for the first experiment, loaded into straws, and assigned to one of 4 treatment groups. Half the straws from each bull were exposed to 90 MPa/30 min, 90 MPa/90 min, 30 MPa/30 min, or 30 MPa/90 min, and then cryopreserved. Controls consisted of straws that were cryopreserved without pressure treatment. Cryopreservation steps were 60 min equilibration at 5°C, followed by 10 min at −110°C, and then plunging into liquid nitrogen. Straws were thawed in a 35°C water-bath for 30 s. Each treatment and control group was replicated 8 times (8 samples per bull). The average post-thaw motility was significantly superior with pressure pre-treatment in each of the pressurized groups compared to the samples frozen without previous pressurization ($P < 0.001$) (Bull I: 2–3% without pressurization vs. 17–33% with pressurization; Bull II: 0% without pressurization vs. 21–35% with pressure pre-treatment). Among the pressure/time parameters used, 30 MPa/90 min proved significantly superior (33 and 35%; $P < 0.05$) for each of the bulls. Expt. 2 clearly demonstrates the beneficial effect of a previous pressure treatment on post-thaw motility of bull semen cryopreserved in our experiment. Further investigations are needed, including samples from different bulls, different freezing protocols, and the biological background of the process.

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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 (Misumi 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 (LN_2) for 15 s and then plunged into LN_2. The pipette tip was broken and the tip and associated frozen microdrop were placed inside an LN_2-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% CO_2 in air environment and scored daily for development. For embryos undergoing cryopreservation, retrieval rates from cryovials 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%; $P < 0.001$; χ^2-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 *v*. 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 ± 1.08 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 ± 3.03%) ($P < 0.05$), 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

	Plasma	GCs	COCs	*P* value
Saturated FA	51.6 ± 1.35	56.2 ± 2.10	87.3 ± 1.00	<0.001
Monounsaturated FA	20.3 ± 0.82	32.7 ± 0.68	11.1 ± 0.66	<0.001
PUFA	28.2 ± 1.80	11.1 ± 2.23	2.1 ± 0.35	<0.001
n-6 PUFA	26.4 ± 1.77	10.2 ± 2.14	1.6 ± 0.26	<0.001
n-3 PUFA	1.8 ± 0.10	0.9 ± 0.13	0.5 ± 0.15	<0.001
n-6/n-3 ratio	16.6 ± 1.06	10.9 ± 1.94	3.8 ± 0.71	<0.001

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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 progestagen 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. Ultrasonographic 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

Oocyte source	Collected oocytes (%)	Oocytes selected for IVM (%)	Fertilized oocytes (%)	Cleaved oocytes (%)	Blastocysts (%)
Group A	151 (57.6)	111 (73.5)	89 (80.1)	37 (41.5)	9 (10.1)*
Group B	161 (62.4)	121 (75.1)	113 (93.3)	55 (48.6)	31 (27.4)*

* Indicates statistical difference.

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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 Claix, 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 *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-*in vitro* fertilization. Total RNA was extracted from batches of 140 (morula) and 200 (4-cell-stage) embryos and the amount of PolyA$^+$ RNAs was estimated according to Duranthon and Renard (in *Biology 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 *Arabidopsis thaliana* (Stratagene, La Jolla, CA, USA) were added either to the Tester only (at three concentrations: 10^{-3}, 5×10^{-3}, 5×10^{-2}) or to both the Tester and the Driver materials (at two concentrations: 5×10^{-3}, 5×10^{-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^{-3}) or moderate (5×10^{-3}) concentrations but not for abundant ones (5×10^{-2}). These conditions are thus beneficial for the isolation of rare zygotic transcripts present at an initial concentration of only 10^{-3} 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.

