transfer program in Brangus cows in a tropical climate. We used the same set of CIDRs in the same location in cows of the same breed, age, and body condition during three consecutive weeks. Cows were randomly allocated to one of three treatment groups. In Group 1, all cows ($n = 44$) were treated with a new 1.9-g CIDR (CIDR-B, InterAg, Hamilton, New Zealand), combined with 2 mg EB on Day 0. In Group 2, all cows ($n = 43$) were treated with a reused (first reuse) 1.9-g CIDR and 2 mg EB on Day 0. In Group 3, all cows ($n = 42$) received a reused (second reuse) 1.9-g CIDR and 2 mg EB on Day 0. CIDR devices were removed on Day 7 and all cows received PGF (0.25 μg cloprostenol) at that time. Estrus was expected to occur 24 h later. Seven days after estrus all cows showing heat were examined by rectal palpation, and those with a CL 15 mm in diameter or larger were selected to receive a frozen/thawed embryo (in 1.5 M ethylene glycol) by nonsurgical direct transfer. PR were determined by rectal palpation 60 days after estrus. In Group 1, a total of 90.9% of the cows displayed signs of estrus (40/44), and 42% of those that received a frozen embryo were pregnant (16/38). In Group 2, a total of 88.4% of the treated cows showed signs of estrus (38/43), and 37% of those recipients became pregnant (13/35). In Group 3, 88% of treated cows showed signs of estrus (37/42), and 36% of cows receiving an embryo were pregnant (13/36). ESR and PR were compared by χ^2 and Fisher's tests, and no differences were found among the three groups studied. This confirms that, in 1.9-g CIDRs used on two previous and consecutive occasions, there is still a sufficient amount of P4 remaining that allows successful estrus synchronization and pregnancy rates in a third use.

166 EFFECT OF TREATMENT WITH hCG OR GnRH AT THE TIME OF EMBRYO TRANSFER ON PREGNANCY RATES IN COWS SYNCHRONIZED WITH PROGESTERONE VAGINAL DEVICES, ESTRADIOL BENZOATE, AND eCG

R. Tribulo[A,B], *E. Balla*[A,B], *L. Cutaia*[A,C], *G.A. Bo*[A,B], *P.S. Baruselli*[D], *and L. Peres*[A]

[A]Instituto de Reproduccion Animal Cordoba, Cordoba, Argentina; [B]Universidad Catolica de Cordoba, Cordoba, Argentina; [C]Agencia Cordoba Ciencia, Cordoba, Argentina; [D]Departamento de Reproducao Animal, FMVZ-USP, Sao Paulo, Brazil.
Email: gabrielbo@iracbiogen.com.ar

Although several studies have investigated the relationship between circulating progesterone and pregnancy rates in cattle, the beneficial effect of treatments that increase progesterone concentrations, by insertion of a progesterone (P4) releasing device or induction of an accessory CL with hCG, GnRH, or LH treatment, has resulted in inconsistent effects on pregnancy rates in embryo recipients. An experiment was designed to evaluate the effect of hCG or GnRH treatment, given at the time of embryo transfer without estrus detection, on pregnancy rates in recipients treated with intrauterine P4-releasing devices, estradiol benzoate (EB), and eCG. The experiment was performed in two replicates; non-lactating *Bos taurus* × *Bos indicus* crossbred beef cows with a body condition score between 2.5 to 3.5 (1-to-5 scale) were used (replicate 1, $n = 180$; replicate 2, $n = 140$). All cows received 1 g of P4 via a P4-releasing device (DIB, Syntex, Argentina) and 2 mg EB i.m. (Syntex) on Day 0, and 400 IU of eCG i.m. (Novormon 5000, Syntex) plus 150 μg D(+)cloprostenol i.m. (Ciclase, Syntex) on Day 5. DIBs were removed on Day 8 and all cows received 1 mg EB i.m. on Day 9. Recipients were not observed for signs of estrus, and those >1 CL, or a single CL with an area >256 mm^2, received 195 Grade 1 and 46 Grade 2 frozen/thawed "direct transfer" embryos on Day 17. At the time of embryo transfer, recipients were randomly allocated to 1 of 3 treatment groups to receive 1500 IU hCG (Ovusyn, Syntex), 50 μg Lecirelina (GnRH, Gonasyn, Syntex), or no treatment (control) at that time. Ovarian ultrasonography was performed on Day 0 to determine ovarian status (only cows with a CL or a follicle >10 mm and uterine tone were used), on Day 17 to measure CL area, and 40 days after embryo transfer to determine pregnancy status. Data were analyzed by logistic regression and the effects of replication, technician, treatment, and embryo quality were considered in the model. From the 320 recipients treated with a DIB plus EB and eCG, 241 (75.3%) were selected to receive an embryo. Nine (3.7%) and 1 (0.4%) of the selected recipients had 2 and 3 CL, respectively. Pregnancy rates did not differ between replicates (replicate 1: 80/140, 57.1%; and replicate 2: 57/101, 56.4%; $P = 0.84$), technicians (technician 1: 65/118, 55.1%; and technician 2: 72/123, 58.5%; $P = 0.64$), or treatments (hCG: 43/80, 53.8%; GnRH: 45/83, 54.2%; and control: 49/78, 62.8% $P = 0.99$). However, pregnancy rates were higher ($P = 0.001$) in recipients receiving Grade 1 embryos (121/195, 62.1%) than in those receiving Grade 2 embryos (16/46, 34.8%). GnRH or hCG treatment at the time of embryo transfer did not increase pregnancy rates in recipients synchronized with P4 releasing devices, EB, and eCG.

Research was supported by Syntex S.A., Estancia El Mangrullo S.A., and Agencia Cordoba Ciencia S.E.

