Developmental Biology

99 COMPARISON OF IN VITRO DEVELOPMENT FOLLOWING CRYOPRESERVATION OF MEISHAN AND WHITE CROSS SWINE EMBRYOS

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Development of improved protocols for cryopreservation of zona pellucida-intact porcine embryos could greatly impact the swine industry. Our aim was to investigate in vitro development following cryopreservation of embryos from Chinese Meishan (M) and occidental white cross (WC) breeds using a modified protocol described previously (Mismu K. et al. 2003 Theriogenology 60, 253–260). First-parity M sows (n = 11) and WC gilts (n = 13) were observed for estrus every 12 h and inseminated at 12 and 24 h after estrous onset within breed using semen from 2 different boars. Females were sacrificed between Days 4.5 and 6 after estrus and embryos were collected using Beltsville embryo culture medium (BECM). Compact morula (CM) or blastocyst stage embryos from each female within breed were randomly allocated either directly into the culture system to serve as controls (68 M and 48 WC embryos) or to undergo cryopreservation. A total of 101 M and 78 WC embryos were cryopreserved using the following protocol: (1) 5 min in BECM + 10% ethylene glycol (EG); (2) 5 min in BECM + 10% EG + 0.27 M sucrose + 1% polyethylene glycol (PEG); and (3) 30 to 45 s in BECM + 40% EG + 0.36 M sucrose + 2% PEG. In the last solution, 5 to 10 embryos in a 5- to 10-μL microdrop attached to a fine glass pipette were exposed to the vapor phase of liquid nitrogen (LN2) for 15 s and then plunged into LN2. The pipette tip was broken and the tip and associated frozen microdrop were placed inside an LN2-submerged 2-mL cryotube containing a hole in the lid for 1 h. Next, embryos were thawed using a 4-step (5 min each) procedure: (1) BECM + 5% EG + 0.57 M sucrose; (2) BECM + 2.5% EG + 0.29 M sucrose; (3) BECM + 0.3 M sucrose; and (4) BECM alone. All procedures were performed with solutions maintained at 37°C. Cryopreserved and control embryos were cultured in 50 μL drops of modified Whitten's medium + 1.5% BSA under oil at 37°C in a 5% CO2 in air environment and scored daily for development. For embryos undergoing cryopreservation, retrieval rates from cryoovials were 92% and 96% for M and WC, respectively. The percentage of embryos surviving 24 h after cryopreservation without lysis or degeneration was higher for M (72%) than for WC (44%; P < 0.001; χ2-test). However, in vitro development of embryos that survived cryopreservation was not different between M and WC at the expanded (64%) or hatched (22%) blastocyst stages. Developmental rates were significantly higher for control embryos than for frozen embryos from both breeds. Rates of expanded blastocyst formation did not differ between M and WC control embryos (98% and 95%, respectively), but more M embryos developed to the hatched blastocyst stage (22% for M vs. 9% for WC; P < 0.05). Our results suggest that M embryos have a higher capacity to survive the vitrification process than WC embryos.

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Developmental Biology

100 DIET AND Fatty Acid Composition of bovine plasma, granulosa cells, and cumulus–oocyte complexes

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The effects of altering dietary carbohydrates and lipids to oocyte donors during superovulation and ovum pickup (OPU) on in vitro embryo production was reported previously (Adamiak et al. 2004 Reprod. Dev. Fert. 16, 193–194). Here we report the effects of these dietary treatments on the fatty acid (FA) composition of plasma, granulosa cells (GCs), and cumulus–oocyte complexes (COCs) from the 32 heifers used in that study. Blood samples were collected by jugular venipuncture. COCs and GCs were harvested from each heifer by OPU as described previously but were pooled between pairs of heifers within treatment to provide adequate material for FA analysis. Both GCs and COCs were washed twice in PBS supplemented with 0.3% (w/v) BSA (FA-free) before being transferred into 2:1 (v/v) chloroform:methanol solution for FA extraction. FA composition was determined using gas chromatography as described previously (Reis et al. 2002, Theriogenology 57, 507). Data were analyzed by ANOVA. Total plasma FA content averaged 1.12 μg/mL and was unaffected by body condition score (BCS). Low BCS heifers had more saturated (54.0 ± 1.76 vs. 49.2 ± 1.74%) and monounsaturated (22.4 ± 1.06 vs. 18.2 ± 0.69%) FA, but less polyunsaturated FA (PUFA) (23.7 ± 1.75 vs. 32.8 ± 2.21%) in plasma than moderate BCS heifers (P < 0.01). Animals fed high relative to low fiber diets had greater plasma FA (1.3 ± 0.15 vs. 1.0 ± 0.12 μg/mL).
and PUFA (31.2 ± 2.56 vs. 25.3 ± 2.19%), but less monounsaturated FA (18.8 ± 1.04 vs. 21.5 ± 1.09%) (P < 0.01). Dietary protected lipid (Ca soaps of FA) increased plasma FA (1.6 ± 0.07 vs. 0.6 ± 0.04 μg/mL) and PUFA (30.0 ± 1.92 vs. 26.4 ± 0.03%) (P < 0.05), but reduced plasma saturated FA (48.9 ± 1.12 vs. 54.2 ± 2.14%; P < 0.01) in the animals fed high compared to low fiber diets, respectively. In contrast to their effects in plasma, BCS and diet had little effect on FA composition of GCs and COCs. Although low BCS and dietary lipids both increased FA in COCs (78.3 ± 2.01 vs. 69.2 ± 2.80, P < 0.01; and 77.1 ± 2.96 vs. 70.5 ± 2.26 ng/COC; P < 0.05, respectively), neither factor significantly altered the FA composition of COCs. Across treatments, the FA composition of GCs and COCs differed markedly from that of plasma (Table 1), confirming the presence of a selective uptake mechanism in the follicle that can moderate dietary induced fluctuations in FA supply from peripheral circulation.

### Table 1. Concentrations of fatty acids in plasma, GCs, and COCs expressed as mean percentages (w/w) of total fatty acids

<table>
<thead>
<tr>
<th>Source</th>
<th>Plasma</th>
<th>GCs</th>
<th>COCs</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saturated FA</td>
<td>51.6 ± 1.35</td>
<td>56.2 ± 2.10</td>
<td>87.3 ± 1.00</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Monounsaturated FA</td>
<td>20.3 ± 0.82</td>
<td>32.7 ± 0.68</td>
<td>11.1 ± 0.66</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PUFA</td>
<td>28.2 ± 1.80</td>
<td>11.1 ± 2.23</td>
<td>2.1 ± 0.35</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>n-3 PUFA</td>
<td>1.8 ± 0.10</td>
<td>0.9 ± 0.13</td>
<td>0.5 ± 0.15</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>n-6/n-3 ratio</td>
<td>16.6 ± 1.06</td>
<td>10.9 ± 1.94</td>
<td>3.8 ± 0.71</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

This work was supported by Defra and The Perry Foundation.

