A209E Cloning, transgenesis, and stem cells

RS-1 increases CRISPR-mediated Knock-in rate in bovine embryos

Ismael Lamas-Toranzo[1], Álvaro Martínez-Moro[1,2], Elena O´Callaghan[3], José María Sánchez[3], Gema Millan[1], Pat Lonergan[3], Pablo Bermejo-Alvarez[1]

[1]1Animal Reproduction Department, INIA, Madrid, Spain; [2]Procreatec, Madrid, Spain; [3]School of Agriculture and Food science, University College Dublin, Belfield, Dublin 4, Ireland.

Keywords: CRISPR, bovine, homologous recombination.

The insertion of genomic sequences at specific loci (targeted Knock-in, KI) has been challenging due to the low efficiency of homologous recombination (HR). This efficiency has been boosted by the use of endonucleases, such as CRISPR, that generate a double-strand break (DSB) at the target locus. However, CRISPR-generated DSB can be repaired by one of two mechanisms: 1) HR, which can lead to the intended targeted KI if a donor DNA is provided or 2) non-homologous end Joining (NHEJ), which generates random mutations. The objective this study has been to test the effect of an enhancer of HR pathway, RS-1, on the KI rates following CRISPR injection in bovine zygotes. A preliminary study (3 replicates) was conducted to evaluate the highest concentration of RS-1 compatible with normal percentages of developmental. Bovine zygotes were incubated in SOF supplemented with 0, 7.5 or 15 µM RS-1 for 24 h and subsequently cultured in the absence of RS-1 for 8 days. RS-1 at 15 µM significantly reduced embryonic cleavage and blastocyst development, whereas 7.5 µM resulted in similar percentages of development to the control group (cleavage: 71.8±3.0; 83.6±1.8; 84.0±3.4 %; blastocysts 16.9±3; 30.3±3; 37.8±6.5 %, for 15, 7.5 and 0 µM groups, respectively; mean±s.e.m. logistic regression and ANOVA $P<0.05$). In a second experiment, zygotes where injected with CRISPR components (300 ng/µl Cas9 mRNA and 100 ng/µl sgRNA) and a single-stranded donor DNA (100 ng/µl) to mediate the insertion of an XbaI restriction site on a target non-coding region. Following microinjection, zygotes were transiently incubated for 24 h in SOF containing no RS-1 or RS-1 at 3.75 µM or 7.5 µM. As expected, the three groups displayed similar percentages of embryonic cleavage (77.8±3.3; 78.8±2.5; 73.7±4.0 %, for 0, 3.75 and 7.5 µM groups, respectively; mean±s.e.m., 5 replicates) and development to blastocyst (25.8±1.7; 27.0±2.0; 23.2±1.8 %, for 0, 3.75 and 7.5 µM groups, respectively; mean±s.e.m., 5 replicates). Resulting blastocysts were genotyped to detect genome edition (Sanger sequencing of PCR product) and targeted KI (XbaI digestion of PCR products). A significantly higher incidence of targeted insertion was achieved in embryos exposed to 7.5 µM RS-1 following microinjection compared with other groups (53.1% -17/32- for 7.5 µM vs. 26.5% -9/34- and -9/39- 23.1 % for 0 and 3.75 µM, respectively; Fisher's exact test $P < 0.05$). In conclusion, transient exposure of bovine embryos to 7.5 µM RS-1 following CRISPR microinjection enhances targeted insertion of genomic sequences, highlighting its potential for animal research.

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Vital tagging of potential naïve pluripotency in a mouse model

Iqbal Hyder[1], Naresh L Selokar[1,3], Wiebke Garrels[2], Sabine Klein[1], Wilfried A. Kues[1]

[1]Friedrich Loeffler Institut - Institute of Farm Animal Genetics, Neustadt, Germany; [2]Institute of Laboratory Animal Science, Medical School Hannover, Germany; [3]Central Institute for Research on Buffaloes, ICAR, Hisar, India.

Keywords: pluripotency, naive cells, endogenous retrovirus.

