Genome-wide inactivation of porcine endogenous retroviruses (PERVs)

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The shortage of organs for transplantation is a major barrier to the treatment of organ failure. While porcine organs are considered promising, their use has been checked by concerns about transmission of porcine endogenous retroviruses (PERVs) to humans. Here, we describe the eradication of all PERVs in a porcine kidney epithelial cell line (PK15). We first determined the PK15 PERV copy number to be 62. Using CRISPR-Cas9, we disrupted all 62 copies of the PERV *pol* gene and demonstrated a >1000-fold reduction in PERV transmission to human cells using our engineered cells. Our study shows that CRISPR-Cas9 multiplexability can be as high as 62 and demonstrates the possibility that PERVs can be inactivated for clinical application to porcine-to-human xenotransplantation.

Pig genomes contain from a few to several dozen copies of PERV elements (*1*). Unlike other zoonotic pathogens, PERVs cannot be eliminated by biosecure breeding (*2*). Prior strategies for reducing the risk of PERV transmission to humans have included small interfering RNAs (RNAi), vaccines (*3–5*), and PERV elimination using zinc finger nucleases (*6*) and TAL effector nucleases (*7*), but these have had limited success. Here we report the successful use of the CRISPR-Cas9 RNA-guided nuclease system (*8–10*) to inactivate all copies of the PERV *pol* gene and effect a 1000-fold reduction of PERV infectivity of human cells.

To design Cas9 guide RNAs (gRNAs) that specifically target PERVs, we analyzed the sequences of publically available PERVs and other endogenous retroviruses in pigs (methods). We identified a distinct clade of PERV elements (Fig. 1A) and determined there to be 62 copies of PERVs in PK15 cells using droplet digital PCR (Fig. 1B). We then designed two Cas9 guide RNAs (gRNAs) that targeted the highly conserved catalytic center (*11*) of the *pol* gene on PERVs (Fig. 1C and fig. S1). The *pol* gene product functions as a reverse transcriptase (RT) and is thus essential for viral replication and infection. We determined that these gRNAs

targeted all PERVs but no other endogenous retrovirus or other sequences in the pig genome (methods).

Initial experiments showed inefficient PERV editing when Cas9 and the gRNAs were transiently transfected (fig. S2). Thus we used a PiggyBac transposon (*12*) system to deliver a doxycycline-inducible Cas9 and the two gRNAs into the genome of PK15 cells (figs. S2 and S3). Continuous induction of Cas9 led to increased targeting frequency of the PERVs (fig. S5), with a maximum targeting frequency of 37% (~23 PERV copies per genome) observed on day 17 (fig. S5). Neither higher concentrations of doxycycline or prolonged incubation increased targeting efficiency (figs. S4 and S5), possibly due to the toxicity of nonspecific DNA damage by CRISPR-Cas9. Similar trends were observed when Cas9 was delivered using lentiviral constructs (fig. S6). We then genotyped the cell lines that exhibited maximal PERV targeting efficiencies. We observed 455 different insertion and deletion (indel) events centered at the two gRNA target sites (Fig. 2B). Indel sizes ranged from 1 to 148 bp; 80% of indels were small deletions (<9 bp). We validated the initial deep sequencing results with Sanger Sequencing (fig. S7).

We next sorted single cells from PK15 cells with high PERV targeting efficiency using flow cytometry and genotyped the *pol* locus of the resulting clones via deep sequencing (*13*, *14*). A repeatable bimodal (Fig. 2A and figs. S8 and S9) distribution was observed with ~10% of the clones exhibiting high levels of PERV disruption (97%-100%), and the remaining clones exhibiting low levels of editing (<10%). We then examined individual indel events in the genomes of these clones (Fig. 2B and figs. S10 and S11). For the highly edited clones (clone 20, 100%; clone 15, 100%; clone 29, 100%; clone 38, 97.37%), we observed only 16-20 unique indel patterns in each clone (Fig. 2B and fig. S11). In addition, there was a much higher degree of repetition of indels within each clone than across the clones (fig. S25), suggesting a mechanism of gene conversion in which previously mutated PERV copies were used as templates to repair wild-type PERVs cleaved by Cas9 (Fig. 2B and fig. S25). Mathematical modeling of DNA repair during PERV elimination (fig. S26)

and analysis of expression data (figs. S22 to S24) supported this hypothesis and suggested that highly edited clones were derived from cells in which Cas9 and the gRNAs were highly expressed.

