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*Fok*I-dCas9 mediates high-fidelity genome editing in pigs

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Running head: High-fidelity genome editing in pigs

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Abstract

Background: Gene editing using CRISPR/Cas9 has great potential for improving the compatibility of porcine organs with human recipients. However, the risk of detrimental off-target mutations in gene-edited pigs remains largely undefined. We have previously generated *GGTA1* knock-in pigs for xenotransplantation using *Fok*I-dCas9, a variant of Cas9 that is reported to reduce the frequency of off-target mutagenesis. In this study, we used whole genome sequencing (WGS) and optimized bioinformatic analysis to assess the fidelity of *Fok*I-dCas9 editing in the generation of these pigs.

Methods: Genomic DNA was isolated from porcine cells before and after gene editing and sequenced by WGS. The genomic sequences were analyzed using *GRIDSS* variant-calling software to detect putative structural variations (SVs), which were validated by PCR of DNA from knock-in and wild type pigs. *Platypus* variant-calling software was used to detect single nucleotide variations (SNVs) and small insertions/deletions (indels).

Results: *GRIDSS* analysis confirmed the precise integration of one copy of the knock-in construct in the gene-edited cells. Three additional SVs were detected by *GRIDSS*: deletions in intergenic regions in chromosome 6 and the X chromosome, and a duplication of part of the *CALD1* gene on chromosome 18. These mutations were not associated with plausible off-target sites, and were not detected in a second line of knock-in pigs generated using the same pair of guide RNAs, suggesting that they were the result of background mutation rather than off-target activity. *Platypus* identified 1,375 SNVs/indels after quality filtering, but none of these were located in proximity to potential off-target sites, indicating that they were probably also spontaneous mutations.

Conclusions: This is the first WGS analysis of pigs generated from *Fok*I-dCas9-edited cells. Our results demonstrate that *Fok*I-dCas9 is capable of high-fidelity gene editing with negligible off-target or undesired on-target mutagenesis.

Keywords

Xenotransplantation, CRISPR, knock-in pig, off-target event.

Abbreviations used

CRISPR	clustered regularly interspaced short palindromic repeats
indel	insertion/deletion
KI	knock-in
mAb	monoclonal antibody
SCNT	somatic cell nuclear transfer
SNV	single nucleotide variation
SV	structural variation
WGS	whole genome sequencing

WT wild type

Authorship statement

N.F. and E.J.S. performed the molecular and cellular biology including data analysis, and contributed to the writing of the manuscript.

M.J.W. designed the genomic and bioinformatic analysis. G.K.P. and M.J.W. performed the bioinformatic analysis and contributed to the writing of the manuscript.

M.B.N. and W.J.H. contributed to the writing of the manuscript.

P.J.C. conceived and designed the study, contributed to data analysis, wrote the manuscript, and carried the main responsibility for the study.

Acknowledgements

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Introduction

CRISPR-Cas9-mediated genome editing has had an extraordinary impact in many areas of research, because it enables relatively simple but precise genetic modification of cells and animals. In xenotransplantation, the list of pigs generated from CRISPR-modified cells or embryos is expanding rapidly (1). One of the concerns with CRISPR technology is the propensity of the widely used Cas9 nuclease from *Streptococcus pyogenes* to introduce off-target mutations into the genome, with unpredictable consequences. Screening for such events in modified animals has generally been

limited to the *in silico* prediction of off-target sites, followed by PCR/sequencing analysis of a variable number of the top 'hits'. Many groups have used this technique to analyze mice produced from Cas9-modified embryos, and have reported a frequency of off-target mutations ranging from negligible (2, 3) to present in up to 29% of mice born (4). Although fewer such studies have been performed in Cas9-modified pigs, the reported rate of off-target events is generally low (5-7), with the caveat that this detection method is somewhat biased and relatively inaccurate (8). Whole genome sequencing (WGS), a less biased and more powerful approach, also suggests a low rate of Cas9 off-target mutagenesis in both mice (9) and pigs (10). However, short-read, high-throughput WGS technology can easily miss large structural rearrangements including deletions and inversions (8). Furthermore, a recent study has shown that these types of genetic alteration may occur as a result of on-target activity of Cas9 (11), i.e. independent of off-target events. Thus it is possible that the frequency of unintended Cas9 mutagenesis in animals has been underestimated.