Embryonic Stem Cells

167 ISOLATION AND COMPARATIVE PROFILING OF HUMAN ADIPOSE-DERIVED ADULT STEM CELLS

A. Boquest[A], *A. Shahdadfar*[B], *K. Fronsdal*[B], *J. Brinchmann*[B], *and P. Collas*[A]

[A]Institute of Basic Medical Sciences, University of Oslo, Oslo, Norway; [B]Department of Immunology, Rikshospitalet, Oslo, Norway.
Email: andrewcb@medisin.uio.no

The stromal compartment of mesenchymal tissues is thought to harbor stem cells that display extensive proliferative capacity and multilineage potential. However, despite their potential impact in the field of regenerative medicine, little is known about the biology of stromal stem cells prior to culture. After removing adipocytes and erythrocytes from collagenase digested human adipose tissue, we identified two cell populations using flow cytometry which shared expression of stem cell markers SH2 and CD34, but lacked the phenotypic characteristics of leukocytes (CD45−). However, they were found to be discernible based on CD31 expression, a marker for endothelial cells. Using CD31 conjugated magnetic beads, we separated these cells (CD45-CD31− and CD45-CD31+) from three patients and compared global gene expression profiles using an Affymetrix platform. The

prominant feature of CD45-CD31+ cells was the up-regulation of genes associated with endothelial cells. By contrast, CD45-CD31− cells were found to overexpress transcripts involved in cell cycle quiescence and cell signaling elements including those of the WNT pathway thought to be important for maintaining the stem cell state. Upon culture in DMEM/F12 with 20% FCS, only CD45-CD31− cells were capable of adhering to plastic and forming colonies. These cells with fibroblastic morphology met the key criterion of stem cells, the ability to proliferate while retaining the capacity to differentiate into mature tissues. Under appropriate inductive conditions, they were found to exclusively form bone, cartilage, adipose and neuronal-like tissues *in vitro*. Clonal cell lines generated from individually cultured CD45-CD31− cells displayed multilineage and proliferative capacity, validating our conclusion that they are true stem cells and not simply committed progenitors. We then undertook extensive comparative profiling of CD45-CD31− cells with their cultured counterparts to examine changes that stromal stem cells undergo during culture. Except for the disappearance of CD34, flow cytometry analysis using 52 antibodies revealed little change in cell surface phenotype as a result of culture. However, comparative global gene profiling revealed extensive down-regulation of many genes during culture. These included cell cycle arresting genes, as expected, and genes encoding elements involved in cell signaling including those belonging to the tumor necrosis factor, interleukin, transforming growth factor and chemokine families. The consequences of these changes remain unknown, but ultimately may affect the potential use of adipose tissue stem cells in regenerative medicine.

168 ESTABLISHMENT AND MOLECULAR CHARACTERIZATION OF PIG PARTHENOGENETIC EMBRYONIC STEM CELLS

T.A.L. Brevini, F. Cillo, and F. Gandolfi

Department of Anatomy of Domestic Animals, University of Milan, Milan, Italy. Email: fulvio.gandolfi@unimi.it

Parthenogenetic embryonic stem cells have been obtained in mouse and in primates. However, it would be desirable to have an alternative experimental model that could be used to investigate the therapeutic potential of these cells. For this purpose, we generated parthenogenetic pig blastocysts from *in vitro*-matured oocytes activated by sequential exposure to 10 μM ionomycin for 5 min and 2 mM 6-DMAP for 3 h. Inner cell masses were isolated by immunosurgery and plated on mitotically inactivated STO fibroblast feeder layers in 4-well dishes. Cells were incubated in 5% CO_2 at 37°C in low glucose DMEM/F10 medium supplemented with 1000 IU/mL of mouse recombinant LIF, 10% Knockout serum replacer (Gibco, Italy), and 5% FBS. Within 3 days, circular colonies with distinct margins of small round cells were observed on both substrates. When a colony enlarged enough to cover half or more of the well surface, cells were trypsinized in clumps never reaching single-cell suspension and passaged to a newly prepared well. The expression of a gene panel was examined by RT-PCR on a portion of the cells at each passage. Oct-4 and nanog were used as markers of pluripotency. Interferon-τ, α-Amilase, Bone Morphogenetic Protein-4, and Neurofilament were used as markers of trophectoderm, endoderm, mesoderm, and ectoderm differentiation respectively. After 4 passages, three colonies expressed Oct-4 and nanog and were negative for all four differentiation markers. Two colonies at the 5th and 7th passages maintained nanog but not Oct-4 expression, while remaining negative to all of the other genes. To induce the formation of embryoid bodies (EBs), cells were cultured in 50-μL droplets of medium without LIF. Initiation of differentiation of EBs was confirmed through both morphological examination and molecular analysis; mesodermal, ectodermal, and endodermal markers were all expressed by Day 9 of culture and Oct-4 and nanog expression was completely down-regulated. Interestingly, when EBs were returned to adherent culture conditions patches of differentiated cells tended to form, spontaneously differentiating into mesodermal, endodermal, or neuroectodermal cell monolayers. The present data suggest that it is possible to establish putative embryonic stem cells from pig parthenotes. Further studies are in progress to determine their ability to stably maintain the undifferentiated state.

This work was supported by MIUR COFIN 20022074357 and Fondazione CARIPLO.

169 CULTURE OF MURINE EMBRYONIC STEM CELLS ON NWPF DISCS

G. Cetinkaya[A], *S. Arat*[B], *H. Odaman Mercan*[B], *M.A. Onur*[A], *and A. Tumer*[A]

[A]Biology Department, Faculty of Science, Hacettepe University, Ankara,Turkey; [B]Research Institute for Genetic Engineering and Biotechnology, TUBITAK, Kocaeli, Turkey. Email: sezen@rigeb.gov.tr

Murine embryonic stem cells derived from the inner cell mass of mouse blastocysts can be maintained in culture for extended periods by using feeder layers and leukemia inhibitory factor (LIF). Maintenance of undifferentiated status occurs via LIF-mediated signalling pathways. In this study we cultured embryonic stem (ES) cells in Knockout-DMEM with serum replacement on a three-dimensional matrix, non-woven polyester fabric (NWPF), which is formed from non-arrayed polyethylene teraphthalate fibers. The surface of the fibers was modified by immobilizing LIF. While stimulating the matrix-bound form of LIF *in vitro*, we also tried to induce LIF-mediated signalling pathways continually. Our goal was to constitute a synthetic microenvironment that would support the undifferentiated growth of murine ES cells. Experimental groups were examined according to colony morphology, alkaline phosphatase activity, SSEA-1 antibody immunoreactivity, and SEM analyses. It was shown that three dimensional macroporous fibrous matrix, NWPF could support growth of undifferentiated ES cells. However, the ratio of undifferentiated colonies was higher on feeder layers than an polymeric surfaces (93% on mouse embryonic fibroblasts; 63,7% on hydrolized polymeric surface, $P < 0,05$). Results showed that LIF-immobilized surfaces supported undifferentiated growth of ES cells better than hydrolyzed surfaces. Colonies cultured on LIF-immobilized surfaces, had higher alkaline phosphatase activity and undifferentiated phenotype ratio than those on hydrolyzed surfaces. When the soluble or the matrix-bound form of LIF was used, the number of undifferentiated colonies increased in the polymeric groups (77.8% soluble LIF; 81.6% matrix bound LIF $P < 0,05$). On NWPF discs, ES cells formed big cell aggregates which had high alkaline phosphatase activity but low SSEA-1 immunoreactivity . When they were passaged to feeder layers, SSEA-1 activity increased. We managed to obtain undifferentiated colonies on NWPF discs by using LIF but the skeletal structure of polymeric matrix would be more convenient for differentiation studies.

