Animal Reproduction

Abstracts - 35th Annual Meeting of the Brazilian Embryo Technology Society (SBTE)

Cloning, transgenesis and stem cells

Delivery of Cas9 protein/gRNA complexes using lipofectamine CRISPRMAX in mammary gland epithelial cells (bMEC)

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Resumo

The bovine mammary gland epithelial cells culture (bMECs) is an efficient strategy to generate recombinant proteins, using the CRISPR-Cas9 gene editing system. The genetically modified bMEC constitutes an in vitro platform for the production of human recombinant proteins, such as human plasma fibronectin (pFN1), involved in the healing process. Therefore, genetically modified bMECs can be used for the evaluation of important factors of the animal bioreactors production, like analysis of the expression pattern of the transgene of interest, in addition to serving as a source of cell nuclei for nuclear transfer of somatic cells experiments. The aim of this study was to insert the pFN1 plasmid using purified Cas9-Nickase protein and gRNA complexes into bMEC and to establish the optimal conditions for transfection using lipofectamine CRISPRMAX. The pFN1 plasmid contains approximately 13kb, in which it includes the promoter of the BLG gene (beta lactoglobulin). For insertion of pFN1 into the bovine genome trough HDR strategy, 3'ARM (834pb) and 5'ARM regions (855pb) of ROSA26 were coupled to the plasmid, that is a common to donor vector in the bovine genome. The lipofection was performed in bMEC in the seventh passage and the pFN1+Cas9+gRNA+lipofectamine complex was prepared according to the manufacturer. Lipofected bMEC remained in culture for 96h and then were trypsinized for incorporation analysis. Qualitative analysis of the pFN1 sequence of interest was performed by RT-PCR and visualized on a 1% agarose gel. In addition to the pFN1 sequence, the housekeeping gene bGAPDH was also analyzed for positive control, and non-lipofected bMECs for negative control. All lipofected bMECs showed incorporation of pFN1 plasmid. Our preliminary results indicate that bMEC can be used to produce recombinant proteins and that delivery of Cas9/gRNA protein complexes using CRISPRMAX lipofectamine is an efficient method for the production of transgenic bMEC. The next steps of the assay are to sequence the lipofected bMECs, in addition to inducing the secretion of milk proteins and isolating the pFN1+ strains.

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Pre-implantation development of in vitro fertilized bovine zygotes injected with CRISPR/Cas9 system targeting the beta-lactoglobulin gene

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Resumo

The beta-lactoglobulin (BLG) protein is one of the main allergen in cow's milk. An interesting approach to eliminate this protein from cow's milk is to use genome editing to knockout the BLG gene in order to generate cows able to produce BLG-free milk. This study aimed to evaluate the pre-implantation development and nucleotide insertion/deletion (indel) rates in embryos derived from zygotes injected with CRISPR/Cas9 system targeting the BLG gene. Synthetic guide RNA (sgRNA) was designed to target exon 2 of the BLG gene. Cytoplasmic injection solution was composed of 100 ng / µL sgRNA and 100 ng / µL Cas9 mRNA (GeneArt CRISPR nuclease mRNA, Invitrogen, Carlsbad, USA) diluted in OptiMEM medium (Invitrogen). In vitro matured oocytes were in vitro fertilized (IVF) and 18 - 19 h after fertilization the cytoplasm of presumptive zygotes were injected with sgRNA and Cas9 mRNA solution using an ICSI needle attached to a micromanipulator mounted on an inverted microscope. Presumptive zygotes (n = 167) were cultured in Synthetic Oviduct Fluid medium supplemented with 1.5% fetal calf serum at 38.5 °C with 5% CO2 in air, for seven days after IVF. Non-injected presumptive zygotes (n = 64) were used as control and cultured in vitro under the same conditions of the CRISPR/Cas9 injected zygotes. Cleavage and blastocysts rates were analyzed by Chi-square. Pools of blastocysts were collected for DNA sequencing analysis. DNA extraction and PCR amplification of the target site were performed in duplicates using two pools of 25 - 30 blastocysts and PCR fragments were submitted to Sanger sequencing. The control group was comprised of non-injected blastocysts. Proportion of sequences with indels within samples was calculated by TIDE (tracking of indels by decomposition) web application (Brinkman et al., Nucleic Acid Research 46: e58, 2018). Characterization of alleles was performed by CRISP-ID web application (Dehairs et al., Sci. Rep. 6: 28973, 2016). Thirteen blastocysts derived from CRISPR/Cas9 injected zygotes were transferred to synchronized recipients (one embryo per recipient). Cleavage (79.6% and 70.0%) and blastocyst (43.7% and 34.7%) rates were similar (P > 0.05) between control and CRISPR/Cas9 injected groups, respectively. The mean percentage of DNA sequences with indel was 42.5 ± 2.0% with a R2 value of 0.96, considering a P-value threshold of < 0.001. Two or more alleles were identified with samples displaying monoallelic or biallelic heterozygous indels. Pregnancy rate was 38.4% (5 out of 13 recipients) but only one gestation went to term, generating a non-edited calf. In conclusion, cytoplasmic injection of CRISPR/Cas9 system targeting the exon 2 of the BLG gene did not impair pre-implantation development of bovine embryos. Although the tested procedure resulted in a reasonable indel rate in the embryo samples, studies are required to generate BLG-edited calves derived from IVF embryos.

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