To address the risk of detrimental off-target mutations, variants of Cas9 with higher fidelity have been developed (12). One such variant, *Fok*I-dCas9, comprises a 'nuclease-dead' Cas9 fused to a subunit of the endonuclease *Fok*I (13). The requirement for two appropriately spaced and oriented target sites to activate *Fok*I-mediated cleavage of DNA accounts for the improved specificity of *Fok*I-dCas9, albeit coming at the cost of reduced flexibility in targeting. We have previously used *Fok*I-dCas9 to generate knock-in pigs in which a 7.1-kb anti-CD2 monoclonal antibody (mAb) transgene was integrated into the *GGTA1* gene responsible for synthesis of the αGal xenoantigen (14). We confirmed correct integration by PCR and sequencing of the transgene junctions. In the current study, we used WGS and variant-calling software to search for off-target and on-target mutations in the *Fok*I-dCas9-edited cells used to generate the knock-in pigs. The bioinformatic analysis was designed to detect not only single nucleotide variations (SNVs) and small insertions/deletions (indels), but also larger off-target and on-target structural variations (SVs). Apart from the transgene knock-in and the previously reported small deletion in the second copy of *GGTA1*, we

identified three SVs (two deletions and one tandem duplication) in the edited cells. Analysis of the wild type (WT) genomic sequence in the vicinity of these rearrangements revealed an absence of plausible off-target sites, suggesting that the mutations were a result of background *de novo* mutation. 1,375 SNVs/indels were also detected, but none of these were located in close proximity to possible off-target sites. We therefore conclude that *Fok*I-dCas9 mediates high-fidelity genome editing in pigs.

Materials and Methods

Whole genome sequencing

Genomic DNA was prepared from WT and *Fok*I-dCas9-edited porcine cells (14) using the Roche High Pure PCR Template Preparation Kit (Roche Diagnostics Australia Pty. Ltd., North Ryde, Australia). Whole genome sequencing was performed at the Australian Genome Research Facility (AGRF, Parkville, Australia) using a TruSeq DNA PCR-free library on an Illumina HiSeq X Ten system at 30x coverage. The genomic sequence data are available from the authors on request.

Bioinformatic analysis

The *Sus scrofa* genome (version 11.1) was downloaded from Ensembl and the anti-CD2 transgene sequence was added as an extra chromosome (*Sus scrofa + transgene* genome). *Trimmomatic* v0.36 (15) was used to trim adapters from the paired-end reads with the TruSeq3-PE_adapters.fa file provided and with parameters ILLUMINACLIP, seedMismatches:1, palindromeClipThreshold:25, simpleClipThreshold:12, minAdapterLength:4 and keepBothReads:true. Trimmed reads were then aligned to the *Sus scrofa + transgene* genome using *BWA mem* v0.7.12 with default parameters, and alignments were co-ordinate sorted with *SAMtools* v1.4 (16, 17). The Genome Rearrangement IDentification Software suite (*GRIDSS*) (18) was used to detect structural variations (SVs). The *GRIDSS* algorithm was chosen to detect SVs because of its relatively high precision and recall for the types of events that would be generated by CRISPR, compared to other SV callers (19). The aligned and sorted reads were

used as input into the *GRIDSS CallVariants* v2.0.1 tool with the default parameter set. Variants were filtered using *bcftools* v1.9 to exclude (i) those that failed *GRIDSS* filters, (ii) where the WT sample differed from the *Sus scrofa* reference genome (VF[WT]>0), and (iii) single break-end calls (VF[Transgene]==0 && VF[WT]==0). Filtered variants were then annotated using *VEP* v94 (20).

