

ABSTRACTS FOR POSTER PRESENTATION

186 SEROCONVERSION OF CALVES TO BOVINE VIRAL DIARRHEA VIRUS (BVDV) FOLLOWING INTRAVENOUS INOCULATION WITH ARTIFICIALLY EXPOSED IN VIVO-DERIVED EMBRYOS

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An early study demonstrated that washing embryos was effective for removal of virus after artificial exposure of in vivo-derived embryos to a cytopathic isolate of BVDV (Singh *et al.*, 1982 Theriogenology 17,437–444). However, a recent study using representative noncytopathic isolates of BVDV demonstrated that washing was less beneficial for removing some isolates of BVDV than for others (Waldrop *et al.*, 2000 Theriogenology 57, 575). Thus, the objective of this study was to determine if the quantity of a high affinity isolate of BVDV that remains associated with single-washed or trypsin-treated embryos is sufficient to cause infection in vivo. Twenty zona pellucida-intact Day 7 morulae and blastocysts (MB) were collected from superovulated cows. After collection, all MB were washed according to International Embryo Transfer Society (IETS) standards, and all but 4 (negative controls) were exposed for 2 h to approximately 106 cell culture infective doses (50% endpoint) per mL (CCID50/mL) of viral strain SD-1. Following exposure, one-half of the MB were washed and one-half were trypsin-treated according to IETS standards. All MB were then individually sonicated, and sonicate fluids were injected intravenously into seronegative calves. Blood was collected from each calf on Days 0, 3, 6, 9, 12, 15, 20, 25, and 30, and sera were assayed for BVDV and anti-BVDV antibodies. All cattle used in the study were determined to be virus- and antibody-negative 30 d prior to and the day of intravenous inoculation of sonicate fluids into calves. Viremia was not detected in any calf following injection, possibly due to intermittent sampling and/or small amount of embryo-associated virus present. However, seroconversion of 38 and 13% of the calves occurred following injection with sonicate fluids from washed and trypsin-treated embryos, respectively. Findings demonstrated that the quantity of a high affinity isolate of BVDV associated with single-washed or trypsin-treated embryos is sufficient to be infective in vivo as evidenced by seroconversion. These results emphasize the need for studies to determine if the virus associated with exposed individual embryos constitutes an infective dose when placed into the uterus.

Embryonic Stem Cells

187 EFFECTS OF OXYGEN TENSION ON ESTABLISHMENT, LDH ISOZYMES, AND mRNA EXPRESSION OF MURINE EMBRYONIC STEM CELLS

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Orchestrated differentiation of embryonic stem cells into specific tissues or cells will be invaluable for xenotransplantation, biomedicine, and pharmacology. However, the lack of a standardized culture environment for establishment and maintenance of cell lines has hindered the application of this technology. In other cell types, O₂ concentration in the culture environment can have a profound effect on proliferation and differentiation. This study, therefore, tested the hypothesis that establishment dynamics, LDH isoforms, and mRNA expression patterns of the resulting cell lines would be affected by the O₂ tension in the culture environment. Blastocysts recovered from mice (C57BL/6) uteri on Day 4 (post coitus) were placed in Speciality Medium (4500 mg L⁻¹ glucose) DMEM (plus 0.01 µg mL⁻¹ LIF) and cultured in a gas environment of 5% CO₂ and either ~20% or 5% O₂. More ($P < 0.05$) blastocysts hatched and produced outgrowths (Day 4 of DMEM culture) in the low ($76.7 \pm 0.1\%$) compared to the high ($58.3 \pm 0.1\%$) O₂ group. Although the number of cells per outgrowth was similar between groups (low = 58.1 ± 5.2 , high = 58.8 ± 5.7), a smaller number of colonies in the high O₂ group (9/15; 64.3%) stained positive for alkaline phosphatase relative to the low O₂ group (14/15; 93.3%). Oxygen treatment had no effect on the patterns of activity of the oxoreductase, lactate dehydrogenase (LDH), in either outgrowths or established stem cell lines. Interestingly, the stem cell lines (both O₂ groups) displayed multiple isoforms (Isoforms 3, 4, and 5) of LDH, whereas the outgrowths displayed only Isoform 5. In contrast, two-cell embryos and blastocysts displayed only Isoform 1, and fibroblasts displayed Isoforms 4 and 5. Expression (mRNA) profiles were developed from blastocyst outgrowths, stem cell colonies, and established stem cell lines cultured under either high or low O₂ tension, using a RT-PCR for LDH (Isozymes α and β) and a key regulatory enzyme of glycolysis, phosphofructokinase-2 (PFK2; Isozyme 1 and 2). There were no differences between the high and low O₂ groups in mRNA expression of LDH α in the outgrowths, or established stem cells. Expression of LDH β was at a very low level regardless of O₂ treatment or cell type. Outgrowths from the low O₂ group expressed Isozyme 1 of PFK2 whereas the outgrowths from the high O₂ group did not. Enhanced expression of PFK2 is suggestive of increased glycolytic capacity. Reduced O₂ environment during the peri-hatching period had significant effects on the resulting embryonic stem cells, supporting the hypothesis that O₂ tension can affect stem cell establishment and maintenance.

