Zhang et al. *Zool. Res.* 2022, 43(6): 1005−1008
https://doi.org/10.24272/j.issn.2095-8137.2022.244

ZR | Zoological Research

Letter to the editor Open Access

Optimization of sgRNA expression strategy to generate multiplex gene-edited pigs

DEAR EDITOR,

The use of CRISPR/Cas9 technology to breed polygenic-modified animals with desired traits holds great promise in agriculture and biomedicine. However, as most studies are based on human and mouse models, whether different single guide RNA (sgRNA) expression strategies affect multiplex gene-editing efficiency in domestic animals remains elusive. Here, taking pigs as a model and targeting porcine CD163 and myostatin (MSTN) genomic loci, we evaluated the gene-editing efficiency of several gRNA expression approaches, including the co-transfection of plasmids carrying different sgRNAs, the transfection of one plasmid with tandem sgRNA expression cassettes, and the transfection of vectors containing polycistronic sgRNAs (PTG strategy). We found that one strategy showed differential editing ability in different species. Notably, although the PTG strategy improved editing efficiency in plant and human cell lines, it was inefficient in porcine fetal fibroblasts (PFFs). Among the three strategies, the tandem sgRNA expression cassettes demonstrated the greatest editing efficiency. Using this approach, double gene-edited PFF lines were obtained. Based on somatic cell nuclear transfer results, this approach generated homozygous multiplex gene-edited pigs economically and precisely, as revealed by whole-genome sequencing. This study provides a safe and effective sgRNA expression strategy for multiplex genetic (CD163 and MSTN) modification in domestic animals. Furthermore, it lays the foundation for high-productivity breeding of pigs resistant to porcine reproductive and respiratory disorder syndrome (PRRS) and promoting muscle growth.

CRISPR/Cas9 is a powerful technology for animal genomic modification. Under the influence of sgRNA, CRISPR-associated endonuclease binds to a target locus for site-specific manipulation of the genome. For example, CRISPR/Cas9-mediated genetically modified pigs are considered ideal models for human disease, as well as organ donors for xenotransplantation, due to their high similarity to humans regarding metabolism, physiology, and organ anatomy (Zhang et al, 2022; Zhu et al, 2021). Knockout of porcine *CD163* is sufficient to induce resistance to porcine reproductive and respiratory syndrome virus (PRRSV) infection, which has caused major economic losses to the global pork industry over the last few decades, and genome editing of myostatin (*MSTN*) can significantly increase the skeletal muscle rate in pigs (Fan et al., 2022; Yang et al., 2018). Nonetheless, simultaneous editing of multiple genes (e.g., *CD163* and *MSTN*) has become increasingly important given the substantial time and resources required to generate gene-edited animals with particular traits via conventional methods.

Previous studies have revealed that multiplex gene-editing efficiency is associated with the gRNA expression strategy. In plants, the polycistronic transfer RNA (tRNA)-sgRNA cassette (PTG) system can significantly improve editing efficiency (Xie et al., 2015). In human cell lines, the application of PTG can increase editing efficiency of the HF-Cas9 (engineered Cas9 protein with a reduced off-target rate) system (Zhang et al., 2017). However, due to the rapid evolution across species (Wu et al., 2021), whether the reported strategies work well in domestic animals remains an open question. In this study, using pigs as a model animal, we explored the optimal sgRNA expression strategy for the generation of multiple gene-edited large animals.

Given that zygote injection-mediated genetically modified animals are often mosaic and heterozygous, and that hybridization and selection of homozygous animals consumes many resources, the generation of gene-edited animals by somatic cell nuclear transfer (SCNT) is considered an economical approach. Here, using porcine fetal fibroblasts (PFFs), commonly used as donors for SCNT, we investigated

