A272 Cloning, Transgenesis and Stem Cells

The morphology-based selection of mouse spermatogonial stem cells (mSSC) provides a cell population with pluripotency characteristics similar to mouse embryonic stem cells (mESC)

A.G. Galuppo, P.C. Chagastelles, L.A. Santos, P. Pranke

UFRGS.

Keywords: mouse embryonic stem cell, mouse spermatogonial stem cell, pluripotency.

The knowledge that mouse spermatogonial stem cells (mSSCs) present pluripotency characteristics similar to mouse embryonic stem cells (mESCs) opens a new path in cell therapy and regenerative medicine research. The aim of this work has been to verify if the morphology-based selection of mSSC provides a cell population with pluripotency characteristics similar to the mESC. The mSSCs were isolated from 5-10 day-old C57Bl/6 mice. The testicles were removed and the seminiferous tubules were treated with collagenase/DNase followed by trypsin. Following this, the cells were passed through a 70 μm nylon mesh and kept in culture in a 0.1% gelatin-coated dish. The cells were submitted to a sequence of incubation. The supernatant of the final incubation was collected and plated on a feeder layer composed of mouse embryonic fibroblasts inactivated with mytomicin C. The culture conditions for the mSSCs were 32°C in a 5% CO2 atmosphere and 90% humidity. The culture medium used was alpha modified MEM, supplemented with fetal calf serum (FCS), penicillin and streptomycin, non-essential amino acids, b-mercaptoethanol, N21, GDNF, EGF and bFGF. A lineage of mESCs (C57Bl/6), previously isolated in the laboratory, was used as a control group. The mESCs were cultured in DMEM high glucose, with FCS, penicillin and streptomycin, LIF, sodium piruvate, L-glutamine, b-mercaptoethanol and non-essential amino acids. The culture conditions for the mESCs were 37°C in a 5% CO2 atmosphere and 90% humidity. To analyze the morphology of the colonies, phase contrast microscopy was used and a DAPI/Phalloidin stain was performed. The pluripotency markers Oct-4 and Sox-2 were analyzed by immunofluorescence and the SSEA-1 expression was analyzed by flow cytometry. The morphology analysis by phase contrast and DAPI/Phalloidin staining showed that the mSSCs were organized in colonies like the mESCs. That can be considered as the first sign of mSSC pluripotency because it is the main morphological mESC characteristic. The immunostaining for Oct-4 and Sox-2 was positive in both groups, showing that two of the most important pluripotency transcription factors are present in the mSSCs. The SSEA-1 expression was only observed in the mESCs. However, in the literature it is related that the SSEA-1 expression in mESCs can vary and that it will probably not be the best pluripotency reference for comparison with the mSSC. These preliminary results showed that the isolation of mSSCs based on morphology leads to cells with pluripotent characteristics when compared with the mESCs. However, a more complete evaluation of pluripotency characteristics of these cells is needed, such as the production of embryoid bodies and the chimeras. Because mSSCs have no ethical problems compared to mESCs, they could be an interesting tool for use in cell therapy and regenerative medicine studies.

Financial support: CNPq, CAPES, FAPERGS and Stem Cell Research Institute.

A273 Cloning, Transgenesis and Stem Cells

Immunofluorescence and flow cytometry on characterization of equine induced pluripotent stem cells

M.D. Guastali[1], M.S. Araújo[1], B.A. Monteiro[1], D.M. Paschoal[1], R.R.D. Maziero[1], T.L. Ikeda[1], F.F. Bressan[2], F.V. Meirelles[2], F.C. Landim-Alvarenga[1]

[1]Universidade Estadual Paulista Botucatu; [2]Universidade de São Paulo.

Keywords: flow cytometry, immunofluorescence, induced pluripotent stem cells.

