Proceedings, 10th World Congress of Genetics Applied to Livestock Production

Improving pig genetic resistance and muscle production through molecular biology

Qianqian Kang, Yiqing Hu, Yunlong Zou, Wenping Hu, Li Li, Fei Chang, Yang Li, Dan Lu, Zhaolin Sun, Ran Zhang, Xiaoxiang Hu, Qiuyan Li, Yunping Dai, *Ning Li*
State Key Laboratory for Agrobiotechnology, China Agricultural University, Beijing, P.R. China, 100193

ABSTRACT: Genome modification technology of livestock has developed rapidly in recent years. We can not only insert foreign genes or DNA into a specific locus or disrupt an endogenous locus by gene targeting, but it is now possible to precisely engineer the genome. The tools for this include Zinc Finger Nucleases (ZFN), TALENs and most recently CRISPR/Cas9 which are highly efficient. In this brief review, we focus on the current status of gene editing technology as well as molecular biology in farm animals, and their application relevant to improvement of production traits and disease resistance, with emphasis on the pig.
Keywords: ZFN; TALEN ; CRISPR/Cas9; Molecular biology; Livestock animals

Introduction

Genome editing is a powerful tool that has been used in gene function research and molecular breeding of animals. In 1980, Gordon and colleagues invented DNA microinjection technology to introduce foreign DNA into the mouse genome (Gordon et al. (1980)). DNA microinjection into fertilized mammalian eggs is the classic method of gene transfer in mice and farm animals. But with the limitation that the foreign DNA integrates randomly and inefficiently (Wall (1996)), microinjection technology is not suitable for the research and development of farm animals. In 1976, Jaenisch infected mice with the exogenous Moloney leukemia virus (M-MuLV). The M-MuLV integrated into the germ line and descended to offspring (Jaenisch 1976). For decades now, genes of interest were integrated into pigs (Hofmann et al. (2003)), cattle (Chan et al. (1998)), chicken and even monkeys (Chan et al. (2001)) using lentiviruses. However, due to the limitation imposed by virus packaging, it was not possible to move gene fragments larger than 10kb in size. In addition, animals produced by lentiviral transgenesis are usually mosaic and the efficiency of transfer to offspring is low (Lillico et al. (2011)). These issues were overcome for mice with the advent of pluripotent stem cells (e.g. embryonic stem (ES) cells), however ES cells are not yet available for livestock. Fortunately the advent of nuclear transfer technology (SCNT) has enabled farm animal transgenic research. In the 1980s, Capecchi and colleagues developed a method for gene targeting which makes site-specific genome modification possible in animals (Thomas and Capecchi (1987)). McCreath et al achieved the first site-specific targeting in livestock when they inserted the human AAT gene within an ovine beta-lactoglobulin (BLG) expression vector and the cassette into the ovine COL1A1 locus (McCreath et al. (2000)). After that, pigs (Dai et al. (2002)) and cows (Richt et al. (2007)) were successfully manipulated through gene targeting. Recently genome editing technology tools have been developed and it has already played an important role in molecular biology as well as genetic engineering. As powerful tools for accurate genomic modification, ZFN, TALEN and CRISPR/Cas9 have been used successfully to produce many examples of knock-out (KO) or knock-in farm animals.

Genetically modified livestock using ZFN and TALEN. With the development of technologies such as zinc-finger nucleases (ZFN) and Transcription activator-like effector nucleases (TALENs), many genetically modified livestock have been generated for agricultural and biomedical applications.

α 1,3-galactosyltransferase (GGTA1) gene was knocked out in pigs in 2011 using ZFN (Hauschild et al. (2011)) and TALEN in 2013 (Xin et al. (2013)). The α 1,3-galactosyltransferase gene encodes for the enzyme responsible for the generation of Gal epitopes on the cell surface of all porcine tissues. Gal epitopes are the major antigen in a pig-to-primate transplantation, resulting in a hyperacute rejection (HAR) response mediated by preformed antibody binding (Platt and Lin (1998)). So, with the inactivation of the GGTA1 gene, xenotransplantation will be more likely to come true in the future.

