GENOME EDITING APPLICATIONS IN ANIMAL RESEARCH

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GENOME EDITING APPLICATIONS IN ANIMAL RESEARCH

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ORIGINAL RESEARCH
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Efficient One-Step Knockout by Electroporation of Ribonucleoproteins Into Zona-Intact Bovine Embryos

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Somatic cell nuclear transfer or cytoplasm microinjection have been used to generate genome-edited farm animals; however, these methods have several drawbacks that reduce their efficiency. This study aimed to develop electroporation conditions that allow delivery of CRISPR/Cas9 system to bovine zygotes for efficient gene knock-out. We optimized electroporation conditions to deliver Cas9:sgRNA ribonucleoproteins to bovine zygotes without compromising embryo development. Higher electroporation pulse voltage resulted in increased membrane permeability; however, voltages above 15 V/mm decreased embryo developmental potential. The zona pellucida of bovine embryos was not a barrier to efficient RNP electroporation. Using parameters optimized for maximal membrane permeability while maintaining developmental competence we achieved high rates of gene editing when targeting bovine OCT4, which resulted in absence of OCT4 protein in 100% of the evaluated embryos and the expected arrest of embryonic development at the morula stage. In conclusion, Cas9:sgRNA ribonucleoproteins can be delivered efficiently by electroporation to zona-intact bovine zygotes, resulting in efficient gene knockouts.

Keywords: embryo, genome editing, CRISPR, Cas9, OCT4 gene

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INTRODUCTION

The rapid advance of CRISPR/Cas9 technology has enabled the efficient generation of gene edited animals by one-step embryo manipulation (Wang et al., 2013). The CRISPR/Cas9 system, consists of a complex formed by Cas9 endonuclease, which cuts the target DNA site creating a double-strand break (DSB) and single guide RNA (sgRNA) which interacts with Cas9 and provides target recognition by simple Watson-Crick sequence complementarity (Jinek et al., 2012). In the presence of the NGG protospacer motif upstream of the sgRNA recognition sequence, SpCas9 introduces a DSB at the specific genomic location. DSBs are typically repaired by cells or embryos using one of two repair mechanisms: non-homologous end-joining (NHEJ) or homologous-directed repair (HDR). NHEJ can sometimes be error prone, often introducing insertion or deletion (indel) mutations in the repaired region, which if resulting in a frame-shift mutation at a protein coding region can effectively generate a loss-of-function mutation or gene knock-out (KO). HDR uses a homologous region of DNA to repair the DSB with high fidelity, which offers the opportunity of

providing the cells with an artificial nucleic acid repair template for introducing a specific mutation, which can range from a single SNP up to introduction of a whole gene (Cong et al., 2013).

Gene editing technologies can find applications ranging from basic research to gene therapy (Doudna and Charpentier, 2014; Knott and Doudna, 2018). In livestock, gene editing could be used to generate genetically engineered animals to synthetize recombinant pharmaceutical drugs (Oishi et al., 2018), or organ donors for xenotransplantation (Niemann and Petersen, 2016; Cowan et al., 2019). Moreover, genome editing can be utilized to increase disease resistance (Burkard et al., 2017), or the frequency of alleles or polymorphisms associated to favorable traits (Jenko et al., 2015; Hickey et al., 2016; Tait-Burkard et al., 2018) such as heat tolerance, milk and/or meat production/composition.

In order to generate genome-edited animals, gene editing systems has been used to edit the genome of somatic donor cells which have then been used to produce live animals through somatic cell nuclear transfer (SCNT). However, this approach has limitations due to the low efficiency of SCNT for generating healthy cloned animals (Akagi et al., 2014; Vajta, 2018). The CRISPR/Cas9 system has also been delivered to *in vitro*-fertilized zygotes by cytoplasmic or pronuclear microinjection, avoiding the issues associated with SCNT. While the efficiency of producing live animals using this approach is higher than SCNT, embryo manipulation requires special skills and expensive equipment, as well as being laborious and time-consuming. Moreover, CRISPR/Cas9 microinjection of zygotes frequently results in genetic mosaicism, which has been reported in several species (Mianné et al., 2017; Lamas-Toranzo et al., 2018), including rabbits (Wan et al., 2019), mice (Yen et al., 2014; Horii and Hatada, 2017), pigs (Sato et al., 2015), and cattle (Bevacqua et al., 2016).

An alternative to cytoplasmic microinjection is zygote electroporation. Electroporation has been shown to deliver genome editing reagents, including Cas9:sgRNA ribonucleoproteins (RNP), to mouse, rat and pig zygotes with reasonable efficiency (Kaneko, 2017; Teixeira et al., 2018; Hirata et al., 2019). Recent reports demonstrated that electroporation could be used to deliver RNP into bovine zygotes; however, this came at the cost of compromised embryo development resulting in a decreased blastocyst rate (Miao et al., 2019; Namula et al., 2019). In this study, we aimed to optimize electroporation conditions to deliver Cas9:sgRNA RNPs to bovine zygotes to introduce gene silencing mutations and to evaluate the resulting embryonic phenotype.

MATERIALS AND METHODS

Experimental Design

This study was composed of five complementary optimization experiments. The first experiment evaluated the effect of increasing voltages (0, 10, 15, 20, 25, and 30 V) on permeability of bovine zygotes to 3 kDa tetramethylrhodamine-labeled dextran (Thermo Fischer Scientific, Walthan, United States). The second experiment evaluated the effect of voltages (0, 15, and 20 V) on embryo development. Zygotes were electroporated in OptiMEM (Thermo Fischer Scientific) and cleavage and blastocyst rates were evaluated. The third experiment assessed the effect of electroporation (15 V) with two different RNPs concentrations (2.15 μM = 100:50 ng/μL and 4.3 μM = 200:100 ng/μL Cas9:sgRNA; 1:2.5 molar ratio) on embryo development and mutation rate. For this experiment, sgRNA targeting the zinc finger protein X-linked (*ZFX*) gene were used. The fourth experiment evaluated the effect of zona drilling (laser ablation of small points of the zona pellucida) before electroporation with RNPs (200:100 ng/μL Cas9:sgRNA *ZFX*) on embryo development and mutation rate. The fifth experiment evaluated the efficiency of the optimized RNP electroporation protocol by targeting an embryo specific gene (octamer-binding transcription factor 4; *OCT4*, a.k.a. POU class 5 homeobox) that allows for phenotypic assessment of the induced mutations. This experiment included three groups: control, representing embryos not subjected to electroporation; Electroporated controls, embryos electroporated with RNPs targeting a gene not required for development (stearoyl-CoA desaturase; *SCD1*); and OCT4-KO, embryos electroporated with RNPs targeting exon 2 of *OCT4* (a gene required for expanded blastocyst formation). In both electroporation groups, the RNP concentration was 200:100 ng/μL Cas9:sgRNA. Cleavage and blastocyst rates were recorded for each group. Embryo genotyping was performed in day 6 morulas. Embryos (32 or more cells) at day 6 and day 8, 144, and 192 post fertilization (hpf), respectively, were fixed and immunostained to evaluate the presence of OCT4 protein. Experiments 1–4 were carried out with parthenogenetic embryos, whereas experiment 5 was carried out with *in vitro*-fertilized embryos.

Single Guide RNAs (sgRNAs)

Single guide RNAs were designed to target *ZFX* (5′- TCTTACAAGGGTGATAGTAC), *SCD1* (5′- CTGACTTACC CGCAGCTCCC) and *OCT4* (5′- GATCACACTAGGATATAC CC) genes. These sgRNA were produced by *in vitro* transcription (*ZFX*) using the AmpliScribe T7-Flash Transcription kit (Lucigen, Palo Alto, CA) and purified using the MEGAclear Transcription Clean-Up kit (Thermo Fischer Scientific, Chicago, IL), or by Synthego Corporation, Redwood City, United States (*SCD1* and *OCT4*).

Oocytes Recovery, *in vitro* Maturation (IVM), Parthenogenesis and *in vitro* Fertilization (IVF)

Ovaries were obtained from a commercial cattle slaughterhouse (Cargill, Fresno, United States) and transported to the laboratory in saline solution at 34–36°C. Follicles with 3–8 mm diameter were aspirated and cumulus-cell oocytes (COC) complexes with homogeneous cytoplasm and compact layers of cumulus cells were selected. IVM was performed for 21–22 h in BO-IVM medium (IVF Bioscience, Fallmouth, United Kingdom) at 38.5°C, 5% CO_2 and humidified air. Parthenogenetic activation for experiments 1–4 was induced in denuded oocytes by 5 μM ionomycin (Sigma Aldrich, Saint Louis, United States)

incubation during 4 min at 38.5°C in air followed by 2 mM 6-(Dimethylamino) purine (6-DMAP; Sigma) for 4 h at 38.5°C, 5% CO_2 in atmospheric air. *In vitro* fertilization was performed by incubating COCs with 1×10^6 spermatozoa/mL in BO-IVF medium (IVF Bioscience) for 17–18 h at 38.5°C, 5% CO_2 in humidified air.

Laser Zona Drilling

Presumptive zygotes were denuded of cumulus cells by vortexing for 3 min and placed in a warmed 20 μL drop of SOF Hepes medium under mineral oil and "zona drilling" was performed using an inverted microscope equipped with laser system (Saturn Laser System, Research Instruments Ltd., Cornwall, United Kingdom). The zona pellucida was ablated at two points using pulses of laser beam set to 0.5–0.6 ms in order to make holes with ∼16 μm diameter. Afterward, zygotes were washed twice in SOF Hepes medium before undergoing electroporation.

Electroporation and Embryo Culture

Electroporation of denuded presumptive zygotes was performed using the Nepa21 electroporator system (Nepagene, Chiba, Japan) and a glass slide with 1 mm gap between electrodes (BEX, Japan). Embryos were electroporated following activation or *in vitro* fertilization. Poring pulses were set to different initial voltage (0, 10, 15, 20, 25, or 30 V/mm, accordingly to the experiment), always including 6 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, 5 pulses of 50 ms at 50 ms interval with 40% decay rate and positive/negative polarity (**Figure 1A**). RNPs solution with 200:100 ng/μL Cas9:sgRNA was prepared with 4.8 μL Cas9 protein (PNA Bio, Thousand Oaks, United States) stock solution (500 ng/μL) and 6 μl sgRNA stock solution (200 ng/μL) plus 1.8 μL OptiMEM to have a final work solution with 12 μL containing 200 ng/μL Cas9 protein + 100 ng/μL sgRNA. That solution was diluted with plus 12 μL OptiMEM to make the 100:50 ng/μL RNP solution. RNP solution was mixed and kept on ice for 5–10 min before using for electroporation. Electrode gap was filled with 3–4 μL and checked the impedance. Oocytes were washed three times in OptiMEM and once in RNP solution before electroporation. Pools of 30–40 zygotes were placed in line between the electrodes using a mouth-pipette and electroporated at room temperature. Afterward, zygotes were collected and washed three times in SOF Hepes followed by two times in BO-IVC medium (IVF Bioscience) and then cultured in BO-IVC medium at 38.5°C, 5% CO_2, 5% O_2, and 90% N_2 in humidified air. Supplementation with fetal bovine serum (2.5%) was performed at 72 h post activation/IVF when cleavage rate was recorded. Blastocyst rate was recorded at 168–192 h post activation/IVF.

Analysis of Zygotes Permeability to Dextran

For experiment 1, parthenogenetic zygotes were electroporated with 2 mg/mL of tetramethylrhodamine-labeled dextran diluted in DPBS and presence of the dye in the cytoplasm was evaluated by epi-fluorescence microcopy 20–30 min after electroporation. Corrected total cell fluorescence (CTCF) was calculated and means compared among treatment groups.

Embryo Lysis and Sequencing

Single embryos were collected at morula (Experiment 5) or blastocyst (Experiments 3–4) stage and lysed in 10 μL lysis buffer (Lucigen, Palo Alto, CA, United States) at 65°C for 6 min and 98°C for 2 min. PCR reactions were performed in two rounds with 35 cycles each. First PCR was composed of 9.2 μL embryo lysis and 10 μL Master Mix (GoTaq Hot Start Green Master Mix, Promega, Madison, United States) at 0.8 μL of 10 μM primers (**Table 1**) in DNAse/RNAse free water. Second round of PCR was composed of 5 μL from first PCR, 4.2 μL of water, 10 μL Master Mix and 0.8 μL of 10 μM primers in DNAse/RNAse free water. PCR conditions included one cycle at 95°C for 3 min followed by 35 cycles of 95°C for 30 s, primer annealing temperature for 30 s (*ZFX*: 60°C; *OCT4*: 54°C) and elongation at 72°C for 30 s, and then 1 cycle at 72°C for 5 min. PCR products were run in a 1% agarose gel and bands were extracted and purified (Qiaquick Gel extraction kit, Qiagen, Hilden, Germany) for Sanger sequencing. Sequencing was performed by services provided by Genewiz (South Plainfield, NJ, United States). Mutations were analyzed by ICE CRISPR Analysis Tool (Synthego) and multiple sequence alignment (SNAPGene, GSL Biotech LLC, Chicago, United States). Indel rate was calculated based on the proportion of embryos with insertions/deletions vs. embryos sequenced.

Embryo Immunostaining

Embryos with 32 or more cells at 144 h post IVF were fixed in 4% paraformaldehyde and permeabilized with 1% Triton X-100 in PBS. Samples were blocked with 1% BSA and 10% normal donkey serum in DPBS and incubated overnight with goat anti-OCT4 primary antibody (1:300; OCT3/4 antibody, Santa Cruz Biotechnology, Santa Cruz, United States). After extensive washing, embryos were incubated for 1 h with anti-goat IgG Alexa 568 secondary antibody (1:500; Invitrogen, United States) and 20 min with 10 μg/mL Hoechst 33342. Samples were observed using an epi-fluorescence microscope (Revolve, Echo, San Diego, United States). Number of cells per embryo showing expression Hoechst and/or Alexa 568 fluorescence was recorded and means compared between treatments.

Statistical Analysis

Each experiment was independently repeated at least three times. The number of embryos analyzed for each experiment is provided in **Supplementary Table 1**. Developmental data, CTCF, number of total cells and cells expressing OCT4, were analyzed by analyses of variance and means compared by Tukey's test. Results are shown as mean ± S.E.M. Proportion of embryos with indels were analyzed by Chi-square. Differences were considered significant at the 95% confidence level ($P < 0.05$).

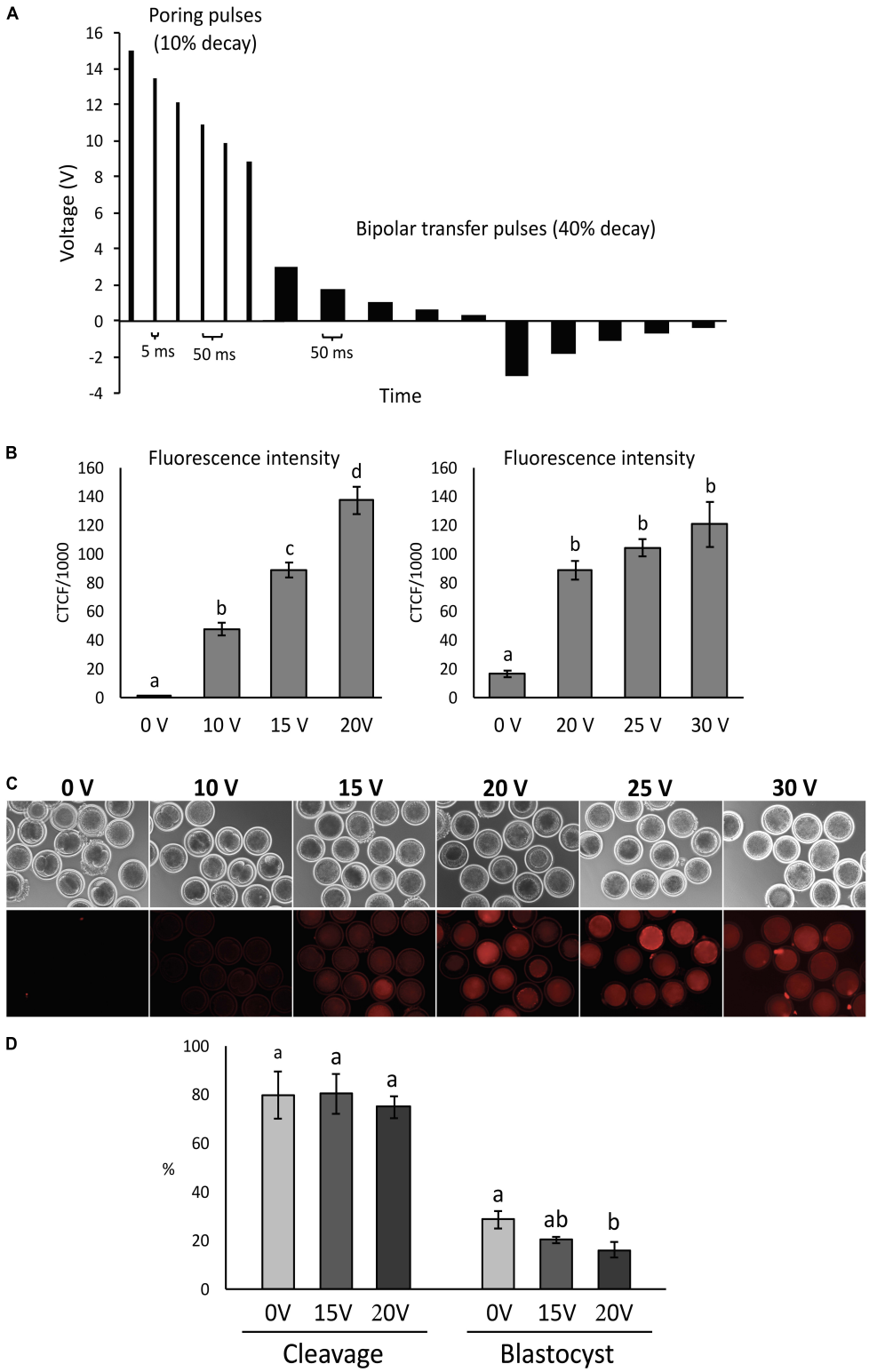


FIGURE 1 | Condition optimization for efficient electroporation of bovine zygotes. **(A)** Diagram depicting the electroporation settings used in the study. **(B)** Fluorescence intensity of parthenogenetic bovine zygotes after electroporation in the presence of tetramethylrhodamine-labeled dextran (n = 54 zygotes). $^{a\text{-}d}$Different letters indicate statistically significant differences ($P < 0.001$). CTCF, corrected total cell fluorescence. **(C)** Representative images of zygotes after electroporation at different voltages. **(D)** Development of parthenogenetic zygotes after sham electroporation at different initial voltages. ^{a,b}Different letters indicate statistically significant Intensity of differences ($P < 0.05$). Experiment replicated four times. Sample size per group: 0 V = 114 zygotes; 15 V = 89 zygotes; and 20 V = 84 zygotes.

TABLE 1 | PCR primer sequences spanning the *OCT4* and *ZFX* sgRNA target sites.

Gene symbol	Primer sequence (5′–3′)	Fragment size (bp)	Gene ID
OCT4	F-AGAGGGGGTGAGGTGGATAG	854	282316
	R-CCAGTATCAGGGGGACAATG		
ZFX	F-AGCAGTGCTTCCAAACTTGAG	520	280961
	R-GATGAGAGCTTATGTAACTGTTGG		

RESULTS

Experiment 1

Zygotes were electroporated with tetramethylrhodamine-labeled dextran and fluorescence intensity was measured to assess the effect of electroporation voltage on membrane permeability. Comparisons were performed at 0, 10, 15, and 20 V, followed by 0, 20, 25, and 30 V.

Fluorescence increased ($P < 0.001$) with increasing voltage up to 20 V, but there was no difference ($P > 0.05$) from 20 to 30 V (**Figures 1B,C**).

Experiment 2

Parthenogenetic zygotes were electroporated with 0, 15, and 20 V in OptiMEM medium only and cleavage and blastocyst rate were compared. There was no significant effect of voltage ($P > 0.05$) on cleavage rates. Blastocyst rates were similar between embryos electroporated at 15 V compared to 0 V controls (28.5 ± 3.6% and 20.2 ± 1.3%, respectively), but were significantly reduced in embryos exposed to 20 V (16.2 ± 3.2%) compared to controls ($P < 0.05$; **Figure 1D**).

Experiment 3

Embryo development and indel rate were evaluated when electroporation at 15 V was performed using two different Cas9:sgRNA RNPs concentrations (100:50 and 200:100 ng/μL of Cas9:sgRNA). The *ZFX* gene was targeted using a previously validated sgRNA (data not shown). There was no effect on cleavage or blastocyst rates between the Cas9:sgRNA concentrations evaluated, nor there was any differences in the achieved indel rate (**Figure 2A**). **Figures 2B,C** show ICE analysis of sequencing data of a representative embryo displaying the insertion of one nucleotide.

Experiment 4

Zygotes were electroporated with 15 V using 200:100 ng/μL of Cas9:sgRNA RNPs targeting the *ZFX* gene in intact and zona-drilled zygotes (**Figure 3A**). No differences in blastocyst rate or CRISPR-induced indel rates were observed between electroporated embryo groups ($P > 0.05$; **Figure 3B**), with electroporated embryo groups presenting similar developmental rates to controls ($P > 0.05$; **Figure 3**).

Experiment 5

Finally, we evaluated the efficiency of CRISPR/Cas9 RNPs electroporation (15 V with 200:100 ng/μL of Cas9:sgRNA) for inducing a loss-of-function mutation to a gene required for blastocyst formation (*OCT4*), thus allowing phenotypic assessment during *in vitro* culture. For this purpose, we used a sgRNA that was previously reported to efficiently knockout bovine *OCT4* after zygote cytoplasm microinjection (Daigneault et al., 2018). A non-electroporated and an electroporated control group, with RNPs targeting a gene not related to early embryo development (*SCD1*), was included in each experiment.

Electroporation with RNPs targeting *OCT4* (KO-OCT4 group) did not affect cleavage rate ($P > 0.05$) but significantly decreased the proportion of morulas at 144 hpf ($P < 0.05$) and blastocysts at 192 hpf ($P < 0.01$; **Figure 4A**). Indeed, only one blastocyst was found in the KO-OCT4 group at 192 hpf from a total of 87 embryos evaluated. There was no effect of control electroporation on cleavage or blastocyst formation ($P > 0.05$; **Figure 4A**).

Of 13 KO-OCT4 morulas evaluated, 12 (92.3%) presented indel mutations, with most of the mutated embryos (11/12) having biallelic mutations (**Figure 4B**). The other mutated morula was considered mosaic based on chromatogram analysis of PCR products. Sequence alignment showed that deletions were more frequent than insertions and ranged from 2 to 450 nucleotides (**Figure 4C**).

No significant difference between total cell number in morulas collected at 144 hpf was observed between control, control electroporation and KO-OCT4 groups ($P > 0.05$; **Figures 5A,B**). OCT4 immunostaining was negative in all morulas evaluated from the KO-OCT4 group (**Figures 5A,B**), whereas controls were OCT4 positive with a similar number of OCT4 positive cells ($P > 0.05$) between control groups (**Figures 5A,B**). The single blastocyst found in the KO-OCT4 group was at an early stage, with only 76 cells and expression of OCT4 was absent (**Supplementary Figure 1**), in contrast to control and control electroporated embryos that averaged 101 ± 8.6 and 102 ± 8.7 cells, respectively, and all expressed OCT4 (**Supplementary Figure 1**).

DISCUSSION

We report an optimized electroporation condition that allowed highly efficient gene KO, as demonstrated by embryo genotyping, lack of gene product, and expected developmental phenotype (embryonic arrest). To limit the detrimental effect of electroporation on embryo development, voltage had to be kept at 15 V/mm, which was sufficient to achieve high membrane permeabilization and efficient delivery of CRISPR/Cas9 RNPs.

Using a 3 kDa tetramethylrhodamine-labeled dextran, we determined effective conditions for membrane permeabilization,

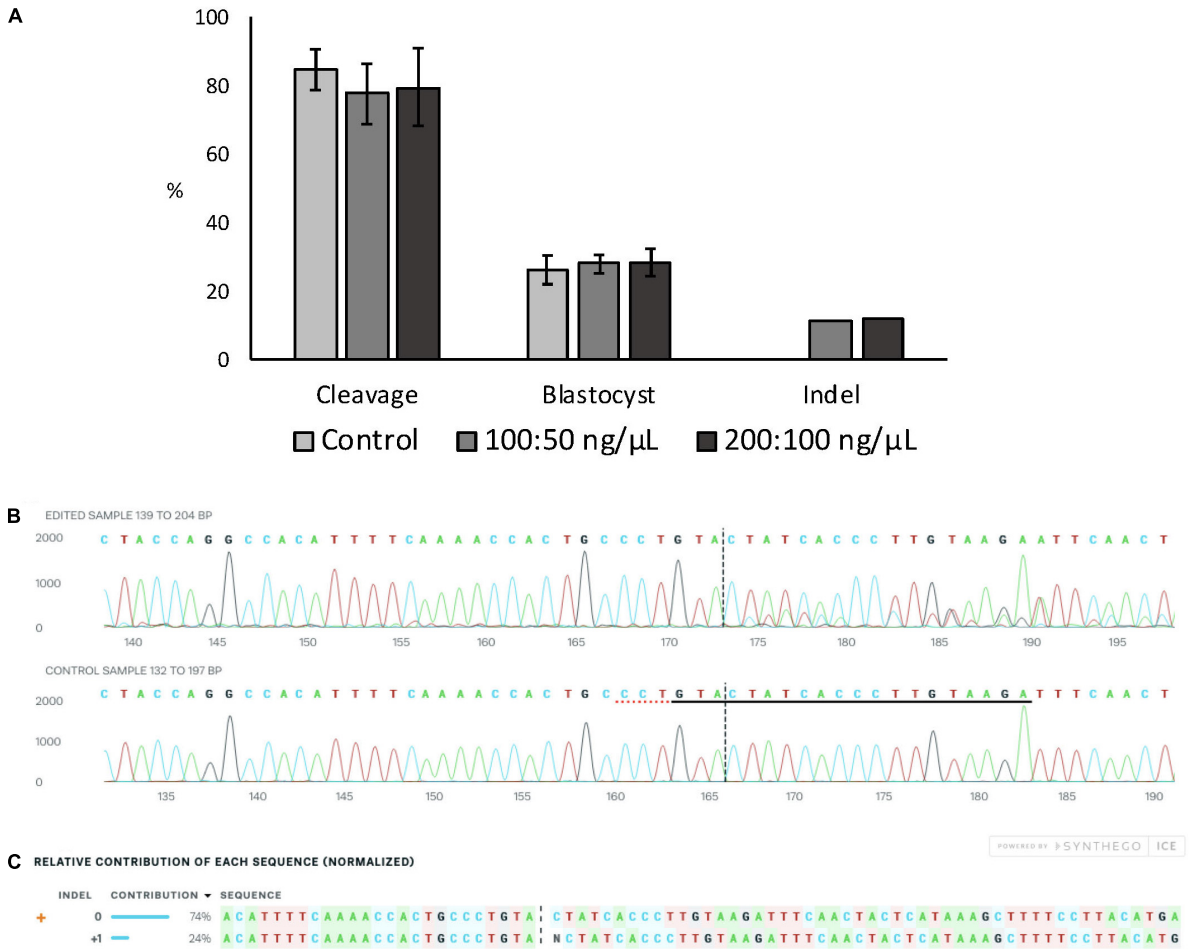


FIGURE 2 | Effect of Cas9:sgRNA concentration on electroporation efficiency of bovine zygotes. **(A)** Cleavage and blastocyst rates (based on number of presumptive zygotes cultured) and indel rate (based on number of blastocysts sequenced) after electroporation of bovine zygotes with RNPs targeting the ZFX gene. No difference between groups was observed ($P > 0.05$). The experiment was replicated three times. Sample size per group: control (no electroporation) = 112 zygotes; 100:50 ng/µL = 103 zygotes, and 200:100 ng/µL = 101 zygotes. Blastocysts sequenced: 100:50 ng/µL = 27; 200:100 ng/µL = 25. **(B)** Trace file provided by ICE software of a representative blastocysts electroporated with 15 V and 200:100 ng/µL Cas9:sgRNA. The sgRNA *ZFX* sequence is underlined in black and the PAM sequence is denoted by a dotted red underline in the control sample. **(C)** Relative contribution of each sequence identified by ICE in the same representative embryo. The insertion of one nucleotide was derived from one sequence with a contribution of 24%. Expected Cas9 cut site is shown by black vertical dotted lines in **(B,C)** figures.

as had previously been done in rat embryos (Kobayashi et al., 2018). We found that voltage as low as 10 V allows delivery of dextran, with membrane permeation to the dye increasing up until 20 V, without further improvement with higher voltage levels. While 20 V pulses maximized membrane permeabilization, this voltage level impaired bovine embryo development to the blastocyst stage. Similar results were previously reported, where pulses of 20, 25, and 30 V resulted in lower bovine blastocyst development (Miao et al., 2019). Under our conditions, 15 V, which achieved significant membrane permeabilization, did not affect embryo development and was chosen as optimal voltage for electroporation. A recent study also observed that 15 V was the highest voltage at which bovine embryos could be electroporated without affecting development to blastocyst stage (Namula et al., 2019).

Electroporation of rat and mouse zygotes has been shown to be effective with 40–50 V (Kaneko, 2017; Kobayashi et al., 2018; Teixeira et al., 2018), which is higher than the 15 V used in bovine zygotes. It has been shown that the size of a cell is an important parameter influencing electroporation (Agarwal et al., 2007). Reversible membrane permeabilization on larger cells can be achieved at lower voltages than what is required for smaller cells (Kandušer et al., 2006). Bovine oocytes and zygotes are larger (∼120 µm diameter) (Fair et al., 1997) than those of rats and mice (∼70 µm) (de Wolff-Exalto and Groen-Klevant, 1980; Eppig, 1996), suggesting that lower voltages could be effective for bovine embryos, as found in our study.

Our electroporation conditions use a series of high-voltage (HV) pulses followed by a series of low-voltage (LV) pulses with polarity inversion (poring and transfer pulses, respectively).

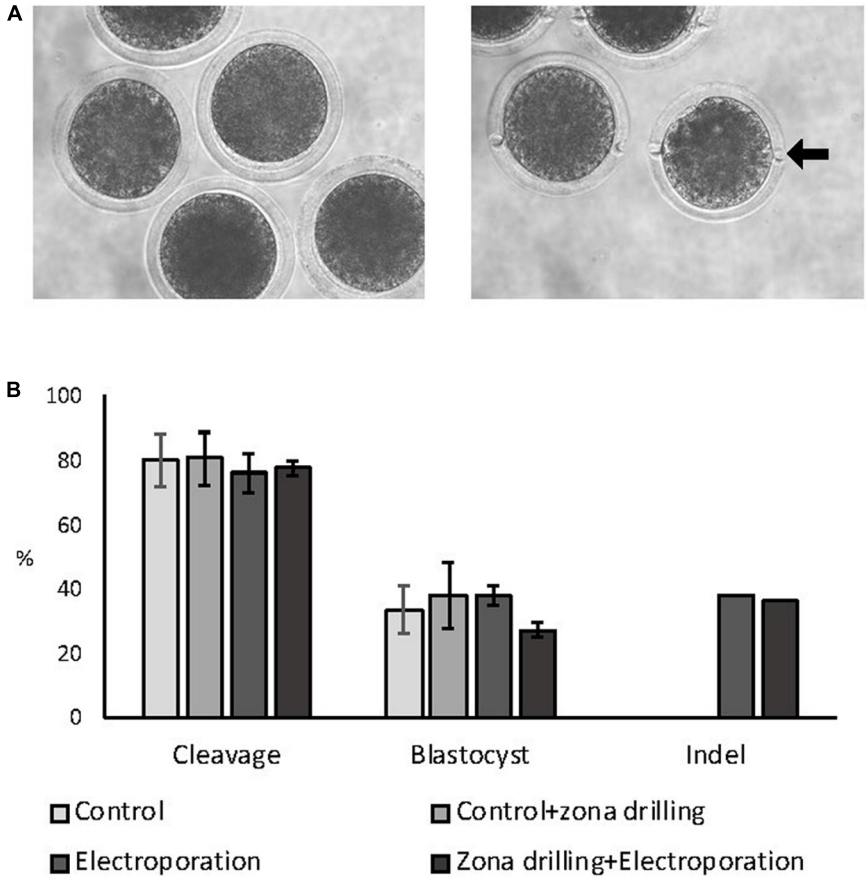
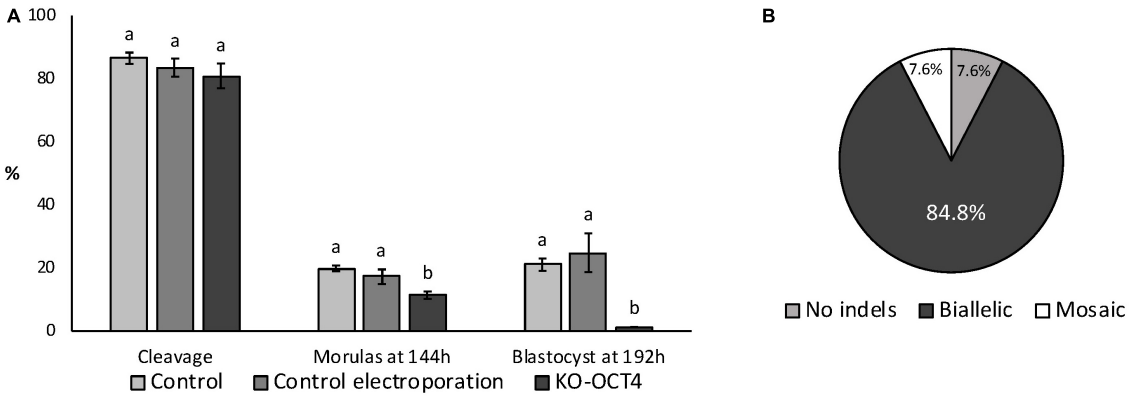


FIGURE 3 | Effect of zona drilling on electroporation efficiency of bovine zygotes. **(A)** Picture of intact (left) and zona drilled (right) embryos. Arrow indicates one of the two holes made in the zona pellucida of each embryo. **(B)** Cleavage and blastocyst rates (based on number of presumptive zygotes cultured) and indel rate (based on number of blastocysts sequenced) after electroporation with RNPs targeting the *ZFX* gene. No difference between groups were observed ($P > 0.05$). The experiment was replicated three times. Sample size: control (no electroporation and no zona drilling) = 69 zygotes; control+zona drilling = 69 zygotes; intact electroporation = 45 zygotes; zona drilling+electroporation = 98 zygotes. Blastocysts sequenced: electroporation = 16; zona drilling+electroporation = 25.

Combination of HV with LV has been shown to increase the transfection of eukaryotic cells with plasmid DNA or siRNA (Stroh et al., 2010), especially when using low DNA concentration (Kandušer et al., 2009; Čepurnienė et al., 2010). While HV pulses are important to create pores for permeabilization, the LV pulses allow the DNA to be electrophoretically dragged into the cell (Sukharev et al., 1992). In addition, bipolar LV pulses can increase the interaction between DNA and the membrane (Faurie et al., 2004) and improve electrotransfer efficiency (Orio et al., 2012). The combination of poring and transfer pulses could in part be responsible for the high rate of biallelic mutations observed in *OCT4* gene (85%) compared to the Namula et al. (2019) study which used only 3 poring pulses and obtained less than 5% biallelic mutations for the 15 V condition.

The concentration of CRISPR/Cas9 RNPs used for microinjection or electroporation often requires optimization to achieve optimal target disruption, where typically higher RNP concentrations being more efficient, while high concentrations can also result in increased toxicity. Cas9 protein concentrations above 100 ng/μL have usually been used for electroporation of mouse and rat zygotes in order to generate NHEJ-mediated indels or HDR-mediated nucleotide substitutions with reasonable efficiency (Chen et al., 2016; Tröder et al., 2018). Remy et al. (2017) reported 60% NHEJ and 25% knock-in efficiency in rats electroporated with 3 μM (∼480 ng/μL) Cas9 protein. One argument to use high concentrations of CRISPR/Cas9 components for genome editing is to reduce the level of mosaicism, despite the fact it may reduce embryo viability (Mehravar et al., 2019). Tanihara et al. (2019) reported that increasing Cas9 protein concentration from 20 to 100 ng/μL for cytoplasmic microinjection of porcine zygotes increased not only mutation efficiency but also the proportion of biallelic mutations. In our study, there was no difference in embryo development when 100:50 ng/μL and 200:100 ng/μL of Cas9:sgRNA were used for electroporation, providing a good range for testing and optimizing reagents for efficient gene editing.

Given that the ZP has been reported to negatively affect CRISPR/Cas9 electroporation efficiency in mouse zygotes (Qin et al., 2015; Chen et al., 2016), we tested whether large laser-drilled holes in the ZP would increase mutation rate by



C

CTCCTAAAGCAGAAGAGGATCACACTAGGATATACCCAGGCCGATGTGGGGCTCACCCTG - WT
CTCCTAAAGCAGAAGAGGATCACACTAGG- - - - - - - - - - - CGATGTGGGGCTCACCCTG (-12 nt) – Em#22
CTCCTAAAGCAGAAGAGGATCACCC - - - - - - - - - - - - - - CCCGATGTGGGGCTCACCCTG (+1;- 14 nt) – Em#25
CTCCTAAAGCAGAAGAGGATCACA- - - - - - - - - - - CCAGGCCGATGTGGGGCTCACCCTG (-11 nt) – Em#32

CTCCTAAAGCAGAAGAGGATCACACTAGGATATACCCAGGCC//CACAGGGACTACATCTG – WT
CTCCTAAAGCAGAAGAGGATCACAC - - - - - - - - - - - - - - - - // - - - AGGGACTACATCTG (-86 nt) – Em#16

AAGAGGATCACACTAGGATATACCCAGGCCGA//CGAGAATCTGCAGGAG- - GTGAGGG - WT
AAGAGGATCACACTAGGAT- - - - - - - - - - - - - -// - - - - - - - - - - - - - - - - - GTGTGAGGG (+2; -450 nt) – Em#18

FIGURE 4 | Developmental capacity of zygotes electroporated with RNPs targeting *OCT4*. **(A)** Embryo development until blastocyst stage. Control: no electroporation; Control electroporation: electroporation with RNPs targeting *SCD1*; KO-OCT4: electroporation with RNPs targeting *OCT4*. ^{a,b}Different letters within developmental stage indicate statistically significant differences ($P < 0.05$). Sample size for cleavage and morulas at 144 h (five replicates): control = 167; control electroporation = 117; and KO-OCT4 = 220. Sample size for blastocyst (three replicates): control = 81; control electroporation = 55; and KO-OCT4 = 87. **(B)** Genotyping of morulas electroporated with RNPs to knockout *OCT4* ($n = 13$). **(C)** Alignment of sequences from representative morula stage embryos targeted for OCT KO. WT, wildtype; Em, Embryo; green sequences, sgRNA; underlined sequence, PAM; red nucleotide, insertion.

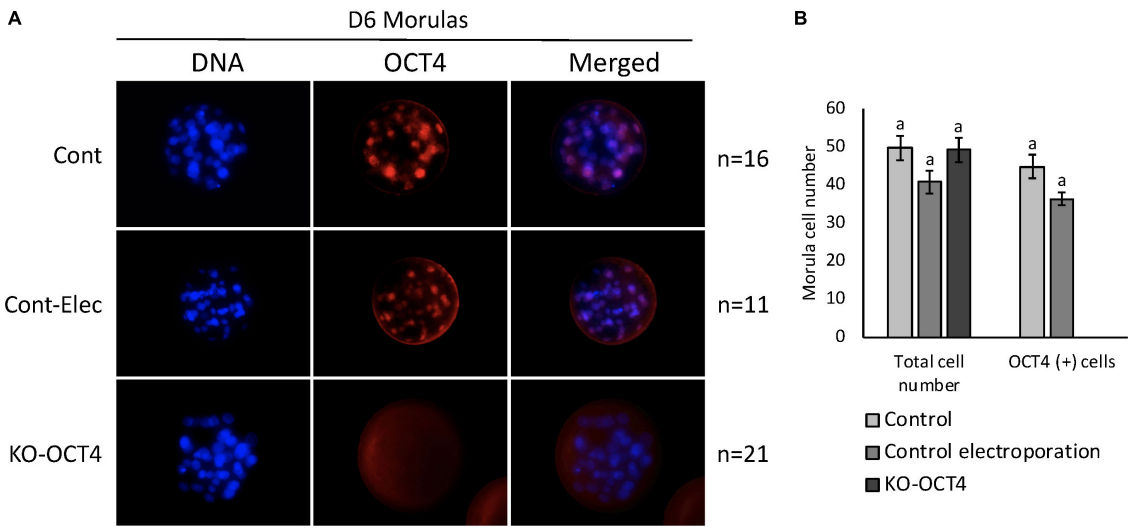


FIGURE 5 | Zygote electroporation with RNPs targeting *OCT4* eliminated OCT4 expression in morula stage embryos. **(A)** Immunofluorescence analysis of OCT4 expression in morulas collected 144 h post *in vitro* fertilization (D6) in control (Cont), control electroporation (Cont-Elect) and OCT4-targeting RNPs electroporation (KO-OCT4) groups. **(B)** Number of total cells and cells expressing OCT4 ($OCT4^+$) in morulas collected 144 h after *in vitro* fertilization. n: number of embryos evaluated in each group. No statistical difference in total cell number detected between groups ($P > 0.05$). No statistical difference in $OCT4^+$ cell between control groups. No $OCT4^+$ cells found in any morula analyzed in KO-OCT4 group.

facilitating the flow of RNPs components into the perivitelline space of bovine zygotes. Zona drilling followed by electroporation did not affect embryo development, nor did it increase indel rates, indicating that the bovine ZP is not an obstacle for RNP components. These results are consistent with successful gene editing after RNP electroporation of zona-intact mouse and rat zygotes (Kaneko, 2017). The zona pellucida is a porous non-charged network structure and in bovine oocytes and zygotes pores range in sizes from 171 to 223 nm in diameter (Vanroose et al., 2000; Báez et al., 2019), whereas Cas9 protein has approximately a 7.5 nm hydrodynamic diameter and the sgRNA has a 5.5 nm hydrodynamic diameter (Mout et al., 2017). Thus, in bovine zygotes, the ZP does not represent a barrier to the efficient electroporation of CRISPR/Cas9 RNPs.

An important factor to consider in CRISPR/Cas9 experiments is the sgRNA efficiency. Despite not making any direct comparisons between sgRNAs in this study, we noticed differences in mutation efficiency between experiments that targeted different genes. While *ZFX* sgRNA achieved up to 37% indel mutation rate, *OCT4* sgRNA resulted in 92.3% mutations. Such differences may be due to features inherent of each individual sgRNA and/or targeted region, which may include characteristics such as GC content, purine residues position, accessibility of seed region, and secondary structure (Doench et al., 2014; Moreno-Mateos et al., 2015; Cui et al., 2018). While bioinformatic tools provide predictions of sgRNA efficiency (Cui et al., 2018; Liu et al., 2020), these predictions are not often accurate *in vivo* and thus testing multiple sgRNA is necessary for optimizing mutation efficiency, regardless of the RNP delivery method.

One-step zygote editing is often associated with high levels of mosaicism resulting from indel introduction after the first round of DNA replication (Yen et al., 2014; Sato et al., 2018; Mehravar et al., 2019). Assessment of mosaicism in preimplantation embryos is complicated given the limited amount of sample from single embryos. To circumvent this limitation, we sought to assess gene editing efficiency and embryo mosaicism using a model in which KO efficiency can be determined at the single cell level by immunostaining for the protein encoded by the targeted gene. OCT4 is expressed from the embryonic genome at morula stage, with all cells presenting positive staining at this stage in development. We previously reported that microinjection of CRISPR/Cas9 RNPs targeting *OCT4* resulted in high mutation efficiency, suppression of the OCT4 protein, as demonstrated by immunofluorescence staining, and developmental arrest at the morula stage (Daigneault et al., 2018). Interestingly, CRISPR/Cas9 RNP microinjection resulted in mosaicism of OCT4 expression in 29% of morula stage embryos. The use of the same sgRNA delivered by electroporation in this study resulted in high rate of gene editing, with most embryos (11/13) presenting biallelic mutations, and evidence of genetic mosaicism observed in only one embryo (1/13), while based on immunostaining, none of the embryos analyzed were positive for OCT4 in any of their cells (100% KO; no mosaicism). The mutation rates assessed by embryo genotyping were higher for electroporation compared to previously reported (Daigneault et al., 2018) microinjection results (92 vs. 84%, respectively). As previously reported, embryos with OCT4 mutations arrested at the morula stage, with a single embryo in this study developing to the early blastocyst stage and presenting a reduced cell number compared to controls. Overall, we show that electroporation of RNPs resulted in efficient OCT4 KO and embryo phenotypic changes consistent with lack of OCT4 function.

In conclusion, Cas9:sgRNA RNPs can be delivered efficiently by electroporation of zona-intact bovine zygotes without affecting embryo development. Electroporation of Cas9/sgRNA RNPs into bovine zygotes can result in highly efficient mutation induction, gene disruption and expected phenotypic changes. The use of electroporation for introducing gene edits in zygotes significantly simplifies the methodology for creating gene edited livestock.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

PR and LC conceived and designed the work. LC and JO collected and analyzed the experimental data. PR, LC, AV, and JO wrote and revised manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2020.570069/full#supplementary-material

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frontiers in Genetics

BRIEF RESEARCH REPORT
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Cytosine Base Editor (hA3A-BE3-NG)-Mediated Multiple Gene Editing for Pyramid Breeding in Pigs

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Pig is an important agricultural economic animal, providing large amount of meat products. With the development of functional genomics and bioinformatics, lots of genes and functional single nucleotide polymorphisms (SNPs) related to disease resistance and (or) economic traits in pigs have been identified, which provides the targets for genetic improvement by genome editing. Base editors (BEs), combining Cas9 nickase and cytidine or adenine deaminase, achieve all four possible transition mutations (C-to-T, A-to-G, T-to-C, and G-to-A) efficiently and accurately without double strand breaks (DSBs) under the protospacer adjacent motif (PAM) sequence of NGG. However, the NGG PAM in canonical CRISPR-Cas9 can only cover approximately 8.27% in the whole genome which limits its broad application. In the current study, hA3A-BE3-NG system was constructed with the fusion of SpCas9-NG variant and hA3A-BE3 to create C-to-T conversion at NGN PAM sites efficiently. The editing efficiency and scope of hA3A-BE3-NG were confirmed in HEK293T cells and porcine fetal fibroblast (PFF) cells. Results showed that the efficiency of hA3A-BE3-NG was much higher than that of hA3A-BE3 on NGH (H = A, C, or T) PAM sites (21.27 vs. 2.81% at average). Further, nonsense and missense mutations were introduced efficiently and precisely *via* hA3A-BE3-NG in multiple pig economic trait-related genes (*CD163*, *APN*, *MSTN*, and *MC4R*) in PFF cells by one transfection. The current work indicates the potential applications of hA3A-BE3-NG for pyramid breeding studies in livestock.

Keywords: base editing, NGN PAM, hA3A-BE3-NG, multiple gene editing, pyramid breeding

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INTRODUCTION

As an agricultural animal, pig is an important meat resource with great economic value. The conventional pig breeding is to pyramid desirable traits by cross breeding with cost and long breeding cycle. The genome-editing technology is an effective approach for pig improvement in growth, meat quality, reproductive capacity, and disease resistance (Song et al., 2020). It is the desired goal to exploit efficient and precise genome-editing tools to achieve rapid pyramid breeding through modifying multiple agriculture-related functional genes simultaneously.

Base editors (BEs), combining Cas9 nickase and cytidine or adenine deaminase, perform efficient and accurate base substitutions (C-to-T, A-to-G, T-to-C, and G-to-A) without double strand breaks (DSBs) at target sites, which provides an alternative strategy for precise genome editing (Komor et al., 2016; Gaudelli et al., 2017). Recently, various versions of BEs were exploited to optimize the specificity, sensitivity, and safety of base conversions (Rees and Liu, 2018). One of the many versions, the hA3A-BE3 system, replaces the rat cytidine deaminase (APOBEC1) with human cytidine deaminase (APOBEC3A), which performs C-to-T conversion more efficiently with expanded activity windows at target sites in human cells, plants, rabbits, and pigs than the original BE3 (Wang et al., 2018; Zong et al., 2018; Liu et al., 2019; Xie et al., 2019). However, the targetable scope of hA3A-BE3 is restricted for use with conventional SpCas9, which recognizes target loci through NGG as its protospacer adjacent motif (PAM) sequence.

This limitation can be overcome by using Cas9 variants with targeting preferences other than NGG PAM, which have been validated using conventional BEs. For example, BE variants, such as VQR-BE3, EQR-BE3, VRER-BE3, SaBE3, and SaKKH-BE3, have been developed to target NGAN, NGA, NGCG, NNGRRT, and NNNRRT PAM sites, circumventing the need for NGG PAM sequences in human cells (Kim et al., 2017). CRISPR-Cpf1-based BEs have even been developed that recognize and target T-rich PAM sequences (TTTV; Li et al., 2018; Kleinstiver et al., 2019). Recently, three newly engineered SpCas9 variants, xCas9, SpCas9-NG, and SpG, were reported to expand the targetable scope of NGN PAM sites in cultured cells, plants, and animals (Hu et al., 2018; Nishimasu et al., 2018; Endo et al., 2019; Fujii et al., 2019; Walton et al., 2020). Further, the SpCas9-NG system has been applied only in bacteria, human cells, plants, and rabbits (Huang et al., 2019; Thuronyi et al., 2019; Wang Y, et al., 2019; Zhong et al., 2019; Li et al., 2020; Liu et al., 2020b).

CRISPR/Cas9 mediated *clusters of differentiation 163* (*CD163*)-deletion conferred the ability of effective resistance to porcine reproduction and respiratory syndrome virus (PRRSVs) infection on pigs (Whitworth et al., 2016; Wang H, et al., 2019). *Aminopeptidase N* (*APN*) gene deletion gave the ability of neonatal piglets to resist infection with the highly virulent transmissible gastroenteritis virus (TGEVs; Luo et al., 2019; Whitworth et al., 2019). For meat production, deletion of the porcine *myostatin* (*MSTN*) gene has been shown to improve muscle growth, resulting in a double-muscled phenotype (Qian et al., 2015). Many of these targeted gene deletions could potentially be achieved by generating a premature terminal codon (iStop-codon) through precise C-to-T mutations *via* cytosine base editors (CBEs; Billon et al., 2017; Kuscu et al., 2017). Precision single-base editing provides a strategy to manipulate functional single nucleotide polymorphisms (SNPs) for accurate genetic improvement in pig production. For example, porcine *melanocortin-4 receptor* (*MC4R*) c.893G>A was reported to be associated with fatness, growth, and feed intake traits (Kim et al., 2000). In the current study, in order to increase the efficiency of base editing at expanded target sites in pigs, hA3A-BE3-NG system was constructed and used to produce C-to-T mutation with high efficiency and expanded editable scope in human cells and porcine cells. Economic related genes including *CD163*, *MSTN*, *APN*, and *MC4R*, were simultaneously targeted *via* hA3A-BE3-NG. To our knowledge, this is the first study to precisely edit multiple genes responsible for economic traits in the porcine genome using BEs, and suggest the incredible potential of using BEs to accelerate molecular pyramid breeding in livestock.

MATERIALS AND METHODS

Plasmid Construction

The hA3A-BE3-NG vector was constructed in this study through in-fusion cloning to transfer the DNA fragment containing VRVRFRR variants of SpCas9-NG from Target-AID-NG (119861#; Addgene, Watertown, MA, United States) to hA3A-BE3 (113410#; Addgene, Watertown, MA, United States). For construction of sgRNAs, oligos were synthesized, annealed, and cloned into the *Bsa*I site of the sgRNA-expressing vector, pGL3-U6-sgRNA-PGK-puromycin (51133#; Addgene, Watertown, MA, United States). The fragment pCAG-tdTomato was cloned into the *Bsp*QI linearized pGL3-U6-sgRNA-PGK-puromycin to construct the sgRNA-tdTomato-expressing vector, pGL3-U6-sgRNA-tdTomato. The sgRNAs used in this study are summarized in **Supplementary Table S1**. The primers used in the construction of hA3A-BE3-NG are listed in **Supplementary Table S2**.

Cell Culture and Transfection

HEK293T cells were cultured in Dulbecco's Modified Eagle's medium (DMEM; Gibco, Grand Island, NY, United States), supplemented with 10% fetal bovine serum (FBS; v/v; HyClone, Logan, UT, United States) and 1% Penicillin Streptomycin (v/v; Gibco, Grand Island, NY, United States). HEK293T cells were seeded 1 day prior to transfection in 24-well plates (Corning, Corning, NY, United States), at a density of 1×10^5 cells per well. Cells were transfected with 1 μg base editor plasmid (hA3A-BE3, Target-AID-NG or hA3A-BE3-NG), 500 ng pGL3-U6-sgRNA-PGK-puromycin, and 50 ng pCMV-GFP (11153#; Addgene, Watertown, MA, United States) per well, using Lipofectamine LTX (Life Technologies, Gaithersburg, MD, United States) according to the manufacturer's recommended protocol. HEK293T cells were cultured at 37°C with 5% of CO_2.

Porcine fetal fibroblast (PFF) cells were isolated from 35-day-old fetuses of Bama pigs. A day before transfection, PFF cells were thawed and cultured in the Minimum Essential Medium (MEM Alpha; Gibco, Grand Island, NY, United States), supplemented with 15% FBS (v/v; HyClone, Logan, Utah, United States), 1% nonessential amino acids (NEAA; v/v; Gibco, Grand Island, NY, United States), 2 mM GlutaMAX (Gibco, Grand Island, NY, United States), and bFGF (Life Technologies, Gaithersburg, MD, United States). PFF cells were seeded 1 day prior to transfection in 6-well plates (Corning, Corning, NY, United States). Four microgram base editor vector (hA3A-BE3 or hA3A-BE3-NG) and 2.73 μg pGL3-U6-sgRNA-tdTomato were co-transfected into 5×10^5 PFF cells by nucleofection with Lonza/Amaxa Nucleofector 2B (Lonza, Basel, Switzerland)

according to the manufacturer's recommended protocol. Cells were harvested approximately 48 h post-transfection. PFF cells were cultured at 38.5°C with 5% of CO_2.

Fluorescence-Activated Cell Sorting

HEK293T and PFF cells were harvested and subjected to flow cytometry 48 h after transfection. A total of 10,000 cell events were collected and analyzed using FlowJo software. Single PFF cell with positive signal was seeded into 96-cell plates and cultured for 8 days to form colonies.

Base Editing Analysis and Single Cell Line Genotyping

Genomic DNA of HEK293T and PFF cells was extracted using One Step Mouse GenoTyping Kit (Vazyme, Nanjing, China). The cell lysate was then used as the PCR template. PCR fragments for Sanger sequencing were generated in one step PCR reaction. The editing efficiency was analyzed by an online tool, EditR 1.0.9.[1] The primers are listed in **Supplementary Table S3**.

Reverse Transcription-PCR

Total RNA was extracted from cultured cells by using TRIzol reagent (Invitrogen, Carlsbad, CA, United States), according to manufacturer's protocol. Complementary DNA (cDNA) was generated by using Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, United States). The PCR reaction with 25 ng cDNA template was performed for 30 cycles. The housekeeping gene, *GAPDH*, was used as an internal control. Relative expression of Cas9 was detected by gel electrophoresis. All the primer sequences were shown in **Supplementary Table S4**.

Statistical Analysis

The statistical data are expressed as mean ± SEM, and at least three individual replicates were conducted in all experiments. Statistical significance was analyzed with unpaired Student's *t*-tests using GraphPad prism software 6.0 (GraphPad Prism, La Jolla, CA, United States). A value of $p < 0.05$ was considered statistically significant.

RESULTS

The Successful Construction of hA3A-BE3-NG Targeting Plasmid

The targetable scope of traditional BEs was restricted for the conventional SpCas9 preferred to recognize the target loci with NGG PAM. Approximately 205,013,891 NGG and CCN sites exist in the pig genome, which accounts for only 8.27% of the total genome sites (**Figure 1A**) within the approximately 2,478,444,698 base pairs estimated by Sscrofa11.1 assembly (Li et al., 2017). Overall, the percentage of NGN and NCN sites in the porcine genome was about 33.04%, which is four times higher than that of NGG and CCN sites (**Figure 1A**). To expand the targeting scope of hA3A-BE3, we fused SpCas9-NG with hA3A-BE3 to generate a new BE named hA3A-BE3-NG by in-fusion strategy (**Figure 1B** and **Supplementary Figure S1**). Our construct incorporated three fragments: a restriction fragment of 5,570 bp digested by *Bsr*GI and *Pme*I from hA3A-BE3, and two PCR fragments amplified from hA3A-BE3 and Target-AID-NG, respectively (**Figures 1B,C**). The successful construction of the vector was confirmed by PCR, gel electrophoresis (**Figure 1C**), and Sanger sequencing (**Figure 1D**).

hA3A-BE3-NG-Mediated Gene Editing at NGN PAM Sites in Human Cells

One study revealed that Target-AID-NG was another superior base editor for introducing C-to-T conversion at NGN PAM sites efficiently in human cells (Nishimasu et al., 2018). To further validate the editing capacity of hA3A-BE3-NG, fused with different cytosine deaminase, four sgRNAs that targeted AGA, GGT, GGG, and AGC PAMs sites in *human empty spiracles homeobox 1* (*EMX1*) loci were designed. The hA3A-BE3, hA3A-BE3-NG, or Target-AID-NG plasmid were co-transfected with sgRNAs- and GFP-expressing plasmids into HEK293T cells, respectively. All GFP-positive cells (no less than 25% of total cells) were isolated *via* flow cytometry for further characterization (**Supplementary Figures S2A**,**B**). The expression of hA3A-BE3-NG was confirmed by reverse transcription PCR (RT-PCR) in 48 h post-transfected HEK293T cells (**Supplementary Figure S2C**). Mutation frequencies by different BEs at NGN PAM sites were quantified using Sanger sequencing and EditR software (**Figures 2A–C** and **Supplementary Figure S2D**). Results showed that hA3A-BE3-NG achieved a C-to-T editing frequency of at least 15% at AGA and GGT PAM sites when compared with the mutation frequency of hA3A-BE3 showed less than 5% (**Figures 2A,B** and **Supplementary Figure S2D**). The hA3A-BE3-NG induced slightly lower C-to-T conversion, compared to those of hA3A-BE3 (14.27 vs. 19.00%) in the activity window (C3, C4, C5, C6, and C12) at GGG PAM (**Figure 2A** and **Supplementary Figure S2D**). This is in line with a recently observed phenomenon, SpCas9-NG shows slightly reduced activity at NGG PAM sites in human cells (Nishimasu et al., 2018). In addition, hA3A-BE3-NG also showed relatively low conversion efficiency at AGC PAM sites (**Figure 2A** and **Supplementary Figure S2D**), which is consistent with a previous report (Nishimasu et al., 2018). Overall, hA3A-BE3-NG-mediated C-to-T conversion was more efficient than hA3A-BE3 at NGH PAM sites (21.27 vs. 2.81% at average; **Figure 2C**). In addition, Target-AID-NG showed efficient editing of C3 and C4 at GGG PAM site (**Figure 2A** and **Supplementary Figure S2D**), highlighting differences in editing windows, base preference, and efficiencies between hA3A- and PmCDA1-derived BEs. However, hA3A-BE3-NG achieved a higher mutation frequency than that of Target-AID-NG at AGA and GGT PAM sites (**Figures 2A,B** and **Supplementary Figure S2D**), indicating that hA3A-BE3-NG could be considered a more efficient BE with an expanded targetable scope for gene editing in the mammalian genome.

[1]https://moriaritylab.shinyapps.io/editr_v10/

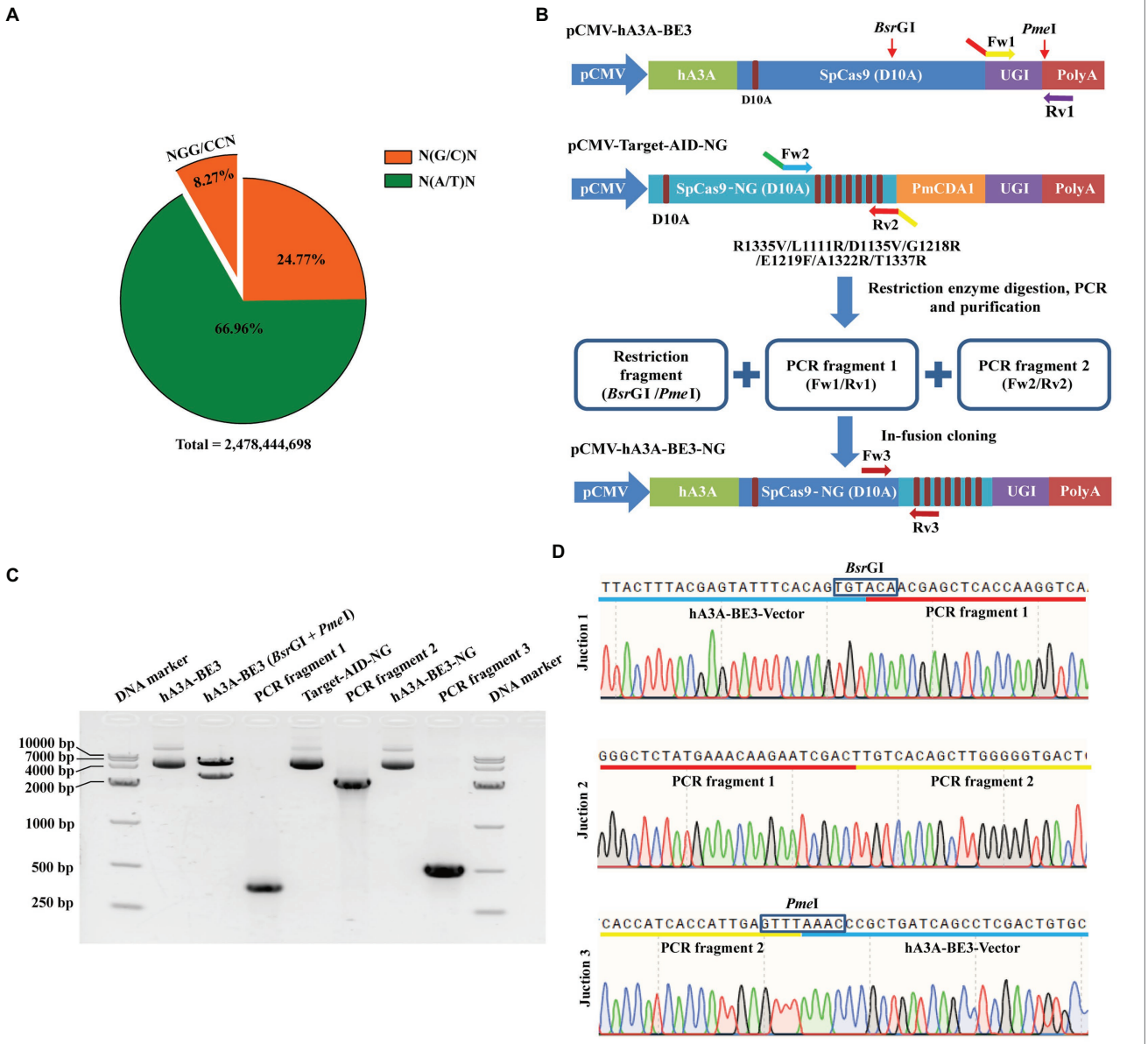

FIGURE 1 | Construction of hA3A-BE3-NG vector for expanded targeting scope. **(A)** Pie chart shows the proportion of porcine genomic sites that can be targeted by SpCas9 or SpCas9-NG with distinct protospacer adjacent motif (PAM) specificities (NGG or NG). Pig reference genome (Sscrofa11.1) was used for analysis. **(B)** Schematic of the pCMV-hA3A-BE3-NG vector. Compared to SpCas9 (D10A) in hA3A-BE3, SpCas9-NG (D10A) in Target-AID-NG contained seven amino acids variants: R1335V, L1111R, D1135V, G1218R, E1219F, A1322R, and T1337R. hA3A-BE3-NG was constructed by in-fusion cloning of a restriction fragment digested *via* *Bsr*GI and *Pme*I from hA3A-BE3, PCR fragment 1 amplified from hA3A-BE3 *via* Fw1/Rv1 primers, and PCR fragment 2 amplified from Target-AID-NG *via* Fw2/Rv2 primers. Overlapping sequences exist in the junction of the three different fragments. **(C)** The gel image indicates that hA3A-BE3 was digested into two fragments by *Bsr*GI and *Pme*I. The PCR fragment 1 (387 bp) was amplified from hA3A-BE3 *via* Fw1/Rv1 primers, and the PCR fragment 2 (2,556 bp) was amplified from Target-AID-NG *via* Fw2/Rv2 primers. The large fragment (5,570 bp) from hA3A-BE3, PCR fragment 1 and PCR fragment 2 were fused into a recombinant vector, hA3A-BE3-NG, which was confirmed by a PCR product (540 bp) amplified *via* Fw3/Rv3 primers. **(D)** The chromatograms of Sanger sequencing show the junctional sequence was accurate among the above three fragments in recombinant hA3A-BE3-NG.

Expanded Editable Scope Through hA3A-BE3-NG to Generate Premature Terminal Codon in PFF Cells

Stop codons (TAG, TGA, or TAA) could be produced by a C-to-T conversion of the CAG, CGA, or CAA codons on the sense strand and the G-to-A conversion of the TGG codon caused by C-to-T mutation on the anti-sense strand (**Figure 3A**). The loss of function mutation in various genes was reported to confer the elite traits in pigs, such as *CD163* gene for PRRSVs resistant (Whitworth et al., 2016; Burkard et al., 2017;

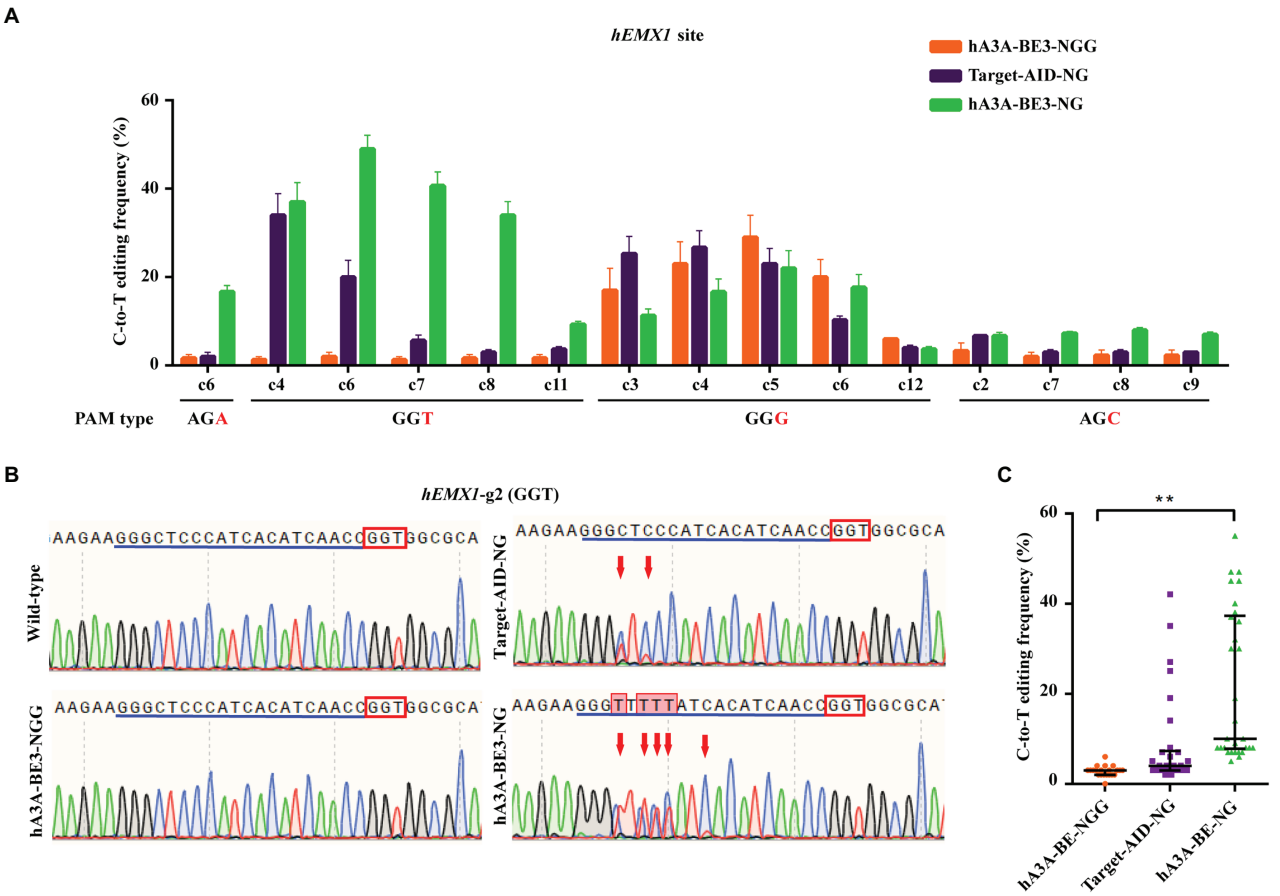

FIGURE 2 | Efficient C-to-T conversion in human HEK293T cells by hA3A-BE3-NG. **(A)** C-to-T editing by hA3A-BE3, hA3A-BE3-NG, and Target-AID-NG at four endogenous *EMX1* gene sites in human HEK293T cells. The target base in the editing window is shown, counting the end distal to the PAM as position 1. Data are represented as the mean ± SEM (n = 3). **(B)** Sanger sequencing results of HEK293T cells transfected with hA3A-BE3, hA3A-BE3-NG, or Target-AID-NG. Red boxes indicate PAMs and blue lines indicate sgRNA sequences. Red arrows indicate substituted nucleotides. **(C)** Statistical analysis of the C-to-T editing frequency induced by hA3A-BE3, hA3A-BE3-NG, or Target-AID-NG at NGH PAM sites in **(A)**. The median and interquartile range (IQR) are shown; $^{**}p < 0.01$.

Wells et al., 2017), *APN* gene for TGEVs resistant (Luo et al., 2019; Whitworth et al., 2019; Zhang et al., 2019), and *MSTN* for increased lean meat production (Qian et al., 2015; Wang K, et al., 2015). Thus, we explore the possibility and editing efficiency of inducing stop codons over these loci at expanded targetable sites by hA3A-BE3-NG in pigs. We designed a total of 32 sgRNAs (A1–19, C1–7, and M1–6) with NGN PAM in porcine *CD163*, *APN*, and *MSTN* genomic loci. Of the 32 sgRNAs, 28 (A2–18, C2–7, and M1–5) could produce premature terminal codons in the targeted activity windows if C-to-T conversion occurs (positions 2–13, counting the PAM as positions 21–23; **Figure 3B**). We firstly evaluated hA3A-BE3-NG-mediated editing efficiency on 32 NGN PAM sites (**Supplementary Figure S3A**). hA3A-BE3-NG showed comparable activity to hA3A-BE3 at 6 NGG PAM sites (A2, A6, A11, A13, A15, and C6) and reduced activity at 2 NGG PAM sites (A3 and M3), suggesting that hA3A-BE3-NG was also a useful BE at NGG PAM sites in pigs (**Figure 3C** and **Supplementary Figure S3B**). With NGH PAM sites, hA3A-BE3-NG showed at least a 3% mutation frequency at 21 of the 24 sites and at least a 10% mutation frequency at half of the 21 sites (**Figure 3C** and **Supplementary Figure S3C**). By contrast, hA3A-BE3 only edited the AGA PAM site (M4) with a low mutation frequency of 4% and had no efficiency at other 23 NGH PAM sites (**Figure 3C**). Interestingly, as shown in **Figure 3C**, hA3A-BE3-NG was editing ineffective at modifying TGA PAM sites (M1 and M2), which might be resulted from sequence signatures and nucleotide preferences (Xue et al., 2019). In brief, compared with hA3A-BE3 that induced C-to-T conversion efficiently at NGG PAM sites, hA3A-BE3-NG showed efficient editing at a variety of PAM sites (**Figures 3C,D** and **Supplementary Figures S3B,C**). Particularly, at 25 of 28 target sites that sgRNAs could generate premature stop codons to knockout target genes, hA3A-BE3-NG achieved detectable C-to-T mutation frequency if 3% was used as the cutoff threshold. By contrast, only 9 of these 28 sgRNAs were functional with hA3A-BE3 (**Figure 3C**). Besides the above three genes that was designed to induce loss-of-function mutations, we further exploited to introduce a beneficial SNP (c.893 G>A) into *MC4R* gene

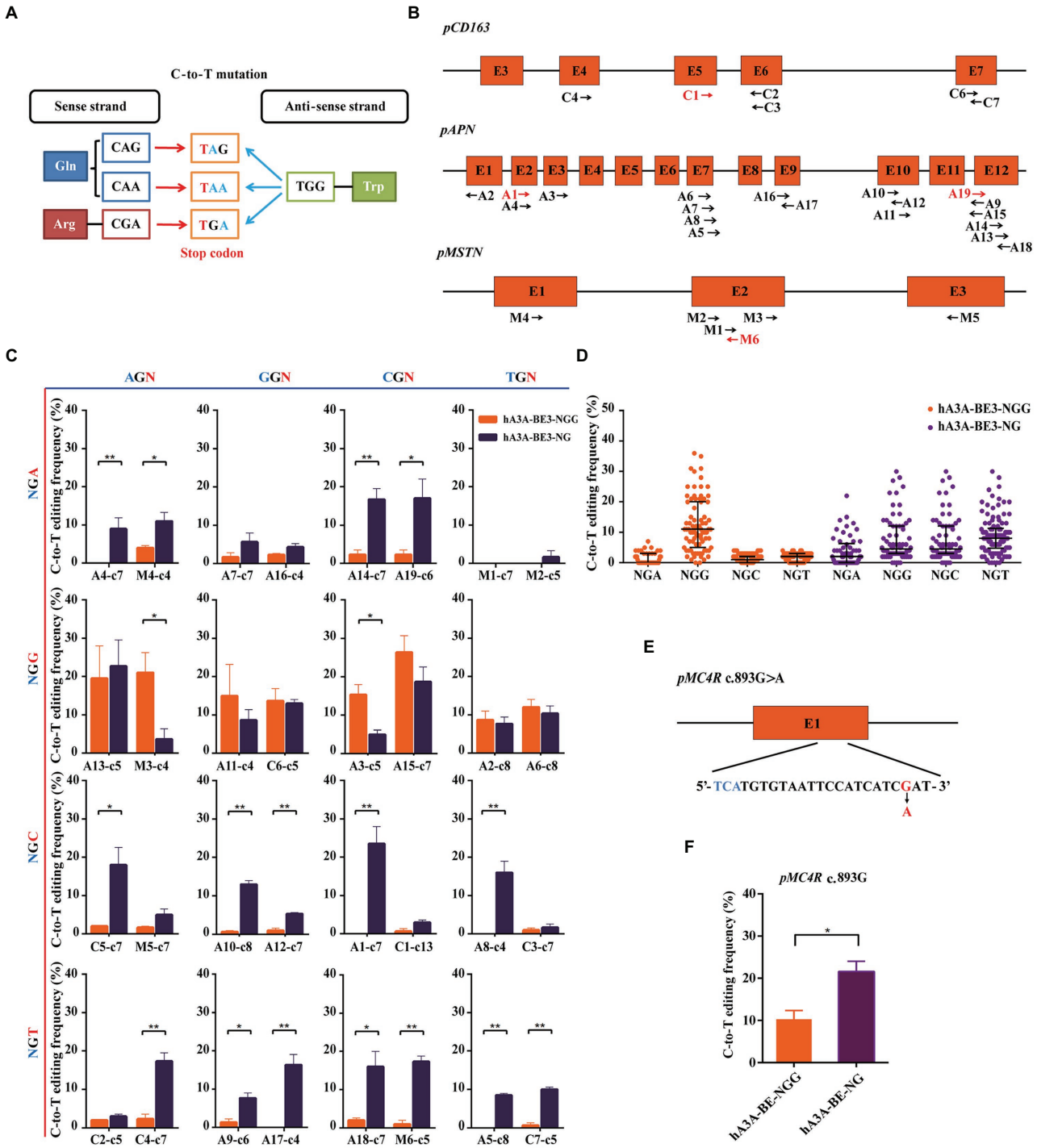

FIGURE 3 | Precision missense mutation using hA3A-BE3-NG to expand the editing scope in porcine fetal fibroblast (PFF) cells. **(A)** Representation of the C-to-T conversion induced by base editors to generate stop codons. The base editors convert CAA, CAG, and CGA codons to stop codons (red) in the sense strand. The TGG codon is converted to stop codons (blue) through G-to-A conversion. **(B)** Schematic of the target sites at the porcine *CD163*, *APN*, and *MSTN* loci. The target sites indicated by the black arrows can generate stop codons using base editors (BEs). The forward direction of arrow indicates sgRNA-matched anti-sense strand, and vice versa. Total of 32 sgRNAs were designed (A1–19, C1–7, and M1–6). **(C)** Base editing at 32 NGN PAM sites by hA3A-BE3 and hA3A-BE3-NG. The target sites covered all 16 possible NGN PAM combinations, counting the end distal to the PAM as position 1. **(D)** Statistical analysis of the C-to-T editing frequency induced by hA3A-BE3 or hA3A-BE3-NG at a total of 32 endogenous target sites. The median and IQR are shown. **(E)** Schematic of the target site at *MC4R* locus. *MC4R* c.893G>A could be produced by hA3A-BE3-NG. The PAM sequence and substituted base are shown in blue and red, respectively. **(F)** Base editing at the *MC4R* locus by hA3A-BE3 and hA3A-BE3-NG. In **(C,F)** values were shown as mean ± SEM ($n = 3$); $^{**}p < 0.01$ and $^{*}p < 0.05$.

that was reported to be a marker for decreased fat deposition trait (Kim et al., 2000; Schroyen et al., 2015). The sgRNA was designed on the reverse strand of TGA PAM site, positioning the targeted cytosine in the activity window of hA3A-BE3-NG to produce *MC4R* c.893G>A on the sense strand (**Figure 3E**). hA3A-BE3-NG mediated higher mutation frequency than hA3A-BE3 (21.67 vs. 10.33%; **Figure 3F** and **Supplementary Figure S3D**).

hA3A-BE3-NG-Mediated Base Editing in Multiple Loci

In livestock, most of the economic traits were considered to be regulated by a massive number of SNPs in various genes (Song et al., 2020). Thus, the ability to create precise and multiple genetic modification in various loci across the pig genome simultaneously is necessary for successful pyramid breeding. To investigate the feasibility of hA3A-BE3-NG for base editing in multiple loci, we simultaneously co-transfected hA3A-BE3-NG and sgRNAs-tdTomato-expressing plasmid that targeted *APN*, *CD163*, *MC4R*, and *MSTN* into PFF cells. After 48 h of transfection, tdTomato-positive single PFF cell was isolated and seeded into 96-cell plates *via* FACS, and then cultured for another 8 days to form single-cell colonies. A total of 54 colonies were obtained and genotyped by Sanger sequencing (**Figures 4A,B**). Results showed that 21 out of 54 (38.89%), 23 out of 54 (42.59%), 3 out of 54 (5.56%), and 25 out of 54 (46.30%) colonies had mutations in the *APN*, *CD163*, *MC4R*, and *MSTN* genes, respectively, and most of them had effective C-to-T conversion at the target sites (**Figure 4A**). Due to the wide activity window of hA3A-BE3-NG, we also found that a number of colonies had bystander mutations with C-to-T substitution existing in the vicinity of the targeted cytosine (35.19, 12.96, and 18.52% colonies in *APN*, *CD163*,

A

Target genes	No. of screened colonies	No. of mutants (%)	No. of target mutants (%)	No. of bystander mutants (%)	No. of proximal off-target mutants (%)	No. of indels (%)	No. of non-C-to-T mutants (%)	No. of single gene mutants (%)	No. of double genes mutants (%)	No. of three genes mutants (%)	No. of four genes mutants (%)
APN	54	21 (38.89)	18 (33.33)	19 (35.19)	2 (3.70)	5 (9.26)	1 (1.85)	11 (20.37)	8 (14.81)	14 (25.93)	2 (3.70)
CD163		23 (42.59)	19 (35.19)	7 (12.96)	12 (22.22)	3 (5.56)	5 (9.26)				
MC4R		3 (5.56)	3 (5.56)	0 (0)	0 (0)	0 (0)	0 (0)				
MSTN		25 (46.30)	25 (46.30)	10 (18.52)	5 (9.26)	1 (1.85)	6 (11.11)				

B

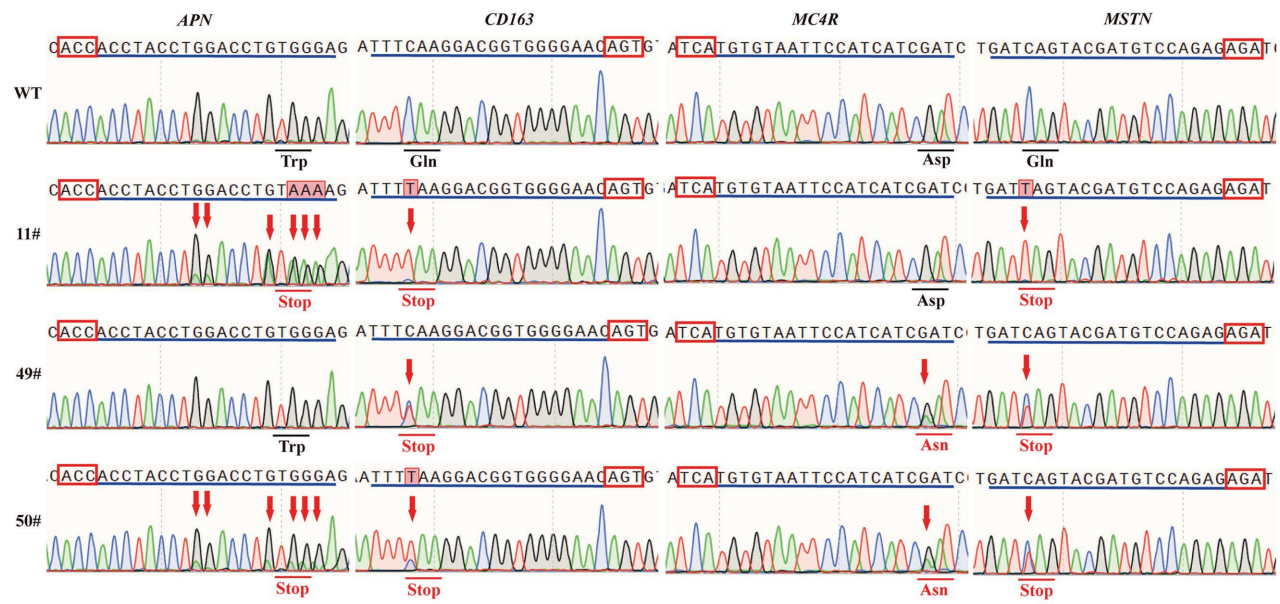

FIGURE 4 | hA3A-BE3-NG-mediated base editing at multiple genes in PFF cells. **(A)** Summary of multiple sites base editing by hA3A-BE3-NG in PFF cells. **(B)** Sanger sequencing results of selected single-cell colonies. 11# and 49# colonies have mutations on three genes, and 50# colony has mutations on all four genes. The red box indicates the PAMs and the blue line indicates the sgRNA sequence. The red arrow indicates the substituted nucleotide. The amino acid in the red line indicates expected substitutions at target sites.

and *MSTN* genes, respectively; **Figure 4A**). Moreover, 12 out of 54 (22.22%) colonies showed a proximal off-target mutation at position −4 (with the base distal from the PAM set as position 1) in *CD163* (**Figure 4A**). Importantly, 35 out of 54 colonies had mutations, and therein, two single-cell colonies (3.70%, 2/54) showed targeted mutations of all four genes (*APN*, *CD163*, *MSTN*, and *MC4R*; **Figures 4A,B**). In addition, we identified 14 colonies (25.93%, 14/54) with triple-gene mutations, 8 (14.81%, 8/54) with double-gene mutations, and 11 (20.37%, 11/54) with single-gene mutation (**Figures 4A,B**).

