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In vitro generation of primordial germ cells (PGCs) from induced pluripotent stem cells (iPSCs) in cattle

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Keywords: iPSCs, PGCs, cellular reprogramming.

The nuclear reprogramming process provides significant contributions for both basic and applied sciences, as for example by enhancing animal production technologies or by the possibility of regenerative medicine with autologous cellular therapies for innumerous disorders. Several studies report that embryonic pluripotent stem cells (ESCs), and more recently, the induced pluripotent stem cells (iPSCs), are capable of differentiating into cells similar to primordial germ cells (PGCs) *in vitro* and even of deriving gametes (oocytes and spermatozoa) in the murine or human models. The present study aimed the generation of PGCs in vitro (iPGCs) from iPS cells in cattle and its characterization through pluripotency and germinative markers. For that, two bovine iPS cell lines previously generated and characterized (Bressan et al., Reprod Fertil Dev 27; 2015) were *in vitro* induced into PGCs (five repetitions) following the protocol previously described for murine iPS cells (Hayashi et al., Cell 146:4; 2011) and adapted herein. Briefly, bovine iPS cells were induced into an epiblast-like stage (EpiLCs) after culture in fibronectin-coated 6-well plates (16.7ug/ml) in N2B27 culture media supplemented with activin A (20 ng/ml), bFGF (12ng/ml) and KSR (1%) for 48 hours and induced into iPGCs by non-adherent culture (Agreewell plates) with GK15 medium (GMEM supplemented with 15% KSR, 0.1mM NEAA, 1mM sodium pyruvate, 0.1mM 2-mercaptoetanol, 2mM L-glutamine and 1% antibiotics) in the presence of cytokines BMP4 (500ng/ml), SCF (100 ng/ml), BMP8b (500ng/ml) and EGF (50ng/ml) for 4 days. The structures obtained were characterized regarding morphology, detection of alkaline phosphatase and specific markers OCT4, DDX4, VASA and c-Kit through immunofluorescence. The structures obtained in vitro were similar to PGCs and positive for the specific markers, an unpublished result worldwide. The *in vitro* production of PGCs from iPS cells represents an important tool for reproductive technologies providing crucial and unprecedented answers regarding molecular and cellular mechanisms involved in both cellular reprogramming and pluripotent germ cells biology.

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Effect of transfection and co-incubation of bovine sperm with exogenous DNA on sperm functionality and quality for its use in sperm-mediated gene transfer (SMGT)

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Keywords: transfection, transgenesis, SMGT.

Sperm-mediated gene transfer (SMGT) is based on the capacity of sperm to bind exogenous DNA and transfer it into the oocyte at fertilization. In bovines its progress has been slow due to the poor reproducibility and low efficiency of this technique for the generation of transgenic embryos. The aim of the present study was to evaluate different commercial transfection reagents on sperm quality and functional parameters. Additionally, the ability of the sperm to bind and incorporate exogenous DNA was also assessed. These analyses were carried out by flow cytometry and confocal fluorescence microscopy and motility parameters were also evaluated by computer-assisted sperm analysis (CASA) system. Transfection was carried out using complexes of FITC-labeled plasmid DNA with i) Lipofectamine (Lipofectamine®LTX-Plus™; Life Technologies, CA, USA), ii) SuperFect (SuperFect®; Quiagen, Hilden, Germany) and iii) TurboFect (TurboFect® ; Thermo Scientific, MA, USA) for 0.5, 1, 2 and 4 h, respectively. The differences between treatments were analyzed using ANOVA after arcsine transformation of the proportional data. To identify differences between groups, Tukey's post-test was performed with a level of significance of $P < 0.05$. The results showed that 100% of transfected sperm bound exogenous DNA, including the control DNA without transfection. Furthermore, SuperFect and TurboFect treatments promoted ($P < 0.05$) the incorporation of exogenous DNA. Sperm viability was affected ($P < 0.05$) only with Lipofectamine (73.4%) and TurboFect (62.9%), after 4 h treatment. All treatments and incubation times significantly affected the motility parameters, except for Lipofectamine and SuperFect after 0.5 h incubation. DNA integrity and Reactive Oxygen Species (ROS) levels were not affected in all treatments. However, transfection using SuperFect and TurboFect negatively affected ($P < 0.05$) the acrosome membrane and TurboFect showed the lowest mitochondrial membrane potential of all treatments. In conclusion, we demonstrated the successful sperm binding and incorporation of exogenous DNA after transfection and confirmed the capacity of sperm to spontaneously incorporate exogenous DNA. Additionally, we showed that the sperm transfection using Lipofectamine enabled the sperm to capture DNA without compromising the viability and motility. Therefore, this method would be more suitable for producing transgenic embryos by IVF-SMGT. Although transfection using SuperFect and TurboFect increased the amount of exogenous DNA present in the sperm, these compounds negatively affected some sperm parameters, including motility and acrosome integrity, which paradoxically, could be beneficial for SMGT procedures via ICSI (Arias et al. Reprod Fertil Dev. 26(6):847-54. 2014). Future studies are still required to confirm the effect of these treatments on the transgenic embryo/animal production by ICSI-SMGT and/or IVF-SMGT.