### 101 OVARIAN RESPONSE AND DEVELOPMENTAL COMPETENCE OF OOCYTES COLLECTED BY OPU IN SHEEP TREATED WITH GnRH ANTAGONIST


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The use of a single dose of GnRH antagonists during the preovulatory treatment prior to superovulatory treatment protocols in sheep increases the number of smaller follicles able to grow and ovulate in response to the exogenous FSH treatment (Lopez-Alonso C et al. 2004 Reprod. Fertil. Dev. 16, 233). The aim of our study was to test if such treatment affects the in vitro developmental competence of oocytes collected by ovum pick up (OPU) from GnRH-antagonist treated sheep during an ovarian by perstimulation protocol. Adult Sarda sheep (n = 18) were synchronized by the insertion of intravaginal sponges (Day 0) which were left in situ for 12 days; on Day 7, group A (n = 10) received a single dose of 3 mg of Antarelix (Teverelix, Europeptides, France) s.c., while group B (n = 8) served as control. All animals received 96 IU of FSH (Ovagen, ICP, New Zealand) administered in 4 equal doses given i.m. every 12 h starting on Day 10. Twelve hours after the last FSH administration oocytes were collected by OPU technique. Follicular growth was monitored by transrectal ultrasonography from Day 7 to Day 11. Collected oocytes were matured, fertilized, and cultured in vitro up to blastocyst stage under standard conditions used in our laboratory (Berlinguer F et al. 2004 Theriogenology 61, 1477–1486). After IVF, uncleaved oocytes were stained with acetolacmoid to evaluate chromatin configuration, while the cleaved ones were cultured in SOF + 0.4% BSA up to the blastocyst stage. Data were analyzed by ANOVA statistical analysis after arcsine transformation of the value percentages. Ultrasonicographic monitoring showed a significant increase in the number of follicles (mean ± SEM) present in the ovaries from Day 8 to Day 11 of treatment in group A compared to group B (Day 8: 19 ± 5.1 vs. 13 ± 3.4, P > 0.05; Day 9: 20.1 ± 4.6 vs. 14.1 ± 2.4, P > 0.001; Day 10: 22.5 ± 6.1 vs. 14.7 ± 2.7, P > 0.001; Day 11: 25.3 ± 5.1 vs. 20.5 ± 4.1, P > 0.05), thus confirming that GnRH antagonist administration enhances ovarian response to exogenous FSH stimulation. On the other hand, oocytes collected from untreated sheep lead to a higher blastocyst output (P = 0.014), as illustrated in the table. These results indicated that although GnRH antagonist administration caused a significant increase in the ovarian response to the hormonal treatment, the final blastocyst output was significantly lower compared to that of the control group. This finding seems to suggest an impairment in the developmental competence of treated sheep oocytes.

### Table 1. In vitro maturation, fertilization, and developmental capacity of oocytes collected from follicles of GnRH antagonist-treated (group A) and untreated (group B) sheep

<table>
<thead>
<tr>
<th>Oocyte source</th>
<th>Collected oocytes (%)</th>
<th>Oocytes selected for IVM (%)</th>
<th>Fertilized oocytes (%)</th>
<th>Cleaved oocytes (%)</th>
<th>Blastocysts (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A</td>
<td>111 (73.5)</td>
<td>89 (80.1)</td>
<td>37 (41.5)</td>
<td>9 (10.1)*</td>
<td></td>
</tr>
<tr>
<td>Group B</td>
<td>121 (75.1)</td>
<td>113 (93.3)</td>
<td>55 (48.6)</td>
<td>31 (27.4)*</td>
<td></td>
</tr>
</tbody>
</table>

* Indicates statistical difference.

This work was supported by funds from the Spanish MEC (projects SC 00-051-C3.1 and HI2002-0004) and the Italian MIUR (cofin).
102 ANALYSIS OF EARLY EMBRYONIC TRANSCRIPTION IN THE BOVINE EMBRYO USING A DEDICATED cDNA LIBRARY

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Early embryonic development is initially dependent on mRNAs that have been transcribed during oocyte growth (maternal transcripts). Newly formed zygotic transcripts then become required during what is called the maternal-to-zygotic transition. In cattle, this transition initiates at the 8-cell stage and ends before the morula stage. Because of its decisive role in the further development of the embryo we are interested in characterizing the regulative functions of those cattle zygotic transcripts that are differentially expressed at the end of this transition. For that reason a subtracted cDNA library corresponding to the first zygotic transcripts was established at the early compacted morula stage using suppressive subtractive hybridization (SSH; Clontech, LePont de Clai, France). Morula derived cDNAs were used as Tester and 4-cell stage cDNAs as Driver materials. Cattle embryos were obtained from slaughterhouse-derived ovaries using standard \textit{in vitro} maturation and fertilization techniques. Since, in cattle, early cleaving (2-cell-stage) zygotes are more likely to develop to the blastocyst stage than their later-cleaving counterparts, all embryos used to establish the cDNA library were selected from zygotes that were already at the 2-cell stage 32 h post-\textit{in vitro} fertilization. Total RNA was extracted from batches of 140 (morula) and 200 (4-cell-stage) embryos and the amount of Poly\textsuperscript{A} RNAs was estimated according to Duranthon and Renard (in \textit{Biologie and Pathology of the Oocyte}, Trounson and Gosden eds, Cambridge Univ. Press, 2003, p. 96). Double-stranded cDNAs were synthesized with the SMART cDNA amplification kit (Clontech) before SSH was undertaken. Upon RNA extraction, exogenous transcripts obtained from \textit{Arabidopsis thaliana} (Stratagene, La Jolla, CA, USA) were added either to the Tester only (at three concentrations: $10^{-3}$, $5 \times 10^{-3}$, $5 \times 10^{-2}$) or to both the Tester and the Driver materials (at two concentrations: $5 \times 10^{-3}$, $5 \times 10^{-2}$). These transcripts allowed us to report on the efficiency of our subtraction procedure and on the quality of the bacterial library in terms of tester-specific transcript enrichment. We found the library to be enriched in specific transcripts of the Tester (morula stage) by a factor of 300. Normalization of the library, as determined from the proportion of exogenous transcripts after bacterial transformation, was effective for those added initially at low ($10^{-5}$) or moderate ($5 \times 10^{-3}$) concentrations but not for abundant ones ($5 \times 10^{-2}$). These conditions are thus beneficial for the isolation of rare zygotic transcripts present at an initial concentration of only $10^{-6}$ of the messengers. Ongoing study using various differential screening of this cattle library with morula- and 4-cell-stage probes will now allow us to identify zygotic transcripts specifically expressed at the onset of genome activation and not present in the pool of maternal transcripts up to the 4-cell stage.

This work was supported by an INRA CIRAD grant (BioDivia) to LCB.

