Matsuo et al. reported that circadian clock genes regulate the timing of cell division in mouse regenerating liver cells (2003). Their results suggested the importance of circadian clock genes for organs or tissues for which functions are characterized by cell division, such as pre-implantation embryos. To obtain basic information on the molecular functions of circadian clock genes in pre-implantation embryos, we investigated the expression profiles of transcripts and proteins of some circadian clock genes, clock, bmal1, cry1, and per2, in mouse germinal vesicle oocytes (GV), MII oocytes (MII), and pre-implantation embryos using real-time PCR and immunocytochemistry (ICC). Germinal vesicle oocytes were collected from ICR females at 48 h after PMSG priming. The mouse at 48 h after PMSG priming was primed with hCG, and MII were collected at 15 h after hCG priming. The pre-implantation embryos were collected at 6, 12, 24, 36, 48, 60, 72, 84, and 96 h after insemination, and they corresponded to early 1-cell, late
This study was supported by a Grant-in-Aid for the 21st Century COE Program of the Japan Mext and by a grant for the Wakayama Prefecture Collaboration of Regional Entities for the Advancement of Technology Excellence of the JST.

119 EARLY BOVINE GESTATIONAL DEVELOPMENT: MANIPULATED (IVF) AND NOT MANIPULATED IN LABORATORY


This work was supported by a Grant-in-Aid for the 21st Century COE Program of the Japan Mext and by a grant for the Wakayama Prefecture Collaboration of Regional Entities for the Advancement of Technology Excellence of the JIST.

High embryonic and fetal death rates in manipulated embryos in laboratory suggest that the process of early placentation can be inefficient. This investigation aimed to evaluate the development of placenta, and organogenesis of Nelore bovine embryos, and fetuses by natural stud and in vitro fertilization (IVF) over the period from 15 to 70 days of pregnancy. Fifty-nine embryos (15 to 50 days of gestation), 9 fetuses in initial period (60 to 70 days of gestation), and 10 embryos originated by IVF technique (35 to 46 days of gestation) were used. The same semen was used for all IVF, except the embryos originated by natural stud. The embryos were prepared by serial sections and the fetal membranes were fixed in 4% paraformaldehyde for light and scanning electron microscopy (SEM) and 2.5% glutaraldehyde for transmission electron microscopy (TEM). All material was routinely processed and stained. The embryos and fetuses originating from natural stud showed an increase in growth based on the weight of the gestational sac, and related with cranio-caudal and dorso-ventral length of the choioallantois and amniotic membrane in periods from 20 to 30 and from 50 to 60 days of the pregnancy. The gross appearance of the first cotyledons in development (9.39 ± 0.73 cm) was quantified at 30 to 40 days of pregnancy. The IVF embryos on days 35 to 46 of gestation showed discreet cotyledons presenting a functional decrease of development compared to embryos derived naturally. Ultrastructurally, the trophoblast showed binucleate trophoblast giant cells with a cytoplasm rich in electrodense vesicles and few mitochondria located in the apical poles suggesting lower cell activity. The yolk sacs of IVF embryos were shorter (1.07 ± 0.55 cm) when compared with the normal group (5.53 ± 3.14 cm) over 30 to 40 days of pregnancy. In both groups, the epithelium of the yolk sac presented cells with round nuclei, hemangiblast cells, and blood islands with a great number of primitive mononuclear cells. Embryos by natural stud (10% of analyzed ones) showed malformations in cephalic and frontal curvature, encephalocele, gastroschisis, and hepatomegaly. In conclusion, these results indicate occurrence of alteration in the organogenesis in bovine embryos originated by natural stud and retardation of fetal membrane development in bovine embryos by IVF.

This work was supported by FAPESP and CAPES.

120 EMBRYO SURVIVAL FOLLOWING LIPID-BASED TRANSFECTION OF 1-CELL STAGE BOVINE EMBRYOS WITH SMALL INTERFERING RNA (siRNA) FRAGMENTS AND/OR DNA


A, B, M. Bertolini, L. R. Bertolini, S. G. Petkov, K. R. Maddoc, and G. B. Anderson

The RNA interference (RNAi) technology is a powerful tool for studies in functional genomics. The aim of this study was to evaluate the effects of a cationic lipid-based small interfering RNA (siRNA) and/or DNA delivery to 1-cell-stage bovine embryos on survival to the blastocyst stage. In vitro-produced (IVP) embryos were generated according to Bertolini et al. 2002 (Theriogenology 58, 973), and cloned embryos were produced by the handmade cloning technique (Vajta et al. 2003 Biol. Reprod. 68, 571) using green fluorescent protein (GFP)-expressing fibroblast cells as nuclear donors. Lipofections were performed on zona-free 1-cell-stage IVP embryos at 24–28 h post-fertilization by exposure to 1% (v/v) Lipofectamine 2000 (Invitrogen Co., CA, USA), 0.002% (w/v) GFP plasmid (pEGFP-N1, Clontech Laboratories, CA, USA) and/or various doses of siRNA GFP-specific siRNA oligonucleotide (Invitrogen) or DNA methyltransferase 1 (Dnmt1)-specific siRNA fragments for 60 min at 39 ºC, according to
In mammals, the successful development of live offspring by somatic cell nuclear transfer (SCNT) has demonstrated the ability of oocyte or egg cytoplasm to reprogram the differentiated status of somatic DNA. However, the efficiency of development is low, and this has been attributed to incomplete or inappropriate reprogramming of epigenetic status. One such epigenetic marker is methylation of genomic DNA at CpG islands. In SCNT, derived embryo abnormal DNA methylation patterns have been reported by a number of groups; in particular, it has been observed that the methylation pattern of embryonic cells resembles that of the donor cell (Santos et al. 2003Curr. Biol. 13, 1116–1121). One strategy to improve reprogramming and, hence, development is to erase or reprogram the epigenetic status of the donor cell prior to nuclear transfer. We have previously reported that Xenopus egg and oocyte extracts show a differential effect on transcription. In oocyte extracts Pol I and II transcripts are maintained in the somatic cells; in egg extracts, these are abolished (Alberio et al. 2005 Exp. Cell. Res. 307, 131–141). To extend these studies, we have investigated the ability of oocyte and egg extracts to demethylate bovine somatic DNA. Preparation of Xenopus oocyte and egg extracts, culture, permeabilization of donor cells, and incubation conditions were all as previously described (Alberio et al. 2005 Exp. Cell. Res. 307, 131–141). Cells were incubated in extracts for 1 and 3 h at 21°C, centrifuged onto glass slides fixed in 4% Para formaldehyde for 15 min, followed by 4 M HCL for 1 h at 39°C, and blocked for 1 h. Cells were stained with mouse monoclonal anti-1MeC (1:50) overnight at 4°C followed by FITC-conjugated goat anti-mouse antibody (1:20) for 1 h at room temperature and mounted in Vectashield containing 10 µg of propidium iodide/mL. Nuclei were scored as positive or negative for 5MeC staining. In control cells, 90% of nuclei stained positively for 5MeC. In both oocyte and egg extracts the number of positive nuclei decreased with time showing demethylation of the somatic DNA 68 and 58% and 38 and 42% positive, respectively, after 1 and 3 h of incubation. Addition of apyrase (2%) to hydrolyze ATP inhibited demethylation in both extracts (90% nuclei positive). High rates of DNA replication were observed in somatic cells in egg extracts in contrast to no replication in oocyte extracts. Aphonidolin (1 µg/20 µL) was added to egg extracts to inhibit DNA replication, and under these conditions, DNA demethylation was abolished, suggesting a passive DNA demethylation mechanism as a result of DNA replication. In conclusion, Xenopus laevis oocyte and egg extracts can demethylate mammalian somatic DNA in an energy-dependent manner. In oocyte extracts, demethylation is independent of DNA replication, suggesting an active mechanism. In egg extracts,
122 UNIQUE EXPRESSION PATTERNS OF DIFFERENTIATION, GROWTH, AND CELL STRUCTURE FACTORS IN THE ELONGATING PORCINE CONCEPTUS

L. A. Blomberg, J. R. Miles, and K. A. Zuelke

USDA-ARS, Biotechnology and Germplasm Laboratory, Beltsville, MD 20705, USA

Elongation of the trophoderm and gastrulation of the embryonic disc, observed during gestational Days 11 (D11) through 12 (D12), denote a critical period of porcine conceptus development. Serial analysis of gene expression identified genes involved in cellular differentiation/structure (cytokeratin-8 and -18) and growth/cell migration/mesoderm-epithelial interaction (stratifin and midkine), which could potentially be regulated by steroids such as estrogen. Characterization of these factors is lacking in porcine conceptuses, therefore, the current study investigated mRNA expression of these factors and primordial tissues as well as protein expression and cellular localization to better define their biological significance. Conceptuses examined were of ovoid (D11; 6–10 mm), tubular (D11; 11–50 mm), or filamentous (D12; >100 mm) morphology. Cells of the conceptus were highly proliferative at all stages and the embryonic disc of the ovoid conceptus was already polarized as indicated by the protein expression of Ki67 and brachyury. Real-time PCR was utilized to determine the transcript expression profiles. Differential expression of cytokeratin-18 and midkine were not apparent; however, cytokeratin-8 was clearly down-regulated in filamentous compared to ovoid conceptuses. In contrast, stratifin mRNA levels were greatest in tubular conceptuses of 42–50 mm size. Transcripts for cytokeratin-8 and -18, stratifin, and midkine were detected in both cell types (endoderm and trophoblast) of the trophoderm. Western blotting and/or immunohistochemistry were utilized to examine protein expression and cellular localization. The embryonic disc of ovoid conceptuses was almost devoid of cytokeratin-18 protein, however, its distribution was uniform throughout the trophoderm at all stages of elongation. Stratifin and midkine proteins demonstrated more unique expression patterns within the conceptus. Distinct cell populations of the embryonic disc and the trophoderm contained stratifin; cellular localization was predominantly cytoplasmic but occasional nuclear translocation was evident. Furthermore, total protein levels of stratifin were not different among ovoid, tubular, and filamentous conceptuses, but proteolysis of the protein was apparent at the filamentous stage. Midkine protein expression was prominent in the embryonic disc of ovoid conceptuses. In tubular conceptuses, midkine was associated with cells that appeared to be migrating away from embryonic disc as well as some concentrating in the tips of the trophoderm. Our findings suggested that cytokeratin-8 and -18 are associated primarily with the trophoderm, as seen in other species. Furthermore, the distribution and localization of stratifin and midkine proteins could reflect attributed functions of these factors, minimal anti-proliferative activity in the rapidly growing conceptuses and cell migration important for gastrulation/trophoderm elongation, respectively.