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Isolation of extracellular vesicles from the conditioned medium of mesenchymal stem cells from bovine adipose tissue

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Keywords: exosomes, microvesicles, transmition electron microscopy.

Extracellular vesicles (EVs) have therapeutic potential because they participate in processes as intercellular communication, immunomodulation, cell proliferation and differentiation (De Jong et al, Front Immunol. 608: 1-13, 2014). A fundamental stage to characterize EVs is the determination of its size and morphology by electron microscopy (Lässer et al, J Vis Exp. 59: 3037e, 2012). The exosomes (exos) and microvesicles (mvs) are the main representatives of EVs. Exos are nanovesicles ranging from 40 to 150nm of size and mvs from 50 to 1000nm (De Jong et al., 2014). Thus, the aim of this study was to test and standardize the EVs isolation technique from the conditioned medium of mesenchymal stem cells from bovine adipose tissue (bAT-MSCs) by analysis using transmission electron microscopy. For this, visceral adipose tissue adjacent of the uterine part from abattoir was collected (n = 3). A pool of three biological replicates was subjected to enzymatic digestion (collagenase solution 4 mg/ml) and stromal vascular portion cultured in maintenance medium consisting of DMEM low glucose/F12 (1:1), 10% fetal bovine serum (FCS), 1% penicillin, 1% amphotericin B at 37.5°C in a humidified atmosphere containing 95% air and 5% CO2. bAT-MSCs were previously characterized in our laboratory using the same protocol for isolation and cultivation. The protocol for obtaining EVs begun using cells in first (P1) and second (P2) passage (Pascucci et al, Vet J. 202: 361-366, 2014). Initially the maintenance medium was switched for FBS-free medium for 24 hours. Then the samples were centrifuged at 2000g for 20 minutes at room temperature. The supernatant was filtered through 0,22µm filter and then subjected to a first ultracentrifugation at 100000G for 60 minutes at 4 °C. The pellet was then resuspended and washed in PBS, and was followed by new ultracentrifugation with the same specifications. The final pellet was resuspended in 1ml PBS and stored at -80C. For electronic microscopy, a nickel grid coated with Formvar was positioned on 20µL of PBS suspension for 60 minutes. The grids were washed sequentially with 3 drops (30 uL) of PBS. Then, the sample was fixed in 2% paraformaldehyde for 10 minutes washing the grid sequentially. Then the sample was incubated in 2% uranyl acetate and 0.13% methyl cellulose plus 0.4% uranyl acetate and placed for drying. In ultrastructural analysis by electron microscopy it was observed in samples from both P1 and P2 many vesicular structures 50 to 150 nm in diameter, mainly. These findings indicate the presence of extracellular vesicles in the conditioned medium from bAT-MSCs studied in both passages. Thus, we conclude that the technique was effective for obtaining extracellular vesicles from bAT-MSCs, opening new possibilities for obtainment, research and future therapeutic application.

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Proteomic analysis of the secretome from bovine endometrial progenitor cells challenged or not with bacterial lipopolysaccharide

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Keywords: endometrium, secretome, bovine.