103 GAP JUNCTIONAL INTERCELLULAR COMMUNICATION IS DISPENSABLE DURING REGULATION OF TIGHT JUNCTION MEMBRANE ASSEMBLY BY CELL CONTACT PATTERN AND PKC SIGNALING

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Contact symmetries are involved in regulating cell lineage segregation during blastocyst biogenesis when tight junction (TJ) membrane assembly is restricted to the epithelial trophoderm (TE). Manipulation of cell contact patterns by immunosurgical isolation of inner cell masses (ICMs) providing a contact-free cell surface serves as a switch to induce TE differentiation upon \textit{in vitro} culture. In this model, protein kinase C (PKC)-mediated signaling up-regulates TJ membrane assembly. Whether signaling via gap junctional intercellular communication (GJIC) affects these processes is controversial. The current study investigates the interrelationship between changes in cell contact pattern, PKC signaling, and GJIC mediated signaling up-regulates TJ membrane assembly. Whether signaling via gap junctional intercellular communication (GJIC) affects these processes is controversial. The current study investigates the interrelationship between changes in cell contact pattern, PKC signaling, and GJIC. GJIC appeared dispensable during cavitation, TJ assembly, and PKC signaling. A better understanding of the interrelationships between different signaling mechanisms may help to improve embryo culture methods and viability.

\textit{Funding by the Wellcome Trust and MRC} is gratefully acknowledged.
Focusing on the role of pluripotency-determining factors Oct-4 and Nanog in goat pre-implantation embryo development, this study examined expression patterns of these factors in domestic animals. OCT-4 protein and mRNA were detected at the morula and blastocyst stages, with Oct-4 protein localization shifting to a more nuclear position at the blastocyst stage. Nanog mRNA was detected in the ICM but not the trophectoderm of expanded goat blastocysts, mirroring expression patterns observed in mice. Sequence alignment (ClustalW) showed high amino acid identity between species, with 87% identity between goat and human and 96% between goat and bovine OCT4 sequences. Oct-4 mRNA expression was detected at the 8-cell, morula, and blastocyst stages, and Nanog mRNA was detected at the morula and blastocyst stages. The expression patterns observed in this study establish Nanog as a candidate marker gene for embryo development and set the stage for further studies on molecular trends necessary for confirming the utility of the PHD system as an alternative embryo culture method.
106 GENOMIC IMPRINTING OF IGF2R IN TISSUES OF BOVINE FETUSES GENERATED BY ARTIFICIAL INSEMINATION OR IN VITRO FERTILIZATION

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The insulin-like growth factor 2 receptor gene (IGF2R) is involved in fetal growth regulation. A study in sheep associated fetal overgrowth after in vivo embryo culture with abnormal DNA methylation and expression of IGF2R (Young et al. 2001 Nat. Genet. 27, 153–154). This suggested that abnormal IGF2R imprinting is a major cause of fetal overgrowth. To test this hypothesis in bovine fetuses, we developed a microsatellite marker for IGF2R from cDNA sequence data and screened 45 Day-80 fetuses generated in vivo, by artificial insemination (AI), or in vitro, by in vitro fertilization (IVF) procedures, for parent-of-origin-specific gene expression. A total of 17 fetuses were heterozygous, but available parental DNA samples showed that only 12 (8 AL 4 IVF) allowed unambiguous discrimination from parental alleles. Parent-of-origin-specific allelic expression patterns indicated that bovine IGF2R was expressed predominantly from the maternal allele and thus imprinted in fetal heart, kidney, liver, lung, muscle, and cotyledon tissue. However, the relative amount of expression from the paternal allele was tissue-specific and ranged from 6.4 ± 0.8% in skeletal muscle up to 27.4 ± 0.9% in cotyledon (SPSS or 11.5, ANOVA, P < 0.001). Tissues that originated from the same germ layer showed similar allelic expression ratios whereas significantly different expression ratios (P < 0.05) were observed between tissues originating from different germ layers. Contrary to expectations from sheep data, there was no evidence for gross abnormalities in IGF2R imprinting in tissues from overgrown (n = 2) or normal sized (n = 2) IVF fetuses. However, relative paternal expression levels in several tissues showed significant relationships (P < 0.05–0.001) with growth parameters and pointed to subtle changes in paternal IGF2R expression in overgrown IVF fetuses.

We thank W. Schols and M. Weppeart for excellent technical assistance.

107 INITIAL RESULTS FROM MALE GERM CELL TRANSFER BETWEEN CATTLE BREEDS

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Male germline cell transfer has produced offspring in mice (Brinster & Zimmermann 1994PNAS 91, 11 298–11 302). Recently the first livestock animal, a goat, was produced (Honaramooz et al. 2003 Reproduction 126, 765–774; Oatley et al. 2002 J. Anim. Sci. 80, 1925–1931). We have assessed the outcome of male germ cell transfer between breeds of cattle and the efficacy of two vital dyes as markers of donor cells following transfer. Testis cells from three Bos taurus (Angus) bull calves were used as donor cells to transfer into six Bos indicus cross (predominantly Brahman bloodline) bull calves. Each of the calves was prepubertal and aged between 5 and 7 months. The calves were castrated; then a single-cell suspension of testis cells was prepared enzymatically using collagenase, DNAase, and trypsin. Prior to transfer into the recipient calves, the testis cell suspensions were dyed with one of two long-term vital dyes (PKH26 or CFDA). Approximately 300 million cells were injected into the rete of each testis under ultrasonographic guidance. In four of the six recipients, CFDA was injected into one testis and PKH26 into the other. These four recipients were castrated at 2, 4, 6, and 8 weeks after transfer.

We were unable to find positive cells with any certainty. We have concluded that PKH26 was more suitable for labeling donor testis cells which indicated either cell division or variable uptake of the stain during the staining procedure. We were disappointed to find no conclusive evidence of PKH positive donor cells. Positive cells were located on the seminiferous tubule basement membrane, which indicates these cells had successfully migrated from the tubule lumen and were likely to be spermatagonia. There was variation in the amount of fluorescence for individual cells, which indicated either cell division or variable uptake of the stain during the staining procedure. We were disappointed to find no conclusive evidence of CFDA stained cells as we encountered high background fluorescence from the majority of testis cells. Although this fluorescence was quenched within 10 s, we were unable to find positive cells with any certainty. We have concluded that PKH26 was more suitable for labeling donor testis cells and that donor cells can be identified for at least 2 months following transfer. Each of the recipients that received PKH26 stained cells retained these cells in the tubule epithelium, which suggests that transfer between different animals, and indeed between breeds, can be achieved. Further studies will aim to demonstrate that donor cells are able to undergo spermatogenesis in the recipient animals.

108 THE ROLE OF NITRIC OXIDE SYNTHASE IN IN VITRO DEVELOPMENT OF BOVINE OOCYTES AND PRE-IMPLANTATION EMBRYOS

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Nitric oxide (NO) is a free radical that serves as a key-signal molecule in various physiological processes including reproduction. Four isoforms of nitric oxide synthase (NOS) have been characterized: endothelial (eNOS), inducible (iNOS), neuronal (nNOS), and mitochondrial (mtNOS). The
The aim of the present study was to examine transient expression of transgene injected into nuclei of rat 2-cell stage embryos. We also investigated demonstrating the presence of lamins A/C in pluripotent cells of pre-implantation embryos. This study characterized lamin A/C expression in bovine embryos. Lamin A/C expression is generally associated with terminally differentiated cell types; however, numerous conflicting reports in the literature indicate that nitric oxide may play an important role as a diffusible regulator of bovine oocyte maturation and preimplantation embryo development.

