Targeted gene knock-in by CRISPR/Cas ribonucleoproteins in porcine zygotes

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The domestic pig is an important “dual purpose” animal model for agricultural and biomedical applications. There is an emerging consensus in the biomedical community for the use of large animal models such as pigs to either serve as an alternative, or complement investigations from the mouse. However, the use of pig has not proven popular due to technical difficulties and time required in generating models with desired genetic modifications. In this regard, the ability to directly modify the genome in the zygote and generate edited animals is highly desirable. This report demonstrates for the first time, the generation of gene targeted animals by direct injection of Cas9 ribonucleoprotein complex and short stretches of DNA sequences into porcine zygotes. The Cas9 protein from Streptococcus pyogenes was pre-complexed with a single guide RNA targeting downstream of the ubiquitously expressed COL1A gene, and co-injected with a single-stranded repair template into porcine zygotes. Using this approach a line of pigs that carry pseudo attP sites within the COL1A locus to enable phiC31 integrase mediated introduction of transgenes has been generated. This new route for genome engineering in pigs via zygote injection should greatly enhance applications in both agriculture and biomedicine.
short palindromic repeat) and CRISPR-associated (Cas) nuclease system (CRISPR/Cas). Of the three widely used editors, the CRISPR/Cas system is emerging as the tool of choice because of the ease of design, delivery, and relatively high fidelity in engineering DSBs. The CRISPR/Cas system has evolved in archaea and eubacteria as an RNA-based adaptive immunity system to detect and cleave invading viruses and plasmids.\(^5^,^6\) The Streptococcus pyogenes (Sp) Type II CRISPR/Cas system has been adapted to successfully modify somatic cells and generate modified pigs\(^9^--^13\). In these studies, a mammalian codon-optimized Cas9 nuclease along with a synthetic single-guide RNA (sgRNA)\(^1^4\) has been used to generate genetically modified pigs\(^9^--^13\). Although, live offspring have been generated using CRISPRs and SCNT, the difficulties in culturing primary somatic cells long enough to edit, clonally propagate, and screen for correct recombinants, coupled with the challenging task of using the somatic cells for nuclear transfer, makes the technique intractable for an average laboratory. An often overlooked and less often discussed difficulty is targeting certain loci that are within heterochromatin regions of the genome in somatic cells requiring additional rounds of targeting or screening more clonal lines. Direct injection of editors into porcine zygotes may overcome many of these difficulties. A few publications have already successfully demonstrated the generation of bi-allelic knock-out pigs by injection of editors into zygotes\(^1^3^,1^5^,1^6\).

A recent report showed the successful introduction of point mutations by microinjection in pig zygotes using zinc finger nucleases\(^1^7\). Additionally, gene targeting has been achieved in vitro by microinjection targeting reagents and CRISPR/Cas reagents into porcine zygotes; although high degree of mosaicism has been observed\(^8\). In the current study, we sought to go a step further and demonstrate that it is possible to knock-in short DNA sequences (or transgenes) into target loci by injection into in vivo derived zygotes and to generate live offspring bearing targeted mutations using the CRISPR/Cas system. As a proof of concept, we sought to introduce LoxP and pseudo-attP sites into sites permissive for the subsequent introduction of functional transgenes. We hypothesise that the genome in early zygote will be in an open conformation and thus ideal for targeting most loci with relative ease. Additionally, in porcine and other large animal models, the pronuclei are often not clearly visible in early zygotes. This work avoids the need to inject into the pronucleus (as is often required for zygote injections). Rather, it is only necessary to inject the CRISPRs and targeting oligonucleotides into the cytoplasm in order to generate targeted offspring.

