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Efficient generation of pLEG1a-/- and pLEG1b-/- double knockout minipig fibroblast cells using the CRISPR/Cas9 system*

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The liver-enriched gene 1 (LEG1), which is conserved from bacteria to mammals, is essential for normal liver development by serving as a secreted signaling molecule/modulator in zebrafish. However, its functions in mammals are still unclear. A minipig model with the LEG1 knockout, which can be constructed by somatic cell nuclear transfer (SCNT), may facilitate studies on the functions of LEG1 in mammals. To achieve this goal, we constructed minipig fibroblast cells with the LEG1 knockout using the CRISPR/Cas9 system. Two single guide RNAs were designed that targeted exon 1s of the two copies of porcine LEG1, pLEG1a, and pLEG1b. Vector pX330 was used to construct the recombinant plasmid pX330-pLeg1a-pLeg1b. Three nucleofector parameters CA-137, CL-133, and DO-113 were tested, with DO-113 showing the highest fluorescence intensity and thus it was selected for subsequent experiments. A total of 39 cell colonies were obtained, of which four were pLEG1a-/-; pLEG1b-/- double knockouts with frameshift mutations including both insertions and deletions. No mutation occurred in the predicted off-target sites and there was no CRISPR/Cas9 plasmid integration in the four colonies. In conclusion, this is the first study of pLEG1a and pLEG1b knockout in minipig fibroblast cells, providing a preliminary basis for the future production of SCNT-cloned pigs and studies on LEG1 function.

KEY WORDS: CRISPR / Cas9 / pLEG1a / pLEG1b / minipig fibroblast cells

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The CRISPR/Cas9 system is a part of the adaptive system in archaea and bacteria. The system acts by recognising target sequences in the DNA by single-guide RNA (sgRNA), after which the Cas9 nuclease cleaves the double strands of DNA [Lau and Davie 2017; Liu *et al*. 2017]. The CRISPR/Cas9 gene-editing technology has been widely applied in various eukaryotic systems, including yeasts, worms, insects, plants and animals [Lau and Davie 2017, Liu *et al.* 2017, Ma *et al*. 2016]. It is superior to traditional gene-editing technologies such as zinc-finger nucleases (ZFN) and transcription activator-like effector nucleases (TALEN), thanks to its simplicity and low cost [Wang *et al*. 2016].

Minipigs are one of the most important laboratory animals because of their similarity to humans [Dalgaard 2015, Vodicka *et al.* 2005] and thanks to these high physiological and anatomical similarities they are widely applied both in disease modeling and xenotransplantation [Bode *et al*. 2010, Suenderhauf and Parrott 2013, Wang *et al*. 2019]. To overcome the immunological rejection associated with xenotransplantation, minipigs are genetically modified in preclinical experiments.

The liver-enriched gene 1 (LEG1) is evolutionally conserved in vertebrates and encodes a novel glycosylated secreted protein LEG1 [Chang *et al.* 2011, Hu *et al.* 2016]. LEG1 has been shown to be essential for normal liver development by serving as a secreted signaling molecule/modulator in zebrafish [Chang *et al*. 2011, Hu *et al.* 2016]; however, its functions in mammals are still unclear. In our previous work, we cloned and characterised the pig LEG1a, LEG1b, and LEG1c genes (pLEG1a, pLEG1b, pLEG1c). We also demonstrated that pLEG1a is molecularly, structurally and evolutionarily closer to human and mouse LEG1a orthologs; meanwhile, eutherian LEG1a genes might be functionally different from zebrafish homologs [Dang *et al.* 2020].

Therefore, in this study we generated pLEG1a-/-;pLEG1b-/- double knockout minipig fibroblast cells using the CRISPR/Cas9 system so that they may be used as donors for somatic cell nuclear transfer (SCNT) in order to generate genetically modified minipigs to study functions of pLEG1a and pLEG1b.

Material and methods

All animal experiments were performed according to guidelines developed by the China Council on Animal Care and Protocol and were approved by the Experimental Animal Welfare Committee of Zhejiang University (Zhejiang, China).