The induced pluripotent stem cells (iPS) are adult cells genetically reprogrammed to a state similar to embryonic stem cells. Lentiviral vectors are used as a safe and effective way of producing iPS. The umbilical cord (UC) is a reserve of multipotent mesenchymal stem cell and therefore may represent an efficient source of cells to be reprogrammed. To characterize reprogrammed cells specific positive markers for pluripotent clones are used such as Oct-4, Nanog, Sox-2, TRA-1-60, TRA-1-81, SSEA-1, and SSEA3 SSEA-4. In this context, the aim of this study was to characterize, by immunofluorescence and flow cytometry methods, cells from equine cord matrix after reprogramming with lentiviral vector. For this, five samples of horses UC were collected at birth. Umbilical matrices were subjected to enzymatic digestion in a solution of 0.004% collagenase diluted in PBS, and cells obtained by filtration were plated into plastic culture flasks with 5 mL DMEM supplemented with 20% fetal bovine serum, antibiotics and antimycotics, followed by incubation at 37°C in a 100% wet, 5% CO2. When cells reached 40% confluence, a concentration of 10^5 cells were transfected with lentiviral human-vector EF1α 50mL STEMCCA (OKSM) (Millipore, SCR544), which contains the transcription factor OCT-4, SOX2, C-MYC and KLF-4 to generate iPS cells, according to the manufacturer's protocol, plus 8 ng/mL polybrene (hexadimethrine bromide, Sigma). The culture medium was renewed 12 hours after incubation. Five days after transduction, the cells were transferred into a subculture of mouse fibroblasts and cultured for 14 days in a specific medium for iPS. The first colonies were visualized after two weeks of the infection. When the clones were well established two mechanical passages and two enzymatic were made. After the re-establishment and proliferation of colonies, they were subjected to immunostaining protocols and analyzed by flow cytometry. Immunocytochemistry was performed in 6-well plates containing arround 20 to 30 colonies per well. For flow cytometry 200.000 cells were analyzed for each of the antibodies. In both techniques the following antibodies were used: Oct-4, Nanog, Sox -2, TRA- 1-60 and TRA- 1-81. We noticed that, in the immunofluorescence, horse umbilical cord cell's colonies scored positive for Oct-4, Sox-2 and TRA-1-81, as in flow cytometry, colonies were positive for Oct-4, Nanog, TRA-1-60 and TRA-1 -81. Although the markers used were specific to pluripotent cells, we did not observe a correspondence between the two techniques. Despite this, we can conclude that the equine umbilical cord cells were successfully reprogrammed and presented characteristics of pluripotent cells.

A274 Cloning, Transgenesis and Stem Cells

Adult stem cells and animal reproduction: potential use of adipose MSCs as alternative to the traditional granullosa co-culture system in bovine IVEP

H.S. Nascimento[1], M.P.R. Costa[1], C.T.A. Lopes[1], F. Brito[1], K.N.L. Brito[1], C. Carvalho[2], S.S.D. Santos[1], O.M. Ohashi[1], M.S. Miranda[1]

[1]Universidade Federal do Pará; [2]Universidade de São Paulo.

Keywords: adipose mesenchymal stem cells, bovine IVEP, co-culture.

In bovine IVEP (b-IVEP), the use of co-culture system with granulosa cell monolayer is still very traditional. Somatic cells in co-culture would protect embryos from toxic metabolites and oxidative stress (Orsi, et al., Theriogenology (67), 441–458, 2007). It has been showed that MSCs are multipotent, secrete growth factors and cytokines, and can be isolated from many tissues of many species (Dimarino. Front. Immunol.(4) 201, 2013), including bovines. In this work we compared bovine adipose derived mesenchymal stem cells (b-AMSCs) and granulosa cells in co-culture system of b-IVEP. B-AMSCs were isolated from an adult female (collagenase type I - 1µg/mL) and cultured in IMDM with 10% FBS. At P3, stemness was evaluated by immunophenotyping (CD73, CD90 and CD105) and three-lineage *in vitro* differentiation (bone, adipose and cartilage). IVEP followed standard protocol. Oocytes were matured for 20 hours (TCM-199 with 10% FBS, FSH and LH) and fertilized for 24h with frozen semen from only one bull. Embryos were cultured in 100µL droplets of SOF medium with 5% FBS and 6 mg/mL BSA for 7 days in 5% CO2 at 38.5 °C. In experiment 1, two concentrations of b-ASCs were tested for co-culture: 10^3 b-AMSCs and 10^4 b-AMSCs. In experiment 2, embryos were cultured with 10^4 b-AMSCs, with IVM derived granulosa cell monolayer or without co-culture (Control). Cleavage and Blastocysts numbers were recorded on day 2 and 7 respectively. Blastocysts were analyzed for total cell number, and gene expression (POU5F1, G6PDHand HSP70). Results were analyzed by "t" test or ANOVA at 5% significance level. Stemness of b-AMSCs was confirmed at P-3 (CD73+, CD90+ and CD105+) as well as stem cells were successfully *in vitro* differentiated into adipose, bone and cartilage tissues. In experiment 1, the use of 10^4 b-AMSCs in co-culture improved ($p<0.05$; t test) blastocyst rate (54.16% ± 8.18) in comparison to the use of 10^3 b-AMSCs (33.11% ± 6.06). In experiment 2, blastocyst rate of co-culture with 10^4 b-AMSCs (45.97% ± 12.5; n= 231) was superior ($p<0.05$) to co-culture with granulosa cells (33,67% ± 14,7; n= 231) and Control (34.70% ± 9.8; n= 232). Moreover, co-culture with 10^4 b-AMSCs increased ($p<0.05$) total cell number of blastocysts (177.4 ±35.3; n=28) compared to co-culture with granulosa (136.2 ± 26; n=22) and Control (115 ± 21; n= 23). Finally blastocysts co-cultured with 10^4 b-AMSCs showed an increased expression ($p<0.05$) of POU5f1 (pluripotency) and G6PDH (glucose metabolism) in comparison to embryos co-cultured with granulosa cells. In conclusion, b-AMSCs (10^4 cells) can be successfully used for co-culture in b-IVEP. Compared to the traditional co-culture system with granulosa cells, co-culture with b-AMSCs improved quantity and quality of obtained embryos. Further studies must be done to evaluate the pregnancy rates of the embryos and to identify factors responsible for the effect of AMSCs on embryo development.