Table 1. Effect of $\cdot$-NAME addition in maturation or culture medium on embryo development

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Maturation medium</th>
<th>Culture medium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cleavage rate (%)</td>
<td>Blastocyst rate (%)</td>
</tr>
<tr>
<td>0 mM</td>
<td>83.7 ± 0.9</td>
<td>12.5 ± 2.0</td>
</tr>
<tr>
<td>1 mM</td>
<td>68.9 ± 6.7</td>
<td>4.9 ± 2.5</td>
</tr>
<tr>
<td>10 mM</td>
<td>69.4 ± 2.9</td>
<td>6.2 ± 1.5</td>
</tr>
</tbody>
</table>

Values with different superscript within columns are significantly different ($P < 0.05$).

109 A BRIDGE OF SPERM TAIL BETWEEN BLASTOMERES ENHANCED PROTEIN MIGRATION IN THE RAT TWO-CELL STAGE EMBRYOS

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The aim of the present study was to examine transient expression of transgene injected into nuclei of rat 2-cell stage embryos. We also investigated the relationship between expression in both blastomeres and tail position of penetrated spermatozoa in rat 2-cell stage embryos. Rat 2-cell stage embryos were recovered from superovulated Wistar females mated with same strain mature males at 48 h after hCG injection. DNA fragments, as the transgene containing the EGFP (enhanced green fluorescent protein) gene controlled under the CMV-IE promoter, were microinjected into one nucleus of 2-cell stage embryos. After microinjection, embryos were cultured in KRB at 37.0°C in a 5% CO₂ and 95% humidified air until observation. First, transient EGFP expression in 151 injected embryos was observed using a fluorescence microscope at 6 h intervals until 48 h after injection. At 6 h after microinjection fluorescence embryos were detected, and the proportion of fluorescent embryos increased over time. The rate reached maximum (84%, 52/62) at 24 h after microinjection, and several fluorescent patterns of fluorescent blastomeres in the embryos were observed. There were blastomeres with the same or different fluorescence levels and a single fluorescent blastomere. Second, to assess tail position of the penetrated sperm in the fluorescent embryos, 75 whole mount specimens were observed by inverted phase-contrast microscopy at 24 h after the injection. Also, parthenogenetic 2-cell stage embryos that never contained sperm tail were microinjected with the transgene and observed in the same manner. To obtain parthenogenetic 2-cell embryos, 80 ovulated ova were collected from non-mated females, and incubated with 2 mM 6-DMAP for 4 h. The ova were additionally cultured for 20 h in KRB at 37.0°C in a 5% CO₂ and 95% humidified air. In embryos with both blastomeres fluorescent (94%, 33/35), the sperm tail existed in both blastomeres like a bridge between blastomeres. In contrast, in one embryo with a single fluorescent blastomere (4%, 1/24), the sperm tail existed in both blastomeres, and in other embryos with a single fluorescent blastomere (75%, 18/24), the sperm tail was positioned in the one blastomere. On the other hand, in 63 parthenogenetic rat 2-cell embryos in which there was no sperm tail, most embryos (86%, 54/63) had a single fluorescentblastomere at 24 h after microinjection. The results indicated that the sperm tail position in the 2-cell embryos makes the protein migration variable. In conclusion, when the CMV-IE/EGFP gene was microinjected into nuclei of rat 2-cell embryos, at 24 h after the microinjection the EGFP was detected in most embryos; however, fluorescent patterns in blastomeres varied. It seems that EGFP derived from the transgene injected into one blastomere may move into another blastomere in rat 2-cell stage embryos, and that the presence of a sperm tail in both blastomeres may influence EGFP distribution.

110 NUCLEAR LAMIN A/C EXPRESSION IN BOVINE IVF EMBRYOS

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Lamin A/C expression is generally associated with terminally differentiated cell types; however, numerous conflicting reports in the literature demonstrate the presence of lamins A/C in pluripotent cells of pre-implantation embryos. This study characterized lamin A/C expression in bovine
pre-implantation embryos using two monoclonal lamin A/C antibodies: anti-A/C IgM (A/C1) and anti-A/C IgG (A/C2) (Santa Cruz, California). Bovine embryos were produced as previously described (Foulad-Nashta et al., 1998 Biol. Rep. 59, 255–262) and collected at various stages for immunofluorescence staining. Embryos were fixed in 100% methanol at −20°C for 20 min and then blocked for 1 h (4% goat serum in PBS) at RT. Samples were then incubated overnight at 4°C with mouse lamin A/C antibodies or with blocking solution as a control. Following the primary incubation, embryos were washed extensively in 1% BSA in PBS and then incubated with rabbit anti-mouse immunoglobulins (1:20) (DAKO, Denmark) for 1 h at RT. Unbound secondary antibody was removed by washing with 1% BSA in PBS, and embryos were counter-stained with 4′,6-diamidino-2-phenylindole (2 μg/mL). Bovine fetal fibroblasts (BFF1) and human embryonic teratocarcinoma cells (EC1 and EC2) were processed identically to the embryos and used as positive and negative controls, respectively (Stewart and Burke 1987 Cell 51, 383–392). Images were viewed using epifluorescence (Leica DMR, Germany) and confocal microscopy (Leica TCS). BFF1 cells reacted with both lamin A/C1 and A/C2 antibodies. EC1 and EC2 stained positively for A/C2 whereas A/C1 was negative in both. All germinal vesicle (GV)-stage oocytes stained strongly for A/C2; however, for A/C1 only 67.5% were positive, and staining intensity was variable. Metaphase II oocytes stained negatively for both antibodies. One-cell zygotes exhibited a variable staining pattern similar to that of GV-stage oocytes. In contrast, all embryos from the 2-cell to blastocyst stage were negative for A/C1 but positive for A/C2. Our observations in embryos and EC cells indicate that the mouse anti-lamin A/C1 is specifically binding to lamin A/C whereas A/C2 is cross-reacting with other nuclear envelope proteins, possibly lamin B1/B2. The cross-reactivity of A/C2 has led to contradicting results in previous reports on lamin A/C expression in pre-implantation embryos. Our results with A/C1 show that lamin A/C is present in GV oocytes and 1-cell zygotes, suggesting that lamin A is important for pronuclear formation after fertilization. These results suggest that active remodelling of the nuclear envelope occurs during the early stages of bovine embryo development.