The U6 promoter-driven gRNA cloning vector pX330 was purchased from Addgene (Plasmid No. 42230; Watertown, MA, USA). The gRNAs targeting exon 1s of pLEG1a and pLEG1b were designed using an online gRNA design tool (http://crispr.mit.edu) and the annealing primers used for gRNA-pLeg1a were forward: 5'-CACCGCATGGCTGGCAATATACTAC-3' and reverse: 5'-AAACGTAGTATATTGCCAGCCATGC-3'; while those for gRNA-pLeg1b were forward: 5'-CACCGCTGGCATTACCTTGAGAGAC-3' and reverse: 5'-AAACGTCTCTCAAGGTAATGCCAGC-3'. Then, a 380-base-pair fragment

containing U6-gRNA-pLeg1b was amplified from pX330-pLeg1b using primers P1 (5'-TCTGTACCTCTAGAGAGGGCCTATTTCCATGAT-3') and P2 (5'-ATCGTCGTCTAGAACTTGCTATTTCTAGCTCTAAAAC-3'), and then inserted into the XbaI site of pX330-pLeg1a, resulting in the recombinant vector pX330-pLeg1a-pLeg1b containing both U6-gRNA-pLeg1a and U6-gRNA-pLeg1b (Fig. S1). Transfection of 32-days porcine fetal fibroblasts (PFFs) with different plasmids (pMax-GFP, pX330-pLeg1a-pLeg1b, pCAG-pLeg1a-3×FLAG, and pCAG-pLeg1b-3×HA) was performed using Lonza Nucleofector™ applying different parameters (CA-137, CL-133, and DO-113). After transfection, single PFF colonies were picked according to the published protocol using the limiting dilution strategy [Chen *et al.* 2020]. Briefly, transfected cells were subcultured in 10 cm Petri dishes at 5000 cells/dish and then were cultured in DMEM (Gibco, Thermo Fisher Scientific, Shanghai, China) containing 20% FBS (Gibco, Thermo Fisher Scientific, Shanghai, China) with 10 ng/mL basic fibroblast growth factor (Gibco, Thermo Fisher Scientific, Shanghai, China) for about 10 days. Individual cell colonies were selected and cultured in 24-well plates. When cells reached confluency, 50% were cryopreserved, and the other 50% were subjected to genome extraction for mutation identification. The pLEG1a and pLEG1b genes were examined with primers for pLEG1a (pLeg1a-F: 5'-ATGGCTTTCCTTCCTCCTTGG-3', pLeg1a-R:5'-GTGAACCTGTAGCGTCATCCA-3') and pLEG1b (pLeg1b-F:5'-ATCTTCCCTGGGCCTGTGTA-3', pLeg1b-R:5'-TCCTTGCAACTTTGAACTTCATT-3'). Genetic modification and exogenous gene integration were detected using PCR, in which the Cas9 gene was used as the indicator of pX330 integration (Cas9-F: 5'-CATCGAGCAGATCAGCGAGT-3' and Cas9-R: 5'-CGATCCGTGTCTCGTACAGG-3'). Off-target cleavage sites were predicted using an online tool (http://crispr.mit.edu) with default parameters.

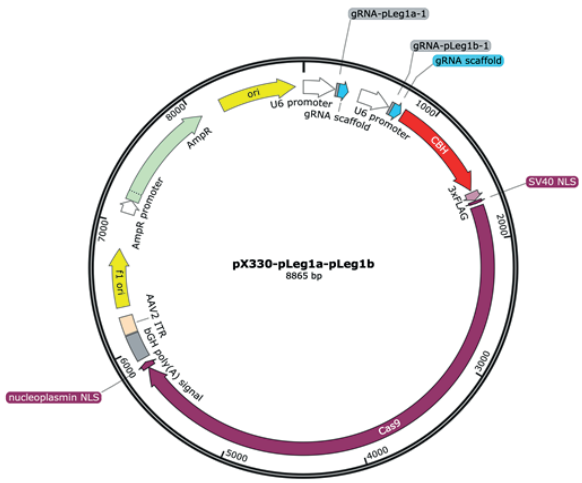

Fig. S1. Schematic representation of pX330-pLeg1a-pLeg1b. gRNA sequences targeting pLeg1a and pLeg1b are shown as grey boxes. U6 promoters are immediately upstream of the gRNAs.

