

Sample time		1st wave		2nd wave	
		T1 <i>n</i> = 11	T2 <i>n</i> = 9	T3 <i>n</i> = 11	T4 <i>n</i> = 9
P <sub>4</sub> (ng/mL)	At GnRH	0.1 ± 0.3 <sup>a</sup>	0.4 ± 0.3 <sup>a</sup>	2.7 ± 0.3 <sup>b</sup>	1.9 ± 0.3 <sup>b</sup>
	At PG	1.8 ± 0.4 <sup>a</sup>	1.7 ± 0.4 <sup>a</sup>	3.7 ± 0.4 <sup>b</sup>	3.9 ± 0.4 <sup>b</sup>
Follicle diam. (mm)	At PG	13.0 ± 0.5 <sup>a</sup>	12.9 ± 0.5 <sup>a</sup>	12.2 ± 0.5 <sup>a</sup>	10.7 ± 0.5 <sup>b</sup>
	At 20 h	15.2 ± 0.4 <sup>ab</sup>	15.3 ± 0.4 <sup>ab</sup>	15.7 ± 0.4 <sup>a</sup>	14.4 ± 0.4 <sup>b</sup>
PG to estrus (h)	Mean	51 ± 2.1 <sup>a</sup>	52 ± 2.4 <sup>a</sup>	62 ± 2.1 <sup>b</sup>	59 ± 2.4 <sup>b</sup>
	Range	41–65 <sup>a</sup>	42–66 <sup>a</sup>	47–71 <sup>a</sup>	54–65 <sup>b</sup>
Estrus to ovulation (h)	Mean	31 ± 1.2 <sup>a</sup>	27 ± 1.4 <sup>b</sup>	30 ± 1.2 <sup>ab</sup>	30 ± 1.4 <sup>ab</sup>
	Range	26–46 <sup>a</sup>	22–38 <sup>ab</sup>	26–34 <sup>c</sup>	26–34 <sup>bc</sup>

<sup>abc</sup> Unlike superscripts within a row differ,  $P < 0.05$ .

This work was supported by USDA-NRI grant 2000-02163; Pfizer Animal Health, New York, NY; and Merial, Athens, GA, USA.

## 209 NORGESTOMET IMPLANTS REDUCE LH RELEASE PATTERN IN ZEBU COWS UNDERGOING REPEATED OOCYTE PICK-UP

J.H.M. Viana<sup>A</sup>, L.S.A. Camargo<sup>A,C</sup>, A.M. Ferreira<sup>A</sup>, W.F. Sa<sup>A</sup>, C.A.C. Fernandes<sup>B</sup>, and A.P. Marques Junior<sup>C</sup>

<sup>A</sup>Emprapa Dairy Cattle Research Center, Federal University of Juiz de Fora, Minas Gerais 36038-330, Brazil;

<sup>B</sup>University of Alfenas, Federal University of Alfenas, 37170-000 Alfenas, Brazil; <sup>C</sup>Federal University of Minas Gerais, Belo Horizonte, Minas Gerais, Brazil. Email: jhmviaana@cnpqgl.embrapa.br

Ultrasound-guided follicular puncture (OPU) has become the most used technique to recover cumulus-oocyte complexes (COCs) from valuable donors for *in vitro* embryo production, because of the low risk and the possibility of collecting COCs at intervals as short as twice-a-week. However, repeated aspiration of ovarian follicles may induce endocrine abnormalities due to partial luteinization of punctured follicles and interference with follicular development. The use of exogenous progestagens is an alternative used to control these side effects, and is under evaluation. The aim of this study was to evaluate whether the effect of norgestomet treatment on intra-follicular and systemic steroid concentrations and on ovarian follicular dynamics is related to changes in LH releasing pattern. Pluriparous non-lactating Gir breed (*Bos indicus*) cows ( $n = 10$ ) were randomly distributed between treatment (norgestomet ear implants, replaced weekly) and control (no hormone used) groups, and had their ovarian follicles larger than 3 mm in diameter aspirated twice a week, during the next two consecutive weeks. Follicular dynamics were evaluated every 12 h between OPU sessions, and the largest follicles present were used to recover samples of follicular fluid. Blood samples were collected daily for progesterone evaluation in all cows, and 3 times within a 4-h window interval, at 24, 48, 72, and 96 h after follicle puncture for LH evaluation, in 6 cows (3 from treated and 3 from control groups). LH was measured by a standardized RIA procedure. Data was analyzed by ANOVA, and means were compared by Tukey's test. Results are presented as means ± SEM. Treatment with norgestomet reduced mean progesterone plasma concentration during the evaluated period ( $36.3 \pm 14.0$  vs.  $250.3 \pm 49.3$  pg/mL;  $P < 0.0001$ ), the incidence of follicles growing above 9 mm (30% vs. 65%;  $P < 0.05$ ) in the intervals between OPU sessions, and intrafollicular estradiol and progesterone concentrations in the largest follicles ( $n = 27$ ) present ( $265.5 \pm 47.4$  and  $34.9 \pm 5.4$  ng/mL vs.  $765.2 \pm 169.1$  and  $173.3 \pm 43.4$  ng/mL, respectively;  $P < 0.05$ ). Plasma LH concentrations were consistently lower during the 3 session intervals in cows treated with norgestomet ( $0.16 \pm 0.04$ ,  $0.22 \pm 0.03$ ,  $0.22 \pm 0.09$  and  $0.17 \pm 0.01$  vs.  $0.44 \pm 0.15$ ,  $0.53 \pm 0.04$ ,  $0.42 \pm 0.05$  and  $0.39 \pm 0.11$  for 24, 48, 72, and 96 h after OPU, respectively;  $P < 0.05$ ). These results confirm the theory that norgestomet treatment is associated with a reduction in the LH-release pattern, as expected due to the reduction in both luteinization of punctured follicles and in the steroidogenic activity of growing follicles observed during the experiment. The use of norgestomet ear implants can be an alternative in the management of donor cows undergoing oocyte pickup.

This work was sponsored by FAPEMIG and CNPq. The authors thank Dr. Robert Douglas for aiding with LH RIA.

## Gene Expression

## 210 COMPARISON OF REAL-TIME PCR AND END-POINT PCR FOR ANALYSIS OF GENE EXPRESSION IN PREIMPLANTATION EMBRYOS

A. Baji Gal<sup>A</sup>, J.W. Carnwath<sup>B</sup>, A. Dinnyes<sup>A,C</sup>, D. Herrmann<sup>B</sup>, C. Wrenzycki<sup>B</sup>, and H. Niemann<sup>B</sup>

<sup>A</sup>Department of Animal Biology, Agricultural Biotechnology Center, Godollo, Hungary; <sup>B</sup>Department of Biotechnology, Institute for Animal Science, Mariensee, 31535 Neustadt, Germany; <sup>C</sup>Research Group on Applied Animal Genetics and Biotechnology, Hungarian Academy of Sciences and Szent Istvan University, Godollo, Hungary. Email: baji@abc.hu

The purpose of this study was to compare real-time PCR and end-point PCR with respect to their suitability for the analysis of gene expression in samples in which the number of cells is limited, for example, in studies of pre-implantation embryonic development. The real-time instrument was a

LightCycler® from Roche Diagnostics (Budaors, Hungary) which is a capillary-based PCR system. Primers for histone H2A (housekeeping gene) were used for all PCR reactions. The end-point PCR system included an MJ Research PT-100 thermocycler, agarose gel electrophoresis, ethidium bromide staining, and image acquisition with a 12-bit CCD camera and densitometry. The sensitivity, dynamic range, and standard error of both PCR systems were compared using a single stock solution of cDNA. The more precise real-time PCR system was then used to determine the precision of a protocol for reverse transcription and the precision of a complete gene expression protocol including mRNA purification and reverse transcription. The real-time system was 100 times more sensitive than the end-point system and had a dynamic range of more than four orders of magnitude. The linear range for end-point PCR was extended for two orders of magnitude using a fixed end-point of 31 cycles. The standard error of the mean based on 30 replicates was 0.14% for the real-time system and 6.8% for the end-point system. The standard deviations for reverse transcription combined with real-time analysis and for the complete gene expression protocol were 0.6% and 1.4%, respectively. The *t* standard deviation was 1.8% for expression analysis of 6 bovine oocytes. In conclusion, real-time PCR system has advantages in sensitivity, dynamic range, and precision of measurement. New research areas which involve subtle changes in expression reprogramming or the analysis of low copy number transcripts (even from single cells and embryos) clearly benefit from the advent of real-time PCR analysis. However, when genes with high transcription levels are analyzed, the amount of cDNA taken from the reverse transcription reaction can be adjusted to lie within the operating range of end-point PCR. Pooling embryos is a valuable approach for both methods when the goal is to determine the behavior of the average embryo rather than variation between embryos. In many cases, the magnitude of biologically significant expression changes is so great that the higher levels of precision afforded by real-time PCR are not essential for the analysis.

*This work was funded by the Bilateral Scientific and Technological Collaboration Agreement (TET) between Hungary and Germany (TET D-6/01) and by the National Office of Research and Technology (NKTH) (BIO-00017/2002).*

## 211 TEMPORAL AND SPATIAL EXPRESSION PROFILE OF THE UTERINE MILK PROTEIN – A MEMBER OF THE SERINE PROTEASE INHIBITOR SUPERFAMILY – IN THE BOVINE ENDOMETRIUM

S. Bauersachs<sup>A,B</sup>, S.E. Ulbrich<sup>C</sup>, K. Gross<sup>A,B</sup>, S. Schmidt<sup>A</sup>, H. Wenigerkind<sup>D</sup>, H.H.D. Meyer<sup>C</sup>,  
H. Blum<sup>B</sup>, and E. Wolf<sup>A,B</sup>

<sup>A</sup>Institute of Molecular Animal Breeding, Ludwig Maximilians University, 81377 Munich, Germany;

<sup>B</sup>Laboratory for Functional Genome Analysis (LaFuGa), Ludwig Maximilians University, 81377 Munich, Germany;

<sup>C</sup>Institute of Physiology, Technical University of Munich, 85350 Freising, Germany; <sup>D</sup>Bavarian Research Center for Biology of Reproduction, 85764 Oberschleissheim, Germany. Email: bsachs@lmb.uni-muenchen.de

A combination of subtracted cDNA libraries and cDNA array hybridization was applied to identify differentially expressed genes in the bovine intercaruncular endometrium at estrus and diestrus. Six cyclic Simmental heifers were cycle-synchronized and slaughtered the morning after standing heat occurred ( $n = 3$ ) or 12 days after estrus ( $n = 3$ ), respectively. The uterus was divided into seven sections: corpus, plus the caudal, middle, and cranial parts of both uterine horns. Samples were collected from the epithelial layer of the lamina propria of the intercaruncular endometrium. Two subtracted libraries were prepared and analyzed by array hybridization with probes produced from the 42 tissue samples. More than 130 differentially expressed genes were identified. Among these genes, the mRNA for the uterine milk protein (UTMP) showed a much higher expression level at estrus versus diestrus. Furthermore, the expression level differed markedly between the uterus sections. Statistical significance was tested using ANOVA and Student Newman Keuls post-hoc test (GeneSpring® version 6.1, Silicon Genetics, Redwood City, CA, USA). There was a pronounced expression gradient from both cranial uterine horns (highest) to the corpus (lowest) at estrus. UTMP mRNA level was highest in the ipsilateral cranial horn. A more accurate quantification was done by quantitative real-time RT-PCR using the same RNA samples as for array hybridization and, in addition, samples from further time points of the estrous cycle and early pregnancy. The results obtained by array hybridization were clearly confirmed. UTMPs, also known as uterine serpins (US), were previously shown to be the major products secreted by the uteri of cattle, sheep, and pigs during pregnancy. The US are glycoproteins belonging to the serpin (serine proteinase inhibitor) superfamily of proteins. However, no inhibitory activity to any serine proteinase has been found. The ovine uterine serpin (OvUS) has been shown to inhibit a wide variety of immune responses, including mixed lymphocyte reaction, T cell-dependent antibody production, and NK (natural killer)-like activity, indicating a major role in protecting the conceptus from maternal immune reactions. The four members of the US family form a clade distinct from other serpins, which have probably adopted important biological functions in the reproductive biology of Artiodactyla. Therefore, the identified expression gradient of the UTMP mRNA at estrus may be of particular importance, e.g. for sperm selection, and deserves further investigation at the protein level and in functional studies.