A275 Cloning, Transgenesis and Stem Cells

Development of pig embryos produced by electrical or chemical parthenogenetic activation

L.C.B. Motta[1], L.C. Magalhães[2], C.F.G. Jesus[2], D.Í.A. Teixeira[2], L.M. Melo[2], V.J.F. Freitas[2], M.H. Bhat[2]

[1]Universidade Federal do Ceara; [2]Universidade Estadual do Ceará.

Keywords: chemical activation, electrical activation, parthenogenesis.

Parthenogenetic embryos produced after artificial activation of oocytes have high importance for the study of embryonic processes, including morphological and biochemical changes essential for development. The objective of this study was to evaluate the effect of parthenogenetic activation method (electrical or chemical) in pig oocytes on embryonic development rates. Cumulus-oocyte complexes (COCs) were recovered from slaughterhouse ovaries and matured in TCM 199 for 48 hours at 38.5°C and 5% CO2. After IVM, the COCs were denuded by repeated pipetting in TCM containing hyaluronidase and vortexed for 3 minutes. Chemical activation was performed with ionomycin (5 mM) for 5 minutes (Group IONO) and the two electric experimental groups, using 20V or 40V (both for 80 μsec), all structures were incubated in 6-DMAP (2mM) for 3 hours, followed by IVC in SOF for eight days. The cleavage and embryonic rates were assessed after three and eight days in culture, respectively. Data analysis was performed Fisher's exact test with $P < 0.05$. Out of 125 slaughterhouse ovaries, 567 COCs were obtained after puncture for IVM. Of these, 392 were matured resulting in maturation rate of 69.1%. These structures were used for parthenogenetic activation, activation being divided into three groups (IONO, 20V and 40V). In IONO, 20 V and 40V groups 153, 115 and 124 oocytes were activated, respectively. The activation rates in IONO, 20V and 40V groups were 74.5% (114/153), 80.9% (93/115) and 78.2% (97/124), respectively without any statistical difference ($P > 0.05$). The embryonic development rates in IONO, 20V and 40V groups were 21.1% (24/114), 17.2% (16/93) and 15.5% (15/97), respectively; showing no statistical differences between groups ($P > 0.05$). In conclusion, pig parthenogenetic embryos can be obtained efficiently by methods tested.

A276 Cloning, Transgenesis and Stem Cells

Effect of trichostatin a in cloned cattle embryo production by nuclear transfer with mesenchymal stem cells

C.G. Silva[1,3], H.C. Bessler[2], Á.M. Fonseca Neto[2], C.F. Martins[2], S.N. Báo[3]

[1]EMBRAPA Cerrados; [2]EMBRAPA Cerrados; [3]Universidade de Brasília.

Keywords: cloning, epigenetic, histone deacetylase.