In parallel, single nucleotide and short insertion-deletion variant detection was performed using *Platypus callVariants* v0.8.1.1 (21) with default settings. The *Platypus* algorithm was chosen to detect SNVs and indels due to its high sensitivity and low rate of false positives and artefacts relative to other callers (22). A bed file of repeat regions in the *Sus scrofa* (v11.1) reference genome was downloaded from the UCSC Table browser (23), and BEDTools (v2.28.0) intersect-v (24) was used to mask variants occurring in the repeat regions. In addition, variants were filtered using *bcftools* v1.9 to exclude (i) calls that failed *Platypus*'s filters, (ii) had missing variants, (iii) where the WT sample differed from the *Sus scrofa* reference genome, and (iv) where there were less than 10 reads in the WT sample. Filtered variants were then annotated using *VEP* v94 (20). The *filter_vep* script was used to filter for variants located in protein-coding genes, with a moderate or high impact.

PCR screening of putative mutations

Genomic DNA was prepared from either peripheral blood mononuclear cells or tail-tip samples from *GGTA1*/anti-CD2 knock-in (14) and WT pigs using the Roche High Pure PCR Template Preparation Kit or Quick Extract DNA extraction solution 1.0 (Gene Target Solutions, Dural, Australia) respectively. CLC Main Workbench software version 7.6.4 (QIAGEN Bioinformatics, Redwood City, CA) was used to design PCR primers to amplify WT and putative mutant sequences. Primer sequences are listed in Supplementary Table 1. PCR was performed on a Bio-Rad T100 Thermal Cycler (Bio-Rad Laboratories Pty. Ltd., Gladesville, Australia), using the following touchdown protocol: 95ºC/3 min; 13 cycles of 95ºC/20 sec, 58ºC/30 sec (reducing by 0.5ºC per

cycle), 72ºC/90 sec; 26 cycles of 95ºC/20 sec, 52ºC/30 sec, 72ºC/90 sec; 72ºC/2 min. PCR products were cloned into pGEM-T Easy (Promega Australia, Alexandria, Australia) and sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Scoresby, Australia), with analysis by AGRF.

Results

Confirmation of knock-in sequence

The experimental outline for the study is shown in Fig. 1. We originally isolated a clonal population (clone #3) of *GGTA1*/anti-CD2 knock-in cells, following co-transfection of WT porcine fetal fibroblasts with an H-2K^{b} promoter-driven anti-CD2 transgene knock-in construct and expression vectors for *Fok*I-dCas9 and two guide RNAs (14). Knock-in pigs were generated from clone #3 by somatic cell nuclear transfer (SCNT) (14). Genomic DNA was isolated from clone #3 and the untransfected WT cells from which it was derived, and sequenced by WGS. Analysis of the sequences using *GRIDSS* SV-calling software confirmed the precise integration of the 7.1 kb anti-CD2 mAb transgene into one allele of *GGTA1* and a 43 bp deletion in the second allele, as reported previously (14). The analysis also confirmed the absence of integration anywhere in the genome of additional copies of the transgene, or of the *Fok*I-dCas9 and guide RNA expression vectors.

Identification of structural variations and SNVs/indels

After filtering, *GRIDSS* SV-calling software identified three putative off-target differences between the genomic sequences of clone #3 and the WT parental cells (Fig. 2 and Supplementary Table 2). A 12,452 bp deletion, coupled with a 3 bp insertion (ATT), was detected in an intergenic region in chromosome 6 in clone #3. The X chromosome in clone #3 contained a 706 bp deletion, also within an intergenic region. Chromosome 18 in clone #3 exhibited a 36,060 bp tandem duplication in a region containing the 3´ end of the *CALD1* (caldesmon 1) gene.

To confirm each SV, PCR primers were designed to amplify WT and putative mutant sequences. The primers are shown as red arrowheads in Fig. 2. Genomic DNA from a knock-in founder pig (14) or a male WT control pig was used as the template. As can be seen in Fig. 3, the chromosome 6 WT primer pairs amplified the expected products from both WT and knock-in DNA (tracks 1-4), indicating the presence of at least one chromosomal copy lacking the putative mutation. The chromosome 6 mutant primer pair amplified the expected product from the knock-in DNA only (track 6), and sequencing of this product confirmed the 12 kb deletion/3 bp (ATT) insertion. These data indicate that the deletion/insertion was present in one copy of chromosome 6 in knock-in pigs generated from the *Fok*I-dCas9-modified cells. Similarly, the chromosome 18 WT primer pair amplified the expected product from both WT and knock-in DNA (tracks 13-14), whereas the mutant primer pair generated a product from the knock-in DNA only (track 16), indicative of the predicted tandem 36 kb duplication on one copy of chromosome 18 in the knock-in pigs.

