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Scalable Production of Genome-Edited Livestock Embryos

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Scalable Production of Genome-Edited Livestock Embryos

By

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THESIS

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Abstract

Genetic improvement of livestock is critical for animal production and wellbeing. Traditional breeding approaches however are slow and often result in unwanted genetic linkage drag. Genome-editing technologies offers an alternative that can quickly and precisely introduce useful traits into animals without linkage drag. Editing of mammalian livestock species has been achieved by somatic cell nuclear transfer of an edited cell or direct microinjection of zygotes. However, the efficiency of cloning in large livestock species is low and perinatal abnormalities are common, and introducing editing reagents through microinjection is a time-consuming task with a high technical barrier. Electroporation is a widely used technique for delivering gene-editing reagents into cells and poses as a possible high throughput approach to generate genome-edited animals through the treatment of early-stage embryos. Electroporators work by directing pulses of electrical current to create transient pores in the lipid bilayer of the plasma membrane, allowing the passage of reagents into cells. Upwards of 100 zygotes can be processed with the push of a button making electroporation a scalable and simple approach to producing genome edited livestock. Here, various electroporation parameters for generating gene-edited bovine, ovine, and caprine embryos were tested for producing targeted mutations.

Targeted genetic knock-ins of over 1kb however have not been produced with electroporation alone as nucleic acids larger than 1kb are unable to pass the zona pellucida. To develop a high throughput approach to producing large template targeted knock-in livestock, another delivery system for large DNA repair templates must be employed.

Non-pathogenic viruses such as rAAV can transport nucleic acid fragments of up to 4.9kb into cells. Here, a scalable approach was developed to transduce large DNA repair templates into bovine zygotes prior to electroporation for the production of 2.7kb knock-in blastocysts.

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Chapter 1. Electroporation-mediated genome editing of livestock zygotes[1]

Abstract

The introduction of genome editing reagents into mammalian zygotes has traditionally been accomplished by cytoplasmic or pronuclear microinjection. This time-consuming procedure requires expensive equipment and a high level of skill. Electroporation of zygotes offers a simplified and more streamlined approach to transfect mammalian zygotes. There are a number of studies examining the parameters used in electroporation of mouse and rat zygotes. Here, I review the electroporation conditions, timing, and success rates that have been reported for mice and rats, in addition to the few reports about livestock zygotes, specifically pigs and cattle. The introduction of editing reagents at, or soon after, fertilization can help reduce the rate of mosaicism, the presence of two of more genotypes in the cells of an individual; as can the introduction of nuclease proteins rather than mRNA encoding nucleases. Mosaicism is particularly problematic in large livestock species with long generation intervals as it can take years to obtain non-mosaic, homozygous offspring through breeding. Gene knockouts accomplished via the non-homologous end joining pathway have been more widely reported and successfully accomplished using electroporation than have gene knock-ins. Delivering large DNA plasmids into the zygote is hindered by the zona pellucida (ZP), and the majority of gene knock-ins accomplished by electroporation have been using short single stranded DNA (ssDNA) repair templates, typically less than 1 kb. The most promising approach to deliver larger donor repair templates of up to 4.9 kb along with genome editing reagents into zygotes, without using cytoplasmic injection, is to use recombinant adeno-associated viruses (rAAVs) in combination with electroporation. However, similar to other

[1] An earlier version of this literature review was published as Lin JC, Van Eenennaam AL. 2021. Electroporation-Mediated Genome Editing of Livestock Zygotes. Frontiers in Genetics. 12:648482. doi: 10.3389/fgene.2021.648482. PMID: 33927751; PMCID: PMC8078910.

methods used to deliver clustered regularly interspaced palindromic repeat (CRISPR) genome-editing reagents, this approach is also associated with high levels of mosaicism. Recent developments complementing germline ablated individuals with edited germline-competent cells offer an approach to avoid mosaicism in the germline of genome edited founder lines. Even with electroporation-mediated delivery of genome editing reagents to mammalian zygotes, there remain additional chokepoints in the genome editing pipeline that currently hinder the scalable production of non-mosaic genome edited livestock.

INTRODUCTION

Genome editing offers an opportunity to introduce targeted genetic alterations into livestock genomes. To be useful in animal breeding, these alterations have to be transmissible through the germline. To date, in livestock this has mostly been achieved by editing somatic cells and subsequently cloning the edited cell line to make an animal (Tan et al. 2016). Somatic cell nuclear transfer (**SCNT**) cloning remains an inefficient process and limits the genetic diversity of the germplasm to specific cell lines. Editing in zygotes offers an opportunity to introduce alterations to the next generation of a breeding program, and has the advantage of producing a diversity of foundation animals as each zygote will produce a genetically distinct animal, as opposed to animals derived from a clonal cell line (Bishop & Van Eenennaam 2020). To date, the standard method of delivering genome-editing components into livestock zygotes has been cytoplasmic microinjection (**MI**). This method requires expensive equipment and is both labor and time intensive, as a highly skilled individual is required to inject zygotes with a precise amount of genome-editing components one-by-one. It can take hours to microinject a large number of zygotes, and this can result in considerable variation in the timing of MI relative to fertilization.

Additionally, varying skill levels introduces operator-dependent variation into editing experiments.

Electroporation offers a high throughput alternative method of delivering genome-editing components into zygotes that carries a low technical barrier. Although electroporation has traditionally been used to introduce reagents into cultured cell lines, it is also effective at introducing editing reagents into mouse and rat zygotes (Peng et al. 2012; Kaneko et al. 2014; Kaneko & Mashimo 2015; Hashimoto et al. 2016). The protocol for electroporation requires only a stereomicroscope, electroporator, and an electroporation vehicle. Zygotes are placed into a cuvette or onto a slide while suspended in a medium containing genome-editing reagents (Takemoto 2020). The electroporator directs pulses of electrical currents through the zygotes via electrodes to create temporary micro-holes in the ZP and plasma membrane to allow the movement of genome editing reagents into zygotes (Figure 1.1). The workflow of delivering genome-editing reagents is considerably accelerated relative to MI, as anywhere from 35 to 100 zygotes can be electroporated simultaneously (Modzelewski et al. 2018).

Due to the potential scalability and ease of use of electroporation, it has the potential to become the platform to enable high throughput genome editing in livestock species. However, species specific optimization of electroporation parameters is necessary to achieve both a high survival-rate and efficient editing of zygotes. Here we review the literature on electroporation-mediated genome editing, with a focus on conditions that maximized zygote survival and editing efficiency in livestock species.

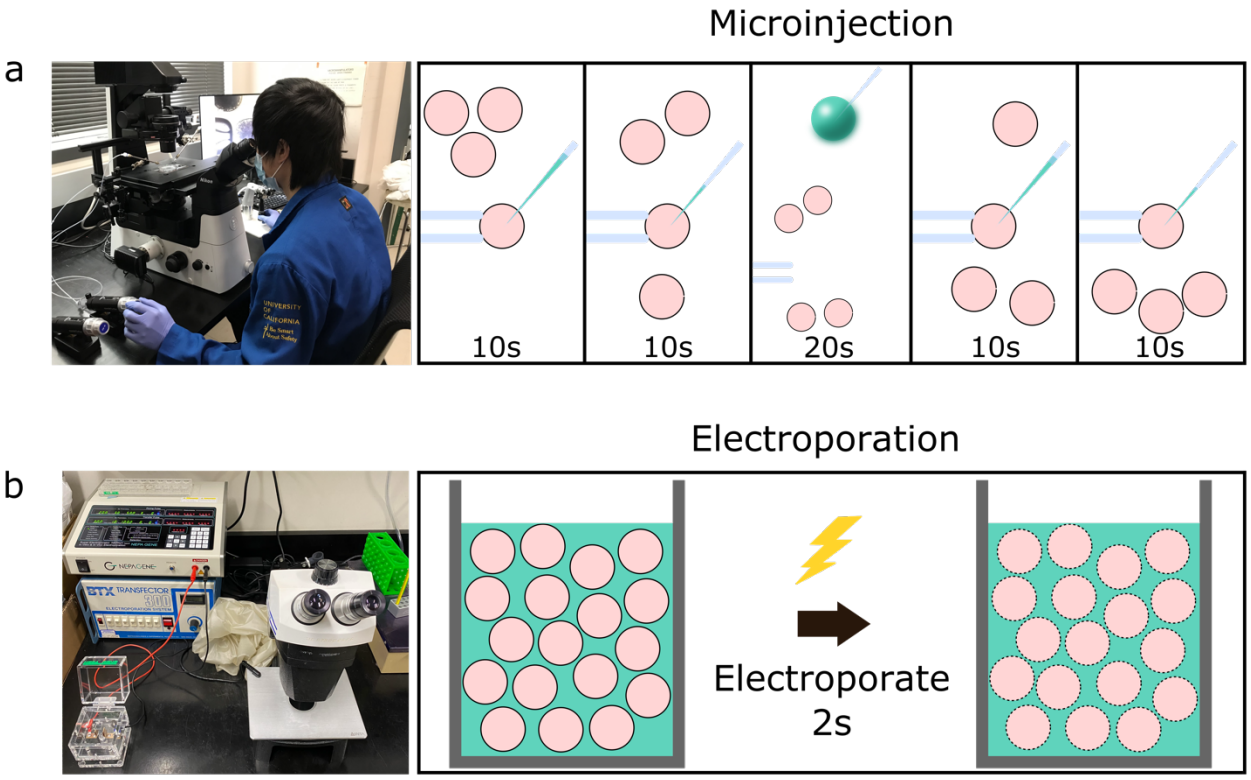


Figure 1.1. Graphical schematic of a comparison between setup and time necessary for the microinjection vs. electroporation of embryos. (A) The equipment necessary for the microinjection of embryos and the workflow involved to introduce editing reagents (green) into four presumptive zygotes (pink) using a holding needle (left) to stabilize the zygote before introducing the injection needle (right). (B) The equipment necessary for the electroporation of embryos and the workflow involved to introduce editing reagents into 30–100 presumptive zygotes via a cuvette. Image from Lin and Van Eenennaam (2021).

ELECTROPORATION CONDITIONS

One of the first studies published on the electroporation of mouse zygotes concluded that the voltage, pulse length and concentration of clustered regularly interspaced palindromic repeat (CRISPR) RNA-guided endonuclease Cas9 (Cas9)/single guide RNA (sgRNA) all play a critical role in the survival of embryos and efficiency of mutations (Hashimoto & Takemoto 2015). The study noted that higher voltages, longer pulse lengths, and higher Cas9/sgRNA concentrations were all positively associated with increased editing efficiency, but negatively correlated with embryo viability. There is a need to strike a balance between the mutation rate and embryo viability when optimizing electroporation conditions. The most efficient parameters for electroporation are

highly dependent both on the species of zygote and type of edit (knockout vs. knock-in), therefore it is necessary to optimize the parameters for each of these variables in order to maximize the generation of live edited animals.

There are several voltage variables to consider when optimizing electroporation conditions including the amplitude of the voltage to be used, how many times that voltage will be applied (number of pulses), and the length (width) of the pulse. There are also two common types of pulses used in electroporation: square-wave, and exponential decay pulses. Square-wave pulses are pulses of a consistent voltage set for a specific amount of time whereas an exponential decay pulse is a continuous pulse with a decaying voltage. In the electroporation of embryos, only square-wave pulses have been reported and there are two sub-types that are commonly used, a "poring" pulse which is a brief mid-level voltage pulse designed to open holes in cell membranes, and a long low voltage "transfer" pulse that is designed to transport negatively charged nucleic acid molecules into cells and nuclei (Sukharev et al. 1992). Combined pulse electroporation uses alternating poring and transfer pulses and can increase the transfection of eukaryotic cells with plasmid DNA or siRNA (Stroh et al. 2010). However, not all electroporators have both pulse types available, and often only the poring voltage is used and reported in many papers.

Poring Pulse Voltage

Increasing the poring voltage has been shown to increase the density of membrane pores (Gowrishankar et al. 2006; Krassowska & Filev 2007; Saulis & Saulė 2012). Studies focused on the electroporation of rat and mouse zygotes have typically reported success in producing genome edited animals when using poring voltages of 25–50 V/mm and anywhere from 2 to 7 pulses (Table 1). A study tested poring voltages of 30, 100, and 300 V/mm to find the optimal conditions and 30 V/mm resulted in the highest development and mutation rate in mice. These electroporation

experiments achieved mutation rates of 13–100%, suggesting the possibility of high efficiency editing with the further optimization of parameters (Qin et al. 2015). Another study tested pulses of 0 - 50V/mm and found that pulses above 40V/mm significantly decreased embryo development and that 30V/mm resulted in the best balance between development and transfection efficiency (Nakano et al. 2021). The studies therefore suggest that higher voltages typically achieved higher mutation rates, although embryo viability was concomitantly decreased.

Studies with livestock zygotes typically report using lower voltages, with porcine zygotes reporting success with 25–30 V/mm and 2–5 pulses; and bovine studies 10–20 V/mm and 2–3 pulses (Table 1). Bovine zygotes appear to be especially sensitive to high voltages; with 20 V/mm (three pulses, 1 ms width) resulting in lower blastocyst rates than 10 V/mm (Namula et al. 2019). Increasing the voltage strength to 45 V/mm (five pulses, 3 ms width) was associated with high rates of bovine zygote lysis suggesting damage to the cell membrane lipid bilayer (Wei et al. 2018). Similar results were also reported by Miao et al. (2019), where pulses of 20, 25, and 30 V/mm had an increasingly negative impact on bovine blastocyst development rates. One study found that 15 V/mm achieved significant membrane permeabilization in bovine zygotes to enable efficient rates of gene knockout using Cas9:sgRNA ribonucleoproteins (RNPs), while maintaining acceptable rates of embryo development (Camargo et al. 2020).

Pulses

Evidence have suggested that pulse number and duration both play a role in the size and density of pores created. Increasing the number of pulses was shown to increase the density of pores, and increasing pulse duration increased the size of the pores created (Gowrishankar et al. 2006; Krassowska & Filev 2007; Saulis & Saulė 2012). To test the effect of increasing the number of pulses, Chinese hamster ovary cultured cells were electroporated with a varying number of

square-wave pulses. A positive linear relationship was found between the number of pulses and the amount of DNA that entered the electroporated cells (Escoffre et al. 2011). Mouse and rat studies found 2–7 pulses of 1–5 ms pulse widths to be effective in generating efficient mutation and developmental rates. Conditions for electroporating intact rat embryos using zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the CRISPR associated (Cas) mRNAs were first optimized for the most efficient editing in a study by Kaneko et al. (2014). Using the voltage magnitude of 45 V/mm, various pulse lengths were examined, and for ZFN, a pulse length of 1.5 ms was the most efficient parameter for generating edited embryos with a survival rate of 91% and editing rate of 73%. Rat embryos electroporated with both TALEN and Cas9 editing reagents showed high survival rates with a pulse length of 2.5 ms, however, the editing rates for these nucleases were only 18 and 9%, respectively, possibly due to the fact that TALEN and Cas9 mRNA are three times larger than that of ZFN mRNA (Kaneko et al. 2014).

Studies with porcine embryos have found 4–5 pulses of 1–2.5 ms pulse widths to be successful, and those with bovine embryos have found 2–6 pulses of 1–3 ms pulse widths to be successful (Table 1). Various pulse numbers and durations were tested in the electroporation of porcine zygotes, and similar to rodent zygotes, mutation rates increased in proportion with increased pulse numbers and duration, however, blastocyst development rates fell to near zero when the parameters were increased to seven pulses of 3 ms (Tanihara et al. 2016). Nishio et al. (2018) tested a range of voltage magnitudes, in addition to unipolar and bipolar pulses, and the results showed that bipolar pulses and voltages over 30 V/mm resulted in significantly lower rates of blastocyst formation, whereas 25 V/mm and unipolar pulses resulted in acceptable rates of embryo survival and editing. Another study by Hirata et al. (2019a) tested the effect of the number of pulses on both blastocyst formation rates and editing efficiency. Both oocytes and zygotes were

electroporated at 30 V/mm in this study, and the authors found that using more than five pulses resulted in a significantly lower blastocyst formation rate. The mutation rate varied between electroporation of matured oocytes and putative zygotes, and additionally by the gene being targeted. The same group later followed up with another publication utilizing five pulses at 25 V/mm to generate edited embryos, however, no blastocysts developed so only two to eight cell embryos were analyzed. The authors found that 80–100% of the analyzed embryos showed the intended mutations (Hirata et al. 2019b).

There are currently only five studies describing the electroporation of bovine zygotes to generate knockout embryos. The first of these five studies targeted the Myostatin (*MSTN*) gene to test the effects of voltage magnitude and electroporation timing on embryo survival and mutation rates. They found that using 20 V/mm considerably lowered the blastocyst formation rate, however, there was a strong correlation between increasing voltage strength and mutation rates. That study also concluded that electroporating bovine zygotes 10 hours post-insemination (hpi) yielded higher mutation rates than electroporating zygotes 15 hpi, regardless of the voltage used (Qin et al. 2015; Namula et al. 2019). Another study utilized in vivo-derived blastocysts and examined the quality of hatched blastocysts and blastocysts with their ZP still intact after electroporation. The authors concluded that the intact status of a blastocysts' ZP played a role in the quality of blastocysts as the diameter of the hatched blastocysts shrank significantly after electroporation indicating a loss of quality, whereas the diameter of ZP intact blastocysts did not change significantly after electroporation (Tanihara et al. 2019a). These results support previous experiments in mice embryos that found the removal of the ZP hindered embryonic development (Bronson & McLaren 1970; Modliński 1970; Chen et al. 2016; Troder et al. 2018; Miao et al. 2019; Tanihara et al. 2019a). Camargo et al. (2020) reported efficient knockout of bovine OCT4

following electroporation at 17 hpi using six 15 V/mm poring pulses of 1.5 ms at 50 ms intervals and a 10% decay rate of successive pulses. Transfer pulses were set at 3 V/mm, with five pulses of 50 ms at 50 ms interval with a 40% decay rate and positive/negative polarity. In that study, 92.3% of the electroporated embryos evaluated contained the intended edit, however, it should be noted that only a single embryo reached the blastocyst stage when subjected to these conditions.

Collectively, these studies suggest that increasing the duration and number of pulses increases the mutation rates of electroporation-mediated genome editing, correlating with an increase in pore density and size allowing for the entry of more genome editing regents into the embryo. However, increasing parameters to increase transfection efficiency, and/or weakening the ZP can negatively affect subsequent embryonic development, further demonstrating the need to strike a balance between editing efficiency and embryo viability when optimizing electroporation parameters.

Concentration of editing reagents

The concentration of editing reagents is yet another parameter that affects the efficiency of electroporation-induced gene editing. Mouse and rat embryos were electroporated with various Cas9 mRNA/gRNA/single-stranded oligonucleotide (ssODN) donor concentrations to optimize conditions for generating knock-in and knockout animals (Kaneko & Mashimo 2015). The study found that increasing the Cas9 mRNA/gRNA/ssODN concentrations to 400/600/300 ng/µl in both mice and rats resulted in editing efficiencies of 67 and 88%, respectively. Qin et al. (2015) also tested different concentrations of Cas9 mRNA/gRNA and found that increasing the concentrations from 200/100 to 600/300 ng/µl, respectively, increased editing efficiency from 3 to 57%. Tanihara et al. (Tanihara et al. 2021) performed a similar experiment with pig embryos and concluded that embryos electroporated with 200ng/µL or 400ng/µL of Cas9 had significantly higher mutation

rates at all three loci targeted than embryos electroporated with 100ng/µL of Cas9. Bi-allelic mutations also increased with the increase of Cas9 concentration.

However, when using ssODN donors to optimize conditions for the delivery of a large donor repair plasmid in rat zygotes, it was found that the electroporation of Cas9 protein/gRNA/ssODN at 950/200/200 ng/µl decreased development and did not improve editing efficiency when compared to 475/150/150 ng/µl (Remy et al. 2017). Increasing Cas9 protein and gRNA concentrations from 20 to 100 ng/µl for MI of porcine zygotes increased not only mutation efficiency, but also the proportion of bi-allelic mutations (Tanihara et al. 2019b). A 2020 publication tested seven different concentrations of Cas9 protein (0, 25, 50, 100, 200, 500, and 1,000 ng/µl) in porcine zygotes without changing the gRNA concentration of 100 ng/µl, and found that neither embryonic development nor non-specific off-target cutting were affected by Cas9 concentration, although the frequency of biallelic edits tended to increase with Cas9 protein concentration. Additionally, the gene editing efficiency, defined as the frequency of indel mutations in each edited blastocyst, was significantly lower with 25 ng/µl of Cas9 protein compared with higher Cas9 protein concentrations (Le et al., 2020). Most recently, a study testing the effects of pulse number and gRNA concentration found that higher pulse numbers increased transfection efficiency while lowering development, and that doubling the gRNA concentration from 12.5ng/µL to 25ng/µL had no effect on mutation rates (Navarro-Serna et al. 2022). Collectively, these results suggest that, as with voltage and number of pulses, increasing the total concentration of editing reagents is associated with an increase in editing efficiency. Moreover, there appears to be an optimum concentration beyond which embryo viability is impaired with no concomitant increase in editing efficiency, and that may vary depending upon the species and target gene.