DISCUSSION

Genome editing technologies have provided a revolutionary strategy for making genetic improvements in pig breeding. Compared to conventional cross breeding in livestock, the molecular breeding to accurately modify the agriculture-related functional genes will save a lot of time, money, and manpower. The focus of recent genome editing research is to modify the genome efficiently, accurately, and safely. In the current study, hA3A-BE3-NG was constructed and proved to be a powerful base editor to improve the editing efficiency and expand the targeting scope in pigs. It has been reported that, 20 endogenous target sites (including *EMX1*, *VEGFA*, *GRIN2B*, etc.) with different PAM have been used to compare C-to-T conversion efficiency between Target-AID and Target-AID-NG in HEK293T cells (Nishimasu et al., 2018). Therefore, we also selected the *EMX1* targets from the study above to analyze the targeting efficiency of hA3A-BE3, Target-AID-NG, and hA3A-BE3-NG in the current study. Here, hA3A-BE3-NG was confirmed to show editing activity comparable with or even higher than Target-AID-NG at the four target sites in human cells. And it could induce C-to-T mutation in a broader activity window in human and porcine cells efficiently, which is consistent with a previous study that hA3A-BE3 had an approximately 12 nucleotides activity window (Wang et al., 2018).

To avoid potential chimeric issues and long-time frame of breeding, the generation of genetically modified large animals was mostly created by genome editing technology combined with somatic cell nuclear transfer (SCNT) instead of embryo injection (Zhao et al., 2019). So how to obtain the cell colonies with desired modification efficiently is one of the key steps. Here, we found that hA3A-BE3-NG could induce C-to-T conversion efficiently not only at NGG PAM sites as hA3A-BE3 but also exhibited expand targeting scope at NGN PAM sites. For the *MC4R* c.893G>A mutation, hA3A-BE3-NG showed more efficient than hA3A-BE3 at the TGA PAM site (21.67 vs. 10.33% at average). Thus, applications of hA3A-BE3-NG could expand the editing scope at NGN PAM sites, possibly facilitating breeding improvements in pigs.

With the development of functional genomics and bioinformatics, more and more SNPs responsible for economic traits have been identified in livestock (Song et al., 2020). And many economic traits are majorly controlled or orchestrated by combinations of SNPs. Therefore, it is of importance to create precise and multiple genome-editing livestock for exploring the function of SNPs and evaluate their potential breeding value. In addition, the potential of chromosomal structural abnormalities would increase when multiplex target loci were cut simultaneously by conventional CRISPR-Cas systems, causing genomic instability, chromosome elimination, and even cell death (Wang T, et al., 2015; Aguirre et al., 2016; Zuo et al., 2017). BEs provided a safe strategy to edit multiple gene sites efficiently and accurately without DSBs. Recently, the multiplex base editing was accomplished by BE3 at NGG PAM sites in pigs (Xie et al., 2019; Yuan et al., 2019). Using BE3 and hA3A-BE3, Xie et al. (2019) simultaneously mutated the porcine *RAG1*, *RAG2*, and *IL2RG* or *DMD*, *TYR*, and *LMNA* triple gene in PFF cells with high efficiency, and subsequently generated a triple gene knockout pig model with immunodeficiency for applications in regenerative medicine. Yuan et al. (2019) prepared *GGTA1/B4GAlNT2/CMAH* triple gene knockout pigs which could be used as organ donors for xenotransplantation by BE4-Gam. hA3A-BE3-NG could simultaneously introduce targeted mutations at multiple sites of four genes, *APN*, *CD163*, *MSTN*, and *MC4R* in PFF cells, suggesting the great potential of hA3A-BE3-NG in animal pyramid breeding.

Previous studies have suggested that CBEs could cause DNA off-target effects in mouse embryos and plants (Jin et al., 2019; Zuo et al., 2019); however, BE variants are continuously being improved and exploited to improve targeted specificity (Doman et al., 2020). In this current study, bystander and proximal off-target mutations were also found at *APN*, *CD163*, and *MSTN* gene sites, resulting from the wide editing window of hA3A-BE3-NG. Some engineered precise hA3A variants have been developed to reduce bystander mutations such as hA3A-Y130F *via* narrowing the width of the editing window and eA3A (hA3A-N57G) according to the preferential target base motif (Gehrke et al., 2018; Wang et al., 2018; Liu et al., 2020a). These off-target effects are less crucial when using base editing to introduce premature terminal codons, generating loss-of-function mutations and inactivating protein function. In agricultural breeding, the unpredicted editing byproducts through BEs might be more tolerated and could provide a new source of mutations with favorable economic characteristics. Recently, it has been reported that some new engineering variant of the Cas9, SpRY, which is free of PAM restriction (Walton et al., 2020). In the future, the combine of BEs and the new Cas9 variant will further expand the editing scope to improve base editing tools for pyramid breeding and genetic improvement in livestock.

In summary, we generated hA3A-BE3-NG, a versatile CBEs, that substantially expands the scope and capability of base editing at NGN PAM sites. To our knowledge, this is the first study to precisely edit multiple genes responsible for economic traits in the porcine genome using BEs, suggesting the incredible potential of using BEs to accelerate molecular pyramid breeding in livestock.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material** and further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by Laboratory Animal Welfare and Ethics Committee Institute of Zoology, Chinese Academy of Sciences.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2020.592623/full#supplementary-material

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frontiers in Genetics

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Generation of Multi-Transgenic Pigs Using PiggyBac Transposons Co-expressing Pectinase, Xylanase, Cellulase, β-1.3-1.4-Glucanase and Phytase

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The current challenges facing the pork industry are to maximize feed efficiency and minimize fecal emissions. Unlike ruminants, pigs lack several digestive enzymes such as pectinase, xylanase, cellulase, β-1.3-1.4-glucanase, and phytase which are essential to hydrolyze the cell walls of grains to release endocellular nutrients into their digestive tracts. Herein, we synthesized multiple cellulase and pectinase genes derived from lower organisms and then codon-optimized these genes to be expressed in pigs. These genes were then cloned into our previously optimized *XynB* (xylanase)- *EsAPPA* (phytase) bicistronic construct. We then successfully generated transgenic pigs that expressed the four enzymes [*Pg7fn* (pectinase), *XynB* (xylanase), *EsAPPA* (phytase), and *TeEGl* (cellulase and β-glucanase)] using somatic cell cloning. The expression of these genes was parotid gland specific. Enzymatic assays using the saliva of these founders demonstrated high levels of phytase (2.0∼3.4 U/mL) and xylanase (0.25∼0.42 U/mL) activities, but low levels of pectinase (0.06∼0.08 U/mL) activity. These multi-transgenic pigs are expected to contribute to enhance feed utilization and reduce environmental impact.

Keywords: transgenic pigs, digestive enzymes, salivary gland, polycistronic, PiggyBac

INTRODUCTION

Ineffective digestion in pigs causes excess nutrients into be released to the environment. This results in soil salinity and potential pollution to water and air (Shirali et al., 2012). Domestic pigs mainly feed on common cereal grains, oil seed meals and their by-products. These contain various anti-nutritional factors such as non-starch polysaccharides and phytic acid (Gilani et al., 2005; Bohn et al., 2008). These anti-nutritional factors have an obvious effect on the digestion and absorption of nutrients. It hinders the contact of endogenous digestive enzymes with chyme and hence slows down the nutritional diffusion rate into the intestines (Shirali et al., 2012).

As a consequence, undigested nutrients containing large amounts of inorganic nitrogen and phosphorus are excreted by pigs. This subsequently stimulates the growth of algae and other aquatic plants when they contaminate rivers and streams. This in turn enhances microbial proliferation to ultimately contribute to air pollution.

Several dietary manipulation strategies have been employed to reduce fecal output and nutrient excretion in swine. The most widely practiced strategy is to introduce phytate or non-starch polysaccharides, which are degrading enzymes in feed formula. These could effectively decrease nitrogen and/or phosphorus emissions and hence reduce environmental impact. However, various factors affect the catalytic activity of these microbial enzymes, such as feed processing and storage, feed components, pH, minerals, and temperature. Recently, genetically engineered pigs that express specific or multiple digestive enzymatic genes have provided an alternative strategy to replace dietary enzyme supplementation in the feed. Recently study demonstrated that transgenic pigs that produce salivary phytase had less than 75% of fecal phosphorus. In addition, these pigs required almost no inorganic phosphate supplementation for normal growth compared to non-transgenic pigs (Golovan et al., 2001). In our previous study, we established transgenic pigs that simultaneously expressed three microbial enzymes, β-glucanase, xylanase, and phytase in their salivary glands. These pigs had significantly enhanced growth and reduced fecal nitrogen and phosphorus levels (Zhang et al., 2018).

Cell wall of cereals is mainly composed of cellulose, hemicellulose, and pectin. These components cannot be digested by pigs, which leads to a part of energy loss. Therefore, the expression of pectinase and cellulase in the digestive tract of pigs seems to have crucial and potential value. Among them, pectinase can separate the cellulose molecules wrapped by pectin and reduce the feed viscosity, which increases absorption and release of nutrients, either by hydrolyzing non-biodegradable fibers or by liberating nutrients blocked by these fibers and reduces fecal output (Hoondal et al., 2002). Additional, cellulose and hemicellulose digested by cellulase become monosaccharides or oligosaccharides, which are then absorbed by the digestive tract. The expression of these enzymes in porcine saliva is more convenient to detect than in other places, such as the pancreas (Lin et al., 2015). In this study, we isolated and characterized several novel digestive enzyme genes, and then generated transgenic pigs that expressed these multiple enzymes. These included pectinase, xylanase, cellulase, β-1.3-1.4-glucanase, and phytase. These genes were expressed using a salivary gland promoter. These transgenic pigs had no adverse reactions and had better feed digestion compared to non-transgenic pigs.

MATERIALS AND METHODS

Ethics Statement

All experimental animal protocols were in accordance with the care and use of laboratory animals issued by the Ministry of Science and Technology of China. The use of animal experiments was approved by the Institutional Animal Care and Use Committee of South China Agricultural University.

Plasmid Construction

Three pectinase genes, *PgaA* (*Aspergillus niger* JL-15) (Liu et al., 2014), *Pg7fn* (*Thielavia arenaria* XZ7) (Tu et al., 2014) and *PGI* (*chaetomium* sp.) (Tu et al., 2013); one xylanase *gene XynB* (*Aspergillus niger*) (Deng et al., 2006; Zhang et al., 2018), one phytase gene *EsAPPA* (*Escherichia coli*) (Zhang et al., 2018) and six cellulase and β-glucanase genes (respectively), *cel5B* (*Gloeophyllum trabeum*) (Kim et al., 2012), *egII* (*Pichia pastoris*) (Akbarzadeh et al., 2014), *AG-egaseI* (*Apriona germari*) (Lee et al., 2004), *TeEGI* (*Teleogryllus emma*) (Kim et al., 2008), *cel9* (*Clostridium phytofermentans*) (Zhang et al., 2010) and *Bh-egaseI* (*Batocera horsfieldi*) (Mei et al., 2016) were synthesized by Genscript (Nanjing, China) that were pig codon-optimized. They were then cloned into pcDNA3.1(+). *Pg7fn*, *XynB*, *EsAPPA* and *TeEGI* genes were then head-to-tail ligated using E2A, P2A, and T2A linkers. The ligated construct was named *PXAT*. *PXAT* was then inserted into pcDNA3.1(+) and enzymatic activity was then evaluated. *PXAT* was also inserted into the tissue-specific vector (pPB-mPSP-loxp-neoEGFP-loxp) to generate the final transgene construct (mPSP-PXAT). The primer sets used for cloning are listed in **Supplementary Table 1**.

Cell Culture and Transfection

The PK-15 cell line (ATCC CCL-33) and porcine fetal fibroblasts (PFFs) were cultured in DMEM (Thermo Fisher Scientific, Suwanee, GA, United States) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Suwanee, GA, United States). To evaluate enzymatic activity, PK-15 were grown to 70% confluence, and then transfected using lipofectamine LTX reagent (Thermo Fisher Scientific, Suwanee, GA, United States). Sixty hours post-transfection, the culture supernatant was collected for enzymatic assays. For transgene cell line selection, PFFs were co-electroporated with a circular transposase plasmid (pCMV-hyPBase) and the circular mPSP-PXAT plasmid using the program A-033 on the Nucleofector 2b Device (Amaxa Biosystems/Lonza, Cologne, Germany). After cell attachment, 400 μg/ml G418 (Gibco) was added to the culture media for transfected cell selection. Clonal cells expressing green fluorescence were selected and identified using PCR and sequencing.

Generation of Transgenic Pigs

The EGFP marker gene and neomycin resistant gene (neoR) were removed from transgenic cells using the Cre enzyme (Excellgen, Rockville, MD, United States) and then mixed multiple positive clones as nuclear donors for somatic cell nuclear transfer. Somatic cell nuclear transfer was described as previously studied (Zhang et al., 2018). The reconstructed embryos were transferred into recipient gilts, and piglets were naturally born after gestation. Afterward, genomic DNA from tail was extracted and sequenced using PCR (**Supplementary Table 2**). Additionally, mRNA was extracted from porcine tissue samples and reversed transcribed to cDNA to be used as the template for reverse transcriptase PCR (RT-PCR) and quantitative Real-time PCR (qPCR) (primers used are listed in **Supplementary Table 3**). Relative qPCR and absolute qPCR were used to identify mRNA expression levels and copy number in transgenic pigs, respectively.

Southern and Western Blot Analysis

Genomic DNA was digested with restriction enzymes *Kpn* I or *Eoc47* III, and then run on an 0.8% agarose gel. The digested fragments were then transferred to a nylon membrane. The membrane was hybridized using digoxigenin-labeled DNA probes (**Supplementary Table 2**) for *mPSP* based on the DIG-High Prime DNA Labeling and Detection Starter Kit II protocol (Roche, Mannheim, Germany). For western blotting, saliva was collected and then ultra-filtrated using a centrifugal filter (Millipore, MA, United States). Total protein from saliva was then electrophoresed on an SDS polyacrylamide gel and subsequently transferred to a polyvinylidene fluoride membrane (Millipore, MA, United States). The membranes were incubated overnight at 4°C with primary rabbit polyclonal antibodies (**Supplementary Table 4**) against XynB or TeEGI (purchased from Genscript, Nanjing, China). The salivary amylase antibody (ab34797, Abcam) was used to confirm equal protein loading and the dilution ratio was 1: 1000. Membranes were then washed and incubated with a secondary IgG antibody. Bands were visualized using the UVP software.

Enzymatic Activity Assay

Cell culture supernatants, porcine saliva and rumen fluid of cattle were centrifuged and used for enzymatic activity assay. Saliva collection has been described in previous study (Zhang et al., 2018). Pectinase, xylanase, β-glucanase and cellulase activities were assayed using 1% (w/v) polygalacturonic acid (and 55∼70% esterified pectin, >85% esterified pectin), 1% (w/v) xylan, 0.8% (w/v) β-d-glucan, and 1% (w/v) sodium carboxymethyl cellulose as the substrates, respectively. Reducing sugar content was measured using the 3,5-dinitrosalicylic acid (DNS) method (Kim et al., 2012; Tu et al., 2013). One unit of enzymatic activity was defined as the rate at which 1 μmol of reducing sugar was released per minute. Phytase activity in saliva was measured as previously described (Zhang et al., 2018).

The optimal pH of these proteins was determined at 39.5°C for 30 min in buffers of pH 1.0∼8.0. The buffers used were 0.2 M potassium chloride (KCl)- hydrochloric acid (HCl) for pH 1.0, 0.2 M glycine-HCl for pH 2.0∼3.0, and 0.2 M citric acid-disodium hydrogen phosphate (Na_2HPO_4) for pH 4.0∼8.0. All protein tolerance tests were measured after buffer treatment for 2 h under optimal conditions (optimal pH, 39.5°C and 30 min).

Feeding Management

Transgenic pigs and wild-type littermates were fed on the same diet (**Supplementary Table 5**). They were raised in the same pens fitted with MK3 FIRE feeders (FIRE, Osborne Industries Inc., Osborne, KS, United States). Individual daily feed intake and body weight were recorded when the pigs accessed the FIRE feeders. All pigs had free access to feed and drinking water throughout the growth phase. Blood was collected in a sterile manner at 90 days of age. Serum biochemical parameters of growing-finishing pigs were determined using a Hitachi 7020 full-automatic biochemical analyzer (Japan).

Statistical Analysis

Data were analyzed using the IBM SPSS Statistics 20 (IBM SPSS, Chicago, IL, United States) or SAS 9.4 (SAS Inst. Inc., Cary, NC, United States). For enzymatic activities analysis and relative gene expression, one-way ANOVA or *t*-test was used. For serum biochemical data, unpaired *t*-test (two-tailed) was used. For growth performance, a total of 3 F1 transgenic pigs (1 boar, 2 gilts) and 6 wild-type littermates (3 boars, 3 gilts) were test. When it comes to statistics, multivariate analysis of variance (MANOVA) was performed using the GLM procedure, with sex and initial weight used as the covariate (Zhang et al., 2018). Data were expressed as mean ± SEM. $P < 0.05$ considered statistically significant.

RESULTS

Characterization of the Three Pectinase Genes Expressed in PK-15 Cells

Based on a previous study, we initially selected three pectinase genes *Pg7fn*, *PgaA*, and *PGI* for our studies. Enzymatic activity assays demonstrated that *Pg7fn* had the highest pectinase activity toward 1% polygalacturonic acid and 55∼70% for esterified pectin when used as the substrates, respectively. *PGI* had the second highest pectinase activity toward 1% polygalacturonic acid. However, the activities of *Pg7fn*, *PgaA*, and *PGI* were lower than 0.1 U/mL for >85% esterified pectin (**Figures 1A–C**). We selected *Pg7fn* and *PGI* to determine their optimal pH in 1% polygalacturonic acid. The enzymatic activity of *Pg7fn* increased with pH between 1.0 and 4.0 and reached the highest pectinase activity at pH 4.0, at approximately 1.15 U/mL. The high enzymatic activity was stable at pH 4.0∼6.0, and then decreased significantly after pH 6.0. *PGI* showed a similar trend as *Pg7fn*, but reached its highest enzymatic activity at pH 6.0 (**Figure 1D**). The relative pectinase activities of *Pg7fn* and *PGI* remained at least 56.8 and 46.8% during the stationary phase, respectively. To simulate the pig's digestive tract, we treated *Pg7fn* and *PGI* at 39.5°C for 2 h with different pepsin and trypsin pH solutions. The results indicated that pectinase activity of *PGI* was significantly decreased after pepsin or pH 6.5 trypsin treatment (**Figure 1E**). However, *Pg7fn* was not affected by treatment with pepsin and trypsin. Hence, *Pg7fn* was selected as the candidate gene.

Characterization of the Six Cellulase Genes Expressed in PK-15 Cells

We selected six endo-β-1,4-endoglucanase genes (*cel5B*, *egII*, *AG-egaseI*, *TeEGI*, *cel9*, and *Bh-egaseI*) to measure cellulase and β-glucanase activities at various pH conditions. *egII* and *TeEGI* cellulase activities were significantly higher (0.27 and 0.28 U/mL, respectively) compared to the other genes for 1% sodium carboxymethyl cellulose (**Figure 2A**). Furthermore, β-glucanase activities of *egII* and *TeEGI* were approximately 0.76 and 0.86 U/mL for 0.8% β-D-glucan as substrate, respectively. The other genes had activities of less than 0.09 U/mL (**Figure 2B**). To further determine the enzymatic characteristics of *egII* and *TeEGI*, we optimized the pH levels of the reaction buffer. We

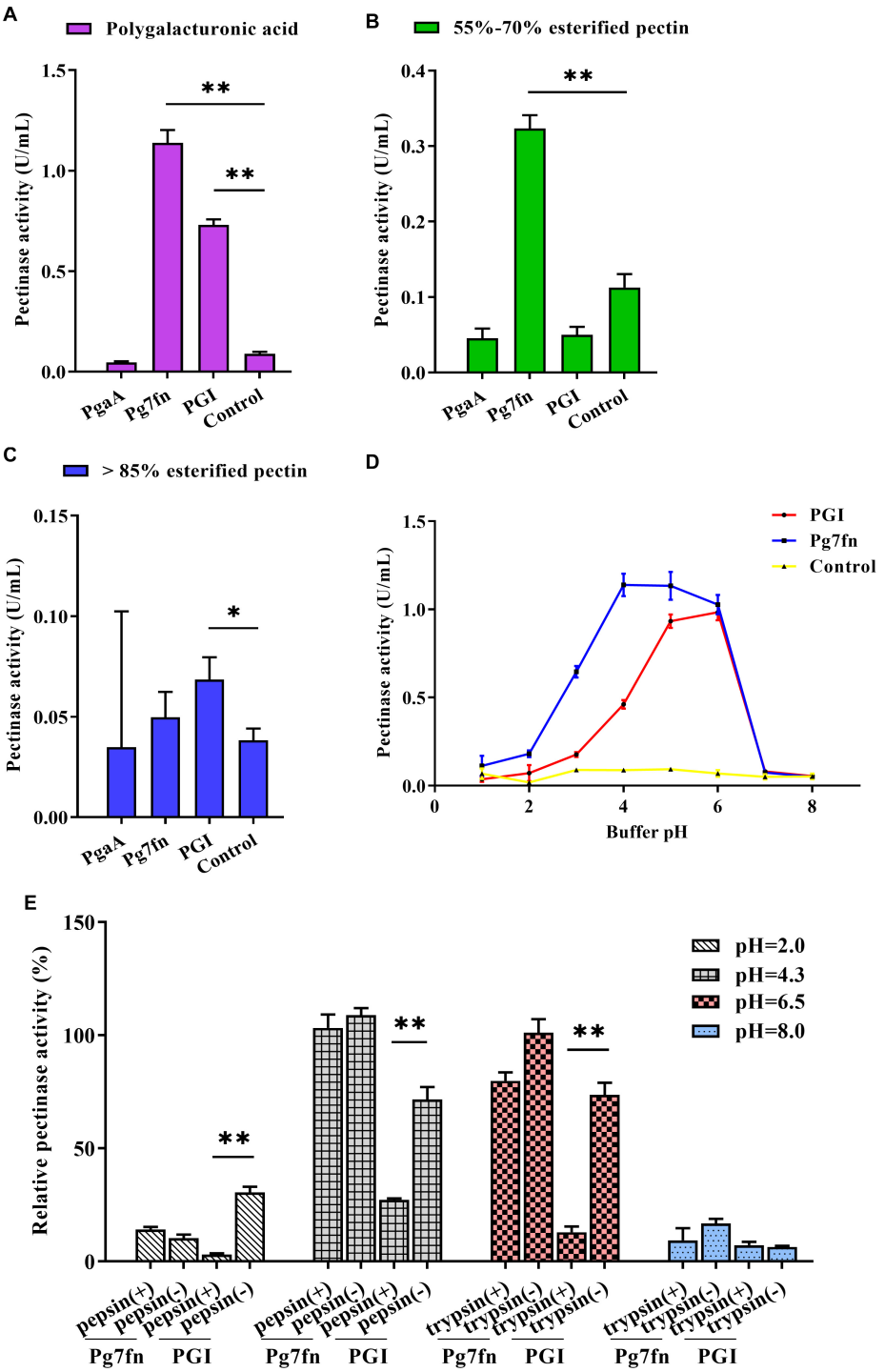

FIGURE 1 | Characterization of the three pectinase genes expressed in PK-15 cells. Pectinase activities of *PgaA*, *Pg7fn*, and *PGI* were evaluated using 1% **(A)** polygalacturonic acid, **(B)** 55∼70% esterified pectin, and **(C)** >85% esterified pectin as substrates at pH 4.5, respectively. **(D)** Pectinase activities of *Pg7fn* and *PGI* at different pH levels (1.0∼8.0). **(E)** *Pg7fn* and *PGI* were incubated with different pepsin and trypsin pH solutions at 39.5°C for 2 h. Control represents the pcDNA 3.1(+) vector. Data were shown as mean ± SEM, $n = 3$ (one-way ANOVA). $^{*}P < 0.05$, $^{**}P < 0.01$.

found that *TeEGI* had the highest cellulase activity at pH 4.5 and had higher residual activity after treatment with pH 3.5∼7.0 (**Figure 2C**). *egII* had similar trends, however, the optimal pH was 5.0. The β-glucanase activity of *TeEGI* was greater than 0.88 U/mL at pH 3.0∼7.0 and reached a maximum of 1.11 U/mL at pH 5.5 (**Figure 2D**). Compared to *TeEGI*, the

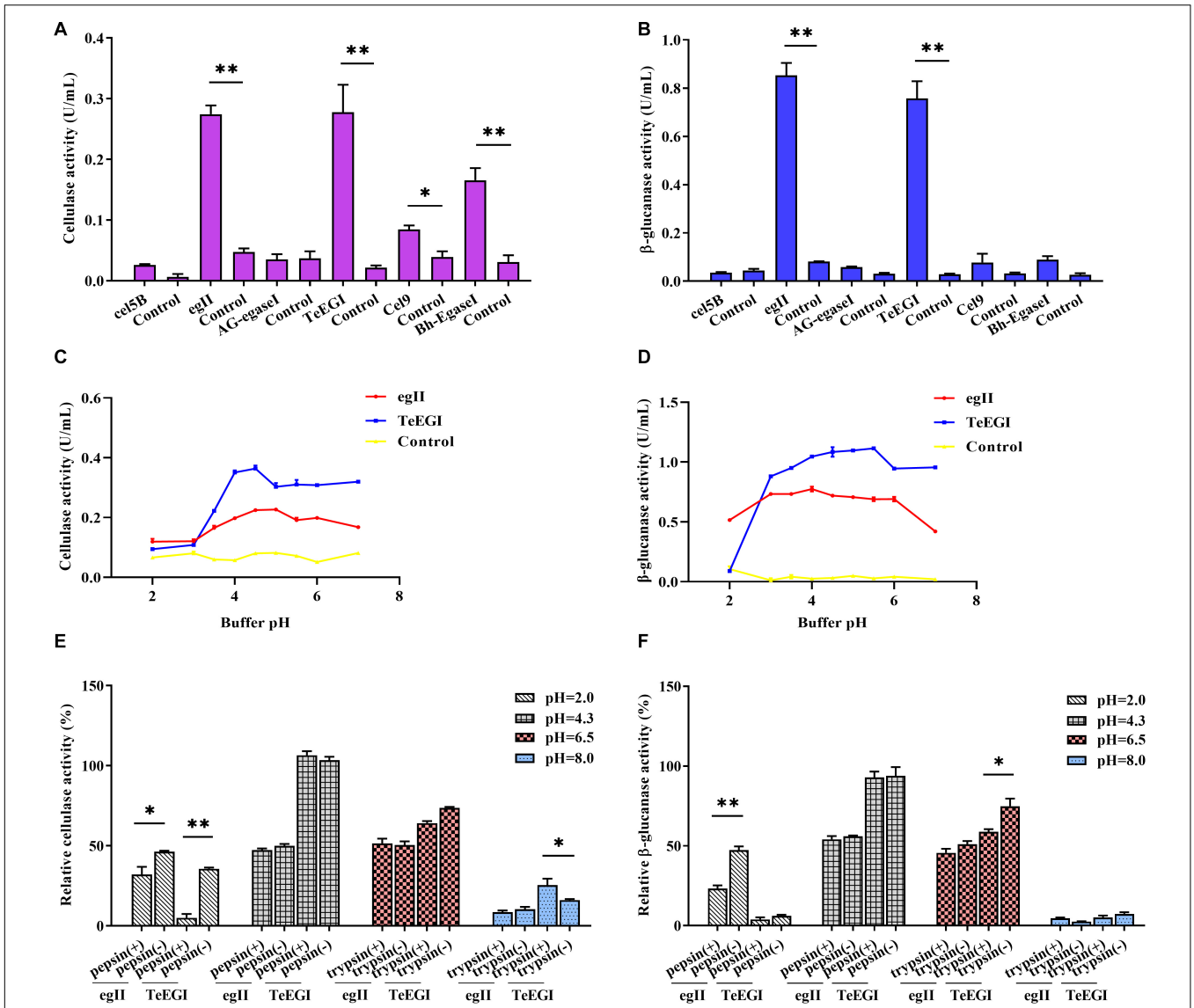

FIGURE 2 | Characterization of six cellulase genes expressed in PK-15 cells. **(A)** Cellulase and **(B)** β-glucanase activities of *cel5B*, *egII*, *AG-egaseI*, *TeEGI*, *cel9*, and *Bh-egaseI* were evaluated at suitable pH conditions. **(C)** Cellulase activities of *egII* and *TeEGI* at different pH levels (2.0∼7.0). **(D)** β-glucanase activities of *egII* and *TeEGI* at different pH levels (2.0∼7.0). **(E)** Cellulase and **(F)** β-glucanase activities of *egII and TeEGI* were measured following incubation with different pepsin and trypsin pH solutions. Control represents the pcDNA3.1(+) vector. Data were shown as mean ± SEM, $n = 3$ (*t*-test). $^{*}P < 0.05$ or $^{**}P < 0.01$.

highest β-glucanase activity of *egII* was 0.77 U/mL and had a residual activity of greater than 50% between pH 2.0∼7.0. We then investigated whether *egII* and *TeEGI* would have high enzymatic activity in different pepsin and trypsin pH buffers. The results indicated that *TeEGI* was resistant to pepsin and trypsin digestion, but *egII* β-glucanase and cellulase were significantly inhibited at pH 2.0 pepsin buffer (**Figures 2E,F**). Hence, we selected *TeEGI* as the candidate cellulase and β-glucanase gene.

Enzymatic Activity Between Polycistronic and Monomeric Constructs

To assess the polycistronic positions of the four genes (*Pg7fn*, *TeEGI*, *EsAPPA*, and *XynB*), we initially included the 2A linker at the end of each corresponding gene. Previous studies had demonstrated that the XynB protein with the P2A residue at the C-terminus still had high xylanase activity in porcine saliva (Zhang et al., 2018). Our results demonstrated that the enzymatic activities of Pg7fn and EsAPPA with 2A residue also kept high relative activity (>77 and >92%, respectively) (**Figures 3A,B**). However, cellulase and β-glucanase activities of *TeEGI* with T2A were significantly reduced to 64.8 and 55.1%, respectively (**Figure 3C**). We fused *Pg7fn*, *XynB*, *EsAPPA*, and *TeEGI* genes head to tail with E2A, P2A, and T2A linkers, and named the final construct *PXAT* (**Figure 3D**). *PXAT* was then ligated into pcDNA3.1(+) to evaluate enzymatic activity. The results showed that using *PXAT,* the pectinase, xylanase, phytase,

cellulase, and β-glucanase activities were significantly reduced to 31.0, 23.5, 30.2, 24.5, and 24.4%, respectively, compared to constructs expressing a single gene (**Figure 3E**). RT-PCR further confirmed that the four co-expressed genes had lower mRNA levels compared to single gene constructs (**Figure 3F**).

Generation and Identification of Transgenic Pigs

PXAT was also inserted into the tissue-specific vector pPB-mPSP-loxp-neoEGFP- loxp to form the final transgene construct (mPSP-PXAT) (**Figure 4A**). The mPSP-PXAT contained the mouse parotid secretory protein (mPSP) promoter, loxp flanking the neo-EGFP marker genes, and the left and right ends of the PiggyBac elements. For transgene cell line selection, PFFs were co-electroporated and G418 was used for selection. The EGFP marker gene was deleted in clonal cells using Cre enzyme prior to somatic cell nuclear transfer (**Figure 4B**). A total of two cell lines were pooled and used as nuclear donors. We transferred a total of 2,096 reconstructed embryos into 10 recipient gilts. Four recipients became pregnant and delivered nine Duroc piglets, of which seven were alive and two were dead (**Supplementary Table 6**). PCR sequencing demonstrated that the five founders were positive for the transgene (**Figure 4C**), but only three of which were alive (**Figure 4D**). Southern blot and quantitative PCR demonstrated that three piglets carried two copies of the transgene (**Figures 4E,F**). A positive boar was euthanized, and tissue samples were collected to determine expression levels of transgenic mRNA at 10 months of age. The results showed that the four genes, i.e., *Pg7fn*, *XynB*, *EsAPPA*, and *TeEGI* were highly expressed in the parotid gland, had low expression in the sublingual and submandibular gland, and not expressed in other tissues (**Supplementary Figures 1,2**). Enzymatic activity assays showed that the saliva from three founders was positive for pectinase, xylanase, and phytase (0.06∼0.08, 0.24∼0.42, 1.9∼3.4 U/mL, respectively) (**Figures 4H–L**). Although we were unable to detect cellulase and β-glucanase activities, interestingly the western blotting analysis indicated that the TeEGI protein was expressed (**Figure 4G**). The F1 pigs (6 transgenic pigs and 6 wild-type littermates) were obtained from 920307 transgenic pig by mating with 2 wild-type gilts (**Supplementary Figure 3**). The results revealed that F1 pigs had pectinase, xylanase, and phytase activities, but no cellulase and β-glucanase activities (**Supplementary Figure 4**), which were consistent with the founders. We also measured serum biochemical markers in both F1 transgenic and wild-type pigs (**Supplementary Table 7**). The results showed that the phosphorus content of transgenic pigs (3.32 mM) was higher compared to wild-type pigs (2.79 mM), which revealed that PXAT pigs maybe promote phosphorus absorption in feed. The growth data of F1 transgenic pigs and wild-type littermates were measured (**Supplementary Table 8**), which shown that PXAT pigs had a tendency to improve growth performance (**Supplementary Figure 5**). Transgenic pigs had better feed conversion ratios compared to wild-type pigs fed the same diet and it took an average of 84 days for transgenic pigs to grow from 30 to 100 kg, whereas wild-type pigs required about 96 days. Compared with wild-type pigs, our transgenic pigs improved feed conversion efficiency by 10.94% and saved an average of 7.08% in feed costs per pig at the 30∼100 kg stage. The growth curve revealed that transgenic pigs grew faster than the littermates, mainly at the 50∼70 kg stage (**Supplementary Figure 6**).

DISCUSSION

Environmentally friendly transgenic pigs could efficiently improve the absorption of anti-nutritional factors, have enhanced growth, and emit lower levels of nitrogen and phosphorus into the environment (Zhang et al., 2018). Previous studies have demonstrated that salivary phytase and xylanase produced from transgenic pigs could effectively reduce phosphorus and nitrogen emissions (Golovan et al., 2001; Zhang et al., 2019). However, no studies to date have investigated cellulase or pectinase transgenic pigs. In this study, we initially selected three pectinase genes (*PgaA, Pg7fn*, and *PGI*) and six cellulase genes (*cel5B, egII, AG-egaseI, TeEGI, cel9*, and *Bh-egaseI*) based on previous studies. Our results demonstrated that *Pg7fn* and *TeEGI* had high enzymatic activities at different pH levels and maintained their stability in different pepsin and trypsin pH buffers. However, several genes had no detectable enzymatic activities. These genes were derived from microorganisms and insects, and the PK-15 cell line that was used to express these genes were unable to properly recapitulate the post-translation modifications needed for enzymatic activity. In addition, the polycistronic order of the four genes (*Pg7fn*, *XynB*, *EsAPPA*, and *TeEGI*) were constructed using the 2A linker at the end of each corresponding gene. In PK-15 cells, our result demonstrated that the target protein with 2A residue at the C-terminus significant reduced enzymatic activity, such as Pg7fn, EsAPPA, and TeEGI, in which, the activities of cellulase and β-glucanase (TeEGI) decline were most pronounced. It seemed that particular protein require special folding compared to the others. The 2A linker was derived from viruses, such as foot-and-mouth- disease virus (F2A), equine rhinitis A virus (E2A), thosea asigna virus (T2A), and porcine teschovirus-1 (P2A). When mRNA is translated, ribosomes jump from Gly to Pro in the 2A sequence. This results in the absence of a peptide bond between Gly and Pro. As a consequence, the upstream protein that is generated has a 17∼19 amino acid peptide that contains Gly at the C-terminus, while the downstream protein that is generated has a Pro residue at the N-terminus, which may affect the spatial folding of the protein. As mentioned previously, the incomplete cleavage of the 2A linker could reduce protein expression (Velychko et al., 2019). There is a parotid gland expression signal peptide in front of each gene, which seems to rule out the reason that the C-terminal protein stays on the endoplasmic reticulum due to the inability of 2A linker to completely cleave (De Felipe et al., 2010). In addition, some proteins may be unable to be completely synthesized due to incomplete translation. This may explain why some of the PXAT enzymatic activities were significantly reduced compared to proteins that were synthesized using the single-gene vector. Finally, the larger size

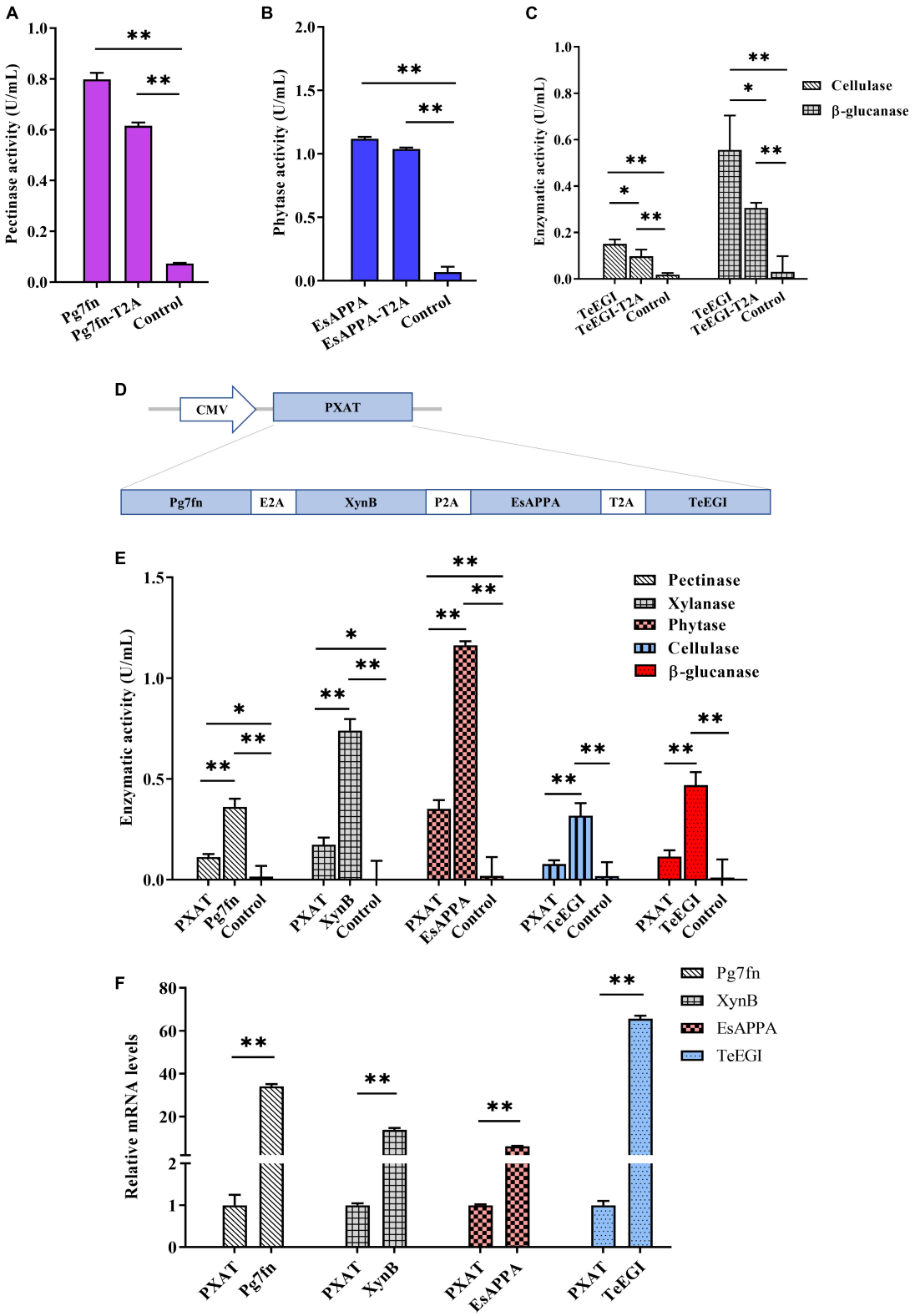

FIGURE 3 | Enzymatic activities between the polycistronic and single gene vector constructs. The effect of 2A linker peptide on **(A)** pectinase, **(B)** phytase, **(C)** cellulase, and β-glucanase activity. **(D)** Schematic of the *PXAT* vector. **(E)** Enzymatic activities between PXAT and its corresponding protein expressed by single gene constructs. **(F)** Relative mRNA expression levels between genes expressed with PXAT and single gene constructs. Control represents the pcDNA3.1(+) vector. Data were shown as mean ± SEM, $n = 3$ (one-way ANOVA). $^{*}P < 0.05$ or $^{**}P < 0.01$.

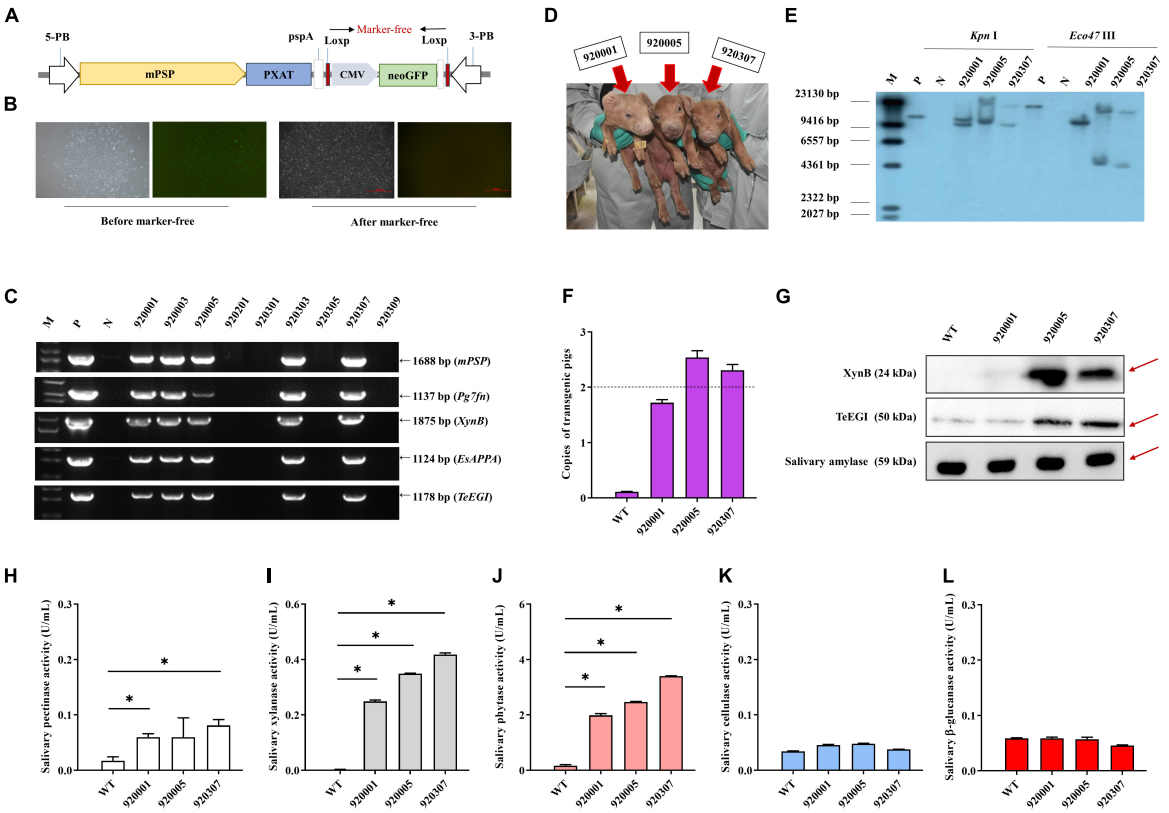

FIGURE 4 | Generation and identification of the transgenic pigs. **(A)** Schematic of the transgenic plasmid mPSP-PXAT. The mPSP-PXAT consisted of the mouse parotid secretory protein (mPSP) promoter, loxp system with the neo-EGFP marker protein, and a PiggyBac transposon. **(B)** EGFP was deleted using Cre recombinase prior to somatic cell nuclear transfer. **(C)** Genomic identification of transgenic piglets using PCR and gel electrophoresis. **(D)** Transgenic piglets at 2-week-old. **(E)** Southern blot analysis of transgene integration in transgenic piglets. Genomic DNA was digested using *Kpn* I and *Eco47* III endonucleases. **(F)** Copy number determination in transgenic piglets by absolute quantification. **(G)** Western blotting analysis of XynB and TeEGI protein expression. Salivary amylase was used as a protein reference. **(H)** Salivary pectinase, **(I)** xylanase, **(J)** phytase, **(K)** cellulase, and **(L)** β-glucanase expression at 4 months. M is the DNA marker, P indicates mPSP-PXAT plasmid; N and WT represent wild-type pigs. Data were shown as mean ± SEM (one-way ANOVA). $^*P < 0.05$.

of the PXAT construct may contribute to lower transfection efficiency compared to constructs having only a single gene (Kreiss et al., 1999).

We successfully generated three transgenic pigs expressing multiple digestive enzymatic genes using the PiggyBac transposon system. Although the transgenic pigs could efficiently express pectinase, xylanase, and phytase, we were unable to detect the enzymatic activity of cellulase and β-glucanase. Western blot analysis indicated that the TeEGI protein was expressed. Previous study suggested that different post-translational modification manners had an effect on protein function (Knorre et al., 2009). Thus, we suspect that TeEGI lacks cellulase activity, possibly due to post-translational modification that alters the folding or function of the protein. Additional, although cellulase was secreted in PK-15 cells, interaction between various cells in an individual could also affect protein function. The polyA tail plays a crucial role in transcription, translation and stabilization of mRNAs (Edmonds, 2002). In our previous work, we used the *bGH-pA* (bovine growth hormone polyadenylation signal) as a termination sequence (Zhang et al., 2018). But in this study, we firstly utilized an unconventional polyA (3′ UTR of parotid secretory protein as a termination sequence *pspA*) to evaluate its effect (**Figure 4A**). We inferred that *pspA* may affect the activity of cellulase and β-glucanase. Due to the unavailability of porcine parotid gland cell lines, we used the PK-15 cell line to express the four enzymatic genes driven by the CMV promoter. However, in animal models, multiple digestive enzymatic genes are driven by the parotid secretory protein promoter. Hence, the low enzymatic activity that was observed may be due to an incompatible promoter.

In previous study, transgenic founders expressed four genes for three digestive enzymes, which produced 0.34∼2.32 U/mL of β-glucanase, 0.40∼2.37 U/mL of xylanase, and 0.40∼5.7 U/mL of phytase in the saliva (Zhang et al., 2018). High β-glucanase was mainly supported by *bg17A* and *eg1314* genes not *TeEGI*. The transgenic founders in this study had a slightly lower xylanase activity (0.25∼0.42 U/mL) than the previous pigs. However, the pectinase (0.07∼0.83 U/mL) and xylanase (0.34∼1.20 U/mL) activities of the F1 generation have been improved, which may be related to age and individuals (Li et al., 2020). In addition, seasonal changes, eating environment and saliva collection

methods may affect the protein content of saliva, thereby affecting the secretion of digestive enzymes (Sanchez et al., 2019). Thus, further exploration of porcine saliva secretion patterns in the future is essential for the study of saliva bioreactors. Moreover, with reference to previous research, we were able to predict that the genetically modified pigs in this study would have a weaker capacity to reduce emissions than the previous ones, mainly because their key enzyme (TeEGI) was inactivated in the salivary glands. Therefore, the establishment of a porcine parotid gland cell line is beneficial for the screening of polycistrons and the model preparation of saliva bioreactors.

The ability of ruminants to digest plant fibers is well known, and the average pH of their rumen tends to be neutral. However, as a monogastric animal, pigs have an average gastric pH of about 4.4 (Merchant et al., 2011), which causes most of the digestive enzymes derived from bacteria and other microorganisms to be inactivated in the acidic stomach. In order to have a clearer understanding of our transgenic pig's ability to digest feed fibers, we measured their ability to digest anti-nutritional factors with bovine rumen. The results revealed that our transgenic pigs had better pectinase, xylanase and phytase activities than cattle, while the cellulase and glucanase activities of cattle were higher than those of our transgenic pigs (**Supplementary Figure 7**). The reason why the activities of pectinase and xylanase of cattle were lower than that of our genetically modified pigs was mainly related to the feed ingredients of cattle (Mendoza et al., 2014), which affected the microbial environment of cattle rumen (McCann et al., 2014). Glucanase or cellulase is an extremely important glycol-hydrolase for improving the digestibility of pig feed. Therefore, it may be more effective to screen out a variety of acid-resistant hydrolases or mutants, and even use acid-resistant molecular chaperones such as HdeA or HdeB on improving porcine digestibility and reducing environmental release in the future.

In summary, pork is a daily necessity of people, with an annual output exceeding 100 million tons. However, a significant amount of food was wasted due to porcine low feed utilization. Additional, nitrogen and phosphorus that can not be absorbed by pigs are discharged into the environment, which will increase the sewage treatment load and even cause water pollution. In this study, we successfully produced an animal model using somatic cell transfer. These transgenic pigs expressed, under the control of the parotid gland specific promoter, four enzymatic genes [*Pg7fn* (pectinase), *XynB* (xylanase), *EsAPPA* (phytase), and *TeEGI* (cellulase and β-glucanase)], although TeEGI was inactive. It offer a valuable experience for the global environmental concerns and the inefficient absorption of feed in livestock.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

All experimental animal protocols were in accordance with the care and use of laboratory animals issued by the Ministry of Science and Technology of China. The use of animal experiments was approved by the Institutional Animal Care and Use Committee of South China Agricultural University.

AUTHOR CONTRIBUTIONS

HW, GL, and XZ wrote the manuscript. XZ, DL, and ZW conceived and designed the project. CZ, YS, JM, GL, and HW collected and organized the data. CZ, HW, GL, and XZ analyzed and interpreted the data. JS, RZ, ZL, ZW, DL, and XZ provided the technology and resources. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2020.597841/full#supplementary-material

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Analysis of Wild Type LbCpf1 Protein, and PAM Recognition Variants, in a Cellular Context

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Nucleases used in genome engineering induce hydrolysis of DNA phosphate backbone in a sequence-specific manner. So far CRISPR-Cas, the RNA-guided nucleases, is the most advanced genome engineering system. The CRISPR nucleases allows recognition of a particular genomic sequence with two distinct molecular interactions: first, by direct interaction between the nuclease and the protospacer-adjacent motif, wherein discrete amino acids interact with DNA base pairs; and second, by hybridization of the guide RNA with the target DNA sequence. Here we report the application of the single strand annealing cellular assay to analyze and quantify nuclease activity of wild type and mutant CRISPR-Cpf1. Using this heterologous marker system based on GFP activity, we observed a comparable PAM recognition selectivity with the NGS analysis. The heterologous marker system has revealed that LbCpf1 is a more specific nuclease than AsCpf1 in a cellular context. We controlled the *in vitro* activity of the Cpf1 nuclease complexes expressed in mammalian cells and demonstrated that they are responsible of the DNA cleavage at the target site. In addition, we generated and tested LbCpf1 variants with several combinations of mutations at the PAM-recognition positions G532, K538 and Y542. Finally, we showed that the results of the *in vitro* DNA cleavage assay with the wild type and mutants LbCpf1 corroborate with the selection of 6TG resistant cells associated to the genomic disruption of *hprt* gene.

Keywords: Cpf1, genome engineering, PAM, specificity, selectivity

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INTRODUCTION

For reverse genetics approaches, genomic modification is often required to establish the functional link between an observed phenotype and a particular gene. Precise DNA modification such as base substitution or introducing insertion/deletion are crucial for those functional validation. Indeed, generating nucleotide substitutions (point mutations) or indels (insertions/deletions) can

Abbreviations: 6-TG, 6-Thioguanine; As, Acidaminococcus; Cas, CRISPR-associated protein; CMV, early enhancer/chicken β actin promoter; Cpf1, CRISPR from Prevotella and Francisella 1; CRISPR, Clustered regularly interspaced short palindromic repeat; crRNA, CRISPR RNA; DMEM, Dulbecco's modified Eagle Medium; DMSO, Dimethyl sulfoxide; DNMT, DNA methyltransferase; DSB, Double strand Break; Fn, Francisella novicida; FokI, Flavobacterium okeanokoites; GFP, Green fluorescent protein; HPRT, Hypoxanthine Phosphoribosyltransferase; HR, Homologous recombination; Indels, Insertion- deletion; Lb, Lachnospiraceae bacterium; MBP, Maltose binding protein; NHEJ, Non-homologous end joining; PAM, Protospacer adjacent motif; sgRNA, single guide RNA; Sp, Streptococcus pyogenes; SSA, Single strand annealing; TALEN, transcription activator-like effector nucleases; tracrRNA, Transactivating CRISPR RNA; Wt, wild type; ZFN, Zinc finger nuclease.

lead to gene knockout by generating a premature stop codon or to truncated gene products by frameshift mutation. Various strategies have been developed to induce genetic alterations, and the resulting cells harboring desired changes are suitable for phenotypic analyses. In some cases, genetically modified cells or organisms can be isolated from the population upon a positive selection pressure. The protein Hypoxanthine Phosphoribosyl transferase (HPRT) is an enzyme that catalyzes conversion of hypoxanthine to inosine monophosphate, and guanine to guanosine monophosphate. The protein is encoded by a unique gene (*hprt*) in human cells, carried by the chromosome X. Disruption of the *hprt* gene allows a survival of the mutated cells upon treatment with the cytotoxic chemical agent 6-Thio-Guanine (6-TG) (Fenwick and Caskey, 1975), thus leading to positive selection of *hprt* homozygote -/- cells in cultures.

In recent years, the zinc finger nucleases (ZFNs) (Bibikova et al., 2001), the transcription activator-like effector nucleases (TALENs) (Christian et al., 2010), and more recently, the bacterial adaptive CRISPR-Cas systems (Gasiunas et al., 2012; Jinek et al., 2012) were efficiently established to generate eukaryotic mutant cells. Those engineered nuclease enzymes induce DNA double strand break (DSB) at genomic target sites. The CRISPR-Cas system has been developed to perform specific genetic modifications by generating precise DSB in the targeted genomic locus. Unlike ZFNs and TALENs that fuses DNA-binding domains to the DNA cleavage domain from the *FokI* restriction endonuclease, the CRISPR-Cas system require a guide RNA that directs the nuclease to the target DNA sequence. The ribonucleoprotein (RNP) complex catalyze the DNA cleavage at target sites. Since DNA is targeted by specific base-pairing with the guide RNA, the CRISPR-Cas system is highly versatile and more specific for the binding to target DNA sequences within the genome (Kim et al., 2015; Yan et al., 2017). More recently, base editors have been developed by fusing Cas9 nickase D10A variant with cytosine deaminase, or engineered adenine deaminase domain. Each fused protein, termed Cytosine Base Editor (CBE) and Adenosine Base Editor (ABE), are enabled to catalyze the conversion of C-G to T-A and A-T to G-C base pairs, in a sequence specific manner (Komor et al., 2016; Gaudelli et al., 2017).

Two of the most common nucleases for CRISPR-Cas systems, SpCas9 and Cpf1 (also named Cas12a) proteins (Zetsche et al., 2015), share similar features when associated with their respective guide RNA. However, they differ in structural organization and DNA cleavage properties. Cpf1 only requires a crRNA to bring the specificity for a target in the RNP complex, whereas SpCas9 requires additional trans-activating RNA (tracrRNA) as well, that bound to the protein for its stabilization. This was simplified by fusing the crRNA and tracrRNA into a chimeric RNA called single guide RNA (sgRNA) (Jinek et al., 2012). In addition to the DNA sequence recognized by the guide RNA, the CRISPR proteins interact with a short DNA region adjacent to the target DNA sequence, termed as Protospacer Adjacent Motif (PAM). The PAM recognized by SpCas9 is 5′-NGG-3′ and is located downstream to the target DNA sequence. The sequence recognized by Acidaminococcus (As) and Lachnospiraceae bacterium (Lb) Cpf1 is 5′-TTTv-3′ and is located upstream of the target DNA sequence. Nevertheless, those two CPF1 proteins have a different specificity and selectivity for the PAM sequence. In addition, the DNA cleavage site itself is different between those complexes, the SpCas9 cleavage site is proximal to the PAM sequence and inside of the nuclease structure. In contrast, the cleavage site of the Cpf1 nucleases complexes is distal to the PAM sequence, and outside of the core structure. The DSB induced by SpCas9 results in blunt ends, whereas Cpf1 generates 5′ overhangs end. The target specificity of SpCas9 and Cpf1 proteins are determined by the crRNA sequence, and the molecular interaction between few amino acids from the PAM Interacting Domain (PID) and the PAM sequence. The crystal structure of SpCas9 (Anders et al., 2014; Nishimasu et al., 2014) in complex with the sgRNA and the target DNA revealed two arginine residues that are directly in contact with the two guanines of the PAM sequence on the major groove side of the DNA. This interaction involves hydrogen bonds that take place on the major groove side of the DNA. By comparison, crystal structure of AsCpf1 (Yamano et al., 2016) in complex with the crRNA and target DNA shows that the PAM sequence recognition requires two lysines (**Figure1**: residues K548 and K607). The two lysine are interacting with the second complementary base (Adenine) of the 5′-TTTv-3′ sequence. Both lysines are in contact from both sides of the double helix, K548 on the major groove side, and K607 on the minor groove side. Two amino acids, N552 and S542, are near the residue K548 and structurally in the vicinity of the DNA. Those amino acids are interacting with the phosphates from the DNA backbone. In LbCpf1 structure, the PAM/PID interaction is similar (Lysines K538 and K595) to the AsCpf1, but the amino acids in vicinity with the DNA backbone are different (G532, Y542). Because of the structural features of Cpf1 proteins, we focused our research on the properties of those proteins in a cellular context.

In this report, we used two strategies to analyze the nuclease activities in eukaryotic cells. First, the genomic modifications were measured by the indels frequency at the target site, which are generated by the non-homologous end joining (NHEJ), using the deep sequencing approach. Secondly, the single-strand annealing (SSA) based on a plasmid harboring the nuclease target site that is between two fragments of the reporter gene with repeated sequences. Inducing DSB at the target site, and the subsequent recombination of the repeated sequences by homology-directed recombination (HR), results in the restoration of a functional reporter gene. Thus, the nuclease activity in the cells is measured by the signal from the heterologous reporter gene (Cathomen et al., 2008; Tóth et al., 2016).

The study is investigating the PAM recognition of Cpf1 proteins in a cellular context using deep sequencing with a crRNA collection that target the exon 3 of *hprt* gene. We focused our tests on the variation of two nucleotides from the PAM sequence (position -2 and -3, 5′-TNNa-3′) that are common to all Cpf1 proteins orthologues. Thereby each selected target sequences requires one of the 16 possible 5′-TNNa-3′ sequence. A 16 pGFP-SSA plasmid library was also designed with the same DNA target sequence but flanked with the different 5′-TNNa-3′ PAM. The indels frequencies observed with AsCPF1 and LbCPF1 are consistent with the SSA assay results from the pGFP-SSA library.

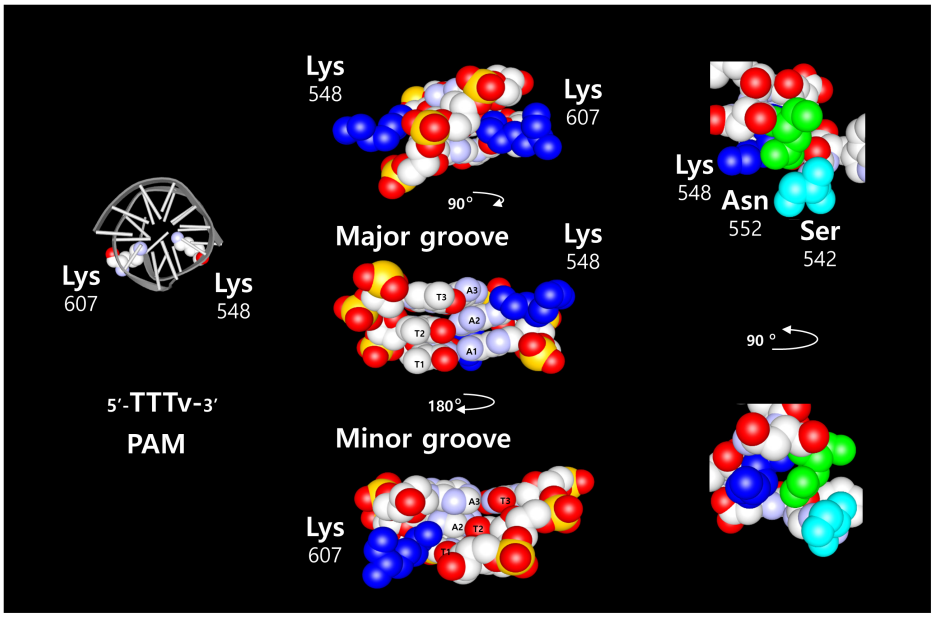

FIGURE 1 | Graphic representation of AsCpf1 amino acids in complex with its DNA sequence. On the left side, schematic structure of the DNA with the two Lysine 548 and 607 required for the AsCpf1 5′-TTTv-3′ PAM sequence recognition. The three T-A base pairs are represented with the two Lysine 548 and 607 (blue) to show the direct contacts between the second Adenine base and the two Lysine. The contact occurs from both side of the DNA helix. Lysine 548 on the major groove and Lysine 607 on the minor groove. Other amino acids in vicinity to the lysine 548 (Asparagine 552: green, and serine 542: light blue), exposed on the major groove side of the DNA, are depicted in the picture on the right side.

In addition, we controlled the *in vitro* nucleases activity with the purified Cpf1 complexes from mammalian cells. We designed, and tested LbCpf1 mutations at position 532, 538 and 542 to modulate the PAM recognition. Using the SSA assay, we observed that the mutant LbCpf1-NS has the same PAM specificity and selectivity than AsCpf1. We found that the mutants LbCpf1 RAR and RSR recognize the three sequences 5′-TTTa-3′, 5′-TATa-3′ and 5′-TACa-3′. Those mutants are suitable to perform the *in vitro* cleavage of DNA substrates with the corresponding PAM sequences. Finally, we controlled their efficacy to target genomic sites, by disrupting the *hprt* gene (selection of 6-TG resistant cells), with guides crRNA associated to the three different types of PAM sequences at the genomic sites.

MATERIALS AND METHODS

Cell Culture

HeLa (ATCC CCL-2) and HEK 293 (ATCC CRL-1573) cells were cultivated at 37 °C, 5 % CO2 in Dulbecco's modified eagle's medium containing 4.5 mg/L D-glucose (DMEM, WelGene, South Korea), Glutamine 2,5 mM, supplemented with 10 % heat inactivated fetal bovine serum (FBS, Gibco, United States) and antibiotics solution (100 U/ml penicillin and 100 µg/mL streptomycin solution, Gibco, United States). Cells were frozen at the density of 1×10^7 cells per mL in DMEM containing 50 % FBS and 10 % dimethyl sulfoxide (DMSO, Sigma-Aldrich, United States). For Isolation of *hprt* −/− cells, the transfected cells were incubated for 4 days with the 6TG (Sigma-Aldrich, United States) at 20 µM final concentration. In order to remove the product of the target gene insight the cells, a minimum of 5 days incubation is required prior the selection with the 6TG. For shorter incubation time, only the nuclease effects on the target DNA can be elucidated. To visualize the cells, the cultures were washed and stained using methylene blue and inspected by microscopy (Pimovert Zeiss, Germany).

Plasmids and Guide RNA

For the design of the target DNA used in this study, the crRNA sequence was established to target the human exon 3 *hprt* gene was selected among a collection of guide sequences (data not shown) and demonstrated a high genomic modification ability. The expression plasmid for the crRNA LbCpf1 was transfected into Hela cells, after 5 days exposure and cell culture, the cells were selected using the 6TG and analyzed by Deep sequencing (See **Supplementary Data 1**). A *dnmt1* crRNA target sequence was used as a negative control to confirm the specificity. All the *hprt* gene target sequences used in this study were cloned in the U6 promoter-driven RNA expression vector pU6-As-crRNA and pU6-Lb-crRNA for AsCpf1 and LbCpf1, respectively (The oligonucleotide sequences cloned are reported in **Supplementary Data 2**).

The pGFP-SSA plasmids were generated by cloning of two *gfp* gene fragments containing the left- and right-repeats (LR and RR, respectively: **Supplementary Data 3A,B**) into pcDNA3 vector. The SSA target DNA fragments, with the corresponding PAM sequences, were inserted between the LR and RR using *EcoRI* and *BamHI* restriction sites. An adenine was placed at the first

position of the PAM sequence adjacent to the target sequence and described as 5′-TNNa-3′ (The 5′-TNNa-3′ sequences cloned in the pGFP-SSA constructs are reported, **Supplementary Data 4**). The assay was optimized, and controlled, by comparison of the Hela cells transfection with the full length *gfp* and the *EcoRI* linearized SSA plasmids (**Supplementary Data 5A**). In order to reduce the background signal in the experiments, the plasmids were purified using anion-exchange silicate resin (Macherey-Nagel, Germany) with bacteria cell cultures at low turbidity (Exponential phase) to prevent the presence of linearized plasmid DNA in the samples. The DNA concentration was characterized by spectrometry (OD260 nm) and controlled by double DNA digest with restriction enzymes and agarose gel stained with ethidium bromide. The quality and the quantity of DNA in the samples were compared by intensity of the bands in the agarose gel. All the DNA sequences used in this study, that target *hprt* exon 3, were tested for their activity by co-transfection with the corresponding 5′-TTTa-3′ PAM pGFP-SSA target reporter and the Wt LbCpf1 protein (**Supplementary Data 6**).

The expression plasmids of human codon-optimized AsCpf1 (pCDNA3-AsCpf1) and LbCpf1 (pCDNA3-LbCpf1) were prepared according to previously reported study (Tóth et al., 2018). The plasmid expressing AsCPF1-MBP and LbCPF1-MBP fusion proteins, the MBP tag coding cDNA was cloned, at the C terminal part of *Cpf1* genes, between *BamHI* and *EcoRI* restriction sites. As described (Bokhove et al., 2016), the MBP tag C terminal fusion allow the purification of full-length proteins in a single step.

LbCpf1 PAM mutants were generated using QuickChange II Site directed Mutagenesis Kit (Agilent, United States) according to manufacturer's instructions. The oligonucleotides used for mutagenesis are reported in **Supplementary Data 4**.

SSA Assay

Cryopreserved HeLa cells were thawed and reconstituted in DMEM with 10 % FBS at a density of 5×10^5 cells per mL, then plated in 24 well plate (1 mL per well) 4 h prior to transfection. Transfection was performed using Lipofectamine 2000 (Invitrogen, United States) under the following conditions: 5 μL Lipofectamine 2000 (1 mg/mL), 300 ng of Cpf1 expression vector, 500 ng of the crRNA plasmid, 500 ng of corresponding pGFP-SSA target construct, were mixed in OptiMEM I (Gibco, United States) to a final volume of 500 μL. The pGFP-SSA 5′-TNNa-3′ PAM plasmid library experiments were performed with a lower quantity of nuclease expression vector (100 ng). After 2 days incubation, image of fluorescent cells, expressing GFP protein, were obtained using a Nikon Eclipse confocal microscope (Nikon, Japan). The cell surviving was measured using Cell-titer-Glo luciferase assay (Promega, United States). The fluorescence was characterized using a Filter max F5 reader Multimode (Molecular Device, United States). For each transfection experiment a pGFP construct was co-transfected under the same conditions as a GFP signal stability control, and a pGFP-SSA *EcoRI* linearized as a reference for the percentage of fluorescence calculation. Duplicates or triplicates transfection were performed during the same experiment for a better quantitative reproducibility.

Deep Sequencing Analysis

Hela cells were seeded (5×10^5 cells /well) in a 24 well plate and were transfected with plasmids expressing crRNA and Cpf1 Wt or mutants (500 ng of each vectors) using lipofectamine 2000 (Invitrogen, United States), according to the manufacturer instructions. Following 3 days exposure and incubation, the cells were collected, and genomic DNA isolated using DNeasy Blood and Tissue kit (Quiagen, Netherlands). The genomic region, encompassing the *hprt* exon3 target sites was amplified using the following primers: NGS1-Fw (5′- CAAGGTCTTGCTCTATTGTCCAG-3′) and NGS1-rev (5′-CCCTTGAGGACACAGAGG-3′). The amplified fragments were analyzed by deep sequencing, according the previously published experimental procedure (Kim et al., 2016; Tóth et al., 2018).

Protein Expression and *in vitro* Nuclease Activity

HEK293 cells were cultivated in a 10 cm dish at 80% confluency and were transfected using lipofectamine 2000 reagent (100 μL) with 50 μg pcDNA3-AsCPF1-MBP or pcDNA3-LbCPF1-MBP, with or without 10 μg of the pU6 guide RNA expression vector. 48 h post transfection, the cells were collected, and Cpf1-MBP fusion protein were purified with amylose resin (New England Biolabs, United States). The cells were washed with PBS and re-suspended in 500 μL of Lysis buffer containing Tris-HCl pH8 100 mM, KCl 50 mM, MgCl2 20 mM, Triton x-100 0.1%, RNase Inhibitor (200 Unit/mL: New England Biolabs, United States) and Protease inhibitor minus EDTA (Merk, United States). The cells were broken by thermal shock (freezing in liquid nitrogen and thawing at 42°C). After centrifugation for 10 min at 15000 rpm, the supernatants containing the recombinant proteins were incubated for 2 h with amylose beads (150 μL). The beads were washed three times with 1 mL of lysis buffer, the recombinant proteins were eluted with a Lysis buffer adjusted at 20 mM maltose (Merk, United States). The *in vitro* cleavage assay was performed in lysis buffer, using *XmaI* linearized pGFP-SSA target vector as a substrate DNA. After 15 min incubation at 37°C, the reactions were arrested by addition of EDTA, proteinase K, and incubated at 60°C for 10 min. The DNA fragments were analyzed on a 1% agarose gel with ethidium bromide.

RESULTS

Analysis of Cpf1 Proteins PAM Selectivity Using Deep Sequencing at Genomic Target Sites

We first investigated the Cpf1 proteins PAM selectivity in a cellular context by analyzing the activity of several crRNA with different PAM sequences that target the same genomic area corresponding to the *hprt* exon 3. We used deep sequencing analysis to quantify indels frequencies that reflect the nuclease activity at the genomic target sites. The Cpf1 proteins need to interact with the PAM adjacent sequence to perform the DNA cleavage. Based on the protein/DNA interactions described in Cpf1 complexes resolved structures, we decided to select

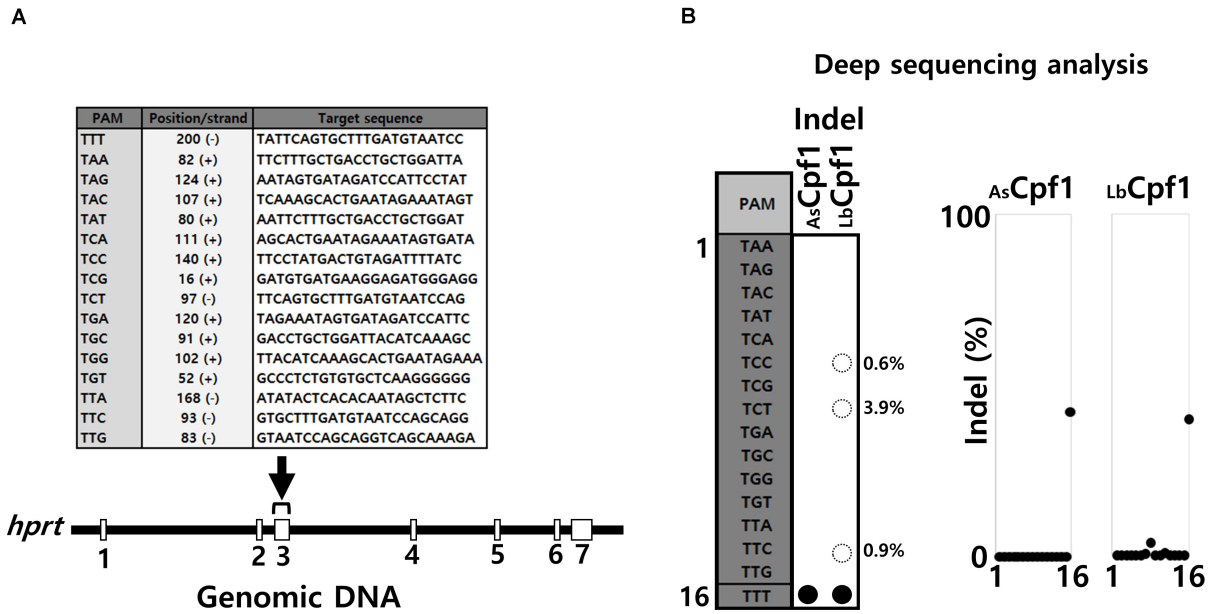

FIGURE 2 | Comparison of AsCPF1 and LbCpf1 PAM recognition by Deep sequencing analysis. **(A)** Target sequences: The sequences of the target *hprt* gene (exon3) are depicted in the table with the corresponding flanked 5′-TNNa-3′ sequences at the genomic sites. **(B)** NGS analysis: Deep sequencing analysis and indels frequency (%) observed for each guide RNA by AsCPF1 and LbCpf1 protein co-expression. The table on the right side summarize the Graph of the activity with the 16 guide RNA/5′TNNa-3′ for each protein. The expression vectors quantity was adjusted for both enzyme (AsCPF1 and LbCpf1) to obtain the same indels frequency with the optimal 5′-TTTa-3′guide RNA.

16 different endogenous target sequences (**Figure 2A**) that are flanked by one of each different permutation of 5′-TNNa-3′ PAM sequences. Hela cells were transfected with AsCpf1 and LbCpf1 expression vectors, together with each crRNA expression plasmids. Following genomic DNA isolation for each sample, indels frequencies induced by the different crRNA were determined by deep sequencing analysis for an identical PCR amplicon. The crRNA targeting the site flanked by the PAM 5′-TTTa-3′ sequence was used as a control, to verify that the activity of AsCpf1 and LbCpf1 are similar under our experimental conditions. The results depicted in **Figure 2B** show the quantification diagram (right panel) and a table summarizing the activity observed with each crRNA (left panel). For both proteins, AsCpf1 and LbCpf1 with the PAM 5′-TTTa-3′ crRNA, the indels frequencies detected are around 40%. All the other crRNAs, analyzed under AsCpf1 protein expression are not able to induce indels at the corresponding target sites, whereas LbCpf1 when combined with crRNA targeting the sequences flanked by the PAM 5′-TCTa-3′, 5′-TTCa-3′ and 5′-TCCa-3′ showed some nuclease activities, represented by low indels frequencies.

Analysis of Cpf1 Proteins Activity Using SSA Assay

We next investigated the usage of the SSA assay with a plasmid to assess the activity of nuclease in a cellular context. We used the GFP reporter gene as a readout marker. This strategy is based on the reconstitution, by homologous recombination (HR), of a functional full-length *gfp* gene. The *gfp* reporter gene is activated because of the cleavage of the plasmid by a nuclease at a specific target site. The target site from *hprt* gene was inserted between two inactive fragments with a repeated sequence (**Figure 3A**). After DNA cleavage, the repeated sequence recombines by HR, thus generating an active gene. This cellular assay allows to analyze the cellular activity of a nuclease upon its co-transfection with the reporter plasmids. Nevertheless, this artificial system reflects a comprehensive nuclease activity if the enzyme is highly specific, and sufficiently selective, based on the fact that unspecific nuclease activities are generating multiple cleavages of the plasmid, and leads to the degradation of the reporter construct. This in turn abolish the reporter gene signal and, non-specific cleavage is generating false negative samples in the SSA analysis. Thus, for a short time of incubation (48 h) after the plasmid transfection, the destabilization of the GFP signal suggest that the nuclease as a high non-specific DNA cleavage activity that leads to the reporter plasmid degradation.

We analyzed the activity of Cpf1 proteins, by co-transfection of the nuclease expressing plasmids (300 ng DNA of CRISPR protein expression vectors) with the corresponding crRNA expression plasmids, and reporter vector. The experiment was performed with the pGFP control, or the pGFP-SSA construct with or without a specific target sites flanked by the optimal 5′TTTv-3′ PAM sequence (**Figure 3B**). Without co-transfection of a nuclease (**Figure 3B**, Cont. Panels), only the pGFP reporter vector show fluorescent cells. Indeed, the pGFP SSA vectors require a plasmid linearization between the repeated sequences to induce GFP expression (**Supplementary Data 5A**, transfection control with a circular and an *EcoRI* linearized pGFP-SSA vector).

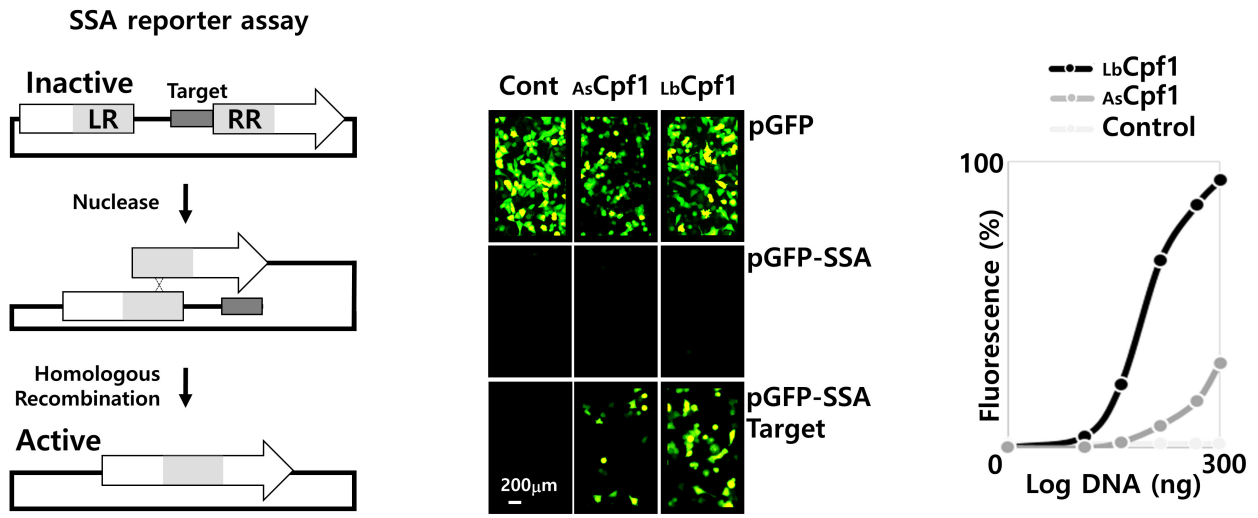

FIGURE 3 | SSA assay. **(A)** Representation of the SSA assay: The two fragments of the inactive reporter gene are represented with the nuclease target site in between. The left and the right repeated sequence are depicted as LR and RR, respectively. Following the nuclease cleavage, at the target site, the fragment is recombined to reconstitute a full-length active reporter gene. **(B)** Pictures of cells using a fluorescent microscope (×20): Fluorescent cells were inspected after transfection and 48 h incubation. Three reporter constructs were transfected with the nucleases, the pGFP native expression vector to control the GFP signal stability, the negative control pGFP-SSA vector without target site, and the pGFP-SSA 5′-TTTa-3′PAM target plasmid. The control panels (Cont.) on the left, are reporter vectors transfected without nucleases. **(C)** Dose response curve of the AsCPF1 and LbCPF1: Cpf1 expression vectors were co-transfected with the guide expression vector and the pGFP-SSA 5′-TTTa-3′PAM reporter construct. A negative control was performed with LbCpf1 and guide expression vector against the *dnmt1* gene.

The pGFP control experiment (upper panels) was performed to observe the stability of the fluorescent signal upon nuclease expression. The result shows that the number of fluorescent cells decreased by AsCpf1 nuclease co-transfection, indicating that the GFP signal is destabilized, whereas the GFP expression remains similar to the GFP control with LbCpf1 (**Figure 3B**). The quantification (**Supplementary Data 5B**, quantification of GFP signal and number of cells) indicated that the nuclease expression did not significantly affect the cell surviving, but the GFP fluorescent signal is dramatically reduced with the AsCpf1 nuclease and crRNA guide expression. The pGFP-SSA target plasmids (**Figure 3B**, lower panels) show fluorescent cells with all nucleases tested, whereas no cells are GFP positive with the plasmid construct without target site (middle panels). The results are demonstrating the requirement of the target site, between the repeated sequences, to induce GFP expression. The GFP positive cells are more abundant with LbCpf1 than with AsCpf1, suggesting that the GFP signal destabilization occur with the pGFP-SSA vector and AsCpf1, which is consistent with the case of pGFP and AsCpf1. The **Figure 3C** show the quantification, and the dose response curves, obtained with both nucleases targeting the same *hprt* sequence, and a negative control corresponding to LbCPf1 co-transfected with a non-specific crRNA targeting *dnmt1* gene. The LbCpf1 nuclease exerts a better SSA activity than AsCPf1. To compare the SSA activity of the Cpf1 nucleases with spCas9, we also analyzed the spCas9 wild type and nickase D10A activity (**Supplementary Data 7**). The results demonstrated that spCas9 has a similar activity to AsCpf1 and show that the Nickase D10A, that require an inverted tandem repeat, is more active than the Wt SpCas9 and do not destabilize the GFP signal in the control experiment.