Next, we examined whether unexpected genomic rearrangements had occurred as a result of the multiplexed genome editing. Karyotyping of individual modified clones (figs. S12 to S14) indicated that there were no observable genomic rearrangements. We also examined 11 independent genomic loci with at most 2 bp mismatches to each of the intended gRNA targets and observed no non-specific mutations (fig. S27). This suggests that our multiplexed Cas9-based genome engineering strategy did not cause catastrophic genomic instability.

Last, we examined whether our disruption of all copies of PERV *pol* in the pig genome could eliminate in vitro transmission of PERVs from pig to human cells. We could not detect RT activity in the cell culture supernatant of the highly modified PK15 clones (fig. S15), suggesting that modified cells only produced minimal amounts of PERV particles. We then co-cultured WT and highly modified PK15 cells with HEK 293 cells to test directly for transmission of PERV DNA to human cells (*15*). After co-culturing PK15 WT and HEK 293 cells for 5 days and 7 days (figs. S16 to S17), we detected PERV *pol, gag,* and *env* sequences in the HEK 293 cells (Fig. 3A). We estimated the frequency of PERV infection to be approximately 1000 PERVs/ 1000 human cells (Fig. 3B). However, PK15 clones with >97% PERV *pol* targeting exhibited up to 1000-fold reduction of PERV infection, similar to background levels (Fig. 3C). We validated these results with PCR amplification of serial dilutions of HEK293 cells that had a history of contact with PK15 clones (Fig. 3D and figs. S18 to S21). We could consistently detect PERVs in single HEK293 cells isolated from the population co-cultured with minimally modified clone 40, but we could not distinctly detect PERVs in 100 human cells from the population co-cultured with highly modified clone 20. Thus, we concluded that the PERV infectivity of the engineered PK15 cells had been reduced by up to 1000 fold.

In summary, we successfully targeted the 62 copies of PERV *pol* in PK15 cells and demonstrated greatly reduced in vitro transmission of PERVs to human cells. While in vivo PERV transmission to humans has not been demonstrated (*16, 17*), PERVs are still considered risky (*18, 19*) and our strategy could completely eliminate this liability. As no porcine embryonic stem cells exist, this system will need to be recapitulated in primary porcine cells and cloned into animals using somatic cell nuclear transfer. Moreover, we achieved simultaneous Cas9 targeting of 62 loci in single pig cells without salient genomic rearrangement. To our knowledge, the maximum number of genomic sites previously reported to be simultaneously edited has been six (*20*). Our methods thus open the possibility of editing other repetitive regions of biological significance.

