protein in the oviduct. Therefore, we studied systematically the changes in gene expression in bovine ipsilateral oviduct epithelial cells at estrus and diestrus. To identify differentially expressed genes, a combination of subtracted cDNA libraries and cDNA array hybridization was used. Two subtracted libraries were produced to enrich cDNAs of upregulated genes at estrus and at diestrus. A total of 1536 cDNA clones of each library were analyzed with radioactively (33-P) labeled probes generated from the oviduct epithelial cells of six Simmental heifers, three of them slaughtered at Day 0 (estrus) and three at Day 12 after standing heat (diestrus). After normalization of the raw data and statistical analysis, all cDNAs showing significant differences in their expression levels at estrus compared to diestrus were sequenced. Sequencing revealed 84 different cDNAs; 42 of them matched bovine genes or their human/mouse homologs with known functions, and 42 matched genes without a known function. Half of the genes ($n = 42$) were expressed at a higher level at estrus; for the other ($n = 42$) expression levels were higher at diestrus. The regulated genes or their products represented a variety of functional classes, such as genes of the secretory pathway, genes involved in transcription regulation, cell-surface proteins, cell–cell interaction proteins, secreted proteins, members of signal transduction pathways, immune-related proteins, and some enzymes. The identification of genes differentially regulated in ipsilateral oviduct epithelial cells at estrus v. diestrus is the first step of a systematic analysis of differential gene expression during the estrous cycle. Further studies will follow, focusing on different compartments of the bovine oviduct and additional times of the estrous cycle.

231 EXPRESSION PATTERN OF CERTAIN DEVELOPMENTALLY IMPORTANT GENES IN BOVINE NUCLEAR TRANSFER EMBRYOS PRODUCED USING CELL LINES OF DIFFERENT EFFICIENCY

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Developmental abnormalities associated with the cloning process suggest that reprogramming of donor nuclei into an embryonic state may not be fully completed in most of the cloned animals. One of the areas of interest in this respect is the analysis of gene expression patterns in nuclear transfer embryos to dissect the processes that failed and to develop means to overcome the limitations imposed by these factors. In this study, we investigated the expression patterns of histone deacetylase-1,-2,-3 (*HDAC-1,-2,-3*), DNA methyltransferase-3A (*DNMT3A*) and octamer binding protein-4 gene (*POU5F1*) in donor cells with different cloning efficiencies (low: no-pregnancy, medium: pregnancy but no live birth and high: live birth) and nuclear transfer embryos derived from these cell lines using a real time reverse transcription-polymerase chain reaction (RT-PCR) assay with SYBR green chemistry. Employing standard protocols, we produced nuclear transfer embryos from three different cell lines categorized as having varying efficiencies in supporting development to term. Embryos were collected at morula, blastocyst and hatched blastocyst stages and total RNA was extracted from pools of 4–5 embryos using Absolutely RNA nanoprep kit (Stratagene, La Jolla, CA, USA). Relative level of expression at these stages was analyzed using $\Delta\Delta$CT method with *HH2A* as the reference gene and in vitro-fertilized embryos as the control samples. Statistical analysis was performed on ranked expression data employing SAS statistical analysis software procedure ANOVA. Same set of genes were also analyzed on

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donor cells using standard curve method. All genes investigated were affected by nuclear transfer and followed somewhat altered expression patterns. In general, expression of HDAC genes was elevated especially at the compact morula stage but became comparable to control embryos at the hatched blastocyst stage. *DNMT3A* expression in NT embryos was lower than in IVF embryos at all stages. *POU5F1* transcript levels were also reduced in nuclear transfer embryos at the compact morula and blastocyst stages. The difference, however, disappeared at the hatched blastocyst stage. There was a cell line effect on the expression patterns of all genes investigated. Cell lines efficient in producing offspring tended to resemble control embryos in gene expression patterns compared to inefficient cell lines. These results agree with several studies reporting altered gene expression patterns for certain genes in cloned embryos. Our data also suggest that cell line differences in developmental competency observed in cloning experiments might be related to physiological differences in transcriptional regulation and nuclear remodeling, DNA methylation, and lineage differentiation in embryos cloned from these cell lines.

232 SEARCH FOR THE BOVINE HOMOLOG OF THE MURINE PED GENE

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The aim of the current study was to identify the bovine homolog of the murine Ped (preimplantation embryo development) gene, which regulates mouse preimplantation embryonic growth, including cleavage rate and embryo survivability, and to characterize the expression pattern of this gene during bovine preimplantation embryo development. Experiment (I): The National Center for Biotechnology Information (NCBI) GenBank/EBI EST databases were searched for bovine-expressed sequence tags (EST) that were homologous with the murine Ped gene (Accession number: NM_010394). The resulting ESTs were aligned and assembled in to one complete sequence (841 bp), which was shown to be homologous with the Murine Ped gene and the Bovine Major Histocompatibility Complex class I 4221.1 gene (Accession No.: AJ010865, length 1090 bp). The expression of the protein product of the Ped gene by bovine tissue was confirmed using Western Blot analysis. Experiment (II): The expression pattern of the Ped gene homolog during in vivo and in vitro bovine preimplantation embryo development was characterized using real time PCR. Embryos at the same stage for age were compared (Day 1: 2-cell; Day 2: 4-cell; Day 3: 8-cell; Day 4: 16-cell; Day 5: early morula; Day 6, compact/late morula; Day 7: blastocyst). The relative transcript abundance was consistently lower in the in vitro-cultured embryos at all stages of preimplantation development the differences were significant on Days 2, 4, 5, 6, and 7. The relative transcript abundance was significantly lower on Days 1, 2, and 3 of in vivo culture than on Days 4, 5, 6, and 7 and was significantly higher in Day 7 blastocysts than in Day 5 early morula. In in vitro-cultured embryos the relative transcript abundance was significantly higher in Day 7 blastocysts compared to all other stages of the preimplantation period. Experiment (III): A quantitative analysis of the Ped gene homolog was carried out on replicates of pools of ten 2-cell embryos collected at 25, 28, 32, and >36 hpi from three different fertilizations. Transcript relative abundance was highest in those embryos that had cleaved by 25 hpi. By 28 hpi abundance had decreased slightly; as time to cleavage increased further to 32 and >36 hpi, the relative abundance decreased significantly. In conclusion, we have successfully identified a potential bovine homolog of the murine Ped gene. Furthermore, we have characterized the expression pattern of this gene during preimplantation embryo development in cattle and we have shown that a greater relative abundance of the gene transcript is associated with embryos of higher quality and greater developmental potential.

233 EXPRESSION OF INSULIN-LIKE GROWTH FACTOR (IGF) MRNA IN BOVINE CONCEPTUSES FROM EMBRYOS PRODUCED IN VIVO OR IN VITRO

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The objective of this study was to determine the effect of in vitro embryo production (IVP) on expression of mRNAs for IGF-1, IGF-2, IGF-1 receptor (IGF-1R), IGF-2R and GAPDH in bovine conceptuses at Day 17 of gestation. In vivo embryos (In vivo) were recovered from superovulated Holstein cows. For IVP, Holstein oocytes were matured, fertilized and then cultured in M199 with 10% serum (IVPS) or 1% BSA (IVPSR) to 72 hpi. All embryos were then transferred to M199 with 10% serum and cultured to 168 hpi. The same Holstein sire was used to produce all embryos. Single grade 1 blastocysts were transferred into recipient heifers. On day 10 after transfer, conceptuses were recovered, measured and stored at −80°C prior to extraction of whole cell (wc) RNA and genomic (g) DNA. wcRNA was assessed for quality based on A260/A280 ratio and visualization of 18S and 28S ribosomal RNA. Only complete (intact) conceptuses with at least 10 μg high-quality wcRNA recovered after extraction were included in the analysis (In vivo, $n = 8$; IVPS $n = 8$; IVPSR $n = 7$). Conceptus sex was determined by PCR analysis of gDNA. Semi-quantitative PCR assays were used to assess relative expression of all mRNAs. For each conceptus, 2 μg wcRNA were mixed with 3 pg rabbit globin mRNA, DNase-treated and reverse-transcribed using random hexamers. Conceptus cDNA samples (140 ng each for IGF-1, IGF-2 mRNAs; 100 ng each for IGF-1R, IGF-2R, GAPDH and globin mRNAs) were assayed in duplicate within single assays. Relative mRNA expression was calculated as the ratio of band intensities representing the mRNA of interest to globin mRNA. All data were analyzed for effects of treatment (In vivo, IVPS, IVPSR or In vivo, IVP), sex, stage of blastocyst development at transfer (early, mid, expanded) and the interactions of treatment sex and treatment stage. Conceptus length (LSM ± SEM mm) did not differ with treatment (In vivo: 182 ± 55; IVPS: 257 ± 47; IVPSR: 310 ± 72). Expression of GAPDH mRNA was greater ($P = 0.02$) in female conceptuses (1.31 ± 0.11) than in males (0.96 ± 0.07) and did not differ ($P = 0.14$) between treatment groups (In vivo: 1.30 ± 0.11, IVPS: 1.00 ± 0.09, IVPSR: 1.10 ± 0.14). However, when the IVP groups were combined and compared to In vivo controls, expression of GAPDH mRNA was both greater ($P = 0.002$) in females (1.38 ± 0.10) than in males (0.94 ± 0.06) and differed ($P = 0.03$) with treatment (In vivo: 1.30 ± 0.10, IVP: 1.01 ± 0.07). Expression of IGF-1 and IGF-2 mRNA was not detected in the majority of conceptuses. Expression of IGF-1R mRNA was greater

($P = 0.02$) in female (1.19 ± 0.10) than in male conceptuses (0.87 ± 0.07) and differed ($P = 0.02$) with stage of blastocyst development at transfer (early: 0.79 ± 0.07; mid: 1.23 ± 0.13; expanded: 1.07 ± 0.11). There was no effect of treatment on expression of IGF-1R mRNA. Expression of IGF-2R mRNA differed ($P = 0.03$) with treatment (In vivo: 0.85 ± 0.10; IVPS: 0.76 ± 0.09; IVPSR: 0.33 ± 0.13). In conclusion, expression of both imprinted and non-imprinted genes is altered in conceptuses resulting from embryos produced in vitro. Supported by the North Carolina Agricultural Research Service.

