

GENOME ENGINEERING

Inactivation of porcine endogenous retrovirus in pigs using CRISPR-Cas9

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Xenotransplantation is a promising strategy to alleviate the shortage of organs for human transplantation. In addition to the concerns about pig-to-human immunological compatibility, the risk of cross-species transmission of porcine endogenous retroviruses (PERVs) has impeded the clinical application of this approach. We previously demonstrated the feasibility of inactivating PERV activity in an immortalized pig cell line. We now confirm that PERVs infect human cells, and we observe the horizontal transfer of PERVs among human cells. Using CRISPR-Cas9, we inactivated all of the PERVs in a porcine primary cell line and generated PERV-inactivated pigs via somatic cell nuclear transfer. Our study highlights the value of PERV inactivation to prevent cross-species viral transmission and demonstrates the successful production of PERV-inactivated animals to address the safety concern in clinical xenotransplantation.

The shortage of human organs and tissues for transplantation represents one of the most substantial unmet medical needs (1). Xenotransplantation holds great promise. Porcine organs are considered to be favorable resources for xenotransplantation because they are similar to human organs in size and function, and pigs can be bred in large numbers (2).

However, the clinical use of porcine organs has been hindered by immunological incompatibilities (2) and by the potential risk of porcine endogenous retrovirus (PERV) transmission (3). PERVs are gamma retroviruses found in the genome of all pig strains and can be vertically transferred through inheritance (4). Although, to date, no study has shown PERV transmission to humans in a clinical setting, it has been demonstrated that PERVs can infect human cells (3, 5) and integrate into the human genome in cell culture (6). PERV integration could potentially lead to immunodeficiency and tumorigenesis, as reported with other retroviruses (7, 8).

We recently demonstrated a method to inactivate all 62 copies of PERVs in an immortalized porcine cell line (PK15) and thus eliminate PERV transmission to human cells (5). In the present study, we adopted a strategy to conduct multiplexed genome engineering to inactivate PERV activity in a primary porcine fibroblast cell line, after which we used the modified fibroblasts to produce embryos through somatic cell nuclear transfer (SCNT) and then transferred the SCNT embryos into surrogate sows. With the use of such an approach, we successfully generated PERV-inactivated pigs.

We previously demonstrated the transmission of PERVs from an immortalized pig epithelial cell line, PK15, to green fluorescent protein (GFP)-labeled human embryonic kidney 293T (HEK293T-GFP) cells after coculturing them for 1 week (5). We wondered whether PERVs remain active and propagate in human cells. To detect this, we monitored PERV copy number in both a population and clones of PERV-infected HEK293T-GFP cells (i-HEK293T-GFP) for more than 4 months and observed that PERV copy number increased over time (Fig. 1A and fig. S1B), as determined by droplet digital polymerase chain reaction (ddPCR). Consistent with previous reports (3, 9), we detected that both subtypes PERV-A and PERV-B were present in the infected human cells (fig. S1B), which confirms that they are human-tropic. We did not detect PERV-C in either PK15 or i-HEK293T-GFP cells (fig. S1, A and B). To determine whether the PERVs integrate into the human genome or stay episomal in the infected human cells, we performed junction capture sequencing of the infected clonal i-HEK293T-GFP cells. We detected novel PERV junctions in the human genome and observed that they are over-represented in intragenic regions and in active chromatin areas (Fig. 1B and fig. S2, A and B).

The increased copy number of PERVs in i-HEK293T-GFP clones can be caused by intracellular transposition or by intercellular PERV transmission among human cells. To clarify this, we examined whether infected human cells could transmit PERVs to wild-type (WT) human cells. We cocultured clonal i-HEK293T-GFP cells with WT HEK293T cells for 2 weeks and subsequently checked PERV elements in the cocultured WT clones via PCR. We detected the robust presence of PERV elements in WT HEK293T cells with no history of contact with porcine cells (Fig. 1C and fig. S2C). The percentage of infected WT HEK293T cells varies from 20 to 97% (Fig. 1D), depending on different parental i-HEK293T-GFP clones in the coculture. We concluded from our observations that infected human cells can transmit PERVs to previously unexposed human cells.