123 HISTONE H3 MODIFICATIONS IN PIG OOCYTES DURING GROWTH, MATURATION, AND ACTIVATION

H.-T. Bui\textsuperscript{A,B}, V. T. Nguyen\textsuperscript{B}, T. Wakayama\textsuperscript{B}, and T. Miyano\textsuperscript{A}

\textsuperscript{A}Department of Life Science, Graduate School of Science and Technology, Kobe University, Kobe, Japan; \textsuperscript{B}Laboratory for Genomic Reprogramming, RIKEN-Center for Developmental Biology, Kobe, Japan

Oocyte growth, maturation, and activation are complex processes that include transcription, heterochromatin formation, chromosome condensation and decondensation, two consecutive chromosome separations, and genomic imprinting for producing the mature egg. The first sign of oocyte maturation is phosphorylation of histone H3, which leads to the chromosome condensation (Bui et al. 2004 Biol. Reprod. 70, 1843–1851). The objective of this study was to investigate the change in chromosome morphology in relation to histone modifications in pig oocytes during growth, maturation, and activation. Growing oocytes were collected from follicles at various diameters (from 0.1 to 6 mm) in pig ovaries. For maturation, oocyte–cumulus–granulosa cell complexes (OCGC) were collected from follicles that were 3 to 6 mm in diameter and cultured in modified TC199 for different periods of time to obtain meiotic stages of oocytes. For activation, oocytes were cultured for maturation in 42 h and were activated using a protocol that was described previously (Nguyen et al. 2003 Theriogenology 59, 719–734). Then, oocytes were examined by immunostaining with antibodies: anti-phospho-histone H3 at serine 10 or serine 28 (S10 or S28), anti-trimethyl-histone H3 at lysine 9 (K9), and anti-acetyl-histone H3 at lysine 9, 14, or 28 (K9, K14, or K28). Some oocytes were examined for double assay of Cdc2 and H3 kinase, which were measured by phosphorylation of histone H1 and myelin basic protein as their substrates. To examine the effects of histone deacetylase (HDAC) inhibition, OCGC were cultured in maturation medium supplemented with or without 100 nM trichostatin A for 42 h. The results show that, during the growth phase, histone H3 became methylated at K9 and is acetylated at K9, K14, and K18. When the fully grown oocytes start maturation, histone H3 becomes phosphorylated at S28 and then S10 and is deacetylated at K9, K14, and K18. After oocyte activation, reacetylation and dephosphorylation of histone H3 correlates to the decondensation of chromosomes. We also found that the activity of histone H3 kinase occurred at a similar time course to that of phosphorylation of histone H3-S28. This suggests that phosphorylation of H3-S28 might be one of the key events initiating meiotic chromosome condensation. The inhibition of HDAC induces maintenance of acetylation of H3-K14 and dephosphorylation of histone H3 at S10 and S28. Therefore, the chromosome could not condense and affect meiotic progression. It is possible that deacetylation is required for the phosphorylation of histone H3. The results suggest that chromatin morphology of pig oocytes is regulated by acetylation/deacetylation and phosphorylation/dephosphorylation of histone H3 and that histone deacetylase activity is essential for the process of chromatin remodeling in pre-ovulatory oocytes. Although histone acetylation and phosphorylation were reversible, histone methylation has energetic stability and is established during the oocyte growth phase. It is also suggested that the ordered phosphorylation of histone H3 at S10 and S28 is influenced by acetylation of neighboring lysines in the histone H3 molecule.
124 KNOCKING-IN OF A FOREIGN GENE ON A BOVINE BETA-CASEIN GENE INTO BOVINE PRIMARY FIBROBLASTS USING HOMOLOGOUS RECOMBINATION EVENTS

M. Chang\textsuperscript{A}, K.-B. Oh\textsuperscript{A}, J.-S. Park\textsuperscript{A}, D.-B. Koo\textsuperscript{A}, S.-T. Shin\textsuperscript{B}, K.-K. Lee\textsuperscript{A}, and Y.-M. Han\textsuperscript{A}

\textsuperscript{A}Korea Research Institute of Bioscience and Biotechnology, Daejeon, 305-106, South Korea; \textsuperscript{B}College of Veterinary Medicine, Chungnam National University, Daejeon, 305-164, South Korea

Knocking-in of a foreign gene on a tissue-specific genomic region has not been reported in livestock. In this study, we constructed two knock-in vector cassettes specific for the bovine beta-casein gene which is expressed only in the mammary gland during lactation. The targeting vector cassettes, pBCK1 and pBCK2, have homology regions for the bovine beta-casein gene and contain 13.1 kb and 9.1 kb targeting arms with different long arm lengths, respectively. The targeting vector cassettes have unique restriction enzyme sites for insertion of foreign therapeutic genes in front of the neo gene which was inserted into the vector as a selection marker. The human thrombopoietin (hTPO) gene was inserted into the restriction enzyme sites of both targeting vectors, which were named pBCTPOKI1 and pBCTPOKI2. When the two targeting vectors were transfected into bovine ear skin fibroblasts using Lipofectamine (Invitrogen, Seoul, South Korea), 6.3% of neo resistant clones (2/32) were homologously targeted with the pBCTPOKI2 vector. Cells from the targeted colonies were nuclear-transferred into enucleated bovine oocytes and cultured to the blastocyst stage. A total of 66 blastocysts were generated of which 46 were transferred into recipients. No offspring have been produced at this time. Here we first describe knock-in vectors specific for a bovine beta-casein gene, which will be employed to generate animal bioreactors that produce therapeutic proteins secreted into the milk.

125 ISOLATION OF MULTILINEAGE STEM CELLS FROM THE PORCINE MAMMARY FAT PAD

T. Davidson\textsuperscript{A}, S. Lane\textsuperscript{A}, C. Ferguson\textsuperscript{A}, M. Wheeler\textsuperscript{A,B}, and W. Hurley\textsuperscript{A}

\textsuperscript{A}Department of Animal Sciences, University of Illinois, Urbana, IL, USA; \textsuperscript{B}Department of Bioengineering, University of Illinois, Urbana, IL, USA

Porcine mammary tissue is a dynamic system that undergoes multiple cycles of growth, differentiation, and regression during the life cycle of a female; however, the mechanisms of mammary morphogenesis are not fully understood. The current hypothesis suggests that epithelial stem cells in the terminal end buds interact with surrounding epithelial and stromal cells during mammary tissue development and regeneration. Because the mammary gland of the virgin animal consists largely of a pad of adipose tissue, we propose that adipose-derived stem cells from the mammary fat pad (MFP) may also play a role in the regeneration process. Therefore, the objective of this experiment was to determine whether multilineage stem cells are present in the MFP of gilts. Adipose tissue from the MFP and back fat (BF) from the loin region were harvested from six nulliparous, cycling Yorkshire gilts, approximately 16 mo of age. Methods for isolation of adipose-derived stem cells were modified from those of Malusky and Wheeler (2004; International Society for Stem Cell Research, Proceedings 2nd Annual Meeting, abstr. 248.124). Presumptive stem cells isolated from each tissue type were maintained in vitro in DMEM supplemented with either 10% newborn calf serum (NCS; Rep 1 and 2) or 10% fetal calf serum (Rep 3). BF and MFP cell types formed colonies of fibroblast-like cells within 3 to 5 d. Growth characteristics are shown in Table 1. When the cells were 80% confluent, they were trypsinized and reseeded into 75-cm\textsuperscript{2} flasks. When cultured in the presence of NCS, both cell types had a progressive decrease in viability, and these cultures could not be maintained past Passage 4. Differentiation of both cell types (Passage 1) into adipogenic, myogenic, and osteogenic lineages are currently underway. Based on morphological evaluations, both cell types are able to differentiate into the aforementioned lineages; however, differentiation of MFP-derived cells occurred at a slower rate and was less pronounced than that of BF-derived cells. These preliminary findings suggest that the MFP likely contains a population of multipotent stem cells; however, at this time, it is not possible to make meaningful statistical comparisons. Further experiments are needed to fully characterize these cells and determine their role in mammary gland morphogenesis.

<table>
<thead>
<tr>
<th>Table 1. Growth of porcine adipose-derived stem cells obtained from the BF or from the MFP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rep 1</td>
</tr>
<tr>
<td>BF</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>Pop DbLgs\textsuperscript{a}</td>
</tr>
<tr>
<td>Pas 1</td>
</tr>
<tr>
<td>Pas 2</td>
</tr>
<tr>
<td>Pas 3</td>
</tr>
<tr>
<td>Hours/Dblg\textsuperscript{b}</td>
</tr>
<tr>
<td>Pas 1</td>
</tr>
<tr>
<td>Pas 2</td>
</tr>
<tr>
<td>Pas 3</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Pop DbLgs = population doublings; number of times the original cell population doubled before passage.  
\textsuperscript{b}Hours/Dblg = hours per doubling; number of hours for one population doubling to occur.