234 IMPRINTING STATUS OF DEVELOPMENTALLY IMPORTANT GENES IN BOVINE PREIMPLANTATION EMBRYOS

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Several imprinted genes have been identified in different species such as mouse and man but in cattle imprinting has only been confirmed for the Insulin-like growth factor 2 receptor gene (Igf2r). DNA methylation is one of the most common mechanisms of imprinting. Imprinting is correlated with the methylation of normally unmethylated CpG islands, and aberrations in methylation are thought to be involved in the Large Offspring Syndrome (LOS) which is frequently observed in offspring derived from in vitro-produced and/or cloned embryos. The imprinting of the bovine Insulin-like growth factor 2 gene (Igf2), the Igf2r gene and the Mammalian achaete scute homologue 2 gene (Mash2) was analyzed in bovine preimplantation blastocysts cultured in SOF + BSA. mRNA levels of the Igf2r gene and the Mash2 gene in in vitro-produced (IVP) and parthenogenetic expanded single blastocysts were analyzed by semi-quantitative RT-PCR (Wrenzycki C *et al.*, 2001 Biol. Reprod. 65, 309–317). Genes expressed exclusively from the maternal allele (i.e. paternally methylated) should be represented by a higher relative abundance of transcriptional products in parthenogenetic embryos whereas paternally expressed genes (i.e. maternally methylated) should be correlated with a higher gene expression in IVP embryos carrying one paternal and one maternal allele. For determination of methylation patterns by bisulfite sequencing, sequences of several DNA fragments from the bovine Igf2 gene were identified in samples from bovine uterus and kidney. The primer pairs were generated from the ovine and bovine Igf2 sequences available in the Genebank database (accession number U00664/X53553). DNA fragments identified were from the 5′ untranslated region and the 3′ translated region of the bovine Igf2 gene. All fragments consist of a high number of CG dinucleotides, and computer analysis using the CpGwin program (Anbazhagan R *et al.*, 2001 BioTechniques 30, 110–114) revealed CpG islands within these fragments. Relative abundances of transcriptional products were statistically analyzed using the SigmaStat 2.0 (Jandel Scientific, San Rafael, CA, USA) software package. After testing for normality, an ANOVA followed by multiple pairwise comparisons using the Tukey test was employed. Results from the semi-quantitative RT-PCR analyses revealed no difference ($P > 0.05$) in gene expression pattern of the Igf2r gene, suggesting that the Igf2r gene is biallelically expressed during bovine preimplantation development. In contrast, there was a significant difference ($P < 0.05$) in the relative abundance of mRNA of the Mash2 gene, with a lower relative abundance of transcriptional products in parthenogenetic expanded blastocysts. Thus, in cattle as reported in mice (Guillemot F *et al.*, 1995 Nat. Genet. 9, 235–242), the paternal allele of the Mash2 gene seems to participate in the expression of the gene prior to implantation.

235 RETINOID-DEPENDENT mRNA EXPRESSION IN BOVINE OOCYTES PREMATURED AND/OR MATURED IN VITRO

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As a transcription factor, retinoic acid (RA) can activate or silence a wide number of genes, thus inducing differentiation in cell systems and playing a role in cell cycle regulation. However, little is known of RA-dependent gene expression in the oocyte. Bovine oocytes and cumulus cells express most RA receptors, and the presence of 9-cis-RA during in vitro maturation (IVM) is beneficial to oocyte development (Duque *et al.*, 2002 Hum. Reprod. 17, 2706–2714; Hidalgo *et al*., 2003 Reproduction 125, 409–416). The present work analyzes the relative abundance of various developmentally important gene transcripts in bovine oocytes during in vitro prematuration and/or maturation. Cumulus-oocyte complexes (COCs) were manipulated in defined medium with polyvinyl-alcohol (DM-PVA). Those COCs undergoing prematuration were cultured for 24 h in DM-PVA with 25 μM roscovitine. For IVM, some prematured COCs were cultured for 24 h in DM-PVA containing pFSH, LH and E2. Incubations were made at 39°C in an atmosphere of 5% CO_2 in air and high humidity. Within experiments, COCs were cultured with nM 9-cis-RA 5, in 1% ethanol (both as vehicle and inhibitor of endogenous RA synthesis), 3% ethanol, 5% ethanol and untreated. Using Real Time PCR (10 oocytes per group) (Rizos *et al*., 2003 Biol. Reprod. 68, 236) we examined the relative mRNA expression of genes involved in protection against free oxygen radicals (Mn-superoxide dismutase, MnSOD), glucose metabolism (glucose-6-phosphate dehydrogenase, G6PDH) and cell cycle events (Cyclin B1 and H1). Data (of 4 replicates) were analyzed by ANOVA and Duncan test ($P < 0.05$). Regarding immature oocytes, prematuration in 1% ethanol increased cyclin B1 expression and decreased cyclin H1, while 9-cis-RA increased G6PDH. Maturation without additives increased cyclin B1 and G6PDH, but decreased cyclin H1 and MnSOD expression; opposite trends were observed under increasing ethanol dosages (3% and 5%). Maturation with 1% ethanol or 9-cis-RA enhanced cyclin B1 and G6PDH, while reducing cyclin H1 and MnSOD expressions. The presence of 9-cis-RA during both prematuration and maturation processes tended to show more prominent effects than the ones observed when it was present only during prematuration or maturation alone. In our study, in presence of 9-cis-RA during both prematuration and maturation processes, the expression of cyclin B1 and G6PDH tended to increase, while cyclin H1 and MnSOD tended to decrease. However, the differences with the control group without additives were not significant. Our study during both prematuration and maturation processes show that beneficial effects of RA on oocyte developmental competence may not be related to the alteration of mRNA expression of the four genes analyzed. Grant support: Spanish Ministry of Science and Technology (AGL-2002-01175; 2003-05783).

236 LOCALIZATION OF INTERFERON-TAU IN BOVINE EMBRYOS AND CUMULUS CELLS BY CONFOCAL MICROSCOPY

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Interferon-tau (IFN-t) is a protein secreted by the conceptus of ruminant species and thought to be the primary signal in maternal recognition of pregnancy. Experiments were conducted to detect IFN-t in bovine oocytes, cumulus cells and embryos by use of immunocytochemistry and laser-scanning confocal microscopy. Embryos were produced by in vitro fertilization of in vitro-matured oocytes. Oocytes and embryos were fixed in formaldehyde at various stages of development, and stored in PBS until staining and microscopy. Cumulus cells were stripped from immature oocytes and cultured in M-199 (10% fetal calf serum) on coverslips treated with poly-D-lysine. They were divided into four treatment groups: (1) without hormones (control), (2) with the addition of FSH, (3) estradiol, or (4) FSH and estradiol. Bovine MBDK cells (ATCC CCL 22) and primary fibroblasts were cultured as controls on coverslips but without addition of hormones. A polyclonal antibody raised against bovine IFN-t was used, followed by a secondary conjugated antibody (AlexaFluor 488, Molecular Probes, Eugene OR). Actin was stained with phalloidin (AlexaFluor 568, Molecular Probes, Eugene OR). Cumulus, MDBK cells and fibroblasts were further stained with propidium iodine to visualize nuclei. Imaging was performed on a Leica laser-scanning confocal microscope. IFN-t was detected in hatched and unhatched Day 7 and Day 9 blastocysts, where its expression was restricted to the trophectoderm. IFN-t was also found in Day 5 and 6 morulae, but not at earlier stages. Furthermore, IFN-t was detected in the cumulus cell masses of oocytes before and after IVM, but not in the oocyte itself. Controls, in which the primary antibody was omitted, were negative regardless of developmental stage. IFN-t was also found in cultured cumulus cells regardless of whether hormones had been added to the medium; however, the protein was localized in the nuclei of cells only if they had been cultured with FSH, whereas in cells cultured with estrogen alone or without hormones IFN-t was restricted to the cytoplasm. In contrast, no IFN-t was detected in MDBK cells or fibroblasts. These results extend previous findings by showing that IFN-t is expressed as early as the morula stage. Moreover, these results demonstrate that IFN-t is also produced by cumulus cells where FSH appears to initiate a translocation of IFN-t into the nucleus, suggesting a role in regulation of gene expression.