The conditioned medium (CM) by the culture of endometrial cells there is the presence of bioactive molecules which have important role in inflammatory modulation besides the paracrine effect. Uterine inflammation/infection causes substantial economic losses to the livestock industry and the use of the CM or mesenchymal stem/progenitor cells (PC) has already been proved in other species for the control of various diseases including those from the uterine environment. The objective of this study was to classify, according to PANTHER (Protein Analysis Through Evolutionary Relationships; http://pantherdb.org/), proteins found in secretome of bovine endometrial progenitor cells (bEPCs) stimulated or not with bacterial lipopolysaccharide (LPS). bEPCs were isolated (Fortier J. Reprod Fert 83:239-248,1988 modified), characterized, grown in third passage on 24 wells plates (1000 cells/cm2) until confluence at which time they were deprived of medium with fetal bovine serum and subjected (treated group-TG) or not (control group-CG) to the challenge with 1µg/ml LPS (E. coli serotype 0111: B4, Sigma). After 12 hours, the CMs of three biological replicates from different animals were collected (TG vs CG) and a pool of TG and CG was subjected to reduction, alkylation, trypsin digestion and analysis by mass spectrometry coupled to liquid chromatography (nanoLC-/MS/MS). Data analyses were done with Proteome Discoverer against Swiss-Prot Mammalia / TREMBL Bos Taurus database. 396 and 300 protein groups were identified, respectively in the TG and CG samples. From these, 155 proteins were unique to the TG. The classification of proteins was done by PANTHER according to the biological processes (BP), molecular function (FM), cellular component (CC) and protein class (PC). In general, we verified positive enrichment (PE) for BP in macrophage activation function (GO: 0042116, EF = 7.87) and receptor-mediated endocytosis (GO: 0006898, EF = 5.23) in TG. Regarding FM, it was seen PE for hydrolase activity (GO: 0016787; FE = 1.87) and inhibitory enzyme (GO: 0004857; FE = 5.15) in the TG, and activity structural molecule (GO: 0005198; FE = 2.73) in the CG. For CC, enrichments were verified in both groups to extracellular matrix (GO: 0031012) and extracellular region (GO: 0005576), as well as intermediate filament cytoskeleton (GO: 0045111) in the CG. Interestingly, with respect to PC we evidenced PE in antibacterial response proteins (PC00051; FE = 5.87) in the TG. Our results, especially based on PE to macrophage activation, allowed us to infer that our in vitro model of stimulation with bacterial LPS of bEPCs is effective to study immunoinflammatory response. Probably, the bEPCs secrete proteins with antibacterial response as a defense mechanism, which is a very interesting feature for use in cell therapy.

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A301 Cloning, Transgenesis and Stem Cells

Proteomics analysis of mesenchymal stem cells from equine umbilical cord: global and functional approach

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Keywords: proteomics, stem cells, equine.

In equine medicine, studies involving proteomic analysis are still scarce, especially in the subject stem cells (SCs). To the best of our knowledge this is the first study describing the proteome of mesenchymal stem cells from intervascular matrix of equine umbilical cord (MSC-UCMI) in a global and functional way. The aim of this work was to analyze the proteome of the previously characterized MSC-UCMI, in order to determine protein abundance and classify the identified proteins according to GO (Gene Ontology,*http://geneontology.org*). Three biological replicates of MSC-UCMI lysates (N = 3 animals) were enzymatically digested and analyzed by mass spectrometry (nanoLC-MS/MS) using a shotgun strategy. Samples were analyzed in three technical replicates. Database search was done against Uniprot Equus protein database. 2118 protein groups were identified with at least two high confidence peptides (FDR<1%) per protein. Protein classification analysis according to biological process, molecular function and cellular component was performed using PANTHER (Protein ANalysis THrough Evolutionary Relationships Classification System, *http://pantherdb.org*). The Panther GO analysis evidenced the enrichment in 42 biological processes, 23 molecular functions and 18 cellular components. Protein abundance was estimated by calculation of emPAI (10^{PAI}-1), in which PAI (Protein Abundance Index) is the number of identified peptides divided by the number of observable peptides per protein. To calculate the protein content in mol% the equation emPAI/Σ (emPAI) x100 was used (Ishihama et al. Mol Cell Proteomics. 4(9):1265-72, 2005). The two most abundant proteins in the proteome of MSC-UCMI are the cytoskeletal proteins actin (4.68 mol%) and vimentin (3.58 mol%), which have important role in cell stability and motility. Additionally, we identified 14 cell surface antigens. Three of them, antigens CD44, CD90 and CD105 had been previously validated by flow cytometry. In the present study we also evidenced important information about the biological properties of MSC-UCMI that reinforces their use for cell therapy. Such information is mainly related to the excellent differentiation potential, low immunogenicity (low MHC-II expression) and chromosomal stability, added to the proteomics finds that allow us to infer an important migration ability and activities related to primary metabolic processes, protein synthesis, coat vesicles, mediated vesicles transport and antioxidant activity. In addition, the identification of different cell surface markers may contribute to the establishment of an immunophenotypic panel more suitable for the characterization of MSCs from equine fetal membranes.