Results and discussion

The pig has three LEG1 gene copies, of which pLEG1a is phylogenetically closer to the human and mouse LEG1a genes [Dang *et al*. 2020]. Transcriptionally, only pLEG1a could be detected in the salivary gland; however, pLEG1b shares many similarities with pLEG1a in terms of protein structure and sequence similarity. pLEG1c is evolutionarily distant to pLEG1a and pLEG1b, and its encoding protein loses the signal peptide, which is a characteristic of LEG1 proteins [Dang *et al.* 2020]. Hence, pLEG1a and pLEG1b might be functionally redundant, which was also observed in zleg1 genes [Chang *et al*. 2011]. Therefore, to avoid pLEG1b functionally complementing the pLEG1a knockout, we concurrently knocked out the pLEG1a and pLEG1b genes in the PFFs.

Plasmid transfection was first optimised in PFFs. Three nucleofector parameters CA-137, CL-133, and DO-113 were tested with 3 μg pMax-GFP. The results showed

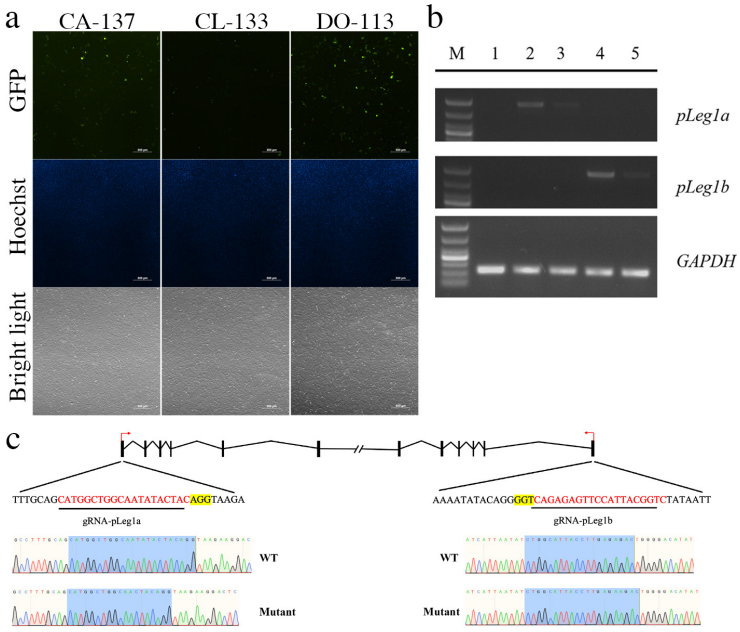

Fig. 1. a – Transfection efficiency using different nucleofector parameters. To determine transfection efficiency, PFFs cells were electroporated with pMax-GFP encoding green fluorescent protein using nucleofector parameters CA-137, CL-133, and DO-113, respectively. Hoechst staining of nuclei and bright light images are also shown in the middle panel and lower panel, respectively. Scale bar = 500 μm. b – RT-PCR detection of endogenous pLEG1a and pLEG1b in PFFs. pLEG1a (upper panel), pLEG1b (middle panel) and GAPDH (lower panel) were amplified using gene-specific primers. M, marker; 1, PFFs without plasmid transfection; 2 & 3, PFFs transfected with 3 μg and 1 μg pCAG-pLeg1a-3×FLAG, respectively; 4 & 5, PFFs transfected with 3 μg and 1 μg pCAG-pLeg1b-3×HA, respectively. c – Two gRNAs targeting exon 1s of pLEG1a and pLEG1b. Red sequence, spacer bases; Yellow highlighted, PAM sequences. The Representative Sanger sequencing results of WT and mutant genotypes are shown below.

that transfection with DO-113 showed the highest fluorescence intensity (Fig. 1a). Therefore, DO-113 was selected for subsequent experiments.