Recent studies suggested that two distinct pluripotent states exist in mouse and human embryonic stem cells that are termed as naïve and primed. The naïve pluripotency constitutes a ground state with full differentiation potential, whereas the primed state has a restricted differentiation potential (Boroviak et al. Nat. Cell Biol. 16, 516–528). The preimplantation epiblast represents a naïve pluripotent state, whereas the post-implantation epiblast reaches a primed state. Recently, it was shown that the expression of particular classes of endogenous retrovirus (ERV) mirror the naïve state in murine and human pluripotent cells (Wang et al. Nat Protoc. 11(2):327-46, 2016). We hypothesized that the naïve pluripotency could be tagged in vivo by a fluorescent reporter construct driven by the long terminal repeat (LTR) promoter of the human ERV7. To achieve this, the LTR7-GFP construct in a Sleeping Beauty (SB) transposon together with the SB transposase vector were co-injected into the cytoplasm of murine zygotes. The zygotes were transferred to surrogate animals, and subsequently the newborn mice were genotyped. A total of four founder transgenic animals were identified and used to establish stable lines. Expression of the LTR7-GFP was restricted to a subpopulation of the inner cell mass of late murine blastocysts. Additionally, expression of the reporter could be reactivated by reprogramming of fetal fibroblasts to induced pluripotent stem (iPS) cells. In somatic cells, no reporter expression was detected by fluorescence microscopy and RT-PCR. Thus the vital reporter labels a subpopulation of pluripotent cells in vivo and in vitro. Currently, additional experiments are ongoing to characterize this subpopulation in detail. This mouse model can be used to understand the development of pluripotency during early ontogenesis, and may provide insights into the regulation of pluripotency especially by temporal divergence in expression patterns of various endogenous retroviruses.

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Generation of vascular deficient porcine embryos

Marta Moya-Jódar[1], Giulia Coppiello[1], Gloria Abizanda[1], Juan Roberto Rodríguez[1], Francisco Alberto García-Vázquez[2], Felipe Prósper[1], Xabier L. Aranguren[1]

[1]Regenerative Medicine Program, Center for Applied Medical Research (CIMA), University of Navarra, Pamplona, Spain; [2]Department of Physiology, University of Murcia, Murcia, Spain;

Keywords: organ generation, CRISPR/Cas9, porcine embryos.

Organ transplantation is, in many cases, the only life-saving treatment for end-stage organ failure, but the main problem is the shortage of these organs. Currently, organs donations are insufficient to cover demand, so other alternatives are needed. Generation of human organs from pluripotent stem cells (PSCs) in animal recipients would provide an endless source of organs for clinical use. Blastocyst complementation is an extraordinarily promising approach to fulfil this unmet medical need. This technique allows the development of an organ/tissue that a genetically modified embryo is unable to form. It consists in the microinjection of PSCs into the cell type/organ-deficient preimplantational embryo and the completion of the embryonic development in the uterus of a foster mother. In this environment, the microinjected PSCs colonize the empty developmental niche and contribute entirely to its formation. Using blastocyst complementation strategy, a cell type/organ deficient pig embryo could be used to generate a humanized/human organ, owing to pigs are physiologically similar to humans as well as the size of their organs. Therefore, as first step, organ/tissue deficient pig embryos need to be generated. Because endothelial cells play a very relevant role in organ rejection upon xenotransplantation, we are generating vascular deficient pig embryos using CRISPR/Cas9 technology to erase ETV2 gene, since it is a master regulator of hematoendothelial lineages. For a preliminary study, we designed 5 different guides (Benchling, USA) against pig's ETV2 gene and were tested *in vitro* on pig's fibroblast. Genome editing was analyzed by Surveyor Mutation Kit (IDT, Spain) and 4 out of 5 guides showed cleavage capacity. Subsequently, the 4 selected guides were individually microinjected with Cas9 protein complex (100 ng/µl Cas9 protein and 50 ng/µl sgRNA) (IDT, Spain) 6h post fertilization into 1 cell-stage pig embryos. The embryos were cultured until blastocyst stage in a humidified atmosphere at 38.5ºC, 5% O_2 and 5% CO_2. Next, DNA amplification by PCR were performed before the deep sequencing analysis. Mutant embryos were obtained with the four microinjected guides, but only two of them achieved biallelic ETV2 disruption, although at low efficiency (Guide 1; 5% KO, Guide 2; 5% heterozygous, Guide 3; 5% KO and 5% Mosaic, Guide 4; 10% Mosaic and 5% heterozygous). Based on the foregoing, future experiments are required in order to optimize the generation of vascular deficient embryos. In the future, efficient ETV2 KO pig embryos could be used as recipient for human chimera-competent cells, that one may generate human vascularized organs. Moreover, this strategy can be expanding to any other organ in a deficient specific model embryo thus permitting the generation of fully humanized organs in a livestock animal and one day, resolve the organ shortage in the clinic.