Clone #3 and the founder knock-in pigs generated from it are male (14) and therefore possess only one copy of the X chromosome. The X chromosome WT primer pairs amplified the expected products from WT DNA only (tracks 7 and 9). The X chromosome mutant primer pair amplified products of different sizes from WT (track 11) and knock-in (track 12) pig DNA, with the difference in size (determined by sequencing) corresponding to the predicted deletion of 706 bp. These data confirmed the presence of this mutation on the X chromosome of the knock-in pigs.

After filtering, 1,375 SNVs/indels were detected by the *Platypus* algorithm (Supplementary Table 3). The background mutation rate in human and mouse somatic cells has been measured at 2.66 x 10^{-9} and 8.1 x 10^{-9} mutations per bp per mitosis, respectively (25). Assuming a similar rate in pigs, and given the size of the porcine genome (approximately 2.7 Gb), one would expect around 7-22 spontaneous mutations per cell doubling for porcine fibroblasts. We estimate that clone #3 cells underwent at

least 25 doublings during their generation from the parental WT cells. It is thus conceivable that the SNVs/indels observed in clone #3 genomic DNA arose due to spontaneous mutation.

Off-target events or background mutation?

Two approaches were taken to investigate whether the three SVs detected by *GRIDSS* were likely to be due to *Fok*I-dCas9 off-target activity, or to background *de novo* mutation during the generation of the modified cells. First, the program *fuzznuc* from the EMBOSS (v6.6.0.0) package (26) was used to scan the WT genomic sequence encompassing each of the mutations for the presence of potential off-target sites for the two guide RNAs. Even with up to 5 mismatches permitted (excluding mismatches in the PAM sequence), no single off-target sites were identified within 100 kb upstream or downstream of the mutations (Supplementary Table 4). Second, genomic DNA from an independent *GGTA1* knock-in pig line, generated using *Fok*I-dCas9 and the same *GGTA1*-targeting guide RNAs (14), was used as template for PCR with the three mutant primer pairs shown in Fig. 2 and listed in Supplementary Table 1. No products were amplified with any of the mutant primer pairs (data not shown). Together these results suggest that background mutation, rather than *Fok*I-dCas9 activity, was the probable source of the three SVs identified in this study. The WT genomic sequence was also scanned using *fuzznuc* for potential off-target sites in proximity to the 1,375 SNVs/indels found by *Platypus*. No sites were detected within 75 bp of any of these events.

Discussion

Fidelity is a key consideration in CRISPR genome editing. There is ongoing debate about the degree of 'collateral damage' mediated by Cas9, both off- and on-target, and how best this damage can be measured and minimized (27). Our WGS analysis demonstrates that it is possible to edit the pig genome with high fidelity using the Cas9 variant *Fok*I-dCas9. Only three structural variations were detected following the editing process: deletions in intergenic regions on chromosome 6 and the X chromosome, and a tandem duplication in chromosome 18 in the region of the *CALD1* gene. *CALD1*

encodes a calmodulin- and actin-binding protein that plays a role in the regulation of smooth muscle contraction (28). The duplication encompasses several exons of *CALD1*, but because pig *CALD1* has five known transcripts it is difficult to predict the effect, if any, of this mutation. The knock-in pigs carrying all three mutations were healthy and developed normally, suggesting that the *CALD1* mutation (at least in the heterozygous state) is harmless. More importantly, our data suggested that the SVs were likely to have arisen *de novo* during the expansion of clone #3, rather than as a result of *Fok*I-dCas9 off-target activity. Similarly, none of the 1,375 SNVs/indels were associated with even a single potential off-target site, let alone appropriately spaced and oriented paired sites. Furthermore, the number of SNVs/indels observed was broadly consistent with previously observed rates of somatic mutation in cell culture (25).

