poor transgene expression due to "position-effect". hTGF-β1 gene targeted integration into the goat genome will be a more efficient strategy for development of an improved transgenic goat model for AF. AAVS1 locus, a common integration site of adeno-associated virus 2, is used as a nonpathogenic "safe harbor" for site-specific integration to achieve persistent and strong transgene expression (Zou et al. 2011 Blood 117: 5561–5572). Here, we report that through TALEN-mediated gene targeting strategy, the hTGF-β1 gene was successfully integrated into the goat AAVS1 locus. Specifically, we first showed that TALEN-AAVS1 can efficiently induce a double-strand break with cleavage efficiency of 2 % in transfected goat fibroblast cells. Then we knocked in the α-MHC-TGF-β1 expression cassette (8.5 kb) into the goat AAVS1 locus by co-transfecting goat fibroblast cells with TALEN-AAVS1 and α-MHC-TGF-β1 donor vector. Single cell fibroblast colonies were isolated from the transfected cells using puromycin-mediated positive selection. Among the 8 puromycin resistant clones, 6 (75 %) were confirmed as correctly targeted by PCR and sequence analysis. These cell clones will be used for transgenic goat production by SCNT. In addition, the target site sequence of goat TALEN-AAVS1 has 100 % homology to that in the sheep and therefore can also be used for developing transgenic sheep by TALENs-mediated safe harbor (AAVS1) targeting.

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## Evaluation of culture and electroporation of feline fetal fibroblasts used to assess the effect of zinc finger nucleases on gene targeting efficiency

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To generate genetically engineered animals by somatic cell nuclear transfer, methods must be established for transfection, selection and culture of primary cells. Parameters for transfection and selection of feline fetal fibroblasts (FFF) are not well established. The objectives of this project were to evaluate conditions for square-wave electroporation, and G418 selection. In the culture conditions used (DMEM supplemented with 12.5 % fetal bovine serum, at 38.6 °C in and atmosphere of 5.0 % $O_2$, 5.5 % $CO_2$), the minimum dose of G418 that produced no background colonies was between 120 and 130 μg/ml. Electroporation was evaluated in 2 mm cuvettes using a single 1 ms pulse of varying voltages (V; 100, 150, 200, 250, 300, 350) and multiple 1 ms pulses (1, 2, 3, 4 or 5) at 300 V for delivery of a red fluorescent protein reporter gene. Cell survival, percent transfected of surviving cells, and total number of transfected cells were evaluated. Survival decreased with increased voltages and with increased pulse number. The percentage of the surviving cells that were transfected increased with voltage and pulse number (maximum of 58–63 %). The maximum number of transfected cells resulted from a single pulse at 300 V (range). FFFs were transfected with a targeting vector alone, or a targeting vector co-transfected with plasmids that encoded gene-specific ZFNs in order to assess targeting efficiency by homologous recombination (HR) with and without ZFNs. Co-transfection of ZFNs increased HR rates by as much as 50 fold. FFFs can be successfully cultured and transfected for genetic modification.

## Effect of lentiviral vectors microinjection into the perivitelline space of bovine early zygotes on embryo development and transgene expression

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Lentiviral vectors are an alternative to deliver transgenes into oocytes and zygotes. Previous study reported higher blastocyst production but lower expression rate when lentiviral vectors were microinjected in bovine zygotes after 18 h of insemination and compared to matured oocytes (Ewerling et al. 2006, Transgenic Res 15:447–454). The present study evaluated embryo development and transgene expression after lentiviral microinjection into perivitelline space of early zygotes (6 h post in vitro fertilization) or matured oocytes. In a preliminary experiment we evaluated the effect of short incubation period and of sperm concentration on embryo development. In vitro matured oocytes (n = 591, five repetitions) were co-incubated with 2 or 4 million sperm/mL for 6 or 20 h and cleavage and blastocyst rate were analyzed by Chi square. Higher ($P < 0.05$) cleavage rate was obtained with 4 million sperm for 20 h whereas the blastocyst rate at day seven post-insemination was similar ($P < 0.05$) between 4 million sperm for 6 and 20 h. At day 8, the blastocyst rate was higher ($P < 0.05$) for 2 and 4 million sperm for 20 h (26.6 and 28.7 %, respectively) than 2 and 4 million sperm for 6 h (13.7 and 17.1 %, respectively). Based on the preliminary results we evaluated the blastocyst rate and the expression of green fluorescent protein (GFP) transgene after perivitelline microinjection of lentiviral vectors in early zygotes (6 h post in vitro fertilization) and in matured oocytes. Lentiviral particles were generated in 293T cells by co-transfection of pMDLg pRRE, pRSV Rev, pMD2.g (Addgene, Cambridge, MA, USA), and a modified pLGW with the GFP gene under the control of the CMV promoter. The supernatant was collected and ultra-centrifuged to concentrate the viral particles, which were microinjected into perivitelline space of oocytes (n = 214, four repetitions) and early zygotes (n = 138, four repetitions). GFP expression in blastocysts was visualized using an epi-fluorescence stereoscope with eGFP filter and GFP transgene incorporation was analyzed by PCR. Data were compared by Chi square. There