It has been suggested that the transcriptional activity of the target gene could affect gene editing efficiency, where gene editing might be delayed for an inactively transcribed gene or heterochromatin [Hockemeyer *et al*. 2009, Kallimasioti-Pazi *et al.* 2018]. Moreover, the previous analysis showed that pLEG1a is expressed exclusively in the salivary gland, while pLEG1b is transcriptionally inactive [Dang *et al*. 2020]. We here tested whether pLEG1a and pLEG1b are expressed in the PFF. RT-PCR was conducted using the RNA extracted from the PFF and the result showed that neither of the genes was detectable in the PFFs (Fig. 1b), thus suggesting that gene editing efficiency targeting both genes may be compromised.

Using the online sgRNA design tool, two gRNAs targeting exon 1s of pLEG1a and pLEG1b were designed (Fig. 1c) and cloned into the pX330 in series to obtain the recombinant plasmid pX330-pLeg1a-pLeg1b. Then PFF cells were transfected with the plasmid and selected using the limiting dilution strategy [Chen *et al*. 2020]. A total of 39 cell colonies were obtained, with bi- and mono-gene mutant colonies being identified. Of them, four colonies (nos. 11, 15, 19 and 24) were pLEG1a-/-;pLEG1b-/- double gene knockouts with frameshift mutations including both insertions and deletions (Tab. 1); 23 colonies showed at least a monoallelic mutant in one gene; while 12 colonies showed no mutation. The double gene knockout rate was approx. 10.3% (4/39), whereas the knockout rate with at least a monoallelic mutant was 69.2% (27/39), being higher than those using TALEN in PFFs. Huang *et al*. [2014] reported gene knockout rates of 7.7% (14/181) for the recombination-activating gene

Table 1. Genotypes of four colonies with *pLEG1a*$^{-/-}$ and *pLEG1b*$^{-/-}$ double gene knockout

Item	Sequence	Genotype
WT(*pLEG1a*)		
No.11	GCCTTTGCAGCATGGCTGGCAATATACTACAGGTAAGAAGGAC[a] GCCTTTGCAGCATGGCTGGCAA....CTACAGGTAAGAAGGAC[b]	del 4bp/ins 1bp
No.15	GCCTTTGCAGCATGGCTGGCAATATACCTACAGGTAAGAAGGAC[c] GCCTTTGCAGCATGGCTGGCAATATACCTACAGGTAAGAAGGAC	ins 1bp/del 2bp
No. 19	GCCTTTGCAGCATGGCTGGCAATAT..TACAGGTAAGAAGGAC GCCTTTGCAGCATGGCTGGCAA.....TACAGGTAAGAAGGAC	del 4bp/del 5bp
No. 24	GCCTTTGCAGCATGGCTGGCAAT....TACAGGTAAGAAGGAC GCCTTTGCAGCATGGCTGGCAATATACTTACAGGTAAGAAGGAC 124bp del...	ins 1bp/del 124 bp
WT(*pLEG1b*		
No.11	TCATTAATATCTGGCATTACCTTGAGAGACTGGGGACATATAA TCATTAATATCTGGCATTACCTTGAGAAGACTGGGGACATATAA	ins 1bp/ins 1bp
No.15	TCATTAATATCTGGCATTACCTTGAGAAGACTGGGGACATATAA TCATTAATATCTGGCATTACCTTGAGAAGACTGGGGACATATAA	ins 1bp/ins 2bp
No. 19	TCATTAATATCTGGCATTACCTTGAGAAAGACTGGGGACATATAA TCATTAATATCTGGCATTACCTT....GACTGGGGACATATAA	del 4bp/del 4bp
No. 24	TCATTAATATCTGGCATTACCTT....GACTGGGGACATATAA TCATTAATATCTGGCATTACCT.....TGGGGACATATAA …41 bp del...TGGGGACATATAA	del 8bp/del 41bp

Protospacers are highlighted in red.
Dots indicate deleted bases.
Blue letters indicate inserted bases.