*This work was supported by the Deutsche Forschungsgemeinschaft (Research Unit "Mechanisms of Embryo-Maternal Communication"; FOR 478/1).*

## 212 HETEROGENEITY OF RIBOSOMAL RNA GENE ACTIVATION AMONG CELLS OF *IN VITRO*-PRODUCED PORCINE EMBRYOS

B. Bjerregaard<sup>A</sup>, F. Strejcek<sup>B,C</sup>, Z. Rasmussen<sup>D</sup>, J. Laurincik<sup>B,C</sup>, H. Niemann<sup>E</sup>, P. Maddox-Hyttel<sup>A</sup>, and P.D. Thomsen<sup>A</sup>

<sup>A</sup>Department of Animal and Veterinary Basic Sciences, Royal Veterinary and Agricultural University, 1870 Frederiksberg C, Denmark;

<sup>B</sup>Constantin the Philosopher University, Nitra, Slovak Republic; <sup>C</sup>Research Institute of Animal Production, Nitra, Slovak Republic;

<sup>D</sup>Department of Small Animal Clinical Science, 1870 Frederiksberg C, Denmark; <sup>E</sup>Department of Biotechnology,

Institute for Animal Science (FAL), Mariensee, 31535 Neustadt, Germany. Email: bol@kvl.dk

*In vitro* production (IVP) of porcine embryos by *in vitro* maturation of oocytes followed by fertilization and culture *in vitro* is hampered by great deficiencies. Initiation of at least the major embryonic genome transcription, which includes activation of ribosomal RNA (rRNA) genes and

the associated formation of a fibrillo-granular nucleolus, is normally seen during the 4-cell stage in pigs. We have investigated the activation of rRNA synthesis and the presence of silver staining nucleolar proteins in porcine IVP embryos as a marker of transcriptional activity and, thus, developmental competence. A total of 205 porcine IVP embryos from the 2-cell to the blastocyst stage were examined using sequential fluorescent *in situ* hybridization (FISH) to the rRNA genes and their transcripts and silver staining of nucleolar proteins as previously described (Viuff *et al.* 2002 Biol. Reprod. 66, 629–634). Briefly, cumulus-oocyte complexes with at least three cumulus cell layers and evenly granulated ooplasm were isolated from 2–5 mm ovarian follicles with stereomicroscopic evaluation. Subsequently, oocytes were matured in NCSU-37 and mechanically denuded followed by fertilization using frozen-thawed epididymal semen. Presumptive zygotes were then cultured in NCSU-23 at 39°C, 5% CO<sub>2</sub>. Around the time of expected cleavage, the embryos were examined every second hour to determine the time of cleavage. Embryos at the 2-cell stage were harvested at 5 h post-cleavage (hpc), 4-cell embryos late during the third cell cycle at 30 hpc, and tentative 8- and 16-cell embryos at 10 hpc. Blastocysts were harvested at Day 5 post-insemination. In general, nuclei of 2-cell embryos displayed 4 small foci of FITC labelling (presumably the rDNA), but no specific silver staining, and were consequently categorized as transcriptionally inactive. At the late 4-cell stage, 58% of the embryos resembled the 2-cell stage. However, in the remaining embryos (42%), some or all nuclei displayed large areas of FISH labelling (presumptive rDNA and rRNA) co-localized with silver staining, and were categorized as transcriptionally active. Among the 8-cell embryos, 64% displayed a majority of transcriptionally active nuclei, whereas this was the case in 83% and 92% of the embryos in the 16-cell embryos and the blastocysts, respectively. In general, the majority of the embryos contained a mixture of transcriptionally active and inactive cells. These findings show that the porcine IVP embryos are often delayed and asynchronous with respect to activation of the rRNA genes.

**Table 1. Categorization of nuclei according to transcriptional activity**

Developmental stage	Total no. of embryos	% of nuclei within an embryo categorized as transcriptionally active			
		<10% n (%)	10–50% n (%)	50–90% n (%)	>90% n (%)
2 cell	34	34 (100)	0 (0)	0 (0)	0 (0)
4 cell	45	26 (58)	7 (16)	10 (22)	2 (4)
8 cell	47	5 (11)	12 (25)	25 (53)	5 (11)
16 cell	42	5 (12)	2 (4)	18 (43)	17 (40)
Blastocyst	37	1 (3)	2 (5)	29 (78)	5 (14)

*This work was supported by grants from “Disease models, disease prevention and animal welfare improvement: The pig embryo as a model.” Danish Research Agency (Grant: 9901178), NATO (Grant: 978658), and Deutsche Forschungsgemeinschaft (DFG).*

## 213 SAGE ANALYSIS OF TRANSITIONS IN THE PORCINE CONCEPTUS TRANSCRIPTOME DURING TROPHECTODERM ELONGATION

*L. Blomberg<sup>A</sup>, T. Sonstegard<sup>B</sup>, C. Van Tassell<sup>B</sup>, and K. Zuelke<sup>A</sup>*

<sup>A</sup>USDA ARS Biotechnology and Germplasm Laboratory; <sup>B</sup>USDA ARS Bovine Functional Genomics Laboratory, Beltsville, MD 20705, USA. Email: kzuelke@anri.barc.usda.gov

Rapid elongation of the pre-implantation conceptus trophoctoderm between gestational days 11 and 12 represents a critical developmental period in swine. Serial analysis of gene expression (SAGE) in ovoid (Ovd; 7 mm diameter) and filamentous (Fil; 150 mm length) conceptuses has identified unique transcriptomes associated with these transitional states. The initial utility of SAGE prompted an investigation of the intermediate tubular conceptus (Tub; 15–20 mm length) as well as the development of micro-SAGE methodologies, small-amplified RNA-SAGE (SAR-SAGE) and PCR-amplified SAGE (m-SAGE), for future use with early pre-implantation conceptus stages. Total RNA from *in vivo*-derived Tub conceptuses was used to construct an unamplified SAGE library (Tub SAGE) and two micro-SAGE libraries (160X less template RNA) by SAR-SAGE and m-SAGE. Comparative analyses of the amplification proportionality between the Tub SAGE and amplified libraries, consisting of 12,000 tags each, demonstrated that SAR-SAGE was a more reliable amplification method. Seventy-five percent of the SAR-SAGE tags, in contrast to 43% of m-SAGE tags, had less than a 2-fold frequency difference when compared to Tub SAGE tags occurring at least 10 times in a library. The Tub SAGE library size was extended to a total of 42,415 tags representing 12,779 unique putative transcripts. Comparing the Tub SAGE tags with previously generated Ovd and Fil SAGE libraries (NCBI GEO Acc. Nos. GSM24470 and GSM24471, respectively) yielded a total of 33,191 unique tags, of which 3,575 tags (10.8%) were common to all three libraries. Chi-square-based analyses (SAGE2000 software; 1997 Gen. Res. 7, 986–995) of the tag frequencies revealed the differential expression of 479 tags between Ovd:Tub libraries and 362 tags between Tub:Fil libraries at a  $P < 0.05$  significance. The Tub SAGE tags were annotated following batch BLASTs against the TIGR porcine index database and then classified according to function. Real-time RT-PCR was used to confirm the differential expression patterns identified between Ovd:Tub, Ovd:Fil, and Fil:Tub. For example, the relative quantity of mRNA for steroidogenic acute regulatory protein, 1.0:6.4:11.1 (Ovd:Tub:Fil), and cytokeratin 8, 1.1:0.6:0.4 (Ovd:Tub:Fil), demonstrated an increase and decrease (ANOVA;  $P < 0.05$ ), respectively, throughout these three developmental stages. Based on their putative functional annotations, the differentially expressed genes revealed by SAGE are potentially involved in regulating embryo growth, cellular differentiation, apoptosis, steroidogenesis, and energy metabolism during embryo elongation. The present SAGE analyses have elucidated the tubular conceptus transcriptome and enabled the identification of genes that are differentially regulated during the 24-h period that encompasses pig embryo elongation. Furthermore, the decreased template requirement of SAR-SAGE makes this technique a suitable option for SAGE analyses of early stage embryos where the sample is small and limited.

## 214 DIFFERENT GENE EXPRESSION OF INDIVIDUAL BLASTOMERES IN EARLY MOUSE EMBRYO DETECTED BY REAL TIME PCR

Sz. Bodo<sup>A</sup>, A. Baji Gal<sup>A</sup>, D. Boonkusol<sup>B</sup>, B. Gorhony<sup>A</sup>, E. Balogh<sup>A</sup>, and A. Dinnyes<sup>A</sup>

<sup>A</sup>Department of Animal Biology, Agricultural Biotechnology Center, Godollo, Hungary; <sup>B</sup>Department of Anatomy, Faculty of Science, Mahidol University, Bangkok, 10400, Thailand. Email: bodo@abc.hu

The objective of this study was to detect gene expression differences among blastomeres from 4- and 8-cell stage mouse embryos by a sensitive method. In earlier studies gene expression was determined in pooled or single embryos. With the higher levels of precision afforded by our method, we could quantify mRNA in single blastomeres. For comparison of gene expression, different inner cell mass specific (Oct-4, Nanog) and trophectoderm-specific (Glut-3) genes and the housekeeping beta-actin gene (for reference) were chosen. Late 4- and early 8-cell stage embryos were obtained from oviducts 58 and 66 h post-hCG, respectively. The embryos were collected in CZB-HEPES and the zonae pellucidae were dissolved by acid Tyrode solution. Blastomeres were separated mechanically from each other by gentle pipetting with a fire-polished glass capillary. Messenger RNA was isolated from the frozen blastomeres using the Dynabeads<sup>®</sup> mRNA DIRECT<sup>™</sup> Micro Kit (Kvalitex Kft., 1136 Budapest, Hungary) following the manufacturer's protocol. The reverse transcription reactions were performed at 25°C for 10 min, and then at 42°C for 1 h followed by 99°C for 5 min. Real-time PCR was performed in an iCycler iQ Real-Time PCR Detection System (Bio-Rad Laboratories, 1094 Budapest, Hungary). Reaction mixtures, which were separated for each gene, consisted of QuantiTect iQ SYBR Green Supermix (Bio-Rad Laboratories), 500 nm of both primers, and a 1/8 aliquot of the blastomere cDNA. The cDNA template was denatured by heating to 95°C for 2 min and amplified by 50 cycles of 95°C for 20 s, 60°C for 20 s, and 72°C for 20 s, with a single fluorescence measurement at each cycle. When the reaction was finished, melting curves were plotted to confirm product purity. By this procedure we could measure the expression level of four genes in six 4- and 8-cell-stage embryos. Glut-3 expression was not detectable in the blastomeres of 4-cell stage embryos. In spite of the 1.3–1.5-fold technical error, Oct-4 and Nanog showed 10-fold and 9-fold differences, respectively. Among the blastomeres of the same embryo, one of the 4-cell-stage showed notably high expression of Oct-4. In the 8-cell stage blastomeres Oct-4 expression showed slight differences (2–8-fold), and Nanog and Glut-3 showed 28-fold and 12-fold differences, respectively. Two of the 8-cell blastomeres showed notably high expression of Nanog. In conclusion, this method was accurate and sensitive enough to measure the expression of different genes in a single blastomere. We found that the mRNA levels of Oct-4 and Nanog have remarkably discrete differences within the blastomeres of a 4-cell-stage embryo. Expression of Nanog gene showed notable differences within blastomeres of an 8-cell-stage embryo. These differences may show a very early sign of the cell fate determination.