Finally, we controlled that the expressed Cpf1 nucleases in the cells are functional and cleave specifically the plasmid at the target site. Because Hela cells do not allow episomal amplification of the plasmids and a high yield protein expression, we expressed AsCpf1-MBP and LbCpf1-MBP proteins in HEK 293 cells, with or without crRNA, and performed the purification using amylose resin (**Figure 4A**, purification of AsCpf1 protein). The purified proteins were tested for their *in vitro* cleavage activity using an *XmaI* linearized pGFP-SSA target (5′-TTTa-3′ PAM sequence) as a substrate. The purified nucleases complexes were preincubated on ice with the DNA substrate prior to the incubation at 37°C in order to observe the reaction products at the equilibrium of the Protein/DNA interaction. Using the same quantity of proteins in the reactions, the DNA cleavage at the target site was only observed with the Cpf1-MBP/crRNA purified complexes (**Figure 4B**: Lanes 3 and 5 versus Lanes 4 and 6). In addition, we observed that the purified LbCpf1-MBP/crRNA activity is better than the AsCpf1-MBP complex. We further tested an increasing quantity of the purified complex. The quantity of the protein was analyzed by acrylamide SDS Page (**Figure 4C**, top panels, 2-fold dilution cascade). We observed a difference around 10-fold in activity between the two proteins. Under the tested *in vitro* reaction conditions,

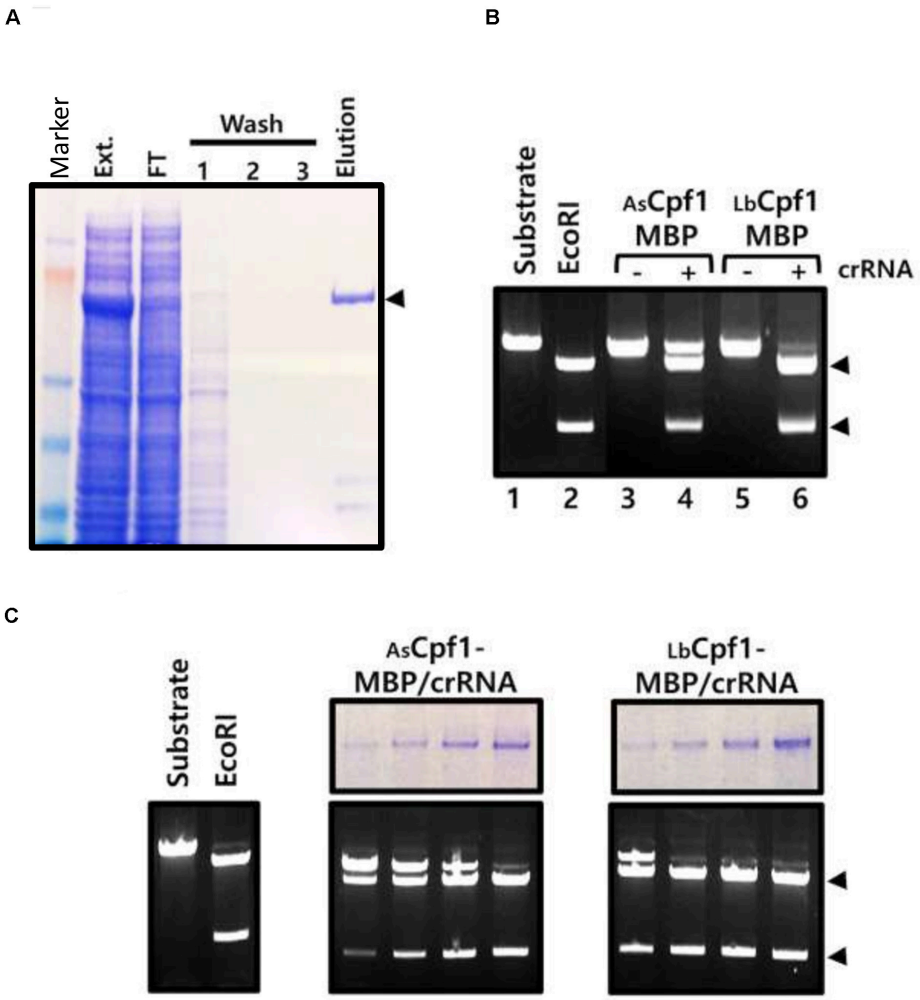

FIGURE 4 | *In vitro* activity of cellular Cpf1 proteins: **(A)** AsCpf1-MBP protein purification: The HEK293 AsCpf1-MBP cellular extract (Ext.) was analyzed on an 8% denaturing acrylamide gel, together with the Flow through (F.T.), washing steps (1, 2, and 3), and Elution of the affinity chromatography with amylose beads. The black triangle indicates the position of the migration of the AsCpf1-MBP protein (170kDa). **(B)** *In vitro* activity of Cpf1-MBP purified proteins: Cleavage activity of Cpf1-MBP proteins expressed or not with the crRNA was performed with pGFP-SSA 5′-TTTa-3′ linearized *XmaI* target plasmid. The plasmid substrate was digested with the *EcoRI* restriction enzyme to control the size of the products. **(C)** Dose response activity of the purified Cpf1-MBP /crRNA complex. The quantity of proteins in the sample was assessed by SDS Page electrophoresis (upper panel) and tested for DNA cleavage activity (lower panel).

we did not observe nonspecific cleavage sites of the reporter pGFP-SSA target plasmid. The *in vitro* cleavage experiment with the ribonucleo-proteinic complexes confirmed that Cpf1/crRNA are active and are able to cleave the DNA specifically at the target site. The stronger cleavage activity observed with LbCpf1-MBP/crRNA complex, compare to AsCpf1-MBP/crRNA can be associated to a better stability of the crRNA expressed in the cells, to a higher stability of the ribonucleo-proteinic complex during the purification process, or to a better catalytic activity of the complex. To observe the *in vitro* cleavage reaction in a similar condition than in a cellular context, the same reactions were performed without preincubation on ice (**Supplementary Data 8**) and demonstrated that the AsCpf1 nuclease cleave the plasmid DNA substrate at unspecific site compare to LbCpf1.

Analysis of LbCpf1 Proteins PAM Selectivity Using SSA Assay

Since the SSA assay show a reproducible and robust activity in Hela cells with LbCpf1, we further developed the assay to analyze the PAM selectivity of Cpf1 proteins. We generated a pGFP-SSA target plasmid library, with the same DNA target sequence, flanked with the 16 different PAM 5′-TNNa-3′ sequences. The same crRNA expressing vectors are used to analyze the cleavage activity of LbCpf1 protein and are also suitable to determine the cellular activity of the mutants. The first LbCpf1 PAM recognition mutant tested in parallel with the Wt protein (**Figure 5A**), is a protein with the amino acid substitutions G532S and Y542N. The lysines K538 and K595, that define the PAM sequence 5′-TTTv-3′ specificity, were not modified. As a consequence, the LbCpf1 mutant G532S /Y542N has the same PID structure

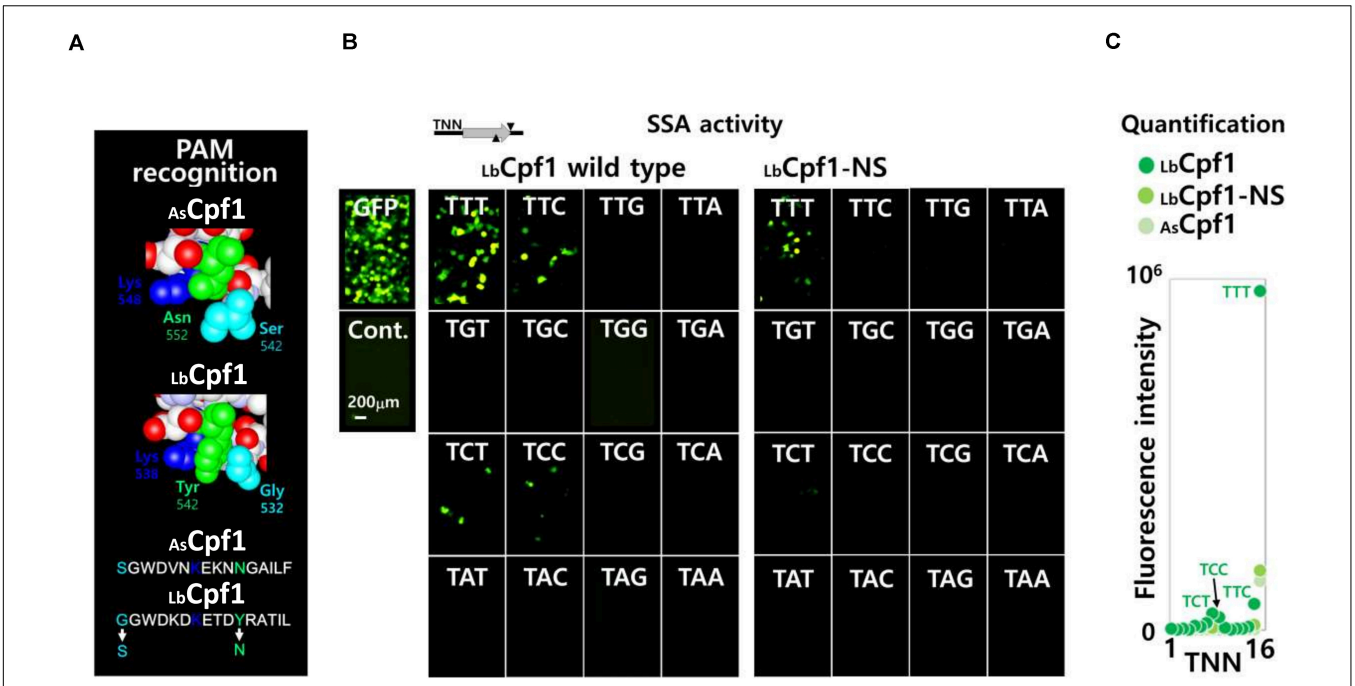

FIGURE 5 | Wt and mutant LbCpf1-NS PAM selectivity analysis using a 5′-TNNa-3′ library. **(A)** Structural representation of the PAM amino acids interacting on the major groove of the DNA: representation of both enzymes AsCpf1 (S542/K548/N552) and LbCpf1 (G532/K538/Y542). The mutant LbCpf1-NS (Y532S/K542N) correspond to an amino acids swap between the two protein species depicted bellow. **(B)** Image of fluorescent cells using a fluorescent microscope (×20): Fluorescent cells were inspected after transfection and 48 h incubation. The pictures show the fluorescent cells observed by co-transfection of the crRNA with Wt or NS mutant LbCpf1, together with the different pGFP-SSA 5′-TNNa-3′ vectors. 300 ng of the Cpf1 expression vectors were transfected for this experiment. The target site, represented on top of the pictures, is the same target sequence with the 16 different 5′TNNa-3′ sequences. The GFP, and a negative control, are depicted on the left side. **(C)** Graph of the quantification (Fluorescence intensity) of the fluorescent cells with the 16 constructs pGFP-SSA 5'-TNNa-3' for each enzyme.

than the AsCpf1 at the DNA major groove/Protein interface. To detect minor PAM interaction activities, AsCpf1 and the LbCpf1 Wt and mutant G532S /Y542N were co-transfected at high concentration (300 ng DNA), together with the crRNA expression vector, and one of the 16 pGFP-SSA 5′-TNNa-3′ target vectors. As previously observed with the Wt LbCpf1 protein, the pictures of fluorescent cells (**Figure 5B**) show a strong GFP expression with the PAM 5′-TTTa-3′ sequence. The presence of fluorescent cells was also observed with the pGFP-SSA target PAM 5′-TCTa-3′, 5′-TTCa-3′ and 5′-TCCa-3′ reporter constructs. The LbCpf1-NS mutant, harboring G532S and Y542N substitutions, show only a moderate GFP induction with the 5′-TTTa-3′ pGFP-SSA target construct. The fluorescent quantification for the three proteins AsCpf1, LbCpf1 and LbCpf1-NS is depicted **Figure 5C**. The mutant LbCpf1-NS activity is identical to the AsCpf1 protein with the PAM 5′-TNNa-3′ library.

We generated further mutations, with substitution of amino acids at the position 532, 538 and 548. The mutations K538S or K538A that dramatically reduce the cellular activity (Data not shown), were associated together with the mutation Y548R. With those mutations, the negatively charged residue Lysine 538, present at the protein/DNA major groove interface of the Wt protein, is structurally replaced by an Arginine at the position 548. In addition, we substituted the amino acid Glycine 532 by an arginine to generate the two triple mutants G532R/K538S/Y548R (RSR), G532R/K538A/Y548R (RAR). The PAM sequence selectivity, analyzed with the pGFP-SSA 5′-TNNa-3′ constructs (**Figure 6A**), show that the mutants RSR and RAR are inducing GFP expression with the PAM sequences 5′-TTTa-3′, 5′-TATa-3′ and 5′-TACa-3′. The mutant RSR show also moderate activities with the 5′-TGCa-3′, 5′-TCCa-3′ PAM constructs.

To control the observed cellular activities, the *in vitro* cleavage assay was performed using the RSR, RAR and the Wt LbCpf1-MBP fusion enzymes with the three *XmaI* linearized target DNA pGFP-SSA plasmid substrates, containing the same target site flanked by the PAM sequences 5′-TTTa-3′, 5′-TATa-3′ and 5′-TACa-3′ (**Figure 6B**). As expected, the Wt LbCpf1 enzyme cleave the 5′-TTTa-3′ PAM only, whereas the mutant RSR and RAR are able to cleave the three substrates.

According to the observed recognition for their PAM sequences, the Wt LbCpf1and the mutants were tested for their efficacy to induce the modification of the *hprt* gene (exon3) at the genomic sites. Hela cells were co-transfected with the Wt or mutants LbCpf1 expression vectors, and three crRNA expression vectors targeting sequences that requires the different types of PAM sequences (5′-TTTa-3′, 5′-TATa-3′ and 5′-TACa-3′). The cells were selected with the 6TG treatment to observe the *hprt* gene disruption. The results of the 6TG resistant cells selection (**Figure 6C**) show the surviving cells when transfected with the PAM 5′-TTTa-3′, 5′-TATa-3′ and 5′-TACa-3′ crRNA and the RSR

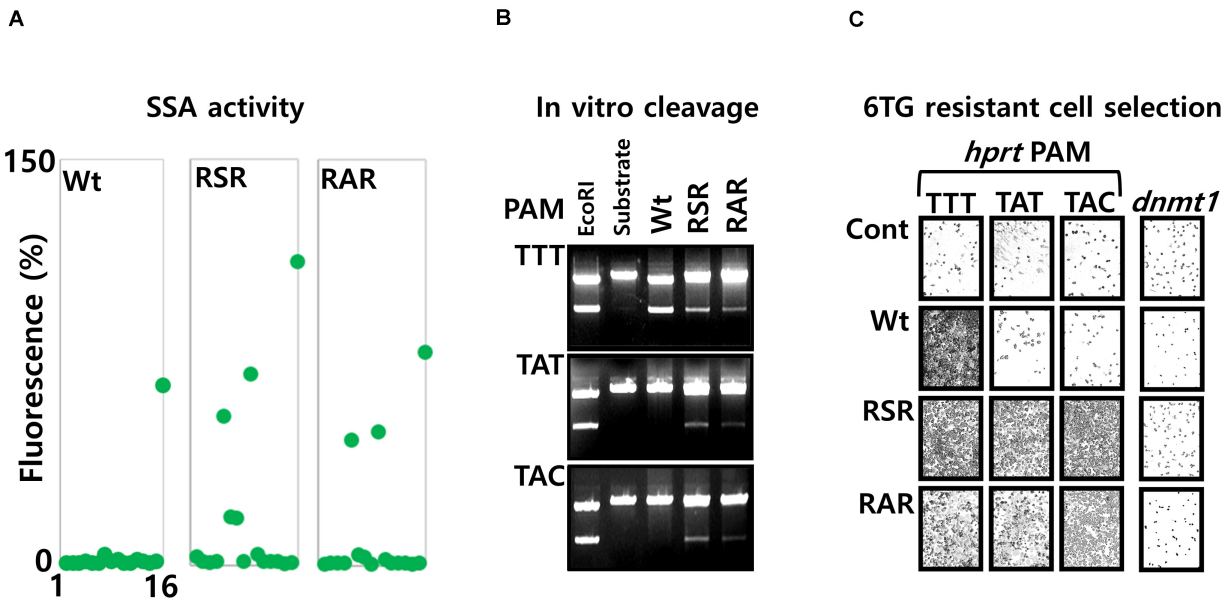

FIGURE 6 | Analysis of LbCpf1 RAR and RSR mutants with the 5′-TNNa-3′ SSA library. **(A)** Graphic representation of the Wt and mutant protein activity: the graph is summarizing the activity observed with the 16 5′-TNNa-3′ reporter vectors for LbCpf1 and each PAM recognition mutant (RSR and RAR). The transfection was performed with 100 ng of LbCpf1 expression vectors. **(B)** Nuclease activity: *In vitro* cleavage activity of Wt LbCpf1-MBP and each mutants RSR-MBP and RAR-MBP with the *XmaI* linearized plasmid pGFP-SSA substrates 5′-TTTa-3′, 5′-TATa-3′ and 5′-TACa-3′. One of the plasmid substrates was digested with the *EcoRI* restriction enzyme to control the size of the products. **(C)** 6TG resistant cell selection: Pictures of Hela cells, after selection with the 6TG, following the co-transfection of the LbCpf1 (Wt or the PAM recognition mutants RSR and RAR) expression vectors, and the crRNAs that target the *hprt* exon3 sequences flanked, respectively by the 5′-TTTa-3′, 5′-TATa-3′ and 5′-TACa-3′ PAM sequences. The left Panel is a control experiment using a guide crRNA against *dnmt1* gene.

and RAR variants, whereas resistant cells were obtained only with the 5′-TTTa-3′crRNA when the Wt LbCpf1 is co-transfected. The guide crRNA targeting *dnmt1* gene was used as a negative control and do not allow the selection of 6TG resistant cells. We performed the same experiment using others crRNA with an upstream 5′-TATa-3′ PAM sequence, and the mutant RAR, in order to control the functionality of this mutant with several sequences (**Supplementary Data 9**).

DISCUSSION

Nuclease Activity in a Cellular Context

In this study, we are reporting the analysis of nuclease activity in a cellular context using two complementary procedures. On the one hand, we used the deep sequencing that allow to quantify the nuclease activity at the genomic target site. This technic is measuring indels generated after NHEJ repair of the DSB generated by the nuclease. This strategy does not take into account other modification, i.e., large genomic deletion, inversion, and chromosomic translocation since they are not amplified. After genomic DNA isolation of the pool of modified cells, and PCR of a small genomic DNA region, amplified fragments are sequenced individually. On the other hand, the SSA reaction using a plasmid cleaved by the nuclease was developed. The DSB induce the recombination of repeated sequences and reconstitute an active reporter gene. This second strategy require a highly specific and selective nuclease. Indeed, the lack of nuclease specificity and selectivity leads to false negative results and wrong quantifications because of the plasmid degradation and the subsequent GFP signal destabilization. The advantage of the plasmid strategy is that the target site can be customized compare to a genomic target. In addition, the accessibility of the target site is identical compare to the variable chromatin states at genomic sites (i.e.: Euchromatin and Heterochromatin). We also favored the SSA assay with a plasmid, because it is difficult and fastidious to perform an equivalent engineering of a cell line with several plasmid constructs. Performing the SSA experiment with a stable and genomic insertion of the target gene is very interesting, however the GFP signal stability assay is not suitable. Indeed, only a *gfp* specific target sequence can disrupt the *gfp* gene inserted in the genome, and the time required (5 days) to remove the GFP protein from the cells is longer than the incubation time to induce the reporter gene. As a matter in fact, non-specific nucleases might also generate false negative samples and wrong quantification with the genomic SSA assay, by generating large genomic deletion, inversion, and chromosomic translocation at the tested locus.

In the analysis of the two Cpf1 enzymes tested using the SSA assay in this study, we showed that LbCpf1 is the nuclease with the most robust SSA cellular activity. Using the same guide RNA sequence with the two orthologue enzymes, that need the same optimal PAM sequence (5′-TTTv-3′), we observed a strong SSA activity and a low GFP signal destabilization with LbCpf1 (**Supplementary Data 5B**, GFP signal stability and cell surviving). This demonstrates the great selectivity of this enzyme

in the cellular assay, at least for the crRNAs against *hprt* (exon 3) used in this study (**Supplementary Data 6**, SSA activity of 16 crRNA target sequence with LbCpf1 protein). We also observed, with the purified Cpf1-MBP/crRNA complexes, that the LbCpf1 enzyme expressed in mammalian cells has a higher *in vitro* DNA cleavage activity than AsCpf1. In addition, we demonstrated that AsCpf1 has an unspecific *in vitro* cleavage activity on the plasmid substrate without preincubation of the nuclease with the DNA on ice (**Supplementary Data 8**). Those observations correlate with the SSA activity of both enzymes. In this study, we performed the cellular assays, and *in vitro* cleavage assay, using Cpf1 proteins expressed from mammalian cells. Those experiments are physiologically equivalent (i.e.: proteins were exposed to the same biological environment and the potential post translational modifications associate to it). Increasing AsCpf1 expression in the SSA experiment do not lead to a better cellular activity of this protein because of the GFP destabilization associated to the non-specific cleavage activity of this enzyme observed *in vitro*. The FnCpf1 nuclease was also analyzed (Data not shown) and exerted a low activity (Zetsche et al., 2015). It was clearly demonstrated that this member of the Cpf1 nuclease family has an increased cellular activity and detectable at a genomic site by alteration (mutation RVR) of its PAM (5′-TTn-3′) recognition (Tóth et al., 2018). In addition, we also demonstrated that the nuclease spCas9 exert the same properties than AsCpf1 in a cellular context (**Supplementary Data 8**), and the usage of a tandem inverted repeated target site is required to obtain an efficient SSA activity without GFP signal destabilization, demonstrating the suitability of the assay to analyze other CRISPR nucleases.

The experiments conducted with the pGFP-SSA 5′-TNNa-3′ PAM sequences clearly confirmed the specificity of the Cpf1 proteins for the PAM 5′-TTTa-3′ sequence, as it was already described (Komor et al., 2016). In fact, the single change of a nucleotide, in the PAM sequence, dramatically reduced or completely abolish the cellular activity. This in turn, demonstrate that the protein must interact with the PAM sequence, prior the hybridization of the guide RNA to the target DNA sequence. This observation was also demonstrated *in vitro* using recombinant enzyme (Kim et al., 2016). Since the amino acids interacting with the PAM sequence is a small region of interaction, a single change at the protein/DNA interface dramatically reduce the affinity, and subsequently the nuclease activity in a cellular context.

Finally, we analyzed genetic modification in mammalian cells, induced by the Wt and the mutants RSR and RAR LbCpf1 proteins. We tested the targeting of the *hprt* gene and the positive selection of resistant cells upon 6TG treatment. The result of this cellular assay with the LbCpf1 Wt and the mutant correlates with the observation of the SSA experiments (PAM 5′-TTTa-3′, 5′-TATa-3′ and 5′-TACa-3′ crRNA). Altogether, our observations are reflecting that LbCpf1 nucleases used with an optimal guide RNA is sufficiently specific and selective to perform a gene disruption in mammalian cells. Nevertheless, selecting the best guide crRNA and studying its off-target effects (Kim et al., 2015; Kanchiswamy et al., 2016; Yan et al., 2017) are required to optimize the genome engineering of a target gene (Strohkendl et al., 2018).

PAM Recognition Mutant Analysis

Since LbCpf1 and AsCpf1 are different in their PAM sequence selectivity, we first investigated the effect of a swap, between AsCpf1 and LbCpf1 amino acids. We mutagenized LbCpf1 amino acids that are different, compare to AsCpf1, on the DNA major groove/Protein interface. The mutant LbCpf1-NS is reacting as the AsCpf1 protein in the pGFP-SSA PAM 5′-TNNa-3′ selectivity assay. The fact that this amino acid swap is sufficient to modified LbCpf1 protein properties, to an AsCpf1, is supporting the central role of the PAM specificity and selectivity for the PAM sequence in the cellular activity.

Based on the observations of this study, and the mutant RR and RVR described so far with AsCpf1 protein (Gao et al., 2017), we designed further mutations of the amino acids responsible of the PAM sequence recognition in the LbCpf1 protein. We investigated the interaction with similar amino acid combination at the protein/major groove DNA interface. We substituted the lysine 538 that is directly in contact with the second Adenine of the 5′-TTTv-3′ sequence, by a Serine or an Alanine residue. We structurally replaced this positively charged residue with two Arginines at position 548 and 532. We observed that both substitutions, corresponding to the R532/A538/R548 (RAR) and R532/S538/R548 (RSR), still interact with the 5′-TTTa-3′ PAM, and allows the interaction with 5′-TATa-3′ and 5′-TACa-3′ sequences as well. Other mutant combinations were tested, such as G532/S538/E548 or E532/S538/E548 but failed to interact with all 5′-TNNa-3′ sequences tested (data not shown). In comparison, the RVR mutant described for AsCpf1 (Nishimasu et al., 2017) interacts mainly with the 5′-TATa-3′ sequences. The Valine at the position corresponding to the Serine 542 in AsCpf1 is more restrictive in the sequence selectivity, most probably because of the size of the isopropyl group of this amino acid. The modification of the second lysine K585 interacting with the PAM sequence on minor groove side of the DNA is crucial for the interaction. As with AsCpf1 (Mutant K607A), substitution of the Lysine 585 by an alanine residue, for the wild type and mutants, dramatically reduce the protein activity (Data not shown).

PAM Specificity and Selectivity of Wt and Mutant LbCpf1

The PAM selectivity of Cpf1 proteins was demonstrated using *in vitro* experimental procedures (Komor et al., 2016), bacterial assay (Kim et al., 2017) and lentiviral expression in mammalian cells (Yamano et al., 2017). A high affinity for the 5′-TTTv-3′ sequence was reported as an optimal sequence for the two nucleases AsCpf1 and LbCpf1. The technics reported are covering larger adjacent sequences libraries and an analysis using statistical methods. Because of the number of plasmids required in both assays tested in this study, we did not cover the full diversity of the 5′ flanked region. We restricted our analysis to the 16 PAM sequences corresponding to 5′-TNNa-3′. The results of both strategies demonstrate a stringent activity for the 5′-TTTa-3′ PAM sequence with AsCpf1. On the contrary, LbCpf1 show a lower selectivity. Indeed, in addition to the high activity observed with the 5′-TTTa-3′ sequence, minor activities were observed

with the PAM sequences 5′-TCTa-3′, 5′-TTCa-3′ and 5′-TCCa-3′ using both assays. This feature of LbCpf1 reflect the structural adaptability of the K538 described in the resolved structures with those particular sequences (Yamano et al., 2017). Recently, it was show that AsCpf1 interacts specifically with the PAM sequences 5′-GTT-3′ and 5′-GCT-3′ (Jacobsen et al., 2019) but we did not investigate such sequences in your study.

To be conclusive, studying independently the AsCpf1 and LbCpf1 interaction with the PAM sequences, in order to characterize and compare their respective *in vitro* affinity is required. The PID domain of the Cpf1 nucleases that interact with the PAM sequence are small regions without a distinguish structure inside the ribonucleo-proteinic complexes. To obtain a stable interaction between the PAM and the few amino acids of the PID, the guide RNA/target DNA hybridization is required. Thus, it is unfortunately not possible to isolate the PID domain and observed the interaction with the PAM sequence *in vitro*. However, it is interesting and remarkable, that despite a lower PAM selectivity than AsCpf1, LbCpf1 exert a stronger cellular activity in the SSA assay, reflecting a better selectivity for the target site. Increasing or reducing the affinity for the PAM sequences influences the cellular activity of the enzymes. A high specificity for the PAM sequence might increase unspecific effects and cleavage at more off-target sites with an upstream 5′-TTTv-3′ PAM sequence, whereas a lower affinity might reduce those undesired activities of the nucleases. Thus, a high specificity and selectivity for the PAM sequence is not required to obtain a selective nuclease for a genomic target.

Unspecific Off-Target Effects Analysis of Nucleases

The analysis of the nuclease's unspecific off-target effects can be addressed using assays with plasmids as demonstrated with the SSA assay and the GFP destabilization experiment. The unspecific activity of the nuclease is observed indirectly by measuring the destabilization of the GFP signal, whereas the off-target effect using deep sequencing analysis is performed on preselected sequences similar to the target site among the genome. Nevertheless, the experiments with plasmids are rapid and cost-effective assays for a primary experiment to demonstrate and characterize the nuclease activity and their unspecific off-target effects. On the contrary, the Deep sequencing is more precise to quantify the off-target activity and analyze the effects of mutations. The deep sequencing experiment is focusing the analysis at similar sites that are selected but not discreet, and do not answer a general view of unspecific activity of the nucleases. In our study, we evaluated the effects of mutations affecting the PAM recognition of LbCpf1, we tested the mutant RSR and RAR that were not evaluated to date but that are similar to the described mutations RVR (Gao et al., 2017) or RRVR (Tóth et al., 2020). The previous studies about those type of mutations demonstrated that they are not reducing the off-target effects, but they are expending the cleavage activity to the all PAM sequences accessible with similar sequences to the target. Reducing the off-target effects need further mutations affecting other domains of the protein Cpf1/RNA complex (Gao et al., 2017).

In fine, our results demonstrate the complementarity of all technologies developed so far, NGS analysis, SSA plasmid assay and *in vitro* DNA cleavage, to characterize the nuclease activity of CRISPR proteins. Altogether, the experimental information of those assays is useful for the genetic manipulation of eukaryotic cells.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

VB designed and performed the experiments, analyzed and interpreted the data. US did the NGS. VB wrote the manuscript. Both authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2020.571591/full#supplementary-material

Supplementary Data 1 | Sequences of the oligonucleotides cloned into the pU6 vectors for the AsCpf1 and LbCpf1crRNA expression in mammalian cells.

Supplementary Data 2 | (A) Representation of the *hprt* exon 3 target sequence used in the disruption cellular assay. **(B)** Deep sequencing analysis of cells 6TG resistance selected after the nuclease transfection with guide RNA: Hela cells were co-transfected with LbCpf1 and the 5′-TTTa-3′ Guide RNA. A control experiment was performed with a guide RNA targeting *Dnmt1* gene. The 6TG selection was performed after 5 days incubation. Following the 6TG exposure, the genomic DNA was isolated. The PCR amplified DNA of the *hprt* gene (exon 3) was analyzed by Deep sequencing (percentage of Indels frequency). The graphic show the positions and the quantification of insertions and deletions at the *hprt* target site

Supplementary Data 3 | (A) Vector map of the pGFP-SSA reporter plasmid. **(B)** Sequence of SSA *gfp* fragments: the 5′ and 3′ sequences of the *gfp* gene fragments are depicted, the dark green sequences are corresponding to the left and right repeat that recombine during the HR reaction.

Supplementary Data 4 | Sequences of the oligonucleotides cloned between *EcoRI* and *BamHI* in the pGFP-SSA reporter plasmid. Sequences of the oligonucleotides used for the mutagenesis of the LbCpf1 expression plasmid.

Supplementary Data 5 | (A) Control of pGFP-SSA activity in transfected Hela cells: Hela cells were transfected with the pGFP positive control and the circular, or *EcoRI* linearized, pGFP-SSA reporter plasmids. The pictures show the fluorescent cells using a fluorescent microscope (×20) after transfection and 48 h incubation. The *EcoRI* linearized pGFP-SSA vector was used as a control for samples normalization of the SSA assay. **(B)** GFP signal stability and cell surviving: Hela cells were transfected with the pGFP control expression vector and the Cpf1 proteins with the guide RNA against the same target sequence (*hprt* gene, Exon 3, 5′-TTTa-3′ PAM sequence). The cells surviving and the GFP fluorescence were quantified after 48 h of incubation. The results indicate that the cells survived to Cpf1 expression, but the GFP signal is lower with AsCpf1 compare to LbCpf1.

Supplementary Data 6 | SSA activity of 16 crRNA target sequence with LbCpf1 protein: The 16 target sequences, used for the deep sequencing analysis in **Figure 2**, were cloned in pGFP-SSA reporter vector. All target sequences are flanked by the optimal 5′-TTTa-3′ PAM sequence, instead of the genomic PAM 5′-TNNa-3′ sequences. The 16 pGFP-SSA 5′-TTTa-3′ target vectors were transfected with the corresponding guide crRNA expression plasmids and the LbCpf1 expression vector at 300 ng. Fluorescent cells were quantified after 48 h of incubation and reported in the graph as a percentage of fluorescence, the calculation was made using *EcoRI* linearized pGFP-SSA as a reference. A negative control was performed with the empty pGFP-SSA vector.

Supplementary Data 7 | Analysis of spCas9 with the SSA assay. **(A)** Representation of the *hprt* exon 3 target sequence used in the disruption cellular assay with SpCas9 nuclease and analysis by deep sequencing of the cells resistant to the 6TG: The Hela cells were selected after the transfection of the plasmid expressing the nuclease and the guide RNA. A control experiment was performed with a guide RNA targeting *Dnmt1* gene. The graphic show the positions and the quantification of insertions and the deletions at the *hprt* target site (percentage of Indels frequency). **(B)** GFP signal stability: The unspecific off-target effects of the Wt SpCas9, the Nickase (N-Cas9: single mutant D10A) and the catalytically inactivated nuclease (D-Cas9, termed dead-Cas9: double mutant D10A/H840A) is tested by co-transfection of the plasmids expressing SpCAS9 (500 ng) and the guide RNA with the pGFP expression vector. The Wt SpCas9 nuclease show a low GFP signal compare to the nickase and the dead nuclease demonstrating the destabilization of the GFP signal. C. SSA activity of SpCas9 nuclease: The activities of the Wt spCas9, N-Cas9 and D-Cas9 proteins with the SSA assay were tested. The Hela cells were co-transfected with the plasmids expressing the proteins, the guide RNA and the pGFP-SSA reporter vectors containing the corresponding target site and an inverted tandem repeat. The two reporters allow to observe the differential activity of the Wt SpCas9 and the mutant N-Cas9 protein. As expected, the catalytically inactivated nuclease and the Nickase are inactive on the target site. The SpCas9 nuclease is active on both SSA reporter constructs whereas N-Cas9 is active on the tandem repeats only.

Supplementary Data 8 | *In vitro* cleavage activity of the Cpf1 nucleases without pre-incubation on ice. **(A)** Schematic representation of the Cpf1 nuclease interactions with the specific target and the off-target site on both conditions, with and without preincubation on ice. **(B)**. *In vitro* activity of Cpf1-MBP purified proteins without preincubation on ice: The cleavage activity of Cpf1-MBP proteins expressed with the crRNA was performed with pGFP-SSA 5′-TTTa-3′ linearized *XmaI* target plasmid. The unspecific cleaved product observed with AsCpf1 is depicted (*) on the side of picture. The substrate was digested with the restriction enzyme *EcoRI* to control the size of the specific products after cleavage.

Supplementary Data 9 | Selection of 6TG resistant cells with the RAR mutant: **(A)** Target sequences: The sequences of the crRNA with a flanked 5′-TATa-3′ PAM within the target *hprt* gene (exon3) are depicted. **(B)** Quantification of the 6TG resistant cells: The graph represent the results of the luminescence observed by cell titer Glo quantification, after selection with the 6TG, following the co-transfection of the LbCpf1 (Wt or the PAM recognition mutant RAR) expression vectors, and the crRNAs that target the *hprt* exon3 sequences flanked by the 5′-TATa-3′ PAM sequence.

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REVIEW
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Improvements in Gene Editing Technology Boost Its Applications in Livestock

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Accelerated development of novel CRISPR/Cas9-based genome editing techniques provides a feasible approach to introduce a variety of precise modifications in the mammalian genome, including introduction of multiple edits simultaneously, efficient insertion of long DNA sequences into specific targeted loci as well as performing nucleotide transitions and transversions. Thus, the CRISPR/Cas9 tool has become the method of choice for introducing genome alterations in livestock species. The list of new CRISPR/Cas9-based genome editing tools is constantly expanding. Here, we discuss the methods developed to improve efficiency and specificity of gene editing tools as well as approaches that can be employed for gene regulation, base editing, and epigenetic modifications. Additionally, advantages and disadvantages of two primary methods used for the production of gene-edited farm animals: somatic cell nuclear transfer (SCNT or cloning) and zygote manipulations will be discussed. Furthermore, we will review agricultural and biomedical applications of gene editing technology.

Keywords: CRISPR/Cas9, agriculture, animal models, livestock, gene editing

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INTRODUCTION

The development of CRISPR/Cas9-based genome editing tool has revolutionized the field, and led to the modification of livestock genomes with much greater simplicity and efficiency (Urnov et al., 2010; Joung and Sander, 2013; Laible et al., 2015; Lillico et al., 2016; Georges et al., 2019). CRISPR technology was first applied to the mammalian genome in 2013 (Cong et al., 2013) and subsequently, expanded to a wide range of cell lines and mammalian species including livestock. This technology allows for modifications that lead to improvements in livestock production traits, animal health, and welfare, generation of more refined large animal models of human diseases, pharmaceutical protein production, and investigating gene function. Since 2014, over 500 research papers have been published using CRISPR gene editing approach in livestock (pigs, cattle, sheep, and goats; based on the October 1st, 2020 PubMed search).

Precise genome editing is based on the ability of engineered nucleases ZFNs (Zinc Finger Nucleases), TALENs (Transcription Activator-Like Effector Nucleases), and CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) to cut the genome in a specific targeted position. Then, the resulting double-stranded break (DSB) triggers the cell repair mechanism to repair

the damage by either non-homologous end-joining (NHEJ) or homology-directed repair (HDR), which introduces a targeted mutation into a specific genomic location (McMahon et al., 2012). The CRISPR/Cas9 system is a simple and versatile method compared to ZFN and TALEN approaches that require the assembly of the associated engineered proteins for each target. The efficiency of CRISPR-based genome editing has increased to the point that the technology allows multiple edits simultaneously (Georges et al., 2019), which has led to this becoming the method of choice for introduction of specific genomic modifications in livestock species. The list of new CRISPR/Cas9-based genome editing tools is constantly expanding. This review will discuss the methods developed to improve efficiency and specificity of gene editing tools as well as approaches that can be employed for gene regulation, base editing, and epigenetic modifications. Advantages and disadvantages of two primary methods used for the production of gene-edited farm animals: somatic cell nuclear transfer (SCNT) and zygote manipulations will also be discussed. We will also review the use of gene editing technology in agriculture and biomedicine.

GENE EDITING TECHNIQUES

Several comprehensive reviews discussing gene-editing technology and its current status in livestock are available (Kalds et al., 2019; McFarlane et al., 2019; Kalds et al., 2020; Lee et al., 2020; Menchaca et al., 2020a; Navarro-Serna et al., 2020), therefore, we provide an overview of critical landmark events and recent improvements in the CRISPR/Cas9 field and include a comprehensive literature review focused on the production of gene edited farm animals with specific application to agricultural and biomedical fields.

ZFNs

The chimeric nucleases, ZFNs, were developed in 2001 (Bibikova et al., 2001) and designed to target and disrupt precise DNA sequences (Qomi et al., 2019). Zinc fingers are small protein (20–30 amino acids) motifs regulated by zinc ion that binds to DNA, recognizing a 3-base pair (bp) sequence. The motifs have been combined with the genetically engineered restriction enzyme *Fok*I to create a programmable nuclease with the ability to identify target sequence sites. The ZFNs are effective when two zinc finger modules bind to the DNA in sites that opose each other with the *Fok*I enzyme in the middle, which forms a homodimer complex. Once the homo-dimerization is established, the nuclease breaks both DNA strands, and mutations are randomly inserted (Adli, 2018). The target site can be designed by changing the residues in a single zinc finger that alters its specificity for DNA recognition, thus, the finger motifs can be customized to recognize many different DNA triplet nucleotides (Carroll, 2017). Although ZFNs were innovative due to their higher specificity to the DNA sequence, they have a few major disadvantages, such as an exhaustive time-consuming process to design a pair of ZFNs against a target sequence. Also, there are a low number of potential targets in the genome, which makes this gene editing molecule not applicable to many studies. In fact, for every 50-bp, only one locus is suitable for this approach (Qomi et al., 2019).

TALENs

In search of more efficient gene editing tools, in 2009, a new generation of nucleases, transcription activator-like effector nuclease emerged. Originally found in the plant pathogenic bacteria *Genus Xanthomonas*, the transcription activator-like effectors (TALEs) are DNA-binding domains containing 33–35 amino acid repeat motifs that identify each of the bps. Its site-specificity is determined by two hypervariable amino acids known as repeat-variable di-residues (Gaj et al., 2013). Similar to ZFNs, TALEs have been engineered to fuse with the DNA-cutting domain of the *Fok*I nuclease to serve as a gene editing tool known as TALENs (Adli, 2018). The difference between the ZFNs and TALENs is related to the number of nucleotides recognized by the protein domains, 3-bp versus 1-bp, thus making TALENs more site-specific and less likely to cause an off-target cleavage (Khan, 2019).

CRISPR/Cas9

Although ZFNs and TALENs have offered vast improvements for gene manipulation, the most significant discovery came in 2013 when Dr. Zhang and colleagues successfully accomplished the first CRISPR/Cas9 genome editing in mammals (Cong et al., 2013). The unusual 29 sequence RNA repeats were initially found in 1987 by Yoshizumi Ishino at Osaka University while studying *Escherichia coli* bacteria. Years later, in 2002, the molecule was named by Drs. Mojica and Ruud Jansen as CRISPR, an abbreviation for Clustered Regularly Interspaced Short Palindromic Repeats (Mojica et al., 2000; Hsu et al., 2014). CRISPR and CRISPR-associated protein (Cas) can be easily customized to effectively introduce mutations at specific locations within genes in mammalian cells (Cong et al., 2013). The CRISPR/Cas9 complex was elucidated as a primitive acquired immune system of some bacteria and most of the archaea species to defend against the foreign DNA of bacteriophage (Humphrey and Kasinski, 2015). This mechanism consisted of two phases: immunization and immunity phases. In the immunization phase, Cas1 and Cas2 endonucleases recognize the viral genome, break it into small fragments and insert them into the bacterial genome as repeat-spacer units. During a subsequent viral invasion (immunity phase), the bacteria produce precursor-CRISPR RNA (pre-crRNA) based on the previously captured repeat-spacer units. The pre-crRNA binds to the Cas9 endonuclease and trans-activating crRNA (tracrRNA) forming the crRNA-Cas9-tracrRNA complex (Marraffini, 2015; Qomi et al., 2019). The complex is then degraded by RNase III, which results in the cleavage of each repeat fragment, turning the long CRISPR precursor into small crRNA guides for targeting the exogenous DNA. This CRISPR-Cas immunity promotes the DSB of invading DNA (Marraffini, 2015).

The CRISPR/Cas9 system consists of the Cas9 endonuclease with putative nuclease and helicase domains bound to a tracrRNA:crRNA duplex. The crRNA region contains 20 customizable nucleotides at 5′ end that forms the guide RNA (gRNA) and a repeat region with 12 nucleotides, whereas the

tracrRNA consists of 14 nucleotides anti-repeat region and three loops (Mei et al., 2016). The duplex RNA is responsible for guiding the Cas9 to the specific sequence on the DNA where the gRNA aligns against the complementary sequence. With the target sequence found, the helicase domain works by opening the double strands while the nuclease sites (RuvC and HNH) perform the DSB of the DNA (**Figure 1**). Subsequently, the crRNA:tracrRNA has been genetically engineered to become a single guide RNA with changeable 5′ nucleotides. In addition to the gRNA identification, the designed target sequence must be located upstream to a protospacer-adjacent motif (PAM) – 5′-NGG-3′ where N can be any of the four known DNA nucleotides to be recognized by the Cas9 nuclease (Yang, 2015). Experiments have shown that the Cas9 starts the target site-searching process by probing a suitable PAM sequence before matching the gRNA complementary to the DNA. The identification of the site occurs through the molecular interactions between the gRNA with the target DNA nucleotides, and once mismatched, the Cas9 rapidly dissociates from the DNA. The Cas9 only triggers the DSB after a precise complementarity between the gRNA and the target DNA have been reached, which provides the energy to the enzyme to break the DNA (Jiang and Doudna, 2017).

DNA REPAIR MECHANISMS: NHEJ AND HDR

Genes can be effectively knocked out by merely producing mutations through a DSB of the targeted gene by engineered nucleases. After the break, the cells naturally attempt to repair the damage by using one of the two main repair mechanisms: the NHEJ and HDR pathways (Riordan et al., 2015).

The NHEJ system is the primary DNA repair mechanism for DNA DSB. It involves a straight ligation of the blunt ends, produced by the symmetric break of the DNA, using a complex of Ku70/80 proteins associated with the DNA Ligase IV (Pannunzio et al., 2018). NHEJ is the homology-independent pathway as it involves the alignment of only one to a few complementary bases for the re-ligation of two ends. It is an error-prone repair mechanism and frequently results in out-of-frame mutations (insertions or deletions – indels) in the repaired sequence. Moreover, even when an appropriate DNA repair takes place, the CRISPR/Cas9 continues to bind and disrupt the DNA sequence increasing the possibility of subsequent mutations. Indels often promote frameshift alteration of the codons, which leads to a disruption of the protein-coding sequence and often a premature stop codon (Dow, 2015). Thus, the strategy of gene inactivation by indels introduction is known as knockout (KO). The CRISPR/Cas9 tool has being successfully used in many organisms and cell types (e.g., human, sheep, goat, cattle, pig, and mouse) (Mei et al., 2016; DeWitt et al., 2017; Xie et al., 2017; Seki and Rutz, 2018; Jin et al., 2019) with efficiency ranging from 10% to over 90%. Initial use of CRISPR/Cas9 relied on plasmid transfection, but since CRISPR/Cas9 ribonucleoprotein (RNP) has become commercially available, RNP delivery provides higher KO efficiency (DeWitt et al., 2017; Perisse et al., 2020) and avoids the pitfalls associated with use of DNA plasmid delivery. The RNP provides fast action to perform DSB and indels are detectible very shortly after CRISPR/Cas9 RNP delivery. RNP is cleared from the cells within 24 h, thus, reducing the risk of off-target mutations. In contrast, plasmid delivery risks unintentional off target mutation and may also result in a vector integration into the host genome (DeWitt et al., 2017).

The second DSB repair mechanism is the HDR pathway, which uses the allelic gene from the sister chromatid as template DNA for reconstitution of the original sequence (Johnson and Jasin, 2000). The template DNA provides information to repair precisely the damaged chromosomes (Yeh et al., 2019). This repair system is highly specific and precise but in eukaryotic cells its occurrence is much lower due to the high prevalence of NHEJ (Riordan et al., 2015). The HDR takes place during synthesis (S) through G2 phases of the cell cycle (Zhao et al., 2017). When a sister chromatid is available, cyclin-dependent kinases 1 and 2 (CDK1/2) phosphorylate *C*-terminal binding

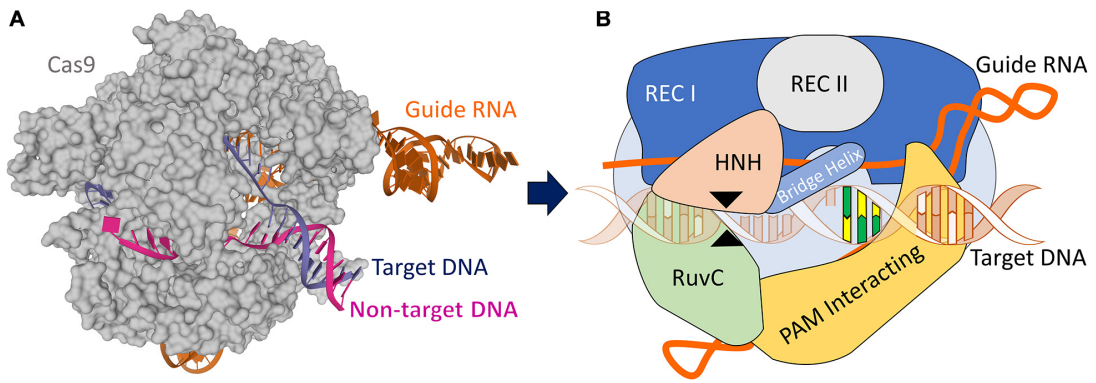

FIGURE 1 | CRISPR/Cas9 structure. **(A)** X-ray structure of the Streptococcus pyogenes (Sp) CRISPR/Cas9 system (5F9R.pdb) in the pre-activated state (Jiang et al., 2016), created using Mol* (Sehnal et al., 2018). Cas9 (gray) is shown in molecular surface. The guide RNA (orange), the target DNA (dark blue), and non-target DNA (pink) strands are shown as cartoons. **(B)** A schematic CRISPR/Cas9 ribonucleoprotein structure formed by six domains: Rec I, Rec II, RuvC, HNH, Bridge Helix, and PAM Interacting domain, and guide RNA targeting DNA. The black arrow heads indicate the cut sites from each RuvC and HNH domains. The yellow/green nucleotides represent the PAM sequence.

protein (CtBP)-interacting protein (CtIP) endonucleases. These nucleases activate with the MRN (Mre11, Rad50, Nbs1) protein complex that binds to the damaged DNA strands (Yeh et al., 2019). Then, CtIP promotes the resection of the damaged DNA, which is crucial for homologous recombination. The resection results in a longer 3′ single-stranded DNA (ssDNA) fragments that are coated by replication protein A (RPA). This protein is replaced by Rad51 to form a nucleoprotein presynaptic filament, which facilitates the search for a homologous DNA sequence. Once the donor DNA is aligned, the new DNA strands are synthesized followed by the dissociation of Rad51 and ligation of the DNA breaks (Pawelczak et al., 2018).

IMPROVEMENTS OF CRISPR/Cas9

Cas9 Nickase (nCas9)

This modified Cas9 endonuclease has been engineered to increase the efficiency of single point-mutation introduction and specificity to the target gene. The enzyme was modified to cut a single strand by either the RuvC or the HNH domain (see **Figure 1B**) and thus, being named Cas9 "nickase." The nCas9 (nickase) contains one inactive domain (inactivated through one amino acid substitution in the protein-coding sequence) along with another functional domain that retains the ability to create a single-strand DNA break providing the opportunity for directed modification. By using two different gRNAs with targets that are close to each other in combination with a nCas9, a process known as "double nicking," the gene editing based on nCas9 increases the specificity and reduces the chances of off-target mutation events without affecting the on-target efficacy (Cho et al., 2014; Adli, 2018). The CRIPSR/Cas9 recognition mechanism typically may tolerate up to three nucleotide sequence mismatches between gRNA and target DNA, though as many as six have been previously reported (Tsai et al., 2015; Tycko et al., 2016). Undesirable off-target mutations could lead to alterations in gene expression or protein function, potentially introduce genotoxicity, and reduce cell viability. It is estimated that off-target activity can be decreased by 50- to 1,500-fold in cell lines when using double nicking (Zhang X. H. et al., 2015; Harrison and Hart, 2018).

Dead Cas9 (dCas9)

Another modified Cas9 is known as nuclease-null deactivated Cas9 or "dead Cas9." The dCas9 is designed to prevent double or single strand DNA breaks. With RuvC and HNH (**Figure 1B**) nuclease domains inactive, the CRISPR/dCas9 is capable to find the target sequence and cause direct transcriptional perturbation of the gene without causing a damage in the DNA. This dCas9 can be fused with proteins in order to inhibit (CRISPRi) or activate (CRISPRa) gene expression. For instance, Cas9 fused with Kruppel-associated box (KRAB) promotes gene repression whereas the enzyme fused with VP16 or VP64 activates gene expression (Gilbert et al., 2013; Lawhorn et al., 2014). This mechanism offers a variety of possibilities to re-write how genes are traditionally expressed and creates the potential for using transcription factors and other enzymes to alter the regulation of epigenetic marks and provides the opportunity to potentially correct epigenetic disorders (reviewed in Mei et al., 2016).

Base Editing

Base editing was the first breakthrough in the gene editing field after CRISPR/Cas9 due to the ability to perform precise point-mutation without a DSB. The first generation of base editor (BE) was BE1, a CRISPR/dCas9 fused at the *N*-terminus with a cytidine deaminase (rat APOBEC1) that produced a direct conversion of cytidine to uridine, thus effecting a C → T or G → A substitution (Komor et al., 2016). The BE1 targets deamination of nucleotides positioned within 4–8 bp that includes the PAM. However, initially BE1 was not highly effective in transitioning the U.G pair to a T.G pair due to the intermediate U.G cell repair mechanism. Dr. Liu and colleagues developed a novel BE2, a uracil DNA glycosylase inhibitor (UGI), a small protein from bacteriophage primer binding site (PBS), fused to the *C*-terminus of BE1 (Rees and Liu, 2018) to increase the efficiency of this transition. The BE2 conversion rate is three-fold higher compared to BE1 in human cells (U2OS and human embryonic kidney (HEK293T) cells), with indels formations below 0.1%. To further improve BE efficiency, the catalytic histidine residue at position 840 was restored in the Cas9 HNH domain of the BE2, creating the third-generation BE (BE3). BE3 is significantly more effective, achieving up to 37% of *C*-to-*T* conversion of total DNA (Komor et al., 2016). Since BE3, many other variants of cytidine BE have been generated resulting in improved *C*-to-*T* editing (Nishida et al., 2016; Kim et al., 2017; Komor et al., 2017; Koblan et al., 2018), including the newest BE4max and AncBE4max (up to 90% base editing efficiency) in HEK293T cells, and YFE-BE4max (up to 98%) (Koblan et al., 2018; Liu et al., 2020). These optimized BEs have been efficiently applied in mouse, rabbit, and pig embryos as well as mouse, rabbit, pig, and human cells (Kim et al., 2017; Zafra et al., 2018; Xie et al., 2019; Liu et al., 2020).

In human cells, spontaneous hydrolytic deamination of cytosine and 5-methylcytosine occurs about 100 to 500 times per day and results in the formation of uracil and thymine, respectively. This alteration may result in a permanent C.G to T.A mutations, which is known to affect about half of all pathogenic single nucleotide polymorphisms (SNPs). Adenosine base editor (ABE) is the new generation of base editor approaches that converts A.T bp to G.C bp, and has potential to revert pathogenic SNPs (Gaudelli et al., 2017). This ABE system uses laboratory-developed TadA tRNA deoxyadenosine deaminases fused with dCas9 to convert adenines into inosines. Ultimately, inosine is interpreted by polymerases as guanine (Anzalone et al., 2020). The first engineered ABE 7.8/9/10 exhibited a modest editing efficiency ranging from 1.7 to 20% in U2OS and HEK293T cells (Gaudelli et al., 2017). Genetically improved versions are able to increase the editing efficiency in HEK293T cells up to 52% using ABEmax (Koblan et al., 2018) and 69% using PAM-expanded *Sp*Cas9 variant (xCas9)-ABE7.10, and also increase the editing scope of this tool (Hu et al., 2018). Additionally, a modified ABE (ABE8e) showed the highest editing efficiency (up to 86%) in HEK293T cells (Richter et al., 2020). This technology has been

applied for efficient generation of mouse model of human disease (Liu et al., 2018) and has potential to develop large animal models.

Interestingly, some studies indicated an unexpected C-to-G edits using ABE at the position 5, 6, and 7 of the protospacer (numbering beginning from the most distal position to the PAM) (Grünewald et al., 2019; Kim et al., 2019). This finding led to a new BE platform, a C-to-G base editor (CGBE1) (Kurt et al., 2020). This is the first known BE capable of introducing a transversion mutation (C→G) without a DSB. The CGBE1 was engineered from BE4max and consisted of an RNA-guided Cas9 nickase, an *E. coli*-derived uracil DNA *N*-glycosylase (eUNG) and a rat APOBEC1 cytidine deaminase variant (R33A). In HEK293T cells, highly efficient C-to-G mutation was observed with an editing frequency ranging from 41.7 to 71.5%. Moreover, they reported that C-to-G edits are more efficiently introduced in AT-rich sequences in human cells (Kurt et al., 2020). Therefore, although some of these BEs need to be improved, they may provide a powerful tool for safe gene editing *in vivo* applications to revert inherited genetic mutations.

Point Mutation Introduction

Here, we defined point-mutation introduction as an intentional modification of target sequence with a very specific programmed mutation using either single-stranded oligodeoxynucleotide (ssODN) or double stranded donor DNA (dsDNA) to insert, delete or replace nucleotides in the target site. Targeted gene point-mutation can be genetically engineered to subvert the HDR system to introduce desired novel and controlled nucleotide modifications (deletion, insertion, or replacement of known single nucleotide or small sequences) using a customized template DNA with homologous arms (HA) to the target site (Maruyama et al., 2015; Riordan et al., 2015). With the high capability of CRISPR/Cas9 to produce DSB, both small and long template DNA can be transfected along with the CRISPR complex to promote the cell to repair the DSB by HDR using the introduced DNA template. ssODN or donor vector plasmid containing target modifications have been commonly used to perform precise alterations in many cell types (Yoshimi et al., 2016; Okamoto et al., 2019). The ssODN is a short single-strand DNA fragments containing the mutation of interest surrounded by 30 to 60 nt long homologous arms. The ssODN contains a homology sequence flanking the DSB of the targeted gene, thus, the gene is altered by knocking-in (KI) the designed mutations in the break. This approach has been successful in inserting/deleting or replacing short nucleotides (<50 bp) within the DSB (Paix et al., 2017). In mammalian cells, ssODN-mediated KIs are more effective to introduce targeted mutation than the donor plasmid approach (Yoshimi et al., 2016).

Cas9 Tethering ssODN

Recently, Aird et al. (2018) developed a Cas9 platform to allow ssODN to be present at the moment when the CRISPR/Cas9 breaks the target sequence. This new modified Cas9 contains a fused nuclease that is a member of the endonuclease superfamily, HUH endonuclease (histidine-U-histidine with the "U" a hydrophobic residue). These endonucleases process ssDNA through a specific reaction mechanism for cleavage and ligation of recognized ssDNA site (Chandler et al., 2013; Nelson et al., 2019). These proteins contain small domains with the ability to form a covalent ligation to ssDNA. While the mechanism of this sequence binding and specificity is poorly understood, it is generally believed that it involves an identification of a DNA hairpin. The covalent bond reaction occurs at room temperature and the phosphotyrosine bond is initiated with the hydroxyl group in the tyrosine amino acid attacking the phosphate group in the ssDNA that forces the release of the nucleotides at 5′ end (Lovendahl, 2018).

Viral HUH-tags endonuclease reacts quickly with ssDNA and requires no chemical modification in their ssDNA (Lovendahl, 2018; Nelson et al., 2019). A specific HUH domain is found in the porcine circovirus 2 rep protein (PCV), a virus known to infect domestic pigs with a plasmid that originated from *Pseudomonas aeuruginosa* (Lovendahl, 2018). Aird et al. (2018) created a PCV-Cas9 that can fuse HUH-domain of PCV to either side of the Cas9 termini. Then, ssODN is designed to contain 13 nucleotides of recognition sequence at 5′ terminus to be covalently bond to PCV domain. The combination has been shown to improve the HDR up to 30-fold in both HEK293T and U2-OS cell lines targeting different genomic sequences. Nonetheless, these researchers found that a PCV fused at the *N*-terminus in the Cas9 (PCV-Cas9), resulted in a much higher point mutation efficiency than the domain fused to the *C*-terminus (Cas9-PCV). The mechanism of such difference is not fully understood. Moreover, they found that lower concentrations of Cas9-PCV RNP (1.5 pmol) enhanced the HDR efficiency up to 15- to 30-fold (Aird et al., 2018). Thus, the tethering between Cas9 to ssODN may significantly improve CRISPR/Cas9 gene editing efficiency.

Prime Editor

The newest gene editing tool known as prime editor (PE), is one of the most accurate approaches for point-mutation introduction with great therapeutic potential to restore human genetic inherited mutations (Anzalone et al., 2019). This new concept of prime editing has been designed to insert point mutations without using a donor DNA template for the HDR pathway, or even performing a DSB in the target sequence. This gene editing tool – PE, is a catalytically impaired nCas9 (H840A) that is fused with a reverse transcriptase (RT-nCas9) with the capacity to be transfected along with a prime editing guide RNA (pegRNA). The molecular mechanism of prime editing involves the regular identification of DNA target with 20 nucleotides at the 5′ end of the pegRNA and a long 3′ end extending to interact with the opposite strand of the target sequence. The RT-nCas9 breaks the single-strand DNA via the RuvC nuclease domain. Then, the tip of the 3′ end of pegRNA, which contains a PBS, aligns against the broken DNA strand. The RT-nCas9 uses the pegRNA template containing the modification site upstream to the PBS to synthesize a brand-new sequence (Anzalone et al., 2019). Dr. David Liu's laboratory has undoubtedly demonstrated the effectiveness of the prime editing for the introduction of targeted insertions and deletions without performing a DSB in cells (Anzalone et al., 2019).

They performed 175 edits in human HEK293T cells typically achieving 20 to 50% editing efficiency, with less than 10% of indels. Prime editing holds remarkable promise for gene editing, but this technology is still immature and additional studies are needed to fully realize the prime editing potential (Yan et al., 2020).

The prime editing has been applied in mouse cells (mouse neuro-2a (N2a) cells) of which the prime editor 3 (PE3) mediated base transversion at three target sites of *Hoxd13* and androgen receptor genes with an efficiency from 8 to 40% (Liu et al., 2020). Moreover, zygote microinjection of pegRNAs, targeting the same *Hoxd13* gene led to successful conversion mutations. G-to-C and G-to-T conversions were found in 8 out of 18 (44%) and 12 out of 16 (75%) blastocysts, respectively, with mutation frequencies ranging from 1.1 to 18.5% in each embryo. Additionally, injected mouse embryos were transferred into surrogate mothers. Eight out of 30 mice contained the conversion mutation (editing efficiency of G-to-C above 1%) as well as two out of 19 mice presented conversion mutation (editing efficiency of G-to-T above 1%) (Liu et al., 2020).

Chemically Modified ssODN

Due to the low rate of homologous recombination in the cell, different approaches were developed to improve the point-mutation efficiency through HDR pathway. Although, chemical reagents have been vastly applied (Maruyama et al., 2015; Robert et al., 2015; Vartak and Raghavan, 2015; Yu et al., 2015; Song et al., 2016; Khan et al., 2018; Kostyushev et al., 2019) to improve KI by either stimulating HDR pathway (e.g., RS-1, L755507, and Brefeldin A) or inhibiting the NHEJ (e.g., SCR7, NU7441, NU7026, KU-0060648, and VX-984), the potential adverse effects caused by these small molecules remains unknown (Okamoto et al., 2019).

Chemically modified donor oligonucleotides have also been developed to increase the KI efficiency. The ssODN has been developed by using the designed donor DNA with chemical modification of its structure. Although the mechanism by which ssODN-mediated DNA repair occurs is still not fully understood, these molecules are very useful tools for precise gene editing (Davis and Maizels, 2016; Kan et al., 2017). Renaud et al. (2016) demonstrated that subtle modifications in the ssODN can not only significantly improve gene editing efficiency but also increase the flexibility of the DNA to insert longer DNA sequences. This approach consists of the replacement of some phosphates in the ssODN sequence structure by phosphorothioate. In this molecule, one of the oxygens not involved in the phosphodiester ligation between two nucleotides is changed to Sulfur atom (S), thus, forming the phosphorothioate (O_3PS^{-3}) bond. Two of these modified phosphates are added in both 5′ and 3′ ends of the ssODN sequence. Renaud et al. (2016) reported that KI using phosphorothioate ssODN may improve gene editing efficiency up to three-fold in cell lines when compared to the conventional phosphodiester ssODN. In another study using phosphorothioate ssODN, Harmsen et al. (2018) investigated the effects of phosphorothioate in sense and antisense ssODN, as well as the presence of a single phosphorothioate in either 5′ or 3′ ends. They evaluated the efficiency of introducing a point-mutation of a single nucleotide replacement located 42 nt away from the DSB site using a 120 nt ssODN. The findings indicate that the 3′ phosphorothioate enhances gene editing by promoting integration of nucleotides away from the DSB. Also, they propose a critical role of the mismatch repair pathway at the 3′ end of ssODN that enables gene editing far away from the break, which removes the mismatch, and ssODN sequence is copied into genome (Harmsen et al., 2018).

In addition to use in ssODN, gRNAs have been adapted to be chemically synthesized as 2′-*O*-methyl-3′-phosphorothioate-modified gRNAs. The phosphorothioate results in an increase in stability and protects against exonucleases, as well as it improves gene editing efficiency of CRISPR/Cas9 to over 90% (Hendel et al., 2015; Hoellerbauer et al., 2020). Moreover, phosphorothioate-modified gRNAs have reduced off-target risk compared to the gRNA from plasmid or viral delivery (Cameron et al., 2017). The chemically modified oligonucleotide concept also led to the development of chemically modified dsDNA, which has recently been applied in HEK293T cells and led to up to 65% targeted-insertion efficiency of long fragments of DNA, discussed in the next section (Yu et al., 2020).

Targeted Integration of Long dsDNA

Transfection or injection of long DNA fragments containing a gene of interest has been used as a strategy to express foreign genes in cells *in vitro* (Kohn et al., 1987; Bayna and Rosen, 1990) and for the production of GE animals. However, targeted integration has been a challenge due to the low rate of HDR in the cells and the high probability of random integration (reviewed by Bischoff et al., 2020). Different approaches to improve the integration of long fragments of DNA have been developed, including CRISPR/Cas9 mediating homologous recombination (HR), microhomology-mediated end-joining (MMEJ) targeted integration, homology-mediated end joining (HMEJ)-based targeted integration, and the NHEJ-mediated KI named homology-independent targeted integration (HITI) (Suzuki et al., 2016; Wu et al., 2016; Yao et al., 2017a,b). Often these approaches aim to accomplish specific targeted integration of genes of interest into what is known as safe harbor' genes, such as Rosa26, adeno-associated virus integration site 1 (AAVS1), and H11 (Ruan et al., 2015; Wu et al., 2016; Xie et al., 2017). These sites in the genome are able to accommodate the transgene integration that ensures its high transcriptional activity in embryonic and adult tissues, and does not suppress critical endogenous genes (Ruan et al., 2015; Oceguera-Yanez et al., 2016; Weber et al., 2016; Wu et al., 2016; Yu et al., 2019; Kelly et al., 2020).

The HR was the first strategy used for targeted integration, and its approach consists of using long homologous sequences copied from the target site to induce DNA repair through the HDR pathway using the DNA template (Capecchi, 1989). The HR allows a precise mechanism for modifications of the genome of cells *in vitro* and has been extensively used to investigate gene function and to generate mouse models of human diseases

(Zwaka and Thomson, 2009). The initial applications aimed to either alter the genes' reading frame, producing gene KO, or introduce exogenous genes (KI) (Rosenthal and Brown, 2007). The ability to generate mice with specific genetic alterations has revolutionized biomedical research (Zwaka and Thomson, 2009). These targeting vectors are commonly constructed using backbone vector, such as MultiSite Gateway® technology. The constructed vector contains the following basic components: either a gene of interest downstream to a constitutive promoter (e.g., cytomegalovirus promoter) or a modified target sequence; a selectable marker, which frequently is an antibiotic resistance gene (e.g., hygromycin and puromycin) or some fluorescence protein (e.g., GFP) for identification of the colonies containing the insert; the last components are homologous sequences (>500 bp each) flanking the insert (Conlon, 2006; Iiizumi et al., 2006). Once assembled, the vector is linearized for transfection into the cells using some transfection-based methods – viral particles, electroporation, lipid-mediated transfection, etc. (Kim and Eberwine, 2010). CRISPR/Cas9 co-transfected with a targeting vector could facilitate HDR by creating the DSB in the target site (Meyer et al., 2010; Sommer et al., 2014; Wu et al., 2016).

Although NHEJ and HDR are well known DNA repair pathways, a third not so popular pathway was discovered over the last decade, MMEJ pathway. MMEJ forms an alternative end-joining to repair DSB via microhomology (5 to 25 bp) between the sequences. This pathway is known to be associated with abnormalities in the cell, such as deletions, translocations, inversions, and other complex rearrangements (McVey and Lee, 2008; Yao et al., 2017b). The MMEJ pathway shares aspects with NHEJ and HDR since it joins the DSB ends without a template, like NHEJ, and MMEJ requires initial DSB end resection, similar to HDR. MMEJ initiation requires short-sequence resection of DSB ends to disclose the homologies, which also initiates HDR (Yeh et al., 2019). Moreover, MMEJ pathway seems to compete with HDR in the DNA repair, as MMEJ is active in the S and early M phases, whereas HDR is activated in late S- to G2 phase (Zhao et al., 2017; O'Brien et al., 2019). MMEJ-mediated targeted integration is also known as PITCh (Precise Integration into Target Chromosome) system (Sakuma et al., 2016) that has been shown to have an increased efficiency for targeted integration. The first studies to successfully introduce a donor plasmid by microhomology PITCh system was mediated by TALENs and CRISPR/Cas9 in silkworms and frogs (Nakade et al., 2014). In another study, PITCh system was used along with CRISPR/Cas9 for a gene cassette KI in human cells and mouse zygotes (Aida et al., 2016). They successfully knocked-in 5 kb gene cassette by MMEJ-based target integration in mice with 10% efficiency. Additionally, co-delivery of the PITCh system with *Exo1* improved KI efficiency in this study to 30%. Yao et al. (2017b) reported that MMEJ-mediated targeted integration has increased KI efficiency up to 10-fold when compared to the standard HR approach in mouse tissue. Thus, MMEJ-mediated integration is a robust approach to KI gene of interest through both *ex vivo* and *in vivo* and may offer broader applications in gene therapy (Yao et al., 2017b).

The CRISPR/Cas9-mediated HMEJ is the third alternative method for insertion of long DNA fragments into a host genome. HMEJ relies on CRISPR/Cas9-mediated cleavage of both constructed transgene vector and target genome site. The donor plasmid contains HAs with approximately 800 bp and the targeted genome gRNA site at the 5′ end of the left HA, as well as the 3′ end of the right HA (Banan, 2020). This strategy may take advantage of HDR pathway as well as a HMEJ pathway (Yao et al., 2017a). Yao et al. (2017a) demonstrates that HMEJ strategy provides the highest targeted integration efficiency (up to 27% KI) when compared to HR, MMEJ, and NHEJ approaches in HEK293T cells, mouse primary astrocytes, and neurons cells, as well as mouse and monkey embryos.

The newest potential approach for targeted integration is the HITI. This method is a NHEJ-mediated KI, which works independent from HDR for targeted insertion and provides a robust donor vector for both dividing and non-dividing cells (Suzuki et al., 2016). This concept has been highly efficient to KI donor vectors with low rates of off-target mutations *in vitro* and *in vivo* (Suzuki and Belmonte, 2018). The method is based on the transfection of a minicircle vector produced from pre-minicircle plasmids containing the target site of CRISPR/Cas9 inside of the minicircle. Suzuki et al. (2016), demonstrated the potential of HITI with 56% efficiency of targeted insertion of IRESmCherry in mouse neurons, while keeping the indels mutations at the same target site at the low level (5 to 10%). Moreover, their findings present high on-target specificity of HITI (90–95%). Among all evaluated cells, 30–50% showed biallelic transgene integration (Suzuki et al., 2016). Shi et al. (2020) applied HITI along with CRISPR/Cas9 targeting to the ovalbumin (OVA) locus in chicken DF-1 and embryonic fibroblast cells. EGFP cassette was introduced into the OVA locus via HITI and the GFP expression activated by endogenous OVA promoter using the dCas9-VPR transactivating approach (Shi et al., 2020). In another study, an efficient transgenesis using HITI was performed in ferret embryos. An 8 kb cassette expressing Tomato/EGFP was inserted into intron 1 of the Rosa26 locus. Zygotes ($n = 151$) were microinjected with the plasmid and CRISPR/Cas9 RNP. Five out of 23 offspring exhibited the reporter expression (Yu et al., 2019). Therefore, HITI method offers a great enhancement over the other methods as it takes advantage of NHEJ for gene insertion.

Gene insertion approaches have received a new endorsement using chemically modified oligonucleotides. Recently, Yu et al. (2020) inserted different types of modifications into dsDNA to evaluate the effect of chemically modified dsDNA to improve gene insertion into target integration site. The recent results demonstrate that using short homologous arms (50 bp) containing 5′-modified double-stranded modification, the KI rates for long inserts (2.5 kb) was up to 40%, whereas for short inserts (0.7 kb) reached an unprecedented rate of 65% in HEK293T cells. Moreover, up to five-fold increase of gene KIs was observed in different loci of human cancer and stem cell genomes. The chemical modification that provided such an improvement was a C6-PEG10 at the 5′ end of each homologous arm (Yu et al., 2020). Although the approach has not been tested in other cell types, including animals, the chemically modified dsDNA may become a solution for insertion of gene of interest in the

target sequence with higher efficiency when compared to the traditional approaches.

PRODUCTION OF GENE EDITED FARM ANIMALS

Zygote Manipulation

The first Genetically Engineered (GE) farm animals were produced 35 years ago by DNA microinjection into the pronucleus of zygotes (Hammer et al., 1985). Transgenic animals were successfully produced in several species including mice (Gordon et al., 1980), rabbits, pigs, sheep, cattle, and goats by injection of genes of interest into the pronucleus of a zygote (review by Wall, 1996). At that time, this technique was suffering from several serious limitations (Wilmut and Clark, 1991; Pursel and Rexroad, 1993). The most profound constraint was that DNA can only be added, not deleted, or modified *in situ*. Also, the integration of foreign DNA was random leading to erratic transgene expression due to the integration site effect. Furthermore, random integration has a risk for the disruption of essential endogenous DNA sequences or activation of cellular oncogenes, both of which could have deleterious effects on the animal's health. Finally, GE animals generated using zygote microinjection are commonly mosaic, i.e., when desired genetic alteration is not present in all cells (Wieland et al., 1990). Therefore, the production of the required phenotype coupled to germ line transmission could require the generation of several transgenic founder lines followed by breeding.

Advances in CRISPR/Cas9 genome editing significantly improved the ability to precisely disrupt genes and/or introduce specific mutations by direct zygote manipulation (pronuclear or cytoplasmic injection, or electroporation; Navarro-Serna et al., 2020). Recently, a high efficiency of generating indels mutations in bovine and porcine zygotes via electroporation was reported (Miao et al., 2019). This method greatly simplifies generation of GE livestock as it does not require micromanipulation expertise. However, genetic mosaicism continues to be a major challenge using zygote manipulation approach (reviewed by Mehravar et al., 2019). Mosaicism emerges when DNA replication precedes CRISPR-mediated genome edition, which greatly reduces the likelihoods for direct KO generation. The impact of mosaicism could be even more devastating if both somatic and germline mosaicism are present in the offspring. One of the approaches proposed to reduce genetic mosaicism is an introduction of CRISPR/Cas9 into either metaphase II (MII) oocyte or a very early zygote stage. Electroporation of Cas9 RNP into an early zygote stage has eliminated mosaic mutants in mice (Kim et al., 2014; Hashimoto et al., 2016). However, injection of CRISPR/Cas9 into MII oocytes did not reduce mosaicism compared to the zygote injection in sheep and cattle (Lamas-Toranzo et al., 2019; O'Neil et al., 2020). Inability of CRISPR to recognize its target locus prior to some degree of chromatin de-condensation took place might be a reason for these somewhat surprising outcomes.

Shortening longevity of Cas9 by accelerating its degradation is another possible tactic for reducing mosaicism. This can be accomplished by tagging Cas9 with ubiquitin-proteasomal degradation signals that facilitate the Cas9 degradation. Alternatively, to completely eliminate the risk of mosaicism nuclear transfer approach using GE cells could be considered.

Somatic Cell Nuclear Transfer – Cloning

Somatic cell nuclear transfer was initially developed in sheep with the birth of Dolly in 1996 (Wilmut et al., 1997). The technology was later established for other key livestock species: cattle (Cibelli et al., 1998), goats (Baguisi et al., 1999), pigs (Polejaeva et al., 2000), and equine (Woods et al., 2003), providing the first cell-mediated platform for livestock genetic engineering. Precise genetic manipulations are introduced in somatic cells (typically fetal fibroblasts), followed by the isolation of single-cell-derived colonies and cell screening to confirm that the desired genetic modifications are present in the cells. Subsequently, the cells are used as donor cells for SCNT (Schnieke et al., 1997; Clark et al., 2000; McCreath et al., 2000; Dai et al., 2002; Phelps et al., 2003). This method has a major advantage compared with zygote manipulation approach for GE animal production, because the entire animal is derived from a single GE donor nucleus, thus the risk of mosaicism is eliminated (Polejaeva and Campbell, 2000). However, this method is more technically challenging and typically has a low term development rate. Additionally, potential cloning related epigenetic alterations might contribute to the GE animal phenotype, thus generation of F1 animals is often desirable for a proper characterization of GE models. Despite these limitations, SCNT continues to be the primary method for the production of the KI gene edited livestock, with nearly 70% of the published work was conducted using this methodology (**Table 3**). Additionally, about half of the published KO farm animals were generated using SCNT (**Tables 1**, **2**). GE animals produced by SCNT often required the use of fewer recipient animals compared to the number of animals needed for the zygote micromanipulations (Schnieke et al., 1997).

GENE EDITING APPLICATIONS IN AGRICULTURE

The global demand for animal products is substantially growing, driven by a combination of burgeoning population, urbanization, and income growth. However, approximately one billion people in the world are still chronically malnourished (Godfray et al., 2010). Global climate change will only exacerbate the lack of animal protein production (McMichael, 2012). Present efforts to satisfy global food needs are degrading an already burdened environment (Foley et al., 2011; Tilman et al., 2011). Improvements in the efficiency of animal production and food safety are becoming more important considerations for protection of the environment and reduction in land usage (Clark and Whitelaw, 2003). The United Nations (UN) predicts world population will reach 9.8 billion by mid-century (United Nations, 2020), and therefore, calls for use of innovative strategies and new technologies to double food production by 2050 in order to meet demand from the world's growing population. According to the UN, this increased production must come from virtually the same

TABLE 1 | CRISPR-meditated gene knockout in livestock: agricultural applications.

Species	*Gene*	Purpose of manipulation	Approach	Mosaicism (%)	References
Sheep	*ASIP*	Coat color pattern	MI	2/5 (40.0%)	Zhang X. et al. (2017)
	FGF5	Wool growth	MI	(6.3–100%)	Hu et al. (2017), Li W. R. et al. (2017), Zhang R. et al. (2020)
	MSTN, ASIP, and BCO2	Economically important traits	MI	2/2 (100%)	Wang X. et al. (2016b)
	MSTN	Meat production	MI or SCNT	(0–100%)	Deng et al. (2014); Crispo et al. (2015), Zhang Y. et al. (2019); Yi et al. (2020)
Goat	*BLG*	Milk quality	MI	3/4 (75.0%)	Zhou et al. (2017)
	MSTN and FGF5	Meat and cashmere production	MI	5/10 (50.0%)	Wang X. et al. (2015a)
	MSTN	Meat production	MI or SCNT	(0–100%)	Ni et al. (2014); Guo et al. (2016), He et al. (2018); Zhang Y. et al. (2019)
	NANOS2	Surrogate sires for genetic dissemination	SCNT	N/A	Ciccarelli et al. (2020)
	EDAR	Cashmere yield	SCNT	N/A	Hao et al. (2018)
Pig	*IGF2 regulatory element*	Meat production	MI (nCas9)	6/6 (100%)	Xiang et al. (2018)
	NANOS2	Surrogate sires for genetic dissemination	MI	6/18 (33.3%)	Park et al. (2017)
	ANPEP	Viral resistance	MI	1/9 (11.1%)	Whitworth et al. (2019)
	CD163	Resistance to PRRS virus	MI, EP, or SCNT	No	Whitworth et al. (2014); Yang et al. (2018), Tanihara et al. (2019)
	IRX3	Reduced fat content in Bama minipigs	SCNT	N/A	Zhu et al. (2020)
	NANOS2	Surrogate sires for genetic dissemination	SCNT	N/A	Ciccarelli et al. (2020)
	MSTN	Meat production	SCNT	N/A	Wang K. et al. (2015), Wang K. et al. (2017), Li R. et al. (2020)
	CD163 and pAPN	Viral resistance	SCNT	N/A	Xu et al. (2020)
	FBXO40	Meat production	SCNT	N/A	Zou et al. (2018)
Cattle	*NANOS2*	Surrogate sires for genetic dissemination	MI	1/3 (33.3%)	Ciccarelli et al. (2020)

SCNT, somatic cell nuclear transfer; MI, zygote microinjection; EP, zygote electroporation; nCas9, Cas9 nickase; N/A, not applicable.

land area as today. Thus, the need for innovation through new technologies is essential for the future of people, communities, and natural resources. The recent development of gene editing combined with the animal production technologies provide the potential for accelerating the genetic improvement of livestock, including alteration of production traits, enhancing resistance to disease, reducing the threat of zoonotic disease transmission, and improvement of livestock welfare (Tan et al., 2013). Genetic-based increases in sustainable animal productivity will be a key to meet the global food demand.

Improving Livestock Production Traits

Examples of gene editing application for livestock production trait improvements are provided in this section. Additionally, a comprehensive summary included in **Tables 1** and **2**. Key interest areas covered under agricultural umbrella include meat and fiber production, improvements in milk quality, and reproductive performance, as well as disease resistance and animal welfare (**Figure 2**).

Myostatin (MSTN), a negative regulator of skeletal muscle mass (McPherron et al., 1997) is the most frequent target of gene editing, as *MSTN* KO offers a strategy for promoting animal muscle growth in livestock production. Myostatin (previously called GDF-8) was originally identified in a screen for new members of the TGF-ß superfamily in mammals (McPherron et al., 1997). In adult tissues, myostatin is expressed almost exclusively in skeletal muscle, but clearly detectable levels of myostatin RNA are also present in adipose tissue (Roberts and Goetz, 2003; Lee, 2004). The function of myostatin was elucidated through gene KO studies, in which myostatin KO mice have about a doubling of skeletal muscle weights throughout the body as a result of a combination of muscle fiber hyperplasia and hypertrophy (McPherron et al., 1997). The myostatin gene has been analyzed in many different species and has been found to be extraordinarily well conserved. Natural gene mutations of MSTN have also been reported in some cattle breeds (Grobet et al., 1997, 1998), sheep (Boman et al., 2009), dogs (Mosher et al., 2007), and human (Schuelke et al., 2004). These animals show a double-muscled phenotype of dramatically increased muscle mass, and still viable and fertile (Grobet et al., 1997, 1998; Mosher et al., 2007; Boman et al., 2009). Moreover, pharmacological agents capable of blocking MSTN activity have been shown to cause significant increases in muscle growth when administered systemically to adult mice (Bogdanovich et al., 2002; Whittemore et al., 2003; Lee et al., 2005), demonstrating that MSTN plays a critical role in regulating muscle homeostasis postnatally by suppressing muscle growth. Successful disruption of the MSTN gene by gene editing was reported in sheep, goats, and pigs that lead to enhance animal growth performance (Deng et al., 2014; Ni et al., 2014; Wang K. et al., 2015).

Another potential candidate gene for improving meat production in livestock and for developing therapeutic

TABLE 2 | CRISPR-meditated gene knockout in livestock: biomedical applications.