This study was performed in TUBITAK-RIGEB and supported by a part of grant from Hacettepe University (0102601001).

170 ASSESSING THE POTENTIAL OF STEM CELLS TO GENERATE CHIMERIC RATS

J. Guo, *S. Fida*, *K. Gou*, *C. Zhang*, *J. Morrison*, *and Z. Du*

Copyrat Pty Ltd., Clayton, Victoria 3168, Australia. Email: jitong.guo@med.monash.edu.au

Embryonic stem (ES) cells are pluripotent cells derived from inner cell masses (ICMs) of blastocysts. The capacity of pluripotency in differentiation is assumed to contribute to embryonic development to form a chimeric individual when these cells are reintroduced into embryos. Chimeric mice can be routinely generated by aggregation of ES cells with morulae or injection into blastocysts, which are then implanted in pseudopregnant foster mothers. Furthermore, recent studies have demonstrated that bone marrow-derived stem cells and neural stem cells can integrate into the embryonic development in mouse (Geiger *et al*. 1998 Cell 93, 1055–1065; Clarke *et al*. 2000 Science 288, 1660–1663). We therefore tried to assess the ability of rat ICMs and neural stem cells to form chimeras by injecting these cells into rat blastocysts. Forty-two rat ICMs from Day 6 blastocysts of Dark Agouti (DA) inbred rat were injected into Day 5 blastocysts of Sprague-Dawleyd (SD) outbred rats; 14 pups were born following embryo transfer of these blastocysts injected into Hooded Wistar (HW) recipients. One male of the 14 pups was coat color-patched and displayed germline transmission. Following embryo transfer of 22 SD blastocysts injected by Day 5 DA ICMs, 7 pups were born and 2 of them were coat color-patched. Nine pups were obtained from 23 DA blastocysts injected by Day 5 SD ICMs; 4 of them were coat color-patched. The ICM cells were isolated and cultured for 6 days. No chimeras were generated by injection of the cultured ICM cells, as assessed by coat color patching. These results suggest that rat embryonic ICMs have potential to develop into chimeras, but the chimeric potential of ICMs was rapidly lost in our culture system. Investigation of potential chimeric development of rat fetal neural stem (rFNS) cells transfected with Lac Z was carried out. Staining was observed in tissues from 2 of 41 E14 fetuses. These results demonstrated that rFNS cells can integrate into the early embryonic environment although the ability of these cells to contribute to chimeric formation was marginal. No coat color chimerism was observed in any of the 88 pups generated from the LacZ-rFNS cell experiments.

171 NORMAL REPROGRAMMING OF IMPRINTING IN PARTHENOGENETIC FEMALE GERM CELLS

T. Horii[A], *Y. Nagao*[B], *M. Kimura*[A,C], *and I. Hatada*[A,C]

[A]Gene Research Center, Gunma University, Gumma 371-8511, Japan; [B]Laboratory of Reproductive Physiology, Kyoto University, Kyoto 606-8501, Japan; [C]PRESTO, Japan Science and Technology Corporation (JST), Kawaguchi City, Saitama Prefecture 332-0012, Japan. Email: horii@showa.gunma-u.ac.jp

Mammalian parthenotes cannot develop normally to term. Mouse parthenogenetic embryos die by Day 10 of gestation. On the other hand, viable parthenogenetic chimeras were produced by normal host embryos, although parthenogenetic cells were observed in a limited number of tissues and organs and, even in these instances, their contribution was substantially reduced. This can be explained by the aberrant expressions of imprinted genes in parthenogenetic cells. In female mice, erasure of imprints occurs around the time that primordial germ cells enter the gonad, and establishment of imprints occurs in the postnatal growth phase of oogenesis. In this study, we investigated whether aberrant imprints in parthenogenetic embryonic stem (PgES) cells can be erased through the germline. Diploid parthenogenetic embryos were produced by activation of (CBA × C57BL/6-EGFP) F1 mouse superovulated unfertilized oocytes by exposure to Sr2+ and cytochalasin B. Ten parthenogenetic blastocysts were plated and three PgES cell lines were isolated. Chimeras were made by injecting 10–15 PgES cells into ICR(CD-1) mouse blastocysts. Chimeras and chimeric tissues were detected by fluorescent microscopy. In all, 173 chimeric blastocysts were transferred to 9 recipient females, and 101 live pups containing 9 female and 21 male chimeras were born. No significant growth retardation was apparent in PgES chimeras, irrespective of their degree of chimerism. In 5 male chimeras killed at 1 day postpartum (dpp), PgES cells showed a restricted tissue contribution. The contribution to lung, liver, and intestine was considerably lower than in the other tissues such as brain, heart, spleen, and kidney. PgES derived or host embryo derived non-growing oocytes were isolated from dissociated ovaries of female chimeras at 1 dpp under fluorescent microscopy. Methylation imprints in non-growing oocytes were analyzed for maternally methylated imprinted genes *Peg1*, *Snrpn*, and *Igf2r* by the combined bisulfite restriction analysis (COBRA). In normal oocytes, imprints are expected to be erased and these genes are unmethylated at this stage. We observed that these genes were unmethylated in both PgES derived and host embryo derived non-growing oocytes. These results suggest that aberrant imprints in PgES cells can also be erased normally through the germline.

172 PRIMORDIAL GERM CELL DIFFERENTIATION FROM ES CELLS *IN VITRO* IN MOUSE

J. Kobolak[A], *E. Deak*[B], *and A. Dinnyes*[A,B]

[A]Micromanipulation and Genetic Reprogramming Group, Agricultural Biotechnology Center; [B]Research Group for Applied Animal Genetics and Biotechnology, Hungary Academy of Sciences and Szent Istvan University, Godollo, Hungary. Email: kobolak@abc.hu