188 FATE OF CHIMERIC EMBRYONIC STEM CELLS RECONSTRUCTED WITH PARTHENOGENETIC MOUSE EMBRYOS

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Mouse parthenogenetic embryos have been known to be unable to develop to term and are absorbed from Day 11. However, Nagy *et al.* (1990 Development 110, 815–821) reported that the aggregated tetraploid with embryonic stem (ES) cell developed to term and survived after birth. Thus, our study investigated the developmental capacity of the aggregated ES cells with mouse parthenogenetic embryos. Oocytes obtained from superovulated female mice (BCF1) were treated with 7% ethanol and 5 $\mu\text{g mL}^{-1}$ cytochalasin B for the production of parthenotes and co-cultured with sperm ($1 \times 10^6 \text{ mL}^{-1}$) for production of fertilized embryos. The reporter vector (pNeoEGFP) was introduced into ES cells (129S4/SvJae) by electroporation. At the 8-cell stage, parthenotes or fertilized embryos, from which the zona pellucida was removed, were co-cultured with ES cells for 4 h. The aggregated parthenotes or fertilized embryos with 5–10 ES cells were cultured to the blastocyst stage, and transferred into the uteri of 2.5-day post-coitum pseudopregnant recipients. In experiment I, 144 parthenogenetic blastocysts were transferred into the uterine horns of 9 pseudopregnant recipients, and 5 recipients became pregnant. At Day 9, all fetuses were observed visible in uteri of pregnant fonder mice. At Days 10–11, many fetuses were observed in the progress of absorption in uteri of pregnant fonder mice, but a few fetuses were still alive. However, parthenogenetic fetuses were not detected alive beyond 11 days. In experiment II, the 171 aggregated fertilized embryos with ES cells were transferred (15–20 blastocysts/recipient) into 10 recipients and successfully produced 5 offspring from a recipient. We found that three newborn were chimeric mice derived from ES cells. In experiment III, the 209 aggregated parthenotes with ES cells failed to produce offspring, but inserted pNeoEGFP gene in ES cells was detected in the parthenogenetic 1 of 7 fetuses at 15-days of post-gestation by polymerase chain reactions. Therefore, this result suggests that the parthenotes show restricted development to fetus stage, but the aggregated parthenotes with ES cells might extend their developmental capacity. In the future, we will characterize the mechanism of this unusual phenomenon to understand the role of ES cells during development of chimeric parthenotes with ES cells.