1 (RAG1) gene and 1.3% (6/454) for the RAG2 gene. In turn, Cheng *et al.* [2016] reported a gene knockout rate of 2.4% (3/126) for its target gene GGTA1. The highest reported knockout rate for TALEN was 42% (8/19) [Shen *et al*. 2017]. However, the CRISPR/Cas9 system always results in a high gene knockout or knock-in rate in PFFs. Zhou *et al*. [2015] reported mutation rates of 49.4%, 66.7%, and 69.9% for tyrosinase, parkin RBR E3 ubiquitin-protein ligase, and PTEN-induced kinase 1, respectively, while Su et al. [Su *et al*. 2018] reported a mutation rate of 87.5% in PFFs. Moreover, Ruan *et al*. [2015] reported a knock-in rate of up to 54%. Our data and those from the above-mentioned studies suggest that the CRISPR/Cas9 system has a high gene-editing efficiency.

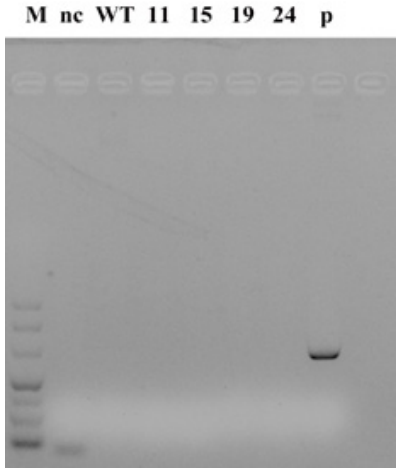

Fig. S2. Detection of exogenous Cas9 DNA integration in cell colonies. M, DNA marker; nc, negative control with water; WT, wildtype PFFs; #11, 15, 19, and 24, four colonies with pLEG1a-/- and pLEG1b-/- double knockout; p, positive control with cloning plasmid pX330.

Table S1. Predicted off-target sequences, locations, and primers for PCR

Item	Sequence	Location	Primers for sequencing
pLEG1a	CATGGCTGGCAATATACTACAGG	chr1(+): 35600757-35600779	CCCCCGCTGTGGAATAAGAG GCCCCACTGCAGTTTTTAGC
OT1	AAAGGCTAGAAATATACTACGAG	chr4(+): 119853139-119853161	TCCGTTCCTGACTACAGGTTG AACTTCATGTGGTGGGATTGC
OT2	CATTAATGGTAATATACTACTGG	chr9(+): 25063892-25063914	TGCTTTCTGGCTGTATGGAGAA GGCTTTCACACATGAATACCGC
OT3	CACAGCTAGCAAAATACTACTGG	chr3(-): 109488572-109488594	GTCCCATCAGCTAAGGCACA CCTGTTGATTTCGCAGAGCC
OT4	GTTTGCTGGCAATATACTCCTAG	chr18(-): 21067271-21067293	GCAGATGTCAAAGTGACTACCA CTAGCTTTGTGGTGGGCAAAC
pLEG1b	CTGGCATTACCTTGAGAGACTGG	chr1(-): 35647065-35647087	ACAAGGGAAGTGCCCTATTACC TGATGTACAGGCAATCCCCA
OT1	CAAGGCATTACCTTGAGAGAATGATGG	chr2(-): 27978102-27078128	TGCACGCTGAATTTACAACAGG CCTTGAGGAAGGGCAATAGC
OT2	CATGGCATTACCTGGACAGCCTGGTGG	chr5(-): 15064818-15064844	CGGCTACTCACCAAGCTTCC AAGCCTCGAGTCAGGTCCTA
OT3	CTGGCCTCACCTCTGAGAGACTGGAGG	chr12(-): 25725124-25725150	AACTGCTGGAGTGGGGATTG CTCTCAAGGCAGACCCCAAG

To exclude exogenous DNA integration in the gene-edited cell colonies, which may affect the phenotype of genetically modified minipigs, PCR identification of the Cas9 gene was performed. The PCR results showed no evidence of CRISPR/Cas9 plasmid integration for all the four pLEG1a-/-;pLEG1b-/- colonies (nos. 11, 15, 19, and 25) (Fig. S2). Off-target effects are the major disadvantage of the CRISPR/Cas9 gene-editing technology. The selected off-target sequences, locations and primers for PCR are shown in Table S1. Direct sequencing was first employed to detect whether off-target editing occurred in our double gene knockout colonies (Fig. 2). Subsequently, TA-cloning was performed to confirm the direct sequencing results. Both assays indicated that no off-target event took place in our selected colonies (nos. 11, 15, 19 and 24. Fig. 2).