Results

Targeted knock-out of alleles by cytoplastmic injection into parthenogenetic and in vitro fertilized embryos. In vitro transcribed SpCas9 and sgRNA targeting PRNP (Prion gene) were injected 5 hours (h) following parthenogenetic activation or in vitro fertilization of porcine oocytes. SpCas9 is a fusion construct with green fluorescent protein (GFP) and nuclear localization signals (NLS) that allows for tracking the expression of full length Cas9 protein and its translocation into the nucleus of the zygote. As shown in Supplementary Fig. S1, expression of full length GFP fused Cas9 protein can be seen in zygotes with some of the protein translocating into the nucleus of the zygote 24 h following injection. Screening of injected embryos at the blastocyst stage demonstrated successful bi-allelic targeting of the PRNP locus as evidenced by large deletions (Supplementary Fig. S1). Injection of in vitro transcribed SpCas9 mRNA and a sgRNA targeting a second gene target, ZBED6, as well as a combination of guides targeting PRNP and ZBED6, generated indels causing knock-out of the ZBED6 gene (Supplementary Fig. S2), or knock-out of both PRNP and ZBED6 genes (Supplementary Fig. S3) respectively, in porcine zygotes. Following the success of targeting a single or a combination of genes by intracytoplasmic injection of porcine zygotes in our preliminary studies, we sought to optimize the concentrations of CRISPR reagents to assess the percentage of gene targeting and developmental rates in porcine embryos following CRISPR injections (Supplementary Fig. S4). Serial 2-fold dilutions of Cas9, sgRNA starting from 100 ng/μl Cas9 and 50 ng/μl sgRNA were injected into porcine zygotes to generate mutations at the target sites. This confirmed a range of concentrations within which CRISPRs can introduce mutations at the target sequences.

Targeting knock-in of alleles by cytoplasmic injection in parthenogenetic and in vitro fertilized embryos. Following the initial success with generating knock-outs in vitro, we sought to systematically optimize conditions for targeted knock-in of short stretches of DNA sequences as well as long transgene constructs into porcine zygotes. Single stranded DNA (ssDNA) oligonucleotides (hereafter referred to as “oligo”) have proven effective in facilitating homology directed repair (HDR) driven knock-in of short stretches of sequences into porcine zygotes\(^1^5\). Here we attempted to knock-in a 34-base pair (bp) LoxP site into the PRNP and ZBED6 genes by co-injection of SpCas9, sgRNA and ssDNA targeting oligo. The targeting oligo were designed with 75 bp of homology arm (HA) on either side of the SpCas9 cut site in each target allele respectively. Additionally, the oligo was designed (with 1 bp modification) to create a new EcoR1 site following HDR knock-in to facilitate screening (Fig. 1A). As shown in Fig. 1B, cloning of ampiclons and Sanger sequencing of 5 bacterial clones each from 10 different blastocysts confirmed reconstitution of EcoR1 site in 6/10 blastocysts (Blastocysts# 1, 3, 4, 6, 7, and 10). No knock-in was evident in 3/10 blastocysts (Blastocysts # 2, 5 and 9), whereas a mutated EcoR1 was identified in one of the blastocysts (Blastocyst# 8). Fidelity of the reconstituted EcoR1 site in the knock-in embryos was further investigated by EcoRI restriction digestion (Fig. 1C). The cloning vector (PCR2.1; Life Technologies) has two EcoR1 sites flanking the cloned fragment. As expected, digestion of plasmids bearing wild type sequence (blastocysts # 2, 5 and 9) with EcoRI resulted in the release of one insert (445 bp; bottom band) from the vector backbone (3.9 kb; top band) when resolved on an agarose gel. However, as anticipated, restriction digestion of representative clones with an EcoRI site knock-in (from blastocysts# 1, 3, 4, 6, 7, and 10) resulted in the release of two fragments (329 bp and 150 bp; bottom bands) from the vector backbone (top band). That said, among the blastocysts with a functional EcoR1 site, only two blastocysts (blastocyst # 1 and 3) showed full length (34 bp) LoxP sequence (Fig. 1B), with the remainder embryos showing 1–2 bp insertions or deletions in the introduced LoxP site potentially due to errors in oligo synthesis or DNA repair.
The ability to site-specifically knock-in transgenes into a specific locus that eliminates the possibility of insertion mutagenesis, positional variegation, expression of transgenes and viability of the resulting animal is highly desired for generating transgenic animals. To achieve this objective, we targeted two constitutively expressing loci (PRNP and COL1A). Injection of a double stranded GFP transgene targeting the PRNP locus with 1000 bp and 500 bp of homology on either side of the CRISPR cut site, along with the CRISPR reagents (Cas9 nuclease or Cas9D10A nickase and sgRNA complex) into the embryos led to the targeted knock-in of the transgene into the PRNP locus (Fig. 2 and Supplementary Fig. S5). As a control, only Cas9:GFP RNA or vector only injections were performed. Even though, residual expression of GFP was noticed in plasmid only controls, site-specific integration of the GFP transgene and persistent expression of GFP transgene was noticed only in embryos derived from