189 ESTABLISHMENT OF BUFFALO EMBRYONIC STEM-LIKE (ES-LIKE) CELL LINES FROM DIFFERENT SOURCES OF DERIVED BLASTOCYSTS

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In the pluripotent embryonic stem (ES) cells in mammalian species are derived from the inner cell mass (ICM) of preimplantation embryos. In the current study we report the successful isolation of pluripotent undifferentiated buffalo ES-like cells from the ICMs of in vitro fertilization (IVF), somatic cell nuclear transfer (NT)-reconstructed and parthenogenetic (PA) embryos. The ICMs were isolated from hatched blastocysts that had spread out after 3–5 days of culture on mouse embryonic fibroblast (MEF) feeder cell layer in the presence of leukemia inhibiting factor (LIF). The production of MEF and ES-like cells were the same as previously described in bovine ES cells (Kitiyanant *et al.*, 2000 Science Asia 26, 81–86). The primary culture and propagation of the cell lines were performed every 2–3 days. The cell lines appeared to be normal diploid karyotype and expressed the cell specific markers of alkaline phosphatase, stage-specific embryonic antigen 3 and 4 (SSEA-3 and SSEA-4), TRA-1-60 and TRA-1-81 similar to those characterized in monkey ES cells (Thomson *et al.*, 1995 PNAS 92, 7844–7848 and Kuo *et al.*, 2003 Biol. Reprod. 68, 1727–1735). The buffalo ES-like cells from these three different sources were able to be cultured in vitro for more than 20 passages on the feeder cell layer without differentiation. They were aggregated to form the embryoid bodies (EBs) in suspension culture. When EBs were plated on 0.1% gelatin-coated culture dishes, the cells differentiated to be neural-, epithelial- and fibroblast-like morphologies. These results suggest success in establishment of buffalo ES-like cells from either IVF, NT or PA preimplantation embryos and also their differentiation in vitro. Buffalo ES-like cells should be a useful source of cells for gene targeting and for the studies of the mechanism of gene expression in the preimplantation embryos obtained from different methods. These results are encouraging for the feasibility of cell transplantation studies in the future. This research was funded by Thai Government and The National Center of Biotechnology and Genetic Engineering.

190 FEEDERS FROM RHESUS MONKEY SUPPORT PROLONGED DIFFERENTIATED GROWTH OF RHESUS MONKEY EMBRYONIC STEM CELLS

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Previous study demonstrated that feeders are important for growth and differentiation of rhesus monkey embryonic stem cells (rES) (Thomson *et al.*, 1995 PNAS 92, 7844–7848). In this study, we developed 5 rhesus monkey feeder cell lines for rES culture: MESF (ear skin fibroblasts from 2-week-old neonatal monkeys), MAF (adult Fallopian tube cells), MGF (adult follicular granulose fibroblast-like cells), MGK (adult follicular granulose kidney-like cells), and MSC (single-cell cloning from MESF), all of which were fibroblast-like cells. Two experiments were designed to investigate rES cell growth and differentiation potentials. In experiment 1, rES (R366.4) was cultured on MESF, MAF, MGF, MGK, and MSC, with MEF (mouse embryonic fibroblasts) as control. In experiment 2, rES was cultured on the mixed feeders: MSC: MGK at 1:1, 7:3, 8:2 and 9:1, depending on the results of experiment 1. Results of experiment 1 showed that the rES underwent undifferentiated growth on MESF, MAF, MGF and MSC (cultured for 15 passages), but not on MGK, with morphology typical of rES on MEFs, and exhibiting positive alkaline phosphatase staining,

positive expression of Oct-4 and GAPDH, and negative expression of PAX6, AFP, BMP4 and hCG of rES as well as normal karyotypes. Embryonic bodies (EBs) were formed on Day 7 to 8 from the rES cultured on MESF, MAF, MGF and MSC. The EBs showed positive expression of hCG, AFP and BMP4. Differentiation of neuron, epithelium and muscle cells was observed after 21 days of continuous culture of the rES. The colonies of rES cultured on MESF, MAF, MGF and MSC were higher than on MEF (1.5 to 3 times). Interestingly, expansion speeds and differentiation rates on MSC were less than on MESF. These results also indicated that fibroblast cells, on the one hand, were alone sufficient for supporting prolonged undifferentiated growth of rES, and on the other hand, other mixed cells except fibroblast cells had the ability to promote the growth and differentiation of R366.4 rES. Results of experiment 2 showed that the mixed feeders (MSC : MGK) at 8 : 2 or 9 : 1 supported undifferentiated growth of rES but not feeders at 7 : 3 or 1 : 1. Our work demonstrated that the rhesus monkey feeders were better able to support undifferentiated growth and maintain differentiation potentials of rES than were MEFs feeders. The rES could grow if the ratio of mixed other cells serving as the feeders exceeded 20%.