The primordial germ cells (PGC) in the genital ridge of the embryo are the progenitors of sperm and eggs. The goal of the present study was to derive PGC cells from embryonic stem (ES) cells and compare their gene expression with that of primary PGC cultures. R1 (Nagy A *et al*. 1990 Development 110, 815–821) and Oct4-GiP (Ying QL *et al*. 2002 Nature 416, 545–548) ES cell lines were differentiated into PGCs. For *in vitro*

differentiation, the modified method of Geijsen N *et al*. (2004 Nature 427, 148–154) was used. In brief, ES cell suspension was put into hanging drops (400 cells per drop) for two days, where they formed embryoid bodies (EBs). The medium consisted of Iscove's Modified Dulbecco's Media (Gibco) supplemented with 10% FBS (Hyclone, Logan, UT, USA), 30 $\mu g\,mL^{-1}$ iron saturated transferrin (Gibco), 1 mM sodium pyruvate (Gibco), 0.1 mM 2-mercaptoethanol (Sigma, Hungary), non-essential amino acids (Sigma), 4.5 mM monothioglycerol (Sigma), 50 $\mu g\,mL^{-1}$ ascorbic acid (Sigma), 2 mM glutamine (Gibco), and antibiotics. The EB clumps were differentiated in suspension culture for 2 or 5 days, and then dissociated with collagenase treatment. Cells positive for SSEA-1 were isolated from dissociated EBs by immunomagnetic bead sorting and plated into gelatinized plates in the presence of 2 μM retinoic acid (Sigma). After 7 days of culture, individual PGC colonies were isolated and subcloned. The subcloned PGCs were cultured in PGC medium consisting of Dulbecco's Modified Eagle Media (Gibco) supplemented with 15% FBS, 2 mM glutamine, 1 mM sodium pyruvate, 1000 U recombinant mouse leukaemia inhibitor factor (ESGRO®, Chemicon International, Inc., Temecula, CA, USA), 20 $ng\,mL^{-1}$ basic fibroblast growth factor (Sigma), 60 $ng\,mL^{-1}$ soluble mouse stem cell factor (Sigma), and antibiotics. The gene expression profile was monitored using semi-quantitative RT-PCR. The gene expression of Oct4, Nanog, Stella, Piwil2, Rnf17, and Tex14 were analyzed during the differentiation. Primary PGC cultures were also isolated from (C57BL/6 × DBA)F1 embryos of age 8.5 and 11.5 days post-coitum, and differentiated *in vitro*. The previously described PGC medium was used to proliferate the isolated cells. The gene expression profile of PGCs and ES-derived PGC lines were compared. There were no great differences between the gene expression profiles of PGCs and ES cell-derived PGC cells. SSEA-1 and alkaline phosphatase staining of cells did not show differences between the two cell populations. We have shown here the two PGC populations do not differ from each other in gene expression of the selected genes. Further investigation is needed to differentiate the PGCs into gametes and to analyze the gene expression of other genes involved in gamete differentiation.

The authors would like to acknowledge Gyorgyi Kungl for the technical help. This research was supported by OTKA T046171 grant.

173 EFFECT OF CULTURE SYSTEM FOR IVM-IVF PIG EMBRYOS ON THE ICMS ABILITY TO PRODUCE OUTGROWTHS FOR EMBRYONIC STEM CELL DERIVATION

G. Lazzari, *I. Lagutina*, *G. Crotti*, *P. Turini*, *S. Colleoni*, *R. Duchi*, *and C. Galli*

Laboratorio di Tecnologie della Riproduzione, CIZ srl, Istituto Sperimentale Italiano Lazzaro Spallanzani, 26100 Cremona, Italy.
Email: giovannalazzari@galli2.191.it

Attempts to derive true embryonic stem cells in large farm animals rely on the supply of good quality embryos. In these species, including the pig, pre-implantation-stage embryos can be produced by *in vitro* techniques from slaughterhouse ovaries. The objective of this study was to evaluate the ability of the inner cell masses (ICMs) of pig embryos, produced *in vitro* by different methods, to provide viable initial outgrowths of ICM cells that could be subsequently subcultured and expanded. Porcine oocytes were recovered from slaughtered donors and matured *in vitro* for 40–44 h in DMEM-F12 supplemented with 10% FCS, 0.05 IU LH and FSH (Menogon, Ferring, Milan, Italy), 0.3 mM cystine, 0.5 mM cysteamine, 50 ng/mL long-EGF, 100 ng/mL long-IGF1, 5 ng/mL bFGF (Sigma-Aldrich, Milan, Italy) in 5% CO_2 at 38.5°C. Boar frozen-thawed semen was separated on a percoll gradient and diluted in TALP medium with PHE (penicillamine, hypotaurine, epinefrine) to a concentration ranging from 0.05 to 0.1 million sperm per mL. Oocytes were partially decumulated, co-incubated with sperm for 24 h, and finally denuded and cultured in microdrops of mSOFaa or NCSU. After cleavage, approximately half of the cleaved embryos were surgically transferred into the sheep oviduct for 4 days of *in vivo* culture and the remaining embryos were left *in vitro* in the two media. On Day +6 *in vivo*-cultured embryos were recovered from the sheep oviduct. Blastocyst formation and quality were comparatively evaluated in the three culture groups. Quality specifically referred to the morphology/size of the ICM according to the following criteria: ICM A (large/prominent), ICM B (flat), and ICM C (non-visible). All embryos with a visible inner cell mass were subjected to microdissection with needles to recover the ICMs that were then plated on feeder-layers of mitomycin-treated STO fibroblasts. Attachment and outgrowth was evaluated 48–72 h post-plating. Results are presented in Table 1. Our data indicate that *in vivo* culture of pig embryos in the sheep oviduct greatly enhance both blastocyst development and ICM quality. As a consequence the efficiency of outgrowth formation, following plating for ES cell derivation, was significantly higher with ICMs derived from IVM-IVF pig embryos cultured *in vivo* as compared to their *in vitro*-cultured counterparts. Within the two culture media tested for *in vitro* culture, SOF and NCSU, the rate of blastocyst formation was similar but the quality of SOF-cultured embryos is higher. In conclusion, embryo/ICM quality represents a fundamental requirement for the derivation of ES cell lines, and *in vivo* culture in the sheep oviduct provides the most efficient source of high quality IVM-IVF pig embryos.

Table 1. Blastocyst development and ICM quality of *in vitro*-produced pig embryos

Culture method	No. oocytes	No. cleaved	No. recov.	No. blast. Day +6	% of cleaved or recov. No.	% ICM A	% ICM B	% ICM C	No. outgrowths (% of blast.)
In vitro-SOf	297	192	–	43	22.4 ± 5.4^{a}	0^{a}	29^{a}	71^{a}	$3\ (7)^{a}$
In vitro-NCSU	297	199	–	51	25.6 ± 3.5^{a}	0^{a}	5^{b}	95^{b}	0^{a}
Sheep oviduct	654	325*	239*	118	$\#49.4 \pm 5.8^{b}$	28^{b}	35^{a}	37^{c}	$37\ (31)^{b}$

* Embryos transferred to/recovered from sheep oviduct.
[abc] Numbers within columns with different letters are different ($P < 0.05$), chi-square test.

This work was supported by the Istituto Superiore di Sanità, Programma Nazionale Cellule Staminali, Rome, Italy, grant No. CS 11.

