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

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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.

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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%

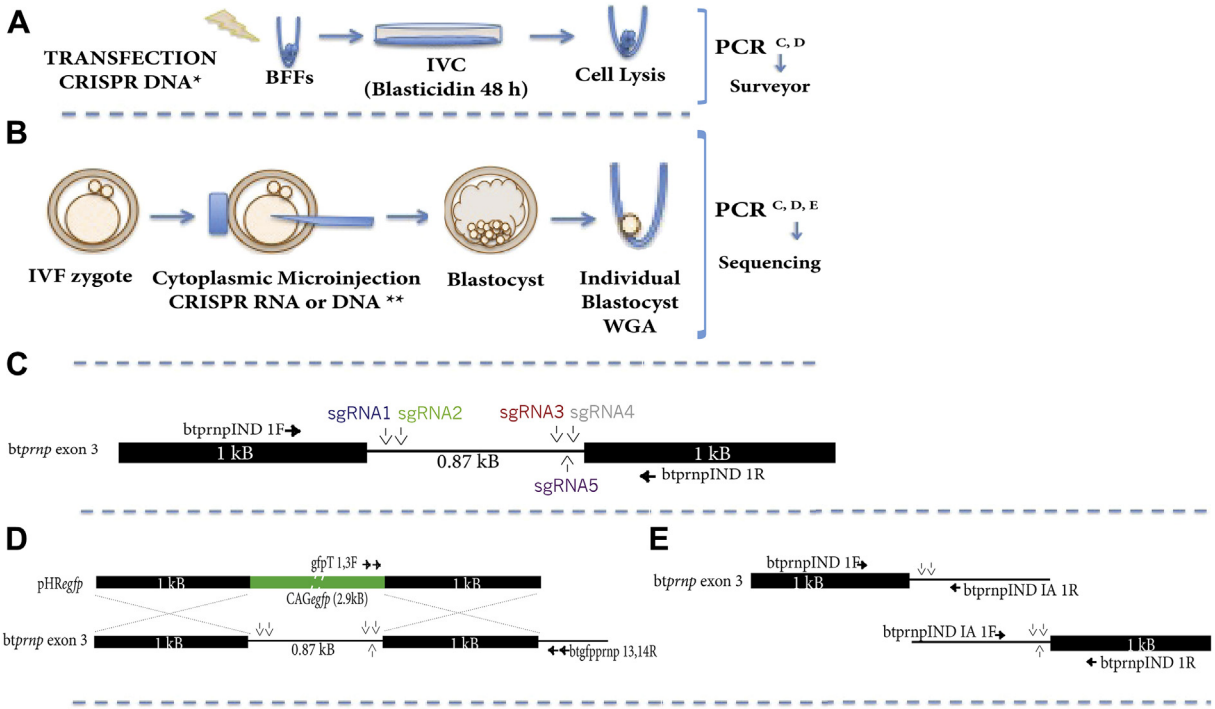

Fig. 1. (A and B) General procedure for *PRNP* gene targeting with the CRISPR/Cas9 system in cattle (A) Bovine fetal fibroblasts (BFFs); (B) IVF embryos. (C) Scheme of bt*PRNP* exon 3, the five sgRNAs target sites (sgRNAs in different colors) and the primers btprnpIND1F/1R (external to all sgRNAs targeted sequences) used to verify occurrence of mutations/deletions. (D) Scheme of HR vector used (pHR*egfp*) and primers, used for HR detection. In this case, nested PCR with primers gfpT1F, gfpT3F (at the terminal sequence of *egfp*), and btgpprnp12 R and btgfpprnp14R (over genomic *PRNP*, external to pHR*egfp* homology arms) were used. (E) Scheme of PCRs performed to detect indels. In this case, one primer in between the sgRNAs targets (btprnpINDia1F or btprnpINDia1R) was used combined with previously described btprnpIND1R of btprnpIND1F, respectively. Primers are indicated with horizontal arrows and the sgRNAs targets with vertical arrows. Cas, CRISPR-associated nuclease; CRISPR, clustered regularly interspaced short palindromic repeat; PCR, polymerase chain reaction.

heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 1% nonessential amino acids, 1 mM sodium pyruvate, 0.05 mM mercaptoethanol, and 100 U/mL penicillin streptomycin. The cells were allowed to grow until 75% confluence. Then, 3.5×10^6 cells were trypsinized, washed in PBS^- and resuspended in electroporation buffer (1652677, BioRad Gene Pulser Xcell). Electroporation conditions were 200 V, single pulse of 10 ms. Two plasmid concentrations were used, both of them with or without pHR*egfp*, constituting four treatments: (1) 1X: 2 μg pCMVCas9 + 0.2 μg of each pSPgRNA plasmid; (2) 1X + pHR*egfp*: 1X +2 μg pHR*egfp*; (3) 2X: 4 μg pCMVCas9 + 0.4 μg of each pSPgRNA plasmid; and (4) 2X + pHR*egfp*: 2X +4 μg pHR*egfp*. In all cases, the five pSPgRNA plasmids were transfected together. After 24 h, cells were subjected to 5 μg/mL blasticidin selection, resistance provided by pCMVCas9, for a 48-h period, followed by analysis schematized in Figure 1A.

Table 1
Sequence of sgRNAs and primers used to target *PRNP* gene and to detect the desired genetic modifications.

sgRNA1	GGGAAAGTAATCCTCTATTA
sgRNA2	GGAAAGTAATCCTCTATTAT
sgRNA3	GTCACACTGCCAGTTGCGGC
sgRNA4	GACTGCCAGTTGCGGCAGGA
sgRNA5	GGAAGCCCTCCTGCCGCAAC
btprnpIND1F	ACAGTCGGGTATACCAGTTG
btprnpIND1R	TCAATGGGTGTTGTCACCAG
btprnpINDia1F	GTTCTTGGTGCAAATGTGTCT
btprnpINDia1R	CCAGATGCACTTTACAATCTTC
gfpT1F	ATTCACTCCTCAGGTGCAGG
gfpT3F	ACATCATGAAGCCCCTTGAG
btgfpprnp12R	GGTAGAGGGGGTTCAAGAGG
btgfpprnpI4R	TGTATGATGCAGGGAAACCA
BGH Reverse	TAGAAGGCACAGTCGAGG
CMV Forward	CGCAAATGGGCGGTAGGCGTG

All sequences are shown in the 5′to 3′direction.
Abbreviation: sgRNA, single-guide RNA.

2.4. Bovine oocytes collection and in vitro maturation

Bovine ovaries were collected from a local slaughterhouse and transported to the laboratory at 25 °C to 30 °C. Cumulus-oocyte complexes were aspirated with 21-gauge needles from follicles with a diameter of 2–5 mm into Hepes-buffered Tyrode's albumin lactate pyruvate (Hepes-TALP). Oocytes covered with at least three layers of granulosa cells were selected for *in vitro* maturation in bicarbonate-buffered TCM-199 (31100–035; Thermo Fisher Scientific, Grand Island, NY, USA), containing 10% FCS (013/07; Internegocios, Buenos Aires, Argentina), 10 μg/mL follicle-stimulating hormone (NIH-FSH-P1, Folltropin, Bioniche, Caufield Junction Caufield North, Victoria, Australia), 0.3 mM sodium pyruvate (P2256), 100 μM cysteamine (M9768), and 2% antibiotic-antimycotic (ATB, 15240–096; Thermo Fisher Scientific, Grand Island, NY, USA). The

oocytes were incubated for 24 h under mineral oil (M8410) in 100 μL droplets, in 6.5% CO_2 in humidified air at 39 °C.