SIZE OF ZYGOTE

Zygote size is another factor that may influence the efficiency of gene editing using electroporation. Agarwal et al. (2007) found that cell diameter was positively correlated with cell transmembrane potential. This suggests that larger embryos may be permeabilized by a lower voltage than is needed for smaller embryos. Figure 1.2 shows the proportional size of embryos from various mammalian species, ranging from mice (80 μm diameter) to cattle (110–120 μm). In the early embryo, the primary repair mechanism for DNA double-strand breaks (DSBs) is the non-homologous end-joining (NHEJ) repair pathway. The homology directed-repair (HDR) pathway is primarily restricted to actively dividing cells (S/G2-phase), and only becomes highly active toward the end of the first round of DNA replication (Hustedt & Durocher 2017) It is worth noting that the long G2 phase resulting from genome activation at the two-cell stage in mice is known to be associated with elevated rates of gene knock-ins, presumably due to both the open-chromatin state during genome activation, and the fact that HDR is predominantly active in the late S-G2 phases (Gu et al. 2018; Plaza Reyes & Lanner 2018). The timing of zygotic genome activation varies among species (Li et al. 2013), ranging from as early as the S/G2 phase in the male pronucleus of the mouse zygote, to the four-cell stage in pigs, the eight-cell stage in goats, and between the eight- and 16-cell stages in cattle and sheep (Sirard 2012; Graf et al. 2014; Deng et al. 2020). It is unclear if the facts that among mammals mice are “early genome activators” while livestock (e.g., bovine) are considered “later genome activators” (Svoboda 2018), means it is more difficult to achieve gene knock-ins in early livestock embryos.

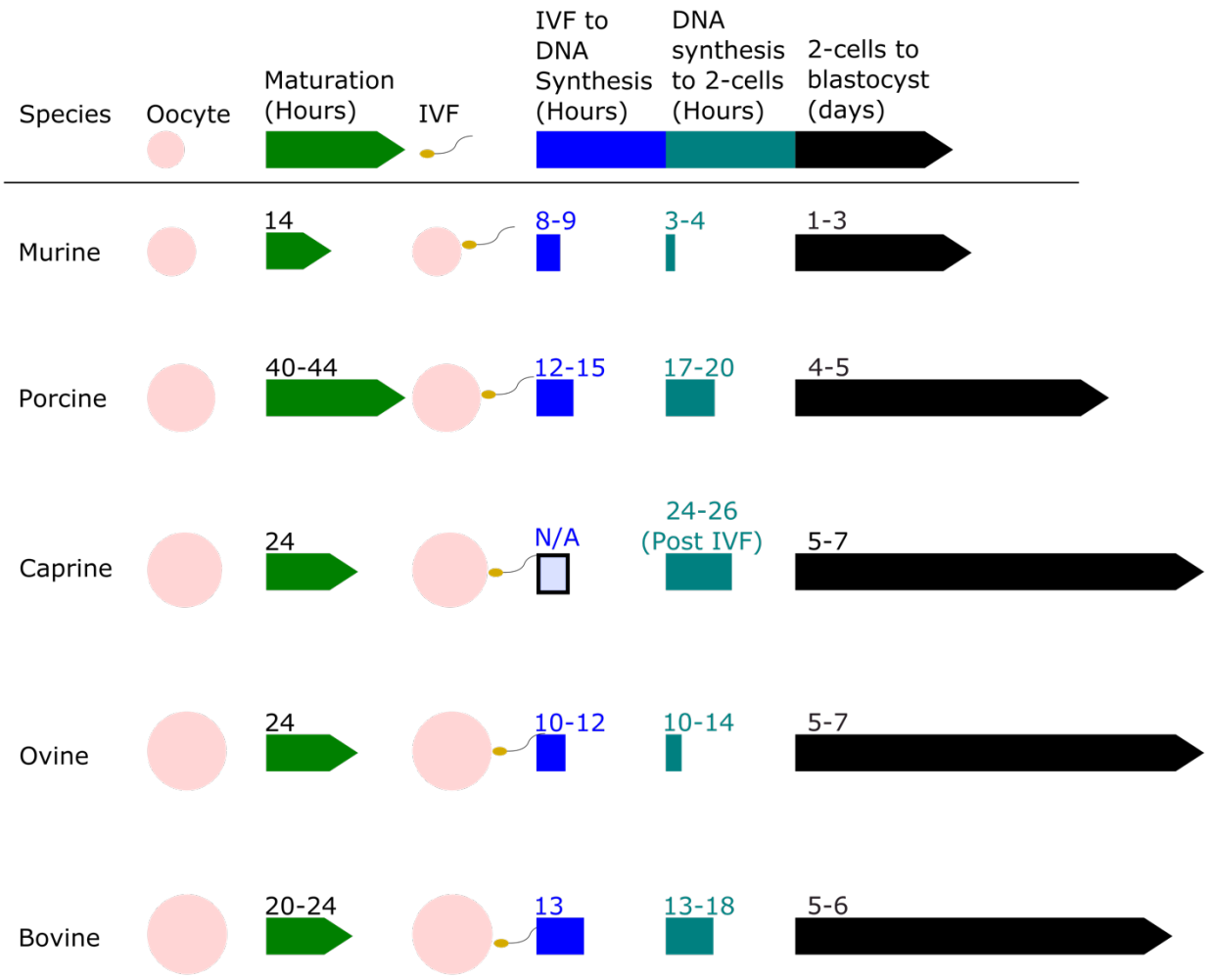


Figure 1.2. Relative oocyte size and a timeline of embryo development for murine, porcine, caprine, ovine, and bovine zygotes. The oocyte size of murine, porcine, caprine, ovine, and bovine species are shown to scale and compared. The relative timeline of embryo development from the oocyte stage to blastocyst stage after in vitro fertilization (IVF) is shown. Data derived from (Harlow & Quinn 1982; Motlik et al. 1984; Crosby et al. 1988; Papaioannou & Ebert 1988; Sakkas et al. 1989; Prather 1993; Campbell et al. 1994; Gardner et al. 1994; Laurincik et al. 1994; Fair et al. 1995; Rath et al. 1995; Serta et al. 1995; Bouniol-Baly et al. 1997; Fair et al. 1997; Otoi et al. 1997; Gómez et al. 1998; Wang et al. 1998; Anderson et al. 1999; Comizzoli et al. 2000; Raghu et al. 2002; Sanfins et al. 2003; Ciemerych & Sicinski 2005; Moon et al. 2005; Griffin et al. 2006; Ptak et al. 2006; Surjit et al. 2006; Zhou & Zhang 2006; Chaves et al. 2010; Catalá et al. 2011; Wang et al. 2012; O'Hara et al. 2014; Paramio & Izquierdo 2014; Morohaku et al. 2016; Cadenas et al. 2017; Yoon et al. 2018; HosseinNia et al. 2019; Mclean et al. 2020; Owen et al. 2020). Image from Lin and Van Eenennaam (2021).

MOSAICISM and the TIMING OF ELECTROPORATION

Mosaicism is the presence of two or more genotypes in the cells of one individual. Mosaicism poses a problem when generating live animals due to false-positive genotyping, non-transmission of mutations to offspring, and complications with phenotyping (Mehravar et al. 2019). Avoiding mosaicism is particularly important in large livestock species, especially uniparous large animals like cattle with a 2-year generation interval. Whereas researchers utilizing mice can breed mosaic founders and practically guarantee the production of non-mosaic animals with the desired mutations in the first generation (mice reach sexual maturity at 7–8 weeks of age), researchers utilizing livestock may have to wait for years. The ability to generate non-mosaic mutations is therefore essential for the efficient development of genetically modified livestock (Mehravar et al. 2019). Previous studies in mice, cattle, goat, sheep, and pig that have produced genome edited animals using CRISPR and MI have noted the prevalence of mosaic individuals (Hai et al. 2014; Ma et al. 2014; Yen et al. 2014; Oliver et al. 2015; Bevacqua et al. 2016; Zhang et al. 2017). Microinjection with the CRISPR Cas9 system in particular results in a high proportion of mosaic animals (Whitworth et al. 2014; Yen et al. 2014; Sato et al. 2015; Vilarino et al. 2017; Sato et al. 2018; Vilarino et al. 2018).

There are two possible explanations for relative high rates of mosaicism from MI of the CRISPR system. Firstly, the nuclease may continue to target and cut DNA even after the first genomic replication and secondly, genome-editing reagents may not be active within the zygote until after the first genomic replication. As MI is a long and tedious task, the high rate of mosaicism when producing genome-edited animals using MI may be due to the fact that the zygotes will continue to develop throughout the injection process and while Cas9 is active. The continuous development of zygotes during the MI process means that the zygotes that are processed late in the batch are more likely to be closer to the DNA synthesis stage of the first genomic replication

when injected, thus resulting in Cas9 being active later in zygotic development and potentially past the one-cell stage (Burkard et al. 2017). Using a Cas9:sgRNA RNP, rather than Cas9 mRNA, was found to decrease mosaicism as the RNP is active immediately upon MI, and does not result in the delay associated with Cas9 mRNA translation and formation of active RNP (Hennig et al. 2020).

A study published in 2016 compared the editing efficiencies of electroporation and MI, and found that electroporation had an 11% lower incidence of mosaicism at an optimized setting when compared to MI, however, the authors electroporated Cas9 protein but injected mRNA, which could have likely played a confounding role in the difference observed (Chen et al. 2016). Another recent study also evaluated the editing efficiencies in addition to the timing of electroporation and MI of porcine embryos, and found that MI significantly decreased the blastocyst rates in one and two cell injected embryos as compared to electroporation of one cell embryos (Le et al. 2021). This paper used Cas9 protein for both procedures and also noted that mutation efficiency and bi-allelic mutation rate were higher when one cell embryos were microinjected. In addition to substituting Cas9 protein for Cas9 mRNA, other approaches to further reduce mosaicism have included editing embryos sooner after fertilization, degrading Cas9 quicker, in vivo germline editing, and co-transfection with other reagents such as a three-prime repair exonuclease to improve gene editing efficiency (Chapman et al. 2015; Hashimoto et al. 2016; Tu et al. 2017; Yamashita et al. 2020).

The timing of electroporation also affects the efficiency of generating bi-allelic mutants. Earlier delivery of gene editing components relative to insemination, whether through electroporation or MI, results in an increased rate of bi-allelic and non-mosaic mutants (Vilarino et al. 2017; Namula et al. 2019). One study reported that electroporation of mouse zygotes at only 5 hpi generated 100% non-mosaic animals whereas the electroporation of naturally bred zygotes

produced mostly mosaic pups (Hashimoto et al. 2016). The authors concluded that electroporation of mouse zygotes 5 hpi allowed the editing of the mouse genome to occur prior to the first genome-replication and eliminated mosaicism

In the case of ovine, porcine, and bovine zygotes, DNA synthesis occurs 10–12 hpi, 12–15 hpi, and 18 hpi, respectively (Figure 1.2). Namula and colleagues utilized electroporation to deliver CRISPR Cas9 genome-editing components to bovine zygotes and found that electroporation 10 hpi increased the bi-allelic mutation rate, as compared to electroporation at 15 hpi (Namula et al. 2019). The authors later electroporated pig oocytes to produce triple mutations prior to insemination, at 40, 42, and 44 hours from the start of in-vitro maturation, and found that oocytes electroporated at 40 and 42 hours from the start of maturation had significantly lower blastocyst and triple mutation rates while oocytes electroporated 44 hours from the start of maturation had improved development and triple mutation rates . It is important to note that electroporating pig oocytes prior to fertilization did not increase bi-allelic mutation rates when compared to control embryos that were electroporated 13 hpi (Namula et al. 2022c). Another study in bovine zygotes found a significant reduction in mosaicism rates from MI of zygotes at 10 hpi compared to 20 hpi, however, the even earlier delivery of CRISPR Cas9 genome-editing reagents into bovine MII oocytes did not eliminate mosaicism (Lamas-Toranzo et al. 2019). Microinjection of MII sheep oocytes before fertilization also did not eliminate mosaicism, but it did produce more bi-allelic mutations compared to MI of zygotes (Vilarino et al. 2017). In pigs, mosaicism was reduced when editing reagents were introduced 18 hours after parthenogenetic activation, prior to the onset of DNA replication (Tao et al. 2016). However, the downside of this early electroporation time is that fertilization rates tend to be decreased if oocytes are co-incubated with cumulus cells and spermatozoa for a shorter period of time (Ward et al. 2002).

ELECTROPORATION-MEDIATED KNOCKOUTS

The primary method for DSB repair in gametes and the early zygote is the NHEJ pathway (Rothkamm et al. 2003). Multiple studies in numerous species have used electroporation to deliver CRISPR Cas9 genome-editing reagents into zygotes to generate knockout embryos and animals. Non-mosaic knockouts have been most efficiently produced in rats and mice (Hashimoto et al. 2016; Chen et al. 2019) targeting a wide range of genes, including *LIF* (Kim et al. 2020), *Rad51* (Iwata et al. 2019), and *Rosa26* (Troder et al. 2018).

As previously noted in the poring voltage section, Kaneko et al. (2014) was one of the first to optimize electroporation conditions for rat embryos and successfully generated knockout embryos with a 9% mutation rate. Qin and colleagues (Qin *et al.* 2015) were able to target 10 different genes in mice and generate 10 different knockout mice with mutation rates from 13 to 100%. Another study published in 2019 utilized Cas12a instead of Cas9 as the site-directed nuclease, and targeted three different genes with electroporation. The authors found knockout mutation rates in mouse embryos ranged from 34 to 70% (Dumeau et al. 2019). Unfortunately, mosaicism rates were not reported. More recently, Kaneko successfully electroporated pronuclear-stage embryos that underwent slow freezing and subsequent warming to generate *Tyr* knockout mice (Nakagawa et al. 2018) and rats (Kaneko & Nakagawa 2020) using Cas9 protein and dual sgRNA. This same group used a combination with electroporation of Cas9 protein and gRNA into rat oocytes following intracytoplasmic sperm injection (ICSI) of frozen or freeze-dried sperm and reported rates of 56% and 50% genome edited offspring for frozen and freeze-dried sperm, respectively (Nakagawa & Kaneko 2019). They demonstrated that although ICSI produces embryos that are sensitive to physical stress, they can still successfully develop with the desired mutations after electroporation.

There are currently only a handful of studies describing the generation of live genome edited livestock following electroporation of editing reagents. To date, only porcine, bovine, and ovine zygotes have been successfully electroporated to produce live knockout animals. Pig researchers have electroporated zygotes and oocytes to generate genome edited blastocysts and live piglets using Cas9 genome editing reagents. A group led by Tanihara has published seven studies describing the electroporation of porcine zygotes 7 to 13 hpi to generate edited blastocysts with at least an 80% success rate in all seven studies (Table 1). They also produced live knockout piglets in six of the studies. The first of the seven studies targeted the *MSTN* gene using five 1 ms pulses at a voltage of 30 V/mm and generated 10 piglets. Nine of the 10 piglets had mutations at the target site, and seven of these piglets were mosaic. The next study targeted the *TP53* gene using the same electroporation parameters which resulted in nine piglets, six of which were genetic knockouts. However, again four out of the six mutated piglets were mosaic individuals, a less than ideal outcome if electroporation is to be widely used for the generation of genetically modified livestock (Tanihara et al. 2018). A third study utilized the same parameters again to produce *PDX1* knockout blastocysts and achieved a success rate of up to 94.1%. That same study also attempted to generate *PDX1* knockout fetuses, however, only one fetus was collected, and it did not carry genetic mutations at the target site (Tanihara et al. 2019c). A subsequent study re-attempted to generate *PDX1* knockout piglets and was successful in producing 10 piglets, nine of which contained the intended knockout. Two of nine piglets with the intended mutations contained no wild-type sequences and another two were mosaic (Tanihara et al. 2020b).

This same group then targeted the *CD163* gene with slightly different parameters, using 25 V/mm instead of 30 V/mm, and was able to successfully produce edited blastocysts with a 90% success rate as well as eight piglets, one of which showed a mutation at the intended target

(Tanihara et al. 2019d). These studies were able to successfully generate edited blastocysts and piglets, however, up to four of the *CD163* blastocysts, four *TP53* piglets, and seven *MSTN* piglets were mosaic. In 2020, this group successfully knocked out *MSTN* and *GGTA1* using electroporation at 12 hpi with five 1 ms transfer pulses at 25V/mm (Le et al. 2020; Tanihara et al. 2020a). Five out of six piglets born in the *GGTA1* study carried a bi-allelic mutation in the targeted region of *GGTA1*, with no off-target events (Tanihara et al. 2020a). More recently, the group attempted to produce triple knockout piglets targeting the *GGTA1*, *B4GALNT2*, and *CMAH* genes for xenotransplantation purposes. One of the two piglets born contained mutations at all three sites, however the mutation at *B4GALNT2* was in frame, resulting in a gene that was still functional (Tanihara et al. 2021).

Another group consisting of many of the same personnel lead by Namula, published 3 studies in 2022 describing the effects of ZP weakening prior to electroporation, and the production of triple knockout pig embryos. To test the effect of ZP weakening, the group treated zygotes with actinase E to either weaken or remove the ZP. They found that ZP weakening lowered blastocyst development rates albeit not statistically significantly, and completely removing the ZP significantly decreased blastocyst development rates (Namula et al. 2022a; Namula et al. 2022b). ZP intact, weakened, and free embryos had mutation rates of 35%, 58%, and 100% respectively suggesting that weakening or removing the ZP will increase mutation rates although with negative development impacts. The group also described the effects of electroporating pig oocytes at 40, 42, and 44 hours post-maturation as mentioned earlier, and the effects of using electroporation of CRISPR RNP in combination with the MI of the same CRISPR RNP for the generation of triple knockout embryos. Blastocyst formation rates were significantly reduced and mutation rates were not significantly improved when MI was used in combination with electroporation (Namula et al.

2022b). The rate of blastocysts carrying bi-allelic mutations in at least one target locus was improved when microinjecting prior to electroporating the same gene-editing reagents, however there was no difference in the rate of blastocysts carrying bi-allelic mutations in at least two target loci.

Another study published in 2020 attempted to address the issue of generating mostly mosaic mutants through the co-transfection of a three-prime repair exonuclease (Trex2), an exonuclease known to digest DNA ends with breaks, into pigs. The authors claim to have increased the production of non-mosaic blastocysts by 70.7% when Trex2 was co-transfected with Cas9. Unfortunately, Trex2 is a known inhibitor of HDR which may result in problems if attempting to generate non-mosaic, knock-in animals (Yamashita et al. 2020).

Two studies used electroporation to introduce multiple gRNAs to target more than one gene in porcine zygotes. Double bi-allelic mutations were obtained when targeting two genes, although at a low frequency (0–25%) depending upon the gRNA combination (Hirata et al. 2020b). Another study by this group targeted four genes simultaneously. Guides for each gene were first tested independently, and the best guide for each gene was combined to target the four loci. Mutations were observed in one (55.8%) and two genes (20.9%), and no blastocysts had mutations in three or more target genes. This was despite the fact that each guide had independently achieved a rate of at least ~ 20% bi-allelic mutations in blastocysts. The majority of the blastocysts were mosaic. Bi-allelic knockouts were identified in six of the 43 (14%) blastocysts in one of the four genes, and none of these contained edits in a second gene. It is possible that larger than expected deletions or translocations may have occurred that were not detected by the screening methods being used in this study. The authors concluded that the technique to deliver gRNA and Cas9

protein to edit multiple genes will require considerable optimization to improve the success rates (Hirata et al. 2020a).

Miao et al. (2019) published a study describing electroporation of Cas9 protein with gRNA targeting the *Nanos2* gene in mice, pigs, and cattle. They were successful in generating knockout embryos for all three species, and pups in mice. They found that the optimal voltage strengths for efficient survival and editing rates were 20 V/mm for bovine and 30 V/mm for mice and porcine. Analysis of mouse embryos and pups found that two cell embryos were 90% mutated and 70% of pups had a *Nanos2* mutation. Analysis of bovine and porcine embryos revealed bi-allelic *Nanos2* edits at a rate of 82 and 73%, respectively. Some of these knockout *Nanos2* bovine embryos were brought to term, and two calves were born alive while one was stillborn (Ciccarelli et al. 2020). The stillborn and one live calf were bi-allelic knockouts, while the other live bull calf was mosaic containing both wildtype and mutated allele sequences in varying proportions depending upon the tissue analyzed. It should be noted that electroporation in this study was done at 18–20 hpi.

ELECTROPORATION-MEDIATED KNOCK-INS

While the electroporation of embryos has been able to efficiently generate knockout animals in several species, the generation of knock-in livestock via zygote electroporation has not been as widely reported. This can be attributed in part to the low rates of HDR in zygotes, as HDR is predominantly active in the late S-G2 phases of the cell cycle (Liu et al. 2019). This makes it difficult to achieve knock-ins of zygotes.

Generation of knock-in animals requires the cleavage of a specific target as well as the integration of donor DNA into the genome. Therefore, in addition to successfully introducing Cas9 and sgRNA and inducing cleavage at the target site, targeted knock-ins also require the successful transfer of template nucleic acid sequences into the zygote. Large supercoiled or linear DNA

requires larger functional pores for its entry in the cell compared to short single stranded DNA (ssDNA). Introducing large nucleic acid templates into embryos may require weakening or removing the ZP. The host genome must then be able to repair the cut with the donor template to successfully generate a knock-in embryo. In an unedited cell, the sister chromatid may be used as the homologous donor for HDR; but when generating a knock-in animal, a donor template with the desired insert flanked by homology arms is necessary to successfully repair the DSB induced by the nuclease and insert the intended sequence (Smirnikhina et al. 2019).