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103 GAP JUNCTIONAL INTERCELLULAR COMMUNICATION IS DISPENSIBLE 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 trophectoderm (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 *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 on TE differentiation and TJ assembly. Eight-cell embryos flushed from MF1 mice were cultured in T6/BSA to time development to early blastocyst stage (<2 h of cavitation). Laser confocal microscopy (BioRad MRC 600, BioRad Laboratories, Inc., Hertfordshire, UK) after immunostaining with antibodies against PKCδ, θ, λ, or ζ isoforms (Transduction Labs, Oxford, UK or Sigma) or junctional proteins (E-cadherin, ZO-2, Occludin, ZO-1α+, Desmoplakin) combined with ALEXA 488 conjugated secondary antibodies (Cambridge Bioscience, Oxford, UK) was used to determine the distribution of PKCs and junctional proteins in intact blastocysts and fully and partially isolated ICMs after immunosurgery and *in vitro* culture in DMEM + 10% FCS (Eckert *et al*. 2004 Reproduction 127, 653). While broad PKC activators (1 μM 12-O-tetradecanoylphorbol-13-acetate or Indolactam; Calbiochem, Nottingham, UK) accelerate membrane assembly of the TJ proteins ZO-2 and ZO-1α+ in fully isolated ICMs this up-regulation was suppressed in intact blastocysts ($n = 32$–47 per treatment and antibody) and in partially isolated ICMs (remnants of lysed TE remaining surrounding the ICM; $n = 17$–21 per treatment and antibody) for up to 24 h with no TJ protein detectable within the ICM, even after two consecutive rounds of TE lysis ($n = 13$–22 per treatment and antibody). When GJIC was inhibited during blastocyst formation *in vitro* and in cultured fully isolatd ICMs by 18 α-glycyrrhetinic acid (AGA, 65 μM; Sigma), cavitation rate and distribution of PKCs or junction assembly were not affected compared to controls (70–80% cavitated with characteristic distribution of junctional proteins and PKCs; $P > 0.05$, ANOVA; $n = 15$–20 per treatment, antibody, and cell contact pattern). When GJIC inhibition by AGA was confirmed by Lucifer yellow (Sigma) injection (no dye transfer in 82–100%, $n = 14$–17 per contact pattern), GJIC was also absent in 50% of fully isolated ICMs without AGA treatment, suggesting that cell contact modulation may affect GJIC. Taken together, our data suggest that cell contact pattern regulates TJ assembly via PKC signaling pathways and may also affect 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.

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104 EFFECT OF FROZEN MEDIA ON IGF_2 EXPRESSION OF BOVINE EMBRYOS CULTURED ENTIRELY *IN VITRO* UNTIL DAY 14

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Freezing and stocking ready to use IVP medium, including hormones and fetal serum, is a practical alternative to rationalize work and reduce costs in *in vitro* embryo production. In our laboratory routine, embryo culture in frozen or fresh medium (produced weekly) has shown equal Day 7 blastocyst rates. Although morphological aspects were also similar, it is known that culture environment may alter gene expression patterns and embryo development on later stages. Thus, a preliminary study of mRNA expression of IGF_2 in Day 14 embryos produced in both culture conditions (frozen and fresh medium) was performed. For that, IVM (TCM 199 with hormones, antibiotics, L-glutamine and 10% fetal bovine serum) and IVC medium (SOFaaci) were split into 2-mL aliquots into Eppendorf tubes and frozen at −80°C temperature four weeks prior to use. The thawing was performed in a 4–5°C refrigerator overnight and the medium was stabilized in the incubator at least 4 h prior to use. Abattoir-derived oocytes were collected and randomly distributed into two culture groups: T1 (fresh IVM, fresh IVC) and T2 (frozen IVM, frozen IVC). On Day 7, blastocysts classified as Grades 1 and 2 ($n = 12$) were selected and continued *in vitro* culture in the Post Hatching Development system; PHD system (Brandao *et al*. 2004 Reprod. Fertil. Dev. 16, 123–124) under 38.5°C, 5% CO_2 in air. On Day 14, elongated embryos from T1 and T2 were removed from culture and analyzed by RT-PCR. Total RNA of each group was prepared from two D14 embryos of two distinct replicates using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol with modifications. The reverse transcription (RT) was done using the EZ-First Strand cDNA Synthesis Kit (Biological Industries, Israel). The β-actin gene was used as a constitutive control and PCR reactions for the two genes were carried out in triplicate using a PTC-100 MJ Research thermocycler. PCR products were electrophoresed on a 1.5% agarose gel. The two genes were detected in both samples; the relative expression of IGF_2 was higher in embryos cultivated in fresh medium (control). The consequences of IGF_2 levels in embryo development are still unknown, but highlight the late effects of culture conditions on embryonic gene expression. The perspective use of the PHD system might be an alternative embryo development for monitoring until further stages. In conclusion, IGF_2 appears to be a candidate marker gene for embryo development and, although frozen ready-to-use medium is a practical strategy, further studies on molecular trends are necessary to confirm its use.