2.5. Bovine IVF

Frozen semen from one bull of proven fertility in our IVF system was thawed in a 37 °C water bath for 30 s. Spermatozoa were then centrifuged twice (490 g × 5 min) in Brackett–Oliphant medium (BO, [55]) and resuspended in BO medium supplemented with 5 mM caffeine (C4144) and 20 IU/mL heparin (H3149). Spermatozoa were adjusted to 40×10^6/mL and diluted to half the concentration (20×10^6/mL) with BO containing 10 mg/mL fatty acid-free BSA (A6003). Cumulus-oocyte complexes were washed twice with BO medium plus 5 mg/mL fatty acid-free BSA and subsequently exposed to the sperm suspension for 5 h in a 100 μL drop at 39 °C under 5% CO_2 in humidified air. Presumptive zygotes were then washed three times in Hepes-TALP. After IVF, cumulus cells were removed from presumptive zygotes by vortexing for 2 min in hyaluronidase (H-4272) (1 mg/mL in Dulbecco's PBS). Finally, presumptive zygotes were washed in Hepes-TALP, selected by visualization of at least one polar body, and immediately used for microinjection.

2.6. In vitro transcription

The Cas9 expression plasmid was polymerase chain reaction (PCR) amplified (1 cycle at 95 °C for 2 min, followed by 95 °C for 20 s, 60 °C for 20 s, and 68 °C for 4 min, during 30 cycles, and a final extension at 65 °C for 5 min with BGH reverse and CMV forward primers, included in Table 1), resulting in a 4.3 kbp PCR product, which was *in vitro* transcribed using the T7 Ultra Kit (Ambion, AM1345). The mRNA was purified with the RNeasy Mini Kit (Qiagen, NV, Germany, 74104). The pUC57-sgRNA expression vector was linearized by DraI and *in vitro* transcribed using the MEGAshortscript Kit (Ambion, AM1354). The sgRNAs were purified with the MEGAclear Kit (Ambion, AM1908) and used for injection. Proper IVT products were confirmed by denaturing gel electrophoresis.

2.7. Cytoplasmic injection of zygotes with plasmids or RNA coding for Cas9/sgRNAs

For cytoplasmic injection, 9 μm pipettes were used (HG-MIC-9UM, Origio Inc., Charlottesville, Denmark), and the plasmid or RNA mixtures were diluted in 10% polyvinylpyrrolidone. For plasmid cytoplasmic injection, the group DNA2X was treated with a mixture consisting of 1 pg pCMVCas9 and 0.1 pg of each pSPgRNA per zygote. In all cases, the five pSPgRNA plasmids were injected. The group DNA2X + pHR*egfp* was also coinjected with 1 pg pHR*egfp* to induce HR in zygotes. For RNA cytoplasmic injection, two conditions were compared, both of them with and without pHR*egfp*, constituting four groups: RNA2X: 1 pg Cas9 mRNA and 0.1 pg of each sgRNA injected per zygote; group RNA2X + pHR*egfp*: 1 pg Cas9 mRNA, 0.1 pg of each sgRNA, and 1 pg pHR*egfp* injected per zygote; RNA1X: 0.5 pg Cas9 mRNA and 0.05 pg of each sgRNA injected per zygote; RNA1X + *p*HR*egfp*: 0.5 pg Cas9 mRNA, 0.05 pg of each sgRNA, and 0.5 pg pHR*egfp* injected per zygote, respectively. In all cases, the five sgRNA were injected.

After injection, presumptive zygotes were cultured as described below and targeted genetic modifications were evaluated as schematized in Figure 1B.

2.8. In vitro embryo culture

Presumptive zygotes were cultured in 50 μL droplets of synthetic oviductal fluid medium [56] supplemented with 2.5% FCS at 39 °C in 6.5% CO_2 in humidified air. The embryos were transferred to a new droplet every 48 h. Cleavage was evaluated on Day 2, and the number of blastocysts was determined on Day 7.

2.9. Evaluation of EGFP fluorescence

Somatic cells transfected with the CRISPR system and pHR*egfp*, as well as *in vitro* produced embryos treated with the CRISPR system and pHR*egfp*, were briefly exposed to blue light using an excitation-filter at 488 nm and an emission-filter at 530 nm to determine *EGFP* expression. Cells were evaluated 24 h after transfection and embryos on Days 4 and 7 postinjection.

2.10. PCR amplification, surveyor assay, and sequencing

Blasticidin-selected somatic cells were subjected to overnight treatment with lysis buffer (0.2% SDS, 0.05 mg/mL Proteinase K and 1X PCR-buffer) at 37 °C. The lysed cells were transferred into eppendorf tubes, and lysis was stopped by incubation at 95 °C for 12 min. Blastocysts were subjected to whole genome amplification (WGA) using the REPLI-g Midi Kit (150045, Qiagen, NV, Germany) according to the "Amplification of Blood or Cells" protocol. Then, 0.3 μL of the WGA product or 5 ng of the cell lysis was used as PCR template. Polymerase chain reaction conditions were 1 cycle at 95 °C for 2 min, followed by 95 °C for 20 s, 62 °C for 45 s, and 68 °C for 45 s, during 40 cycles, and a final extension at 68 °C for 5 min (primers btprnpIND1F–btprnpIND1R), resulting in a 1.5 kbp PCR product (Fig. 1C). For somatic cells, the surveyor assay was performed according to the manufacturer's instructions. Briefly, 25 ng/μL of the PCR product was subjected to hybridization for heteroduplex formation and surveyor treatment for 1 h at 42 °C (Surveyor Mutation Detection Kit for Standard Gel Electrophoresis). The PCR and surveyor product were run on 1.5% agarose gel for 1 h at 100 V. A 100-bp ladder was run in parallel to all cell transfected PCR and surveyor samples (SM0241, GeneRuler 100 bp DNA Ladder, Thermo Scientific).

For embryos, btprnpIND1F/1R purified PCR products (28051; MinElute 96 UF PCR purification kit; Qiagen, NV, Germany) of whole embryos were sequenced from both primers. Some embryo PCR products smaller than 1.5 kbp were subcloned into TOPO cloning system (K4575–01SC; Thermo Fisher Scientific, Grand Island, NY, USA). Colonies were picked randomly and sent for sequencing. The PCR products were run on 1% agarose gel, and a 1 kB size marker was run in parallel in all cases (SM0313, GeneRuler 1 kb DNA ladder, Thermo Scientific).

In addition, to assess indels occurring independently from large deletions, two additional PCRs were performed, with one of the primers described before and primers btINDia1R and btINDia1F between the sgRNAs 1–2 and 3–5, as shown in Figure 1E. As before, 3 μL of a 1/10 dilution of the WGA product was used as PCR template. Polymerase chain reaction conditions were 1 cycle at 95 °C for 2 min, followed by 95 °C for 20 s, 62 °C for 45 s, and 72 °C for 45 s, during 30 cycles, and a final extension at 68 °C for 5 min (PCRia1: btIND1F–btINDia1R; PCRia2: btINDia1F–btIND1R). The PCRia1 resulted in a PCR product of 710 bp and the PCRia2, in a 666 bp PCR product. Sequencing in this case was performed from primer btINDia1R, for PCRia1 and from primer btIND1R, for PCR2.

For amplicon abundance analysis, the band intensities were measured using the ImageJ1.50f program (http://imagej.nih.gov/ij), and the percentage of each amplicon was estimated, considering 660 g as the molecular weight (MW) of a base pair.