Donor molecules for gene knock-ins include double stranded DNA (dsDNA) as well as ssDNA (Smirnikhina et al. 2019). Double stranded templates have traditionally been used for gene knock-ins; however, single-stranded oligonucleotides (ssODN) has gained popularity due to the more rapid construction, higher efficiency, and lower possibility of off-target or plasmid backbone integration (Chen et al. 2011). Additionally, ssODN are able to efficiently integrate into the target locus with homology arms as short as 20 nucleotides, whereas dsDNA donors typically require homology arms around 1–2 kb (Chen et al. 2011; Zhao et al. 2020; Wittayarat et al. 2021). Long ssDNA has been used to knock-in large fragments varying from 800 nucleotides to 1.4 kb with efficiencies ranging from 25 to 67% (Quadros et al. 2017). This group used a strategy called efficient additions with ssDNA inserts-CRISPR or Easi-CRISPR (Miura et al. 2018). The homology arms used in that study were 60–105 nucleotides in length. The disadvantage of this approach is that synthesis of long ssDNA greater than 1.5 kb is challenging, and secondary structures could be a problem with long ss templates.

There are also end joining-based techniques that can be used to introduce template sequences into targeted genomic locations. Although NHEJ is the prominent DSB repair pathway, other repair pathways join, anneal, and ligate resected homologous DNA ends. The homology-

independent targeted integration method utilizes a donor template containing a gene of interest flanked by the CRISPR Cas9 target sites, but without the use of homology arms. The target sites within the donor template are cleaved alongside the genomic target site, and the gene of interest is inserted by blunt end ligation using the NHEJ repair pathway (Suzuki et al. 2016).

Microhomology-mediated end-joining (MMEJ) is typically defined by homologous joining of sequences less than 25–50 bp in length. A technique called CRISPR/Cas9-based precise integration into the targeted chromosome, or CRIS-PITCh, used an MMEJ donor plasmid containing the knock-in fragment flanked with 40 base pair homology arms and Cas9:sgRNA RNPs in mouse zygotes to generate knock-ins with efficiencies as high as 40% (Aida et al. 2016).

Targeted integration of linearized dsDNA-CRISPR or tild-CRISPR, uses a linear dsDNA donor template flanked with 800 base pairs of homology arms (Yao et al. 2018). Donor plasmids where the CRISPR target sites are placed outside of 800 bp homology arms so that in vivo cleavage by Cas9 generates a linear dsDNA template for homology mediated end joining (HMEJ) have shown robust DNA knock-in efficiency in embryos of several species (Yao et al. 2017). A HMEJ donor plasmid with 800 bp homology arms flanked by the CRISPR Cas9 target site microinjected into bovine zygotes significantly increased the knock-in efficiency of a 1.8 kb fragment when compared to a donor plasmid with the knock-in fragment flanked by 800 bp arms alone (37.0 and 13.8%; $p < 0.05$), and additionally more than a third of the knock-in embryos (36.9%) were non-mosaic. All told, using the HMEJ approach resulted in 7% of total injected embryos being non-mosaic, bi-allelic knock-ins (Owen et al. 2020).

A downside of the HMEJ approach is that the linear dsDNA template, containing the gene of interest and flanking homology arms, generated by Cas9/sgRNA directed cleavage can be inserted into the cleaved genome by blunt end ligation. The lack of control over copy number and

orientation of the insert when it is repaired in this way, and the resultant potential presence of random indels and insertion of plasmids into the genome, limits the use of this approach as a precise genome engineering strategy (Salsman & Dellaire 2017).

ELECTROPORATION OF DONOR NUCLEIC ACID SEQUENCES

Grabarek et al. (2002) was the first to demonstrate that nucleic acids can be delivered to isolated oocytes and zygotes by electroporation if the ZP was weakened by exposure to acid Tyrode's solution. Of relevance to this review is the size of the donor template that can be introduced into zygotes using electroporation. Larger donor plasmids have traditionally been delivered to the zygote via MI. There have been only a few studies describing the successful delivery of ssODN donors of 30–200 nucleotides, and even fewer describing the successful delivery of large plasmids into an embryo when using electroporation alone (Kaneko & Mashimo 2015; Chen et al. 2016; Hashimoto et al. 2016; Wang et al. 2016; Remy et al. 2017; Bagheri et al. 2018; Troder et al. 2018; Chen et al. 2019; Sentmanat et al. 2022).

The majority of knock-in animals created through electroporation have been mice or rat zygotes electroporated with Cas9/gRNA/ssODN. Hashimoto and Takemoto (Hashimoto & Takemoto 2015) were able to use an ssODN donor template of 117 nucleotides to disrupt the expression of mCherry in mice. All 11 of the surviving embryos did not fluoresce suggesting a successful knock-in. However, further sequencing did reveal some mosaicism in the edited embryos as up to three distinct alleles were found (Hashimoto & Takemoto 2015).

Electroporation of an ssODN donor enabled successful genome editing of both mice and rats harboring a single amino acid substitution, with a success rate of 33% in both species (Kaneko & Mashimo 2015). Other successful electroporation mediated knock-ins include a 92 nucleotide ssODN targeting the *Tyr* gene in mice. In this study, a pulse width of 1 ms produced 47% *Tyr*-

edited mice of which 42% were mosaic while a pulse width of 3 ms produced 97% *Tyr*-edited of which 9.4% were mosaic (Chen et al. 2016). Others include a 103 ssODN donor targeting the *Fgf10* gene (Hashimoto et al. 2016), and a 128 bp oligonucleotide targeting the *Aicda* gene (Wang et al. 2016).

Sakurai et al. (2020) utilized oocytes from transgenic mice expressing maternal Cas9 (maCas9) to generate gene-edited embryos and pups. The group compared mutation rates between embryos and pups following zygote transfections either with gRNA alone or with both Cas9 and gRNA. They found that the electroporation of Cas9-expressing transgenic zygotes with gRNA alone was able to generate indels at the target region in nearly 100% of the embryos analyzed, and no off-target mutations were observed. They also found that the electroporation of zygotes expressing maCas9 with gRNA alone showed significantly lower mosaicism rates when compared to wild-type zygotes electroporated with Cas9/gRNA. Most notably, the authors found that the electroporation of maCas9 zygotes with gRNA to disrupt Et1 resulted in 40% genome-edited pups, compared to wild-type zygotes electroporated either with Cas9 mRNA/gRNA (21%) or Cas9 protein/gRNA (23%).

In this same study, birth rates were also higher following electroporation of maCas9 zygotes. The authors attempted a knock-in mutation at the *Klf5* locus either into maCas9 zygotes with gRNA/ssODN which gave a 48% rate of live pups, as compared to 20–21% for wild-type zygotes electroporated with Cas9/gRNA and ssODN. Similarly, when knock-in mutations were attempted at the *Ar* locus, blastocyst rates for maCas9 zygotes were higher (69%) when compared to wild-type zygotes electroporated with Cas9/gRNA/ssODN (8–15%). Actual knock-in rates at the *Klf1* locus were similar between maCas9 zygotes (46–48%) and wild-type zygotes (41–44%); and knock-in rates at the *Ar* locus were 8% in maCas9 zygotes and 0% in control zygotes.

There is one publication reporting a successful knock-in with bovine zygotes using electroporation, however, it is unknown what the target locus was, or the size of the ssODN template. The publication only details that an ssODN was used as a donor template and that one of 16 blastocysts (6%) collected and analyzed showed a successful knock-in. The authors concluded this result demonstrated that knock-ins are possible with the electroporation of bovine zygotes albeit at a low rate (Wei et al. 2018). The authors also found that a 4.7 kb pEGFP plasmid could only be introduced into bovine zygotes following removal of the ZP using pronase. They reported that only zona-free zygotes generated EGFP-positive blastocysts following electroporation, indicating that the ZP presents a strong barrier for large dsDNA-uptake following electroporation. They concluded that the bovine ZP effectively blocked the delivery of plasmids to the cytoplasm.

In rat and mouse embryos, a 5.1 kb plasmid was successfully delivered into the cytoplasm by electroporation but only following MI of the plasmid, along with all of the CRISPR Cas9 genome-editing reagents, into the sub-ZP space (Bagheri et al. 2018). All mutant blastocysts were found to be mosaic. Although MI of all CRISPR components prior to electroporation allows the donor plasmid to bypass the ZP and integrate into the host genome, this method does not eliminate the technical barrier and time required to perform MI. A different study attempted to knock-in a 3.1 kb plasmid into the *Rosa26* locus of rats without the use of prior MI but failed to generate any embryos with successful integration (Remy et al. 2017).

Laser zona drilling (LZD) is another method of facilitating movement across the ZP that may be able to help in the transfection of larger plasmids into zygotes. LZD generates a hole in the membrane of the ZP allowing larger molecules to enter the sub-ZP space and was previously used to assist in the MI of CRISPR Cas9 genome-editing components (Bogliotti et al. 2016).

Additionally, LZD has been shown to have minimal effects on embryo viability when used in conjunction with MI. LZD in conjunction with electroporation may be able to better facilitate the movement of large plasmids into embryos where the ZP presents a barrier to transfection. However, LZD again requires handling each zygote individually and requires a high level of skill.

Recombinant adeno-associated viruses (rAAV) offer an opportunity to overcome the size limitation of ssODN donors for knock-in animals. They are relatively small viruses of about 20 nm belonging to the family Parvoviridae that do not incorporate into the host chromosomes. They can however transduce across the ZP to transiently deliver genes to fertilized mammalian zygotes with intact ZP (Mizuno et al. 2018; Romeo et al. 2020; Oikawa et al. 2022b). They have been used to successfully generate genome edited mouse pups with both high embryo survival and editing rates, without the need for micromanipulation (Yoon et al. 2018; Hyunsun et al. 2021; Oikawa et al. 2022b). A 2019 study used rAAV to transduce large HDR donors of up to 4.9 kb into mouse zygotes, prior to electroporation with genome editing reagents (Chen et al. 2019). Known as CRISPR RNP electroporation and AAV donor infection (CRISPR READi), the authors generated large DNA fragment knock-in mice by incubating rAAV packaged with ssDNA with flushed zygotes for 6 h prior to electroporation, then cultured and transferred the edited embryos into surrogate mothers (Chen et al. 2019). This technique achieved up to 50% knock-ins, however, the animals had high rates of mosaicism. rAAV-serotypes 1, 2, and 6 have all been used to transduce mammalian embryos of various species, with serotype 6 appearing to be useful in a variety of mammals (Mizuno et al. 2018). Since the AAV genome can be episomally maintained for an extended period, mosaicism might result from insertions that occur after the one-cell stage of embryo development (Mizuno et al. 2018), posing a potential mosaicism issue for livestock applications.

DISCUSSION

The studies done in rodents show that electroporation has the potential to streamline the process of generating genetically modified livestock and making this technology more accessible to laboratories lacking MI expertise. However, the limited number of studies done in cattle and pigs shows much work still remains to optimize these experimental protocols to improve both editing and survival efficiency, and eliminate the production of mosaic animals. There are several chokepoints in the pipeline from the collection of oocytes to the production of non-mosaic blastocysts homozygous for the intended edit, that need to be streamlined and optimized before this technique can become routine (Figure 1.3).

It is perhaps not obvious to those not working in the field, but a source of livestock oocytes must be readily available to perform zygote editing, often obtained from ovaries collected at a local slaughter facility. To produce viable mammalian offspring, it is also necessary to have a ready supply of synchronized recipient or surrogate females. This is not an inexpensive undertaking in the case of large livestock species, and due to seasonal breeding and other climatic factors, it is almost impossible to conduct this work during certain times of the year. To improve the efficiency of the process, ideally only blastocysts carrying the desired edits would be transferred to surrogate females. Although studies have shown that taking a biopsy from the trophectoderm of in vitro matured bovine embryos can result in live, healthy offspring (de Sousa et al. 2017), a high level of skill is required. Another problem with preimplantation biopsies is that mosaicism decreases the usefulness of these results (Vilarino et al. 2018) as the trophectoderm may have a different genetic composition compared to the inner cell mass.

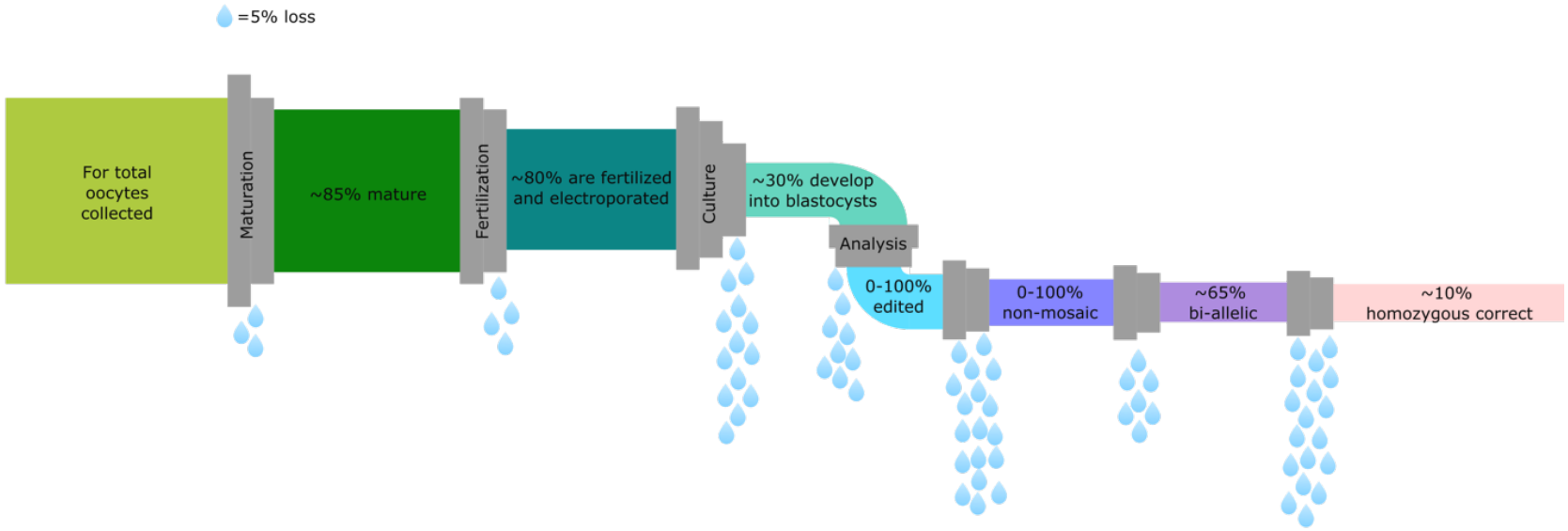


Figure 1.3. Graphical representation of the losses in the genome editing pipeline from collection of oocytes to the percentage of blastocysts that are non-mosaic homozygotes for the intended edit. Data derived from (Remy et al. 2017; Teixeira et al. 2018; Miao et al. 2019). Image from Lin and Van Eenennaam (2021).

It is perhaps ironic given the important role that sheep played in the development of livestock genetic engineering and SCNT cloning techniques, that there are currently no published studies detailing electroporation-mediated genome editing of sheep zygotes. All small ruminant edits have been accomplished by either SCNT or embryonic MI (Kalds et al. 2020). An unpublished dissertation reports the production of electroporation-mediated genome edited sheep (Mahdi 2021). Future sheep and goat experiments will first need to optimize electroporation conditions prior to generating genetic knockouts and knock-ins, but previous work, especially in cattle, should help pave the way. There are already many targets in the sheep and goat genome that have previously been edited using MI of CRISPR Cas9 genome-editing reagents, so the transition to electroporation should be relatively straightforward.

Gene knockouts using the NHEJ pathway have been the most successful type of embryo-mediated genome edit, to date, and there are several experiments documenting very high rates of bi-allelic mutation using electroporation. Although it should be noted that gene compensation through exon skipping has been observed to reinitiate transcription and translation, which can result in partial gain-of-function alleles rather than the predicted nonsense or missense alleles (Lalonde et al. 2017; Smits et al. 2019; Hosur et al. 2020). When the editing reagents are working well and producing 100% bi-allelic knockouts, transferring edited embryos carries little downside. However, if rates decrease below this, the probability of transferring mosaic, hemizygous, or wild type animals increases. Obtaining a high proportion of bi-allelic knockouts of multiple genes in a zygote is still extremely challenging. Likewise obtaining targeted gene knock-ins in zygotes is very inefficient, especially for large DNA insertions. Undoubtedly, further improvements in editing reagents such as base pair editors, and improved repair templates will be forthcoming. Viral

transduction using rAAV offers an opportunity to introduce single-stranded DNA of up to 4.5kb in length (Kaulich et al. 2015), although this approach has not yet been applied to livestock zygotes.

Other approaches to increasing the production of non-mosaic edited animals include editing embryonic stem cells (ESCs). The production of porcine (Gao et al. 2019), bovine (Bogliotti et al. 2018), and ovine (Vilarino et al. 2020) stable, pluripotent ESCs have recently been reported. The advantage of using ESCs is that multiple sequential edits could be performed due to their perpetual ability to self-renew. It may be that cloning ESCs increases the efficiency of cloning success relative to SCNT (Mclean et al. 2020). Alternatively, embryo complementation or injecting donor totipotent edited stem cells into genome edited knockout, germline ablated host embryos (Ciccarelli et al. 2020; Miura et al. 2020), or edited primordial germ cells in the case of poultry (Woodcock et al. 2019), may provide an alternative approach to produce animals that transmit gametes derived solely from an edited cell line. This could help to resolve the problem of mosaicism that is frequently associated with electroporation-mediated genome editing of mammalian zygotes. The downside of ESCs is similar to SCNT in that they represent a limited genetic pool, and they may accumulate mutations during culture. Delivery of genome editing components into the zygote edits the next generation of a livestock breeding program, and avoids the inefficiencies associated with SCNT. It has been successfully used to achieve targeted knockouts in embryos, although mosaicism can reduce germline transmission, and efficient gene knock-ins have proven difficult. Although electroporation provides an improved approach over MI to rapidly introduce editing reagents into developing zygotes of mammalian food animal species, further development and optimization of enabling methodologies will be required to routinely obtain non-mosaic knockout and targeted-gene insertion founders in livestock at scale.

Such developments will be required before genome editing can be seamlessly introduced into livestock genetic improvement programs

Table 1.1 Summary of experiments using electroporation to introduce genome editing reagents into mammalian zygotes. Summary includes: Species, form and type of nuclease used for editing, method of fertilization, electroporation conditions and timing relative to fertilization, type of edit, survival rate, developmental rates and editing efficiency. RNP, ribonucleoprotein; ND, not discussed; IVF, in vitro fertilization; Mate, natural mating; Settings, Electroporation Voltage (V/mm) Pulse length (msec) # pulses; Reagent, medium used for electroporation. Modified from Lin and Van Eenennaam (2021).