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A302 Cloning, Transgenesis and Stem Cells

Genetically modified ovine embryos by Sperm Mediated Gene Transfer and in-vitro fertilization

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Keywords: IVF in-vitro fertilization, IULAI intra-uterine laparoscopic insemination , SMGT Sperm Mediated Gene Transfer.

Transgenesis in farm animals has, in Sperm Mediated Gene Transfer (SMGT) an option that exceeds efficiency to other methods such as gene microinjection in male pronucleus, retro-viral factors, or transfected STEM cells. Our goal has been to develop a protocol in sheep that combines the techniques of in-vitro fertilization (IVF) and Intra-Uterine Laparoscopic Insemination (IULAI), with transfer of heterologous genes to the egg at the time of fertilization, by mediation of intact sperm. The first effort was aimed at the development of the IVF technique in combination with sperm cells transfected with reporter genes based on the results set forth above for both sheep IVF as transfection and capacitation of intact sheep sperm (Raffaelli, P. Proc . Ann. Meet. SBTE 2011, p.410). Starting from abattoir sheep ovaries, 48 Complexes Cumulus Oocyte (COCs) were obtained by follicular puncture which were matured in vitro for 24H in TCM199 supplemented with 20% heat inactivated sheep serum (HISS) and antibiotic, in gassed stove with 5% CO^2 in air at 38.5°C at moisture saturation. Two semen samples, one treated and one control were processed. In the sample a) (treated) $1,2X10^6$ sperm cells were co-incubated with 400 ng of a reporter gene encoding red fluorescence. At the sample b) (control) the same quantity of sperm were not co-incubated. Sperm capacitation was induced by resuspension of both samples in Synthetic Oviductal Fluid (SOF) plus 20% HISS and 10 mg / ml heparin, and cultured for 3H at 38.5 °C in sealed tube. Fertilization was performed in SOF medium supplemented with 2% HISS. The batch a) received 21 oocytes, adding $1,2x10^6$ co-incubated sperm. Batch b) 27 oocytes were seeding with $1,2x10^6$ not co-incubated spermatozoa. After 15H of culture presumptive zygotes were removed and placed in TCM199 plus HISS and antibiotic under the same conditions of temperature and moisture that were referred in maturation. After 120H the collected embryos were classified and recorded by confocal fluorescence micrograph. On sample a) 11 blastocysts, 6 unfertilized oocytes and 4 empty zona pellucidae were collected. All embryos (51.4%) showed red fluorescent signal in all blastomeres. In contrast, unfertilized and degenerated eggs (48.6%) showed no fluorescence signal. In the sample b) (control) the reading was difficult by presence of fibrinosis, however 4 blastocysts and 3 unfertilized eggs were collected, none of which (100%) showed fluorescence signal. It was concluded that transfection by sperm and trangene integration into the embryo was effective, since both the control sample “b)” (4 embryos, 3 unfertilized eggs) and the unfertilized eggs (6) from sample “a)”, showed no fluorescence, while all fertilized eggs (11) from the sample "a)" showed intense fluorescent signal in all blastomeres. Although eloquent results were obtained, the samples were submitted to a X^2 statistical análisis, showing significant difference ($P < 0.05$).

A303 Cloning, Transgenesis and Stem Cells

Cell cycle synchronization analysis of skin fibroblasts from domestic cat (*Felis silvestris catus*) and kodkod (*Leopardus guigna*)

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Keywords: Cell cycle synchronization , domestic cat, wild felids.