Generating PERV-inactivated pigs involves construction of primary porcine cells devoid of PERV activity, which can be cloned via SCNT to produce porcine embryos. We tested whether we could use the same strategy that we used previously in PK15 (5) to inactivate the PERV activities in a primary porcine fetal fibroblast cell line (hereafter denoted as FFF3). We first aimed to map and characterize the PERVs present in the FFF3 genome. We detected 25 copies of functional PERVs, as determined by ddPCR on the reverse transcriptase (*pol*) gene (fig. S3). The detected PERV copy number was close to the sum of 10 copies of the PERV-A *env* gene, 14 copies of the PERV-B *env* gene, and 0 copies of the PERV-C *env* gene that we identified in the genome (fig. S3) by ddPCR. We used whole-genome sequencing to further detect one additional copy of truncated PERV-B that was not detectable by ddPCR. We used hybridization capture followed by sequencing to map PERVs copies into the genome (Fig. 2A and fig. S4). To target these PERVs for inactivation, we designed two CRISPR guide RNAs (gRNAs) specific to the catalytic core of the PERV *pol* gene (fig. S5). After treating a population of FFF3 cells with CRISPR-Cas9 and the two gRNAs for 12 days, we observed 37% PERV *pol* inactivation. Notably, we observed a bimodal distribution of targeting efficiency among single FFF3 cells after treatment, resembling our previous results obtained with PK15 cells (5). About 35% of single cells had high editing efficiency (>90%) and 61% had low editing efficiency (<20%) (Fig. 2B). Unfortunately, despite the presence of highly modified cells in the population, we could not grow the single-cell clones with >90% PERV editing efficiency.

We hypothesized that simultaneous DNA cleavages by Cas9 at multiple PERV sites in the FFF3 genome trigger DNA damage-induced senescence or apoptosis; hence, we could not obtain the highly modified FFF3 clones. Through screening of different anti-apoptotic strategies (figs. S6 and S7), we observed that, during genetic modification, the application of a cocktail containing p53 inhibitor, pifithrin alpha (PFT α), and basic fibroblast growth factor (bFGF) significantly increased the average targeting efficiency of the resulting FFF3

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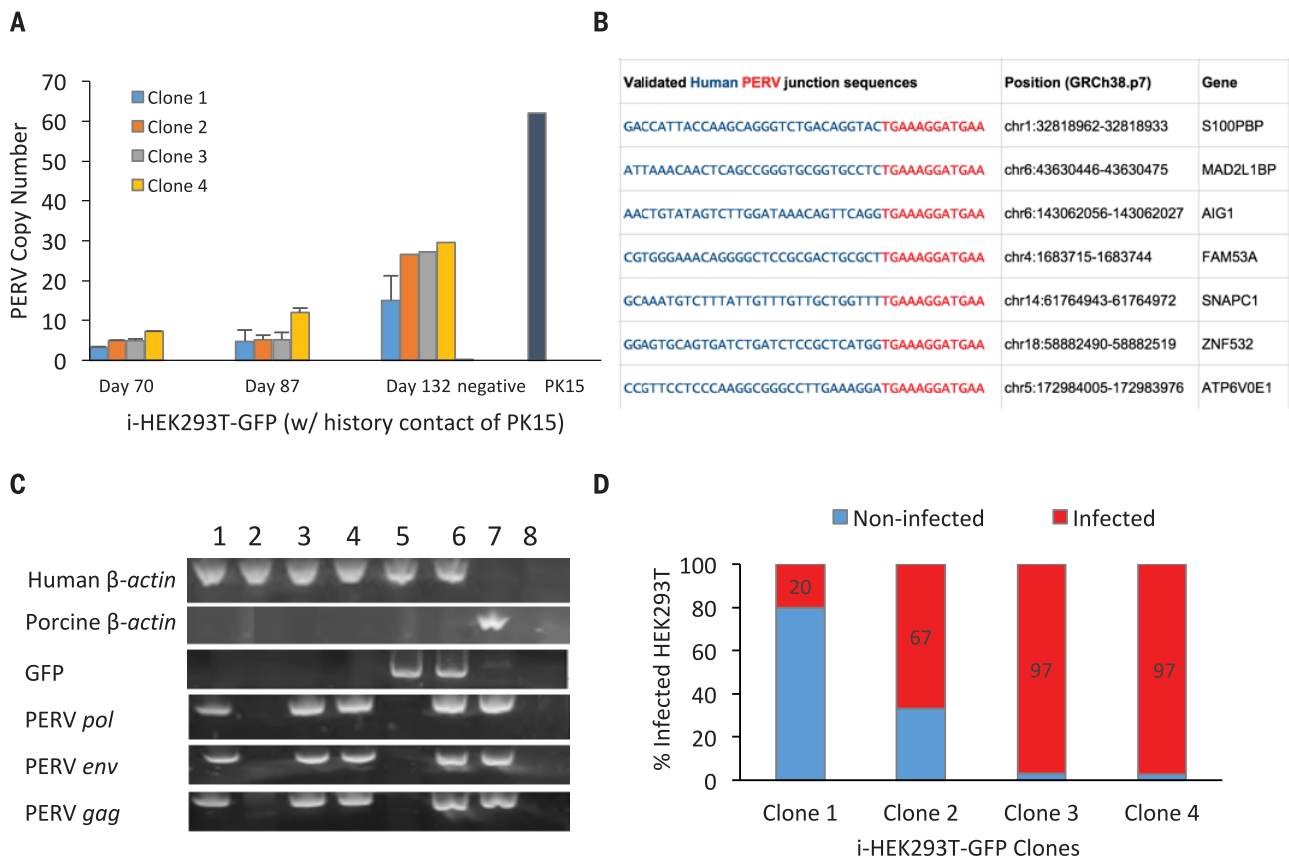