Species (Oocyte = *) (Embryo = †)	**Nuclease**	**RNP, mRNA**	**Electroporation timing Fertilization**	**Settings** (V/mm) **Reagent**	**Target Type of edit (Size of donor or insert bp or nt)**	**Zygote survival rate**	**Development rate (2-cell = *) (blast = †)**	**Edited rate (live animal = *) (Embryo = †)**	**Reference**
Mouse†	Cas9	Protein	ND: Mate 6hr: IVF	30V 3msec 2-6 pulses Opti-MEM	69 targets KI (Various)		10-26% (Live Birth)	0.7-14%*	(Sentmanat *et al.* 2022)
Rat†	Cas9	Protein	ND Mate	Rat: 30V 3msec 4 pulses Opti-MEM	Tfap2c KI (ND)	99-100%	94%*	40%*	(Oikawa *et al.* 2022b)
Mouse†	Cas9	Protein/ mRNA	ND IVF frozen vitrified	Poring: 0-50V 0-4msec 4 pulses	Psmb11 Tyr Rosa26 KI/KO		51-99%*	4.7-100%*†	(Nakano *et al.* 2021)
Mouse†	Cpf1	Protein							(Hyunsun *et al.* 2021)

Mouse† Rat†	Cas9	Protein/ dual RNA	ND Mouse: IVF Rat: Mate	Poring: 40V 3.5msec 4 pulses Opti-MEM	*Tyr* KO	Mouse:100% Rat: 100%	Mouse: 100%* Rat: 92%*	Mouse: 18%* Rat: 100%*	(Kaneko & Nakagawa 2020)
Mouse†	Cpf1	Protein/ mRNA	ND Mate	ND Opti-MEM	Leukemia Inhibitory Factor *Lif* KO	Protein: 71.6% mRNA: 76.4%	Protein: 84.6%† mRNA: 68.1%†	Protein: 45.5%* mRNA: 33.3%* Protein: 18.1%† mRNA: 13.3%†	(Kim *et al.* 2020)
Mouse† In-vivo	Cas9	protein	ND IVF	Poring: 50V 5msec 3 pulses Opti-MEM	*Adamts20* PN-locus *Rad51* Inversion Deletion			Adamts20: 10%* PN-locus: 50%* Rad51: 50%*	(Iwata *et al.* 2019)
Mouse†	Cas9	Maternal	IVF	20V 3msec 5 pulses	Et-1, Tyr KOKO *Klf5*, *Ar* KIKI *Adm, Amy,* *Aldh2* *Cyp1a1* *Hprt* *Npr3* *Ramp1* *Ramp3*		32-58%* 8-69%†	46-48%*	(Sakurai *et al.* 2020)

Rat† 	Cas9 	Protein/dual RNA 	5hr IVF 	Poring: 20, 30, 40V 3.5msec 4 pulses Opti-MEM	*Tyr* KO 	94-100% 	0-25% (Offspring)	0-100%* 	(Nakagawa & Kaneko 2019)
Mouse† Both in-tact ZP and weak ZP	Cas12a 	Protein 	ND Mate 	Poring: 30V 3msec 6 pulses Own Mix	*UBN1* *UBN2* *RBM 12* KO	72-100% 	21-46%† 	34-70%† 	(Dumeau *et al.* 2019)
Mouse† Weakened ZP	Cas9 	Protein 	ND Mate 	30V 3msec 6 pulses Opti-MEM	*Tyr* *Sox2* *Rosa 26* KI (Up to 4.9kb)		41% (morula)	18-40%* 33-69%†	(Chen *et al.* 2019)
Mouse† Rats† 	Cas9 	Protein 	ND Mate 	Mouse: 25V 3msec 3-4 pulses Rat: 30V 3msec 4 pulses Opti-MEM	*Rosa26* *KI* *(1.8kb)*		58-86%† 	21-43%† 	(Mizuno *et al.* 2018)
Mouse† Both intact and weakened ZP	Cas9 	Protein 	ND Mate/ IVF 	30V 3msec 2 pulses	*NPHS2* *ATP1a1* *GTRosa26Sor*			23%* 	(Troder *et al.* 2018)

				Opti-MEM	*Tmem218* KI/ KO (150nt)			(Unable to knock-in large plasmid)	
Mouse†	Cas9	Protein	ND Mate	Poring: 40V 3.5msec 4 pulse Opti-MEM	*BRCC3* *Vash1* *Vash2* *CTSE* *MCT8* KO/KI		89-97% (Transferred embryos)	60-100%*	(Teixeira *et al.* 2018)
Mouse†	Cas9	Protein	6.5-7.5hr IVF	25/30V 3msec 7 pulses Opti-MEM	*Tyr* *IL11* *SPP1* KI/ KO		90-100%*	50-100%*	(Nakagawa *et al.* 2018)
Mouse† ZP pierced, injected	Cas9	Protein	ND Mate	25V 3msec 7 pulse Own Mix	*Nanog* *RP113a* KI inject 9.7kb/7.17kb	90%		2.3-3%† KI	(Bagheri *et al.* 2018)
Rat†	Cas9	Protein	ND Mate	Various PBS	*EPHX2* *FLNA* *Rosa26* KI (100nt; 119nt)	30-100%		0-100%†	(Remy *et al.* 2017)
Mouse† Weakened ZP	Cas9	Protein	ND IVF/ Mate	30V 1msec 2 pulses ND	*Aicda* *Smc1b* *Rosa 26* KI (34bp insertion)			14-100%*	(Wang *et al.* 2016)

Mouse† Cas9 Protein 5hr IVF/ Mate 30V 3msec 7 pulses Opti-MEM *FGF10* KI/ KO (103nt) 93-96% 2-cell 63.7-100% 44.4%† (Hashimoto *et al.* 2016)									
Mouse† Weakened ZP Cas9 Protein ND Mate 30V 3msec 2 pulses Opti-MEM *Tyr* *Cdh1* *Cdk8* *Kif11* *MecP2* *Sox2* KI/ KO (92nt donor) 18-63% (morula) 27-88%* 54-100%† (Chen *et al.* 2016)									
Mouse† Weakened ZP Cas9 mRNA ND Mate 30V 1msec 2 pulses Opti-MEM *Tet1, Tet2* *Cd69, Cd226Cd226* *Clec16a* *Cyp27b1* *Fut2* *Ormdl3* *Rgs1* *Tlr7* *Tlr8* *Tnfsf9* KO KI (126nt donor) 0-100%* (Qin *et al.* 2015)									

Mouse† Rat† Pronuclear Embryo No ZP	Cas9	mRNA	ND Mouse: IVF Rat: Mate	Poring: 45V 2.5msec 4 pulses PBS	*Il2rg* KI/ KO		73-98%*	33-88%*	(Kaneko & Mashimo 2015)
Mouse†	Cas9	mRNA	ND Mate	30V 3msec 7 pulses Opti-MEM	*FGF10* *Rosa26* KI/ KO (117nt donor)	94-95%	51-72% (Embryo)	12-97%†	(Hashimoto & Takemoto 2015)
Rat* Embryo	ZFN TALEN CRISPR	mRNA	ND	Various PBS	*Il2rg* KO	24-97%	6-55% (offspring)	4-75%*	(Kaneko *et al.* 2014)
Mouse various embryonic stages Weakened ZP			ND Mate	Various Opti-MEM	*OCT4*	85-94%			(Peng *et al.* 2012)
Pig† Weakened ZP	Cas9	Protein	7hr IVF	25V 1msec 5 pulses Nuclease-Free-Duplex-Buffer	*GGTA1* *KO*	81-86%	3-11%†	35-100%	(Namula *et al.* 2022a)
Pig†	Cas9	Protein	10hr IVF	25V 1msec 5 pulses Nuclease-Free-Duplex-Buffer	*PDX1* *GGTA1* *CMAH* *KO*	47-94%	6-26%*	95-97%†	(Namula *et al.* 2022b)

				(Injected + electroporated)					
Pig*	Cas9	Protein	13hr IVF	25V 1msec 5 pulses Opti-MEM	*KDR* *PDX1* *SALL1* *KO*	57-90%	15-44%†	80-95%†	(Namula *et al.* 2022c)
Pig*	Cas9	Protein	ND IVF	30V 1msec 2-6 pulses	*CAPN3* *KO*	43-79%	11-38%†	10-69%†	(Navarro-Serna *et al.* 2022)
Pig†	Cas9	Protein	7hr IVF	25V 1msec 5 pulses Nuclease-Free Duplex Buffer	*KRAS* *Point Mutation*		8-22%†	15-95%†	(Wittayarat *et al.* 2021)
Pig†	Cas9	Protein	7hr IVF	25V 1msec 5 pulses Nuclease-Free Duplex Buffer	*GGTA1* *CMA* *B4GALNT2* *KO*		20-27%†	85-95%†	(Tanihara *et al.* 2021)
Pig†	Cas9	Protein	20hr IVF	Poring: 45V 2.5msec 4 pulses Opti-MEM	*GHR* KO		C9: 38%† T: 45%†	C9: 37%† T: 45%†	(Yamashita *et al.* 2020)
Pig†	Cas9	Protein	12 & 24hr IVF	Poring: 25V 1msec 5 pulses	*B4GALNT2* KO		8.1-32.6%†	20-90%†	(Le *et al.* 2021)

				Nuclease-Free Duplex Buffer					
Pig†	Cas9	Protein	12hr IVF	25V 1msec 5 pulses Opti-MEM	*CD163* KO			12.5%* 84.6-90%†	(Tanihara *et al.* 2019d)
Pig†	Cas9	protein	18-20hr IVF	30V 3 pulse 3msec Opti-MEM	*Nanos2* KO		9-36%†	63-90%†	(Miao *et al.* 2019)
Pig†*	Cas9	protein	13hr IVF	30V 1msec Various pulses Opti-MEM	*MSTN* *FGF10* KO	52.6-90.7%	8.8-27.6%†	12.5-60%†	(Hirata *et al.* 2019a)
Pig†	Cas9	Protein	13hr IVF	20V 1msec 5 pulses Opti-MEM	PERV pol KO	74-88.9%	0-22%†	80-100%†	(Hirata *et al.* 2019b)
Pig†	Cas9	Protein	7hr IVF	25 V 1msec 5 pulses Opti-MEM	*IL2RG/GHR* KO		20-30%†	85-95%†	(Hirata *et al.* 2020b)
Pig†	Cas9	Protein	13hr IVF	25 V 1msec 5 pulses Opti-MEM	*CMAH/GHR/* *GGTA1/PDX1* KO		29.7%	76.7%† 20.9%† (in two target genes)	(Hirata *et al.* 2020a)

Pig†	Cas9	Protein	12hr IVF	30V 1msec 5 pulses Opti-MEM	*PDX1* KO		10.3-11.9%†	0%* 37.8-94.1%†	(Tanihara *et al.* 2019c)
Pig†	Cas9	Protein	12hr IVF	30V 1msec 5 pulses Opti-MEM	*TP53* KO			66.67%* 72.7-100%†	(Tanihara *et al.* 2018)
Pig†	Cas9	mRNA	13hr IVF	Various Opti-MEM	*FGF10* KO	61.1-91.4% cleaved	4.1-26.4%†	3.6-7.7%†	(Nishio *et al.* 2018)
Pig†	Cas9	mRNA Protein	13hr IVF	30V 1msec 5 pulses	*FGF10* *MSTN* KO		10-16%†	90%* 23-100%†	(Tanihara *et al.* 2016)
Pig†	Cas9	Protein	12hr IVF	25V 1msec 5 pulses Nuclease-Free Duplex Buffer	*GGTA1* KO		Approx. 18%†	83.3%* 37.5%†	(Tanihara *et al.* 2020a)
Pig†	Cas9	Protein	5hr IVF	30V 1msec 5 pulses Nuclease-Free Duplex Buffer	*PDX1* KO		Approx. 15%	90%* 76.5-77.8%†	(Tanihara *et al.* 2020b)
Pig†	Cas9	Protein	12hr IVF	25V 1msec 5 pulses Nuclease-Free Duplex Buffer	*MSTN* KO		65.6-78.9%* 13.3-23.1%†	Approx. 70-90%	(Le *et al.* 2020)

Cattle In-vivo derived blastocysts			8 d in vivo–derived blastocysts	20V 1msec 3-10 pulses Opti-MEM					(Tanihara *et al.* 2019a)
Cattle†	Cas9	Protein	10/15hr IVF	10, 15, and 20V 3 pulse 1 msec nuclease- free duplex buffer	*MSTN* KO	60-92%	14-38%†	4.8-16.7%†	(Namula *et al.* 2019)
Cattle†	Cas9	Protein	18-20hr IVF	20V 2 pulse 3msec	*Nanos2* KO		9-36%†	63-90%†	(Miao *et al.* 2019)
Cattle†	Cas9	Protein	18-20hr IVF	20V 2 pulse 3msec Opti-MEM	*Nanos2* KO			16 embryos, 8 pregnancies, 3* born (1 stillborn); 2 KO; 1 mosaic	(Ciccarelli *et al.* 2020)
Cattle†	Cas9	Protein	8hr IVF	30V 6 pulses Opti-MEM	ND Undisclosed KI		33%†	6%†	(Wei *et al.* 2018)
Cattle†	Cas9	Protein	17-18 hr IVF	15V (0-30) 1.5msec 6 pulses Opti-MEM	*ZFX* *OCT4* KO		Approx. 80%*	92.3%†	(Camargo *et al.* 2020)

CHAPTER 2. Optimization of Electroporation Conditions for the Generation of Targeted Mutations in Livestock Embryos.

Abstract

The production of genome-edited livestock has traditionally relied on either somatic cell nuclear transfer cloning of an edited cell, or microinjection of gene-editing reagents into developing embryos. Both of these methods are inefficient as somatic cell nuclear transfer results in a lack of genetic diversity and cloning artifacts, while microinjection is time intensive, and requires a highly skilled operator. Electroporation of zygotes offers an alternative that is relatively quick and easy to use. The objective of this study was to optimize electroporation conditions for bovine, ovine, and caprine zygotes for the efficient generation of genome-edited blastocysts. Various electroporation conditions were tested on the three species to identify mutation and blastocyst development rates. Electroporation of bovine zygotes with two 3 msec 20V/mm bipolar pulses and ovine zygotes with two 3.5 msec 40V/mm bipolar pulses, gave satisfactory rates of survival to the blastocyst stage, and high rates of editing efficiency. Electroporation of caprine activated oocytes will need to be further optimized to produce blastocysts with high mutation and embryo viability. Additionally, rates of mosaicism were evaluated for electroporated bovine, ovine, and porcine blastocysts under optimal conditions.

Introduction

Genome editing provides an opportunity to quickly introduce useful traits into animals that bypasses the multi-generational process of selective breeding (Bishop & Van Eenennaam 2020). Previously, editing has been achieved by somatic cell nuclear transfer (SCNT) of an edited cell which constrains editing to the genetic diversity of cell-lines, and cloning artifacts are common (Keefer 2015). The introduction of genome editing reagents into mammalian zygotes has

traditionally been accomplished by cytoplasmic or pronuclear microinjection. This time-consuming procedure requires expensive equipment, and a high level of skill. Electroporation is a technique widely used in biotechnology and medicine for the delivery of drugs and genes into living cells. The electroporator directs "poring" pulses of electrical current to create temporary (msec to minute range) pores in the lipid bilayer of the plasma membrane which allows the transport of genome editing reagents into zygotes. Electroporation has been used to introduce genome editing reagents into early-stage murine and porcine embryos (Peng et al. 2012; Remy et al. 2017; Nakagawa et al. 2018; Teixeira et al. 2018; Troder et al. 2018; Dumeau et al. 2019; Hirata et al. 2019a; Miao et al. 2019; Tanihara et al. 2019c; Hirata et al. 2020b; Tanihara et al. 2020a). Additionally, there are a small number of papers describing the electroporation of bovine embryos (Wei et al. 2018; Miao et al. 2019; Namula et al. 2019; Tanihara et al. 2019a; Camargo et al. 2020), and to our knowledge, one unpublished Ph.D. dissertation describing the electroporation of ovine and caprine embryos (Mahdi 2021).

The aim of this study was to optimize electroporation parameters for the efficient targeted mutation of the *H11* safe harbor locus in bovine and ovine zygotes, in addition to activated ovine and caprine oocytes. Based on previous literature where available, a range of parameters including poring pulse voltage, number, polarity, and more were tested to electroporate clustered regularly interspaced palindromic repeat (CRISPR) RNA-guided endonuclease Cas9 (Cas9)/single guide RNA (sgRNA) reagents into zygotes and oocytes targeting the *H11* locus. This locus was selected due to its ability to incorporate and express exogenous pieces of DNA without posing adverse health risks to the host organism which makes it an excellent candidate for potential future knock-in studies (Papapetrou & Schambach 2016; Owen et al. 2021). Electroporated zygotes and oocytes that developed to the blastocyst stage were collected and analyzed for mutation rates. The best

settings were then used to target an alternate locus in bovine and sheep (the *Rosa26* locus in bovine, and the bone morphogenetic protein receptor type II (*Bmpr2*) gene in sheep) to confirm mutation and development efficiency. Additionally, a subset of embryos electroporated with these settings targeting the *H11* locus underwent next generation sequencing (NGS) to identify levels of genetic mosaicism. For the NGS mosaicism analysis, 33 porcine blastocysts that had previously been electroporated with Cas9:gRNA RNP targeting the *H11* locus were also analyzed.

Results

Optimization of electroporation conditions for bovine zygotes

Various electroporation parameters were tested on bovine zygotes for the delivery of Cas9:gRNA RNP to induce targeted mutations at the *H11* locus. The blastocyst development and mutation rates of treated bovine blastocysts were then evaluated for the different electroporation conditions (Table 2.1). First, five 1 msec bipolar pulses at 15 and 20V/mm was examined based on previous publications (Wei et al. 2018; Miao et al. 2019; Namula et al. 2019; Camargo et al. 2020). A significant reduction in embryo development was observed when comparing 20V/mm to 15/mm, but the latter was associated with a low rate of mutation (7%) (Table 2.1). The number of pulses was then decreased while the length of the pulse was increased to 3 msec at 20V/mm, and comparisons were made between two pulses to three pulses, and unipolar to bipolar pulses. A unipolar pulse resulted in a higher blastocyst development rate ($P < 0.05$) as compared to a bipolar pulse. However, the opposite was noted in the mutation rate. Of note, mutation rates of 96% and 93% ($P = 0.30$) were observed when using 2 and 3 bipolar pulses respectively. The electroporation conditions of 20V/mm, and three bipolar 3 msec pulses were then used to electroporate bovine zygotes with Cas9:gRNA RNP targeting the *Rosa26* locus. Similar results were obtained with a

mutation rate of 84% (n=19), and a blastocyst development rate of 12% (n=146; control 25%, n=20).

Table 2.1. The electroporation parameters to introduce Cas9:gRNA RNP into bovine oocytes, and their development and mutation rates. Values in the same column with same superscripts do not differ significantly ($p < 0.05$).

Group	Volts V/mm	Pulse Length msec	Number of Pulses	Polarity	Embryos Treated	Blastocysts Formed % (n)	Blastocysts Analyzed	Mutation % (n)	Untreated Blastocyst Formed	Treated/ Untreated Blastocyst development ratio
A	20V	3	2	Uni	246	47 (115)	90	79 (71) [a]	48%	.98 [a]
B	20V	3	2	Bi	703	27 (191)	84	96 (81) [b]	43%	.63 [b]
C	20V	3	3	Uni	55	25 (14)	14	50 (7) [c]	28%	.89 [c]
D	20V	3	3	Bi	270	31 (83)	74	93 (69) [b]	43%	.72 [d]
E	20V	1	5	Bi	58	5 (3)	0	-	28%	.18 [e]
F	15V	1	5	Bi	154	23 (35)	15	7 (1) [d]	38%	.61 [b]

Optimization of electroporation conditions for ovine zygotes and activated oocytes

Various electroporation parameters were also tested on ovine zygotes and activated oocytes for the delivery of Cas9:gRNA RNP to induce targeted mutations at the *H11* locus (Table 2.2). First, four unipolar pulses at 40V/mm, followed by 5 bipolar 5V/mm transfer pulses was trialed based on previous experiments at UC Davis (Mahdi 2021). However, this resulted in a significant reduction in blastocyst formation rate to 1.8% 9 (n=220) ($P < 0.05$). The number of pulses was therefore decreased to two 3.5 msec unipolar pulses at 40V/mm. This improved the blastocyst formation rate to 50% of the untreated blastocyst rate, but the mutation rate was only 74%. Two 3.5 msec 40V/mm bipolar pulses increased resulted the rate to 89% (40/45), but reduced development to 39% of the untreated blastocyst rate. These same parameters at 30V/mm resulted in a slightly lower, albeit not significant, blastocyst development rate. It should be noted that the untreated control development rates were lower for this set of 30V/mm bipolar experiments, and although development rates are expressed as a proportion of controls, this can be a confounding factor in experiments when oocyte collection occurs at different times of the year with seasonal breeders like sheep. Based on these data, electroporation conditions of 40V/mm, and two bipolar 3.5 msec pulses was used with Cas9:gRNA RNP targeting the *Bmpr2* locus. With the *Bmpr2* gRNA, similar results of a 100% (n=8) mutation rate and 18% (n=461; control 18%, n=50) blastocyst development rate was observed.

Table 2.2. The electroporation parameters used for ovine zygotes and activated oocytes and their development and mutation rates. If a transfer pulse was also included, the parameters are noted after a comma. Values in the same column with same superscripts do not differ significantly ($p < 0.05$).

Group	Volts V/mm	Pulse Length msec	Number of Pulses	Polarity	Embryos Treated	Blastocysts Formed % (n)	Blastocysts Analyzed	Mutation % (n)	Untreated Blastocyst Formed	Treated/ Untreated Blastocyst development ratio
A	40V	3.5	2	Uni	83	30 (25)	18	72 (13) [a c]	60%	.50 [a]
B	40V	3.5	2	Bi	364	18 (64)	45	89 (40) [a b]	46%	.39 [a b]
C	30V	3.5	2	Bi	537	14 (76)	37	97 (36) [b]	38%	.37 [b]
D	30V	3.5	3	Bi	507	6 (30)	21	95 (20) [b]	30%	.20 [c]
E	40V	3.5	3	Bi	163	9 (14)	7	57 (4) [c]	26%	.35 [b]
F	40V, 5V	1.5, 50	4, 5	Uni, Bi	220	2 (4)	0	-	39%	.05 [d]

Optimization of electroporation conditions for Caprine activated oocytes

In goat oocytes, two and three bipolar 3.5 msec pulses at 40V/mm was tested first based on the sheep electroporation data (Table 2.2). These parameters however resulted in a significant reduction in blastocyst formation rate of 4.1% (n=169) for 2 pulses and 2.7% (n=75) for 3 pulses, and a targeted mutation rate of 80% (n=5) for 2 pulses and 100% (n=2) for 3 pulses. Various voltage parameters, pulse numbers, decay rates, and polarities, were trialed as outlined in Table 2.3, however these resulted in either high blastocyst development but low mutation rates, or low blastocyst development with high mutation rates. The parameters yielding the best mutation rates were three bipolar pulses at 30V/mm and 40V/mm, however the treated/untreated blastocyst development rate was low, only 17% and 0.04%, respectively suggesting that like bovine embryos, the development of goat embryos is impaired by electroporation with a 40V/mm poring voltage in contrast to sheep embryos.

Mosaicism Analysis

Bovine and ovine blastocysts electroporated with optimized electroporation parameters were prepared for next generation sequencing (NGS) to evaluate rates of mutation and mosaicism. Additionally, previously electroporated porcine blastocysts were also prepared for NGS analysis. Bovine zygotes electroporated with three bipolar 3 msec pulses at 20V/mm were observed to harbor an average mutation rate of 89.87% per embryo, with many harboring more than two genetically distinct alleles (Figure 2.1) suggesting that genetic mosaicism is a common outcome from our approach. There are instances of blastocysts that appear to be homozygous mutations with very few (<5) reads showing other mutant alleles, compound heterozygotes with approximately 50% of the sequences in each of two different mutant allele types (two orange colored alleles in one column), some with 75% of the reads with one allele and 25% with the other

suggesting an edit at the two-cell stage, and some with more than 2 alleles at frequencies > 10% suggesting mosaicism.