REFERENCES AND NOTES

1. D. Lee, J. Lee, J. K. Yoon, N. Y. Kim, G. W. Kim, C. Park, Y. K. Oh, Y. B. Kim, Rapid determination of perv copy number from porcine genomic DNA by real-time polymerase chain reaction. *Anim. Biotechnol.* **22**, 175–180 (2011). Medline doi:10.1080/10495398.2011.595294
2. H.-J. Schuurman, The International Xenotransplantation Association consensus statement on conditions for undertaking clinical trials of porcine islet products in type 1 diabetes—chapter 2: Source pigs. *Xenotransplantation* **16**, 215–222 (2009). Medline doi:10.1111/j.1399-3089.2009.00541.x
3. J. Ramsoondar, T. Vaught, S. Ball, M. Mendicino, J. Monahan, P. Jobst, A. Vance, J. Duncan, K. Wells, D. Ayares, Production of transgenic pigs that express porcine endogenous retrovirus small interfering RNAs. *Xenotransplantation* **16**, 164–180 (2009). Medline doi:10.1111/j.1399-3089.2009.00525.x
4. M. Semaan, D. Kaulitz, B. Petersen, H. Niemann, J. Denner, Long-term effects of PERV-specific RNA interference in transgenic pigs. *Xenotransplantation* **19**, 112–121 (2012). Medline doi:10.1111/j.1399-3089.2012.00683.x
5. U. Fiebig, O. Stephan, R. Kurth, J. Denner, Neutralizing antibodies against conserved domains of p15E of porcine endogenous retroviruses: Basis for a vaccine for xenotransplantation? *Virology* **307**, 406–413 (2003). Medline doi:10.1016/S0042-6822(02)00140-X
6. M. Semaan, D. Ivanusic, J. Denner, Cytotoxic effects during knock out of multiple porcine endogenous retrovirus (PERV) sequences in the pig genome by zinc finger nucleases (ZFN). *PLOS ONE* **10**, e0122059 (2015). Medline doi:10.1371/journal.pone.0122059
7. D. Dunn, M. DaCosta, M. Harris, R. Idriss, A. O'Brien, Genetic modification of porcine endogenous retrovirus (PERV) sequences in cultured pig cells as a model for decreasing infectious risk in xenotransplantation. *FASEB J.* **29**, LB761 (2015).
8. M. Jinek, K. Chylinski, I. Fonfara, M. Hauer, J. A. Doudna, E. Charpentier, A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* **337**, 816–821 (2012). Medline doi:10.1126/science.1225829
9. P. Mali, L. Yang, K. M. Esvelt, J. Aach, M. Guell, J. E. DiCarlo, J. E. Norville, G. M. Church, RNA-guided human genome engineering via Cas9. *Science* **339**, 823–826 (2013). Medline doi:10.1126/science.1232033
10. L. Cong, F. A. Ran, D. Cox, S. Lin, R. Barretto, N. Habib, P. D. Hsu, X. Wu, W. Jiang, L. A. Marraffini, F. Zhang, Multiplex genome engineering using CRISPR/Cas systems. *Science* **339**, 819–823 (2013). Medline doi:10.1126/science.1231143
11. S. G. Sarafianos, B. Marchand, K. Das, D. M. Himmel, M. A. Parniak, S. H. Hughes, E. Arnold, Structure and function of HIV-1 reverse transcriptase: Molecular mechanisms of polymerization and inhibition. *J. Mol. Biol.* **385**, 693–713 (2009). Medline doi:10.1016/j.jmb.2008.10.071
12. M. H. Wilson, C. J. Coates, A. L. George Jr., PiggyBac transposon-mediated gene transfer in human cells. *Mol. Ther.* **15**, 139–145 (2007). Medline doi:10.1038/sj.mt.6300028
13. L. Yang, M. Guell, S. Byrne, J. L. Yang, A. De Los Angeles, P. Mali, J. Aach, C. Kim-Kiselak, A. W. Briggs, X. Rios, P. Y. Huang, G. Daley, G. Church, Optimization of scarless human stem cell genome editing. *Nucleic Acids Res.* **41**, 9049–9061 (2013). Medline doi:10.1093/nar/gkt555
14. M. Güell, L. Yang, G. M. Church, Genome editing assessment using CRISPR Genome Analyzer (CRISPR-GA). *Bioinformatics* **30**, 2968–2970 (2014). Medline doi:10.1093/bioinformatics/btu427
15. C. Patience, Y. Takeuchi, R. A. Weiss, Infection of human cells by an endogenous retrovirus of pigs. *Nat. Med.* **3**, 282–286 (1997). Medline doi:10.1038/nm0397-282
16. W. Heneine, A. Tibell, W. M. Switzer, P. Sandstrom, G. V. Rosales, A. Mathews, O. Korsgren, L. E. Chapman, T. M. Folks, C. G. Groth, No evidence of infection with porcine endogenous retrovirus in recipients of porcine islet-cell xenografts. *Lancet* **352**, 695–699 (1998). Medline doi:10.1016/S0140-6736(98)07145-1
17. J. H. Dinsmore, C. Manhart, R. Raineri, D. B. Jacoby, A. Moore, No evidence for infection of human cells with porcine endogenous retrovirus (PERV) after exposure to porcine fetal neuronal cells. *Transplantation* **70**, 1382–1389 (2000). Medline doi:10.1097/00007890-200011150-00020
18. D. Butler, Last chance to stop and think on risks of xenotransplants. *Nature* **391**, 320–324 (1998). Medline doi:10.1038/34749
19. Xenotransplantation: Science, ethics, and public policy. *ILAR J.* **38**, 49–51 (1997). doi:10.1093/ilar.38.1.49
20. H. Wang, H. Yang, C. S. Shivalila, M. M. Dawlaty, A. W. Cheng, F. Zhang, R. Jaenisch, One-step generation of mice carrying mutations in multiple genes by