The kodkod is the smallest wild cat in South America and its population is in constant decrease. The SCNT might be a valuable tool for preserving the genetic pool of the kodkod. The cell cycle synchronization of donor cells plays a crucial role in the SCNT. The objective of this research was to evaluate the effect of two different treatments, serum starvation (SS) and contact inhibition (CI), for induction of cell quiescence, on skin fibroblast derived from domestic cat and kodkod. All experiments were performed with fibroblasts in the passes 5 to 6. SS was achieved by replacing culture medium with 10% FBS by medium with 0.5% FBS. CI was performed by allowing the cells to reach 100% confluence. Both treatments were evaluated at day 1, 3 and 5, and growing cells (60-80% confluence; GC) were used as control. Three replicates of each treatment were performed. For cell cycle analysis, fixed cells were incubated with 50 µg/ml of propidium iodide and 100 µg/ml of RNase for 30 min at 37ºC. Flow cytometry was conducted on the Attune® NxT Acoustic Focusing Cytometer (Thermo Fisher Scientific). At least 10,000 events were scored using the BL2-A channel (filter 574/26 nm). Results were analysed using the Attune® NxT SW v1.1 software. Apoptosis was evaluated by gene expression analysis of BAX and BCL2 by RT-qPCR, SDHA was used as internal control. Flow cytometry results were analyzed by ANOVA using the GLM procedure in the SAS software, means comparison was made by Tukey's test. RT-qPCR results were analyzed by the Kruskal-Wallis test using the Infostat software. In all experiments, differences were considered at $P < 0.05$. Flow cytometry analysis revealed that in fibroblasts of domestic cat, SS and CI, both at 3 and 5 days of treatment, increased the percentage of cells in G0/G1 and reduced the fraction of cells in S and G2/M stages compare to GC ($P < 0.05$). On the other hand, only SS for 3 and 5 days and CI for 1 and 3 days increased the percentage of cells in G0/G1 in fibroblasts of kodkod ($P < 0.05$). Furthermore, only SS for 5 days reduced the percentage of cells in G2/M stage compare to GC, in kodkod fibroblasts ($P < 0.05$). Regarding to gene expression analysis, in fibroblasts of domestic cat, no statistical differences were found in the BAX/BCL2 ratio between both SS and CI (at 1, 3 and 5 days) compared to GC. However, in kodkod cells BAX/BCL2 ratio was high in CI at 3 and 5 days of treatment compared to SS at 3 and 5 days ($P < 0.05$). In SS the relative expression of BCL2 increases with the time of treatment, while in CI the relative expression of BCL2 is not enough to reduce the levels of BAX. In conclusion, fibroblasts of domestic cat and kodkod respond differently to different methods of cycle synchronization. In kodkod fibroblasts CI had a negative effect after 3 days, which was related to a high apoptosis incidence. We suggest SS for 3 or 5 days for cell cycle synchronization in kodkod fibroblasts. These results are relevant for production of kodkod embryos by SCNT.

A304 Cloning, Transgenesis and Stem Cells

Characterization of stem-cells derived from bovine endometrium during the folicular phaseof the oestral cycle

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Keywords: stem cells, endometrium, bovine.