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111 CLONING AND CHARACTERIZATION OF PIG VASA HOMOLOG GENE AND ITS SPECIFIC EXPRESSION IN GERM CELL LINEAGE

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All of the vasa homologue genes in C. elegans (Caenorhabditis elegans, a free-living soil nematode), Xenopus, zebrafish, mouse, human, chicken, trout, and rat exhibited a germ line-specific expression and are used as specific molecular probes to distinguish the developmental profile of germ cells. In order to determine a useful marker for the research of germ cell commitment and development in pigs, we investigated the cloning and expression profile of porcine vasa homolog gene (Pvh). A Pvh cDNA gene of size 2172 bps (submitted to NCBI gene Bank No. AY626785) was cloned from pig ovary by reverse transcription-polymerase chain reaction (RT-PCR) amplification. The amplification was repeated three times and each RT-PCR product was sequenced. The isolated cDNA had 724 deduced amino acids with significant homology to mouse (85%) or human (91%) vasa. The Pvh sequence presents five copies of the RGG motifs and the DEAD box. By RT-PCR amplification, the expression of Pvh mRNA was restricted to the ovary and testis and was undetectable in somatic tissues including brain, whole blood, heart, lung, kidney, spleen, intestine, and liver. When analyzed by RT-PCR amplification, during pre-implantation embryo development, Pvh was transcribed in oocytes and fertilized 2-cell embryos (no difference in the expression levels between oocytes and fertilized 2-cell embryos), but not in 4-cell, 8-cell, morula and blastocyst stages. Using mouse vasa antibody (kindly donated from Dr. Noce, Japan; tested in porcine cells with porcine oocytes and mouse oocytes as positive control), immunohistochemical analysis of fetal Day 100 and adult gonad sections revealed that Pvh protein was specifically expressed in proliferating primordial germ cells (PGC), oocytes and spermatocytes. Interestingly, Pvh protein was not expressed in embryonic germ cells, but it was strongly expressed in freshly isolated PGC. Our results indicate that Pvh gene is specifically transcribed in pig germ cells.

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112 THE PRESENCE OF LAMIN A/C ANTIGENS IN PORCINE EMBRYOS

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Nuclear lamins are components of the nuclear lamina, and their primary role is to support the nuclear envelope and provide anchorage sites for the chromatin. While type B lamins are expressed in all cells, type A lamins (including lamins A and C) are developmentally regulated and expressed in differentiated cells only. There are conflicting results about the presence of lamin A/C in early mammalian embryos. Lamin A/C was found to localize in the nuclear envelope of bovine, pig, and mouse embryos, while recently it has been reported that early mouse and bovine embryos lacked lamin A/C antigens. It has also been suggested that the existence of lamin A/C in the pronuclei of mouse and bovine nuclear transfer embryos indicated faulty reprogramming. The aim of this study was to investigate the presence of lamin A/C in porcine embryos of different origins (in vivo, parthenogenetic, and nuclear transfer). Embryos of various developmental stages were collected from inseminated gilts. For the production of parthenogenetic embryos, mature oocytes were electropropulated and cultured for up to seven days. Fibroblast cells served as differentiated controls; progenitor cells from the olfactory bulb of a porcine fetus were used as undifferentiated controls. Lamin A/C was visualized by immunocytochemistry. Olfactory bulb progenitor cells lacked lamin A/C (0 out of 50 cells showed staining) while all fibroblast nuclei (n = 50) reacted positively with...
the antibody. GV-stage oocytes, being terminally differentiated cells, also possessed lamin A/C antigens (30/30). Lamin A/C was not detectable in any of the mature oocytes examined (n = 30), but it was found in early cleavage-stage embryos [both in vivo (20/20) and parthenogenetic (30/30)] with the signal becoming weaker in blastocysts (15/15). After nuclear transfer, the lamin A/C signal from fibroblast nuclei disappeared (20/20), consistent with nuclear envelope breakdown. Later it became detectable again; all nuclear transfer embryos reconstructed with either fibroblast or progenitor cells displayed lamin A/C staining in their pronuclei and at all stages examined (n = 65). This suggests that recipient oocytes remodel the donor nuclei and reassemble the nuclear envelopes of both differentiated and undifferentiated cells with type A lamins. Activated oocytes (n = 30) and early embryos (n = 40) were then incubated in the presence of actinomycin D (an inhibitor of RNA polymerase II) or cycloheximide (a protein synthesis inhibitor) for 14h. Lamin A/C assembly was not perturbed by either treatment, indicating that the assembly did not result from de novo gene transcription but rather from solubilized lamins already in the cytoplasm. The results imply that lamin A/C is present in early pig embryos and that its presence after nuclear transfer is not an indicator of erroneous reprogramming, unlike that reported in cattle and mouse.

113 THE DISTRIBUTION OF THE LEPTIN PROTEIN WITHIN BOVINE OOCYTES AND PRE-IMPLANTATION EMBRYOS MATURED AND FERTILIZED IN VITRO

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It has recently been documented that leptin regulates processes linked to reproduction including preimplantation development, embryo implantation (trophoblast invasion), and fetal growth. Transcripts for the leptin gene (LEP) and the leptin receptor gene (LEPR) have been identified in ovary, testis, placenta, endometrium, ovarian follicles, and oocytes, and also in mouse, rat, human, and bovine pre-implantation embryos. Moreover, the leptin protein was detected in mouse and human oocytes and embryos, and its localization was polarized. The distribution of regulatory proteins within oocytes and pre-implantation embryos is critical for early mammalian development, such as determination of the animal pole and the establishment of the trophoblast and the inner cell mass cells (ICM). So far there is no published evidence concerning this phenomenon in bovine oocytes and embryos. Therefore, the aim of this work was to analyze the leptin protein distribution within bovine oocytes and preimplantation embryos matured and fertilized (in vitro). The material for this work consisted of oocytes collected from slaughterhouse ova and sperm collected from AI bulls. In vitro oocyte maturation and fertilization were carried out according to the method described by Makarevich and Markkula (2002 Biol. Reprod. 66, 386–392). The preliminary experiment of leptin protein localization by immunofluorescent staining included immature and matured oocytes and blastocysts. Oocytes and embryos were fixed in PBS containing 4% paraformaldehyde and reacted with affinity-purified polyclonal rabbit primary antibody directed against leptin (0.1 mg/mL; Ob (Y20), Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA); and then exposed to secondary goat-anti-rabbit antibody (1.0 mg/mL; Santa Cruz Biotechnology Inc.)-fluorescein isothiocyanate (FITC) conjugate. Finally, chromatin was visualized by propidium iodide staining (0.5 µg/mL). Slides were examined under a conventional fluorescence microscope (Nikon) and confocal microscope (Zeiss). The preliminary results demonstrate that the distribution of leptin differed between immature and mature oocytes: it was spherical in immature oocytes (a rim beneath the oolemma) whereas it became evenly distributed after maturation. In blastocysts, leptin signals were present in both the trophoblast cells and in the ICM cells. This is in contrast with studies on mouse embryos which showed the presence of the LEP protein in the trophoblast only. Future experiments will include studies of embryos at the 2-cell, 4-cell, 8–16-cell, and morula stages. The present study for the first time shows the pattern of leptin protein distribution within bovine oocytes and preattachment embryos.