Received: 14 August 2022; Accepted: 09 October 2022; Online: 09 October 2022

Foundation items: This work was supported by the National Natural Science Foundation of China (32072806), Shaanxi Province Science and Technology Innovation Team (2019TD-036), and Key Program of Shaanxi Province Science and Technology (2022NY-044)

the optimal sgRNA expression method for efficient multiplex gene-editing in domestic animals. Porcine MSTN and CD163 were selected for polygenic modification. We designed sgRNAs targeting the exon related to the MSTN start codon and exon 7 of CD163, both of which are considered important domains for PRRSV infection (Fan et al, 2022; Wang et al, 2019) (Figure 1A). The top-ranked sgRNAs for the MSTN locus and CD163 locus with low off-target rates were selected. Vectors carrying the sgRNAs targeting MSTN and CD163 were then constructed and transfected into the PFFs. After 48 h of puromycin selection, the editing efficiencies of the two sgRNAs for each locus were measured and compared. Based on this, sgRNAs with editing efficiencies of 47.5% for MSTN and 52.2% for CD163 were selected and subjected to off-target tests (Supplementary Figure S1A). Sanger sequencing demonstrated that editing occurred at the predicted loci (Supplementary Figure S1B). Importantly, no mutations were detected in the top three potential off-target sites, demonstrating that both sgRNAs were efficient and safe (Supplementary Figure S2A, B).

We next explored whether the different sgRNA expression strategies affect multiple gene editing in large animals. According to previously published reports, three main strategies are used for multiplex gene-editing in plants and animals (Minkenberg et al., 2017; Ren et al., 2021; Zhu et al., 2020), including co-transfection of plasmids carrying different sgRNAs, transfection of one plasmid with tandem sgRNA expression cassettes, and transfection of vectors containing PTG-mediated polycistronic sgRNAs. In this study, the sgRNAs obtained by the three strategies for targeting porcine MSTN and CD163 were introduced into the PFFs (Figure 1B). Through transfection of control vectors containing green fluorescent protein (GFP) and puromycin expression cassettes, we monitored whether the vectors were successfully transfected and measured the optimal puromycin screening conditions (Supplementary Figure S1C). As a result, we chose 2.5 μg/mL puromycin for 48 h to screen positively transfected cells.

Next, we quantified and compared the editing efficiencies of MSTN and CD163 using Sanger sequencing and TIDE analysis. Among the three strategies, the tandem U6 promoter-driven sgRNA expression cassettes achieved the greatest efficiency for MSTN (27.3%) and CD163 (50.4%), while the PTG strategy failed to edit genes in the PFFs as efficiently as reported in plants and humans (Supplementary Figure S3A, B), indicating differential editing abilities in different species (Figure 1C). We speculated that the relatively low editing efficiency of the strategy involving co-transfection of plasmids carrying different sgRNAs may have resulted from fewer MSTN or CD163 sgRNA-positive cells compared to the tandem sgRNA expression cassette strategy. We also hypothesized that the inefficiency of the PTG strategy in porcine cells may be due to codon preference across species, with most nascent tRNAs-gRNAs not maturing over such a short period. Furthermore, although the sgRNAs were produced via different strategies, they displayed similar editing propensities in PFFs. The sgRNA selected to target MSTN tends to result in a one-base-pair deletion, while that targeting CD163 tends to result in a one-base-pair insertion. Our results indicated that relatively high multiplex gene-editing efficiencies can be established in pig cells using tandem sgRNA expression cassettes.

We next asked whether the tandem sgRNA expression cassette strategy can produce high-quality multiplex gene-edited cell lines. Plasmids containing tandem sgRNA expression cassettes were transfected into PFFs. After electrotransfection, the cells were treated with 2.5 μg/mL puromycin for 48 h. We then performed limiting dilution to obtain single-cell-derived clones (Figure 1D). After 12 days of incubation, we harvested nine single-cell-derived clones with good cell states (Figure 1E). Four of the nine single-cell clones were positive for gene-editing, consistent with the editing efficiencies assessed by TIDE analysis. One of the four editing-positive clones was MSTN-edited only, and one was CD163-edited only, indicating that the genome-editing events were probabilistic. Two of the editing-positive clones showed mutations at both the MSTN and CD163 genomic loci (Supplementary Figure S4). Clone 9 displayed homozygous MSTN and CD163 modification compared to the wild-type PFFs. Clone 1 showed homozygous base-pair deletion of MSTN and heterozygous knockout of CD163, suggesting that the editing of each allele may be inconsistent (Supplementary Figure S4). Importantly, the genotype of each edited clone was stable over three consecutive cell passages, indicating that our results were reliable. Therefore, we confirmed that the of tandem sgRNA expression cassette strategy is sufficient to produce single cell lines with multiplex gene-editing.