The aim of this research was to identify/characterize mesenchymal stem cells derived from bovine endometrium during the follicular phase of the oestral cycle. Endometrial biopsies were taken from 4 healthy cows ipsilaterally to the pre-ovulatory follicle, during the follicular phase (18 days post-ovulation), according to morphological evaluation of the ovaries and quantification of serum progesterone and 17b-estradiol by radioimmunoassay. Primary cell cultures were collected, expanded and frozen and cellular assays were performed in cells at passage 3. Total RNA and proteins were isolated and screened for markers of embryonic (OCT4, NANOG, SOX2) and mesenchymal stem cells (CD44 and CD117) using RT-qPCR, or protein assays (OCT4, SOX2, CD44) such as immunohistochemistry in fixed tissues and/or Western blot (WB) for both tissues and cells. Primary cell cultures were isolated and characterized in terms of morphology and induced into chondrogenic, osteogenic, and adipogenic in vitro differentiation during 7 and 14 days. The evaluation of the differentiation potential was performed by alcian blue, alizarin red and red oil staining, respectively. Alliquots in first passage (P1) were seeded at low density (30 cells/cm2) and cultured for 30d, when colonies were counted by Giemsa staining. Cloning efficiency was calculated as (n° of clones/n° of cells seeded) x100. Statistical analysis of the quantitative real-time PCRs was conducted using Wilcoxon non parametric test on Infostat 3.0 software (Infostat, Buenos Aires, Argentina). Embryonic stem cell markers OCT4 and SOX2, but not NANOG were detected in tissues and cells via RT-qPCR and protein determination. OCT4 protein was not found in the Western Blot in thawed cells, but it was localized in immunohistochemistry in fixed tissues, displaying perinuclear, and cell membrane expression in stromal and glandular tissue. A similar pattern was found for Sox2 protein in tissues. Mesenchymal markers CD44 and CD117 were detected by qPCR in both tissue and cells, while protein was found only in tissue (WB). Primary cultures were able to plastic adherence, showed in vitro potential to differentiation into chondrogenic and osteogenic, but not to adipogenic lineage, as judged by both staining and RT-qPCR. The colony forming unity CFU were observed for the tested cell line, at an average of 5,3 colonies in 3 replicates (cloning efficiency: 0,63%) and a doubling time of 30hs. Our findings indicate that bovine endometrium bears undiferentiated progenitor/stem cells capable of self-renewal, colony formation, and specific stem cells gene and protein expression and able to differentiate into at least two mesenchymal lineages that agrees with our previous similar findings for bovine endometrial tissue at the early and late luteal phases of the estrus cycle.

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A305 Cloning, Transgenesis and Stem Cells

Gene transfection of primary bovine fibroblast cells through carboxylate multi-walled carbon nanotubes

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Keywords: carbon nanotubes, gene delivery, transgenic.

The insertion of exogenous DNA into primary mammalian cells is an important step towards production of transgenic animals. But, primary cell lines are difficult to be transfected (Gresh et al., 2012. Methods Mol Biol, 801:65-74). Recently, carbon nanotubes have been explored as an efficient novel non-viral gene delivery system to cells due to their unique structure and properties (Cheung et al., 2010. Adv Drug Deliv Rev,62: 633-649. However, their potential to transfect primary fibroblast cells has not been assayed. Thus, the aim of the present study was to evaluate the potential using of carboxylated multiwall carbon nanotubes (COOH-MWNTs) as a gene delivery vector for gene transfection of bovine fibroblast primary cells. COOH-MWCNTs were complexed directly with the GFP plasmid, which encodes the green fluorescent protein (GFP) to form COOH-MWCNT/pDNA carries. Bovine fibroblasts collected from Gyr cows were frozen at -80ºC. The stocked cells at 2th passage were thawed and cultured in DMEM (1x104 cell/well) supplemented with 10% fetal calf serum and incubated at 37°C, 5% CO2 and 95% humidity in air. Fibroblast cells were transfected with COOH-MWCNT/pDNA complexes (rate 5:1) or polyethylenimine (PEI) 25KDa (rate 1:1) (Control). GFP gene expression in the transfected cells was examined by flow cytometry with excitation at 488 nm from an argon-ion laser in bandpass filter of the 530/30 nm (FL1 channel) (FACScalibur; Becton Dickinson, San Jose, CA) and the detection of GFP gene by real time PCR analysis (ABI Prism 7300 Sequence Detection Systems, Applied Biosystem, Foster City, CA, USA). Statistical analysis was performed by ANOVA and compared by Student Newman Keuls. Analysis by flow cytometry showed no difference in transfection efficiency between COOH-MWCNT and PEI($P > 0.05$). Flow cytometry showed 3.30% GFP-positive cells in fibroblast transfected with COOH-MWCNT and 3.96% GFP-positive cells with PEI. To verify the results obtained from flow cytometry analysis, real time PCR examination was performed to ensure the presence of GFP in fibroblasts. The band of PCR fragment of GFP gene was also detected by electrophoresis analysis, confirming the presence of the transgene in the transfected fibroblast. Our results demonstrate that COOH-MWNTs can be an alternative transporter for gene delivery into primary bovine fibroblast.