Species	*Gene*	Purpose of manipulation	Approach	Mosaicism (%)	References
Sheep	*PDX1*	Pancreas-deficient model development	MI	2/2 (100%)	Vilarino et al. (2017)
	BCO2	b-carotene metabolism research	MI	2/6 (33.3%)	Niu Y. et al. (2017)
	CFTR	Cystic fibrosis model	SCNT	N/A	Fan et al. (2018a)
Goat	*IGHM*	Human polyclonal antibody production	SCNT	N/A	Fan et al. (2018b)
Cattle	*GGTA and CMAH*	Xenotransplantation	SCNT	N/A	Perota et al. (2019)
Pig	*SCD5*	Chronic Maxillary Sinusitis and Dysostosis diseases	MI	No	Carey et al. (2019)
	CMAH	Viral resistance	MI	3/5 (60.0%)	Tu et al. (2019)
	Ig-J$_H$	Hepatitis E virus pathogenicity	MI	No	Yugo et al. (2018)
	ULBP1	Xenotransplantation	MI (nCas9)	No	Joanna et al. (2018)
	TMPRSS2	Resistance to influenza viruses	MI	5/12 (41.7%)	Whitworth et al. (2017)
	PDX1	Lack of pancreas, regenerative medicine	MI	2/3 (66.6%)	Wu et al. (2017)
	DMD	Duchenne muscular dystrophy model	MI	1/1 (100%)	Yu et al. (2016)
	PARK2, DJ-1, and PINK1	Parkinson's disease model	MI	2/2 (100%)	Wang X. et al. (2016a)
	RAG2 and IL2RG	Model for severe combined immunodeficiency	MI	3/17 (17.6%)	Lei et al. (2016)
	NPC1L1	Human cardiovascular and metabolic diseases	MI	5/11 (45.5%)	Wang Y. et al. (2015)
	MITF	Human Waardenburg and Tietz syndromes	MI	No	Wang X. et al. (2015b), Hai et al. (2017)
	vWF	Model of von Willebrand disease	MI	Most pigs	Hai et al. (2014)
	EDA	Lung disease model	MI	No	Ostedgaard et al. (2020)
	GRB10	GRB10 role in insulin resistance and obesity	MI or EP	No	Sheets et al. (2016)
	GGTA1	Xenotransplantation	MI or EP	0–40.0%	Petersen et al. (2016); Chuang et al. (2017), Tanihara et al. (2020)
	TP53	Model with tumor phenotypes	EP	5/6 (83.3%)	Tanihara et al. (2018)
	IL2RG	Immunodeficiency model	SCNT	N/A	Ren et al. (2020)
	SIX1 and SIX4	Kidney-deficient model	SCNT	N/A	Wang J. et al. (2019)
	B2M	Xenotransplantation	SCNT	N/A	Sake et al. (2019)
	GGTA1,β4GalNT2, CMAH	A source of Bioprosthetic heart valves	SCNT	N/A	Zhang R. et al. (2018)
	ApoE	Models of atherosclerosis	SCNT	N/A	Fang et al. (2018)
	INS	Diabetes research	SCNT	N/A	Cho et al. (2018)
	TPH2	5-HT deficiency and behavior abnormality	SCNT	N/A	Li Z. et al. (2017)
	Hoxc13	Ectodermal dysplasia–9 disease	SCNT	N/A	Han et al. (2017)
	GGTA1 and CMAH	Xenotransplantation	SCNT or sSCNT	N/A	Fischer et al. (2016); Gao et al. (2016)
	PERV	PERV-inactivated animals, xenotransplantation	SCNT	N/A	Niu D. et al. (2017)
	C3	Roles of C3 in human diseases	SCNT	N/A	Zhang W. et al. (2017)
	IL2RG	Severe combined immunodeficiency	SCNT	N/A	Kang et al. (2016a)
	RUNX3	Cancer model	SCNT	N/A	Kang et al. (2016b)
	Ig-J$_H$	B cell-deficient model for h Ab production	SCNT	N/A	Chen et al. (2015)
	TYR	Oculocutaneous albinism type 1 disease	SCNT (nCas9)	N/A	Zhou et al. (2015)
	PARK2 and PINK1	Parkinson's disease	SCNT	N/A	Zhou et al. (2015)
	GGTA1, CMAH & iGb3S	Xenotransplantation	SCNT	N/A	Li et al. (2015)
	CD1D	Models for biomedicine	SCNT	N/A	Whitworth et al. (2014)
	Class I MHC	Model for immunological research	SCNT	N/A	Reyes et al. (2014)
	ApoE and LDLR	Human cardiovascular disease	SCNT	N/A	Huang et al. (2017)

SCNT, somatic cell nuclear transfer; sSCNT, serial SCNT; MI, zygote microinjection; EP, zygote electroporation; nCas9, Cas9 nickase; N/A, not applicable.

interventions for muscle diseases is *FBXO40* protein coding gene, a member of the F-box protein family. Expression of FBXO40 is restricted to muscle, and mice with an *Fbxo40* null mutation exhibit muscle hypertrophy. FBXO40 KO pigs have been recently produced but exhibited only marginal increase in muscle mass (4%) compared to WT controls (Zou et al., 2018). The KO pigs developed normally, and no pathological changes were found in major organs.

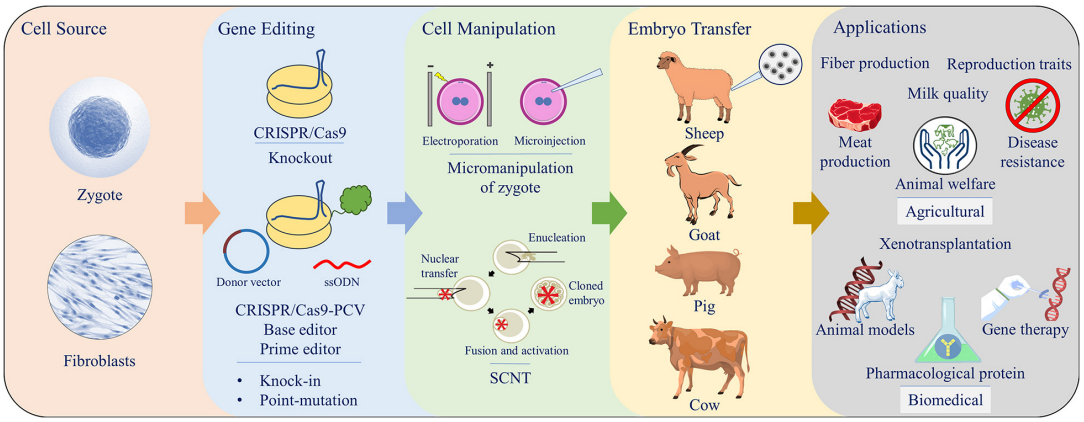

FIGURE 2 | Schematic summary of CRISPR/Cas9 gene editing using either zygote micromanipulation (electroporation or microinjection) or somatic cell nuclear transfer (SCNT) for generation of livestock animals for various applications.

The whey protein β-lactoglobulin (BLG) is a major milk allergen which is absent in human milk. *BLG* KO goat and cows have been produced by CRISPR/Cas9 and zygote microinjection and ZFNs gene editing and SCNT, respectively (Zhou et al., 2017; Sun et al., 2018). Western blot results showed that the BLG protein had been abolished in the milk of the BLG KO goat. In comparison with WT goats, BLG KO goats have exhibited a decreased level of fat, protein, lactose, and solid not fat in the milk by 5.49, 7.68, 7.97, and 7.7%, respectively.

In several studies two or three genes were targeted simultaneously leading to double or triple gene KOs (Wang X. et al., 2016b). For instance, MSTN and FGF5 KO goats were produced to improve meat production and cashmere yield (Wang X. et al., 2015a). Fibroblast growth factor 5 (FGF5), a secreted signaling protein that inhibits hair growth by blocking dermal papilla cell activation and is regarded as the causative gene underlying the angora phenotype (long hair coat). The efficiency of disrupting MSTN and FGF5 in 98 tested animals was 15 and 21%, respectively, and 10% of the animals had double gene KOs.

A concept of "surrogate sires" was recently validated for pigs, goats, and cattle (Ciccarelli et al., 2020) by demonstrating that the *NANOS2* gene KO males generated by CRISPR/Cas9 editing have testes that are germline ablated but otherwise structurally normal. Subsequent, spermatogonial stem cell transplantation (SSCT) with allogeneic donor stem cells led to sustained donor-derived spermatogenesis. This prove of principle study has great potential for dissemination of elite livestock genetics.

Improving Health and Welfare

Porcine reproductive and respiratory syndrome virus (PRRSV) causes severe economic losses to current swine production worldwide. Highly pathogenic PRRSV (HP-PRRSV), originated from a genotype 2 PRRSV, is more virulent than classical PRRSV and further exacerbates the economic impact. Several groups successfully generated CD163 KO pigs using CRISPR/Cas9 gene editing (Whitworth et al., 2014; Yang et al., 2018; Tanihara et al., 2019). Challenge with either the NVSL 97-7895 PRRSV virulent virus isolate (Whitworth et al., 2016) or the HP-PRRSV strain (Yang et al., 2018) showed that CD163 KO pigs are completely resistant to viral infection manifested by the absence of viremia, antibody response, high fever or any other PRRS-associated clinical signs. By comparison, wild-type (WT) controls displayed typical signs of PRRSV infection (Whitworth et al., 2016; Yang et al., 2018). More recently, Whitworth et al. showed that amino peptidase N (APN) deficient pigs are fully resistant to transmissible gastroenteritis virus (TGEV), but not porcine epidemic diarrhea virus (PEDV) (Whitworth et al., 2019). Additionally, porcine alveolar macrophages derived from the APN-deficient pigs showed resistance to porcine deltacoronavirus (PDCoV). However, lung fibroblast-like cells derived from these animals supported a high level of PDCoV infection indicating that APN is a dispensible receptor for PDCoV (Stoian et al., 2020).

Double-gene-knockout (DKO) pigs containing KOs for known receptor proteins CD163 and pAPN are reported to be completely resistant to genotype 2 PRRSV and TGEV (Xu et al., 2020). Additional infection challenge experiments have shown that these DKO pigs exhibit decreased susceptibility to PDCoV, thus providing *in vivo* evidence that pAPN as likely to be one of PDCoV receptors.

Prion diseases, such as scrapie in goats or sheep, bovine spongiform encephalopathy (BSE) in cattle and Creutzfeldt-Jakob disease (CJD) in humans, are a group of fatal and infectious neurodegenerative disorders of the central nervous system (CNS) (Prusiner, 1998). There is considerable evidence that the prion diseases are caused by propagation of misfolded forms of the normal cellular prion protein (PrP) (Aguzzi et al., 2008). The pathogenic form of this protein appears to be devoid of nucleic acids and supports its own amplification in the host. This self-propagating process allows for the exponential increase and accumulation of misfolded PrP in cells, resulting in a disruption of cell function and ultimately cell death (Aguzzi et al., 2008). Prion diseases have had important economic impact, resulting in billions of dollars in lost earnings in many countries due to trade embargos and weakened consumer confidence.

This has energized efforts to understand prion diseases as well as to develop tools for disease detection, prevention, and management. More interestingly, while the cellular PrP is absolutely required for disease pathogenesis, it is dispensable for normal animal development. Disruption of PrP expression in mice resulted in no apparent developmental abnormalities (Bueler et al., 1993; Manson et al., 1994). Moreover, cattle devoid of PrP are clinically, histopathologically, immunologically, and physiologically normal, and the brain tissue homogenates from PrP KO cattle are resistant to prion propagation *in vitro* (Richt et al., 2007). PrP KO livestock will improve food safety, which will potentially relieve food crisis in the future (Ni et al., 2014).

Disease causing mutations can also be effectively corrected using gene editing techniques. Ikeda et al. were able to repair a recessive mutation responsible for isoleucyl-tRNA synthetase (IARS) syndrome in Japanese Black cattle (Ikeda et al., 2017). Selective breeding for more than 60 years has yielded high meat quality famous for its distinctive marbling but has also resulted in the accumulation of recessive mutations that cause genetic diseases. The c.235G > C (p.Val79Leu) substitution in the *IARS* gene causes a 38% reduction in the aminoacylation activity of the IARS protein, which impairs protein synthesis. Homozygous mutant calves exhibit neonatal weakness with intrauterine growth retardation.

In modern livestock, daily management of horned cattle pose a high risk of injury for each other as well as for the farmers. Dehorning is associated with stress and pain for the calves and raises concerns regarding animal welfare. Naturally occurring structural variants causing polledness are known for most beef cattle. Polled Celtic variant from the genome of an Angus cow was isolated and integrated into the genome of fibroblasts taken from the horned bull using the CRISPR/Cas12a system, followed by SCNT (Schuster et al., 2020). The study successfully demonstrated practical application of CRISPR/Cas12a in dairy husbandry.

BIOMEDICAL APPLICATIONS

GE livestock models play a critical role in advancing our understanding of disease mechanisms due to their anatomical and physiological similarity to humans, and thus, are likely to open new clinically relevant mechanism-based targets for the prevention and treatment of numerous diseases. Livestock models have undoubtedly made a significant contribution in translational medicine. They effectively represent the complexity of outbred species and often have more similar pathogenesis of genetic, metabolic, infectious, and neoplastic diseases to those in human compared with the mouse model equivalents (Roth and Tuggle, 2015; Polejaeva et al., 2016). Similar organ size and function make them more suitable than a mouse for many biomedical applications, such as tissue recovery, serial biopsies, and blood sampling, device development, whole-organ manipulations, cloning, and the development of surgical procedures (Reynolds et al., 2009). Current availability of genome sequences and efficient gene-editing techniques are increasing accessibility of GE livestock models for biomedical research, xenotransplantation, and gene therapy. Numerous review papers discussing the topic of engineering large animal models are available (Whitelaw et al., 2016; Hamernik, 2019) including reviews on gene-editing for xenotransplantation (Meier et al., 2018; Cowan et al., 2019). GE swine models have been made available to researchers through institutions such as the National Swine Resource and Research Center at the University of Missouri–Columbia (http://www.nsrrc.missouri.edu, accessed 29 September 2020) and the Meiji University International Institute for Bio-Resource Research (MUIIBR) in Japan (http://www.muiibr.com, accessed 30 September 2020). Here, we provide a list of livestock models recently generated by CRISPR/Cas9 (**Tables 2**, **3**). The pig is increasingly gaining approval and it is the most frequently used large biomedical model (Gutierrez et al., 2015). Porcine gene-edited models represent aproximaly 80% of all GE livestock models (**Tables 2**, **3**).

Cattle are commonly used as a model for human female reproduction, including ovarian function, the effect of aging on fertility, and embryo–maternal communication (reviewed in Polejaeva et al., 2016). Similarities between sheep and humans in the physiological parameters of lung function, such as airflow, resistance, and breathing rates, have made sheep a valuable model for asthma research (Van der Velden and Snibson, 2011). Furthermore, preterm and term lambs have similar pulmonary structure, including airway branching, submucosal glands, and a dual oxidase (Duox)–lactoperoxidase (LPO) oxidative system, as well as prenatal alveologenesis that make them an ideal model to study respiratory distress syndrome in preterm infants (Liggins and Howie, 1972) and respiratory syncytial virus (RSV) infection (Derscheid and Ackermann, 2012). Ovine model of Cystic Fibrosis (CF) could be also very valuable to study developmental progression of CF (Fan et al., 2018a). Advancement in gene editing technology will further accelerate development of new more sophisticated large animal models allowing to study different aspects of various human diseases.

DISCUSSION

Initial studies in livestock have primarily utilized CRISPR/Cas9 NHEJ mechanism for disruption of genes of interest (KO) via indels introduction (**Tables 1**, **2**). More recently, farm animals with point mutations and gene insertions (KI) have been successfully produced using ssODN donor sequences, CRISPR/Cas9 base editing and CRISPR/Cas9 nickase approaches (**Table 3**). The applications of gene editing technologies for generation of livestock are very diverse, ranging from enhancing important production traits such as meat, milk, and fiber production (Deng et al., 2014; Crispo et al., 2015; Hu et al., 2017; Zhou et al., 2017; Zhang R. et al., 2020) to improving disease resistance, health, reproductive efficiency, facilitating animal welfare, and developing new biomedical models to better understand the etiology of diseases and develop novel mechanism-based therapeutic approaches (Vilarino et al., 2017; Fan et al., 2018a,b; Tu et al., 2019; Tanihara et al., 2020).

Newly developed gene editing tools (cytosine base editor, CBE and ABE) facilitate the generation of point-mutations without DSB. They can introduce four types of transition mutations (C → T, A → G, T → C, and G → A), which cover approximately 30% of all known human pathogenic variants (Anzalone et al., 2020), so the use of these tools could be increasingly beneficial for gene therapy. The CRISPR/Cas9 platform can also be used to modulate gene expression and impact epigenetics (Gilbert et al., 2013; Lawhorn et al., 2014). This mechanism offers a variety of possibilities to re-write how genes are traditionally expressed and provides the opportunity to use transcription factors and other enzymes in the regulation/modification of epigenetic marks and correcting epigenetic disorders (reviewed in Mei et al., 2016). Prime Editing technology has shown that all 12 combinations of base changes (transition and transversion) are possible without performing a DSB in cells (Anzalone et al., 2019). This gene editing tool is a catalytically impaired nCas9 (H840A) fused with a reverse transcriptase (RT-nCas9) that is transfected along with a pegRNA. Several strategies have been developed to improve the integration efficiency of long DNA fragments, including

TABLE 3 | CRISPR-mediated gene knockin in livestock.

Species	*Gene*	Purpose of manipulation	Type of KI	Approach	SCNT or MI	KI Animals produced	Mosaicism (%)	References
		Agriculture: improvements in						
Sheep	*SOCS2*	Reproductive traits	Point mutation	Crispr/Cas9 BE	MI	3/4 (25%)	3/3 (100%)	Zhou et al. (2019)
	BMPR1B	Reproductive traits	Point mutation	Crispr/Cas9	MI	5/21 (23.8%)	Not stated	Zhou et al. (2018)
Goat	*Tβ4*	CCR5-targeted KI, cashmere yield	Gene insertion	Crispr/Cas9	SCNT	1	N/A	Li X. et al. (2019)
	FGF5	Cashmere yield	Point mutation	Crispr/Cas9 BE	MI	5/5 (100%)	5/5 (100%	Li G. et al. (2019)
	GDF9	Reproductive traits	Point mutation	Crispr/Cas9	MI	4/17 (23.5%)	2/4 (50.0%)	Niu et al. (2018)
	FAT-1	Disease resistance	Gene insertion	Crispr/Cas9	SCNT	1 from 8 pregnancies	N/A	Zhang J. et al. (2018)
Cattle	*Pc*	Generation of a polled genotype	Gene insertion	Crispr/Cas12a	SCNT	1, died on D1 after birth	N/A	Schuster et al. (2020)
	NRAMP1	Tuberculosis resistance	Gene insertion	Crispr/Cas9n	SCNT	9	N/A	Gao et al. (2017)
	IARS	Correction of IARS syndrome	Gene insertion	Crispr/Cas9	SCNT	5 viable fetuses	N/A	Ikeda et al. (2017)
Pig	*PBD-2*	Disease-resistant pigs	Gene insertion	Crispr/Cas9	SCNT	5 pigs	N/A	Huang et al. (2020)
	MSTN	Meat production	Gene insertion	Crispr/Cas9	SCNT	2 pigs	N/A	Zou Y.-L. et al. (2019)
	UCP1	Reproduction traits	Gene insertion	Crispr/Cas9	SCNT	12 piglets	N/A	Zheng et al. (2017)
	MSTN	Meat production	Point mutation	Crispr/Cas9	SCNT	1 stillborn piglet	N/A	Wang K. et al. (2016)
	MSTN	MSTN-KO without selectable marker	Gene insertion	Crispr/Cas9	SCNT	2 piglets	No	Bi et al. (2016)
	RSAD2	Generation of pigs with viral resistance	Gene insertion	Crispr/Cas9	SCNT	1 pig	No	Xie et al. (2020)
		Biomedical applications:						
Sheep	*ALPL*	Model of hypophosphatasia	Point mutation	Crispr/Cas9	MI	6/9 (66.6%)	No	Williams et al. (2018)
	PPT1	Infantile neuronal ceroid lipofuscinoses	Point mutation	Crispr/Cas9	MI	6/24 (25.0%)	Not stated	Eaton et al. (2019)
	tGFP	Rosa26-targeted KI	Gene insertion	Crispr/Cas9	MI	1/8 (12.5%)	Not stated	Wu et al. (2016)
	OTOF	Hearing loss phenotype	Point mutation	Crispr/Cas9	MI	8/73 (11.0%)	2/8 (25.0%)	Menchaca et al. (2020b)
Cattle	*CMAH*	Xenotransplantation	Point mutation	Crispr/Cas12a	SCNT	2	N/A	Perota et al. (2019)
Pig	*hF9*	Gene therapy for hemophilia B pigs	Gene insertion	Crispr/Cas9	SCNT	5 pigs	N/A	Chen et al. (2020)
	BgEgXyAp	Salivary gland as bioreactor	Gene insertion	Crispr/Cas9	SCNT	4 piglets (1/4 alive)	N/A	Li G. et al. (2020)
	hIAPP	Type 2 diabetic miniature pig model	Gene insertion	Crispr/Cas9	SCNT	24	N/A	Zou X. et al. (2019)
	SNCA	Parkinson's disease model	Gene insertion	Crispr/Cas9	SCNT	8 piglets	N/A	Zhu et al. (2018)
	HTT	Huntingtin KI model	Gene insertion	Crispr/Cas9	SCNT	6 piglets	N/A	Yan et al. (2018)
	GGTA1	Xenotransplantation	Gene insertion	*Fok*I-dCas9	SCNT	2 piglets	N/A	Nottle et al. (2017)
	tdTomato	porcine Oct4 reporter system	Gene insertion	Crispr/Cas9	SCNT	2 piglets	N/A	Lai et al. (2016)
	hALB	Tg animals as bioreactors	Gene insertion	Crispr/Cas9	MI	16/16 (100%)	1/16 (6.3%)	Peng et al. (2015)
	GFP	H11-targeted KI	Gene insertion	Crispr/Cas9	SCNT	1 piglet	N/A	Ruan et al. (2015)

SCNT, somatic cell nuclear transfer; MI, zygote microinjection; BE, base editing; N/A, not applicable.

CRISPR/Cas9 mediating HR, MMEJ targeted integration, HMEJ targeted integration, and the NHEJ-mediated KI named HITI. HITI has the highest on-target specificity (90–95%) with biallelic integration of transgene ranging between 30 and50% *in vitro* in several cell types including dividing (HEK293) and non-dividing (mouse primary neurons) cells. Furthermore, HITI approach led to the successful DNA KI *in vivo* demonstrating the efficacy of HITI in improving visual function using a rat model of retinitis pigmentosa (Suzuki et al., 2016). The robustness of this approach is likely to be translatable to the livestock species. The use of chemically modified ssODN, such as phosphorothioate, is highly efficient method for the introduction of point mutations and/or single nucleotide replacements that could be very useful for correction of pathogenic mutations in livestock, and developing animal models of human disease or testing gene therapy strategies.

While editing scope and efficiency of CRISPR/Cas9 and its variants continue to improve, potential introduction of off-target mutations remains the major concern when producing animals for agriculture or using them in biomedical applications (Zhang X. H. et al., 2015). These off-target sites are sequences similar to the gRNA sequence except for up to four mismatched mutations that can be tolerated by CRISPR/Cas9 (Haeussler, 2020). The tolerance for mismatch pairing may cause attack by CRISPR/Cas9 during gene editing, which ultimately may lead to an introduction of unintended mutations. Off-target mutations may result in a silent mutation or produce a loss of function in coding regions. Nonetheless, the concerns are in the formation of an aberrant form of protein that induces food allergenicity or affect animal health if unintended genetic modifications could lead to tumor formation due to disruption of mechanisms such as a tumor suppressor gene (Ishii, 2017). Up to thousands off-target mutations have been found in previous studies in gene edited cells, embryos, and animals (Crispo et al., 2015; Kim et al., 2015; Tsai et al., 2015; Wang X. et al., 2015a; Carey et al., 2019; Zuo et al., 2019; Haeussler, 2020; Zuccaro et al., 2020), which raise the importance on investigating in-depth the gene editing approaches for reduction of those mutations. For instance, the use of CRISPR/Cas9 RNP instead of a plasmid vector, reduced the risk of off-target mutations as RNP is cleared from the cells within 24 hours after transfection (DeWitt et al., 2017). Furthermore, other methods are in development to minimize the off-target effects such as CRISPR Guide RNA Assisted Reduction of Damage (GUARD) that protects off-target sites by co-delivering short gRNAs directed against off-target loci by competition with the on-target gRNA without affecting on-target editing efficiency (Coelho et al., 2020). Nonetheless, it would be appropriate to investigate off-target mutations in animals, embryos or somatic cells as deeply as possible using methods for identification of off-target sites, such as whole genome sequencing (WGS) and whole-exome sequencing (WXS) (Ishii, 2017).

Currently, SCNT is the main technique for the production of KI gene edited livestock (**Table 3**). Furthermore, about half of the published KO farm animals were produced by SCNT (**Tables 1**, **2**). The primary advantage of this cell-mediated gene editing approach is the ability to verify that the gene-edited cells contain the desired genetic modification prior to live animal production takes place. This approach eliminates the occurrence of genetic mosaicism and has a potential to decrease the timeframe for generating the desired genotype and reducing the overall cost of animal production. These aspects are especially critical for application in large domestic animals that have particularly long generation intervals. While mosaicism resulting from CRISPR/Cas9 genome editing is typically regarded as an undesirable outcome, in certain cases, it may be valuable especially in animal models. These include assessments of candidate gene function *in vivo* where direct comparison of mutant and wild-type cells can be performed in the same organ of mosaic animals (Zhong et al., 2015). Mosaic animal models could also help us better understand the effect of gene dosage in congenital disorders. One example involves mosaicism of the Pax6 gene in mice. This gene plays an important role in eye development. CRISPR/Cas9-mediated mutation of Pax6 in mice have resulted in somatic mosaicism and variable developmental eye abnormalities in founder animals (Yasue et al., 2017). Thus, certain mosaic animal models could provide insights into the complexities of human congenital diseases that appear in mosaic form. Derivation of Bovine Embryonic Stem Cells (bESCs) was recently reported, and these cells could potentially be used as donor cells for nuclear transfer (Bogliotti et al., 2018). bESCs may offer some advantages compared to somatic cells such as greater *in vitro* longevity and potentially higher efficiency of homologous recombination. However, these hypothetical benefits will need to be further validated. Direct zygote manipulation, especially the zygote electroporation technique, is much less technically challenging compared to SCNT (Miao et al., 2019). Advancements in gene editing precision and efficiency, as well as developing strategies for reducing mosaicism have the potential to greatly enhance the accelerated and widespread utilization of gene editing technology in domestic animals, regardless of the specific application. This also assumes the technology receives favorable regulatory allowance, which will allow rapid integration of this high-value technology to contribute to the goal of increasing world-wide food security, and broad application as an important research tool.

AUTHOR CONTRIBUTIONS

IVP, ZF, and IP wrote the manuscript. All authors contributed to the review of appropriate literature, preparation, and review of the manuscript. All authors contributed to the article and approved the submitted version.

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Production of CFTR-ΔF508 Rabbits

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Cystic Fibrosis (CF) is a lethal autosomal recessive disease caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR). The most common mutation is the deletion of phenylalanine residue at position 508 (ΔF508). Here we report the production of CFTR-ΔF508 rabbits by CRISPR/Cas9-mediated gene editing. After microinjection and embryo transfer, 77 kits were born, of which five carried the ΔF508 mutation. To confirm the germline transmission, one male ΔF508 founder was bred with two wild-type females and produced 16 F1 generation kits, of which six are heterozygous ΔF508/WT animals. Our work adds CFTR-ΔF508 rabbits to the toolbox of CF animal models for biomedical research.

Keywords: CRISPR/Cas9, cystic fbrosis, CFTR-ΔF508, rabbits, gene edit

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INTRODUCTION

The cystic fibrosis transmembrane conductance regulator (CFTR) is a cyclic adenosine monophosphate (cAMP)-dependent chloride (Cl) channel at the apical membranes of most epithelial cells. Loss of CFTR function causes cystic fibrosis (CF), a fatal autosomal recessive disorder with a disease frequency of 1 in 2,000 live births and a carrier rate of approximately 5% in the Caucasian population (Cutting, 2015).

More than 2,000 mutations on the CFTR gene have been identified,[1] with the most common one being the deletion of phenylalanine residue at position 508 (ΔF508 or ΔF). Approximately 70% CF patients are ΔF508/ΔF508 homozygous. In addition, another 20% patients are of heterozygous compound mutations with ΔF508 on one allele and a different mutation on the other allele.

In 2019, the US Food and Drug Administration (FDA) approved Trikafta, marking a breakthrough in the CF drug development journey. Trikafta is a combination of three drugs: two CFTR correctors (VX-445 and VX-661) and a CFTR potentiator (VX-770). While Trifafta provides benefits to the majority of CF patients including those carrying one or two alleles of the dF508 mutation (Heijerman et al., 2019; Middleton et al., 2019), the consensus in the community is that CF is far from being cured and continued efforts should be dedicated to the development of novel therapeutics, for example gene editing mediated correction of CFTR mutations.

Clustered regularly interspaced short palindromic repeats/CRISPR Associated Protein 9 (CRISPR/Cas9) is originally discovered as a core member in the bacterial adaptive immune system (Price et al., 2016). It is now most known as the gene editing nuclease of choice (Khalil, 2020).

[1] https://cftr2.org/

In action, the CRISPR/Cas9 uses a guide RNA (gRNA) to locate the target sequence, where it efficiently generates double stranded breaks (DSBs), which are repaired by the error-prone non-homologous end joining (NHEJ) pathway or the homology directed repair (HDR) pathway. In recent years, CRISPR/Cas9 has become a mainstream tool in biomedical research. For example, it can be employed to generate gene knockout and knock-in animals as disease models. Our team has established a robust platform in generating knockout and knock-in rabbit models (Yang et al., 2014; Song et al., 2016; Yang et al., 2016; Song et al., 2017; Yang et al., 2019; Song et al., 2020). Furthermore, CRISPR/Cas9 can be used to correct disease causing mutations hence holds the promise for gene editing based therapeutics. In genetic diseases such as CF, the hope has been that CRISPR/Cas9 may enable a permanent correction of the intrinsic defect (i.e., the CFTR mutation).

Several groups including us have reported efficient gene editing of the CFTR gene in stem cells and in stem cell-derived organoids (Ruan et al., 2019; Geurts et al., 2020; Vaidyanathan et al., 2020). However, no one has reported successful gene editing therapy in a preclinical animal model system. Toward this goal, in the present work, we generated CFTR-ΔF508 rabbits by CRISPR/Cas9. These animals are useful not only as a model for the study of CF pathogenesis, but also for the development of gene editing strategies to correct the most prevalent ΔF508 mutation of CF.

MATERIALS AND METHODS

The animal maintenance, care and use procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Michigan, an AAALAC International accredited facility. All procedures were carried out in accordance with the approved guidelines.

CRISPR/Cas9 Construction and sgRNA Synthesis

The Cas9 expression plasmid JDS246 was obtained from Addgene. Single guide RNAs (sgRNAs) were designed using the CRISPOR software (Concordet and Haeussler, 2018) and synthesized as chemically modified sgRNAs by Synthego (Menlo Park, CA, United States).

Cas9 mRNAs were transcribed *in vitro*, capped and polyadenylated using the T7 mScript™ Standard mRNA Production System (C-MSC100625, CELLSCRIPT, Madison, WI, United States). Cas9 mRNA and sgRNA were diluted in RNase-free TE buffer (1 mM Tris–Cl pH 8.0, 0.1 mM EDTA), stored in −80°C in 10 μl aliquots, and were thawed and kept on ice before microinjection.

Microinjection and Embryo Transfer

Sexually matured female New Zealand White (NZW) rabbits were superovulated by subcutaneous injection of follicle-stimulating hormone (FSH, Folltropin-V, Bioniche Life Sciences, Canada) twice/day with a dosage of 3 mg for the first two injections, 5 mg for the next two injections and 6 mg for the last two injections. Seventy-two hours after the first FSH injection, a single intravenously injection of 200 IU human chorionic gonadotropin (hCG, Chorulon, Intervet, Holland) was administered to induce ovulation. The superovulated females were mated with a male rabbit immediately after hCG injection. Sexually matured recipient female rabbits were synchronized by stimulate mechanically in the vagina and intravenous injection 200 IU hCG. Eighteen hours post insemination (psi), the superovulated rabbits were euthanized. The oviduct ampullae were recovered, flushed with 10 ml of Hepes buffered manipulation (HM) medium containing 25 mM TCM 199 (#12350039, Life Technologies, Grand Island, NY, United States) supplemented with 10% fetal bovine serum (FBS, #12003C, Sigma, St. Louis, MO, United States), and the recovered oocytes were observed under a microscope for the occurrence of fertilization, and then kept in the HM medium at 38.5°C in air.

Microinjection was performed on pronuclear stage embryos 19–21 h psi using a micromanipulator under an inverted microscope equipped with a differential interference contrast (DIC) device. Rabbit embryo was held with a holding glass pipette (120–150 μm diameter) in the HM medium. A mixture containing 150 ng/μl Cas9 mRNA and 50 ng/μl sgRNA, and 100 ng/μl donor oligo or plasmid DNA were used for cytoplasm microinjection. Injected embryos were washed three times in embryo culture medium, which consisted of Earle's Balanced Salt Solution (E2888, Sigma) supplemented with non-essential amino acids (M7145, Sigma), essential amino acids (B-6766, Sigma), 1 mM L-glutamine (25030-081, Life Technologies), 0.4 mM sodium pyruvate (11360-070, Life Technologies), and 10% FBS. The injected embryos were surgically transferred into the oviducts of a synchronized recipient doe. Twenty to thirty embryos were transferred to one recipient doe. For *in vitro* validation, instead of transferring to a recipient doe, the injected embryos were washed and cultured *in vitro* for additional 3–4 days until they reach blastocyst stage.

Confirmation of Gene Targeting Events

For *in vitro* validation of gRNAs, blastocyst stage embryos were pooled and lysed, genomic DNA extracted, and the whole genome was replicated using a REPLI-g® Mini Kit (Qiagen, Germantown, MD, United States) following the manufacturer's protocol with slight modification. Briefly, for harvesting denatured DNA, 3.5 μl Buffer D2 was added to the embryos, mixed by vortexing and centrifuged briefly. The samples were incubated on ice for 10 min. After that 3.5 μl Stop Solution was added, mixed by vortexing and centrifuged briefly. For replication, 2 μl of the denatured DNAs were added to 8 μl master mix and incubate at 30°C for 10–16 h. Then REPLI-g Mini DNA Polymerase was inactivated by heating at 65°C for 3 min. The PCR products were purified, and Sanger sequenced at the University of Michigan DNA Sequencing Core. The sequences were analyzed by using the online software ICE Analysis by Syntheco[2] to determine the efficiencies of gRNAs, indicated by the rates of insertions and deletions (indels) at or close to the target locus (**Supplementary Figure 1**).

[2]https://ice.synthego.com

To determine the genotypes of animals, ear skin tissues were biopsied, and genomic DNA extracted. For animals produced in Condition (i) and (ii) where short length oligo donors were used, genomic DNAs were PCR amplified using primer set F/R (F: CCTCCAACCCTATCCCAACACTCTG; R: ATGATGGGCTAGGTTGGTGTATTAAA, **Supplementary Figures 2**, **3**). For animals produced in Condition (iii) where a long length ds-donor was used, genomic DNAs were PCR amplified using primer sets LF/LR (LF: ACCATCT TTAATCATGTAGTTTCA; LR: AATCTTCCAATACCTTTCT GCTCATAA) and RF/RR (RF:GTTTCCAGACTTCGCTTC; RR: AATTTCCCAAACAACTACT) (**Supplementary Figure 4**). PCR products were purified, and Sanger sequenced at the University of Michigan DNA Sequencing Core. The sequences were analyzed by using the online software ICE Analysis by Syntheco.[3] Animals carrying the desired knock-in sequences are considered knock-in animals.

Off-Target Analyses

Potential off-target loci associated with sg02 in the rabbit genome were predicted by using an online off-target analysis tool CRISPOR (Concordet and Haeussler, 2018). Top 8 potential off-target loci that fall on an exon or an intro (**Supplementary Table 1**) were selected for off-target analysis, by using corresponding primer sets (**Supplementary Table 2**) to PCR amply the sequence, followed by T7EI (see below) assays to determine any indel events.

T7EI Assay

The T7 endonuclease I (T7EI) assay was conducted as previously described (Xu et al., 2018). Briefly, the purified PCR products were denatured and re-annealed and digested with T7EI (M0302L, New England BioLabs, Ipswich, MA, United States) for 30 min at 37°C, and then run in an agarose gel. Non-perfectly matched DNA (presumably indel sites) would be recognized and cleaved by T7EI leading to two cleaved bands; whereas the perfectly matched DNA would not be recognized and cleaved by T7EI hence leading to only one band (the unedited band).

Necropsy and Histology

Tissues were collected at necropsy following humane euthanasia, and immersion fixed in 10% neutral buffered formalin. Tissues were trimmed and processed through graded alcohols, cleared with xylene, and embedded in paraffin. Tissues were sectioned at 5 μm, mounted on microscope slides, and stained with hematoxylin and eosin (H&E) for microscopic analysis by a board-certified veterinary pathologist.

RESULTS

Validation of Guide RNAs

We first analyzed the F508 proximal sequence of the rabbit CFTR gene (NCBI GeneID: 100009471) and designed two guide RNAs: sg01 and sg02 (**Figure 1A**).

[3]https://ice.synthego.com

We then microinjected sg01 or sg02 into pronuclear stage embryos to test their efficiencies *in vitro*. Similar blastocyst developmental rates were achieved in both groups (**Figure 1B**). To determine the rates of insertions or deletions (indels), we pooled 12 blastocytes from each group, extracted genomic DNAs, followed by PCR and Sanger sequencing. The sequencing results were analyzed by ICE online software,[3] which estimated that the indel rate was 75% or 33% by sg01 or sg02, respectively (**Figure 1B** and **Supplementary Figure 1**).

Because both gRNAs passed our quality control threshold of 30% indel generating capacity, both were chosen for knock-in experiments.

Production of ΔF508 Founder Rabbits

To knock-in the ΔF508 mutation, we designed two single stranded oligonucleotide (ssODN) donor templates (donor-oligo-01 and donor-oligo-02) and one double stranded donor template (ds-donor-01). Donor-oligo-01 is 130 nucleotides (nt) long and carries the ΔF508 mutation as well as 6 silent mutations (**Supplementary Figure 2**). Donor-oligo-02 is 120 nt long that carries the ΔF508 mutation but without any other mutations (**Supplementary Figure 3**). Ds-donor-01 is 3.9 kilobases (kb) long with 1.5 and 2.4 kb homology arms on each side, and carries the ΔF508 mutation and 4 silent mutations (**Supplementary Figure 4**).

We then used three conditions: Condition (i) sg01 + donor-oiligo-01; Condition (ii) sg02 + donor-oligo-02; or Condition (iii) sg02 + ds-donor-01, along with Cas9 encoding mRNA for embryo microinjection, followed by embryo transfer.

For Condition (i), we transferred 110 embryos to five pseudopregnant recipients, and obtained 14 kits. Genotyping of the ear skin biopsy samples revealed that eight kits carried indel mutations, however none had the ΔF508 mutation (**Figure 2A**).

For Condition (ii), we transferred 90 embryos to three recipients, and obtained 20 kits, of which five possessed undesired indel alleles only, and three (15%) possessed the ΔF508 allele (**Figure 2A**).

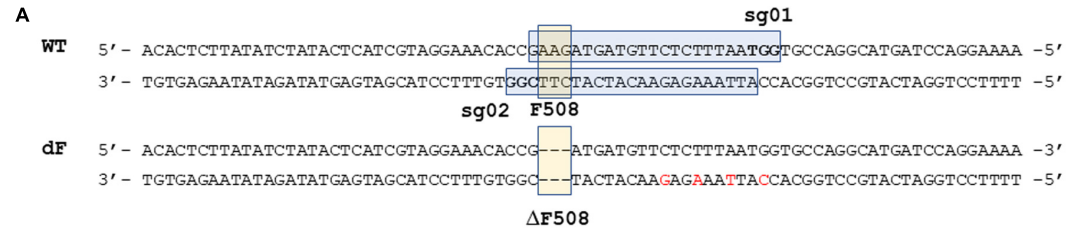
For Condition (iii), we transferred 185 embryos to seven recipients, and obtained 43 kits, of which 25 possessed undesired indel alleles only, and two (4.7%) possessed the ΔF508 allele (**Figure 2A**).

Together, we produced multiple ΔF508 founder rabbits (exampled in **Figure 2B**) from Condition (ii) and Condition (iii) but none from Condition (i).

Germline Transmission of the ΔF508 Allele to the F1 Generation Rabbits and Off-Target Analysis

We next worked to test the germline transmission capacity of the founder ΔF508 rabbits. One male animal from Condition (ii) was bred with two wild-type females. A total of 16 kits were born, and six were confirmed as heterozygous ΔF508/WT animals (**Figure 2C** and **Supplementary Figure 5**). These F1 generation ΔF508/WT animals look indistinguishable from their WT littermates.

A



B

gRNA	#embryos injected	#blastocysts	#embryos pooled for sequencing	% estimated indel
sg01	22	13	12	75
sg02	22	15	12	33

FIGURE 1 | Design and validation of guide RNAs. **(A)** illustration of gRNA design. Blue box: sgRNA sequence. Bold letters within the blue box: PAM sequence. Yellow box: the F508 or the ΔF508 sequence. **(B)** *In vitro* validation results of sg01 and sg02.

A

Condition	gRNA and donor	# Embryos	# Recipients	# Kits born	# WT	# Indel only	# ΔF508 (%)
Condition (i)	sg01+donor-oligo-01	110	5	14	6	8	0 (0)
Condition (ii)	sg02+donor-oligo-02	90	3	20	12	5	3 (15)
Condition (iii)	sg02 + ds-donor-01	185	7	43	16	25	2 (4.7)

B

C

Litter #	# Kits born	#dF kits (%)
01	8	3 (37.5)
02	8	3 (37.5)

FIGURE 2 | Production of ΔF508 founder rabbits. **(A)** Summary of embryo transfer and genotyping results. **(B)** One founder ΔF508 kit. **(C)** Summary of breeding outcome to generate F1 generation ΔF508 rabbits. #Indel only refer to number of animals that carry non-ΔF508 indel mutations. #ΔF508 refers to number of animals that carry ΔF508 allele.

One concern for CRISPR/Cas9 based gene editing is the potential off-target mutations. We therefore evaluated the top potential off-target mutations that fall on the exon or intron regions in one founder (#163) and five F1 generation ΔF508/WT animals (#254, 255, 263, 264, 265). In the selected top eight loci, no off-target mutations were detected (**Supplementary Table 1** and **Supplementary Figure 6**), indicating that there are minimal off-target mutations in these dF508 animals.

Production of a Compound Heterozygous ΔF508/KO Rabbit

Many CF patients carry heterozygous compound mutations. We previously produced CFTR knockout (CFTR-KO) rabbits and recently reported the phenotypes of these CFTR-KO rabbits (Xu et al., 2020). To test if heterozygous compound CF rabbits can be produced, we bred one male ΔF508 founder from Condition (iii) with a heterozygous CFTR knockout female rabbit and successfully produced a compound heterozygous ΔF508/KO rabbit. One allele of this animal has the ΔF508 allele while the other allele has the deletion of one nucleotide (Δ1) mutation (**Figure 3A**).

This ΔF508/KO CF rabbit survived 58 days, a lifespan that is similar to those of CFTR-KO rabbits (Xu et al., 2020). Postmortem examination revealed severe intestinal obstruction (**Supplementary Figure 7**). This is also similar to the observations in CFTR-KO rabbits, in which gut obstructions is the primary cause of mortality (Xu et al., 2020).

Formalin fixed samples of lung and trachea of this animal were subjected for histopathology. In H&E stained sections of trachea, the tracheal epithelium diffusely was attenuated and flattened, with scant cytoplasm (**Figures 3B,C**). There was moderate to marked congestion and edema within the underlying submucosa. In H&E stained sections of lung, there was mild to moderate edema within alveoli multifocally, alveoli contained variable amounts of fibrin, and there was loss of detail of alveolar walls (**Figure 3D**). There were increased numbers of heterophils within the vasculature or within alveolar capillaries. There were numerous rod-shaped bacteria multifocally (**Figure 3E**) within alveolar spaces, without associated inflammation.

These results demonstrate the feasibility of generating heterozygous compound ΔF508/KO rabbits, and suggest that ΔF508/KO rabbit may manifest typical CF phenotypes including intestinal obstruction and airway inflammation and infections.

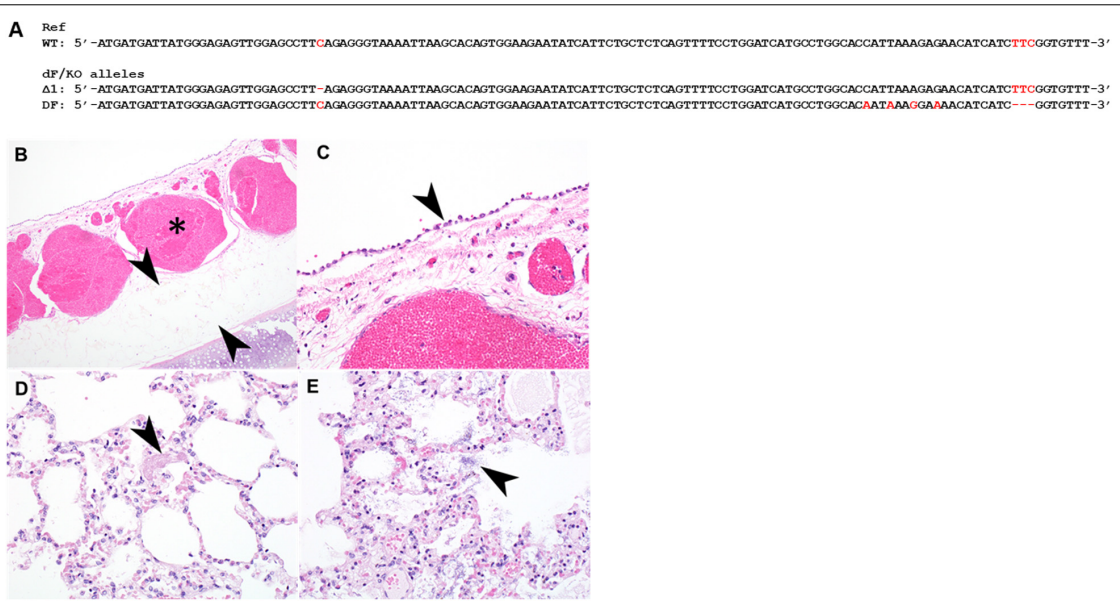


FIGURE 3 | Histopathology of the tracheal and lungs of a heterozygous compound ΔF508/KO rabbit. **(A)** Illustration of allele sequence of the ΔF508/KO rabbit. Red letters indicate positions of mutations. **(B)** The submucosa of the trachea was thickened with moderate to marked amounts of edema (arrowheads) and submucosal blood vessels were markedly congested (asterisk). **(C)** tracheal epithelium was markedly flattened and attenuated (arrowhead). **(D)** Lung contained variable amounts of fibrin within alveoli (arrowhead), variable amounts of edema, and multifocal rod-shaped bacterial colonies [**(E)**, arrowhead].

DISCUSSION

In the present work, we produced CFTR-ΔF508 rabbits by using the CRISPR/Cas9 system. Importantly, one founder animal transmitted the ΔF508 mutant allele to its offspring, thereby satisfying the gold standard of transgenic animal production. Follow-up work is needed to establish the homozygous ΔF508 rabbits and comprehensive characterize their CF phenotypes.

Interestingly, although sg01 was shown to have higher indel generating capacity than sg02, all ΔF508 founder rabbits were produced from the groups that used sg02 but none from the sg01 group. We reason that the most possible explanation to the differential outcome between sg01 and sg02, as suggested by an early report (Paquet et al., 2016), is the distances between the PAM (protospacer adjacent motif) location and the targeted mutation site. Sg01's PAM is further away from the F508 locus than that of sg02. Consequently, sg01's cutting site is 13 base pairs (bps) from the F508 locus; whereas sg02's cutting site is right at the F508 locus. This result underscores the importance of PAM location in Cas9 mediated knock-in applications.

The ΔF508 rabbits are a new addition to the CF mammalian animal model family, which currently consist of two rodent species, mouse (Grubb and Boucher, 1999) and rat (McCarron et al., 2020), and four non-rodent species: pig (Stoltz et al., 2015; Yan et al., 2015), ferret (Sun et al., 2010; Yan et al., 2015; Sun et al., 2019), sheep (Fan et al., 2018), and rabbits (Xu et al., 2020). CF mouse models were the first developed (Semaniakou et al., 2018). Different CF mice, including knockout, ΔF508, G551D and others, have made significant contributions toward our understanding of the disease and the development of therapies. However, unlike human patients, CF mice rarely show pulmonary pathophysiology nor obvious pancreatic pathology and liver problems. Similar to mice, CF rats, both knockout and ΔF508, develop gut obstructions but are otherwise normal in the pancreas, liver and lungs (Dreano et al., 2019). In the non-rodent models, CF ferrets (knockout and G551D), CF pigs (knockout and ΔF508) and CF sheep (knockout) were generated by nuclear transfer. CF ferrets and pigs, have been shown a closely mimicking pathology that is observed in CF patients, including lung, pancreatic and liver phenotypes that are not often found in CF mice. However, neither pig, ferret nor sheep is a convenient laboratory species, and they are associated with high maintenance cost and require special animal handling skills. Most recently, we reported the production of CFTR knockout rabbits that show many typical CF phenotypes (Xu et al., 2020). However, the specific knockout genotypes in these rabbits are not found in CF patients, hence not optimal for the development of mutation specific gene editing strategies.

The ΔF508 rabbits therefore may represent a useful model by offering several desirable features. Comparing to the non-rodent CF models (i.e., pigs, ferrets, and sheep), rabbit is a classic animal species that can be easily housed in most research facilities, and many experimental procedures are well established. Furthermore, rabbit has a short gestation time (30 days) and large litter size, making herd expansion very efficient. Comparing to CF rodent models (i.e., mice and rats), observations from the ΔF508/KO rabbit generated from the present work highly suggest that ΔF508 rabbits may be more clinically relevant, i.e., manifesting some typical CF phenotypes that rodent models failed to demonstrate, in line with our recent findings in the CFTR-KO rabbits (Xu et al., 2020). While this is only a

single case, which needs follow-up studies in large number of homozygous ΔF508/ΔF508 rabbits to verify, the finding is exciting and promising.

One other advantage of the ΔF508 rabbit model over that of the mouse model is the relatively longer lifespan. A laboratory NZW rabbit can live beyond 6 years; whereas the lifespan of a mouse is 1–2 years. This is particularly important in the development of gene editing therapy strategies, as the longer lifespan allows a longer observation window for potential side effects, which do not always manifest in short term. For example, in the late 1990s, gene therapy for primary immunodeficiency was conducted using a gamma-retroviral vector. Strikingly, 5 of 20 patients developed leukemia 2–6 years after the gene therapy due to the integration of the vector in the vicinity of oncogenes (Hacein-Bey-Abina et al., 2003; Howe et al., 2008). Such long-term safety risks are beyond the lifespan of a rodent, but are legitimate considerations for gene editing therapies including those for CF. In this context, the ΔF508 rabbit provides a tool to monitor long-term efficacy and safety of novel therapies.

In summary, we successfully produced CFTR-ΔF508 rabbits by CRISPR/Cas9. These animals add a valuable tool to facilitate the study of CF pathogenesis and the development of novel therapies including gene editing therapeutics.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee (IACUC) of the University of Michigan.

AUTHOR CONTRIBUTIONS

YC, FS, and JX conceived the idea. JX, YC, J-PJ, and FS designed the experiments. DY, XL, BP, MH, JZ, ZR, RH, and JX conducted the experiments and analyzed the data. JX, YC, J-PJ, and FS wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Targeted Gene Editing in Porcine Spermatogonia

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To study the pathophysiology of human diseases, develop innovative treatments, and refine approaches for regenerative medicine require appropriate preclinical models. Pigs share physiologic and anatomic characteristics with humans and are genetically more similar to humans than are mice. Genetically modified pigs are essential where rodent models do not mimic the human disease phenotype. The male germline stem cell or spermatogonial stem cell (SSC) is unique; it is the only cell type in an adult male that divides and contributes genes to future generations, making it an ideal target for genetic modification. Here we report that CRISPR/Cas9 ribonucleoprotein (RNP)-mediated gene editing in porcine spermatogonia that include SSCs is significantly more efficient than previously reported editing with TALENs and allows precise gene editing by homology directed repair (HDR). We also established homology-mediated end joining (HMEJ) as a second approach to targeted gene editing to enable introduction of larger transgenes and/or humanizing parts of the pig genome for disease modeling or regenerative medicine. In summary, the approaches established in the current study result in efficient targeted genome editing in porcine germ cells for precise replication of human disease alleles.

Keywords: pig, spermatogonia, gene targeting, CRISPR/Cas9, homology directed repair, homology-mediated end joining

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INTRODUCTION

Applicable preclinical models are needed to investigate the pathophysiology of human diseases, develop novel treatments and medical devices, and improve approaches for regenerative medicine. While rodent models are currently the standard for early preclinical studies, pigs are physiologically, anatomically, and genetically more similar to humans than are mice and are delivering increasing value to biomedical research. Genetically modified pigs, such as pig models of cystic fibrosis (Rogers et al., 2009), neurofibromatosis type I (Isakson et al., 2018), and diabetes (Kleinwort et al., 2017), are essential where rodent models fail to recapitulate the full pathophysiological spectrum of a disease. The generation of biomedical pig models primarily relies on somatic cell nuclear transfer (SCNT) using cells genetically modified with engineered nucleases such as Zinc Finger nucleases (ZFNs), Transcription activator-like Effector Nucleases

(TALENs), and Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated-9 (CRISPR/Cas9) (Tan et al., 2012).

Even though SCNT is a well-established process, it is inefficient and associated with abnormal fetal and placental development and neonatal mortality due to incomplete reprogramming of the somatic cell nuclei (De Sousa et al., 2001; Hill et al., 2002). Microinjection and electroporation of TALENs, ZFNs, and CRISPR/Cas9 into *in vitro* fertilized pig zygotes have been used to more efficiently produce gene-edited piglets that are free of SCNT (reprogramming)-associated defects (Armstrong et al., 2006; Bonk et al., 2008; Tian et al., 2009). However, microinjection and electroporation of engineered nucleases often result in genetic mosaicism that requires the time-consuming process of outcrossing of mutants to generate isogenic animals to investigate alleles of interest. Moreover, all of the current approaches for generating pig models require expensive specialized equipment and considerable expertise and time. An alternative approach for generating genome-edited animals is through the use of spermatogonial stem cells (SSCs) (Hamra et al., 2002; Orwig et al., 2002). SSCs, a subpopulation of undifferentiated type A spermatogonia, are unipotent stem cells that reside in the stem cell niche at the basement membrane of seminiferous tubules where they undergo a highly coordinated process of self-renewal and differentiation to form sperm (De Rooij, 2001). Hence, SSCs are the genetic basis of future generations. When cell populations containing SSCs are transplanted to a recipient testis, SSCs establish donor-derived spermatogenesis, making them an ideal target for genetic modification (Brinster and Avarbock, 1994; Brinster and Zimmermann, 1994). Currently, there are no molecular markers that allow prospective identification of SSCs within the population of undifferentiated type A spermatogonia.

Engineered nucleases have been utilized in cultured mouse and rat spermatogonia to produce progeny with targeted gene knockout or gene-corrected alleles after transplantation of gene-edited SSCs and *in vitro* fertilization or natural breeding (Chapman et al., 2015; Sato et al., 2015; Wu et al., 2015). Although viral-mediated transgenesis and transplantation of pig spermatogonia containing SSCs resulted in transgenic embryos after *in vitro* fertilization (Zeng et al., 2013), the lack of site-specific targeting due to random integration and use of viral vectors limits the application of this approach for production of biomedical pig models. Introduction of site-specific TALENs in pig spermatogonia using nucleofection resulted in non-homologous end joining (NHEJ) with indel efficiencies of up to 18% but at the expense of low cell viability (Tang et al., 2018). Moreover, optimization of gene-editing efficiency with cell viability was insufficient to facilitate homologous recombination when a single-strand oligo donor (ssODN) repair template was introduced with the TALENs (unpublished). Here we report the site-specific genetic engineering of porcine spermatogonia using the CRISPR/Cas9 ribonucleoprotein (RNP) system resulting in efficient generation of custom indel and single nucleotide polymorphism (SNP) alleles through homologous recombination of an ssODN repair template. In addition, we demonstrate integration of a ubiquitin-driven EGFP cassette/transgene into the safe harbor *ROSA26* locus of spermatogonia using CRISPR/Cas9 RNP and plasmid donors linearized within the cell to provide a template for homology-mediated end joining (HMEJ) repair.

MATERIALS AND METHODS

Isolation and Enrichment of Germ Cells

Testes were obtained from 8-week-old pigs by surgical castration. Single-cell suspensions were prepared by a sequential enzymatic digestion protocol (Sakib et al., 2019). Briefly, the tunica albuginea and visible connective tissue were removed, and the exposed seminiferous tubules were dissociated with Type IV collagenase (2 mg/ml; Sigma–Aldrich Cat# C5138, RRID:AB_008988) in Dulbecco modified Eagle medium (DMEM, Sigma–Aldrich Cat# D6429, RRID:AB_008988) at 37°C for 20–40 min with occasional agitation, followed by incubation at 37°C for 30 min in DMEM with Type IV collagenase (2 mg/ml; CEDARLANE Laboratories Limited LS004189, RRID:AB_004462) and hyaluronidase (1 mg/ml; Sigma–Aldrich Cat# H3506, RRID:AB_008988). The digested tubules were rinsed three times in Dulbecco phosphate-buffered saline (DPBS, $Ca^{2+}$and Mg^{2+} free; Sigma–Aldrich Cat# D8537, RRID:AB_008988) and further digested with 0.125% (w/v) trypsin and 0.5 mM ethylenediaminetetra-acetic acid (EDTA) (Sigma–Aldrich Cat# T4049, RRID:AB_008988) at 37°C for 15–20 min. DNase I (7 mg/ml in DMEM; Sigma–Aldrich Cat# DN25, RRID:AB_008988) was added during the digestion process as needed. After trypsin digestion, the cell suspension was filtered through 70 μm and 40 μm cell strainers sequentially (BD Biosciences). The single cells were then collected by centrifugation at 500 *g* for 5 min at room temperature (RT) and the cell pellet was resuspended in DMEM/F-12 (Life Technologies Cat# 11330032, RRID:AB_008817) with 5% fetal bovine serum (FBS, Life Technologies Cat # 12483020, RRID:AB_008817) for differential plating.

Differential Plating

Immediately after tissue digestion, 2.5×10^7 cells in 8 ml DMEM/F-12 with 5% FBS were plated onto 100 mm tissue culture plates and incubated at 37°C in 5% CO_2. Three sequential rounds of differential plating were performed (1.5 h, 1 h, and overnight). At the second and third round of plating, cell suspensions from two plates were combined and plated onto a new 100 mm culture plate. Attached cells were discarded. After overnight incubation, supernatant from all the plates was pooled. To collect loosely adhered germ cells, 2–3 ml of diluted Trypsin/EDTA (1:5 or 1:20 dilution with PBS) was added to each plate. Plates were incubated at 37°C for 2 min and then at RT for 3 min with constant agitation to release attached germ cells without disturbing somatic cells. The reaction was stopped by adding an equal volume of DMEM/F12 with 10% FBS. Cell suspensions were pooled from all plates, combined with cells collected from the supernatants, pelleted by centrifugation at 500 *g* for 5 min, and washed twice with PBS. After washing, cells were plated again onto 100 mm plates in DMEM/F12 with 5% FBS for 8 min at RT, and cell debris and contaminating red blood

cells were gently and slowly collected from the top and discarded, and GSCs were collected from the bottom of the plates.

Fluorescence-Activated Cell Sorting (FACS)

Germ cells were further enriched by sorting for light scatter properties as described (Tang et al., 2018). Briefly, enriched cell fractions collected after differential plating containing 58.6 ± 0.61% UCH-L1 + spermatogonia (mean ± SEM, $n = 3$; **Figure 1C**) were resuspended in PBS with 1% BSA (Sigma–Aldrich Cat# A7906, RRID:AB_008988) and subjected to sorting on a FACSAria III (Becton Dickinson, BD FACSARIA III cell sorter, RRID:AB_016695). A gate was drawn around the distinctive germ cell population on the forward and side light scatter dot plot, and cells within this gate were sorted. Sorted cells were pelleted and washed once with PBS. The viability of sorted cells was assessed by Trypan Blue staining. A sample was fixed with 2% paraformaldehyde (PFA; Thermo Fisher Scientific Cat# 41678-5000, RRID:AB_008452) and assessed for enrichment by immunocytochemistry with antibodies against UCH-L1 and Vimentin. UCH-L1 is a spermatogonia-specific marker that was used to assess the enrichment efficiency and to determine the percentage of germ cells present in a given cell population (Luo et al., 2009; **Figure 1**). Vimentin was used to label somatic cells. For each sorting experiment, 1000 cells were evaluated. As reported previously (Tang et al., 2018), cells enriched by fluorescence-activated cell sorting (FACS) contained 88.7 ± 4.36% UCH-L1 + spermatogonia (mean ± SEM, $n = 3$; **Figures 1D–F**).

Immunocytochemistry

Cells were fixed in 2% PFA for 30 min at RT and washed twice with PBS. Cells were then transferred onto slides for immunostaining by cytospin centrifugation (800 *g* for 5 min at RT) (Thermo Fisher Scientific Cat# A78300002, RRID:AB_008452), permeabilized in PBS with 0.1% Triton-X (EMD4Biosciences Cat# 9410, RRID:AB_008441), and washed three times in PBS prior to 1 h blocking with 3% BSA. Cells were incubated with the following primary antibodies overnight at 4°C: rabbit-anti-human UCH-L1 (Abcam Cat# ab108986, RRID:AB_10891773) at 1:500, mouse anti human DDX4 (Abcam Cat# ab27591, RRID:AB_11139638) at 1:100, and mouse-anti-pig vimentin-Cy3 at 1:400 (Sigma–Aldrich Cat# C9080, RRID:AB_259142). Three washes were performed after overnight primary antibody incubation and secondary antibodies donkey-anti-mouse IgG Alexa Fluor 488 (1:1000) or donkey-anti-rabbit IgG Alexa Fluor 594 (1:1000) were added onto samples. After 1 h RT incubation, cells were washed three times and mounted in VECTASHIELD Mounting Medium with DAPI (Vector Laboratories Cat# H1200, RRID:AB_000821) for imaging. For each cell prep experiment, images from five to six randomly chosen fields were collected and >1000 cells were evaluated.

CRISPR Design and RNP Complexing

CRISPR gRNAs were designed using Cas-Designer (CRISPR RGEN Tools; Bae et al., 2014; Park et al., 2015), selected for minimal predicted off-target sites, and purchased as Alt-R® CRISPR-Cas9 crRNAs with Alt-R® CRISPR-Cas9 tracrRNA from Integrated DNA Technologies (IDT) (Coralville, IA, United States) or as sgRNAs from Synthego (Redwood City, CA, United States). Guide RNAs for each locus are listed in **Table 1**. Cas9 protein, Alt-R® S.p. HiFi Cas9 Nuclease V3, or sNLS-SpCas9-sNLS Nuclease were purchased from IDT (Coralville, IA, United States) or Aldevron (Madison, WI, United States), respectively. To anneal the crRNA and tracrRNA, equimolar concentrations of each were combined and heated to 95°C for 5 min and then cooled to 22°C at −0.1°C/s. To form RNP complexes, crRNA:tracrRNA duplex was incubated at a ratio of 1.14:1 with Cas9 protein and incubated at RT for 10–15 min.

Germ Cell Nucleofection

Nucleofection was performed with the Amaxa Nucleofector II device (Lonza Cat# AAD-1001S RRID:AB_000377) essentially as described (Tang et al., 2018). Enriched cells were resuspended in solution V and transfected with the program X-005. Each transfection included RNP complexes formed by incubation of 200 pmol guide RNA with 175 pmol Cas9 protein for 15 min at RT. RNP complexes were introduced to 1×10^6 cells and transferred onto six-well plates in αMEM Advanced culture medium (Life Technologies Cat# 12492013, RRID:AB_008817) supplemented with 1% FBS, 0.1% BSA, 1X non-essential amino acids (Life Technologies Cat# 11140-050, RRID:AB_008817), 1 mM sodium pyruvate (Life Technologies Cat# 11360-070, RRID:AB_008817), 15 mM HEPES (Life Technologies Cat# 15630-080, RRID:AB_008817), 2 mM L-glutamine (Life Technologies Cat# 25030-081, RRID:AB_008817), 10 μM beta-mercaptoethanol (Sigma–Aldrich Cat# M7522, RRID:AB_008988), 100 U/ml Penicillin-100 μg/ml Streptomycin (Sigma–Aldrich Cat# P4333, RRID:AB_008988), and 10 ng/ml glial cell line-derived neurotrophic factor (GDNF; R&D systems Cat# 212-GD-010, RRID:AB_006140) for cell recovery and short-term cell culture. After overnight recovery, the medium was replaced with fresh culture medium, and cells were incubated at 30 or 37°C for 3–5 days depending on the experimental design. At the end of incubation, cells were harvested by gentle trypsinization (1:5 dilution of 0.25% Trypsin/EDTA). The number of cells collected was counted by hemocytometer and the viability was assessed by Trypan Blue (Thermo Fisher Scientific Cat#15250061, RRID:AB_008452) staining. Collected cells were used for further analysis.

Transfections With ssODNs

Single-stranded DNA templates for homology directed repair (HDR) were manufactured by IDT, Coralville, IA, United States, selecting the 100 nmol synthesis and standard desalting options. Transfections with ssODNs were performed as above including 168 pmol of ssODN template specific for each gene. Single-stranded DNA templates for each locus are listed in **Table 1**.

HMEJ Transfections

For HMEJ insertion, enriched germ cells were transfected with Universal and *ROSA26* RNP (**Table 2** and **Supplementary Figure S1**) complexes in the quantities indicated above, along

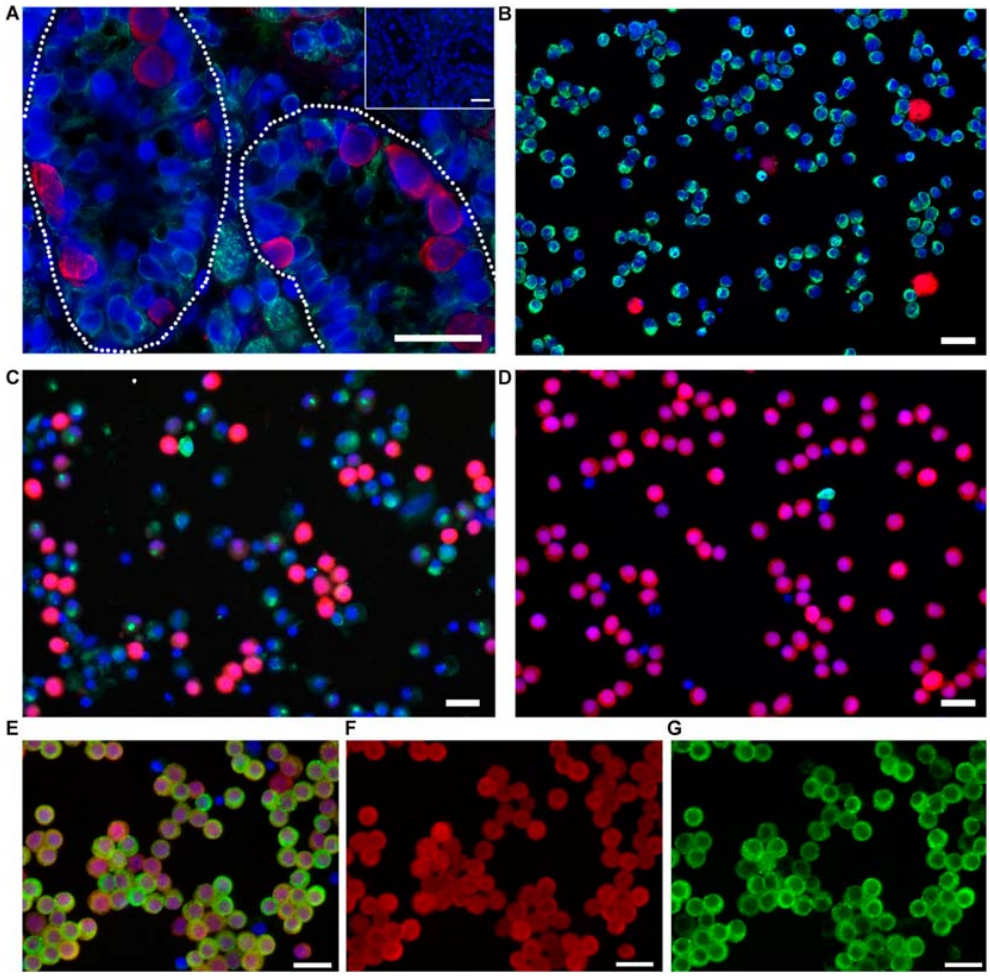

FIGURE 1 | Undifferentiated spermatogonia express UCH-L1. **(A)** Testis tissue from 8-week-old pig. Broken line outlines seminiferous tubules. Inset: negative control. **(B)** Testicular cells after enzymatic digestion of testis tissue. **(C)** Enriched spermatogonia after differential plating. **(D–F)** Highly enriched spermatogonia after FACS for light scatter properties. UCH-L1 red, vimentin green **(A–D)**, DDX4 green **(E,G)**, DAPI blue, bars = 25 μm.

with 1.7 μg of the eGFP plasmid cassette and electroporation enhancer (IDT, Coralville, IA, United States, Cat# 1075916).

Assessment of Targeted Mutagenesis and Homology Directed Repair

Genomic DNA was extracted from spermatogonia using PCR-safe lysis buffer [10 mM Tris-Cl, pH 8.0; 2 mM EDTA; 2.5% (vol/vol) Tween20; 2.5% (vol/vol) Triton-X 100; 100 mg/ml Proteinase K (Sigma–Aldrich Cat# P2308, RRID:AB_008988)] followed by incubation at 50°C for 60 min and 95°C for 15 min. The genomic region flanking the gRNA target site was PCR amplified with gene-specific primers (**Table 1**) and AccuStart^{TM} Taq DNA Polymerase HiFi (QuantaBio, Cat# 95085, Beverly, MA, United States) according to the manufacturer's recommendations. To analyze the frequency of NHEJ mutation in a population, the Surveyor mutation detection kit (Cat# 706020; IDT) was used according to the manufacturer's recommendations using 10 μl of the PCR product as described above. To analyze the frequency of HDR mutations in a population, restriction endonuclease digest was performed. Briefly, 6 μl of PCR product was digested with 6–10 units of enzyme, *ROSA26* and *HNF1a-Hin*dIII, *INS-Spe*I, in recommended buffer (Cat # R3144 and R0133, New England BioLabs, Ipswich, MA, United States; RRID:AB_013517). Surveyor and restriction digest reactions were resolved on a 10% TBE polyacrylamide gels and visualized by ethidium bromide (Fisher Scientific Cat# BP102-5) staining. Densitometry measurements of the bands were performed using ImageJ (ImageJ, RRID:AB_003070). The mutation rate of Surveyor reactions was calculated as described previously (Guschin et al., 2010) and the HDR rate of the restriction digest reactions was calculated as [(sum of RFLP bands/sum of wildtype and RFLP bands)*100].

Analysis of HMEJ Insertions

After genomic DNA extraction from GFP positive and negative GSC-enriched populations, the 5′ and 3′ junctions

TABLE 1 | Guide RNAs, ssOligos, and primers for HDR at three loci.

Locus	Guide RNA (5′–3′)	HDR oligo (5′–3′)	Primers (5′-3′)	Amplicon size (bp)	Cut band sizes (bp)
HNF1a	ssHNF1a g4.3 GGCGCAAGGA AGAAGCAUUU	ssHNF1A g4.3 HD3-KO GTCTACAACTGGTTTGCCAATC GGCGCAAGGAAGAAGCATAAA GCTTTTTCGGCACAAGTTGGC CATGGACACGTACAGTGGGCC ACC	ssHFN1A E4 NJ F3: GAGGGTTCTTCTGTGCCTGG ssHFN1A E4 NJ R3: GAGTGGAGAAAGCCAGGAGG	NHEJ: 415 HDR: 423	166 + 249 171 + 252
ROSA26	ssROSA g2 GGAUUUUUCU AGGCCCAGGG	ssROSA g2 HD3 ATGACGAGATCGCGGGGGAG GGAGGGATTTTTCTAGGCCAT AAAGCTTGGGCGGTCCTTAGG AAAAGGAGGCAGCAGAGAAC TCCCATA	ssROSA g2 F2: GCCTGAAGGACGAGACTAGC ssROSA g2 R2: AACACGCAGTCTCAATGCAT	NHEJ: 530 HDR: 538	254 + 276 257 + 281
INS	ssINS g2: CUGGUAGAGG GAACAGAUGC	ssINS g2 C94Y *Spe*I CCTAGTSDTGCAGTAGTTCTCC AGCTGGTAGAGGGAACAGATA CTAGTGTAGCACTGCTCCACG ATGCCACGCTTCTGCGGGGGC CCCTCC	ssINS E3 NJ F2: GTGGCTGTCTCTGTGTGACC ssINS E3 NJ R2: GGAAGCTTAGAGCAGCCGAT	NHEJ: 361 HDR: 361	136 + 225 132 + 229

from the endogenous gene to the exogenous cassette were PCR amplified with 2X AccuStart™ II PCR Supermix (Cat# 95136, QuantaBio) according to the manufacturer's recommendations. Universal gRNA and primer sequences for the 5′ and 3′ junctions are shown in **Table 2**. PCR products were resolved on an agarose gel and products in the region of the precise integration expected product sizes (5′ junction 585 bp, 3′ junction 589 bp) were excised and gel purified with the Qiagen Gel Extraction Kit (Qiagen, Cat# 28706, RRID:AB_008539) per the manufacturer's instructions. Purified PCR products were TOPO cloned into the pCR4-TOPO sequencing vector (ThermoFisher Scientific Cat#K457502, RRID:AB_008452); 5–20 clones per junction were sequenced via Sanger sequencing.

Statistical Analysis

Data were analyzed using GraphPad Prizm 4.0 (GraphPad Software Inc., La Jolla, CA, United States; RRID:AB_000306). A Student's *t*-test and ANOVA were performed to compare groups. Data were expressed as means ± SEM, and $P < 0.05$ was considered significant.

TABLE 2 | Guide RNAs and primers for HMEJ.

Name	Sequence (5′–3′)	Purpose
Universal gRNA	GGGAGGCGUUCGGGCCA CAG	Liberate repair cassette from plasmid
ss*ROSA26* g2 F2	GCCTGAAGGACGAGACT AGC	5′ Junction screening (585 bp amplicon)
ss*ROSA26* HDR Test R3	GAGATCCCTCCGCAGAA TCG	
bt*ROSA26* Ins F1	CACATGGTCCTGCTGGA GTT	3′ Junction screening (589 bp amplicon)
ss*ROSA26* g2 R2	AACACGCAGTCTCAATG CAT	

Bioethics

All animal experimentation was conducted with approval and oversight of the Animal Care and Use Committee at the University of Calgary.

RESULTS

Germ Cell Gene Editing by HDR

As the initial live offspring produced by GST would be heterozygote due to modification of the male germline only, we chose to evaluate gene editing at three biomedically relevant loci associated with either dominant forms of diabetes mellitus, *Hepatocyte nuclear factor-1 alpha* (*HNF1a*) and *insulin* (*INS*), or a common safe-harbor locus for transgene insertion, *ROSA26* (**Figure 2A**). Initially, we compared gene-editing efficiency of two gRNA structures, duplexed and single guides, delivered as RNP complexes followed by culture at either 30 or 37°C. We found that neither guide structure nor temperature had an effect on indel formation by Surveyor assay at two loci, *HNF1a* (not shown) or *ROSA26* (**Supplementary Figure S1**). Next, we evaluated the efficiency of indel formation at all three loci and found editing ranging from 20 to 35%, depending on locus (**Figure 2B**). Of note, cell recovery at 37 versus 30°C was slightly higher (82.8 ± 2.73 versus 76.9 ± 0.61%, $n = 6$, $p < 0.5$), and since temperature did not influence editing efficiency by CRISPR/Cas9 RNPs, further studies were performed using recovery at 37°C.

The high recovery and efficiency of CRISPR/Cas9 RNP mediated editing in spermatogonia provided the basis for more complex modifications. To test this, we designed a series of 90-mer ssODN templates to stimulate homology-directed repair (HDR) (**Figure 3A**). The templates for *ROSA26* and *HNF1a* were designed to insert a novel 8-base-pair sequence containing a *Hin*dIII restriction endonuclease site, intended to replicate a premature termination codon as observed in common maturity onset diabetes of the young (MODY) alleles. For the *INS* gene,

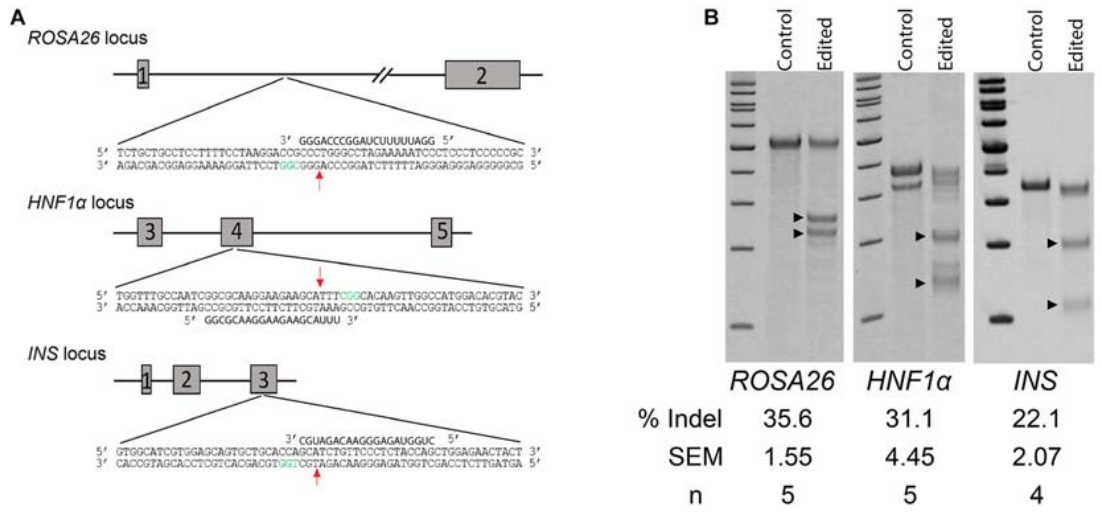

FIGURE 2 | Non-homology mediated end joining (NHEJ) in germ cells edited with CRISPR/Cas9 RNPs. **(A)** Schematic of targeted loci and guide RNAs. Red arrows indicate location of double-strand breaks (DSBs). Green nucleotides represent the Protospacer Adjacent Motive (PAM). **(B)** Efficiency of indel formation (NHEJ) at three targeted loci. Arrow heads indicate digested fragments (see **Table 1**).

we designed the template to replicate the C96Y mutation (C94Y in pigs; Renner et al., 2013) known to cause permanent neonatal diabetes mellitus (PNDM) in humans, a type 1 diabetes like disease. In contrast to the insertion designs of *HNF1a* and *ROSA26*, the *INS* repair template introduces three SNPs to alter a codon, mutate the protospacer motif to prevent re-cleavage of homology repaired sequences, and introduce a silent *Spe*I restriction endonuclease site (**Figure 3A**). Each of the templates was delivered into spermatogonia by CRISPR/Cas9 RNPs using the conditions used for NHEJ. The efficiency of HDR was measured by restriction fragment length polymorphism (RFLP) analysis revealing robust HDR at each locus (**Figure 3B**). As observed previously (Tan et al., 2013), the insertion HDR alleles, *HNF1a* and *ROSA26*, was more efficient than the SNP allele, *INS*.

Germ Cell Gene Editing by HMEJ

While efficient, HDR with ssODN templates is limited to creation of small (<50 bp) changes to the genome. To expand the utility of GSC editing, we evaluated whether HMEJ could be used to integrate cargo into the *ROSA26* safe harbor locus. The plasmid template was designed to integrate an eGFP expression cassette under control of the ubiquitin C (UbC) promoter (**Figure 4A** and **Supplementary Material**). The cassette was designed based on the pGTag vector series with universal gRNA target sites with no predicted off targets in the swine genome and short, 48-base-pair homology arms flanking the insertion cassette (**Figure 4A**) (Wierson et al., 2020). When the cassette is introduced into cells with CRISPR/Cas9 RNPs targeted to the universal gRNA site, the insertion cassette is liberated from the plasmid and integrated into the cut target site via a HMEJ mechanism. After transfection of enriched spermatogonia with each component, the cells were cultured at 37°C and sampled at 4 and 11 days post transfection, the latter time point to reduce non-integrated transient eGFP expression prior to FACS. FACS analysis revealed that about 8% of spermatogonia stably expressed eGFP after 11 days in culture (13.9 ± 3.77% eGFP+ cells after 4 days in culture, and 7.9 ± 1.07% eGFP+ cells after 11 days in culture; mean ± SEM, $n = 3$). Molecular analysis was performed on populations of cells 4 and 11 days post transfection. PCR junction products were observed for the 5′ and 3′ of cells when all components of the transfection were included, but not in controls missing any one component (**Figure 4B**). Although not quantified, band intensity appears greater in eGFP positive populations compared to non-sorted cells. Interestingly, light banding could also be observed in eGFP negative cell populations (**Figure 4B**). Sequencing of cloned junction amplicons from the eGFP positive populations showed that precise HMEJ is a frequent repair mechanism, but variants with imprecise HMEJ or NHEJ integration junctions were also observed (**Figure 4C**). Precise and imprecise HMEJ along with NHEJ insertions were also observed in eGFP negative cells by sequencing (**Figure 4C**). HMEJ and NHEJ junctions in the eGFP negative cells either indicate partial insertion events where the entire cassette was not integrated or cases where eGFP expression was silenced.