105 EXPRESSION OF PLURIPOTENCY-DETERMINING FACTORS Oct-4 AND NANOG IN PRE-IMPLANTATION GOAT EMBRYOS

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The objective of this study was to determine the expression patterns of the pluripotency-determining factors, Oct-4 and Nanog, in pre-implantation goat embryos. The POU octamer-binding domain transcription factor Oct-4 and the homeobox transcription factor Nanog have been shown to play key roles in the maintenance of pluripotency in the inner cell mass (ICM) of pre-implantation mouse embryos and in embryonic stem cells. As Oct-4 protein has been observed in human, monkey, bovine, and porcine pre-implantation embryos, its role in embryonic development and differentiation may be conserved across these species. The patterns of mRNA expression for Oct-4 and Nanog have not been reported for ruminant embryos. In this study, total RNA was extracted from 10 *in vivo*-derived goat embryos at each stage (8-cell, morula, and blastocyst) using an Absolutely RNA Nanoprep Kit (Stratagene, La Jolla, CA, USA). The first-strand cDNAs were synthesized using Superscript III (Invitrogen, Carlsbad, CA, USA) and cDNAs were amplified with PfuUltra hotstart PCR master mix (Stratagene). Oct-4 primers were designed based on bovine Oct-4 open-reading sequence, while Nanog primers were designed based on the human Nanog open-reading sequence. Expression screening by PCR was performed. Oct-4 mRNA expression was detected at the 8-cell, morula and blastocyst stages. Sequencing of the 1.1-kb PCR product with Oct-4 primers revealed 87% homology to human cDNA sequence and 96% homology to the bovine sequence. Protein localization of Oct-4 as observed by immunocytochemistry was diffuse at the morula stage, but moved to a more nuclear location at the blastocyst stage. Oct-4 protein and mRNA expression were detected in both the ICM and trophectoderm of expanded blastocysts. This pattern of protein expression is similar to that reported by others in the pig and cow. As caprine, bovine, and porcine embryos all show extensive proliferation and elongation of the trophectoderm, continued expression of Oct-4 protein in the trophectoderm may be necessary to prevent premature differentiation of the trophectoderm. Nanog mRNA was detected at the morula and blastocyst stages. Nanog mRNA was detected in the ICM but not the trophectoderm of expanded goat blastocysts, a pattern that follows the expression observed in mice. Sequencing of the 698 bp PCR product obtained by RT-PCR from goat blastocysts confirmed that the mRNA detected was Nanog. Sequence alignment (ClustalW) showed that the cDNA sequence identities were 96% between goat and human and 70% between goat and mouse. The amino acid identities were 93% between goat and human and 52% between goat and mouse. To our knowledge this is the first report of detection of Nanog in domestic animals. These results are supportive of the premise that core components involved in the control of pluripotency are analogous across vertebrate species.

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 vitro* 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 AI, 4 IVF) allowed unambiguous discrimination of 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 $6.4 \pm 0.8\%$ in skeletal muscle up to $27.4 \pm 0.9\%$ 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.

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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 & Zimmerman 1994 PNAS 91, 11 298–11 302). Recently the first livestock animal, a goat, was produced (Honaramooz *et al*. 2003 Mol. Reprod. Dev. 64 422–64 428.), and early results in cattle are promising (Izadyar *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. The other two recipients received either CFDA or PKH26 into both testes and were castrated at 8 weeks after transfer. Following castration, PKH positive donor cells were found in freshly isolated tubules of each of the five recipients that received PKH-dyed cells, while no CFDA-positive donor cells were conclusively identified in any of the recipients. In the freshly isolated tubules, clumps of PKH-positive donor cells were observed, which indicated either cell division or substantial local colonization of certain areas of the tubules. Frozen sections were used to further localize the 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 spermatogonia. 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