Acetylation of histones is a major mechanism of genome epigenetic reprogramming of the gametes in order to establish a totipotent state to the normal development (Ikeda et al., Zygote 17, 209-215, 2009). The deacetylation is catalysed by histone deacetylases (HDAC) which remove acetyl groups and causes chromatin compaction and DNA segment silencing at this location (Johnstone, Nature Reviews Drug Discovery 1, 287-299, 2002). The trichostatin A (TSA) is an HDAC inhibitor that increases the amount of acetylated histones and the demethylation of DNA (Lee et al., Journal of Reproduction and Development 57, 34-42, 2011). In this sense, the drug has been used in an attempt to increase the production efficiency of embryos by nuclear transfer (NT). The objective of this study was to test the effect of TSA in exposure times of 20 and 25 hours in the culture of bovine embryos cloned by NT with mesenchymal stem cells (MSCs) derived from adipose tissue. A biopsy of skin and adipose tissue was collected from the perineal region of a female bovine Gir, at two months of age. The cells were isolated by the explant and cultured in Dulbeccos Modified Eagle Medium added with 10% fetal bovine serum. Cumulus-oocyte complexes recovered from slaughterhouse ovaries were matured for 18 h at 38.5°C and 5% CO_2. The NT was then performed with the adipose tissue and the MSCs reconstructed embryos were subjected to culture with 50 nM TSA for 20 and 25 h, for 4 h in activation medium containing 6-DMAP and further for 16 or 21 h in medium cultivation. Subsequently, the embryos continued in culture in synthetic oviductal fluid (SOF) medium without TSA and the parthenogenetic control was performed in every manipulation. Five NT procedures were performed for each treatment (20 h, 25 h and without TSA). Fusion rates, cleavage and blastocyst production were compared by Tukey test ($p < 0.05$). The cleavage rate of parthenogenetic embryos (93.45 ± 7.97) was higher than the cleavage rate of embryos without treatment (82.45 ± 5.59) but was statistically similar to embryos treated with 20 and 25 h (87.25 ± 8.41 and 85.54 ± 3.88, respectively). Still, there was no difference in cleavage rate between treated and untreated embryos. The blastocyst production rate on the seventh day of culture was superior to the parthenogenetic control (59.24 ± 11.75) compared to treatments for 20 h (36.22 ± 16.80), 25 h (33.66 ± 12.84) and without the use of TSA (32.70 ± 9.11), which did not differ. It can be concluded that the use of TSA had no significant effect in improving the rates of cleavage and development of bovine embryos by nuclear transfer with MSCs from adipose tissue. However, additional studies to evaluate the quality and embryos of the methylation pattern should be conducted to better understand the effects of TSA in embryos cloned with this cell type.

A277 Cloning, Transgenesis and Stem Cells

Cell cycle effect and inhibition of deacetylation on development of swine embryos produced by nuclear transfer

V.B. Rissi[1], W.G. Glanzner[1], L.K. Serrano[1], M.W. Manta[1], M.P. Macedo[1], A.Q. Antoniazzi[1], P.B.D. Gonçalves[1], V. Bordignon[2]

[1]UFSM; [2]Mcgill University.

Keywords: cell cycle, cloning, scriptaid.

