



A204 Cloning, transgenesis and stem cells

### **Generation of myostatin knock-out cow embryos using crispr/cas9-assisted gene editing and somatic cell nuclear transfer**

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Myostatin (MSTN) is a member of the transforming growth factor- $\beta$  superfamily that inhibits muscle growth. In the European cattle breeds Charolais, Marchigiana and Maine Anjou, natural mutations on the exon 2 of MSTN gene result in greater muscle mass than other cattle breeds. Previous studies on MSTN knock-out (KO) embryos have shown that blocking the MSTN gene expression causes double muscle phenotype, increasing bovine commercial value. In this study we aimed to disrupt the MSTN gene in bovine fetal fibroblasts using CRISPR-Cas9 nuclease and generate cloned embryos with the modified genotype. First, we evaluated four different single guide RNA (sgRNAs), targeting exon 1 (sgRNA-1), exon 2 (sgRNA-2) or exon 3 (sgRNA-3 and sgRNA-4) of the *Bos taurus* MSTN gene. Experimentally, two sets of  $5 \times 10^4$  bovine fetal fibroblasts were nucleofected with 500 ng of the plasmid hspCas9-2A-PuroV2.0 which encodes for Cas9 nuclease, one of the above mentioned sgRNAs and Puromycin resistance. The two sets of nucleofected cells were then cultured in the same well of a 12 multi-well plate for 48 h and then treated with 3  $\mu\text{g/ml}$  of Puromycin for another 48 h to select those cells that incorporated the plasmid. Afterwards, we isolated the genomic DNA from the Puromycin-surviving cells and amplified the sequence of the MSTN gene targeted by each sgRNA by PCR to be further sequenced by Sanger method. Sequencing results were evaluated with ICE-Synthego software to determine de gene edition efficiency. The percentage of modified sequences was analyzed with respect to the control sample. Different edition efficiencies were obtained for each sgRNA: sgRNA-1: 6 %, sgRNA-2: 96 %, sgRNA-3: 15 % and sgRNA-4: 5 %. In view of these results, the sgRNA-2-edited cells were used for embryo production by somatic cell nuclear transfer (SCNT). Embryo development for the MSTN-KO group and the wild-type control group was 67% (n=122/182) vs. 70% (n=98/139) cleavage, and 7.7% (n=14/182)<sup>a</sup> vs. 15.8% (n=22/139)<sup>b</sup> blastocysts (p<0.05 Chi-Squared test), respectively. Ten blastocysts were individually genotyped by PCR amplification of MSTN-exon 2 and further Sanger sequencing. The results of the analysed blastocysts were: 100% bi-allelic mutations (4/10 *homozygous* and 6/10 *heterozygous*). In conclusion, we produced cloned bovine embryos edited on exon 2 of the MSTN gene by CRISPR-Cas9 with a very high efficiency using sgRNA-2. Although the bovine embryos generated with MSTN-edited cells showed a lower blastocyst rate than control fibroblasts of the same cell line, it was probably due to the cellular stress caused by the nucleofection/puromycin treatments and higher cell culture passages. Our future main goal is to achieve the birth of healthy calves with double-muscle phenotype. This represents a step forward towards the production of animals with increased commercial value.



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## Successful generation of induced pluripotent stem cells (iPS) derived from skin fibroblasts of an aged equine

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Cellular aging is a limitation in cellular reprogramming since it is associated with cell senescence. As the cell ages, an upregulation of pathways such as p53, p16<sup>INK4A</sup>, and p21<sup>CIP1</sup> occurs leading to cell cycle arrest along with alterations in cell morphology and metabolism. Considering the difficulty on reprogramming of aged cells, the objective of the work was to achieve reprogramming into pluripotency of a more than 20 years old horse. Therefore, a skin fragment was collected from the dorsal lateral metacarpophalangeal region, taken to the lab and fibroblasts were recovered after a 3 hours digestion period with Collagenase IV (#C2674 Sigma Aldrich). The fibroblasts were then seeded in a 6 well plate ( $2 \times 10^4$  cells per well) and the lentiviral vector STEMCCA containing the human sequences of OCT4, SOX2, KLF4, and c-MYC was used for transduction. Six days after transduction cells were seeded in mouse embryonic fibroblast (MEF) layer ( $4,75 \times 10^4$  cells per well). The reprogramming efficiency was calculated by dividing the number of formed colonies by the number of seeded cells. The iPS colonies were evaluated regarding their morphology and detection of alkaline phosphatase, immunocytochemistry for Oct4 (#sc8628, Santa Cruz), Sox2 (#ab97958, Abcam), Nanog (#ab21624, Abcam), SSEA-1 (MAB 4301, Millipore), TRA-1-60 (Mab 4360, Millipore) and TRA-1-81 (Mab 4381, Millipore). The transcript levels were determined by RT-qPCR, for pluripotency genes OCT4, REX-1, NANOG, and SOX2. Therefore, the cycle threshold (Ct) values of the target genes were normalized by the average of Ct values of the housekeeping genes (HPRT1 and PPIA) and the fold changes were then calculated using the  $2^{(-\Delta CT)}$  equation. After 16 days of the transduction, colonies were visualized, being primarily identified by their typical morphology: tightly packed cells with a high nuclear/cytoplasm ratio. The efficiency of the reprogramming process was 0,059% (28 colonies from  $4,75 \times 10^4$  seeded cells). Colonies were positive for alkaline phosphatase at passages 4 and 12. Immunocytochemistry revealed that cells were found to be positive for OCT4, NANOG, SSEA-1, and TRA-1-81. Cells showed endogenous expression of the pluripotency genes OCT4 ( $0,3670 \pm 0,1032$ , n=3), REX-1 ( $0,0391 \pm 0,0005$ , n=3), NANOG ( $0,1421 \pm 0,2903$ , n=3) and SOX2 ( $0,0034 \pm 0,0020$ , n=3), being the Ct values all minor than 31,8, using specific equine primers. Herein we conclude that although age is considered as a great barrier to the reprogramming of somatic cells, it was possible to achieve successful reprogramming in an animal in advanced age in our conditions. Financial Support: FAPESP (2018/04009-6; 2015/26818-5) and CAPES.