191 IN VIVO AND IN VITRO DIFFERENTIATION OF EMBRYONIC STEM CELLS DERIVED FROM PARTHENOGENETIC EMBRYOS IN MICE

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Availability of embryonic stem (ES) cells opens the prospect for regenerative medicine. However, ES cells genetically mismatched to diseased individuals cause immunological rejection. In this study, we established ES cells from parthenogenetic embryos in mice and examined their pluripotency. Oocytes were collected from (C57BL/6xDBA)F1 mice (BDF1) by superovulation. Parthenogenetic diploid embryos were produced by activation treatment in 5 mM SrCl₂ in Ca²⁺-free KSOM medium for 2 h, followed by cultivation in 5 µg mL⁻¹ cytochalasin B for 6 h. The zona pellucidae of embryos developed to the blastocyst stage in vitro were removed by a 5-min incubation in 0.5% pronase. Inner cell masses (ICMs) isolated immunosurgically were seeded on the feeder layers (mitomycin C-treated mouse embryonic fibroblasts) in DMEM supplemented with 15% Knock-Out Serum Replacement (Invitrogen), 2 mM L-glutamine, non-essential amino acids, β-mercaptoethanol and 10³ U mL⁻¹ of Leukemia inhibitory factor (LIF) at 37°C in a humidified atmosphere with 5% CO₂ in air. The attached ICM cells were mechanically disaggregated and seeded on the fresh feeder layers. After several passages, parthenogenetic ES (PnES) cell lines were established. The efficacy of establishing PnES cell lines was 66% (37/56). To examine the characteristics of PnES cell lines, seven lines were subjected to histochemical and immunohistochemical analysis. All showed alkaline phosphatase activity and immunoreactivity to anti-SSEA-1 and anti-Oct4 antibodies. They maintained euploid sets of chromosomes at 29; 59%. PnES cells from two of the seven lines were injected into 59 host blastocysts obtained from ICR mice, resulting in 16 chimeric offspring (27%). In another experiment, injection of ICM cells and ES cells obtained from fertilized BDF1 blastocysts and ICM cells obtained from BDF1 parthenogenetic blastocysts also produced chimeric offspring (35%, 7/20; 46%, 6/13; and 53%, 10/19, respectively). However, no chimeric mouse with germline transmission was obtained from PnES cells. Injection of 1 × 10⁷ of PnES cells into SCID mice formed teratocarcinomas. Immunohistochemical analysis showed cells positive for nestin (specific to neuroepithelial stem cells), Tu-J (class III β-tubulin), NF-M (neurofilament), desmin (muscle), and albumin (hepatocytes), which indicated their differentiation potency to the cells derived from all three germ layers. Simple embryoid bodies produced from these cell lines were plated on tissue culture dishes under conditions for induction of differentiation. Immunohistochemistry and RT-PCR analysis showed their differentiation into neurons (NF-M, nestin), cardiomyocytes and hepato-like cells (albumin, α-fetoprotein). Our results indicate that PnES cells are pluripotent similar to the ES cells from fertilized embryos except for germline transmission and should be tested in cell replacement animal models.