237 EFFECTS OF IN VITRO V. IN VIVO CULTURE ON EXPRESSION OF EMBRYO DERIVED GENE TRANSCRIPTS INVOLVED IN APOPTOSIS IN SINGLE BOVINE EMBRYOS

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Earlier studies reported that the level of apoptosis in in vitro-produced bovine blastocysts is higher then in in vivo developed blastocysts (Gjorret *et al.*, 2003: Biol. Reprod, in press). The molecular basis for this difference has not yet been studied. The regulation and execution of apoptosis is dependent on a cascade of events in which many proteins are involved. The aim of the present study was to analyze expression of BAX, a pro-apoptotic, and BCL-XL, a anti-apoptotic member of the BCL-2 family, and heat shock protein (HSP 70.1) transcripts in blastocysts cultured in vitro or in vivo. Furthermore, to verify if these transcripts detected in the blastocysts stages were newly expressed from the embryonic genome, a RNA polymerase II specific inhibitor, α-amanitin, was added to the culture medium from the zygote stage onwards, to block transcription. For the in vitro group, embryos were obtained from abattoir oocytes after IVM/IVF and IVC in SOF medium. For the in vivo group, embryos were collected from normally cyclic cows, superovulated with 3000 IU eCG (Intergonan; Intervet, Tönisvorst, Germany) at day 7 po by non-surgical uterine flushing. The developmental stage of the embryos was determined with stereomicroscopy, and early blastocysts (eb), blastocysts (b), and expanded blastocysts (xb) were collected and frozen at −80°C. For transcription inhibition experiment, embryos were cultured in vitro as for the in vitro group until the 8- to 16-cell stage at 100 h after the start of fertilization, one group with 10 mM α-amanitin added to the culture medium (α-amanitin group) and the other group without (control group). A highly sensitive semi-quantitative RT-PCR assay (Wrenzycki *et al.*, 1999) Mol. Reprod. Dev. 53, 8–18) was used to determine the relative levels of gene transcripts in single blastocysts and pooled 8- to 16-cell embryos. The relative abundance was calculated on a per cell basis per embryo. Assays were repeated on average eight times. Data on the relative expression of transcripts in blastocysts were analyzed by Anova followed by multiple pairwise comparisons using the Tukey test. No significant differences in relative abundance between in vitro and in vivo cultured embryos, for any of the developmental stages, were found for the three apoptosis related genes. The molecular basis for the difference in level of apoptosis between in vitro and in vivo cultured blastocysts is not related to the level of expression of BAX, BCL-XL and HSP transcripts but other genes involved in the apoptotic cascade may be responsible for the reported differences. The expression of BCL-XL and HSP 70.1 transcripts in the blastocysts was from embryonic origin as no expression of these transcripts was detected in the 8- to 16-cell stage embryos treated with α-amanitin. The expression of BAX gene transcripts was not affected by α-amanitin. Probably the maternally derived transcripts are very stable and not yet degraded at the 8- to 16-cell stage.

Table 1. Relative abundance (±SEM) of BAX, BCL-XL, and HSP 70.1 transcripts in bovine single in vitro or in vivo-produced eb, b, and xb and in vitro produced pooled 8- to 16-cell stage embryos treated with or without α-amanitin

	BAX		BCL-XL		HSP 70.1	
	In vitro	In vivo	In vitro	In vivo	In vitro	In vivo
Early blastocyst	1.4 ± 0.5	1.2 ± 0.4	1.2 ± 0.3	1.3 ± 0.5	0.4 ± 0.1	0.3 ± 0.1
Blastocyst	1.3 ± 0.2	1.1 ± 0.4	1.9 ± 0.3	1.1 ± 0.3	0.3 ± 0.1	0.3 ± 0.1
Expanded blastocyst	1.0 ± 0.3	0.9 ± 0.3	1.5 ± 0.3	1.1 ± 0.6	0.3 ± 0.1	0.5 ± 0.2
8–16 cell control	0.6 ± 0.1		1.0 ± 0.3		1.9 ± 0.5	
8–16 cell α-amanitin	0.7 ± 0.2		0		0	

238 ESTROGEN RECEPTOR ALPHA AND PROGESTERONE RECEPTOR EXPRESSION FROM REPRODUCTIVE TISSUE AND IN VITRO PRODUCED EMBRYOS OF THE DOMESTIC CAT

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The in vitro production of cat embryos has been reported by several laboratories, and kittens have been born after transfer of embryos derived by IVF, ICSI, and NT. However, evidence accumulating in other species indicates that in vitro-derived embryos exhibit altered gene expression of developmentally important genes. Because the domestic cat genome is not well defined, the lack of primer sequence information poses a challenge for gene expression profiling. Estrogen, in addition to its essential role in the development and function of the female reproductive tract, is important for maturation of the oocyte. The aim of this preliminary study was to evaluate the expression profile of estrogen receptor alpha (ERα) and progesterone receptor (PR) in domestic cat female reproductive tissue and in vitro-produced (IVP) embryos. Embryos were produced in vitro as described by Gomez *et al.* (2003 Theriogenology 60, 239–251). mRNA was isolated from pools ($n = 35$–50) of IVP domestic cat embryos at the morula and blastocyst stages (Days 7 and 8) using a modified protocol of the PolyAT Tract System (Promega, Madison, WI, USA). Uterus and ovaries were collected from hystorectomized cats and total RNA was isolated from these estrogen-targeted organs (RNeasy standard protocol, QIAGEN, Valencia, CA, USA). All RNA was reverse-transcribed and subjected to real-time PCR to evaluate expression of ERα and PR. The housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was used as a control for expression in all tissues. Using primers designed to amplify human sequences or multispecies primers, we successfully amplified all three genes from as little as ~100 ng of ovarian or uterine mRNA. We were also able to detect ERα and GAPDH from pools of IVP embryos. Transcripts were cloned and confirmed by sequencing to be homologous to known sequences of the respective genes in a variety of species, including human, mouse, and pig. A 234-bp transcript of ERα (accession #AY349164; GenBank) corresponding to exons 5 through 7 of the human ERα gene (hormone-binding domain) was identified along with a 110-bp sequence of PR and 98-bp sequence of GAPDH. To our knowledge, this is the first description of ERα or PR cloning in the domestic cat. In summary, we have demonstrated that highly sensitive real-time PCR is effective for the assessment of gene expression in cat ovarian and uterine tissue, as well as in embryos, the latter, of which, are known to have a low transcript copy number. Furthermore, these experiments provide the basis for future studies on the effect of exogenous estrogen on gene expression in cat IVM oocytes and IVP embryos.

239 IDENTIFICATION OF DIFFERENTIALLY EXPRESSED GENES IN PORCINE PARTHENOTES AT 2-CELL AND BLASTOCYST STAGES USING ANNEALING CONTROL PRIMER TECHNOLOGY

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Identification of embryo-specific genes will provide insight into early embryonic development. However, the current methods employed to identify genes expressed at specific stages are labor intensive and produce a high degree of false positives. In the present study we employed an accurate PCR technology controlled by an annealing control primer (ACP, SeeGene, Seoul, Korea) to identify differentially expressed genes in presumptive porcine parthenotes. In vitro-matured porcine oocytes were parthenogenetically activated by square electrical direct current pulses. After 3 h of culture in North Carolina State University (NCSU) 23 medium with 0.4% BSA containing 7.5 mg mL^{-1} cytochalasin B, embryos were washed and cultured in NCSU 23 medium with 0.4% BSA. Total RNA was prepared from a pool of 200 2-cell stage and a pool of 100 blastocyst-stage porcine parthenotes using a Dynabeads mRNA DIRECT Kit. First-strand cDNA synthesis was carried out using dT-ACP1, wherein the 3′-end core portion comprised a hybridizing sequence complementary to a poly (A) region of mRNA transcripts. Second-strand cDNAs were synthesized using PCR with an arbitrary ACP whereby the 3′-end core portion of the dT-ACP2 was prevented from annealing to the first-strand. The PCR products were electrophoresed on 2% agarose gel and stained with ethidium bromide. The differentially expressed bands were extracted and cloned into a TOPO TA cloning vector (Invitrogen, Seoul, Korea), sequenced and analyzed by BLAST search. To date we have identified 21 differentially expressed genes using 28 arbitrary ACPs. BLAST analysis revealed that most of these genes have strong homology (more than 95%) with known mouse and human genes. Differentially expressed genes in the 2-cell stage include GDP dissociation, putative translation initiation factor (SUI1) and succinate dehydrogenase iron-protein subunit. The blastocyst specific genes include rennin binding proteins, ATPase inhibitor, catenin, ribosomal protein S15a, ribosomal protein large P1, puromycin sensitive aminopeptidase, BMDP gene, arginine N-methyltrasferase p77 isoform, acylamino acid-releasing enzyme, ribosomal protein S14 and KIAA0630 protein. RT-PCR confirmed the expression of these genes in porcine parthenotes during preimplantation development. In conclusion, ACP technology is a specific and sensitive method to locate differentially expressed genes in preimplantation mammalian embryos.

240 OVINE PREPUBERTAL OOCYTE SHOWS ALTERATE GENE EXPRESSION AND LOW DEVELOPMENTAL COMPETENCE

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The aim of this work was to evaluate developmental competence and gene expression of prepubertal and adult ovine oocytes. GV prepubertal and adult oocytes were matured, fertilized and cultured in vitro until blastocyst stage; the time (days) needed to reach this stage was recorded. Blastocysts

developed on different days were cultured for hatching to evaluate their quality in relation to cleavage rate. Adult and prepubertal GV oocytes and blastocyst-stage embryos produced, respectively, at 6 and 7 days were compared for quantitative expression of poly(A) polymerase (polyA-P), glucose transporter I (Glut-I), desmocollin II (desmoII), plakofilin (plako) and heat shock protein 70.1 (HSP70) genes. Confirming previous results (Ledda *et al.*, 1996 Zygote 4, 343–348), fertilized prepubertal ovine oocytes developed to blastocyst stage at lower rates than the adult ones (19.9 v. 51.3%, respectively, $P < 0.001$) and this stage was delayed 24 h in prepubertal compared to adult embryos ($P < 0.01$), reflecting a lower quality (Fenwick *et al.*, 2002 Hum. Reprod. 17, 407–412) of the former. In fact, 44.7, 25.0, 30.3 and 0% of adult blastocysts were obtained after 6, 7, 8 and 9 days, respectively, of postfertilization culture compared to 0, 48.4, 34.3 and 17.2% of prepubertal ones. Faster-developed blastocysts showed higher hatching rate in both prepubertal (54.8%, 7 days of culture) and adult (89.8%, 6 days). Hatching rate dropped to 18.2% when blastocysts were obtained at 8–9 days in prepubertal and to 54.5% and 32.5% at 7 and 8 days, respectively, in adult embryos. Analysis of gene expression showed that HSP70, plako and desmo genes were not expressed in GV oocytes, and Glut-I mRNA was lower in prepubertal GV oocytes than in the adult ones ($P < 0.01$). All genes were expressed in blastocysts; we found that Glut-I was at lower levels ($P < 0.01$) in prepubertal-derived blastocysts whereas HSP70 was highly expressed ($P < 0.05$) in prepubertal blastocysts than in those derived from adult oocytes. In conclusion this work shows that prepubertal ovine oocytes have a lower developmental competence compared to the adult ones, correlated to an altered gene expression during the growth phase of the oocyte and early embryonic development. Supported by MIUR (cofin).