*This work was funded by the Wellcome Trust International Senior Fellowship and Bio-Rad Laboratories.*

## 215 COMPARISON OF STRONG ENDOTHELIAL CELL-SPECIFIC PROMOTERS FOR EXPRESSION OF HUMAN COMPLEMENT REGULATORY PROTEINS IN PORCINE XENOGRAFT ENDOTHELIAL CELLS

T.W. Choi<sup>A</sup>, J.H. Kim<sup>A</sup>, H.S. Choi<sup>A</sup>, S.J. Uhm<sup>A</sup>, C.S. Park<sup>B</sup>, H.T. Lee<sup>A</sup>, and S.G. Cho<sup>A</sup>

<sup>A</sup>Department of Animal Biotechnology, Konkuk University, Seoul 143-702, South Korea; <sup>B</sup>Research Center for Transgenic Cloned Pigs, Chungnam National University, Daejeon, 305-764, South Korea. Email: ssangoo@konkuk.ac.kr

The complement system is composed of a complex group of soluble proteins that have important roles in the immune response against foreign cells such as xenografted tissue cells. Activation of complement pathways results in the formation of the membrane attack complex (MAC). Several cell surface regulators known as membrane complement regulatory proteins (mCRPs) prevent the formation of MAC. For blocking the formation of MAC in the xenotransplanted cells, we tried to overexpress mCRPs in the endothelial cells to prevent the hyperacute rejection (HAR), because one of the major obstacles to xenotransplantation of vascularized organs was reported to be HAR. For this purpose, we isolated human MCP (membrane cofactor protein, CD46) (1.5 kb) and DAF (decay-accelerating factor, CD55) (1.1 kb) promoter regions from human genomic DNA isolated from the human embryonic kidney cell line HEK293, and cloned into pGL3 plasmid for luciferase reporter assay. We also inserted other known endothelial cell-specific promoters, such as Flk-1 (fetal liver kinase-1) and ICAM-2 (intercellular adhesion molecule-2), thrombomodulin promoters, into pGL3 vector to compare their promoter activities. We transfected the plasmids into several endothelial cell lines such as bovine aortic endothelial cells (BAEC), human umbilical vein endothelial cells (HUVEC), and mouse pancreatic microvascular endothelial cells (MS1). We also used two epithelial cell lines, human embryonic kidney epithelial cells (HEK293) and human epidermal keratinocytes (HaCaT). The endothelial specific expression of the promoters were compared with those of SV40, CMV, and EF-2a (elongation factor) promoters which are generally used in mammalian gene expression. Luciferase assays showed that among the endothelial cell-specific promoters, the 1.1 kb DAF promoter was the strongest, followed by the Flk-1 promoter. The promoter activity of 1.1 kb DAF was about 2-fold that of the Flk-1 promoter. Moreover, the 1.1 kb DAF and Flk-1 promoters were chosen as the best endothelial specific promoters, as both showed about 5 times more luciferase activity in the endothelial cells compared to that in the epithelial cell lines. We also found that the 5'-flanking region between -1126 and -968 of the 1.1 kb DAF promoter was very important for the endothelial-specific strong gene expression. On the 0.2 kb region of DAF promoter, we could detect several conserved nucleotide sequences interacting with the specific transcription factors, such as GATA-1, USF, CdxA, SRY, Sox-5, and Sp1. Interestingly, deletion of the GATA-1 motif between -1113 and -1104 reduced the promoter activity of the DAF promoter by about 25%. We conclude that the 1.1 kb DAF promoter is a suitable candidate promoter for strong endothelial cell-specific expression of mCRP genes.

## 216 IDENTIFICATION OF DIFFERENTIALLY EXPRESSED GENES IN BOVINE EMBRYOS CULTURED *IN VIVO* OR *IN VITRO*

D. Corcoran<sup>A</sup>, T. Fair<sup>A</sup>, D. Rizos<sup>A</sup>, G.W. Smith<sup>B</sup>, P.M. Coussens<sup>B</sup>, O.V. Patel<sup>B</sup>, J.J. Ireland<sup>B</sup>, M.P. Boland<sup>A</sup>, A.C.O. Evans<sup>A</sup>, and P. Lonergan<sup>A</sup>

<sup>A</sup>Department of Animal Science and Centre for Integrative Biology, Conway Institute for Biomolecular and Biomedical Research, University College Dublin, Newcastle Co. Dublin, Ireland; <sup>B</sup>Department of Animal Science and Center for Animal Functional Genomics, Michigan State University, East Lansing, MI 48824, USA. Email: pat.lonergan@ucd.ie

The post-fertilization culture environment of the bovine embryo is known to influence the quality of the resulting blastocyst, manifested in terms of morphology, cryotolerance, and the relative gene transcript abundance of several candidate genes. This may have consequences for the pregnancy rate following embryo transfer. The objective of the current study was to take a broader approach toward identifying differentially expressed genes in bovine blastocysts derived from either *in vivo* or *in vitro* culture. Presumptive zygotes, produced by *in vitro* maturation and fertilization, were randomly assigned to one of two groups and cultured for 6 days, either *in vitro* in SOF medium, or *in vivo* in the ewe oviduct following transfer by mid-ventral laparotomy. Blastocysts were recovered from both systems on Day 7 after insemination, snap frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$ . Total RNA was extracted from 50 blastocysts for each culture group from each of four replicates using the PicoPure<sup>TM</sup> RNA Isolation Kit (ARCTURUS, Mountain View, CA 94043, USA). The RiboAmp<sup>TM</sup> RNA Amplification Kit (ARCTURUS) was used to linearly amplify the mRNA fraction of total RNA using double-stranded cDNA as template in a T7 RNA polymerase-catalyzed amplification. Samples from both culture environments were differentially labelled using N-hydroxysuccinimide (NHS)-activated fluorescent Cy3 or Cy5 dyes (Amersham Pharmacia Ltd., Piscataway, NJ, USA) and were hybridized onto a cDNA microarray. Each microarray contained 3888 total spots, with 932 bovine EST clone inserts developed from a normalized bovine total leukocyte (BOTL) cDNA library and an additional 459 amplicons representing additional genes including cytokines, receptors, signal transduction molecules, transcription and growth factors, enzymes, cell cycle regulators, and cellular components. Data were normalized and an expression ratio calculated between the two groups. This was compared to 1 within each of the 4 replicates by Student's *t*-test. Microarray analysis identified 15 gene transcripts that were differentially expressed  $P \leq 0.05$  between blastocysts produced *in vivo* or *in vitro*. Among these, four genes involved in transcription (nuclear receptor co-repressor 1, zinc finger protein 22, CCR4-NOT transcription complex, DOT1) and two genes involved in intracellular signalling (proteasome 26S subunit non-ATPase 13 and guanine nucleotide binding protein) had a higher mRNA expression level in blastocysts produced *in vivo* compared to those produced *in vitro*. In addition, Connexin 43, a gene involved in gap junction formation, was down regulated following *in vitro* culture which is consistent with our previous studies. Among the genes up-regulated following *in vitro* culture were WNT2B wingless-type MMTV integration site, CD103 integrin, and tumor necrosis factor superfamily member 8. In conclusion, we have identified previously uncharacterized, differentially expressed genes involved in cell communication, intracellular signalling, and regulation of transcription in bovine blastocysts cultured *in vivo* or *in vitro*.

This work was supported by Science Foundation Ireland under Grant No. 02/IN1/B78.

## 217 PROMOTER-SPECIFIC EXPRESSION OF THE IMPRINTED *Igf2* GENE IN CATTLE

C. Curchoe<sup>A</sup>, S. Zhang<sup>B</sup>, Y. Bin<sup>B</sup>, L. Yang<sup>A</sup>, and X.C. Tian<sup>A</sup>

<sup>A</sup>Department of Animal Science, Center for Regenerative Biology, University of Connecticut, Storrs, CT 06269, USA;

<sup>B</sup>College of Animal Science, South China Agricultural University, Guangzhou 510642, China.

Email: carolcurls@hotmail.com

Insulin-like growth factor II (*Igf2*) is an imprinted gene crucial to fetal development and maternal/fetal nutrient transfer. Studies in the pig have demonstrated that this quantitative trait locus controls muscle growth, fat deposition, and heart size. Additionally, studies have shown that four splice variants transcribed from promoters (P1–P4) regulate the complex temporal and spatial expression of *Igf2* in mice, humans, pigs, and horses. Cattle have a cotyledonary placenta and therefore a distinct type of maternal/fetal interaction. Here we have studied for the first time the promoter-specific expression of *Igf2* in cattle, an economically valuable livestock species. Five naturally reproduced animals obtained from an abattoir were used in this study (2 mid-gestation fetuses, 1 calf, and 2 adults) of which all major internal organs were tested. Here we used RT-PCR to show that, like that of the pig and the human, the bovine *Igf2* is expressed from four different promoters in a temporal and spatial manner. However, unlike for pigs and humans, we have found that transcripts from promoter P1 were present in several bovine fetal and adult tissues including the liver, heart, kidney, lung, placenta, and spleen. Promoter P2 was expressed only in mid-gestation fetal tissues including the liver, bladder, lung, and kidney. Promoter P2 was not detected in the brain. Promoter P3 was expressed ubiquitously throughout fetal and adult life; however, expression appears to be lower in the heart. Promoter P4 was expressed in all mid-gestation fetal tissues. Transcription from P4 decreased with age until transcripts were detected only in the kidney, lung, heart, and spleen. Using single-stranded conformational polymorphism polyacrylamide gel electrophoresis and a single nucleotide polymorphism in exon 10 of *Igf2*, we have confirmed that a loss of imprinting occurs with age from all transcripts (P1–P4) and biallelic expression is observed in most adult tissues studied.

This work was funded by a grant from the USDA.

## 218 SELECTIVE REDUCTION OF p66<sup>shc</sup> mRNA IN BOVINE OOCYTES/EMBRYOS BY RNA INTERFERENCE

*L.A. Favetta, G.F. Mastromonaco, E.J. St. John, W.A. King, and D.H. Betts*

Department of Biomedical Sciences, Ontario Veterinary College, University of Guelph, Guelph, Ontario, N1G 2W1, Canada.

Email: lfavetta@uoguelph.ca

High embryo loss occurs during the first week of *in vitro* bovine embryo development with a high percentage of embryo death and arrest. These early stages of development are regulated by stored maternal mRNAs that accumulate in the oocyte cytoplasm during its growth phase. In our *in vitro* production (IVP) system approximately 13.5% of embryos arrest at the 2–4-cell stage, displaying no characteristics of apoptosis. We hypothesized that these arrested embryos enter an oxidative stress-induced senescence-like state. We previously showed that elevated levels of reactive oxygen species and increased levels of the stress-adaptor protein p66<sup>shc</sup> were associated with this early embryonic arrest. The aim of this study was to selectively down-regulate p66<sup>shc</sup> mRNA levels in bovine oocytes, using post-transcriptional gene silencing by RNA interference (RNAi), to study the effects of p66<sup>shc</sup> mRNA “knock-down” on early arrest of IVP bovine embryos. Approximately 12,000 short hairpin (sh) RNAi molecules specific for p66<sup>shc</sup> were microinjected into bovine germinal vesicle (GV) oocytes. Experiments consisted of a control group undergoing IVF alone, and two groups microinjected with and without p66<sup>shc</sup> shRNAi molecules. Quantification of p66<sup>shc</sup> mRNA by real-time PCR was used to assure down-regulation of p66<sup>shc</sup> mRNA in 2-cell embryos collected at 35 hours post-insemination (hpi). The amount of p66<sup>shc</sup> mRNA detected in control IVF, vehicle-injected, and p66<sup>shc</sup> RNAi-injected groups was  $0.92 \pm 0.02 \times 10^{-5}$  pg,  $1.2 \pm 0.20 \times 10^{-5}$  pg, and  $0.46 \pm 0.06 \times 10^{-5}$  pg, respectively. This statistically significant ( $P < 0.001$ ) reduction in p66<sup>shc</sup> mRNA levels by 54% upon p66<sup>shc</sup> shRNAi microinjection was selective for p66<sup>shc</sup> mRNA, as both histone H2a and p53 mRNA levels were not altered. Percentage of 2–4-cell arrest was evaluated at Day 8 post-insemination and related to p66<sup>shc</sup> mRNA down-regulation. While there were no significant differences in the percentage of 2–4-cell arrested embryos between the control ( $13.3 \pm 0.8\%$ ) and vehicle-microinjected ( $10.8 \pm 0.7\%$ ) embryos, there was a significant decrease ( $P < 0.001$ ) in the incidence of arrest in p66<sup>shc</sup> shRNAi-microinjected embryos ( $0.9 \pm 0.9\%$ ). Quantification by real time PCR in blastocysts from the three groups showed no significant differences in p66<sup>shc</sup> mRNA levels ( $P = 0.314$ ) among control IVF, vehicle, or p66<sup>shc</sup> hairpin RNAi microinjected at  $0.59 \pm 0.1 \times 10^{-5}$  pg,  $0.57 \pm 0.2 \times 10^{-5}$  pg, and  $0.51 \pm 0.2 \times 10^{-5}$  pg, respectively. This suggests that p66<sup>shc</sup> down-regulation by RNAi might be temporary. Thus, using this novel approach of RNAi and microinjection of oocytes at the GV stage, we were able to selectively down-regulate the expression of p66<sup>shc</sup> mRNA and correlate this down-regulation with a significant decrease in 2–4 cell arrest. These data reinforce our hypothesis that p66<sup>shc</sup> is involved in a stress-induced pathway that executes a senescent-like embryonic arrest at the 2–4-cell stage, thereby postulating the possible role of p66<sup>shc</sup> as a new molecular marker for developmental competence. Statistics performed using ANOVA with Fisher LSD test for multiple comparisons.

