Transgenesis

355 COMPARISON OF TN5 AND SLEEPING BEAUTY SYSTEMS IN BOVINE EMBRYOS AND IN OVINE OFFSPRING

R. J. Bevacqua[A,B], *R. Fernandéz Martín*[A], *A. Gibbons*[C], *D. Teixeira*[D], *N. G. Canel*[A], *F. Lange*[B], *M. I. Hiriart*[A], *W. A. Kues*[E], *S. Ferraris*[B], *and D. F. Salamone*[A]

[A]Laboratorio de Biotecnología Animal, FAUBA, Buenos Aires, Argentina;
[B]Laboratorio de Clonación y Transgénesis, Universidad Maimónides, Buenos Aires, Argentina;
[C]Estación Experimental Bariloche, INTA, Bariloche, Río Negro, Argentina;
[D]Laboratorio de Fisiologia e Controle da Reprodução, FAVET, UECE, Fortaleza, Brazil;
[E]Friedrich-Loeffler-Institut, Neustadt, Germany

Current techniques for the production of transgenic domestic animals remain inefficient. Only recently, DNA transposons resulted in improved efficiencies for mouse and pig transgenesis. In this work, we evaluated Tn5 and Sleeping Beauty systems for transgenesis in bovine and ovine species. First, both transposon systems were assessed *in vitro* in bovine embryos employing transposons carrying fluorescent reporter genes. *In vitro*-produced bovine zygotes were microinjected with either 1) a complex of Tn5:*egfp* transposon (20 ng μL^{-1}) (protein: transgene with mosaic ends recognised by Tn5, in Mg+2 free medium), or 2) two plasmids carrying Sleeping Beauty 100X (pSB100X, 5 ng μL^{-1}) and pT2/Venus transposon (10 ng μL^{-1}). *In vitro* results for Tn5 transgenesis in bovine showed that blastocysts, Day 4 *egfp* embryos and *egfp* blastocysts rates for the group injected with Tn5:*egfp* did not differ from the group injected with the *egfp* transposon alone (73/145, 50%; 86/145, 59%; and 65/145, 45% *v*. 65/129, 50%; 87/129, 67%; and 57/129, 44%, respectively). For SB transgenesis, blastocysts, D4 Venus embryos, and Venus blastocysts rates did not differ between co-injection

of pSB100X and pT2/Venus or injection with pT2/Venus alone (46/99, 46.5%; 64/99, 64.6%; and 33/99, 33.3% *v.* 41/83, 49.4%; 52/83, 62.7%; and 26/83, 31.3%, respectively). However, Venus intensity in blastocysts was markedly higher for the group co-injected with pSB100X and pT2/Venus respective to pT2/Venus alone. Both systems were assessed *in vivo* for the production of transgenic lambs employing a functional transposon (hrFIX, recombinant human factor IX driven by a Beta-lactoglobulin promoter). Laparoscopic artificial insemination of donor sheep was performed, and presumptive zygotes were flushed from the oviducts. The microinjections were done identically as described for the bovine embryos. A total of 24 presumptive zygotes were recovered and injected with the Tn5:hrFIX complex. Then, 21 zygotes were transferred to 5 synchronized ewes; one pregnancy of siblings was obtained, and one animal was born. Genomic DNA from skin, placenta, and blood was genotyped by PCR, but the hrFIX gene could not be detected. For the SB approach, 64 presumptive zygotes were recovered from 4 superovulated ewes, microinjected with the SB plasmids, and 21 of them were transferred to 7 oestrous synchronized recipients. The remaining zygotes were cultured *in vitro* and blastocysts ($n = 7$) were vitrified. Currently, 3 donor ewes are pregnant, one with siblings (4 total fetuses). Deliveries are expected by the end of August of this year. Our results indicate that both Tn5 and SB systems are capable of resulting in the production of transgene expressing embryos, and the presence of the transposases does not affect embryo viability. However, phenotyping of blastocyst stages does not seem to be predictive for stable transgene integration. The *in vivo* results will help to better address the suitability of Tn5 and SB approaches for the production of transgenic sheep.