There is evidence from a number of studies in different species that treatment of somatic cell nuclear transfer (SCNT) embryos with inhibitors of histone deacetylase enzymes (HDACi) facilitates cell reprogramming and improves development. The aim of this study was to evaluate if the positive effect of HDACi treatment on SCNT embryos is affected by the cell cycle stage of nuclear donor cells and host oocytes at the time of embryo reconstruction. SCNT embryos were produced with MII or TII cytoplasts and G0-1 or G2/M stage nuclear donor cells. To obtain cells at G0-1 stage, fibroblasts were maintained in culture for at least 48h after reaching confluence and fixed for cell cycle analysis. The proportion of cells in G0-1 (88.2 ± 4.3%), S (2.7 ± 1.9%) and G2/M (9.1 ± 2.8%) confirmed that most of the cells were at the expected phase of the cell cycle. To obtain G2/M cells, confluent cells were trypsinized and plated in a non-confluent density, which allows for a synchronized wave of cells resuming their cell cycle and progressing to S and then G2-phases. The cells were then trypsinized and fixed for cell cycle analysis at 0, 16, 20, 24, 28, 32 and 36 h after plating. We confirmed that more than 30% of cells have reached the G2/M phase at 24 to 28 h post-passage from confluent cultures. Thus, we selected for nuclear transfer only the larger cells (mean diameter = 25.9µm) collected between 24 and 28h after plating from confluent cultures. Embryos reconstructed with the different cell cycle combinations were treated or not with the HDACi Scriptaid (500 nM) for 15 h, and then cultured in vitro for 7 days. A total of 568 embryos were reconstructed using G0-1 cells and MII or TII cytoplasts. Development to the blastocyst stage was significantly higher in MII (19.8%) than TII (4.6%) cytoplasts. Interestingly, Scriptaid treatment enhanced embryo development in MII cytoplasts (MII 19.8% vs. MII+S 32.6%; p=0.09), but not in embryos reconstructed with TII cytoplasts (TII 4.6% vs TII+S 6.2%; p=0.73). A total of 448 embryos were reconstructed using G2/M donor cells. Development to the blastocyst stage was significantly lower in MII (8.7%) compared to TII (22.2%) cytoplasts. Scriptaid treatment enhanced development in embryos reconstructed with MII (MII 8.7% vs. MII+S 16.6%) but not TII (TII 22.2% vs. TII+S 22.4%) cytoplasts. In summary, embryos reconstructed with MII-G0-1 and TII-G2/M developed to the blastocyst stage in higher frequency compared to the other groups, which confirms the importance of cell cycle interactions on cell reprogramming and SCNT embryo development. Treatment of reconstructed embryos with HDACi improved development of embryos produced with MII but not TII. Our findings indicate that: i) cell cycle interactions between the host cytoplast and the nuclear donor cell affect nuclear reprogramming; and ii) the response of SCNT embryos to HDACi treatment depends on factors present in MII-stage cytoplasts.

A278 Cloning, Transgenesis and Stem Cells

Potential of completed conditioned medium (CM), supernatant (SN), microvesicles (MV) and lysed microvesicles (L-MV) derived from mesenchymal stromal cells (MSC) of equine amnion in suppressing allogeneic lymphocyte proliferation: preliminary results

B. De Vita[1], A. Lange-Consiglio[2], P. Romele[3], C. Perrini[2], P.B. Signoroni[3], F.C. Landim-Alvarenga[1], F. Cremonesi[2], O. Parolini[3]

[1]FMVZ/UNESP; [2]Universidade de Milão; [3]Centro di Ricerca E. Menni/Fondazione Poliambulanza.

Keywords: cell, immunity, mesenchymal.

MSCs have been shown to secrete molecules with interesting paracrine effects and immunomodulatory properties that can be found in the medium where they are cultured. In addition to soluble factors, recent studies have suggested that some of the regenerative effects of MSCs are mediated by MVs. MVs can be classified as shedding vesicles released from plasma membrane or exosomes (endosomal membrane origin), and represent an important way of intercellular communication. The aim of this study was to evaluate the potential of the complete CM (soluble factors + MV), SN (soluble factors), MV and L-MV (internal content) derived from equine amnion MSCs (EqAMSC) in suppressing allogeneic peripheral blood mononuclear cells (PBMC) proliferation. Conditioned medium (CM) was obtained from the culture of $1x10^6$ equine amnion cells/ml after overnight serum privation culture. Microvesicles (MV) were obtained by ultracentrifugation of the CM. After centrifugation of the microvescicles, the supernatant (SN) was also recovered. Lysed Microvesicles (L-MV) samples were obtained after MVs' sonication. Three samples of each group were evaluated in triplicate. The different preparations mentioned above were added at different volumes and concentrations: CM and SN were plated 170µL, 120µL and 80µL, and MV was plated at $250x10^6$, $50x10^6$, $10x10^6$ and $2x10^6$ MV/well added to a culture of equine PBMC cells ($2x10^5$ in a final volume of 200µL of DMEM completed medium) in the presence of the mitogen phytohemagglutinin (PHA) at a final concentration of 2 µg/mL. Lymphocyte proliferation was assessed after 3 days of culture by adding 0.67 µCi/well of [3H]-thymidine for 16–18 hours and thymidine incorporation was measured using a microplate scintillation and luminescence counter. Next, to evaluate the effect of the internal content of MV on PBMC proliferation, L-MV samples were plated in the correspondent volume of three different concentrations of the entire MV resuspended in DMEM plated at $100x10^6$, $50x10^6$ and $5x10^6$ MV/well. Then, the PBMC proliferation assay was performed as already described. Paired T-test, baseline corrected unpaired T-test and ANOVA plus Tukey's test were used with GraphPad Prism software version 6 (Significance $P \leq 0.05$). The preliminary results obtained indicate that CM and SN were both able to significantly suppress more than 50% the PBMC proliferation when 170 µl and 120 µl were used; whereas none of the MV concentrations seemed to have any effect on lymphocyte proliferation, even after lysing. Based on these results, we conclude that MV and their internal content did not impact on lymphocyte proliferation, as shown by the CM and SN. However the immunomodulatory effects of MV cannot be ruled out since more studies are required regarding MV and L-MV influence on the different lymphocytic subpopulations.

