Developing the laboratory conditions for bovine zygote-mediated genome editing by electroporation

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Summary

The current strategy for animal breeding by marker-assisted selection in livestock is inherently slow and unsuitable to respond in time to contemporary challenges arising from rapid changes in global climates and increasing human population. Genome editing with programmable nucleases allows for the efficient introduction of beneficial natural sequence variants or entirely novel traits known from other breeds or species into elite livestock genotypes, essentially within a single generation. In combination with traditional selection schemes, it can accelerate genetic gain by increasing the numbers of desirable variants that are assembled in an individual high merit animal. Thus, it offers an exciting new targeted breeding approach with the ability to quickly respond and adapt livestock to the changing conditions.

Genome editing relies on the introduction of site-specific DNA double strand breaks by chimeric designer nucleases that triggers the cellular repair machinery. In the presence of a 'repair' template the cell can repair the damage by a homology driven mechanism and enables precise control of the repair and the introduction of specific sequence variants specified by the repair template.

Presently, the main laboratory approach uses microinjection of the editing tools into individual zygotes which is labour- and cost-intensive. Our study investigates an emerging approach of simultaneously introducing the genome editing tools into numerous bovine zygotes by electroporation, greatly increasing throughput. Using an EGFP reporter plasmid we established suitable laboratory conditions for the electroporation of bovine zygotes. These conditions were then applied for Cas9/gRNA ribonucleoprotein-mediated genome editing. Our results showed that through electroporation of Cas9/gRNA ribonucleoproteins site-specific mutations, induced by the non-homologous end joining repair mechanism, could be introduced with high efficiency into bovine zygotes. Furthermore, we demonstrated the feasibility of homology-directed repair (HDR)-mediated precision editing according to an exogenous repair template in electroporated bovine zygotes.

Keywords: CRISPR, electroporation, genome editing, zygotes, bovine

Introduction

CRISPR-mediated mammalian genome editing is typically accomplished by microinjection of Cas9 nuclease together with guide RNA (gRNA) that provides the target specificity, into 1-cell embryos to generate modified animals in a single step (Yang *et al.*, 2014). We are routinely using this method and have successfully generated precisely genome edited embryos and live calves with this approach (Wei *et al.*, 2015). However, this standard practice relies on microinjection of Cas9 and gRNAs into 1-cell zygotes, causing physical

damage and reducing embryo viability. Microinjection of individual zygotes is also labour- and cost-intensive and requires high technical skills in micromanipulation of embryos, introducing operator-dependent experimental variation as a confounding parameter in editing experiments. This makes it difficult to distinguish between biological and technical reasons underlying low editing efficiency.

It is therefore important to develop simplified procedures which increase throughput, ease of operation and reproducibility. Electroporation-based approaches have been reported to deliver Cas9/gRNA ribonucleoproteins (RNPs), Cas9 mRNA and gRNAs into rodent embryos, achieving high biallelic editing efficiencies (Kaneko *et al.*, 2014; Hashimoto *et al.*, 2016). This offers a simple, economic, high-throughput and efficient alternative to microinjection in bovine. In the present study we used a plasmid-encoded EGFP reporter gene as indicator aimed to establish the condition for the successful electroporation of bovine embryos. Suitable electroporation conditions were then used to demonstrate successful CRISPR-mediated genome editing with electroporated bovine embryos.

Materials and Methods

In vitro zygote production

Abattoir-sourced oocytes were matured in vitro for 22-24h and fertilized with sperm (1 million/ml) from a single sire. After 8 h post in vitro fertilization, the cumulus cells were dispersed by hyaluronidase (1 mg/ml, Sigma, in holding media H199, Gibco) and stripped by vortexing for 3 min. After washing in H199 containing 10 % FBS (Moregate Biotech), zygotes with a visible 2nd polar body were collected. For removal of the zona pellucida, the zygotes were transferred to culture drops of pronase (5mg/ml in HSOF with 1mg/ml PVA) and incubated until the zona dissolves (approximately 5-10 sec). The denuded zygotes were washed in H199/BSA (3 mg/ml, Gibco) to remove pronase and transferred to drops of AgResearch IVF medium until electroporation.