We next performed SCNT and embryo transfer to generate MSTN- and CD163-edited embryos and pigs simultaneously. To avoid homologous recombination between alleles during embryonic development, we subjected clone 9 to further experiments. Cells derived from single-cell clone 9 with good growth status were selected and injected into enucleated pig oocytes. According to previous reports, CRISPR/Cas9-mediated gene-editing may randomly affect the genome, leading to unknown risks that may reduce the rates of development to the blastocyst stage (Feng et al., 2020). Our *in vitro* results demonstrated that the genome from these gene-edited cells retained the ability to direct the development of reconstructed embryos into blastulas (Figure 1F). We transferred the reconstructed embryos into three recipient sows to generate gene-edited pigs. Approximately 300 reconstructed embryos at the two-cell stage were surgically transferred into both oviducts of the estrus-synchronized recipient gilts. Two of the three recipients had successful pregnancies and two piglets were obtained after 117 days (Figure 1G, H). Sanger sequencing confirmed that the piglets showed the same genotype as their donor cells, demonstrating that our strategy was economical and sufficient to direct the generation of polygenic-improved pigs (Figure 1I).

Subsequently, we performed whole-genome sequencing to confirm the accuracy of our strategy for generating genome-edited pigs. Single-nucleotide variants (SNVs) and small indels were identified in the two pigs and porcine primary cells. The density distributions of SNVs and indels in each chromosome of the gene-edited pigs and porcine primary cells were approximately comparable to the reference genome, suggesting that our editing had limited impact on the genome