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First production of Calpain3 KO pig embryo by CRISPR/Cas9 technology for human disease modelling: efficiency comparison between electroporation and intracytoplasmic microinjection

Sergio Navarro-Serna[1], Raquel Romar[1], Martxel Dehesa-Etxebeste[2], Jordana S. Lopes[1], Adolfo López de Munain[2], Joaquin Gadea[1]

[1]University of Murcia Dept. Physiology, Murcia, Spain. International Excellence Campus for Higher Education and Research "Campus Mare Nostrum" and Institute for Biomedical Research of Murcia (IMIB-Arrixaca), Murcia, Spain; [2]IIS Biodonostia, Neuroscience, San Sebastián, Spain.

Keywords: transgenesis, CRISPR/Cas9, porcine.

Limb-Girdle Muscular Dystrophy Type2A is an autosomal recessive myopathy caused by mutations in the Calpain3 gene. Currently the disease has not treatment and lacks good animal models. Thus, the study of this disease in pig would offer a great valuable tool to understand the disease and its possible treatments. In porcine species, somatic cell nuclear transfer and CRISPR microinjection (MI) are the main techniques to produce genetically modified embryos (GME). Previous studies have demonstrated that electroporation (EP) allows the production of GME embryos and further offspring (Tanihara, Sci. Adv. 2, e1600803, 2016). In this work we compare the use of MI and EP to produce GME for Calpaine3 evaluating embryo quality and mutation rate. In vitro matured porcine oocytes were treated before insemination with 100ng/µl of CRISPR/Cas9 ribonucleoprotein (RNP) by using two Calpain3 RNAguides. Five groups were used to compare mutation efficiency and embryo development achieved by MI and EP: oocytes microinjected with RNP (MI group), oocytes microinjected without RNP (sham group); oocytes electroporated with 4 (EP4 group) or 6 (EP6 group) pulses (30mV, 1 ms); and non-treated oocytes (Control group). All treatments were performed before IVF. Oocytes were inseminated in TALP medium with frozen-thawed boar spermatozoa selected with NaturARTs-PIG sperm swim-up medium (EmbryoCloud, Murcia, Spain). Eighteen hours after insemination, putative zygotes were cultured (NCSU-23 medium) for additional 156 h to evaluate the blastocyst yield, regarding the total number of oocytes, and the gene deletion by PCR previous digestion of zona pellucida to remove bound spermatozoa. Experiment was repeated 4 times with 50-55 embryos per group and variables were analyzed by one-way ANOVA. Results showed similar cleavage rate among groups (63.2-72.9%) except for EP6 group which was the lowest (42.9; $p<0.01$). Blastocyst yield decreased in all treatment compared to control group (32.9±3.2%), being similar between MI (22.5±2.9%), sham (21.1±2.8%) and EP4 groups but again the EP6 was the lowest (11.1±2.2%; $p<0.01$). Regarding mutation rate, 41.5% (17/41) of MI derived blastocysts had a large gene deletion whereas EP4 showed 20.7% (6/29) and EP6 was 17.65% (3/17). As for the biallelic KO, it was similar in all blastocysts independently of the treatment applied: 14.6% (6/41) for MI; 17.2% (5/29) for EP4 and 11.8% (2/17) for EP6. These results confirm that EP is a valuable technique to produce KO embryos using CRISPR/Cas9 technology. Despite the low efficiency, the easiness to produce a greater number of embryos in a shorter time as well as the requirement of less high-qualified personnel and high-value equipment than with MI increase the possibility of its use to generate KO embryos. This is the first report about production of Calpain3 KO pig embryos, opening the doors to generation of KO big animals and promising further advances in the relevant field of human disease study. Supported by MINECO-FEDER (AGL 2015-66341-R), Fundación Séneca 20040/GERM/16 and FPU fellowship (FPU16/04480) from the Spanish Ministry of Education, Culture and Sport.

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Generation of a polled phenotype in cattle using CRISPR/Cas

Felix Schuster[1], Patrick Aldag[1], Antje Frenzel[1], Petra Hassel[1], Maren Ziegler[1], Andrea Lucas-Hahn[1], Heiner Niemann[2], Björn Petersen[1]

[1]Institute of Farm Animal Genetics, Friedrich-Loeffler-Institute, Mariensee, Germany; [2]TWINCORE, Hannover Medical School, Hanover, Germany.