*This work was funded by NSERC, CIHR, OGS, and OMAFRA.*

## 219 METHYLATION STATUS OF A DIFFERENTIALLY METHYLATED REGION (DMR) WITHIN THE BOVINE Igf2 GENE IN PREIMPLANTATION EMBRYOS

*C. Gebert<sup>A</sup>, C. Wrenzycki<sup>A</sup>, D. Herrmann<sup>A</sup>, R. Reinhardt<sup>B</sup>, D. Gröger<sup>B</sup>, A. Lucas-Hahn<sup>A</sup>, J.W. Carnwath<sup>A</sup>, and H. Niemann<sup>A</sup>*

<sup>A</sup>Department of Biotechnology, Institute for Animal Breeding (FAL), 31535 Neustadt, Germany;

<sup>B</sup>Max Planck Institute for Molecular Genetics, 14195 Berlin, Germany. Email: gebert@tzv.fal.de

Specific DNA regions within imprinted genes become differentially methylated on the maternal and paternal chromosomes during germ cell development. These DMRs play a crucial role in the regulation of imprinted gene expression. The murine insulin-like growth factor2 gene (Igf2) is imprinted and contains an intragenic DMR within the last exon. Recently it became known that the bovine Igf2 gene is also imprinted (Dindot *et al.* 2004 Biol. Reprod. 71, 470–478) where we have now identified an intragenic DMR in the last exon with the paternal allele being methylated. Aberrant methylation patterns within the bovine Igf2 gene could result in deregulated gene expression and could therefore be involved in the development of fetal abnormalities such as the large offspring syndrome (LOS) in cattle. We have studied the methylation status of 27 CG dinucleotides within this DMR in bovine pre-implantation embryos of different origin by bisulfite sequencing. DNA was isolated from expanded blastocysts collected *in vivo* and generated by *in vitro* fertilization (IVF), somatic nuclear transfer (NT), and parthenogenesis (PA). Additionally, DNA was obtained from fibroblasts derived from a female and a male adult animal and used as donor cells for NT and from zygotes and 4-cell embryos both produced by IVF. After PCR amplification of the bisulfite-treated DNA, PCR products were cloned and sequenced. Methylation percentages were calculated for each individual clone by division of the 27 CpGs with the number of methylated CpGs per sample. The methylation levels (%) from each sample were then used to obtain the global methylation levels of the analyzed region. Methylation decreased during the transition from the zygote ( $28.4\% \pm 3.8$  SEM) to the 4-cell embryo ( $6.3\% \pm 2.2$  SEM) indicating that the DMR is demethylated after fertilization. An increased methylation level was observed in expanded blastocysts (*in vivo*:  $10.2\% \pm 1.2$  SEM; IVF:  $10.1\% \pm 0.7$  SEM; female NT:  $12.4\% \pm 1.4$  SEM). Thus, remethylation starts before the blastocyst stage. The higher methylation level of male NT blastocysts ( $22\% \pm 1.9$  SEM) in comparison to their *in vivo* and IVF counterparts could be due to an insufficient reprogramming of the donor cells after nuclear transfer. Female and male donor cells were both heavily methylated ( $77\% \pm 2.2$  SEM;  $72\% \pm 2.9$  SEM, respectively). Parthenogenetic expanded blastocysts were less methylated ( $2.3\% \pm 1$  SEM), probably due to their diploid maternal genome. Results show for the first time that the methylation status at this DMR is associated with the origin of the embryo. Analysis of methylation patterns in pre-implantation embryos could provide a diagnostic tool to unravel mechanisms involved in fetal malformations often observed after the use of *in vitro* fertilization and/or nuclear transfer.

## 220 AN EXPRESSION PROFILE OF GENES CRUCIAL FOR PLACENTAL DEVELOPMENT IN SINGLE *IN VIVO*, *IN VITRO* AND CLONED BOVINE BLASTOCYSTS

V. Hall, N. Ruddock, R. Tecirlioglu, M. Cooney, and A. French

Monash Institute of Reproduction and Development, Monash University, Clayton, Victoria 3168, Australia.

Email: vanessa.hall@med.monash.edu.au

Abnormalities of the placenta are a major factor contributing to early death in cloned bovine conceptuses. This is primarily due to incomplete chromatin remodeling and reprogramming of the donor nucleus. It is unknown whether genetic aberrations of genes crucial for placental development can be detected in pre-implantation cloned bovine embryos. This study looked at the expression profile of four genes in single bovine blastocysts derived from *in vivo*, *in vitro* produced (IVP), or cloning techniques, including handmade cloning (HMC) and serial HMC (SHMC). The genes studied included acrogranin, caudal type homeobox 2 (*cdx2*), estrogen-receptor-related receptor beta (*essrb*), and the mammalian relative of DnaJ (*MRJ*). These genes play a role in trophoblast regulation and placental development. Messenger RNA expression was analyzed by using PCR following cDNA amplification by means of SMART cDNA synthesis (Clontech, Palo alto, CA, USA). Primers were designed from homologous human and mouse sequence. PCR products were sequenced for verification. Five single blastocysts were analyzed from each of the following treatments: *in vivo*, IVP, HMC, and SHMC. Pooled ( $n = 10$ ) IVP blastocyst cDNA produced by standard RT was used as a positive control. Grade 1 Day 7 blastocysts were selected for all treatments. Amplified cDNA was tested using control genes polyA, IFN- $\tau$  and GDF9. *In vitro*-produced embryos were matured, fertilized and cultured as published by Ruddock *et al.* (2004 Biol. Reprod. 70, 1131). Cloned HMC embryos were produced as described by Tecirlioglu *et al.* (2003 Reprod. Fertil. Dev. 15, 361). Serial HMC embryos were produced as per the HMC embryos, followed by a second round of nuclear transfer at the pronuclear stage. The pooled IVP, *in vivo*, and IVP blastocysts expressed all four genes of interest. In the HMC-cloned embryos, all four genes were expressed. However, in the SHMC cloned embryos, although MRJ was found to be expressed in all blastocysts, three of the five blastocysts did not express acrogranin. Similarly, two SHMC embryos did not express *cdx2*, and *essrb* was weakly expressed in three of the five embryos analyzed. Initial pregnancy rates of HMC and SHMC embryo transfers are similar. Further pregnancy results are pending. These results indicate that aberrations of genes crucial for placental development can be detected in single cloned blastocysts. It also suggests that failed implantation and/or placental defects may stem from patterned genetic defects in the pre-implantation embryo. An increase in the number of embryos analyzed would further strengthen results. These genes could act as markers to identify cloning techniques that produce more embryos with normal genetic profiles. The benefits of developing a screening tool to assess abnormalities in single pre-implantation embryos would be significant.

## 221 TYPICAL HIF1-REGULATED GENES ARE UNALTERED BY OXYGEN IN BOVINE BLASTOCYSTS

A. Harvey, K. Kind, and J. Thompson

Research Centre for Reproductive Health, Department of Obstetrics and Gynaecology, The University of Adelaide, Adelaide, South Australia, Australia. Email: alexandra.harvey@adelaide.edu.au

Oxygen-regulated gene expression in the bovine embryo contrasts markedly with that observed in the mouse. Under low (2%) post-compaction oxygen conditions moderate changes in gene expression are observed in the bovine blastocyst (Harvey *et al.* 2004 Biol. Reprod. 71, in press), compared with 3–4 fold increases in the mouse (Kind *et al.* 2004 Mol. Reprod. Dev., in press). Specifically, GLUT-1 (Harvey *et al.* 2004), myotrophin, and anaphase-promoting complex 1 (Harvey *et al.*, unpublished) mRNAs are increased in bovine blastocysts following 2% oxygen culture, compared with those cultured under 20% oxygen. These oxygen-mediated differences in gene expression in the bovine are most likely regulated by hypoxia-inducible factor (HIF)2 transcription factor activity, as we have previously observed that HIF1 $\alpha$  protein is not detectable in bovine embryos whereas HIF2 $\alpha$  is readily detectable (Harvey *et al.* 2004). The aim of this study was to determine the effect of post-compaction oxygen concentration on the expression of typically HIF1-regulated and potential HIF2-regulated (suggested from a mouse knockout study; Scortegagna *et al.* 2003 Nat. Genet. 35, 371) genes in bovine blastocysts. *In vitro*-produced bovine embryos were generated using standard protocols. Compact morulae were randomly allocated to treatments under 2%, 7%, or 20% oxygen for 72 h from Day 5. Blastocyst RNA was isolated using TriReagent (Molecular Research Center, Inc., Cincinnati, OH, USA) and samples were reverse-transcribed using Superscript II (Invitrogen, Melbourne, Australia). Amplification and analysis of cDNA was achieved by real-time PCR using specific primers and Sybr green PCR master mix (Applied BioSystems, Melbourne, Australia). Statistically significant differences in gene expression were analyzed by ANOVA,  $P < 0.05$ . Examination of expression of genes known to be regulated by HIF1 in somatic cells (reviewed by Semenza 2002 Biochem. Pharm. 64, 993) revealed no oxygen-mediated alteration in expression of aldose reductase, cyclooxygenase 2, or inducible nitric oxide synthase. However, the expression of lactate dehydrogenase A (LDHA) displayed a 4-fold increase under 2% oxygen, compared with 7% and 20% oxygen ( $P < 0.001$ ). Expression of glutathione peroxidase, and CuZn- and Mn-superoxide dismutase (putative HIF2-regulated genes) was not influenced by oxygen concentration post-compaction. This study suggests that typical HIF1-regulated genes are not influenced by alterations in the external oxygen environment in the bovine embryo. These results complement previous observations that HIF1 $\alpha$  protein is not detectable in blastocyst-stage bovine embryos, and suggest that LDHA may be an HIF2 target gene in the bovine embryo. As embryo development is influenced by oxygen concentration, levels of LDHA at the blastocyst stage may be used as a marker of oxygen responsiveness.