356 SLEEPING BEAUTY TRANSGENESIS IN CATTLE

W. Garrels[A,B], *T. R. Talluri*[A], *R. Bevacqua*[C], *A. Alessio*[D], *A. Fili*[D], *D. Forcato*[D], *N. Rodriguez*[D], *M. F. Olmos Nicotra*[D], *Z. Ivics*[E], *D. F. Salamone*[C], *P. Bosch*[D], *and W. A. Kues*[A]

[A]Friedrich-Loeffler-Institut, Institut für Nutztiergenetik, Neustadt, Germany;
[B]Institute for Laboratory Animal Sciences, Medical School Hannover, Hannover, Germany;
[C]Departamento de Producción Animal, Universidad de Buenos Aires, Argentina;
[D]Departamento de Biología Molecular, FCEFQyN, Universidad Nacional de Río Cuarto, Córdoba, Argentina;
[E]Paul-Ehrlich-Institute, Langen, Germany

Transposon-mediated transgenesis is a well-established tool for genome modification in small animal models. However, translation of this active transgenic method to large animals warrants further investigations. Here, the Sleeping Beauty (SB) transposon system was assessed for stable gene transfer into the cattle genome. The transposon plasmids encoded a ubiquitously active CAGGS promoter-driven Venus reporter and a lens-specific α A-crystallin promoter driven tdTomato fluorophore, respectively. The helper plasmid carried the hyperactive SB100x transposase variant. In total, 50 *in vitro*-derived zygotes were co-injected (Garrels *et al.* 2011 PLoS ONE 6; Ivics *et al.* 2014 Nat. Protoc. 9) and cultured up to blastocyst stage (Day 8). Two blastocysts were Venus-positive and were transferred to synchronized heifers, resulting in one pregnancy. The resulting calf was normally developed and vital; however, it died shortly after cesarean section due to spontaneous bleeding from an undetected aneurism. Phenotypic analysis suggested that the calf was indeed double-transgenic, showing widespread expression of Venus and lens-specific expression of tdTomato. Genotyping and molecular analyses confirmed the integration of both reporter transposons and the faithful promoter-dependent expression patterns. Subdermal tissue of an ear biopsy was used to culture fibroblasts, which were employed in somatic cell nuclear transfer experiments. In total, 39 embryos were reconstructed, of which 34 underwent cleavage, and at the end of culture 12 morulas and 12 blastocysts were obtained. Ten of the blastocysts were Venus positive, and embryo transfer of Venus-positive blastocysts is planned. In summary, we showed that the cytoplasmic injection of SB components is a highly efficient method for transgenesis in cattle. Due to the modular composition of SB plasmids, even double transgenic cattle can be generated in a one-step procedure. Importantly, the SB-catalyzed integration seems to favour transcriptionally permissive loci in the genome, resulting in faithful and robust promoter-dependent expression of the transgenes. The transposon constructs carry heterospecific *loxP* sites, which will be instrumental for targeted insertion of functional transgenes by Cre recombinase-mediated cassette exchange.

Financial support of DFG (Ku 1586/3-1), UNRC, CONICET and Agencia Nacional de Promoción Científica y Tecnológica de la Argentina (ANPCyT) is gratefully acknowledged.