For HR detection, nested PCR was performed, using primers that specifically matched the 3′ end of CAGegfp sequence (gfpT1F and gfpT3F) and primers 5′ to the end of the HR arm (btgfpprnp12R and btgfpprnpI4R) (Fig. 1D). Briefly, PCR1 conditions were 1 cycle at 95 °C for 2 min, followed by 95 °C for 20 s, 55 °C for 45 s, and 72 °C for 45 s, during 40 cycles, and a final extension at 72 °C for 5 min, with primers ogfpT1F and ogfpprnp14 R. For PCR2, the same conditions were used, but annealing was at 58 °C for 45 s with primers ogfpT3F and ogfpprnp12 R. The nested PCR product was 1661 bp. All primer sequences are included in Table 1.

2.11. Statistical analysis

For statistical analyses, SAS program was used [57]. *In vitro* embryo development and fluorescent expression were compared by Fisher's exact test analysis. Differences were considered to be significant at $P < 0.05$.

3. Results

3.1. Evaluation of the CRISPR/Cas9 system for genomic editing of PRNP exon 3 in bovine fetal fibroblasts

To evaluate the efficiency of the CRISPR/Cas9 system to target *PRNP* exon 3, five sgRNA plasmids (pSPgRNA) were cotransfected with the Cas9 plasmid (pCMVCas9) with or without the plasmid pHR*egfp*. When pHR*egfp* was also transfected, high *EGFP* expression rates were detected (Fig. 2A, B). For the 2X concentration, shorter PCR products were also identified in 10/15 (66.6%) PCRs performed on lysates from two independent transfection events (Fig. 2D). By surveyor assay, it was possible to identify indels for

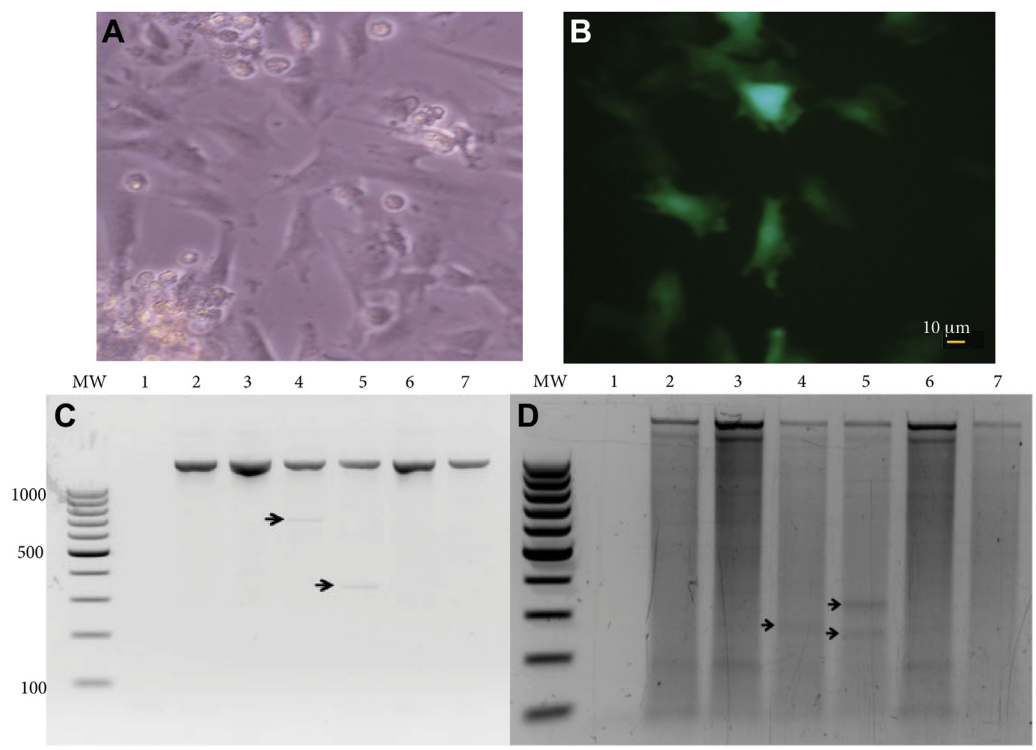

Fig. 2. (A and B) BFFs transfected with plasmids coding for the five sgRNAs, Cas9 + pHR*egfp* (DNA1X + pHR*egfp*). (A) Under bright light. (B) Under blue light (488 nm); (C) 1.5% agarose gel of btprnpIND1F/1R PCR product: lane 1, negative control; lane 2, DNA1X (transfection event 1); lane 3, DNA1X (transfection event 2); lane 4, DNA2X; lane 5, DNA2X + pHR*egfp*; lane 6, DNA1X + pHR*egfp*; and lane 7, wild-type control. Arrows indicate large deletions. (D) Surveyor performed on PCR samples shown in (C) in the same order. Arrows indicate surveyor nuclease digested products. A GeneRuler 100 bp size marker was run in parallel to samples in 1.5% agarose gels (C) and (D); MW of reference bands is indicated. BFF, bovine fetal fibroblast; Cas, CRISPR-associated nuclease; PCR, polymerase chain reaction. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 2
In vitro development and gene edition of embryos treated with plasmids or RNA coding for the CRISPR/Cas9 system targeting *PRNP* exon 3.

Injection group	pHR*egfp*	n	Blastocysts (%)	*EGFP* blastocysts (%)	*PRNP* edited embryos (%)	Type of modification[f]	
					Total/analyzed[e]	Large deletion/s[c]	Indels[d]
DNA2X	–	140	26 (18.6)[a]	n.a.	9/20 (45)[a,b]	9 (45)	0 (0)[a]
	+	110	18 (16.4)[a]	17 (94.4)[a]			
RNA1X	–	103	46 (44.6)[b]	n.a.	5/20 (25)[a]	5 (25)	0 (0)[a]
	+	105	45 (42.8)[b]	33 (73.3)[b]			
RNA2X	–	116	55 (47.4)[b]	n.a.	12/16 (75)[b]	5 (31.3)[f]	10 (62.5)[b]
	+	105	40 (38.1)[b]	38 (95.0)[a]			
Control	–	114	44 (38.6)[b]	n.a.	n.a.	n.a.	n.a.

Abbreviation: n.a., not applicable.
[a,b]Different superscripts indicate significant differences (Fisher test; $P < 0.05$). Control group: noninjected IVF.
[c] The edition involved loss of over 200 bp among the sgRNAs.
[d] The edition involved very short deletions/insertions and shift of the open reading frame.
[e] Total number of *PRNP* modified/total analyzed embryos.
[f] Some embryos harbored more than one type of genetic modification. Three replicates of each treatment were performed.