241 POLYADENYLATION STATUS OF mRNAs CODING FOR PRDX-6, GDF-9, G6PDH AND CYCLIN B1 BEFORE AND AFTER IN VITRO MATURATION OF BOVINE OOCYTES: REAL-TIME PCR RESULTS AFTER REVERSE TRANSCRIPTION WITH OLIGO(dT) OR WITH HEXAMERS

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Changes in length of the polyA tail of an mRNA is determinant for its stability and translation. During oocyte maturation, although some genes are specifically polyadenylated, an important decrease of polyadenylated mRNA is generally observed. Both degradation and de-adenylation can be responsible for this decline but little information about these processes is available yet for in vitro-matured bovine oocytes. Total RNA was extracted from 2 pools of 50 immature and 2 pools of 50 in vitro matured bovine oocytes, after addition of 1 pg of polyadenylated rabbit globin mRNA to each pool. The RNA of each pool was then divided in 8 samples, half of them were reverse-transcribed using hexamers and the other half using oligo(dT). Six genes were amplified in every sample using real-time PCR and specific molecular beacon probes: (1) rabbit globin, an exogenous gene added as a reporter for the efficiency of extraction and RT; (2) histone H2A, an endogenous gene reporter for RNA quantity and quality in each pool [This housekeeping gene, that is not adenylated, should be maintained at a constant level through the maturation process (Robert *et al.*, 2002 Biol of Reprod. 67, 1425–1472)]; (3) two antioxidant enzymes, peroxyredoxin 6 (PRDX-6) and G6PDH preventing oxidative stress during maturation [PRDX-6 also has a phospholipase A2 activity that could be implicated in prostaglandins synthesis during maturation]; (4) GDF-9, an oocyte-specific gene involved in cumulus expansion during maturation; and (5) cyclin B1, one subunit of the MPF factor whose activity is required for meiosis resumption. The PCR result of each gene was normalized with the globin value of the same sample. After this standardization, the variability of the results among the 4 samples of a same pool similarly reverse transcribed did not exceed 15%. Within a pool, the mean PCR signal obtained for a specific mRNA using oligo(dT) was compared with the mean signal obtained with hexamers. Results obtained for immature and mature oocytes are reported in the Table 1. Before maturation, the PCR signals were higher when reverse transcription was performed using oligo(dT) instead of hexamers but that was not the case after maturation. Most messengers coding for these genes probably lost their polyA tails during maturation. This loss was much less pronounced for cyclin B1 messengers. Furthermore, for all genes studied, the PCR results obtained after reverse transcription using hexamers and normalized with the histone H2A value did not show a decrease during the maturation process. In conclusion, deadenylation, more than degradation, would be responsible for the disappearance of maternal polyadenylated mRNA during maturation.

Table 1. Ratio of the PCR signals obtained after RT with oligo(dT) v. hexamers

Oocyte status	Cyclin B1	PRDX-6	GDF-9	G6PDH
Immature	2.9 ± 0.65*	3.75 ± 0.7	3.05 ± 1.06	5.48 ± 1.23
Mature	1.6 ± 0.02	0.71 ± 0.13	0.51 ± 0.15	0.69 ± 0.13

*Values ± SEM.

242 GLUTATHIONE CONTENT AND EXPRESSION OF ANTIOXIDANT ENZYME mRNAs IN SHEEP OOCYTES

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Reactive oxygen species (ROS) are normal products of embryo metabolism; however, oxidative injury may negatively impact development if ROS production exeeds antioxidant defense mechanisms. Protection of the embryo against ROS is dependent, in part, upon the pool of antioxidant enzyme mRNA and products stored in the oocyte. Previous work has demonstrated that retinol administration to superovulated ewes, followed by natural service, resulted in embryos with improved competence to develop in vitro. In other cell systems, retinoids have been shown to participate in the antioxidant network and redox system. The objectives of this study were to analyze glutathione content and characterize antioxidant enzyme mRNA

expression in mature ovine oocytes and test the hypothesis that retinol administration affects levels of these products. Ewes were superovulated and administered retinol on the first and last day of FSH injection. Oocytes were collected from oviducts 60 h later and either prepared for glutathione (GSH) analysis or subjected to RNA isolation. Glutathione was quantified by the recycling assay of the enzyme 5,5-dithiobis-(2-nitrobenzoic acid)-glutathione reductase using a total of more than 260 oocytes. Transcripts encoding for manganese superoxide dismutase (Mn-SOD), copper zinc superoxide dismutase (Cu-Zn SOD), glutathione synthetase (GS) and glutathione transferase pi (GSTp) were analyzed in a total of 86 single oocytes by semi-quantitative RT-PCR using a histone H2a transcript as the internal control. Chi-square analysis was performed to detect differences due to treatments. Glutathione content did not differ significantly between oocytes collected from retinol-treated (6.78 ± 3.81 pmol/oocyte) and control (6.38 ± 1.58 pmol/oocyte) ewes. Transcripts encoding for Mn-SOD, Cu-Zn SOD, GS and GSTp were detected in greater than 95% of the oocytes analyzed. Relative abundance of these transcripts did not differ between treatments. For the first time, glutathione content and antioxidant enzyme mRNA expression has been analyzed in in vivo-matured sheep oocytes. Retinol treatment did not appear to affect the expression of these products. Results demonstrate the existence of endogenous antioxidant defense products, stored as mRNA and protein, in sheep oocytes.

243 TEMPORAL DIVERGENCE IN THE PATTERN OF mRNA EXPRESSION IN BOVINE EMBRYOS CULTURED FROM THE ZYGOTE TO BLASTOCYST STAGE IN VITRO OR IN VIVO

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The objective of this study was to examine the time during the post-fertilization culture period that gene expression patterns of in vitro cultured bovine embryos diverge from those of their in vivo cultured counterparts. Presumptive bovine zygotes were produced by IVM/IVF of immature oocytes collected from the ovaries of slaughtered animals. At approximately 20 h post-insemination (hpi), presumptive zygotes were randomly divided into two culture groups, either in vitro in synthetic oviduct fluid or in vivo, and transferred into the ewe oviduct. Embryos were recovered from both systems at approximately 30 hpi (2-cell), two (4-cell), three (8-cell), four (16-cell), five (early morula), six (compact morula) or seven (blastocyst) days pi and snap-frozen for the analysis of transcript abundance using real-time PCR. The transcripts studied were interferon-tau, apoptosis regulator box-a (Bax), connexin 43, sarcosine oxidase, glucose transporter 5, mitochondrial Mn-superoxide dismutase, insulin-like growth factor II, and insulin-like growth factor-I receptor, most of which are known from our previous work to be differentially transcribed in blastocysts derived from culture in vitro or in vivo. Analysis was done on pools of 10 embryos. Data were analyzed using one-way repeated measures ANOVA. The relative abundance of the transcripts studied varied throughout the preimplantation period and was strongly influenced by the culture environment. For example, transcripts for interferon-tau were detected from the 8-cell stage onwards in in vitro-cultured embryos but not until the early morula stage in those cultured in vivo. Levels of this transcript increased significantly at the compact morula and blastocyst stages in both groups but were significantly higher ($P < 0.05$) in in vitro-cultured embryos at both stages. mRNA for Bax was not detected before the 8-cell stage in in vitro cultured embryos and not until the 16-cell stage in in vivo cultured embryos. The abundance of this transcript increased significantly thereafter up to the blastocyst stage in both groups. The level of expression was significantly higher ($P < 0.05$) at all stages of development in in vitro-cultured embryos than those cultured in vivo. The relative abundance of Cx43 transcripts decreased in both in vitro- and in vivo-cultured embryos at the 8- to 16-cell stage. Levels remained low thereafter in the in vitro-cultured embryos but significantly increased in those cultured in vivo. Transcript abundance was significantly higher in in vivo cultured embryos from Day 4 onwards with a ten-fold difference presence at the blastocyst stage. Differences also existed for the other transcripts studied. These data demonstrate that changes in transcript abundance in blastocyst stage embryos are in many cases a consequence of perturbed transcription earlier in development. Depending on the transcript, these differences may be evident in as short as 10 h of culture.