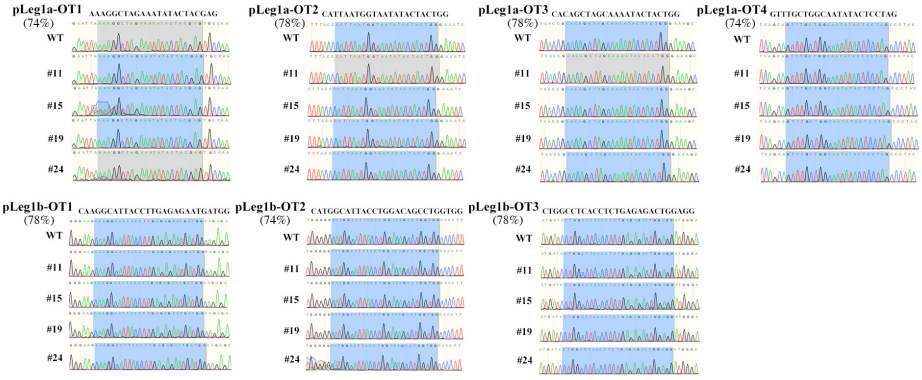

Fig. 2. Detection of off-target events in pLEG1a-/-;pLEG1b-/- double knockout colonies. A total of seven off-target sites (four for pLEG1a and three for pLEG1b) similar to our target sequences were selected for PCR analysis. The percentages in brackets represented the identities of the target sequence and its respective off-target sequences.

In conclusion, this was the first study to generate pLEG1a-/-;pLEG1b-/- double knockout PFFs with a high mutation rate of 10.3% under no antibiotic selection. These gene edited PFFs may be used as donor cells for further SCNT to generate genetically modified minipigs to study functions of pLEG1a and pLEG1b.

REFERENCES

1. BODE G., CLAUSING P., GERVAIS F., LOEGSTED J., LUFT J., NOGUES V., SIMS J., and Steering Group of the Rethink Project. 2010 – The utility of the minipig as an animal model in regulatory toxicology. ***Journal of Pharmacological and Toxicological Methods*** 62, 196-220.
2. CHANG C., HU M., ZHU Z., LO L.J., CHEN J., PENG J., 2011 – Liver-enriched gene 1a and 1b encode novel secretory proteins essential for normal liver development in zebrafish. ***PLoS One*** 6:e22910.

