A194 Cloning, Transgenesis and Stem Cells

Effect to cell type and transfection method in production of transgenic bovine cells

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Keywords: genetic modification, GFP, transgene.

In spite of the advances in the field and the availability of methodologies, the production of transgenic cattle by nuclear transfer can still be considered a low efficiency biotechnology. Among other factors, this can be occasioned due to the way that exogenous DNA is integrated into the donor cell nucleus. In general, the insert integration into chromosomal DNA occurs spontaneously, but it is an uncommon and random process, which makes gene expression, when present, unpredictable. The lentiviral transduction, however, allows more precise transgene integration. Another factor that may influence transgene expression and thus, the success of the production of a transgenic bovine, is the cell type used as donor cell nucleus, since each cell type may have a different pattern of transgene expression due to epigenetic factors. Therefore, the aim of this study was evaluate the cell type influence [fetal fibroblasts (FF), adults fibroblasts (FA), or cumulus cells (CC)] for incorporation of the GFP (Green Fluorescent Protein) transgene derived from plasmid FUGW genetically modified by lentiviral transduction (G1) (ViraPower Lentiviral Expression System, Invitrogen, Carlsbad, USA) or by lipofection (G2) (Lipofectamine 2000, Invitrogen, Carlsbad, USA). The samples were analyzed 48 hours post transfection by flow cytometry (FACSAria, Software: FACSDIVA). 10,000 cell/repetition were count and two repetitions were made. Transfections were performed following the manufacturers' instructions. Since significant interactions were observed ($P < 0.05$), the analyses were made for each cell type in relation to each treatment. Means were compared by SNK test (Student-Newman-Keuls, $P<0.05$). The lentiviral system (G1) showed to be the most efficient transfection method independent of cell type analyzed (FF: 88.8 % ± 0.98, FA: 91.6 ± 2.96% CC: 60.7 % ± 14.7) and differed significantly from G2 (FF : 17.8 ± 2.82; FA: 10.66 ± 0.65, CC: 3.9 ± 1.97). When the lipofection was used, fetal fibroblasts showed a higher number of GFP+ cells being followed by adult fibroblasts and the cumulus cells. With respect to fluorescence intensity, which is related to levels of expression, there was no difference between G1 (FF: 4273 ± 118.79; FA: 7957.5 ± 1120 CC: 6020.5 ± 310.42) and G2 (FF: 4542 ± 497.09; FA: 9367.5 ± 3490; CC: 3486 ± 2638). With regard to cell type, adult fibroblasts showed higher fluorescence intensity, followed by granulosa cells and fetal fibroblasts, in boot transfection methods. Thus, it can be concluded that the efficiency of gene transfer differs between cell types according to the transfection method used and that the lentiviral transduction resulted in enhanced transfection rates independent of cell type analyzed.

A195 Cloning, Transgenesis and Stem Cells

Immunophenotypic differences between embryonic-like stem cells and induced pluripotent cells (IPS) in the horse

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Keywords: immunostaining, pluripotents, stem cells.

Differently from other usual cell cultures which aim to obtain protein or virus products from cells, pluripotent stem cells culture presents a challenge: a culture whose aim is to improve the number of cells in an undifferentiated state. Despite the high biomedical potential, the maintenance of in vitro pluripotency is still considered a problem in domestic species. There are many studies involving primates and murines embryonic stem cells (ESCs). However, there is still little information for the equine species. ESCs are pluripotent cells derived from inner cell mass, obtained from pre-implanted blastocysts. On the other hand, iPS cells are adult cells genetically reprogramed to an embryonic state, expressing genes and important factors for the maintenance of pluripotents properties. The aim of this work is to compare the expression of pluripotent markers between equine ESCs and iPS cells. For ESCs culture an equine fibroblast monolayer was used, and a mouse fibroblast monolayer was used for iPS cells culture, both properly blocked with Mitomycin C. The ESCs were obtained from inner cell masses from fresh and cooled equine blastocysts. For iPS transfection, lentiviral vector STEMCCA, composed with the combination of four transcriptions factors (Oct-4, Sox-2, Klf4 and c-Myc) was used, through equine umbilical cord cells. The immunofluorescence was made in embryonic-like stem cells colonies and iPS. The cultures were fixed and immunostained according to the manufacturer's recommendation. For the evaluation of pluripotents markers immunofluorescence, ESC and iPS cells colonies were cultured in 24 wells coated plated dishes and stained with monoclonal antibodies of ESC Chemicon® kit (Milipore, SCR 002). The immunofluorescence protocol utilized was effective, once equine ESCs obtained from fresh embryos were positively stained for Oct-4, SSEA-1 and SSEA-4, although TRA-1-60 and TRA-1-81 showed less labeling. The equine ESC from cooled embryos were positively stained for Oct-4, SSEA-1, -3 and -4. iPS cells only expressed Oct-4 marker. The negative expression for the other pluripotents markers on iPS cells can be explained by the utilization of nonspecific markers (mouse), once the viral vectors were built based in human DNA. The pluripotent characterization is necessary to confirm the equine ESC and iPS lineage, with marker standardization for equine species.