This study was partially supported by the Council for Food and Agricultural Research (C-FAR) Sentinel Program and USDA Multistate Project (W-1171).
126 NUCLEAR TRANSLOCATION OF NUCLEAR FACTOR KAPPA B AND ITS ROLE IN MOUSE PRE-IMPLANTATION DEVELOPMENT

Y. Fumiwa, H. Imai, and M. Yamada

Laboratory of Reproductive Biology, Graduate School of Agriculture, Kyoto University, Kyoto, Japan

In mouse pre-implantation development, it has been reported that RelA, one of the subunits of nuclear factor kappa B (NF-κB), is expressed in eggs and embryos from the Metaphase II oocyte to the blastocyst stage. However, the role of NF-κB in the pre-implantation development has not yet been elucidated in detail. In this study, we examined (1) the activation of NF-κB during mouse pre-implantation development and (2) the effect of a synthetic peptide inhibitor of NF-κB, SN-50, which inhibits nuclear translocation of NF-κB on the pre-implantation development. Fertilized one-cell embryos were collected 17 h post-hCG from the ampulla of oviducts of superovulated ICR mouse females that had been mated with the same strain of males and then were cultured in KSOM medium at 37°C under 5% CO2 in air for 4 d. To elucidate the timing of NF-κB activation, we examined the localization of NF-κB in the nucleus by an immunofluorescence approach using RelA antibody with a laser confocal microscope. RelA was distributed mainly in the cytoplasm of embryos from the one-cell stage through the blastocyst stages. The presence of RelA in the nucleus, evidence for NF-κB activation, was observed in embryos from the one-cell to the compacted 8-cell stages. Moreover, we observed RelA punctate localization in nucleoplasm of embryos from the one-cell to the 4-cell stages, and nuclear dots were enriched conspicuously in the one-cell embryos and the late 2-cell embryos. These results suggest that NF-κB is activated in embryos from the one-cell to the compacted 8-cell stages and that its activation seems to be particularly strong at the developmental stage when RelA appeared to be concentrated in nuclear dots, as it has been reported that NF-κB and other transcription factors and co-activators form punctate structures called ‘enhancersomes’ in association with particular promoters in the nucleus. Next, we examined the effect of SN-50 on the pre-implantation development of mouse embryos. When embryos were treated with SN-50 at 20 μg/mL from the 2-cell stage, 63% (33 of 52) of the embryos developed to blastocysts, but 55% (18 of 33) of the blastocysts showed abnormal morphology, such as poor cavitation, and many degenerating cells extruded into the perivitelline space. The percentages of 2-cell embryos that formed morphologically normal blastocysts were significantly lower in the SN-50 treatment group (29%; 15 of 52) than in the untreated control group (76%; 35 of 46) and in the SN-50M (inactive analogue of SN-50, 20 μg/mL treatment group (72%; 38 of 53). These experiments were done in 4 replicates, and the statistical analyses of the data were done by ANOVA and Fisher’s PLSD test. Nuclear location of RelA was not observed in the embryos at the 4-cell stage when treated with SN-50 from the 2-cell stage, although observed in control and SN-50M-treated embryos. Furthermore, it was found that most of embryos (23 of 37) treated with SN-50 from the compacted 8-cell or morula stages developed normally to the blastocyst stage as control embryos (25 of 36). These results suggest that morphological aberration at the blastocyst stage is elicited by inhibiting NF-κB activation.

127 BINDING RETINOID RECEPTORS BY SPECIFIC AGONISTS AFFECTS THE BOVINE BLASTOCYST DEVELOPMENT INVITRO


A Genética y Reproducción, SERIDA, Gijón, Asturias, Spain; B Livestock Farm of Graduate School of Agriculture, Kyoto University, Tamba, Kyoto Prefecture, Japan

Production of embryos in vitro with improved inner cell mass (ICM) and high ICM per total cell rate is a major objective in reproductive biotechnology. Exogenous all-trans retinoic acid (ATRA), a vitamin A metabolite, and endogenous retinoid regulate development and differentiation during bovine morula to blastocyst transition in vitro. ATRA binds to retinoic acid-receptor (RAR), and the ATRA isomere 9-cis-retinoic acid (9-cis-RA) binds to both RAR and the retinoid X receptor (RXR). The unspecific binding of 9-cis-RA to receptors makes it difficult to study RXR transactivation. Therefore, in this work we studied blastocyst development and cell counts by using a specific synthetic RXR agonist [LG100268 LG; a gift of Ligand Laboratories] as opposed to the effect exerted by ATRA upon RAR binding. Cumulus–oocyte complexes from slaughterhouse ovaries were matured and fertilized in vitro. Presumptive zygotes were cultured in B2 medium with Vero cells until 139 h post-insemination (Day 6), the time at which embryos [morulae (≥90%) + early blastocysts] underwent treatments for 48 h in 400 μL of SOFaaci with 5% FCS. Data (5 replicates per experiment) were analyzed by CATMOD for effects, processed by GLM and Duncan’s test, and expressed as LSM ± SE (a,b,c P ≤ 0.05). After a LG dose-response experiment (n = 480 morulae), blastocysts rates from LG 1 μM on Day 7 were higher than LG 10 μM, LG 0.1 μM, and LG 0 μM (Day 7: 42.8 ± 4.1 vs. 34.4 ± 3.7, 36.8 ± 3.7, and 32.4 ± 3.7, respectively). On Day 8, LG 1 μM also yielded more blastocysts than LG 0.1 μM (50.4 ± 4.2 vs. 44.4 ± 3.7, respectively). By differential cell counting (n = 113 blastocysts), hatched blastocysts with LG 10 μM showed proliferation in the ICM, while trophectoderm (TE) cells decreased conversely to LG concentration. These effects were not obvious in expanded blastocysts. In a subsequent experiment (n = 340 morulae), ATRA led to blastocysts rates on Day 8 that were higher than negative, untreated controls, but not different from LG 1 μM (42.4 ± 2.4 vs. 33.1 ± 2.0 and 36.0 ± 2.4, respectively). ATRA and LG 1 increased TE in expanded blastocysts (n = 42) (102 ± 13.2 and 96.23 ± 13.2, respectively vs. 72.8 ± 10.9 in the untreated group) but not in their hatched counterparts (n = 44). There were no differences in the ICM; but percentages of ICM per total cells were higher in hatched blastocysts cultured with ATRA than in expanded LG 1 μM blastocysts and expanded controls (39.5 ± 5.5 vs. 24.2 ± 5.7, and 20.9 ± 4.7, respectively). Manipulation of retinoid receptor-specific pathways make it possible to control blastocyst development and differentiation, leading to embryos of improved quality and viability. Work is in progress to analyze gene expression in these blastocysts.

This work was supported by grant MCYT, project AGL–2005–04479.
128 MITOCHONDRIAL DNA DELETIONS IN RHESUS MACAQUE OOCYTES, EMBRYOS, AND ADULT AND EMBRYONIC STEM CELLS

T. Gibson, T. Quebedeaux, S. Rajasekaran, and C. Brenner
University of New Orleans, New Orleans, LA, USA

Mitochondria are the most abundant organelles in mammalian oocytes and early embryos. Previous data have shown that mitochondrial DNA (mtDNA) deletions are present both in human oocytes and in embryos from in vitro fertilization (IVF) patients and suggest that accumulation of these deletions may contribute to mitochondrial dysfunction and impaired ATP production. In addition, high levels of mitochondrial mutations are present in skeletal muscle fibers from aged rhesus macaques. The specific aims of this study were to determine whether the mitochondrial common deletion is present in non-human primate oocytes and embryos generated by IVF and to determine whether mtDNA mutations are already present in immature oocytes from rhesus ovaries. Using a nested primer polymerase chain reaction (PCR) strategy, we determined the frequency of the rhesus common deletion in immature oocytes compared with stimulated oocytes and embryos. There was a low incidence (21%) of the rhesus common deletion present in immature, unstimulated oocytes derived from necropsied ovaries of 2 to 10-yr-old rhesus macaques. However, there was >3-fold increase (71.4%) in the frequency of deleted mtDNA in stimulated oocytes and IVF embryos from age-matched fertile monkeys. We postulated that, in addition to skeletal muscle, a similar time-dependent accumulation of mtDNA deletions occurs in fertile rhesus macaque oocytes and embryos. We are now investigating the effects of culture and passage number on mtDNA deletions in primate adult and embryonic stem cells. We propose the rhesus monkey to be an excellent model to assess the quality of gametes and embryos, as well as stem cells, and their developmental competence in human and non-human primates.

This study was supported by National Institutes of Health grants RR15395 and HD045966.

129 INSULIN-LIKE GROWTH FACTOR-I PROMOTES BLASTOCYST DEVELOPMENT OF HEAT-SHOCKED BOVINE EMBRYOS INDEPENDENT OF ITS ANTI-APOPTOTIC EFFECTS REQUIRING PI3K SIGNALING

F. D. Jousan and P. J. Hansen
University of Florida, Gainesville, FL, USA

Insulin-like growth factor-I (IGF-I) reduces effects of heat shock on blastocyst development and induction of apoptosis. The present objective was to test whether IGF-I would allow for blastocyst development following heat shock because of its anti-apoptotic effects. Because anti-apoptotic actions of IGF-I require signaling through the phosphatidylinositol 3-kinase (PI3K) pathway, an inhibitor of PI3K (LY 294002) was used in Exp. 1 to determine whether it would prevent the thermoprotective effects of IGF-I on development. Embryos were produced in vitro in KSOM-BE2 medium ±100 ng mL⁻¹ of IGF-I. Embryos ≥16 cells at 5 d post-insemination (dpi) were placed in fresh drops containing the same IGF-I treatment as well as LY 294002 (100 µM) or vehicle (0.1% DMSO) and cultured at either 38.5°C or 41°C for 15 h. All groups were then cultured at 38.5°C until 8 dpi when blastocyst development was assessed (10 replicates; 112 to 142 embryos per treatment). For embryos in DMSO, IGF-I did not increase the percentage of blastocysts because of culture conditions. However, for IGF-I embryos, heat shock reduced blastocyst development (P < 0.01), and IGF-I blocked this decrease (percent blastocyst: 42.0 and 54.7% for control and IGF-I embryos, respectively; SEM = 3.6%). Similar results were obtained for embryos cultured with LY 294002. The percentage of embryos becoming blastocysts for control embryos was 53.5 and 35.5%, respectively, at 38.5 and 41°C, development for IGF-I-treated embryos was, respectively, 55.8% and 49.4% at 38.5°C and 54.7% and 48.6% for IGF-I embryos at 38.5°C and 41°C, respectively (SEM = 4.8%). Analysis of the entire data set revealed an IGF-I × temperature interaction (P < 0.05) but no interactions with inhibitor treatment. Thus, IGF-I protected embryos from heat shock in the presence and absence of LY 294002. In Exp. 2, procedures were similar, except that embryos were cultured with a caspase-3 inhibitor (z-DEVD-fmk, 100 µM) instead of LY 294002 (12 replicates; 114 to 137 embryos per treatment). For embryos in DMSO, blastocyst development was reduced by heat shock (P < 0.06) and increased by IGF-I (P < 0.06). The percentage of embryos becoming blastocysts was 67.0 and 55.8% for control embryos at 38.5 and 41°C, respectively, vs. 74.7 and 70.8% for IGF-I embryos at 38.5 and 41°C, respectively (SEM = 5.2%). For the embryos cultured with z-DEVD-fmk, heat shock reduced blastocyst development (P < 0.01), and IGF-I was no longer effective in blocking the reduction in blastocyst development caused by heat shock (IGF-I × inhibitor treatment; P < 0.01). Blastocyst development was 66.4 and 58.7% for control embryos at 38.5 and 41°C, respectively, vs. 61.8 and 46.8% for IGF-I embryos at 38.5 and 41°C, respectively (SEM = 4.6%). Note that z-DEVD-fmk did not protect embryos from the anti-developmental effects of heat shock but exacerbated heat shock effects for IGF-I-treated embryos. In conclusion, the ability of IGF-I to allow heat-shocked embryos to continue development to the blastocyst stage is independent of its anti-apoptotic effects involving the PI3K pathway but may depend on active caspase-3.

