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Efficient edition of the bovine *PRNP* prion gene in somatic cells and IVF embryos using the CRISPR/Cas9 system

R.J. Bevacqua [a], R. Fernandez-Martín [a], V. Savy [a], N.G. Canel [a], M.I. Gismondi [b], W.A. Kues [c], D.F. Carlson [d], S.C. Fahrenkrug [d], H. Niemann [c], O.A. Taboga [b], S. Ferraris [e], D.F. Salamone [a,*]

[a] *Animal Biotechnology Laboratory, INPA UBA-CONICET, Buenos Aires, Argentina*
[b] *Instituto de Biotecnología, CICVyA, INTA-CONICET, Hurlingham, Argentina*
[c] *Institute of Farm Animal Genetics (FLI), Mariensee, Hannover, Germany*
[d] *Recombinetics Inc., St Paul, Minnesota, USA*
[e] *Cloning and Transgenesis Laboratory, Maimonides University, Buenos Aires, Argentina*

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ABSTRACT

The recently developed engineered nucleases, such as zinc-finger nucleases, transcription activator-like effector nucleases, and clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated nuclease (Cas) 9, provide new opportunities for gene editing in a straightforward manner. However, few reports are available regarding CRISPR application and efficiency in cattle. Here, the CRISPR/Cas9 system was used with the aim of inducing knockout and knock-in alleles of the bovine *PRNP* gene, responsible for mad cow disease, both in bovine fetal fibroblasts and in IVF embryos. Five single-guide RNAs were designed to target 875 bp of *PRNP* exon 3, and all five were codelivered with Cas9. The feasibility of inducing homologous recombination (HR) was evaluated with a reporter vector carrying *EGFP* flanked by 1 kbp *PRNP* regions (pHR*egfp*). For somatic cells, plasmids coding for Cas9 and for each of the five single-guide RNAs (pCMVCas9 and pSPgRNAs) were transfected under two different conditions (1X and 2X). For IVF zygotes, cytoplasmic injection was conducted with either plasmids or mRNA. For plasmid injection groups, 1 pg pCMVCas9 + 0.1 pg of each pSPgRNA (DNA2X) was used per zygote. In the case of RNA, two amounts (RNA1X and RNA2X) were compared. To assess the occurrence of HR, a group additionally cotransfected or coinjected with pHR*egfp* plasmid was included. Somatic cell lysates were analyzed by polymerase chain reaction and surveyor assay. In the case of embryos, the *in vitro* development and the genotype of blastocysts were evaluated by polymerase chain reaction and sequencing. In somatic cells, 2X transfection resulted in indels and large deletions of the targeted *PRNP* region. Regarding embryo injection, higher blastocyst rates were obtained for RNA injected groups (46/103 [44.6%] and 55/116 [47.4%] for RNA1X and RNA2X) than for the DNA2X group (26/140 [18.6%], $P < 0.05$). In 46% (26/56) of the total sequenced blastocysts, specific gene editing was detected. The total number of genetic modifications (29) was higher than the total number of gene-edited embryos, as three blastocysts from the group RNA2X reported more than one type of modification. The modifications included indels (10/56; 17.9%) and large deletions (19/56; 33.9%). Moreover, it was possible to detect HR in 1/8 (12.5%) embryos treated with RNA2X. These results report that the CRISPR/Cas9 system can be applied for site-specific edition of the bovine genome, which could have a great impact on the development of large animals resistant to important zoonotic diseases.

* Corresponding author. Tel.: +541145248000 ×8188; fax: +541145148737.
E-mail address: salamone@agro.uba.ar (D.F. Salamone).

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1. Introduction

Site-specific genetic engineering is a valuable tool for pharmaceutical research, development of biomedical models, and also for accelerated breeding. However, until a few years ago, knockout and knock-in in mammal cells and embryos comprised a complex challenge, especially when applied to large domestic species.

The recent advent of engineered nucleases has enabled the precise modification of genomes of different species, through simple introduction of site-specific double-strand breaks, which can be repaired either by the non-homologous end joining machinery or by homology-directed repair, in the presence of a homologous template [1]. Although the first reports on the use of engineered nucleases for precise genetic engineering of domestic species relied on zinc-finger nucleases [2–6] and transcription activator-like effector nucleases [7–10]; more recently, clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated nuclease (Cas) 9 emerged as the tool of choice, mainly due to its simple design and construction [11–19].