Electroporation of zygotes

Recombinant CAS9 protein (0.75 μM, supplier) was incubated with the gRNA (0.75 μM or 1.5 μM) for 10 min at room temperature followed by the addition of single stranded oligonucleotide (ssODN) repair template (1.0 μM). The CRISPR/ssODN mixture and the EGFP plasmid (1200 ng/μl, pEGFP-N1, Clontech Laboratories) were then diluted (1:1) in opti-MEM solution. Bovine zygotes were combined with 10 μl of the CRISPR/ssODN mixture or EGFP plasmid solution and transferred to a microfabricated co-planar film electrode, positioned under a stereo microscope and connected to a square wave generator for open-access electroporation (Figure 1). Zygotes were electroporated with a range of voltages and number of pulses, as detailed in the results section, using a pulse width of 3 ms.

Analysis of electroporation results

After in vitro embryo culture to the blastocyst stage, electroporated embryos were individually analyzed. Blastocysts derived from zygotes electroporated with the EGFP plasmid were examined under a fluorescent microscope for the emission of green fluorescence. Blastocysts derived from zygotes electroporated with CRISPR/ssODN were evaluated by digital droplet PCR for non-homologous end joining (NHEJ) and homology-directed repair (HDR) events using a fluorescent NHEJ and HDR probe, respectively.

Results and discussion

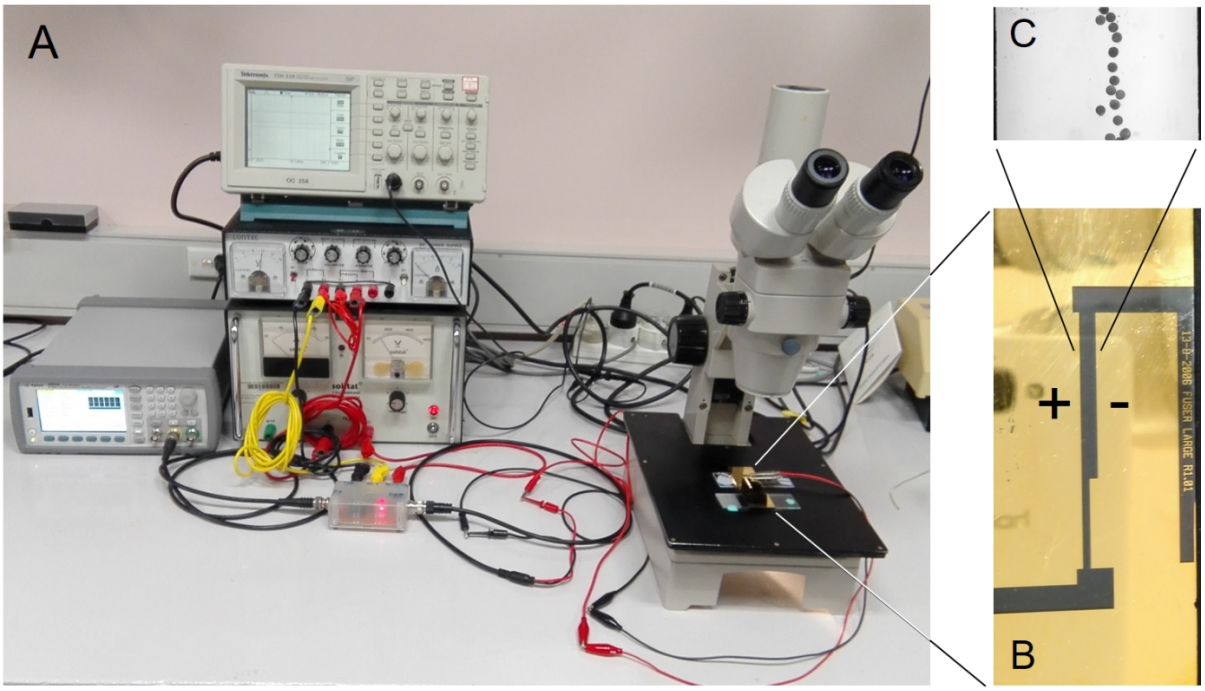

Figure 1: Set up for the electroporation of bovine zygotes. A) Shown is the general set-up for electroporation with a co-planar electrode placed under a stereo microscope and connected to a square wave generator. B) Magnified view (10X) of the co-planar electrode. The polarity is indicated. C) Shown (100X magnification) are bovine zygotes that are loaded in the main channel of the co-planar electrode prior to electroporation.

Following electroporation, embryos were cultured in vitro to the blastocyst stage to determine the developmental potential and efficiency of plasmid uptake dependent on different treatments. Initially we assessed the ability for DNA-uptake by zona-intact and zona-free zygotes. At the blastocyst stage, embryos were examined for the emission of green fluorescence as an indirect measure of DNA-uptake. Only zona-free zygotes generated EGFP-positive blastocysts indicating that the zona pellucida presents a strong barrier for DNA-uptake following electroporation (Table 1).