192 ISOLATION AND CULTURE OF EMBRYONIC STEM CELL-LIKE CELLS FROM IN VITRO FERTILIZED PORCINE BLASTOCYSTS

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The establishment of porcine embryonic stem (ES) cell lines should be useful for the production of transgenic pigs and studies of developmental gene regulation. Recent development of techniques for production of embryos in vitro could be a useful source for the isolation of ES cells. Therefore, to establish porcine ES cells, this study was conducted to isolate and culture inner cell mass (ICM) from in vitro-fertilized (IVF) porcine blastocysts. Cumulus-oocyte complexes were collected from prepubertal gilt ovaries, and matured in vitro. Oocytes were then fertilized using a modified swim-up method to prevent polyspermy and cultured to the blastocyst stage. Initial culture of ICM was conducted after either culture of whole embryos or isolation of ICM by immunosurgery. Developing IVF embryos were continuously cultured in 50% DMEM and 50% F-10 with 15% fetal bovine serum, 1% non-essential amino acids, 1.7 mM L-glutamine, 1% penicillin/streptomycin, 0.1 mM α-mercaptoethanol, 1000 unit recombinant human LIF, 40 ng mL⁻¹ recombinant human SCF and 20 ng mL⁻¹ recombinant human basic FGF on a mytomycin-C-inactivated murine embryonic fibroblast (MEF) feeder layer. Antibodies against porcine cells were produced in rabbit. After removal of zona pellucida, ICMs were isolated by immunosurgery and cultured on feeder cells the same as described above. After IVF, the rates of 2-cell embryos and blastocysts were 70.8% and 20.4%, respectively. Results from the isolation and culture of ICMs of porcine blastocysts are shown in following table. ICM isolated by immunosurgery showed better attachment to feeder cells and ES cell colony formation than cultured whole blastocysts. Morphology of colonies was similar to that of mouse ES cells, showing compact colonies with delineated boundary. Also, these colonies showed alkaline phosphatase activity. Porcine ES-cell like colonies were passed 3 times through physical separation on fresh feeder layers. These results indicated that porcine ES-like cell line can be established from IVF porcine blastocysts. Further characterization of these porcine ES-like cell lines is required.

Table 1. Isolation and culture of ICM from porcine blastocyst produced by IVF

Treatment	No. of blastocysts	No. of attached (%)	No. of ES-like cell colonies (%)
Immunosurgery	49	4 (1)	2 (0.5)
Whole blastocysts	28	2 (0.7)	0 (0)

193 CHARACTERIZATION OF PORCINE TROPHECTODERM-DERIVED CELLS IN THE PRESENCE OF hBMP4 IN THE ABSENCE OF A FEEDER LAYER

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A porcine trophoblastic cell line could provide a powerful model for understanding trophoblast cell biology as well as placental gene expression and proteomics in vitro. In this experiment, we derived porcine trophoblastic cells from trophoctoderm tissue and assessed their growth on three different extracellular matrix substrates and in three different concentrations of human recombinant bone morphogenetic protein 4 (hBMP4). Human BMP4 has been shown to induce differentiation of human embryonic stem cells into trophoblast lineages. Elongated embryos were flushed using DPBS supplemented with 1% fetal calf serum and penicillin-streptomycin (1X) from the hysterectomized uteri of superovulated and bred prepuberal gilts 15 days post-insemination. The embryonic disc was visualized with a dissecting microscope. The trophoctoderm tissue was cut 2–3 mm away from the embryonic disc with a scalpel and the trophoctoderm tissue was manually dissected into cell aggregates. These aggregates were plated on collagen type IV, Matrigel, and human extracellular matrix (laminin, collagen type IV and heparan sulfate proteoglycan derived from human placenta) in culture medium (DMEM with 15% FCS, 0.1 mM 2-mercaptoethanol, 4 ng mL⁻¹ basic FGF4 and 1X P/S) in the presence or absence of hBMP4 at 0, 10, or 20 ng mL⁻¹. Cell outgrowth was observed within 24 hours of culture. After three days of culture, various cell types (based on size and morphology) were present. Among cultures of predominant large cells were colonies of smaller cells with epithelial-type morphology that had a prominent nucleus and a high nuclear-to-cytoplasmic ratio. The epithelial-type cells grew in tight colonies with definite borders and contained cytoplasmic structures resembling lipid-containing vesicles. These colonies initially appeared on all matrices across all hBMP4 concentrations. After seven days in culture the colonies developed distinct differences across groups. Cell growth on collagen was comprised of tight colonies having definite borders among large cells. Colonies on collagen were larger and more pronounced in both the hBMP4-supplemented groups than when cultured without hBMP4. The Matrigel coated plates contained large sheets of epithelial-type cell growth instead of compact colonies. This type of growth characteristic was present in all hBMP4 treatments on Matrigel. In contrast, few cells survived and propagated on human extracellular matrix. Only small colonies having the desired morphology were among the large cells on human extracellular matrix when cultured in medium containing 10 ng mL⁻¹ hBMP4. Cells were passaged and only cells growing on Matrigel could be further cultured. These data suggest that both the cell substrate and hBMP4 affect initial trophoblast outgrowths. Further analysis including immunocytochemistry and RT-PCR is currently being performed to better characterize these cells.