A196 Cloning, Transgenesis and Stem Cells

Isolation, characterization and differentiation of bovine derived mesenchymal stem cells from amniotic fluid, Wharton's jelly and adipose tissue

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Keywords: assisted reproduction, bovine, mesenchymal stem cells.

Mesenchymal stem cells (MSC) are multipotent stem cells with well-defined characteristics and intrinsic capacity for self-regeneration and differentiation into functional cell types (Baksh D, Journal of Cellular and Molecular Medicine, v. 8, p. 301-16, 2004). These cells can be used in regenerative medicine in humans (Wagner W, Experimental Hematology, v. 33, p. 1402-16, 2005) and domestic animals (Cremonesi F, Theriogenology, v. 75, p. 1400-15, 2011). The objective of this study was to isolate, characterize and differentiate in MSC, different cellular types from amniotic fluid, adipose tissue and Wharton jelly. Firstly, transvaginal amniocentesis by ultrasound-guided was performed in a Guzerá cow with 65 days of pregnancy for recovery of amniotic fluid. The fluid obtained was centrifuged at 135g for 10 min and the supernatant was discarded. The sediment was resuspended in 3 mL Amniomax Complete II Medium (Gibco-BRL/Life Technologies, Rockville, USA). The umbilical cord was collected during parturition of this cow, and Wharton's jelly cells (WJC) was isolated by explant and cultured in Dulbecco's Modified Eagle Medium (DMEM/Invitrogen Life Science, Rockville, USA). The adipose tissue collection was realized in the same animal with seven months old. The biopsy of skin and adipose tissue was collected from perineal region and immediately immersed in phosphated-buffered saline solution (PBS) 37°C. Cells from skin and adipose tissue were culture of the same form that WJC. The morphological analysis was performed by brightfield and scanning electron microscopy (SEM) during cellular culture. For phenotypic and genotypic characterization of these cells, flow cytometry, immunohistochemistry, RT-PCR and differentiation induction in different cell lineages were performed. SEM micrographs revealed that adipose cells (AC) presented fusiform morphology; amniotic fluid cells (AFC) and WJC did not present defined form under culture. It was demonstrated that bovine WJC, AC and AFC can differentiate into osteogenic, chondrogenic and adipogenic lineagesas confirmed by Alizarin Red, Safranin O and Oil Red stains, respectively. Moreover, these cells were positive for CD105, CD73, CD29, CD90 and H2A markers and negative for CD45, CD34 and CD44 markers. As reported by the International Society of Cellular Therapy, MSC populations have to express CD73, CD90 and CD105. Both CD45 and CD34 are considered hematopoietic surface markers and are not expected their expression in MSC, as confirmed in the present study. The results showed that cells from amniotic fluid, adipose and Wharton jelly tissue are mesenchymal stem cells and can be used in the field of regenerative veterinary medicine and reproduction by nuclear transfer.

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A197 Cloning, Transgenesis and Stem Cells

Inability to control and synchronize the cell cycle of bovine induced pluripotent stem cells (biPS) after starving or inhibition of cyclin-dependent kinases

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Keywords: cell cycle, IPS, nuclear transfer.