Our study has some limitations. We tested only one target site, with one pair of guide RNAs, and we did not compare the fidelity of *Fok*I-dCas9 with that of Cas9 or other gene-editing nucleases. However, we believe that the study provides useful data on the precision of *Fok*I-dCas9-mediated gene editing, which to our knowledge has not previously been examined by WGS methods. Furthermore, in the field of xenotransplantation, it gives confidence in the use of the *GGTA1*-targeted guide RNAs with *Fok*I-dCas9 to knock other transgenes into this critical target gene with a very low risk of off-target or undesired on-target mutagenesis. Finally, we have refined the analytical bioinformatic tools to allow more accurate detection of large-scale rearrangements in genome-edited cells.

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Figure Legends

Figure 1. Experimental outline. Solid lines indicate work performed previously (14); dashed lines indicate work performed in this study.

Figure 2. Mutations in *Fok*I-dCas9-modified porcine cells (clone #3), identified by whole genome sequencing. In each case, the WT chromosome is shown above, with bp coordinates indicated, and the putative mutant chromosome is shown below. WT sequence that is deleted is shaded grey, and tandemly repeated sequence is shaded blue. PCR primers used to confirm WT and mutant sequences are indicated by red arrowheads. Note that the 'mutant' (MUT) PCR primers will also bind to WT DNA, but will either generate a different sized product to mutant DNA or no product at all. [Not drawn to scale.]

Figure 3. PCR analysis of mutations in *GGTA1*/anti-CD2 knock-in pigs. Template genomic DNA is denoted as WT or KI (knock-in). Primer sets are indicated below each pair of reactions. Expected product sizes are as follows. 6WT-F1/R1: 183 bp; 6WT-F2/R2: 694 bp; 6MUT-F1/R1: 620 bp (KI only); XWT-F1/R1: 590 bp; XWT-F2/R2: 438 bp; XMUT-F1/R1: 1,179 bp (WT), 473 bp (KI); 18WT-F1/R1: 615 bp; 18MUT-F1/R1: 474 bp (KI only). All PCR products were confirmed by sequencing. MW = molecular weight markers (Lambda/*Hin*dIII – PhiX 174/*Hae*III).

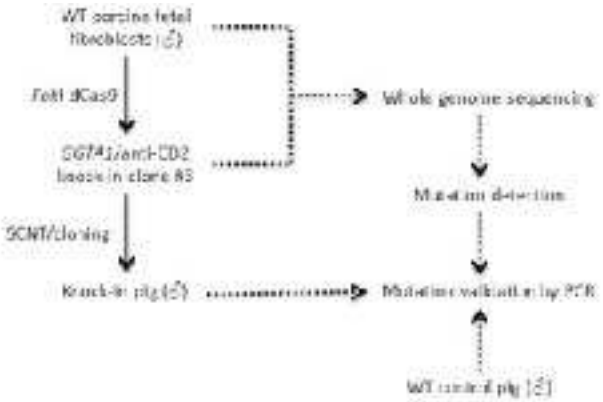

Figure 1: Experimental outline. Solid lines indicate work performed previously (14); dashed lines indicate work performed in this study.