CRISPR/Cas-mediated genome engineering. *Cell* **153**, 910–918 (2013). Medline doi:10.1016/j.cell.2013.04.025

SUPPLEMENTARY MATERIALS

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Methods
Figs. S1 to S27
References
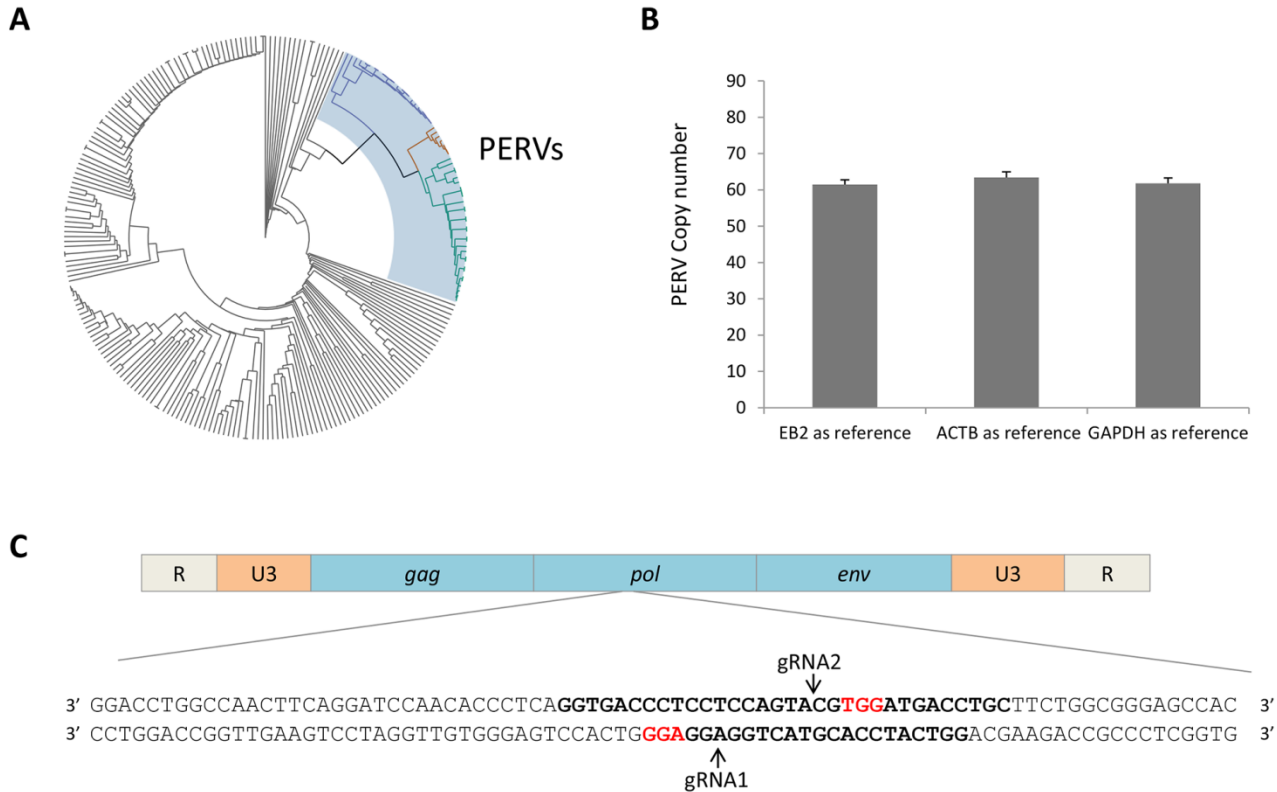
Fig. 1. CRISPR-Cas9 gRNAs were designed to specifically target the *pol* gene in 62 copies of PERVs in PK15 cells. (A) Phylogenetic tree representing endogenous retroviruses present in the pig genome. PERVs are highlighted in blue. (B) Copy number determination of PERVs in PK15 cells via digital droplet PCR. The copy number of *pol* elements was estimated to be 62 using three independent reference genes: *ACTB*, *GAPDH*, and *EB2*. $n = 3$, mean ± SEM. (C) We designed two CRISPR-Cas9 gRNAs to target the catalytic region of the PERV *pol* gene. The two gRNA targeting sequences are shown below a schematic of PERV gene structure. Their PAM sequences are highlighted in red.