first two isoforms are reported to be expressed in mouse follicles, oocytes, and pre-implantation embryos (Nishikimi A *et al*. 2001 Reproduction 122, 957–963). However, the role of any of these isoforms have not yet been investigated in bovine embryos. Here we aimed to examine the role of NOS in *in vitro* development of bovine embryos by treating embryos with NOS inhibitor, N-omega-L-nitro-arginine methyl esther (L-NAME), and examining the localization of the protein in pre-implantation embryos. Oocytes and embryos were grown in the media with NOS inhibitor added at a level of 0 mM (control), 1 mM, and 10 mM to either maturation or culture medium. Each experiment was conducted in four replicates each containing 100 oocytes for IVP. Cleavage and blastocyst rate were recorded at Days 2 and 7, respectively. Data were analyzed using the General Linear Model in SAS version 8.02 (SAS Institute, Inc., Cary, NC, USA) with the main factors being the level of L-NAME and the point of application. Pairwise comparisons were done using the Tukey test. Protein localization in bovine oocytes and embryos was performed by immunocytochemistry using eNOS- and iNOS-specific antibodies. Embryos were fixed in 3.7% paraformaldehyde, permeabilized in 0.1% Triton-X100, and washed three times in PBS supplemented with BSA. They were incubated with eNOS and iNOS primary antibody (1:200 dilutions) and washed before incubation with secondary antibody conjugated to FITC. After washing they were mounted on glass slides and examined under a confocal laser scanning microscope (Carl Zeiss Jena, Carl Zeiss AG, Oberkochen, Germany). In the controls the primary antibodies were omitted. As shown in the table below, the presence of L-NAME in the maturation medium significantly reduced the cleavage and blastocyst rate independent of the dosage applied. However the presence of L-NAME in the culture medium had an influence only on the blastocyst rate. The immunocytochemical staining results showed that both eNOS and iNOS are expressed in the cytoplasm of the MII oocytes, and during the pre-implantation stage the fluorescence signal was observed in nuclei and cytoplasm. However, the nuclear signal was much weaker. In conclusion, the present study is the first to determine the role of NO and to detect NOS protein in bovine oocytes and pre-implantation embryos. These results indicate that nitric oxide may play an important role as diffusible regulator of bovine oocyte maturation and preimplantation embryo development.

Table 1. Effect of L-name addition in maturation or culture medium on embryo development

Concentration	Maturation medium		Culture medium	
	Cleavage rate (%)	Blastocyst rate (%)	Cleavage rate (%)	Blastocyst rate (%)
0 mM	83.7 ± 0.9^{a}	12.5 ± 2.0^{A}	67.2 ± 4.1^{1}	$11.3 \pm 4.5^{\alpha}$
1 mM	68.9 ± 6.7^{b}	4.9 ± 2.5^{B}	65.0 ± 5.0^{1}	$0.8 \pm 1.6^{\beta}$
10 mM	69.4 ± 2.9^{b}	6.2 ± 1.5^{B}	60.3 ± 2.6^{1}	$0.0 \pm 0.0^{\beta}$

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% CO_2 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 fluorescent 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% CO_2 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 parthenogenic rat 2-cell embryos in which there was no sperm tail, most embryos (86%, 54/63) had a single fluorescent blastomere 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 (Fouladi-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 elegens*, 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 and with porcine brain cells as negative 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 electroporated 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 14 h. 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 ovaries 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 50 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.

Stage of development at 7 dpi	% (ratio) of embryos with fragmentation	Average fragmentation (%)	% (ratio) of embryos with apoptosis	Average apoptotic cell ratio (%)
Arrested 1- to 4-cell	50[a] (4/8)	10.0[a]	25[a] (2/8)	8.3[ab]
Arrested 5-cell to morula	77[a] (41/53)	10.8[a]	72[b] (38/53)	16.1[b]
Blastocyst	14[b] (10/71)	2.5[b]	14[a] (10/71)	3.4[a]

[ab] Values within a row with a different superscript differ significantly ($P < 0.05$).

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

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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%CO_2 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 1×10^3 U mL^{-1} 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 inductivity of MHC molecules and the expression of hepatic genes, from hAECs.

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

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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 nucleus and sperm embryo (about 25% Mgen, about 75% Pgen). Each experiment was repeated five times to obtain more than 120 embryos per group. Our results show that: (1) in the ICSI embryo, histone H3 methylation occurs in Mgen but not in Pgen at the first and second mitotic divisions; (2) in the parthenogenetic embryo, histone H3 methylation occurs in both nuclei at the first and second mitotic divisions; (3) in the somatic nuclear transfer embryo, histone H3 is methylated in all of the nuclei at the first and second mitotic divisions; (4) in the androgenetic embryo, methylated H3-K9 is detected weakly in the heterochromatin enclosed around the nucleolus of the pronuclei of the one-cell embryo, and methylated in the entire nuclei of the two-cell embryo; and (5) in the 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

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.

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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/6J × 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% CO_2 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 compared to B6P1F1 control embryos. These results indicate that the source of the cytoplasm strongly influences the development of reconstructed embryos containing SAMP1/Yit karyoplasts.

Table 1.