244 REAL TIME PCR EVALUATION OF EXPRESSION PROFILES OF SOME MATERNAL-SOURCED GENE TRANSCRIPTS IN IN VITRO PRODUCED PRE IMPLANTATION STAGE BOVINE EMBRYOS

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Gene expression profiling data collected in a time series and quality related parameters are important for understanding the developmental mechanisms carried out in a developing embryo, and are also a source to enrich the knowledge base of embryo development. However, such data are frequently constrained by limitation and handling of the sample as well as cost associated with generating such data. Cumulatively, these factors have contributed to the existing insufficient data compared to the large need stemming from a drive to control and guide optimum embryo development. In this ongoing study, with objectives to quantify and evaluate gene transcripts identified from certain developmental stages, expression profiles of two ESTs (C256 and C112), derived from an oocyte cDNA library, were analyzed from the above perspectives to understand the change in the level of these gene transcripts throughout the pre-implantation stage of embryo development. For this analysis, pools of oocytes and embryos were prepared by balancing the amount proportional to the number of cells present. mRNA was isolated separately from each pool of matured oocytes, 2-cell, 4-cell, 8-cell, and 16-cell stages, as well as morula and blastocyst stages by using Dynal beads Oligo $(dt)_{25}$ (Dynabeads, Dynal Biotech, Oslo, Norway) following the manufacturer's recommendations. These mRNAs were checked for DNA contamination and, when proved free, first-strand cDNA was synthesised by reverse transcribtion at 42°C for 2 h following standard laboratory procedures. Transcript quantification was performed by real-time PCR using gene-specific primers, equal amounts of cDNA from each sample and SYBR Green universal master mix. Following this analysis, both transcripts were found to be expressed in a wave-like manner being highly expressed in mature oocytes, declining gradually as the development stage advanced, with the lowest level at the 16-cell stage, and then reviving in level thereafter until it reached blastocyst stage. Taking the 16-cell stage as calibrator

for both, C256 was 26.4, 23.2, 8.5, 1.7. 2.4 and 2.7 times more expressed in oocyte, 2-cell, 4-cell, 8-cell, morula and blastocyst stages, respectively. Similarly, C112 was 110.7, 169.2, 9.8, 2.5, 4.1 and 7.3 times more expressed in oocyte, 2-cell, 8-cell, morula and blastocyst stages, respectively. These expression patterns suggest the probable origin of these transcripts initially to be maternal. C256 is strongly similar to human retinoid X receptor beta (RXRb) gene (NM_021976.3), which is involved in transcriptional functions and in increasing DNA binding, whereas C112 is strongly similar to TATA box-binding protein-associated factor gene (AY189986.1), which is also involved in transcriptional functions. As seen from their functions, these transcripts can be vital for developing the embryo and the variations at different developmental stages shows their most probable role as part of genes contributing to developmental competence in pre-implantation development stages.

245 RELATIVE ABUNDANCE OF HSP 70 mRNA IN BOVINE EMBRYOS PRODUCED IN VITRO USING DIFFERENT EMBRYO/VOLUME RATIOS IN CULTURE

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In spite of in vitro embryo production systems having been greatly improved over recent years, employing a variety of culture conditions (media, protein sources, gas atmosphere, etc.), we still do not know much about the real necessity of embryos to develop under the same conditions as occur in vivo. These differences between in vivo and in vitro culture at preimplantation embryonic stages can produce deviations in gene expression and in normal fetal development (large offspring syndrome). Heat shock proteins (Hsp) are engaged in cell response to regulatory signals or perturbations in the microenviroment and can be used as a sensitive indicator of stress caused by suboptimal culture conditions (Wrenzycki *et al.*, 2001 Hum. Reprod. 16, 893–901). Hsp act as chaperones in facilitating protein folding and assembly and stabilize damaged proteins to prevent aggregation of fragments, thereby allowing repair or degradation. The aim of the present study was to investigate the effects of different embryo/volume ratios on bovine embryo development and the relative abundance of Hsp 70.1 gene transcripts. In this experiment, oocytes were isolated from slaugterhouse ovaries and matured, fertilized and cultured in groups of 5, 10, 20 or 30 per each drop of 100 μL. The oocytes were matured in TCM 199 supplemented with 0.4% BSA. After maturation, oocytes were fertilized in TALP medium, using frozen/thawed sperm, selected using a percoll density gradient. The zygotes were cultured to the morula or Day 7 blastocyst stage employing SOF supplemented with 0.4 % BSA. Developmental check points were cleavage rate (Day 3 pi), blastocyst formation (Day 8 pi) and hatching (Day 11 pi). A semi-quantitative RT-PCR assay was used to determine the relative levels of gene transcripts in single embryos at morula (Day 6) and blastocyst (Day 7) stages (Wrenzycki *et al.*, 2001 Biol. Reprod. 65, 309–317). Data of cleavage, blastocyst formation and hatching rates were analyzed using chi-square test. Relative abundance (RA) of Hsp 70.1 mRNA were compared in tested groups using ANOVA followed a Tukey test. Differences at $P < 0.05$ were considered significant. Results show that no significative difference in hatching rate per blastocyst produced was detected among the four groups. Cleavage rate and blastocyst formation were significantly higher in groups with 5, 10 and 20 embryos compared with drops containing 30 embryos. Hsp transcripts were detected in morula and blastocyst stages in all groups. In morula stage, no differences were observed in the RA of Hsp 70.1 mRNA among groups with 5, 10, 20 and 30 embryos cultured per drop. However, in blastocyst stage, the RA was significantly increased in the group with 20 embryos per drop as compared to the group with 5 embryos. The results show that different embryo/volume ratios in culture influence not only cleavage rate, blastocyst formation and hatching rate, but also expression of Hsp 70.1 gene. Further studies changing other culture conditions and using in vivo-derived bovine embryos will aid in elucidating which culture systems are ideal to produce bovine embryos in vitro. This research was supported by CAPES/DAAD program and CNPq.

246 IMPROVEMENT OF THE DEVELOPMENTAL CAPACITY OF OOCYTES FROM PREPUBERTAL CATTLE BY INTRAOVARIAN IGF-I APPLICATION

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The developmental potential of oocytes from prepubertal cattle is lower compared to their adult counterparts. Differences between oocytes from calves and cows have been found with regard to size, ultrastructural characteristics and metabolism. GH and IGF-I receptor mRNA have been identified in the cumulus cells and in oocytes (Izadyar F *et al.* 1997 Mol. Reprod. Dev. 47, 175–180; Armstrong D. *et al.*, 2002 Reproduction 123 (6), 789–797). It is known that IGF-I and GH affect mRNA expression of glucose transporter 1 (Glut1) as well as glucose uptake by embryos. The goal of this study was to improve the developmental capacity of oocytes from prepubertal cattle and to determine whether an induced increase of GH and IGF-I levels affects the mRNA expression pattern of Glut1, UBF and eIF1A. Holstein calves ($n = 30$), 6–7 months old, were randomly divided into three groups. One group received a single s.c. injection of 500 mg of somatotrophin (rbST Posilac, Monsanto Company, St. Louis, MO, USA) and during the subsequent 2 weeks their follicles were aspirated 4 times via transvaginal ultrasound-guided ovum pick-up (OPU). The second group received an intraovarian injection of 6 μg rhIGF-I (R&D Systems, Wiesbaden-Nordenstadt, Germany). The third group served as control and received an intraovarian injection of 0.6 mL of 10 mM acetic acid. All animals were i.m. injected with 60 mg FSH (Folltropin®; Vetrepharm Inc., Ontario, Canada) 48 h prior to aspiration. The treatments were repeated with the same animal groups at the ages of 9–10, 11–12 and 14–15 months. Five adult cows were i.m. injected each with 100 mg FSH. For mRNA expression analysis (RT-PCR), embryos were collected with 2–4 cells, 8–16 cells and as blastocysts. The relative abundance of mRNA for Glut 1 and eIF1A was higher ($P < 0.05$) in 8–16 cell embryos from IGF-I-treated calves and cows than in control and rbST treated calves. The in vitro embryo production (IVP) results are shown in the Table 1. Our data show that IGF-I increased mRNA expression of Glut1 and eIF1A which may improve the developmental capacity of embryos produced from calves.

Table 1. OPU/IVP-Results for calves (6–15 months old) in comparison with cows

Groups	*COC/aspiration	*COC usable/aspiration	(%) dividing to 2 cell stage	No. blastocysts (%)
Control ($n = 80$)	(622) 7.7^{ab}	(343) 4.3^{a}	55^{a}	38 (11^{a})
IGF-I ($n = 79$)	(670) 8.4^{a}	(351) 4.4^{a}	65^{ab}	92 (26^{b})
rbST ($n = 77$)	(442) 5.7^{b}	(218) 2.8^{b}	75^{b}	34 (16^{a})
Cows ($n = 50$)	(444) 8.9^{a}	(270) 5.4^{a}	76^{b}	68 (25^{b})

*Tukey Test ($^{a,b}P < 0.05$), chi square ($^{a,b}P < 0.05$), n = total number of aspirations.