Fig. 1. Pig-to-human and human-to-human PERV transmission.

(A) PERV copy number in infected human cells increases over time when cocultured with PK15 cells. Human HEK293T-GFP cells were cocultured with equivalent numbers of porcine PK15 cells for 1 week. HEK293T-GFP cells that did not have any contact with PK15 cells were used as a negative control (negative). Error bars indicate SD. (B) Detection of PERV insertion sites in the human genome. Among the 22 PERV insertion sites detected by inverse PCR, 15 were mapped to the intragenic region. We tested a portion of the intragenic hits and validated 7 out of 12 by junction PCR (shown here). The 30–base pair human genomic sequences are shown in blue, whereas the PERV long terminal repeats are shown in red. (C) Detection of human-to-human PERV transmission. Individual clones of HEK293T cells were grown from the single cells isolated from the coculture of

i-HEK293T-GFP cells with HEK293T cells through flow cytometry. The PCR gel image shows that three out of four randomly tested HEK293T clones were infected and contained PERV sequences (PERV *pol*, *env*, and *gag*) but no sequence of GFP or pig genomic DNA (tested by pig-specific GGTA). Sample order is as follows: (1) HEK293T clone 1; (2) HEK293T clone 2; (3) HEK293T clone 3; (4) HEK293T clone 4; (5) HEK293T-GFP control; (6) i-HEK293T-GFP; (7) PK15 WT; and (8) negative control. (D) Four different i-HEK293T-GFP clones have different infectious potential. Four infected parental i-HEK293T-GFP clones are cocultured with WT HEK293T cells. The PERV copy numbers of the four parental i-HEK293T-GFP clones are 15, 28, 27, and 28, respectively. The percentages of the infected WT HEK293T clones from the coculture of i-HEK293T-GFP and WT HEK293T cells varied from 20 to 97%. Primers used are listed in table S1.

populations {fig. S6A [ANOVA (analysis of variance), $P = 0.00002$] and fig. S6B}. Using this optimized cocktail, we were able to grow 100% PERV-inactivated FFF3 cells (PERV-inactivated FFF3) from the population treated with CRISPR-Cas9 (Fig. 2, C and D).

Having confirmed that we genetically mutated PERV *pol* in the genome, we performed RNA sequencing (fig. S8) on PERV-inactivated FFF3 clones and confirmed that all *pol* transcripts had been mutated. Furthermore, we examined whether the genome-wide disruption of PERV *pol* would eliminate in vitro production of PERVs from FFF3. We could not detect reverse transcriptase activity of PERVs in the cell culture supernatant of the PERV-inactivated FFF3 (fig. S9), suggesting that no viral particles are secreted by the modified cells.