Figure 1. Targeted knock-in of a LoxP site into ZBED6 allele. (A) Schematic of the microinjection of targeting mixture consisting of in vitro transcribed Cas9: GFP mRNA, sgRNA targeting ZBED6 and a single stranded targeting oligo containing 75 bp homology arm (HA) on either side of the CRISPR cut-site and 34 bp LoxP site (184 bp total). One of the nucleotides on the left HA has been modified to facilitate an A:C substitution (arrow) and generation of a novel EcoR1 site following successful targeting. (B) Following microinjection, the embryos were cultured toblastocyst stage, lysed, PCR amplified, cloned into PCR2.1 vector (Life Technologies), and plasmid DNA from five bacterial colonies sequenced by Sanger sequencing. Representative colonies that showed knock-in into one of the two alleles were shown (wildtype allele not shown). Sequencing of clones from 10 blastocysts revealed that 7/10 embryos had efficient knock-in of the LoxP site (highlighted in blue), however blastocyst# 8 had mutations in the EcoR1 site. Blastocyst # 9 had no knock-in of LoxP site. (C) Agarose gel electrophoresis of EcoR1 digested plasmids from each blastocyst that were confirmed by sequencing. The cloning vector has two EcoR1 sites, and the knock-in alleles will have reconstituted a third EcoR1 site. Consequently, a clone with wild type sequence is expected to release 445 bp insert (Blastocysts# 2, 5, and 9), whereas a successful knock-in of EcoR1 site is expected to release 2 fragments of 329 bp and 150 bp (Blastocysts# 1, 3, 4, 6, 7, and 10), in addition to the 3.9 kb vector backbone (top band). Blastocyst # 8 was successfully knocked-in, however, the EcoR1 site was mutated resulting in 2 bands being generated, and a shift in the size of the top band due to knock-in of a truncated (32 bp) LoxP site (477 bp).
injection of targeting vector and Cas9 or Cas9D10 nickase RNA (Fig. 2B,C and Supplementary Fig. S5). That said, full length amplicons were identified in the nickase injections, whereas truncations in the 3′ end around the cut-site was identified in Cas9 nuclease injections. This is because, the PRNP targeted sequence has intact guide

Figure 2. CRISPR-mediated knock-in of GFP transgene into the PRNP locus. (A) Schematic of the targeting vector showing targeting homology arms (HA) to the pig PRNP locus and strategy for knock-in. In the targeting vector, the upper arm is 1000 bp and the lower arm 500 bp in length. The linearized targeting vector, Cas9 mRNA and sgRNA targeting PRNP were co-injected into the cytoplasm of porcine zygotes. As shown in the schematic, Cas9 induces double strand break in exon 3 of PRNP gene. The cut DNA is then repaired by HDR using the targeting vector, resulting in the targeted allele. The use of Cas9D10A (nickase) introduced a single stranded nick as compared to DSB by Cas9, triggering HDR mediated knock-in into the PRNP locus. Dark grey boxes represent exons of the PRNP gene. (B) PCR amplification using primers one within the targeting vector and another outside of the targeting vector produced specific bands of 1050 bp confirming targeting to the intended locus. In the figure, the 1000 bp and 500 bp markers with corresponding bright bands on the ladder are shown as a reference. (C) Representative knock-in embryos that have developed to the blastocyst stage showed varied GFP expression in both Cas9D10A and Cas9 injections.
RNA sequence following knock-in of GFP transgene that resulted in subsequent targeting by Cas9 resulting in cleavage and truncation.

In a second experiment, we targeted downstream of the polyA site of the COL1A locus to knock-in two pseudo attP sites (50 bp each; 100 nt total). Injection of a targeting complex containing Cas9 mRNA or protein, in the presence or absence of DNA ligase IV inhibitor (SCR7) at two different time points has been investigated. Injected zygotes in batches of 25 were cultured for 6 days and the blastocysts were screened with primers spanning the targeted region. As shown here, the use of Cas9 protein was effective in introgressing pseudo attP sites into the COL1A locus as evident from 100 bp shift in the product size (two 50 bp attP sites) denoted by asterix. In the figure, the bottom band represents the wildtype allele. The final concentrations of the reagents are: Cas9 RNA: 25 ng/μl; Cas9 protein: 25 ng/μl; sgRNA: 12.5 ng/μl; Oligo: 50 ng/μl; and SCR7: 10 μM.