247 GENE EXPRESSION OF MAMMALIAN RELATIVE OF DNAJ IN BOVINE ENDOMETRIUM DURING EARLY PREGNANCY

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The mammalian relative of DNAJ (MRJ), a member of the molecular chaperones that are known as heat shock proteins, plays an important role in the process of murine chorioallantoic fusion. The objective of this study was to determine the expression pattern of MRJ in the bovine endometrium during early and mid-pregnancy using quantitative RT-PCR. Twenty-eight Japanese Black cows, aged between 1.2 and 15.2 years, with normal estrous cycles, were used in this study. Twenty-one cows were used for study of the level of MRJ during pregnancy. They were artificially inseminated, and their endometrial tissues were collected on Days 16 to 21 ($n = 7$), 30 to 36 ($n = 6$), 48 to 49 ($n = 4$) and 74 to 140 ($n = 4$) of pregnancy. Seven cows were used as controls for the study of cyclic level of MRJ, and their endometrial tissues were collected on Days 13 to 14 ($n = 4$) and 17 to 20 ($n = 3$) of the estrous cycle. The caruncles and the intercaruncles were isolated from the endometrial tissues. All tissues were frozen immediately using liquid nitrogen. Total RNA from these samples was extracted from the tissue using Trizol (Invitrogen Corp., Carlsbad, CA, USA). The first strand of cDNA was synthesized from the total RNA by oligo $(dT)_{12–18}$ and SuperScript™ II RNase H- Reverse Transcriptase (Invitrogen Corp.) according to the manufacturer's instructions. Primers and the TaqMan probe for MRJ and glyceraldehyde-phosphate-dehydrogenase (GAPDH) were designed using the primer design software Primer Express™ (Applied Biosystems, Foster City, CA, USA). Bovine GAPDH was used as an internal standard. All PCR reactions were performed using a TaqMan™ PCR Reagent Kit and a MicroAmp Optical 96-Well Reaction Plate and Cap (Applied Biosystems). The assay used an ABI Prism 7700 Sequence Detector (Applied Biosystems). Signals were detected according to the manufacturer's instructions. The relative level of MRJ expression was calculated on the basis of GAPDH quantity (the method of calculation: relative level = MRJ quantity/GAPDH quantity). Data were analyzed by one-way ANOVA, and means were conpared by Tukey-Kramer's HSD test. Mammalian relative of DNAJ genes were expressed in all samples examined; the levels in intercaruncle tended to be greater than those in caruncle. Although MRJ expression level at Days 16 to 21 of pregnancy was greater than at other days during pregnancy, there were no significant differences between the levels at Days 16 to 21 of pregnancy and those of the estrous cycle. These results suggest that MRJ is produced in the endometrium and may play a role in early and mid-pregnancy and the estrous cycle.

248 EXPRESSION AND LOCALIZATION OF VASCULAR ENDOTHELIAL GROWTH FACTOR AND VASCULAR ENDOTHELIAL GROWTH FACTOR RECEPTOR 2 IN EQUINE FOLLICLES

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Ovarian follicles undergo pronounced morphological changes, alternating between periods of growth and regression. The equine follicle will grow to an average of 45 mm in diameter at ovulation, and during the phase of growth, there is an increase in blood supply to the follicle. Vascular endothelial growth factor (VEGF) is a cytokine that interacts with tyrosine kinase receptors to stimulate angiogenesis, endothelial cell proliferation and vascular permeability. The aim of the study was to evaluate the expression and localization of VEGF and the VEGF-receptor 2 (VEGF-R2) in equine follicles. Ovaries were collected from a slaughterhouse. Granulosa cells from follicles were pooled regardless of the size of the follicles. Western blots were performed using protein extracted from granulosa cells and follicular fluid. Blots were probed with rabbit anti-human VEGF and rabbit anti-mouse VEGF-R2 antibodies and visualized with chemiluminescence. Total RNA was extracted from granulosa cells and integrity of the RNA samples was tested by the amplification of β-actin. Complementary DNA was synthesized by reverse transcription, followed by polymerase chain reaction amplification of cDNA encoding with bovine primer sequences for VEGF and VEGF-R2. The PCR product was resolved on 1% agarose gel and the resulting VEGF and VEGF-R2 bands were sequenced. Immunostaining for VEGF and VEGF-R2 was performed on fixed, paraffin-embedded sections of follicle wall from follicles larger than 30 mm. Western blot analysis of granulosa cell lysates revealed 22 kDa bands for VEGF, and 210 kDa bands for VEGF-R2. VEGF protein was present in follicular fluid, whereas VEGF-R2 was not detectable. RT-PCR experiments revealed the presence of VEGF and VEGF-R2 mRNA in isolated granulosa cells. Sequencing demonstrated 93% and 99% homology to known sequences of equine VEGF and VEGF-R2, respectively. Immunofluorescence experiments performed on dissected equine follicles localized VEGF to the granulosa cell layer and sporadically to the theca cell layer. VEGF-R2 co-localized with VEGF in the granulosa cells, and was relatively absent in the theca layer. The present study detected novel expression patterns for VEGF and VEGF-R2 in equine ovarian follicles. The results of these experiments suggest an extra-vascular role for the VEGF family in follicle development. Future studies will be directed at studying the genomic and proteonomic profiles of follicles during the selection of the dominant follicle in mares.

249 BOVINE GRANULOSA CELLS mRNA EXPRESSION OF PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR-α AND THE PROTO-ONCOGENE c-Fos

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PPARα and c-Fos are involved in regulation of gene expression and are known to be dependent on retinoic acid (RA), which in turn influences oocyte growth and developmental competence (Duque *et al*., 2002 Hum. Reprod. 17, 2706–2714; Hidalgo *et al*., 2003. Reproduction 125, 409–416), probably acting in part through granulosa cells. Peroxisome proliferator-activated receptor-α (PPARα) heterodimerizes with the retinoid receptor X (RXR), while c-Jun/c-Fos heterodimerizes with liganded retinoic acid receptors (RARs), then preventing formation of transcription factor activator protein 1 (AP-1) complexes capable of DNA binding. Cellular retinoic acid binding protein (CRABP) limits RA excess and regulates the transcriptional potential of RA; CRABPII has been detected in rat granulosa cells from mature follicles and luteal cells. The aim of this study was to investigate PPARα, c-Fos and CRABPII mRNA expression in bovine granulosa cells. In parallel, other genes whose expression can be influenced by RA were analyzed: luteinizing hormone receptor (LHr), follicle stimulating hormone receptor (FSHr), aromatase and growth hormone (GH). Ovaries were collected at a local abattoir and kept in saline at 30–35°C. Granulosa cells were obtained by aspirating 2- to 7-mm antral follicle contents, pelleted at 700g for 4 min and resuspended in RNA-later (Ambion®). Total RNA was isolated with a NucleoSpin® RNAII kit (Macherey-Nagel), and mRNA was reverse transcribed into single-stranded cDNA using a 1st Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche). A PCR standard method was made using 1 μL of the cDNA as a template. All PCR primer couples were designed on the basis of the bovine sequence, but c-Fos and CRABPII primers were designed based on the human-murine sequences. Primers within the couple were located in different exons to distinguish DNA from RNA amplification. CRABPII was further investigated in bovine whole ovary, corpus luteum (CL) and liver, in a search for positive controls. Bovine β-actin, 18S and 28S were examined in each sample as positive controls for RNA isolation and cDNA synthesis efficiency. Ten μL of product were loaded into an agarose 2% gel in TBE buffer containing ethidium bromide, and were separated by horizontal electrophoresis. Gels were visualized with ultraviolet light and photographed using a digital camera. Gene expression in granulosa was demonstrated for PPARα, c-Fos, LHr, FSHr, aromatase, GH and controls (β-actin, 18S and 28S) but CRABPII gene did not express in granulosa cells, whole ovary, CL or liver under our experimental conditions. While lacking CRABPII expression remains intriguing, the expressed genes support a role of retinoid pathway within granulosa cells under both in vivo and in vitro conditions, because granulosa cells used in the present experiments were derived from follicles providing oocytes for IVM-IVF. Grant support: Spanish Ministry of Science and Technology (AGL-2002-01175).

250 SPECIFIC GENE KNOCK DOWN OF OCT-4 IN MOUSE PREIMPLANTATION EMBRYOS USING SHORT INTERFERENCE RNA

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RNA interference is used to specifically and effectively inhibit the expression of cognate genes. In the present study we investigated the inhibitory effect of gene expression in mouse embryos developing in vitro by injecting short interference RNA (siRNA). Fertilized mouse zygotes were obtained from mated females 20–24 h after hCG injection. Chemically synthesized 21-nt siRNA was commercially obtained and injected into mouse zygotes. The zygotes were then cultured in KSOM medium supplemented with 4% BSA at 37°C. Semi-quantitative RT-PCR was used to examine Octamer-binding transcription factor (Oct-4) gene expression in a single mouse embryo developing in vitro following siRNA-injection. In order to determine the expression and distribution of Oct-4 in mouse embryos, the mouse embryos were fixed in 4% paraformaldehyde for 20 min and permeabilized with 0.2% triton x-100 for 10 min. Embryos were then incubated with rabbit Oct-4 polyclonal antibody for 1 h and with FITC-labeled goat anti-rabbit antibody. Propidium iodide was used for DNA staining. siRNA injection did not retard the development of mouse zygotes. The number of blastocyst cells and the ICM/TE ratio did not differ in the siRNA injected blastocysts and the non-injected control group. Semi-quantitative RT-PCR revealed that Oct-4 expression was decreased at the 4-cell embryo stage and was significantly high at the morula and blastocyst stages. Injection of siRNA into oocytes inhibited RNA expression of Oct-4 and Nanog, but not of E-cadherin and Heat shock protein 70.1. Immunocytochemical staining showed inhibition of Oct-4 synthesis of the morulae and blastocysts following injection of siRNA. After culture of the embryos in the ES cell-derived conditioned medium, the embryos were stained for alkaline phosphatase (AP), a marker specific to pluripotent cells. AP was not detected in the inner cell mass of blastocysts following siRNA injection. These results suggest that siRNA injection into a mouse zygote specifically inactivates Oct-4 in mouse embryos developing in vitro.