xen_12551_f1.jpg

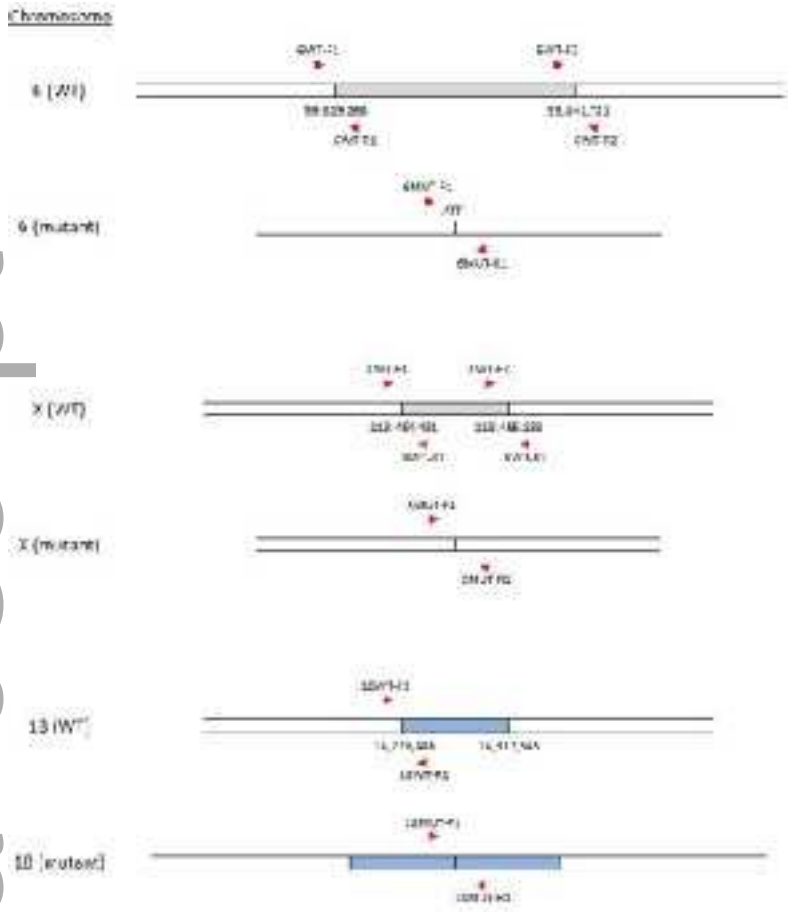

Figure 2: Mutations in fast-cCas9-modified pachytene cells (n=45), identified by whole-genome sequencing. In each case, the WT chromosome is shown above, with bp coordinates indicated, and the putative mutant chromosome is shown below. WT sequence that is deleted is shaded grey, and tandemly repeated sequence is shaded blue. PCR primers used to confirm WT and mutant sequences are indicated by red arrowheads. Note that the 'mutant' (MUT) PCR primers will also bind to WT DNA, but will either generate a different sized product to mutant DNA or no product at all. [Not drawn to scale.]

xen_12551_f2.jpg

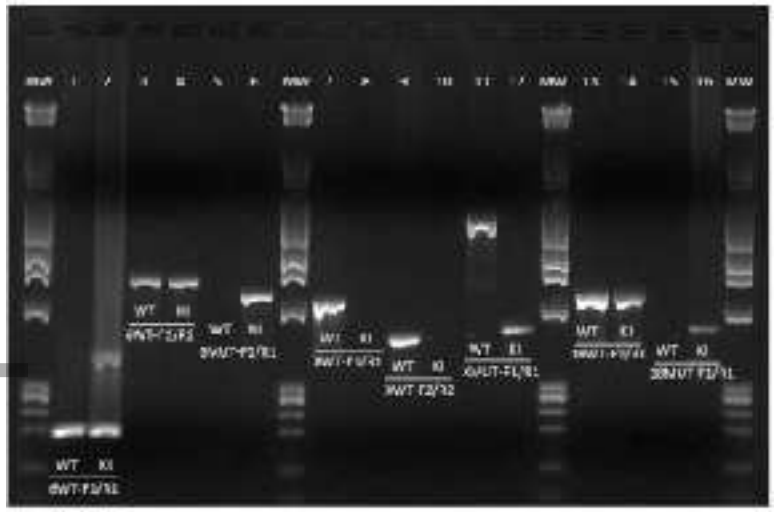

Figure 3: PCR analysis of mutations in Gt(ROSA)26Sor-LUZ knock-in pigs. Template genomic DNA is denoted as WT or KI (knock-in). Primer sets are indicated below each pair of reactions. Expected product sizes are as follows: RWT-F1/R1: 187 bp; RWT-F2/R2: 494 bp; RMUT-F1/R1: 620 bp (KI only); RWT-F1/R1: 590 bp; RWT-F2/R2: 454 bp; RMUT-F1/R1: 1,179 bp (WT); 815 bp (KI); HWT-F1/R1: 515 bp; HMUT-F1/R1: 474 bp (KI only). All PCR products were confirmed by sequencing. MW = molecular weight markers (Lambda/HindIII + PhiX 174/HaeIII).

xen_12551_f3.jpg