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### **Transfection of swine oocyte with polyethyleneimine (PEI): a low cost and convenient method to produce genetically modified swine**

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Oocytes are excellent candidates to produce genetically modified pigs due to their physiology and absence of nuclear envelope, which favors the incorporation of DNA. However, the presence of the zona pellucida and the sensitivity to stressors make transfection a challenge, since the techniques available are labor-intensive and expensive. Therefore, the objective of this work was to develop a protocol for transfection of porcine oocytes using a cationic polymer, polyethyleneimine (PEI). Thus, the branched PEI 25 KDa (100mL, Sigma Aldrich, Saint Louis, USA) was used. Oocyte maturation and in vitro embryo production procedures were performed according to Marques *et al.*, 2011 (Zygote, 19: 331-337). The data (mean minimum squares  $\pm$  SE) were evaluated using PROC MIXED (SAS®) with 5% significance. In the 1<sup>st</sup> experiment, the ability of PEI to overcome the zona pellucida and the cytoplasmic membrane of oocytes matured in vitro was evaluated. For that, PEI was labeled with FITC, and oocytes were incubated (30 min) with 4 concentrations of PEI-FITC (10, 20; 40 and 80  $\mu$ g/mL). The internalization of the PEI-FITC was evaluated by fluorescence microscopy and the pixel quantification performed using the software Image J 1.40g®. It was observed that all concentrations of PEI were able to reach the cytoplasm. The internalization rate was significant ( $p < 0.001$ ) and concentration dependent, and the concentration 10  $\mu$ g/mL resulted in the lowest internalization as the concentration of 80  $\mu$ g/mL provided the highest one ( $19.60 \pm 0.25 \times 10^3$  and  $22.69 \pm 0.23 \times 10^3$  pixels, respectively). In the 2<sup>nd</sup> experiment, transfection rates were evaluated using two preparations containing PEI (20 or 80  $\mu$ g/mL) complexed with the pmhyGENIE-5 vector at 2 N/P ratio, and then incubated with oocytes matured in vitro. Incubations with the respective vector concentration were also performed in the absence of PEI (INC20 and INC80) and a Control group. After 30 min of incubation, the oocytes were fertilized and cultured in vitro until day 7 of development. No effect of the treatments on the cleavage rates ( $p = 0.8307$ ) and blastocysts ( $p = 0.9780$ ) were observed. The cleavage rates ranges from  $41.19 \pm 10.55\%$  to  $54.83 \pm 7.46\%$  and the blastocyst rates from  $16.96 \pm 7.81\%$  to  $23.68 \pm 7.81\%$ . Besides, only the PEI20 group presented blastocytes with GFP expression ( $3.2 \pm 1.91\%$ ). The data suggest that PEI, unlike other transfectant agents, has the ability to pass the zona pellucida, and the protocol described herein is capable of producing transgenic blastocysts expressing GFP, so that it could be used as a cheap and easy tool for transfection of swine oocytes.



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## **Induced pluripotent stem cells (iPSCs) derived from urine progenitor cells in the swine: a novel non-invasive method for regenerative medicine**

