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ABSTRACTS FOR POSTER PRESENTATION

Student Competition Finalists

1 DEVELOPMENT OF BOVINE NUCLEAR TRANSFER EMBRYOS CLONED FROM FOLLICULAR DONOR CELLS IN SEQUENTIAL STAGES OF DIFFERENTIATION

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Efficient production of cloned embryos and live offspring is dependent on the ability of the nuclear-donor cell to be reprogrammed to direct normal conceptus development. Results of comparative studies indicate that embryonic and fetal cells are more successful candidates for nuclear transfer (NT) than terminally differentiated cells. Comparison of donor-cell efficiency is difficult to interpret across laboratories and from donor animals of varying genetic backgrounds and tissues of origin. This study was undertaken to determine the effect of the stage of differentiation of adult somatic donor cells derived from one-cell lineage of an individual donor animal. The follicular cell lineage including preantral follicle (PAFC), cumulus (CC), granulosa (GC), and luteal cells (LC) was chosen as several cell types in the lineage have previously proven successful for NT. Donor cell cultures were established from a 3-year-old Holstein cow. Embryos were reconstructed using confluent, early passage cultures. For each replicate, embryos were produced from two donor cell types in randomized block design (14 trials). Viable embryos were transferred to recipient females after 7 days of in vitro culture. Pregnancy and fetal viability were monitored weekly by ultrasonography from Days 30–100 of gestation and by rectal palpation thereafter. Embryos reconstructed from PAFC were less likely to develop to the blastocyst stage on Day 7 than embryos derived from CC or LC (Table 1, $P < 0.05$). Pregnancy rates at Day 30 were similar across donor cell types. A greater proportion of PAFC embryos were viable at Day 60 of gestation than embryos derived from CC and GC ($P < 0.05$). To date, normal cloned calves have been delivered at term from CC and GC, and two pregnancies ($n = 3$ fetuses) are ongoing from PAFC. The fetus cloned from LC, the terminally differentiated cell type in the lineage, was spontaneously aborted at day 211 with congenital abnormalities. Results from comparative studies of development of mouse embryos cloned from embryonic stem (ES) cells v. somatic donor cells indicate that ES-derived clones are less efficient in blastocyst formation, but survival to term is greater (Humpherys D *et al.*, 2002 PNAS 99, 12 889–12 894). Likewise, our results in cattle suggest that PAFC, the least differentiated cells in the lineage, result in fewer cloned blastocysts, but blastocysts that do develop are more likely to progress through implantation and into later stages of pregnancy.

Table 1. Development of NT embryos reconstructed from follicular donor cells at sequential stages of differentiation

	Preantral follicle	Cumulus	Granulosa	Luteal
Day 7/total NT (%)	19/175[a] (11)	31/104[b] (30)	24/103[a,b] (23)	29/118[b] (25)
Day 30/Day 7	4/19	10/31	11/24	9/29
Day 60/Day 30	3/4[a]	1/10[b]	1/11[b]	1/9[a,b]
Term calves	3 in progress	1	1	0

Contrasts made with 95% confidence intervals on survival proportions.
[a,b]Within row, values with different superscripts differ significantly ($P < 0.05$).

2 POSTHATCHING DEVELOPMENT SYSTEM: A NOVEL IN VITRO CULTURE OF BOVINE EMBRYOS

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Although high blastocyst rates can be achieved in somatic cell nuclear transfer, abortions and developmental abnormalities still hamper advancement. Reliable and practical methods to evaluate early embryonic development and differentiation are required to understand and overcome the problem. Our aim was to establish an in vitro culture system for monitoring posthatching development (PHD). Slaughterhouse-derived bovine oocytes were matured in vitro, fertilized (Day 0) and cultured (Holm *et al.*, 1999, Theriogenology, 52, 683–700). On Day 8, degenerated embryos were removed from each well and 400 L of modified culture medium (SOFaaci plus 0.5% glucose and 10% fetal bovine serum) were added. At Day 11, hatched blastocysts were selected by scoring them as Quality 1 (Q1: > 1.0 mm , clear trophoblast, compact inner cell mass), Quality 2 (Q2: 0.5 mm , dark spots in the trophoblast, less compact inner cell mass), or Quality 3 (Q3: <0.5 mm , many dark spots in the trophoblast, spread inner cell mass). The resulting 304 blastocysts in 12 replicates were then loaded into 15 mm × 1.2 gel tunnels of 2.4% agarose in PBS, supplemented with either 5% (Agar5) or 10% (Agar10) fetal bovine serum, covered with the modified culture medium, and then incubated at 38.5°C in 5% CO_2, 5% O_2, 90% N_2. Embryo morphology and length were evaluated using a stereomicroscope on Days 12, 13, 14 and 15. On Day 14, 75 embryos were removed, biopsed (1 mm) for sex determination of each embryo, and processed for light and transmission electron microscopy. Qualitative and quantitative data were analyzed by χ^2 test and GLM procedure of SAS, respectively, with P level of 0.05. A total of 170 embryos (56% of total) initiated