DISCUSSION

Here, we report significantly improved gene editing in porcine spermatogonia by demonstrating efficient site-specific indel generation and HDR from ssODN and plasmid cassette donors using the CRISPR/Cas9 RNP system. This is the first application of CRISPR/Cas9 in pig spermatogonia, and rates of editing by NHEJ were nearly double of what we reported previously using TALENs (Tang et al., 2018). Compared with somatic cells, spermatogonia, including SSCs, are more refractory to transfection (Zheng et al., 2017). SSCs are also very sensitive to double-strand breaks (DSBs) and are more prone to undergo apoptosis in response to DSBs than somatic cells (Zheng et al., 2018). The cell recovery after transfection when

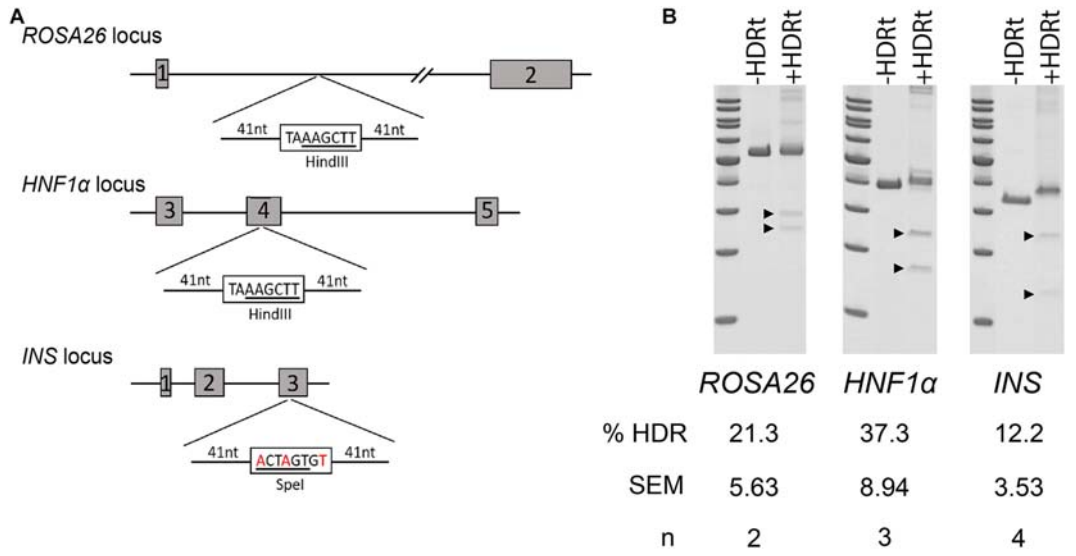

FIGURE 3 | Homology directed repair (HDR) in germ cells edited with CRISPR/Cas9 RNPs. **(A)** Schematic of targeted loci with HDR templates and restriction sites. Boxed nucleotide sequences represent insertions in the *ROSA26* and *HNF1a* loci, and red nucleotides represent SNPs in the *INS* locus. **(B)** Efficiency of HDR measured by restriction fragment length polymorphism (RFLP) analysis at three targeted loci. ±HDRt indicates transfection with or without the HDR template. Arrow heads indicate cut fragments (see **Table 1**).

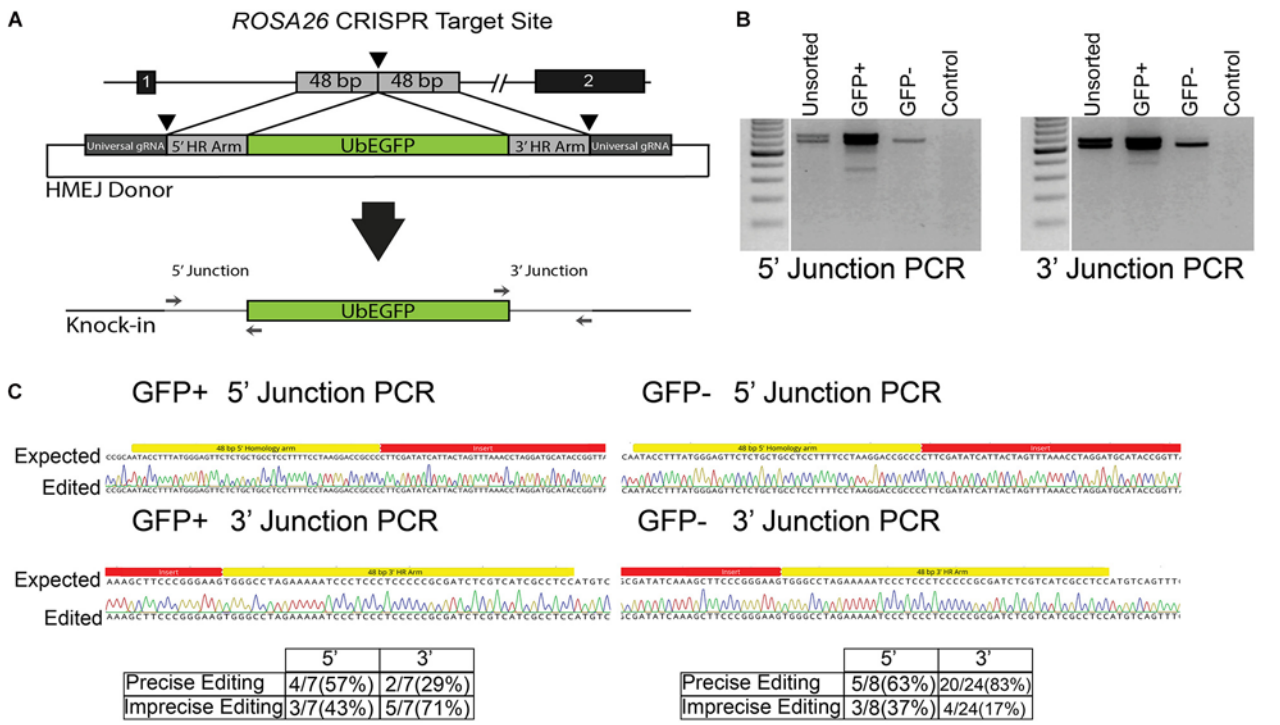

FIGURE 4 | Homology mediated end joining (HMEJ) in germ cells edited with CRISPR/Cas9 RNPs. **(A)** Schematic of HMEJ at the *ROSA26* locus with the UbEGFP donor. The genome is cut with *ROSA26* gRNA while the donor template is liberated from a plasmid with the universal gRNA. **(B)** HMEJ-mediated knock-in of the UbEGFP reporter into the *ROSA26* locus with CRISPR/Cas9 RNPs induced genomic double-strand breaks (DSBs). PCR analysis at 11 days revealed 5′ and 3′ integration junctions in the unsorted, GFP+ and GFP- germ cell populations but no non-DSB stimulated integration of the ubiquitin-EGFP donor (Control). **(C)** Sequencing of junction amplicons at 11 days indicated precise and imprecise HMEJ in GFP+ and GFP- sorted germ cells.

using CRISPR/Cas9 RNPs was greatly improved compared to transfection with TALEN plasmids, almost twofold. This difference in efficiency and cell recovery could be due to multiple factors. First, our highest editing rates using TALENs required delivery of 25–50 μg of TALEN expressing plasmid. This quantity is 10–20-fold more plasmid DNA than required to achieve a similar editing rate in pig fibroblasts under similar conditions (Carlson et al., 2012), and much greater than the 1.7 μg of plasmid used here as a template for HMEJ. This high quantity of plasmid could alone account for the lower cell recovery rates. Second, CRISPR/Cas9 RNPs are an active complex and do not rely on the cell's transcriptional and translational machinery to produce active editing reagents. Last, optimal TALEN editing occurred in spermatogonia at 30°C where cell recovery was reduced. In contrast, CRISPR/Cas9 RNPs performed well at 37°C where cell recovery is at its highest.

The ability to use HDR to generate precise mutations greatly expands the versatility of SSC gene editing. Whereas attempts to stimulate HDR with TALENs had previously failed (unpublished), we were encouraged to revisit HDR considering the higher rates of cell editing and recovery with CRISPR/Cas9 RNPs. To our surprise, HDR editing using ssODNs was achieved at rates of 10–40%. As with our previous results in fibroblasts, insertional HDR edits were more efficient than edits that introduced SNPs (Tan et al., 2013), presumably by enhancing the stability of resulting edited alleles. This high rate of editing unlocks the potential to directly model dominant or gain of function alleles identified in humans in founder pigs produced by SSC editing, exemplified by our choice to engineer *HNF1a* and *INS* to model dominant forms of diabetes.

Single-stranded DNA templates longer than standard oligonucleotides (60–200 bases) are difficult and expensive to produce in the quantities required for HDR. This restricts the application of ssDNA to introduction of small alleles in the range of 1–150 base pairs. However, several biomedical applications benefit from introduction of transgeneses and/or gene replacements in a site-specific manner. Precisely integrated transgenes are useful for a diversity of applications such as cell reporting, cell-specific ablation, and immune modulation (Ruan et al., 2015; Carneiro D'Albuquerque et al., 2018). Our results demonstrate that as observed in pig fibroblasts (Wierson et al., 2020), HMEJ insertion is effective in porcine spermatogonia. Based on GFP expression, our integration rate of ∼8% is encouraging, but this is a relatively small (3 kb) expression cassette, and it will be interesting to determine if transgenes with much larger cargos can be introduced. The approaches established in the current study for efficient targeted genome editing in porcine spermatogonia have been used in other species and cell types. However, to our knowledge, this is the first example of CRISPR/Cas9 RNP-mediated HDR and HMEJ transgene insertion in primary spermatogonia of any species, further expanding the SSC editing toolbox.

Since germline-competent embryonic stem cells (ESCs) are not well established in pigs, the generation of engineered pigs currently relies on SCNT and zygote injection or electroporation. These established approaches require manipulation of embryos, which can result in chimerism, incomplete reprogramming of the somatic cell nuclei, abnormal fetal and placental development, or neonatal mortality. Due to their reliance on oocytes obtained from commercial pigs at slaughter, zygote injection is not applicable to smaller strains of pigs that are more suitable for biomedical research than large commercial breeds. The generation of pig models using gene-edited SSCs and germline stem transplantation is advantageous in that it avoids the production of mosaic mutant progeny, can be applied to diverse strains of pigs, and shortens the timeline to production of gene-edited spermatozoa (Tang et al., 2015). As the genetic change is introduced into the male germline just before the onset of spermatogenesis, the approach is more broadly applicable to disease models where gene dosage and epigenetics play a role. The production of rodent progeny with targeted genetic modifications following transplantation of gene-edited SSCs and *in vitro* fertilization or natural breeding has been achieved (Chapman et al., 2015; Sato et al., 2015; Wu et al., 2015).

In pig, cattle, sheep, and goats with functional immune systems, transplantation of germ cells including SSCs isolated from unrelated donors demonstrated that the recipient testes is immunotolerant, simplifying the approach by eliminating the need to identify and use genetically related donors or induce immune suppression before transplantation (Honaramooz et al., 2002, 2008; Herrid et al., 2006; Rodriguez-Sosa et al., 2006; Zeng et al., 2012, 2013). To improve the outcome of germ cell transplantation, effective chemical and radiological approaches to ablate a recipient's endogenous SSCs and expand the availability of the stem cell niche for the transplanted SSCs to colonize have been developed in pigs (Honaramooz et al., 2005). Recently, pig models with genetically impaired spermatogenesis have been generated to overcome the drawbacks associated with chemical and radiological SSC ablation (Nicholls et al., 2019; Ciccarelli et al., 2020). We are optimistic that the high rates of editing reported here along with transplantation into germline ablated pigs will enable efficient production of gene edited founders.

We also recognize that success in germ cell gene-editing followed by transplantation is dependent on characteristics of isolated spermatogonia including purity and cell viability. To obtain a pure population of pig spermatogonia, we recently refined a differential plating protocol for pig germ cell enrichment (Sakib et al., 2019). Further enrichment was achieved using flow activated cell sorting and light scatter properties (Tang et al., 2018). These approaches allowed for efficient gene editing in primary spermatogonia in the current study. *In vitro* culture conditions that promote proliferation and long-term culture of mouse SSCs are well established facilitating the process of obtaining a highly enriched and robust population of SSCs for gene editing with engineered nucleases. Culture conditions for mouse germ cells have not translated to pig germ cell culture where proliferation and long-term culture remain limited. However, recent advances in improved culture systems for porcine spermatogonia (Zhang et al., 2017; Zheng et al., 2020) may allow for gene targeting in porcine germ cells at a larger scale.

A specific limitation of producing gene-edited animals by germline stem cell transplantation is that founder offspring will carry only one engineered allele. This limits the ability to directly produce biomedical animals where homozygosity is required to achieve a desired phenotype. However, there are numerous dominant disorders and biomedical applications that can be produced in the heterozygous state. The results reported here are critical to unlock the potential of dominant disease modeling or site-specific transgene integration, and represent an attractive alternative to SCNT or zygote manipulation for this purpose.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Care and Use Committee at the University of Calgary.

AUTHOR CONTRIBUTIONS

DW, SS, AB, NL, and LS designed and performed the experiments under the direction of DC and ID. All authors contributed to data analysis. SS, TG, DC, and ID wrote the manuscript.

FUNDING

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2020.627673/full#supplementary-material

Supplementary Figure 1 | Analysis of indel formation (NHEJ) in the *ROSA26* locus by Surveyor assay after editing with duplexed or single gRNAs at two different temperatures. Arrowheads indicate digested fragments (see **Table 1**).

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REVIEW
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Genetically Modified Rabbits for Cardiovascular Research

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Rabbits are one of the most used experimental animals for investigating the mechanisms of human cardiovascular disease and lipid metabolism because they are phylogenetically closer to human than rodents (mice and rats). Cholesterol-fed wild-type rabbits were first used to study human atherosclerosis more than 100 years ago and are still playing an important role in cardiovascular research. Furthermore, transgenic rabbits generated by pronuclear microinjection provided another means to investigate many gene functions associated with human disease. Because of the lack of both rabbit embryonic stem cells and the genome information, for a long time, it has been a dream for scientists to obtain knockout rabbits generated by homologous recombination-based genomic manipulation as in mice. This obstacle has greatly hampered using genetically modified rabbits to disclose the molecular mechanisms of many human diseases. The advent of genome editing technologies has dramatically extended the applications of experimental animals including rabbits. In this review, we will update genetically modified rabbits, including transgenic, knock-out, and knock-in rabbits during the past decades regarding their use in cardiovascular research and point out the perspectives in future.

Keywords: atherosclerosis, CRISPR-Cas9, hypercholesterolemia, knock-out rabbits, transgenic rabbits

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INTRODUCTION

Rabbits were first used for disclosing the pathogenesis of human atherosclerosis a century ago. In 1908, a Russian physician, Alexander I. Ignatowski (1875–1955) fed rabbits with a diet supplemented with animal proteins (milk, meat, and eggs) and found that these rabbits developed pronounced aortic atherosclerosis.

Later, a Russian experimental pathologist, Nikolai N. Anichkov (or Anitschkow) (1885–1964) further demonstrated that it was dietary cholesterol rather than proteins that play the critical role in the pathogenesis of atherosclerosis in rabbits and proposed a causal role of cholesterol in the development of atherosclerosis (Fan et al., 2015). Now, a consensus has been widely hold in this field that, in both humans and experimental animals, high levels of plasma cholesterol carried by apolipoprotein (apo)-B-containing particles such as low density lipoproteins (LDL) initiate the development of atherosclerosis (Steinberg, 2004). These pioneering studies derived from rabbit experiments not only provided the first evidence but also established a theory basis of the "lipid hypothesis" of atherosclerosis (Steinberg, 2004). Since then, cholesterol-fed rabbits along with Watanabe heritable hyperlipidemic (WHHL) rabbits, a mutant rabbit with genetic deficiency of LDL receptor

functions, have been extensively used to elucidate multiple facets of the pathophysiology of human atherosclerosis, leading to the discovery of the LDL receptor functions in familial hypercholesterolemia (Goldstein et al., 1983) and the development of the most-prescribed lipid-lowering drug, statin (Brown and Goldstein, 2004). On the other hand, transgenic rabbits with overexpression of various genes were generated from early 90's and served as an alternative tool for investigating the gene functions in cardiovascular disease. Moreover, recent genome editing technology has provided enormous opportunities to create knock-out (KO) and knock-in (KI) rabbits. Important roles of rabbits in studying human atherosclerosis have been extensively reviewed in the previous reviews (Fan et al., 1999a, 2015, 2018; Fan and Watanabe, 2000, 2003). In this review, we will focus on genetically modified rabbits for their applications in cardiovascular research.

"NATURALLY" GENETICALLY MODIFIED RABBITS

Spontaneous mutations in rabbits can be found accidentally and they can be used in controlling the coat color for commercial purposes such as tyrosinase and the melanocortin 1 receptor (Aigner et al., 2000; Xiao et al., 2019). However, some spontaneous mutations in rabbits can cause a pronounced phenotype that can mimic human diseases, such as Watanabe heritable hyperlipidemic (WHHL) rabbits (Watanabe, 1980), St. Thomas hyperlipidemic rabbits (Laville et al., 1987; Seddon et al., 1987) and complement 6 deficient rabbits (Rother, 1986; Liu et al., 2007a). WHHL rabbits were originally established by Dr. Yoshio Watanabe (1927–2008) at Kobe University, Japan, through serial inbreeding (Watanabe, 1980). Homozygous WHHL rabbits exhibit spontaneous hypercholesterolemia characterized by high levels of LDLs and severe atherosclerosis and often serve as a human familial hypercholesterolemia model (Watanabe et al., 1985). Genetic analysis revealed that WHHL rabbits have defective LDL receptor functions due to a deletion of 12 nucleotides in exon 4 of the LDL receptor gene, which leads to a 4-amino acid deletion in the cysteine-rich ligand-binding domain of the LDL receptor protein (Yamamoto et al., 1986). LDL receptor mutations can be easily detected by PCR analysis (Sun et al., 2002a); however, high levels of plasma LDL-cholesterol are the major manifestation observed in homozygous WHHL rabbits. In addition to hyperlipidemia and aortic atherosclerosis, some WHHL rabbits (later designated as WHHL-MI) show coronary atherosclerosis and myocardial infarction (Shiomi et al., 2003; Shiomi and Fan, 2008). Using WHHL rabbits, Tomoike et al. further developed a subline of WHHL designated a hereditary hypertriglyceridemic rabbit after selected in-breeding. This model exhibited postprandial hypertriglyceridemia along with insulin resistance and visceral obesity although polygenetic loci for these pathophysiogical changes have not been determined (Kawai et al., 2006). In addition to WHHL rabbits, the St. Thomas hospital hyperlipidemic rabbits were developed by La Ville et al. in London (Laville et al., 1987; Seddon et al., 1987). Different from WHHL rabbits which have high plasma LDL levels due to LDL receptor dysfunctions, the St. Thomas hospital hyperlipidemic rabbits showed high levels of very low density lipoproteins (VLDL) and intermediate density lipoproteins (IDL), and LDL, thus this rabbit model resembles human familial combined hyperlipidemia. Elevated plasma cholesterol levels in these rabbits were caused by overproduction of these apo-B-containing lipoproteins in the liver although the genetic mutations responsible for hyperlipidemia have not been examined in details. There is a complement-6 (C6) deficient rabbit originally reported by Rother in 1986 (Rother, 1986). C6 deficiency in these rabbits arises from a single gene defect and is not known to be associated with other genetic abnormalities. In spite of this, C6 deficient rabbits are protective against cholesterol diet-induced atherosclerosis (Schmiedt et al., 1998).

TRANSGENIC RABBITS

Because spontaneous mutant rabbits with obvious phenotypes resembling human disease phenotypes are rare and accidently discovered by experimental animal staff, it is necessary to make genetically modified rabbits according to one's own research purposes. The technology for producing transgenic (Tg) rabbits was almost concurrently reported by German (Brem et al., 1985) and US (Hammer et al., 1985) groups in 1985, but the actual use of Tg rabbit technology as an experimental tool in the field of cardiovascular diseases was not realized until 1994 when John Taylor's laboratory at the Gladstone Institute of Cardiovascular Disease in San Francisco created the first Tg rabbit expressing human hepatic lipase (Fan et al., 1994). Later on, they also produced Tg rabbits expressing human apoB-100 (Fan et al., 1995), apoE (Huang et al., 1997; Fan et al., 1998), and apoB mRNA editing protein (Yamanaka et al., 1995). Until now, more than 20 kinds of Tg rabbits expressing different genes that are involved in lipid metabolism and atherosclerosis have been reported and studies using these Tg rabbits have provided considerable insights into the molecular mechanisms of these gene functions in lipoprotein metabolism and atherosclerosis (Fan and Watanabe, 2003; Peng, 2012; Fan et al., 2015). The transgenes expressed in Tg rabbits for the study of lipoprotein metabolism and atherosclerosis can generally be classified into three categories: (1) those proteins that constitute lipoprotein structures such as apo(a) (Rouy et al., 1998; Fan et al., 1999b), apoAI (Duverger et al., 1996a), apoAII (Koike et al., 2009a; Wang et al., 2013), apoB-100 (Fan et al., 1995), apoCIII (Ding et al., 2011), and apoE (Huang et al., 1997; Fan et al., 1998); (2) those enzymes or transfer proteins that participate in the lipid metabolism such as hepatic lipase (Fan et al., 1994), lipoprotein lipase (Fan et al., 2001a), phospholipid transfer protein (Masson et al., 2011), apoB-100 mRNA editing enzyme catalytic polypeptide protein (Yamanaka et al., 1995), lecithin:cholesterol acyltransferase (Hoeg et al.,

Abbreviations: Apo, Apolipoprotein; Cas9, CRISPR-associated (Cas) protein 9; CETP, Cholesteryl ester transfer protein; CRISPR, Clustered regularly interspaced short palindromic repeat; FH, Familial hypercholesterolemia; HDL, High density lipoproteins; IDL, Intermediate density lipoproteins; LDL, Low density lipoproteins; KI, Knock-in; KO, Knock-out; TALEN, Transcription activator-like effector nuclease; Tg, Transgenic; VLDL, Very low density lipoprotein; WHHL, Watanabe heritable hyperlipidemic; ZFN, Zinc finger nuclease.

1996), endothelial lipase (Wang et al., 2017); and (3) those proteins that may exert some functions on the arterial wall cells which participate in the pathogenesis of atherosclerosis including matrix metalloproteinase-1,9,12 (Liang et al., 2006; Niimi et al., 2019; Chen et al., 2020), 15-lipoxygenase (Shen et al., 1996), C-reactive protein (Koike et al., 2009b), and vascular endothelial growth factor (Kitajima et al., 2005) (**Table 1**). In addition, Tg rabbits have also been used for the investigation of human heart diseases, including LQT syndrome (Brunner et al., 2008), hypertrophic cardiomyopathy (Marian et al., 1999) and tachycardia-induced cardiomyopathy (Suzuki et al., 2009). This is because, in comparison with mice and rats, the rabbit heart is similar to that of humans in both structure and function (Bers, 2002; Marian, 2006; Pogwizd and Bers, 2008). For example, like human heart in which β-myosin heavy chain (β-MyHC) accounts for 90% of total myofibrillar myosin, rabbit heart is composed of 80% β-MyCH which is different from the mouse heart predominated by 95% α-MyHC (Marian, 2005; Bosze et al., 2016). Tg rabbits can be generated by microinjecting a transgenic DNA construct into the pronuclei of fertilized embryos (Fan et al., 1999a; Kitajima et al., 2003). The transgenic constructs are typically composed of the transgene (either cDNA or genomic DNA) under the control of a tissue-specific promoter such as liver- and macrophage-specific promoter. In addition to the pronuclear microinjection method, other methods such as sperm vector (Wang et al., 2003; Li et al., 2006, 2010; Shen et al., 2006), ICSI-mediated transgenesis (Li et al., 2010; Zhang et al., 2016), somatic cell nuclear transfer (SCNT) (Li et al., 2009) or chimeric SCNT (Matsuda et al., 2002; Skrzyszowska et al., 2006), lentiviral vectors (Hiripi et al., 2010), transposon-mediated transgenesis (Katter et al., 2013; Ivics et al., 2014), and novel genome editing technology (Song J. et al., 2016; Yang et al., 2016; Li et al., 2019) have been reported to produce Tg rabbits. In spite of this, the pronuclear microinjection is still the most common method even though transgene integration rate is low.

RABBIT EMBRYONIC STEM CELLS AND GENOME INFORMATION

Because of the lack of both rabbit embryonic stem (ES) cells and the genome information, it has been considered impossible to create KO rabbits by homologous recombination-based genomic modification as to generate KO mice. Unavailability of KO rabbits also constitutes another obstacle that hampers researchers to study loss-of-functions of genes in rabbits. We strived to use somatic cell nuclear transfer technique to generate KO rabbits after Chesne et al. reported the first cloned rabbit about 17 years ago (Chesne et al., 2002). However, after enormous attempts, we got to the conclusion that the production of KO rabbits by somatic cell nuclear transfer is far remote from reality. As a research tool, nuclear transfer technique is unworkable owing to the extraordinarily low efficiency of gene transfer into somatic cells and the possibility in generating cloned rabbits (Song J. et al., 2020). Many groups reported that they could obtain rabbit ES-like cells, but none of these so-called ES-like cells have been proved to be able to generate chimera rabbits (Fan et al., 2015). Rabbit genome has long been an empty area mainly because of budget insufficiency and narrow research communities. In 2014, Carneiro et al. successfully reported a high-quality reference genome using the European rabbit with references to domestication and speciation (Carneiro et al., 2014a,b). Almost at the same period, we along with researchers from the US, Japan and China organized an International Rabbit Genome Sequencing Project Consortium aiming at implementing more extensive whole-genome sequencing of three kinds of common laboratory rabbits: Japanese white rabbits, New Zealand white rabbits and WHHL rabbits. In addition, we performed deep transcriptome sequencing of the aortas, livers, hearts, and kidneys of cholesterol-fed and WHHL rabbits (Wang et al., 2016). After a 2-year collaborative work, we were able to completed whole-genome sequencing of 10 male rabbits for each line with coverage of 13x for each individual after alignment to the reference genome. With the successful completion of rabbit genome sequencing (Carneiro et al., 2014a,b; Wang et al., 2016), researchers now can easily not only design PCR primers to study gene expression in rabbits but also to generate KO rabbits using genome editing techniques as described below. Rabbit genome information is now available from the NCBI database and a comprehensive rabbit transcriptome information established by the Chinese Academy of Sciences in Shanghai (Zhou et al., 2018) is also available at http://www.picb.ac.cn/RabGTD/.

KNOCK-OUT AND KNOCK-IN RABBITS BY GENOME EDITING TECHNIQUES

In the past decade, the emergence of three powerful genome editing technologies has dramatically enhanced the application of genetically modified rabbits (Song J. et al., 2020). The first one is the zinc finger nuclease (ZFN)-mediated genome editing method by which KO rats were successfully created in 2009 (Geurts et al., 2009). Two years later after the birth of KO rats, Flisikowska et al. generated the first immunoglobulin KO rabbits in an attempt to produce humanized antibodies (Flisikowska et al., 2011). Almost at the same time, we successfully created apoCIII KO rabbits with ZFN-mediated genome editing technology (Yang et al., 2013). ZFNs are engineered DNA-cleaving enzymes made by fusing a tailor-made DNA-binding domain to the DNA cleavage domain of Fok1, a type II restriction enzyme. ZFNs generate site-specific double-strand breaks in the DNA at researcher-assigned sites, thus resulting in targeted modification of the genome. However, while ZFNs were not extensively applied in this field, the second generation of the genome editing tool, transcription activator-like effector nucleases (TALEN) were shown up to make the first KO rats in 2011 (Tesson et al., 2011). TALENs are considered much simpler to design and assemble than ZFNs. The DNA binding domain in TALENs was derived from *Xanthomonas spp. Bacteria* (Christian et al., 2010; Miller et al., 2011). While TALENs utilize the same Fok I endonuclease domain as ZFNs, its DNA binding domain contains a repeated highly conserved 33–34 amino acid sequence with divergent 12th and 13th amino acids which called Repeat Variable

TABLE 1 | Transgenic rabbits for the study of human lipoproteins and atherosclerosis.

Genes	Expression cells	Major phenotypes	References
Apolipoproteins (apo)			
Apo(a)	Liver	Atherogenic	Rouy et al., 1998; Fan et al., 2001b; Ichikawa et al., 2002; Sun et al., 2002b; Kitajima et al., 2007
Apo(a) and apoB	Liver	Not determined	Rouy et al., 1998
ApoA-I	Liver	Athero-protective	Duverger et al., 1996a,b
ApoA-II	Liver	Athero-protective	Koike et al., 2009a; Wang et al., 2013
ApoA-I/C-III/A-IV	Liver and intestine	No effect on atherosclerosis	Recalde et al., 2004
ApoB-100	Liver	LDL↑, HDL↓	Fan et al., 1995
ApoCIII	Liver	VLDL↑	Ding et al., 2011
ApoE2	Liver	Atherogenic	Huang et al., 1997
ApoE3	Liver	Atherogenic	Fan et al., 1998; Huang et al., 1999
Enzymes or transfer proteins			
APOPEC1	Liver	LDL↓, liver carcinoma	Yamanaka et al., 1995
APOPEC1	Knockdown by RNAi	Lean	Jolivet et al., 2014
CETP	Liver	HDL↓	Gao et al., 2017
Endothelial lipase	Liver	Atheroprotective	Wang et al., 2017; Yan et al., 2020b
Hepatic lipase	Liver	Athero-protective	Fan et al., 1994
LCAT	Liver	Athero-protective	Hoeg et al., 1996
Lipoprotein lipase	Universal	Athero-protective	Fan et al., 2001a
PLTP	Universal	Atherogenic	Masson et al., 2011
Vascular cell factors			
C-reactive protein	Liver	Thrombogenic	Matsuda et al., 2011
Lipoprotein lipase	Macrophage	Atherogenic	Ichikawa et al., 2005
15-lypooxygenase	Macrophage	Athero-protective	Shen et al., 1996
MMP-1	Macrophage	Aortic aneurysm↑	Niimi et al., 2019
MMP-9	Macrophage	Vascular calcification	Chen et al., 2020
MMP-12	Macrophage	Atherogenic	Liang et al., 2006; Yamada et al., 2008
Urotensin II	Macrophage	Atherogenic	Zhao et al., 2015
VEGF	Liver	Hemangiomas and impaired glomerular functions	Kitajima et al., 2005; Liu et al., 2007b

APOPEC1, apolipoprotein B mRNA editing enzyme, catalytic polypeptide 1; CETP, cholesteryl ester transfer protein; LCAT, lecithin:cholesterol acyltransferase; PLTP, phospholipid transfer protein; MMP, matrix metalloproteinase; VEGF, vascular endothelial cell growth factor. ↑, increase; ↓, decrease.

Diresidue (RVD) for recognizing one specific nucleotide, for example, NN for guanine, NI for adenine, HD for cytosine, and NG for thymine. This direct relationship between amino acid sequence and DNA recognition has made engineering sequence specific binding domains much easier than ZFNs (Boch et al., 2009; Moscou and Bogdanove, 2009). Using TALEN technology, Lai's laboratory at GIBH, immediately generated two kinds of KO rabbits: an immunodeficent KO rabbit with deficiency of Rag1 and Rag2 genes (Song et al., 2013) and fumarylacetoacetate hydrolase deficient rabbits (Li et al., 2017), which mimics human genetic disease tyrosinemia type I, an autosomal recessive disorder caused by mutations in the both copies of the gene encoding the enzyme. Although TALENs are considered superior to ZFNs in terms of fewer off-target effects, easy design and production, it was soon replaced by the CRISPR-Cas9 based genome editing technology, which is even more rapid and modular than the TALEN platform. Cas9 is an endonuclease playing a protective role against foreign nucleic acids in the adaptive immune system in bacteria. The feature of bacterial CRISPR immune system is that genetic materials taken up from previous invasive elements are expressed in crRNA, which could direct the Cas9 endonuclease to cut foreign DNA elements containing the same sequences (Jinek et al., 2012). Therefore, the CRISPR/Cas9 system has been remolded from bacterial immune system to the genome editing tool, using a designed RNA to guide Cas9 nuclease to the specific DNA sequence (Hsu et al., 2014). Binding of Cas9 nuclease on a specific protospacer adjacent motif (PAM) sequence on the genome (NGG for spCas9) will unwind the adjacent sequence, allowing the RNA:DNA pairing, which activates the nuclease domains in Cas9 to cut DNA and make double-strand breaks. While ZFNs and TALENs rely on protein-DNA recognition, which is less predictable for design and more labor and time consuming for assembly, the CRISPR/Cas9 system relies on the RNA–DNA recognition, which is much simpler and more predictable.

Because CRISPR-Cas9 technique is so efficient and powerful, it was quickly adopted to generate KO rabbits. In this respect, Chen's laboratory at the University of Michigan first established a number of KO rabbits aiming at studying human cardiovascular disease (Yang et al., 2014) and then KO rabbit boom started. Lai's

laboratory at GIBH and Li's laboratory at Jilin University made more than 30 KO rabbits using CRISPR-Cas9 along with base-editing (Liu et al., 2018) and CRISPR/Cpf1 (Wu et al., 2018). Most KO rabbits were created in attempt to recapitulate human genetic or congenital disorders or immunodeficient rabbits (Song J. et al., 2017, 2018) as shown in **Table 2**. In addition, this technique has been tried to target the tyrosinase gene to modify rabbit coat colors (Honda et al., 2015; Song Y. N. et al., 2016, 2017, 2018). The standard protocol for generation of KO rabbits using CRISPR-Cas9 has been recently published (Yang et al., 2019). The techniques have been further refined (Liu et al., 2018, 2020) so we can predict that in the next few years, more and more KO or KI rabbits will be made using this technology. Here we will briefly review some valuable KO rabbits created recently to discuss their usefulness in disclosing the molecular mechanisms of atherosclerosis.

APOCIII KO RABBITS

ApoCIII is a major component of plasma chylomicrons and VLDLs, and is a minor component of high density lipoproteins (HDLs) and was first reported by Brown et al. 50 years ago (Brown et al., 1969). It is generally believed that physiological functions of apoCIII is to mediate the triglyceride(TG)-rich lipoprotein metabolism thereby maintaining the plasma TG homeostasis and high plasma levels of apoCIII are positively associated with plasma TG and increases the risk of ischemic heart disease (Huff and Hegele, 2013; Norata et al., 2015; Ramms and Gordts, 2018). However, for a long time, it is not clear whether apoCIII was directly involved in the pathogenesis of atherosclerosis because mouse models failed to provide a clear answer (Yan et al., 2020a). Yang et al. first generated apoCIII KO rabbits using ZNF (Yang et al., 2013) and after several years efforts to breed enough numbers of homozygous apoCIII KO rabbits, we were able to examine the hypothesis whether apoCIII may participate in atherosclerosis. Recently, we have shown that that genetic deletion of the apoCIII gene in KO rabbits significantly accelerates catabolism of TG-rich lipoproteins in the liver and apoCIII deficiency leads to the resistance of KO rabbits to a cholesterol diet-induced hyperlipidemia and inhibits atherosclerosis (Yan et al., 2020a). These results indicate that therapeutic inhibition of apoCIII expression may become a novel strategy for the treatment of hyperlipidemia and atherosclerosis.

APOE KO RABBITS

ApoE is a ligand for both LDL receptor and LRP and plays an important role in the catabolism of remnant lipoproteins in the liver and genetic deficiency of apoE is a cause of human type III hyperlipoproteinemia (Mahley, 1988; Mahley et al., 1999). Deletion of apoE in mice even on a normal chow diet exhibited hyperlipidemia along with spontaneous aortic atherosclerosis (Plump et al., 1992; Zhang et al., 1992). ApoE KO rabbits were produced at University of Michigan using CRISPR-Cas9 (Yang et al., 2014) and Sage Company using ZFN (Ji et al., 2015), respectively. Even though different techniques were adopted, apoE KO rabbits generated by these two methods exhibit the same phenotypes (Niimi et al., 2016). Homozygous apoE KO rabbits on a normal diet only showed mild hyperlipidemia and their plasma total cholesterol levels reached ∼200 mg/dL, similar to human type III hyperlipoproteinemia patients, whose cholesterol levels are elevated to 300∼350 mg/dL (Mahley et al., 1999). Because plasma levels of cholesterol in apoE KO rabbits on a normal diet are not high to be atherogenic, there are not spontaneous atherosclerosis, which is different from apoE KO mice. However, when apoE KO rabbits were fed a cholesterol diet, they developed more prominent hypercholesterolemia than WT rabbits, which is basically caused by the remarkable accumulation of intestinally-derived remnant lipoproteins, β-VLDLs (Niimi et al., 2016). Recently, we found that apoE KO rabbits are highly susceptible to a cholesterol diet-induced atherosclerosis. Therefore, apoE KO rabbits will serve as a new model for human hyperlipidemia.

LDL RECEPTOR KO RABBITS

In humans, genetic deficiency of LDL receptor functions causes severe hypercholesterolemia and atherosclerosis at early ages, called familial hypercholesterolemia (FH). FH is an autosomal dominant genetic disorder characterized by elevated plasma LDL levels due to LDL receptor dysfunctions (Soutar and Naoumova, 2007). Two laboratories have successfully generated LDL receptor KO rabbits using CRISPR-Cas9 (Yang et al., 2014; Lu et al., 2018). Similar to human FH, homozygous LDL receptor KO rabbits develop spontaneous hypercholesterolemia and atherosclerosis (Lu et al., 2018). Therefore, like WHHL rabbits, LDL receptor KO rabbits can be used for the study of human FH.

CHOLESTERYL ESTER TRANSFER PROTEIN KO RABBITS

Cholesteryl ester transfer protein (CETP) is a glycoprotein that transfers plasma lipids between HDLs and apoB-containing particles therefore plays an important role in lipoprotein metabolism. However, it is not known whether inhibition of CETP activity can prevent cardiovascular disease because four CETP inhibitors (torcetrapib, dalcetrapib, evacetrapib, and anacetrapib) failed to prove their efficacy in terms of reduction of cardiovascular risk by clinical trials(https://en.wikipedia.org/wiki/CETP_inhibitor). Since CETP is genetically absent in rodents (mice and rats) and pigs, rabbits are considered the best model for investigation of CETP functions because rabbits have high levels of CETP in the plasma as humans. Taking this advantage, Zhang et al. created CETP KO rabbits and found that CETP KO rabbits showed higher plasma levels of HDL-cholesterol (Zhang et al., 2017). When fed a cholesterol-rich diet, CETP KO rabbits still exhibited higher HDL-cholesterol levels accompanied by lower total cholesterol levels than wild-type (WT) rabbits (Zhang et al., 2017). CETP KO rabbits had significant less atherosclerosis in both aorta and coronary arteries than WT rabbits (Zhang et al., 2017). These results indicate

TABLE 2 | Human congenital disease models of KO rabbits recently created by CRISPR-Cas9 or TALEN.

Human diseases	Targeted genes	Major phenotypes	References
Congenital cataracts	αA-Crystallin	Cataracts, microphthalmia, obscurity	Yuan et al., 2017
	GJ48	Microphthalmia, small lens size, and cataracts	Yuan et al., 2016
Muscular dystrophy/hypertrophy	ANO5	Muscular dystrophy with increased serum creatine kinase	Sui et al., 2018a
	DMD	Impaired physical activity, elevated serum creatine kinase	Sui et al., 2018b
	Myostatin	Hyperplasia or hypertrophy of muscle	Lv et al., 2016 (base editing)
Metabolic diseases			
	ATP7B	Wilson disease, Death at 3 mon	Jiang et al., 2018 (Precision point mutation)
	Dentin matrix protein 1	Mineralization defects	Liu et al., 2019
	GADD45G	Congenital defects cleft palate	Lu et al., 2019
	Glucokinase	Maturity-onset diabetes of the young 2 (MODY2)	Song Y. et al., 2020
	Fumarylacetoacetate hydroxylase	Hereditary tyrosinemia type 1	Li et al., 2017 (TALEN)
	HOXC13	Hair and nail ectodermal dysplasia	Deng et al., 2019
Syndromes			
	FBN1	Marfanoid-progeroid-lipodystrophy syndrome	Chen et al., 2018
	SRY	Sex reversal syndromes and hermaphroditism syndromes	Song Y. et al., 2017, 2018
	LMNA	Premature aging syndrome	

that genetic ablation of CETP gene inhibits the development of atherosclerosis in cholesterol-fed rabbits.

APOAII KI RABBITS

ApoAII is the second major apolipoproteins in HDLs. However, its physiological functions are largely unknown compared with apoAI. Interestingly, WT rabbits are genetically deficient in apoAII so their HDLs only contain apoAI. This unique feature makes WT rabbits as a "natural" apoAII KO model. We first made Tg rabbits expressing human apoAII gene and found that hepatic expression of human apoAII inhibits cholesterol diet-induced atherosclerosis (Wang et al., 2013). To examine the apoAII specific functions in the absence of apoAI, we further replaced the rabbit endogenous apoAI with human apoAII gene through knock-in (KI) using TALEN technology (Koike et al., 2021). In this way, apoAII KI rabbits expressed exclusively human apoAII without apoAI in HDL particles, which enables us to compare the net functions of apoAI-only-HDLs in WT rabbits with apoAII-only-HDL in KI rabbits in terms of HDL metabolism and atherosclerosis. In the latest study, we found that apoAII KI rabbits showed consistently lower TG and higher HDL-cholesterol levels and developed significantly less aortic atherosclerosis on a cholesterol diet (Koike et al., 2021).

CONSIDERATIONS AND FUTURE PERSPECTIVES

Although genetically modified rabbits are an important experimental model in cardiovascular research, they should not be simply used as a substitute of mice and rats, as discussed above. Because rabbits are more expensive, require larger space, and need more time to breed compared with mice, the generation of genetically modified rabbits should be carefully planned to solve those specific problems that cannot be well-examined in other experimental animal models, such as the development of lipid-lowering drugs (Niimi et al., 2020). However, off-target effects in these animals remain a concern as the genome editing is extremely productive and efficient. So far, almost all studies claimed that off-targets in genetically modified rabbits through genome editing are either none or negligible as comprehensively discussed in the recent review (Song J. et al., 2020); nevertheless, there is a need to performed careful genotyping, including sequencing, and expression validation of genetically modified rabbit models. It can be expected that more and more genetically modified rabbits will be made and used in a variety of medical sciences which will certainly expand our knowledge to explore new mechanisms of human diseases. Genome editing technique may eventually replace the pronuclear microinjection for the generation of Tg rabbits. However, complicated gene manipulation in rabbits, such as conditional KO in an organ- or cell-specific and time-controlled manner using the Cre/LoxP system is still lacking, thus it will be absolutely necessary to build such a platform in future. Finally, the preservation of valuable strains of genetically modified rabbits is an urgent task with increased number of rabbit models produced. In this aspect, various procedures for cryopreservation of rabbit sperm (Vicente and Viudes-de-Castro, 1996; Dalimata and Graham, 1997; Nishijima et al., 2015) and embryos (al-Hasani et al., 1992; Kasai et al., 1992; Marco-Jimenez et al., 2016) have been reported but have not been standardized. In the future, it may be necessary to establish an international rabbit bio-resource center or sperm and embryo bank to stock and share valuable rabbit models worldwide.

AUTHOR CONTRIBUTIONS

JF, YW, and YC wrote the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Recent Advances in the Application of CRISPR/Cas9 Gene Editing System in Poultry Species

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CRISPR/Cas9 system genome editing is revolutionizing genetics research in a wide spectrum of animal models in the genetic era. Among these animals, is the poultry species. CRISPR technology is the newest and most advanced gene-editing tool that allows researchers to modify and alter gene functions for transcriptional regulation, gene targeting, epigenetic modification, gene therapy, and drug delivery in the animal genome. The applicability of the CRISPR/Cas9 system in gene editing and modification of genomes in the avian species is still emerging. Up to date, substantial progress in using CRISPR/Cas9 technology has been made in only two poultry species (chicken and quail), with chicken taking the lead. There have been major recent advances in the modification of the avian genome through their germ cell lineages. In the poultry industry, breeders and producers can utilize CRISPR-mediated approaches to enhance the many required genetic variations towards the poultry population that are absent in a given poultry flock. Thus, CRISPR allows the benefit of accessing genetic characteristics that cannot otherwise be used for poultry production. Therefore CRISPR/Cas9 becomes a very powerful and robust tool for editing genes that allow for the introduction or regulation of genetic information in poultry genomes. However, the CRISPR/Cas9 technology has several limitations that need to be addressed to enhance its use in the poultry industry. This review evaluates and provides a summary of recent advances in applying CRISPR/Cas9 gene editing technology in poultry research and explores its potential use in advancing poultry breeding and production with a major focus on chicken and quail. This could aid future advancements in the use of CRISPR technology to improve poultry production.

Keywords: CRISPR/Cas9 system, genome editing, transgenic, gene editing, poultry species, primordial germ cells

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INTRODUCTION: GENE EDITING TOOLS

The poultry industry is undergoing a gene editing revolution that will change the poultry genome in the near future through targeted gene editing of the poultry species (Hwang and Han, 2018). The application of genome editing technology in the poultry industry, as well as livestock production in general, has improved over the last decade due to the availability of precision genome engineering tools (Petersen, 2017; Cooper et al., 2018). There are three commonly used genome-editing techniques for the production of animals, including poultry.

The first is the zinc finger nuclease (ZFNs), which is used for binding specific DNA domains that complement the target DNA sequences. Secondly, transcription activator-Like effector nucleases (TALENs) are another gene and genome editing technology that employs the nuclease domain to produce double strands breaks (DSBs). Finally, yet importantly, the clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein 9 (CRISPR/Cas9), is the most common and advanced technique for genome editing. The similarity between these three techniques is that they all require the two domains for accurate and defectless gene and genome editing. ZFN and TALEN differ from CRISPR/Cas9 since both use proteins that are fused together as a DNA binding domain while the CRISPR/Cas system requires the use of a specific RNA sequence molecule for DNA binding instead of the fused proteins (Kim and Kim, 2014; Razzaq and Masood, 2018). ZFNs and TALENs also require more time to produce an effective system, making the two more-time consuming. ZFNs and TALENs have been found to have more off target effects as opposed to CRISPR/Cas9 system (Hwang and Han, 2018; Bahrami et al., 2020). This is because of the availability of computational tools while using the CRISPR/Cas9 system that help in designing sgRNAs. Therefore, predictability of guide specificity is achieved, and this minimizes off-target effects. There is also a chance that the design of successful sgRNAs with the available CRISPR/Cas9 computational tools has a strong on-target activity hence reducing off-target effects (Wilson et al., 2018). The CRISPR/Cas9 technology uses a specific RNA sequence called guide RNA which binds to another target sequence of DNA (target DNA) followed by the cleavage of Cas9 where binding has occurred. This makes the CRISPR/Cas9 system stand out as the most suitable gene editing tool as it improves the frequency of precise genome modifications in creating genetically edited animals (Chu et al., 2015). The CRISPR-based system is continuously undergoing improvement. The most recent development of the CRISPR system employs coexpression of CRISPR-associated nucleases 9 and 12a hence having the ability to edit multiple target sites in the genome at the same time to help study how different genes cooperate in functions (Pennisi, 2013). Therefore, this system is very important in interrogating gene functions (Cong et al., 2013; Yang et al., 2013; Najm et al., 2018; Gonatopoulos-Pournatzis et al., 2020).

CRISPR is a family of DNA sequences found in the genomes of prokaryotic organisms such as bacteria and archaea. These sequences are derived from DNA fragments of bacteriophages that had previously infected the prokaryote. The CRISPR tool together with Cas endonuclease is a powerful programmable nuclease system (Barrangou et al., 2007). Studies conducted by Jinek et al. (2012) unveiled a double RNA, known as a guide RNA (gRNA) which consisted of a 20-bp CRISPR RNA (crRNA) and universal trans-activating crRNA (tracrRNA). This RNA coupled with *Streptococcus pyogenes* type II Cas9 protein can induce cleavage of specific target DNA sequences in virtually any organism. The Cas9 nuclease activity is initiated by protospacer adjacent motif (PAM) sequence NGG, which is usually located next to the target site (Anders et al., 2014). It is possible to engineer DNA Cas9-mediated DSBs at a specific genomic locus. Non-homologous end-joining (NHEJ) can induce DSB repair that disrupts the target gene, generating insertions and deletions. Another way of repairing Cas9-mediated DSBs is by homologous directed repair (HDR), which allows specific gene editing by integrating genetic modifications into the target template (Thomas and Capecchi, 1987; Salsman and Dellaire, 2017).

THE STATUS OF CRISPR/Cas9 TECHNOLOGY IN THE POULTRY INDUSTRY

The CRISPR/Cas9 system is among the gene editing technologies that are creating a rapid change in poultry genomics for both poultry breeding and food production purposes (Doran et al., 2017). To date, substantial progress in using CRISPR/Cas9 technology has been made in only two poultry species (chicken and quail), with chicken taking the lead. The CRISPR technology is not aimed at replacing the traditional breeding system, but it provides a complementary option by giving the breeder more genetic variation to select from since the use of traditional breeding for genetic gain has limitations of introducing genetic variation within a given population of the poultry flock. The introduction of genetic variations using the CRISPR/Cas9 system can be used to improve the performance of livestock animals such as poultry.

The CRISPR/Cas9 system has several benefits that could be used to improve poultry growth and production performance. These benefits include increased bird performance by improving the digestibility and overall growth, increased egg production, increased bird's immunity and disease resistance, producing birds that are leaner with little or no fat deposition in poultry meat for better nutritional profiles. A good example is the recent attempt to create chickens that have decreased accretion of abdominal fat and increased lean percentage of carcass meat by altering the percentage of fatty acid composition (Park et al., 2019). The CRISPR/Cas9 has also been employed in animal welfare improvements through in-ovo sexing (Lee et al., 2019b). There is an increased need to produce birds that meet the benefits of both commercial producers and consumers in the poultry industry. Several strategies have been proposed for the generation of transgenic birds to meet several demands in the poultry industry. This review discusses various applications of the CRISPR/Cas9 technology for genome editing in poultry, with a focus on recent and current advances in CRISPR/Cas9-mediated gene editing technology to produce genetically modified birds for various purposes. This review also provides a summary and discussion of the challenges, possible approaches, and future perspectives on applying CRISPR/Cas9 technology for gene and genome engineering in poultry species.

GENERATION OF GENETICALLY MODIFIED CRISPR/Cas9-MEDIATED BIRDS

CRISPR/Cas9 has gained traction as an efficient method for precise gene editing and modification of genomes in various

organisms including the avian species (Bai et al., 2016; Oishi et al., 2016; Wang et al., 2017b). Various methods have been proposed to produce genetically modified animals. In mammals, germ-line modification was used in the generation of the first transgenic animals such as mice, rabbits, sheep, and pigs, by microinjection of the target DNA into the pro-nucleus of a fertilized embryo (Gordon et al., 1980; Hammer et al., 1985). Another method that has been used to modify the germ line in animals uses embryonic stem cells (ESCs). ESCs are genetically modified, then cells are injected into the recipient blastocyst to produce germ-line chimeras. Unlike mammals, the microinjection of avian ESCs into the zygote in avian species is very difficult because the avian zygote is surrounded by a large amount of yolk and a small germinal disc. Therefore, the first transgenic chicken was produced *via* retroviral injection into the sub-germinal cavity of Eyal-Giladi and Kochav (EGK; Eyal-Giladi and Kochav, 1976) stage X embryos (Salter et al., 1986). Salter et al. (1987) created the first retrovirus-mediated transgenic chickens by insertion of retroviral genes into the chicken germ line. Their transmission frequencies varied from 1 to 11%. McGrew et al. (2004) produced germline transgenic chickens using lentiviral vectors with transmission efficiencies between 4 and 45%. Lillico et al. (2007) generated the first oviduct-specific expression of transgenes in hens but there was very low efficiency in the rate at which transgenic birds were generated. Various strategies such as the viral infection of stage X embryos (Thoraval et al., 1995; Sherman et al., 1998), microinjection of transgenes into fertilized eggs (Love et al., 1994; Sherman et al., 1998), and embryonic stem cells (Zhu et al., 2005) have been used to produce transgenic birds. In van de Lavoir et al. (2006) generated the first inter-individual transfer of chicken primordial germ cells (PGCs). As compared to the use of ESCs in mammals, PGCs have been used widely in the generation of transgenic birds to overcome the limitation of low efficiency germ-line transmission. Transgenes can be introduced into the cultured genomes of PGCs using transfection reagents to produce transgenic birds (Han and Park, 2018). Transgenic birds have been generated by injection of transgenes into the embryonic blood vessel to transfect the circulating PGCs to produce germline chimera, although these birds had a lower transgenic efficiency (Zhang et al., 2012; Tyack et al., 2013; Lambeth et al., 2016). Just before the onset of the CRISPR technology, Schusser et al. (2013) created the first knock-out in chickens using efficient homologous recombination in primordial germ cells.

With the advent of the CRISPR/Cas9 system, an *in vitro* culture system for PGCs can be combined with this efficient genome-editing system to produce programmable genome-edited poultry. First, the PGCs in poultry can be obtained from embryonic blood or gonads. The delivery of the CRISPR/Cas9 system is followed by the establishment of genome-edited poultry by the microinjection of directly isolated or *in vitro* cultured PGCs into the blood vessels of recipient embryos to produce a chimera that hatches and grows into mature avian poultry. Oishi et al. (2016) used the CRISPR/Cas9 system to efficiently generate ovomucoid gene-targeted chickens by transferring transiently drug-selected PGCs into recipient embryos using gamma-ray irradiation to deplete endogenous PGCs. In one of their most recent works, CRISPR/Cas9-mediated knock-in of human interferon beta (hIFN-β) was created into the chicken exon 2 of the ovalbumin gene (Oishi et al., 2018). Since the generation of the first CRISPR/Cas9-mediated chicken in 2015 by Veron and his group (Véron et al., 2015) through electroporation of chicken embryos, many more studies involving transgenic poultry-related species have been published as discussed in the next section. The current trend in using the CRISPR/Cas9 system in poultry species is incorporating this genome editing tool with genomic analysis software such as CRISPR to increase target specificity, efficiency, and lower off-target effects. **Figure 1** shows a workflow using the CRISPR/Cas9 system of programmable genome editing in avian species.

CRISPR/Cas9-MEDIATED GENOME EDITING IN SELECTED POULTRY SPECIES

Many researchers are studying the potential use of CRISPR/Cas9 for genome editing in the avian species. There is substantial progress in using CRISPR/Cas9 technology in chicken and quail, with chicken taking the lead as far as the poultry industry is concerned. Véron et al. (2015) published the first CRISPR/Cas9-mediated chickens 5 years ago. This study coupled the use of electroporated chicken embryos with Cas9 and guide RNAs encoded plasmids against the transcription factor paired box 7 (PAX7). In another recent study, the CRISPR/Cas9 system was used to produce chicken using ovalbumin and ovomucoid (OVM) genes. In this study, puromycin-selected CRISPR-induced mutant-ovomucoid PGCs were transiently transplanted into recipient chicken embryos with gamma-ray irradiation (Oishi et al., 2016). Their results indicated that the CRISPR/Cas9 system was used to induce OVM mutation getting a high efficiency (93%) in most donor PGCs with an average mutant semen efficiency of 93%. Another study in chicken by Dimitrov et al. (2016) shows a successful germline gene editing by efficient CRISPR-mediated homologous recombination in primordial germ cells. In this study, an additional *loxP* site was inserted into the variable region segment of a *loxP* by homology directed repair (HDR). This segment had been previously inserted into the chicken immunoglobulin heavy chain (IgH) locus gene. Their results showed variable germline transmission rates (0–90% efficiency) for the different PGC lines used.

As studies, PGC lines show different germline competencies for genetic modification and gene editing using CRISPR/Cas9 technology (Naito et al., 2015). More recently, Cooper et al. (2017) also reported a very successful method of avian genome editing known as "sperm transfection-assisted gene editing." This method involves the delivery of CRISPR gRNA and Cas9 mRNA mixture directly into a mature chicken sperm cell. This method was able to achieve a targeting efficiency of 26.6% and about 3% mutation in the green fluorescent protein (GFP) and, double sex and mab-3 related transcription

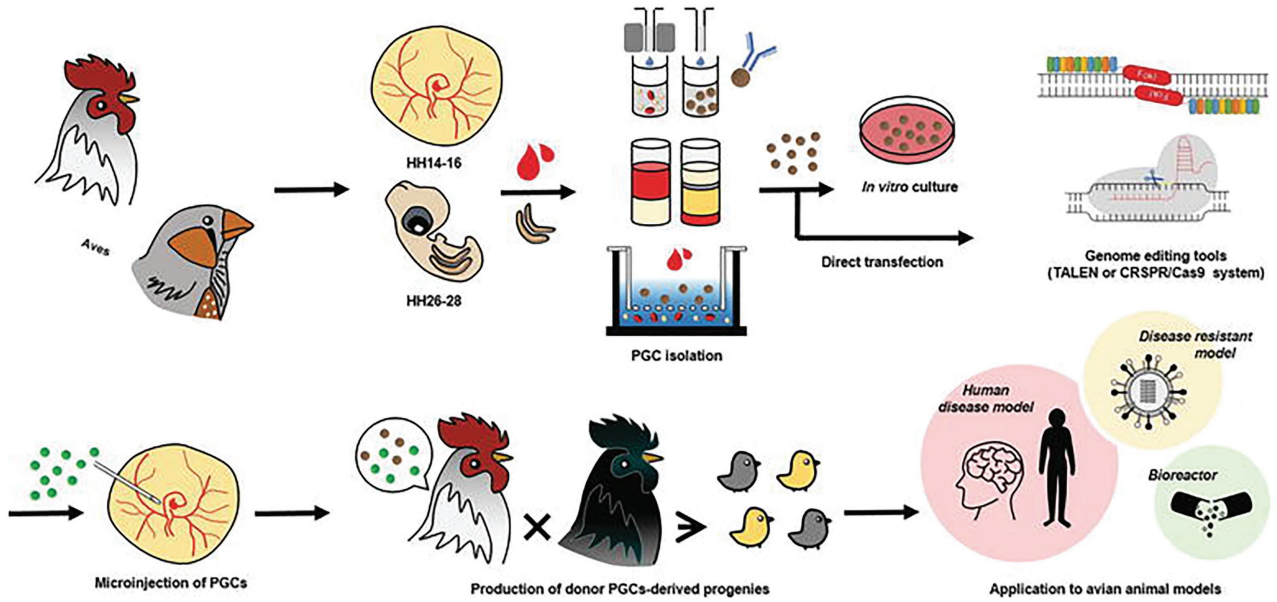

FIGURE 1 | Genome editing in poultry species. Primordial germ cells (PGCs) in poultry can be obtained from embryonic blood and embryonic gonads. Delivery of genome editing tools such as the CRISPR/Cas9 system is followed by the establishment of genome-edited poultry by microinjection of directly isolated or *in vitro* cultured PGCs into the blood vessels of recipient embryos. Avian genome editing systems can be applied to produce various avian models and poultry. This figure is reproduced from an earlier publication (Han and Park, 2018, p. 19) after obtaining the permission from Journal of Animal Science and Biotechnology and the corresponding author (Jae Yong Han, Seoul National University, Seoul, Korea).

factor 1 (DMRT1) genes, respectively. Morin et al. (2017) have recently described a technique that combines the CRISPR/Cas9 system with *in vivo* electroporation hence inhibiting the gene functions of target genes in the somatic cells of developing chicken embryos.

Abu-Bonsrah et al. (2016) worked on projects that targeted genes in the DF-1 and DT-40 cell lines. The genes targeted are highly important in embryonic progression for targeted genetic manipulation of the chicken genome using the CRISPR/Cas9 system. These genes included EZH2, CDKN1B, DROSHA, MBD3, KIAA1279, HIRA, TYRP1, among others. Many methods for CRISPR/Cas9-mediated gene modifications in avian species are based on genome modification of PGCs *in vitro* followed by in-ovo injection of modified PGCs into the embryonic blood vessels. There is however a possibility of using adenoviral vectors for delivery of CRISPR/Cas9 into the bird blastoderm in eggs resulting in chimeras that generate offspring having targeted mutations (Lee et al., 2019c). This technique of generating genome-edited poultry could fast-track many avian research studies with potential applications in poultry production. The use of poultry-specific CRISPR/Cas9 designed vectors containing inserted avian-specific promoters for the expression of guide RNA and Cas9 protein can efficiently introduce targeted gene modifications in poultry species (Ahn et al., 2017). This type of CRISPR vector can be applied in many poultry species to generate efficient knockout avian cell lines and knockout birds for various purposes.

Quail is an important avian species due to its value in the poultry food industry and its use as a research model for various research areas, especially avian transgenesis and genome editing. Currently, the use of CRISPR/Cas9 genome editing technology is more widely used in chicken than quail since chicken has been the most valuable avian model in developmental biology and immunology. Quail is however gaining tract as an alternative model to chicken in genome-editing studies due to their short generation time, high level of producing eggs, and small size (Poynter et al., 2009; Lee et al., 2019c). Ahn et al. (2017) designed a poultry-specific CRISPR/Cas9 system that introduces targeted deletion mutation in chromosomes of the quail muscle cell lines using a customized quail CRISPR vector. In this study, quail 7SK promoter and CBh promoter were cloned into a CRISPR vector for the expression of gRNA and Cas9 protein. The gRNA was designed to target the quail melanophilin (MLPH) locus. Lee et al. (2019c) reported CRISPR/Cas9-mediated gene knockouts in quail targeting the MLPH gene. In this study, CRISPR/Cas9 adenoviral vector was directly injected into the quail blastoderm. The offspring obtained from the quail chimeras were found to have mutations in the MLPH gene. Lee et al. (2020) targeted the myostatin (MSTN) gene to generate mutations in quail *in vivo* using an adenoviral CRISPR/Cas9 system-mediated method. This study showed that the mutation in MSTN resulted in the deletion of cysteine 42 in the MSTN propeptide region and the homozygous mutant quail showed significantly increased body weight and muscle mass decreased fat percentage weight and increased heart weight as compared to heterozygous mutant and wild-type quail.

APPLICATIONS OF CRISPR/Cas9 SYSTEM IN POULTRY-RELATED SPECIES

CRISPR/Cas9-mediated genetically modified poultry-related species have many applications in agricultural and biomedical research. There is a steady upward trend in the number of published reports on the use of CRISPR/Cas9 gene editing technology in poultry species since its introduction a few years ago. **Table 1** contains a selective list of the advances of CRISPR/Cas9-mediated gene edited poultry species and avian cells. This list was selected from recently published reports partly because of their significance on various aspects of CRISPR/Cas9-mediated genome editing in avian species, which is described in this review. **Figure 2** shows a summary of various applications of the CRISPR/Cas9 system in animals many of which are yet to be tested in avian species.

Agricultural Applications of CRISPR/Cas9 System in Poultry

Various agricultural traits can be achieved using CRISPR/Cas9-mediated gene editing approaches in poultry. Disease outbreaks

TABLE 1 | A selective list in advances of CRISPR/Cas9-mediated gene editing in poultry species and avian cells for different purposes.

Genetic Modification in Avian Cells	Target gene/Receptor	References
CRISPR mediated somatic cell genome engineering in the chicken	Paired Box 7 (PAX7)	Véron et al., 2015
Site-directed genome knockout in chicken cell line and embryos using CRISPR/Cas gene editing technology	C2EIP	Zuo et al., 2016
CRISPR/Cas9-mediated genome modification in chicken cell lines (B cell and DT40 cell lines)	DROSHA, DICER, MBD3, KIAA1279, CDKN1B, EZH2, HIRA, TYRP1, STMN2, RET, and DGCR	Abu-Bonsrah et al., 2016
Chicken cell line (DF-1) expressing edited PPAR-γ, OVA, ATP5E using CRISRP/Cas9 vectors	Peroxisome proliferator-activated receptor-γ (PPAR-γ), ATP synthase epsilon subunit (ATP5E), and ovalbumin (OVA)	Bai et al., 2016
Chicken DF-1 cells expressing myostatin gene knockout mediated by Cas9-D10A nickase without off-target effects	Myostatin	Lee et al., 2016
Targeted deletion mutation using poultry-specific CRISPR/Cas9 system in quail muscle cell line	Melanophilin (MLPH) locus	Ahn et al., 2017
Induced loss-of-function *via* a frameshift mutation in the CXCR4 gene in chicken PGCs	C-X-C chemokine receptor type 4 (CXCR4)	Lee et al., 2017c
CRISPR/Cas9-mediated chicken Stra8 gene knockout in male germ cell differentiation	Stimulated by retinoic acid 8 (Stra8) gene	Zhang et al., 2017
CRISPR/Cas9-mediated genome modulation of cis-regulatory interactions and gene expression in the chicken embryo	Msx1, Pax7, Sox9, c-Myb and Ets1	Williams et al., 2018
Chicken DF-1 cells expressing eGFP under control of the chicken GAPDH promoter	Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) gene	Antonova et al., 2018
Genetic resistance to Avian Leukosis Viruses induced by CRISPR/Cas9 editing of specific receptor genes in chicken DF-1 cells	tva, tvc, and tvj receptor genes	Koslová et al., 2018
CRISPR/Cas9-Mediated TBK1 gene knockout chicken DF-1 cells	TANK binding kinase 1 (TBK1)	Cheng et al., 2019
HMEJ-mediated efficient site-specific gene integration in chicken DF-1 cells	Deleted in AZoospermia-Like (DAZL) gene	Xie et al., 2019
Direct delivery of adenoviral CRISPR/Cas9 vector into the blastoderm for generation of targeted gene knockout in quail	Melanophilin (MLPH) gene	Lee et al., 2019c
Sequential disruption of ALV host receptor genes in chicken DF-1 cells	tva, tvb, and chicken Na^+/H^+ exchange 1 (chNHE1) genes	Lee et al., 2019a
Functional study of the ANP32A genes mediated by the CRISPR/Cas9 system in chicken cell lines	Acidic (Leucine-Rich) Nuclear Phosphoprotein 32 Family, Member A (ANP32A)	Park et al., 2020
Genetic Modification in Poultry Species		
Chicken expressing CRISPR/Cas9-mediated OVA and OVM mutations	Ovalbumin (OVA) and ovomucoid (OVM)	Oishi et al., 2016
Chicken expressing CRISPR-targeted locus in PGCs	Immunoglobulin heavy chain locus of EGFP gene	Dimitrov et al., 2016
Chick embryo optimized for early loss-of-function using CRISPR/Cas9	Pax7 and Sox10	Gandhi et al., 2017
Chicken Embryo expressing CRISPR/Cas9	Somatic cells genes	Morin et al., 2017
Induced loss-of-function *via* a frameshift mutation in the CXCR4 gene in chicken PGCs	C-X-C chemokine receptor type 4 (CXCR4)	Lee et al., 2017c
Chickens overexpressing human IFN-β	Ovalbumin (OVA)	Oishi et al., 2018
Chicken primordial germ cells expressing gene insertion into Z chromosome for avian sexing model development	Z chromosome	Lee et al., 2019b
Efficient knock-in at the chicken ovalbumin locus using adenovirus as a CRISPR/Cas9 delivery system	Ovalbumin (OVA)	Qin et al., 2019
Precise CRISPR/Cas9 editing of the NHE1 gene renders chickens resistant to the J subgroup of avian leukosis virus	NHE1 gene	Koslová et al., 2020
Single amino acid deletion in myostatin propeptide of Japanese quail using CRISPR/Cas9	Myostatin (MSTN) gene	Lee et al., 2020
Acquiring resistance against a retroviral infection *via* CRISPR/Cas9 targeted genome editing in a commercial chicken line	Chicken Na^+/H^+ exchanger type 1 (chNHE1) receptor	Hellmich et al., 2020

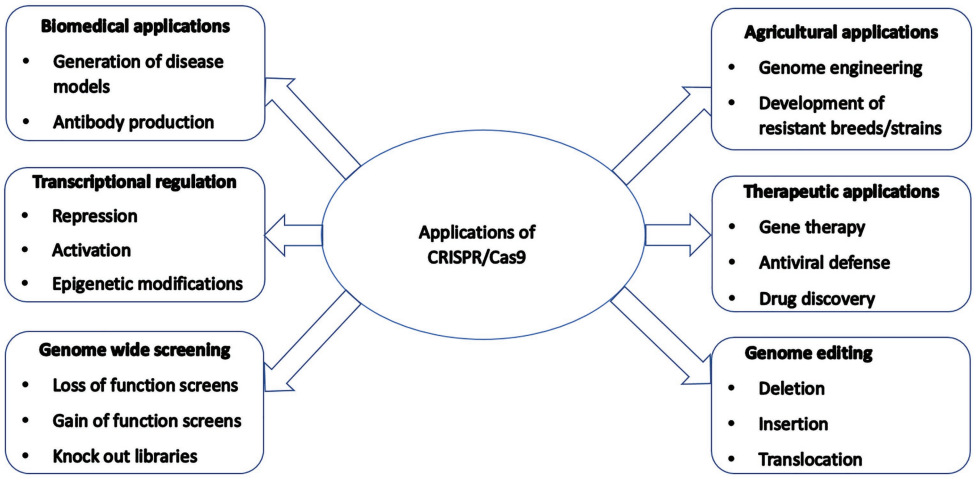

FIGURE 2 | A summary of various applications of the CRISPR/Cas9 system in animals many of which are yet to be tested in poultry species.

in poultry pose a significant risk to the commercial poultry industry causing an increased cost of production for commercial poultry producers. There is a high demand for genetically modified chickens that are highly resistant to a specific disease-causing microorganism, and the available genome editing tools could help in this endeavor (Sid and Schusser, 2018). Avian influenza virus (AIV) is a poultry disease with high hypervirulence that causes sporadic pandemic events that lead to a high mortality rate (Suarez, 2000). Most vaccination strategies to control AIV are ineffective hence the need to breed resistance to AIV (Doran et al., 2017). There have been several recent attempts to suppress the transmission of AIV in genetically modified chickens. Lyall and his group generated transgenic chickens expressing a short-hairpin RNA (shRNA) that targets the viral genome. The shRNA is designed to inhibit and block influenza virus polymerase hence interfering with virus propagation, (Lyall et al., 2011).

Recent findings on the species-specific host co-factor polymerase activity of avian influenza viruses in chickens show that adding approximately 33 amino acid inserts in the chicken acidic nuclear phosphoprotein 32 family member A (chANP32A) protein enhances avian polymerase activity in avian cells. CRISPR/cas9 can also be used to substitute the chANP32A gene with huANP32A that has enhanced avian polymerase activity in avian cells. This could impair the enhanced polymerase activity of the avian influenza virus in chicken cells, thereby providing resistance to poultry species against influenza (Long et al., 2016). More recently, Park et al. (2020) conducted a study targeting chicken ANP32A using CRISPR/Cas9-mediated genome editing to examine the functional roles of ANP32A and other members of the ANP32 family using avian cell lines. The absence of the retinoic acid-induced gene I (RIG-I) in avian species has been shown to increase the susceptibility of chickens against AIV infection as compared to ducks where it is present hence making the ducks more resistant to influenza viruses (Barber et al., 2010). CRISPR/Cas9 can be used to introduce RIG-I-like disease-resistant genes in the genomes of poultry related species then breed these birds having higher resistance to AIV (Smith et al., 2015; Blyth et al., 2016). More recent studies conducted by Byun et al. (2017) have established the possibility to suppress AIV transmission in genetically modified birds that express the 3D8 single chain variable fragment (scFv).

Another poultry disease that causes economic losses in the poultry industry is the avian leukosis virus (ALV). ALV is a retrovirus that causes tumors in avian species by inserting a copy of their genome DNA into the host cell. Kučerová et al. (2013) identified W38 as the critical amino-acid residue in chicken Na^+/H^+ exchange 1 receptor (NHE1), whose deletion might confer the resistance to subgroup J avian leukosis virus. Lee et al. (2017a) were able to induce acquired resistance to ALV-J infection by using the CRISPR/Cas9-mediated homologous recombination in cultured chicken DF-1 cells. Lee et al. (2017c) modified critical residues of chicken NHE1 in cultured cells to induce resistance to viral infection and create mutations of the tryptophan residue at position 38 (Trp38) using single-stranded oligodeoxynucleotide (ssODN) recombination to confer resistance to ALV-J. In another research by Koslová et al. (2018), genetic resistance to ALV was successfully induced using the CRISPR/Cas9-mediated approach. Some frame-shifting mutations were introduced into tva, tvc, and tvj loci encoding receptors for the A, C, and J ALV subgroups, respectively. Therefore both Lee et al. (2017a) and Koslová et al. (2018) successfully produced KO or gene edits of NHE1 in the chicken DF-1 cell line. Lee et al. (2019a) used a CRISPR/Cas9-based disruption strategy of exon 2 within the tumor virus locus A gene (tva) of DF-1 fibroblasts to confer resistance to infection by ALV subgroup A. More recently, Koslová et al. (2020) prepared CRISPR/Cas9-mediated gene-edited chickens and found out that gene editing of the NHE1 gene renders chickens' resistance to the J subgroup of avian leukosis virus. Therefore, Koslová et al. (2020) were able to produce an ALV-J-resistant

chicken line as the first example of true site-specific gene editing. Hellmich et al. (2020) corroborated this strategy in commercial chicken lines by precise deletion of chicken NHE1 W38 using CRISPR/Cas9-system in combination with homology directed repair to induce ALV-J resistance. These examples show that CRISPR/Cas9 genome editing technology can be used widely to modify poultry species to produce a line of birds that exhibit desired resistance characteristics to viral infection. This might be the initial step in developing a virus-resistant line of birds in poultry. The use of such CRISPR-mediated genome edited poultry could substantially reduce a lot of economic losses as well as decreasing the cost of production in the poultry industry.

Increasing the performance of birds by enhancing muscle growth is another important agricultural application of CRISPR/Cas9-mediated gene editing in poultry species. MSTN suppresses skeletal muscle development and growth in animals (McPherron et al., 1997). A mutation in myostatin has resulted in increased muscle mass in mammals and fishes. In poultry, the increasing growth performance of birds can be enhanced by targeting MSTN to suppress its inhibitory effects on muscle growth. For example, a non-frameshift mutation in the MSTN of Japanese quail resulted in a significant increase in body weight and muscle mass (Lee et al., 2020). A disruption or removal of MSTN by genetic mutations using CRISPR/Cas9 inhibits its anti-myogenic function resulting in increased muscle mass in MSTN knockdown chickens (Bhattacharya et al., 2019). This is an important agricultural application in the poultry industry that could enhance bird performance and increase productivity, and help solve food shortage problems.

Applications of CRISPR/Cas9 in Biomedical Research

Genome editing is a major development in biomedical research, with the current trend of innovative approaches providing directions for the treatment of various genetic and non-genetic diseases in the future. The availability of the CRISPR/Cas9-mediated gene and genome editing system has enabled the advent and use of more efficient strategies in gene targeting and the creation of gene edited avian species. This has guided recent and on-going advancements in biomedical research in the animal biotechnology field.

CRISPR/Cas9 technology has ushered in an innovative era in genome editing technology for the manipulation of invaluable avian models such as chickens. By applying CRISPR/Cas9 gene editing technology, researchers will be able to create an efficient bioreactor system for producing valuable proteins in poultry species. In chickens, the bioreactor system will enable efficient production and easy purification of egg white protein in large amounts (Lillico et al., 2005). The development of chickens as bioreactors for the production of target proteins has mostly utilized ovalbumin promoters (Park et al., 2015). The development of transgenic hens for protein production in eggs is highly necessary for the expression of therapeutic proteins which has resulted in significant advances in the generation of transgenic chicken models in this advancing era of genome editing. Oishi and colleagues have shown recently that the human interferon beta (hIFN-β) can be integrated into the chicken ovalbumin locus used in the production of hIFN-β in egg white (Oishi et al., 2018). Oishi et al. (2016) used CRISPR/Cas9 technology to demonstrate that disruptions of ovalbumin and ovomucoid genes had the potential to produce low allergenicity in eggs, which allowed a reduced immune response in egg white sensitive individuals. Therefore CRISPR/Cas9-mediated genome editing is expected to be key in the mitigation of allergic reactions caused by chicken eggs in some individuals by ensuring that chicken meat and eggs are allergen-free. This can be achieved by knocking out allergen-related genes such as ovalbumin and ovomucoid. This type of progress is important in the production of safe food products as well as the production of vaccines in the pharmaceutical industry.

The production of therapeutic antibodies against antigens is now possible through humanized chicken for therapeutic applications. The *loxP* site was inserted into the variable region of the immunoglobulin heavy chain using the CRISPR/Cas9-mediated approach (Dimitrov et al., 2016). Production of these genome-edited chickens will provide numerous opportunities for the discovery of therapeutic antibodies: a game-changer in biomedical research.

LIMITATIONS OF USING CRISPR/Cas9 SYSTEM IN POULTRY PRODUCTION

Despite the many advantages and breakthroughs that CRISPR/Cas9 system offers the poultry industry, several concerns touch on the ethical, legal, and social issues that affect the use of this powerful genome editing tool. One big concern of using the CRISPR/Cas9 technology is that this system generates off-target effects that can be very harmful. Off-target effects could play a critical role in the recognition and destruction of hypervariable viral nucleic acids or the plasmid DNA of beneficial bacteria that can potentially alter the microbiome profiles of a bird. With the newly developed ways of delivering the DNA-editing tool CRISPR-Cas9 into microorganisms, there is a possibility of altering the birds' microbiome composition just like in other organisms (Hamilton et al., 2019; Ramachandran and Bikard, 2019). The cutting frequency determination (CFD) score of up to 0.28 has been found in some cases (Oishi et al., 2016; Koslová et al., 2020). The CFD score range from 0 to 1, with a higher off-target score, has much off-target potential that should be avoided. Off-target effects create unfavorable mutations at random sites that impact the precision of genome modification which raises concerns about safety and efficacy especially when the birds are raised for meat and egg production (Zhang et al., 2015; Chira et al., 2017).

There are high chances of having targeted alleles carrying additional modified and integrated targeted vectors through deletions and duplications because the DNA repair system has a scope that cannot integrate DNA fragments in the genetic makeup of an organism. This is based on the fact that the molecular mechanism that is used in the insertion of DNA fragments is highly mediated

by the DNA repair mechanism that is turned on by the DSB created by the Cas9 enzyme (Li et al., 2015).

Decreasing the off-target effects may cause an upward trend in future applications of CRISPR/Cas9 gene-editing technology, especially in the generation of food animals such as poultry (Kleinstiver et al., 2016; Lee et al., 2017b). This goal could be achieved through studies that develop understanding of off-target mechanisms. The advent of transcriptome sequencing technology and the availability of high-throughput sequencing technology screening of gene edited animals can be enhanced to provide critical information about the potential off-targets associated with the use of CRISPR/Cas9 system in food animals (Roy et al., 2018).

Another major disadvantage of using the CRISPR/Cas9 system in poultry production is the low transfection efficiency (<2%) of avian cells in genome editing (Tyack et al., 2013; Lambeth et al., 2016) and the low germ-line transmission efficiency of less than 10% (Cooper et al., 2017; Hwang and Han, 2018). Just like other genome editing tools (TALENs and ZFNs), CRISPR/Cas9 system needs much more improvement to increase transfection efficiency and germ-line transmission. In the years before the advent of CRISPR technology, there were attempts to generate transgenic chickens but the germ-line transmission rate from one generation to another was very low. In Mozdziak et al. (2003) research group reported the first credible study of a genetically modified line of chickens that express a protein ubiquitously (Mozdziak et al., 2003). In Mozdziak et al. (2006) and his colleagues evaluated germline transmission rates of PGCs using fluorescence-activated cell sorting (Mozdziak et al., 2006). Many studies discussed earlier involving in ovo electroporation of chicken embryo proved to be very inefficient for germline transmission. There is a high possibility that the issue of low germline transmission efficiency in the production of genetically modified birds can be improved through PGC-mediated transgenesis and genome editing. First, PGCs are transfected then followed by subsequent injection into a host animal. The germline transmission rates obtained here are quite acceptable though they are variable from 0–90%. This could be an alternative strategy for improving germline transmission efficiency (Dimitrov et al., 2016).

Trends in the current meat market show that there are difficulties in the commercialization of transgenic poultry products generated by CRISPR/Cas9 technology in various countries around the world. This is mainly because of the high cost of developing this system and the major constraints of regulatory agents on genetically modified organisms (Manghwar et al., 2019).

CURRENT STRATEGIES FOR MINIMIZING OFF-TARGET EFFECTS IN CRISPR/Cas9-MEDIATED GENOME EDITING

Improved Cas9 Variants

The most broadly utilized Cas9 is the *Streptococcus pyogenes* Cas9 (SpCas9), but it has been found to generate genome-wide off-target mutations. In the last 5 years, scientists have been working to develop Cas9 variants and other Cas9 orthologous that show minimized off-target effects and increased specificity to solve this issue. Among these, the available Cas9 variants include SaCas9, SpCas9-Nickase, dCas9, dCas9-FokI, xCas9, Cas9-NG, evoCas9, SpCas9-HFI, eSpCas9, Hypa-Cas9, Sniper-Cas9, HiFi Cas9, SpG, and PAM-less SpRY.

SaCas9 is a nuclease derived from *Streptococcus aureus*. It is widely used for ex vivo or *in vivo* gene therapy instead of SpCas9 due to its small size, which allows packaging in adeno-associated-virus (AAV) vectors. The saCas9 also recognizes a longer PAM sequence (5'-NNGRRT-3') as opposed to the shorter 5'-NGG-3' sequence recognized by SpCas9. Using SaCas9 for genome editing may therefore have very minimal off-target mutations (Kumar et al., 2018). Genome-wide unbiased identification of DSBs enabled by sequencing (GUIDE-seq) performed to detect off-targets show that the on-target activity was higher in the saCas9 than the wild type SpCas9 (Ono et al., 2019). SpCas9 nickase which is engineered through deactivation of the RuvC domain of SpCas9 through mutation has shown to have reduced off-target effects by more than 1,500 folds when compared with the wild type SpCas9 (Frock et al., 2015). dCas9-FokI which is deactivated or simply dead SpCas9 fused with the catalytic domain of FokI has shown decreased off-target sites and increased on-target activity by 140-fold when compared with the wild type SpCas9 (Wyvekens et al., 2015). XCas9, Cas9-NG, and evoCas9 is another set of engineered variants of spCas9 that have shown minimized off-target effects minimized and increased specificity in both animals and plants. The variant xCas9 recognizes a broad range of PAMs including GAT, GAA, and NG. Therefore, compared to SpCas9, xCas9 has a higher specificity and low off-target effects in animal cells (Liang et al., 2015; Hu et al., 2018). The GUIDE-seq has been used to assess the efficiency of Cas9-NG and evoCas9 at different loci. The on-target activity was significantly higher than off-target activity in both Cas9-NG (Nishimasu et al., 2018) and evoCas9 (Kleinstiver et al., 2015) than the wild type SpCas9. Other SpCas9 variants such as SpCas9-HFI (Kleinstiver et al., 2016), eSpCas9 (Slaymaker et al., 2016), Hypa-Cas9 (Chen et al., 2017), Sniper-Cas9 (Lee et al., 2018), HiFi Cas9 (Vakulskas et al., 2018), SpG and PAM-less SpRY (Walton et al., 2020) have been used more recently to minimize genome-wide off-target effects with exceptional accuracy.

Improved Viral and Non-Viral CRISPR Delivery Methods

Viral vector delivery systems have been extensively used to deliver the components of gene-editing in gene therapy. In the CRISPR/Cas9 gene-editing system that uses viral based delivery methods, the Cas9 and gRNA are packaged into plasmid DNA, which is delivered *via* the viral vector to the target cell. This delivery increases the chances of off-target effects since the CRISPR/Cas9 components exist persistently in the target cell resulting in elevated Cas9 levels. Adeno viruses (AdV) have been used in viral vector delivery systems to minimize off-target

effects since AdV show very minimal potential to integrate into the target cell genome (Gaj et al., 2017; Lino et al., 2018).