This work was supported by USDA NRICGP 2002–35203–12664, BARD US–3551–04, and USDA TSTAR 2004–34135–14715.

130 ANTI-APOPTOTIC pTEGT SHOWS A DIFFERENTIAL mRNA TRANSCRIPT LEVEL DURING IN VITRO MATURATION AND EARLY DEVELOPMENT OF PORCINE IVP EMBRYO

Department of Animal Biotechnology, BMIC, and IBST, Konkuk University, Seoul 143-101, South Korea

It is well known that very early development of the mammalian pre-implantation embryo is regulated by gene transcripts and proteins stored in the oocyte and that the embryonic genome gains control of development following 1 to 3 cleavage divisions. An active transcription and translation
is required for chromatin condensation and germinal vesicle breakdown in pig oocyte. The transition from maternal to embryonic control of development is a gradual event, and following this transition, the maternally derived transcripts and proteins are gradually degraded. Successful embryonic development is dependent on the temporal and stage-specific expression of proper genes, but information on specific gene expression during early stages before zygotic gene activation (ZGA) is limited. Before activation of the embryonic genome, mRNA and proteins synthesized during oocyte growth and maturation contribute to early development. In this study, we compared the mRNA transcripts level among porcine immature, in vitro-matured and cleaved 2- to 4-cell stage embryos after in vitro fertilization to identify genes that show differential mRNA transcript levels during maturation and very early embryonic development. For the first strand cDNA synthesis, oligo (dT) primers were added to the total RNA isolated from each sample. Using annealing control primer (ACP)-based GeneFishing PCR, we detected tens of different genes showing differential mRNA transcript level (DRTL) and nine DRTL genes were identified to be KCRF, CAMSAP1, SMP1, FLJ20647, LOC132321, NADH1, NADH6, HERC3, and TEGT. Of 9 DRTL genes, TEGT showed higher mRNA transcript level at the immaturity stage, and mRNA transcript levels of the other 8 genes were increased after in vitro maturation. Therefore, we focused on TEGT (tests enhanced gene transcript), which is highly expressed in testis and also in oocytes before in vitro maturation. Differential mRNA transcripts pattern of CAMSAP1 and TEGT were confirmed using RT-PCR and real-time RT-PCR. Porcine TEGT (pTEGT) was cloned and sequenced to have an ORF of 714 bp nucleotides and to encode an integral membrane protein. When overexpressed in HEK293 cells, pTEGT suppressed apoptosis induced by etoposide. We found that pTEGT, but not TEGT-C (C-terminal deletion mutant), inhibited etoposide- and staurosporine-induced cell death. Next, we found that introduction of TEGT siRNA suppressed the anti-apoptotic effect of TEGT. Interestingly, expression of TEGT suppressed etoposide-induced ERK activation, suggesting that ERK phosphorylation is involved in the anti-apoptotic function of the gene. Several reports showed that apoptosis and MAP kinase signaling pathways play important roles in oocyte maturation and early embryo development. Therefore, the anti-apoptotic effect of TEGT was suggested to play a key role in the normal oocyte maturation and early embryo development.

This work was supported by the Research Project on the Production of Bio-organs, Ministry of Agriculture and Forestry, Republic of Korea.

131 DYNAMICS OF HISTONE H3 METHYLATION AT POSITIONS K4 AND K9 IN MOUSE, RABBIT, AND BOVINE PRE-IMPLANTATION EMBRYOS


A University of Saarland, Natural Sciences-Technical Faculty III, FR 8.3, Biological Sciences, Genetics/Epigenetics, Saarbrücken, Germany; B Department of Molecular Animal Breeding and Biotechnology, Ludwig-Maximilian University, Munich, Germany; C Institute for Animal Breeding (FAL), Department of Biotechnology, Neustadt/Mariensee, Germany

In mammals, upon the penetration of sperm into the oocyte, the parental genome undergoes dramatic epigenetic changes. Proteins packaging of DNA is replaced by histones that acquire specific modifications. In mouse zygotes, paternal DNA gets rapidly demethylated by an active mechanism. In bovine zygotes the methylation from parental DNA is erased only partially, and in rabbit zygotes it persists at the initial level. To understand whether these reprogramming differences are also reflected in histone modifications, we examined the dynamic changes of histone H3 methylation at positions K4 and K9 in mouse, bovine, and rabbit zygotes and in preimplantation embryos using an immunofluorescence staining procedure (Lepikhov and Walter 2004 BMC Dev. Biol. 4, 12). In zygotes, maternal chromatin contains both types of histone H3 methylation. After fertilization protamines in sperm are very quickly replaced by histones. After the formation of nucleosomes, histone H3 acquires methylation at position K4 in a stepwise manner: first as mono-methylated form and later as tri-methylated. In the late zygote, both maternal and paternal pronuclei show equal levels of histone H3 methylation at position K4. Regardless of the differences in DNA reprogramming in these 3 species, H3/K9 di-methylation is not detected on paternal genomes and is only associated with maternal genomes. During the subsequent cleavage stages, H3/K9 di-methylation decreases gradually and becomes hardly detectable in 4-cell bovine and rabbit embryos. In mouse embryos, it is detectable through all the stages. Bovine embryos reacquire this type of modification at the 8–16 cell stage, and it remains at the very low levels in rabbit, embryos until the blastocyst stage. In conclusion, mouse, rabbit and bovine zygotes show similar patterns of H3/K4triMe and H3/K9diMe distribution despite the difference in paternal DNA demethylation. The dynamics of H3/K9diMe distribution patterns in cleavage stage embryos from all embryos do not correlate with embryonic genomic activation events.

132 DIFFERENCES IN RESPIRATION RATES BETWEEN IN VIVO- AND IN VITRO-PRODUCED BOVINE EMBRYOS


A Danish Institute of Agricultural Sciences, 8830, Tjele, Denmark; B Royal Veterinary & Agricultural University, 1870, Frederiksberg C, Denmark; C Trans - Embryo Genetics, 8740 Bradstrup, Denmark; D Fertilitech ApS, 8000 Aarhus C, Denmark

In vitro-produced (IVP) bovine embryos differ (e.g. morphology and physiology) from their in vivo counterparts. Oxygen consumption is an indicator of the overall metabolic activity of a single embryo. Therefore, the aim of this study was to determine and compare respiration rates of in vivo- and in vitro-produced bovine day 7 embryos. Diameters of these two embryo types were also compared. In vivo embryos (n = 28) were recovered from 8 superovulated Holstein Frisian cows on day 7 following AI, while IVP embryos (n = 160; Holm et al. 1999 Theriogenology 52, 683–700) were used on day 7 after fertilization. Embryos were measured (outer diameter) and morphologically evaluated (Quality 1 to 4, IETS Manual, 1998). Only
transferable in vivo embryos were used (i.e. excluding Quality 4). Respiration rates were measured on each embryo by Nanorespirometer technology (Lopes et al. 2005 Reprod. Fertil. Develop. 17, 151). Data were analyzed using Proc Mixed, and values are presented as mean ± SEM. Values with different superscripts differ significantly (P < 0.05). The average respiration rates were 0.82 ± 0.06 nL/h for in vivo vs. 1.37 ± 0.06 nL/h for IVP embryos. The average respiration rates for the different morphological qualities were as follows (nL/h, numbers in brackets): IVP: 2.1 ± 0.08a (38), 1.37 ± 0.07a (55), 1.08 ± 0.07a (48) and 0.62 ± 0.11d (19) for Quality 1, 2, 3, and 4, respectively. In vivo: 1.17 ± 0.21b,c,e (6), 0.80 ± 0.15c,d,e (12), and 0.64 ± 0.16d,e (10) for Quality 1, 2, and 3, respectively. The average diameter (mm) of in vivo and IVP embryos was 0.157 ± 0.002a and 0.176 ± 0.002b, respectively. Respiration rates were directly related to embryo diameter; larger embryos were associated with higher respiration rates (y = 17.55 ± 1.32 nL/h x mm, n = 188). Respiration rates of in vivo embryos were significantly lower than those of IVP embryos, regardless of quality. This difference could reflect an effect of the culture conditions on IVP embryos because media components affect embryo metabolism. Moreover, the different ages (day 7 for IVP vs. approximately Day 6.5 for in vivo embryos, because in vivo embryos are less than 7 days after fertilization at recovery) and stages (IVP: up to expanded blastocyst stage; in vivo: morula or early blastocyst stage) could have influenced the results and also partly explain the smaller diameter of the in vivo embryos. Finally, respiration rates decreased proportionately to the morphological quality within embryo type, indicating that morphological differences are reflected at the physiological level. In conclusion, this study further outlines metabolic differences between in vivo and IVP bovine embryos. Whether such differences are a manifestation of metabolic stress associated to the separation from the natural environment or reflect suboptimal culture conditions is yet to be determined.

ASL is supported by FCT, Portugal.