357 EARLY FETAL DEVELOPMENT OF NUCLEAR TRANSFER BOVINE EMBRYOS GENERATED FROM FIBROBLASTS GENETICALLY MODIFIED BY *PIGGYBAC* TRANSPOSITION

A. Alessio[A], *A. Fili*[A], *D. Forcato*[A], *M. F. Olmos-Nicotra*[A], *F. Alustiza*[A], *N. Rodriguez*[A], *R. V. Sampaio*[B], *J. Sangalli*[B], *F. Bressan*[B], *P. Fantinato-Neto*[B], *F. Meirelles*[B], *J. Owens*[C], *S. Moisyadi*[C], *W. A. Kues*[D], *and P. Bosch*[A]

[A]Department of Molecular Biology, FCEFQyN, National University of Río Cuarto, Río Cuarto, Córdoba, Argentina;
[B]Department of Veterinary Medicine, Faculty of Animal Sciences and Food Engineering, University of São Paulo, São Paulo, Brazil;
[C]Department of Anatomy, Biochemistry and Physiology; John A. Burns School of Medicine, Honolulu, HI, USA;
[D]Friedrich-Loeffler-Institut, Institut für Nutztiergenetik, Mariensee, Germany

Transposon-mediated transgenesis is a well-established tool for genome manipulation in small animal models. However, translation of this active transgenesis method to the large animal setting requires further investigation. We have previously demonstrated that a helper-independent *piggyBac* (PB) transposon system can efficiently transpose transgenes into the bovine genome [Alessio *et al.* 2014 Reprod. Domest. Anim. **49** (Suppl. 1), 8]. The aims of the current study were a) to investigate the effectiveness of a hyperactive version of the PB transposase, and b) to determine the ability of

the genetically modified cells to support early embryo and fetal development upon somatic cell nuclear transfer (SCNT). Bovine fetal fibroblasts (BFF) were chemically transfected with either p*m*GENIE-3 (a helper-independent PB transposon conferring genes for hygromycin resistance and enhanced green fluorescent protein (EGFP); Urschitz *et al.* 2010 PNAS USA **107**, 8117–8122), p*mhy*GENIE-3 (carrying an hyperactive version of the PB transposase; Marh *et al.* 2012 PNAS USA **109**, 19 184–19 189), or p*m*GENIE-3/Δ PB (a control plasmid lacking a functional PB transposase). Upon transfection, cell cultures were subjected to 14 days of hygromycin selection. Antibiotic-resistant and EGFP+ colonies were counted and data analysed by ANOVA and Tukey's test. For SCNT, p*mhy*GENIE-3 and p*m*GENIE-3 polyclonal cell lines were selected by FACS and individual cells used as nuclear donors. Day 7 blastocysts were nonsurgically transferred to synchronized recipients. Conceptuses were recovered by Day 35 of gestation, observed under fluorescence excitation, and genotyped. The mean number of colonies in p*mhy*GENIE-3 group was significantly higher than those in p*m*GENIE-3 and the control group (324.0 ± 17.8 *v.* 100.0 ± 16.1 and 2.8 ± 0.8 respectively, $n = 4$–7; $P < 0.05$). The hyperactive transposase increased transgene integration efficiency 3.24 times compared with the conventional PB transposase. The SCNT and early fetal development data are summarised in Table 1. Phenotypic analysis revealed that both transgenic fetuses and the extraembryonic membranes expressed EGFP with no macroscopic evidence of variegated transgene expression. Molecular analysis by PCR confirmed that both fetuses carried the transposon DNA. Here, we demonstrate that a hyperactive version of the PB transposase is more active in bovine cells than the conventional PB transposase. In addition, SCNT embryos generated from genetically modified cells by the pGENIE transposon system can progress to early stages of fetal development.

Table 1. SCNT and early fetal development of bovine fibroblasts transposed with piggyBac[1]

Experimental group	Oocytes	Fused couplets (%)	Cleavage (%)	Blastocysts (%) [no. of blastocysts expressing EFGP]	No. of transferred embryos	No. of pregnant/total recipients	No. of recovered fetuses (Day 35)/ no. of transgenic fetuses
p*m*GENIE-3	58	26 (44.8)	26 (100)	4 (15.4) [4]	4	0/2	0/0
p*mhy*GENIE-3	58	36 (62.0)	33 (91.7)	9 (25.0) [9]	9	2/8	2/2
PA	30	–	28 (93.3)	19 (63.3) [0]	n.a.	n.a.	n.a.