174 ISOLATION, CULTURE AND POTENTIAL USE OF THE PORCINE NEURAL AND EPIDERMAL STEM CELLS

J. Motlik[A,B], *P. Vodicka*[A,B], *J. Klima*[A,B], *K. Smetana*[B,C], *F. Liu*[D] *and H. Gabius*[E]

[A]Institute of Animal Physiology and Genetics, Czechoslovak Academy of Sciences, 277 21 Libechov, Czech Republic; [B]Center of Cell Therapy and Tissue Repair, 2nd Faculty of Medicine, Charles University, Prague, Czech Republic; [C]1st Faculty of Medicine, Institute of Anatomy, Charles University, Prague, Czech Republic; [D]Department of Dermatology, School of Medicine, University of California-Davis, Davis, CA 95616, USA; [E]Faculty of Veterinary Medicine, Institute of Physiological Chemistry, Ludwig-Maximilian University, Munich, Germany. Email: motlik@iapg.cas.cz

The mammalian brain and epidermis contain stem cells, so-called neural stem cells (NSC) and epidermal stem cells (EpSC). To achieve the full therapeutic potential of stem cells, appropriate animal models have to be used to establish the sequence of pathological changes and to test potential therapies to block these changes. In the following studies miniature pigs were used as a biomedical model. We isolated multipotent cells from brains of porcine fetuses for future use in allotransplantation experiments in the inbred miniature pig strain. Brain tissue from 40- and 80-day-old porcine fetuses was mechanically dissociated, and cells were cultured in serum-free F12/DMEM medium with B27 and N2 supplements, EGF and bFGF. In 3–5 days some cells divided and formed floating spheres that were dissociated to single cell suspension and formed secondary spheres in culture. At all time points tested, the spheres represented mixtures of undifferentiated cells stained with nestin and Ki-67 antibodies and already differentiated neurons (Tu-20, MAP2) and glia (GFAP). After being plated on laminin/fibronectin coated coverslips and cultured in medium containing 2% FBS or 1 μM retinoic acid, the spheres adhered to the surface, and flattened, and cells started to migrate out. After immunofluorescence staining with antibodies to neuronal markers Tu-20 and MAP2, glial marker GFAP and oligodendrocyte marker CNPase showed that all the three cell types were present among differentiated cells. The EpSC are characterized by a slow and unlimited proliferation rate and, therefore, they retain labelled precursors of DNA more extensively than other keratinocytes. The main pool of EpSC is located in the bulge region of the hair follicle root sheath. A new procedure to isolate porcine hair follicles including their root sheaths was developed. The keratinocytes that migrated from hair follicles in the presence of feeder cells were poorly differentiated and specifically expressed galectin-1 or galectin-1-binding sites in their nuclei in co-localization with ΔNp63α. The exclusion of feeder cells from experimental system induced formation of spheroid bodies from these keratinocytes. Approximately one-third of these spheroids were able to adhere to a surface precolonized with feeder cells and to start forming normally growing colonies. Porcine hair follicles represent an excellent model for study of the functional phenotype of hair follicle-originated keratinocytes, and the endogenous lectin Gal-1 seems to be a potential marker of the porcine stem cell compartment of the hair follicle under *in vitro* conditions.

175 ESTABLISHMENT OF PORCINE EMBRYONIC STEM CELL LINE DERIVED FROM *IN VIVO* BLASTOCYSTS

S.-A. Ock, *B. Mohana Kumar*, *H.-F. Jin*, *L.-Y. Shi*, *S.-L. Lee*, *S.-Y. Choe*, *and G.-J. Rho*

College of Veterinary Medicine, Gyeongsang National University, Chinju, 660-701, Republic of Korea. Email: jinrho@nongae.gsnu.ac.kr

A porcine embryonic stem (ES) cell line was established from an *in vivo*-flushed blastocyst. The present study evaluated the effectiveness of IVP, parthenotes and *in vivo*-produced embryos on establishment of an ES cell line. IVP blastocysts were produced from slaughterhouse ovaries based on the previously reported protocols (2000 Theriogenology 54, 787–797) with minor modifications. Parthenote blastocysts were produced by activation of oocytes matured *in vitro* with electric stimulation of 2 DC pulses at 2.0 kV/cm for 30 μsec in 0.3 M mannitol solution containing 100 μM $CaCl_2$ and 100 μM $MgCl_2$ *in vivo* blastocysts were recovered on Day 7 after AI (Day = 0) by flushing the uterus with D-PBS containing 10% FBS from three females. After removal of zona pellucida with 0.2% pronase, the blastocysts were subjected to immunosurgical treatment with 10% rabbit anti-pig serum to isolate the inner cell mass (ICM) as previously reported (1975 PNAS 72, 5099–5102). The ICM was seeded onto the feeder layer of STO which was inactivated by treatment with 10 μg/mL mitomycin for 2.5 h and cultured in DMEM with 0.1 mM β-mercaptoethanol, 100 IU/mL penicillin, 0.05 mg/mL streptomycin, 0.1 mM MEM non-essential amino-acid, 20 ng/mL rh-bFGF, 40 ng/mL rh-LIF, 0.03 mM adenosine, 0.03 mM guanosine, 0.03 mM cytidine, 0.03 mM uridine, 0.01 mM thymidine, and 15% FBS. The culture was maintained by changing the medium every day after initiation of ICM attachment onto the feeder layer. Any ES-like colonies were individually picked off the feed layer, dissected with 0.25% trypsin-0.02% EDTA for 3–5 min and reseeded on to new STO feed layer. Out of 140 blastocysts (25, *in vivo*; 55, IVF; 60, parthenotes) used, attaching rates of the ICMs onto the feeder layer were 88% (22/25, *in vivo*), 56.4% (31/55, IVF), and 58.3% (35/60, parthenotes). A total of 15 primary ES-like colonies was formed in *in vivo* (3, 12%), IVF (5, 9.1%), and parthenote (7, 11.7%). However, only one ES cell line from *in vivo* blastocyst was established, which was confirmed as positive by AP activity (Promega, Madison, WI, USA), and was maintained through four passages. In conclusion, for establishment of an ES cell line in pig, the *in vivo* blastocyst method is superior to currently available methods utilizing IVF or parthenotes.

This work was supported by grant No. 1000520040020000 from Biogreen 21, Republic of Korea.