114 PORCINE EMBRYO FRAGMENTATION, DEVELOPMENT AND APOPTOSIS: A CONFOCAL MICROSCOPY STUDY

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The relationship between embryonic fragmentation, embryonic arrest, and apoptosis has been the subject of some controversy (Hardy K 1999 Rev. Reprod. 4, 125–134). In order to investigate possible links, in vivo-produced, in vitro-cultured porcine embryos (n = 132) were scored for developmental stage and fragmentation at 7 days post insemination (dpi) and processed for propidium iodide and annexin V labelling. After fixation, embryos were processed for terminal deoxynucleotidyl transferase mediated dUTP nick end labelling (TUNEL). Using confocal microscopy, a cell was categorized apoptotic if (i) it had a fragmented or condensed nucleus, (ii) the cell membrane was annexin V-positive, and (iii) the nucleus was TUNEL labelled. An apoptotic cell ratio (ACR) was determined as the percentage of apoptotic cells per embryo. Differences in the % of fragmented and apoptotic embryos and correlations were analyzed using chi-square. Logistic regression was used to compare the average fragmentation % and the ACR. Sixty-one embryos (46%) arrested during the culture period, with 8 embryos arresting before or at the 4-cell stage. Significantly more arrested embryos were fragmented compared to embryos that were blastocysts at 7 dpi. Also, the average fragmentation percentage was significantly higher for arrested embryos compared to blastocysts. The correlation detected between developmental arrest and fragmentation was 0.60 (P < 0.05). None of the embryos without fragmentation had cells categorized as apoptotic, whereas 30 out of 55 embryos with fragmentation possessed apoptotic cells, which led to a correlation of 0.87 (P < 0.01) between fragmentation and apoptosis. The percentage of embryos with apoptotic cells was significantly higher for embryos arrested during the 5-cell to the morula stage compared to embryos that arrested before or at the 4-cell stage and embryos with blastocyst development at 7 dpi. The average ACR of embryos arrested during the 5-cell to the morula stage was significantly higher compared to the average ACR of blastocysts at 7 dpi. The correlation detected between the developmental arrest, during the 5-cell to the morula stage period and apoptosis was 0.57 (P < 0.01). Taken together, significant correlations between fragmentation, developmental arrest and apoptosis were detected. However, the association between embryonic arrest and apoptosis could be established only for embryos arrested after embryonic genome activation.
Human amniotic epithelial cells (hAECs) have been reported to have unique properties. They express almost no class I and class II MHC antigens and lack response to interferon-γ (IFN-γ) which mediates the expression of those MHC molecules. Moreover, hAECs express some genes characteristic of hepatic cells. Therefore, hAECs seem to have multipotency and are expected to substitute for hepatic tissues in part. We aimed to develop the experimental model for investigating AECs in mice (mAECs). In this study, we examined the induction of MHC molecules by IFN-γ and the hepatic gene expression in mAECs. Murine amniotic membranes were collected from C57BL/6J females at 17.5 days of gestation. They were digested by 0.03% hyaluronidase followed by 0.2% collagenase treatment. Dissociated mAECs were cultured on dishes in DMEM supplemented with 10% FBS at 37˚C under 5%CO2 in air. Embryonic fibroblasts (EFs) collected from C57BL/6J fetuses at 13.5 dpc were cultured in the same condition as mAECs. In Experiment I, the effect of IFN-γ on induction of MHC molecules in mAECs was examined. mAECs and EFs cultured in the presence or absence of IFN-γ at 1 × 103 U/ml for 72 h were recovered and incubated with FITC-conjugated antibodies against mouse H-2 MHC class I or I-A/I-E MHC class II antigens. The cells were analyzed by flow cytometry. In Experiment II, the expression of the genes in mAECs was examined by RT-PCR. mRNA was purified from adult liver, EFs, fresh mAECs, and mAECs cultured for 5 days. As the genes characteristic for hepatic cells, HNF-3α, HNF-3β, HNF-3γ, HNF-4, transthyretin (TTR), albumin, α-fetoprotein (AFP), glucose-6-phosphatase (G6P), and asialoglycoprotein receptor-1 (Asgr1) were examined. In Experiment I, cell-surface expression of class I and class II MHC antigens in response to IFN-γ was observed weakly in mAECs as compared to EFs, suggesting different property in hAECs which lack the expression of those antigens. In Experiment II, RT-PCR analysis showed that all of the genes except G6P were expressed in fresh mAECs. However, the expression of transcription factors such as HNF-3α, HNF-3β, HNF-4, and TTR, serum proteins such as albumin and AFP, and Asgr1 decreased after in vitro culture, contrary to the case of hAECs in which, for example, albumin appeared after cultivation. In conclusion, it was evident that mAECs have quite different properties, both in the inducibility of MHC molecules and the expression of hepatic genes, from hAECs.

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

### 115 CYTOLOGICAL ANALYSIS OF HEPATIC GENE EXPRESSION AND IMMUNOLOGICAL RESPONSE OF MHC ANTIGENS IN MOUSE AMNIOTIC EPITHELIAL CELLS

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Epigenetic modification of parental genomes plays a prominent role in regulating genome expression in the early development of embryos. In general, histone H3 of the paternal genome is demethylated at lysine 9 (H3-K9) during the first and second mitotic divisions in fertilized embryos, while the maternal genome is methylated. We investigated the effects of maternal genomes (Mgen) and paternal genomes (Pgen) on H3-K9 methylation and acetylation during the early development of murine embryos. Histone H3-K9 methylation and acetylation were detected by anti-trimethyl histone H3-K9 and anti-triacetyl histone H3-K9 antibodies. The following embryos were used in this study: (1) intracytoplasmic sperm injection (ICSI) embryos (50% Mgen, 50% Pgen); (2) parthenogenetic diploid embryos (100% Mgen, 0% Pgen); (3) somatic nuclear transfer embryos (50% Mgen, 50% Pgen from previous generation); (4) androgenetic diploid embryos (0% Mgen, 100% Pgen); and (5) haploidized somatic and sperm embryo, the pattern of histone H3-L9 methylation resembles that of the ICSI embryo. While histone H3-K9 acetylation occurs in both paternal and maternal genomes during interphase, even when the nuclear membrane is completely degraded and the chromosome is condensed, it disappears rapidly when the chromosome enters the real metaphase, and reappears at the early stage of pronuclear formation in all types of embryo. These results suggest that the absence of maternal genomes results in histone H3-K9

### 116 ACTIVE METHYLATION AND ACETYLATION OF HISTONE H3-K9 IN MOUSE EMBRYO WITH DIFFERENT PROPORTIONS OF MATERNAL AND PATERNAL GENOME

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<table>
<thead>
<tr>
<th>Stage of development</th>
<th>% (ratio) of embryos with fragmentation</th>
<th>Average fragmentation (%)</th>
<th>% (ratio) of embryos with apoptosis</th>
<th>Average apoptotic cell ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arrested 1- to 4-cell</td>
<td>50(^{a}) (4/8)</td>
<td>10.0(^{a})</td>
<td>25(^{a}) (2/8)</td>
<td>8.3(^{ab})</td>
</tr>
<tr>
<td>Arrested 5-cell to morula</td>
<td>77(^{a}) (41/53)</td>
<td>10.8(^{b})</td>
<td>72(^{a}) (38/53)</td>
<td>16.1(^{b})</td>
</tr>
<tr>
<td>Blastocyst</td>
<td>14(^{b}) (10/71)</td>
<td>2.5(^{b})</td>
<td>14(^{a}) (10/71)</td>
<td>3.4(^{a})</td>
</tr>
</tbody>
</table>

\(^{a}\)Values within a row with a different superscript differ significantly (P < 0.05).
methylation in the paternal genomes during the first and second mitotic divisions of embryos in mice. In addition, histone H3-K9 acetylation is independent of the presence or absence of maternal or paternal genomes during pre-implantation development in mice.

This study was supported by grants-in-aid for Creative Scientific Research (13GS0008) and a project for the realization of regenerative medicine (the research field for the technical development of stem cell manipulation) to T.W. from MEXT, Japan.

