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Establishment of a humanized swine model for COVID-19

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Dear Editor,

Currently, wild-type (WT) hamsters, ferrets, cats, and non-human primates are being used as COVID-19 animal models. However, no severe clinical symptoms develop in these animals^{1–5}. Similarly, most of the human ACE2 (hACE2) transgenic mouse models develop only mild COVID-19 disease with only a few recent transgenic mouse models developing severe and fatal respiratory diseases^{6–10}, calling for a better large animal model that could mimic the full spectrum of COVID-19 symptoms. Although the pig is thought to be a better model for human diseases in general¹¹, due to its similarity to human anatomy, physiology, and immunology, previous studies have shown that WT pigs are not susceptible to SARS-CoV-2^{2,12}. Here we report our attempt to create the first humanized pig expressing the hACE2 receptor for COVID-19 research, speculating that humanization of the pig ACE2 receptor could make pigs susceptible to SARS-CoV-2.

To create a COVID-19 pig model with the targeted insertion of *hACE2* at the pig *ACE2* locus, we constructed a homologous recombination donor vector with homology arms of ~1 kb on each side. We used the CRISPR/Cas9 system to increase the chance of homologous recombination. Single-guide RNAs (sgRNAs) closest to the start codon of exon 1 were selected for the construction of sgRNA-expressing vector pX459 (Fig. 1a). To optimize the efficiency of the sgRNA, 9 sgRNAs were synthesized and assembled. IBRS-2 porcine kidney cells

were electroporated with plasmids of *pACE2*-sgRNAs and Cas9. Sanger sequencing was used to identify the indels and evaluate the targeting efficiency for these sgRNAs. The cleavage efficiency of sgRNA3 was higher than that of other sgRNAs. Therefore, we chose sgRNA3 for subsequent experiments.

Porcine fetal fibroblasts (PFFs) isolated from embryos of Bama mini-pigs were electroporated with plasmids of *pACE2*-sgRNA3, Cas9, and donor linearized by in vitro cleavage of the donor vector by restriction enzymes (*SpeI* and *NotI*). We used puromycin selection (1 µg/mL for 2 days) to enrich positive cell colonies, and their genotype was identified using PCR and Sanger sequencing (Supplementary Fig. S1a, b). Among the 85 single cell colonies, three of them (3/85, 3.53%) were identified as positive for *hACE2* insertion.

Next, the verified colonies were used as donor cells for somatic cell nuclear transfer (SCNT) into three surrogates. After about four months of pregnancy, these surrogates gave birth to nine genetically modified piglets (Fig. 1b). Genomic DNA was extracted from a variety of tissues of these piglets one day after birth, and their genotype was identified by PCR. The results showed that all of the piglets were positive for *hACE2* insertion (Supplementary Fig. S1a).

The transcriptional level of *hACE2* in several tissues of the knock-in piglets was quantified by qPCR, with *GAPDH* serving as the reference gene. The *hACE2* mRNA levels in the kidney, liver, small intestine, and lung of the knock-in pigs were significantly higher than their WT littermates. In contrast, only low mRNA levels of *hACE2* were detected in the brains of knock-in pigs (Fig. 1c).

To further determine whether the CRISPR/Cas9-mediated knock-in of *hACE2* could increase the expression of *hACE2*, the protein was isolated from different organs in the WT and knock-in pigs. Western blot showed high

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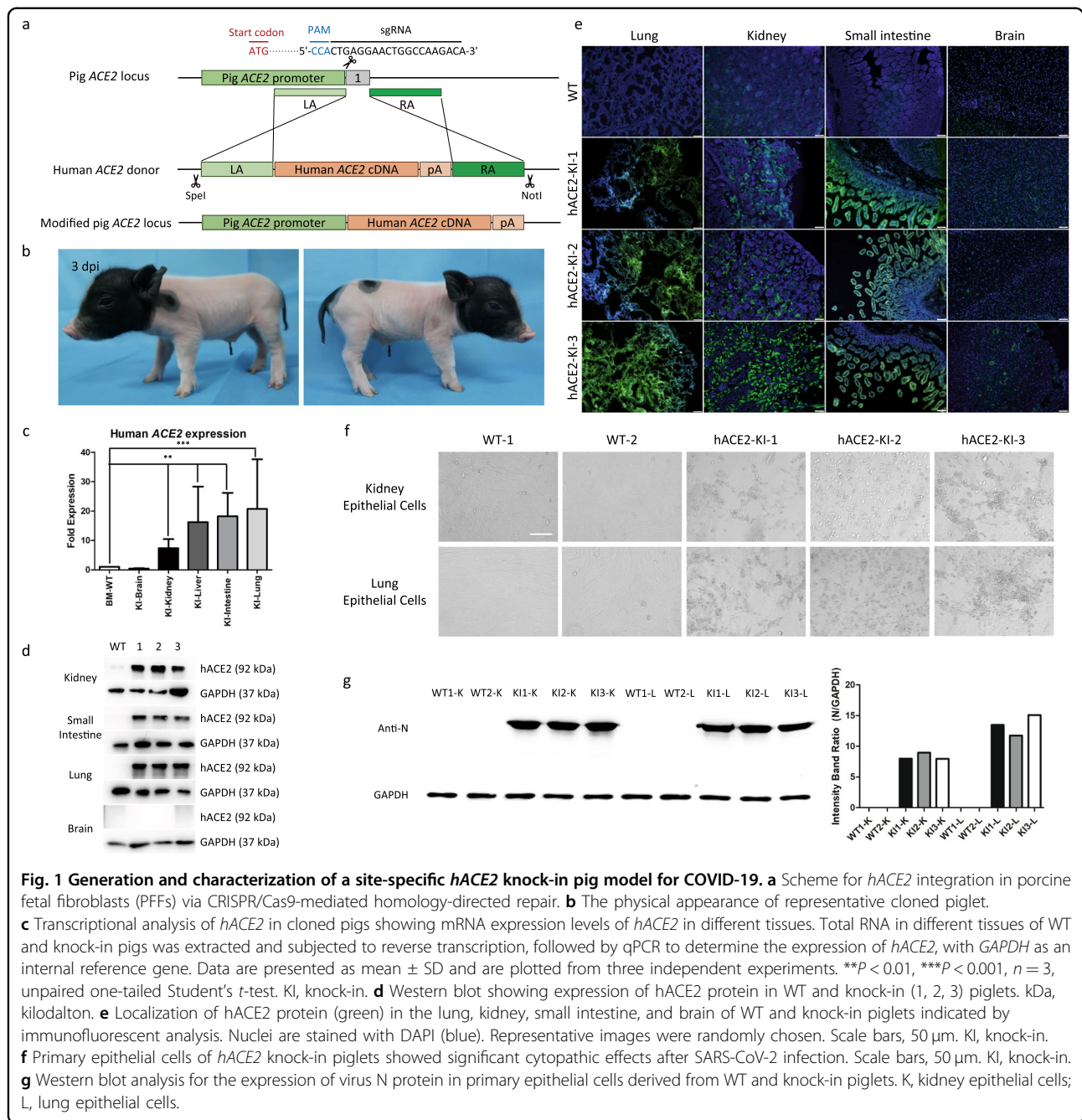
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expression of hACE2 in the knock-in pigs in all tissues except the brain (Fig. 1d).