The recent generation of bovine iPS cells (biPS) represent an important tool for improving the production of cloned animals since the use of undifferentiated nuclei donor cells (NDCs) have already shown to enhance nuclei reprogramming after nuclear transfer (NT). The efficient cell cycle synchronization of NDCs is crucial for successful developmental rates after NT. The most common protocols involve the serum starvation for 24 to 72h or else the use of cell-cycle controlling drugs, followed by nuclear transfer of the nuclei donor to the metaphase-II ooplast. Herein biPS cells were generated and the rates of cell cycle synchronization aiming its use as NDCs at NT was evaluated. For that, biPS cells were transduced with murine lentiviral pluripotency-related transcription factors (Oct4, Sox2, c-Myc and Klf4 - OSKM) in bovine fibroblasts, characterized regarding its pluripotency and submitted to the treatments: knockout serum starvation for 24h (starving 24h), supplementation with roscovitine at 15μM for 12h (rcv 12h) and at 15μM for 24h (rcv 24h), and a control group. The cell cultures were treated following recommendations of the BD Cycletest Plus DNA Reagent Kit commercial kit and the cell cycle was analyzed by flow citometry (FACSCalibur, BD) in six replicates; the data was obtained with the CellQuest Pro Software and further analyzed by the ModfitLT V3.0 software. The percentage of cells in G1 was analyzed by Tukey test at 5% significance. The analysis on fibroblasts showed that the three treated groups were synchronized in G1, and the starving 24h group (average of 92.12% ± SD of 3.70) was more efficient than the rcv 12h (85.20±3.08) and rcv 24h (83.18±0.59) groups, when compared to the control group (68.24±5.18). In biPS cells, starving 24h (37.71±4.58) and rcv 24h (41.54±2.22) groups were similar to control (37.52±5.15), and however rcv 12h group (47.58±1.65) in the biPS was different from the other groups, the synchronization rate (inferior to 50%) is not satisfactory for its use as NDCs at TN. Moreover, serum starvation longer than 24h, as well as roscovitine treatment longer than 24h or in a concentration of 50μM or higher led to the loss of typical iPS morphology and high rates of cell death. The three treated groups were used as NDCs at NT in at least one replicate, and preliminary results showed no difference on embryo production between groups. In conclusion, the non-synchronization of the iPS cell cycle hampered the observation of a possible beneficial effect of its use in NT when ooplasts in metaphase-II were used. Other methodologies allowing the use of non-synchronized iPS cells in NT should be explored.

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A198 Cloning, Transgenesis and Stem Cells

Multiwalled carbon nanotubes vehicles for gene delivery into bovine embryos

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Keywords: carbon nanotubes, embryo, transfection.

The *zona pellucida* (ZP) is a protective barrier of embryonic cells against chemical, physical and biological substances. For this reason, usual transfection methods are not as efficient for mammalian oocytes and embryos as they are for somatic cells. Carbon nanotubes (CNTs) have emerged as a new method for gene delivery and they can be an alternatively used for embryos transfection, but its ability to cross the PZ and mediated gene transfer is still unknown. The aim of the present study was to determine whether multiwall carbon nanotubes (MWNTs) could pass through the PZ and delivery pDNA for *in vitro*-produced bovine embryos and their effects on apoptosis in bovine blastocysts. Oocytes obtained from ovaries collected at a local slaughterhouse were *in vitro* matured with TCM 199 (Invitrogen, California, USA) supplemented with 20 µg ml-1 FSH (Sigma, St. Louis, USA) and 10% fetal calf serum (FCS; Invitrogen) in high humidity, under 5% CO2 in air at 38.5°C for 24h. Maturated oocytes were subjected to *in vitro* fertilization in 100-µl drops of Fert-TALP supplemented with heparin and 2x106 spermatozoa/ml-1 for 6h in a humidified atmosphere of 5% CO2 and 38.8 ºC. Presumptive zygotes were distributed randomly in the following groups: G1 (control group - without MWNT); G2 (0.2 µg pDNA: 0.2 µg ml-1 MWNT), G3 (0.2 µg pDNA: 2 µg ml-1 MWNT) and G4 (0.2 µg pDNA: 4 µg ml-1 MWNT). Transfection was allowed to proceed in serum-free CR2aa medium for 12 h at 38.5°C in an atmosphere of 5% CO2. Subsequently, the embryos were transferred to CR2 medium containing 2.5% FCS under 5% O2, 90% N2 and 5% CO2 at 38.5°C in air and high humidity for 7 days. GFP expression in the transfected embryos at day 3 (72h post-fertilization) was observed under fluorescent microscope. Real time PCR examination (Applied Biosystems Prism 7300 Sequence Detection Systems, Foster City, EUA) was performed using the genomic DNA of the transformed embryos (n=30 per group). Blastocysts at eight day post-fertilization from G1 (n=19), G2 (n=23), G3 (n=16) and G4 (n=16) were fixed and permeabilized for TUNEL assay (DeadEndTM Florimetric TUNEL System-PROMEGA). Statistical analysis was performed by chi-square test or ANOVA ($P<0.05$). GFP expression was observed as in-situ fluorescence at 3 days post-transfection (embryos at 2 to 8-cell stage) for G2, G3 and G4. However, the expression GFP was not observed at blastocyst stage. The rate of positive-GFP embryos by PCR analysis in G4 (46.67%, n=14/30) was higher ($P<0.05$) than in G2 and G3 (3.33%, n=1/30). Apoptosis index was higher ($P<0.05$) in blastocysts from G2 (15.12±1.21), G3 (19.48±2.30) and G4 (18.20±1.90) than those in G1 (10.28±2.83). In conclusion, we showed that MWNTs are able to deliver the GFP gene into embryos. However, further studies are required to identify non-harmful exposure period and MWNT concentration.