117 CYTOPLASMIC FACTORS INFLUENCE DEVELOPMENTAL POTENTIAL OF SAMP1/Yit MOUSE EMBRYOS

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Nuclear transplantation is an efficient means to investigate nucleo-cytoplasmic interactions of mammalian embryos during early development. A recent study has shown that the developmental potential of embryos is affected by the type of cytoplasm. The SAMP1/Yit mouse, an inbred strain that develops spontaneous chronic ileitis resembling Crohn’s disease (Matsumoto S 1999 Bioscience Microflora 18, 1–9), has poor reproductive performance, and the developmental ability of embryos is low (unpublished data). Therefore we need to enhance productivity of the SAMP1/Yit mouse.

Recently it was reported that cytoplasm of F1 mouse egg supported the development of embryos which have low developmental ability (Muggleton-Harris A et al. 1982 Nature 299, 460–462). In the present study, we examined the influences of the nucleus and cytoplasm on the development of reconstructed embryos in vitro and in vivo, using reciprocal nuclear transplantation between SAMP1/Yit and B6P1F1 (C57BL/6 × SAMP1/Yit) mouse embryos. We evaluated the developmental ability of reconstructed embryos by the development rate into blastocysts in vitro and by the rate of offspring after transfer of blastocysts to recipient mice. Pronuclear transplantation was carried out as reported previously (McGrath J and Solter D 1983 Science 220, 1300–1302). Briefly, karyoplasts from one-cell SAMP1/Yit embryos were introduced into enucleated B6P1F1 zygotes (SAMP1/B6P1F1) and fused by addition of inactivated HVJ (2700 U L−1). The other group of reconstructed embryos (B6P1F1/SAMP1) was manipulated similarly. After fusion, reconstructed embryos were cultured in drops of KSOM medium for 120 h at 37°C in 5% CO2 in humidified air. Some reconstructed and control (unmanipulated) embryos that developed to the blastocyst stage were transferred to the uteri of recipient mice. Data were compared using chi-square test; differences were considered significant at P < 0.01. The development rate of [SAMP1/B6P1F1] embryos to the blastocyst stage was significantly (P < 0.01) higher (75.0%) than that of SAMP1/Yit controls (39.1%). The rate of offspring in [SAMP1/B6P1F1] was also significantly (P < 0.01) higher (47.5%) than that of SAMP1/Yit controls (22.1%). On the other hand, [B6P1F1/SAMP1] embryos showed low developmental potential. The data indicate that the source of the cytoplasm strongly influences the development of reconstructed embryos containing SAMP1/Yit karyoplasts.

Table 1.

<table>
<thead>
<tr>
<th>Group</th>
<th>Karyoplast</th>
<th>Cytoplasm</th>
<th>No. of blastocysts developed (%)</th>
<th>No. of offsprings transferred (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (unmanipulated)</td>
<td>SAMP1</td>
<td>SAMP1</td>
<td>77/197 (39.1)</td>
<td>15/68 (22.1)</td>
</tr>
<tr>
<td>Control (unmanipulated)</td>
<td>B6P1F1</td>
<td>B6P1F1</td>
<td>118/138 (85.5)</td>
<td>57/87 (65.5)</td>
</tr>
<tr>
<td>Reconstructed</td>
<td>SAMP1</td>
<td>B6P1F1</td>
<td>69/92 (75.0)*</td>
<td>29/61 (47.5)*</td>
</tr>
<tr>
<td>Reconstructed</td>
<td>B6P1F1</td>
<td>SAMP1</td>
<td>56/87 (64.4)</td>
<td>20/56 (35.7)</td>
</tr>
</tbody>
</table>

* Significantly different from corresponding column of control (SAMP1) at P < 0.01.

118 TRANSFER OF IRON FROM MOTHER TO FETUS IN WATER BUFFALO: ERYTROPHAGOCYTOSIS AND UTEROFERRIN


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The transplacental transport of iron by uteroferrin (Uf), and the hemophagous areas in the water buffalo placenta were analyzed to clarify the mechanism of blood extravasation in the maternal-fetal interface with consequent transfer of iron to the fetus through the trophoblastic erythrophagocytosis (Murai and Yamauchi 1986 Nippon Juigaki Zasshi 48, 75–88) and in the endometrial glands (Bazer et al. 1991 Exp. Hematol. 19:910–19:915). In the water buffalo placenta this mechanism remains unclear; uteroferrin is very important in this process because it is both an iron transporter and a progesterone-induced hematopoietic growth factor. Our objective was to characterize these hemophagous areas and the endometrium of the water
This work was funded by FAPESP.

A chimaera is an organism composed of cells derived from two (or more) zygotes. Spontaneously originated diploid-triploid (2n-3n) chimaeric embryos were obtained from females F1 (C57Bl/6 × CBA) crossed with F1 males as a result of “delayed mating.” The triploidy was induced by suppression of the extrusion of the second polar body with cytochalasin D (1 µg/mL). Triploid-chimaeric embryos were created by aggregation of diploid embryos with triploid embryos at 4-8 cell stage. In chimaeras created according to this procedure, the triploid component was agouti and produced the 1B1B isoform of glucose phosphate isomerase (GPI) and the diploid component was albino and produced the GPI-1A1A isoform. Electrophoresis of GPI was performed in order to determine the contribution of both populations of cells in tissues of embryos and individuals. Over a thousand oocytes were subjected to triploidization. A total number of 201 diploid-triploid aggregates developed into blastocysts and were transplanted to the oviducts of 30 recipients. Our experiment yielded 23 living and 6 dead embryos.

The presence of MBD4, a member of the methyl binding domain family, was investigated in both murine and bovine oocytes and pre-implantation embryos. MBD4 is the only MBD family member that is involved in DNA repair but not active in transcriptional repression or in the formation of complexes with histone deacetylase complexes (HDACs). It contains a mismatch-specific glycoalyase domain that acts to repair G:T mismatches within a CpG context. Bovine cumulus oocyte complexes were collected from abattoir-derived ovaries, matured in vitro and used for IVF as described previously (Ruddock et al. 2004 Biol. Reprod. 70, 1131–1135). Samples were analyzed at all steps in this process. Murine oocytes were collected from superovulated mice (C57Bl6 × CBA) and subjected to conventional IVF. A polyclonal antibody derived in the rabbit against human peptides from specific regions of MBD4 (Imgenex, San Diego, CA, USA) was used to localize MBD4 protein. This antibody was tested at a variety of concentrations against both human HL60 leukemia cells and bovine embryos. Staining of HL60 cells was optimum at 32–64 µg/mL and embryos at 64 µg/mL. Briefly, the staining protocol consisted of fixing cells and zona-free oocytes or embryos in 4% paraformaldehyde for 15 min, followed by 15 min in 0.1% Triton X-100. Primary antibody incubation was performed overnight at 4°C. Embryos were then washed in blocking buffer for 1 hr prior to incubation at 4°C in mouse anti-rabbit IgG conjugated to FITC in blocking buffer for 30 min in the dark. Lastly, embryos were incubated in 10 µg/L Hoescht 33342 for 15 min, and then washed and mounted with Vectashield (Vector Labs, Burlingame, CA, USA). Negative controls contained no primary antibody. Mounted cells/embryos were viewed by epifluorescence microscopy. MBD4 was found to be expressed in both murine and bovine oocytes and pre-implantation embryos. In the cow, faint nuclear expression was detected at 2-cell stage, but was then concentrated in the nucleus from the 2-cell stage onward. It will be interesting to determine if this is due to the different timing of embryonic genome activation between the two species, hence implying a role for MBD4 in this important biological process. Further investigations are underway to compare the subcellular localization of the other MBD proteins in both species during preimplantation development and to identify a role for MBD4 in embryonic genome activation.