**Figure 3.** Knock-in of pseudo attP sites downstream of the ubiquitously expressed COL1A locus. Targeted knock-in of two pseudo attP sites (50 bp each; 100 nt total) downstream of the polyA site of the COL1A locus. Injection of a targeting complex containing Cas9 mRNA or protein, in the presence or absence of DNA ligase IV inhibitor (SCR7) at two different time points has been investigated. Injected zygotes in batches of 25 were cultured for 6 days and the blastocysts were screened with primers spanning the targeted region. As was shown here, the use of Cas9 protein was effective in introgressing pseudo attP sites into the COL1A locus as evident from 100 bp shift in the product size (two 50 bp attP sites) denoted by asterix. In the figure, the bottom band represents the wildtype allele. The final concentrations of the reagents are: Cas9 RNA: 25 ng/μl; Cas9 protein: 25 ng/μl; sgRNA: 12.5 ng/μl; Oligo: 50 ng/μl; and SCR7: 10 μM.
**Table 1. Summary of experiments and embryo transfer outcomes.** *All zygotes are in vivo matured. All transfers into pre-ovulatory recipients except ZBED6.

| Experiment | Pregnancy outcome | # Embryos transferred | # of offspring/fetuses | % of transferred embryos that generated fetuses/offspring | % knock-in | Type of fertilization | Time (h) embryos transferred after injection |
|------------|-------------------|------------------------|------------------------|----------------------------------------------------------|------------|-----------------------|------------------------------------------|
| 1. PRNP    | Not Pregnant      | 100                    | NA                     | NA                                                       | NA         | IVF                   | 15                                       |
| 2. PRNP    | Not Pregnant      | 90                     | NA                     | NA                                                       | NA         | IVF                   | 15                                       |
| 3. ZBED6   | Not Pregnant      | 68                     | NA                     | NA                                                       | NA         | IVF                   | 48                                       |
| 4a. COL1A  | Not pregnant     | 34                     | NA                     | NA                                                       | 9.40%      | In vivo               | 36                                       |
| 4a. COL1A  | Pregnant          | 32                     | 3                      | 9.30%                                                    | 0          | In vivo               | 36                                       |
| 4b. COL1A  | Pregnant          | 54                     | 5                      | 8.90%                                                    | 57%        | IVF                   | 36                                       |
| 5. COL1A   | Pregnant          | 78                     | 7                      | 7.10%                                                    | 57%        | IVF                   | 36                                       |
| 6. COL1A   | Pregnant          | 42                     | 3                      | 7.10%                                                    | 33%        | In vivo               | 36                                       |

**Generation of attP knock-in pigs by direct injection of CRISPR reagents into porcine zygotes.** Following *in vitro* validation, we performed embryo transfers to generate animals with knock-in of a LoxP site into the PRNP or ZBED6 loci, or pseudo attP sites downstream of the COL1A locus. The summary of embryo transfer experiments is shown in Table 1. In initial trials, the use of *in vitro* fertilized embryos and zygotic injections proved to be unsuccessful in generating edited animals. However, using *in vivo* matured oocytes that were fertilized *in vitro*, or the use of *in vivo* derived zygotes resulted in successful pregnancies. As shown in Fig. 4 and Supplementary Figs S6 and S7, these experiments resulted in litters ranging from 3 to 7 piglets, and observed knock-in efficiencies between 0% and 66% of the resulting fetuses carrying a targeted knock-in. Following the birth of the first batch of live animals, pregnancies that have progressed further and that ensure a successful pregnancy outcome were terminated at day 60 of pregnancy. At this stage, the piglets have not started respiration, and the fetuses will not have to be euthanized, but can be collected to assess targeting efficiencies. Among the live offspring that have been generated, evidence for any mosaicism was investigated at 6 months of age. As shown in Supplementary Fig. S8, no evidence of mosaicism (only 2 alleles noted) was evident in tissues from the 3 germ layers that were investigated at the time of euthanasia at 6 months of age. Additionally, in the offspring, no evidence for off-targeting was found in the top two exonic and non-exonic off-target regions (Supplementary Fig. S9). To test the functionality of the pseudo attP sites, a GFP donor vector containing a complementary attB site was co-transfected with a plasmid expressing mammalian codon optimized phiC31 integrase. The resulting cells were selected for 2 weeks with G418, the GFP positive cells flow sorted and screened for targeted knock-in. As shown in Supplementary Fig. S10, only cells knock-in for pseudo attP sites in the COL1A locus showed site-specific integration of the GFP transgene and not the control wildtype cells.