Ovine zygotes electroporated with two bipolar pulses at 30V/mm resulted in an average mutation rate of 89.78% per embryo. The distribution of types of edits was similar to the cattle with evidence of homozygous mutations, compound heterozygotes, some with 75% of the reads with one allele and 25% with the other suggesting an edit at the two-cell stage, and a considerable number of embryos containing more than two alleles (Figure 2.2). Porcine zygotes were electroporated with two separate gRNAs targeting different regions of the H11 locus with five 1 msec 30V/mm pulses. Zygotes electroporated with gRNA1 resulted in an average mutation rate of only 27.8% per embryo and zygotes electroporated with gRNA2 resulted in a 53.93% mutation rate per embryo. Again, many embryos targeted with gRNA1 and gRNA2 were observed to contain more than two distinct alleles (Figure 2.3).

A large percentage of amplicons from our NGS analysis did not amplify with the unique barcode preventing the analysis of allele variants at the individual embryo level. However, we were able to analyze the types of mutations in these blastocysts for each species and guide-RNAs as a group. The total mutation efficiency in the unmatched bovine, ovine and porcine blastocysts was 90% (n=8,455,161), 94.7% (n= 2,210,754), and 31% (n=1,571,722), respectively. The types of allele variants that were found in bovine, ovine and porcine blastocysts are illustrated in Figure 2.4, 2.5 and 2.6, respectively. It is evident that the porcine electroporation parameters and guides were much less efficient at producing mutations than were the sheep and cattle optimized conditions.

Table 2.3. The electroporation parameters used for activated caprine oocytes and their development and mutation rates. If a transfer pulse was also included, the parameters are noted after a comma. Values in the same column with same superscripts do not differ significantly ($p < 0.05$).

Group	Volts V/mm	Pulse Length msec	Number of Pulses	Decay	Polarity	Embryos Treated	Blastocysts Formed % (n)	Blastocysts Analyzed	Mutation % (n)	Untreated Blastocyst Formed	Treated/ Untreated Blastocyst development ratio
A	40v	3.5	2	0%	+/-	169	4 (7)	5	80 (4) [a]	73%	.05 [a, f]
B	40v	3.5	3	0%	+/-	75	3 (2)	2	100 (2) [a]	77%	.04 [a, f]
C	30v	3.5	2	0%	+/-	98	0 (0)	-	-	57%	0 [a, b, e]
D	30v	3.5	3	0%	+/-	35	6 (2)	1	100 (1) [a]	36%	.17 [c, d]
E	30v	3.5	2	0%	+	61	0 (0)	-	-	69%	0 [a, b, e,]
F	20v	3.5	3	0%	+/-	104	12 (12)	8	38 (3) [a]	69%	.17 [c]
G	20v	3.5	3	0%	+	61	23 (14)	6	17 (1) [b]	69%	.33 [d]
H	25v	3.5	2	0%	+/-	46	4 (2)	2	0 (0) [a]	34%	.12 [f, c]
I	25v	3.5	3	0%	+/-	94	0 (0)	-	-	34%	0 [a, b, e]
J	15v	3.5	3	0%	+/-	42	33 (14)	14	14 (4) [b]	36%	.92 [g]
K	15v, 3v	1.5, 50	6, 5	10%, 40%	+, +/-	69	49 (34)	14	14 (2) [b]	69%	.71 [g]
L	30v, 3v	1.5, 50	6, 5	10%, 40%	+, +/-	81	0 (0)	-	-	69%	0 [a, b, e]

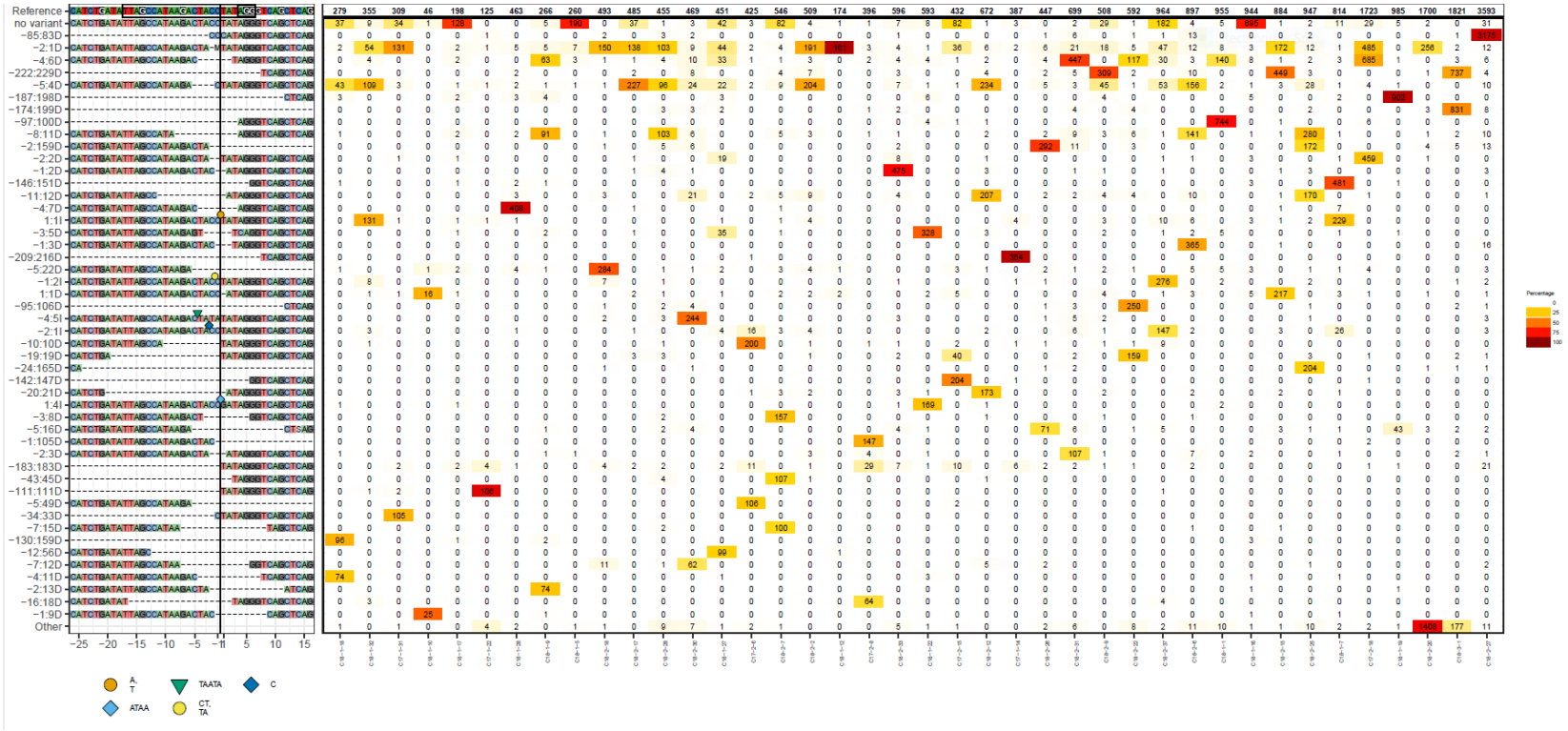


Figure 2.1. Graphical representation of the different allele variants are displayed for 40 electroporated bovine blastocysts with the most NGS reads. The majority of the bovine blastocysts had very low levels of wildtype sequence, and many contained more than two genetically distinct alleles, suggesting genetic mosaicism. X-axis displays each treated embryo. Y-axis displays allele variants detected.

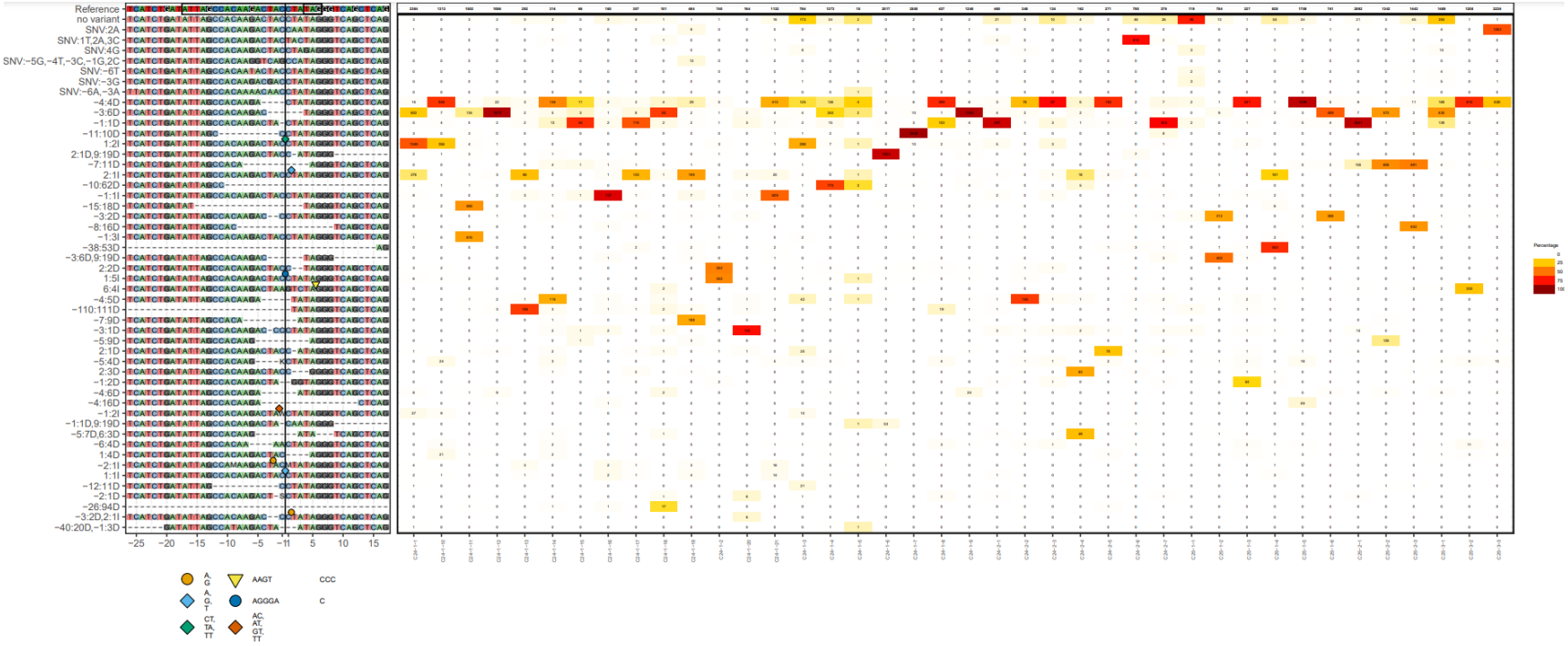
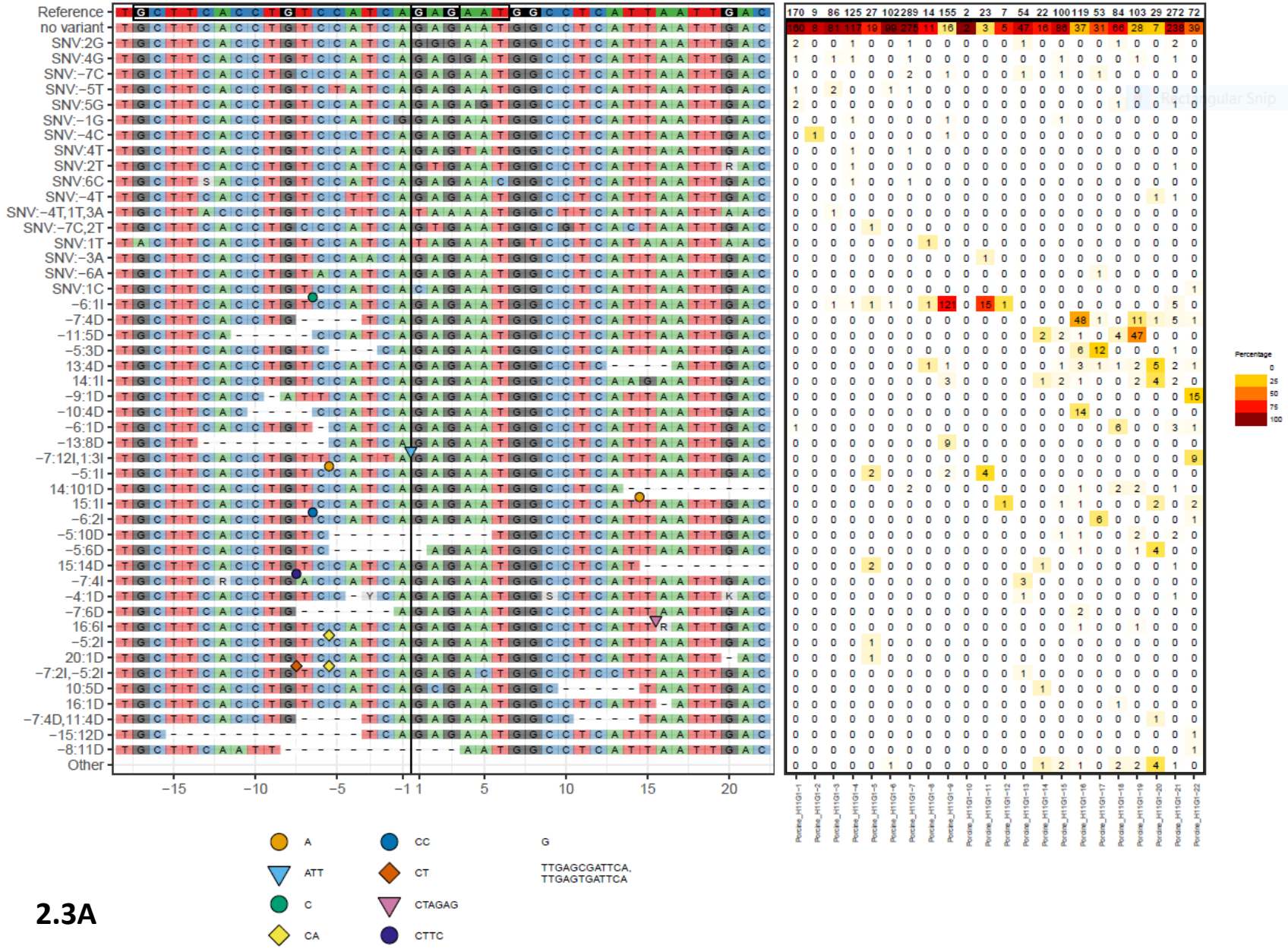


Figure 2.2. Graphical representation of the different allele variants are displayed for 40 electroporated ovine blastocysts. The majority of the ovine blastocysts contained more than two genetically distinct alleles, suggesting genetic mosaicism. X-axis displays each treated embryo. Y-axis displays allele variants detected.


Reference
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SNV:4G
SNV:−7C
SNV:−5T
SNV:5G
SNV:−1G
SNV:−4C
SNV:4T
SNV:2T
SNV:6C
SNV:−4T
SNV:−4T,1T,3A
SNV:−7C,2T
SNV:1T
SNV:−3A
SNV:−6A
SNV:1C
−6:1I
−7:4D
−11:5D
−5:3D
13:4D
14:1I
−9:1D
−10:4D
−6:1D
−13:8D
−7:12I,1:3I
−5:1I
14:101D
15:1I
−6:2I
−5:10D
−5:6D
15:14D
−7:4I
−4:1D
−7:6D
16:6I
−5:2I
20:1D
−7:2I,−5:2I
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16:1D
−7:4D,11:4D
−15:12D
−8:11D
Other
−15
−10
−5
−1 1
5
10
15
20
170 9 86 125 27 102 289 14 155 2 23 7 54 22 100 119 53 84 103 29 272 72
130 8 81 117 19 99 275 11 16 2 3 5 47 16 86 37 31 66 28 7 238 39
Percentage
0
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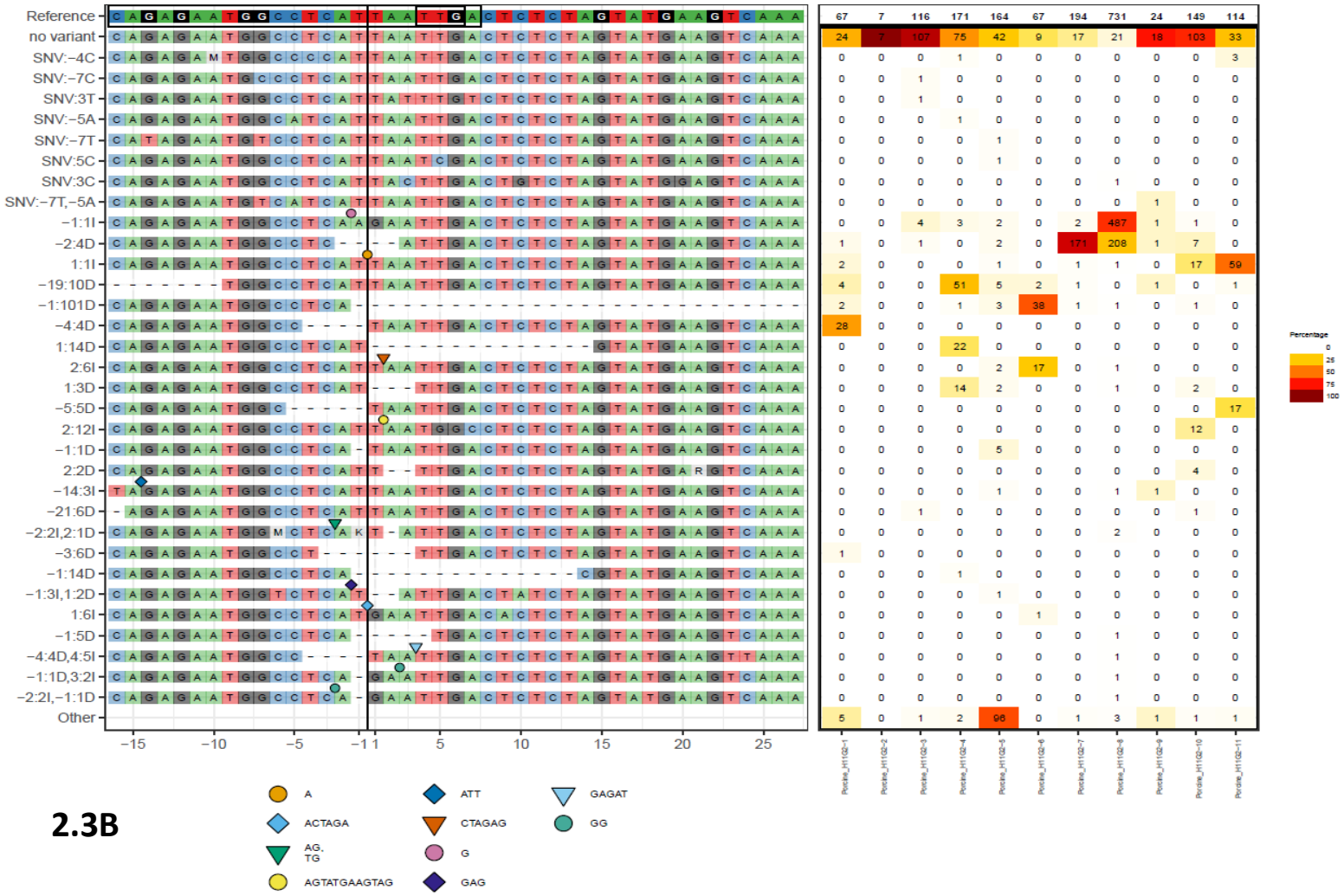
TTGAGTGATTCA

2.3A



2.3B

Figure 2.3. Graphical representation of the different allele variants are displayed for 33 electroporated porcine blastocyst. 2.3A). The first 22 blastocysts were electroporated with gRNA1, and 2.3B) eleven with gRNA2. It can be seen that the majority of blastocysts had a high percentage of wildtype sequence especially with gRNA1, suggesting these guides were not cutting efficiently. X-axis displays each treated embryo. Y-axis displays allele variants detected.