[1]PA = parthenogenetic activation; n.a. = not applicable.

The financial support of UNRC, CONICET and ANPCyT from Argentina is gratefully acknowledged.

358 EXCESS EXPRESSION OF 11B-HSD1 CONTRIBUTES TO LETHALITY THROUGH DYSFUNCTION IN ENERGY BALANCE BETWEEN ANABOLIC PROCESS AND ENERGY RECOVERY PROCESS

H. Y. Kang and E.-B. Jeung

College of Veterinary Medicine, Chungbuk National University, Cheongju, Chungbuk, South Korea

11β-Hydroxysteroid dehydrogenase type 1 (11β-HSD1) effectively amplifies glucocorticoid action in liver, adipose tissue, and brain. This enzyme converts the inactive 11-keto form into a glucocorticoid (e.g. cortisol). Glucocorticoids (GC) are steroid hormones found in the body and produced by the adrenal cortex, the outer layer of the adrenal glands. Glucocorticoids are lipophilic and readily access their intracellular receptors. Glucocorticoids regulate carbohydrate, fat, and protein metabolism. In a previous study, we constructed a vector composed of 2 parts: the 11β-HSD1 expression cassette and the selection cassette containing EGFP and Neo resistant genes. Porcine fibroblasts overexpressing 11β-HSD1 under the control of adipose tissue-specific aP2 promoter were established and used in somatic cell nuclear transfer (SCNT). Somatic cells from the resulting stillborn transgenic piglets were used in a second round of SCNT. Non-obese, transgenic piglets overexpressing 11β-HSD1 were obtained and were identified through PCR-based methods using specific primers for the targeting cassettes from the genomic DNA of piglets. Six live piglets, 1 stillborn piglet, and 3 mummies were born. Integration of target gene into the genomic DNA was confirmed for all of them. However, all 6 live piglets died within 1 month. All of the piglets had displayed hypoglycemia. Increased expression of 11β-HSD1 in metabolic tissues induced up-regulation of gluconeogenesis-related genes (G6PT, G6Pase, PEPCK, HNF4a, FOXO1) in liver and kidney, and showed up-regulation of lipogenesis-related genes (SREBP1c, FASN, DGAT, ACC, SCD) in muscle. The AMPK and SIRT signalling, which controls energy balance and mitochondrial biogenesis, was also stimulated. We propose that overexpression of 11β-HSD1 evokes the excess production and action of glucocorticoid or its receptors, and activates gluconeogenic and lipogenic pathways. For this reason, AMPK and SIRT1 signalling was induced. Also, in compensation of energy loss by anabolic processes, the expression of mitochondrial biogenesis-related genes was increased. Finally, the constitutive expression of 11β-HSD1 might continuously activate complementary energy-gaining processes, and these problems could develop into more fatal diseases that resulted in the piglets' death.

359 A NONINVASIVE APPROACH TO DIAGNOSE TRANSGENIC CONCEPTI DURING PREGNANCY IN GOATS

K. C. S. Tavares, C. R. Lazzarotto, C. M. Calderon, L. T. Martins, S. G. Neto, L. H. Aguiar, A. M. Miranda, M. Bertolini, and L. R. Bertolini