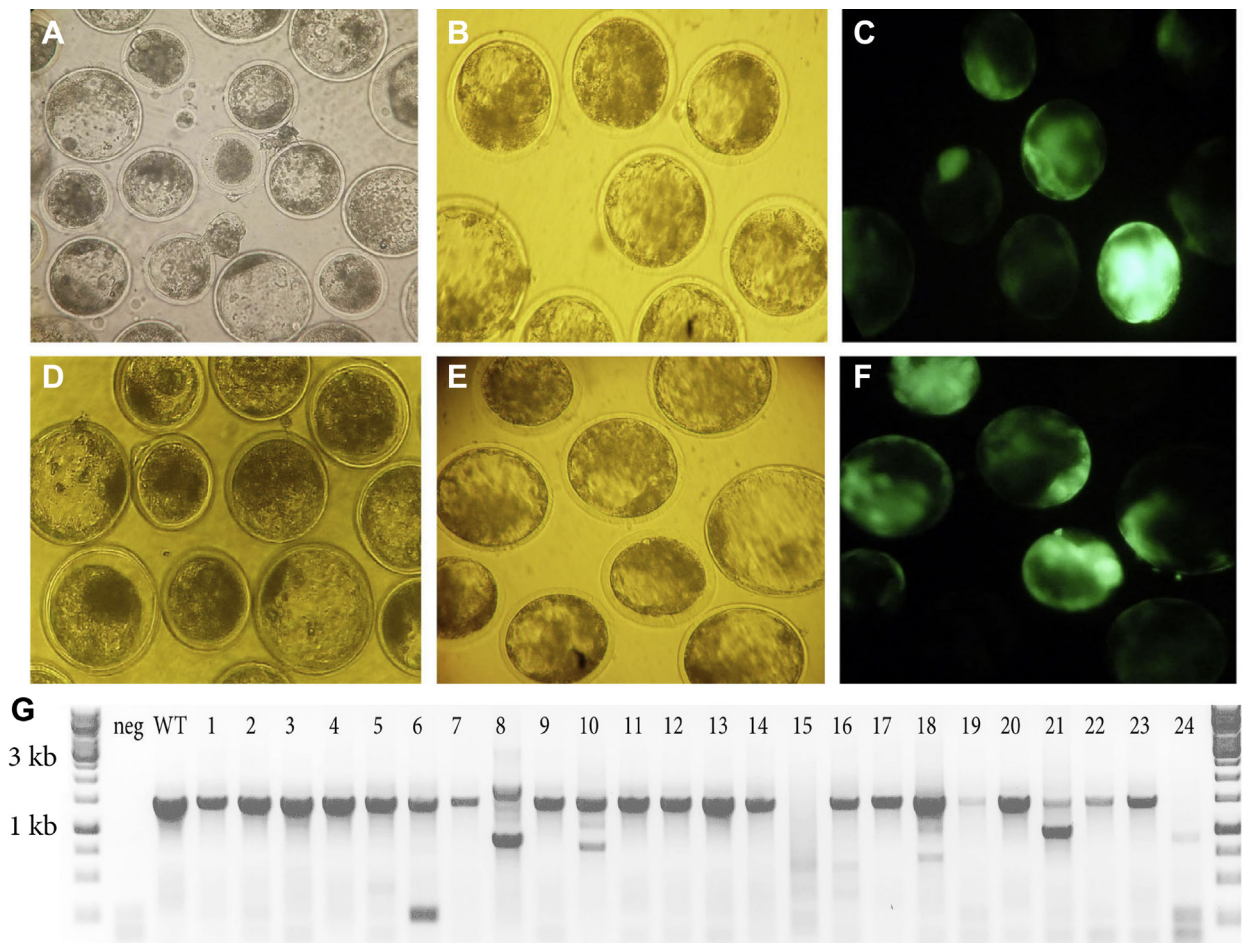

Fig. 3. Bovine blastocysts produced by CRISPR/Cas9 injection in the conditions: (A) RNA1X; (B–C) RNA1X + pHR*egfp*; (D) RNA2X; (E and F) RNA2X + pHR*egfp*; (A, B, D, and E) Under bright light. (C and F) Under blue light (488 nm); (G) 1% agarose gel of btprnpIND1F/1R PCR product on some representative WGA-treated blastocysts from the conditions: WT, wild-type control; 1–4, DNA2X; 5–8, DNA2X + pHR*egfp*; 9–12, RNA2X; 13–16, RNA2X + pHR*egfp*; 17–20, RNA1X; and 21–24, RNA1X + pHR*egfp*. The GeneRuler 1 kb DNA size marker was run in parallel in all cases; MW of reference bands is indicated. Cas, CRISPR-associated nuclease; CRISPR, clustered regularly interspaced short palindromic repeat; PCR, polymerase chain reaction. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

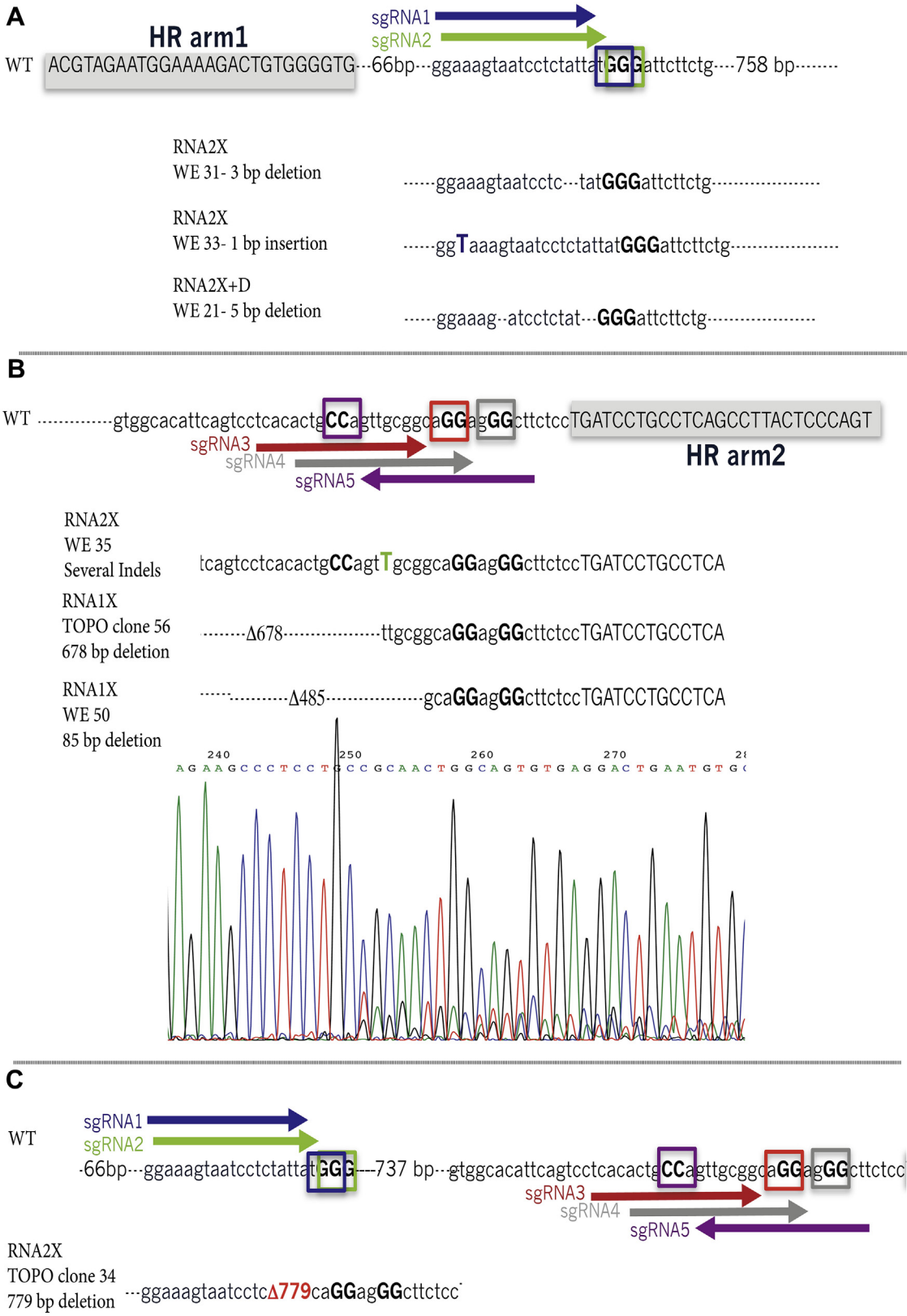

Fig. 4. Representative sequence analysis of the CRISPR-/Cas9-targeted bovine blastocysts. (A) Evaluation of mutations induced by sgRNA1 and sgRNA2 (primers btprnpIND1F/btprnpINDia1R or btprnpIND1F/1R) in three example embryos. (B) Evaluation of mutations induced by sgRNA3, sgRNA4, and sgRNA5 (primers

DNA2X and DNA2X + pHR*egfp*. For the DNA2X condition, a small band (approx. 250 bp), compatible with indels generated by the 3 sgRNAs next to the right HR arm, was detected in 25% of loci. For the DNA2X + pHR*egfp* condition, two small bands were evident (approx. 250 bp and 320 bp) in 35% and 23% of the loci (Fig. 2E). On the other hand, it was not possible to amplify the product expected as a result of HR (data not shown).