We next sought to examine the off-target effects of CRISPR-Cas9 in the PERV-inactivated FFF3. We performed karyotyping of eight PERV-inactivated

FFF3 clones and observed that five carried chromosomal abnormalities. Of note, the translocation sites in the genome tend to correlate with presence of PERV cutting sites, which suggests that CRISPR-Cas9 on-target toxicity may contribute to the translocations observed (fig. S10A). The remaining three PERV-inactivated FFF3 clones carry normal chromosomal structures (fig. S10B). To examine chromosomal integrity with higher resolution, we performed PERV genomic junction sequencing on the three normal karyotype clones to examine potential deletions between Cas9-induced double-strand breaks. All 21 tested junctions remained intact (fig. S11), which indicates no Cas9-induced macrodeletions in these regions. Therefore, we concluded that we could obtain PERV-inactivated FFF3 clones without detectable structural variations.

Having obtained PERV-inactivated FFF3 cells, we attempted to produce PERV-inactivated em-

bryos via SCNT. For every round of embryogenesis, ~20 to 40% of the constructed PERV-inactivated embryos reached the blastocyst stage after being cultured for 6 days (materials and methods), which is within the normal range of porcine SCNT efficiency. We observed normal 64–cell stage blastocyst structure and validated the pluripotency of inner cell mass (detected by SOX2 antibody) on day 6 (fig. S12A). We performed genomic deep sequencing to check the PERV *pol* genotypes in embryos and confirmed 100% PERV-inactivation efficiency (fig. S12B).

Next, we transferred the PERV-inactivated embryos into surrogate sows that are free of PERV-C and present a total PERV copy number of 12 to 30 (fig. S13A). We detected pregnancy rates of 75% [33 of 44 (33/44)], 63% (28/44), and 52% (23/44) for sows with embryos created using PERV-inactivated FFF3 cells at day 23, 51, and 70, respectively, and 100% (5/5) for sows with embryos