**Discussion**

The importance of the domestic pig as a model for agricultural and biomedical applications is well documented. From a biomedical standpoint, arguably the pig (and other livestock species) is better suited for investigating diabetes, cardiovascular and metabolic disorders than the mouse or other phylogenetically distant animal models. However, the widespread use of pigs in biomedical studies has not been realized despite a broad consensus on the utility of pig models within the biomedical community. Apart from considerations such as costs associated with housing and maintaining large animals and long generation interval, the time and effort it takes in generating genetically modified pigs carrying targeted modifications was very inefficient, laborious and often unpredictable. The advent of genome editors has been a game changer for genetic engineering of pigs and other livestock, which have not witnessed a major breakthrough since cloning or nuclear transfer two decades ago. The CRISPR/Cas system is ushering in a revolution in this field. The CRISPRs are easy to engineer for an average laboratory as they require the use of just two reagents, Cas9 mRNA or a commercially available Cas9 protein preparation, and a chimeric single guide RNA that can be designed and *in vitro* transcribed or purchased commercially. The CRISPRs engineer double strand breaks at the target site with high reliability. This double strand break is a precursor for targeted modifications, including disruption of the open reading frame and generation of knock-out animals, introduction of short or long stretches of sequences, and more importantly point mutations. The main objective of this study is to showcase the utility of these tools in the context of zygotes and the advantages they offer, such as being able to engineer targeted mutations directly in the embryos and generate edited animals in one generation. In this study, the introduction of DSBs coupled with the use of ssDNA oligos has engineered targeted mutations in 33–60% among viable piglets. An added advantage to performing gene targeting in zygotes is the elimination of the need to engineer mutations in somatic cells and subsequent SCNT. This work relieves the difficulties in achieving targeted mutations in somatic cells, especially those genomic regions that are highly refractory to modification (regions that are transcriptionally silent in somatic cells and potentially within heterochromatic regions (not shown)). SCNT is a challenging technology beyond the capability of most laboratories. That said the use of zygotes also has its own set of limitations. As shown in Table 1, our use of *in vitro* fertilized embryos failed to establish a successful pregnancy even though we could show that IVF embryos had undergone gene targeting *in vitro*. In pigs, *in vitro* matured oocytes fail to mount an effective block to polyspermy, resulting...
in fertilization from more than a single sperm, and consequently a failure to generate viable embryos and establish pregnancies\textsuperscript{25,26}. A second concern is zona hardening, where the expanded embryos fail to hatch from the hardened zona and establish pregnancy. The use of \textit{in vivo} matured oocytes that have been \textit{in vitro} fertilized has generated a healthy litter. Genotyping of genomic DNA via PCR from tail (T) or ear (E) samples showed that 3 of the 5 piglets carried targeted knock-in of the pseudo attP sites (denoted by asterix). Control genomic DNA from SCNT derived fetus (NT), targeted fibroblasts (C), and wildtype (WT) pigs is shown for comparison. (B) Photograph of the litter generated from embryo transfer. (C) Sanger sequencing confirmed the knock-in of 100 bp of pseudo attP sites (Piglet # 60 and 63). Interestingly, animals that did not undergo targeted knock-in via HDR, had indels at the cut site, diagnostic of NHEJ repair (Piglets # 60 second allele; 61 and 62).

Figure 4. Piglets generated by targeted knock-in of pseudo attP sites into the \textit{COL1A} locus using a Cas9 ribonucleoprotein complex. Injection of targeting mixture consisting of Cas9 protein complexed with sgRNA and a targeting oligo into \textit{in vivo} matured, \textit{in vitro} fertilized embryos has generated a healthy litter. Genotyping of genomic DNA via PCR from tail (T) or ear (E) samples showed that 3 of the 5 piglets carried targeted knock-in of the pseudo attP sites (denoted by asterix). Control genomic DNA from SCNT derived fetus (NT), targeted fibroblasts (C), and wildtype (WT) pigs is shown for comparison. (B) Photograph of the litter generated from embryo transfer. (C) Sanger sequencing confirmed the knock-in of 100 bp of pseudo attP sites (Piglet # 60 and 63). Interestingly, animals that did not undergo targeted knock-in via HDR, had indels at the cut site, diagnostic of NHEJ repair (Piglets # 60 second allele; 61 and 62).
Materials and Methods