3.2. Evaluation of CRISPR/Cas9 for genomic editing of PRNP exon 3 in bovine zygotes

Zygotes were cytoplasmically treated with CRISPR/Cas9 plasmids or RNA, with pHR*egfp* (DNA2X + pHR*egfp*, RNA1X + pHR*egfp*, and RNA2X + pHR*egfp*) or without it (DNA2X, RNA1X, and RNA2X). Results are summarized on Table 2. Although injection with RNA1X or RNA2X did not significantly affect blastocyst yields, injection with plasmids was detrimental for development ($P < 0.05$). On the other hand, when the HR plasmid pHR*egfp* was included, both 2X conditions (RNA2X + pHR*egfp* and DNA2X + pHR*egfp*) resulted in higher *EGFP* blastocysts rates than RNA1X + pHR*egfp* ($P < 0.05$; Table 2; Fig. 3A–F).

After PCR with external primers to all sgRNAs target sites (btprnpIND1F/1R), large deletions were detected in some CRISPR/Cas9 injected embryos (Table 2; Fig. 3G; Fig. 4C). The putative deletion amplicons were gel purified, TOPO cloned, and sequenced. It was possible to identify three deletions of 678, 677, and 779 bp, the first one for RNA1X, interrupting the target site of sgRNA 3, 4 and 5 and the remaining two for RNA2X, interrupting the sequence of sgRNA 3, 4, and 5 and the sequence of all five sgRNAs, respectively (Fig. 4C). To evaluate only indels, two additional PCRs were performed with one primer among the sgRNAs target sites and an external one (Fig. 1E). In this case, it was possible to detect indels in embryos from the group RNA2X (4/6) and RNA2X + pHR*egfp* (6/10). Indels were detected as a result of the activity of the sgRNAs on both sides of the 875 bp *PRNP* sequence (Fig. 4A, B). A summary of all genetic modifications detected is included on Table 2. Most of the gene-edited embryos were mosaic, and mosaicism was due either to higher percentage of the wild type than the modified sequence or to the presence of more than one type of gene modification per embryo. All the gene-edited embryos of the group DNA2X group were mosaic. In this group, 6/9 (66.7%) embryos reported more than one deletion, and the remaining three reported the edited sequence in lower proportion than the wild type one. For the RNA1X group, 1/5 (20%) embryo was homozygous for the gene edition, as only the modified sequence was detected by sequencing. The remaining four embryos were mosaic, one of them due to the presence of two deletions. For the RNA2X group, 2/12 (16.7%) embryos were homozygous, 1/12 (8.3%) heterozygous, 3/12 (25%) embryos were mosaic due to the presence of both indels and deletions and the remaining 6/12 (50%), mosaic as a result of higher percentage of the wild type than the modified sequence (Supplementary Fig. 1).

Finally, the PCR product expected as a result of HR could be amplified by nested PCR in 1/8 (12.5%) of the blastocysts produced by RNA2X + pHR*egfp*. On the other hand, HR events could not be detected in blastocysts produced by injection with pHR*egfp* alone (0/175 *egfp* blastocysts).

4. Discussion

Until recently, specific gene modification was very difficult to achieve in livestock species. Since the introduction of engineered nucleases, a revolution in gene targeting begun. In this report, we found that *Bos taurus PRNP* gene can be efficiently mutated with the CRISPR system both in somatic cells and in zygotes. The design of five sgRNAs targeting 875 bp on PRNP exon 3, contiguous to sequences homologous to a HR donor vector, is a simple strategy for the simultaneous detection of deletions, indels, and HR with large templates, induced by the CRISPR system in the bovine genome.

Although *PRNP* knockout calves were first reported in 2007 [51], their production involved one round of cell transfection with a vector harboring an antibiotic resistance cassette, cloning to rejuvenate the cell line through fetuses production, and a second round of cell transfection, with a second vector harboring another antibiotic resistance cassette. The lack of true ES cells in domestic species hampered the possibility of gene targeting in domestic species for many years [58]. In this report, we found that the CRISPR system efficiently induced indels and also deletions of the fragment in between CRISPR target sites by simple cytoplasmic injection in cattle zygotes, avoiding use of antibiotic resistance and difficulties associated to the SCNT process. Interestingly, the targeting efficiency was dependent on concentration, both in somatic cells and embryos. Though RNA2X condition resulted in the higher gene edition and blastocysts rates, all CRISPR conditions tested in embryos were capable of inducing at least some level of gene editing. It was possible to identify mutations occurring as a consequence of the activity of the CRIPSR system on both targeted sequences of *PRNP* gene. Moreover, HR could also be detected in one of the embryos of the group RNA2X + pHR*egfp*.

In the first experiment, the CRISPR/Cas9 system proved to effectively cleave *PRNP* exon 3 in *Bos taurus* fetal fibroblasts. The CRISPR system had previously shown to efficiently knockout [26] and also knock-in bovine fetal fibroblasts [36] and induced pluripotent stem cells [39]. Interestingly, doubling the concentration of the plasmids resulted in PCR amplifications compatible with deletions, not observed after transfection with the lower concentrations. For the 2X condition, it was possible to detect indels by surveyor, in agreement with these observations.

btprnpIND1F/btprnpINDia1R or btprnpIND1F/1R) in three example embryos, including one TOPO clone. The Sanger sequence of purified PCR product of whole embryo 30 (primers btprnpIND1F/btprnpIND1R) is included. (C) TOPO clone of embryo 34 (RNA2X) showing a deletion of the complete sequence in between the sgRNAs. Each sgRNA is identified with a different color arrow, and their corresponding PAM sequences are shown in boxes. Cas, CRISPR-associated nuclease; CRISPR, clustered regularly interspaced short palindromic repeat; PAM, proto-spacer adjacent motif; PCR, polymerase chain reaction. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

In the second experiment, the use of the CRISPR/Cas9 system was evaluated for gene editing of *in vitro* derived IVF cattle zygotes. Although this approach was successfully used in mice, goats, monkey, sheep, and pigs [18,19,33,34], only one report is available for cattle so far [41]. The high rates of gene editing observed in our work support the notion that CRISPR/Cas9 system might be adapted to target different genes in different species by simple zygotic injection. In this report, we used an injection procedure previously described by our group [59–61] and more recently adapted by Yang et al. [62] for the CRISPR technology. In this system, larger pipettes can be used for injection, making use of polyvinylpyrrolidone to control the system obviating the need for embryo-toxic high pressure injection to expel high concentration DNA or RNA solutions.

While RNA2X reported higher rates of gene edition than RNA1X, injection with DNA2X had a detrimental effect on development. We previously detected that ectopic plasmid DNA persist at least 7 days postinjection [59], and this could have caused the observed drop in development. Many reports support the notion that engineered nucleases expression should be at low concentration and transient, as an excessive protein concentration exacerbates cytotoxic effects [63–65].

Regarding the kind of gene modifications, deletions were detected for the three conditions tested, in 25%–45% of the embryos evaluated, in agreement with recent reports in mice [66,67]. Indels, on the other hand, could only be detected for the groups treated with RNA2X. The HR of a large template was also detected in one of the embryos of the group treated with RNA2X.