Keywords: genome editing, somatic cell nuclear transfer, cattle.

In modern livestock farming horned cattle pose an increased risk of injury for each other as well as for the farmers. Dehorning without anesthesia is associated with stress and pain for the calves and raises concerns regarding animal welfare. Naturally occurring mutations causing polledness are known for most beef cattle but are rarely distributed within dairy populations such as Holstein-Friesians and Brown Swiss. The propagation of polled Holsteins and Brown Swiss is limited due to the rather low genetic merit of the offered polled bulls which originate from a few founder bulls. In beef cattle, a mutation consisting of a 210 bp insertion and an 8 bp deletion (*Celtic mutation*) causes the polled phenotype while in Holsteins an 80 kbp duplication accompanied by several single point mutations is causative (Medugorac, Seichter et al. 2012). In this project, we used the CRISPR/Cpf1 system (Cas12a) to introgress the *Celtic mutation* into the *horned locus* of Holstein-Friesian and Brown Swiss fibroblasts derived from horned individuals with the aim of producing polled clones from originally horned bulls. The *Celtic mutation* was isolated from an Angus cow via PCR and cloned into a transfection vector as a knock-in template. Editing efficiencies in this locus were low, so multiple CRISPR/Cpf1 target sites were evaluated in order to improve knock-in efficiencies. Furthermore, we used the CRISPR/Cas9 system to create a novel knock-out mutation in the *horned locus* to examine whether also a deletion in this genomic area causes a polled phenotype. For this purpose, two target sites flanking a 300 bp sequence were used to create a large knock-out mutation. Cell clones carrying the desired mutation were propagated further to serve as donor cells for the somatic cell nuclear transfer (SCNT). In the fetus, horn buds are histologically detectable from day 90 of the gestation (Allais-Bonnet et al. 2013), hence the first pregnancy of each experiment will be aborted prematurely to examine the development of horn buds. All other pregnancies will be carried to term. Sequencing data and PCR results showed the desired integration for both the knock-in and knock-out experiment. First cloning experiments showed that development rates of edited embryos (blastocyst rate: 23,68 %; (9/38)) were comparable with those of wild type embryos (blastocyst rate: 20,29 %; (14/60)). The edited embryos were recently transferred into surrogate mothers. In conclusion, we successfully edited the genome of bovine fibroblasts by using different variants of the CRISPR system to introgress a complex mutation (*Celtic mutation*) and also create a novel knock-out mutation. Furthermore, we were able to reliably produce embryos from edited cell lines using SCNT. Once the embryo transfers result in pregnancies the fetuses and offspring, respectively, will be examined for polledness.

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Establishment of several cloned pregnancies of buffalo breeding bulls

Naresh Selokar, Monika Saini, Rasika Rajendran, Seema Dua, Dharmendra Kumar, Rakesh Sharma, Prem Singh Yadav

ICAR-Central Institute for Research on Buffaloes, India.

Keywords: buffalo, cloning, breeding bull, embryo.

Buffalo cloning is a valuable tool to improve the genetic potential of buffalo. Despite reported births of cloned buffaloes worldwide, the birth of several clones (more than 5 clones) of an individual buffalo have not yet been reported. Thus, in the present study, we attempted to produce multiple clones of an individual buffalo. The skin-derived fibroblast cells of two buffalo breeding bulls, namely M-29 and NR-480, were used as nuclear donors. The cloned embryos were produced using optimized handmade cloning (HMC) of our laboratory. The blastocyst production rate ranges from 35-40% in each experiment for both bulls. We transferred one or two cloned blastocysts on day 7 and 8 post oestrus into recipient buffaloes. We used transrectal ultrasonography to confirm the pregnancies at Day 30 post embryo transfer, and reconfirmed it again at day 60. We established 13 pregnancies (13 pregnant/56 recipient buffaloes, represents 23% conception rate) of M-29 bull, of which three were aborted at the first trimester. For NR-480 bull, we established 3 pregnancies (3 pregnant/7 recipient buffaloes, represents 42% conception rate). These established pregnancies are continuing at six to two months of gestation. To establish more cloned pregnancies, the embryo production and embryo transfer experiments are on-going. In conclusion, we established multiple cloned pregnancies of an individual buffalo using HMC embryos. HMC can be used to multiply elite buffalos in short period.