Production of Cas9 mRNA and sg RNA. Expression plasmid for Cas9 nuclease (pMJ920) was a gift from Jennifer Doudna (Addgene plasmid # 42234)27, and Cas9_D10A nickase was a gift from George Church (Addgene plasmid # 41816)28. The SpCas9 and Cas9D10A plasmids were gel purified and used as the template for in vitro transcription using mMESSAGE T7 ULTRA kit (Life Technologies). Targeting guide RNAs were designed based on the software available from MIT (http://www.genome-engineering.org/crispr/). Two complementary sgRNA oligo DNAs (19–22 nucleotides in length depending on the guide sequence) were synthesized by IDT (IDT DNA technologies), annealed to form double-strand DNA and cloned into a BsaI restriction enzyme digested in-house T7 promoter driven vector. The cloned fragments were DNA sequenced to confirm their fidelity and in vitro transcribed using MEGA shortscript T7 kit (Life Technologies) to generate chimeric single guide (sg) RNAs. In vitro transcribed Cas9, Cas9_D10A mRNA and the sgRNA were purified with MEGAClear kit (Life Technologies) and eluted in RNase-free water for embryo injections.

Assembly of targeting vector. A gene targeting vector was generated consisting of 1000 bp and 500 bp upper and lower arms homologous to the targeted cut site bordering exon-3 of PRNP gene. Using primers bearing unique restriction sites for Ascl, and Xhol, 1000 bp of sequence of upper arm was PCR amplified, restriction enzyme digested, gel purified and ligated into a similarly cut GFP expressing piggyBac vector (Sanger Institute)29. Likewise, using primers consisting of unique BsiWI and MluI sites, 1000 bp of sequence downstream of the cut site were amplified and cloned into the same GFP piggyBac vector. Standard molecular techniques were used to select for positive clones and all clones were confirmed with DNA sequence analysis. The Ascl, HpaI fragment carrying 1000 bp upper arm and 500 bp lower homology arm was used for microinjection and gene targeting.

In vitro maturation, in vitro fertilization and embryo culture. All chemicals were obtained from Sigma Chemical Company (St. Louis, MO) unless stated otherwise. Cumulus oocyte complexes (COCs) from abattoir sows were purchased from ART Inc. (Madison, WI) or DeSoto Biosciences Inc. (Seymour, TN) and shipped to the lab overnight in commercial Maturation medium #1 (provided by ART or DeSoto) at 38.5°C. Twenty-four hours (h) after being placed in the Maturation medium #1, 50 to 75 COCs were placed in commercial Maturation medium #2 (provided by ART or DeSoto) and cultured for an additional 20 h at 38.5°C and 5% CO2 in air, 100% humidity. COCs were vortexed in 0.1% hyaluronidase in HEPES-buffered medium containing 0.01% PVA for 4 minutes to remove the cumulus cells following maturation. Groups of 30–35 mature, denuded oocytes were placed in 100 μl of a modified Tris-buffered medium (mTBM) and fertilized according to established protocol30 using fresh extended boar semen. One ml of extended semen was mixed with Dulbecco’s Phosphate Buffered Saline (DPBS) containing 1 mg/ml BSA to a final volume of 10 ml and centrifuged at 1000 × g, 25 °C for four minutes, and spermatozoa were washed in DPBS three times. After the final wash, spermatozoa were re-suspended in mTBM medium and added to oocytes at a final concentration of 5 × 105 spermatozoa /ml, and co-incubated for 5 h at 38.5°C and 5% CO2. Presumptive zygotes were used for microinjection and subsequently placed in Porcine Zygote Medium 3 (PZM3) supplemented with 3 mg/ml fatty acid-free BSA31 and cultured at 38.5 °C, 5% CO2, 5% O2 and 100% humidity.

Parthenogenetic activation. Cumulus-oocyte complexes (COCs) following culture and maturation in Maturation medium II for 20 hours were electro-activated for generation of parthenogenetic embryos. The oocytes were placed in activation medium (300 mM Mannitol, 0.1 mM CaCl2, 0.1 mM MgSO4, 0.5 mM Hepes, 0.01% BSA) between two platinum electrodes of an Electrocell manipulator (BTX, San Diego, CA) and activated by two DC pulses of 1.2 kV/cm for 30 μs. After 2 minutes of equilibration in activation medium, the embryos were transferred into PZM3 medium and cultured for 7 days. The resulting blastocysts were screened for targeted knock-ins. Parthenogenetic embryos were used for Cas9 and sgRNA titration experiments and for optimization of methodologies for targeting of COL1A locus (Fig. 3 and Supplementary Fig. S4).