The number of *egfp* expressing blastocysts from the groups coinjected with pHR*egfp* greatly exceeds the number of blastocysts modified by HR. The episomal expression of pHR*egfp* appears to be the reason for the high rates of *egfp* expression observed in this work. The possibility to achieve high transgene expression rates through cytoplasmic injection of covalently closed circular plasmids was proposed by Iqbal et al. [68]. In addition, previous results from our group support those observations [59].

While a large frequency of embryos was positive for editing events, a high rate of mosaicism was observed. The detection of up to three different genotypes in several embryos suggests that the CRISPR-/Cas9-mediated cleavage occurred after the first cell division. In addition, the detection of embryos harboring modifications only in some of the cells seems to be associated to the induction of double-stranded breaks after the first cell stage. In bovine embryos, transcription activity is limited before major genome activation, at the 4-8-cell stage [69,70]. For this reason, injection with RNA is usually preferred because it can be readily translated into protein while DNA must be first transcribed. While a recent report in mice suggests that allelic mosaicism could correlate with high cleavage efficiency at the single-cell stage [71], our results suggest persistent activity beyond the 1-cell stage. A recent report in cattle found that transcription activator-like effector nucleases had fairly low mosaicism, especially when compared to zinc-finger nucleases [72]. Presumably, optimized RNA conditions could further reduce mosaicism for the CRISPR system.

In summary, the present results report that the CRISPR/Cas9 system is efficient to induce indels and also deletions at the *PRNP* gene in bovine cell lines and embryos. Though HR still needs improvement, our results indicate that both KO and KI cattle zygotes can be produced by simple cytoplasmic injection with the CRISPR/Cas9 system, opening new horizons for genetic engineering of large animals.

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Appendix A. Supplementary data

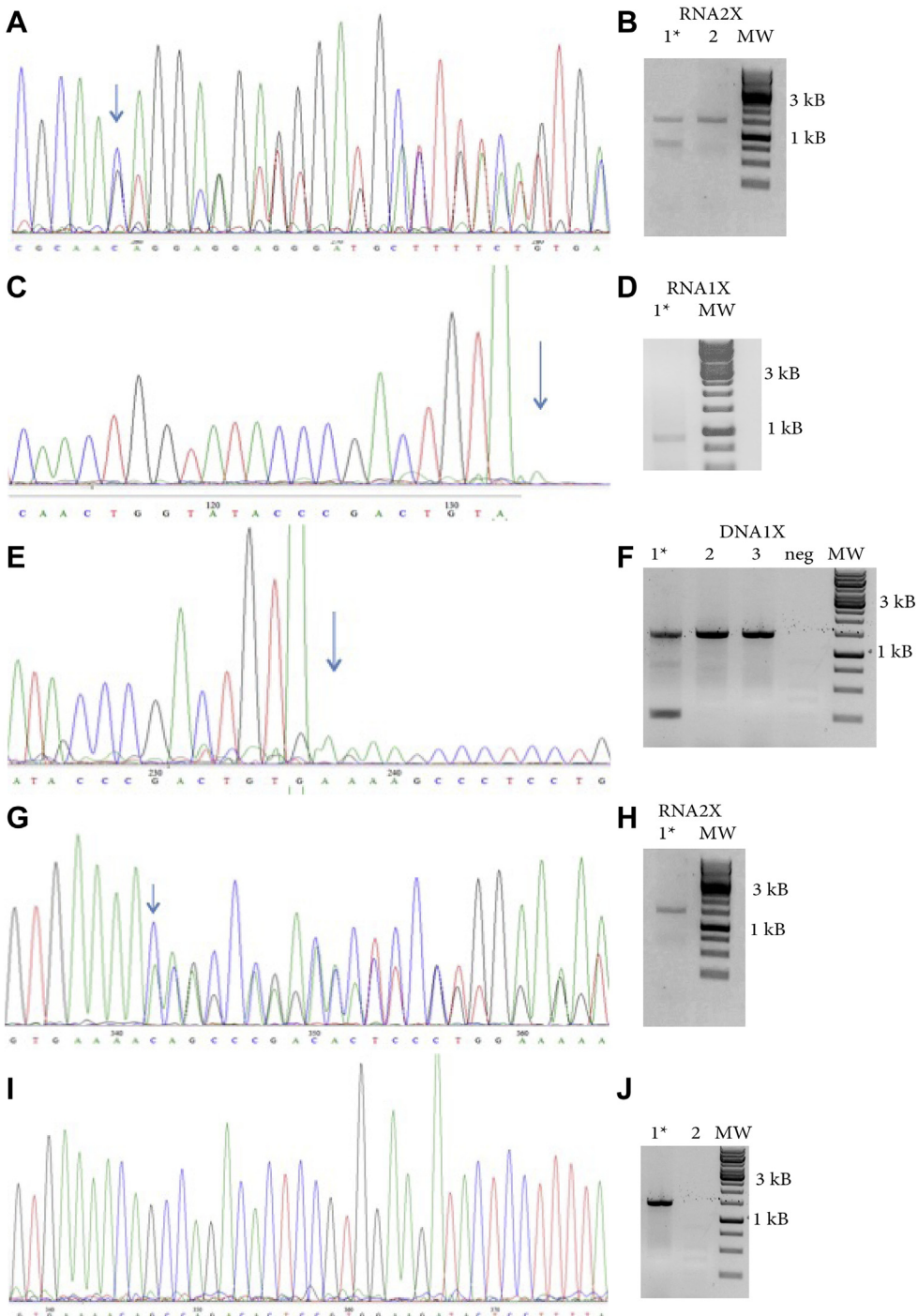
Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.theriogenology.2016.06.010.

References

[1] Johnson RD, Jasin M. Double-strand-break-induced homologous recombination in mammalian cells. Biochem Soc Trans 2001;29:196–201.
[2] Santiago Y, Chan E, Liu PQ, Orlando S, Zhang L, Urnov FD, et al. Targeted gene knockout in mammalian cells by using engineered zinc-finger nucleases. Proc Natl Acad Sci U S A 2008;105:5809–14.
[3] Hauschild J, Petersen B, Santiago Y, Queisser AL, Carnwath JW, Lucas-Hahn A, et al. Efficient generation of a biallelic knockout in pigs using zinc-finger nucleases. Proc Natl Acad Sci U S A 2011;108:12013–7.
[4] Yang D, Yang H, Li W, Zhao B, Ouyang Z, Liu Z, et al. Generation of PPARc mono-allelic knockout pigs via zinc-finger nucleases and nuclear transfer cloning. Cell Res 2011;21:979–82.
[5] Yu S, Luo J, Song Z, Ding F, Dai Y, Li N. Highly efficient modification of beta-lactoglobulin (BLG) gene via zinc-finger nucleases in cattle. Cell Res 2011;21:1638–40.
[6] Liu X, Wang Y, Guo W, Chang B, Liu J, Guo Z, et al. Zinc-finger nickase-mediated insertion of the lysostaphin gene into the beta-casein locus in cloned cows. Nat Commun 2013;4:2565.
[7] Hockemeyer D, Wang H, Kiani S, Lai CS, Gao Q, Cassady JP, et al. Genetic engineering of human pluripotent cells using TALE nucleases. Nat Biotechnol 2011;29:731–4.
[8] Bedell VM, Wang Y, Campbell JM, Poshusta TL, Starker CG, Krug 2nd RG, et al. In vivo genome editing using a high-efficiency TALEN system. Nature 2012;491:114–8.
[9] Carlson DF, Tan W, Lillico SG, Stverakova D, Proudfoot C, Christian M, et al. Efficient TALEN-mediated gene knockout in livestock. Proc Natl Acad Sci U S A 2012;109:17382–7.
[10] Proudfoot C, Carlson DF, Huddart R, Long CR, Pryor JH, King TJ, et al. Genome edited sheep and cattle. Transgenic Res 2015;24:147–53.
[11] Ran FA, Hsu PD, Lin CY, Gootenberg JS, Konermann S, Trevino AE, et al. Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. Cell 2013;154:1380–9.
[12] Mashiko D, Young SA, Muto M, Kato H, Nozawa K, Ogawa M, et al. Feasibility for a large scale mouse mutagenesis by injecting CRISPR/Cas plasmid into zygotes. Dev Growth Differ 2014;56:122–9.
[13] Mizuno S, Dinh TT, Kato K, Mizuno-Iijima S, Tanimoto Y, Daitoku Y, et al. Simple generation of albino C57BL/6J mice with G291T mutation in the tyrosinase gene by the CRISPR/Cas9 system. Mamm Genome 2014;25:327–34.
[14] Hai T, Teng F, Guo R, Li W, Zhou Q. One-step generation of knockout pigs by zygote injection of CRISPR/Cas system. Cell Res 2014;24:372–5.
[15] Fujihara Y, Ikawa M. CRISPR/Cas9-based genome editing in mice by single plasmid injection. Methods Enzymol 2014;546:319–36.
[16] Crispo M, Mulet AP, Tesson L, Barrera N, Cuadro F, Dos Santos-Neto PC, et al. Efficient Generation of Myostatin Knock-Out Sheep Using CRISPR/Cas9 Technology and Microinjection into Zygotes. PLoS One 2015;10:e0136690.