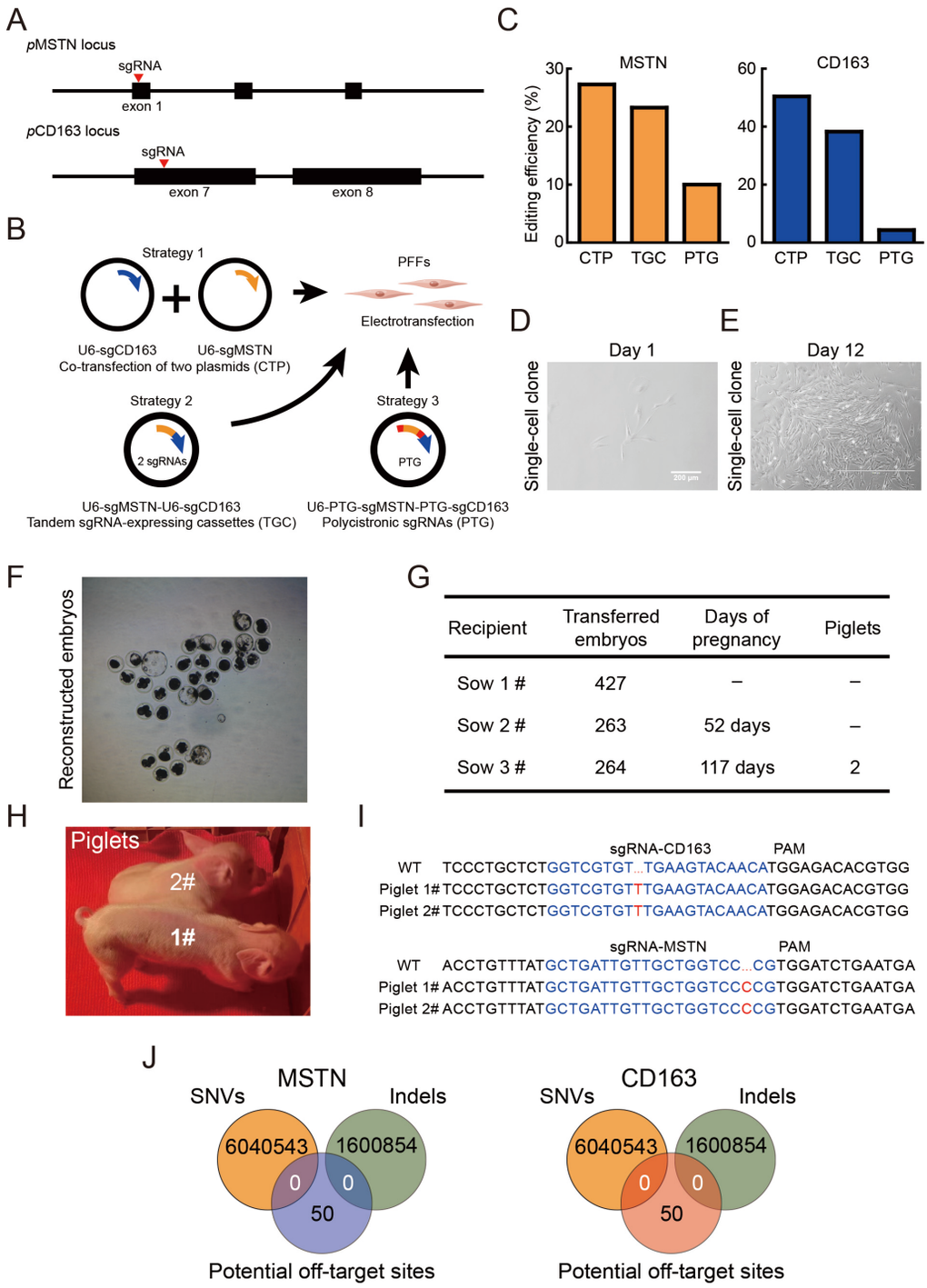

Figure 1 Different sgRNA expression strategies affect multiplex gene-editing efficiency

A: Schematic of sgRNAs targeting MSTN and CD163 loci, respectively. Exons near sgRNAs are indicated by black squares. B: Schematic of three strategies used for multiplex gene-editing in this study. Constructed vectors were transfected into PFFs. The sgRNA targeting MSTN is marked in blue and that targeting CD163 is marked in orange. PTG element is marked in red. CTP, co-transfection of two plasmids. TGC, tandem sgRNA-expressing cassettes. PTG, polycistronic sgRNAs. C: Histograms of MSTN and CD163 indels by different sgRNA expression strategies. Editing efficiency was quantified by TIDE analysis. D: Representative images of single-cell-derived clones after 1 day of limiting dilution. Scale bar: 200 μm. E: Representative images of single-cell-derived clones after 12 days of limiting dilution. Scale bar: 1 000 μm. F: Representative images of blastulas developed from reconstructed embryos. G: Statistics of the developmental competence of the reconstructed embryos *in vivo*. –: Not available. H: Genetically modified piglets carrying MSTN and CD163 mutation. I: Genotypes of genetically modified piglets. J: Overlap of all whole-genome sequencing-identified SNVs and indels with potential off-target sites predicted by CRISPOR.

(Supplementary Figure S5A, B). In addition, most SNVs and indels occurred in noncoding regions, indicating that most mutations should be nonsense (Supplementary Figure S5C, D). Compared with the porcine primary cells, the unique SNVs and indels in the genome-edited piglets were considered as genomic variant regions defined by whole-genome sequencing. We selected the top 50 CRISPOR-predicted off-target sites as potential off-target sites and observed no variations surrounding these regions based on whole-genome sequencing (Figure 1J). These findings suggest that the strategy involving tandem sgRNA expression cassettes is safe and effective for generating genetically modified domestic animals.

In summary, by systematic comparison of multiplex gene-editing efficiency using different sgRNA expression approaches in PFFs, we found that the different strategies indeed affected multiplex gene-editing efficiency in pigs, with transfection of a single plasmid containing tandem sgRNA expression cassettes showing the greatest efficiency. Importantly, Sanger sequencing and whole-genome resequencing demonstrated that the strategy involving tandem sgRNA expression cassettes was accurate, with no off-targets. The generation of two homozygous genome-modified pigs using only three recipient sows also indicated that this strategy was an economical and straightforward way to achieve genome improvement in large animals. This study provides insights into the polygenic modification of large animals using CRISPR/Cas9 to efficiently produce piglets as important breeding resources anti-PRRSV and promoting muscle growth.

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

J.L.H., H.J.W., and J.Q.Z. designed the study and wrote the manuscript. J.L.H., H.J.W., J.Q.Z., Y.W., Z.H.L., W.W., J.X.G., X.J.W., W.Y., X.L.W., Q.Y.S., A.M.L., and X.F.W. performed the experiments and analyzed the data. All authors read and approved the final version of the manuscript.

ACKNOWLEDGMENTS

We thank Dr. Yong Tang (University of Connecticut) and Dr. Shuai Yu (Yangzhou University) for revising the manuscript.