## 222 METHYLATION OF THE 5'-UPSTREAM REGION OF THE *H19* GENE IN MOUSE SOMATIC CELL, GAMETES, WILD TYPE AND ANDROGENETIC ES CELLS

H. Kato<sup>A</sup>, H. Murakami<sup>B</sup>, M. Kawasumi<sup>B</sup>, T. Kunieda<sup>B</sup>, M. Okuno<sup>B</sup>, M. Kishimoto<sup>B</sup>, M. Soma<sup>B</sup>, D. Iwai<sup>B</sup>, M. Anzai<sup>A</sup>, T. Mitani<sup>A</sup>, K. Matsumoto<sup>A,B</sup>, K. Saeki<sup>A,B</sup>, Y. Hosoi<sup>A,B</sup>, and A. Iritani<sup>A,B</sup>

<sup>A</sup>Institute of Advanced Technology, Kinki University; <sup>B</sup>Department of Biology-Oriented Science and Technology, Kinki University, Wakayama, Japan. Email: kato@gene.waka.kindai.ac.jp

In mammals, several genes influenced by the phenomenon of genomic imprinting are critical during development. Recently, Kono *et al.* (2004 Nature 428, 860–864) reported the production of intact female mouse individuals that had only two haploid sets of maternal genome. They obtained

these mice by combining a normal haploid maternal genome and a mutant haploid maternal genome with a 13 k base deletion in the *H19* gene and its 5'-upstream region. This genomic combination resulted in the appropriate expression of the *Igf2*, *H19*, and other imprinted genes. In the mouse genome, there are four CTCF-binding sites in the 5'-upstream region of the *H19* gene. The binding of CTCF to these binding sites regulates the expression of the *Igf2* and *H19* genes. The binding of CTCF to its binding sites is regulated by methylation of CpG sites in binding sites. In this study, as the first step to elucidate the role of the paternal genomic imprinting during development, we investigated the methylation of CpG sites in the 5'-upstream region of the *H19* gene in mouse somatic cells, gametes, and two types of ES cells. Genomic DNA was isolated from BDF1 (C57BL/6N × DBA/2N) mouse's tail (male and female somatic tissue, mST and fST, respectively), spermatozoa (S), oocytes (O), and wild type and androgenetic embryonic stem cells (wtES and agES, respectively). The methylation of CpG sites was evaluated by using the bisulfite sequencing assay. There were 13 CpG sites and a CTCF-binding site in the region from -4413 to -3976 in the *H19* gene relative to the transcription start site. The percentages of CpG sites in this region that were methylated were 88% (160/182), 79% (27/130), 93% (230/247), 8% (10/130), 77% (10/13) and 89% (314/351) for mST, fST, S, O, wtES, and agES, respectively. In the CTCF-binding site core motif (CCGCGTGGTGGCAG), the percentages of methylated CpG sites were 93% (26/28), 80% (16/20), 95% (36/38), 0% (0/20), 50% (1/2) and 96% (52/54) for mST, fST, S, O, wtES, and agES, respectively. The CpG sites in the sequence of agES were highly methylated similar to the finding in spermatozoa. However, an aberrant methylation pattern was observed in some clones of agES. From these results, it was concluded that the methylation of CpG sites in the genomic sequence of agES was well conserved and, therefore, agES is useful to elucidate the role of the paternal genomic imprinting during development.

*This work was supported by Wakayama Prefecture Collaboration of Regional Entities for the Advanced of Technological Excellence, Japan, and by a Grant-in-Aid for the 21st Century COE Program of the Japan MEXT.*

## 223 ABERRANT TIMP-2 GENE EXPRESSION IN CLONED BOVINE NEONATAL PLACENTA IS DUE TO ABNORMAL EPIGENETIC MODIFICATIONS

H.R. Kim<sup>A</sup>, J.K. Kang<sup>A</sup>, J.T. Yoon<sup>B</sup>, J.K. Jung<sup>C</sup>, H.H. Seong<sup>C</sup>, C.S. Park<sup>A</sup>, T. Wakayama<sup>D</sup>, and D.I. Jin<sup>A</sup>

<sup>A</sup>Research Center for Transgenic Cloned Pigs, Chungnam National University, Daejeon, 305-764, South Korea;

<sup>B</sup>Genetic Engineering Institute, Hankyong National University, Ansong, South Korea; <sup>C</sup>National Livestock Research Institute, RDA, Suwon, South Korea; <sup>D</sup>Center for Developmental Biology, RIKEN Institute, Kobe, Japan. Email: dijin@cnu.ac.kr

A global proteomics approach by 2-D gel electrophoresis and mass spectrometry was used to analyze the differential protein patterns of three placentae obtained after postnatal death of fetuses from SCNT of Korean Native cattle and three normal placentae obtained after birth of AI fetuses, as previously reported (Kim *et al.* 2004 Reprod. Fertil. Develop. 16, 145). One differentially up-regulated proteins in SCNT placenta was identified as TIMP-2 protein that is related to extracellular matrix degradation and tissue remodeling processes. To ensure that the identified protein was truly up-regulated in SCNT placenta, a small portion of the protein lysates were subjected to Western blotting with the antibodies against TIMP-2 and  $\beta$ -actin protein. Band images were scanned with a GT-6000 Scanner (Epson-Seiko, Suwa, Nagano, Japan) and densitometric analyses were performed using NIH Image (version 1.56). Proteins were normalized against the levels of  $\beta$ -actin protein. Indeed, Western blot analysis revealed a significant increase in TIMP-2 protein level (about 5-fold) in SCNT placenta compared with normal. Epigenetic programming of the genome by DNA methylation and histone modification ensures proper gene expression during development. To examine how the TIMP-2 gene was programmed to be active in SCNT placenta, we first investigated the status of DNA methylation in the promoter region of the TIMP-2 gene. Sodium bisulfite mapping revealed that most CpG sites in SCNT placenta cells were unmethylated in agreement with expression of TIMP-2 compared with those in normal placenta cells, suggesting that TIMP-2 gene programming by hypomethylation of CpG sites in the promoter region was responsible for the expression in SCNT placenta. We then examined histone modification of the nucleosome in the coding region of the TIMP-2 gene of SCNT placenta cells and normal placenta cells. Chromatin immunoprecipitation (ChIP) assay revealed that H3-K9/K14 acetylation of the TIMP-2 locus in SCNT placenta cells was about 2-fold higher than in normal placenta cells. Therefore, it is postulated that the SCNT placenta at the end of gestation seems to be unusual in tissue remodeling via improper expression of TIMP-2 by abnormal epigenetic modification, and that may affect placental abnormality such as enlarged and dysfunctional placentae as reported in cloned bovine and mouse.

## 224 TETRACYCLINE-INDUCIBLE GENE EXPRESSION WITH RETROVIRUS VECTOR

B.C. Koo<sup>A</sup>, M.S. Kwon<sup>B</sup>, I. Jeon<sup>C</sup>, W. Chang<sup>C</sup>, and T. Kim<sup>B,D</sup>

<sup>A</sup>Department of Animal Sciences, Chungbuk National University, Cheongju, Chungbuk, 361-763, South Korea;

<sup>B</sup>Department of Physiology, Catholic University of Daegu School of Medicine, Daegu, South Korea; <sup>C</sup>Animal Biotechnology Division, National Livestock Research Institute, RDA, Suwon, 441-706, South Korea; <sup>D</sup>Center for Transgenic Cloned Pigs, Chungnam National University, Daejeon, 305-764, South Korea. Email: takim@cnu.ac.kr

One of the critical problems to be solved in transgenic animal production is non-controllable constitutive expression of the foreign gene, which usually results in serious physiological disturbances in the transgenic animal. To circumvent this problem, we constructed and tested two retrovirus vectors designed to express the GFP (green fluorescent protein) gene under the control of tetracycline-inducible promoters. To maximize the GFP gene expression at turn-on state, the WPRE (woodchuck hepatitis virus post-transcriptional regulatory element) sequence was also introduced into the retrovirus vector downstream of either the GFP gene or the sequence encoding rtTA (reverse tetracycline-controlled transactivator). Transformed cells derived from porcine fetus were cultured in the medium supplemented with or without doxycycline (tetracycline derivative) for 48 h, and induction efficiency was measured by comparing the GFP gene expression level using fluorometry and western blotting. Higher GFP expression was observed from the vector carrying the WPRE sequence at the 3' side of the GFP gene, while tighter expression control (up to 20-fold) was



obtained from the vector in which the WPRE sequence was placed at the 3' side of the rtTA sequence. Encouraged with these data, we substituted the hPTH (human parathyroid hormone) gene for the GFP gene in the retrovirus vector. The porcine fetal fibroblast cells transformed by the modified retrovirus vector secreted hPTH into the medium under the tight control of doxycycline as observed in GFP expression. The resulting porcine cells secreting hPTH will be used in nuclear transfer experiment.

*This study was financially supported by the National Livestock Research Institute RDA (Suwon 441-350, Korea), ARPC (Agriculture R & D Promotion center, 2002–2005), and by grant No. R11-2002-100-01000-0 from the ERC program of the Korea Science & Engineering Foundation.*

## 225 RECOMBINANT HUMAN ERYTHROPOIETIN PRODUCED IN THE MILK OF TRANSGENIC MICE: FUNCTIONAL CHARACTERIZATION AND PHARMACEUTICAL APPROACH

D.N. Kwon, J.Y. Park, S.Y. Lee, S.J. Kang, and J.H. Kim

Division of Applied Life Science, College of Agriculture, Gyeongsang National University, Chinju, 660-701, South Korea.

Email: sun-76@hanmail.net

The proper post-transcriptional modification of recombinant human erythropoietin (rhEPO) is critical to retain its biological functions, either *in vivo* or *in vitro*. The major glycosyltransferases for the determinant of glycosylation patterns of rhEPO are N-acetylglucosaminyltransferase (GnT) and  $\alpha$ -1-3/4 fucosyltransferase (Fut). GnT-III expression ( $388 \pm 19.09$ ) in the mouse mammary gland has been shown to be dramatically different from that in CHO cells, although Fut-VIII expression in CHO cells ( $1970 \pm 255.9$ ) is comparable to mouse mammary gland ( $272 \pm 14.8$ ), suggesting that the mammary gland may proceed with the proper glycosylation of rhEPO as shown in CHO cells. To identify this hypothesis and establish the rhEPO bioreactor system for mass production of protein in transgenic animals, we have generated two transgenic mouse lines that express rhEPO in milk. Both lines of transgenic mouse express only rhEPO in the lactating mammary gland, and the protein yield of rhEPO in lactating milk is comparable to that in CHO cells. After determining the protein expression in lactating milk, using three different methods – enzymatic release of oligosaccharide analysis, two-dimensional electrophoresis, and 2-aminobenzamide-labeled analysis – we report that the rhEPO produced by the animal bioreactor system has the proper glycosylation patterns as shown in CHO cell-derived Epoietin  $\alpha$ , and has more tetra-acidic oligosaccharide structures than Epoietin  $\alpha$ , which is the widely used rhEPO for therapeutic purposes. The *in vitro* biological property of transgenic mouse milk-derived rhEPO has been tested by measuring luciferase activity in MCF-7 cells, indicating that rhEPO from mammary gland up-regulates the EPO-receptor-mediated STAT5 gene expression in a dose-dependent manner the same as Epoietin  $\alpha$  does. In addition, *in vivo* biological activity demonstrated that direct injection of rhEPO into a mouse vein increases blood components such as RBC and HCT. In light of these findings, we suggest that high levels of tetra-acidic structures observed in transgenic mouse milk-derived rhEPO may be related to the high level of expression of glycosyltransferases (GnT-III and Fut-VIII) in mammary gland; thus the bioreactor system using the mammary gland of a transgenic animal could be a good candidate for production of rhEPO for pharmaceutical purposes.

*This work was supported in part by a grant program from RDA(Biogreen21) and Cho-A, Republic of Korea. D.N. Kwon is the recipient of a scholarship from the BK21 program, granted by the Ministry of Education, Korea.*

## 226 GREATER DYSREGULATION OF GENE EXPRESSION IN PRE-IMPLANTATION CLONED OVINE COMPARED WITH CLONED BOVINE CONCEPTUSES

N. Li, T. Xiang, A. Ledgard, J. Peterson, D. Wells, and R. Lee

AgResearch Ltd., Ruakura, Hamilton, New Zealand. Email: rita.lee@agresearch.co.nz

In our experience, the cloning of sheep by somatic cell nuclear transfer has been less successful than with cattle (5 v. 10% live births from embryo transfer). Here, we compare data collected over many years on the pre-implantation development of nuclear transfer (NT) ovine and bovine embryos with contemporary *in vitro* produced (IVP) embryos at the elongation (bovine Day 16–18 v. ovine Day 14) and allantois formation (bovine Day 26/27 v. ovine Day 21) stages. Sheep NT conceptuses were generated from three fibroblast types (skin, kidney, and lung) derived from a Day 100 female fetus. Bovine NT embryos were generated from a granulosa cell line derived from a mature dairy cow. The expression of a subset of genes (*IGF2*, *H19*, *IGF2R*, *IGFBP-2*, *PEG1/Mest* and trophoblast-expressed genes such as *IFN- $\tau$* , *TKDP-1*, *COX-2*, and placental lactogen) was examined in both species at the two stages of development, using either Northern or slot-blot analysis. Pairwise comparisons between groups were carried out using Student's *t*-test. The proportion of ovine NT embryos that developed into blastocysts from the three cell types combined was 11% (117/1052) compared with 79% (86/109) for bovine NT from the one cell line. After transfer to synchronized recipients, 50–60% developed to the elongated conceptus stage for both species. Day 14 ovine NT ( $n = 19$ ) and IVP ( $n = 26$ ) conceptus lengths were similar whereas Day 16 bovine NT ( $n = 9$ ) lengths were slightly shorter than those of IVP ( $n = 3$ ) conceptuses ( $P > 0.05$ ). However, only 30% (16/52) of ovine NT embryos developed to Day 21 compared with 64% (9/14) for Day 26/27 bovine NT. Bovine NT and IVP embryo and allantoic lengths were not significantly different; however, both lengths were shorter in ovine NT than in IVP controls ( $P < 0.05$  and  $< 0.005$ , respectively). In each species, there was considerable individual variation in expression levels of most genes at each stage, probably due to variation in developmental stage of individuals even from the same day of gestation. The difference may be due to the timing of onset or termination of gene expression. In Day 16–18 bovine NT conceptuses, only the mean level of *PEG1/Mest* expression was significantly different ( $P < 0.05$ ) from that of IVP conceptuses. In contrast, mean expression levels for *TKDP-1* ( $P < 0.0001$ ) and *COX-2* ( $P < 0.05$ ) were higher in ovine Day 14 NT, and *PEG1/Mest* was higher ( $P < 0.05$ ) in Day 21 NT when compared with contemporary IVP controls. *H19* and *IGF2* expression was coordinately regulated in bovine NT and in ovine and bovine IVP conceptuses (*IGF2* and *H19* mRNA abundance was directly correlated). This coordinate regulation was disrupted in certain Day 21 ovine NT conceptuses, where high *H19*

levels occurred in the presence of low levels of *IGF2*, but not the converse. Thus, greater dysregulation in gene expression and loss of coordinate regulation of two key imprinted genes may explain the lower survival rate of ovine NT embryos to the early fetal stage when compared with bovine NT embryos. The effect of donor cell type on the species difference cannot be discounted.