[17] Sato M, Koriyama M, Watanabe S, Ohtsuka M, Sakurai T, Inada E, et al. Direct injection of CRISPR/Cas9-related mRNA into cytoplasm of parthenogenetically activated porcine oocytes causes frequent mosaicism for indel mutations. Int J Mol Sci 2015;16:17838–56.
[18] Wang X, Yu H, Lei A, Zhou J, Zeng W, Zhu H, et al. Generation of gene-modified goats targeting MSTN and FGF5 via zygote injection of CRISPR/Cas9 system. Sci Rep 2015;5:13878.
[19] Honda A, Hirose M, Sankai T, Yasmin L, Yuzawa K, Honsho K, et al. Single-step generation of rabbits carrying a targeted allele of the tyrosinase gene using CRISPR/Cas9. Exp Anim 2015;64:31–7.
[20] Sorek R, Kunin V, Hugenholtz P. CRISPR–a widespread system that provides acquired resistance against phages in bacteria and archaea. Nat Rev Microbiol 2008;6:181–6.
[21] Westra ER, Buckling A, Fineran PC. CRISPRCas systems: beyond adaptive immunity. Nat Rev Microbiol 2014;12:317–26.
[22] Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science 2012;337:816–21.
[23] Deltcheva E, Chylinski K, Sharma CM, Gonzales K, Chao Y, Pirzada ZA, et al. CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. Nature 2011;471:602–7.
[24] Hsu PD, Lander ES, Zhang F. Development and applications of CRISPR-Cas9 for genome engineering. Cell 2014;157:1262–78.
[25] Sander JD, Joung JK. CRISPR-Cas systems for editing, regulating and targeting genomes. Nat Biotechnol 2014;32:347–55.
[26] Choi W, Yum S, Lee S, Lee W, Lee J, Kim S, et al. Disruption of exogenous eGFP gene using RNA-guided endonuclease in bovine transgenic somatic cells. Zygote 2015;23:916–23.
[27] Tan W, Carlson DF, Lancto CA, Garbe JR, Webster DA, Hackett PB, et al. Efficient nonmeiotic allele introgression in livestock using custom endonucleases. Proc Natl Acad Sci U S A 2013;110:16526–31.
[28] Zhou X, Xin J, Fan N, Zou Q, Huang J, Ouyang Z, et al. Generation of CRISPR/Cas9-mediated gene targeted pigs via somatic cell nuclear transfer. Cell Mol Life Sci 2015;72:1175–84.
[29] Li P, Estrada JL, Burlak C, Montgomery J, Butler JR, Santos RM, et al. Efficient generation of genetically distinct pigs in a single pregnancy using multiplexed single-guide RNA and carbohydrate selection. Xenotransplantation 2014;21:376–84.
[30] Whitworth KM, Lee K, Benne JA, Beaton BP, Spate LD, Murphy SL, et al. Use of the CRISPR/Cas9 system to produce genetically engineered pigs from *in vitro*-derived oocytes and embryos. Biol Reprod 2014;91:78.
[31] Ni W, Qiao J, Hu S, Zhao X, Regouski M, Yang M, et al. Efficient gene knockout in goats using CRISPR/Cas9 system. PLoS One 2014;9:e106718.
[32] Shao Y, Guan Y, Wang L, Qiu Z, Liu M, Chen Y, et al. CRISPR/Cas-mediated genome editing in the rat via direct injection of one-cell embryos. Nat Protoc 2014;9:2493–512.
[33] Chen Y, Zheng Y, Kang Y, Yang W, Niu Y, Guo X, et al. Functional disruption of the dystrophin gene in rhesus monkey using CRISPR/Cas9. Hum Mol Genet 2015;24:3764–74.
[34] Wang Y, Du Y, Shen B, Zhou X, Li J, Liu Y, et al. Efficient generation of gene-modified pigs via injection of zygote with Cas9/sgRNA. Sci Rep 2015;5:8256.
[35] Sung YH, Kim JM, Kim HT, Lee J, Jeon J, Jin Y, et al. Highly efficient gene knockout in mice and zebrafish with RNA-guided endonucleases. Genome Res 2014;24:125–31.
[36] Niu Y, Shen B, Cui Y, Chen Y, Wang J, Wang L, et al. Generation of gene-modified cynomolgus monkey via Cas9/RNA-mediated gene targeting in one-cell embryos. Cell 2014;156:836–43.
[37] Yen ST, Zhang M, Deng JM, Usman SJ, Smith CN, Parker-Thornburg J, et al. Somatic mosaicism and allele complexity induced by CRISPR/Cas9 RNA injections in mouse zygotes. Dev Biol 2014;393:3–9.
[38] Jeong YH, Kim YJ, Kim EY, Kim SE, Kim J, Park MJ, et al. Knock-in fibroblasts and transgenic blastocysts for expression of human FGF2 in the bovine β-casein gene locus using CRISPR/Cas9 nuclease-mediated homologous recombination. Zygote 2015;22:1–15.
[39] Heo YT, Quan X, Xu YN, Baek S, Choi H, Kim NH, et al. CRISPR/Cas9 nuclease-mediated gene knock-in in bovine-induced pluripotent cells. Stem Cells Dev 2015;24:393–402.
[40] Prusiner SB. Molecular biology of prion diseases. Science 1991;252:1515–22.
[41] Biacabe AG, Laplanche JL, Ryder S, Baron T. Distinct molecular phenotypes in bovine prion diseases. EMBO Rep 2003;5:110–5.
[42] Yamakawa Y, Hagiwara K, Nohtomi K, Nakamura Y, Nishijima M, Higuchi Y, et al. Atypical proteinase K-resistant prion protein (PrPres) observed in an apparently healthy23-month-old Holstein steer. Jpn J Infect Dis 2003;56:221–2.
[43] Casalone C, Zanusso G, Acutis P, Ferrari S, Capucci L, Tagliavini F, et al. Identification of a second bovine amyloidotic spongiform encephalopathy: molecular similarities with sporadic Creutzfeldt-Jakob disease. Proc Natl Acad Sci U S A 2004;101:3065–70.
[44] Sakaguchi S, Katamine S, Nishida N, Moriuchi R, Shigematsu K, Sugimoto T, et al. Loss of cerebellar Purkinje cells in aged mice homozygous for a disrupted PrP gene. Nature 1996;380:528–31.
[45] Nico PB, de-Paris F, Vinade ER, Amaral OB, Rockenbach I, Soares BL, et al. Altered behavioural response to acute stress in mice lacking cellular prion protein. Behav Brain Res 2005;162:173–81.
[46] Bueler H, Fischer M, Lang Y, Bluethmann H, Lipp HP, DeArmond SJ, et al. Normal development and behavior of mice lacking the neuronal cell-surface PrP protein. Nature 1992;356:577–82.
[47] Büeler H, Aguzzi A, Sailer A, Greiner RA, Autenried P, Aguet M, et al. Mice devoid of PrP are resistant to scrapie. Cell 1993;73:1339–47.
[48] Manson JC, Clarke AR, Hooper ML, Aitchison L, McConnell I, Hope J. 129/Ola mice carrying a null mutation in PrP that abolishes mRNA production are developmentally normal. Mol Neurobiol 1994;8:121–7.
[49] Sutou S, Kunishi M, Kudo T, Wongsrikeao P, Miyagishi M, Otoi T. Knockdown of the bovine prion gene PRNP by RNA interference (RNAi) technology. BMC Biotechnol 2007;7:44.
[50] Wongsrikeao P, Sutou S, Kunishi M, Dong YJ, Bai X, Otoi T. Combination of the somatic cell nuclear transfer method and RNAi technology for the production of a prion gene-knockdown calf using plasmid vectors harboring the U6 or tRNA promoter. Prion 2011;5:39–46.
[51] Richt JA, Kasinathan P, Hamir AN, Castilla J, Sathiyaseelan T, Vargas F, et al. Production of cattle lacking prion protein. Nat Biotechnol 2007;25:132–8.
[52] Shen B, Zhang J, Wu H, Wang J, Ma K, Li Z, et al. Generation of gene-modified mice via Cas9/RNA-mediated gene targeting. Cell Res 2013;23:720–3.
[53] Perez-Pinera P, Kocak DD, Vockley CM, Adler AF, Kabadi AM, Polstein LR, et al. RNA-guided gene activation by CRISPR-Cas9-based transcription factors. Nat Methods 2013;10:973–6.
[54] Shen B, Zhang W, Zhang J, Zhou J, Wang J, Chen L, et al. Efficient genome modification by CRISPR-Cas9 nickase with minimal off-target effects. Nat Methods 2014;11:399–402.
[55] Brackett BG, Oliphant G. Capacitation of rabbit spermatozoa in vitro. Biol Reprod 1975;12:260–74.
[56] Tervit HR, Whittingham DG, Rowson LE. Successful culture in vitro of sheep and cattle ova. J Reprod Fertil 1972;30:493–7.
[57] SAS Institute Inc. SAS/STAT. User's Guide. Version 6. Fourth edition, Volume 1. Cary, NC: SAS Institute Inc; 1989. p. 943.
[58] Nowak-Imialek M, Niemann H. Pluripotent cells in farm animals: state of the art and future perspectives. Reprod Fertil Dev 2012;25:103–28. Review.
[59] Bevacqua RJ, Canel NG, Hiriart MI, Sipowicz P, Rozenblum GT, Vitullo A, et al. Simple gene transfer technique based on I-SceI meganuclease and cytoplasmic injection in IVF bovine embryos. Theriogenology 2013;80:104–13.
[60] Pereyra-Bonnet F, Bevacqua R, La Rosa I, Sipowicz P, Radrizzani M, Fernandez-Martin R, et al. Novel methods to induce exogenous gene expression in SCNT, parthenogenic and IVF preimplantation bovine embryos. Transgenic Res 2011;20:1379–88.
[61] Bevacqua RJ, Pereyra-Bonnet F, Olivera R, Hiriart MI, Sipowicz P, Fernandez-Martín R, et al. Production of IVF transgene-expressing bovine embryos using a novel strategy based on cell cycle inhibitors. Theriogenology 2012;78:57–68.
[62] Yang H, Wang H, Jaenisch R. Generating genetically modified mice using CRISPR/Cas-mediated genome engineering. Nat Protoc 2014;9:1956–68.
[63] Beumer K, Bhattacharyya G, Bibikova M, Trautman JK, Carroll D. Efficient gene targeting in Drosophila with zinc-finger nucleases. Genetics 2006;172:2391–403.
[64] Pruett-Miller SM, Connelly JP, Maeder ML, Joung JK, Porteus MH. Comparison of zinc finger nucleases for use in gene targeting in mammalian cells. Mol Ther 2008;16:707–17.
[65] Cho SW, Kim S, Kim Y, Kweon J, Kim HS, Bae S, et al. Analysis of off-target effects of CRISPR/Cas-derived RNA-guided endonucleases and nickases. Genome Res 2014;24:132–41.
[66] Wang L, Shao Y, Guan Y, Li L, Wu L, Chen F, et al. Large genomic fragment deletion and functional gene cassette knock-in via Cas9 protein mediated genome editing in one-cell rodent embryos. Sci Rep 2015;5:17517.
[67] Zhang L, Jia R, Palange NJ, Satheka AC, Togo J, An Y, et al. Large genomic fragment deletions and insertions in mouse using CRISPR/Cas9. PLoS One 2015;10:e0120396.