133 ROLE OF CASPASE-9 AND STAGE OF DEVELOPMENT IN INDUCTION OF APOPTOSIS BY HEAT SHOCK AND TUMOR NECROSIS FACTOR-α IN BOVINE PRE-IMPLANTATION EMBRYOS

B. Loureiro A,B, A. M. Brad a, and P. J. Hansen A

A Dept. of Animal Sciences, University of Florida, Gainesville, FL, USA; B Departamento de Medicina Veterinária, Universidade Federal Rural de Pernambuco, Recife, PE, Brasil

Heat shock and tumor necrosis factor-α (TNF-α) can increase apoptosis in bovine embryos in a developmental-dependent manner. It was hypothesized that addition of the caspase-9 inhibitor, z-LEHD-fmk, would block induction of apoptosis caused by heat shock of 41°C and TNF-α. Furthermore, it was hypothesized that the magnitude of induced apoptosis would increase with stage of development. Embryos were collected on day 4, 5, and 6 after in vitro insemination and were cultured for 24 h in the presence of either 100 µM z-LEHD-fmk reconstituted in 0.5% (v/v) dimethyl sulfoxide or vehicle dimethyl sulfoxide at either (1) 38.5°C for 24 h (control), (2) 41°C for 15 h followed by 38.5°C for 9 h, or (3) 38.5°C for 24 h with 10 ng/mL murine TNF-α. Embryos were then fixed, and the proportion of blastomeres undergoing apoptosis was determined using TUNEL labeling. Heat shock did not increase the percentage of blastomeres that were TUNEL-positive (% apoptosis) at day 4 (n = 100 embryos total). In contrast, heat shock increased % apoptosis at day 5 and day 6 (P < 0.04) and this effect was blocked by z-LEHD-fmk (temperature × inhibitor, P < 0.04). At day 5, % apoptosis in the absence and presence of z-LEHD-fmk was 3.8 ± 1.9% and 3.7 ± 1.7% at 38.5°C vs. 8.9 ± 1.5% and 4.1 ± 1.7% at 41°C (n = 75 embryos total). At day 6, % apoptosis in the absence and presence of z-LEHD-fmk was 3.6 ± 1.1% and 3.7 ± 1.2% at 38.5°C vs. 11.1 ± 1.1% and 6.1 ± 1.2% at 41°C (n = 168 embryos total). Mean cell number at the end of culture ranged from 21 to 26 cells at day 4, 43 to 73 cells at day 5, and 101 to 114 cells at day 6. Treatment with TNF-α also increased apoptosis at all days (P < 0.01), and z-LEHD-fmk blocked this effect (TNF-α × inhibitor, P = 0.05; n = 361 embryos total). Across days, % apoptosis was 3.6 ± 1.4% (control), 3.3 ± 1.3% (inhibitor), 11.1 ± 1.3% (TNF-α), and 6.0 ± 1.4% (TNF-α + inhibitor). Mean cell number at the end of culture ranged from 21 to 27 cells at day 4, 59 to 74 cells at day 5, and 105 to 115 cells at day 6. In conclusion, activation of caspase-9 dependent pathways is involved in the induction of apoptosis by heat shock and TNF-α. Moreover, the magnitude of induced apoptosis increases as embryos advance in development.

This work was supported by USDA Grant No. 2004–34135–14715 and BARD Grant No. US–3553–04.

134 BRAHMA-RELATED-PROTEIN 1 OVEREXPRESSION IN PARTHENOGENETIC PORCINE EMBRYOS

L. Magnani and R. Cabot

Animal Sciences Department, Purdue University, West Lafayette, IN 47907, USA

The chromatin remodeling protein Brahma-related-protein 1 (Brig1) has been implicated in several cellular processes that affect chromatin structure and transcription. Brig1 is a member of the SWUSNF family, a large group of chromatin remodeling proteins, all of which have the characteristic of utilizing the energy from ATP hydrolysis to catalyze the repositioning of nucleosomes on DNA templates. Various members of this class of proteins are known to be differentially expressed throughout vertebrate development. We hypothesize that the balance of chromatin remodeling factors present in cleavage stage porcine embryos has an impact on embryonic developmental potential. As a first step to testing this hypothesis, we attempted to overexpress a wildtype version of Brig1 in cleavage stage porcine parthenogenetic embryos. The Brig1 construct was a gift from Dr. Anthony Imbalzano (University of Massachusetts, Worster, MA). The Brig1 cassette ligated into the Nhel site of pIRE2-eGFP, Clontech. This construct was sequenced to verify the orientation. Oocytes were matured in vitro in defined culture medium (TCM199 supplemented with 0.1% polyvinyl chloride, 10 ng/mL epidermal growth factor, 0.5 IU/mL LH, 0.5 IU/mL FSH, 1 mmo cysteine) for 42–44 h at 39°C in 5% CO2. Following in vitro maturation, oocytes were denuded and activated by electroproporation and cultured in NCSU-23 supplemented with BSA (4 µg/mL) at 39°C.
The production of pig embryos in vitro is still relatively inefficient compared with results obtained with oocytes matured and fertilized in vivo. The main reasons for this limited performance are polyspermy after IVF and the poor developmental ability of embryos produced by IVM–IVF (Kikuchi et al. 2002 Biol. Reprod. 103:1–1041). Between factors affecting polyspermy are the sperm procedures before IVF. Usually, these procedures including centrifugations that increase reactive oxygen species (ROS) formation in spermatozoa. ROS play an important role in sperm physiology including capacitation. Physiological concentrations of ROS have been proposed to enhance sperm capacitation by increasing cAMP synthesis and by inhibiting protein tyrosine phosphatases whilst activating tyrosine kinases. In general, epididymal spermatozoa appear to be able to capacitate and fertilize eggs in vitro much more easily than ejaculated spermatozoa (Yanagimachi Mammalian Fertilization. In: The Physiology of Reproduction, Raven Press 1988; 135–182). In this study, we investigated how different sources (ejaculated spermatozoa vs. epididymal spermatozoa) and sperm capacitating methods, usually employed in porcine IVF, could affect ROS generation. Sperm-rich fractions from five fertile boars and sperm from five different epididymides were used. The semen samples were then: (i) washed in Dulbecco’s phosphate-buffered saline (DPBS) supplemented with 0.1% BSA, (ii) left unwashed, or (iii) washed on a Percoll (Pharmacia, Uppsala, Sweden) gradient (Matás et al. 2005 J. Androl. 26, 396–404). ANOVA analysis revealed a significant effect of sperm treatment on the ROS generation ($P < 0.001$). The highest value was obtained in sperm washed on a Percoll gradient and the lowest in unwashed semen. When ejaculated vs. epididymal semen was analyzed, the same tendency was observed in both. However, the values were always lower in epididymal semen than in ejaculated semen ($P < 0.001$). As a conclusion, ROS generation is different between treatments and between semen procedures for the time interval studied, and this finding may help to explain the different outcome in IVF among laboratories.

This work was supported by Ministerio de Educación y Ciencia, AGL2003–03144.
from the 1-cell stage to the 2-cell stage ($P < 0.05$). The IF analysis revealed localization of Rhophilin-2 and GABARAP at the nucleolus of all follicle stage in the ovary. Moreover, Rhophilin-2 and GABARAP were found to be localized on the microtubules of 1-cell and 2-cell embryos, but no signal of Rhophilin-2 was detected in 4-cell embryos. These results suggest that Rhophilin-2 protein regulates the cytoskeletal organization in 1-cell to 2-cell embryos and is involved in the molecular mechanism of cell division by coupling with GABARAP.

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

137  **IRON TRANSFER ACROSS THE LLAMA PLACENTA (LAMA GUANICOE GLAMA)**


$^A$Universidade de São Paulo/USP, São Paulo, Brazil; $^B$Faculdade de Zootecnia de Dracena/UNESP, Dracena, São Paulo, Brazil; $^C$Center for Animal Biotechnology and Genomics, Texas A&M University, College Station, TX 77843, USA

The placenta of the llama has been described as epitheliocorial in type, but recent studies have not shown extensively the fetal nutrition aspects in this animal. In epitheliocorial placentaion there is development of structures called areolae, as well as inter-microvillous attachment of the trophoblast, with irregular contact, to the uterine epithelium. This attachment is interrupted and the transfer of substances between the mother and the fetus takes place across the areolar cavity. These areolae appeared as small rounded or dome-shaped elevated areas of the chorioallantoic membrane over the narrow uterine gland openings. In order to detail their mechanisms of iron transfer in the llama placenta, we collected the samples of nine uteri between 28 to 36 weeks of pregnancy in association with fetal membranes. These samples were fixed in 4% paraformaldehyde in PBS, processed, and stained for light microscopy (HE, picrosirius, and Masson’s trichrome), histochemistry (Perls, acid phosphatase, and PAS reactions) and immunohistochemistry with rabbit anti pig uteroferin antibody to confirm the iron transfer, because the uteroferin is an iron transporter and a progesterone-induced hematopoietic growth factor. The trophoblast formed a columnar-type single layer that was comprised of cells of various sizes and shapes with basal nuclei, including the giant binnucleate cells. The trophoblast formed chorionic projections which presented ramifications in number from 4 to 5. A great quantity of blood vessels were found in the materno-fetal interface, between the cells of uterine epithelium and around of the chorionic projections. A PAS-positive reaction was observed with diffuse cytoplasmic PAS staining at the apical region of the trophoblast at the materno-fetal interface as well as in the endometrial glands. Collagen fibers were observed in the mesenchyme and inside the chorionic projections. In the areolae we confirmed the positive reaction of the acid phosphatase enzyme that detects phagocytic activity. In the basal region of the uterine gland epithelium, which is columnar type, and in the gland lumina, this reaction demonstrated a strong positive stain. The Phosph histochemical reaction that reveals ferric iron was positive in the areola, as well as in the uterine glands. The uteroferin immunohistochemistry showed a strong stained in the areolae and in the epithelium and lumina of the uterine glands. Our findings suggest that the areola region and the endometrial glands play an important role in histiotrophic nutrition in llamas, and in fetal red blood cell formation by iron transfer from mother to the fetus.

This work was supported by FAPESP CNPq, CAPES, PRONEX, Brazil.