*This work was supported by FRST C10X0303 programme.*

## 227 CONSTRUCTION OF STAGE-SPECIFIC cDNA MICROARRAY, AND ANALYSIS OF *IN VITRO* PRODUCED PRE-IMPLANTATION STAGE BOVINE EMBRYOS FOR DEVELOPMENTAL COMPETENCE

S. Mamo<sup>A</sup>, C.A. Sargent<sup>B</sup>, N.A. Affara<sup>B</sup>, K. Wimmers<sup>C</sup>, S. Ponsuksili<sup>C</sup>, M. Gilles<sup>A</sup>, and K. Schellander<sup>A</sup>

<sup>A</sup>Institute of Animal Breeding Sciences, University of Bonn, Bonn, Germany; <sup>B</sup>Department of Pathology, University of Cambridge, Cambridge, UK; <sup>C</sup>Molecular Biology Research Division, Research Institute for the Biology of Farm Animals, Dummerstorf, Germany. Email: lilykacha@hotmail.com

Microarray technology currently has wide acceptance as a research tool in the study of gene expression profiling, mainly as a result of its use for monitoring the expression profiles of thousands of genes in a single experiment. However, its use in analyzing gene expression in the pre-implantation stage of bovine embryo development has been limited for reasons such as the large amount of RNA required and the lack of bovine specific cDNA clone collections (Smith L and Greenfield A 2003 Hum. Mol. Genet. 12, 1–8). In this study, with the objectives of producing pre-implantation-stage-specific bovine cDNA clones and examining the developmental competence, eighty-two selected target clones of pre-implantation-stage-specific genes were prepared and spotted on the glass slide. Embryos were produced *in vitro* and mRNAs were isolated from contrasting probes of good quality matured oocytes and blastocyst-stage embryos using a Dynabead mRNA isolation kit by following the manufacturer's instructions. First-strand cDNA syntheses were primed with T7 Oligo d(T)21 primer, followed by random primed second-strand syntheses using a DOP master kit (Roche Diagnostics, Mannheim, Germany) and global amplification using the same primers used for the first- and second-strand syntheses. *In vitro* transcription was performed to amplify the RNA by using the AmpliScribe T7 transcription kit (EPICENTRE Technologies, Oldendorf, Germany), and the amplified RNA (aRNA) was purified using a RNeasy Mini kit (Qiagen, Hilden, Germany). Finally, the results of different RNA amplifications (aRNA) were tested by hybridization on microarrays and also using real-time PCR techniques. With these analyses, the sufficiency of the yield and linearity of amplification procedures were confirmed. Three micrograms each of aRNA were labelled with Cy3 and Cy5 dyes and hybridized to the array. After overnight incubation at 42°C, the slides were sequentially washed and scanned using an ArrayWorx biochip reader (Applied Precision, Marlborough, UK), and quantifications as well as all analyses were carried out using different TIGR software modules (Saeed AI *et al.* 2003 Biotechniques 34(2), 374–378). Analyses of the results of repeated hybridizations showed that 35 genes (43%), which belong to different functional groups, were differentially expressed between the two stages. Further independent analyses using real-time PCR confirmed the results of 25 genes. Hence, it is possible to conclude that the established methods can be used for large scale gene expression analysis, and the identified genes can be potential candidates for characterizing developmental competence.

## 228 EXPRESSION OF *zag1* IN MOUSE PRE-IMPLANTATION EMBRYOS

K. Matsumoto<sup>A,B</sup>, A. Uenoyama<sup>A</sup>, T. Matsuoka<sup>A</sup>, K. Saeki<sup>A,B</sup>, Y. Hosoi<sup>A,B</sup>, and A. Iritani<sup>A,B</sup>

<sup>A</sup>Department of Genetic Engineering, Kinki University; <sup>B</sup>Institute of Advanced Technology, Kinki University, Wakayama, Japan. Email: kazum@gene.waka.kindai.ac.jp

Embryonic gene activation (EGA) first occurs during the second half of the mouse 1-cell embryo (Latham KE 1999 Int. Rev. Cytol. 193, 71–124). Moreover, precise regulation of EGA is considered to be essential for normal embryo development. To understand the molecular basis for the regulation of EGA, we have focused on the identification and functional characterization of genes activated at the late 1-cell stage of the mouse embryo. Recently, we have identified and isolated a novel gene, termed *zag1* (zygotic activating gene 1), transcribed specifically at the EGA, using a fluoro-differential display method with oocytes and embryos at 15 h post-insemination. Messenger RNA of *zag1* expressed at lower level in the oocyte than that in the embryo at 15 h post-insemination. In this study, we investigated the potential function of *zag1* by analysis of mRNA expression and protein distribution in mouse tissues and pre-implantation embryos. Nucleotide sequence analysis of *zag1* cDNA revealed that the open reading frame of 1726 bps encodes a protein of 575 amino acids with a predicted molecular mass of 66 kDa. The deduced amino acid sequence indicated that *zag1* protein might be a soluble protein with a bipartite nuclear targeting sequence, a NACHT NTP domain, and an APT/GTP binding site motif as a predicted functional domain. Two µg of Poly(A)<sup>+</sup> RNA from various tissues of adult mice were subjected to Northern blot analysis using the mouse *zag1* cDNA probe. We detected this gene abundantly expressed in mouse testis and ovary by approximately 2- to 3-fold compared with one in other mouse tissues (heart, liver, kidney, lung, brain, skeletal muscle, and spleen). *zag1* transcript and protein, as assessed by RT-PCR and immunoblotting, respectively, were slightly present in ovulated oocytes, gradually decreased in the early 1-cell embryos, but re-expressed in the late 1-cell and early 2-cell stage embryos which coincided with the mouse EGA. Subsequent to microinjection of an expression vector encoding *zag1*-enhanced green fluorescent protein (EGFP), fused protein into male pronucleus of 1-cell embryos was detected in the nuclei of 2-cell embryos. These findings suggest that *zag1* may be functionally associated with early embryonic development.

*This study was supported by a Grant-in-Aid for the 21st Century COE Program of the Japan MEXT, and by a grant from the Wakayama Prefecture Collaboration of Regional Entities for the Advancement of Technological Excellence of the JST.*

## 229 EFFECT OF CULTURE CONDITIONS ON AQUAPORIN mRNA ABUNDANCE IN MOUSE BLASTOCYSTS

H. Offenberger and P.D. Thomsen

Animal and Veterinary Basic Sciences, The Royal Veterinary and Agricultural University, 1870 Frederiksberg C, Denmark. Email: pdt@kvl.dk

It is known that culture conditions can alter gene expression of the pre-implantation embryo. We have previously shown that aquaporins (AQPs) are expressed in the mouse embryo and that they are involved in the passage of water across the trophoblast cells during blastocyst formation. This study was conducted to investigate whether AQP mRNA abundance is altered by culturing embryos *in vitro* compared to *in vivo* developed embryos. Furthermore we wanted to investigate if AQP mRNA abundance was influenced by the osmolality of the media. It is possible to compare the effect of hyperosmolality that the embryo may be able to compensate for by adding glycerol which can cross some AQPs, compared to the addition of sucrose which can not cross the membranes. Mouse embryos were obtained by superovulating B6D2F1 mice followed by culture of the flushed presumptive zygotes in KSOM to the blastocyst stage (*in vitro*) or by flushing blastocysts from the uterus (*in vivo*). For the study of the influence of osmolality on AQP mRNA abundance, zygotes were flushed and cultured to the compacted 8-cell stage and then placed in media of increasing osmolality, using either glycerol or sucrose. The osmolalities of the media were 243 (control), 300, 350, and 400 mOsm. Embryos were cultured to the blastocyst stage and frozen in liquid nitrogen. Embryonic RNA was extracted using a Dynabeads mRNA Capture kit (Dyna, Oslo, Norway). Real time PCR was performed on embryonic cDNA on a Lightcycler (Roche Diagnostics, 2650 Hvidovre, Denmark) using aquaporin-specific primers and primers for  $\beta$ -actin and GAPDH. The results of the quantitative RT-PCR analysis showed that *in vitro*-cultured embryos had a lower mRNA abundance for AQP 8, 9, and 11 compared to the *in vivo*-developed embryos but that the AQP 3 mRNA abundance was unaltered. Analysis of the housekeeping genes showed that GAPDH mRNA levels were unchanged *in vitro*, whereas  $\beta$ -actin was up-regulated *in vitro*. The osmotically challenged embryos showed the following blastocyst rates compared to the controls: glycerol 300: 100%; glycerol 350: 100%; glycerol 400: 100%; sucrose 300: 100%; sucrose 350: 78%; and sucrose 400: 0%. Thus, glycerol up to 400 mOsm had no effect on blastocyst rates, whereas addition of sucrose reduced blastocyst formation, with a total inhibition at 400 mOsm. Analysis of the mRNA abundance showed a reduction of AQP 8 in the glycerol solutions. The level was reduced to 30% of the control group at 300 mOsm, to 27% at 350 mOsm and to 8% at 400 mOsm. There was no corresponding reduction of AQP 8 mRNA abundance in sucrose solutions. Further, AQP 3, 7, 9, and 11 mRNA levels as well as  $\beta$ -actin and GAPDH mRNA levels were unaltered in the osmotically challenged embryos. In conclusion, this study shows that embryonic culture affects the abundance of several AQPs and that compensation of a glycerol-induced osmotical challenge induces down-regulation of AQP 8 expression. Embryos tolerate high glycerol concentrations better than high sucrose concentrations but the possible role of AQP 8 in this process is unclear at present.