A306 Cloning, Transgenesis and Stem Cells

Bovine blastocysts produced from cloned sperm genome and either mature or pre-activated oocytes

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Keywords: sperm genome cloning, diploid hemiclones, bovine.

Sperm genome cloning has great potential in livestock production. The availability of several copies of a single sperm genome would allow genetic screening for valuable yield traits. Moreover, several oocytes from the same or different donors could be fertilized with the exact same paternal genome. This is possible by production of androgenetic haploid embryos (AHe) followed by fusion of their blastomeres with oocytes, to obtain diploid embryos. As in cloning, cell cycle stage of the recipient oocyte at the time of fusion may affect embryo development. Here, we evaluated the effect of the oocyte´s nuclear stage at the time of fusion with the androgenetic haploid blastomere on in vitro development of bovine embryos. To this aim, zygotes were produced using a short in vitro fertilization (IVF) protocol and zona pellucida was removed. To generate the AHe, zygotes were stained with Hoechst-33342 to visualize DNA and the maternal nucleus was aspirated by micromanipulation using a blunt pipette. A non-enucleated group was used as IVF control. All embryos were individually cultured in microwells covered by SOF medium. After 30-35h of culture, 4 cell stage AHe were disaggregated and the blastomeres were individually fused with either mature zona free oocytes followed by activation (group FA), or with zona free pre-activated oocytes (group AFA). For fusion, a double direct current pulse of 65 V, each pulse of 30 µs duration, 0.1 s apart was applied. The activation protocol consisted on 5µM Ionomycin for 4min followed by 50µM Roscovitine for 5h (in group AFA fusion was performed during the third hour of Roscovitine incubation). For diploid activation control, the same activation protocol was performed on MII oocytes, but Roscovitine was supplemented with 5µg/ml Cytochalasin B to avoid second polar body extrusion (group PA). Moreover, a group of AHe was evaluated for embryo development. Groups FA, AFA and PA were cultured as described above. Data were analysed by Fisher test ($P < 0.05$). Cleavage, morulae and blastocyst rates were: 82.8, 8.2 and 4.1% for FA (n = 122); 86.4, 1.5 and 1.5% for AFA (n = 66); 86.3, 8.4 and 0.9% for AHe (n = 226); 91.4, 42.3 and 30.1% for IVF (n = 163); and 86.9, 23.2 and 18.1% for PA (n = 99). Cleavage rates differed only between IVF and FA, but not between other groups. Interestingly, development to morulae stage dropped significantly in both FA and AFA compared to controls. Group PA development was lower than IVF. Blastocysts were obtained in FA and AFA, although in lower rates than PA and IVF. In conclusion, it was possible to produce diploid blastocysts by fusion of androgenetic haploid blastomeres with both MII oocytes or pre-activated oocytes and the nuclear stage of the recipient oocyte did not significantly affect embryo development. However, synchronization of both donor and recipient cells is still being explored and their ability to develop in vivo after embryo transfer will be evaluated. These results hold promising prospect in sperm genome cloning.

A307 Cloning, Transgenesis and Stem Cells

Ultrastructure comparison of adipogenic differentiation of mesenchymal stem cells from equine Adipose Tissue and Bone Marrow

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Keywords: cell morphology, adipogenesis, cell differentiation.