Collection of in vivo matured oocytes and zygotes for production of gene-targeted pigs via zygote injection with spCas9 protein/sgRNA/ssDNA. All experiments involving live animals were performed in accordance with the approved guidelines of the Beltsville ARS Institutional Animal Care and Use Committee (IACUC). All experimental protocols involving live animals were approved by the IACUC committee. Pubertal gilts were estrus synchronized using Alternogest (Regumate or Matrix, Merck, PA), and checked daily for the onset of estrus (Day 0)15. Gilts displaying estrus were inseminated with extended boar semen (Progenes, SD). Zygotes were recovered surgically 24 h later by mid-ventral laparotomy. Briefly, gilts were anesthetized with an intravenous injection of telazol, ketamine, xylazine mixture, and anesthesia was maintained under 5% isofluorane inhalation. The reproductive tract was exposed and the oviducts were flushed with 25 ml of Hepes-buffered medium. The recovered medium was examined under a dissecting microscope to collect the embryos. All recovered in vivo zygotes were immediately placed into a drop of TL-Hepes medium and used for microinjection.

Microinjection of embryos. In vitro fertilized, parthenogenetic, or in vivo fertilized embryos were microinjected with a mixture of Cas9 mRNA or protein, target sgRNA mixture alongside targeting plasmid or ssDNA using a Femtojet microinjector (Eppendorf; Hamburg, Germany). The micro-injected parthenogenetic embryos were cultured to blastocyst stage in PZM3 medium for 144 h at 38.5°C, 5% CO2, 5% O2 and 100% humidity for screening. The Cas9 protein is purchased from PNA Bio (Newbury Park, CA) and precomplexed as per manufacturers’ recommendations. Briefly 3 μl of 250 ng/μl Cas9 protein is mixed with 3 μl of 250 ng/μl sgRNA and incubated for 10 minutes at room temperature. The resulting Cas9 ribonucleoprotein complex mixture is diluted by 10 fold to a final concentration of 25 ng/μl for microinjection. The in vitro fertilized or in vivo collected zygotes were subjected to cytoplasmic microinjection with the spCas9 protein/sgRNA/ssDNA mixture as described above.
Genotyping of embryos, fetuses and edited animals. In vitro cultured single blastocysts cultured for 144 h were washed three times with PBS-PVA (pH 7.4) medium and transferred into 9 μl of blastocyst lysis buffer (50 mM KCl, 1.5 mM MgCl2, 10 mM Tris pH 8.0, 0.5% NP-40, 0.5% Tween-20 and 100 μg/ml proteinase K) and incubated for 1 h at 65 °C. The digestion was terminated by heating the mixture at 95 °C for 10 min, and 2 μl of supernatant was used as a PCR template. Tissue biopsies (ear notch and tail dock) from fetuses and offspring were digested in a tissue lysis buffer (50 mM Tris pH 8.0, 0.1 M NaCl, 20 mM EDTA, 1% SDS, 50 μg/ml RNase A, 100 μg/ml proteinase K) overnight at 65 °C. Following overnight digest, genomic DNA was extracted using phenol-chloroform, and recovered by resuspension in 100 μl of 10 mM Tris- HCl pH 7.4 buffer following ethanol precipitation. Genomic DNA from embryos above or purified genomic DNA from tissues was amplified using PCR, cloned into PCR2.1 vectors (Life Technologies) and transformed into E. coli DH5α maximum competent cell (Life Technologies). Ten colonies were picked, cultured, plasmid DNA extracted, and sequenced (Macrogen). Sequences were aligned by Bio-Edit software for comparison with wild-type alleles.

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Author Contributions
K.P. and B.T. conceived and planned experiments. K.P. performed in vitro culture and embryo injections. C.K. assisted K.P. in screening targeted embryo modifications. A.P., S.S., and B.T. synchronized the donor and recipient animals and performed embryo transfers. D.D. provided technical discussions and support for both animal space and technical support. A.M. provided germplasm and assisted in design of experiments. K.P., A.P. and B.T. analyzed and evaluated data. B.T wrote the manuscript with critiques from all other authors. All authors reviewed the manuscript.

Additional Information
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