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A199 Cloning, Transgenesis and Stem Cells

Evaluation of TN5 mediated transgenesis in vivo in ovine

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Keywords: ovine, transgenesis, transposon.

Transposable elements are DNA segments with the unique ability to move within the genome. In particular, transposons can be applied as vectors for gene transfer in vertebrates, and have already proven efficient to produce transgenic mice and pigs. Among them, the Tn5 transposition system allows the use of the commercial Tn5 transposase protein to generate in vitro the transposome complex with the transgen. Previously, we demonstrated that simple Tn5 cytoplasmic injection was efficient to produce high rates of transgene expressing embryos in vitro and confirmed integration in embryos by southern blot. In this work, Tn5 transposition mediated transgenesis was evaluated for the first time to produce transgenic ovine capable to express human recombinant factor IX (hrFIX) in milk. With this aim, adult Hampshire Down donor sheep were superestimulated (n=4) by insertion of intravaginal sponges for 14 days and during days 12-14, were injected with decreasing doses of FSH im every 12 h, administered twice daily for up to 12 h before sponge removal. On day 14, sponges were removed and a single dose of eCG (200 IU) was applied. The estrus was detected by males, and the ewes were inseminated by laparoscopy with frozen/thawed semen. Afterwards (16 h post insemination), presumptive zygotes were collected from the oviducts and cytoplasmically microinjected with the complex Tn5: hrFIXtransposon (20 ng/ul; protein: transgene with mosaic ends recognized by the transposon), in Mg+2 free medium. Immediately after microinjection, zygotes were transferred into receptor ewes oviducts (synchronized as donors, without the addition of FSH). As a result, a total of 28 presumptive zygotes were recovered and microinjected with the complex Tn5:hrFIX. A total of 12 microinjected presumptive zygotes were transferred to 5 donor ewes. It was obtained 1 pregnancy of siblings, but only one animal was born. The tissues and blood of the newborn were analyzed by nested PCR and it was determined that the animal was not transgenic. It is necessary to repeat the experience in order to determine the efficiency of Tn5 transposon transgenesis technique in ovine.

A200 Cloning, Transgenesis and Stem Cells

Attempting recovery of transgenic line of goat founder female by use of somatic cell nuclear transfer (SCNT)

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Keywords: cloning, goat, transgenesis.