Mesenchymal stem cell (mSC) have promoted advances in cell therapy, Veterinary Medicine and Cell Biology research. Adipose tissue (AT) and bone marrow (BM) are important mSC sources. This study aimed to compare the ultrastructure of equine MSCs derived from AT and MO and differentiated in vitro in adipogenic lineage. AT were harvested from tail base of a horse, and about 16 mL of BM were harvested with myelogram needle, according to Protocol 758 approved by Ethics Committee of the Institute of Biosciences - Unesp. mSC isolation from AT was performed by enzymatic digestion with collagenase, and mSC-BM were isolated by a Ficoll gradient density. Culture was performed in medium containing DMEM high glucose, penicillin/ streptomycin and amphotericin, and supplemented with 10% FBS until second passage, when samples received adipogenic differentiation medium (StemPro® Adipogenesis Differentiation Kit - Gibco CA USA), during 18 days. Adipogenic differentiation was confirmed by Oil Red satinning which showed abundant lipid droplets on cytoplasm of mSC-TA and less amount on mSC-BM. Ultrastructural analysis showed similarity in morphology of MSC-BM and AT, as surface covered by protrusions and vesicles, indicating high cellular activity. Nucleus was centrally located, showing irregular appearance. Large amount of rough endoplasmic reticulum were observed in cytoplasm, which in some places presented dilated cisterns and large amount of amorphous material. Vesicles and phagosomes of different sizes were also observed, especially in MSC-BM. Mitochondria showed higher electrondensity with evident crests and tubular aspect in both samples, and elongated aspect highlighted in MSCs-AT. Lipids were observed in cytoplasmic vesicles of MSC-AT and practically nonexistent in MSCs-BM. In conclusion, adipogenic differentiation of mSC-AT showed similar characteristics to preadipocytes more evident than mSC-BM.

A308 Cloning, Transgenesis and Stem Cells

Quality assessment of goat oocytes and effects on goat cloning efficiency after *in vivo* or i*n vitro* maturation

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Oocyte quality plays a key role in nuclear reprogramming after cloning by somatic cell nuclear transfer (SCNT), with the oocyte source having a significant effect on the developmental outcome. Although the use of *in vivo*-matured oocytes for goat cloning appears to be more efficient, despite the variation in maturation, the use of *in vitro*-matured oocytes provides more homogeneous and synchronous cytoplasts for cloning. The refinement of evaluation criteria for oocyte quality may improve *in vitro* and *in vivo* survival after cloning. The aim of this study was to evaluate the effect of the ooplasm morphologic quality, chromatin configuration and polar body/MII nearness between *in vivo*- and *in vitro*-matured oocytes on the *in vitro* survival and pregnancy outcome after goat cloning by SCNT, following our established procedures (Martins et al., 2016, Cellular Reprogramming, in press). *In vivo*-matured or immature oocytes were obtained from 65 and 94 FSH-stimulated does by aspiration of pre-ovulatory follicles from abattoir-derived ovaries (*in vivo* group) or by laparoscopic ovum pick up (*in vitro* group). Immature oocytes were *in vitro*-matured for 22 h. Following polar body selection, MII oocytes from both maturation groups were individually classified during the enucleation procedure regarding ooplasm quality as good, regular, or poor, chromatin configuration as normal or abnormal, and polar body/MII nearness as far or near. After reconstruction using transgenic goat fibroblast cells, and following embryo activation (Day = 0) and a 12-h long *in vitro*-culture, cloned embryos from both maturation groups were surgically transferred to the oviduct of synchronous recipients (13 embryos/recipient). Pregnancy diagnosis was performed by ultrasonography on Day 23. Data on maturation, ooplasm quality, chromatin configuration, polar body/MII nearness, survival upon reconstruction and pregnancy rates were compared between groups by ANOVA or chi-square tests ($P < 0.05$). After 10 (*in vivo*) and 15 (*in vitro*) replications, maturation rate was higher for *in vitro*- (996/1682, 59.2%) than *in vivo*-matured (580/1526, 35.7%) oocytes, with no differences observed between groups for the mean number of MII oocytes per doe (10,6 vs. 8,9), for oocytes evaluated as good (23.7 ± 2.8 vs. 14.8 ± 2.8%), medium (38.1 ± 2.4 vs. 37.0 ± 2.4%) or poor (38.2 ± 4.1 vs. 48.2 ± 4.1%), with chromatin abnormalities (98/858, 11.4% vs. 53/562, 8,9%), or for the *in vitro* survival following embryo reconstruction (243/562, 90.4% vs. 797/858, 92.9%), respectively. Although the near polar body/MII rate was higher in *in vivo*- (243/501, 47.2%) than *in vitro*-matured (92/724, 12.8%) oocytes, pregnancy rates were higher in the *in vitro* (14/41, 34.1%, for 539 transferred embryos) than the *in vivo* group (4/29, 13.7%, for 379 transferred embryos). Under our conditions, a higher goat cloning efficiency was obtained using *in vitro*-matured oocytes.