was no difference ($P > 0.05$) in blastocyst rate (12.3 vs 13.1 %) and proportion of embryos expressing GFP (29.4 vs 28.5 %) between early zygotes and oocytes, respectively. However, PCR analysis revealed that 100 % of the embryos generated from microinjected oocytes had the transgene, in contrast to 50 % of those from early zygotes. Despite decreasing blastocyst production, short oocyte-sperm incubation period can be used to generate early zygotes for lentiviral perivitelline microinjection, but the efficiency of transgene incorporation is inferior to microinjected oocytes. Moreover, the difference between embryos emitting green fluorescence and embryos with the transgene incorporated suggests that silencing of GFP expression may occur at blastocyst stage. Financial support: CNPq 402607/2010-4 and FAPEMIG.

### Evaluation of Lentivirus-mediated multi-point shRNA targeting foot-and-mouth disease virus both in vitro and in vivo

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Foot-and-mouth disease virus (FMDV) causes an economically important and highly contagious disease of cloven-hoofed animals that is not adequately controlled by vaccination or biosecurity measures. To develop genetically modified FMDV resistant animals, a three shRNAs simultaneously expression cassette was constructed with lentivirus vector (LV-3shRNA). Under regulation of buffalo and bovine pol III promoters, the three different shRNAs were designed, one targeted to non-structural protein 3B gene, the other two targeted to viral polymerase protein 3D gene of FMDV respectively. First, FMDV replication inhibitory effect of LV-3shRNA on BHK-21 cells and sucking mice were determined, and the results showed that LV-3shRNA could reduce virus growth by threefold (24 h post-infection) when cells were challenged with $10^7$ $TCID_{50}$/ml of O serotype FMDV. The pretreated sucking mice with LV-3shRNA were protected 100 % with 5 $LD_{50}$ of FMDV. Furthermore, we generated transgenic mouse models integrating LV-3shRNA. When examined at the 4th passage in transgenic mice, the target gene was still found by PCR to be integrated in the genome. Compared to the control mice, the transgenic mice showed some delay of the onset of disease when they were infected with the FMDV serotype O, and the higher survival rate in the 5 $LD_{50}$ treatment group. These results facilitate the permanent introduction of novel disease-resistance traits into the genome of buffalo and bovine in the future.

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### IRES mediated bicistronic vector expression in normal mouse epithelial mammary cell line

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In order to test the functionality of an IRES mediated bicistronic vector for human lactoferrin and lysozime, an in vitro culture system of normal murine mammary gland cell line (NMuMG) was used. The pIRES2-EGFP vector (Clontech Inc., USA) was used as the backbone of the construct. CMV promoter was replaced by the goat β-casein obtained from pBC-1 milk expression system (Invitrogen corp., CA, USA). Kozak sequence was added to each oligonucleotide used to amplified human genes to achieve better ribosomal reconnaissance and their correct expression. Vector was spread and kept in *E. coli* Top Ten (Invitrogen, CA, USA). NMuMG cells cultured at 70 % confluence were exposed to a mix of 5 μg DNA of bicistronic vector and 2 μL Lipofectamin 2000 (Invitrogen, CA, USA) in 100 μL OptiMEM (Gibco) without antibiotics for 4 h at 37 °C. After transfection culture wells were covered with 50 mg/cm$^2$ of *Matrigel*$^{TM}$ Basement Membrane Matrix (Becton–Dickinson, Franklin Lakes, NJ, USA). Lactogenic hormones (5 μg mL$^{-1}$ Insulin, 10 μg mL$^{-1}$ prolactin and 1 μg mL$^{-1}$ Dexamethasone) were added to culture media to induced vector expression. Culture media (100 microl) obtained from transfected NMuMG cells was mixed with cracking buffer, resolved in SDS-PAGE and developed by western blotting. Primary antibodies were either rabbit anti human lactoferrin or lisozyme (Dako, Cytomation, USA) diluted 1/3,000 in TBS solution with 5 % milk. Secondary antibodies were goat anti rabbit IgG Horseradish Peroxidase conjugate (Gibco, CA, USA) and developed with Supersignal West Pico Chemiluminescent (Pierce, USA). The reaction was revealed using an X ray film (Kodak, Japón), according to the manufacturer's instructions. Bands corresponding to the molecular weight of human lactoferrin (80 kDa) and lysozyme (14.4 kDa) proteins were detected. The use of NmuMG cell line transfected with a bicistronic vector showed its functionality, previous to expensive developing of bitransgenic animals.

### Injection of recombinant adeno-associated virus (AAV) in the mouse, rat and rabbit mammary gland for the production of recombinant proteins

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The production of recombinant pharmaceutical proteins in the mammary gland of transgenic animals is an attractive option compared to their synthesis in cell culture. However, the