A

B

Parental population (37%)

Single cell derived clones

Clone 15 (100%)

Clone 40 (0%)
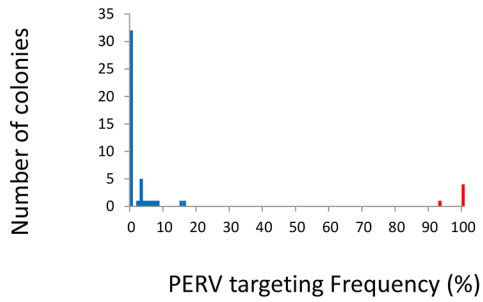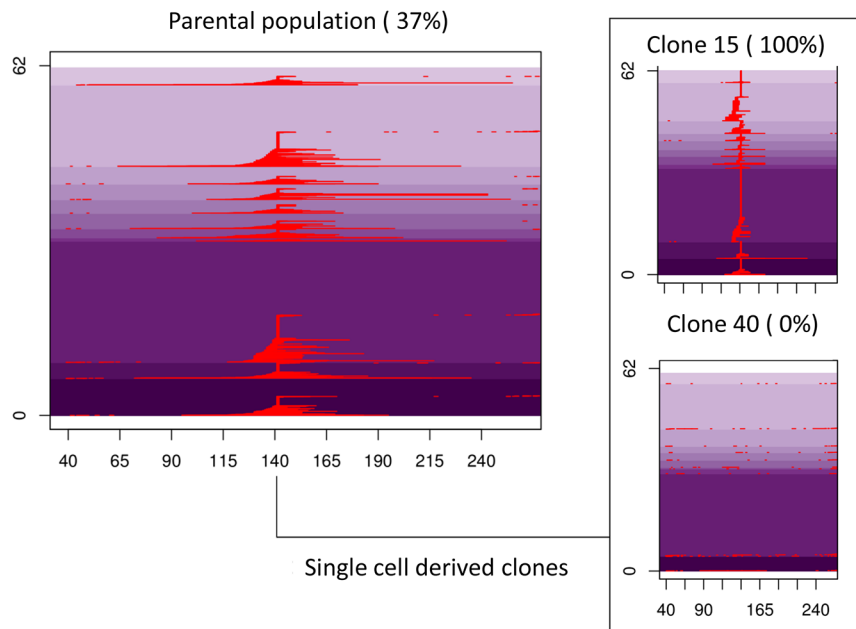
Fig. 2. Clonal PK15 cells with inactivation of all copies of PREV *pol* genes after Cas9 treatment. (A) A bimodal distribution of *pol* targeting efficiencies was observed among the single-cell-derived PK15 clones after 17 days of Cas9 induction. 45/50 exhibited <16% targeting efficiency; 5/50 clones exhibited >93% targeting efficiency. (B) PK15 haplotypes at PERV *pol* loci after CRISPR-Cas9 treatment. In red, indel events in the PERV *pol* sequence are represented. Shades of purple indicate endogenous PERVs.
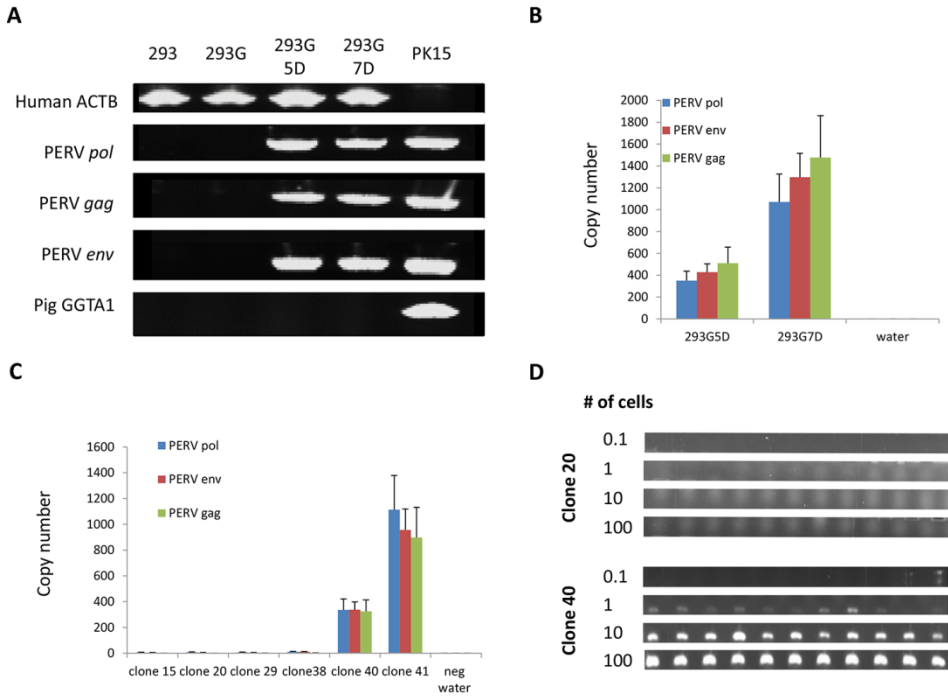
Fig. 3. (A) Detection of PERV *pol, gag*, and *env* DNA in the genomes of HEK-293-GFP cells after co-culturing with PK15 cells for 5 days and 7 days (293G5D and 393G7D, respectively). A pig GGTA1 primer set was used to detect pig cell contamination of the purified human cells. (B) qPCR quantification of the number of PERV elements in 1000 293G cells derived from a population co-cultured with wild type PK15 cells using specific primer sets. (*n* = 3, mean ± SEM.) (C) qPCR quantification of the number of PERV elements in PK15 clones 15, 20, 29, and 38, with high levels of PERV *pol* modification, and minimally modified clones 40 and 41. (*n* = 3, mean+/−SEM.) (D) Results of PCR on PERV *pol* on genomic DNA from various numbers of HEK 293-GFP cells (0.1, 1, 10, and 100) isolated from populations previously cultured with highly modified PK15 clone 20 and minimally modified clone 40. See figs. S18 to S21 for a full panel of PCR reactions.