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

* 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 materno-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

buffalo, focusing in the materno-fetal transfer of iron. Small pieces of the placentomes and interplacentomal region of water buffaloes (*Bubalus bubalis bubalis*, $n = 51$) in all of the gestation periods were fixed in 4% paraformoldehyde, and 2.5% glutaraldehyde in PBS, processed, and stained for light and transmission electron microscopy to characterize the hemophagous areas and endometrial glands morphologically, histochemically (Perls, acid phosphatase reaction), and immunohistochemically with rabbit anti-pig uteroferrin antibody to confirm the iron transfer. The hemophagous areas were present in the placentome from 4–10-months-pregnant placentae. The Perls reaction for ferric iron staining was negative in the placentome and positive in the endometrial glands, and the acid phosphatase reaction to detect phagocytic activity was positive in the placentome as well as in the interplacentomal region. The uteroferrin immunohistochemical reaction was positive in the trophoblast, mainly in determined regions of the materno-fetal interface and in other points deep in the placentome, and the endometrial glands showed a strong reaction in the epithelium and in the lumina. The ultrastructure of the hemophagous areas revealed ingested erythrocytes inside the epithelial cells of trophoblasts, endocytic vesicles, and caveolae. The endometrial gland epithelium is of the columnar type with microvilli and basal nuclei. The results obtained mainly by histochemistry and immunohistochemistry indicated that the hemophagous areas and endometrial glands are very important sites for iron transfer in water buffalo, and are thus involved in the regulation of fetal hematopoiesis.

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119 THE LOCALIZATION OF A METHYL BINDING DOMAIN PROTEIN (MBD4) IN MURINE AND BOVINE OOCYTES AND PRE-IMPLANTATION EMBRYOS

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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 glycosylase 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 the 2-cell stage, followed by exclusion of the protein from the nucleus until the blastocyst stage of development. At this stage, staining was primarily nuclear and quite intense. In the mouse, staining was cytoplasmic at the 2 pronuclear 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.

120 EMBRYONIC AND POSTNATAL DEVELOPMENT OF DIPLOID-TRIPLOID MOUSE CHIMAERAS

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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 (C57Bl/6 × 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 $\mu g\,mL^{-1}$, 5 h). Diploid-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

(age: 8th–19th day) out of which 22 proved to be chimaeric and 3 were adults. Two of these animals were albino but had the triploid component in several internal tissues; both were fertile. The third animal, a male, was an overt chimaera. It turned out to be infertile (no sperm in the ejaculate; testes small and deprived of germ cells). The infertility of this individual is puzzling because the FISH studies with the help of X and Y chromosome painting probes proved that the diploid component was XY and the triploid component was XXX. The results of our study indicate that the rate of postimplantation development of 2n-3n chimaeric embryos is normal or only slightly retarded. Developmental stage of chimaeric embryos was assessed by comparison of their external morphology with normal diploid embryos of equivalent post-coital age according to the descriptions given by Theiler K (1972 *The House Mouse*, Springer-Verlag, Berlin). With the exception of one embryo lacking both eyes (but otherwise looking quite normal) no other morphological abnormalities were observed. Comparison of the contribution of both components to the fetal and extra-embryonic tissues at the consecutive foetal stages has shown that participation of triploid cells slightly but steadily decreased in all tissues examined. However, the presence of triploid cells in mouse chimaeras was compatible with their normal postnatal development to adulthood.

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

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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-dimethyaminopurine treatment (6-DMAP, 2.5 μM, 4 h, Sigma). The cleavage rates in HS2h ($43 \pm 29\%$) and HS4h ($35 \pm 28\%$) decreased ($P < 0.05$) compared to those in C0h ($62 \pm 12\%$) and C4h ($66 \pm 8\%$). In addition, the blastocyst formation rates and total cell numbers reduced ($P < 0.05$) after 2 h ($11 \pm 10\%$, 20 ± 16) and 4 h ($11 \pm 8\%$, 19 ± 8) of HS treatments compared to those in C0h ($23 \pm 14\%$, 32 ± 22) and C4h ($21 \pm 11\%$, 27 ± 17), all 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.

Early Pregnancy/Pregnancy Recognition

122 USE OF A DAY-14 EMBRYONIC ARRAY TO STUDY THE ELONGATION PHASE OF THE BOVINE EMBRYO

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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, Rusin, 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 ^{33}P-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, Osyme, 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 phosphoscreens and scanned after 7 days. Quantifications were done using ImaGene 5.1 (BioDiscovery, El Segundo, CA 90245, USA) and statistically analyzed