[68] Iqbal K, Barg-Kues B, Broll S, Bode J, Niemann H, Kues W. Cytoplasmic injection of circular plasmids allows targeted expression in mammalian embryos. Biotechniques 2009;47:959–68.
[69] Memili E, First NL. Control of gene expression at the onset of bovine embryonic development. Biol Reprod 1999;61:1198–207.
[70] Graf A, Krebs S, Zakhartchenko V, Schwalb B, Blum H, Wolf E. Fine mapping of genome activation in bovine embryos by RNA sequencing. Proc Natl Acad Sci U S A 2014;111:4139–44.
[71] Parikh BA, Beckman DL, Patel SJ, White JM, Yokoyama WM. Detailed phenotypic and molecular analyses of genetically modified mice generated by CRISPR-Cas9-mediated editing. PLoS One 2015;10:e0116484.
[72] Wei J, Wagner S, Lu D, Maclean P, Carlson DF, Fahrenkrug SC, et al. Efficient introgression of allelic variants by embryo-mediated editing of the bovine genome. Sci Rep 2015;5:11735.



Supplementary Fig. 1. Example of different genotypes generated by the CRISPR/Cas9 system in cattle embryos, including Sanger sequences and PCRs. (A and B) RNA2X blastocyst showing a deletion at *prnp* gene in heterozygous fashion. (C and D) RNA1X blastocyst showing an homozygous deletion at the *prnp* gene, evident by the complete elimination of the wild-type sequence in the chromatogram and PCR. (E and F) DNA1X blastocyst showing a deletion in a mosaic fashion. The wild-type sequence can be detected. Sequences in (A, C, and E) were obtained by PCR with primers btprnpIND1F/1R. (G and H) RNA2X blastocyst showing an indel in a mosaic fashion. In this case, the sequenced PCR was performed with primers btprnpIND1F/btprnpINDia1R. (H) The PCR was run with primers btprnpIND1F/1R. (I and J) Wild-type embryo. A unique Sanger sequence and PCR band is detected. All PCRs were run in 1% agarose gels. Arrows indicate start of gene-edited sequence. * indicates the PCR sample for which Sanger sequencing is shown. The GeneRuler 1 kb DNA size marker was run in parallel in all cases in 1% agarose gels; MW of reference bands is indicated. Cas, CRISPR-associated nuclease; CRISPR, clustered regularly interspaced short palindromic repeat; PCR, polymerase chain reaction.