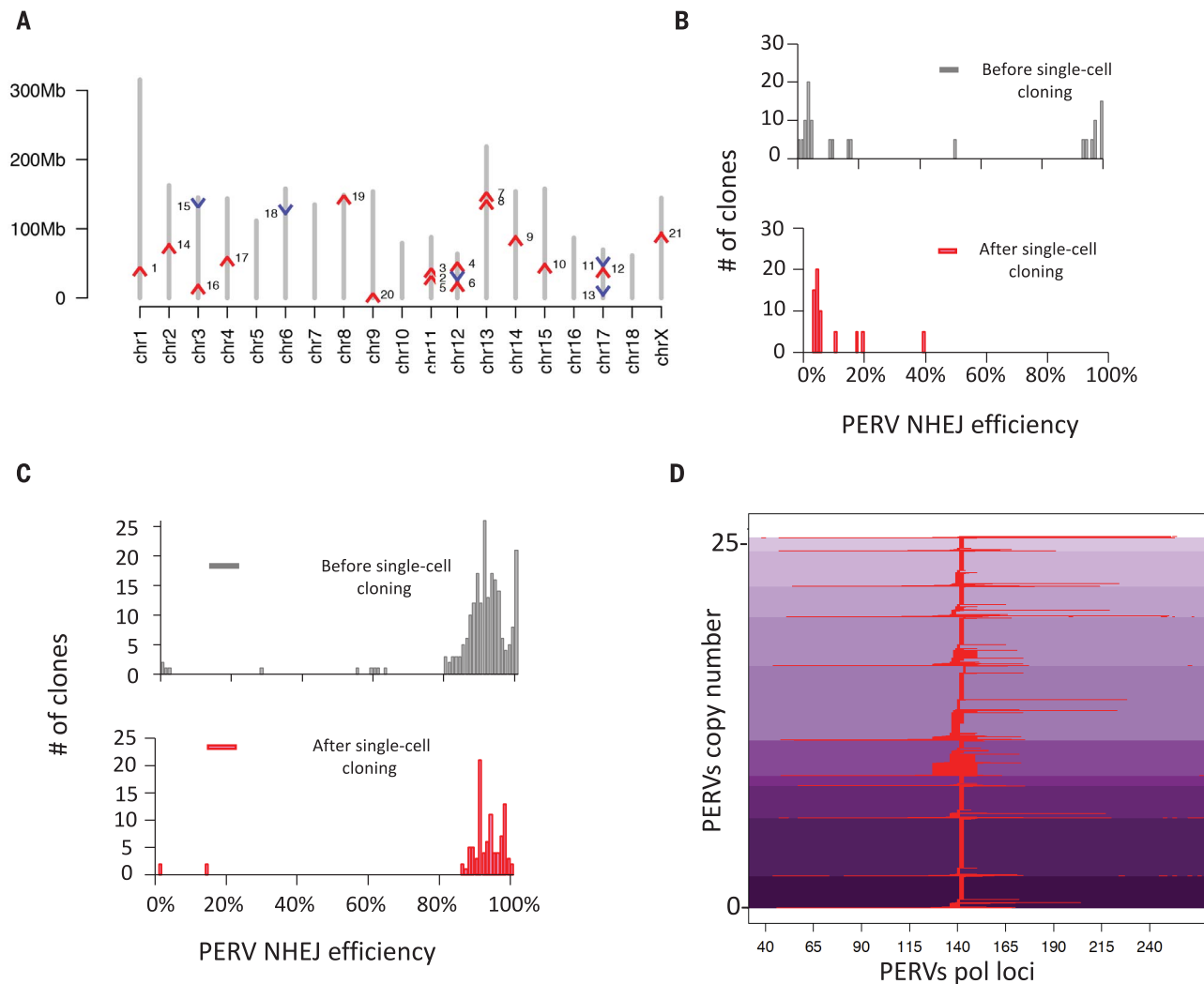


Fig. 2. PERVs insertion site mapping and genome-wide inactivation.

(A) Chromosome mapping of PERV locations in the FFF3 cell line. Chromosomal scaffolds are shown in gray. Red arrows represent PERVs in the forward or positive chain of chromosome; blue arrows denote PERVs in the reverse or negative chain. The y axis represents chromosomal coordinates. Two additional copies were mapped to repetitive regions, and two could not be mapped to the current pig genome assembly and are not shown (11% gaps, *Sus scrofa* build 10.2) (11). (B) Failure to obtain 100% PERV-inactivated FFF3 clones using CRISPR-Cas9. After targeting the PERVs in FFF3, single cells were sorted and immediately genotyped. We observed a bimodal distribution of PERV targeting frequencies among single cells (top), similar to that seen for the PK15 clones (5). 100% PERV-inactivated FFF3 cells were present among the single cells that we directly genotyped. However, this pattern changed after expansion of the single cells (bottom). Among the single-cell clones, we only obtained those with lower efficiency ($\leq 39\%$; the average targeting efficiency in

the population was 37%), not the ones with 100% PERV inactivation (bottom). NHEJ, nonhomologous end joining. (C) Treatment with PFT α and bFGF sustained the growth of highly modified FFF3 clones. The combined use of a p53 inhibitor, PFT α , and a growth factor, bFGF, rescued the highly modified cells. A population of FFF3 was treated with PFT α and bFGF during the gene editing experiment (materials and methods), after which single cells were sorted for direct genotyping and colony growth, followed by genotyping. Both the single cells and expanded clones showed similar distribution in PERV targeting efficiency, and highly modified clones survived under this condition. (D) Genotype of 100% PERV-inactivated clones. Several 100% PERV-inactivated clones were achieved from the PFT α - and bFGF-treated FFF3 population. The figure shows haplotypes of one of the 100% PERV-inactivated clones at PERV *pol* loci after CRISPR-Cas9 treatment. The y axis indicates PERV copy number; the x axis indicates the relative locations of the indels within the PERV loci. Aligned indel events in the PERV *pol* sequence are shown in red. Shades of purple indicate different haplotypes of PERVs.

created using WT cells at all the three time points. Fetuses at pregnancy date 50 were analyzed; all PERV-inactivated fetuses showed 100% PERV inactivation (fig. S12, C and D) and similar PERV copy numbers to those of WT FFF3 cells (fig. S13B). Despite the lower pregnancy efficiency, which is commonly observed in transgenic pig production (10), we successfully produced putative PERV-inactivated pigs (Fig. 3A and fig. S14).