Universidade de Fortaleza, Fortaleza, Ceara, Brazil

The discovery of cell-free fetal DNA (cffDNA) circulating in the blood of pregnant women, and more recently in cows, ewes, and mares, paves the road towards the development of molecular tools to explore genetic features of embryos and/or fetuses before term. Albeit a wide range of analyses are in current use and development in humans, genetic diagnostic targets other than sex determination are still not described for other mammalian species. The aim of this study was to detect cffDNA from transgenic goat concepti for the human lysozyme (*hLZ*) gene in the blood of nontransgenic dams. Blood was collected from 3 nontransgenic goats carrying hLZ-transgenic concepti on Days 40–50, 80–90, and 110–120 of gestation. Also, blood was drawn 8 and 12 days after parturition from two other nontransgenic goats that delivered hLZ-transgenic offspring. Blood samples (10 mL) were spun at 1200 rpm for 10 min; resulting serum or plasma were stored at −20°C (serum) or 4°C (plasma). The DNA was extracted by mixing 350 μL of serum or plasma with an equal volume of TE buffer and 5 μL of proteinase K (20 mg mL^{-1}). The mixtures were incubated at 55°C for 3 h, followed by phenol extraction and DNA precipitation by sodium acetate and 100% ethanol, with further incubation at −20°C overnight and centrifugation at 12 000 × *g* for 10 min. The DNA pellets were washed with 70% ethanol and eluted in 20 μL of ultrapure water. For the PCR, primer sets for the hLZ transgene (hLZ-i1-F 5′ CGGTCCAGGGCAAGGTCTTTGA 3′ and hLZ-i1-R 5′ ACTGCTCCTGGGGTTTTGCC 3′) and for GAPDH as the endogenous control were used. Reactions contained 3 μL of DNA, 200 nM of each primer, and 45 μL of PCR Mastermix (Quatro G Pesquisa & Desenvolvimento, Porto Alegre, Brazil). The DNA from serum and plasma of nontransgenic goats were used as negative controls. The cycling conditions were 95°C for 10 min, followed by 55 cycles of 95°C for 30 s, 58°C for 30 s and 72°C for 30 s, plus a final extension at 72°C for 10 min. The PCR products were analysed by electrophoresis in 2% agarose gel. As expected, GAPDH was amplified in most of the samples (12/13). The 200-bp PCR product corresponding to hLZ was detected in the dam's serum in all 3 gestational phases, with 2 out of 3 animals being positive on 40 to 50 and 80 to 90 days, and all 3 on 110 to 120 days of pregnancy. Furthermore, the transgene was amplified from dam's plasma in all samples after parturition. Only GAPDH amplification was detected in the blood of nontransgenic goats. These results suggest that cffDNA is present in the goat's blood circulation at the fetal phase during pregnancy and at least during the first 2 weeks after parturition. This method can be safely applied as a useful tool in zygote-DNA microinjection experiments, providing an early and preterm diagnostic of transgenic concepti through the dam's blood.

Research was supported by FINEP.

360 DOUBLE KNOCKOUT OF GOAT MYOSTATIN AND PRION PROTEIN GENE USING CLUSTERED REGULARLY INTERSPACED SHORT PALINDROMIC REPEAT (CRISPR)/CAS9 SYSTEMS

S. Hu, M. Yang, and I. Polejaeva

Department of Animal, Dairy & Veterinary Sciences, Utah State University, Logan, UT, USA

Myostatin (MSTN) acts as a negative regulator of skeletal muscle development and growth. Inhibition of MSTN expression may be applied to enhance animal growth performance in livestock production. Prion protein (PrPc) is associated directly with the pathogenesis of the transmissible spongiform encephalopathies occurring in variety of species including human, cattle, sheep, goats and deer. Prion protein-deficient livestock may be a useful model for prion research and producing animal conferring potential disease resistance. The goal of this study was to generate MSTN/PrPc double knockout goat by using CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 system. We generated 2 CRISPR/Cas9 plasmids targeting MSTN and PrPc genes, respectively. The CRISPR/Cas9 plasmids targeting each gene were respectively transfected into goat fibroblasts, and the efficiency of gene modification was determined at Day 3 using restriction fragment length polymorphism (RFLP) assay. The RFLP assay showed that CRISPR/Cas9 plasmids targeting MSTN and PrPc induced precise gene mutations with efficiency of 59 and 70%, respectively. Single cell-derived colonies were further isolated by limiting dilution after co-transfection of 2 CRISPR/Cas9 plasmids targeting MSTN and PrPc. The RFLP assay and DNA sequence analysis indicated that 9 out of 45 colonies (20%) carried simultaneous disruption of both target genes. Moreover, 5 of 9 mutant colonies (55%) had mutations in all 4 alleles of 2 genes. These double-gene knockout fibroblast cells will be used as nuclear donors for developing double knockout goat deficient in MSTN and PrPc. The CRISPR/Cas9 system represents a highly effective and facile platform for multiplex editing of large animal genomes, which can be broadly applied to both biomedical and agricultural applications.