176 IMPROVED GROWTH OF HUMAN EMBRYONIC STEM CELLS IN A REDUCED OXYGEN ATMOSPHERE

T. Peura, *A. Bosman*, *and T. Stojanov*

Sydney IVF, Sydney, New South Wales 2001, Australia. Email: teija.peura@sivf.com.au

Traditional cell culture conditions entail the use of gas atmosphere consisting of 5–6% CO_2 in air. These same conditions have also been used universally for the culture of human embryonic stem cells (hES), despite the natural milieu of the embryos, from which these cells are derived, being

slightly hypoxic. The aim of this work was to examine if human embryonic stem cells would benefit from the reduced oxygen culture environment, as used for human pre-implantation embryos. In Experiment 1, the relative growth in high and low oxygen atmosphere was compared by cutting undifferentiared hES-colonies into equal-size pieces and transferring them to two dishes, one into the conventional incubator in 6% CO_2 in air (HIGH), the other into K-MINC-1000 (Eight Mile Plains, Queensland) mini-incubator in 5% O_2, 6% CO_2 and 89% N_2 (LOW). After 8 days the colony sizes and differentiation status were measured. In Experiment 2, the absolute growth in high and low oxygen atmosphere was compared by cutting exactly same size fragments from undifferentiated hES-colonies and distributing them to two treatments as described. All colonies were measured and passaged at Day 7 and final measurements taken at Day 14. In Experiment 1, improved growth was observed in reduced oxygen, mean percentage of undifferentiated growth from original colony (assigned as 100%) being 217% for HIGH and 482% for LOW over three replicates. In Experiment 2, the total of 16.9 mm^2 of undifferentiated colonies in both treatment groups (24 colonies per group over 3 replicates) had by Day 7 grown to total of 51.1 and 79.5 mm^2 ($P < 0.001$), and by Day 14 to 216.8 and 373.3 mm^2 ($P < 0.0001$) in HIGH and LOW, respectively (see Table 1). In neither experiment were there differences in the differentiation status of the colonies between the treatments (mean 6% and 5% at Day 7, and 9% and 9% at Day 14 in HIGH and LOW, respectively). We conclude that culture in reduced oxygen improves growth of human embryonic stem cells.

Table 1. Growth of hES colonies on high and low oxygen atmosphere (data from 3 replicates)

Group	*n* of colonies	Total area of undiff. Day 0	Colonies (mm^2) Day 7	(mean size; range) Day 14
HIGH	24	16.9 (0.7; 0.3–1.2)	51.1 (2.1; 1.1–4.1)	216.8 (9.0; 8.8–20.8)
LOW	24	16.9 (0.7; 0.3–1.2)	79.5 (3.3; 1.4–7.6)	373.3 (15.6; 6.2–28.1)

We like to acknowledge Bresagen Ltd. for the supply of hesBGN-01 cell line.

177 GENERATION OF ES CELLS AND TRANSGENIC MICE EXPRESSING MTERT-GFP AS A MARKER OF PLURIPOTENTIAL CELLS

M.A. Ramírez, R. Fernández-González, P.N. Moreira, M. Pérez-Crespo, J. de la Fuente, B. Pintado, and A. Gutiérrez-Adán

Departamento de Reproduccion Animal, Instituto Nacional de Investigación y Tecnologia Agraria y Alimentaria, 28040 Madrid, Spain. Email: ramirez@inia.es

There is not a simple system that allows us to identify stem cells in adult tissues. Cells of adult tissues arise from dividing progenitor cells, which themselves are derived from multipotential stem cells. Telomerase is the enzyme that maintains the ends of linear chromosomes in eukaryotic cells. Recently, a segment of the promoter sequence of the reverse transcriptase of murine telomerase (mTert) has been characterized. mTert is expressed with greatest abundance during embryogenesis and becomes widely expressed in adult tissues at low levels. This low expression level in adult tissues may be due to the presence of pluripotent stem cells present in those tissues. To examine the relationship between telomerase activity and multipotential of adult cells we have generated three constructs (1k-, 2k-, and 5k-mTert-GFP) comprising different segments of the mTert promoter sequence coupled to the coding sequence of the green fluorescent protein (GFP). These constructs were electroporated into R1 and B6D2 (generated in our laboratory) ES cells and were used to produce transgenic mice. The generation and identification of transgenic mice (C57BL6 × CBA) has been previously described (Gutierrez-Adan and Pintado 2000 Trangenic Res. 9, 81–89). Transgenic founders were backcrossed to C57BL6 × CBA mice to obtain transgenic lines. The three constructs were able to mimic the mTert expression, which was coupled to green fluorescence. The mTert-GFP transfected ES cells were initially maintained in medium supplemented with LIF, which was subsequently removed to allow differentiation of embryoid bodies (EBs) and other cell types. GFP expression was higher during the first two days after LIF removal (period of enhanced cell proliferation), decreasing in the following days as a result of EB differentiation. Both ES cell lines showed reduced GFP expression upon differentiation, suggesting that mTert is the principal determinant of telomerase activity; moreover, different degrees of expression and down regulation were reported with the different constructs. Using these constructs we have also generated transgenic mice. Eight lines of transgenic mice carrying the 1kmTert-GFP transgene, four with the 2kmTert-GFP, and three with the 5kmTert-GFP, were obtained. There were no significant differences between the proportions of transgenic founder generates. The transgenic mice express and GFP during the fetal development, indicating their telomerase activity. We are now analyzing the expression of mTert-GFP in adults tissues. Our results suggest that telomerase-GFP transgenics are an important tool to assess the role of telomerase in adult multipotential cells as well as to select these pluripotent cells in adult tissue. It will be interesting to see if different levels of mTert-GFP expression are associated with different levels of pluripotency.

178 ESTABLISHMENT OF MOUSE PLURIPOTENT STEM CELLS GENERATED FROM PRIMORDIAL GERM CELLS

S.W. Shim[A,B], *S.J. Song*[C], *H.S. Shim*[D], *S.J. Uhm*[A], *B.Y. Lee*[B], *S.B. Kim*[B], *H. Song*[A], *S.-G. Cho*[A], *H.M. Chung*[E], *K.S. Chung*[A], *and H.T. Lee*[A]

[A]Laboratory of Animal Reproduction, ARRC, Konkuk University, Seoul, Korea; [B]Infertility Clinic, Department of OB/GY, Kyung-Hee Medical Center, Seoul, Korea; [C]Laboratory of Reproductive Biology & Infertility, Samsung Cheil Hospital, Seoul, Korea; [D]Department of Physiology, School of Medicine, Dankook University, Cheonan, Korea; [E]College of Medicine, Pochon CHA University, Seoul, Korea. Email: sjuhm@hanmail.net