Next, we tested a series of different pulse voltages up to 60 V for the best conditions to electroporate zona-free bovine zygotes. With increasing voltages we observed a higher percentage of EGFP-positive blastocysts. With the highest voltage (60 V), all blastocysts generated were EGFP-positive. The percentage of EGFP-positive blastocysts sequentially increased from 0 % for the control without a pulse to 51.4 % (30 V), to 87.5 % (45 V) and 100% for 60 V (Table 1). However, the voltage was negatively correlated with the potential of electroporated zygotes to develop to the blastocyst stage. The non-electroporated controls and 30 V treatment group had comparable in vitro development rates (38.7 % and 36.8 %, respectively). This was approximately halved when 45 V pulses were applied (16.7 %) and even lower at 7.2 % with 60 V pulses. A major contributing factor to the reduced in vitro development rates was the increase in zygotes that lysed with increasing voltages (Table 1).

Table 1: Electroporation of an EGFP plasmid into bovine zygotes

Reporter	Zona pellucida	Plasmid concentration	Pulses	Voltage	Lysed zygotes (%)	Blastocysts (%)	EGFP +ve blastocysts (%)
EGFP	Yes	600 ng/µl	10	20-60	35/80 (44)	20/45 (44)	0/20 (0)
EGFP	No	600 ng/µl	10	20-60	34/80 (43)	19/46 (41)	4/30 (13.3)
EGFP	No	600 ng/µl	0	0 V	1/62 (1.5)	24/62 (38.7)	0/24(0)
EGFP	No	600 ng/µl	5	30 V	5/95 (5.3)	35/95 (36.8)	18/35(51.4)
EGFP	No	600 ng/µl	5	45 V	35/96 (36.5)	16/96 (16.7)	14/16(87.5)
EGFP	No	600 ng/µl	5	60 V	66/97 (68.0)	7/97 (7.2)	7/7(100)

Table 2: Electroporation of CRISPRs to genome edit bovine embryos

Editors	Zona pellucida	Pulses	Voltage	Lysed zygotes (%)	Blastocysts (%)	NHEJ +ve blastocysts (%)	HDR +ve blastocysts (%)
CAS9p:gRNA:ssODN (0.75uM:0.75uM:1uM)	No	6	30 V	5/26(19)	7/21(33)	4/7(57)	0
CAS9p:gRNA:ssODN (0.75uM:1.5uM:1uM)	No	6	30 V	5/50(10)	15/45(33)	15/15(100)	1/15(6)

Taken together, this experiment demonstrated that the electroporation of bovine zygotes can provide an efficient means for the uptake of plasmids. The application of 30 V appeared to be the most effective as it was compatible with uncompromised in vitro embryo development while still providing efficient uptake of exogenous plasmid resulting in more than 50% EGFP expressing blastocysts.

After establishing the electroporation conditions with the EGFP reporter plasmid we then switched to electroporating ribonucleoprotein complexes comprising the Cas9 nuclease and two different amounts of a site-specific guide RNA (gRNA) together with an oligonucleotide as a site-specific repair template (Table 2). The in vitro development rates to the blastocyst stage were similar and comparable to normal in vitro development rates of untreated zygotes for both treatments. The efficiency of genome editing by electroporation of bovine zygotes was assessed by detecting mutations at the target sites introduced by the NHEJ repair mechanism (Salsman & Dellaire, 2017). More than half of the blastocysts (57 %) treated with lower amounts of gRNA were successfully edited at the target site. When the dosage of the gRNA was doubled, the editing rate increased to 100% indicating that the gRNA at the lower dose was limiting the overall editing activity (Table 2).

We then evaluated the blastocysts for the presence of a precise mutation specified by the repair template and introduced by HDR. No HDR events were detected in the blastocysts electroporated with the lower amount of gRNA. By contrast, at the higher dosage of gRNA we were able to identify one blastocyst that had successfully undergone HDR repair resulting in precise genome editing.

Conclusion

The bovine zona pellucida effectively blocked the delivery of plasmids to the cytoplasm. Five pulses of 30 V are suitable electroporation parameters for zona-free bovine zygotes to allow for efficient NHEJ editing using ribonucleoprotein CRISPR complexes. We demonstrated that it is feasible, although with a much lower efficiency, to use electroporation for precision editing of bovine embryos by HDR.

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