## 230 EFFECT OF *IN VITRO* CULTURE TREATMENT AND DONOR EWE DIET ON *IGF2R* EXPRESSION IN FETAL TISSUES

K.A. Powell<sup>A</sup>, K. Mackie<sup>A</sup>, T.G. McEvoy<sup>A</sup>, K.D. Sinclair<sup>A,B</sup>, J.J. Robinson<sup>A</sup>, C.J. Ashworth<sup>A</sup>, L.E. Young<sup>B</sup>, I. Wilmut<sup>C</sup>, and J.A. Rooke<sup>A</sup>

<sup>A</sup>Sustainable Livestock Systems Group, Scottish Agricultural College, Edinburgh, UK; <sup>B</sup>University of Nottingham, Sutton Bonington, Loughborough, LE12 5RD, UK; <sup>C</sup>Roslin Institute, Edinburgh, UK. Email: j.rooke@ab.sac.ac.uk

In separate experiments, elevation of donor ewe plasma urea levels and IVC of embryos in serum have been associated with occurrence of large offspring syndrome (LOS). Fetuses displaying LOS also have abnormal expression of the imprinted gene *IGF2R*. The present study, therefore, examined the effect of variation in donor ewe nitrogen metabolism and embryo culture on fetal development. Zygote-donor ewes were offered a diet with either a 0% (LN) or 3% urea supplement (HN) for two weeks during which superovulation and artificial insemination (AI) took place. Zygotes were recovered 36 h after AI and cultured for 5 days in synthetic oviductal fluid (SOF) to which either steer serum (10%; SOFS) or albumin (0.4%; SOFA) was added. *In vivo* control (C) blastocysts were recovered (Day 5) from superovulated ewes offered diet LN. All embryos were transferred singly to recipient ewes; fetal tissues were recovered and snap-frozen at Day 125 of gestation. *IGF2R* expression was measured by semi-quantitative PCR in fetal heart and kidney and expressed as the ratio to 18S mRNA. Data were analyzed by ANOVA with donor ewe plasma urea-N prior to (preUN) and mean plasma urea-N during (meanUN) feeding as covariates. Correlation analysis was used to test relationships between variables. Heart *IGF2R* expression (see Table 1) was lower in SOFA than in SOFS fetuses ( $P = 0.003$ ; preUN,  $P = 0.007$ ), while kidney *IGF2R* expression was lower in HN than in LN fetuses ( $P = 0.04$ ; preUN,  $P = 0.015$ ; meanUN,  $P = 0.032$ ). Overall the range of both heart and kidney *IGF2R* expression was greater for fetuses from cultured (1st/3rd quartiles; heart, 0.87–1.05; kidney, 0.77–1.10) than from C embryos (heart, 1.03–1.16; kidney, 0.84–1.00). In IVC fetuses, heart *IGF2R* expression was negatively associated with fetal body ( $r = -0.31$ ;  $P = 0.003$ ) and heart weight ( $r = -0.35$ ;  $P = 0.008$ ) whereas kidney *IGF2R* expression was not associated ( $r = -0.23$ ;  $P = 0.089$ ) with fetal body weight and not related to kidney weight. Heart and kidney *IGF2R* expression were only weakly associated ( $r = 0.26$ ;  $P = 0.054$ ). In conclusion, changes in *IGF2R* expression in both fetal heart and kidney were associated with donor ewe N status and IVC conditions. In this study, decreases in heart *IGF2R* expression were reflected in increased heart weight, but kidney *IGF2R* expression was not related to kidney weight.

Table 1. Fetal (kg), organ weights (g), and *IGF2R* expression

	Control	LN/SOFA	LN/SOFS	HN/SOFA	HN/SOFS
Fetal wt (kg)	3.33 $\pm$ 0.374	3.65 $\pm$ 0.669	3.70 $\pm$ 0.54	3.70 $\pm$ 0.712	3.58 $\pm$ 0.141
Heart wt (g)	18.5 $\pm$ 2.11	21.9 $\pm$ 5.81	22.7 $\pm$ 6.85	21.7 $\pm$ 5.83	22.9 $\pm$ 4.23
Heart <i>IGF2R</i>	1.11 $\pm$ 0.132	0.87 $\pm$ 0.207	1.11 $\pm$ 0.191	0.93 $\pm$ 0.233	0.98 $\pm$ 0.106
Kidney wt (g)	19.0 $\pm$ 2.41	27.2 $\pm$ 9.04	33.7 $\pm$ 29.4	25.2 $\pm$ 6.99	35.4 $\pm$ 23.2
Kidney <i>IGF2R</i>	0.91 $\pm$ 0.119	0.97 $\pm$ 0.311	1.01 $\pm$ 0.261	0.84 $\pm$ 0.241	1.01 $\pm$ 0.179
n	10	15	12	14	6

This study was funded by Defra.

## 231 TEMPORAL AND SPATIAL GENE EXPRESSION ANALYSIS OF THE BOVINE OVIDUCT EPITHELIUM

S. Rehfeld<sup>A</sup>, S. Bauersachs<sup>A</sup>, H. Blum<sup>B</sup>, S. Mallok<sup>B</sup>, H. Wenigerkind<sup>C</sup>, and E. Wolf<sup>A,B</sup>

<sup>A</sup>Institute of Molecular Animal Breeding and Biotechnology of the Ludwig Maximilians University, 81377 Munich, Germany;

<sup>B</sup>Laboratory for Functional Genome Analysis (LaFuGa), Gene Center of the Ludwig Maximilians University, 81377 Munich, Germany;

<sup>C</sup>Bavarian Research Center for Biology of Reproduction (BZFZ), 85764 Oberschleissheim, Germany. Email: rehfeld@lmb.uni-muenchen.de

The fallopian tube plays a central role in reproduction, providing the appropriate environmental conditions for oocyte maturation as well as for sperm capacitation. Furthermore, fertilization and the first cleavage stages of embryonic development take place in the oviduct. At the molecular level, only fragmentary data are available regarding the physiological changes in the oviduct epithelium during the estrous cycle. Therefore, we performed a systematic study of gene expression changes in bovine ipsilateral oviduct epithelial cells derived from either the ampulla or the isthmus part of the oviduct at four different time points of the estrous cycle. A cDNA array consisting of approximately 400 candidate genes, primarily identified in different studies in the context of gene expression regulation in the oviduct, was designed and hybridized with <sup>33</sup>P-labeled cDNA probes prepared from 28 different tissue samples. These tissue samples were collected from cyclic Simmental heifers at Day 0 ( $n = 3$ ), Day 3.5 ( $n = 3$ ), Day 12 ( $n = 4$ ) and Day 18 ( $n = 4$ ) of the estrous cycle. Ipsilateral epithelial cells were separately collected from ampulla and isthmus. After array evaluation (AIDA Image Analyzer, version 3.41, Raytest, Straubenhardt, Germany), the raw data were normalized to internal reference cDNAs on the arrays. Statistical analysis was done using ANOVA and the Tukey post-hoc test (GeneSpring<sup>®</sup> version 6.1, Silicon Genetics, Redwood City, CA, USA). For selected genes, differential expression was verified by real-time RT-PCR. A simplified Gene Ontology was built for the genes present on the array and a pathway analysis was performed to elucidate gene networks involved in the regulation of oviduct epithelial cell function. The expression patterns of two functional groups of genes are presented here: genes that are related to immune functions and genes of the secretory pathway or encoding secreted proteins. Messenger-RNA levels of immune-related genes were higher in epithelial cells of the ampulla compared to the isthmus part of the oviduct. This implies that certain immune functions may be differentially regulated in ampulla and isthmus. Furthermore, mRNAs of genes of the secretory pathway showed highest levels mainly in the ampulla around time of estrus, which may be explained with the increase of the secretory activity in the epithelium of the ampulla beginning at pre-estrus. In general, this study shows the importance of a separate analysis of the oviduct compartments and the influence of the estrous cycle on the expression level of a variety of genes. In the context of fertilization and early embryo-maternal communication, these results could provide an insight into the physiological changes during the estrous cycle, which are the bases for these processes.

*This work was supported by the Deutsche Forschungsgemeinschaft (Research Unit "Mechanisms of Embryo-Maternal Communication"; FOR 478/1).*

## 232 SPECIES-RELATED DIFFERENCES IN BLASTOCYST QUALITY ARE ASSOCIATED WITH DIFFERENCES IN RELATIVE mRNA EXPRESSION

D. Rizo<sup>A</sup>, A. Gutierrez-Adan<sup>B</sup>, P. Moreira<sup>B</sup>, C. O'Meara<sup>A</sup>, T. Fair<sup>A</sup>, A.C.O. Evans<sup>A</sup>, M.P. Boland<sup>A</sup>, and P. Lonergan<sup>A</sup>

<sup>A</sup>Department of Animal Science and Centre for Integrative Biology, Conway Institute for Biomolecular and Biomedical Research, University College Dublin, Newcastle, Co. Dublin, Ireland; <sup>B</sup>Departamento de Reproducción Animal y Conservación de Recursos Zootécnicos, INIA, Madrid, Spain. Email: pat.lonergan@ucd.ie

We have previously reported a species-related qualitative difference (in terms of ultrastructural morphology and cryotolerance) between bovine and ovine blastocysts produced under identical conditions of *in vitro* culture in synthetic oviduct fluid (SOF1, Rizo *et al.* 2002 Mol. Reprod. Dev. 62, 320–327). The overall objective of this study was to see if these differences were reflected at the transcript level. From each of five IVF replicates, groups of 10 bovine and 10 ovine blastocysts were used. The objective of Experiment 1 was to compare the relative transcript abundance of eight candidate genes between ovine and bovine blastocysts cultured in SOF1. Following real-time quantitative RT-PCR, transcript levels for MnSOD, survivin, and Glut-5 were significantly higher in ovine than in bovine blastocysts (ANOVA,  $P < 0.05$ ), while transcripts for Cx31, IFN-tau and SOX were significantly more abundant in bovine blastocysts ( $P < 0.01$ ). For the two remaining transcripts, E-cad and Na/K, there was no difference. The objective of Experiment 2 was to examine the possibility of modifying the pattern of expression in both types of blastocysts by changing the culture medium. Culture took place in SOF1 or SOF2 (Holm *et al.* 1999 Theriogenology 52, 683–700). Culture of bovine embryos in SOF2 resulted in a significant increase in the level of expression of MnSOD and Glut-5 ( $P < 0.05$ ) compared to culture in SOF1. For all the other transcripts except survivin, there was a significant decrease in the relative abundance. Culture of ovine embryos in either SOF1 or SOF2 did not have a major influence on transcript abundance; of the eight transcripts examined, the relative abundance of only one, SOX, was significantly altered. Based on the above, the objective of Experiment 3 (3 replicates) was to determine whether the changed pattern of expression in bovine blastocysts produced in SOF2 was associated with an improvement in cryotolerance. Bovine blastocysts produced in both culture media were vitrified and warmed, and survival was assessed by re-expansion and hatching. Blastocysts produced in SOF2 had significantly higher survival rates at 24, 48, and 72 h and significantly higher hatching rates following vitrification and warming than those produced in SOF1 ( $P < 0.001$ ). In conclusion, we have demonstrated that the apparent differences between ovine and bovine embryos in their adaptability to culture conditions, manifested in differences in embryo morphology and cryotolerance, are related to differences in mRNA relative abundance.

*This work was supported by Science Foundation Ireland under Grant No. 02/IN1/B78 and by Ministerio de Ciencia y Tecnología from Spain under Grant No. AGL2003-05783.*

## 233 EXPRESSION AND LOCALIZATION OF DNMT1 DURING EARLY BOVINE DEVELOPMENT

D.F. Russell and D.H. Betts

Department of Biomedical Sciences, Ontario Veterinary College, University of Guelph, Guelph, Ontario, N1G 2W1, Canada.  
Email: fischerd@uoguelph.ca

DNA methylation of CG motifs is an important mechanism of transcriptional regulation. During embryonic development DNA methyltransferase 1 (DNMT1) has been implicated in the maintenance of gametic and embryonic epigenetic patterns. Here we report the characterization of DNMT1 expression patterns within *in vitro*-produced (IVP) bovine embryos. Cumulus-oocyte complexes were recovered from slaughterhouse ovaries and either denuded (germinal vesicle, GV) or matured *in vitro* for 24 h (metaphase II, MII). Embryos at the 1-, 2-, 4-, 8-, 16-cell stage, and morula (Mo, Day 6 post-insemination, p.i.) and blastocyst (Days 7, 8, and 9 p.i.) stages were produced by *in vitro* fertilization and cultured in SOFm for appropriate times under a 5% O<sub>2</sub>, 5% CO<sub>2</sub>, and 90% N<sub>2</sub> atmosphere. Oocytes/embryos were either snap frozen (−80°C) for DNMT1 mRNA and protein expression profiles analysis or wholemount fixed and permeabilized for DNMT1 immunostaining followed by laser confocal microscopy. DNMT1 RNA, determined by real-time PCR analysis, was present throughout bovine embryonic development (replications = 3, *n* = 10 embryos/pool and normalized by Histone H2A expression). Growing oocytes accumulated DNMT1 transcripts until the MII stage (20-fold increase), whereas after fertilization DNMT1 RNA levels decreased and remained constant until the 16-cell stage when DNMT1 RNA levels decreased further and then remains constant until the blastocyst (Day 8 p.i.) stage. Confocal analysis of DNMT1 immunostained oocytes/embryos (*n* = 20/stage) revealed that DNMT1 is localized in the cytoplasm of the oocyte and pre-implantation embryo with the exception of the 16-cell stage, when the enzyme is translocated to nucleus (confirmed by Hoechst co-localization). Moreover, a punctuate staining pattern was observed for DNMT1, which could be due to its association with the mitochondria or endoplasmic reticulum. Interestingly, in GV oocytes DNMT1 was present in localized areas of the nucleus, suggestive of nucleoli association. DNMT1 was observed in the majority of the nuclei in early blastocysts, while after expansion, DNMT1 accumulated in the cytoplasm of the trophectoderm and was localized in both the cytoplasm and the nucleus of the majority of cells within the inner cell mass. Western blot analysis revealed low levels of DNMT1 protein in oocytes and pre-implantation embryos with the exception of the 16-cell embryos and Mo stages during which a significant (*P* < 0.05) increase in the levels of DNMT1 protein was observed. Specificity of primers and conditions for the real-time PCR assay were confirmed by cDNA sequencing whereas specificity of the antibody used for immunofluorescence and western blot analysis was confirmed by amino acid sequencing. These results suggest the participation of DNMT1 in the bovine embryonic genome activation process, supporting a passive DNA demethylation process during the early cleavage stages in the bovine. The low expression profile of DNMT1 after morula stage indicates that other methylases are required for the maintenance of DNA methylation during blastocyst formation and expansion.