The non-viral delivery system involves directly delivering a ribonucleoprotein (RNP), which consists of the Cas9 protein in complex with a targeting gRNA to the target cells. The main advantage of this method is that RNPs may limit the potential for off-target effects since the Cas9-gRNA RNP is degraded over time (Vakulskas and Behlke, 2019). Minimized off-target mutations are possible when RNP complexes are delivered by liposome-mediated transfection as opposed to plasmid DNA transfection (Liang et al., 2015).

Base Editing

NHEJ can introduce DSBs at unintended positions to the target gene hence generating insertions and deletions that are off targets. This causes off-target effects. Recently, a new genome-editing technique has been developed for base editing. This technique can change specific nucleotides in the genome without the introduction of double-stranded (ds) DNA breaks (Komor et al., 2016, 2018; Naeem et al., 2020). Base editing technique comprises of dCas9, catalytic base modification enzyme (deaminase), and sgRNA. The two categories of base editors developed recently are Cytosine base editors (CBE) and Thymine base editors (TBE) which can change C/G to T/A and A/T to G/C, analogously. The use of base editing has enabled new capabilities and applications in the genome editing world despite its recent introduction because it shows significant gene editing efficiency (Rees and Liu, 2018). An efficient base editing delivery system enhances the reduction of off-target mutations (Zhou et al., 2019).

Prime Editing

Recently, Anzalone et al. (2019) reported that the development of a novel genome editing experimental approach that mediates all possible base-to-base conversions, "indels," and combinations in mammalian cells without the need of a double-strand break or donor DNA (dDNA) templates. This new gene-editing method is called prime editing. Transition mutations by base editing are limited to installing four transition mutations efficiently, that is, C to T or G to A, A to G, and T to C. This strategy can therefore only make four of the 12 possible base pair changes. However, Prime editing can install all 12 possible transition changes (C/A, C/G, G/C, G/T, A/C, A/T, T/A, and T/G) in the genome. The prime editing system offers a new approach to minimizing off-target effects and increasing target specificity in genomes but requires more research on animal models to move it into therapeutic gene editing or for human consumption (Anzalone et al., 2019).

Anti-CRISPR Proteins

The recent discovery of the protein inhibitors of CRISPR/Cas systems, called anti-CRISPR (Acr) proteins, has enabled the development of more efficient, controllable, and precise CRISPR/Cas tools in animal cells (Marino et al., 2020). More than 50 anti-CRISPR proteins have now been characterized up to date, each with its own means of blocking the cut-and-paste action of CRISPR systems (Dolgin, 2020). AcrIIA2 and AcrIIA4 proteins have been found to inhibit the CRISPR/Cas system and are hence desired to decrease off-target modifications without decreasing on-target activities in cells (Shin et al., 2017; Basgall et al., 2018).

FUTURE PERSPECTIVES

CRISPR/Cas9 technology has increased significantly the efficiency of the gene editing process when compared to the other modern existing processes of homolog recombination. CRISPR/Cas9-mediated gene editing is more advanced in small mammals such as mice and big mammals such as pigs than in avian species such as chickens, but very soon gene editing in poultry will enter into a highly competitive era of genome editing. In the future, the generation of poultry species expressing Cas9 will be beneficial to the study of biological processes. Studies of biological processes that enable us to understand the functions of the genes that may be involved in growth will be faster and easier in the future. This is already being done in pigs (Wang et al., 2017a) and can be utilized in poultry. In addition, the use of CRISPR/Cas9 to target PGCs offers a promising method of generating genetically engineered avian species with any desired gene characteristics (Abu-Bonsrah et al., 2016).

We predict that the future of the poultry meat industry will involve the production of birds that are highly efficient in feed utilization and lean meat which make them even more attractive for human consumption. Although the possibility of decreasing feed to gain ratio in poultry may be very minimal, this could change with the production of CRISPR-mediated transgenic chickens. There has been tremendous progress in the production of other meat animals such as pigs, with decreased fat deposition using the CRISPR/Cas9 system. For example, Zheng and his research group in China reconstructed the uncoupling protein 1 (UCP1) gene using CRISPR/Cas9 technology in the white adipose tissue of swine species, hence decreasing the accretion of fat (Zheng et al., 2017). In their study, Zheng and colleagues efficiently inserted a mouse adiponectin-UCP1 into the porcine endogenous UCP1 locus. The UCP1 knock-in pigs that were generated showed a decreased deposition of fat and increased carcass lean percentage. In poultry, the use of the CRISPR/Cas9 system has only recently taken off and is currently being used in targeting candidate avian genes in poultry species to produce birds that have higher lean meat and less fat which may lead to increased consumption by consumers (Park et al., 2019).

The production of foreign proteins in eggs can be utilized for industrial and therapeutic applications. Novel methods such as site-directed integration have been used by biotechnology companies such as AviGenics Incorporated (Athens, Georgia) and Crystal Bioscience Incorporated (Emeryville, California) to successfully create transgenic poultry for use in the production of biopharmaceutical proteins. Newer and innovative technologies such as CRISPR/Cas9 can further improve the efficiency of the production of these proteins. With the availability of CRISPR/Cas9 technology, cell and

animal transgenesis providing a more efficient strategy through gene targeting and the creation of transgenic birds that will lead to advancements in biomedical research applications. Antibody-producing companies can purify overexpressed human antibodies from the eggs of poultry species such as chicken and quail to produce recombinant proteins and vaccines using CRISPR/Cas9-mediated approaches (Farzaneh et al., 2017). Furthermore, the production of antibodies using poultry eggs by utilizing the CRISPR/Cas9 system represents an economical and stress-free method of producing specific antibodies for therapeutic applications (Amro et al., 2018).

A great deal of time and resources are required before the CRISPR-Cas9 system becomes 100% safe and effective in the generation of food animals. If the remaining safety and efficiency concerns are fully addressed, then the CRISPR/Cas9 system could be effectively used to improve food quality and production. Diversity among the poultry species should be strongly encouraged and pursued using gene editing technologies. However, because the resulting birds will be genetically engineered and modified, the Food and Drug Administration (FDA) will have to review and approve the use of such poultry birds after guaranteeing that the meat and eggs produced are safe for human consumption. It is expected that in the near future, CRISPR/Cas9-mediated genome editing research will extend to other categories of poultry species such as turkeys, geese, ducks, and guinea fowl across the world since major progress has been made in chicken and quail.

Several recent trends might fast-track the generation of transgenic birds in the near future. First, *in vitro* genetically manipulated PGCs could be re-introduced not only into the embryonic blood but also into the testes of sterilized adult recipients. After such transplantation, donor PGCs colonize the spermatogenic epithelium and mature into fertile sperm. This method was recently described by Trefil et al. (2017). Compared with existing approaches, this procedure will become the method of choice in the future because it is more efficient, faster, requires fewer animals, and could broaden PGC technology in other poultry species. Secondly, genetic sterility might be a very useful tool for CRISPR/Cas9-assisted gene editing. Genetically sterile chickens can be used as surrogate hosts for germ line transfer (Woodcock et al., 2019) or, in the future, for efficient transgenesis. Finally, the use of adenoviral vectors for CRISPR/Cas9 delivery could bring the technique of virus subgerminal injection back into routine use (Lee et al., 2019c). The implementation of this method could accelerate avian knockout studies and lead to the advancement of future agricultural applications.

CONCLUDING REMARKS

The development and improvement of CRISPR technology over the years has enabled access to generate transgenic lines of birds for meat or egg production, mainly for food. The impact of CRISPR technology could potentially lead to the efficient improvement and sustainability of poultry products, which will help address challenges associated with universal food security. Birds raised for meat and egg production using the CRISPR technology could have an immense impact on the advancement of poultry related traits such as feed conversion, digestibility, increased egg production, growth, and overall improved performance of birds. Innovations resulting from CRISPR technology could also lead to developments in fields such as disease resistance, immune function, and vaccine delivery. This will in turn enhance poultry health, increase the safety of vaccines produced using chicken eggs, and increase food safety and production.

The future applications of CRISPR technology in poultry have promising and tremendous potentials in biomedical research that could benefit humankind due to vast opportunities for disease treatment and prevention. Most of these applications have been focused on chickens that show great potential for biomedical research. Finally, yet importantly, the latest progressions in CRISPR/Cas9 gene editing technologies might assist in scaling down or abolishing barriers such as the difficulties of gaining regulatory approval and the public perception and acceptability of CRISPR technology in the production of food animals.

AUTHOR CONTRIBUTIONS

CK wrote the first draft of the manuscript. CK and SN revised and approved the manuscript. All authors contributed to the article and approved the submitted version.

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Adaptation of Gut Microbiome to Transgenic Pigs Secreting β-Glucanase, Xylanase, and Phytase

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We previously generated transgenic pigs with enhanced growth rate and reduced nutrient loss. However, the composition of their gut microbiome is unknown. In this study, we successfully generated *EGFP* marker-free transgenic (MF-TG) pigs with high expression levels of microbial β-glucanase, xylanase, and phytase in the parotid gland. We collected intestinal contents from the ileum, cecum and colon of five MF-TG and five wild-type (WT) sows and investigated the gut microbiome of the transgenic pigs via metagenomic analysis. Results showed that the levels of probiotics, such as *Lactobacillus reuteri* and *Streptococcus*, were more abundant in the cecum of the MF-TG pigs and higher than those of WT pigs. By contrast, the levels of harmful microorganisms, such as *Campylobacter*, *Chlamydia trachomatis*, and *Campylobacter fetus*, and various unidentified viruses, were higher in the cecum of the WT pigs than those of the MF-TG pigs. By comparing unigenes and the eggNOG database, we found that the microorganisms in the colon of the MF-TG pigs had high fractional abundance in DNA (cytosine-5)-methyltransferase 1 and serine-type D-Ala-D-Ala carboxypeptidase, whereas the aspartate carbamoyltransferase regulatory subunit and outer membrane protein pathways were enriched in the WT pigs. Moreover, the microorganisms in the cecum of the MF-TG pigs were active in GlycosylTransferase Family 8 (GT8), Glycoside Hydrolase Family 13 (GH13), and Glycoside Hydrolase Family 32 (GH32). Furthermore, the levels of numerous carbohydrases, such as glucan 1,3-beta-glucosidase, xylan 1,4-beta-xylosidase and exo-1,3-1,4-glucanase, were higher in the cecum of the MF-TG pigs than those of the WT pigs. The results indicated that intestinal microbes can change adaptively to the secretion of transgenic enzymes, thereby forming a benign cooperation with their host. This cooperation could be beneficial for improving feed efficiency.

Keywords: transgenic pigs, microbial enzymes, gut microbiome, metagenomics, feed efficiency

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INTRODUCTION

Livestock industries pursue maximum animal growth rate to utilize the full genetic potential of animals. However, anti-nutrient factors, such as non-starch polysaccharides (NSPs) and phytates, adversely affect feed efficiency, resulting in inefficient feed digestion and substantial rates of nutrient leaching into the environment (Xianwei et al., 2018). Pigs produce considerable amounts

of nitrogen (N) and phosphorus (P) because they are inherently incapable of digesting NSPs and phytates, which are present in feed grain (Golovan et al., 2001). Various methods have been developed to improve pig nutrient utilization (Swiatkiewicz et al., 2016). Dietary supplements with phytate- or NSP-degrading enzymes can effectively reduce P and N emissions and improve feed utilization. However, the effects of supplements are limited by feed production processes, enzyme activity stability and cost. Genetically engineered pigs expressing phytate- or NSP-degrading enzymes in their salivary glands offer an alternative and useful strategy for dietary supplementation. Golovan et al. (2001) reported that transgenic (TG) pigs that produce salivary phytase can remarkably improve P digestion from soybean meals. Several other researchers also successfully generated these types of TG pigs (Lin et al., 2015). In our previous studies, we successfully generated TG pigs expressing three microbial enzymes, namely, β-glucanase, xylanase, and phytase, in their salivary glands. These enzymes considerably enhanced the digestion of NSPs and phytates in feedstuff (Xianwei et al., 2018). However, these TG pigs also systemically express the enhanced green fluorescent protein (EGFP) maker, which may have negative effects on pig health and food safety (Bi et al., 2016).

The mammalian gut microbiome plays critical roles in normal digestive functions, nutrient utilization, antibiotic resistance, and defense against pathogens (Sommer and Bäckhed, 2013). Dyspeptic gut microbiota are associated with several intestinal and extraintestinal diseases and poor animal growth performance. These conditions increase the risk of food safety and public health hazards and result in low profitability of animal production (Wang et al., 2019). Therefore, intestinal microbiome and their interactions with animal hosts have long been a notable research interest. Previous studies adopted various animal models, including TG and knock out (KO) animals, to explore the relationship of host genes to the functions of the gut microbiome, but none of them directly focused on feed efficiency (Qingqing et al., 2016). Thus, whether or not the endogenous phytate- or NSP-degrading enzymes (phytase, β-glucanase, and xylanase) of TG pigs would alter the composition and activity of intestinal microbiome remains unanswered. In the present study, we successfully produced *EGFP* marker-free transgenic (MF-TG) pigs by deleting the *EGFP*-coding gene in our previously generated TG pigs via the cyclization recombination enzyme (Cre)–LoxP recombination system. The transgenes were expressed efficiently in the salivary gland and not expressed in other tissues of the MF-TG pigs. We further tested whether or not the MF-TG pigs would affect the functional contributions and biological roles of intestinal microbes via metagenomic analysis.

MATERIALS AND METHODS

Ethics Statement

The protocol implemented in this study was in accordance with the Instructive Notions with Respect to Caring for Laboratory Animals issued by the Ministry of Science and Technology of China. This study was approved by the Animal Care and Use Committee of the South China Agricultural University.

Deletion of the *EGFP* Marker by Cyclization Recombination Enzyme

The TG pigs we raised in a previous study (Xianwei et al., 2018) were mated with wild-type (WT) sows and then slaughtered on day 30. The fetuses in the womb were removed, and primary pig fetal fibroblasts (PFFs) were isolated by adherence. The *EGFP*-tag was deleted by cyclization recombination enzyme (Excellgen, United States). The PFFs were cultured for another 24 h. Half of the cells were placed into a new 24-well plate. The steps above were repeated twice until the cells grew into a monoclone.

Generation of Cloned Pigs by Somatic Cell Nuclear Transfer

The marker-free PFFs derived from a single colony were used as nuclear donors for somatic cell nuclear transfer, and the embryos were cultured *in vitro* overnight. Afterward, the embryos were transferred to the oviducts of recipient sows. Antibiotics were injected for four consecutive days to reduce inflammation, and the physiological conditions of the recipient sows were recorded daily. In addition, 1000 IU of pregnant mare serum gonadotropin (PMSG) was injected into the recipient sows on the 10th day after embryo transfer, and 800 IU of human chorionic gonadotrophin (hCG) was injected on the 13th day to maintain pregnancy.

PCR and Southern Blot Analyses of Founder Pigs

Genomic DNA of founder (F0) cloned pigs were isolated using a DNA tissue kit (OMEGA, United States) in accordance with the manufacturer's protocol. Primer pairs of P1-F/R, P2-F/R, and P3-F/R were designed to amply the mPSP promoter, bg17-eg1314 dual tansgenes, and marker-free region, respectively. TG and KO genes were amplified via PCR. The PCR condition and procedure were set in accordance with the manufacture's protocol of PrimeSTAR Max DNA Polymarase (TaKaRa, Japan). The PCR products were analyzed by 1.5% agarose gel. For Southern blot, probe 1 and probe 2 were designed to target bg17-eg1314 dual transgenes and marker-free region, respectively. A total of 20 μg of genomic DNA was digested and then analyzed by 0.8% agarose gel at 30 V for 16 h. Subsequently, the gel was washed with alkaline solution, neutralization solution and 20 × SSC solution and then transferred onto nylon membranes. Genomic DNA was hybridized with digoxigenin-labeled DNA probe. After hybridization, the nylon membranes were washed and detected with a buffer by using a DIG-high prime DNA labeling and detection starter kit II (Roche, Germany) following the manufacturer's instructions. Finally, the membranes were imaged using UVP software. The sequences of the primers and probes are shown in **Supplementary Table 1**.

Enzymatic Activity Assay and Western Blot Analysis

Saliva samples of adult MF-TG pigs were collected using non-fat cotton balls. A portion of the samples was tested for enzymatic activity assay at the optimal pH. Enzymatic activity assays were

performed as described in our previous work (Xianwei et al., 2018). In detail, β-glucanase and xylanase activity assays were based on estimating the amount of reducing sugars released from the relevant substrates in the reactions using 3,5-dinitrosalicylic acid (DNS) reagent. One unit of activity was defined as the quantity of enzyme that releases reducing sugar at the rate of 1 mmol/min. Phytase activity was determined by means of vanadium molybdenum yellow spectrophotometry. The reaction was performed in a final volume of 600 mL solution containing 0.25 M acetate buffer (pH 5.5), 5 mM sodium phytate, and 50 mL enzyme preparation at 39°C for 30 min, followed by termination of the reaction by adding 400 mL of an ammonium molybdate-ammonium vanadate-nitric acid mixture. After mixing and centrifugation, the absorbance was measured at a wavelength of 415 nm. One unit of phytase activity was defined as the amount of activity that liberates one micromole of phosphate per minute at 39°C.

The other portion of the saliva samples was centrifuged using Amicon Ultra-15 (Millipore, United States) for Western blot detection. In brief, a total of 20 μg of protein was subjected to sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and then transferred onto PVDF membranes (Millipore, United States). The membranes were blocked with 5% non-fat dry milk for 2 h and then incubated overnight at 4°C with the primary antibodies of HA (Hemagglutinin) protein tag (ab137838, Abcam) or GAPDH (ab8245, Abcam). The membranes were thoroughly washed and then further incubated with a secondary antibody for 2 h at room temperature. Finally, the membranes were imaged using UVP software.

Recording of Production Performance

The MF-TG pigs (F0) were crossed with WT Duroc sows, and F1 generation MF-TG pigs were propagated and grown until they reached 35 kg in weight (about 130 days of age). A total of 14 MF-TG boars (36.93 ± 4.77 kg) and 11 WT littermate boars (39.6 ± 9.57 kg) were divided into two measuring stations. Ten MF-TG gilts (34.71 ± 6.06 kg) and 10 WT littermate gilts (37.88 ± 5.97 kg) were allocated to three pens according to their initial weight; among them, six MF-TG gilts and eight WT littermate gilts were raised in individual pens. All pigs were allowed free access to water and fed the same experimental diet formulations made in accordance with the Nutrient Requirements of Swine (NRC, 2012) (**Supplementary Table 4**). The experimental formula was designed with 2% protein reduction, no mineral P additives, and low energy level to evaluate the functional efficiency of the MF-TG pigs. During the experiment, production performance was recorded using MK3 Fire feeders (Fire, United States). In addition, a total of 11 WT boars and 11 gilts were separately tested and fed complete formula feed as the WT2 (**Supplementary Table 11**). Fresh dung samples of the pigs were randomly collected every morning toward the end of the last 5 days of the trial. The dung samples were stored in a refrigerator at 4°C. Finally, the samples were mixed well and dried at 80°C. Measurement of growth performance was ended when the body weight of the pigs reached 115 kg (about 220 days of age).

Dietary Treatment Experiments

Fresh pig manure was collected at the time of flushing the hog house (08:00 and 17:00) for five consecutive days. Multipoint collection was adopted with consistent sampling quantity of each point. Manure samples were frozen immediately at −20°C. Afterward, the manure of each group was uniformly and thoroughly mixed. Sampling was performed through the quartile method. Total nitrogen was measured using fresh dung samples. The remaining samples were dried at 80°C. The samples were air-dried and then crushed to particles <0.425 mm in size for Ca and P content measurements.

Metagenomics Sequencing and Statistical Analyses

Five MF-TG sows (115.76 ± 1.57 kg) and five WT sows (116.04 ± 0.55 kg) were selected for metagenomic analysis. During the fattening stage, the pigs were raised with the same experimental diet under human-controlled farm conditions and similar management schemes. The intestinal contents were collected from the ileum, cecum and colon and then immediately transferred to liquid nitrogen for temporary storage. The genomic DNA of the samples was extracted using Magnetic Soil and Stool DNA Kit (TIANGEN®, China) in accordance with the manufacturer's protocol, and used to construct the sequencing library. The NEBNex^{R} UtraTM DNA Library Prep Kit for Illumina (NEB, United States) was used to prepare the DNA library. The Raw data was obtained from the Illumina HiSeq sequencing platform. Clean data was generated via removal of low-quality reads in raw data. The specific processing steps are as follows: (a) remove the reads which contain low quality bases (default quality threshold value ≤38) above a certain portion (default length of 40 bp); (b) remove the reads in which the N base has reached a certain percentage (default length of 10 bp); and (c) remove reads which shared the overlap above a certain portion with Adapter (default length of 15 bp).

Clean data was blast to the pig genome database as default using Bowtie2.2.4 software to filter the reads that are of host origin. The parameters are as follows: -end-to-end, -sensitive, -I 200, -X 400. The sequences of the transgenes were also removed from the sequencing data.

The sequencing data was assembled by single sample assembly and mixed assembly. For the single-sample assembly, the Clean Data was assembled and analyzed by using SOAPdenovo (V2.04) software. The parameters are as follows: -d 1, -M 3, -R, -u, -F, -K 55. Then, the assembled Scaftigs were interrupted from N connection and left the Scaftigs without N. All Clean Data of the samples were compared to each Scaffolds by using Bowtie2.2.4 software to acquire the PE reads not used. The parameters are as follows: -end-to-end, -sensitive, -I 200, -X 400. All reads not used in the single assembly of all samples were combined. SOAPdenovo (V2.04) software was used to conduct the mixed assembly under the same parameters used in the single assembly. Then, the fragments shorter than 500 bp were filtered in all of Scaftigs for statistical analysis of data generated from the single or mixed assembly.

Scaftigs (≥500 bp) assembled from single and mixed samples were used to predict the open reading frames (ORFs) by using the MetaGeneMark software. ORFs with length shorter than 100 nt were filtered from the predicted results with default parameters. For ORF prediction, CD-HIT software was used to remove redundancies and obtain unique initial gene catalogs. Clean data of each sample were mapped onto an initial gene catalogs by using Bowtie2.2.4. The number of reads to which genes mapped in each sample was obtained under the following parameters: end-to-end, sensitive, I 200 and X 400. In each sample, the gene with ≤2 reads was filtered to obtain the gene catalog (unigenes) for subsequent analysis. On the basis of the number of mapped reads and gene lengths, the abundance information of each gene in each sample was analyzed.

DIAMOND software was used to BLAST the unigenes to the sequences of bacteria, fungi, archaea, and viruses, which were extracted from the NR database of NCBI with the parameter settings blastp-e 1e-5. For the aligned results of each sequence, the result with the e value ≤ the smallest e value × 10 was selected, considering that each sequence may have multiple aligned results. The LCA algorithm was applied to the system classification of MEGAN software to ensure the species annotation information of the sequences. A table containing the number of genes and the abundance information of each sample in each taxonomic hierarchy was constructed on the basis of LCA annotation results and the gene abundance table. LEfSe analysis was performed to determine differences in species composition among groups. Permutation test between groups was used for the Metastats analysis of each taxonomic group and to obtain the *P* value. The Benjamini and Hochberg False Discovery Rate were utilized to correct the *P* value and acquire the *q* value. LEfSe analysis was conducted by using LEfSe software. Finally, random forest was implemented to construct a random forest model. Important species were screened by Mean Decrease Accuracy and Mean Decrease Gin.

DIAMOND software was used to BLAST the unigenes to the functional database with the parameter settings blastp, -e 1e-5. The functional databases included the KEGG, eggNOG, and carbohydrate enzyme (CAZy). For each sequence's BLAST result, the best BLAST Hit was utilized for subsequent analysis. The relative abundance of different functional hierarchies was analyzed. In this study, the relative abundance of each functional hierarchy was equal to the sum of relative abundance annotated to that functional level. On the basis of functional annotation results and the gene abundance table, the gene number table of each sample in each taxonomic hierarchy was obtained. The gene number of a function in a sample was equal to the gene number annotated to this function and the abundance was non-zero.

For resistance gene annotation, resistance gene identifier (RGI) software was employed to align the unigenes to the CARD database (version 2.0.1) under the parameter settings blastp, e value ≤ 1e-30. The relative abundance of ARO was counted from the aligned results. On the basis of the abundance of ARO, abundance bar charts and abundance cluster heat maps were created, and differences in the number of resistance genes between groups were determined. Furthermore, analyses of the abundance distribution of the resistance genes in each sample, species attribution of resistance genes and resistance mechanism of resistance genes were conducted via comparing the gene catalog and CARD database.

Statistical Analyses

Data was analyzed by using the GLM procedure (SAS, United States). For growth performance, covariance analysis was performed, and initial body weight and experimental period were used as the covariates. For apparent fecal nutrient emission, one-way ANOVA followed by Duncan's multiple comparison were conducted. Unpaired two-sample *t*-test (two-tailed test) was used for enzymatic activity analyses. Statistical significance was set to $P < 0.05$.

RESULTS

Generation of MF-TG Pigs

The *EGFP*-tag was deleted by the Cre-Loxp recombination system in the porcine fetal fibroblasts derived from a single colony, and the MF-TG pigs were generated by somatic cell nuclear transfer technique (SCNT) to remove the potential effect of EGFP protein on the gut microbiome. A total of 2001 reconstructed embryos were transferred to the oviduct of eight recipient sows and four sows became pregnant successfully. Seventeen alive piglets and two stillborn piglets were born (**Supplementary Table 13**). Eight piglets grown healthily and were selected for subsequent experiments. The primers of PCR and Southern blot were used to identify TG fragments (**Figure 1A**). Results of PCR and Southern blot showed that all of the eight pigs were MF-TG pigs (**Figures 1B,C**). Different tissues and organs of the MF-TG pigs were collected. Western blot revealed that the MF-TG pigs efficiently expressed β-glucanase, xylanase, and phytase only in the parotid gland and not in other tissues and WT pigs (**Figures 1D,E**). During the feeding period, we collected saliva samples from 6-month-old MF-TG and WT pigs for enzymatic activity assays. The assays detected enzymatic activity in all MF-TG pigs, among which 903 had the highest enzyme activity; 2.5, 0.98, and 2.07 U/mL of β-glucanase, xylanase, and phytase were detected, respectively (**Figure 1F**).

MF-TG Pigs Had Improved Feed Utilization and Reduced Nutrient Emission

MF-TG boars (803, 903, and 907) were crossed with WT Duroc sows. A total of 48 offspring were born, of which 24 were MF-TG pigs and 24 were WT littermate pigs. A total of 24 MF-TG pigs (14 boars and 10 gilts) and 21 WT littermates (11 boars and 10 gilts) were raised together and fed nutrition-deficient experimental diets to measure the growth performance of the MF-TG pigs (**Supplementary Table 4**). Although the difference in average daily feed intake (ADFI) between the MF-TG and WT pigs was not significant ($P = 0.95$ and 0.05 for male and female, respectively), the MF-TG pigs had better feed consumption, higher average daily gain (ADG), better feed conversion rate (FCR) and shorter day to market those of

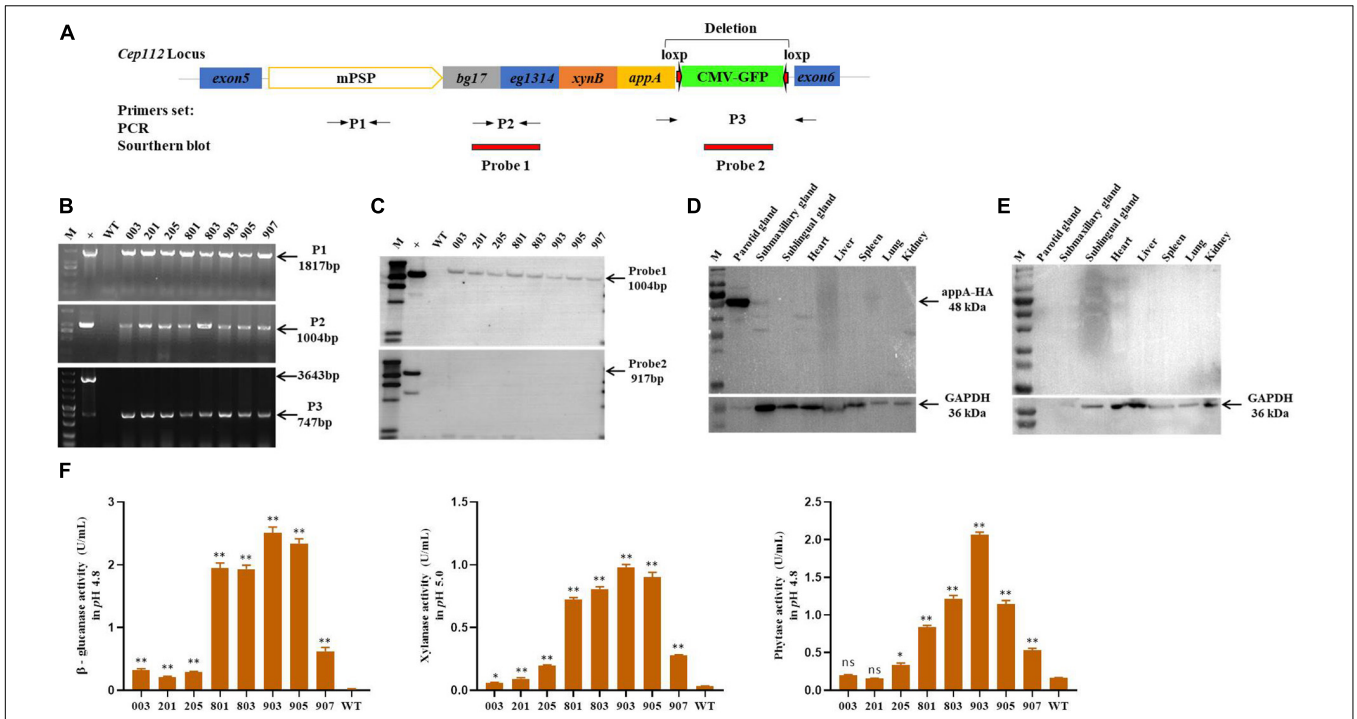

FIGURE 1 | Characterization of transgene and expression in founder MF-TG pigs. **(A)** Four microbial enzymes were integrated into porcine *CEP112* intron 5 after deleting the *EGFP*-tag. Different primers (P1, P2, and P3) were used to confirm the occurrence of transgene, Probe 1 and probe 2 were designed for Southern blot analysis. **(B)** Genomic DNA of the cloned pigs was amplified by PCR and analyzed by gel electrophoresis. **(C)** Southern blot analysis revealed that multiple enzyme transgenes were integrated into porcine *CEP112* intron 5 without the *EGFP* maker. **(D)** Western blot analysis demonstrated that the microbial enzymes were specifically expressed in the parotid gland of the MF-TG pigs. **(E)** Western blot analysis demonstrated that microbial enzymes were not detected in the WT pigs. **(F)** MF-TG pigs could efficiently express β-glucanase, xylanase, and phytase in their salivary gland. Values are shown as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control.

the WT pigs. Feed consumption decreased by 11.85–21.57 kg, ADG increased by 131.34–162.88 g, FCR declined by 0.18–0.42, and day to market shortened by 9.42–15.07 days, respectively (**Table 1**). We further investigated the effects of the microbial enzymes secreted by MF-TG pigs on nutrient emission. Results showed that the P emission of the MF-TG pigs significantly decreased by approximately 26.45–26.52%, but their N and Ca excretion did not significantly change compared with that of the WT1 group. Compared with WT2 boars fed commercial diets for breeding pigs (**Supplementary Table 11**), the fecal N, P and Ca contents of the MF-TG boars decreased by 17.10, 51.95, and 72.65%, respectively (**Figure 2A**), and those of the MF-TG gilts declined by 15.10, 52.42, and 67.15%, respectively (**Figure 2B**). The MF-TG pigs were as good as or even better than the WT2 control group in terms of growth performance (**Supplementary Table 12**).

Comparison of the Gut Microbial Communities of MF-TG and WT Sows

We collected intestinal contents from the ileum, cecum, and colon of five MF-TG sows and five WT sows and investigated their gut microbiome via metagenomic analyses. A total of 1,832,628 predicted genes (71.26%) were annotated into the NR database, and their proportions at the kingdom, phylum, class, order, family, genus, and species levels were 82.85, 79.04, 72.80, 72.25, 60.07, 55.22, and 42.53%, respectively (**Supplementary Table 5**). We selected the top 10 microorganisms with the largest relative abundance in each sample and integrated the clustering results with the relative abundance at the phylum level by using the Bray–Curtis distance for cluster analysis. At the phylum level, the abundance of ileal (IL) microorganisms was substantially lower than those of cecal (Ce) and colonic (Co) microorganisms. Nevertheless, individual samples were highly variable. The main Ce and Co microorganisms were *Firmicutes*, *Bacteroidetes*, and *Euryarchaeota*, whereas the primary IL microorganisms were *Proteobacteria* and *Firmicutes*. Furthermore, the difference in the microbiome of the same intestinal parts of MF-TG pigs and WT pigs was not significant at the phylum level (**Figure 3A** and **Supplementary Table 6**). We also detected no difference in relative abundance at the class, order, family, genus, and species levels of the top10 microorganisms in the same intestinal parts between the MF-TG pigs and WT pigs (data not shown). Principal component analysis (PCA) revealed that the distance between IL and Co/Ce was far and clustered into a different category, but Co and Ce could not be separated (**Figure 3B** and **Supplementary Table 7**). We analyzed the differences in species composition among different groups via LEfSe. We evaluated the abundance of different species by using LDA scores. Results showed that the WT pigs had significantly

TABLE 1 | Comparison of growth performance between F1 MF-TG and their WT littermates fed experimental diets during the growing period from 30 to 100 kg.

	Male					Female				
Items	**TG ($n = 14$)**	**WT ($n = 11$)**	**Pooled SEM**	**P-value**	**Change**	**TG ($n = 9$)**	**WT ($n = 10$)**	**Pooled SEM**	***P*-value**	**Change**
ADFI	1.86	1.87	0.06	0.95	−0.01	9.22	7.85	0.44	0.05	1.37
DVISITS	6.62	8.14	0.51	0.0788	−1.52	2.01	1.93	0.06	0.325	
Total feed	122.68	144.25	2.92	0.0002	−21.57	138.92	150.77	2.22	0.0023	−11.85
Consumption, kg										
Days to market, day	65.94	81.01	2.3	0.0013	−15.07	69.67	79.09	1.99	0.0054	−9.42
ADG, g/d	971.11	808.23	35.41	0.009	162.88	948.12	816.78	29.93	0.0085	131.34
FCR	1.94	2.36	0.05	< 0.0001	−0.42	2.17	2.35	0.03	0.0025	−0.18

ADFI, average daily feed intake; DVISITS, feeding frequency; ADG, average daily gain; FCR, feed conversion rate. Pens effect was defined as random effect. Use covariance analysis for statistics, covariates: initial weight and batch, when P < 0.01, data was correct by covariates.

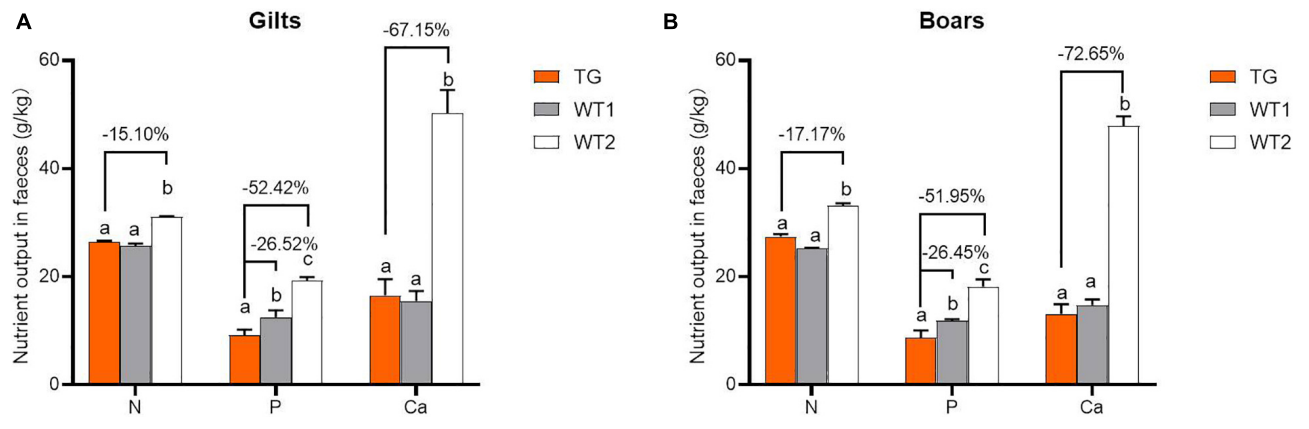

FIGURE 2 | Comparison of nutrient emissions of the MF-TG pigs and WT littermates. Utilization of fecal calcium (Ca), nitrogen (N), and phosphorus (P) was detected in the MF-TG gilts **(A)** and boars **(B)**. Data are shown as mean ± SEM. MF-TG ($n = 5$) and WT1 ($n = 6$) pigs were fed experimental diets with low nitrogen, no mineral phosphorus, and low energy level. WT2 pigs ($n = 11$) were fed commercial diets. Different letters represent $P < 0.05$.

higher proportions of harmful microorganisms, including *Campylobacter*[causes diarrhea (Burnham and Hendrixson, 2018)], *Chlamydia trachomatis* (associated with pneumonia (Lu et al., 2012), and *Campylobacter fetus* [causes septicemia (Sachse and Grossmann, 2002)] and various unidentified viruses, than the MF-TG pigs. By contrast, the MF-TG pigs had higher levels of probiotics, such as *Lactobacillus reuteri* and *Streptococcus*, in cecum than the WT pigs (**Figure 3C**).

Comparison of Functions and Abundance of Microbial Genes of MF-TG and WT Pigs

For resistance gene annotation, unigenes were compared with the CARD database by using the RGI software. Results showed that the difference in the abundance of resistance genes of the MF-TG and WT pigs was not significant (**Figure 4A** and **Supplementary Table 8**). Moreover, common veterinary drugs corresponded with resistance genes, such as tetracycline resistance protein, aminoglycoside antibiotic kinase, lincomycin resistance, tetracycline efflux gene, erythromycin resistance and florfenicol resistance gene, in the pig intestines. The abundance of Fox-5, a cephalosporin resistance gene, was the highest in the ileum, whereas tetW was the highest in the colon and cecum (**Figure 4B** and **Supplementary Table 9**). The microorganism carrying resistance genes of common antibiotics may acquire from external environment where these type of microorganism exist pervasively due to long history of antibiotics abuse. We analyzed the different KEGG functions of MF-TG and WT pigs. Results showed that the microorganisms in the colon of the MF-TG pigs had high fractional abundance in DNA-methyltransferase 1 (K00558, cysteine and methionine metabolism) and serine-type D-Ala-D-Ala carboxypeptidase (K07258, peptidoglycan biosynthesis), whereas the aspartate carbamoyltransferase regulatory subunit (K00610, nucleotide metabolism) and outer membrane protein (K06142, signaling and cellular processes) pathways were enriched in the WT pigs (**Figure 4C**). A comparison of the unigenes and CAZy database revealed that the gene abundance of transgenic cecal microorganisms was active in GT8 (Glycosyl Transferase Family 8), GH13 (Glycoside Hydrolase Family 13), and GH32 (Glycoside Hydrolase Family 32) (**Figure 4D**). We then analyzed the CAZy database by using DIAMOND software. Results showed that a total of 50 carbohydrate enzymes were significantly enriched in the cecal microorganisms of MF-TG pigs (**Figure 5** and **Supplementary Table 10**). Among them,

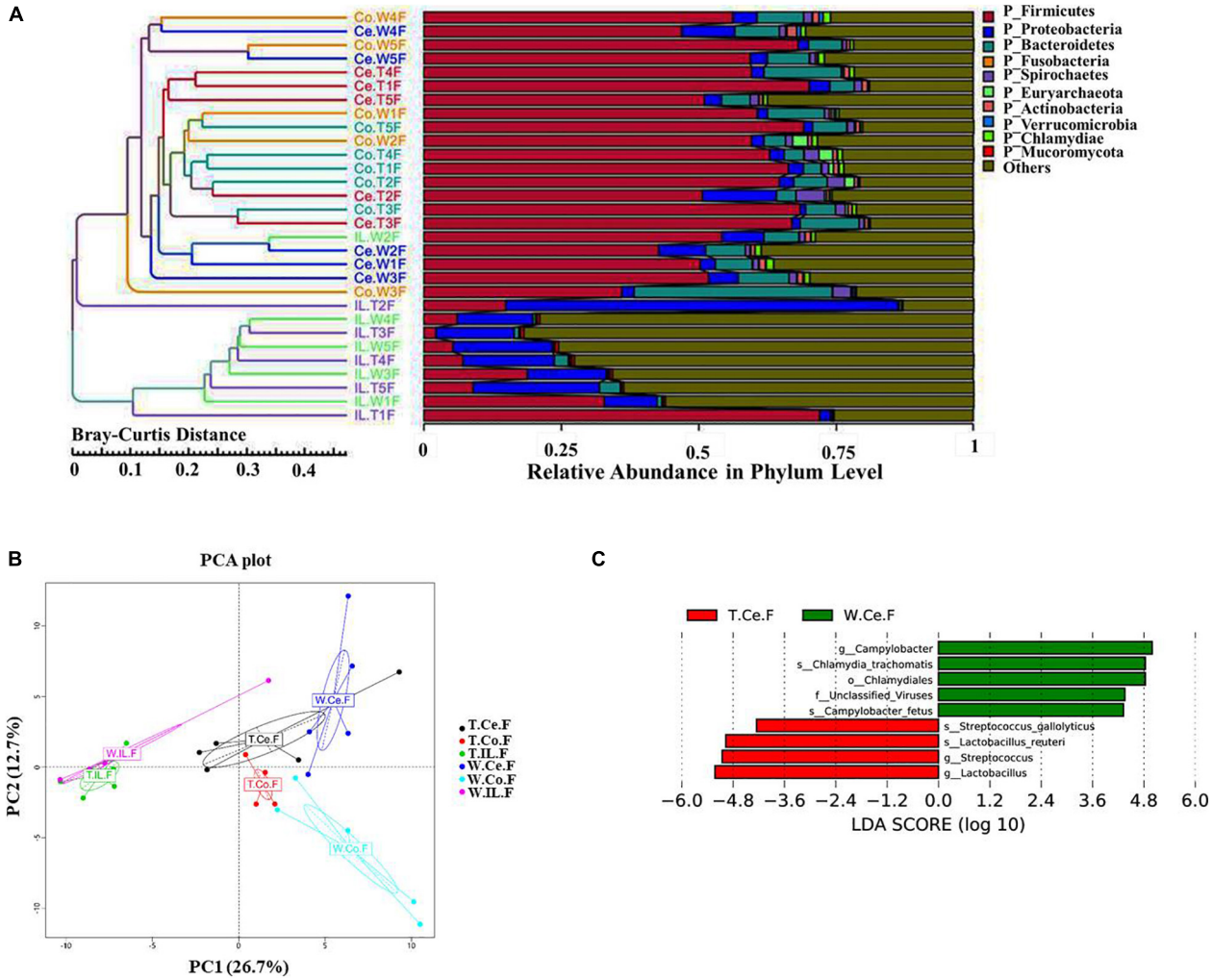

FIGURE 3 | Comparison of intestinal microbial communities of the MF-TG pigs and WT littermates. **(A)** Community composition at the phylum level in MF-TG females and WT littermates. "IL," "Ce," and "Co" stand for ileum, cecum, and colon, respectively. MF-TG and WT female pigs stand for TF and WF, respectively. The Bray–Curtis distance was used for cluster analysis of relative abundance at the phylum level to detect similarities in different samples. **(B)** Principal component analysis (PCA) results of the intestinal microbiota at the phylum level. **(C)** LDA value distribution of different species shown as a histogram. Length of the histogram represents the relative effect of different species.

enzymes involved in the hydrolysis of NSPs, such as glucan 1,3-beta-glucosidase (EC 3.2.1.58), exo-1,3-1,4-glucanase (EC 3.2.1.-), xylan 1,4-β-xylosidase (EC3.2.1.37), and coniferin β-glucosidase (EC3.2.1.126), and starch hydrolases participated in starch and limit dextrins hydrolysis, such as α-amylase (EC3.2.11), oligo-1,6-glucosidase (EC3.2.1.10), and glucan 1,4-α-maltotetraohydrolase (EC3.2.1.60), were most enriched (**Figure 5**). In addition, the level of levan hydrolases, sucrose hydrolases, trehalose hydrolases and many phosphorylases related to metabolism of carbohydrates were up-regulated. Surprisingly, the relative abundance of some polysaccharide synthases, including 1,4-α-glucan branching enzyme (EC2.4.1.18), 4-α-glucanotransferase (EC2.4.1.25), trehalose synthase (EC5.4.99.16), malto-oligosyltrehalose synthase (EC 5.4.99.15), isomaltulose synthase (EC 5.4.99.11), and cyclic beta-1,2-glucan synthase (EC 2.4.1.-), were also induced.

DISCUSSION

Generation of genetically modified pigs is an efficient strategy for improving various indicators of pig performance, such as feed utilization, piglet survival rate and pork nutritional composition. In our previous study, we generated transgenic pigs expressing four microbial enzymes, namely, *bg17A*, *eg1314*, *xynB*, and *eappA*, in the salivary glands specifically (Xianwei et al., 2018). During the feeding process, we found that the N and P emissions of the TG pigs substantially reduced, whereas their nutrient intake and absorption from the feed increased. However, the TG pigs carry the *EGFP* gene. This gene has potential animal and human health hazards (Stepanenko et al., 2008). In the present study, we developed TG pigs without the *EGFP* marker. The MF-TG pigs were found to efficiently secrete the microbial enzymes phytase, β-glucanase, and xylanase.

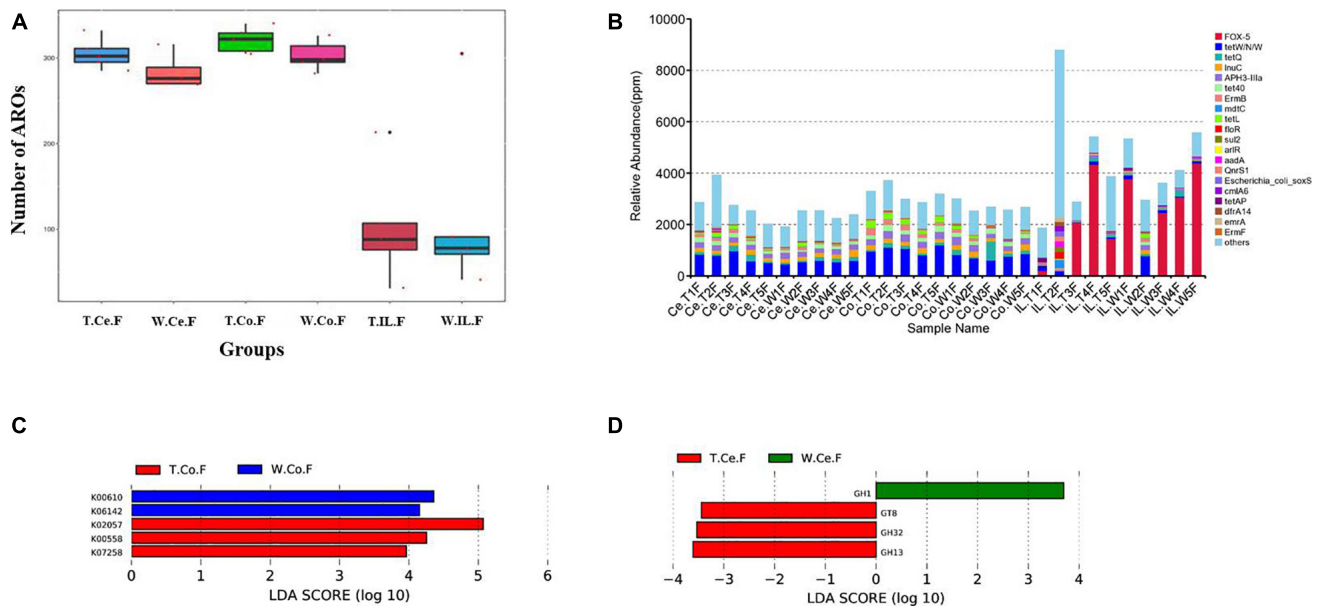

FIGURE 4 | Functional annotation and abundance of microbial genes in different intestinal microorganisms. **(A)** Number of ARO in the MF-TG and WT pigs was not significantly different. **(B)** Relative abundance of the top 20 ARO is shown as a histogram. "Ppm" stand for parts per million. LEfSe analysis revealed the different functions in KEGG **(C)** and CAZy **(D)** databases.

The expression levels of the transgenes varied between different MF-TG pigs. Our previous work (Xianwei et al., 2018) showed that the expression levels of the transgenes were high during feeding time and 10 min before and after feeding time and significantly reduced during rest time in the TG pigs. These results indicated that feeding behavior induced the expression of transgenes. In the present study, pigs were given free access to feed. Some pigs showed high gene expression level when the saliva samples were collected during their feeding time and the others showed low gene expression levels when the saliva samples were collected during their rest time. This phenomenon may explain the wide expression levels of the transgenes in the MF-TG pigs. We compared the MF-TG pigs fed nutrition-deficient experimental diets, which contain lower nitrogen level, no mineral phosphorus additive, and lower energy level than commercial diet, with the WT pigs fed commercial diets. Results showed that the genetically modified pigs considerably reduced their N, P, and Ca emissions in the manure without compromising production performance. When compared with the WT pigs fed the same nutrition-deficient experimental diets, these genetically modified pigs had substantially improved ADG and feed utilization efficiency but only reduced fecal P emissions. This result was not consistent with that of our previous trial in metabolic cage under restrictive feeding and movement (Xianwei et al., 2018). The main difference between the previous and the present studies was that all pigs herein were measured in cages without feeding and movement restrictions. Other prior studies reported that supplementary enzymes in the feed have a positive effect on the digestibility of feed nutrients (Swiatkiewicz et al., 2016; Recharla et al., 2019). Phytase can liberate P from phytate by step-wise dephosphorylation of phytate. β-glucanase and xylanase can effectively degrade glucan and xylan, respectively. Hence, β-glucanase, xylanase, and phytase are both nutritionally and ecologically beneficial because they enhance P/N absorption while reducing P/N excretion (Xianwei et al., 2018).

The issue of whether or not the digestive enzymes secreted by genetically modified pigs would affect their intestinal microbiome remains unclear. In recent years, metagenomic methods based on high-throughput sequencing have rapidly promoted the study of the composition and function of intestinal microorganism floras (Costea et al., 2018). In the present study, all experimental pigs were selected from populations with a similar genetic background, of the same gender and raised under the same environmental, nutritional, and management conditions to minimize the variability caused by genetic, gender and external factors. Nevertheless, results showed that the gut microbiomes of the MF-TG and WT pigs were different, consistent with the results of previous studies (Nicholson et al., 2012; Parks et al., 2013; Quan et al., 2019). The MF-TG pigs had higher levels of probiotics, such as *L. reuteri* and *Streptococcus*, in the cecum than the WT pigs. *L. reuteri* strongly adheres to the intestinal mucosa; thus, this bacterium can improve the distribution of intestinal microbes, antagonize the colonization of other harmful bacteria and prevent the development of intestinal diseases (Kleerebezem and Vaughan, 2009). In addition, *L. reuteri* can produce reuterin, a non-protein broad-spectrum antibacterial substance that can greatly inhibit the growth of Gram-positive/negative bacteria, yeast, fungi and pathogens (Kleerebezem and Vaughan, 2009). *Streptococcus* is generally considered a health-promoting microorganism because of its role in regulating human health. Numerous *Streptococcus* species are involved in carbohydrate fermentation, starch hydrolysis and

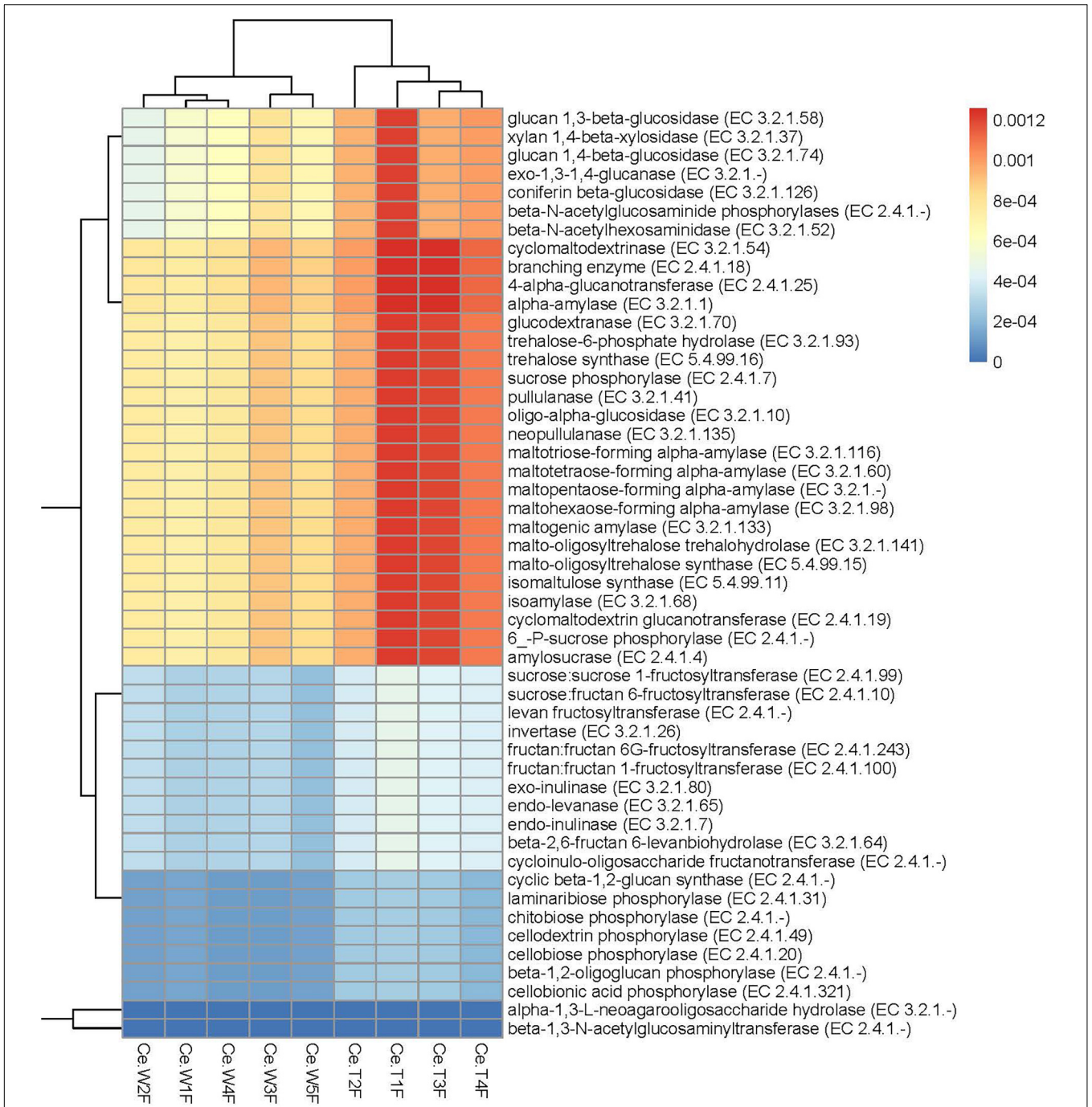

FIGURE 5 | Significantly enriched carbohydrases in the cecum of MF-TG pigs. Color code stands for relative abundance.

glucan production from sucrose (Quan et al., 2019). *Streptococcus gallolyticus* can ferment mannitol, trehalose, and inulin and produce acids from starch and glycogen. Therefore, the presence of these bacteria suggest that the MF-TG pigs were healthier than the WT pigs because they have more probiotics to promote gut health or degrade carbohydrates in their diet. By contrast, the levels of *Campylobacter* and *Chlamydia* in the WT pigs were higher than those in the MF-TG pigs. *Campylobacter* and *Chlamydia* are common pathogenic bacterium in the digestive tract of numerous livestock, such as cattle, sheep, pig, and poultry, and often cause diarrhea and enteritis (Sachse and Grossmann, 2002; Lu et al., 2012; Burnham and Hendrixson, 2018). The presence of these pathogenic bacteria indicated that the WT pigs were more susceptible to diarrhea and enteritis than the MF-TG pigs. Other studies also suggested that the levels of NSP-degrading enzymes tend to increase the population of beneficial

bacteria, thereby enhancing gut physiology, as evidenced by reducing relative weight of organs in the digestive system and increasing villus height (Zijlstra et al., 2010).

We also compared the functions and abundance of microbial genes of the MF-TG and WT pigs. Results showed that the abundance of K07258, K00610 and K06142 in the cecum of the MF-TG pigs was more active than that of WT pigs. These genes are associated with cysteine and methionine metabolism, peptidoglycan biosynthesis and nucleotide metabolism. Prior studies reported that pigs with high feed utilization have high abundance of methionine metabolism, peptidoglycan biosynthesis and nucleotide metabolism pathways. These features seem to verify that the gut microorganisms in the MF-TG pigs can adapt to multiple digestive enzymes and evolve new mechanisms to proliferate despite altered metabolic conditions (Quan et al., 2019). In addition, the relative abundances of carbohydrate enzymes involved in the hydrolysis of NSPs, starch, limit dextrins, fructans, sucroses, and trehaloses, and many phosphorylases related to carbohydrate metabolism in the cecal microbes of MF-TG pigs were high, which may be due to the high concentration of phosphates and oligosaccharides in the intestinal tract of the MF-TG pigs. These results on the functions of microbial genes indicated that the microorganisms promoted the adaptability of transgenic enzymes and increased the feed efficiency of the MF-TG pigs.

Moreover, our results showed that common veterinary drugs were associated with their corresponding resistance genes in the pig intestine. During the experiment, all of the pigs were not treated with any antibiotics. We speculate that the gut microorganisms carrying antibiotic resistance genes were obtained from the external environment where antibiotics resistant microorganisms exist pervasively because of the long history of antibiotics abuse. Nevertheless, the relative abundance and composition of the antimicrobial resistance genes were not significantly different between the MF-TG and WT pigs, suggesting that the development of antibiotic resistance has no relationship to the expression of exogenous digestive enzymes in the MF-TG pigs.

CONCLUSION

The MF-TG pigs secreting NSP-degrading enzymes and phytase in the salivary glands can greatly promote nutrient absorption, improve growth performance and reduce pollutant emissions. Moreover, the intestinal microbiome exhibited adaptive changes to the transgenic enzymes, which may be beneficial to animal nutrient utilization and health.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Care and Use Committee of the South China Agricultural University.

AUTHOR CONTRIBUTIONS

JM conducted the animal work, performed most of the laboratory work, and revised the manuscript. GL conducted part of the laboratory work and wrote the manuscript. GH, HW, JS, RZ, and GC helped to conduct the animal trial and part of the laboratory work. ZW and XZ designed the experiment, oversaw the development of the study, and wrote the last version of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2021.631071/full#supplementary-material

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Practical Approaches for Knock-Out Gene Editing in Pigs

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Pigs are an important resource for meat production and serve as a model for human diseases. Due to their physiological and anatomical similarities to humans, these animals can recapitulate symptoms of human diseases, becoming an effective model for biomedical research. Although, in the past pig have not been widely used partially because of the difficulty in genetic modification; nowadays, with the new revolutionary technology of programmable nucleases, and fundamentally of the CRISPR-Cas9 systems, it is possible for the first time to precisely modify the porcine genome as never before. To this purpose, it is necessary to introduce the system into early stage zygotes or to edit cells followed by somatic cell nuclear transfer. In this review, several strategies for pig knock-out gene editing, using the CRISPR-Cas9 system, will be summarized, as well as genotyping methods and different delivery techniques to introduce these tools into the embryos. Finally, the best approaches to produce homogeneous, biallelic edited animals will be discussed.

Keywords: CRISPR-Cas9, knock-out, electroporation, microinjection, porcine zygotes, SCNT

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INTRODUCTION

In the last few years, there has been a huge impact on porcine biotechnology evolution, evidenced by the numerous pig models developed in this short period of time. Several reviews have been published about gene editing in pigs, from biomedical and agricultural standpoint (Burkard et al., 2018; Yang and Wu, 2018; Lee et al., 2019). In this regard, models to recapitulate human diseases such as arteriosclerosis (Wang et al., 2020), diabetes (Renner et al., 2020) or to test new cancer therapeutics (Kalla et al., 2020) have been developed. Furthermore, gene editing is bringing closer the possibility to use pigs as organ donors for patients on the waiting list for organ transplantation (Lu et al., 2020).

For a long time, the ability to introduce a precise genetic modification in pigs was limited by the available tools. Nowadays, it is possible to induce point mutations in the porcine haplotype, of approximately 2.5×10^9 nucleotides long, through a reverse genetic mechanism. The evolution of genetic modification tools has come a long way. It was initially limited to mice, and later on found a solution in simple bacterial immune mechanisms, the CRISPR-Cas systems. These new molecular tools have been so groundbreaking that have marked the beginning of a new era in genetic manipulation (Doudna and Charpentier, 2014), dividing the history of the generation of modified mammals into "Before and After CRISPR" (BC and AC).

EVOLUTION OF GENETIC MODIFICATION TOOLS

The development of genetically engineered animal models was hampered in most species by the lack of appropriate technologies. In BC times, the conventional gene targeting approaches were based on homologous recombination (HR) that are extremely infrequent and whose uses were mostly restricted to mouse model development. With the advent of the CRISPR-Cas system the AC era began, offering novel opportunities to produce genetically engineered animal models. The relevant techniques to enable gene editing in pigs through the years will be discussed in this section.

Genetic Modification of Animals in BC (Before CRISPR) Times

Several attempts have been made to modify mammalian genomes in the last decades. The first genetically modified mammals were generated by injection of DNA fragments into the male pronucleus (Gordon and Ruddle, 1981; Hammer et al., 1985; and revised in Clark, 2002), where exogenous DNAs were randomly integrated at preexisting double-strand breaks (DSBs), a consequence of the extreme compaction of sperm DNA. Soon after, sperm-mediated gene transfer by *in vitro* fertilization (IVF) was also used to generate genetically modified mammals (Lavitrano et al., 1989); however, this technique could not be replicated by other groups (Brinster et al., 1989). Despite these polemic results, it was later shown that sperm-mediated gene transfer can result in transgenic mammal production when spermatozoa were directly injected into the cytoplasm of the oocyte by intracytoplasmic sperm injection-mediated transgenesis (ICSI-MTG) (Perry et al., 1999; Moreira et al., 2007; Pereyra-Bonnet et al., 2008). However, this technique exhibited limitations related to ICSI species-dependent variable efficiency (reviewed in García-Roselló et al., 2009; Salamone et al., 2017). Later on, it was reported that the cytoplasmic injection of transposon efficiently resulted in transgenic offspring in rodents, pigs, and other large mammals (Sumiyama et al., 2010; Garrels et al., 2011; Furushima et al., 2012; Bevacqua et al., 2017).

Although precise genetic modifications were performed by HR or specific locus integration, their frequencies are usually two or three orders of magnitude lower than a random integration. Thus, the isolation of homologous recombinant cell clones requires long and complex protocols of enrichment, independently of the target locus, based on a combination of positive and negative selections (Thomas and Capecchi, 1987). Nevertheless, these protocols were practically restricted to mouse embryonic stem (ES) cells (Evans and Kaufman, 1981). Cells could be injected into blastocysts generating chimeras with the colonizing the germline (Bradley et al., 1984). Finally, by mating these chimeric animals, it was possible to obtain homogeneous transgenic progeny. The application of this technology in domestic species was limited, because only recently, ES cells from cow were isolated (Bogliotti et al., 2018) and porcine expanded potential stem cells were developed thanks to an exhaustive effort of several groups that tested around 400 combinations of 20 small molecule inhibitors and cytokines (Gao et al., 2019); however, to date no large domestic animals have been obtained with a total or partial contribution of any kind of stem cells yet.

Dolly's birth (Wilmut et al., 1997) brought the attention to somatic cell nuclear transfer (SCNT) as a new possibility to generate transgenic animal models, such as sheep (Schnieke et al., 1997), cows (Cibelli et al., 1998) and pigs (Onishi et al., 2000), since fetal or adult somatic donor cells can be genetically modified prior to nuclear transfer. Although SCNT could also theoretically allow the generation of knock-out animal models, the complex selection protocols to generate specific integrations resulted in very few gene knock-outs produced by this method in pigs: two monoallelic (Dai et al., 2002; Lai et al., 2002) and two biallelic pigs (Rogers et al., 2008; Prather et al., 2013) have been reported.

Another strategy proposed to induce genetic modifications involves the use of endonucleases that can recognize more than 16 bases and make a single cut per genome (by hazard one cut every 4^{16} bases, approx. every 4×10^9 bases or 1 cut per haploid mammalian genome). The first genome-editing strategy was based on the use of *I-Sce*I, a yeast meganuclease with a recognition site of 18 base pairs (Jacquier and Dujon, 1985). In this regard, Choulika et al. (1995) demonstrated an increase in HR in mammalian chromosomes when donor DNA carrying homology regions flanking an endogenous *I-SceI* site was previously inserted in the mouse genome. Moreover, the microinjection of *I-SceI* together with a transgene flanked by meganuclease sites, increased the transgene integration efficiency in bovine embryos (Bevacqua et al., 2013). Lastly, a modified version of this meganuclease containing nuclear localization sequence (NLS) was successfully used to generate transgenic pigs by cytoplasmic injection (Wang et al., 2014).

The most recent developments have been the programmable endonucleases that resulted from the fusion between *Fok1* (Li et al., 1992), and DNA recognition domains such as the zinc finger (ZFN, Kim et al., 1996) and the transcription activator-like effector (TALEN, Christian et al., 2010). Initially, they were used as an efficient modification method to obtain edited somatic cells prior to SCNT (Hauschild et al., 2011; Carlson et al., 2012), and later on, both ZFN and TALEN were directly injected into the zygote as mRNA (Lillico et al., 2013; Tan et al., 2013) to induce specific genetic modifications allowing the expansion of knock-out pig models. However, before these efficient techniques could be spread throughout the scientific community, a much simpler technique was developed.

Genetic Modification of Animals in the New AC (After CRISPR) Era

CRISPR-Cas systems were the most recently programmable endonuclease-based genetic engineering tools developed, practically monopolizing the gene editing field, since these new systems are more efficient, cheaper and simpler than the previous ones (Knott and Doudna, 2018). The year 2013 is considered to be the first year of a new era, the AC era.

Although the discovery of the CRISPR systems can be deemed to be serendipitous, because rare repeat sequences were observed by sequencing bacterial genes (Ishino et al., 1987;

Mojica et al., 1993), the CRISPR-Cas systems were developed after a decade of combined efforts of many researchers who translated their knowledge into a revolutionary molecular biology tool, with a huge impact on many scientific fields (Mojica et al., 2005; Barrangou et al., 2007; Jinek et al., 2012; Cong et al., 2013; reviewed by Lander, 2016).

In almost all archaebacteria and half of bacteria, a huge diversity of CRISPR-Cas systems has been found, described and classified (Makarova et al., 2020). The CRISPR-Cas9 of *Streptococcus pyogenes* (SpCRISPR-Cas9) is one of the most used tool (Cong et al., 2013; reviewed by Marraffini, 2016), both in its original version with two RNAs, the CRISPR RNA and the transactivating CRISPR RNA (crRNA and tracrRNA, respectively) (Cong et al., 2013) or with just one RNA, known as single-guide RNA (sgRNA), a synthetic chimera between crRNA and tracrRNA (Jinek et al., 2012). In the CRISPR-Cas systems, where a single protein is used, target specificity is given by the sequence present in the crRNA or sgRNA (of 20 bp long); therefore, by simultaneously introducing different sgRNAs, several locus modifications are possible at the same time (Cong et al., 2013). Although other CRISPR-Cas systems have been described and used (Kotani et al., 2015), we will focus on SpCRISPR-Cas9, whose only genomic sequence requirement is the presence of an NGG sequence known as the protospacer adjacent motif (PAM), close to the cut site. Considering the CCN triplet in the antiparallel strand, and a random distribution of the four nucleotides, a PAM will be found every 8 nucleotides.

Moreover, following the completion of the Human Genome Project (Green et al., 2015) an accelerated development of cheaper and faster methods converted the Next Generation Sequence (NGS) techniques in standard tools for many applications in clinical and agronomical research (van Dijk et al., 2018). Along with the huge availability of sequences, there are a lot of *in silico* tools that allow the identification of homologous genes between species (Chen and Coppola, 2018).

The available sequence data embraced the development of many online programs that allow for the design of the most convenient guides to perform double-strand breaks at a specific locus, reducing the chances of off-target or undesired breaks (Cui et al., 2018). However, around 10% of the designed guides are not able to drive a precise DSB in mouse zygotes (Yuan and Hu, 2017). This can be explained by a more complex chromatin DNA structure in mammals than in bacteria or phages which are natural substrates for this nuclease. Therefore, the simple screening of guides is required (shown below).

Programmable endonuclease can also facilitate the insertion of exogenous sequences in a specific locus (Mali et al., 2013), producing transgenic animals; however, this strategy will not be discussed in this review.

THE ROAD TO OBTAIN AN EDITED PIG IN THE NEW AC ERA

The easy application of the CRISPR-Cas editing tools promoted the generation of many animal models that were impossible to develop before, such as domestic animals and even, unfortunately, humans. However, the "*replacement*" principle, one of the 3Rs principles of animal welfare, does suggest looking for alternative approaches, such as the use of *in vitro* cell cultures or the generation of rodent models, to answer some biological questions.

Nevertheless, pigs are considered a great promise in biomedical research, since they are interesting models for human diseases and the best option as an organ supply for xenotransplantation. Thus, gene-edited pigs have become an effective and, in some cases, irreplaceable tool. In order to produce them it is necessary to complete the following three stages: (a) the design of efficient programmable nucleases, (b) the generation of edited single-cell embryos, and (c) the subsequent editing analysis of the piglets produced.

Efficiency of the CRISPR-Cas9 System

As it has been already mentioned, the specificity of CRISPR-Cas9 depends on the crRNA or the sgRNA, and there are several publications describing how to synthesize them (Ran et al., 2013; Fujihara and Ikawa, 2014; Jacobi et al., 2017). In this section, different strategies to evaluate the efficiency of CRISPR-Cas9 will be discussed.

The simplest assays are based on the use of DNA plasmids, as a binary system, encoding for Cas9 and for the sgRNA, respectively (Mali et al., 2013). Mashiko et al. (2013) described a tool for quantifying the efficiency of CRISPR-Cas9 based on the reconstitution of *gfp* functionality after a DSB in episomal plasmid constructions. However, in order to mimic the real conditions, an analysis of editing efficiency should be conducted in the porcine genome, using cell lines or *in vitro*-produced embryos (see below in the Porcine zygote production section). Although there are few exceptions, the use of CRISPR-Cas9 plasmids is normally limited to somatic cell cultures (Wang K. et al., 2015). In embryos, due to the transcription arrest until the first mitotic cycles, the use of RNAs or the RNP (ribonucleoprotein) complex is preferred (Hai et al., 2014). In addition, CRISPR-Cas9 *in vitro* digestion can be used as a pre-validation of the system to induce a DSB in a target site. This assay is only applicable for RNP format, and consists of the *in vitro* assembly of the Cas9 protein with the *in vitro* transcribed or chemically synthetized sgRNA or crRNA: tracrRNA duplex, followed by the digestion reaction with the fragment that contains the target site (Mehravar et al., 2019). In cell culture assays, the selection marker commonly carried by the Cas9 coding plasmids can be used after transformation to enrich the culture for transformed cells (Zhou et al., 2015; Bevacqua et al., 2016; Yin et al., 2019). The analysis of these results, which tend to have a high background level, are complex because the obtained cells have different editing events. On the contrary, the *in vitro*-produced embryos have a small number of cells (around 50) derived from a few editing events. These results tend to be clearer and allow the study of features such as mosaicism or heterozygosity (Sakurai et al., 2014; Whitworth et al., 2014; Bevacqua et al., 2016). An animal or embryo is mosaic when not all of its cells have the same genotype, and this happens when gene editing occurs after the first embryonic mitotic divisions. In these cases, more than two alleles per locus can be detected.

In all these assays, the genotype characterization of the resulting cells initiates with an amplification of the edited locus through a PCR reaction. The primers should be designed so that they flank the target site. Since deletions produced in the process of DSB repair can involve hundreds of nucleotides, a primer design far enough from the target sites is recommended to ensure a correct hybridization, even within large deletion events. Optimal primers anneal at least 200 nt. away from the intended cutting sites (Mianné et al., 2017). Moreover, nested PCR is a good choice when the amount of DNA in the samples is limited. The second step is the analysis of the amplified DNA. Although the PCR amplicons could be screened directly by Sanger sequencing, some indirect strategies have been developed allowing massive and inexpensive tests. When the efficiency is low, these assays are an excellent alternative for sorting samples prior to Sanger sequencing.

The use of two sgRNA flanking an essential element in the targeted gene (dropout knock-out, Chen et al., 2014; Low et al., 2016) allows a simple evaluation of the designed sgRNA. In this case, the double cut induces an internal deletion that can be verified by a change in electrophoresis mobility of the new smaller resulting amplicon. However, these tests underestimate the rates of non-functional allele formation, because single or double cuts repaired with indels occur without the internal deletion, and therefore, these cases are indistinguishable from the wild type on an agarose gel electrophoresis.

Single cuts (indels) can also be analyzed by heteroduplex formation assays. These techniques distinguish between amplicons that carry mutations from those which do not. However, these methods do not provide information about the number or the composition of the alleles present. Heteroduplex formation assays consist in denaturing and annealing together wild-type and mutant amplicons (or amplicons that carry two different mutations), creating a bubble due to the mismatched chains. Heteroduplex DNAs can be analyzed by using nucleases such as T7 endonuclease 1 (T7E1) (Mashal et al., 1995) or Surveyor nuclease (an enzyme from the CEL nuclease family, Qiu et al., 2004). These nucleases recognize a mismatch site and, consequently, cleave both DNA strands. Then, the products of enzyme digestion are resolved by agarose gel electrophoresis showing a full-length amplicon (due to the presence of homoduplexes) and the expected-size cleavage products, if Cas9 cleavage occurred producing indels (Harms et al., 2014). Heteroduplex DNAs could also be analyzed by the heteroduplex mobility assay (HMA) (Ota et al., 2013, 2014). Since heteroduplexes have an open single-strand configuration surrounding the mismatched region, they can be separated from homoduplexes by polyacrylamide gel electrophoresis because of changes in complex migration patterns. In some cases, when an induced mutation is well represented in an allele pool, it is necessary to introduce a wild-type amplicon before heteroduplex formation to increase the accuracy of the method (Sentmanat et al., 2018). Another indel detection assay is the high-resolution melting analysis (HMRA) (Bassett et al., 2014). HMRA uses the different melting temperatures of the wild-type and a mutant amplicon to distinguish one from another, using a melting curve analysis with a fluorescent dye that fluoresces brightly when specifically bound to double-stranded DNA (Wittwer et al., 2003).

The Indel Detection by Amplicon Analysis (IDAA) is a sensitive and accurate technique that provides detailed information on cleavage efficiency, size and nature of the allelic variants generated (Yang et al., 2015). The technique is based on a single-step tri-primer PCR, where a universal 6-FAM 5-labeled primer (FamF) designed to target the forward primer in a specific extension is used. This technique results in the labeling of FAM amplicons that can be detected using standard DNA fragment analysis by the capillary electrophoresis methodology (Andersen et al., 2003).

In the case of defined nucleotide changes or specific point mutations, additional silent mutations, which do not alter the amino acid sequence of the encoded protein, can be included in the donor DNA to create new restriction sites. In this way, the amplified DNA at the target locus can be digested with the corresponding new restriction enzyme to detect point mutations by homology-directed repair (HDR) events (Wang et al., 2013). Nevertheless, imperfect or incomplete HDR events can occur, leading to undesired sequence modifications near the target site (Mianné et al., 2017). Similarly, if the chosen sgRNA cuts in a restriction enzyme site when the indels are generated, the restriction site could be lost.

Finally, the sequencing of the target regions of the alleles present in the sample is necessary to obtain a complete characterization. Chromatograms from direct Sanger sequencing of PCR products can be easily analyzed when samples contain only one or two possible alleles, such as clonal cell cultures and F1 animals. In samples that could contain more than two alleles (mosaicism), such as F0 animals, or polyclonal cell cultures, it is often difficult to determine the sequences of the alleles present. In this regard, different algorithms were developed to help in these analyses. The Tracking of Indels by Decomposition (TIDE) is an algorithm that analyzes. Sanger sequence traces, identifies the major induced mutations in a target site, and determines their frequency in a cell population (Brinkman et al., 2014; Ryczek et al., 2020). It is a simple, rapid and cost-effective method compared to sub-cloning individual amplicons of the target region and sequencing enough numbers of them to obtain an accurate characterization of the indel spectrum, which is more labor-intensive and expensive. A modified version of TIDE, the Tracking of Insertion, DEletions, and Recombination events (TIDER), estimates the frequency of targeted small nucleotide changes introduced by CRISPR in combination with HDR using a donor template (Brinkman et al., 2018).

Generation of Single-Cell Edited Embryos

One of the first decisions to be made for the generation of pigs with specific gene modifications is whether to edit somatic cells to be used for cloning or directly introduce the CRISPR-Cas9 components into the zygotes. The advent of this new genome editing technology promotes the use of both strategies and the choice of one over the other will depend on the laboratory capacities.

SCNT

The development of pig cloning (Onishi et al., 2000) opened the possibility of generating homogeneous animals with modifications incorporated into somatic cells (Lai et al., 2002). In addition, in order not to depend on specific equipment and to be able to increase the number of reconstituted embryos, the handmade cloning (HMC) technique has been useful (Vajta et al., 2001; Du et al., 2007). The main distinctive feature of HMC is the use of sharp blades for bisection of zona-free oocytes under stereomicroscope instead of using a micromanipulator to enucleate them.

In either methodology, traditional cloning (TC) and HMC, the results obtained still show a low efficiency to produce cloned piglets (with only 0.3–2% of transferred embryos developing to term; Du et al., 2007; Zhang et al., 2012; Liu T. et al., 2015; Gadea et al., 2020). In this regard, the aggregation of three zona-free reconstructed cloned embryos was proposed as a strategy to improve embryo development, quality (Buemo et al., 2016) and deliveries (Siriboon et al., 2014) in TC and HMC, respectively. Despite the limitation of both techniques, they are used to generate edited pigs with CRISPR/Cas9. Somatic cells, such as fetal fibroblasts, are transformed with plasmids encoding for the Cas9 and the sgRNAs, along with a reporter gene and/or an antibiotic resistance gene; allowing the screening and/or selection of the modified cells (Ren et al., 2019). Once the edited cells are obtained, they are used to generate founder pigs, which will present a predictable genotype avoiding mosaicism (Chen et al., 2015; Wang K. et al., 2015; Kumbha et al., 2020). Furthermore, the multi-targeting capacity of the CRISPR-Cas9 system allows to edit many target genes simultaneously, a feature used by Niu et al. (2017), to produce porcine retrovirus PERV-free pigs by SCNT, where 62 copies of this retrovirus were edited.

Another interesting alternative is to retrieve fetuses generated by CRISPR-Cas9 delivery into porcine zygotes and screen the fetal fibroblasts for the specific modifications. These selected cells will then be used for performing SCNT carrying the desired modifications, avoiding mosaic animal generation and the laborious enrichment and selection process of edited cells from primary cultures (Kang et al., 2016).

Porcine Zygote Production

The new genetic editing tools are now so efficient that allow zygotes direct modification. For this reason, besides cloning, other embryo production techniques, such as IVF or *in vivo* zygote retrieval, are promoted as good alternatives for the generation of genetically modified pigs. The different methodologies to obtain the porcine embryos, as well as the delivery options to introduce the CRISPR-Cas9 system into them, will be further discussed in this section.

Production of parthenogenetic embryos

Parthenogenetic activation is an alternative to *in vitro* embryo production since embryos are capable of developing to blastocysts, like fertilized oocytes (Kure-Bayashi et al., 1996), avoiding variations due to the sperm factor (Gupta et al., 2008). These embryos have been proposed to evaluate *in vitro* the efficiency of gene editing tools (Tao et al., 2016), although these embryos are not viable to generate offspring.

Oocyte activation can be artificially induced by simulating the effects produced by the sperm. The protocols commonly used for this procedure are based on the exposure of oocytes to agents that promote the increase in cytoplasmic levels of Ca 2 +. Following exposure to Ca 2 + inducing agents, oocytes are often treated with inhibitors of protein synthesis (e.g., cycloheximide – CHX) or kinase activity (e.g., 6-dimethylaminopurine – 6-DMAP) generating a diploid parthenogenetic embryo that will be able to develop to the blastocyst stage (Alberio et al., 2001).

Electrical stimulation is commonly used to activate pig oocytes and, in order to optimize this method, the combination of electrical and chemical activation protocols have been proposed to produce transgenic embryos (More details of these protocols are described in Liu S. et al., 2015).

Another important application of parthenogenetic embryos is as a supplementary source to improve maternal recognition, pregnancy and implantation rates of SCNT in pigs (De Sousa et al., 2002; Kawarasaki et al., 2009).

In vitro fertilization (IVF)

Despite the enormous effort and progress, the current *in vitro* fertilization system remains inefficient giving as a result low embryo development and low-quality blastocysts compared to the *in vitro* systems from other species such as bovine or mouse (reviewed by Gil et al., 2010; Grupen, 2014). This is mainly due to the high incidence of polyspermy that occurs during IVF. Over the last 2 decades, many groups have been working to find a methodology to improve IVF and reduce polyspermy (reviewed by Funahashi, 2003; Romar et al., 2016). More recently, Li et al. (2018) showed that by simply reducing sperm concentration in the presence of cumulus cell, an improvement in fertilization (monospermy rate and normal pronuclear formation) and blastocyst formation were obtained. Moreover, IVF systems based on some *in vivo* conditions, such as a higher pH, and the presence of oviductal and follicular fluid and cumulus cell secretions, reduce polyspermy and increase the final embryo production (Soriano-Úbeda et al., 2017). A reason for this improvement may be due to the presence of extracellular vesicles in the porcine oviductal fluid (Alcântara-Neto et al., 2020). Nevertheless, several gene-editing studies use *in vitro* derived embryos, since they are less costly and time-consuming, and a large number of oocytes can be recovered from slaughterhouse ovaries. Considering the incidence of polyspermia, a method to isolate monospermic zygotes to avoid editing and transfer of polyspermic embryos is very useful. This can be achieved by identifying normal pronuclear formation by visualization in presumptive zygotes. A problem is that porcine zygotes exhibit a large amount of cytoplasmic lipid droplets. Therefore, zygote centrifugation after IVF was proposed as a simple non-invasive method to visualize pronuclei to identify two and poly-pronuclear zygotes (Wall et al., 1985). This technique allowed Gil et al. (2013) to identify 2 pronuclear zygotes, and to improve blastocyst quality and pregnancy efficiencies (number of live piglets per total transferred embryos) when these embryos were transferred to recipient gilts

in comparison to non-centrifuged, non-selected zygotes in the control group.