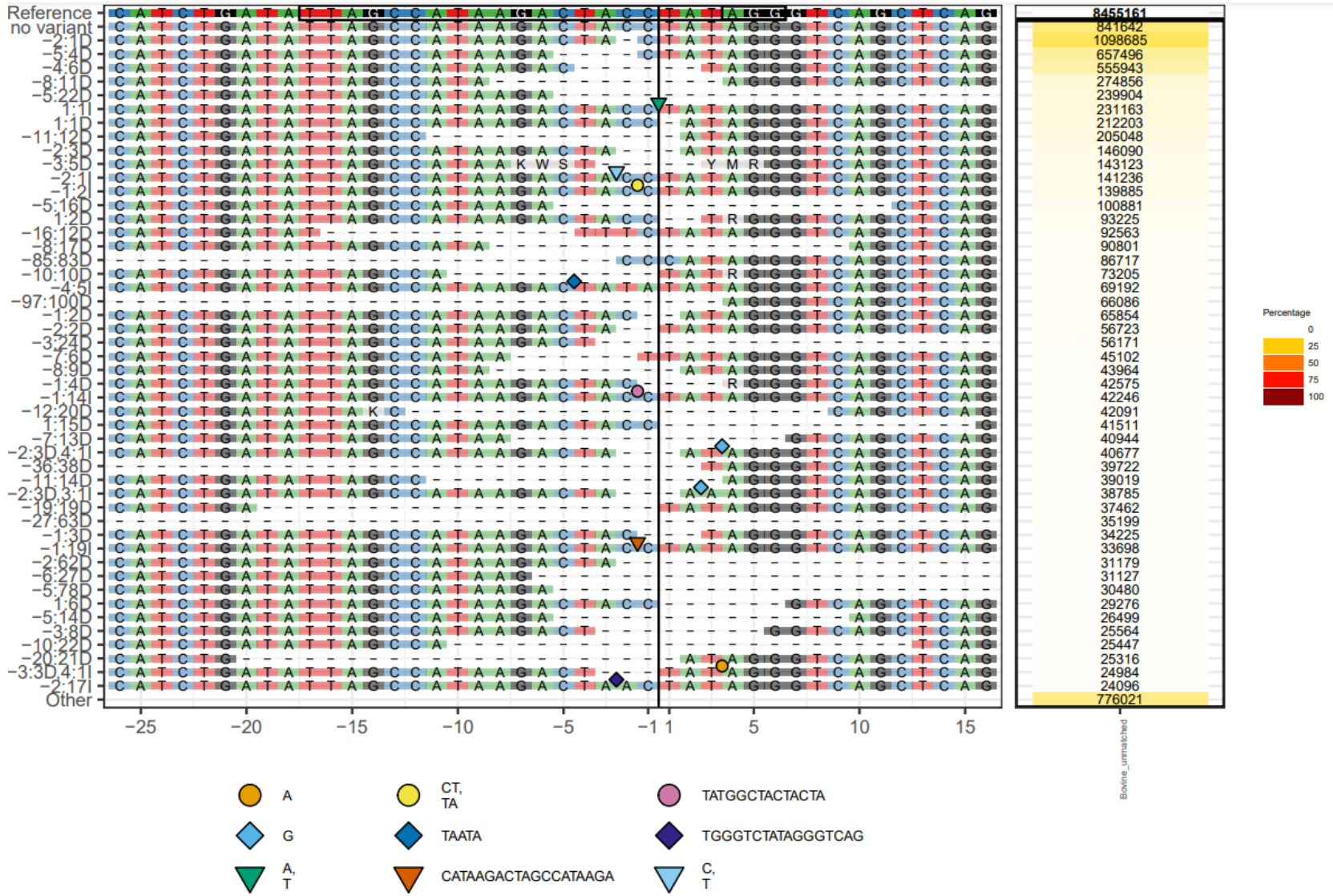


Figure 2.4. Graphical representation of the different allelic variants for unmatched electroporated bovine blastocysts. There were 10% of reads with wildtype sequence. The most common variants were deletions ranging from 1-22 bp around the target cut site.

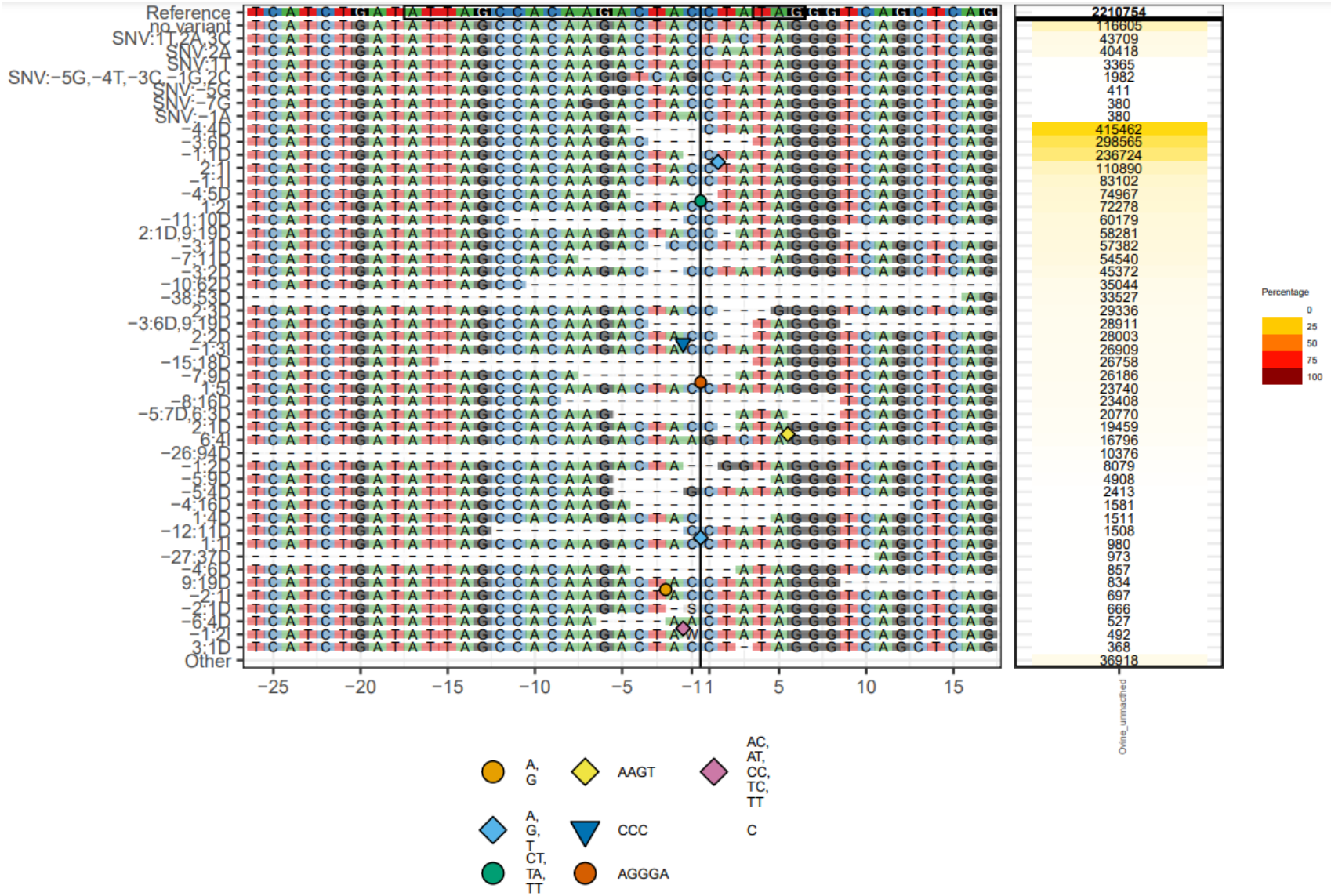


Figure 2.5. Graphical representation of the different allelic variants for unmatched electroporated ovine blastocysts. There were 5.3% of reads with wildtype sequence. The most common variants were small deletions around the target cut site.

Figure 2.6. Graphical representation of the different allelic variants for unmatched electroporated porcine blastocysts. There were 69.0% of reads with wildtype sequence. The most common variants were small insertions and deletions around the target cut site.

Discussion

In this bovine zygotes, high rates of mutation and acceptable embryo viability were observed when electroporating Cas9:gRNA RNP targeting the *H11* locus using three bipolar 3msec 20V/mm poring pulses. Bovine zygotes appear to be especially sensitive to high voltages; with 20 V/mm (3 pulses, 1 msec) resulting in lower blastocyst rates than 10 V/mm (Namula et al., 2019). Increasing the voltage strength to 45 V/mm (5 pulses, 3 msec) was associated with high rates of bovine zygote lysis suggesting damage to the cell membrane lipid bilayer (Wei et al., 2018). Similar results were also reported by Miao et al (2019), where pulses of 20, 25 and 30V/mm had an increasingly negative impact on bovine blastocyst development rates. Three bipolar 3msec 20V/mm poring pulses were also used to target the *Rosa26* locus which similarly yielded high mutation and acceptable development rates.

In sheep embryos, high rates of editing and good embryo viability were obtained using two 3.5 msec 40V/mm bipolar poring pulses. This is twice the poring voltage that was found to be optimal for bovine zygotes which could be influenced by the size of the zygote. Bovine oocytes and zygotes are larger (~150 μm diameter) than those of sheep and goat (~120 μm) (Catalá et al. 2011), which are in turn larger than those of rats and mice (~70 μm). Electroporation of rat and mouse zygotes has been shown to be efficient with high poring voltages around 40–50 V/mm, with acceptable development rates. It is known that membrane permeabilization can be achieved at lower voltages on larger cells as compared to what is required for smaller cells, which may play a role in embryo viability (Camargo et al., 2020).

More work is needed to optimize electroporation of Cas9:gRNA RNP into goat embryos. The parameters that were tested resulted in either a high mutation rate with a low blastocyst development rate, or conversely a low mutation rate with high blastocyst development rate. Both

outcomes unfortunately cannot become scalable approaches for producing gen-edited goats. To be successful, electroporation parameters for transfecting goat zygotes will need to balance both a high mutation rate and acceptable embryo viability.

NGS analysis of electroporated bovine, ovine, and porcine zygotes revealed high rates of mutation in bovine and ovine zygotes, suggesting that electroporation can be used as a high throughput approach to generating genome-edited livestock, however many embryos were found to contain more than two genetically distinct alleles suggesting mosaicism. This is likely due to nuclease activity after the first cell division, which can be caused by the delivery of editing reagents after the first cell division or prolonged nuclease activity after various cell divisions. Genetic mosaicism is not an issue when producing animals with short generational intervals such as mice since the unwanted alleles can be quickly bred out. Mosaicism within livestock species however poses an issue as long generational intervals make breeding unwanted alleles out at a large scale an unrealistic task. There were particularly low rates of editing efficiency seen in porcine blastocysts, especially with gRNA1, as compared to bovine and ovine blastocysts.

The unbarcoded blastocysts could not be analyzed for mutation rates and alleles individually, however they could still be analyzed as a group based on species. The failure of the attachment of DNA barcodes could be due to the overamplification of the first PCR amplification when using target specific primers, the presence of target specific primers in the second PCR amplification, or the failure of barcoding primers to anneal. The cattle and sheep unmatched sequences revealed similar results, with 90% or more of the reads containing mutations. This is in contrast to the porcine unmatched results which showed the majority of the reads as wild-type, and a number of alleles with a single base pair change which were likely sequence errors as these types of mutations were rarely seen in the cattle and sheep blastocysts.

Conclusions

There is a need to strike a balance between the mutation rate and maintaining embryo viability when optimizing electroporation conditions to introduce genome editing reagents into mammalian zygotes. In this study electroporation of bovine and ovine zygotes resulted in the efficient production of genome-edited blastocysts, however they were often mosaic. Further optimization of electroporation conditions and technologies to produce large targeted knock-ins will be needed to routinely obtain non-mosaic, genome-edited mammalian embryos at scale. Such developments will be required before genome editing can be seamlessly introduced into livestock genetic improvement programs.

Materials and Methods:

gRNA design. Guide-RNAs targeting the bovine *H11* locus (TAGCCATAAGACTACCTAT) were designed as described in Hennig et al. 2020 (Hennig *et al.* 2020). Guide-RNAs targeting the *Rosa26* locus in the bovine genome (TGTCGAGTCTCGATTATGGG) were designed as described in Yuan et al. 2021 (Yuan et al. 2021). Guide-RNAs targeting the *H11* locus in the porcine genome (gRNA1: GAGGCCATTCTCTGATGGAC) (gRNA2: TACTAGAGAGTCAATTAATG) and guide-RNAs targeting the *H11* locus in the ovine and caprine genome (TAGCCACAAGACTACCTAT) and *Bmpr2* (CAATTCAGAATGGAACGTAC) were designed using CHOPCHOP (Labun et al. 2019), with no less than 3 mismatches in the guide sequence for off-target sites and at least 1 mismatch in the seed region (8–11 bp upstream of the PAM sequence) when compared to the reference genome. Unmodified guides were then commercially synthesized (Synthego, Redwood City, CA, USA) and confirmed to cut in vivo by cytoplasmic microinjection of in vitro fertilized embryos with 6

pL of a solution containing 67 ng/μL of gRNA alongside 167 ng/μL of Cas9 protein (PNA Bio, Thousand Oaks, CA, USA) incubated at room temperature for 30 min prior to injection.

Embryo production. Bovine, ovine, caprine, and porcine ovaries were collected from a local slaughterhouse and transported to the laboratory in 38.5°C sterile saline. Upon arrival, cumulus-oocyte-complexes (COCs) were aspirated from follicles, washed and placed into 400μL of equilibrated BO-IVM medium (IVF Biosciences, Falmouth, United Kingdom). Bovine, ovine, and caprine COCs were incubated in BO-IVM media for 20 hours at 38.5 °C in a humidified 5% CO2 incubator. Porcine COCs were incubated in IVM-STD for 24 hours, then IVM2 (same as IVM-STD but without FSH and LH) for another 24 hours at 38.5 °C in a humidified 5% CO2 incubator. For bovine and ovine, groups of 25 matured COCs were then transferred into 50μL drops of SOF-IVF and incubated with 2×10^6 sperm per mL for 6 hours at 38.5 °C in a humidified 5% CO2 incubator for fertilization. After 6 hours of incubation with sperm, presumptive zygotes were denuded by vortex in SOF-HEPES for 5 minutes, electroporated, then cultured in BO-IVC medium (IVF Biosciences, Falmouth, United Kingdom) at 38.5 °C in a humidified atmosphere of 5% CO2, 5% O2, and 90% N2 for 7 days. For ovine and caprine parthenogenetic activation, matured COCs were denuded by vortex in SOF-HEPES for 3 minutes prior to activation. Denuded oocytes underwent parthenogenetic activation and were incubated in BO-IVC medium supplemented with 6-dimethylaminopurine (DMAP) for 4 hours. Oocytes were electroporated immediately following the 4-hour incubation then cultured in BO-IVC medium at 38.5 °C in a humidified atmosphere of 5% CO2, 5% O2, and 90% N2 for 7 days. For porcine IVF, groups of 20 COCs were placed in 90 μL drops of SOF-IVF medium and incubated with 2,000 spermatozoa per COC for 6 hours at 38.5 °C in a humidified 5% CO2 incubator. Presumptive zygotes were then incubated for 5 hours in

500 µL drops of PZM-5. After incubation, zygotes were denuded by vortex, electroporated, and cultured in the PZM-5 medium at 38.5 °C in a humidified atmosphere of 5% CO2 for 7 days.

Electroporation of bovine, ovine, and porcine zygotes and parthenogenetically activated ovine and caprine oocytes. Groups of 30-100 presumptive zygotes or activated oocytes were washed 3 times in Opti-MEM (Thermo Fisher Scientific, Waltham, MA, USA) solution and transferred into a 1mm electroporation cuvette (Bulldog Bio, Portsmouth, NH, USA) along with 20µL of electroporation solution containing sgRNA-Cas9 RNP complexes, and Opti-MEM. sgRNA and Cas9 protein concentrations were 100ng/µL and 200ng/µL respectively. Electroporation was performed using the Super Electroporator NEPA 21 (NEPA GENE Co. Ltd., Chiba, Japan) with various parameters as listed in tables. Following electroporation, presumptive bovine, ovine, and caprine zygotes were recovered and washed with SOF-HEPES then equilibrated BO-IVC and left to incubate in 400µL of BO-IVC medium at 38.5 °C in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 for 7 days. Presumptive porcine zygotes were recovered and washed, then left to incubate in the previously used 500µL drops of PZM-5 medium at 38.5 °C and 5% C for 7 days.

Analysis of targeted gene sequence. Blastocysts were collected and lysed in 10 µL of Epicenter DNA extraction buffer using a thermal cycler at 65 °C for 6 min, 98 °C for 2 min and then held at 4 °C. PCR primers were designed using Primer Blast (NCBI) to target each gRNA cut site (Table 2.4) and used for nested PCR to detect gene editing events in lysed blastocysts. The target DNA region for the *H11* locus in bovine was amplified through 2 rounds of PCR using primers H11F2 and H11R2. PCR was performed on a thermal cycler with 10 µL GoTaq Green Master Mix, 0.4 µL of each primer at 10 mM and 9.2 µL of DNA in lysis buffer for 5 min at 95 °C, 35 cycles of 30s at 95 °C, 30s at 59 °C, and 30s at 72 °C, followed by 5 min at 72 °C. The second round of

PCR was run with 10 µL GoTaq Green Master Mix, 4.2 µL of water, 0.4 µL of each primer at 10 mM and 5 µL of first round PCR with the same settings as the first round. The target DNA region for the *Rosa26* locus in bovine was amplified through 2 rounds of PCR using primers bRosa26F1 and bRosa26R1. PCR was performed under the same conditions as above. The target DNA region for the *H11* locus in ovine was amplified through nested PCR using primers oH11F1 and oH11R1 in the first round and ocH11F2 and ocH11R2 in the second round. PCR was performed with an annealing temperature of 58 °C, and 1 min extension, followed by a final 10 min extension. The second round of PCR used an annealing temperature of 57 °C, and 30s extension, followed by a final 10 min extension. The target DNA region for the *Bmpr2* locus in ovine was amplified through nested PCR using primers Bmpr2F1 and Bmpr2R1 in the first round and Bmpr2F2 and Bmpr2R2 in the second round. PCR was performed with an annealing temperature of 60 °C, and 1 min extension, followed by a final 10 min extension. The second round of PCR used an annealing temperature of 57 °C, and 30s extension, followed by a final 5 min extension. The target DNA region for the *H11* locus in caprine was amplified through nested PCR using primers cH11F1 and cH11R1 in the first round and ocH11F2 and ocH11R2 in the second round. PCR was performed with an annealing temperature of 58 °C, and 2 min extension, followed by a 10 min final extension. The second round of PCR used an annealing temperature of 57 °C, and 30s extension, followed by a final 5 min extension. All PCR products were stained with sybr safe (Thermo Fisher Scientific, Waltham, MA, USA) and visualized on a 1% agarose gel using a gel imager, purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and Sanger sequenced. Sequences were analyzed for mutations with Tracking of Indels by Decomposition (TIDE).

Evaluation of Genetic Mosaicism Electroporated bovine, ovine, and porcine blastocysts were collected, lysed, and underwent whole-genome amplification using the Repli-G Mini kit (Qiagen,

Inc., Valencia, CA, USA). Whole-genome amplified samples were used for PCR amplification of cut-sites using a dual round PCR approach described above to barcode each sample with a reduction from 35 to 5 cycles in the first round of PCR. Primers were designed to amplify each region using Primer3 with a 15 bp adapter sequence attached to the forward (AGATCTCTCGAGGTT) and reverse (GTAGTCGAATTCGTT) (Table 2.4). The second round of PCR amplified off the adapters adding an independent barcode for each sample to identify reads for pooled sequencing (Table 2.4). PCR samples underwent library preparation and were sequenced on an Illumina MiSeq600 sequencer by the UC Davis DNA Tech Core (Davis, CA, USA). Consensus sequences were called, reads sorted by barcode and BAM converted to individual FASTQ files. Reads were aligned to each target site using BWA v0.7.16a40. SAM files were converted to BAM files, sorted and indexed using SAMtools v1.941. Samples that could not be sorted by barcode were sorted based on alignment to species reference genomes. Number and types of alleles were determined for each sample using CrispRVariants v1.22.0.

Statistical Analysis. Mutation and blastocyst development outcomes for each electroporation parameter were analyzed using the generalized linear models logistic regression in R. Pairwise comparisons between different electroporation parameters were analyzed for statistical significance. Groups with ($P < 0.05$) were considered significantly different from each other.

Table 2.4. Sequence of primers used for PCR amplification of target region. For indexes and barcodes, index and barcode sequences are highlighted.