This work was supported by the Utah Science Technology and Research Initiative and Utah Agricultural Experiment Station project #31294.

361 GROWTH HORMONE RECEPTOR MUTANT PIGS PRODUCED BY USING THE CLUSTERED REGULARLY INTERSPACED SHORT PALINDROMIC REPEATS (CRISPR) AND CRISPR-ASSOCIATED SYSTEMS IN *IN VITRO*-PRODUCED ZYGOTES

M. Kurome[A], *M. Dahlhoff*[A], *S. Bultmann*[B], *S. Krebs*[A], *H. Blum*[A], *B. Kessler*[A], *H. Leonhardt*[B], *and E. Wolf*[A]

[A]Chair of Molecular Animal Breeding and Biotechnology, and Laboratory for Functional Genome Analysis, Gene Center, LMU Munich, Munich, Germany;
[B]Chair of Human Biology and BioImaging, BioCenter, LMU Munich, Munich, Germany

Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) technology is considered as an efficient strategy for generating gene edited large animals, such as pigs. Compared to somatic cell nuclear transfer, this new technology offers a relatively simple way to generate mutant pigs by direct injection of RNA into the cytoplasm of zygotes. Moreover, the use of *in vitro* produced zygotes would provide a highly effective and practical method for the production of porcine disease models for biomedical research. Here we examined the production efficiency of growth hormone receptor (*GHR*) mutant pigs by the combination of the CRISPR/Cas system and *in vitro* produced zygotes. *In vitro* maturation (IVM) of oocytes was performed as described previously (Kurome *et al*., Meth. Mol. Biol., in press). In all experiments, the same batch of frozen sperm was used. After IVM, around 20 oocytes with expanded cumulus cells were incubated with 5×10^4 spermatozoa in a 100-μL drop of porcine fertilization medium for 7 h. *In vitro*-produced embryos were assessed by the ratio of normal fertilization (eggs with 2 pronuclei) and blastocyst formation at Day 7. The Cas9 mRNA and a single guide RNA, recognising a short sequence of 20 base pairs in exon 3 of the *GHR* gene, were injected directly into the cytoplasm of the embryos 8.5 to 9.5 h after IVF. Injected embryos were transferred laparoscopically to recipient pigs, and 86.4% (57/66) of sperm-penetrated oocytes (66/96) exhibited normal fertilization. Incidence of polyspermy was relatively low (9/66, 13.6%). Developmental ability of *in vitro*-produced embryos to the blastocyst stage was 17.4% (24/138). In total, 426 RNA-injected embryos were transferred into 2 recipients, one of which became pregnant and gave birth to 8 piglets. All piglets were clinically healthy and developed normally. In 3 out of 8 piglets (37.5%), mutations were introduced. Next-generation sequencing revealed that all of them were mosaics: one with a single mutation (22% wild-type/78% mutant) and 2 piglets with 2 different mutations (80% wild-type/2% mutant_1/18% mutant_2 and 94% wild-type/4% mutant_1/2% mutant_2). Four out of 5 mutations caused a frameshift in the *GHR* gene. Our study reports for the first time generation of *GHR* mutant pigs by the use of the CRISPR/Cas system in *in vitro*-produced zygotes. Because all *GHR* mutant offspring were mosaic, Cas9 activation probably occurred after the 1-cell stage under our experimental conditions. The founder animal with the highest proportion of mutant *GHR* alleles will be used for breeding to establish a large animal model for Laron syndrome.

This work is supported by the German Research Council (TR-CRC 127).