In vivo zygote production

It is known that the development of *in vitro* pre-implantable mammalian embryos is compromised compared to those produced *in vivo*, presenting a delay in blastocyst development and fewer cells in the embryos (Macháty et al., 1998; Holm et al., 2002). Unfortunately, the available data on the effectiveness of *in vivo*-derived porcine zygote collection procedures remain limited to date. In this regard, some key aspects to take into account are the formation of pronuclei, which occurs between 3 and 5 h after fertilization and the first mitotic division that occurs 14–16 h later (Hunter, 1974). Therefore, the window for the collection of zygotes to be edited turns out to be very narrow. To perform this procedure, it is necessary to previously synchronize the estrus and ovulation of multiparous sows. Weaning is an effective physiological method, obtaining a fertile estrus between 3 and 5 days after weaning. To increase the number of fertilized oocytes, superovulation can be induced with equine chorionic gonadotropin (eCG) 24 h after weaning followed by human chorionic gonadotropin (hCG) administration. Then, females are submitted to post cervical insemination twice, at 6 and 24 h after the onset of estrus. For zygotes collection, sows are submitted to a surgical procedure in which they are anesthetized, their genital tracts are exposed through mid-ventral laparotomy, and zygotes are finally retrieved by flushing each oviduct. In this regard, Martinez et al. (2020) managed to recover a range between 69.0 and 73.3% of zygotes. However, the above-described procedures involve the need for specialized technicians and veterinarians and adapted facilities with sterile operating rooms, which for some groups could mean a budgetary limitation.

CRISPR-Cas Delivery Methods in Zygotes

Initially, the traditional procedures to deliver the editing tools into the zygotes involved microinjection. Several scientists have tried to develop newer, simpler and cheaper methods and some of these developments have been partially successful. A recent approach includes an electroporation-based method that bypasses microinjection with promising results obtained by numerous groups. In either case, the ultimate goal is to produce biallelic and homogeneous edited animals and, for this reason, timing for CRISPR-Cas9 system action, relative to DNA replication in the zygote, may be the most relevant event to be taken into account to reduce or eliminate mosaicism. The most commonly used methods will be compared in this section.

Intracytoplasmic microinjection

This technique is the most widely used for the generation of different animal models through the years. It consists in the microinjection of editing tools into presumptive one-cell stage embryos produced *in vivo* or by IVF. This technique requires the use of expensive micromanipulation equipment and skilled personnel to operate it. Additionally, it is time-consuming, the reason why the number of zygotes microinjected per repetition will be limited. As has already been mentioned, microinjection of mRNA for CRISPR-Cas9 or RNP is preferable to edit porcine zygotes (Sato et al., 2015; Jacobi et al., 2017; Lamas-Toranzo et al., 2019; Tanihara et al., 2019b). However, plasmids encoding for Cas9 nuclease and for sgRNA have also been used for this purpose (Petersen et al., 2016). The main problem of using plasmid DNA is that it lasts longer inside the cells, potentially increasing off-target mutations.

Considering the IVF limitations already described, some groups prefer to directly collect and microinject CRISPR-Cas9 tools into *in vivo*-produced presumptive zygotes close to insemination; and transfer the embryos into the oviduct immediately after microinjection to improve viability and pregnancy rates very good results (Hai et al., 2014; Wang Y. et al., 2015; Yu et al., 2016).

The main advantage of combining *in vitro*-produced embryos with microinjection as delivery technique of choice, is the possibility to exploit the narrow time window between gamete fusion and first embryo cell division to deliver editing tools. Thus, in order to reduce mosaicism without affecting embryo viability, several studies have been performed to evaluate the best timing to introduce the CRISPR-Cas9 system throughout the *in vitro* embryo production procedure. Tanihara et al. (2019b) concluded that the optimal moment to microinject CRISPR-Cas9 components as the RNP complex into zygotes was 6 h after the start of IVF, when the highest mutation rates were obtained without compromising embryo viability. Furthermore, a higher RNP complex concentration was shown to increase efficiency and biallelic mutations (although still low: 16.7%) in the resulting blastocysts (Tanihara et al., 2019b). Another group reached similar conclusions working with parthenogenetically activated oocytes. They observed that the best moment to microinject the CRISPR-Cas9 components as RNA was 6 h after activation, regarding blastocyst and mutation rates. However, no improvement in mosaicism was observed in this case (Sato et al., 2018). In contrast, Tao et al. (2016) showed a much significant improvement in the rates of biallelic mutation (93%) in embryos when CRISPR-Cas9 mRNA was microinjected 8 h after parthenogenetic activation.

In addition, in a recent study by Su et al. (2019), a microinjection of the CRISPR-Cas9 components as RNA into germinal vesicle porcine oocytes was proposed as a solution to reduce mosaicism. These oocytes were then *in vitro* maturated and parthenogenetically activated or fertilized by IVF. By applying this strategy, up to 83% of the mutant embryos obtained were non-mosaic, having no detrimental effect on embryo viability. Another particular approach is the injection of CRISPR-Cas9 system in reconstituted presumptive zygote (Sheets et al., 2016). In this case, without any selection, 6 out of 6 piglets carried a biallelic modifications.

Although this technique is widely applied for the generation of edited animal models, it requires the use of expensive micromanipulation equipment and skilled personnel to operate it. Additionally, it is time-consuming, the reason why the number of zygotes microinjected per repetition will be limited.

Embryo electroporation

More recently, this technique was developed for embryos, and it has grown in importance, proving to be cheaper and simpler

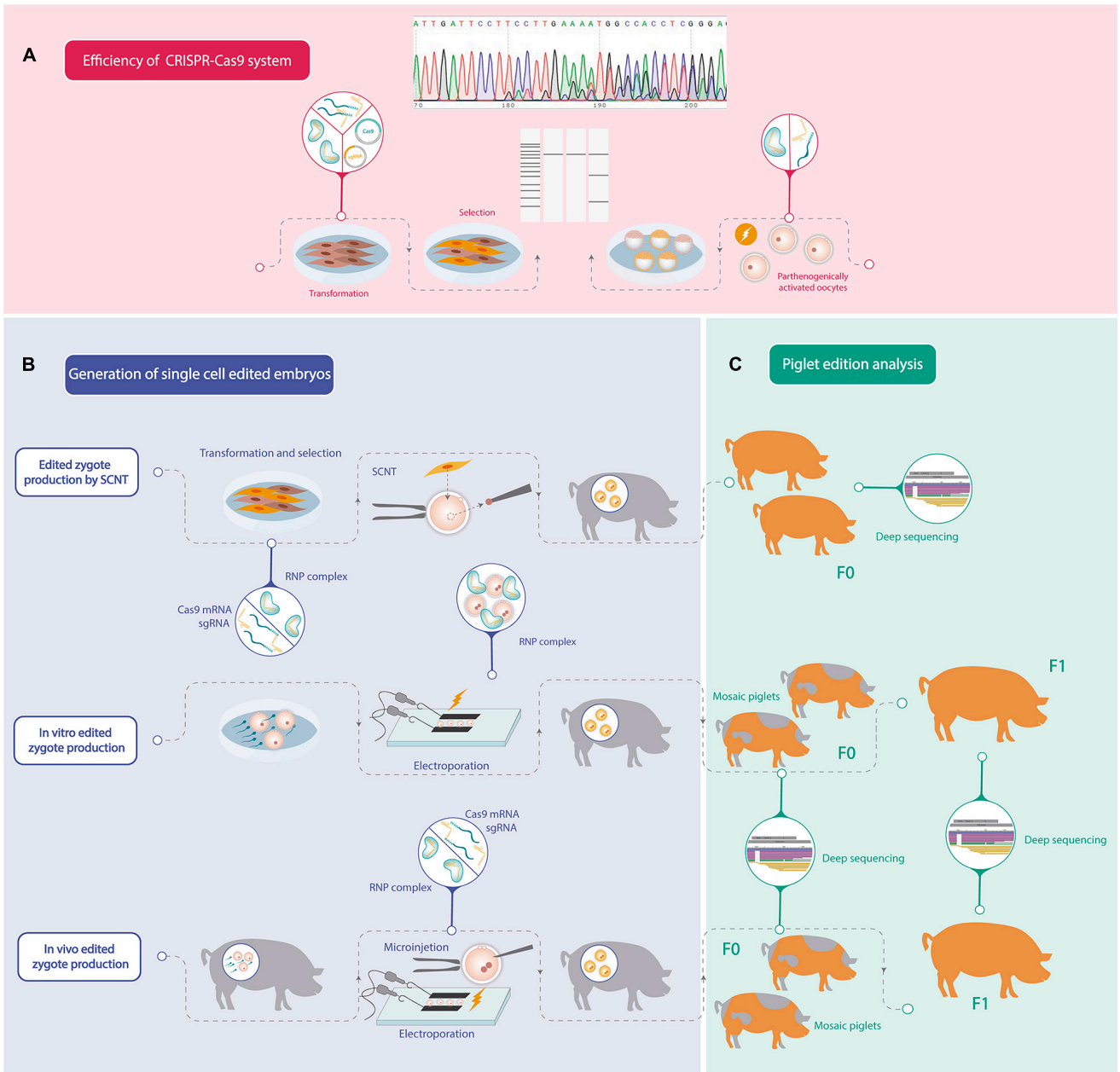

FIGURE 1 | Schematic workflow of the different steps needed to generate a gene edited pig. **(A)** Efficiency analysis of mutations induced by CRISPR-Cas9 system. **(B)** Different strategies to generate one-cell stage porcine edited embryos. **(C)** Gene editing analysis of the founder pigs (F0) and offspring produced by crossbreeding F0 pigs (F1).

than embryo microinjection for introducing indel mutations, large deletions, and small insertions (Kaneko and Mashimo, 2015; Kaneko, 2018). Recent studies have demonstrated that zona pellucida weakening is not necessary to achieve porcine zygotes gene editing by electroporation; preserving the integrity and viability of the embryo. There are mainly two different electroporators that have yielded good results, the CUY21EDIT II electroporator (BEX) (Nishio et al., 2018; Tanihara et al., 2019a,c) and the NEPA21 electroporator. The latter proposes to reduce the damage to embryos by using a three-step electrical pulse system. The first pulse, the poring pulse, makes micro-holes in the zona pellucida and oolemma of the embryos. The second pulse, the transfer pulse, transfers the endonucleases into the cytoplasm of the embryos. The third pulse, the polarity-changed transfer pulse, increases the opportunity of introducing the endonucleases into the embryos (Kaneko, 2017).

Although this technique is yet to generate sufficient data, it has shown good results allowing a faster gene

editing of a bigger number of oocytes or zygotes at the same time, in contrast to the IP microinjection. The combination of a massive embryo production by IVF with the GEEP (gene editing by electroporation of Cas9 protein) technique compensates the poor IVF results with the fast editing rate by electroporation. This permits to transfer up to 200 embryos per recipient, finally obtaining living offspring with the intended gene target modifications (Tanihara et al., 2019a,c).

In addition, the success of this technique is in part due to its combination with Cas9 as protein, since the compact nature of the RNP complex seems to easily enter through the pores generated in zygotes in contrast to large Cas9 mRNA or other editing tools.

Piglet Gene Editing Analysis

Except for edited animals by SCNT, where mosaicism is not an issue, the analysis of F0 is not a simple task (Teboul et al., 2017; Mehravar et al., 2019). It is very likely that F0 individuals could be mosaics; therefore, theoretically, the result of whether they are edited or not may depend on the tissue analyzed. Mosaicism in F0 animals could be responsible for differences between biopsied tissue and its germline; thus, producing F1 offspring without the expected genotype. The most obvious negative consequence will be a non-edited progeny after breeding. One of the first works to study mosaicism was carried out in mice, taking advantage of the *Tyr* gene whose loss of function generates albino phenotypes (Yen et al., 2014). Using CRISPR-Cas 9 in $Tyr^{\pm}$ heterozygous zygotes with a mutation in a different exon, 6/12 pups were albinos (50%), 4/12 were pigmentation mosaics (33%), and 2/12 were fully pigmented (∼17%), and by analyzing DNA tail biopsies, more than 2 different alleles (up to 5) were found, even in homogenous animals (Yen et al., 2014). The backcrosses with homozygous albinos gave F1 homogeneous albino animals for all three mosaic phenotype animals, and unexpectedly for one of the phenotypically homogeneous colored animals too (Yen et al., 2014).

For this reason, in mice, there are authors who suggest analyzing, due to genotypic mosaicism, both the tail and the germline to track down false positives or negatives and to save time and money (Oliver et al., 2015). However, the risk of affecting the reproduction of these animals has slowed down the biopsies of gonads, especially in females. As an alternative to gonadal biopsies, performing ear biopsies is suggested, combined with a TIDE analysis of their sequences (Brinkman et al., 2014), or with deep sequencing by generating a DNA sequencing library with labeled primers to perform thousands of reads for each locus (Yen et al., 2014; Wei et al., 2018).

A good characterization of the founder animals allows to save money and time, and it is important in order to facilitate the decision of which animals to cross to obtain the correct F1. Breeding two F0 edited individuals can reduce the time to obtain a homozygous and homogeneous animal; nevertheless, the analysis can be more complex. In spite of the characterization, genotyping all F1 animals is recommended for the expected modification through Sanger sequencing of the targeted loci (Mianné et al., 2017).

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Recent advances in genome editing technology have accelerated the production of genetic modified pigs for different purposes by using several strategies. Although remarkable progress has been achieved in porcine gene editing, further improvements could still be achieved in order to increase biallelic mutation efficiency. In addition, since porcine reproduction is highly efficient, the application of assisted reproductive technologies has not been developed enough, and consequently protocols for oocyte *in vitro* maturation, IVF or embryo culture can still be improved. For these reasons, porcine gene editing strategies continue to be challenging, and each group should find its own road to generate an edited pig considering their strengths. The steps to follow in order to obtain an edited pig are summarized in **Figure 1**.

Cloning allows to obtain homogeneous animals with biallelic modifications; however, the birth rates of cloned piglets are still low. In addition, the successful generation of porcine expanded potential stem cells opens up new possibilities to simplify future strategies for the generation of edited pigs.

Direct zygote gene editing is a widely used approach because of the higher rates of healthy piglets, although some of them are mosaic. Another alternative is gene editing of *in vitro*-produced embryos by IVF, in combination with electroporation to deliver CRISPR-Cas9 components that seem to be a good and simple strategy, allowing to work with a larger number of embryos that compensate for the poorer development rates of these zygotes. A promising alternative is to obtain *in vivo* zygotes, which exhibit higher viability than *in vitro* embryos, followed by electroporation or microinjection of CRISPR-Cas9 components to ensure higher rates of viable edited embryos. However, this procedure involves additional costs related to the donor animals.

Recently, as a future perspective, some modifications of the CRISPR-Cas9 system are emerging (reviewed by Anzalone et al., 2020). A new chimera Cas9 protein that is capable of editing nucleotide conversions without DSB, has also been used to edit pigs (Xie et al., 2019). Moreover, epigenetic modifications are now possible by using dCas9 (Xu et al., 2020) that have been proposed to improve the viability of cattle embryos *in vitro* (Savy et al., 2020) and could be an effective tool to apply in porcine embryo production. Finally, by improving ICSI technique in pigs, ICSI mediated-gene editing would be an interesting option for the generation of edited piglets since it was demonstrated that when the delivery of the CRISPR-Cas9 system was done during ICSI in humans, mosaicism was reduced in the resulting embryos (Ma et al., 2017).

Nowadays, the simplicity of the new editing tools allowed the democratization of their use for the generation of edited pigs in laboratories around the world. These gene-edited animals cannot be differentiated from spontaneous mutants, since no exogenous genes are introduced and they should not be regulated at all or

their regulation should be less strict than for transgenic animals (Van Eenennaam et al., 2019). However, few national regulatory agencies distinguish between genetically modified organisms and edited organisms. This distinction could greatly impact on the edited animal research, especially for agricultural purposes.

AUTHOR CONTRIBUTIONS

LR and RF-M took part in manuscript writing and editing. DS took part in the manuscript edition. All authors took part in the conceptualization of the review.

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frontiers in Genetics

REVIEW
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Electroporation-Mediated Genome Editing of Livestock Zygotes

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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, we 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 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.

Keywords: gene editing, zygote, embryo, CRISPR, mosaicism, electroporation

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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 and 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 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 an alternative method of delivering genome-editing components into zygotes. 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 and Mashimo, 2015; Hashimoto et al., 2016). The protocol for electroporation requires only a stereomicroscope, an electroporator, and an electroporation cuvette. 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 zona pellucida (ZP) and plasma membrane to allow the movement of genome editing reagents into zygotes (**Figure 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.

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 and 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 variables to consider when optimizing electroporation conditions including 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 that are often 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 and Filev, 2007; Saulis and 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 (**Supplementary Table S1**). Poring voltages of 30, 100, and 300 V/mm were tested 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). It was noted 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 15–20 V/mm and 2–3 pulses (**Supplementary Table S1**). Bovine zygotes appear to be especially sensitive to high voltages; with 20 V/mm

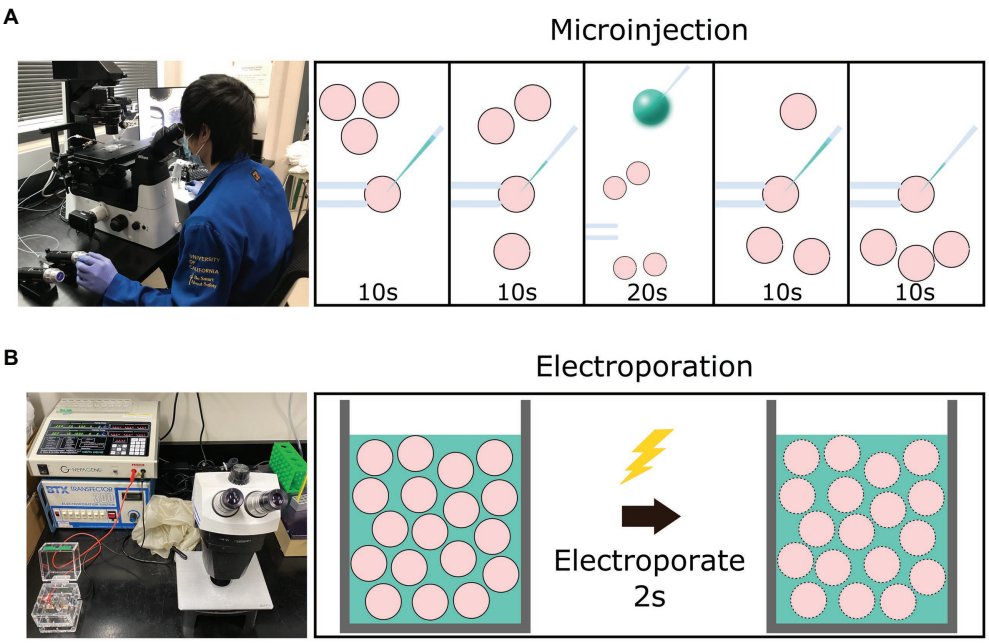

FIGURE 1 | Graphical schematic of a comparison between setup and time necessary for the microinjection vs. electroporation of embryos. **(A)** The equipment necessary for the microinjection of embryos and the workflow involved to introduce editing reagents (green) into four presumptive zygotes (pink) 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.

(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 and Filev, 2007; Saulis and 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 strength 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).

Porcine studies have found 4–5 pulses of 1–2.5 ms pulse widths to be successful, and bovine studies have found 2–6 pulses of 1–3 ms pulse widths to be successful (**Supplementary Table S1**). 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 voltages as well as 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 embryo survival and editing. Another study by Hirata et al. (2019a) tested the effect of the number of pulses on the blastocyst formation rate and successfully generated edited blastocysts with much higher efficiencies. 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 strength 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). The result supports previous experiments in mice embryos that found the removal of the ZP to potentially hinder embryonic development (Bronson and 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 under these conditions.

Together, these findings 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 greater amounts of genome editing components to enter the cells. However, increasing parameters to increase transfection efficiency, and/or weakening the zona pellucida 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 used 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 and 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% (Qin et al., 2015).

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 very recent paper 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 was 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 (Le et al., 2020). 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 AND TIMING OF GENOME ACTIVATION

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 cells. **Figure 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 and 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

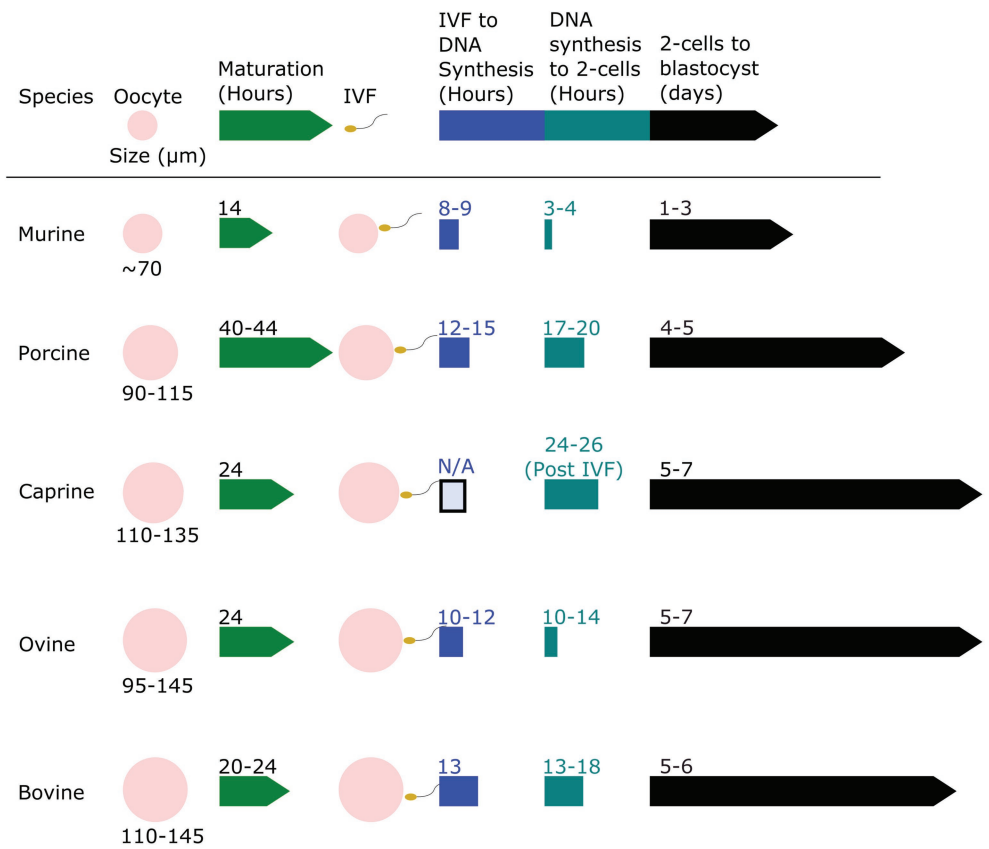

FIGURE 2 | Relative oocyte size and a timeline of embryo development for murine, porcine, caprine, ovine, and bovine zygotes. The oocyte size of murine, porcine, caprine, ovine, and bovine species are shown to scale and compared. The relative timeline of embryo development from the oocyte stage to blastocyst stage after *in vitro* fertilization (IVF) is shown. Data derived from Harlow and Quinn (1982), Motlik et al. (1984), Crosby et al. (1988), Papaioannou and Ebert (1988), Sakkas et al. (1989), Prather (1993), Campbell et al. (1994), Gardner et al. (1994), Laurincik et al. (1994), Fair et al. (1995, 1997), Rath et al. (1995), Serta et al. (1995), Bouniol-Baly et al. (1997), Otoi et al. (1997), Gómez et al. (1998), Wang et al. (1998, 2012), Anderson et al. (1999), Comizzoli et al. (2000), Raghu et al. (2002), Sanfins et al. (2003), Ciemerych and Sicinski (2005), Moon et al. (2005), Griffin et al. (2006), Ptak et al. (2006), Surjit et al. (2006), Zhou and Zhang (2006), Anguita et al. (2007), Chaves et al. (2010), Catalá et al. (2011), O'Hara et al. (2014), Paramio and Izquierdo (2014), Morohaku et al. (2016), Cadenas et al. (2017), Yoon et al. (2018), HosseinNia et al. (2019), McLean et al. (2020), and Owen et al. (2020).

during genome activation, and the fact that HDR is predominantly active in the late S-G2 phases (Gu et al., 2018; Plaza Reyes and 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.

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 has produced a high rate 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 have failed to be injected into 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 results in the later-injected zygotes developing more toward the synthesis stage of the first genomic replication when injected, thus resulting in Cas9 being active later in the zygote stage and past the one-cell stage while the target site remains unmutated (Burkard et al., 2017). Using a gRNA/Cas9 RNP rather than Cas9 mRNA decreases mosaicism as the RNP is active immediately, and does not require the time for 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 role in the difference (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 when compared with electroporation of one cell embryos. The 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 (Le et al., 2021). Additional attempts to further reduce mosaicism have included substituting Cas9 protein for Cas9 mRNA, speeding up the editing process, degrading Cas9 sooner, *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 porcine, ovine, and bovine zygotes, DNA synthesis occurs 12–15 hpi, 10–12 hpi, and 18 hpi, respectively (**Figure 1**). Namula 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). Another study in bovine zygotes found a significant reduction in mosaicism rates from MI of zygotes at 10 hpi compared to 20 hpi, however, even the 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 did not eliminate mosaicism, but 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 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 et al. (2015) was able to target 10 different genes in mice and generate 10 different knockout mice with mutation rates from 13 to 100% (Kaneko et al., 2014). Another study published in 2019 utilized Cas12a instead of Cas9 as the 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 studied. More recently, Kaneko explored the possibility of electroporating frozen-warmed pronuclear-stage embryos to generate *Tyr* knockout mice (Nakagawa et al., 2018) and rats (Kaneko and Nakagawa, 2020) using Cas9 protein and dual sgRNA introduced by electroporation after slow freezing. 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 to produce 56 and 50% genome edited offspring for frozen and freeze-dried sperm, respectively (Nakagawa and Kaneko, 2019).

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 and bovine zygotes have been successfully electroporated to produce knockout live 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 six studies describing the electroporation of porcine zygotes and efficient editing of blastocysts with at least an 80% success rate in all six studies. They also produced live knockout piglets in three of the studies. The first of the six 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 expressed mutations at the target site, seven of which were mosaic. The next study targeted the *TP53* gene using the same parameters which resulted in nine piglets, six of which were genetic knockouts. However, 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).

The next porcine study 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 40% of the *CD163* blastocysts, four *TP53* piglets, and seven *MSTN* piglets were mosaic. In 2020, this group successfully knocked out (Le et al., 2020; Tanihara et al., 2020a) *MSTN* and *GGTA-1* using electroporation at 12 hpi with five 1 ms transfer pulses at 25 V/mm. 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).

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. 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, and 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.

Knock-in animals require 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 zona pellucida. 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 homologous 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, ssODN has gained in 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 is able to efficiently integrate into the target locus with homology arms as short as 40 nucleotides, whereas dsDNA donors typically require homology arms around 1–2 kb (Chen et al., 2011; Zhao et al., 2020). 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 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 and Dellaire, 2017).

ELECTROPORATION OF DONOR REPAIR 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 zona pellucida 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 (Kaneko and 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).

The majority of knock-in animals created through electroporation have been mice or rat zygotes electroporated with Cas9/gRNA/ssODN. Hashimoto and 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 and 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 and 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 zona pellucida using pronase. They reported that only zona-free zygotes generated EGFP-positive blastocysts following electroporation, indicating that the zona pellucida presents a strong barrier for large dsDNA-uptake following electroporation. They concluded that the bovine zona pellucida 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 high skill 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 microinjection 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 zona pellucida presents a barrier to transfection. However, LZD again requires handling each zygote individually and a high level of skill.

Recombinant adeno-associated viruses 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 diffuse across the zona pellucida to transiently deliver genes to fertilized mammalian zygotes with intact zona pellucida (Mizuno et al., 2018; Romeo et al., 2020). They have been used to successfully generate genome edited mouse pups without the need for micromanipulation, with both high embryo survival and editing rates (Yoon et al., 2018). A 2019 study used rAAV to transfect large HDR donors of up to 4.9 kb, 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 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 the potential that electroporation has 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 3**).

It is perhaps not obvious to those not working in the field, but a source of livestock oocytes has to 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

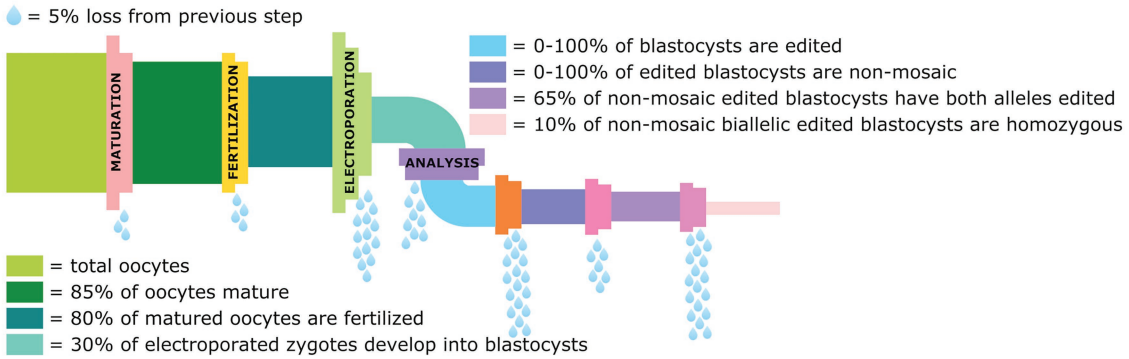

FIGURE 3 | Graphical representation of the losses in the genome editing pipeline from collection of oocytes to the percentage of blastocysts that are non-mosaic homozygotes for the intended edit. Data derived from Remy et al. (2017), Teixeira et al. (2018), and Miao et al. (2019).

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.

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 microinjection (Kalds et al., 2020). 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 a number of 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 4.5 kb 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.

AUTHOR CONTRIBUTIONS

JL performed the literature review and authored the first draft of the manuscript. AV edited and provided suggestions. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2021.648482/full#supplementary-material

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REVIEW
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Advances in Genome Editing and Application to the Generation of Genetically Modified Rat Models

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The rat has been extensively used as a small animal model. Many genetically engineered rat models have emerged in the last two decades, and the advent of gene-specific nucleases has accelerated their generation in recent years. This review covers the techniques and advances used to generate genetically engineered rat lines and their application to the development of rat models more broadly, such as conditional knockouts and reporter gene strains. In addition, genome-editing techniques that remain to be explored in the rat are discussed. The review also focuses more particularly on two areas in which extensive work has been done: human genetic diseases and immune system analysis. Models are thoroughly described in these two areas and highlight the competitive advantages of rat models over available corresponding mouse versions. The objective of this review is to provide a comprehensive description of the advantages and potential of rat models for addressing specific scientific questions and to characterize the best genome-engineering tools for developing new projects.

Keywords: CRISPR-Cas9, rat, knockout, knockin, transgenesis, genetic diseases, immune genes

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INTRODUCTION

Genetically modified animal models are essential to answering questions in biology, modeling human and non-human animal diseases, and generating therapeutic recombinant proteins. Among animal models, small laboratory mammals are often used because they share many biological features with humans, housing them is easy and relatively inexpensive compared to maintenance of large animals, and ethical issues are less prominent than with species such as non-human primates. Among the small laboratory animal models, the rat has been used since at least 1856 (Philipeaux, 1856) and still is an important experimental model (between 9 and 18% of all laboratory models in the EU, The Commission to the European Parliament and the Council, 2015-2017).

Certain intrinsic characteristics of the rat, such as its larger size (10 fold) compared to the mouse, allow easier and more rapid microsurgery, multiple sampling of larger blood and tissue volumes, precise injection of substances into the brain, and *in vivo* and *ex vivo* organ function analysis. Additionally, mice and rats differ in their physiology and more sophisticated traits in the rat have made it a model of choice for toxicology, complex human diseases and neurobehavioral as well as cardiovascular studies among several others (Jacob, 2010).

Such differences have been supported by comparative analyses of the rat and mouse genomes. The rat genome is 2.75 gigabases (Gb), smaller than the human genome (2.9 Gb) but larger than the mouse genome (2.6 Gb) (Gibbs et al., 2004). Overall, rats show enrichment of genes involved

in immunity, metabolic detoxification and chemosensation, as well as conservation of many genes involved in human diseases (Dewey et al., 2004; Gibbs et al., 2004).

Despite these advantages, the use of rats has lagged behind the use of mice in research, mainly because genetically modified mice were generated earlier than genetically modified rats (**Figure 1**). In mice, DNA microinjection was used in the early 1980s and embryonic stem (ES) cells in the late 1980s (Gordon et al., 1980; Palmiter et al., 1982; Doetschman et al., 1987). In contrast, in rats, DNA microinjection and ES cells began in the early 1990s and 2010, respectively (Mullins et al., 1990; Kawamata and Ochiya, 2010). In the meantime, researchers used classical breeding approaches to develop a variety of rat strains that model human diseases (Szpirer, 2020). The need for genetic engineering tools for the rat and the continuous use of zygote pronuclei microinjection of DNA in the rat, explain why gene-specific nucleases were applied in rats in 2009, earlier than in mice (2010) (Geurts et al., 2009; Carbery et al., 2010). These gene-specific nucleases quickly facilitated the exponential generation of knockout (KO) rats for many genes. In synergy with these technological advances, sequencing of the rat genome (Dewey et al., 2004; Gibbs et al., 2004) and characterization of genetic quantitative trait loci (QTLs) linked to diseases (Aitman et al., 2010, 2016) further accelerated the use of models of genetically modified rats.

In this regard, different rat strains are prone to different diseases present in humans and reproduce better than mice some of these diseases. These rat strains have been used to introduce genetic modifications to analyze the role of genes (Aitman et al., 2010, 2016). For example, Wistar Kyoto, Dahl/SS, and spontaneously hypertensive strains develop hypertension and have extensively used to analyze the role of many genes (Moreno et al., 2011; Rudemiller et al., 2014; Nayak et al., 2015; Aitman et al., 2016; Lerman et al., 2019; Szpirer, 2020). The diabetes-prone biobreeding rat strain is another model that has been used to genetically modify genes involved in diabetes

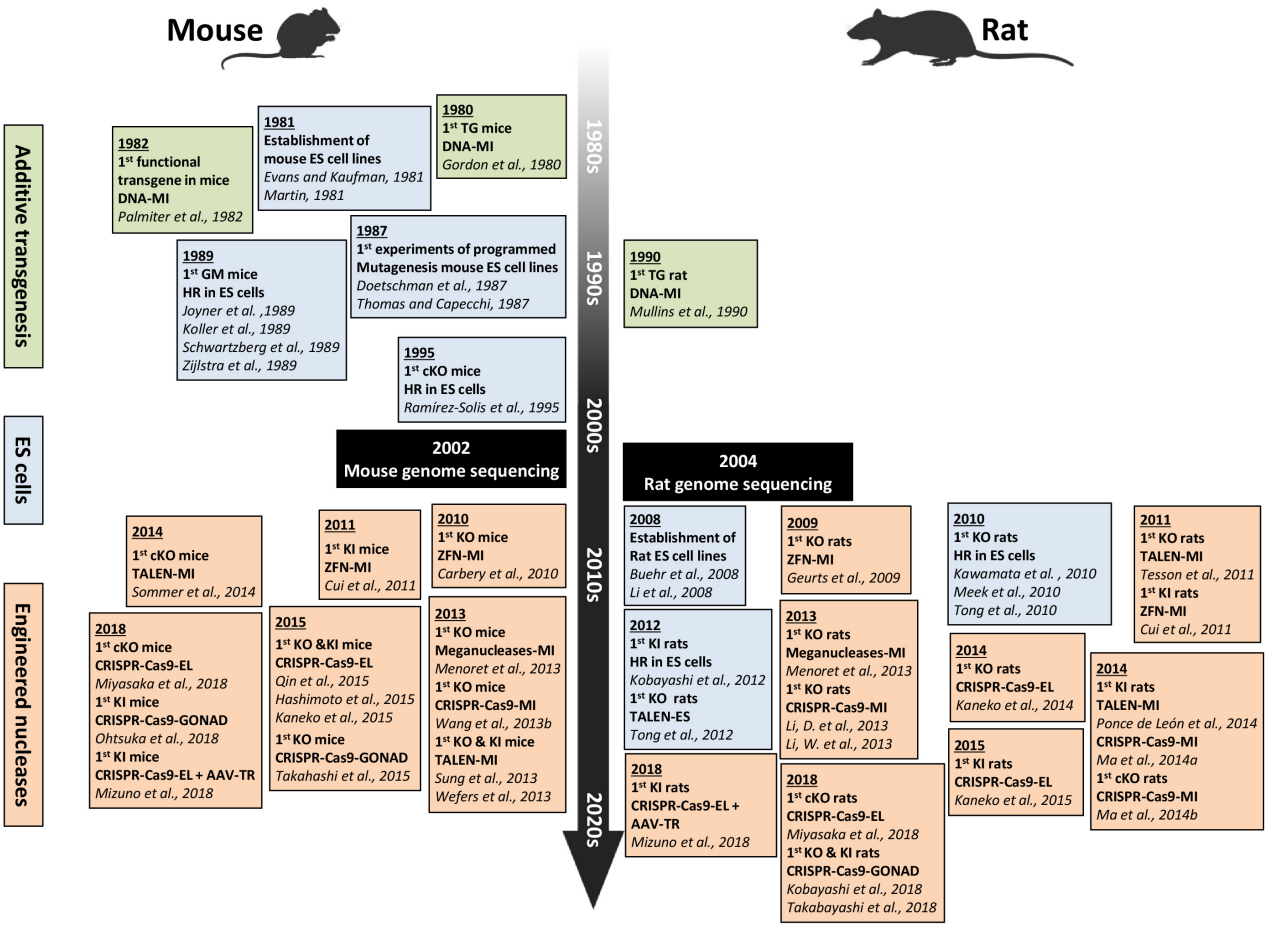

FIGURE 1 | Timeline showing the major technical advances in genome editing and delivery in mice and rats from the 1980s to today. The *green frames* encompass the 1st transgenic mice and rats generated by DNA microinjection. The *blue frames* contain the 1st ES cells-based mouse and rat models, and the *orange frames* contain the 1st mouse and rat models generated using engineered nucleases delivered by different methods. Figure created with BioRender.com. AAV-TR, AAV transduction; cKO, conditional KO; DNA-MI, DNA microinjection; EL, electroporation; ES, embryonic stem cells; GM, genetically modified; GONAD, genome-editing via oviductal nucleic acids delivery; HR, homologous recombination; KI, knockin; KO, knockout; LV-MI, lentiviral microinjection; TALEN-MI, TALE nucleases microinjection; TG, transgenic; ZFN-MI, ZFN microinjection.

(Michalkiewicz et al., 2004; Pandey and Dvorakova, 2020). Lewis rats are more susceptible than mice to the induction of Th1-mediated autoimmune diseases, whereas Brown Norway rats are highly susceptible to Th2-mediated immune diseases. Genomic linkage analysis allowed identification of a region on chromosome 9 that controls these phenotypes (Bernard et al., 2010). Additionally, the rat has been extensively used to analyze autoimmune diseases involving multiple genes (Aitman et al., 2010; Bernard et al., 2010).

In this review, we first describe the evolution and advances in genome editing and in delivery optimization of CRISPRs for producing genetically modified models. Further details are given on the rat to highlight needs and future research paths. The second part of the review focuses on the advantages of genetically modified rat models compared to mouse to mimic human situation, in particular in genetic diseases and immunology studies. Rats differ from mice in several characteristics, manifesting different phenotypes for the same genetic alteration. Rats also can sometimes better reproduce clinical features observed in humans who carry these gene variants (Hammer et al., 1990; Larcher et al., 2014). Our final aim is thus to inform researchers about major progresses in rat genome editing and advantages of rats as model organisms, to give researchers the choice of the best experimental system to answer their scientific questions. To facilitate rat models access and development, major rat resources for finding existing models or designing new ones with the latest gene editing tools, are described in **Table 1**.

GENE-EDITING ADVANCES AND DELIVERY SYSTEM OPTIMIZATION

The last four decades have brought major advances in genome editing allowing for generation of animal models that harbor targeted genetic modifications. Efforts have focused on increasing the precision of these modifications, production efficiency and on simplifying procedures to make them easier and cheaper. The evolution of genome editing approaches and tools is discussed in this section, illustrated in **Figure 1** and nucleases compared in **Table 2**. Clustered, regularly interspaced short palindromic repeat (CRISPR)-associated (Cas) systems applied to rodents are detailed in **Table 3**, with details of specifics regarding rats given in this section. More particularly, *Streptococcus pyogenes* (SpCas) system components are described in **Figure 2** and compared in **Table 4**. Published advances for enhancing knockin (KI) generation rate are also detailed here and illustrated in **Figure 3**. Finally, delivery systems and the evolution of their practice are detailed and compared in **Table 5**.

Historical Overview of Major Gene-Editing Techniques Developed in Mice and Rats

Random Additive Transgenesis and Mutagenesis

The first transgenic rodents were successfully generated in the early 1980s and 1990s (Gordon et al., 1980; Palmiter et al., 1982; Mullins et al., 1990), by microinjection of exogenous donor DNA into the pronucleus of one-cell embryos. The reported efficiencies are quite low in rodents, ranging from 0.5 to 10% of injected embryos in mice and 0.5–5% of injected embryos in rats (Brinster et al., 1985; Charreau et al., 1996b; Hirabayashi et al., 2001). Other problems include random integration, a high copy number of integrated DNA sequences in *cis* and uncontrollable transgene expression. These challenges make this approach labor intensive and time-consuming and require considerable expertise.

N-ethyl-*N*-nitrosurea (ENU) is a highly potent mutagen that was first administered into adult male mice (Bode, 1984) and later into rats (Zan et al., 2003). Several ENU-induced mutant rat (van Boxtel et al., 2010) (for a review see Huang et al., 2011) and mouse models (for a review see Justice et al., 1999) have been described. This method presents some advantages: it requires no embryos or ES handling and the sperm of mutant offspring can be cryopreserved. Disadvantages include uncontrolled and random mutations in multiple loci throughout the genome, which must be identified and localized using high-throughput and time-consuming screening methods.

Transposon-mediated insertional transgenesis is an alternative tool developed to increase the integration frequency of the transgene into the host genome. Transposons are simple and mobile elements, consisting of a DNA sequence encoding transposase and a transgene flanked by binding sites (inverted terminal repeats, ITR) for the transposase, promoting integration into the genome. Transposon systems, such as *Sleeping Beauty* (SB), piggyBac (PB) or Tol2, have demonstrated their efficiency in rapidly producing stable lines of transgenic mice (Carlson et al., 2003; Horie et al., 2003) and rats (Kitada et al., 2007; Lu et al., 2007). The number of transgene insertions is, however, difficult to control.

Targeted Mutagenesis

The derivation of germline-competent mouse ES cells in the early 1980s (Evans and Kaufman, 1981; Martin, 1981) and the first experiments of targeted mutagenesis (Doetschman et al., 1987; Thomas and Capecchi, 1987), allowed introducing mutations into the host genome with a high precision (Joyner et al., 1989; Koller et al., 1989; Schwartzberg et al., 1989; Zijlstra et al., 1989) making mice a privileged model for genetic studies for two decades. Rat ES cells were described in 2008 (Buehr et al., 2008; Li et al., 2008) allowing generation of KO (Kawamata and Ochiya, 2010; Meek et al., 2010; Tong et al., 2010) and KI rats (Kobayashi et al., 2012; Yamamoto et al., 2015) with similar homologous recombination (HR) efficiencies to those observed in mice. Nevertheless, rat ES cells are less robust than mouse ES cells and maintaining their stability in culture and germline competence continues to be challenging.

The development of meganucleases, engineered zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and more recently the CRISPR-Cas system, has unquestionably revolutionized genome editing, opening new possibilities especially in the rat and other species in which ES cells were not available (Fernández et al., 2017). Each of these nucleases have their own properties of DNA-binding, recognition type/site specificities, their own advantages and limitations,

TABLE 1 | Resources on rat genomics and genome edited animals.

Resources	Name	Website and references	Proposed resources
Genomic databases	National Center for Biotechnology Information (NCBI) including Gene, Protein, Nucleotide, Blast, and others	www.ncbi.nlm.nih.gov/ (Sayers et al., 2019)	Comprehensive suite for molecular analysis from rat genome to protein expression and functionality
	The European Bioinformatics Institute (EMBL-EBI) including Ensembl, UniProt, Clustal Omega and others	https://www.ebi.ac.uk/services (Madeira et al., 2019)	From rat genome to protein databases a full suite with analysis tools and multiple sequence alignments
	The University of California, Santa Cruz Genome Browser	https://genome.ucsc.edu/ (Lee et al., 2020)	Genome browser, multiple sequence alignments and others
	Model organism Aggregated Resources for Rare Variant exploration (MARRVEL)	http://marrvel.org/ (Wang et al., 2019b)	Comparison of human genes with model oragnisms' genes such as the rat in a physiologic or pathologic context
Genomic databases and strains repository	Rat Genome Database (RGD) in the United States	https://rgd.mcw.edu (Smith et al., 2020)	Repository of hundreds or rat strains and genome edited rats, mostly for genes involved in hypertension and cardiovascular function. Genetic, phenotype and disease data, sequences, QTLs, mapping data, software tools.
Rat strains repository	Rat Resource and Research Center (RRRC) in the United States	http://www.rrrc.us/	Repository of hundreds or rat strains, genome edited lines, cryopreserved embryos, sperm, and ES cells.
	National Bioresource Project for the rat (NBPR) in Japan	http: //www.anim.med.kyoto-u.ac.jp/nbr/	Repository of hundreds or rat strains, ENU and genome edited lines, cryopreserved embryos and sperm, BAC libraries
	Rat Resource Database in China	http://www.ratresource.com	Repository of rat strains and genomic data.
	Rodent Model Research in Taiwan	https://www.nlac.narl.org.tw/	Strain depository of lines or rats including genome edited ones.
Academic platforms producing genome-edited rat models	Wisconsin Gene Editing Rat Resource Center and The Michigan University Transgenic Animal Core facility in the United States	https://rgd.mcw.edu/wg/gerrc/ https://brcf.medicine.umich.edu/cores/transgenic-animal-model/	Distribution of already available models and generation of new ones on demand
	Transgenic Rat ImmunoPhenomic (TRIP) facility in France	http://www.itun.nantes.inserm.fr/Core-facilities/TRIP-Transgenic-Rats-ImmunoPhenomic	
Commercial vendors for rat models	Charles River laboratories	https://www.criver.com/	Distribution of already available models and generation of new ones on demand
	Janvier Labs	https://www.janvier-labs.com/	
	Envigo (include Horizon discovery models)	https: //www.envigo.com/research-models	
	Taconic Biosciences	https://www.taconic.com	
	genOway (include Axenis models)	https://www.genoway.com/	
	Cyagen	https://www.cyagen.com/us/en/	Custom rat model generation
	Hera Biolabs	https://www.herabiolabs.com/ SRG OncoRats (Noto et al., 2020)	Proprietary gene editing technologies and SRG OncoRats for oncology studies
	Ligand pharmaceuticals	https://www.ligand.com/technologies/omniab OmniRat (Joyce et al., 2020) OmniFlic (Harris et al., 2018)	OmniRat and OmniFlic for human antibodies generation
Software for the use of CRISPR	CRISPOR	http://crispor.tefor.net/ (Concordet and Haeussler, 2018)	On and off target scores
	CHOPCHOP	https://chopchop.cbu.uib.no/ (Labun et al., 2019)	
	E-CRISPR	http://www.e-crisp.org/E-CRISP/ (Heigwer et al., 2014)	
	CCTOP	https://cctop.cos.uni-heidelberg.de:8043/index.html (Stemmer et al., 2015; Labuhn et al., 2018)	
	CRISPRscan	https://www.crisprscan.org/ (Moreno-Mateos et al., 2015)	
	CRISPRdirect	http://crispr.dbcls.jp/ (Naito et al., 2015)	Off-target prediction only
	CRISPR RGEN tools	http://www.rgenome.net/	Cas-OFFinder, Microhomology, Cas-designer, base-editing, prime-editing...
Private company webtool for design of gRNA targeting rat genome	Integrated DNA Technologies	https://eu.idtdna.com/pages/products/crispr-genome-editing	Include on and off target scores
	Synthego	https://www.synthego.com/products/bioinformatics/crispr-design-tool	
	Horizon Discovery	https://horizondiscovery.com/en/ordering-and-calculation-tools/crispr-design-tool	
	Benchling	https://www.benchling.com/crispr/	

TABLE 2 | Comparison of engineered endonucleases.

Specificities, advantages, limitations	Meganucleases	ZFN	TALEN	CRISPR-Cas
DNA binding determinant	Protein	ZF protein	TAL protein	crRNA/sgRNA
Binding specificity	Long sequences of nucleotides[a]	3 nucleotides	1 nucleotide[b]	1/1 nucleotide pairing
Endonuclease	I-CreI and I-SceI[a]	FokI[c]	FokI[c]	Cas9
Function specificity	Monomer	Dimer	Dimer	Monomer
Design/Engineering	Very difficult	Difficult	Simple	Very simple
Restriction in target site	Chromatin compaction	G-rich sequence	Start with T and end with A	End with a NGG sequence
Target site length	18–44 bp	18–36 bp[d]	24–40 bp	22–25 bp
Targeting frequency	Low	High (one/100 bp)	High (one/bp)	High (one/4 or 8 bp)
Specificity	High	Moderate[e]	High	High
Sensitivity to DNA methylation	Yes	Yes	Yes	No[f]
Off-targets	Variable	Low[e]	Very low	Variable
Size	Small size	Small size (∼1 kb/monomer)	Large size (∼3 kb/monomer)	Large size (4.2 kb Cas9)
Commercially available, Cost	Yes, high	Yes, high	Yes, moderate	Yes, low
Patents concern	Yes	Yes	Yes	Yes
Type of editing				
Gene KO (Indels and frameshift)	Yes	Yes	Yes	Yes
Multiplex KO	No data[h]	Very limited	Limited	Yes (up to eight alleles)[g]
Gene correction/point mutagenesis (repaired basepairs)	No data[h]	Yes	Yes	Yes
Gene addition/sequence replacement (integrated gene cassette)	No data[h]	Yes	Yes	Yes
Gene deletion (deleted gene fragments)	No data[h]	No data	No data	Yes
Prime and base editing	No data[h]	No data	No data	Yes

[a] *DNA-binding specificities and cleavage mechanism combined in the same protein (Galetto et al., 2009). I-CreI and I-SceI are the main endonucleases used but a few others have been applied to genome editing.*
[b] *TALE protein consist of 34 amino acid repeat domains, each one recognizing a single DNA nucleotide; highly conserved, excepting two hypervariable residues at positions 12 and 13, which confer the specificity of TALE.*
[c] *FokI cleaves only in its dimeric form*
[d] *Association of 3–6 ZF DNA binding domains fused to the FokI catalytic domain. Binding of two ZFN-FokI heterodimers to two contiguous DNA sequences and separated by a 5–7 bp gap.*
[e] *Specificity depends on number and selected ZF modules.*
[f] *No direct effect of methylation on Cas9 binding or effectivity (Verkuijl and Rots, 2019).*
[g] *Difficult on same chromosome. Limitations overcome by Prime and base editing (cf **Table 3**).*
[h] *The difficulty in designing meganucleases has limited their application in creating new model organisms.*

which are listed in **Table 2**. Injection of these nucleases directly into rat or mouse zygotes allows creation of a double-strand break (DSB) at a targeted locus, repaired thereafter mainly by non-homologous end-joining (NHEJ) or HR (these mechanisms are reviewed in detail in a later section). Careful design of the associated tools makes it possible to better control repair outcome at any targeted locus of the genome with high efficiency and much faster than with ES cells. Several reports demonstrated the high efficiency of ZFN and TALEN in quickly generating different types of modifications in mice and rats, ranging from KO (Geurts et al., 2009; Carbery et al., 2010; Mashimo et al., 2010, 2013; Tesson et al., 2011; Tong et al., 2012; Sung et al., 2013; Sommer et al., 2014), simple point mutations, to large KI by homology-directed repair (HDR) (Sung et al., 2013; Wang et al., 2013a; Wefers et al., 2013; Ponce de León et al., 2014; Remy et al., 2014). Meganucleases, although less used than the other nucleases, were also applied to generate KO mouse and rats (Ménoret et al., 2013). Nevertheless, the design complexity and associated costs made these techniques accessible to only few laboratories, leading to a search for alternative approaches.

The simplicity and rapidity of guided RNA design, compared to complex protein engineering needed for ZFNs and TALENs, made the CRISPR-Cas system largely accessible at low cost, without sacrificing the specificity and reproducibility already observed with ZFNs and TALENs. Nevertheless, the success of CRISPR-Cas, especially in the generation of the first CRISPR mouse (Wang et al., 2013b) and rat (Li D. et al., 2013; Li W. et al., 2013), depended on knowledge gathered using the previous

TABLE 3 | CRISPR variants applied to genetically modified mouse and rat models.

Application	Type – Variant - Name	PAM 5′-3′	Cleavage	GM mice	GM rats
Classical GE	II- SpCas9	NGG	Blunt DSB	Wang et al., 2013b	Li D. et al., 2013; Li W. et al., 2013
Specificity enhancement	II- E -Hypa SpCas9	NGG	Blunt DSB	Ikeda et al., 2019	–
	II- E -SpCas9 nickase	NGG	Nick	Ran et al., 2013	–
Enlarge targeting possibilities	II- E -SpCas9 VQR	NGA	Blunt DSB	Robertson et al., 2018	–
	II- E -SpCas9 VRER	NGCG	Blunt DSB	Robertson et al., 2018	–
	II- E -SpCas9-NG	NGN	Blunt DSB	Fujii et al., 2019	–
	II- SaCas9	NNGRRT	Blunt DSB	Zhang X. et al., 2016	Zheng et al., 2020
	II- E -SaCas9 KKH	NNNRRT	Blunt DSB	Robertson et al., 2018	–
	II- St1Cas9	NNAGAAW	Blunt DSB	Fujii et al., 2016	–
	II- CjCas9	NNNVRYM	Blunt DSB	Kim et al., 2017	–
	II- NmCas9	NNNNGATT	Blunt DSB	Xia et al., 2018	–
	II- FnCas9	NGG	5′ staggered	Hirano et al., 2016	–
	V-A- AsCpf1 (Cas12a)	TTTV	5′ staggered	Hur et al., 2016; Kim et al., 2016	Lee J. G. et al., 2019; Yeo et al., 2019
	V-A- LbCpf1 (Cas12a)	TTTV	5′ staggered	Kim et al., 2016	Lee J. G. et al., 2019
	V-A- ErCas12a CRISPR-Mad7	TTTN, CTTN	5′ staggered	Liu Z. et al., 2020	Liu Z. et al., 2020
	V-A- CRISPR-Mb3Cas12a	TTV	5′ staggered	Wang Z. et al., 2020	–
	V-B- AaCas12b (C2c1)	TTN	5′ staggered	Teng et al., 2018	–
Alternative editing	**Cytosine base editing**				
	II- E -SpBE2	NGG	None	Lee et al., 2018	–
	II- E -HF2-SpBE2	from NGG/A to NGG	None	Liang P. et al., 2017	–
	II- E -SpBE3	NGG	Nick	Zhang H. et al., 2018	–
	II- E -Sp-BE4	NGG	Nick	Lee et al., 2018	–
	II- E -Sp-VQR-BE3	NGA	Nick	Lee et al., 2018	–
	II- E -SaBE3	NNGRRT	Nick	Liu et al., 2018	–
	Adenosine base editing				
	II- E -SpABE7.10	NGG	Nick	Liu et al., 2018	Yang L. et al., 2018
	II- E -SpVQR-ABE	NGA	Nick	Yang L. et al., 2018	–
	II- E -SaKKH-ABE	NNNRRT	Nick	Yang L. et al., 2018	–
	Prime editing				
	PE3	NGG	2 Nicks	Liu Y. et al., 2020	–

GE, genome editing; E, engineered Cas; GM, genetically modified model; DSB, double strand break; St1Cas9, Streptococcus thermophilus Cas9; CjCas9, Campylobacter jejuni Cas9; NmCas9, Neisseria meningitidis Cas9; FnCas9, Francisella novicida Cas9.

gene-specific nucleases in terms of DNA cleavage outcomes, repair pathways mechanisms (molecules involved and forms of DNA donors) and genotyping techniques.

CRISPR-Cas Systems

The CRISPR-Cas9 system is originally based on a ribonucleoprotein (RNP) complex composed of a nuclease (Cas9) driven by a dual-guide RNA (dgRNA) duplex (Jiang and Doudna, 2017). Cas9 cleavage capacity relies on its two nuclease domains, each cleaving one strand of the genomic DNA. Inactivation of either nuclease domain (nickase) generates a nick on the corresponding strand (Jinek et al., 2012), whereas inactivation of both domains (dead Cas9 or dCas9) completely abolishes its cleavage capacity. The native dgRNA (Deltcheva et al., 2011) is formed from a trans-activating CRISPR RNA (tracrRNA) harboring a complex secondary structure to interact with Cas9 and a CRISPR RNA (crRNA), that mostly encodes the 20 nucleotides that give the system its specificity. When formed, this RNP complex quickly interrogates genomic DNA for its specific protospacer adjacent motif (PAM). The PAM is a key factor because it defines the possibilities of DNA targeting sequences. For SpCas9, the targets are limited to a G-rich genomic region with a 5′-NGG-3′ PAM (Jinek et al., 2014; Nishimasu et al., 2014). PAM recognition is followed by specific gRNA (guide RNA) spacer (20 nucleotides) matching. A perfect match creates a targeted blunt DSB three nucleotides away from the PAM. A few mismatches between the gRNA and the targeted genomic DNA are tolerated at certain positions and may lead to off-target editing (Peng et al., 2018). Design of gRNA with the highest homology specificity possible for the targeted DNA sequence is essential to limit off-target edits (Ayabe et al., 2019). Available tools for rat genome editing with CRISPRs are described in **Table 1**. Off-target is less of an issue for animal model generation when compared to the use of gene editing as a therapeutic tool. Indeed, animals require multiple breeding, clearing lines from off-targets on chromosomes different from the one harboring the mutation of interest.

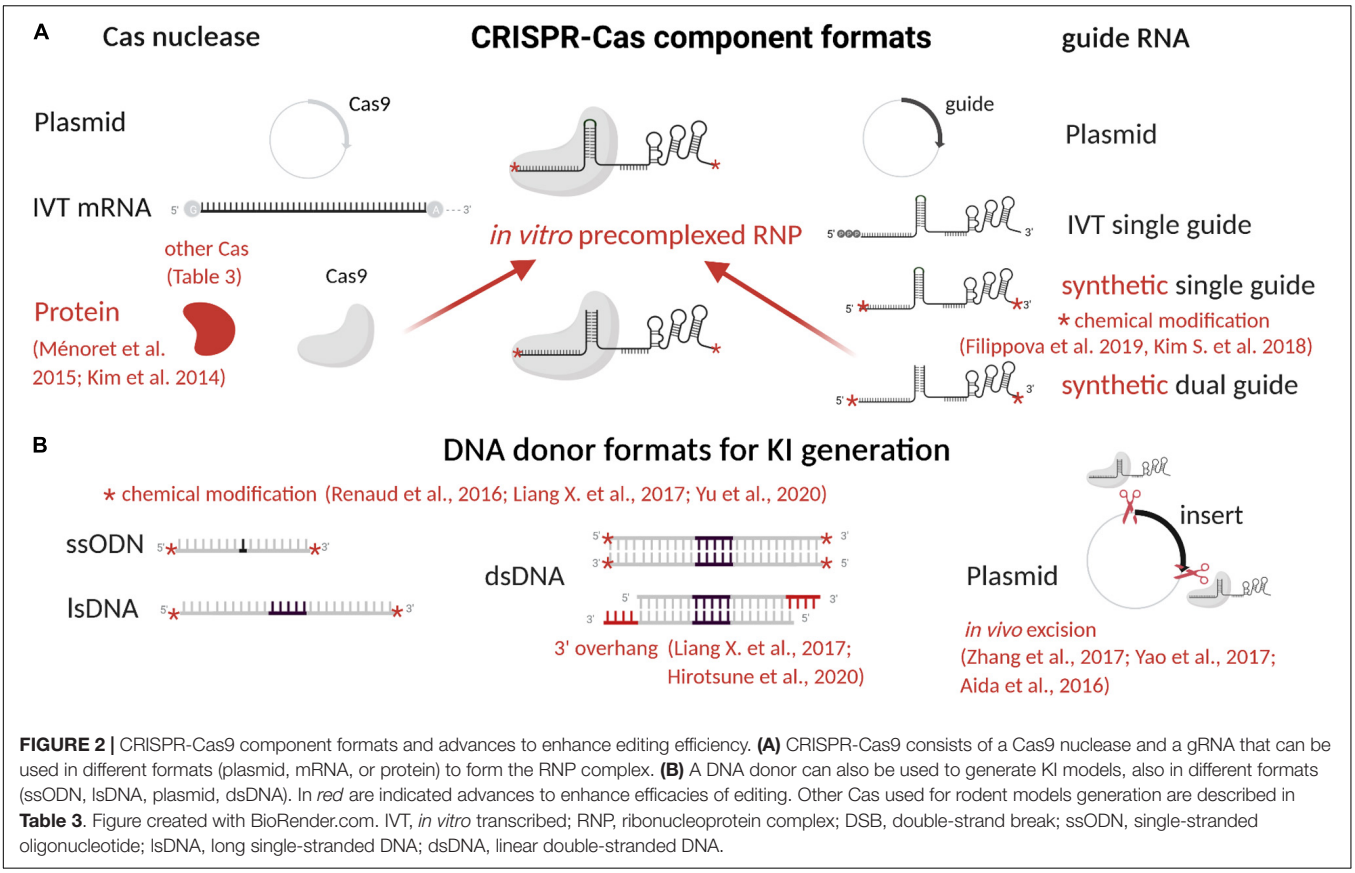

FIGURE 2 | CRISPR-Cas9 component formats and advances to enhance editing efficiency. **(A)** CRISPR-Cas9 consists of a Cas9 nuclease and a gRNA that can be used in different formats (plasmid, mRNA, or protein) to form the RNP complex. **(B)** A DNA donor can also be used to generate KI models, also in different formats (ssODN, lsDNA, plasmid, dsDNA). In *red* are indicated advances to enhance efficacies of editing. Other Cas used for rodent models generation are described in **Table 3**. Figure created with BioRender.com. IVT, *in vitro* transcribed; RNP, ribonucleoprotein complex; DSB, double-strand break; ssODN, single-stranded oligonucleotide; lsDNA, long single-stranded DNA; dsDNA, linear double-stranded DNA.

To expand the CRISPR toolbox, many variants of SpCas9 have been engineered and bacterial strains screened to either enhance specificity or broaden PAM opportunities. Variants (Pickar-Oliver and Gersbach, 2019) and SpCas9 ortholog classification (Makarova et al., 2020) have been recently reviewed. Many of these options have been used at least once to edit mouse embryos, but only a few have been applied to the rat. Those already applied to rodent genome editing are summarized in **Table 3**. Type V Cas have T-rich PAMs and other interesting features, such as staggered DSB generation, that make them complementary to SpCas9. For this reason, some orthologs of Cpf1 (Cas12a) are the most used after SpCas9, including *Acidaminococcus* sp. (AsCpf1) (Lee J. G. et al., 2019; Yeo et al., 2019) and *Lachnospiraceae bacterium ND2006* (LbCpf1) (Lee J. G. et al., 2019).

Classical genome editing, alternatives and their context of application have been recently reviewed in detail (Anzalone et al., 2020). Two of these, namely base editing and prime editing, have been used for rodent genome editing and are summarized in **Table 3**. Cytosine base editor has been engineered using either dCas9 or nickase to transform cytosine into a thymine (Komor et al., 2016; Nishida et al., 2016) and was further improved (Rees and Liu, 2018; Schatoff et al., 2019). Adenine base editor was engineered to mutate adenine into guanine more efficiently than Cas9 genome editing in human cells (Gaudelli et al., 2017). Several base editor variants have been applied to mouse embryos for single (Liang P. et al., 2017) or multiple (Liu et al., 2018; Zhang H. et al., 2018) base editing, whereas only the SpABE7.10 system has been applied in rats (Ma Y. et al., 2018; Yang L. et al., 2018). The main advantage of base editing is its capacity to generate targeted indels or a particular mutation without a DNA donor, enhancing its efficiency compared to classical genome editing. By avoiding DSBs, this system also allows multiplex editing on the same region of a chromosome (Lee H.K. et al., 2019). Its major limitations are bystander effect on non-targeted bases, cytosine and adenine limitations, targeted precision that restrict possibilities, and off-target effects as with classical genome editing. Prime editing is overcoming some of these limitations (Anzalone et al., 2019). This system allows mutation, short insertion and short deletion editing with limited indels generation in contrast to classical Cas genome editing. The first two versions of this system relied on a Cas9 nickase fused to a reverse transcriptase and a prime editing gRNA (pegRNA). This system induces nicking on the non-target strand and reverse transcription of the template encoded in the pegRNA to specifically modify the targeted locus. Prime editing 3 and 3b have been enhanced by the use of a second nickase with its own guide RNA, to target the strand that was not nicked by the pegRNA. Very recently, prime editing 3 has been successfully applied to genetically modify mouse embryos for the first time (Liu Y. et al., 2020). This particularly interesting approach will be applied eventually to generate genetically modified rat models.

TABLE 4 | CRISPR-Cas9 component format advantages, limits and advances.

Format	Advantages	Limitations	Advances demonstrated in any species (rat in bold)
Cas9			
Plasmid	No limit on insert size Easy engineering High expression	Delayed activity Mosaicism Increased off-targets Delayed activity	**Cas9 protein allowing rapid and more efficient editing** (Kim et al., 2014; Ménoret et al., 2015) Large editing toolbox variants **(Table 3)** Improved chromatin accessibility (Chen F. et al., 2017; Ding et al., 2019) **Cas9 engineered to activate repair pathways** (Charpentier et al., 2018; Tran et al., 2019) Cas9 engineering to be degraded in G1 (Gutschner et al., 2016; Charpentier et al., 2018; Lomova et al., 2019)
mRNA	Expression faster than plasmid Limit mosaicism and off-targets	Delayed activity *In vitro* transcription efficiency/toxicity	
Protein	Ready to cut Limit mosaicism and off-targets Affordable and high quality	Crystallization at high dose *In vivo* stability potentially immunogenic	
gRNA			
Plasmid	No limit on insert size Easy to engineer	Delayed activity	Chemical modification (Kim S. et al., 2018; Filippova et al., 2019) Essential sequence, secondary structures and functional modules of gRNA (Briner et al., 2014; Kartje et al., 2018) Overlapping gRNA (Jang et al., 2018) gRNA engineering to activate repair pathways (Nakade et al., 2018; Tran et al., 2019)
IVT sgRNA	Easy to produce and use Flexible in sequence and length Efficient	Time-consuming production Induced immune responses Limited in chemical modification	
Synthetic sgRNA	Affordable and high quality Chemical modifications Ready to use Efficient	Order full sgRNA for each project Long RNA synthesis Difficulties in adding fluorophore for tracking	
Synthetic dgRNA	Short RNA synthesis Low cost and high quality Same tracrRNA for all project Chemical modifications Fluorophores added for tracking Efficient	crRNA & tracrRNA hybridization *in vitro*	
DNA donor			
ssODN	Low cost synthesis High efficacy for mutation or short KI	Limited in length to 200nt	DNA synthesis progresses (Hao et al., 2020) **Chemical modification** (Renaud et al., 2016; Liang X. et al., 2017; Yu et al., 2020) Insertion close to cut site (Inui et al., 2014; Liang X. et al., 2017) 3′ overhang DNA donor (Liang X. et al., 2017; Hirotsune et al., 2020) Carry to cut site by Cas9 (Ma et al., 2017; Aird et al., 2018; Gu et al., 2018; Ling et al., 2020; Wang Z. et al., 2020) Carry to cut site by gRNA (Carlson-Stevermer et al., 2017; Lee et al., 2017) Carry to cut site by DNA donor engineering (Nguyen et al., 2020) DNA donor in vivo excision from plasmid (Aida et al., 2016; Yao et al., 2017; Zhang et al., 2017)
lsDNA	Usable for long KI	Limited in length Difficult to produce Mutated KI Expensive to synthesize	
dsDNA	Usable for long KI Easy to produce and engineer No limit on insert size	Few random insertions	
Plasmid	Usable for long KI Easy to produce and engineer No limit on insert size	Few random insertions	

IVT, in vitro–transcribed; gRNA, guide RNA; sgRNA, single gRNA; dgRNA, dual gRNA; ssODN, single-stranded oligonucleotides; lsDNA, long single-stranded DNA; dsDNA, linear double-stranded DNA.

Advances in CRISPR-Cas Production and Design for Rodent Genome Editing

The components of the CRISPR-Cas system, both for KO or KI, have been closely studied and enhanced to increase efficiency, decrease side effects, and offer better control over repair outcomes, as reviewed below. In particular, we summarized CRISPR-Cas9 component formats and their evolution in **Table 4** and **Figure 2**, and advances to increase KI efficiency are illustrated in **Figure 3**.

RNP Complex

KO and KI model's generation mainly depends on RNP complex cleavage efficiency. Many studies have been done to find RNP complex best settings. It has been clearly demonstrated

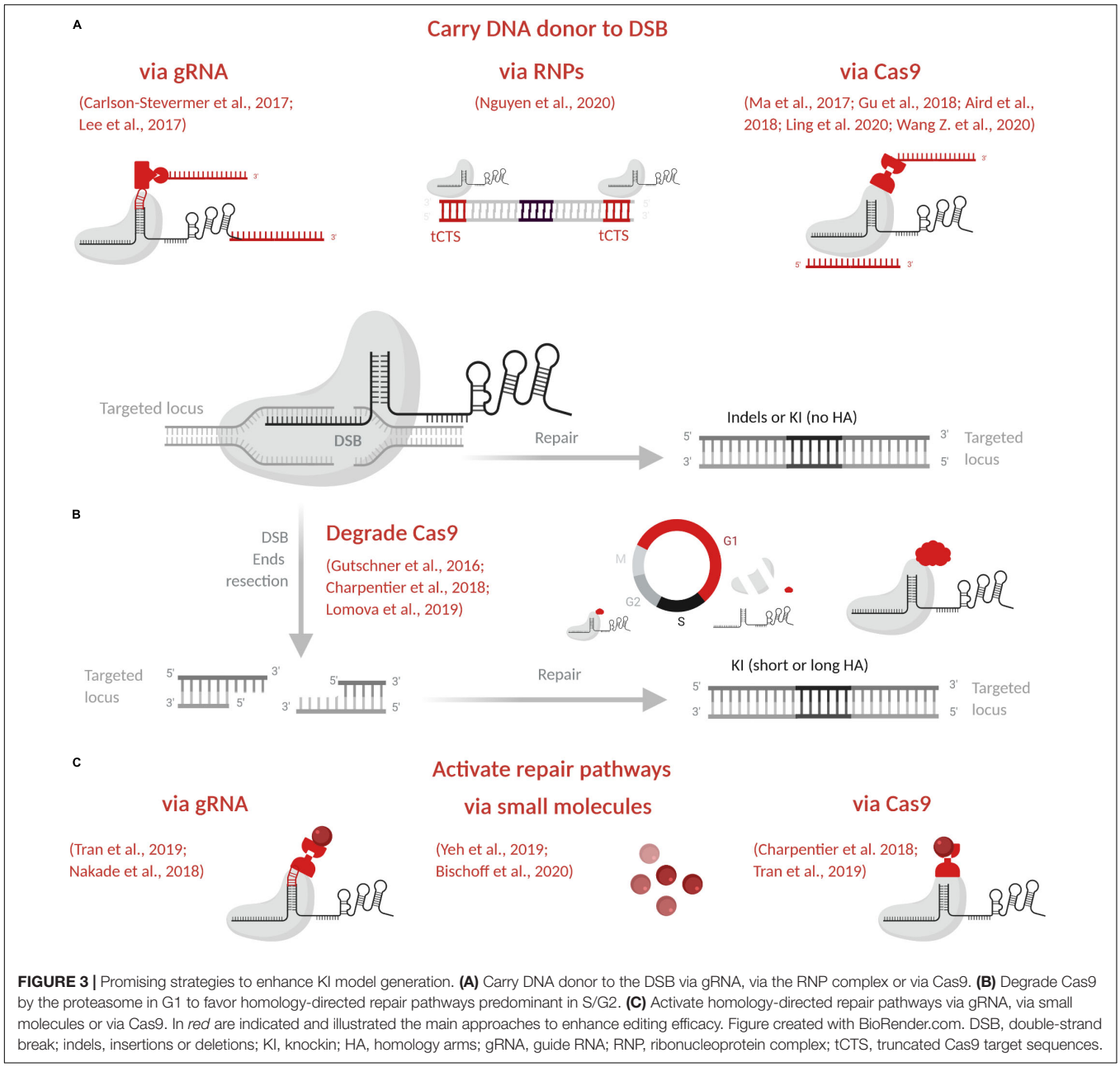

FIGURE 3 | Promising strategies to enhance KI model generation. **(A)** Carry DNA donor to the DSB via gRNA, via the RNP complex or via Cas9. **(B)** Degrade Cas9 by the proteasome in G1 to favor homology-directed repair pathways predominant in S/G2. **(C)** Activate homology-directed repair pathways via gRNA, via small molecules or via Cas9. In *red* are indicated and illustrated the main approaches to enhance editing efficacy. Figure created with BioRender.com. DSB, double-strand break; indels, insertions or deletions; KI, knockin; HA, homology arms; gRNA, guide RNA; RNP, ribonucleoprotein complex; tCTS, truncated Cas9 target sequences.

that the use of Cas9 protein allows transient and faster editing (Kim et al., 2014) necessary for proper animal model generation and increases efficiency of the RNP complex in mouse and rat zygotes (**Figure 2A** and **Table 4**) (Ménoret et al., 2015). Guide RNA's sequence has been extensively studied to better understand its flexibility and structure (**Table 4**) (Briner et al., 2014; Kartje et al., 2018) for improved efficacy. In cells, the 5′ triphosphate group on *in vitro*–transcribed gRNA induces the cell immune system and reduces editing efficacy. This reaction can be limited by phosphatase treatment or prevented by chemical modification of synthetic gRNA (Kim S. et al., 2018). Chemical modifications and gRNA optimization have been recently reviewed (Filippova et al., 2019) and offer a clear advantage for synthetic gRNA (**Figure 2A** and **Table 4**). Regarding their format, both dgRNA and single gRNA (sgRNA) display similar efficiency (Terao et al., 2016; Shapiro et al., 2020). Chromatin state can influence editing efficiency (Janssen et al., 2019; Verkuijl and Rots, 2019) and even prevent editing of gRNA with predicted high on target score. Two main strategies have been developed in cells only to open chromatin locally and increase editing efficiency with SpCas9 and other orthologs (**Table 4**). The first approach uses one or multiple dCas molecules to open chromatin in close proximity to the targeted locus (Chen F. et al., 2017). The second approach relies on fused chromatin-modulating peptides on SpCas9 and other Cas proteins (*Streptococcus pasteurianus* Cas9, *Campylobacter jejuni* Cas9, and others) (Ding et al., 2019). This field is still

TABLE 5 | Delivery methods.

Delivery methods	Cargo	Species /cell target	Location	Advantages	Limitations	References
				Physical delivery		
Microinjection	DNA donor - dsDNA (linear/plasmid) - dsDNA encoding gene-specific nucleases - lsDNA (>200nt) - ssODN (~100nt)	Mouse and rat zygote	Pronucleus or cytoplasm	- Delivery of large DNA fragments - Stable DNA in cell	- Time-consuming method - Expertise required (less for Cyt-MI) - Poor visualization pronucleus, flexibility of the oolemma and nuclear membranes in rat - Variability in efficiency depending on size, DNA quality or purity - Persistent expression and depending on host transcriptional/transductional machinery	1st description (Gordon et al., 1980; Palmiter et al., 1982; Mullins et al., 1990) dsDNA-ZFN (Geurts et al., 2009) dsDNA-TALEN (Tesson et al., 2011) dsDNA-Meganuclease (Ménoret et al., 2013) Efficiency (Charreau et al., 1996b; Hirabayashi et al., 2001) Complex/invasive method (Brinster et al., 1985; Charreau et al., 1996b)
	mRNA encoding gene specific nucleases	Mouse and rat zygote	Pronucleus or cytoplasm	- Moderate efficiency - Transient expression - Cyt-MI more efficient than PN-MI - Off-target reduced - Independent expression dependency of host transcriptional/transductional machinery (mRNA)	- Time-consuming - Expertise required (less for Cyt-MI) - Variation among batches of IVT mRNA - mRNA liable to degradation	mRNA-ZFN (Geurts et al., 2009) mRNA-TALEN (Tesson et al., 2011; Remy et al., 2014) mRNA-CRISPR (Ménoret et al., 2015) Meganucleases (Wang et al., 2014)
	Protein (RNP)	Mouse and rat zygote Mouse/ES	Pronucleus or cytoplasm	- Higher efficiency than using DNA or mRNA encoding gene specific nucleases - Short half-life within cells - Less mosaicism - Off-target cleavage reduced	-*In vivo* stability -Potentially immunogenic	(Ménoret et al., 2015; Wang et al., 2015; Jung C. J. et al., 2017)
Electroporation	DNA donor - dsDNA (linear/plasmid) - ssODN - lssDNA (600–1.5 kb)	Mouse and rat zygote	Uncontrolled cytoplasm (long DNA) Pronucleus (short lsDNA/ssODN)	- Easier delivery than DNA-MI - Processing simultaneously 50–60 zygotes in a short time - Efficient to deliver ssODN or lsDNA (<1 kb)	- Inefficient nuclear transport - Transient nuclear envelop breaking or cell-division required - Inefficient to deliver DNA > 1 kb	ssODN (Hashimoto and Takemoto, 2015; Kaneko and Mashimo, 2015; Qin et al., 2015; Chen et al., 2016; Wang et al., 2016; Remy et al., 2017) lsDNA (Miyasaka et al., 2018) Inefficient delivery dsDNA (Takabayashi et al., 2018)
	mRNA encoding Cas9 + sgRNA	Mouse and rat zygote	Uncontrolled	- Easier delivery than mRNA-MI	- Embryos are quite sensitive to pulse and toxicity is observed	Rat/mRNA encoding Cas9+sgRNA (Remy et al., 2017) CRISPR/mice/KO/HDR-KI (Qin et al., 2015) Mice/CRISPR/KO (Hashimoto and Takemoto, 2015; Hashimoto et al., 2016) Rat/ZFN/TALEN/Crispr/KO (Kaneko et al., 2014; Kaneko and Nakagawa, 2020) Rat/mice/Crispr/KO/KI (Kaneko and Nakagawa, 2020)
	Protein (RNP)	Mouse and rat zygote	Uncontrolled	- Easier delivery than RNP-MI	- High amount of cargo - Uncontrolled delivery amount	Cas9-RNP/mice/indels/large KO/HDR-KI/ssODN-KI (Wang et al., 2016) Cas9-RNP/mice/KO (Hashimoto et al., 2016)

(Continued)

TABLE 5 | Continued

Delivery methods	Cargo	Species /cell target	Location	Advantages	Limitations	References
GONAD	DNA - ssODN - lsDNA (<1 kb)/ Cas9 mRNA/sgRNA RNP	Mouse and rat	Oviduct	- *Ex vivo* embryo handling steps not required - Fewer animals used (e.g., recycling females possible)	- Not yet applicable to deliver long donor DNA (db or long ss DNA)	CasSmRNA + sgRNA/mice/KO (Takahashi et al., 2015) RNP/lsDNA/mice/KO/ssODN and lsDNA-based KI (Ohtsuka et al., 2018) Rat/ssODN based KI (Kobayashi et al., 2018; Takabayashi et al., 2018)
			Viral delivery methods			
AAV vectors (Non-enveloped, lsDNA)	DNA encoding Cas9/sgRNA (separate AAV or all-in-one AAV) – KI DNA cassette	Mouse and rat zygote (transduction)	Uncontrolled	- minimal immunogenicity - low toxicity - wide-range serotypes - No incorporation into the host genome	Low capacity (<5 Kb)	KO/Mice/separate AAV (Yoon et al., 2018) KO/KI/Mice/Rat/RNP Electroporation/AAVtransduction (Mizuno et al., 2018; Chen et al., 2019) (Edraki et al., 2019)
	DNA (expression cassette)	Mouse zygote microinjection	Cytoplasmic injection			(Yu et al., 2015)

MI, microinjection; Cyt-MI, cytoplasmic microinjection; PN-MI, pronuclear microinjection; DNA-MI, DNA microinjection; KI, Knockin; ssODN, single-stranded oligonucleotides; lsDNA, long single-stranded DNA; dsDNA, linear double-stranded DNA; HDR-KI, homology directed repair knockin; RNP, ribonucleoprotein complex.

emerging and requires further studies. There is a need for better understanding of genome editing hurdles to allow edits at any locus with high efficiency.

DNA Donor

DNA donors have been used in different formats to generate KI models: plasmids, single-stranded oligonucleotides (ssODNs), long single-stranded (ls)DNA, and linear double-stranded (ds)DNA (**Figure 2B** and **Table 4**). These formats and their design are important to direct repair toward KI. Because efficient KI generation is the most important issue currently, here we review the main aspects and advances regarding the DNA repair template and pathways.

Historically, transgenesis (Gordon and Ruddle, 1982; Palmiter et al., 1982; Mullins et al., 1990; Charreau et al., 1996b) and targeted mutagenesis using nucleases have been achieved using circular plasmids or an excised dsDNA, to introduce a complete expression cassette in rat and mouse genome (Cui et al., 2011; Brown et al., 2013). DNA synthesis advances in recent decades (Hao et al., 2020) have supported progress in genome editing (**Table 4**), allowing efficient synthesis of dsDNA, ssODNs and lsDNA, with increasing size and purity from commercial vendors. Nevertheless, yield issues persist with synthesis of long DNA fragments. Today, short sequence insertion and precise mutations are mostly generated using ssODNs. Its current synthesis limit is 200 nucleotides or fewer for most providers. A few years ago, lsDNA emerged as a new and efficient way to generate complex KI mouse (Miura et al., 2015; Miyasaka et al., 2018) and rat (Yoshimi et al., 2016; Miyasaka et al., 2018) models. Different production strategies have been developed, including *in vitro* transcription and reverse transcription (Miura et al., 2015), plasmid excision by nicking endonucleases (Yoshimi et al., 2016) and synthesis. High yield and purity are difficult to achieve for lsDNA production, leading to unexpected mutations in addition to the desired KI genotypes (Codner et al., 2018). Synthesis is quite expensive and limited to some kilobases depending on vendors (**Figure 2B** and **Table 4**). Chemically modified ssODNs, in cells and rodents, generally lead to higher editing efficiency (Renaud et al., 2016; Liang X. et al., 2017). A study on human cells showed increased KI efficacy using 5'-end–modified dsDNA (Yu et al., 2020). The proof of concept of this protection has clearly been demonstrated and will probably be tested for all DNA donor formats.