Pluripotent stem cells have been generated from two embryonic sources: ES cells generated from ICM of blastocyst stage embryos, and embryonic germ (EG) cells generated from primordial germ cells (PGCs). Both ES and EG cells are pluripotent and exhibit important characteristics such as

high alkaline phosphatase (AP) activity, multicellular colony formation, normal and stable karyotype, continuous passaging ability, and capacity to differentiate into three embryonic germ layers. This study was performed to establish the culture system for mouse EG cells derived from mouse PGCs. PGCs collected from the genital ridge of Day 11.5, 12.5, and 13.5 mouse embryos (C57BL/6 × DBA/2) were cultured and subsequently passaged on mitotically inactivated STO feeder cell layer. Cells were grown in Dulbecco's modified eagle medium (DMEM) supplemented with 15% fetal bovine serum (FBS), 0.1 mM nonessential amino acids, 0.1 mM 2-mercaptoethanol, 2 mM glutamine, 100 IU/mL of penicillin, 100 μg/mL of streptomycin, 1,000 units/mL of leukemia inhibiting factor (LIF), 6 ng/mL of SCF, and 10 ng/mL of bFGF at culture conditions of 5% CO_2 in air, 95% relative humidity, 37°C temperature. Cells were routinely passaged every 3–4 days. Over a period of 7–10 days in primary culture, PGCs proliferated to form small, densely packed, multicellular colonies consisting of AP-positive cells that morphologically resembled undifferentiated ES cells. RT-PCR analysis confirmed mRNA expression of transcription factors Oct-4 and Nanog in these cells. Cultured cells could be maintained on the feeder cell layer for at least 10 passages and still retain normal karyotype. These results suggest that cell lines derived from mouse primordial germ cells are presumably EG cell lines and could be useful for transgenic animal production and ES cell study.

179 ISOLATION AND CULTURE OF EMBRYONIC GERM-LIKE CELLS FROM PORCINE MESONEPHROS

H.-Y. Son[A,B], *M.-G. Kim*[A,B], *J.-I. Yun*[A,B], *J.-E. Kim*[A,B], *H.-S. Kim*[A,B], *S.-K. Kang*[C], *B.-C. Lee*[C], *W.-S. Hwang*[A,C], *and C.-K. Lee*[A,B]

[A]School of Agricultural Biotechnology, College of Agriculture and Life Sciences, Seoul National University, Suwon, 441-744, South Korea; [B]Xenotransplantation Research Center, Seoul National University, Suwon, 441-744, South Korea; [C]College of Veterinary Medicine, Seoul National University, Seoul, 151-742, South Korea. Email: shy916@hanmail.net

Many attempts to establish embryonic stem (ES) cells from pre-implantation stage embryos in pigs have failed. An alternate source of pluripotent stem cells is embryonic germ (EG) cells derived from primordial germ cells (PGCs) of the genital ridge, which is developed from the mesonephros. Mesonephros is a vestigial, transient renal organ that functions only during embryonic development. It is believed to be a source of multiple stem cells including somatic cells in the gonad, vascular endothelial cells, and hematopoietic stem cells. Therefore, we tried to obtain putative stem cells from cells isolated from porcine mesonephros under culture conditions used to establish EG cells. Porcine fetuses from crossbred gilts were collected by hysterectomy between Days 25 and 30 of pregnancy (estrus = Day 0). Mesonephros and genital ridges were separated from each other, and cells from the mesonephros and PGCs from genital ridges were isolated by a physical method. Isolated cells were cultured in PES medium [50:50 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F10 medium supplemented with 15% fetal bovine serum (FBS), L-glutamine (1.7 mM), β-mercaptoethanol (0.1 mM), 1% MEM non-essential amino acids and 1% antibiotic-antimycotic] containing the cytokines, soluble recombinant human basic fibroblast growth factor (bFGF; 20 ng/mL), and human leukemia inhibitory factor (hLIF; 10 ng/mL). Isolated cells were cultured on fresh primary murine embryonic fibroblast feeder cells (PMEF) in a humidified environment of 5% CO_2 in air at 38°C. The colonies with EG-like morphology were dissociated with 0.25% trypsin/1 mM EDTA for 10 min, and passed to fresh feeders. After 5–8 days, colonies started to grow with typical EG-like morphology. More colonies were obtained from the culture of cells from mesonephros than from culture of PGCs. Porcine EG-like cells from mesonephros (pMN-EG-like cells) were passed to fresh feeder every 6–8 days and have been cultured up to 9 passages while maintaining typical EG-like morphology. pMN-EG-like cells were stained for alkaline phosphatase throughout the culture. Furthermore, these cells reacted with antibodies against Oct-4 and SSEA-1 by immunocytochemistry, indicating that these cells have characteristics of pluripotential stem cells. In order to characterize the pMN-EG-like cells with respect to their potential for differentiation, embryoid body (EB) formation was induced. EBs started to form in 4 days and cystic structures in 2 weeks. EBs were then attached to the dish and cultured without cytokines. Spontaneously, EBs from pMN-EG-like cells could give rise to differentiated cell types such as neuronal-like, epithelial-like, and fibroblast-like cells. Further studies to characterize differentiated cells from pMN-EG-like cells by immunocytochemistry and for teratoma formation by injection into SCID mice will be performed. In conclusion, EG-like cells could be obtained from culture of mesonephric cells from porcine fetus and further characterization of these cells is required.

180 EFFICIENT GENE SILENCING USING siRNA IN MOUSE AND MONKEY ES CELLS AND DIFFERENTIATION

T. Takada[A], *K. Nemoto*[A], *A. Yamashita*[A], *M. Katoh*[B], *Y. kondo*[C], *and R. Torii*[A]

[A]Research Center for Animal Life Science, Shiga University of Medical Science, Shiga, Japan; [B]Central Research Institute, Ishihara Sangyo Kaisya, Ltd., Kusatsu, Japan; [C]Discovery Research Laboratory, Tanabe Seiyaku Co., Ltd., Osaka, 532-8505, Japan. Email: ttakada@belle.shiga-med.ac.jp