138  **IN VITRO CULTURE OF CD9-EXPRESSING CELLS ENRICHED BY MAGNETIC CELL SORTING FROM TESTES OF CRYPTORCHID ADULT AND PUP IN MICE**


$^A$Institute of Advanced Technology, Kinki University, Kainan, Wakayama 642-1017, Japan; $^B$Gene Control Corporation, Kainan, Wakayama 642-1017, Japan; $^C$Department of Genetic Engineering, Kinki University, Uchiha, Wakayama 649-1493, Japan

Recently, studies on cell surface markers of spermatogonia in combination with germ cell transplantation techniques have made possible the functional analysis of germline stem cells (GS cells). The GS cells are downstream of the stem cells such as ES cells and embryonic germ cells (EG cells), which are derived from primordial germ cells (PGCs). Therefore, GS cells are expected to be useful in the production of genetically modified animals. In this study, we examined the enrichment and cultivation of mouse GS cells by magnetic cell sorting (MACS). Testicular cell suspensions were collected from C57BL/6J cryptorchid adult testes at 2 to 3 months after surgery and ICR pup (6 to 8 dpp) testes. They were digested by 0.1% collagenase followed by 0.25% trypsin with gentle shaking. Dissociated cell suspensions were filtered through a glass-wool column followed by a Falcon cell strainer (40-μm mesh). They were then treated with biotin-conjugated anti-mouse CD9 antibody, whose antigen, CD9, is localized on the GS cell surface, followed by streptavidin-microbeads treatment. The cell suspension was passed through a MACS-separation column. In Experiment I, MACS-treated fractions were analyzed by flow cytometry (FCM) on the rates of recovery and enrichment and their cellular characteristics. In Experiment II, CD9-positive (CD9$^+$) cells were cultured on gelatin-coated MultiDish (176740, Nunc) with 4–5 x 10$^2$ cells/well in StemPro34-SFM supplemented with 1% fetal bovine serum, leukemia inhibitory factor, GDNF, bFGF, EGF, insulin, transferrin, putrescine, MEM vitamin solution, MEM-NEAA and some other reagents at 32°C or 37°C under 5% CO$_2$ in air. They were examined for their proliferation and cytological changes such as CD9, u6-integrin and Oct-1 expression by immunohistochemistry. In Experiment I, MACS selection effectively enriched CD9$^+$ cells from mouse testes. However, FCM analysis revealed that the CD9-negative (CD9$^-$) cells partially remained in MACS-selected fraction from cryptorchid adult testes. In contrast, the CD9$^+$ subpopulation could be successfully separated from CD9$^-$ subpopulation from pup testes. Therefore CD9$^+$
subpopulation from pup testes was used for the following cultivation. In Experiment II, the cells proliferated in the first few days in suspension. Then they attached to the dish and formed colonies after 5 days or 3 days of culture at 32°C or 37°C, respectively. Immunohistochemical analysis showed that the cells maintained the expression of CD9 for at least 14 days, but their expression of α6-integrin gradually diminished. It was demonstrated by immunohistochemistry and FCM analysis that the cells in colonies expressed Oct-1, and its expression level was stronger in culture at 37°C than at 32°C. These findings indicate that the CD9+ cells collected from mouse pup testes have stem cell properties.

This work was supported by the Wakayama Prefecture Collaboration of Regional Entities for the Advanced Technological Excellence, JST; by a Grant-in-Aid for the 21st Century Center of Excellence Program of the MEXT, Japan; and by a Grant-in Aid for Scientific Research from the Japan Society for the Promotion of Science.

139 INJECTION OF SOMATIC CELL CYTOPLASM INTO OOCYTES BEFORE ICSI IMPAIRED FULL-TERM DEVELOPMENT AND INCREASED PLACENTA WEIGHT IN MICE


A Center for Developmental Biology, RIKEN-Kobe, Kobe, Hyogo, Japan; B Graduate School of Science and Technology, Kobe University, Kobe, Hyogo, Japan

During the process of somatic cell nuclear transfer, cytoplasm is introduced into the enucleated oocytes, in addition to the genomic material, regardless of the electrofusion methods (Wilmut et al. 1997) or direct injection of somatic nucleus by the Honolulu method (Wakayama et al. 1998). Only 1 to 2% of cloned embryos, however, develop to term with many incidences of developmental anomalies. These cloning failures may be explained by incomplete reprogramming of the donor cell genome, although it is not yet clear whether cytoplasmic materials of the somatic cell also have an effect on development of the cloned embryo. In an attempt to answer this question, this study investigates the effects of somatic cytoplasm of different mouse strains and cytoplasm of fertilized embryos at different stage by injecting them into intact mouse oocytes before intracytoplasmic sperm injection (ICSI). Mature oocytes collected from B6D2F1 female after 14 to 16 h of hCG injection were injected with (1) B6D2F1 cumulus cell cytoplasm with different volumes (collected by 2 to 3 µm of injection pipette and piezo pulses), (2) cumulus cell cytoplasm from different mouse strains (B6D2F1, ICR, C57BL/6), (3) cytoplasm of 1- to 8-cell embryos. After subsequent culture for 1 h, B6D2F1 sperm were injected into those oocytes and examined for preimplantation development competence. The total number of cells, inner cell mass (ICM), and expression of Oct4 in expanded blastocysts were also examined. In order to examine the effects of somatic cytoplasm on full-term development, we transferred 2-cell embryos at 24 h or morula and blastocysts at 72 h after ICSI to the oviduct or uterus of surrogate mothers (ICR) on Day 1 or 3 of pseudopregnancy. The control group received a sham injection with PVP before ICSI. The results showed that an increase in the volume of cytoplasm from 1-fold to 4-fold (equivalent with the volume of 1 cumulus cell) resulted in impairing full-term development (28 and 7%, respectively, vs. 56 to 63% in the control group, P < 0.01). There was no difference in the frequency of embryos developing to the blastocysts stage between B6D2F1 and ICR somatic cytoplasms at the same volume. However, C57BL/6 somatic cytoplasm induced the 2-cell block to B6D2F2 embryos. Fertilized embryo cytoplasm did not reduce the frequency of blastocyst stage and full-term development. Interestingly, we found that somatic cytoplasm increased the placenta weight of ICSI embryo (0.2002 ± 0.03, n = 32; vs. 0.1198 ± 0.02 in control group, n = 87; P < 0.01). We also obtained placenta with no fetus when the volume of somatic cytoplasm was the same size as cumulus cell. We found that an increase in the volume of somatic cytoplasm led to low expression of Oct4 in expanded blastocysts. These findings indicated that injection of somatic cytoplasm into oocytes before ICSI decreased the preimplantation development, clearly impaired full-term development, and caused placental overgrowth in fertilized embryos. This study suggested that somatic cell cytoplasmic material is one cause of the low rate of full-term development of cloned animals.

140 IMMUNOLOCALIZATION OF INDOLEAMINE 2,3-DIOXYGENASE IN PLACENTA FROM BOVINE CLONED CONCEPTUS PREGNANCY


A University of Sao Paulo, FMVZ-USP, Sao Paulo, SP, Brazil; B IQ-USP, Sao Paulo, SP, Brazil; C FZ-Unesp, Dracena, SP, Brazil; DFZEA-USP, Pirassununga, SP, Brazil

The catabolism of tryptophan by indoleamine 2,3-dioxygenase (IDO) activity is strongly correlated to maternal-fetal tolerance in hemochorial placentation. Several studies have been conducted on the role of IDO on maternal-fetal tolerance in human and mice placentation. Changes in IDO expression and activity are related to fertility problems in humans and lower IDO activity was found in women who had recurrent abortions. In mice, IDO was localized in blastocyst, cytotrophoblast, placental macrophages, stromal cells and invasive trophoblast cells, but not in the syncytiotrophoblast. Also, IDO staining was detected on villous trophoblast cells; probably due to the fact that this is the site where fetal antigen is most exposed to the maternal immune system during pregnancy. Our previous studies in bovine showed that IDO activity is present throughout placentation. Several studies have been conducted on the role of IDO on maternal-fetal tolerance in human and mice placentation. Changes in IDO expression and activity are related to fertility problems in humans and lower IDO activity was found in women who had recurrent abortions. In mice, IDO was localized in blastocyst, cytotrophoblast, placental macrophages, stromal cells and invasive trophoblast cells, but not in the syncytiotrophoblast. Also, IDO staining was detected on villous trophoblast cells; probably due to the fact that this is the site where fetal antigen is most exposed to the maternal immune system during pregnancy. Our previous studies in bovine showed that IDO activity is present throughout placentation. Changes in IDO expression and activity are related to fertility problems in humans and lower IDO activity was found in women who had recurrent abortions.
10 normal conceptuses (control) at parturition. Samples were fixed in 10% buffered formalin and embedded in paraffin. Sections were made and processed for immunohistochemistry using a commercial monoclonal antibody against IDO. Placenta from two cloned conceptuses showed IDO staining on fetal mesenchyma, differing from normal pregnancy where the staining of this enzyme was absent in all control samples. IDO staining was absent at the fetomaternal interface in one sample from cloned placenta and present in all normal pregnancy animals. It was difficult to establish a staining profile for giant and binucleate cells in cloned placenta, since IDO staining varied greatly among individuals. IDO immunolocalization in placenta from cloned conceptus was altered in all animals studied. It may raise the question that cloning could interfere in maternal immune modulation during pregnancy and IDO misexpression in fetomaternal tolerance could be involved in a decreased maintenance of cloned pregnancies.

141 EXPRESSION OF GENE PRODUCTS ENCODING CNN PROTEINS DURING THE FIRST WEEK OF DEVELOPMENT

A. Pin\textsuperscript{A,B} and A. Watson\textsuperscript{A,B}  
\textsuperscript{A}OB/GYN and Physiology and Pharmacology, The University of Western Ontario, London, Ontario, Canada;  
\textsuperscript{B}Children’s Health Research Institute, Lawson Health Research Institute, London, Ontario, Canada

CCNs (cysteine-rich 61 (Cyr61), connective tissue growth factor (CTGF), and nephroblastoma overexpressed (Nov)) are a newly characterized gene family encoding 30–40 kDa proteins that are cysteine-rich and associated with the extracellular matrix. CCNs bind integrins and activate intracellular signaling, resulting in kinase activation and gene transcription. They are involved in events essential to development, including implantation and placenta. For example, CTGF is increased during decidualization and both CTGF and Cyr61 are produced by trophoblast giant cells. Furthermore, Cyr61-null mice display early embryonic lethality due to placental defects. Although CTGF protein has been reported in mouse 4.5-day embryo, no studies to date have focused on defining the expression of CCN mRNAs during pre-implantation development. The objective of this study was to characterize the expression of CCN mRNAs during the first week of \textit{in vivo} mouse and \textit{in vitro} cow development. CD-1 mice were superovulated by i.p. injection of pregnant mare serum gonadotrophin (10 U) and human chorionic gonadotrophin (10 U) 22 hours later. Following mating of the mice with CD-1 males, embryos were flushed from oviducts and uteri at 2-cell, 4-cell, 8-cell, morula, and blastocyst stages. Cow oocytes were obtained from slaughterhouse ovaries. Cow embryos were produced by aspiration of early antral follicles followed by \textit{in vitro} oocyte maturation, fertilization, and embryo culture methods, and collected at 2–5-cell, 6–8-cell, morula, and blastocyst stages. Groups of five embryos of each stage were pooled for mRNA extraction using a magnetic bead protocol. Reverse transcription-polymerase chain reaction was used to detect CCN mRNAs at each time point, and product identity was verified by sequencing. CCN mRNAs are detectable during the first week of development in both species. In the mouse, Cyr61 mRNAs were present in all embryo stages investigated. CTGF mRNAs were variable at the 2-cell stage, but were consistently present from the 4-cell stage to the blastocyst stage. Interestingly, Nov appeared transiently at the 4- to 8-cell stage only. In the cow, CTGF and Cyr61 mRNAs were also detectable. Nov was undetectable in the cow. This is the first report of CCN mRNA expression in mouse or cow pre-implantation development. It may raise the question that cloning could interfere in maternal immune modulation during pregnancy and IDO misexpression in fetomaternal tolerance could be involved in a decreased maintenance of cloned pregnancies.