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The swine model is of special interest as a biomedical model due to its immunological and physiological similarity with the human model, and non-invasive collection of cells for iPSCs generation would facilitate its use. Herein we aimed to derive urine progenitor cells (UPCs) *in vitro* cultures from urine samples, still unpublished for species other than human, and to reprogram them *in vitro* into pluripotency. For that, urine samples (approximately 250ml) were collected from three females. Isolation and culture were performed following human UPCs protocol (Steichen et al., 2017). Briefly, the urine was centrifuged at 300 x g, the pellet washed in DPBS (Sigma), resuspended and cultured in 45% DMEM high glucose (Life Technologies), 5% FBS, 50% REBM media (renal epithelial basal media, Lonza) supplemented with 1% glutamine, 1% MEM neaa, 1% penicillin/streptomycin (all Life Technologies) and REGM supplements : hEGF, Insulin, Hydrocortisone, GA-1000, FBS, Transferrin, Triiodothyronine, Epinephrine (Lonza) and 10ng/mL bFGF (Peprotech). After approximately one week, epithelial-like cells were observed in colonies. The cells from one female were submitted to transduction of murine OSKM (OCT4, SOX2, KLF4 and C-MYC - STEMCCA lentiviral vector, Millipore). After 4-5 days, cells were plated onto MEFs and cultured in KnockOut DMEM/F12, 20% KnockOut Serum Replacement, MEM neaa, L-Glutamine, 2-Mercaptoethanol and penicillin/streptomycin (all Life Technologies) supplemented with 10ng/ml bFGF (Peprotech). At approximately 12 days after transduction, colonies presenting typical pluripotent morphology were observed and evaluated regarding efficiency of colony formation and alkaline phosphatase detection. Three clonal lineages (C1, C4 and C6) were further maintained *in vitro* and characterized regarding pluripotency markers for more than 20 passages. The overall reprogramming efficiency observed was 8,455% (percentage of colonies observed in relation to the number of transduced cells plated). All three colonies were positive for alkaline phosphatase in passages 22, 21 and 21, respectively. Immunocytochemistry analysis revealed that C6 was positive for the pluripotency markers OCT4 (1: 100, cat # SC8628), SOX2 (1: 500, cat # ab97959; Abcam), SSEA1 (1: 50, cat # SC21702, Santa Cruz), TRA1- 81, and NANOG (1: 100 # catab77095, Abcam), meanwhile the colonies C1 and C4 were positive only for OCT4 and SOX2. In conclusion, it was possible herein to reprogram cells derived from urine samples into iPSCs that were maintained in culture *in vitro* for at least 20 passages. Further analysis is still needed to prove the complete reprogramming of these cells; however, these results already open a new possibility to generate models of *in vitro* diseases from a non-invasive source in an unprecedented way.

Financial Support: FAPESP (2019/02811-2, 2015/26818-5), CNPq 433133/2018-0 and CAPES.



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## **Isolation, culture and in vitro proliferation of canine mesenchymal stem cells derived from adipose tissue**

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Stem cells (SC) are undifferentiated cells that can be used in various pathologies promoting the healing of chronic patients. The use of adipose surgical waste is a good alternative for the extraction of adipose mesenchymal stem cells (AMSCs) and with few bioethical implications. With differentiation potential similar to that of bone marrow SC, AMSCs are more efficient in terms of ease of collection, abundance of tissue extracted and rate of expansion in vitro. The objective of this study is to describe and validate a simplified method of isolating and primary culture of SC extracted from surgical fat adipose tissue of dogs. Samples of abdominal subcutaneous adipose tissue were collected from surgical dogs rests (n = 4, sample 4.5g each) transported in PBS + amikacin solution (75µg / mL). The AMSCs isolation method consisted of sample washing in the PBS solution, mechanical maceration (clamp and scalpel) and subsequent enzymatic digestion in a conical tube containing 7.5 mL of PBS solution with trypsin (1000U / mL), vortex homogenized (1 min) and incubated at 38.5 ° C for 30 minutes. They were then filtered through filters (75 microns), and centrifuged (10 min at 3000 RPM). The supernatant was discarded and the pellet was resuspended in 1 mL of culture medium customized in the laboratory, called TCM-cell. The TCM-cell medium consists of TCM-199 Earle Salts with bicarbonate (Gibco®) plus 75 µg / ml amikacin, 0.2 mM pyruvate, 5% FBS, 20 µl / ml essential amino acids and 10 µl / ml of non-essential amino acids (both from SIGMA®) and centrifuged again for 10 minutes (6200 RPM). The pellet was resuspended and cells cultured in plate (60mm) containing the TCM-cell medium (38.5 ° C, 5% CO2 atmosphere in air). The TCM-cell medium was renewed every 72 to 96 hours. Cell growth and expansion were monitored under inverted microscopy. The cultures were evaluated according to the minimum criteria for characterization of TCM of the International Society of Cell Therapy (ISCT): to present fibroblastoid format, to adhere to plastic substrate and auto renew. All samples (n = 4) presented adherence to the plastic within the initial 24 hours and presence of colonies with fibroblastoid morphology in 96 hours. The cell confluence in the plates was reached in the 13th day in all samples, in which the first passage was performed and part of the samples were cryopreserved for later characterization study. The primary culture of TCM extracted from surgical abdominal fat adipose tissue in dogs met the minimum criteria for ISCT characterization presenting fibroblastoid format, adhesion to plastic and auto renew, important evaluative aspects in the first stage of TCM characterization. The expansion occurred progressively, being observed already in the third day of culture indicating that the simplified methodology in customized environment was satisfactory for the isolation, cultivation and expansion in vitro.