Ju-Qing Zhang[1,#], Jian-Xiong Guo[2,#], Xiao-Jie Wu[1,#], Yu Wang[3,#], Wei Yue[1], Zhuo-Hui Li[3], Wei Wang[4], Xin-Fang Wei[1], Xiao-Long Wu[1], Qiao-Yan Shen[1], An-Min Lei[1], Hong-Jiang Wei[2,*], Jin-Lian Hua[1,*]

[1] *College of Veterinary Medicine*, *Shaanxi Centre of Stem Cells Engineering & Technology*, *Northwest A&F University*, *Yangling*, *Shaanxi* 712100, *China*

[2] *State Key Laboratory for Conservation and Utilization of Bio-Resources in Yunnan*, *Yunnan Agricultural University*, *Kunming*, *Yunnan* 650201, *China*

[3] *College of Animal Sciences & Technology*, *Northwest A&F University*, *Yangling*, *Shaanxi 712100 China*

[4] *National Institute of Biological Sciences*, *Beijing* 102206, *China*

[#]Authors contributed equally to this work

*Corresponding authors, E-mail: hongjiangwei@126.com; jinlianhua@nwsuaf.edu.cn

REFERENCES

Fan ZY, Liu ZG, Xu K, Wu TW, Ruan JX, Zheng XM, et al. 2022. Long-term, multidomain analyses to identify the breed and allelic effects in *MSTN*-edited pigs to overcome lameness and sustainably improve nutritional meat production. *Science China Life Sciences*, **65**(2): 362−375.

Feng T, Li ZF, Qi XL, Liu J, Gao F, Ma Z, et al. 2020. Measuring targeting specificity of genome-editing by nuclear transfer and sequencing (NT-seq). *Cell Discovery*, **6**(1): 78.

Minkenberg B, Wheatley M, Yang YN. 2017. CRISPR/Cas9-enabled multiplex genome editing and its application. *Progress in Molecular Biology and Translational Science*, **149**: 111−132.

Ren C, Liu YF, Guo YC, Duan W, Fan PG, Li SH, et al. 2021. Optimizing the CRISPR/Cas9 system for genome editing in grape by using grape promoters. *Horticulture Research*, **8**(1): 52.

Wang H, Shen L, Chen J, Liu X, Tan T, Hu Y, et al. 2019. Deletion of CD163 exon 7 confers resistance to highly pathogenic porcine reproductive and respiratory viruses on pigs. *International Journal of Biological Sciences*, **15**(9): 1993−2005.

Wu XL, Zhu ZS, Xiao X, Zhou Z, Yu S, Shen QY, et al. 2021. *LIN28A* inhibits *DUSP* family phosphatases and activates MAPK signaling pathway to maintain pluripotency in porcine induced pluripotent stem cells. *Zoological Research*, **42**(3): 377−388.

Xie KB, Minkenberg B, Yang YN. 2015. Boosting CRISPR/Cas9 multiplex editing capability with the endogenous tRNA-processing system. *Proceedings of the National Academy of Sciences of the United States of America*, **112**(11): 3570−3575.

Yang HQ, Zhang J, Zhang XW, Shi JS, Pan YF, Zhou R, et al. 2018. CD163 knockout pigs are fully resistant to highly pathogenic porcine reproductive and respiratory syndrome virus. *Antiviral Research*, **151**: 63−70.

Zhang DB, Zhang HW, Li TD, Chen KL, Qiu JL, Gao CX. 2017. Perfectly matched 20-nucleotide guide RNA sequences enable robust genome editing using high-fidelity SpCas9 nucleases. *Genome Biology*, **18**(1): 191.

Zhu HC, Li C, Gao CX. 2020. Applications of CRISPR-Cas in agriculture and plant biotechnology. *Nature Reviews Molecular Cell Biology*, **21**(11): 661−677.

Zhang J, Zhou Y, Yue W, Zhu Z, Wu X, Yu S, et al. 2022. Super-enhancers conserved within placental mammals maintain stem cell pluripotency. *Proceedings of the National Academy of Sciences of the United States of America*, **119**(40): e2204716119.

Zhu Z, Wu X, Li Q, Zhang J, Yu S, Shen Q, et al. 2021. Histone demethylase complexes KDM3A and KDM3B cooperate with OCT4/SOX2 to define a pluripotency gene regulatory network. *FASEB J*, **35**(6): e21664.