Transgenic animals expressing recombinant protein of interest have an important economic value and its death may represent a big loss of investments. Our group obtained by DNA microinjection (Freitas et al., Small Rum. Res., 105:105-113, 2012) one transgenic female goat for human Granulocyte Colony Stimulating Factor (hG-CSF) which died after septicemia. The aim of this study was to use the somatic cell nuclear transfer technique for recovering the genetic material of this animal. Therefore, oocytes were collected by laparoscopic ovum pick-up from crossbred goats (n = 9) previously treated with progestagen/luteolytic/FSH. The oocytes were evaluated and selected for in vitro maturation (IVM) in supplemented TCM199 media for 21-23 hours in humidified atmosphere of 5% CO2. After IVM, the oocytes were denuded and stained with Hoescht 33342 for performing the oocyte enucleation by the use of inverted microscope (Nikon, TE2000, Japan) and micromanipulators (Narishige, Japan) under ultraviolet light. Caryoplasts were obtained from fibroblast culture from the transgenic founder female. The embryos were reconstructed by eletrofusion (Multiporator, Eppendorf, Germany) and subjected to culture in supplemented SOF in humidified atmosphere with 5% CO2, 5% O2 e 90% N2. For embryo transfer, four recipients with synchronized estrus were used. Pregnancy diagnosis was performed at 30 days after embryo reconstruction by ultrasonography (FalcoVet, Piemedical, Netherlands). A total of 195 follicles were punctured and 162 oocytes were obtained, resulting in a recovery rate of 83.1%, with 158 of quality compatible for IVM (97.5%). The maturation rate was 93.7% (148/158) and from matured oocytes, 106 were reconstructed, 102 were fused and 70 activated and subjected to culture, leading to an efficient rate of 43.2% (70/162). After culture, the development rate was 54.3% (38/70). Embryo transfer was performed in three sessions: a) one day post-reconstruction, in which 18 presumable zygotes were transferred to two recipients; b) two days post-reconstruction, in which, eight zygotes were transferred to two recipients and c) reconstructed zygotes were cultured for seven days but they were not transferred, due to the absence of response in recipients. No pregnancy was obtained during the pregnancy diagnosis by ultrasonography. In conclusion, despite a satisfactory number of oocytes obtained for further manipulations, it is imperative more repetitions with a higher number of recipients in order to be possible the recovery of this transgenic line.

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A201 Cloning, Transgenesis and Stem Cells

Efficiency of bovine commercial cloning

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Keywords: bovine, cloning, efficiency.

Animal cloning is a biotechnology with emerging commercial application that has been improving its efficiency despite low productivity. The low birth rates of healthy clones are attributed to high incidences of placental abnormalities leading to embryonic and fetal losses (Md Munir Hossain, BMC Genomics, 43, 1-15, 2014). Aiming to demonstrate the indexes obtained by the CENATTE Embriões/SEMEX Team, we monitored 885 cloned embryo transfers (ET) from 21 fibroblasts donors (20 female and 1 male), from October 2009 to May 2013 (Breeds: Gir, Brahman, Guzerá, Nelore and Tabapuã). The fibroblast were obtained by explants from caudal fold and the cloning technique utilized was done with micromanipulators according to Campbell (reviewed by Campbell et al, Cloning and Stem Cells, 3, 201-208, 2001). The efficiency rates were evaluated: ET, gestation and healthy clones delivered at 3 months. From the 885 ETs diagnosed by ultrasound, 41.8% (370/885) pregnancies were observed at 30 days gestation, and confirmed 21.7% (192/885) at 60 days. The gestational losses reached 48.1% (178/370) between 30 and 60 days and 80.2% (297/370) between 30 days to term. In addition, 8.3% births occurred (73/885), 67 clones were alive and 6 stillborn. The data of pregnancy and gestational losses are close to those presented by Y. Heyman, which obtained 33.5% (45/133) of pregnancy at 35 days, 57.7% (26/45) gestational losses between 35 and 70 days and 80.0% (36 /45) between 35 days until birth (Heyman Y et al., Biology of Reproduction, 66, 6-13, 2002). The postnatal survival was equivalent 71.6% (48/67). However, 28.4% (19/67) had some form of physical defect. Considering the total of 885 ETs, 1 male clone and 28 female healthy clones were delivered to customers up to the third month of life, representing an efficiency index in the entire process of commercial bovine cloning of 3.3% (29/885). This rate comes close to literature that states 5% of cloned embryos come to birth (Palmieri C, Vet Pathology, 45, 865-880, 2008). Considering breed, the cloning efficiency was 4.3%, 4.4%, 1.2%, 2.4% and 9.4% to Gir, Brahman, Guzerá, Nelore and Tabapuã, respectively. Donors that produced clones had an efficiency variation between 0.9% and 12.5%. However, 24.3% (7/21) never produced healthy calves. The data stated is similar to the one cited by Panarace, which 9% of embryos transferred resulted in clones with an efficiency variation of 1 to 10%, but 24% of the cell cultures never produced healthy calves (Panarace M et al., Theriogenology, 67, 142-151, 2007). Raising the viability of healthy clones may be associated improving the techniques of nuclear transfer, as well as, improving neonatal care. This would allow greater productivity of the entire bovine commercial cloning technique.