We also performed immunofluorescent analysis to detect hACE2 in different organs of both WT and knock-in pigs. The results showed a high expression of hACE2 in the lung, kidney and small intestine of knock-in pigs, based on a strong green fluorescent signal. In contrast, very faint signal was seen in WT tissues and the brain of knock-in pigs, which was consistent with the results identified by qPCR and western blotting (Fig. 1e).

To determine the replication efficiency of SARS-CoV-2 in primary cells of the hACE2 knock-in pigs, lung and kidney epithelial cells were isolated and infected with SARS-CoV-2 (MOI = 0.01). After 72 h of infection, these primary cells of the hACE2 knock-in pigs showed significant cytopathic effects in contrast to wide-type swine cells (Fig. 1f).

At 72 h post-inoculation (hpi), the cells were collected for the detection of SARS-CoV-2 N protein by western blotting (Fig. 1g) and immunofluorescence assay (IFA)

(Supplementary Fig. S2). Obvious fluorescent signal was detected in the cells of the knock-in pigs compared to the cells from WT pigs. As shown by western blot analysis, significant expression of viral N protein was detected in the cells from the knock-in pigs, but not in the cells from WT pigs. These results indicated that the primary epithelial cells from the *hACE2* knock-in piglets are susceptible to SARS-CoV-2 infection.

In conclusion, we have shown that healthy *hACE2* knock-in pigs can be generated by targeted insertion of *hACE2* to the pig *ACE2* locus. The expression of *hACE2* protein is regulated by the endogenous swine promoter and appears to recapitulate the in vivo expression pattern. The expression patterns of *hACE2* in the lung, kidney, testis, and intestine of our *hACE2* pig models are similar to human and the humanized *ACE2* mice^{8,13}. In humanized *ACE2* mice, the small intestine showed higher levels of *hACE2* than other tissues by qPCR. In contrast, in our humanized *hACE2* pigs the lungs show higher *hACE2* expression levels than other organs.

Since the *hACE2* knock-in pigs are created in this study by SCNT, they have a uniform genetic background and can be generated in large quantities in a short time. Also, severe cases of COVID-19 are often accompanied by underlying diseases, and pig models offer a unique opportunity to combine these existing diseases to reproduce severe cases of COVID-19. In addition to acute lung injury, some COVID-19 patients also experience organ damage, including acute kidney, heart, and liver dysfunction^{14–16}, in particular for people with comorbidities such as hypertension, cardiovascular disease, and diabetes¹⁵. The humanized *hACE2* pigs can be readily combined with other available pig models to accelerate the research of basic disease mechanisms, biomarkers, and treatment methods for high-risk people of COVID-19.

As SARS-CoV-2 mutants are rapidly emerging, scientists are scrambling to design and develop new vaccines, and are making continuous efforts to find antiviral drugs. The immediate urgency of treating severe disease and reining in viral escape variants calls for optimal animal models. Our *hACE2* knock-in pigs have great potential to fulfill this need.

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Author contributions

S.W. conceived and designed the study. X.D., Z.G., C.C., and Y.Q. performed the cell culture and gene targeting experiments. F.G. and P.L. performed gene construction. W.F., P.J., and Y.B. performed virus challenge experiments. D.Y., T.H., X.D., C.C., Z.H., and J.R. performed nuclear transfer experiments. Z.G., W.F., P.J. and Y.Q. designed and performed histological experiments. S.W., D.Y., Y.B., and W.L. supervised the project. S.W., X.D., Z.G., and Y.B. analyzed the data and wrote the manuscript with input from all authors.

Competing interests

The authors declare no competing interests.

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