In terms of SCNT efficiency, we did not observe any difference between PERV-inactivated cells and WT cells. The ratios of piglets born to the number of embryos transferred are similar for PERV-inactivated cells (0.9%) and WT cells (0.8%). To test PERV inactivation in these pigs, we isolated genomic DNA from both the bulk cells and single-cell clones derived from these pigs and observed that all pigs exhibited $\sim 100\%$ PERV inactivation at

the genomic DNA level (Fig. 3B and fig. S15, A and B). In addition, we observed that the copy number of PERVs in the generated pigs stays close to 25, reconfirming that there is no reinfection (fig. S15C). We further isolated total RNAs from a variety of tissues of the pigs and confirmed $\sim 100\%$ PERV inactivation at the mRNA level (Fig. 3C). We performed karyotyping and did not detect abnormal structural changes in the PERV-inactivated

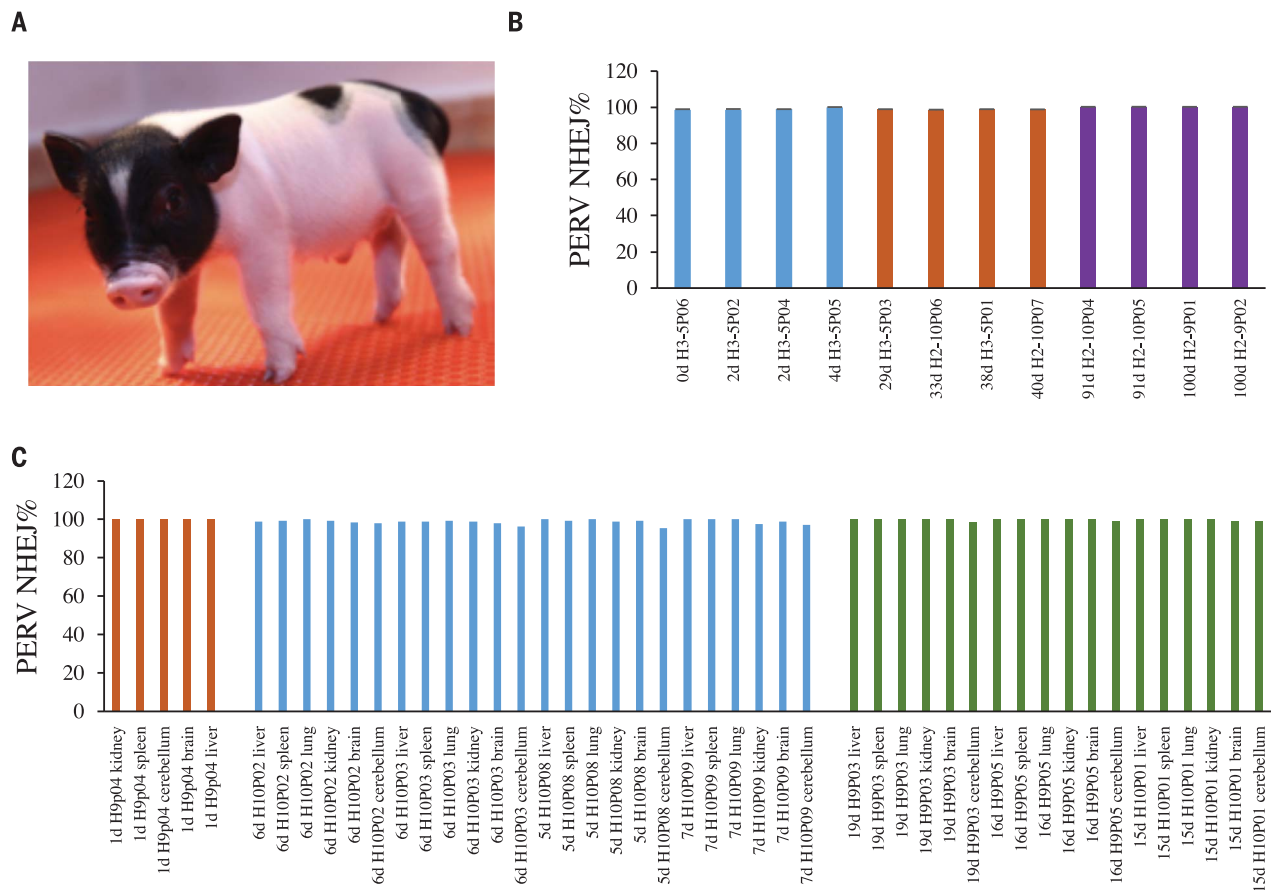


Fig. 3. PERV-inactivated pigs. (A) Image of the first-born PERV-inactivated pig (Laika), 2 days after birth. (B) PERV inactivation at genomic DNA level. We genotyped PERV-inactivated pigs at different ages (up to 100 days) by deep sequencing of the PERV *pol* loci. All examined pigs showed ~100% PERV inactivation efficiency, which demonstrates that there is no detectable PERV reinfection from surrogate sows to cloned pigs. Blue, 0- to 4-day-old piglets;

brown, 29- to 40-day-old piglets; purple, 91- to 100-day-old piglets. (C) PERV inactivation at the mRNA level. Total mRNA-generated cDNA was used to detect the PERV inactivation efficiency of the PERV-knockout pig transcripts. All pigs exhibited ~100% PERV eradication efficiency at the mRNA level. Brown, 1-day-old piglets; blue, 5- to 7-day-old piglets; green, 15- to 19-day-old piglets.

pigs (fig. S16). The PERV-inactivated pig production is robust in that, to date, we have produced 37 PERV-inactivated piglets from 17 sows (200 to 300 embryos transferred per sow), 15 piglets remain alive, and the oldest healthy animals are 4 months old. We are conducting long-term studies to monitor the effect of PERV inactivation and gene editing on these animals as they age and mature.

We observed in our studies that PERVs can be transmitted from pig to human cells and also transmitted among human cells in vitro. These results substantiate the risk of cross-species viral transmission in the context of xenotransplantation. To work toward eliminating this risk, we used a combination of CRISPR-Cas9, apoptosis inhibitor, and growth factor to generate PERV-inactivated primary porcine cell lines. With these cell lines, we produced PERV-inactivated porcine embryos, fetuses, and live pigs.