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elongation. This percentage was higher (LSmeansSD, $n = 12$; $P < 0.05$) in Agar10 v. Agar5 in both Q1 (889 v. 637), Q2 (667 v. 485) and Q3 embryos (529 v. 278). Mean embryo length (mm; LSmeansSEM) on Day 13 was higher ($P < 0.05$) in Q1 (2.10.2, $n = 49$) and Q2 (1.71.4, $n = 98$) than Q3 (1.20.3, $n = 23$). On Day 14, Q1 embryos (3.50.2) were longer ($P < 0.01$) than Q2 and Q3 embryos (2.70.1 and 2.00.3). On Day 15, Q1, Q2 and Q3 embryos (4.40.5, $n = 24$, 4.00.3, $n = 45$ and 2.90.6, $n = 14$, respectively) had similar length, probably influenced by the low number of Q3 embryos. The percentage of males was higher ($P < 0.001$) in Q1 (95%; $n = 40$), but similar in Q2 (39%; $n = 26$) and Q3 (71%; $n = 7$). Light microscopy confirmed hypoblast and epiblast formation. Ultrastructural analysis revealed that the latter had penetrated the trophoblast (Rauber's layer), forming an embryonic disc including many degenerative cells. In conclusion, this culture system represents the first model for rapid growth, elongation, and initial differentiation of bovine posthatching embryos.

3 P66^{SHC}, BUT NOT P53, IS INVOLVED IN EARLY ARREST OF IN VITRO PRODUCED BOVINE EMBRYOS

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High embryo losses occur in the first week of bovine embryo development, before the activation of the embryonic genome, with a high percentage of embryo death and arrest. Arrested embryos appear morphologically normal and do not exhibit any characteristic sign of apoptosis, including DNA fragmentation. We hypothesized that these embryos enter a senescence-like state and that both the cell cycle regulatory protein p53 and the stress-related protein p66^{shc}, which are involved in the onset of senescence in somatic cells, are responsible for this early embryonic arrest of development. The aim of this study was to characterize the expression of p53 and p66^{shc} in 2–4 cell-arrested bovine embryos. Our experimental model consists of in vitro-produced bovine embryos, co-cultured with oviductal cells. In our in vitro production system 86.8 ± 41.4% of embryos cleave, 13.5 ± 0.5% arrest at the 2–4 cell stage and 24.5 ± 0.7% develop to the blastocyst stage. Cleavage occurs between 26 hours post-insemination (hpi) and 48 hpi. Embryos that cleave by 28 hpi show only 0.6 ± 0.3% of 2–4 cell arrest and 41.2 ± 2.1% of blastocyst development, whereas 14.2 ± 0.9% of later-cleaving embryos arrest at the 2–4 cell stage and only 26.5 ± 1.7% develop into blastocysts. We compared 2–4 cell embryos collected at 28 hpi with those arrested at the 2–4 cell stage and collected at Day 8 post-insemination. Quantification by Real Time PCR showed significantly higher p66^{shc} mRNA levels ($P < 0.001$), but no changes in p53 mRNA levels ($P = 0.860$) in arrested embryos v. 28-hpi embryos ($n = 3$ pools of 100 embryos each). We obtained the same pattern of p53 and p66^{shc} mRNA expression when we compared 28-hpi embryos with later-cleaving embryos (28 hpi to 48 hpi, $n = 3$ pools of 100 embryos each), and higher p66^{shc} mRNA levels ($P < 0.050$), or similar p53 mRNA levels ($P = 0.960$). We also confirmed higher p66^{shc} protein levels ($P < 0.001$), but no changes in p53 protein levels ($P = 1.000$), in later cleaving embryos compared with 28 hpi-cleaving embryos by semi-quantitative immunocytochemistry ($n = 70$). Statistical analysis was carried out using 2-sample *t*-test or the equivalent nonparametric test (Mann-Whitney test). Taken together, these results demonstrate that the developmental potential of in vitro-produced embryos is related to the time of first cleavage and that p66^{shc}, but not p53, plays a role in early developmental arrest of in vitro-produced bovine embryos. Further experiments are required to investigate the functional role of p66^{shc} in early embryo arrest. Funded by NSERC, CIHR, OGS and OMAFRA.