This work was funded by CFIA, NSERC, CIHR, and OMAF.

## 234 REGION SPECIFIC ABUNDANCE OF INDUCIBLE NITRIC OXIDE SYNTHASE (iNOS) IN THE BOVINE OVIDUCT DURING THE ESTROUS CYCLE

S.E. Ulbrich<sup>A</sup>, S. Rehfeld<sup>B</sup>, R. Rottmayer<sup>B</sup>, S. Hiendleder<sup>B</sup>, E. Wolf<sup>B</sup>, H.H.D. Meyer<sup>A</sup>, and R. Einspanier<sup>A,C</sup>

<sup>A</sup>Physiology Weihenstephan, Technical University Munich, Freising, Germany; <sup>B</sup>Institute of Molecular Animal Breeding, Gene Center of the Ludwig-Maximilian-University Munich, Munich, Germany; <sup>C</sup>Present address: Institute of Veterinary Biochemistry, Free University Berlin, Berlin, Germany. Email: ulbrich@ww.tum.de

Nitric oxide synthases (NOS) are involved in a large number of biochemical processes. By generating inorganic free NO radicals, which are important modulatory agents, NOS are supposed to contribute to the regulation of mammalian reproduction. We have, therefore, studied the expression and localization of inducible NOS (iNOS) in the bovine oviduct during the estrous cycle. Simmental heifers were synchronized and slaughtered at Days 0, 3.5, 12, or 18 after standing heat. Bovine oviduct epithelial cells (BOEC) of ampulla and isthmus were collected separately for ipsi- and contralateral oviducts, as determined by the ovulatory follicle or the functional corpus luteum. In addition, a BOEC *in vitro* suspension culture from heifers of day 3.5 was established and stimulated with physiological doses of progesterone and estradiol-17β (10 ng/mL and 10 pg/mL, respectively). Total RNA was extracted and iNOS mRNA was absolutely quantified using real-time RT-PCR. For statistical analysis, the MIXED procedure of the SAS program package (SAS Institute, Inc., Cary, NC, USA) was used to model the experimental design (*n* = 4). Differences were indicated as statistically significant in the case of *P* < 0.05. Furthermore, immunoreactive iNOS protein was localized on serial oviductal cryosections. Transcripts of iNOS mRNA were detected in bovine oviducts throughout the estrous cycle. Since the level of 18S rRNA did not differ significantly the data were not normalized. Positive immunohistochemical staining for iNOS in the luminal epithelium gave evidence for the presence of iNOS protein in the bovine oviduct. At estrus (Day 0) and Day 18, the mRNA abundance of iNOS in epithelial cells of both ipsi- and contralateral isthmus was significantly lower than in epithelial cells of the ampulla. At Day 3.5, iNOS expression was significantly reduced in the contralateral but not the ipsilateral isthmus, whereas the abundance in the ipsilateral isthmus reached levels similar to those in the ampulla. At Day 12 the mRNA abundance was low without region specific differences. A down-regulation of iNOS could be associated with an accelerated oviductal transport due to higher contractility and increased ciliary motion in the luminal epithelium of the oviduct. This might be of special importance around ovulation time for gametes as well as for the formation of a homogeneous oviductal fluid. A modulated expression pattern of iNOS during the estrous cycle points towards a possible hormonal regulation. Preliminary *in vitro* results showed that in BOEC iNOS responded to progesterone, while no such up-regulation was observed after estradiol-17β stimulation. As Day 3.5 coincides with early embryonic development shortly after fertilization, the possible progesterone-dependent increase of iNOS transcripts in the ipsilateral isthmus might lead to a necessary local quiescence. Due to region-specific different transcript abundance, the presence of a local NO-system in the bovine oviduct can be postulated. Therefore, these findings point out a possible important role of iNOS in the oviduct for reproductive success.

This work was supported by the DFG Ei 296/10-1 and the Ev. Studienwerk eV.

### 235 THE INVESTIGATION OF mRNA EXPRESSION OF SEVERAL CHROMATIN REMODELLING GENES DURING BOVINE PREIMPLANTATION DEVELOPMENT

K. Wilson<sup>A,B</sup>, M. Cooney<sup>A,B</sup>, A. French<sup>A,B</sup>, M. Holland<sup>A,B</sup>, P. Verma<sup>A,B</sup>, and N. Ruddock<sup>A,B</sup>

<sup>A</sup>Monash Institute of Reproduction and Development, Monash University, Clayton, Victoria 3168, Australia;

<sup>B</sup>Cooperative Research Centre for Innovative Dairy Products, Melbourne, Victoria 3000, Australia.

Email: nancy.ruddock@med.monash.edu.au

The efficiency of obtaining live calves from somatic cell nuclear transfer remains quite low. One factor implicated in this failure is inadequate chromatin remodelling of the donor nucleus. Five Polycomb group (PcG) genes were investigated as potential remodelling factors in bovine oocytes and preimplantation embryos. These genes (Cbx6, Eed, Edr1, Yy1, and Zfp144) are involved in transcriptional activation and cell cycle regulation. We hypothesize that inadequate expression may cause the developmental abnormalities seen following cloning. This study is aimed at characterizing normal expression, prior to comparative studies with cloned embryos. Three single abattoir-derived *in vitro*-matured (IVM) oocytes or *in vitro*-produced (IVP) embryos from each of the following stages: 2-cell, 4-cell, 8-cell, 16–32 cell, morula, Day 7 blastocyst, and Day 8 hatched blastocyst, were studied. Messenger RNA was isolated from individual samples with Dynabeads (Dynal, Inc., Lake Success, NY, USA) and then cDNA was created and amplified with a SMART cDNA synthesis kit (BD Biosciences Clontech, Palo Alto, CA, USA). Products were diluted 1:10 and used to amplify target genes by PCR. PCR products were sequenced for confirmation of identity. All amplified embryo samples expressed the housekeeping genes Poly(A) polymerase and actin. Similarly, all embryos up to the 8-cell stage expressed GDF9, an oocyte-specific gene, while IFN-tau, involved in maternal recognition of pregnancy, was expressed in one morula and all blastocyst samples. Cbx6 was not expressed at any stage. The other four genes were all expressed fairly consistently throughout the pre-implantation period. Eed transcript was detected in all samples, with the exception of one oocyte, one 4-cell embryo, and one 8-cell embryo. Edr1 transcript was detected in all samples except for two oocytes, one 16–32-cell embryo, and one Day 7 blastocyst. Yy1 was expressed in all but one oocyte, one 2-cell embryo, two 4-cell embryos, and one Day 7 blastocyst. Finally, Zfp144 transcript was detected in one oocyte and in all embryos until the 16–32 stage, and then was not detected until seen in one Day 7 and all Day 8 blastocysts. Cbx6, yet to be fully characterized in any species, is also known as the neuronal pentraxin receptor, and is involved in transport and clearance of synaptic debris. It is known to have the characteristic chromobox domain of the Cbx family, of which several family members play a role during pre-implantation development. Eed, Edr1, Yy1, and Zfp144 are expressed throughout the pre-implantation period, although levels of Zfp144 mRNA appear to drop during the embryonic genome activation and then reappear by the late blastocyst stage. This consistent expression may suggest a role for the proteins in chromatin remodelling or modulating patterns of gene expression in early development. Further studies are required to determine if these factors are expressed incorrectly in cloned embryos, potentially providing a link to the abnormalities observed post nuclear transfer.

### 236 SPATIAL GENE EXPRESSION PATTERNS OF Dnmt1, Dnmt3a, AND Hdac2 IN PREIMPLANTATION EMBRYOS

C. Wrenzycki, D. Herrmann, A. Lucas-Hahn, K. Korsawe, E. Lemme, and H. Niemann

Department of Biotechnology, Institute for Animal Breeding (FAL), 31535 Neustadt, Germany.

Email: Wrenzycki@tzv.fal.de

In mammals, DNA methylation and the modification of histones account for the major epigenetic alterations. Usually DNA methylation is associated with transcriptional silencing, which is catalyzed by two important classes of DNA methyltransferases. DNA methyltransferase 1 (Dnmt1), a maintenance enzyme, methylates hemi-methylated DNA after DNA replication. Dnmt3a and Dnmt3b are required for *de novo* methylation *in vivo* and for establishing new DNA methylation marks during development. In addition to DNA methylation, post-translational modifications of the amino termini of core histones are thought to affect the expression or repression of transcription. Histone deacetylation catalyzed by histone deacetylases (Hdac) often results in transcriptional repression. Perturbed epigenetic reprogramming of the genome is a likely cause of developmental abnormalities and epigenetic diseases introduced by assisted reproduction technologies. The objective of the present study was to determine the relative abundance of Dnmt1, Dnmt3a, and Hdac2 transcripts in ICM and TE cells of pre-implantation bovine embryos of different origin. Embryos were generated with standard protocols of *in vitro* production (IVP) and parthenogenetic activation using SOF medium supplemented with BSA (SOF d7/d8, Parth d7/d8) or TCM medium supplemented with estrus cow serum (TCM d7/d8; Wrenzycki *et al.* 2001 Hum. Reprod. 16, 893–901; Wrenzycki *et al.* 2002 Biol. Reprod. 66, 127–134). Expanded blastocysts were collected at Days 7 and Day 8 (d7/d8) of culture (Day 0 = IVF). *In vivo*-generated blastocysts collected from superovulated animals were included as controls (Wrenzycki *et al.* 1996 J. Reprod. Fert. 108, 17–24). A highly sensitive RT-PCR assay (Wrenzycki *et al.* 2003 Biol. Reprod. 68, 2073–2080) was used to determine the relative abundance (RA) of gene transcripts in isolated ICM and TE cells. The allocation of ICM and TE cells was analyzed using a differential staining technique (Eckert and Niemann 1998 Mol. Hum. Reprod. 4, 957–965) to calculate the RA of each transcript on a per cell basis. RT-PCR assays were repeated at least six times. Significant differences in the RA of Dnmt1 (*In vivo*, TCM d7, Parth d7/d8, SOF d8), Dnmt3a (*In vivo*, SOF d7), and Hdac2 (TCM d7) transcripts were detected between ICM and TE cells. No differences were detected for all transcripts in ICM cells of embryos collected at either Day 7 or Day 8. In TE cells, the RA of Dnmt1 transcripts was significantly reduced or increased in embryos generated in SOF or TCM medium at Day 7, respectively, and the RA of Hdac2 transcripts was significantly higher in embryos generated in TCM medium at Day 8. These results suggest that *in vitro* culture alters the spatial expression pattern of genes related to epigenetic modifications in the cell lineages critical for a normal embryonic and fetal development.

*This work was supported by the Deutsche Forschungsgemeinschaft (DFG Ni 256/18-1).*