In this study, we discovered that treatment with p53 inhibitor can mitigate the stress from multiplex DNA damage during multiplexable genome engineering and support clonal expansion of 100% PERV-inactivated cells. Although in this

paper we have focused on the applications to xenotransplantation, we envision, more generally, that the synergistic combination of CRISPR-Cas technology with anti-apoptosis treatment may also be used to enable large-scale genome engineering in primary cells for a broad range of applications, including pathway engineering and modifications of other genetic repetitive elements of biological interest.

Although it is still unclear whether PERVs infect humans in vivo, our study shows that PERV-infected human cells pass the PERVs robustly to fresh human cells that have no prior exposure to pig cells. Therefore, our data substantiates the value of PERV inactivation for safe xenotransplantation practice. The physiological functions of endogenous retrovirus, which exists in all mammalian species, remain largely unknown. Further studies on our PERV-inactivated pigs will shed light on the endogenous retrovirus functionalities in relation to the hosts. Most importantly, the PERV-inactivated pig can serve as a foundation pig strain, which can be further engineered to provide safe and effective organ and tissue resources for xenotransplantation.

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scientific advisor of eGenesis. L.Y. is an inventor on provisional U.S. patent applications 62/487,898 and 62/527,702 that are submitted by eGenesis and cover PERV-inactivated pig production.

SUPPLEMENTARY MATERIALS

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Materials and Methods

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Taking the PERVs out of pigs

With the severe shortage of organs needed for transplants, xenotransplantation (transplantation of nonhuman organs to humans) offers an alternative source. Some pig organs have similar size and function to those of humans. The challenge is that the pig genome harbors porcine endogenous retroviruses (PERVs) that can potentially pass to humans with possibly damaging consequences. Niu *et al.* generated pigs in which all copies of PERVs were inactivated by CRISPR-Cas9 genome engineering (see the Perspective by Denner). Not only does this work provide insights into PERV activity, but it also opens the door to a safer source of organs and tissues for pig-to-human xenotransplantation.

Science, this issue p. 1303; see also p. 1238

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