Clustered regularly interspaced short palindromic repeat/Cas is a simple and effective tool for genome edition on the basis of the defense mechanism against viruses used by bacteria and archea [20,21]. The main advantage of CRISPRs is that a single-guide RNA can direct the Cas to the target sequence in the genome by base complementarity, at sites demarcated by conserved sequences called protospacer adjacent motifs [22]. To form a functional DNA-targeting complex, Cas9 requires two distinct RNA transcripts: CRISPR RNA and trans-acting CRISPR RNA [22,23]. Jinek et al. [22] reconfigured this dual RNA as a single-guide RNA (sgRNA), including sequences that are sufficient to program Cas9 to introduce double-stranded breaks in target DNAs of 20 nucleotides. Initial reports with this system were promising [24,25], and it was rapidly adapted for the genome edition of cells of many different species, including large animals [26,27]. Soon thereafter, gene-edited pigs and goats were efficiently produced by somatic cell nuclear transfer, using CRISPR/Cas9 edited cells as donors [28–31]. More recently, a more straightforward approach, consisting on cytoplasmic injection of one-cell embryos, resulted in genome-edited mice, rat, sheep, monkeys, pigs, goats, and rabbits [12,16,18,19,32–34]. Efficiency rates obtained so far were variable, ranging from 63% in pigs [14] to 15%–21% in goats [18]. In addition, CRISPR/Cas9 RNA injection in zygotes can result in mosaicism [17,35–37].

Despite the potential that the CRISPR technology could have in cattle, only few reports are available so far [26,38,39]. Here, we tested the feasibility of inducing genetic modifications on *Bos taurus* prion gene (*PRNP*), responsible for mad cow disease *via* CRISPR/Cas9 application. The *PRNP* gene encodes the PrP^{C} glycoprotein; however, a misfolded isomer (PrP^{BSE}) of the normal cellular prion protein is accumulated in affected brains [40]. Prion diseases include transmissible spongiform encephalopathies such as Creutzfeldt-Jakob disease in humans, scrapie in sheep, and bovine spongiform encephalopathy in cattle. Although nowadays, the bovine spongiform encephalopathy epidemics is contained through a ban on feeding cattle with ruminant derived bone meal, spontaneous misfolding of the PrP^{C} protein could originate some PrP^{BSE} strains [41–43]. In mice, *PRNP* homozygous ($^{-/-}$) knockout were healthy and resistant to scrapie, and *PRNP* heterozygous ($^{-/+}$) mice expressed PrP^{C} at about half of the normal level [44–48]. In addition, in cattle, *PRNP* knockdown animals, generated by RNAi [49,50], and *PRNP* knockouts, produced by SCNT with donor cell lines subjected to two rounds of traditional cell modifications, were described [51]. However, with inefficiencies of traditional systems, the introgression of *PRNP* knockout genetics into cattle comprises a significant and costly challenge.

This report takes advantage of the CRISPR–Cas9 system adaptability to specifically modify bovine *PRNP* coding exon 3 both in bovine fetal fibroblasts and in early embryos. In particular, sgRNAs were designed not only to induce indels, but also to delete 875 bp of exon 3. The feasibility of inducing homologous recombination (HR) was also evaluated. Our results reported that this strategy could be efficiently applied to provoke deletions in bovine cell lines and embryos. However, most embryos were mosaic, and HR of large constructs was achieved at low efficiencies.

2. Materials and methods

2.1. Chemicals

Except where otherwise indicated, all chemicals were obtained from Sigma Chemical Company (St. Louis, MO, USA).

2.2. Cas9/sgRNA design

Mammalian codon-optimized recombinant human Cas9 under transcriptional control of the CMV promoter pST1374-NLS-flag-linker-rhCas9 (pCMVCas9) was a gift from Xingxu Huang (Addgene plasmid 44758) [52]. The five sgRNAs were designed to target both ends of a 875 bp sequence on *PRNP* exon 3 (Fig. 1C). All possible sgRNAs (5′-N_{20}NGG-3′) were identified and blasted to detect possible off-target sequences (5′-N_{20} A/T/C or GGG-3′) elsewhere in the bovine genome. The pSPgRNA was a gift from Charles Gersbach (Addgene plasmid # 47108) [53]. pUC57-sgRNA expression vector was a gift from Xingxu Huang (Addgene plasmid # 51132) [54]. The sgRNAs were cloned into pSPgRNA for cell transfection or plasmid embryo injection and into pUC57-sgRNA for RNA embryo injection. The sequences of the sgRNAs are shown in Table 1. The correct sequence of the sgRNAs was confirmed by capillary Sanger sequencing with optimized fluorescent terminator protocols (Genomic Unit, Biotechnology Institute, INTA, Hurlingham, Argentina). The HR plasmid (pHR*egfp*) had two 1 Kbp homologous arms flanking the 875 bp targeted sequence on *PRNP* exon 3, adjoining the *EGFP* gene under CAG promoter (Fig. 1D).

2.3. Somatic cell culture and DNA transfection

Bovine fetal fibroblasts were cultured in Dulbecco's modified Eagle's medium supplemented with 10%