This research was funded by NSERC, Canada.

142 INTRACELLULAR CALCIUM UPTAKE AS A RESPONSE TO DIFFERENT CAPACITY TREATMENTS IN EJACULATE AND EPIDIDYMAL BOAR SPERMATOZOA

M. Sansegundo\textsuperscript{A}, S. Ruiz\textsuperscript{A}, A. Gonzalez\textsuperscript{A}, N. T. Atucha\textsuperscript{A}, and C. Matás\textsuperscript{A}  
\textsuperscript{A}Department of Physiology, University of Murcia, Murcia, Spain; \textsuperscript{B}Department of Physiology, School of Veterinary Sciences, University of Extremadura, Caceres, Spain

Both cauda epididymal and ejaculated spermatozoa are considered fully mature, although these two types of spermatozoa do not necessarily show the same behavior \textit{in vitro}. It has been reported that both types of spermatozoa fertilize eggs \textit{in vitro} at the same rate, but, in general, epididymal ones achieve this objective easier than the ejaculated ones. Intracellular Ca\textsuperscript{2+} influx is one of the crucial biochemical events occurring capacitation and Ca\textsuperscript{2+} requirements for capacitation varies among different species. In this study, we investigated how different source of spermatozoa (ejaculated vs. epididymal) and commonly employed sperm capacitating methods can affect calcium uptake. Sperm-rich fractions from seven fertile boars and sperm from seven different epididymides were used. Semen samples were kept unwashed (method A), washed in Dulbecco’s phosphate-buffered saline (DPBS) supplemented with 0.1% BSA (method B), or washed on a Percoll gradient (method C) (Matás et al. 2003 Reproduction 125, 133–141). To measure intracellular free Ca\textsuperscript{2+}, spermatozoa, treated as described above, were incubated with 2.5 μM fura-1/AM in a non capacitating medium (Tardif et al. 2003 Biol. Reprod. 68, 207–213) for 45 min at 37°C. Then, spermatozoa were resuspended in TALP medium, incubated (5% CO\textsubscript{2}, 38.5°C) for a further 60 min and then analyzed in a fluorescence spectrophotometer with excitation wavelength set at 340–880 nm and emission held at 510 nm. The calculation of intracellular free Ca\textsuperscript{2+} was performed according to the equation of Grynkiewicz et al. (1985 J. Biol. Chem. 260, 3440–3450). Results showed that calcium uptake is affected by treatment and semen source (P < 0.001). The intracellular free Ca\textsuperscript{2+} concentrations (nM) in ejaculated semen and in epididymal spermatozoa were 269.52 vs. 208.52, 1134.58 vs. 137.37 and 1224.79 vs. 216.54 for A, B, and C methods, respectively. As a conclusion, it can be stated that sperm capacitation treatment affects calcium uptake. In addition, capacitation pathways may be modified or regulated in some way by seminal plasma, thus increasing intracellular calcium levels in ejaculated sperm (methods B and C) in comparison to those in epididymal spermatozoa.

This work was supported by Ministerio de Educación y Ciencia, AGL2003–03144.
143 PRESENCE OF PROSTAGLANDIN F2α RECEPTOR IN IN VITRO-DERIVED MORULA AND BLASTOCYST STAGE BOVINE EMBRYOS

F. N. Scenna, J. L. Edwards, G. M. Pighetti, and F. N. Schrick
Department of Animal Science, University of Tennessee, Knoxville, TN, USA

Culture of in vitro and in vivo-derived embryos in medium containing prostaglandin F2α (PGF) decreased embryonic development to blastocyst stage and reduced hatching rates (Scenna et al. Prostaglandins 73, 215–226). Moreover, administration of an inhibitor of PGF synthesis at the time of embryo transfer in bovine recipients improved pregnancy rates (Schrick et al. 2001 Theriogenology 59, 335 abstr.). These findings indicate a direct negative effect of PGF on embryonic development. However, to our knowledge, no evidence of PGF receptor expression in morula or blastocyst stage bovine embryos is available in the literature. Therefore, the objective of the current study was to determine the presence of PGF receptor mRNA using real-time RT-PCR and protein expression by Western blotting in morula or blastocyst stage in vitro bovine embryos. Briefly, isolated total RNA from compact morula or blastocyst stage embryos and from bovine tongue epithelium (positive control for PGF receptor mRNA) were reverse-transcribed into cDNA. A volume from the RT reaction equivalent to 10 embryos per tube was utilized to determine transcripts for PGF receptor and Histone H2A (standard PCR control). Polymerase chain reaction was performed, and identity of PCR fragments was confirmed by ethidium-bromide-stained 2% agarose gel electrophoresis and by DNA sequencing. To determine protein expression, morula and blastocyst stage embryos were lysed in lysis buffer (10% SDS, 1 m Tris pH 7.5, 1 m NaF, 1 m DTT, 0.1 m EGTA with protease inhibitors) and stored at ~20°C. Crude proteins isolated from bovine corpora lutea (positive control for PGF receptor protein), embryo samples, and prestained standards were separated by 12% SDS-PAGE under reducing conditions. Proteins were electrotransferred onto a PVDF membrane. Non-specific binding sites in the PVDF membrane were blocked with 10% nonfat dry milk, and the blot was washed and incubated for 1 h at room temperature with a 1:1000 dilution of the primary antibody (rabbit polyclonal antibody against PGF receptor protein). Subsequently, the blot was washed and incubated for 1 h at room temperature with 1:1000 dilution of mouse anti-rabbit IgG conjugated with horseradish peroxidase. Finally, the blot was washed and revealed by chemiluminescence in a CCD camera. Results indicated that transcripts as well as the protein for PGF receptor were present in early stage bovine embryos. Identification of PGF receptor in morula and blastocyst stage bovine embryos may, in part, explain the increase in pregnancy rates after administration of a PGF synthesis inhibitor at the time of embryo transfer, which opens up the possibility to develop new strategies to prevent detrimental effects of PGF during early embryonic development.

144 EPIGENETIC ASYMMETRY IN HISTONE H3 LYSINE 9 DIMETHYLATION STATUS IN PRONUCLEAR STAGE PORCINE EMBRYOS

M. Sega and R. Cabot
Purdue University, Department of Animal Sciences, West Lafayette, IN, USA

Covalent modification of specific residues on the tail regions of core histone proteins has been shown to play a key role in regulation of the genome. Methylation of lysine 9 on histone H3 (H3K9) is associated with repression of transcription and formation of heterochromatin domains. The maternal- and paternal-derived pronuclei from pronuclear stage murine embryos have an asymmetric distribution of H3K9 dimethylation between the 2 pronuclei; maternal pronuclei have the H3K9 dimethylation, whereas the paternal pronuclei lack this modification. The aim of this study was to characterize the H3K9 dimethylation pattern in cleavage stage porcine embryos. Indirect immunocytochemical staining was performed using a commercially available antibody from Upstate (Charlottesville, VA) that recognizes the dimethylated form of lysine 9 on histone H3 and fluorescence isothiocyanate (FITC)-conjugated secondary antibody on germlinal vesicle (GV) stage porcine oocytes and cleavage stage porcine embryos. Germinal vesicle stage oocytes were matured in vitro for 44 h in a chemically defined maturation medium (TCM 199 supplemented with 0.1% PVA, 0.069 mg/mL cysteine, 10 mg/mL EGF 0.5 IU/mL LH, and 0.5 IU/mL FSH) at 39°C in 5% CO2, 5% O2. Embryos were fixed in 3.7% paraformaldehyde for 2 h and washed in PBS and 0.1% Tween20. H3K9 dimethylation is present in the nuclei of GV-stage oocytes (n = 24) and pronuclear (n = 13), 2-cell (n = 4), 4-cell (n = 9), blastocyst (n = 8) stage embryos. The analysis revealed 2 interesting findings. First, examination of thin optical sections through the nuclei of processed embryos on a confocal microscope revealed that in GV-stage oocytes and pronuclear, 2-cell- and 4-cell stage embryos the dimethylated form of H3K9 was distributed throughout the nuclei at these developmental time points, whereas in blastocyst stage embryos, the dimethylated form of H3K9 was restricted to the nuclear periphery. Second, not all pronuclei within pronuclear stage embryos were positive for the dimethylated form of H3K9. To determine if differential H3K9 dimethylation pattern observed among pronuclei in 1-cell stage embryos was due to parental origin, we produced parthenogenetic porcine embryos by electrically activating porcine oocytes in calcium containing activation medium and processed them as indicated above for fertilized oocytes (n = 13). All pronuclei found in pronuclear stage parthenogenetic porcine embryos were positive for dimethylation of H3K9. Chi square analysis revealed this pattern to be different from that observed in pronuclear embryos produced by fertilization (P < 0.05). In summary we conclude that the pronuclei in 1-celled embryos produced by in vitro fertilization are differentially dimethylated at H3K9 and that the localization of dimethylated H3K9 changes during cleavage development.