4 CHROMOSOMAL STABILITY OF AFRICAN WILD CAT (*FELIS SILVESTRIS LIBICA*) SOMATIC CELLS AND CLONED EMBRYOS

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Nuclear transfer (NT) procedures are generally inefficient and chromosomal abnormalities have been suggested as one causative factor. Embryos derived by somatic cell NT show a high incidence of aneuploidy, which seems to be reflective of the donor cell line with high chromosomal abnormalities (Bureau *et al*., 2003, Theriogenology 59, 239). Also, aneuploidies in some cell lines increase progressively with the number of passages (Denning *et al*., 2001, Cloning 3, 221–231). Therefore, the aim of the present study was to analyze the chromosomal stability of donor cells at different passages as well as that of reconstructed embryos derived from these cells. A primary culture of African wild cat (AWC) fibroblast cells was established from a skin sample and cultured until cells stopped dividing at passage 9. Chromosome numbers were determined in cells using a karyotyping technique (Iwasaki *et al*., 1992 J Exp Zool 261, 79–85) at passages 1 and 3 through 9. At passages 1, 3, 4 and 9, NT of AWC fibroblast cells into domestic cat cytoplasts was done (Gomez *et al*., 2003, Biol Reprod 69, 1032–1041). Reconstructed blastocysts were treated with colcemid ($0.28\ \mu g\ mL^{-1}$) and fixed for karyotyping to evaluate chromosome numbers in the blastomeres. Blastomere metaphases were often highly contracted or overlapping, so that only 1 to 7 sets of metaphase chromosomes per embryo were spread sufficiently to allow determination of ploidy. No attempt to produce NT embryos using cells at passages 5 through 8 was done because the percentages of aneuploidies of these passages were not significantly different ($P > 0.05$). Data were analyzed by chi-square test ($P < 0.05$). As shown in the Table, the percentage of aneuploidy in the somatic cells increased progressively with duration of culture. Furthermore, the percentage of chromosomal abnormalities in reconstructed embryos was similar to that of the cells from which they were derived. Accordingly, for future NT, we propose to use donor cells at early passages when the percentage of cells with chromosomal abnormalities is still relatively low. Research was funded partially by the John & Shirley Davies Foundation.

Chromosomal abnormalities in AWC cells and cloned embryos

Cell passage no.	Cell no.	Aneuploid cells no. (%)	Blastocysts no.	Blastomeres no.	Aneuploid blastomeres no. (%)
1	50	18 (36.0)[a]	2	7	0 (0.0)[a]
3	37	13 (35.2)[a]	6	20	9 (45.0)[a]
4	52	22 (42.3)[ac]	7	16	5 (31.0)[a]
5	43	27 (62.8)[b]	–	–	–
6	51	29 (56.8)[bc]	–	–	–
7	45	35 (77.7)[d]	–	–	–
8	45	40 (88.8)[d]	–	–	–
9	45	44 (97.7)[de]	5	16	15 (93.0)[b]
			6 (IVF)	22	2 (0.9)[a]

[a,b,c,d,e]Different superscripts within the same column indicate significant differences (chi-square, $P < 0.05$).

5 MPF AND MAP KINASES IN OVINE OOCYTES: EFFECTS OF ENUCLEATION AND CAFFEINE ON ACTIVITY AND DEVELOPMENT OF NUCLEAR TRANSFER EMBRYOS

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In nuclear transfer (NT) embryos, exposure of the donor chromatin to the MII cytoplasm results in premature chromatin condensation (PCC) which may be beneficial for nuclear reprogramming (Campbell KHS and Alberio R 2003 Reprod. Suppl. 61, 477–494). Following enucleation, maturation promoting factor (MPF) activity in murine oocytes is primarily associated with the meiotic spindle. This reduced MPF activity in the cytoplast may result in decreased PCC and reprogramming. Conversely, increasing cytoplast MPF activity may increase reprogramming. The aims of this study were to perform quantitative analysis of MPF and MAPK activities in ovine oocytes: 1. at anaphase/telophase I (A/TI) or MII; 2. following enucleation; 3. following treatment with caffeine (an inhibitor of Myt1/Wee1 activity). The development of ovine NT embryos reconstructed using caffeine-treated oocytes as cytoplast recipients was then determined. Oocytes were matured in TCM 199, 10% FBS, 5 $\mu g\,mL^{-1}$ FSH, 5 $\mu g\,mL^{-1}$ LH, 1 $\mu g\,mL^{-1}$ estradiol, 0.3 mM sodium pyruvate and 100 μM cysteamine. 15 h post-onset of maturation (hpm) oocytes were stripped of cumulus cells and enucleated in HSOF containing 5 $\mu g\,mL^{-1}$ Hoechst 33342 and 7.5 $\mu g\,mL^{-1}$ cytochalasin B (CB). Control oocytes were sham-enucleated by removing an equal volume of cytoplasm. Oocytes were cultured in SOF $\pm$ 10 mM caffeine. Groups of 10 oocytes were sampled and analyzed for MPF and MAPK activities as previously described (Ye JP *et al.*, 2003 Reproduction 125, 645–656). For NT, primary foetal fibroblasts were quiesced in DMEM containing 0.1% FBS for 2–3 days. Cell fusion was induced with two DC pulses of 25V cM-1 for 80 μs. 3 methods of NT were compared: A. fusion 20 hpm, activation 21 hpm; B. fusion 24 hpm, activation 25 hpm; C. 10 mM caffeine 18–24 hpm, fusion 24 hpm, activation 25 hpm. All oocytes were activated in HSOF containing 5 $\mu g\,mL^{-1}$ calcium ionophore (A23187), cultured in SOF with 10 $\mu g\,mL^{-1}$ of cycloheximide and 7.5 $\mu g\,mL^{-1}$ CB for 5 h, and then transferred to mSOFaaBSA medium, all at 5% CO_2, 5% O_2 and 90% N_2 at 39°C. On Day 2 cleavage was assessed and 10% FBS added to the medium. Development to blastocyst was assessed on Day 7. All data were analyzed by chi-square test. Both MPF and MAP kinase activities were increased at MII compared to A/TI ($P < 0.05$). There were no differences in activities of both kinases between intact and enucleated oocytes. Following enucleation, both kinase activities were identical in all groups, reaching maximum activities 24 hpm followed by a slow decline. Caffeine increased the activity of both kinases (MPF in particular) in all groups. Following 5 replicates (total oocytes 145, 143, 144 for NT methods A,B,C, respectively), no significant differences were observed between fusion (82.1%, 67.8%, 67.4%), cleavage (90.8%, 88.7%, 89.7%) or development to blastocyst (20.2%, 18.6%, 25.8%). Analysis of total cell numbers on limited numbers of blastocysts (7, 6, 7) were NS (70.9 $\pm$ 38.5, 69.3 $\pm$ 25.4, 93.3 $\pm$ 17.8).