3. CHEN J., AN B., YU B., PENG X., YUAN H., YANG Q., CHEN X., YU T., WANG L., ZHANG X., WANG H., ZOU X., PANG D., OUYANG H., TANG X., 2020 – CRISPR/Cas9-mediated knockin of human factor IX into swine factor IX locus effectively alleviates bleeding in hemophilia B pigs. ***Haematologica*** 2019.224063.
4. CHENG W., ZHAO H., YU H., XIN J., WANG J., ZENG L., YUAN Z., QING Y., LI H., JIA B., YANG C., SHEN Y., ZHAO L., PAN W., ZHAO H., WANG W., WEI H., 2016 – Efficient generation of GGTA1-null Diannan miniature pigs using TALENs combined with somatic cell nuclear transfer. ***Reproductive Biology and Endocrinology*** 14, 77.
5. DANG Y., WANG J.Y., LIU C., ZHANG K., JINRONG P., HE J., 2020 – Evolutionary and molecular characterization of liver-enriched gene 1. ***Scientific Reports*** 10, 4262.
6. HOCKEMEYER D., SOLDNER F., BEARD C., GAO Q., MITALIPOVA M., DEKELVER R.C., KATIBAH G.E., AMORA R., BOYDSTON E.A., ZEITLER B., MENG X., MILLER J.C., ZHANG L., REBAR E.J., GREGORY P.D., URNOV F.D., JAENISCH R., 2009 – Efficient targeting of expressed and silent genes in human ESCs and iPSCs using zinc-finger nucleases. ***Nature Biotechnology*** 27, 851-857.
7. HU M., BAI Y., ZHANG C., LIU F., CUI Z., CHEN J., PENG J., 2016 – Liver-enriched gene 1, a glycosylated secretory protein, binds to FGFR and mediates an anti-stress pathway to protect liver development in zebrafish. ***PLoS Genetics*** 12:e1005881.
8. HUANG J., SOLDNER F., BEARD C., GAO Q., MITALIPOVA M., DEKELVER R.C., KATIBAH G.E., AMORA R., BOYDSTON E.A., ZEITLER B., MENG X., MILLER J.C., ZHANG L., REBAR E.J., GREGORY P.D., URNOV F.D., JAENISCH R., 2014 – RAG1/2 knockout pigs with severe combined immunodeficiency. ***Journal of Immunology*** 193, 1496-1503.
9. KALLIMASIOTI-PAZI E.M., THELAKKAD C.K., TAYLOR G.C., MEYNERT A., BALLINGER T., KELDER M.J.E., LALEVÉE S., SANLI I., FEIL R., WOOD A.J., 2018 – Heterochromatin delays CRISPR-Cas9 mutagenesis but does not influence the outcome of mutagenic DNA repair. ***PLoS Biology*** 16:e2005595.
10. Lau V., Davie J.R., 2017 – The discovery and development of the CRISPR system in applications in genome manipulation. ***Biochemistry and Cell Biology*** 95, 203-210.
11. LIU C., ZHANG L., LIU H., CHENG K., 2017 – Delivery strategies of the CRISPR-Cas9 gene-editing system for therapeutic applications. ***Journal of Controlled Release*** 266, 17-26.
12. MA X., ZHU Q., CHEN Y., LIU Y.G., 2016 – CRISPR/Cas9 platforms for genome editing in plants: developments and applications. ***Molecular Plant*** 9, 961-974.
13. RUAN J., LI H., XU K., WU T., WEI J., ZHOU R., LIU Z., MU Y., YANG S., OUYANG H., CHEN-TSAI R.Y., LI K., 2015 – Highly efficient CRISPR/Cas9-mediated transgene knockin at the H11 locus in pigs. Scientific Reports 5, 14253.
14. SHEN Y., XU K., YUAN Z., GUO J., ZHAO H., ZHANG X., ZHAO L., QING Y., LI H., PAN W., JIA B., ZHAO H.Y., WEI H.J., 2017 – Efficient generation of P53 biallelic knockout Diannan miniature pigs via TALENs and somatic cell nuclear transfer. ***Journal of Translational Medicine*** 15, 224.
15. SU X., CUI K., DU S., LI H., LU F., SHI D., LIU Q., 2018 – Efficient genome editing in cultured cells and embryos of Debao pig and swamp buffalo using the CRISPR/Cas9 system. ***In Vitro Cell and Developmental Biology-Animal*** 54, 375-383.
16. Suenderhauf C., Parrott N., 2013 – A physiologically based pharmacokinetic model of the minipig: data compilation and model implementation. ***Pharmaceutical Research*** 30, 1-15.
17. VODICKA P., SMETANA K. JR, DVORÁNKOVÁ B., EMERICK T., XU Y.Z., OUREDNIK J., OUREDNIK V., MOTLÍK J., 2005 – The miniature pig as an animal model in biomedical research. ***Annals of the New York Academy of Sciences*** 1049, 161-171.

18. WANG H., LA RUSSA M., QI, L.S., 2016 – CRISPR/Cas9 in genome editing and beyond. ***Annual Reviews of Biochemistry*** 85, 227-264.
19. WANG Y., LEI T., WEI L., DU S., GIRANI L., DENG S., 2019 – Xenotransplantation in China: Present status. ***Xenotransplantation*** 26:e12490.
20. ZHOU X., XIN J., FAN N., ZOU Q., HUANG J., OUYANG Z., ZHAO Y., ZHAO B., LIU Z., LAI S., YI X., GUO L., ESTEBAN M.A., ZENG Y., YANG H., LAI L., 2015 – Generation of CRISPR/Cas9-mediated gene-targeted pigs via somatic cell nuclear transfer. ***Cell and Molecular Life Science*** 72, 1175-1184.