Acknowledgments: FAPESP, Milan University and Poliambulanza Foundation.

A279 Cloning, Transgenesis and Stem Cells

Clones embryo production in deer *(Mazama gouazoubira)* using goat oocytes: preliminary results

C.F.G. Jesus, L.C. Magalhães, L.C.B. Motta, D.I.A. Teixeira, L.M. Melo, V.J.F. Freitas, M.H. Bhat

UECE.

Keywords: deer, goats, iNTSC.

The brocket deer (*Mazama gouazoubira*) is a species of deer present in northeastern Brazil. Due to the decrease of its territory and poaching, among others, this species may be threatened in the coming years. Furthermore, it can be used as a model for the maintenance of species already endangered by the use of interspecific reproductive cloning. The aim of this study was to produce interspecific Nuclear Transfer Somatic cell (iNTSC) clones embryos (deer and goats) and evaluate their in vitro embryo development. Intraspecific cloned embryo (goat and goat) were used as control. Fourteen goats were used as oocyte donors after hormone treatment consisting progestin / luteolytic and FSH shortly after the end of treatment. After ovarian puncture, the oocytes were evaluated for IVM and placed in TCM 199 supplemented with 10% FBS for 22-24 hours in humidified atmosphere at 38.5°C and 5% CO2. After IVM, oocytes were denuded and stained with Hoechst 33342 for enucleation using an inverted microscope (Nikon TE2000, Tokyo, Japan) equipped with micromanipulators (Narishige, Tokyo, Japan), under UV light. Previously, goats and deer fibroblasts were grown to 95% confluence, and used as karyoplastos to NTSC and iNTSC respectively, in the passage 3-7. The embryos were reconstructed by electrofusion (Multiporator, Eppendorf, Hamburg, Germany) using ionomycin-DMAP activation procedure. Those in which the reconstruction was confirmed were cultured in vitro for eight days in mSOF supplemented with 5% FCS in a humidified atmosphere at 38.5°C with 5% CO2, 5% O2 and 90% N2 (Eve, WTA, Craven , Brazil). The embryonic development rates were evaluated by Fisher's exact test with $P < 0.05$. From 28 ovaries 124 oocytes were obtained, which resulted in an average of 4.4 oocytes per ovary. Out of oocytes harvested 56 (45.0%) were considered grade I and placed to IMV. The maturation rate was 75.0% (42/56). Out of the matured oocytes, 24 were used for iNTSC and 13 for NTSC. Cleavage rate of 38.4% and 25.0% for iNTSC and NTSC embryos was obtained, respectively, ($P > 0.05$). The blastocysts rate in iNTSC group was 50.0% (3/6) and in NTSC was 40.0% (2/5), with no significant difference ($P > 0.05$). These preliminary results show that goat oocytes have the potential to be used as cytoplasts for deer in future conservation programs.

A280 Cloning, Transgenesis and Stem Cells

A reliable protocol to synchronize fibroblast cells at the G2/M phase

J.R.M. Souza[1], V.B. Rissi[1], W.G. Glanzner[1], K. Campagnolo[1], R.M. Freitas[1], F. Ceni[1], A.Q. Antoniazzi[1], P.B.D. Gonçalves[1], V. Bordignon[2]

[1]Universidade Federal de Santa Maria; [2]Mcgill University.

Keywords: Cdk1, cell cycle, ro-3306.