6 ACETYLATION OF HISTONE H4 LYSINE-5 AND LYSINE-8 DURING DEVELOPMENT OF IN VITRO-PRODUCED BOVINE EMBRYOS

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The oocyte is remarkable in its ability to remodel the parental genomes following fertilization and to reprogram somatic nuclei as in nuclear transfer. While significant research has been carried out on DNA methylation patterns in the early embryo, increased interest in histone acetylation is more recent. The objective of this study was to characterize the pattern of acetylation of histone H4 lysine-5 (H4L5) and lysine-8 (H4L8) in the early pre-implantation bovine embryo. Bovine embryos were produced as previously described (Fouladi Nashta AA *et al.*, 1998 Biol. Rep. 59, 255–262) and collected at different developmental stages, 1-cell (20 h), 2-cell (30 h), 4- and 8-cell (Day 2), 16-cell (Day 4), and blastocyst (Days 7–8) with an average of 6 embryos per group in two replicates. Embryos were fixed in 2.5% paraformaldehyde, 15 min at room temperature (RT), stained with polyclonal rabbit antibodies against H4L5 (1 : 800) and H4L8 (1 : 600) residues (Serotec, UK) at 4°C overnight. A polyclonal swine anti-rabbit (1 : 200; Dako, Denmark) was used as secondary antibody for 40 min at RT. Images were examined using a fluorescent microscope (Leica DMR, Germany). Image analysis and quantification were performed using Simple PCI software (Compix Imaging Systems, USA). Changes in intensities

within and between different embryo stages were recorded as a ratio of red stain to blue counterstain. Data were corrected for confounding area and absorbance and analysed using a multivariate linear regression model. The intensity of staining for H4L5 appeared higher in 8-cell embryos than 2- and 4-cell embryos but not to a significant level ($P \geq 0.05$); 8-cell embryos also appeared higher in stain intensity than 16-cell but of borderline significance ($P = 0.073$). Staining intensity decreased between the 8-cell and blastocyst stage ($P \leq 0.05$). In contrast, the intensity of acetylation staining for H4L8 residue decreased slightly between the 1- and 4-cell stages and then decreased significantly between the 4- and 8-cell stages ($P \leq 0.05$), increasing significantly by the 16-cell stage ($P \leq 0.05$). A significant decrease in staining intensity was observed at the blastocyst stage ($P \leq 0.05$). In blastocyst-stage embryos both lysine-5 and lysine-8 showed a differential staining of inner cell mass (ICM) and trophectoderm (TE) cells. ICM cells showed intense staining and TE cells stained very weakly. The intensity results presented are cumulative of ICM and TE intensities, which explains the overall low levels of acetylation in blastocysts when compared to the earlier stages. Acetylation of H4L5 starts high in 1-cell embryo, as it is necessary for protamine replacement (Adenot *et al.*, 1997 Development 124, 4615–4625), decreases when methylation is high and increases when methylation is low (as in the 8-cell stage which corresponds with zygotic gene activation). Acetylation of H4L8 decreases between the 1- and 8-cell stages; however, its association with changes in DNA methylation has yet to be determined.