A chimaera is an organism composed of cells derived from two (or more) zygotes. Spontaneously originated diploid-triploid (2n-3n) chimaeric embryos and adults have been described in many species of mammals. In man, between 1960 and 2002 over 30 cases of chimaerism were discovered (van de Laar I et al. 2002 Clin. Genet. 62(5), 376–382). A deeper insight into the developmental consequences of this rare and odd phenomenon requires experimental production of 2n-3n embryos and animals. The present study is the first and successful attempt to produce diploid-triploid chimaeric embryos, fetuses, and postnatal animals in the mouse. Diploid embryos originated from BAMIZ females crossed with BAMIZ males. The zygotes that were the source of triploid embryos were obtained from females F1 (C57Bl6 × CBA/H) crossed with F1 males as a result of “delayed mating.” The triploidy was induced by suppression of the extrusion of the second polar body with cytochalasin D (1 µg/mL). Triploid-triploidal chimaeric embryos were created by aggregation of diploid embryos with triploid embryos at 4-8 cell stage. In chimaeras created according to this procedure, the triploid component was agouti and produced the 1B1B isoform of glucose phosphate isomerase (GPI) and the diploid component was albino and produced the GPI-1A1A isoform. Electrophoresis of GPI was performed in order to determine the contribution of both populations of cells in tissues of embryos and individuals. Over a thousand oocytes were subjected to triploidization. A total number of 201 diploid-triploid aggregates developed into blastocysts and were transplanted to the oviducts of 30 recipients. Our experiment yielded 23 living and 6 dead embryos.
Oocytes are susceptible to heat shock (HS) during the maturation process. It has been demonstrated that HS induces apoptosis and/or the expression of HS protein 70 (hsp 70) in in vitro-produced oocytes and embryos. The objectives of this study were to analyze the effects of HS on the development and apoptosis of pig oocytes and embryos. Porcine ovaries were collected from a local slaughterhouse and the cumulus-oocyte complexes (COCs) were aspirated from follicles 3–6 mm in diameter and subjected to standard in vitro maturation procedures at 39°C for 42 h. The in vitro matured oocytes were then randomly allocated to different HS treatments at 41.5°C for 0 (control, C0h), 1 (HS1h), 2 (HS2h), or 4 h (HS4h). An additional control group of oocytes was cultured for 4 h without HS (C4h). Data were analyzed by chi-square test. In Experiment 1, anti-hsp 70 (SPA-810AP, Stressgen, San Diego, CA, USA) and Western blotting were used to examine the expression of hsp 70. Results indicated that no significant difference of hsp 70 expression in metaphase II porcine oocytes occurred between controls and HS groups (P > 0.05, 7 replicates). In Experiment 2, apoptosis of metaphase II oocytes after HS was identified by annexin V-FITC (Sigma, St. Louis, MO, USA) staining and TUNEL (Roche, Indianapolis, IN, USA). No significant apoptotic signal was detected in the HS groups compared to the controls. The intensity of annexin V staining was not affected by HS, but it increased with the time of culture (P < 0.05, n = 24–37). In Experiment 3, the apoptotic rate and developmental competence of the HS-oocytes were evaluated by TUNEL assay (n = 123–137, 4 replicates). Parthenogenetic activation (n = 123–137) was performed by an electric pulse (2.2 kV cm⁻¹) combined with 6-dimethylaminopurine treatment (6-DMAP, 2.5 μM, 4 h, Sigma). The cleavage rates in HS2h (43 ± 29%) and HS4h (35 ± 28%) decreased (P < 0.05) compared to those in C0h (62 ± 12%) and C4h (66 ± 8%). In addition, the blastocyst formation rates and total cell numbers reduced (P < 0.05) after 2 h (11 ± 10%, 20 ± 16) and 4 h (11 ± 8%, 19 ± 8) of HS treatments compared to those in C0h (23 ± 14%, 32 ± 22) and C4h (21 ± 11%, 27 ± 17), respectively. The numbers of blastocysts with TUNEL-positive signals were not significantly different between the HS and control groups, but the signals increased (P < 0.05) before the 8-cell stage in HS groups (22–24%) compared to the C0h and C4h controls (16 and 11%), respectively. These results indicate that reduction in developmental competence of in vitro-matured pig oocytes after heat shock is not closely correlated to the expression of hsp 70 in the oocytes and to the apoptotic cell numbers in the blastocyst. Whether detection of apoptosis by TUNEL or annexin V-FITC in oocytes is a good indicator requires further investigation.

121 HEAT SHOCK TO PIG OOCYTES DOES NOT INDUCE APOPTOSIS BUT REDUCES EMBRYO DEVELOPMENT

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Early Pregnancy/Pregnancy Recognition

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In cattle, more than 30% of embryonic losses observed after artificial insemination (AI) have an early origin, coincident with a marked elongation of the trophoblast which occurs before implantation, between the 13th and 19th days of pregnancy. During this exponential growth phase, physiological interactions essential for pregnancy are established between the embryo and the uterus. Our work focuses on the identification of transcripts that regulate this key developmental period in several domestic species. For that, we generated a nylon membrane that contained 1920 gridded inserts originating from a Day-14 bovine embryo cDNA library (dbEST ID.15979; Hue et al., in preparation). Gene expression profiles in trophoblasts of increasing sizes were compared using ovoid (10–18-mm), tubular (50–60-mm), and early filamentous (140–150-mm) stages as complex probes. Trophoblasts were collected and immediately snap-frozen. RNA extractions were performed using RNaPlus (Quantum Appligene, Illkirch 67402, France). Due to the scarce amount of mRNA per embryo, amplified material was used to hybridize the array. For that, antisense-RNA (aRNA) and cDNA were generated starting from 1 μg of total RNA, as described by the MessageAmp aRNA kit instructions (Ambion,ustin, TX 78744, USA) and according to Revel et al. (1995 Zygote 3, 241–250). Five hundred nanograms of aRNA or cDNA were random-primed and labelled with ³²P-alpha-dATP [aRNA, according to the procedure of DeCraene et al. 1999 BioTechniques 27, 962–966; cDNA using the Atlas SMART Probe Amplification kit, (Clontech, Ozyme, Saint Quentin Yvelines 78053, France)]. For each protocol, two probes were generated independently and each of these probes was hybridized to four identical membranes according to Clontech instructions. These were then exposed to phosphorscreens and scanned after 7 days. Quantifications were done using ImaGene 5.1 (BioDiscovery, El Segundo, CA 90245, USA) and statistically analyzed.