	Primer	Sequence	Species	Target	Product Size (bp)
PCR	H11F2	CCCCAGTGTTGTGCATGTAG	Bovine	H11	505
	H11R2	GTGAATGCCACTGCTGTGTT	Bovine	H11	
	oH11F1	CATGCTCAATCCACAAAGCCA	Ovine	H11	1105
	oH11R1	TGTCTTCACCAAAAGGTGGC	Ovine	H11	
	ocH11F2	TTGGACTGGGAGGAATGAAG	Ovine/Caprine	H11	628
	ocH11R2	GGGCTGTTTCTTTTGGTTGA	Ovine/Caprine	H11	
	cH11F1	GTGTCTTCACCAAAAGGTGGC	Caprine	H11	938
	cH11R1	TGGTCTCATTGTTTGAGCCTCT	Caprine	H11	
	bRosa26F1	GGGAGGTGCATGTTCTCCAA	Bovine	Rosa26	608
	bRosa26R1	TCTGTTTTGGCGGTGTAGCA	Bovine	Rosa26	
	Bmpr2F1	TGGCTCATGTGCTTAGTTGC	Ovine	Bmpr2	1891
	Bmpr2R1	GAACAAGGGCCCTCAAGAAT	Ovine	Bmpr2	
	Bmpr2F2	ACAGCAGAAGGACTTAGCCAT	Ovine	Bmpr2	1414
	Bmpr2R2	TGCTTTGAGTTTGGAATTGCAC	Ovine	Bmpr2	

Indexing	bH11Fb	AGATCTCTCGAGGTTCCCCAGTGTTGTGCATGTAG	Bovine	H11	505
	bH11Rb	GTAGTCGAATTCGTTGTGAATGCCACTGCTGTGTT	Bovine	H11	
	oH11Fb	AGATCTCTCGAGGTTATCGGAGCGGAGGAGTAAGA	Ovine	H11	473
	oH11Rb	GTAGTCGAATTCGTTTGAAGCGAATGGCACTGTTG	Ovine	H11	
	pH11Fb	AGATCTCTCGAGGTTCCATGCTCAATCCACAAAGCC	Porcine	H11	468
	pH11Rb	GTAGTCGAATTCGTTGTGTGTTGTATCTTACTCCTCAGC	Porcine	H11	
Barcode	BC1F	TCAGACGATGCGTCATAGATCTCTCGAGGTT			
	BC1R	TCAGACGATGCGTCATGTAGTCGAATTCGTT			
	BC17F	CATAGCGACTATCGTGAGATCTCTCGAGGTT			
	BC17R	CATAGCGACTATCGTGGTAGTCGAATTCGTT			
	BC29F	GCTCGACTGTGAGAGAAGATCTCTCGAGGTT			
	BC29R	GCTCGACTGTGAGAGAGTAGTCGAATTCGTT			
	BC34F	ACTCTCGCTCTGTAGAAGATCTCTCGAGGTT			
	BC34R	ACTCTCGCTCTGTAGAGTAGTCGAATTCGTT			
	BC38F	TGCTCGCAGTATCACAAGATCTCTCGAGGTT			
	BC38R	TGCTCGCAGTATCACAGTAGTCGAATTCGTT			

	BC40F	CAGTGAGAGCGCGATAAGATCTCTCGAGGTT			
	BC40R	CAGTGAGAGCGCGATAGTAGTCGAATTCGTT			
	BC48F	TCACACTCTAGAGCGAAGATCTCTCGAGGTT			
	BC48R	TCACACTCTAGAGCGAGTAGTCGAATTCGTT			
	BC52F	GCAGACTCTCACACGCAGATCTCTCGAGGTT			
	BC52R	GCAGACTCTCACACGCGTAGTCGAATTCGTT			
	BC54F	GTGTGAGATATATATCAGATCTCTCGAGGTT			
	BC54R	GTGTGAGATATATATCGTAGTCGAATTCGTT			
	BC62F	GACAGCATCTGCGCTCAGATCTCTCGAGGTT			
	BC62R	GACAGCATCTGCGCTCGTAGTCGAATTCGTT			
	BC70F	CTGCGCAGTACGTGCAAGATCTCTCGAGGTT			
	BC70R	CTGCGCAGTACGTGCAGTAGTCGAATTCGTT			
	BC9F	CTGCGTGCTCTACGACAGATCTCTCGAGGTT			
	BC9R	CTGCGTGCTCTACGACGTAGTCGAATTCGTT			
	bc1001F	CACATATCAGAGTGCGAGATCTCTCGAGGTT			
	bc1001R	CACATATCAGAGTGCGGTAGTCGAATTCGTT			

	Bc1002F	ACACACAGACTGTGAGAGATCTCTCGAGGTT			
	Bc1002R	ACACACAGACTGTGAGGTAGTCGAATTCGTT			
	Bc1003F	ACACATCTCGTGAGAGAGATCTCTCGAGGTT			
	Bc1003R	ACACATCTCGTGAGAGGTAGTCGAATTCGTT			
	Bc1004F	CACGCACACACGCGCGAGATCTCTCGAGGTT			
	Bc1004R	CACGCACACACGCGCGGTAGTCGAATTCGTT			
	Bc1006F	CATATATATCAGCTGTAGATCTCTCGAGGTT			
	Bc1006R	CATATATATCAGCTGTGTAGTCGAATTCGTT			
	Bc1007F	TCTGTATCTCTATGTGAGATCTCTCGAGGTT			
	Bc1007R	TCTGTATCTCTATGTGGTAGTCGAATTCGTT			
	Bc1008F	ACAGTCGAGCGCTGCGAGATCTCTCGAGGTT			
	Bc1008R	ACAGTCGAGCGCTGCGGTAGTCGAATTCGTT			
	Bc1009F	ACACACGCGAGACAGAAGATCTCTCGAGGTT			
	Bc1009R	ACACACGCGAGACAGAGTAGTCGAATTCGTT			
	Bc1010F	ACGCGCTATCTCAGAGAGATCTCTCGAGGTT			
	Bc1010R	ACGCGCTATCTCAGAGGTAGTCGAATTCGTT			

CHAPTER 3. Electroporation and adeno-associated virus mediated generation of 2.7 kb knock-in bovine blastocysts.

Abstract

Transfer of large (>1 kb) nucleic acid fragments into mammalian zygotes is hindered by the thick extracellular zona pellucida (ZP) glycoprotein layer. Due to this barrier, large homology directed repair (HDR) donor nucleic acid repair templates are unable to enter mammalian zygotes to produce large template knock-in embryos. A potentially scalable approach to deliver HDR templates of up to 4.9 kb into zygotes, without using cytoplasmic injection, is to use recombinant adeno-associated viruses (rAAVs). The objective of this project was to generate bovine blastocysts with a 2.7 kb knock-in at the *H11* locus using electroporation and rAAV transduction. A panel of six natural AAV serotypes packaged with a CMV-eGFP reporter (Charles River, Rockville, MD) was incubated with bovine zygotes to test transduction efficiency. Serotype AAV6 was identified to efficiently transduce the ZP of bovine zygotes. We constructed a 3.9 kb donor cassette including 600 bp *H11* homology arms with gRNA target sites at each end, the CAG promoter driving superfolder GFP with a nuclear localization signal, and AAV inverted terminal repeat arms. This cassette was packaged into serotype AAV6 and incubated with matured, denuded bovine oocytes at various concentrations for 6 hours during fertilization. Transduced zygotes were then electroporated to transfect Cas9 (100ng/μL)-single guide RNA (200ng/μL) ribonucleoprotein complexes targeting the *H11* locus. The highest knock-in rate was 38.1% (n=14), which was observed when using AAV6 at 8 x 10^{10} vgc. The blastocyst formation rate was 5.1% (n=209). The data observed here demonstrates that AAV6 transduction of DNA repair templates in addition to electroporation of CRISPR Cas9 reagents is a viable and scalable approach to producing large knock-in livestock embryos.

Introduction

Electroporation of genome editing reagents into murine and porcine zygotes for the production of edited animals has already been described (Peng et al. 2012; Remy et al. 2017; Nakagawa et al. 2018; Teixeira et al. 2018; Troder et al. 2018; Dumeau et al. 2019; Hirata et al. 2019a; Miao et al. 2019; Tanihara et al. 2019c; Hirata et al. 2020b; Tanihara et al. 2020a). There are also a few publications describing the electroporation of bovine embryos (Wei et al. 2018; Miao et al. 2019; Namula et al. 2019; Tanihara et al. 2019a; Camargo et al. 2020), and one unpublished Ph.D. dissertation describing the electroporation of ovine and caprine embryos (Mahdi 2021).

There are no papers reporting large (>1kb) targeted insertions in mammalian livestock embryos using electroporation alone. This may be in part due to the presence of the zona pellucida (ZP), a hard glycoprotein matrix surrounding zygotes which has been shown to impede the movement of large nucleic acid fragments into embryos (Romeo et al. 2020). This complicates the production of gene-edited animals harboring useful exogenous genes as homology directed repair (HDR) templates containing a gene and promoter often result in DNA cassettes that are larger than 1kb. Previous efforts to produce mammalian embryos harboring targeted insertions of more than 1 kb have required either the removal of the ZP, or microinjection of donor template prior to electroporation (Remy et al. 2017; Bagheri et al. 2018; Wei et al. 2018). Removal of the ZP prior to electroporation reduces development and requires a strict protocol that results in sticky and damaged embryos which become difficult to work with, while the microinjection of the donor template prior to electroporation defeats the purpose of using electroporation as a scalable and high-throughput approach to generating genome edited animals.

Recombinant adeno associated viruses (rAAV) have been employed to deliver nucleic acids to various cell types for many years. They are favored for their non-pathogenic and low immunogenic nature, ability to package either single stranded or self-complimentary DNA, and 4.9kb capacity to efficiently transduce mammalian cells (Nonnenmacher & Weber 2012; Naso et al. 2017; Chen *et al.* 2019). The genome of wild type adeno associated viruses contains only four genes (*rep, cap, aap, maap*) flanked by inverted terminal repeats (ITRs) on both sides. The *rep* gene is required for viral genome replication and packaging, the *cap* gene produces viral capsids, the *aap* gene promotes capsid assembly, and the *maap* gene helps facilitate viral replication (Naso et al. 2017; Galibert et al. 2021). Conversely, rAAV does not contain these genes and only requires the presence of 130bp AAV ITR arms flanking a DNA fragment of up to 4.9kb on either side for packaging (Aponte-Ubillus et al. 2018). The ITRs are the only cis-acting components necessary for the packaging and replication of DNA fragments (Hacker et al. 2020). Gene therapy vectors using AAV can infect both dividing and quiescent cells and persist in an extrachromosomal state without integrating into the genome of the host cell. These qualities make rAAV an ideal vector to transduce embryos to deliver HDR templates for producing targeted knock-ins (KI).

rAAV has been used to successfully transduce DNA fragments into fertilized rat and mouse zygotes in the absence of ZP treatment prior to electroporation (Mizuno et al. 2018; Chen et al. 2019; Romeo et al. 2020; Oikawa et al. 2022a). The protocols for utilizing rAAV and electroporation to generate KI embryos have proven to be high throughput and easy to use, however such methods have not been utilized in livestock species. To date, the largest donor cassette used to produce a targeted insertion with rAAV and electroporation was a 4.3kb template in mice (Chen et al. 2019). There are currently no reports on the production of KI mammalian livestock embryos with the use of electroporation and rAAV.

The aim of this study was to generate bovine blastocysts harboring a 2.7kb green fluorescent protein (GFP) reporter gene KI at the *H11* locus using electroporation and rAAV infection. First, a panel of eight natural rAAV serotypes (1, 2, 5, 6, 8, and 9) packaged with a CMV-eGFP reporter (Charles River, Rockville, MD, USA) at various concentrations were tested for transduction efficiency into early bovine embryos. Then a 3.9kb HDR template was packaged into the most efficient rAAV serotype and incubated with bovine oocytes and sperm for 6 hours prior to electroporation with Cas9:gRNA RNP, to produce bovine blastocysts harboring a 2.7kb targeted insertion at the *H11* locus.

Results

rAAV serotype optimization

rAAV serotypes 1, 2, 5, 6, 8, and 9 were tested for transduction efficiency in bovine zygotes during fertilization. Based on the methods by Chen et al. (2019), matured oocytes were incubated for six hours with sperm as well as various concentrations of rAAV for the delivery of a CMV-eGFP reporter plasmid. Serotype 6 at a concentrations of 10^{10} viral genome copies (vgc) and 10^{11} vgc showed efficient transduction as evidenced by both GFP expression (Figure 3.1. 3.2), and PCR amplification of the CMV-eGFP reporter. Approximately 38% (n = 32) for 10^{10} vgc and 33% (n = 27) for 10^{11} vgc of the treated zygotes expressed bright fluorescence and the CMV-eGFP reporter plasmid was present in all 27 embryos at 10^{11} vgc as confirmed by PCR amplification. All other serotypes tested did not result in expression of GFP, but they did result in variable levels of PCR amplification of the GFP reporter plasmid. It was not possible to differentiate whether the GFP DNA that was amplified by PCR was present inside the embryo or outside of the ZP. Based on these results, rAAV6 was used for further experiments.

Targeted insertion of GFP

To produce bovine blastocysts containing large template knock-in using rAAV6 and electroporation, a 3.9kb HDR donor template containing 600bp *H11* homology arms with gRNA target sites at the ends, the CAG promoter, GFP gene with a nuclear localization signal, and rAAV2 ITR arms was packaged into rAAV6 (Figure 3.3).

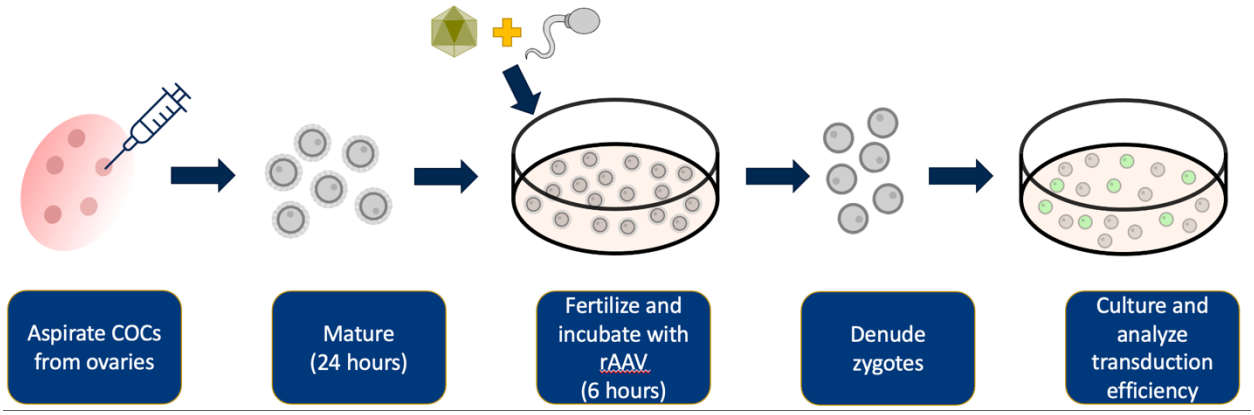


Figure 3.1. Workflow testing rAAV serotypes (1, 2, 5, 6, 8, and 9) packaged with a CMV-eGFP reporter (Charles River, Rockville, MD) for transduction efficiency of bovine zygotes at various concentrations.

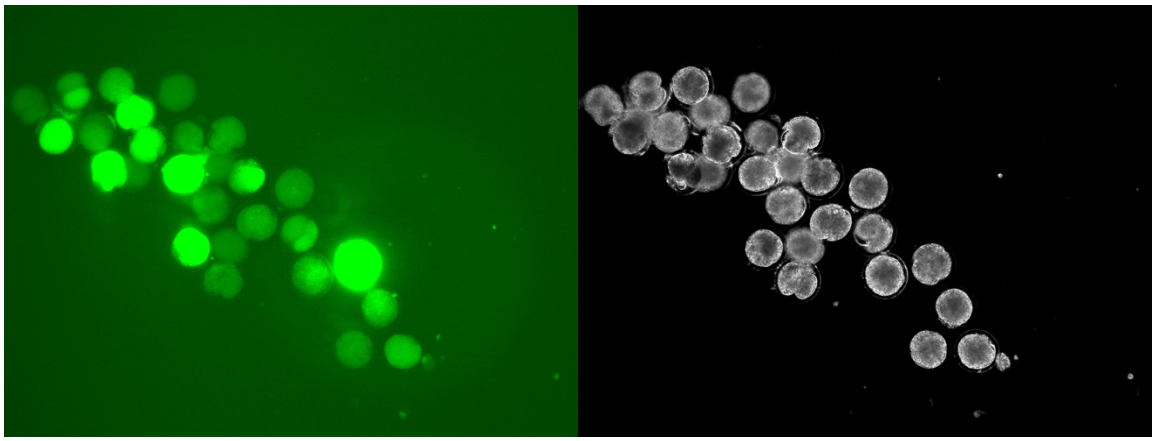


Figure 3.2. Image of bovine blastocysts after transduction with rAAV6 serotype reported plasmid at a concentrations of 10^{10} viral genome copies (vgc). Fluorescent image using FITC and transluscent filter.

Figure 3.3. Donor template for gene KI. The 3.9kb HDR donor template contained 600bp *H11* homology arms (blue) with gRNA target sites at the ends (orange), the CAG promoter (grey), GFP gene with a nuclear localization signal (light green) and rAAV2 ITR arms (dark green).

Initially, matured cumulus cell-oocyte complexes (COCs) were incubated with rAAV6 containing the HDR donor template at different concentrations, and sperm for 6 hours prior to denuding and electroporating with Cas9:gRNA RNP targeting the H11 locus. However, no blastocysts expressing GFP were observed (Figure 3.4, 3.5). Additionally, PCR amplification of blastocyst DNA using primers targeting the 5' and 3' junction of the targeted insertion did not result in a product, and primers amplifying the bovine *H11* locus target site produced wild-type sized amplicons. It was observed that cumulus cells that remained after the post-fertilization denuding were expressing GFP (Figure 3.4), suggesting that the cumulus cells surrounding the oocytes during incubation with rAAV6 were being transduced. To improve the transduction into the oocytes instead of the cumulus cells, an approach that required denuding the cumulus cells from the oocytes prior to incubation with rAAV6 and sperm was trialed (Figure 3.6). Additionally, and importantly, ~5 undenuded COCs for each 25 denuded oocytes were added to each drop to provide the environment to help ensure acceptable fertilization and subsequent blastocyst development rates.

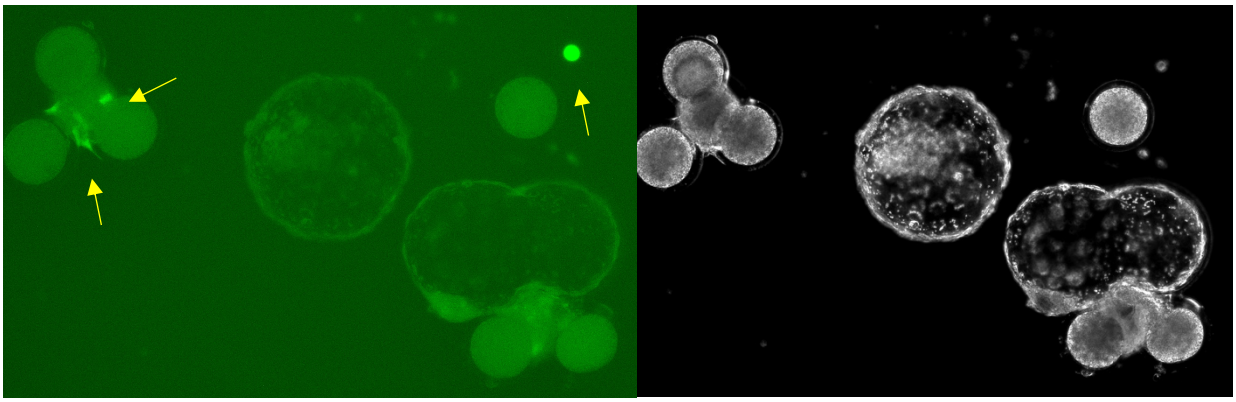


Figure 3.4. Bovine blastocysts that underwent rAAV6 incubation and electroporation imaged on day 7 post-fertilization using a FITC and translucent filter. Blastocyst did not express GFP however cumulus cells that remained in culture expressed GFP.

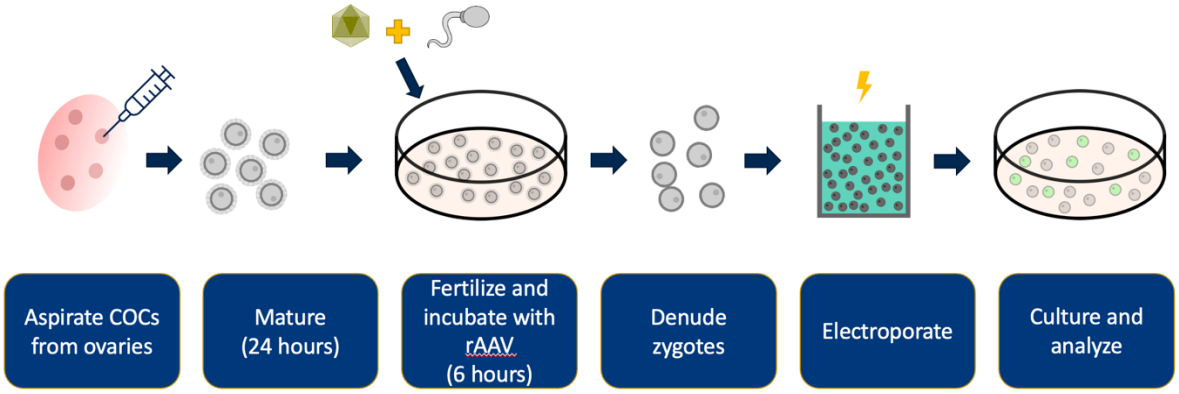


Figure 3.5. Initial workflow to attempt to produce transduced, transfected and edited bovine blastocysts.

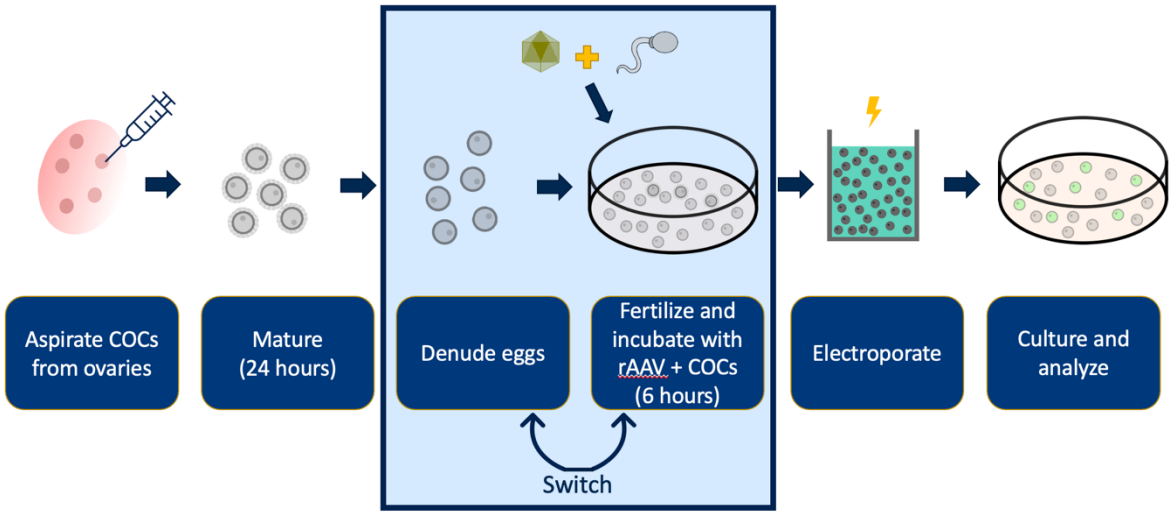


Figure 3.6. Modified workflow to successfully produce transduced, transfected and edited bovine blastocysts.