Several approaches have been developed to optimize DNA donor design, but no clear consensus has emerged regarding impact on KI efficiency. In human cells, some donors have shown better KI efficiency with ssODN complementary to the non-target strand (Richardson et al., 2016), but others have shown similar efficacy for both designs (Liang X. et al., 2017). In the same way, studies on human cells suggest better efficiency with asymmetric ssODNs (Richardson et al., 2016), whereas others report similar KI efficiency with both asymmetric and symmetric donors in mouse embryos (Lanza et al., 2018). Furthermore, in human cells (Liang X. et al., 2017) and mouse embryos (Hirotsune et al., 2020), dsDNA with 3' overhangs displays better KI efficiency (**Figure 2B** and **Table 4**). This improvement could be explained by necessary genomic DNA end resection for KI generation

during repair pathways, as discussed later. The only consensus regarding DNA donor design is that the inserted sequence should be as close as possible to the Cas9 cut site (**Table 4**) to yield efficient KI (Inui et al., 2014; Liang X. et al., 2017). To avoid multiple cleavages on the KI inserted sequences, silent mutations are introduced in the DNA donor close to the PAM.

Major hurdles remain for large (long donor) or complex KI (several ssODNs with complex sequence). One clear way to increase KI efficiency is to use the RNP complex to carry the DNA donor to the DSB (**Figure 3A** and **Table 4**). In this way, all KI components will be present at the same time and concentrate at the cut site. The stable and high affinity between biotin and streptavidin (Le et al., 2019) and the easy production of biotinylated DNA donor have inspired several approaches. Cas fused with avidin and a biotinylated DNA donor has been tested to generate modified mice (Ma et al., 2017; Gu et al., 2018; Wang Z. et al., 2020). The sgRNA has also been engineered to insert a specific S1M aptamer of streptavidin and improve KI generation in human cells (Carlson-Stevermer et al., 2017). To ensure tight linkage, guide RNA and the ssODN donor have also been chemically linked to crRNA (Lee et al., 2017). Covalent attachment of the DNA donor to a Cas9 fused to porcine circovirus 2 Rep protein has been also described (Aird et al., 2018). Recently, Cas9-ssODN conjugates generated chemically or via an adaptor complementary to part of the ssODN, have been used to enhance HDR-mediated genome editing in mouse zygotes (Ling et al., 2020). Another team has used the RNP complex itself in human cells, without modifying it, but by inserting 16-nucleotide truncated Cas9 target sequences (tCTSs) in the linear dsDNA donor (Nguyen et al., 2020). This tCTSs allows RNP recognition without cleavage or use of a dCas9.

Repair Pathways

NHEJ is the most used pathway for DSB repair which produces indels alleles by ligase IV direct ends ligation through well-described mechanisms (Frit et al., 2019). When a DNA repair template is available at the DSB, other pathways may be induced, based on homology recognition. In contrast to NHEJ, other repair pathways, i.e., HR, microhomology-mediated end joining (MMEJ), and single-strand annealing (SSA), depend on a DNA template and are predominant in S/G2 phases. To favor KI, different strategies with small molecules have been used to arrest cells at different phase of the cycle (Yeh et al., 2019; Bischoff et al., 2020) but these strategies are difficult to apply to embryos. To favor HDR pathways predominant in S/G2, Cas9 can be degraded by the proteasome in G1 phase (**Figure 3B** and **Table 4**) by fusion to geminin degron (Gutschner et al., 2016; Charpentier et al., 2018; Lomova et al., 2019). Mouse two-cell embryos have a long G2 phase (Palmer and Kaldis, 2016) and open chromatin state that is favorable for KI model generation. Gu et al. (2018) have taken advantage of these features to develop the two-cell homologous recombination (2C-HR)-CRISPR in mouse, to increase large KI efficiency with WT Cas9 or Cas9 fused to monomeric streptavidin coupled with a biotinylated donor. This approach has been reproduced in mouse using Mb3Cas12a (Wang Z. et al., 2020).

All of these repair mechanisms except NHEJ have a key first step in common: DSB end resection (for a review, see Ranjha et al., 2018). The MRE11-RAD50-NBS1 complex must first be recruited to DSB ends, where it drives CtIP and other resection molecules (Ranjha et al., 2018). Exo1 can further resect DSB ends to produce 3′ overhangs that will be coated by replication protein A (RPA). For HR, RPA will later be replaced by Rad51 to promote strand exchange, whereas for SSA, RPA-coated resected ends are recognized by Rad52 for processing by end annealing. Factors unique for MMEJ are still unclear, but it requires short resection, necessitating the inhibition by RPA end coating. The size of this resection is linked to the repair pathway that is active. Short resection will leave a short sequence for homology-driven repair, as with MMEJ (5–25 bp) and SSA (>20 bp), whereas long resection will allow for long homology recognition, as with HR (>500 bp), and no resection will trigger NHEJ. These features drive the design of DNA donor homology arms (Yao et al., 2017).

To favor KI, small inhibitors of NHEJ or essential molecules carried to the DSB via gRNA, via Cas9 (**Figure 3C** and **Table 4**) have been used. NHEJ inhibitors have mainly been tested on cells (for reviews, see Yeh et al., 2019; Bischoff et al., 2020) and SCR7, an inhibitor of ligase IV, has led to KI increase in mouse (Maruyama et al., 2015; Singh et al., 2015) and rat embryos (Ma et al., 2016). Cas9 in fusion with a domain of CtIP has shown increased KI efficiency in human cells and rats (Charpentier et al., 2018; Tran et al., 2019). In the same way, the use of a MS2 aptamer on the gRNA to carry CtIP showed better KI efficiency in cells than other molecules (Nakade et al., 2018; Tran et al., 2019). Small molecules treatments to increase KI efficiency have been reviewed (Yeh et al., 2019; Bischoff et al., 2020). No data was reported to date in rats or mice, and only two studies showed that RS-1 enhances KI efficiency in rabbit (Song et al., 2016) and bovine embryos (Lamas-Toranzo et al., 2020). Finally, tests on cells and mouse embryos have shown that ExoI overexpression enhances KI activity (Aida et al., 2016).

CRISPR-Cas9 has a repair profile closer to the environmental DSB's one compared to other nucleases with a high frequency of insertions of one nucleotide (Trimidal et al., 2019) and mainly repairs using out-of-frame indels (>70%) and microhomologies (Guo et al., 2018; Taheri-Ghahfarokhi et al., 2018).

One study on mouse embryos showed that multiple overlapping (at least > 5 bases) sgRNAs with ssODNs increase KI efficiency, probably by inducing shorter deletions (Jang et al., 2018) (**Table 4**). Several studies have designed plasmid donors with inserts flanked by gRNA recognition sites to excise it within a cell or zygote (**Figure 2B** and **Table 4**). This strategy may coordinate DSB and DNA donor availability at the cut site but can also create the same ends on both the DNA donor and the genomic DNA. It has led to increased KI in cells with various lengths of the homologous arms (Zhang et al., 2017), in mouse and monkeys embryos with HMEJ arms of 800 bp (Yao et al., 2017) or in cells and mouse embryos MMEJ homology arms of 40 bp (Aida et al., 2016). The results of these studies suggest that repair outcomes can be influenced or used to favor KI. Further experiments should be done in the rat to confirm these results.

Delivery Strategy Overview and System Optimization

Gene-editing efficiency by targeted-mutagenesis approaches, unquestionably depends on the delivery system used. In the following section, we describe the commonly used methods and recently developed strategies, which are summarized in **Table 5**. Latest methods are reported in **Figure 1**.

Microinjection

Since its development in mice in the early 1980s (Gordon et al., 1980; Palmiter et al., 1982), microinjection has become the most commonly used method to introduce different cargos into mouse and rat zygotes. Pronuclear injection, is a well-established method and allows the delivery of purified nucleic acid in any form (plasmid or dsDNA, lsDNA or ssODN, mRNA, gRNA, RNP) and any size (for review, see Giraldo and Montoliu, 2001). Nevertheless, the efficiency of the method is variable, depending in particular on the quality and size of DNA sources, and also the skill of the manipulator (Charreau et al., 1996b; Hirabayashi et al., 2001). In some cases, the pronucleus is hard to visualize and the flexibility of the oolemma and nuclear membranes, as in the rat, make delivery of DNA constructs more complex and invasive (Brinster et al., 1985; Charreau et al., 1996b). Cytoplasmic injection (CI) is an alternative to overcome these technical problems and has been described to deliver linearized DNA (Brinster et al., 1985), mRNA-encoding nucleases or sgRNA (Geurts et al., 2009; Tesson et al., 2011; Remy et al., 2014; Wang et al., 2014; Ménoret et al., 2015; Doe et al., 2018), allowing for a transient expression of nucleases and thus reducing off-target events. TALEN and CRISPR-Cas in the form of proteins can also be directly injected into the zygote pronucleus, cytoplasm, or both sequentially to achieve gene modifications (KO and/or KI). For proteins, efficiencies are higher for CRISPR and lower for TALEN than those observed with delivery in their DNA or mRNA forms (**Table 5**; Ménoret et al., 2015; Wang et al., 2015; Jung C. J. et al., 2017).

Electroporation

Delivery of ZFN, TALEN, or CRISPR-Cas9 nucleic acids or protein components using zygote electroporation enables generation of mice (Hashimoto and Takemoto, 2015; Qin et al., 2015; Hashimoto et al., 2016; Wang et al., 2016) or rats (Kaneko et al., 2014; Kaneko and Mashimo, 2015; Remy et al., 2017) carrying various genetic modifications (**Table 5**). These modifications include NHEJ-mediated indels (Kaneko et al., 2014; Hashimoto and Takemoto, 2015; Kaneko and Mashimo, 2015; Qin et al., 2015; Hashimoto et al., 2016; Wang et al., 2016; Remy et al., 2017), large segment deletions (Hashimoto et al., 2016; Wang et al., 2016), conditional KO (Miyasaka et al., 2018), double-KO (Teixeira et al., 2018), HDR-mediated precise nucleotide substitutions (Kaneko and Mashimo, 2015; Qin et al., 2015; Wang et al., 2016) or short sequence insertions using ssODNs (typically < 200 bp) (Hashimoto and Takemoto, 2015; Chen et al., 2016; Wang et al., 2016; Remy et al., 2017) and lsDNA (from 600 bp to 1.5 kb) (Miyasaka et al., 2018). In some studies, electroporation was done in mouse zygotes that were denuded of the zona pellucida (ZP) by a Tyrod's acid treatment (Qin et al., 2015; Chen et al., 2016; Wang et al., 2016), without affecting the early development unlike data reported in rats (Okuyama and Funahashi, 2012). Electroporation also can be applied to mouse and rat frozen zygotes for efficient introduction of CRISPR RNP complexes, without affecting embryo viability or development (Nakagawa et al., 2018; Kaneko and Nakagawa, 2020).

Electroporation is thus an excellent alternative to microinjection for genome editing in mice and rats, with similar or sometimes higher success rates. It also allows the simultaneous processing of many zygotes in a short time (e.g., a batch of 50 zygotes in few seconds) without requiring expensive equipment and operators with extensive training and expertise. Nevertheless, a major limitation is the low efficiency or even absence of efficacy of this method for introducing a large DNA fragment (>500 bp) using dsDNA; even if entry into the zygote cytoplasm is achieved, the migration into the nucleus is blocked (Remy et al., 2017). LsDNA (up to 1.5 kb) has been described as an alternative (Miyasaka et al., 2018) but with lower KI yields than those observed using short ssODNs. These results have not always been reproducible, probably because of an inefficient migration into the zygote pronucleus (Remy et al., 2017).

Genome Editing via Oviductal Nucleic Acid Delivery (GONAD)

GONAD has the advantages of electroporation without requiring sacrifice of embryo donor animals or *ex vivo* embryo manipulation. In this technique, the RNP complex is directly injected into the oviduct of a pregnant mouse or rat, followed by *in situ* electroporation. It was first described to generate NHEJ using Cas9 mRNA (Takahashi et al., 2015; Gurumurthy et al., 2016, 2019b) and then the improved GONAD (iGONAD) was reported by Ohtsuka et al. (2018) in mice to efficiently generate indels mutations, large deletions, and ssODN and lsDNA-based KI (up to 1 kb), by replacing Cas9 mRNA by Cas9 RNP. Other groups have demonstrated the efficiency of iGONAD in rats for gene disruption and ssODN-based KI (Kobayashi et al., 2018; Takabayashi et al., 2018) and in mice by substituting Cas9 with AsCpf1 (Ohtsuka et al., 2018) (for review see Sato et al., 2020).

Viral Vectors

Since efficacy of KI using long DNA donors is still low, AAV vectors have been used to deliver DNA cargo. Although AAV has a reduced packaging capacity (∼5.2 Kb), that limits their use in delivering large functional components of TALEN and SpCas9, some studies have reported AAV-mediated delivery (mainly with the serotype 6) (Ellis et al., 2013) to generate mutations in mouse and rat zygotes, by using either a dual-AAV system carrying SpCas9 and sgRNA in separate vectors (Yoon et al., 2018) or sgRNA and a shorter Cas9 ortholog in an "all-in-one" vector (Edraki et al., 2019). Two groups have also managed to generate KI mice (Mizuno et al., 2018; Chen et al., 2019) and rats (Mizuno et al., 2018) by combining zygote electroporation to deliver the RNP complex and AAV transduction to introduce a large donor dsDNA (up to 4.9 kb) with efficiency ranging from 6 to 100% depending on the viral

concentration (Mizuno et al., 2018). The method has not been rigorously compared with other methods and requires generation of high-purity AAV vectors.

Sleeping Beauty and PiggyBac transposons systems have been optimized to deliver CRISPR-Cas system into cells to increase gene editing efficiency and allow multi-allele targeting (Weber et al., 2015; Xu et al., 2017; Hu et al., 2018; Ye et al., 2019). Note, however, that CRISPR-Cas integration by transposon into the genome and its long-term expression in the cells could lead to off-target effects.

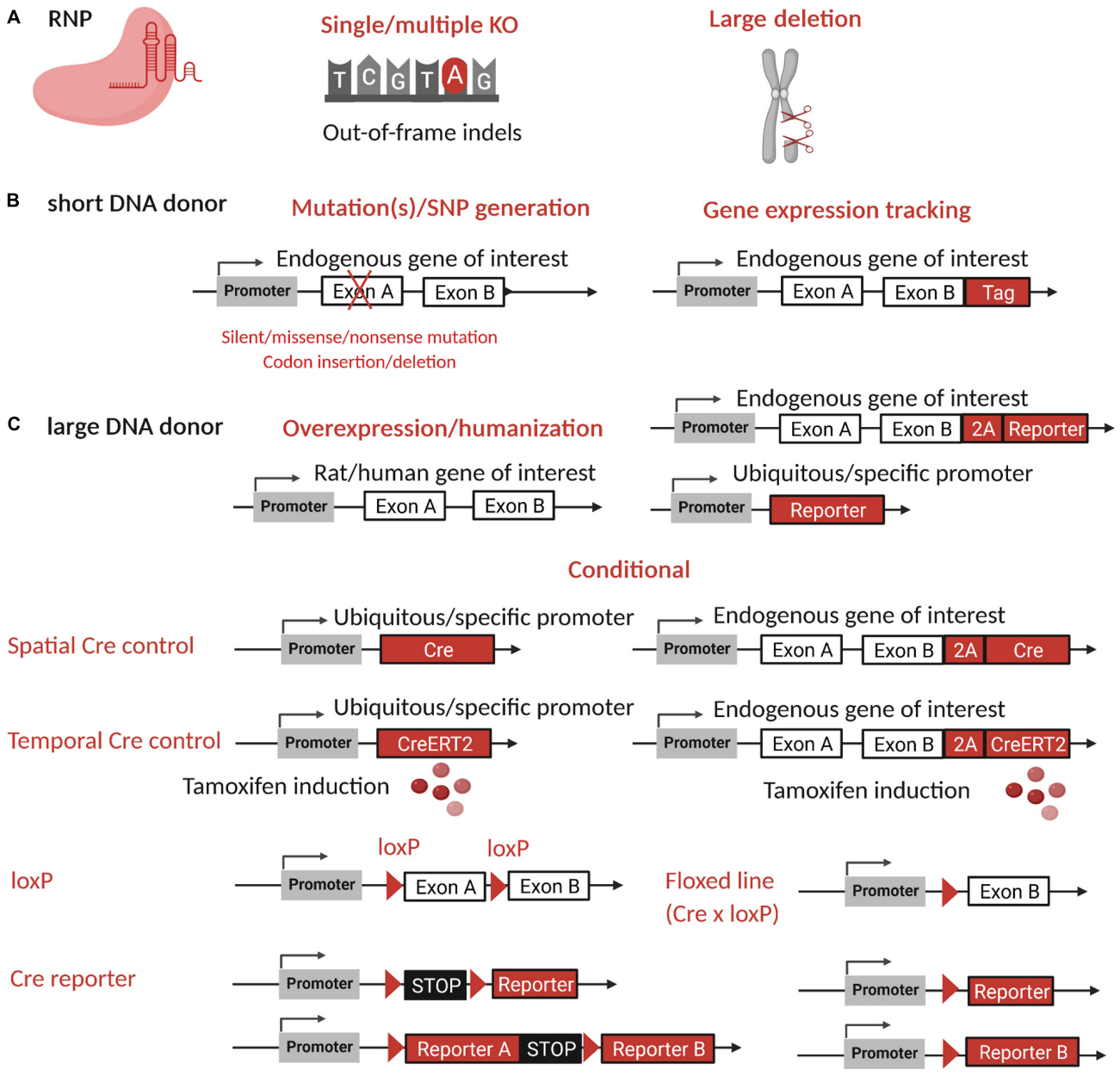

FIGURE 4 | Rat research model generation by CRISPR-Cas9 and applications. Strategies to generate research models by CRISPR-cas9 are multiple and very helpful for studies of gene function and diseases or to generate a reporter model. **(A)** The RNP alone can be used to create indels at one or more loci to generate single or multiple KO or a large deletion. **(B)** RNP with a short DNA donor (ssODN) can be used to generate a stop codon or mutations or to insert a Tag in the reading frame of the endogenous gene of interest. **(C)** A large DNA donor (either lsDNA, dsDNA, or plasmid) can be used to express a reporter gene in the reading frame of the endogenous targeted gene with a self-cleaving peptide, to generate conditional or inducible Cre/lox models with or without a reporter, or to overexpress the rat or human gene of interest or a reporter gene in a safe harbor locus. For expression of inserted genes, an endogenous or ubiquitous promoter or a specific promoter can be used to restrict expression to tissues or cell types. Figure created with BioRender.com. SNP, single nucleotide polymorphism; RNP, ribonucleoprotein complex; 2A, self-cleaving peptide; KO, knockout; indels, insertion or deletion; Cre, Cre recombinase.

Rat Research Models and Applications

Today, it is possible to generate a broad range of genetically modified models, from simple KOs with precise mutations or gene overexpression, to conditional or reporter models. Below, we describe the main strategies to develop these models, which also are illustrated in **Figure 4**. Main resources available to find and develop rat models are available in **Table 1**. **Table 6** describes models already developed to study genes of the immune system. Genome editing application in genetic disease studies is also explained and illustrated by the existing models listed in **Table 7**. Advantages of the rat as a model for those two applications are highlighted in this section.

Strategies to Develop Genetically Modified Models

Single, Multiple or Large Modifications

A KO model can be efficiently generated through out-of-frame indels (**Figure 4A**) by careful design of gRNA. Some of these will lead to a reading frame shift with a premature termination codon followed by mRNA degradation and no translation of the protein. All mechanisms of premature termination codon followed by mRNA degradation are not fully understood on mammals and exceptions exist (Dyle et al., 2020). Most often, the CRISPR-Cas system is designed to target one of the first exons of the gene, but another approach is to generate a promoter-less allele that can lead to a more severe phenotype than the KO model (El-Brolosy et al., 2019). In that case, KO can be easily confirmed by detection at the mRNA level. This strategy has not been used commonly, but it could be particularly useful in the rat, for which protein detection tools are limited. Mainly, these models have been developed by nuclease DSB induction, but adenosine-base editor is also an alternative with mouse and rat (Ma Y. et al., 2018; Yang L. et al., 2018; Wang X. et al., 2020).

Multiple KO models can be generated using multiple RNP complexes (Ma et al., 2014a,b), but to avoid large deletions, they should not be located on the same chromosome (**Figure 4A**). Translocation between chromosomes is also a risk that can be reduced using ssODNs and different Cas (Bothmer et al., 2020). Outcomes analysis for multiple KO can be challenging and should be carefully considered when designing CRISPR tools.

For large genomic KOs involving several consecutive genes, two DSBs can be induced by designing gRNA on both sides of the region of interest (**Figure 4A**). If both DSBs occur at the same time, the result will be a large deletion of this region of interest. To our knowledge, the biggest deletion achieved to date in rats is 24,499 Kb (Birling et al., 2017).

ssODNs that include a STOP codon can be used to create a nonsense mutation and inactivate a specific gene (**Figure 4B**). The rate of KI is usually lower than the frequency of indels, but because both the KI and a large fraction (>70%) of indels (Guo et al., 2018; Taheri-Ghahfarokhi et al., 2018) induce out-of-frame mutations, this increases the chance of obtaining a KO animal.

ssODNs containing a mutation observed in a human disease have been used to generate animal models (**Figure 4B**) such as for cystic fibrosis (Dreano et al., 2019; **Table 7**). The use of ssODNs will allow inclusion of specific features, such as restriction sites, to facilitate KI genotyping. Base- and prime-editing, are particularly fitting tools for generating mutations. Base editing has already been applied in the rat (Yang L. et al., 2018) but prime editing only in the mouse for now (Liu Y. et al., 2020).

Gene Overexpression

Overexpression of the gene of interest might be useful for gaining a better understanding of its role. The gene can be overexpressed by its insertion with its promoter or with an ubiquitous promoter (**Figure 4C**, right panel). In the past, this effect has been achieved through transgenesis, but expression of a randomly inserted cassette is affected by the genomic locus where it is inserted. Advances in genome-editing tools have made it possible to target a permissive locus, also called a "safe harbor," to overcome this issue (Saunders, 2020). *Rosa26* and *Hprt* are the most commonly used safe harbors that have been targeted in rat embryos (Kobayashi et al., 2012; Remy et al., 2014).

Humanized animal models are of great value to better study human diseases by insertion of the human gene into the animal genome (**Figure 4C**, right panel). For some projects, cDNA of the gene of interest is enough and can be used to generate humanized models, as it was done for a humanized model of cystic fibrosis (Birket et al., 2020).

Conditional Models

Site-specific recombinase systems (SSR) are used for conditional excision or inversion of the targeted site. Their application requires the generation of two lines, one expressing the specific SSR and one displaying the two specific DNA sites flanking the locus of interest (**Figure 4C**, lower panel). These lines are then crossed to combine both mutations in a single animal line (Birling et al., 2009). The Cre/lox system is the most commonly used SSR system option for mouse conditional models, even though other variants and other systems (FLP-FRT, Dre-rox, Nigri-nox, and others) have been used and combined. To the best of our knowledge, Cre/lox is the only SSR system that has been used to generate conditional rat models. The use of targeted nucleases permits precise insertion of Cre behind the endogenous promoter (**Figure 4C**, lower panel), allowing reliable and relevant tissue or cell specific expression of Cre (for a review see Kim H. et al., 2018). To achieve temporal control of the gene of interest, drug-inducible systems are used (Navabpour et al., 2020). Fusion of Cre with estrogen receptor 2 (Cre-ERT2) leads to sequestration of Cre in the cytoplasm, and the addition of tamoxifen at a certain time point induces Cre-ERT2 translocation into the nucleus, allowing Cre to recombine loxP sites (**Figure 4C**, lower panel). These animal lines should be carefully bred and analyzed to limit toxicity and leakage (Song and Palmiter, 2018). Cre/CreERT2 models characterization at some point requires the use of Cre reporter models expressing a floxed STOP before a reporter gene (**Figure 4C**, lower panel). After Cre recombination, reporter expression is turned on and specific expression can be characterized. Validation of loxP models requires Cre or CreERT2 models (**Figure 4C**, lower panel). The observed phenotype will then be specific to the Cre expressing tissues and the loxP line tested.

TABLE 6 | Genetically engineered rat models for genes of the immune system.

(A)

Immunology domain	Gene/genetic modification	Genomic tool used	References	Phenotype and rats vs. mice	Depository or breeder company ID
Immuno-deficient models	*Rag1/KO* or *Rag2*/KO	Meganuclease CRISPR	Zschemisch et al., 2012; Ménoret et al., 2013; Tsuchida et al., 2014; Chang et al., 2015; Noto et al., 2018	T-B-NK+. *Rag1*/KO or Rag2/KO rats and mice show similar phenotypes	*Rag2* KO; NBRP Rat #0894
	Foxn1/KO	CRISPR	Goto et al., 2016	T-B+NK+. *Foxn1*/KO rats and mice show similar immune and albino phenotypes	RGD #10053598 #10053601
	Il2rg/KO	TALENs CRISPR	Mashimo et al., 2010; Samata et al., 2015; Kuijk et al., 2016	T-B+/-NK-. *Il2rg*/KO rats and mice show similar phenotype	#0585
	Rag1/KO *or Rag2*/KO *or Prkdc*/KO *or and Il2rg*/KO	ZFNs TALEN CRISPR	Mashimo et al., 2012; Ménoret et al., 2018; He et al., 2019	T-B-NK-. KO rats and mice show similar phenotypes	IL2Rg-Rag2 KO; NBRP Rat #0895 RRG (TRIP)
	Human SIRPa/Tg	BAC microinjection	Goto et al., 2016; Jung et al., 2016; Yang X. et al., 2018; Ménoret et al., 2020	↓ phagocytosis human cells. hSIRPa/Tg rats and mice show similar phenotype	
	Rag1/KO or Rag2/KO *or Prkdc*/KO *or* +*Il2rg*/KO+*human SIRPa*/Tg	ZFNs, TALENs, CRISPR	Yang X. et al., 2018; Ménoret et al., 2020	T-B- NK-, ↓ phagocytosis human cells Similar phenotypes in KO and Tg rats and corresponding mice as well in KO NOD mice which have a spontaneous mutation in Sirpa	RRGS (TRIP)
	Ighm, Iglc, Igkc /KO	ZFNs	Ménoret et al., 2010; Panzer et al., 2018	T+B-NK+. *Ighm*/KO and *IgKc*/KO rats and mice show similar phenotype	IgM KO (Ligand)
	Human Ig heavy and/or light chain loci/Tg	BAC microinjection	Osborn et al., 2013; Ouisse et al., 2017; Xu et al., 2018	Production of human IgG binding domains for the generation of fully human mAbs *Human Ig heavy and/or light chain loci*/Tg rats and mice show similar phenotype	Ligand
	C3/KO	CRISPR	Xu et al., 2018	Role of complement in neuropathy during chemotherapy model not available in mice because of defects in complement activation in mice	RGD #19165133
CDs and membrane molecules	*HLA-B27* + *hb2m*/Tg	DNA microinjection	Hammer et al., 1990	*HLA-B27* + *hb2m*/Tg rats are a much better model of spondyloarthropathy than are *HLA-B27* + *hb2m*/Tg mice	*HLA-B27 RGD #7387221*
	hCD55 + *hCD59*/Tg	DNA microinjection	Charreau et al., 1996a, 1999	*hCD55* + *hCD59/Tg* rat hearts were heterotopically grafted in primates Not possible for corresponding mice	/
	hCD46/Tg	DNA microinjection	Niewiesk et al., 1997	Model of measles infection and complement control. *hCD46*/Tg rats and mice show similar phenotypes	/
	hCD4/hCCR5/Tg	DNA microinjection	Keppler et al., 2002	*hCD4/hCCR5*/Tg rats are a closer model to human *hCD4/hCCR5*/Tg mice exhibited very little or no productive infection	/
	hFasL/Tg	DNA microinjection	Tesson et al., 1999; Bouchet et al., 2002	Expression in endothelial cells Model not available in mice	/
	hCD21/Tg	DNA microinjection	Yang et al., 2003	Model of EBV infection *hCD21*/Tg rats and mice show similar phenotypes	/
	hCD64/Tg	DNA microinjection	van Vuuren et al., 2006	Depletion of macrophages a CD64-immunotoxin and inhibition of arthritis Transgenic rats and mice have similar expression	/
	hP2Y2R/Tg	Lentiviral vector	Agca et al., 2009	Tissue inflammation, increase in certain leukocyte populations No hP2Y2R transgenic mouse line generated	/
	Cd247 (CD3 ζ chain)/KO*	ZFNs	Rudemiller et al., 2014	Fewer kidney lesions in a model of hypertension similar immune phenotype in *Cd247*/KO rats and mice in T cell signaling and depletion of T cells No model of hypertension analysis in *Cd247*/KO mice	*RGD #6484582 #6484564 #6484568*

(Continued)

TABLE 6 | Continued

(A)

Immunology domain	Gene/genetic modification	Genomic tool used	References	Phenotype and rats vs. mice	Depository or breeder company ID
	Tlr4/KO	TALENs	Ferguson et al., 2013	*Tlr4*/KO rats and mice show similar decreased pro-inflammatory cytokine secretion upon lipopolysaccharide stimulation	*RRRC* #694
	Cd40/KO*	CRISPR	Haller et al., 2017	*Cd40*/KO rats have fewer kidney lesions in a model of hypertension than mice No model of hypertension analysis in *Cd40*/KO mice	*RRRC #840*
	Adora2b/KO*	ZFNs	Nayak et al., 2015	Adora2b/KO rats but not mice showed decreased pro-inflammatory cytokine secretion and less cardiac and renal injury/fibrosis in response to hypertension	RGD #6484715
	Clec1/KO	ZFNs	Lopez Robles et al., 2017	*Clec1*/KO rats but not mice showed increased inflammatory responses by DCs	*(TRIP)*
	Cd59/KO	CRISPR	Yao and Verkman, 2017b	Cd59/KO rats and not mice (showed mild hemolytic anemia and a faithful model of neuromyelitis optica	RGD #13792606
	Kv1.3/KO	ZFNs	Chiang et al., 2017	Kv1.3 KO rats are a better and closer model to human. Mouse T cells, unlike rat or human T cells, co-express additional redundant Kv1 channels	/
Cytokines/ secreted products and their receptors	*Avp*/Tg	DNA microinjection	Jessop et al., 1995	A model for the study of thymic arginine vasopressin in T cell differentiation No analysis of AVP expression in thymus of transgenic mice	/
	Ifng/Tg	DNA microinjection	Egwuagu et al., 1999a,b	IFNgamma expression in the eye in a model of uveitis Conflicting results: IFN-g exacerbates uveitis in the rat and confers protection in the mouse	/
	TGFb1/KO*	ZFNs	Chen et al., 2013	Rats and mice TGFb1/KO with a T cell-specific deletion of the Tgfb1 gene developed lethal immunopathology in multiple organs	RGD #5131989
	Il22bp/KO	CRISPR	Martin et al., 2016	IL22BP protective in models of colitis and psoriasis	(TRIP)
	Ifnar1/KO	CRISPR	Qaisar et al., 2017	Absence of IFN-I responses *Ifnar1*/KO rats and mice not analyzed in the same way	RGD #12910493 #12910494
	Il15/KO	ZFNs	Renaud et al., 2017	A genetic model of NK-cell deficiency in rats *Il15/KO* rats and mice show similar phenotypes	RRRC #769
	Tbet/KO	ZFNs	Ma Z. G. et al., 2018	T-bet can direct Th1 lineage commitment *Tbet*/KO rats and mice show similar phenotypes	/
	Csf1r/KO	ES cells	Pridans et al., 2018	Absence of most macrophages in most tissues. Macrophages effects in development of multiple organ systems in rats were distinct from those reported in mice	/
	Csf1r-GFP/KI	DNA microinjection	Irvine et al., 2020	*Csf1r-GFP/KI* rats and mice show similar phenotypes	/
Intracellular molecules	*HMOX1*/Tg	DNA microinjection	Braudeau et al., 2003	*HMOX1*/Tg only described in rats	/
	Hmox1/KO	ZFNs	Atsaves et al., 2017	*Hmox1*/KO rats and mice show similar phenotype with generalized inflammation and kidney lesions and lethality	
	Ian5/Tg	PAC microinjection	Michalkiewicz et al., 2004	A model that shows the essential role of IAN5 for lymphoid development. IAN5 rescues lymphopenia in BB rats with a mutation in the *Ian5* gene	/
	Notch1/Tg	DNA microinjection	van den Brandt et al., 2005	Blockade of thymic development and T cell lymphopenia *Notch1*/Tg rats and mice show similar phenotypes	/
	Selenoprotein M/Tg	DNA microinjection	Hwang et al., 2008	Maintenance of a high level of antioxidant status Selenoprotein M/Tg rats and mice show similar phenotypes in brain	/
	Bcl2/Tg	DNA microinjection	Iscache et al., 2011	Increased B cells and immunoglobulins *Bcl2*/Tg rats and mice show similar phenotypes	/
	Cyp2j4/KO	ZFNs	Behmoaras et al., 2015	Cyp2j4 determines a profibrotic macrophage transcriptome Implications in various inflammatory conditions Similar results in *Cyp2j4/KO* rats and mice	RGD #12904679

(Continued)

TABLE 6 | Continued

(A)

Immunology domain	Gene/genetic modification	Genomic tool used	References	Phenotype and rats vs. mice	Depository or breeder company ID
	Ahr/KO	ZFNs TALENs CRISPR	Harrill et al., 2013; Phadnis-Moghe et al., 2016	A variety of T and B cell alterations. *Ahr*/KO rats are more analyzed than Ahr/KO mice Rats showed other organ alterations	RGD #12903250 (Horizon Discovery); RGD #12903272 (Horizon discovery) RGD #13838845 (not available) RRRC#831 (CRISPR) RGD #15090819 #15090817 (TALEN, not available)
	Aire/KO	ZFNs	Ossart et al., 2018	Autoimmunity in several organs *Aire*/KO rats not observed in *Aire*/KO mice	(TRIP)
	Prox1 promoter-EGFP/Tg	BAC microinjection	Jung E. et al., 2017	Visualization of all lymphatic vessels *Prox1 promoter-EGFP/Tg* rats and mice show similar phenotypes	/
	Eogt/KO	TALENs	Hao et al., 2018	*O*-GlcNAc glycosylation deficiency with defect in Notch signaling in autoimmune hepatitis *Eogt/KO* rats and mice show similar phenotypes	/
	Paraoxonase 1/KO	CRISPR	Bai et al., 2018	Thymocyte blockade at the CD4/CD8 double-negative to double-positive transition stage No mouse model reported	RGD #12790692 #12790698 #12790695
	S100A8 transgenic rats/Tg	DNA microinjection	Okada et al., 2018	Altered macrophage function in a colitis model *S100A8/Tg* rats and mice show similar phenotypes	/

(B)

	Gene/KO	
Miscellaneous	*Snx25*/KO, *Axl*/KO*, *Cd14*/KO*, *Cd55*/KO, *Cd226*/KO, *Cyba*/KO*, *Cybb*/KO*, *Fyn*/KO*, *Gpr183*/KO*, *Ifnar1/KO*	Unpublished, available at MCW RGD

**Performed in the Dahl/S strain. WCM RGD, Wisconsin Medical College Rat Genomic Database. EBV; Epstein Barr virus.*

Other systems have been used in mouse and rat for spatiotemporal control. Tetracycline (Tet) on or off systems, like SSR systems, require two lines, one carrying a Tet (or doxycycline, its derivative)-sensitive transcriptional activator and one on the targeted locus carrying the Tet-responsive promoter element (Kim H. et al., 2018). The use of Tet systems for the development of transgenic mice has been reviewed previously (Sun et al., 2007) and applied to the generation of inducible rat models (Tesson et al., 1999; **Table 6**). For cell specific depletion, the diphtheria toxin receptor can be expressed under a cell specific promoter such as CX3CR1 for microglia depletion in rat (Vichaya et al., 2020).

Rat research is long way behind mouse studies for development of conditional models because of the decades-long use of mouse ES cells (Ramírez-Solis et al., 1995). Use of ES cells remains time consuming in mouse and technically challenging in rat. Efforts have currently been deployed to generate conditional models using CRISPR-Cas9 with all the difficulties previously discussed for large and complex insertion. Overcoming these hurdles is a major issue for both mouse and rat but it is required for the rat. A multicenter study in mice showed that loxP KI using two ssODNs and RNP complexes is less efficient than using a single long DNA donor (Gurumurthy et al., 2019a). Sequential insertion of each loxP ssODN by microinjection and electroporation of one and two-cell embryos has also been tested but is technically demanding (Horii et al., 2017).

Reporter and Tagged Rat Models

Transgenic ubiquitous reporter models have been generated with different fluorophores and promoters. The most developed and used models are animals that express fluorogenic proteins in different tissues, such as CAG-GFP rats (Remy et al., 2014; Ménoret et al., 2015). Today, with CRISPR-Cas systems, a reporter gene or a tag can directly be inserted at the end of the reading frame by replacing the stop codon of the endogenous locus of interest (**Figures 4B,C**, upper left panel). A fusion protein or two separated molecules expressed at the same level can be generated using self-cleaving peptides. Our team has generated a KI IL22bp-T2A-eGFP rat model to identify cells expressing this gene (submitted). For advanced reporter models, conditional

TABLE 7 | Genetically modified rat models of human genetic diseases.

System/organ affected	Human genetic disease	Gene/genetic modification	Genomic tool used	References	Rats vs. mice	Depository or breeder company ID
Cardiovascular	pulmonary arterial hypertension	*BMPR2/KO*	ZFN	Ranchoux et al., 2015; Hautefort et al., 2019; Manaud et al., 2020	*Bmpr2* KO rats showed pulmonary vascular cell phenotypes closer to human patients than in *Bmpr2* KOmice	*RGD#38501086 (not available) RGD #14975305 #14981588*
	Primary pulmonary hypertension 4 (PPH4)	*Kcnk3/KO*	CRISPR-Cas9	Lambert et al., 2019	Rats have a *Kcnk3* gene as humans do but mice do not	/
	Atrial fibrillation, familial, 18 (ATFB18)	*Myl4/KO*	CRISPR-Cas9	Peng et al., 2017	This model reproduces the human disease No *Myl4/KO* mouse model is reported	/
	Familial hypertrophic cardiomyopathy and myocardial genetic diseases	Myh7b/KO	CRISPR-Cas9	Chen et al., 2020	This model reproduces the human disease No *Myh7b/KO* mouse model is reported	/
	Danon disease	*Lamp2/KO*	TALEN	Wang et al., 2017; Ma S. et al., 2018	*Lamp2*-KO rats could be a more valuable animal model for DD than *Lamp2*/KO mice	*RGD #13703119*
Nervous system	Epileptic encephalopathy, early infantile, 63 (EIEE63)	*Cplx1/KO*	CRISPR-Cas9	Xu et al., 2020	*Cplx1*/KO rats and mice show different phenotypes Rat model reproduces the disease better	
	Dystonia 25 (DYT25)	*Gnal/KO*	CRISPR-Cas9	Yu-Taeger et al., 2020	*Gnal/KO* rats show early symptoms as in patients not seen in *Gnal*/KO mice	/
	Cockayne syndrome	*Ercc6/KO (KI R571X)*	CRISPR-Cas9	Xu et al., 2019	The brain is more affected in CSB-deficient rats vs. mice	/
	Neonatal hydrocephalus	*L1cam/KO*	CRISPR-Cas9	Emmert et al., 2019b	*L1cam/*KO rats and mice show similar phenotypes similar to those of patients	*RRRC #850 + 851*
		Ccdc39/KI point mutation c.916+2T	CRISPR-Cas9	Emmert et al., 2019a	*Ccdc39* KO rats and mice show similar phenotypes Rats are more suitable for imaging and surgical experiments	/
	Schizophrenia	*Drd2/KI reporter*	CRISPR-Cas9	Yu et al., 2016	Inter-species difference of DRD2 expression between rats and mice	/
	Amyotrophic lateral sclerosis	*Fus/KI point mutation R521C*	CRISPR-Cas9	Zhang T. et al., 2018	Fus/KI rats and mice show an altered phenotype with subtle differences	/
	Neurofibromatosis type 1	*Nf1/KO*	CRISPR-Cas9	Moutal et al., 2017; Dischinger et al., 2018	*Nf1*/KO rats have a more pronounced phenotype than *Nf/* KO mice	/
	Cystic leukoencephalopathy	*RNaseT2/KO BigDel*	CRISPR-Cas9	Sinkevicius et al., 2018	No *RNaseT2*/KO mice reported	RGD #13781890, not available
	Epileptic encephalopathy, early infantile, 24 (EIEE24)	*Hcn1/KO*	TALEN	Nishitani et al., 2019	*Hcn1*/KO rats but not *Hcn1*/KO mice exhibited epilepsy	*NBRP Rat #0821 #0820 #0819 #0822*
	MECP2-related severe neonatal encephalopathy, Rett-like syndrome (RTT)	*Mecp2/KO*	ZFN	Engineer et al., 2015	*Mecp2/KO* rats displayed more symptoms of RTT than KO mice	*RGD #11567272; Horizon Discovery*
	Fragile X syndrome/ Asperger syndrome, X-linked, 1 (ASPGX1)	*Fmr1/Nlgn3/DKO*	ZFN	Hamilton et al., 2014	Similar phenotype for Fmr1/Nlgn3/DKO rats and mice. Rats more suitable than mice for analysis of complex behavioral and social activities	RGD #11568700; Horizon Discovery; Nlgn3) RGD #11568040; Horzon Discovery; Fmr1 KO; RGD #11553873
	Phelan-McDermid syndrome	*Shank3/KO* *Shank3/KO BigDel*	ZFN CRISPR-Cas9	Harony-Nicolas et al., 2017 Song et al., 2019	*Shank3*-KO rats showed normal social interaction and self-grooming behaviors whereas *Shank3*-KO mice do not	/
	Angelman syndrome	*Ube3A/KO BigDel*	CRISPR-Cas9	Dodge et al., 2020	As in patients, *Ube3A/KO rats* bear a large deletion of the gene whereas *Ube3A/KO* mice not	/

(Continued)

TABLE 7 | Continued

System/organ affected	Human genetic disease	Gene/genetic modification	Genomic tool used	References	Rats vs. mice	Depository or breeder company ID
	Intellectual deficiency from genetic origin	*Cplx1/KO*	CRISPR-Cas9	Xu et al., 2020	*Cplx1/KO* rats showed ataxia, dystonia, exploratory deficits, anxiety and sensory deficits but normal cognitive function	/
	Essential tremor	*Aspa and Hcn1/KO*	TALEN	Nishitani et al., 2020	*Aspa and Hcn1/KO* rats developed tremor	*NBRP Rat #0806 #0805 (Aspa KO)*; Cf **Table 6** *pour Hcn1 KO*
	Ataxia-telangiectasia	*Atm/KO*	ZFN	Quek et al., 2017	*Atm*/KO rats show cerebellar atrophy and neurodegeneration which are poorly recapitulated in *Atm*/KO mice	*NBRP #0627 #0649*
	Autism spectrum disorder	*Cntnap2/KO*	ZFN CRISPR	Scott et al., 2018	*Cntnap2/KO* rats better recapitulate certain behavioral symptoms than *do Cntnap2*/KO mice	*RGD #11568646; Horizon Discovery; RGD #25330087 (CRISPR);*
		Shank2/KO	ZFN	Modi et al., 2018	*Shank2/KO* rats show behavior and electroencephalography abnormalities not seen in *Shank2*/KO mice	/
	Canavan disease	*Aspa/KO*	TALEN	Nishitani et al., 2016	*Aspa*/KO rats and mice show similar phenotypes similar to those of patients	*NBRP Rat #0806 #0805*
	Familial focal epilepsy	*Depdc5/KO*	TALEN	Marsan et al., 2016	Homozygous Depdc5/KO rats and mice have similar phenotypes but heterozygous Depdc5/KO rats and not mice had altered neuron excitability and firing patterns	NBRP Rat #0739
	Parkinson's disease	*Lrrk2/KO*	ZFN	Ness et al., 2013	*LrrK2*/KO rats and mice show similar phenotypes similar to those of patients	*RGD #7241053; Lrrk1/Lrrk2 KO Horizon Discovery RGD #7241047; Lrrk1/Lrrk2 KO Horizon Discovery RGD #7241050; Lrrk2/KO; Horizon discovery RGD #7241056; Lrrk2/KO; Horizon Discovery*
	Alpha-synuclein autosomal dominants forms of Parkinson's disease	*SNCA-A53T-A30P/Tg*	DNA microinjection	Lelan et al., 2011	*SNCA-A53T* transgenic rats and mice have similar phenotypes	/
	Familial Parkinson's disease	*DJ-1 and Pink1/KO*	ZFNs	Sun et al., 2013	*DJ-1 and Pink1/KO* rats and mice show similar phenotypes similar to those of patients	*DJ-1 RGD #7241054 + RGD #7241049 Pink1/KO; Horizon discovery*
	congenital generalized lipodystrophy	*Bscl2/KO*	ENU	Ebihara et al., 2015	*Bscl2/KO* rats have brain reduction and azoospermia as in patients, *Bscl2/KO* mice do not reproduce these pathologies	*NBRP Rat #0763*
	Autosomal-dominant lateral temporal lobe epilepsy	*LGI1/KO*	ENU	Baulac et al., 2012	Rats reproduce the human disease and are complementary to the KO mice	NBRP Rat #0656
Gastrointestinal	Hereditary tyrosinemia type I	*Fah/KO*	CRISPR	Zhang et al., 2016	*Fah/KO* rats developed liver fibrosis and cirrhosis, not observed in Fah/KO mutant mice	*RGD #10002791 (TALEN; PhysGenKO) RGD #14398825 (CRISPR) RGD #14398828 (CRISPR*

(Continued)

TABLE 7 | Continued

System/organ affected	Human genetic disease	Gene/genetic modification	Genomic tool used	References	Rats vs. mice	Depository or breeder company ID
	Hirschsprung disease	*Ednrb/KO*	CRISPR-Cas9	Wang et al., 2019a	*Ednrb*/KO rats in a particular strain caused embryonic lethality and megacolon as in certain strains of *Ednrb*/KO mice	/
	Rotor syndrome	*OATP1B2 /KO*	CRISPR-Cas9	Ma et al., 2020	*OATP1B2 /KO* rats reproduce the hyperbilirubinemia observed in patients	/
	Atypical hereditary non-polyposis colorectal cancer	*Msh6/KO*	ENU mutagenesis	van Boxtel et al., 2008	*Msh6/KO* develop a spectrum of tumors	/
	familial colon cancer	*Apc/KO*	ENU mutagenesis	Amos-Landgraf et al., 2007	Apc/KO recapitulates pathology better than mouse models	RRRC#00782 + RRRC#718 (Amos-Landgraf) NBRP Rat #0443
Muscle	Muscular dystrophy (Duchenne and Becker forms)	*Dmd/KO and BigDel*	TALENs and CRISPR-Cas9	Larcher et al., 2014; Nakamura et al., 2014	*Dmd/KO* rats better recapitulate the pathology than *Dmd*/KO mice	*NBRP Rat #0779 NBRP Rat #0780 NBRP Rat #0781 RGD #12880037; (TRIP)*
	Myostatin-related muscle hypertrophy	*Mstn/KO*	ZFN	Mendias et al., 2015; Gu et al., 2016	In contrast to Mstn/KO mice, Mstn/ KO rats showed higher muscle fiber contractibility and lifelong increase in weight in male but not female	RGD #5131964 (PhysGen KO) RGD #5143985 (PhysGenKO) RGD #5131954 (PhysGen KO)
Lung	Cystic fibrosis	*Cftr/KO*	ZFN	Tuggle et al., 2014	*Cftr*/KO rat and mice show similar phenotypes that are mostly similar to those in patients. Rats but not mice have tracheal and bronchial submucosal glands.	*RGD #14392817 (SAGE, not available) RGD #14392813; Horizon discovery RGD #14392815; Horizon discovery*
		Cftr/KO and DF508	CRISPR-Cas9	Dreano et al., 2019	*Cftr*/KO and *DF508* rats and mice show similar phenotypes. *DF508* rats and mice show phenotypes that are milder than in their *Cftr*/KO counterparts. Rats but not mice have tracheal and bronchial submucosal glands	/
		CFTR/KI and G5551D	ZFN	Birket et al., 2020	CFTR/KI G5551D humanized rats display normalization of several pulmonary parameters after ivacaftor treatment	/
Endocrine	Glucocorticoid resistance	*Nr3c1/cKO*	CRISPR-Cas9	Scheimann et al., 2019	*Nr3c1/cKO* in CNS specific brain regions using injection of AAV-Cre vectors not possible in mice	/
	Estrogen resistance (ESTRR)	*Esr1/KO* and *Esr2/KO*	ZFN	Rumi et al., 2014; Khristi et al., 2019	*Esr1/KO* rats and mice show similar phenotypes similar to those of patients	*RRRC#701 (Esr1 KO) RRRC#849 (Esr1 KO) RRRC#742 (Esr2 KO) RRRC#677 (Esr2 KO)*
	Congenital hypothyroidism	*Tshr/KO*	CRISPR-Cas9	Yang et al., 2018	*Tshr*/KO rats and certain strains of *Tshr* KO mice show similar phenotypes similar to those of patients	/
	Allan-Herndon Dudley-syndrome	*Mct8/KO*	CRISPR-Cas9	Bae et al., 2020	*Mct8/KO* rats showed growth and reduced sperm motility and viability *Mct8/KO* mice did not show growth retardation	/
Metabolic	Congenital leptin deficiency	*Lep/KO*	CRISPR-Cas9	Guan et al., 2017	*Lep*/KO rats and mice show similar phenotypes similar to those of patients	/
	Leptin receptor deficiency	*Lepr/KO*	CRISPR-Cas9 and TALEN	Bao et al., 2015; Chen Y. et al., 2017	*Lep*/KO rats and mice show similar phenotypes similar to those of patients	/
	Aceruloplasminemia	*Cp/KO*	CRISPR-Cas9	Kenawi et al., 2019	*Cp*/KO rats show similar plasma biochemical alterations and profile of iron overload in liver and spleen as in humans *Cp*/KO mice showed different results	*RGD #38501060 #38501061 #38501059; not available*

(Continued)

TABLE 7 | Continued

System/organ affected	Human genetic disease	Gene/genetic modification	Genomic tool used	References	Rats vs. mice	Depository or breeder company ID
	Multiple mitochondrial dysfunctions syndrome, among them pulmonary artery hypertension	*Nfu1/KI point mutation G206C*	CRISPR-Cas9	Niihori et al., 2020	*Nfu1/KI point mutation G206C* is only reported in rats. The model shows both mitochondrial dysfunction, and pulmonary artery hypertension with more prevalence in females than in males, as in patients	/
	Generalized arterial calcification of infancy and pseudoxanthoma elasticum	*Abcc6/KO*	ZFN	Li et al., 2017	*Abcc6/KO* rats allowed ex vivo perfusion of liver and spleen and definition of the liver as the primary site of the disease	*RGD #13792683 #13792682 #10413850 #10413852 #10413854 #10413858 #10413856*
	Diabetes mellitus, non-insulin-dependent, 5 (NIDDM5)	*AS160 (TBC1D4)/KO*	CRISPR-Cas9	Arias et al., 2019	*AS160-KO* rats and mice showed similar alterations in whole body assessment Rats' bigger size allowed measurements using single myofibers	*RGD #38596327*
	multiple mitochondrial dysfunctions syndrome	Isca1/KI-mCherry-Cre	CRISPR-Cas9	Yang et al., 2019	Developmental block in embryos at 8.5 days Not reported in mice	/
	Primary hyperoxaluria type 1 (PH1)	*Agxt/KO*	CRISPR-Cas9	Zheng et al., 2020	*Agxt*/KO rat model better recapitulate the disease than the *Agxt/KO* mice	/
		Agxt/KI mutation D205N	CRISPR-Cas9	Zheng et al., 2018	*Agxt/KI mutation D205N* model recapitulates the disease in rats Not reported in mice	/
	Familial hypercholesterolemia	*Ldlr-ApoE/DKO*	CRISPR-Cas9 and CRISPR-Cpf1	Zhao et al., 2018; Lee J. G. et al., 2019	Double *Ldlr-ApoE/DKO* rats better recapitulate the pathology than do double *Ldlr-ApoE/DKO* mice	/
	Dwarfism	*Ghsr/Tg Ghsr/KO*	DNA microinjection ENU mutagenesis	Flavell et al., 1996 Shuto et al., 2002	Dwarfism in rats as in *GshR*/KO mice Analysis of the role of GSHR in behavioral pathologies including eating disorders	RGD #12910127 RGD #1642278 (PhysGen) RRRC#421RRRC #405
		Ghsr/KO	CRISPR-Cas9	Zallar et al., 2019		RRRC#827
	Hyaline fibromatosis syndrome	*Antxr2/KO*	CRISPR-Cas9	Liu X. et al., 2017	*Antxr2*/KO rats and mice show similar phenotype *Antxr2*/KO rats did not develop hypertension	/
	Obesity (OBESITY)	*Mc3R-Mc4R/DKO*	CRISPR-Cas9	You et al., 2016	Double *Mc3R-Mc4R/DKO* rats better recapitulate the pathology than do double *Mc3R-Mc4R/DKO* mice	RGD #13825199 (Mc4R KO) (Hubrecht Laboratory, Centre for Biomedical Genetics, 3584 CT Utrecht, The Netherlands. Hera Biolabs, Taconic.)
	Congenital hyperinsulinism	*Sur1/KO*	TALEN	Zhou et al., 2019	*Sur1*/KO rats and mice reproduce the disease Rats showed a particular glucose control profile	/
	Fumarase deficiency	*Fh/KO*	TALEN	Yu et al., 2019	*Fh*/KO rats and mice show similar phenotype and reproduce the disease	*RGD #13792795 #13792794 (not available)*
	Fabry disease	*Gla/KO*	CRISPR-Cas9	Miller et al., 2018	*Gla/KO* rats better recapitulate the pathology than do *Gla/KO* mice	*RGD #10054398*
	Oculocutaneous albinism type 1	*Tyr/KO*	TALEN	Mashimo et al., 2013	*Tyr*/KO rats and mice show similar phenotype and reproduce the disease	*NBRP Rat #0666*
	Wolfram syndrome	*Wfs1/KO*	ZFN	Plaas et al., 2017	*Wfs1/KO* rats better recapitulate the pathology than *Wfs1/KO* mice	/
Nephrology	Focal segmental glomerulosclerosis 2 (FSGS2)	*Trpc6/KO BigDel*	CRISPR-Cas9	Kim E. Y. et al., 2018	*Trpc6*/KO rats and mice were protected from FSGS2	*RGD #11553908 #11553912 #11553902*

(Continued)

TABLE 7 | Continued

System/organ affected	Human genetic disease	Gene/genetic modification	Genomic tool used	References	Rats vs. mice	Depository or breeder company ID
	C3 glomerulopathy	*C3/KO* *C3/KO*	ZFN CRISPR-Cas9	Negishi et al., 2018) Xu et al., 2018	C3/KO rats and mice display a similar phenotype Most mouse strains have a defective complement system downstream of C3	/ RGD #19165133
	REN-related kidney disease	*Ren/KO*	ZFN	Moreno et al., 2011	Rats like humans have 1 copy of the Ren gene whereas mice have 2 copies Rats faithfully recapitulate the disease	RGD #4139880 (PhysGen)
Ophthalmology	Autosomal dominant congenital stationary night blindness and retinitis pigmentosa	*Rho s334ter/Tg*	DNA microinjection	Liu et al., 1999	This is a unique widely used model of this disease	
	Retinitis pigmentosa 85 (RP85)	*Ahr/KO*	ZFN	Harrill et al., 2013	Ahr/KO rats and mice showed distinct phenotypes in the eye, liver and kidneys during normal development and toxic responses	Cf **Table 6**
	Autosomal dominant congenital stationary night blindness	*Pde6b/KO*	CRISPR-Cpf1	Yeo et al., 2019	*Pde6b* /KO rats and mice reproduce the disease Slower progression and larger anatomic architecture in rats are advantages versus the mouse model	/
	Familial exudative vitreoretinopathy	*Lrp5/KO*	CRISPR-Cas9	Ubels et al., 2020	*Lrp5/KO* rats show retinal and bone abnormalities Similar phenotype in*Lrp5/KO* mice	/
Cancer	Li-Fraumeni syndrome	*Tp53*	ES ZFN	McCoy et al., 2013	*Tp53/KO* rats developed more diverse tumors and more frequently than *Tp53/KO* mice	*RGD #12904897 (Horizon Discovery) RGD #11553886NBRP Rat #0726 RRRC #00485 (ES)*
Immune and hematological systems	Von Willebrand disease	*Vwf/KO BigDel*	CRISPR-Cas9	Garcia et al., 2020	*Vwf*/KO rats and mice display a similar phenotype	*RGD #18182946 #39128242 #18182944*
	Hemophilia A	*F8/KO*	ZFN	Nielsen et al., 2014	*F8*/KO rats and mice show similar phenotype	*RGD #11531094 (Novo Nordisk, Maaloev, Denmark)*
		F8/KO (gene inversion)	CRISPR-Cas9	Shi et al., 2020		RGD #13800746
	ALSP	*Csf1r/KO*	ES cells	Pridans et al., 2018	*Csf1r/KO* rats showed a more severe phenotype than patients and *Csf1r/KO* mice an even stronger one	/
	SCID	*Rag1/KO*	Meganucleases and CRISPR-Cas9	Tsuchida et al., 2014; Zschemisch et al., 2012; Ménoret et al., 2013	*Rag1*/KO rats and mice show similar phenotype	Cf **Table 6**
		Rag2/KO	CRISPR-Cas9	Liu Q. et al., 2017; Noto et al., 2018	*Rag2*/KO rats and mice show similar phenotype	Cf **Table 6**
		Prkdc/KO	CRISPR-Cas9	Mashimo et al., 2012; Ma et al., 2014a	*Prkdc*/KO rats and mice show similar phenotype	Cf **Table 6**
	X-linked SCID	*Il2Rg/KO*	ZFN, TALEN and CRISPR-Cas9	Mashimo et al., 2012; Samata et al., 2015; Kuijk et al., 2016; Ménoret et al., 2018	*Il2rg*/KO rats and mice show similar phenotype	Cf **Table 6**
	APECED	*Aire/KO*	TALEN	Ossart et al., 2018	*Aire*/KO rats showed a more pronounced phenotype than *Aire*/KO mice	Cf **Table 6**
	Agammaglobulinemia non-Bruton type	*Ighm/KO*	TALEN CRISPR-Cas9	Ménoret et al., 2010; Panzer et al., 2018	*Ighm*/KO rats and mice show similar phenotype	Cf **Table 6**

tools can be used and combined, in particular for genetic lineage tracing (Liu K. et al., 2020).

Models to Study Genes of the Immune System

In general terms, rats share more immune characteristics with humans than mice do (Wildner, 2019). As an example, complement levels in humans and rats are comparable (Ong and Mattes, 1989; Ménoret et al., 2020), whereas in most inbred mouse strains, they are undetectable or very low because of different genetic mutations (Ong and Mattes, 1989; Wetsel et al., 1990; Shultz et al., 1995).

The roles of genes identified in different immune pathophysiological processes, as well as others involved in normal immune responses, also have been analyzed and are listed in **Table 6**. For the sake of space and relevance of the rat model, only some of these generated genetically modified models are described in more detail below.

Immunodeficient Rat Strains

KO of genes involved in early rearrangements of immunoglobulin in B cells and of the T cell receptor genes in T cells, such as *Rag1* (Zschemisch et al., 2012; Ménoret et al., 2013; Tsuchida et al., 2014), *Rag2* (Kuijk et al., 2016; Liu Q. et al., 2017; Noto et al., 2018), and *Prkdc* (Mashimo et al., 2012; Ma et al., 2014a; Beldick et al., 2018) have resulted in defective development of B and T cells (**Tables 6**, **7**). KO of the gamma chain receptor of the IL-2 receptor (*Il2rg*) results in defects of differentiation of T, B, natural killer (NK), and innate lymphoid cells (Mashimo et al., 2010; Samata et al., 2015; Kuijk et al., 2016). Additionally, rat lines combining several genetic modifications, such as with the *Rag1*, *Rag2*, *Il2rg*, *Prkdc*, and *Foxn1* genes, have been developed (Mashimo et al., 2012; Goto et al., 2016; Ménoret et al., 2018; He et al., 2019). Transgenic rats for human SIRPa to inhibit phagocytosis in human cells have been described in recent years (Goto et al., 2016; Jung et al., 2016; Yang X. et al., 2018; Ménoret et al., 2020). These rats have been used in humanization of their immune system and/or other tissues in transplantation and regenerative medicine settings (for a review, see Adigbli et al., 2020) and in cancer research (He et al., 2019). In these models as in others, the larger size of the rat allows to do analysis of human cells of the blood more frequently than in mice. Furthermore, the normal complement levels in rats allow to analyze the effector function of different anti-human antibodies, not possible to do in mice (Ménoret et al., 2020). Other genetic modifications to improve immune or liver humanization that have been developed in mice, will probably also be applied to the present generation of immunodeficient rats (Adigbli et al., 2020).

B cell–deficient rats have been described (Ménoret et al., 2010; Panzer et al., 2018) and used in organ transplantation models, and the rat may better recapitulate lesions mediated by complement activation through antibodies in the transplantation setting (Platt and Cascalho, 2018). One of these B cell–deficient strains (Ménoret et al., 2010) was obtained by disrupting the J sequence of the immunoglobulin heavy chain and further rendered deficient for both immunoglobulin light chains (Osborn et al., 2013). With the objective of generating fully human monoclonal antibodies (mAbs), these immunoglobulin-deficient rats were humanized for immunoglobulins by transgenesis using BACs (Osborn et al., 2013). These animals can generate human mAbs with diversity and affinity (Osborn et al., 2013) and different versions of these animals have been generated (Harris et al., 2018; Clarke et al., 2019).

Inactivation of the C3 complement gene has allowed confirmation of a new role for complement in a model of polyneuropathy following chemotherapy. As stated earlier, the fact that complement levels in humans and rats are comparable (Ong and Mattes, 1989; Ménoret et al., 2020), makes the rat a model of choice for exploring the role of complement in different pathological situations (Xu et al., 2018).

Cluster of Differentiation (CD) or Other Cell Membrane Molecules

In model of neuromyelitis optica induced by passive administration of human IgG autoantibodies targeting aquaporin-4, rats deficient in the cell membrane inhibitor of complement activation CD59 showed a much more pronounced neurological pathology than CD59 KO mice (Yao and Verkman, 2017a,b). This model emphasizes the role of complement in this pathology and the availability of a more relevant model of the disease than mice.

CLEC-1 is a cell membrane receptor expressed by dendritic cells (DCs) that reduces immune responses and plays a role in immune tolerance models (Thebault et al., 2009). CLEC-1 KO rats show enhanced *Il12p40* subunit mRNA expression in DCs and an exacerbation of downstream *in vitro* and *in vivo* $CD4^+$ Th1 and Th17 responses (Lopez Robles et al., 2017).

Human and rat (Maruoka et al., 2004) but not mouse cells express the Fc receptor for IgA (FcaRI, CD89; mice bear only a *FcarI* pseudogene) (Launay et al., 2000). CD89 KO rats have been generated and have provided interesting new information on a model of IgA-induced nephropathy a frequent pathology in humans (submitted).

Similarly, human and rat DCs display quite similar profiles of Toll-like receptor (TLR) expression in different DC subsets, allowing to better explore their role in infectious and inflammatory diseases. DCs from both species express the TLR10, whereas mouse DC subsets do not show a particular profile of TLR expression and TLR10 is not expressed (mice bear only a *Tlr10* pseudogene) (Hubert et al., 2006). Rats deficient for TLR10 have been generated and are being characterized (in preparation).

A human CD4/CCR5 transgenic rat model (Keppler et al., 2002) has been extensively used to analyze different aspects of HIV infection and treatment with more relevant results as compared to mice with similar transgenes (Goffinet et al., 2007).

In humans, HLA-B27 is strongly associated with a series of inflammatory diseases grouped together under the term "spondyloarthropathies." In contrast to the negative results in transgenic mice, transgenic HLA-B27 rats spontaneously develop inflammatory disease in the same organs as those involved in humans (Hammer et al., 1990). This model has been extensively used and is the model of choice in this pathology (for a review, see Braem and Lories, 2012).

Cytokines and Their Receptors

Il22bp KO rats show that IL22-binding protein is protective in models of inflammatory colitis (Martin et al., 2016) and psoriasis (Martin et al., 2017). *Il22bp*-GFP KI rats have facilitated precise definition of cell subsets that express IL22bp by different subsets of DCs in different tissues (submitted).

Viral infections can trigger autoimmune diabetes in rats and type I IFN α/β receptor (IFNAR1) KO rats have a significantly delayed onset and frequency of diabetes. These findings support the idea that innate immunity influences autoimmune diabetes and encourage the use of targeted strategies to inhibit type I IFN α/β (Qaisar et al., 2017).

NK cells could play a role in placenta generation, and IL-15 KO rats showed an absence of NK cells and several abnormal placental characteristics, supporting a role for NK cells (Renaud et al., 2017).

A *Csf1r* reporter gene (Irvine et al., 2020) and *Csf1r KO* (Pridans et al., 2018) lines are useful tools for the analysis of macrophages and of CSF1R biology (Hume et al., 2020). CSF1R is also the receptor for IL-34, and *Il34*-mutated rats exhibit depletion of microglia and Langerhans cells, as well as defects in tolerogenic immune responses (submitted).

Intracellular Molecules

Certain molecules that regulate metabolic functions in many cell types, including in immune cells, have been analyzed using genetically modified rats. Transgenic rats for heme oxygenase-1 (HO-1) under the control of the ubiquitous H-2Kb promoter (Braudeau et al., 2003) and HO-1 KO rats (Atsaves et al., 2017) have facilitated dissection of different aspects of HO-1 effects, particularly in kidney, where the lesions observed in rats differ from those in mice.

The hydrocarbon receptor (AHR) is a transcription factor with an essential role in mediating toxic responses to environmental pollutants and in regulating many cellular pathways involving endogenous ligands. In *Ahr* KO rats, the percentages of T CD3+, T CD8+, and CD11c+ cells in the spleen and the activation of T cells are decreased, whereas the percentage of NK T cells and the activation of B cells is increased compared to wild-type rats (Phadnis-Moghe et al., 2016).

The lymphopenia observed in diabetic biobreeding rats results from a spontaneous mutation in the immune-associated nucleotide gene 5 (*Ian5*), a protein expressed in the mitochondria membrane where it regulates apoptosis. Lymphocyte numbers are normalized when a normal *Ian5* gene is transgenically expressed (Michalkiewicz et al., 2004).

Some of the most commonly used immune system models developed in rats are based on intrinsic characteristics of the species. For example, the rat has always been an important model of autoimmune arthritis (Holmdahl et al., 2001) and HLA-B27 transgenic rats recapitulate spondyloarthropathies much better than do HLA-B27 transgenic mice.

Certain immune reagents, such as antibodies recognizing leukocyte differentiation antigens, are less abundant in rats than in mice but more so than in other experimental species. High-density flow cytometry techniques have not yet been applied in the analysis of the rat immune system and will clearly be of great interest when coupled with modification of rat immune system genes.

Genetic Diseases Models

For 150 years, spontaneous or induced (ENU) genetic mutations in the rat have been used as models of human genetic diseases. For a decade, the advent of genetic engineering tools such as ZFN, TALEN, and CRISPR-Cas have led to a real revolution in obtaining specific and targeted genetic mutations in rats for the study of human genetic diseases. These advances, coupled with historical knowledge and use of the rat in many research fields, have increased the generation of rat models of human genetic diseases. More than 6000 genetic diseases have been described, and several databases have recorded variants that are associated with or responsible for genetic diseases. Several important genetic diseases have been modeled in rats. A complete list is presented in **Table 7**, and a brief description of the most useful models is provided below.

Cardiovascular Diseases (CVD)

Because of its larger size allowing catheterization, lower cardiac frequency versus mice, and historical use in CVD, the rat has been an important model for a series of genetically modified rat models of CVD.

Pulmonary arterial hypertension (PAH) results from a reshaping and thickening of the walls of medium and small caliber pulmonary vessels. By their frequencies and effects, the mutations in the BMPR2 gene are the main variants responsible for inheritable forms of isolated PAH. *Bmpr2* KO rats show some of the critical clinical, cellular, and molecular dysfunctions described in human PAH both in the heart and vessels (Ranchoux et al., 2015; Hautefort et al., 2019; Manaud et al., 2020). Although rarer, mutations in the KCNK3 gene encoding a potassium channel have also been described as causative in PAH. *Kcnk3* KO rats develop age-dependent PAH associated with characteristic electrophysiological and molecular alterations in the myocardium and vessels (Lambert et al., 2019). Because the *Kcnk3* gene is not functional in mice, this rat model offers new insights into the mechanisms of PAH and in the testing of therapeutics.

To investigate the role of the MYL4 gene in atrial cardiomyopathy, Myl4-KO or mutated rats have been generated. Both show a phenotype similar to affected patients and are new models for further mechanistic analysis (Peng et al., 2017).

Danon disease (DD) is a metabolic disease caused by mutations in the LAMP2 gene, and the most common symptom is cardiomyopathy. Recently generated *Lamp2* KO rats show similarities to DD patients at the heart tissue level and with multisystem lesions, constituting an important new animal model of DD (Ma S. et al., 2018).

Neurological Diseases

In neurobiology and cognitive studies, the rat, because of its larger size and more complex and richer behavior, is preferred as a rodent model. Genetically modified rats have provided several important models for neurological disorders with a genetic component.

Mutations in complexin-1 (CPLX1) gene lead to epileptic encephalopathy with onset on infancy. *Cplx1* KO rats have different phenotypes from mice. Both show profound ataxia, but in rats, behavior is more affected, and they have more abnormal histomorphology of the stomach and intestine, resulting in early death (Xu et al., 2020).

A nonsense mutation in the Cockayne syndrome B gene, *Ercc6*, more profoundly affects the rat brain than the mouse KO for the same gene (Xu et al., 2019). In these rats, RNA-seq analysis has revealed transcription dysregulation that contributes to the neurologic disease.

Neonatal hydrocephalus has been analyzed using two different models of mutated rats, one with an invalidation of the *L1cam* gene (Emmert et al., 2019b) and the other with a KI of a specific mutation in the *Ccdc39* gene (Emmert et al., 2019a). These models allow for neurosurgery procedures that are difficult to perform in mice, with resulting characterization of the lymphatic-mediated cerebrospinal fluid circulation and inflammation in this disease.

As a model for familial amyotrophic lateral sclerosis, rats with a FUS point mutation KI via CRISPR-Cas9 express a physiological level of this mutant, along with cognitive impairment and neuromuscular signs. In this rat model, FUS KI highlighted sleep–wake and circadian disturbances as early alarm signals (Zhang T. et al., 2018).

Neurofibromatosis type 1 is an autosomal dominant disease arising from mutations in the NF1 gene that results in the development of tumors in the nervous system, neurological disorders and chronic idiopathic pain (Dischinger et al., 2018). *Nf1* KO rats show increased nociceptor excitability and hyperalgesia. These models are important in the search for a potential key target (CRMP2) for therapeutic intervention (Moutal et al., 2017).

RNASET2 deficiency in humans is associated with cystic leukoencephalopathy. *RnaseT2* KO rats are the only rodent model of this disease. Despite a less severe neurodegeneration phenotype than in patients, this model is useful for studying RNASET2 function, especially for hippocampal neuroinflammation (Sinkevicius et al., 2018).

A group of neurodevelopmental diseases, gathered under the name of autism spectrum disorders (ASDs), are characterized by heterogeneous capabilities in social interactions and by stereotyped behaviors. One subtype of ASD is associated with mutations in the MECP2 gene, causing an X-linked neurodevelopmental disorder named Rett syndrome. *Mecp2* KO rats clearly show both motor and behavioral deficits early in development, more pronounced than in mice (Patterson et al., 2016). Another subtype of ASD is ASD/Fragile X syndrome. Two KO rat models have been generated for this condition, one syndromic (*Fmr1*) and one non-syndromic (*Ngln3*) (Hamilton et al., 2014). These KO rats show some ASD-relevant phenotypes for investigations at the genetic level. Phelan–McDermid syndrome is another ASD-associated condition, caused by mutations in the SHANK3 gene. In contrast to *Shank3* KO mice, *Shank3* KO rats showed normal social interaction but impaired social memory (Harony-Nicolas et al., 2017; Song et al., 2019). Similarly, *Shank2* KO rats better recapitulate the condition than the KO mice (Modi et al., 2018). Angelman syndrome results from mutations in the *UBE3A* gene, which in most cases is a large gene deletion, and in a small fraction with mutations in exon 2. The *Ube3A* mouse model bears a null mutation of exon 2, whereas the rat model is closer to the human condition with a large deletion of the *Ube3a* gene. The rat model mimics human Angelman syndrome with abnormalities in motor coordination and cognitive function (Dodge et al., 2020).

Muscular Diseases

Myopathies are a set of neuromuscular diseases, the most common of which is Duchenne's muscular dystrophy (1 in 3300 newborn babies) resulting from mutations in the dystrophin gene (DMD). As in humans, *Dmd* KO rats show decreased muscle strength as well as a degradation/regeneration phenotype in skeletal muscles, heart, and diaphragm (Larcher et al., 2014; Nakamura et al., 2014). Of note, *Dmd* KO rats but not mice present cardiovascular alterations close to those observed in humans, which are the main cause of death in patients. All of these clinical signs and pathological features are much more pronounced than in *Dmd* KO mice. Rats are becoming an increasingly used model for the study of different aspects of Duchenne's and Becker's myopathies, including biomarkers, neurological abnormalities, and immune/inflammatory responses (Robertson et al., 2017; Ouisse et al., 2019; Caudal et al., 2020; Szabó et al., 2021).

Pulmonary Diseases

Cystic fibrosis is one of the most common genetic diseases in western populations (approximately 1 in 4000 newborns) and is caused by mutations in the *CFTR* gene. The most common mutation in humans is the missense mutation DF508, which leads to abnormal CFTR function and mucus accumulation. Cystic fibrosis is characterized by airway and digestive pathology with a reduced life expectancy. Mice do not have submucosal glands, in contrast to humans and rats. Rats with the DF508 mutation (Dreano et al., 2019), as well as with a complete KO for *Cftr*, have been generated (Tuggle et al., 2014; Dreano et al., 2019). *Cftr* KO rats showed a very severe digestive phenotype and lung lesions in surviving older animals, and reduced weight and life expectancy, although milder in DF508 rats. Very recently, a humanized model of cystic fibrosis was created by inserting the human CFTR cDNA sequence harboring a G551D mutation by KI into the rat genome, downstream of the endogenous *Cftr* promoter (Birket et al., 2020).

Metabolic Diseases

To study disorders of metabolism, leptin, a cytokine-like hormone principally produced by white adipose tissues, was deleted in rats. Microarray analysis has been performed in *Lep* KO rats to evaluate alterations in white adipose gene expression and to explore pathways involved in metabolic diseases with leptin deficiency (Guan et al., 2017). The leptin receptor (*Lepr*) has also been deleted in rats, and these animals show hyperphagia, obesity, hyperglycemia, and dyslipidemia. This model could complement the existing models (db/db mice and Zucker rats) and be useful for research in obesity and diabetes (Bao et al., 2015; Chen Y. et al., 2017).

Hereditary aceruloplasminemia is a genetic disease characterized by progressive iron overload (liver and brain)

and is related to mutations in the ceruloplasmin (*CP*) gene. In contrast to *Cp* KO mice, *Cp* KO rats mimic the human phenotype with hepatosplenic iron load and could be more appropriate for providing information to understand and treat the disease (Kenawi et al., 2019).

Abnormal calcification and phosphate deposition are the basis of generalized arterial calcification of infancy and pseudoxanthoma elasticum, both caused by mutations in the *ABCC6* gene. These mutations lead to generalized arterial calcification through the body in infancy. Because ABCC6 is expressed in liver and kidney, an important question is the respective role of these organs in the generalized disease. Given their small size, mice KO for *Abcc6* are not suitable for *ex vivo* perfusion experiments. *Ex vivo* perfusion of liver and kidneys from *Abcc6 KO* rats has revealed that the liver is the primary site of molecular pathology in these process and points to a preferential target of the liver to treat them (Li et al., 2017).

The low-density lipoprotein receptor (LDLR) and apolipoprotein E (APOE) genes control normal levels of cholesterol and other forms of fat in the blood. A deficiency in LDLR is the cause of familial hypercholesterolemia and a deficiency in APOE is involved in several age-related fatty acid diseases. Recently, two reports (Zhao et al., 2018; Lee J. G. et al., 2019) described double-KO for *Ldlr* and *Apoe* genes in rats. These rats mimic more closely than KO mice the pathological changes observed in hyperlipidemia and atherosclerosis in humans with genetic deficiencies and in normal individuals.

Melanocortin-3 and -4 receptors (MC3R and MC4R) regulate energy and body weight. *Mc3R-Mc4R* double-KO rats exhibit worse phenotypic features than single-KO rats and *Mc3R-Mc4R* double-KO mice (You et al., 2016).

Fabry disease is an X-linked lysosomal storage disease caused by α-galactosidase A (α-Gal A) deficiency resulting from mutations in the GLA gene. *α-Gal A* KO mouse models do not recapitulate the cardiorenal findings observed in humans and *Gla* KO rats more closely mimic the disease phenotypes observed in patients (Miller et al., 2018).

Wolfram syndrome (WS) is a genetic disorder caused by mutations in the *WFS1* gene. Previous mouse models of WS involved only partial diabetes and other symptoms of the disease, whereas *Wfs1* KO rats developed diabetes as well as neuronal degeneration, as do patients (Plaas et al., 2017).

Kidney Diseases

Renin (REN) mutations are involved in REN-related kidney disease and tubular dysgenesis. The role of RAS in the regulation of blood pressure and kidney function has been extensively analyzed in rats (Jacob, 2010), including the generation of one of the first transgenic rat models (Mullins et al., 1990). Although humans and rats have only one copy of the renin gene, mice have two genes and thus increased renin expression levels (10-fold higher than their one-copy counterparts) (Hansen et al., 2004). *Ren* KO rats have lower blood pressure and severe kidney underdevelopment, reproducing the kidney lesions observed in REN-related kidney disease and tubular dysgenesis (Moreno et al., 2011).

Ophthalmology Diseases

Retinitis pigmentosa (RP) is a group of inherited mutations causing photoreceptor degeneration, loss of night vision, and blindness. Rhodopsin mutations comprise an important fraction of autosomal dominant RP. Transgenic rats harboring the *Rho s334ter* mutation are a widely used model for this pathology (Liu et al., 1999).

As noted, AHR is a ligand-activated transcription factor involved in the development of multiple tissues and activated by a large number of exogenous toxic compounds and endogenous ligands, such as kynurenines. *Ahr* KO rats and mice show ophthalmologic lesions as well as different renal and hepatic developmental and homeostatic lesions (Harrill et al., 2013).

Cancer

The tumor suppressor TP53 is a central player in cancer biology, and mutations in the TP53 gene are the most frequent mutations observed in human cancers. *Tp53* KO rats develop a wide variety of tumors, most frequently sarcomas, which are rarely observed in mice. These rats have been used in carcinogenicity assays for drug development (McCoy et al., 2013).

Immune and Hematological Systems

For hemophilia A, *FvIII* KO rats have no detectable FVIII activity, and their activated thromboplastin time and clotting time are significantly prolonged. Episodes of spontaneous bleeding requiring treatments were observed in 70% of the *FvIII* KO rats (Nielsen et al., 2014; Shi et al., 2020). In the rat genome, it is interesting to note that the *F8* gene is situated on chromosome 18, rather than the X chromosome as in humans, mice, dogs, and sheep (Lozier and Nichols, 2013).

Monocyte colony-stimulating factor (CSF-1) is, along with IL-34, a regulator of macrophages and myeloid DC development, acting through the CSF-1R (Ma et al., 2012). Humans with point mutations or less frequently deficiency for CSF-1R develop adult-onset leukoencephalopathy with axonal spheroids and pigmented glia, likely because of a decrease in the number of microglia (Hume et al., 2020). *Csf1r* KO rats (Pridans et al., 2018) develop some or all of the symptoms and lesions of the disease, but with greater severity and more bone lesions than in humans, whereas *Csf1r* KO mouse models show an even more severe phenotype (Hume et al., 2020).

AIRE plays a key role in central tolerance by regulating the expression of peripheral tissue antigens in epithelial cells of the thymus and by eliminating autoreactive T cells. Patients with the autoimmune polyendocrinopathy-candidiasis-ectodermal-dystrophy syndrome have genetic defects in AIRE. *Aire* KO rats show signs of generalized autoimmunity and clinical signs of disease that are much more pronounced than in *Aire* KO mice and closer to manifestations in humans (Ossart et al., 2018).

CONCLUSION AND PERSPECTIVES

CRISPR-Cas system is now the tool of choice for genome editing, particularly for the rat for which ES cells are limited compared to the mouse. In the last decade, efforts have been made to improve

this tool and its delivery but two main hurdles persist. Some loci are still difficult or impossible to edit, and the efficiency of large or complex KI is still too low. Although many advances have been developed in the application of the CRISPR-Cas system to human cells and sometimes in mice, many remain to be applied in rat model generation.

Rats often proved to be better mimics of human situation than mice. It is particularly evident in CVD, neurobiology, ophthalmology, muscular diseases, and immunology, but few of the large number of genetic diseases in these or other organ systems have been modeled in rats. It is difficult to predict when the rat will be better than the mouse, nevertheless, it seems reasonable to try to generate new genetically modified rats in these areas. Moreover, to the best of our knowledge and among the models that can be compared, there are no mouse genetic or immune models that better reproduce human disease than rat. Future work using the CRISPR-Cas system will likely generate new rat models of genetic diseases and to study genes functions. Extensive work in QTLs associated with major polygenic diseases has been performed in rats (Gauguier, 2016; Shimoyama et al., 2017). Within these QTLs, the genes that could be responsible for a given disease will likely be targets of choice in future studies.

Other genes that would be logical to target in rats are those that are absent in mice and present in humans, given that 78 out of the 2544 Mb of the rat genome is common between humans and rats but not humans and mice (Gibbs et al., 2004). Examples within the immune system include *Tlr10* and *Cd89*.

A limitation of rats versus mice that cannot be resolved is also one of its advantages: its bigger size, which brings higher breeding costs.

The rat will continue to be a critical experimental model based on its bigger size and its inherent physiological characteristics, as well as a large and growing body of physiology and genomic data. Tools for modifying the rat genome as well as analyzing the genome are key to the development of new models for understanding biology and diseases.

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