Gene silencing by RNA interference (RNAi) using small interfering RNAs (siRNAs) and short hairpin RNAs (shRNAs) has become a valuable genetic tool for silencing specific genes in various organisms. As compared with transgene-based RNAi using shRNA expression vectors, chemically synthesized siRNAs have an advantage in that they do not modify genome organization. This nature is ideal as a differentiation method for embryonic stem (ES) cells. The objective of this work was to develop an efficient method to repress a specific gene expression in mouse and monkey ES cells using chemically synthesized siRNA, and to investigate whether this transient gene silencing can be used as a new differentiation method of ES cells. First, we tried to knock down the expression of enhanced green fluorescent protein (EGFP) gene in GFP-expressing mouse ES cells. We found that lipofection was effective to deliver siRNA into mouse ES cells. Suppression of EGFP expression was observed by fluorescence microscopy after 24 h of transfection and the silencing continued at least for 5 days. FACS analysis revealed that 86% of cells showed suppression of EGFP at 48 h

after transfection. Then we tried to suppress endogenous gene Oct4 that plays an important role in the maintenance of pluripotency and the lineage commitment in mouse ES cells. We performed RT-PCR analysis and western blotting to assay for Oct4 mRNA and protein at 24, 48, 72, and 96 h after transfection. Hand1 and Cdx2, transcription factors implicated in trophoblast differentiation, were also analyzed. RT-PCR analysis showed a reduced level of Oct4 mRNA at 24–96 h. Reduction of Oct4 protein was confirmed by western blotting and the reduced level was still maintained at 96 h after transfection. RT-PCR analysis also showed up-regulation of Hand1 and Cdx2 concomitant with the suppression of Oct4. Furthermore, ES cells changed their morphology into a large and flattened shape that is characteristic of trophoblast cells. These results suggested that the transient suppression of Oct4 induced differentiation of mouse ES cells to trophectoderm cells as expected. Therefore our data imply that the chemically synthesized siRNA can be used to differentiate ES cells. Next, we tried to suppress EGFP and Oct4 in monkey ES cells. In monkey ES cells, we found that the Sendai virus (hemagglutinating virus of Japan, HVJ) envelope was suitable to deliver siRNA into cells. With this method, we detected efficient silencing of EGFP and Oct4 by fluorescence microscopy, RT-PCR analysis, and western blotting. In the case of monkey ES cells, however, no morphological change was observed by Oct4 suppression at 96 h after transfection. These results suggest possible diversity between murine and primate ES cells in the differentiation process.

This work was supported by grants from the Ministry of Education, Science, Sports, and Culture (14580798, 13358014 and 14380382).

181 Oct-4: A POTENTIAL MARKER FOR PLURIPOTENCY IN CATTLE

M. Vejlsted, B. Avery, J. Gjoerret, M. Schmidt, T. Greve, and P. Maddox-Hyttel

Division of Reproduction, Royal Veterinary and Agricultural University, 1870 Frederiksberg C, Denmark. Email: mov@kvl.dk

The POU (Pit-Oct-Unc) domain transcription factor Oct-4 is one of the most acknowledged markers for pluripotency in murine and primate embryonic cells. At the blastocyst stage, expression of Oct-4 has been shown to remain high in the inner cell mass (ICM) while being rapidly down regulated in the trophectoderm (TE). Furthermore, in these species, expression of Oct-4 is maintained in pluripotent derivatives of the ICM and in embryonic stem (ES) cells, but lost upon differentiation. In the bovine embryo, a marker with similar qualities has long been sought. The aim of this study was to investigate, using a commercially available antibody for immunohistochemistry (IHC), whether Oct-4 might serve this role. *In vitro* produced (IVP) embryos were transferred to synchronized recipients at Day 6 post insemination (p.i) and flushed at Day 12; *in vivo*-derived embryos were flushed at Day 14. Day 8 IVP embryos ($n = 20$) were fixed and processed for IHC in paraffin sections together with Day 12 ($n = 18$) and Day 14 ($n = 3$) embryos. From Day 8 IVP embryos, outgrowth colonies (OCs) were formed by intact blastocyst culture on mouse SNL feeder cells. OCs were photographed using a stereomicroscope on Days 12, 14, and 16 p.i., and were examined for Oct-4 expression by *in situ* IHC at Day 16 p.i. ($n = 94$). From isolated embryonic discs of Day 12 embryos, OCs were derived by similar culture and were either processed for IHC on paraffin sections at Days 16, 18, and 20 p.i. ($n = 9$) or used for establishment of ES-like cell lines. Of colonies formed, representative specimens from each of the initial 5 passages ($n = 18$) were examined for Oct-4 expression either in paraffin sections or *in situ*. In Day 8 embryos, Oct-4 expression was demonstrated in all nuclei of both ICM and TE cells except for presumptive apoptotic ones. Approximately one-fifth of the OCs presented a substantial amount of Oct-4 positive cells of putative ICM, but also of TE origin. Apparently, the formation of Oct-4 positive OCs was favored by initial attachment of the embryonic pole to the feeder cells. In Day 12 and 14 embryos, specific and exclusive Oct-4 staining of nuclei of the complete epiblast, but not the hypoblast and the TE, was revealed. All OCs derived from Day 12 embryonic discs showed specific staining for Oct-4 in nuclei of putative epiblast origin only. On subsequent culture of these isolated epiblast derivatives, loss of Oct-4 staining from colonies was observed by passage 3. This study has, for the first time, shown expression of Oct-4 to be limited to pluripotent cells of bovine Day 12 and 14 embryos. Compared with murine and primate embryos, down-regulation of Oct-4 expression in bovine TE cells appears to be delayed. Findings indicate that Oct-4 may be used as a marker for pluripotency in bovine ES-like cells, although TE derivatives may maintain Oct-4 expression when isolated from Day 8 embryos.

Epidemiology/Diseases

182 DISINFECTION OF DRY (VAPOR) SHIPPERS ("DEWARS") FROM MICROBIAL CONTAMINATION ASSOCIATED WITH CRYOPRESERVED GERMPLASM

A. Bielanski

Animal Diseases Research Institute, Germplasm Centre of Expertise, CFIA-Ottawa Laboratory, Ottawa, Ontario, Canada.
Email: bielanskia@inspection.gc.ca

Cryopreservation, storage, and transport of cryopreserved germplasm without the risk of disease transmission is of great concern to animal and human health authorities. Here we report on the efficacy of microbial decontamination of liquid nitrogen (LN) dry (vapor) shippers used for short-term storage and transportation of germplasm and other biological specimens. Dry shippers containing either a hydrophobic or a nonhydrophobic LN absorbent were experimentally contaminated with high titers of cultures of *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococus aureus*, bovine viral diarrhea virus (BVDV), and bovine herpesvirus-1 (BHV-1). Biocidals with broad-spectrum antimicrobial activity and gas vapors of formalin and ethylene oxide were used for disinfection of the shippers. All biocide solutions were diluted with milli-Q water to the concentrations recommended by the manufacturer and poured directly into the chamber of the dry shippers. The dry shipper was filled with the disinfectant for 30 min, drained, washed three times with sterile water, and drained before testing the residue for microbial contaminant. Among the biocidals used, treatment with sodium hypochlorite solution (30% of household bleach), a quaternary ammonium-based disinfectant (100% Expel), and peracetic acid (30%) were the most effective and useful for dry shippers with a hydrophobic LN absorbent. None of the bacterial or viral microorganisms were detected in samples of semen and embryos stored in dry shippers following their disinfection with these biocides. Other disinfectants (Virkon, Roccal,