145 STAGE-SPECIFIC EFFECT OF OXIDATIVE STRESS ON DEVELOPMENTAL COMPETENCE, ROS GENERATION AND DNA DAMAGE OF PORCINE PARTHENOGENETIC EMBRYOS

A National Agricultural Center for Kyushu Okinawa Region, Kumamoto, Japan; B Meiji University, Kawasaki, Kanagawa, Japan

We investigated the effect of oxidative stress on stage specific developmental ability, reactive oxygen species (ROS) generation and DNA damage of parthenogenetically activated porcine embryos. Cumulus–oocyte complexes (COCs) were aspirated from follicles on the surface of ovaries. The
COCs were matured in NCSU-23 containing 10% (vol/vol) porcine follicular fluid and 10 IU/mL hCG during the first 22 h followed by an extra 22 h of culture in the hormone free NCSU-23. After 44 h of maturation, oocytes were denuded of cumulus cells and used for activation. Oocytes were activated by a 100-μsec pulse of 1.5 kV/cm DC with 1-mm electrodes in 0.3 mM mannotiol, 0.1 mM MgSO₄, and 0.05 mM CaCl₂. Activated oocytes were then cultured for 5 h in NCSU-23 containing 5 mg/mL BSA, 10 μg/mL EGF and 7.5 μg/mL cytochalasin B. Embryos were then cultured for 6 days in PZM-5. In Experiment 1, after parthenogenetic activation, embryos were cultured at 38.5°C under 5% O₂, 5% CO₂ and 90% N₂ (defined as 5% O₂) or 5% CO₂ in air (20% O₂). The oxygen concentration for embryo culture was changed from 5% to 20% on day 1, 2, 3, 4, and 5 post-activation, respectively. Embryos were also cultured throughout 6 days in 5 and 20% O₂. About 100 embryos were used in each experiment. The number of embryos cleaved and developed to blastocyst stage was observed on day 2 and 6, respectively. In Experiment 2, 10 to 20 embryos cultured in 5 and 20% O₂ were collected on Days 2, 4, and 6 for the detection of ROS, intracellular glutathione (GSH) levels and DNA damage. Intracellular ROS and GSH levels, were measured with fluorescent dyes (2',7'-dichlorodihydrofluorescein diacetate for ROS and Cell Tracker™ Blue for GSH). DNA damage of individual embryos was detected with a comet assay. DNA damage was quantified by measuring the length of the streak of DNA comet tail between the edge of the zona pellucida and the end of the visible comet tail by image analysis software. The rate of migrated DNA area per total DNA was also quantified. In Exp. 1, the rate of blastocyst formation was significantly decreased (P < 0.001) when embryos were cultured for 6 days under 20% O₂ (17.8 ± 4%) than 5% O₂ (38.5 ± 5%). The rates of blastocyst formation were significantly decreased (P < 0.05) when O₂ concentration was changed from 5 to 20% before Day 3. After Day 4, high O₂ concentration did not affect the development. In Exp. 2, relative ROS levels were significantly higher (P < 0.05) on Day 2 (1.5 ± 0.03) and Day 4 (1.4 ± 0.06) in embryos cultured under 20% O₂ than in those cultured under 5% O₂ (1.0). No difference was observed in GSH level. DNA damage was significantly increased (P < 0.05) in Day 2 embryos cultured under 20% O₂ (161 ± 54 μm) than 5% O₂ (65 ± 8 μm). These results indicate that the oxidative stress to embryo development by high O₂ concentration is stage specific, that embryos are more sensitive in early stages, and that the oxidative stress has correlation with the increase of intracellular ROS and DNA damage.

### 146 EFFECT OF MATERNAL AGE AND PARITY ON THE PRESENCE AND DISTRIBUTION OF CADHERIN-E IN HAMSTER EMBRYOS DURING PRE-IMPLANTATION DEVELOPMENT AND TRANSPORT

**A. Trejo, C. Navarro-Maldonado, F. Jimenez, and A. Rosado**

Universidad Autonoma Metropolitana-Iztapalapa, Mexico City, Iztapalapa, Mexico

Our knowledge of the factors that contribute to in vivo embryo development and transport are still incomplete. Although there is solid evidence that the hormonal state of the female regulates ovum transport in mammals, recently, because of significant differences between the rate of oviduct transport of fertilized and unfertilized eggs in several mammalian species, there have been some reports showing that the conceptus itself may auto regulates its own transport. During the initial phases of embryonic development the distribution of E-cadherin, a calcium dependent cell adhesion molecule, changes from a general distribution over the whole surface of the blastomeres to become almost exclusively localized over the points of contact between blastomeres. This change on E-cadherin localization has been related to the beginning of embryo compaction and is considered as the initial stage of differentiation. We have recently shown that pre-implantation embryo development and transport is highly synchronous in nuliparous young females (NYF), but not in adults. Nuliparous adult (8 months) females (NAF) and multiparous adult females (MAF) show significant asynchrony and retard on early embryo development. In NYF, all 8 cells embryos reached the uterus by 62 h after coitus. In adult females, both nuliparous and multiparous, a considerable number of 8 cells embryos fail to migrate into the uterus even at 67 h after coitus. E-cadherin distribution was studied by indirect immunofluorescence and confocal microscopy during preimplantation embryo development in three groups, NYF, MAF, and NAF, of regularly cycling golden hamster (*Mesocricetus auratus*) females. Females were mated with males of proven fertility. Only 15 minutes were allowing for mating. Time of ejaculation was registered. Females were sacrificed from 60 to 69 h after coitus. Corpora lutea were counted in both ovarian surfaces. Oviducts and uterine horns were flushed separately and embryo number, stage of development, and distribution were recorded. Cell numbers, cadherin distribution and the number of cadherin-dependent cell to cell contacts were established in at least 20 embryos per group and at each of the different hours of the study. Presence and distribution of E-cadherin on 4-, 6-, and 8-cell blastomeres embryos differs accordingly with the parity and age of the females. Differences are particularly important at the eight-cell stage 80 to 93% of the embryos from NYF showed normal cadherin dependent cell contacts with all neighboring cells. In the case of multiparous adult females (MAF) only 52 to 68% of the embryos showed normal cell to cell contacts. These results will be discussed in relation to the respective role that the female hamster and the conceptus itself play in early embryo development and transport. Our results also indicate that in spite of the significantly higher ovulation rate of old females, their reproductive efficiency were significantly lower than that of NYF.

### 147 IDENTIFICATION OF PRIMORDIAL GERM CELLS IN PORCINE EMBRYOS FROM THE PRIMITIVE STREAK STAGE

**M. Vejlsted, H. Offenberg, and P. Maddox-Hyttel**

Department of Animal and Veterinary Science, Royal Veterinary and Agricultural University, 1870 Frederiksborg C, Denmark

In embryonic stem cell research, Oct-4 is one of the most widely used markers of pluripotency. Moreover, at least in the mouse, this marker is restricted to primordial germ cells (PGCs) after gastrulation. Vimentin is often used as a marker of mesoderm/mesenchyme in embryonic tissues and appears to localize to the same embryonic cells as Oct-4, at least in the bovine epiblast. The expression of neither of these markers has been completely addressed in the pig. Therefore, the purpose of the present study was to examine the expression of Oct-4 and vimentin in the porcine epiblast during differentiation and establishment of the three germ layers, i.e. the process of gastrulation. A total of 410 porcine embryos were collected at 8 to 17 days post-insemination from 29 sows of the Danish Landrace breed. Embryos were categorized based on stereo-microscopic observations into the following stages: pre-streak stages 1 and 2, primitive streak stage, neural groove stage, and somite stage. Specimens were
fixed at all stages, dehydrated and embedded in paraffin wax. Selected embryos at each stage \((n = 28)\) were completely cut into serial sections for immunohistochemical evaluation of Oct-4 and vimentin. Pre-streak stage 1 embryos were defined by lack of polarization of the embryonic disk, whereas in pre-streak stage 2 embryos a crescent shaped thickening was seen at the posterior pole of the disk. This thickening, marking the first morphological anterior-posterior polarization of the embryo proper, was shown to be a site of incipient ingestion of cells from the epiblast. Immunohistochemical analyses localized Oct-4 to nuclei and vimentin to cytoplasm of both founding and ingressing epiblast cells. During formation of mesoderm and endoderm, at the primitive streak stage, solitary Oct-4 positive cells, i.e. potential PGCs, were seen scattered in the endoderm. Cells of the epiblast displayed positive labeling for Oct-4 until specification for the ectoderm cell lineage at the subsequent neural groove stage. In mesoderm, Oct-4 likewise disappeared by the time of formation of the first somites, defining the following somite stage. Thus, at this stage the only cells labeled for Oct-4, i.e. potential PGCs, were seen solitarily scattered in the endoderm. By the 15-somite stage, such cells were no longer visible in the endoderm but were seen located in the mesoderm, spreading from the stalk of the yolk sac and allantois and extending through the mid- and hindgut areas into the incipient genital ridge. Vimentin localized to the mesenchyme and most other derivatives of neural crest and mesodermal origin.

In conclusion, based on Oct-4 labeling and distribution pattern, we strongly believe that we have identified the porcine PGCs from the primitive streak stage.

148 INDUCTION OF HYPMETHYLATION BY EPIGENETIC ALTERATION OF SOMATIC NUCLEI IN CLONED BOVINE BLASTOCYSTS


Laboratory of Development and Differentiation, Korea Research Institute of, Daejon, Korea

Epigenetic reprogramming such as DNA methylation is incomplete in cloned embryos during early development as compared with normal embryos. The increased methylation levels of cloned bovine blastocysts are showed in centromeric heterochromatin. The aim of the present study was to investigate the change of methylation state by treatment of trichostatin A (TSA), a specific inhibitor of histone deacetylase in somatic donor nuclei and cloned blastocyst reconstructed with TSA-treated cells or nontreated cells. Bovine ear skin fibroblast cells (bESF) were used as donor cell and treated with TSA for 60 h at a final concentration of 1 \(\mu\)M. To methylation analysis of satellite I as specific DNA sequence, genomic DNA from \(7 \times 10^4\) cells and a blastocyst were isolated, and then the genomic DNA was analyzed by bisulfite sequencing. The reduction of HDAC1, 2 and Dnmt family such as Dnmt1, Dnmt3a, Dnmt3b, and Dnmt3L after TSA treatment were shown by Western blot in bESF, but histone acetyltransferases such as Tip60 and HAT1 were not changed. Satellite I DNA in nontreated cells was highly methylated in CpG sequences, whereas methylation level of TSA-treated cells was significantly decreased (64 vs. 48%, \(P < 0.05\)). After nuclear transfer using normal or altered donor cells, methylation levels of satellite were measured at the blastocyst stage of NT and TSA-NT cells compared with IVF embryos. In nontreated NT blastocysts, methylation levels were significantly higher than IVF blastocysts (66 vs. 29%, \(P < 0.05\)) and were similar to that of nontreated bESF cells. The reduction of methylation levels in TSA-NT blastocysts were showed and were significantly lower than NT blastocyst derived with nontreated cells (37 vs. 66%, \(P < 0.05\)), but no significant differences were found between TSA-NT and IVF blastocysts. Also, the levels of methylation were similar to that of TSA-treated donor cells. In blastocyst formation, TSA-NT embryos were improved significantly compared with NT or IVF embryos (45.9 vs. 31.7 or 28%, \(P < 0.05\)). These results demonstrated that somatic methylation status after epigenetic alteration affect in early cloned embryo development, suggesting epigenetic control may help to solve of inherent problems in cloning.