Cell cycle coordination is important to investigate nucleus-cytoplasmic interactions after cell fusion and nuclear transfer in basic research. However, there are no reliable protocols that result in higher rates of cells synchronized at the G2/M phase during in vitro culture. The aim of this study was to evaluate the efficacy of a CDK1 inhibitor (RO3306) to synchronize fibroblasts cells at G2/M phase. Porcine fibroblast cells were maintained in culture until reaching confluence and then used to prepare non-confluent cultures by plating 50,000 cells per well of a 6-well plate in 2 ml of culture media. Cells were then trypsinized and fixed for cell cycle analysis at 0, 16, 20, 24, 28, 32 and 36 h after plating. A non-confluent density of cells starting from a confluent culture allows a synchronized wave of cells resuming their cell cycle and progressing to S- and then G2-phases. Indeed, we observed that more than 30% of the cells reached the G2/M phase at 24 to 28 h post-passage from confluent cultures. Based on these results, we tested the effect of two doses (5µM and 9µM) of RO3306 starting at 16 or 20h post-passage from confluent cultures. Cells were fixed to determine their cell cycle stage at 24, 28, 32, 36 and 40h after plating. For cell cycle analysis, cells were trypsinized (0.25% trypsin-EDTA) and re-suspended in ice-cold fixation solution (70% ethanol and 30% PBS) for 15 minutes. Fixed cells were then pelleted by centrifugation and re-suspended in PBS. Before flow cytometry analysis, cells were re-suspended in 1ml PBS containing 50µg propidium iodide and 100µg RNAse at 37˚C for 40 minutes. DNA content of 10,000 cells was determined by fluorescence-activated cell sorting (FACS) using a FACSVerse system (BD Biosciences, San Jose, CA). The percentage of cells at G0-1, S or G2/M was calculated using the FACSsuite Software (BD Biosciences). When treatment started at 16h post-passage, the highest proportion of cells synchronized at the G2/M phase (41.9%) was observed in the group exposed to 5µM RO3306 until 32h, which was significantly higher than control cells fixed at the same time (22.2%). When treatment started at 20h post-passage, higher percentages of G2/M cells were obtained in the groups treated with 9µM RO3306 and fixed at 36h (61.7%) and 40h (56.1%) post-passage. The diameter of cells that were at the different cell cycle stages was determined in cells treated with 9µM RO3306 and fixed at 36 or 40h post-passage. The mean diameter of cells in G1-0 and G2/M phase was 13.9µm and 25.9µm, respectively. Our results provide a reliable methodology based on CDK1 inhibition and cell diameter for selection of G2/M cells.

A281 Cloning, Transgenesis and Stem Cells

Somatic cell nuclear transfer in the equine with donor cells in G2 and oocytes in telophase II

M.P. Maserati Jr, P. Fleury

In Vitro Clonagem.

Keywords: clone, equine, somatic cell.

Equine production by somatic cell nuclear transfer (SCNT) has only been reported by a few labs due to the lack of available equine oocytes and the high cost of maintaining and working with equine. The protocol commercially used for cloning equine by our competitors was adapted from the procedure used to clone Dolly the sheep. In 2005 In Vitro Clonagem (IVC) developed a novel procedure for cloning bovine which we later adapted for use in equine. The Dolly protocol uses donor cells in G1 or G0 of the cell cycle and enucleated metaphase II oocytes whereas the In Vitro Clonagem protocol uses donor cells in G2 of the cell cycle paired with enucleated oocytes in Telophase II of meiosis. Here we compare the procedure used to clone Dolly with the IVC novel cloning technique, both adapted for use in the equine. Our results demonstrate a similar rate of cleavage and a superior rate of blastocyst production and initial pregnancy in comparison with the Dolly cloning method. Overall, we observed: 1. Cleavage rates of embryos were similar between both protocols. 2. The observed increase in blastocyst rate of the IVC over the Dolly method of cloning was statically significant. 3. The observed increase in pregnancy rate of the IVC cloning method over the Dolly cloning method was statically significant. 4. We lost the pregnancy established using the Dolly method of cloning whereas we have four foals born from the IVC cloning method. We have successfully adapted our bovine cloning method using oocytes in Telophase II and donor cells in G2 phase of the cell cycle for use in the equine. The adapted protocol outperforms the method used to clone Dolly in terms of embryo development to the blastocyst stage and establishing pregnancies. Although we are still waiting for live birth results, we have already delivered four cloned equine and are waiting for more to be born in the year ahead.