GFP-expressing blastocysts were successfully produced with this approach as confirmed by fluorescent imaging. Incubation of denuded oocytes with rAAV6 at concentrations of 7 x 10^{10} vgc, 8 x 10^{10} vgc, 9 x 10^{10} vgc, and 10^{11} vgc produced GFP expressing blastocysts (Figure 3.7). There were no GFP expressing blastocysts produced at concentrations below 5 x 10^{10} vgc, or above 3 x 10^{11} vgc (Table 3.1). PCR amplification of the right and left junctions of the targeted KI and Sanger sequencing of the resulting PCR amplicons confirmed a targeted KI of the donor template (Figure 3.8). Knock-in rates for treated embryos were observed between 23% and 38% for AAV6 concentrations between 7 x 10^{10} vgc and 10^{11} vgc (Table 3.1). Primers targeting the bovine genome outside of the targeted gene insertion (bH11WTF2, bH11WTR2) were also used in a subset of the GFP expressing blastocysts to identify whether wild-type sized sequence remained (Table 3.2). Wild-type sized alleles were identified in 100% (n=5) of the samples analyzed, suggesting that the fluorescent blastocysts were mosaic containing both non-KI *H11* sequence, and the targeted 2.7 kb GFP knock-in. Approximately 60% of the wild-type sized *H11* sequence contained small indels indicating cutting at the target site in addition to the targeted knock-in, and 40% contained only wild-type DNA sequence in addition to the targeted knock-in.

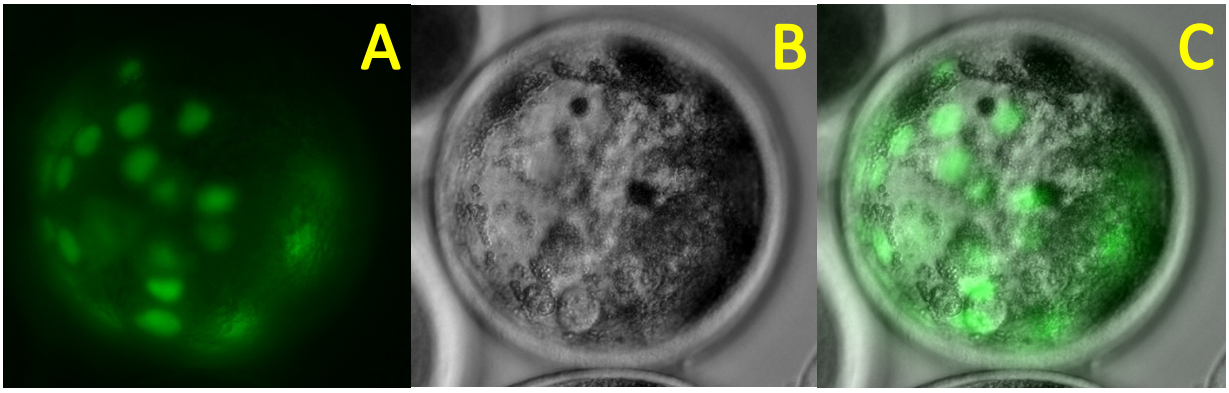


Figure 3.7. Give a title of the figure here. A) GFP expressing bovine blastocyst that underwent rAAV6 incubation and electroporation imaged on day 7 post-fertilization using a FITC filter. B) Same blastocyst imaged using translucent. C) Same blastocyst with FITC and translucent images overlaid.

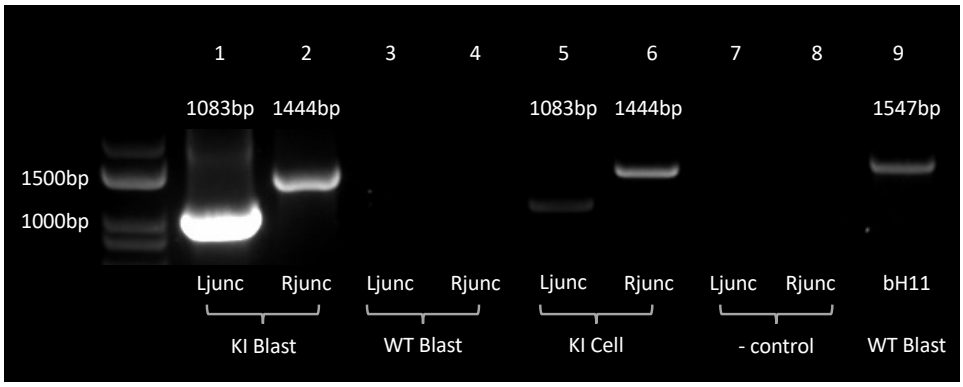


Figure 3.8. Genotyping of blastocysts. PCR genotyping of a treated GFP expressing blastocyst (lanes 1-2), untreated wild type blastocyst (lanes 3-4), treated granulosa cell DNA (lanes 5-6), water (lanes 7-8), and untreated wild-type blastocyst (lane 9). Lanes 1-8 were PCR amplified using primers flanking the 5' (left, Ljunc) and 3' (right, Rjunc) junctions of the targeted knock-in. Junction primers should only amplify genomic DNA so unintegrated vector DNA would not be amplified. The treated GFP blastocyst and treated cell DNA had confirmed targeted knock-ins as seen in lanes 1-2 and 5-6. The wild-type blastocyst and water as seen in lanes 3-4 and 7-8 did not harbor the targeted knock-ins, as expected. Lane 9 was PCR amplified using primers bH11WTF2, bH11WTR2 targeting the bovine genome outside of the HDR template shows the wild-type *H11* sized amplicon as expected.

Table 3.1: rAAV6 concentration in relation to blastocyst development and targeted knock-in rates

rAAV6 Concentration	Embryos Treated	Blastocysts	Green Blastocysts	PCR knock-in	Blastocysts/ Embryos Treated	Knock-in/ Blastocysts	Control Blastocyst Rate	Treated/ Untreated Blastocyst development ratio
7×10^{10}	81	7	3	2	8.6%	28.6%	42.1%	0.20
8×10^{10}	412	21	8	8	5.1%	38.1%	39.0%	0.13
9×10^{10}	222	19	5	5	8.6%	26.3%	38.7%	0.22
1×10^{11}	351	13	3	3	3.7%	23.1%	42.8%	0.09

Discussion

Genome editing technologies offer an approach to introduce targeted genetic alterations in livestock genomes to augment traditional selective breeding approaches (Bishop & Van Eenennaam 2020). Livestock embryos harboring large targeted knock-ins have been produced by SCNT and MI, however the efficiency of SCNT cloning in large livestock species is low and perinatal abnormalities are common, and MI is still a time-consuming procedure that requires expensive equipment and a high level of skill as operators must manipulate each embryo individually. These approaches are therefore unscalable and inaccessible for laboratories without specialized equipment or personnel (Keefer 2015; McFarlane et al. 2019).

Serotype rAAV6 was found to transduce DNA fragments into zygotes without treatment to weaken the ZP, in agreement with murine studies (Mizuno et al. 2018; Chen et al. 2019; Romeo et al. 2020). Additionally, rAAV transduction in combination with electroporation of editing reagents into zygotes was sufficient for the generation of large targeted KI bovine blastocysts. The ability of rAAV to package DNA fragments of up to 4.9kb and transduce various cell types while being non-pathogenic makes it an attractive vector for delivering HDR templates into early-stage embryos. It significantly lowers the technical barrier and conceptually reduces the amount of specialized equipment required for producing large KI animals. However, it should be noted that denuding the oocytes prior to incubation with rAAV6 and sperm was necessary to successfully produce targeted knock-ins. When rAAV was incubated with non-denuded oocytes during fertilization, the cumulus cells were transduced by rAAV apparently reducing the amount of HDR template introduced into the oocyte. As a result, cumulus cells expressed GFP, but no GPF-expressing blastocysts were observed.

To circumvent transduction of the cumulus cells, they were removed by denuding the COCs prior to rAAV incubation. Unfortunately, this negatively impacted embryo development as denuding prior to incubation with sperm has been shown to significantly decrease fertilization and embryo development (Zhang et al. 1995). To improve the embryo development of denuded oocytes, 5 COCs were added to each drop of 20 denuded oocytes to provide factors secreted by cumulus cells (Owen et al. 2020). Rates of embryonic development to the blastocyst stage were significantly decreased in cumulus-denuded oocyte. The blastocyst development rate for denuded, transduced embryos was 3.7-8.6% (Table 1) as compared to 39% for control embryos.

Targeted knock-ins were confirmed by PCR and Sanger sequencing. However, when looking at the fluorescent images of knocked-in blastocysts, it was observed that they were not uniformly green (Figure 3.7). To test whether the cause of uneven GFP expression was due to mosaicism, the presence of wild-type sized *H11* alleles in a subset of the GFP expressing blastocysts was examined using PCR. Wild-type sized alleles were observed in all (n=5) of the GFP expressing blastocysts analyzed, suggesting that the blastocysts were mosaic. Of the various concentrations tested, 8 x 10^{10} vgc resulted in the most efficient KI rate of 38.1%. Further optimization would be needed to optimize the large KI approach to improve blastocyst development rates and reduce mosaicism.

Conclusions

A protocol to quickly and easily produce bovine blastocysts harboring a 2.7kb targeted knock-in using rAAV6 transduction of a 4.9 kb template combined with electroporation of gRNA/Cas9 RNP was developed in this study. With this described approach, there was no need to remove or weaken the ZP and of the blastocysts that developed, a knock-in rate of 38.1% was observed. However, this was only achieved after denuding the oocytes prior to transduction to

ensure rAAV transduced the oocytes rather than the more numerous cumulus cells surrounding the oocyte. Further optimization to improve embryo viability will be necessary before this approach can be used at scale.

Materials and Methods

gRNA design. Guide-RNAs targeting the *H11* locus (TAGCCATAAGACTACCTAT) were designed as described in Hennig et al. 2020 (Hennig et al. 2020). The guide was then commercially synthesized (Synthego, Redwood City, CA, USA) and confirmed to cut in vivo by cytoplasmic microinjection of in vitro fertilized embryos with 6 pL of a solution containing 67 ng/μL of gRNA alongside 167 ng/μL of Cas9 protein (PNA Bio, Thousand Oaks, CA, USA) incubated at room temperature for 30 min prior to injection.

Embryo production. Bovine ovaries were collected from a local slaughterhouse and transported to the laboratory in 38.5°C sterile saline. Upon arrival, cumulus-oocyte-complexes (COCs) were aspirated from follicles and washed and placed into 400μL of equilibrated BO-IVM medium (IVF Biosciences, Falmouth, United Kingdom). COCs were incubated in BO-IVM media for 20 hours at 38.5 °C in a humidified 5% CO2 incubator. Groups of 25 matured COCs were then transferred into 50μL drops of SOF-IVF and incubated with 2×10^6 sperm per mL for 6 hours at 38.5 °C in a humidified 5% CO2 incubator for fertilization. After 6 hours of incubation with sperm, presumptive zygotes were denuded by vortex in SOF-HEPES for 5 minutes and cultured in BO-IVC medium (IVF Biosciences, Falmouth, United Kingdom) at 38.5°C in a humidified atmosphere of 5% CO2, 5% O2, and 90% N2 for 7 days.

Electroporation of bovine zygotes. Groups of 30-100 presumptive zygotes were washed 3 times in Opti-MEM (Thermo Fisher Scientific, Waltham, MA, USA) solution and transferred into a 1mm electroporation cuvette (Bulldog Bio, Portsmouth, NH, USA) along with 20μL of electroporation

solution containing sgRNA-Cas9 RNP complexes, and Opti-MEM. sgRNA and Cas9 protein concentrations were 100ng/μL and 200ng/μL respectively. Electroporation was performed using the Super Electroporator NEPA 21 (NEPA GENE Co. Ltd., Chiba, Japan) using 20V, 3 bipolar pulses, 3.5msec pulse length, 50msec intervals, 0% decay rate. Following electroporation, presumptive zygotes were recovered and washed with SOF-HEPES then equilibrated BO-IVC and left to incubate in 400μL of BO-IVC medium at 38.5 °C in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 for 7 days.

rAAV serotype optimization. Oocytes were collected and matured as described above. Groups of 25 matured COCs were transferred into 50μL drops of SOF-IVF to be incubated with sperm and various concentrations of rAAV serotypes 1, 2, 5, 6, 8, and 9 containing a CMV-eGFP reporter plasmid (Figure 3.9, Charles River, Rockville, MD, USA) for 6 hours at 38.5 °C in a humidified 5% CO2 incubator. Presumptive zygotes were then denuded and cultured for 7 days as described above. Embryos were imaged under a fluorescent microscope with a FITC filter throughout the culturing process to identify transduction efficiency. Embryos were collected on day 7 and lysed in 10 μL of Epicenter DNA extraction buffer (Lucigen, Teddington, United Kingdom) using a thermal cycler at 65 °C for 6 min, 98 °C for 2 min and then held at 4 °C. Polymerase chain reaction (PCR) using primers aavGFPF, aavGFPR (Figure 3.9) targeting the reporter plasmid developed using Primer Blast (NCBI) was performed on a thermal cycler with 10 μL GoTaq Green Master Mix (Promega, Madison WI, USA), 4.2 μL of water, 0.4 μL of each primer at 10 mM and 5 μL of DNA in lysis buffer for 5 min at 95 °C, 35 cycles of 30s at 95 °C, 30s at 60 °C, and 30s at 72 °C, followed by 5 min at 72 °C. The second round of PCR was run with 10 μL GoTaq Green Master Mix, 4.2 μL of water, 0.4 μL of each primer at 10 mM and 5 μL of first round PCR with the same settings as the first round.

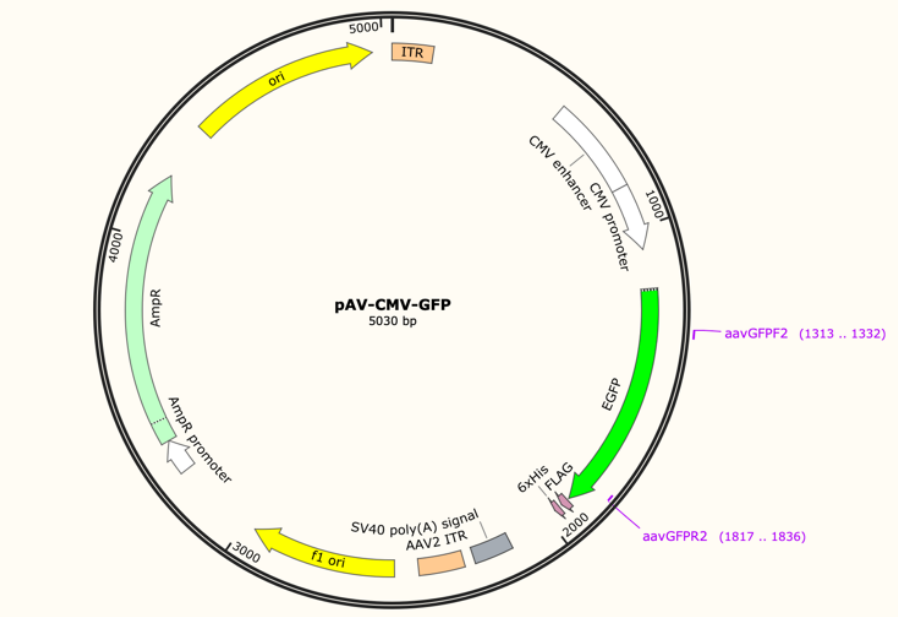


Figure 3.9. CMV-eGFP reporter plasmid (Charles River, Rockville, MD).

rAAV6 transduction with matured COCs. Oocytes were collected and matured as described above. Groups of 25 matured COCs were transferred into 50µL drops of SOF-IVF to be incubated with sperm and various concentrations of rAAV6 containing our HDR template for 6 hours at 38.5 °C in a humidified 5% CO2 incubator. Presumptive zygotes then immediately underwent electroporation as described above.

rAAV6 transduction with denuded oocytes. Oocytes were collected and matured as described above. Matured COCs were then denuded by vortex in SOF-HEPES for 5 minutes and groups of 20 denuded eggs and 5 COCs were transferred into 50µL drops of SOF-IVF to be incubated with sperm and various concentrations of rAAV6 containing our HDR template for 6 hours at 38.5 °C in a humidified 5% CO2 incubator. Presumptive zygotes then immediately underwent electroporation as described above.

Analysis of targeted gene sequence. Resulting blastocysts were analyzed under a fluorescent microscope with a FITC filter to identify GFP expression. Blastocysts were then collected and

lysed in 10 µL of Epicenter DNA extraction buffer using a thermal cycler at 65 °C for 6 min, 98 °C for 2 min and then held at 4 °C. PCR primers were designed using Primer Blast (NCBI) to target each gRNA cut site. The target region was amplified through 2 rounds of PCR using primers flanking the 5' (left) junction and 3' (right) junction of the targeted insert (Figure 3.10, Table 3.2). PCR was performed on a thermal cycler with 10 µL GoTaq Green Master Mix, 4.2 µL of water, 0.4 µL of each primer at 10 mM and 5 µL of DNA in lysis buffer for 5 min at 95 °C, 35 cycles of 30s at 95 °C, 30s at 59 °C, and 3 min at 72 °C, followed by 10 min at 72 °C. The second round of PCR was run with 10 µL GoTaq Green Master Mix, 4.2 µL of water, 0.4 µL of each primer at 10 mM and 5 µL of first round PCR with the same settings as the first round. Wild type alleles were amplified with the same mix for 5 min at 95 °C, 35 cycles of 30s at 95 °C, 30s at 59 °C, and 4 min at 72 °C, followed by 10 min at 72 °C. Like the junction PCR, the second round of PCR was run with 10 µL GoTaq Green Master Mix, 4.2 µL of water, 0.4 µL of each primer at 10 mM and 5 µL of first round PCR with the same settings as the first round. PCR products were stained with sybr safe (Thermo Fisher Scientific, Waltham, MA, USA) and visualized on a 1% agarose gel using a gel imager, purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and Sanger sequenced. The blastocyst DNA was then analyzed for integration of the donor template with DNA sequence alignment using Snapgene (Dotmatics, San Diego, CA, USA).

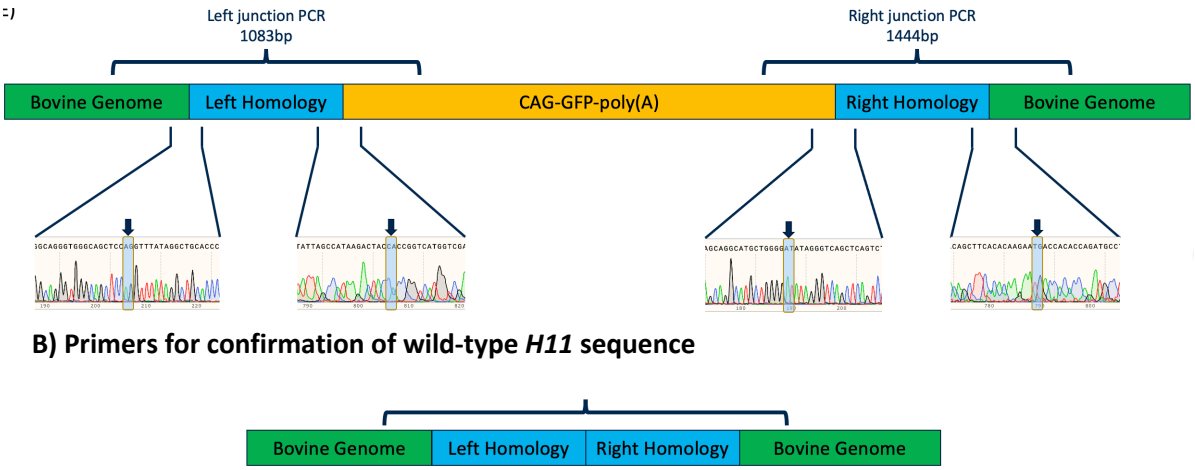


Figure 3.10. Design of PCR primers. A) If there is a KI, left junction primers (bH11LjuncF2, bH11LjuncR2) result in a 1083bp amplicon and right junction primers (bH11RjuncF2, bH11RjuncR2) result in a 1444bp amplicon. Sanger sequencing for both the left and right junctions confirmed the presence of a targeted knock-in. Chromatograms for a GFP expressing bovine blastocyst at the left junction and right junction are shown. Arrows point to the junction of the bovine genome and homology arm, and the homology arm and KI insert. B) Conversely, wild-type genotype at the *H11* locus in the bovine genome using primers bH11WTF2/bH11WTR2 result in a 1547bp amplicon.

Table 3.2. Sequence of primers used for PCR amplification of the bovine H11 region and HDR template.

	Region	Primer	Sequence	Target	Product Size
Knock-in	H11	bH11LjuncF2	TGCCACTGTTGCTTGAGACT	5' Junction	1083
	H11	bH11LjuncR2	CCAAGTGGGCAGTTTACCGT		
	H11	bH11RjuncF2	TGCTGGGATTACACATGGCA	3' Junction	1444
	H11	bH11RjuncR2	AAGCACGGCCTAGTGGAGAA		
Wild type	H11	bH11WTF2	AGGCAGACCTCATGCTCAAT	H11	1547
	H11	bH11WTR2	CTCCATGCCCACCAAAGTCA		
Insert	Donor Template	aavGFPF	ATGGTAATCGTGCGAGAGGG	GFP	560
	Donor Template	aavGFPR	GGCCACGGAACAGGTAGTTT		

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