251 SEARCH FOR GENES INVOLVED IN DEVELOPMENTAL COMPETENCE IN MOUSE OOCYTES USING SUPPRESSION SUBTRACTIVE HYBRIDIZATION

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During the first month after birth, synchronous follicular growth occurs in the ovary of immature mice (first wave). Previously, we showed that mouse oocytes during the first wave were more competent developmentally in older females (Suzuki O *et al.*, 2002 Theriogenology 57, 628 abst), although the numbers of mature oocytes did not differ with female age (17, 18, and 24 days old). In this study, we examined the genetic factors that affect the developmental competence of mouse oocytes during the first wave using suppression subtractive hybridization (SSH). Oocytes collected from 17- and 24-day-old B6D2F1 females (D17 and D24, respectively) without hormonal treatment were matured in Waymouth medium supplemented

with pyruvate (0.23 mM), antibiotics, bovine fetuin (1 mg mL^{-1}), and polyvinylpyrrolidone (3 mg mL^{-1}). After 17-h culture at 37°C in an atmosphere of 5% CO_2, 5% O_2, and 90% N_2, total RNA was isolated from oocytes whose germinal vesicles had broken down (mature oocytes), separately, in three independent culture groups per age (each group contained oocytes from four animals) using Cell-to-cDNA Cell Lysis Buffer (Ambion, Austin, TX, USA). Some of the total RNA from each independent group was pooled by age (total of RNA from approximately 100 oocytes per age) and used for SSH. A SMART cDNA Synthesis Kit (Clontech, Palo Alto, CA, USA) was used to reverse-transcribe total RNA to cDNA. SSH was performed with a PCR-Select cDNA Subtraction Kit (Clontech). The subtracted PCR products were cloned into pGEM-T vector (Promega, Madison, WI, USA). Clones from the subtracted library (D24−D17) were sequenced and their identities were examined using the NCBI BLAST search. The differential expression of candidate genes preferentially expressed in mature D24 oocytes suggested by SSH was confirmed with cDNA transcribed separately in the three independent culture groups per age using real-time quantitative PCR with an ABI Prism 7900HT with TaqMan technology (Applied Biosystems, Foster City, CA, USA). Of 513 clones sequenced, the top six preferentially-expressed candidate genes in more developmentally-competent D24 oocytes were spindlin (20 clones), bmi-1 (4 clones), cyclin B1 (4 clones), E330034G19Rik (4 clones), Jagged1 (4 clones), and Ndfip2 (4 clones). The expression of spindlin in mature D24 oocytes (relative threshold cycle: -3.8 ± 0.7, mean ± SD) was confirmed to be approximately 11-fold higher than in D17 oocytes (-0.3 ± 1.5) when GAPDH was used as an internal control ($P < 0.05$, *t*-test). Quantitative analyses of mRNA expression of the remaining genes are now under way. Our results suggest that spindlin is one of the key factors leading to the acquisition of developmental competence in mouse oocytes during folliculogenesis. Supported by JSPS KAKENHI (No.145716000) and MHLW.

252 A COMPARATIVE EXPRESSION ANALYSIS OF GENES IN PREIMPLANTATION DEVELOPMENTAL STAGES OF BOVINE EMBRYOS PRODUCED IN VITRO OR IN VIVO

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A comparative analysis of mRNA expression patterns between embryos produced under different in vitro and in vivo culture systems allows the isolation of genes associated with embryo quality and investigation of the effect of culture environment on the embryonic gene expression. In this study, expression analysis of four known (PSCD2, TCF7L2, NADH-subunit and PAIP1) genes and one novel transcript, derived from differential display PCR, was performed in in vitro (Ponsuksili *et al.*, 2002, Theriogenology 57, 1611–1624) or in vivo- (Moesslacher *et al.*, 2001 Reprod. Dom. Anim. 32, 37) produced bovine 2-, 4-, 8-, 16-cell, morula and blastocyst stage embryos using real time PCR technology. Poly(A) RNA was isolated from four separate individual embryos from each developmental stage and embryo group (in vitro or in vivo) using Dynabeads mRNA kit (Dynal, Oslo, Norway). After reverse transcription, quantitative PCR was performed with sequence specific primers in an ABI PRISM® 7000 Sequence Detection System instrument (Applied Biosystems, Foster City, CA, USA) using SYBR® Green as a double-strand DNA-specific fluorescent dye. Standard curves were generated for target and endogenous genes using serial dilutions of plasmid DNA. Final quantification was done using the relative standard curve method, and results were reported as relative expression or *n*-fold difference to the calibrator cDNA (i.e., the blastocyst stage) after normalization with the endogenous control (Histone2a). Data were analyzed using SAS version 8.0 (SAS Institute Inc., NC, USA) software package. Analysis of variance was performed with the main effects being the developmental stage and embryo source (in vitro or in vivo) and their interactions followed by multiple pairwise comparisons using Tukey's test. No significant difference was observed in the relative abundance of the PSCD2 gene between the two embryo groups. However, its expression was higher (20-fold) ($P < 0.05$) at the 8-cell stage than the other developmental stages among in vitro embryos. Higher expression ($P < 0.05$) of NADH-subunit mRNA was detected in vivo than in vitro at the 2-cell stage of development. The TCF7L2 mRNA was expressed in the in vitro embryos but not in the in vivo ones. PAIP1 mRNA was higher ($P < 0.05$) in in vitro (1500-fold) than in the in vivo embryos (500-fold) at the 2-cell developmental stage compared to the calibrator. The novel transcript was also detected at higher level ($P < 0.05$) in the in vitro than in the in vivo embryos at the 2-cell stage of development. However, the PAIP1 and the novel transcript showed no significant difference in their expression between the two embryo groups beyond the 2-cell developmental stage. Both PAIP1 and the novel transcript were detected only up to 8-cell stage in both embryo groups, suggesting their maternal origin. In conclusion, the variations in the expression of studied genes between in vitro and in vivo may reflect the effect of the two culture systems on the transcriptional activity of early embryos.

253 BOVINE OOCYTE CYCLIN B1 mRNA UNDERGOES CYTOPLASMIC POLYADENYLATION BEFORE THE BEGINNING OF IN VITRO MATURATION

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Maternal oocyte Cyclin B1 mRNA is known to be stored in the cytoplasm with a short poly(A) tail and be translationally dormant at GV stage. During maturation, Cyclin B1 poly(A) tail is elongated by a process called cytoplasmic polyadenylation and driven by A/U-rich cis-acting elements in its 3′ untranslated region (UTR) known as cytoplasmic polyadenylation elements (CPEs). The objective of this study was to elucidate whether GV-stage bovine oocytes possess a stockpile of Cyclin B1 mRNA stored with a short a poly(A) tail that is elongated during maturation by CPE regulation. The mRNA poly(A) tail length was measured by Rapid Amplification of cDNA Ends Polyadenylation test (Race-PAT) on oocytes ($n = 100$) at the GV stage and 3, 5, 8, 10, 15, 20, and 25 h of in vitro maturation. The mRNA poly(A) tail length was also measured in triplicate ($n = 20$) on cold oocytes in GV (all manipulations on ice), warm oocytes in GV (ovaries transported in warm saline and manipulations on ice) and warm + 2 h 30 min oocytes in GV (oocytes left for an additional 2 h and 30 min at room temperature). To assess for variation in mRNA quantity, Cyclin B1 mRNA level was quantified by real-time PCR (Lightcycler, Roche, Indianapolis, IN, USA) in cold, warm or warm + 2 h

30 min GV oocytes ($n = 20$). The data were treated as factorial design, using treatment and type of RT as factors, and analysed by ANOVA (SAS Inst., Cary, NC, USA). Differences between means were checked using Tukey's test. Oocyte Cyclin B1 transcript show two different 3′ UTRs. These transcripts had the same ORF but different 3′ UTR lengths because of an alternative nuclear polyadenylation element AAUAAA (NPE). The longest form (Cyclin B1L) that possessed a putative CPE (UUUUAAUAAA) fused to the last NPE was studied. In warm GV oocytes, Cyclin B1L had a long poly(A) tail of 100 adenosine residues, and this length did not change during in vitro maturation. Interestingly, we found that Cyclin B1L showed an expected short poly(A) tail when the ovaries and the oocytes were transported and manipulated on ice. We showed that Cyclin B1L mRNA is cytoplasmically polyadenylated (addition of 75 adenosine residues) between the time of collection and the end of manipulation. This lengthening is most probably sufficient to promote translation. There was no significant difference between the Cyclin B1 mRNA quantity of cold oocytes or warm oocytes when the oligo used for the reverse transcription was either dt or decamers. Therefore, we believe that the increase in poly(A) tail length is not the result of Cyclin B1L mRNA degradation in cold oocytes or de novo transcription in warm oocytes. We report for the first time that Cyclin B1L cytoplasmic polyadenylation is carried out well before the beginning of in vitro maturation in bovine oocytes when ovaries are transported from the slaughterhouse in warm saline. Studying the real early mechanisms leading to resumption of meiosis in bovine oocytes is complicated by Cyclin B1 polyadenylation occurring prior to in vitro maturation. (Supported by NSERC.)

254 IDENTIFICATION OF OOCYTE SPECIFIC GENES USING SSH AND MICROARRAY ANALYSIS

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The main objective of this project was to isolate and identify important genes specifically expressed in oocytes. These genes are characterized and may uncover the molecular mechanisms related to the unique functions found in the oocyte. Total RNA (1 μg) from denuded germinal vesicle-stage (GV) oocytes and somatic tissues were used for cDNA production. The mRNAs were reverse-transcribed and the cDNAs were amplified using the Smart cDNA amplification kit (Clontech, BD Biosciences, San Jose, CA, USA). SSH was performed with the PCR-Select cDNA Subtraction Kit (Clontech). Briefly, pools consisted of bovine oocytes for the tester and bovine somatic tissues for the driver. The same procedure was repeated with mouse and *xenopus* tissues. All of the 3500 subtracted PCR products generated by SSH were cloned, PCR amplified and sequenced. The resulting sequences were compared against the GenBank database using online computer BLASTn program. For microarray analysis, purified PCR products were spotted on GAPS II glass slides (Corning, Corning, NY, USA) using a VersArray Chip WriterPro robot (BioRad, Hercules, CA, USA). Forward- and reverse-subtracted PCR products were used as probes labeled with Cy-3 and Cy-5 dyes (Amersham, Piscataway, NJ, USA) using the Amino Allyle cDNA Labeling Kit (Ambion, Austin, TX, USA). Slides were scanned and analyzed using the ChipReader and ArrayPro Analyser software (Media Cybernetics, Carlsbad, CA, USA). Detection of the oocyte-specific zona pellucida (ZP), growth differentiation factor-9 (GDF-9), bone morphogenetic protein 15 (BMP15), H1 histone family, member O, oocyte-specific (H1oo), and cyclin B1 transcript in the oocyte subtracted library increased confidence in the validity of the subtraction procedure. All of these transcripts account for 12 % of the clones for the mouse subtracted oocyte library. Microarray analysis performed with the mouse arrays revealed that 33% (382) of the clones were differentially expressed (ratio > 10) in the oocyte for the mouse library. Of the 139 clones (12%) that seemed to be present only in the oocyte, 65 were found to be expressed in both the bovine and the *xenopus* subtracted oocyte library. The most interesting clones to date are #1518, a gene associated with pluripotency; #1776, a cDNA associated with stem cells in mouse, and #1906, a protein that interacts with chromatin. Since they are conserved across species, the chances that their function is important are quite high. A validation step with RT-PCR analysis will need to be performed on genes identified as oocyte-specific, and the functionality of these genes will be determined with the RNAi technique. WEB page http://www.begc.crbr.ulaval.ca/ (Supported by NSERC).

255 EXPRESSION OF TRANSCRIPTION FACTORS PRIOR TO THE MATERNAL-TO-ZYGOTIC TRANSITION IN BOVINE EMBRYOS

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During the first stages of bovine embryonic development, until the 8- to 16-cell stage, the zygote is maintained by the mRNA and proteins stored in the oocyte. New embryonic transcription is reported to begin only at the 8- to 16-cell stage even if some minor transcription is detected from the 2-cell stage. In order for this to occur, several factors are required to remodel the chromatin and activate the transcription machinery. Some regulating transcription factors are possibly present in the oocyte in their mRNA form, and their translation could enhance the maternal-to-zygotic transition (MZT). In our study, we observed the expression patterns of five transcription factors (ATF2, HMGN2, HMGB2, HUEL and MSY2) in bovine in vitro-produced embryos. Embryos were produced in vitro using selected cumulus-oocyte complexes from 3-5-mm follicles of slaughterhouse ovaries. Pooled GV or MII oocytes, and 2-, 4-, 8-cell and blastocyst-stage embryos ($n = 40$/stage) were washed in PBS and frozen at −80°C. Each pool was spiked with 1 pg of GFP RNA containing a poly(A) tail. The RNA was extracted using the Absolutely RNA Microprep Kit (Stratagene, La Jolla, CA, USA), co-precipitated with linear acrylamide (Ambion, Austin, TX, USA) and reverse-transcribed with Omniscript (Quiagen). The quantitative amplification of the transcription factors was performed in triplicate using the equivalent of 1 oocyte or embryo per reaction on a Lightcycler (Roche, Indianapolis, IN, USA). Data were normalized with the GFP levels found in each pool and a Least-Significant-Difference method was used for statistical analysis. Immunocytochemistry studies were performed on oocytes and embryos fixed and permeabilized in a solution of paraformaldehyde and Triton X-100, and results were observed on a confocal microscope. Our results show that the transcripts of the transcription factors studied are found at higher levels in pre-MZT embryos and at lower levels in subsequent stages. For HMGN2 and MSY2, there

is a decrease in mRNA during oocyte maturation. For both genes, the residual mRNA remains constant up to the 4-cell stage before another loss in transcript levels in the 8-cell stage. In the case of ATF2, HMGB2 and HUEL, the maternal transcript levels are maintained until the 4-cell stage, suggesting that the mRNA is protected from degradation until its possible translation at the MZT. These results, combined to immunolocalization of the proteins, suggest a possible implication of some of these factors in the bovine MZT.

256 CONSTRUCTION OF A BOVINE cDNA ARRAY TO MONITOR GENE EXPRESSION PROFILES IN BOVINE PREIMPLANTATION EMBRYOS

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Array technology is a widely used tool for gene expression profiling, providing the possibility to monitor expression levels of an unlimited number of genes in various biological systems including preimplantation embryos. The objective of the present study was to develop and validate a bovine cDNA array and to compare expression profiles of embryos derived from different origins. A bovine blastocyst cDNA library was generated. Poly(A^+)RNA was extracted from in vitro-produced embryos using a Dynabead mRNA purification kit. First-strand synthesis was performed with SacIT21 primer followed by randomly primed second-strand synthesis with a DOP primer mix (Roche) and a global PCR with 35 cycles using SacIT21 and DOP primers. Complementary DNA fragments from 300 to 1500 bp were extracted from the gel and normalized via reassoziation and hydroxyapatite chromatography. Resulting cDNAs were digested with SacI and XhoI, ligated into a pBKs vector, and transfected into competent bacteria (Stratagene). After blue/white colony selection, plasmids were extracted and the inserts were subjected to PCR using vector specific primers. Average insert size was determined by size idenfication on agarose gels stained with ethidium bromide. After purification via precipitation and denaturation, 192 cDNA probes were double-spotted onto a nylon membrane and bound to the membrane by UV cross linking. Amplified RNA (aRNA) probes from pools of three or single blastocysts were generated as described recently (Brambrink *et al.*, 2002 BioTechniques, 33, 3–9) and hybridized to the membranes. Expression profiles of in vitro-produced blastocysts cultured in either SOF plus BSA or TCM plus serum were compared with those of diploid parthenogenetic ones generated by chemical activation. Thirty-three probes have been sequenced and, after comparison with public data bases, 26 were identified as cDNAs or genes. Twelve out of 192 (6%) seem to be differentially expressed within the three groups; 7/12 (58%) were down-regulated, 3/12 (25%) were up-regulated in SOF-derived embryos, and 2/12 (20%) were up-regulated in parthenogenetic blastocysts compared to their in vitro-generated counterparts. Three of these genes involved in calcium signaling (calmodulin, calreticulin) and regulation of actin cytoskeleton (destrin) were validated by semi-quantitative RT-PCR (Wrenzycki *et al.*, 2001 Biol. Reprod. 65, 309–317) employing poly(A^+) RNA from a single blastocyst as starting material. No differences were detected in the relative abundance of the analysed gene transcripts within the different groups. These findings were confirmed employing the aRNA used for hybridization in RT-PCR and showed a good representativity of the selected transcripts. Results indicate that it is possible to construct a homologous cDNA array which could be used for gene expression profiling of bovine preimplantation embryos. Supported by the Deutsche Forschungsgemeinschaft (DFG Ni 256/18-1).

257 DIFFERENTIAL GENE EXPRESSION IN GERMINAL VESICLE AND METAPHASE II MOUSE OOCYTES.

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The present study was conducted to identify oocyte-specific genes according to developmental stage. We used an accurate Annealing Control Primer (ACP; SeeGene, Seoul, Korea)-based GeneFishing technology. This new and innovative method has developed 120 ACPs so far to locate differentially expressed genes. This method is more specific and sensitive than differential display-PCR that is labor-intensive and results in a high degree of false positives. To identify genes differentially expressed in germinal vesicle (GV)- and metaphase II (MII)-stage oocytes, fully grown GV-intact and MII oocytes were obtained. The addition of 0.2 mM IBMX in the collection medium inhibited GV breakdown. The mRNA samples were prepared from the same number of GV and MII oocytes using a Dynabeads mRNA DIRECT Kit. GeneFishing system utilizes a two-stage PCR to fish out authentic differentially expressed genes by regulating binding of each portion of ACP at a different stage of PCR. The differentially expressed bands were extracted and cloned into a TOPO TA cloning vector (Invitrogen, Carlsbad, CA, USA), sequenced, and analyzed by BLAST search. To confirm the differential expression of selected transcripts, RT-PCR and immunofluorescence staining were conducted. Using 12 different ACPs, we isolated 27 differentially expressed bands from GV and MII oocytes. BLAST results indicated that most of these bands had strong homology with known genes. We confirmed that pleckstrin homology, protein kinase D2 (PKD), and COP9 (constitutive photomorphogenic) homologs were GV-specific, while minichromosome maintenance deficient 2 mitotin (MCM2), malate dehydrogenase, soluble Mor2, growth arrest specific 6 (Gas-6), Bcl-2 homolog (Diva), and suppressor of cytokine signaling 4 were expressed highly in MII. Immunofluoresence staining results confirmed higher expression of Gas-6 protein in MII oocytes compared to GV oocytes. Immunohistochemistry results with ovarian tissues demonstrated that MCM2 protein was highly expressed in the nucleus of developing oocytes and granulosa cells, and Diva was highly expressed in the oocytes of primordial follicles. Immunofluoresence staining for these proteins is under investigation. The functions of many of these genes have been determined in systems other than gamete systems. Those data provide insight about their function in oocyte maturation. For example, PKD, COP9, and pleckstrin homology are genes in the axis of a newly identified PKD signaling system. Therefore, further investigation of the functions of each gene identified in this study will provide a basis for understanding the mechanism of oocyte maturation.