Pig, has a relatively long life and is similar in anatomy and physiology to humans, and it is therefore acquiring increasing attention in biomedical research. So, many genes have been modified in pigs for biomedical purposes. ZFN technology was combined with SCNT to produce monoallelic knockout pigs with a defined mutation in peroxisome proliferator-activated receptor-gamma (Ppar-γ). Pig is often considered an ideal animal model for studying human cardiovascular disease (CVD), and the Ppar-γ KO pig model is an invaluable tool for studying the role of PPAR-γ in development of CVD (Yang et al. (2011)). Toward the goal of producing a large animal model of atherosclerosis, Fahrenkrug has used TALENs to develop Ossabaw miniature swine containing inactivating alleles of the LDL receptor (LDLR) gene (Carlson et al. (2012)). Pigs with knockout alleles in the DAZL and APC genes were also generated to model infertility and colon cancer using TALENs (Tan et al. (2013a)). Fahrenkrug and his colleagues have done much work with livestock fibroblast clones. Single nucleotide alterations or small indels were introduced into 14 genes in pig, goat, and cattle fibroblasts using TALEN mRNA and oligonucleotides (Tan et al. (2013a)).

Table1. Single nucleotide alterations or small indels were introduced by HR into genes in pig, goat, and cattle

Tool	Species	Gene	Mutation type
ZFN	pig	*GHR*	Deletion and frame shift
ZFN	pig	*PPAD*	Deletion
ZFN	pig	*Leptin*	Deletion and frame shift
ZFN	cow	*BLG*	Deletion and frame shift
TALEN	pig	*LDLR*	Insertion and frame shift
TALEN	pig	*DAZL*	Insertion and frame shift
TALEN	pig	*APC*	Insertion and frame shift
TALEN	pig	*Tp53*	Insertion and frame shift
TALEN	pig	*RAG*	Insertion and frame shift
TALEN	cow	*Rosa26*	Insertion of mloxp sequence
TALEN	pig	*SRY*	Insertion of mloxp sequence
TALEN	pig	*KissR*	Insertion and frame shift
TALEN	cow	*GDF8*	Deletion and frame shift
TALEN	Pig	*EIF4GI*	SNPs
TALEN	cow	*GDF8*	SNPs
TALEN	cow	*GDF8*	SNP
TALEN	pig	*P65*	SNP
TALEN	pig	*P65*	SNP
TALEN	pig	*GDF8*	SNP
TALEN	goat	*FecB*	SNP
TALEN	goat	*CLPG*	SNP
CRISPR	pig	*P65*	SNP
CRISPR	pig	*P65*	SNP
CRISPR	Pig	*APC*	Insertion and frame shift
CRISPR	Sheep	*GDF8*	Deletion and frame shift

The interleukin-2 receptor gamma (IL2RG) gene was knocked out in pig using ZFN. The resulting IL2RG KO pigs completely lacked a thymus and were deficient in T and NK cells, similar to human X-linked SCID patients (Watanabe et al. (2013)). The porcine Rosa26 locus was genetically manipulated using TALEN and a Cre-inducible EGFP reporter pig line was created, which could be used as a reliable porcine reporter model for lineage tracing studies and provided a master pig line for stable gene overexpression (Li et al. (2014)). In contrast to pigs gene-edited cattle were generated mainly for agricultural applications. The beta-lactoglobulin (BLG) gene was disrupted via zinc-finger nucleases to humanize milk from cattle: milk from dairy cows contains the protein β-lactoglobulin (BLG), which is not present in human milk and is a major milk allergen (Yu et al. (2011)). Through ZFNickases mediated homology-directed repair, the lysostaphin gene was inserted into the endogenous β-casein (CSN2) locus in bovine fetal fibroblasts. The gene-targeted cows secrete lysostaphin in their milk and in vitro assays demonstrate the milk's ability to kill *Staphylococcus simulans* (Liu et al. (2013)).

Generating genetically modified animals with the robust tool CRISPR/Cas9. The newly developed genome editing technology CRISPR/Cas9, derived from the microbial immune system, has been applied to several species.

Ma et al. (2014) used the CRISPR/Cas9 system to simultaneously disrupt four genes (ApoE, B2m, Prf1, and Prkdc) in rats in one-step, by co-injection of Cas9 mRNA and sgRNAs into fertilized eggs. Zhou et al. (2014) reported that simultaneous use of dual sgRNAs to target an individual gene significantly improved the Cas9-mediated genome targeting with a bi-allelic modification efficiency of up to 78% in mouse. And they further observed that the target gene modifications were characterized by efficient germline transmission and site-dependent off-target effects, and also that the apolipoprotein E gene knockout-mediated defects in blood biochemical parameters were recapitulated by CRISPR/Cas9-mediated heritable gene modification (Zhou et al., 2014). Bassett et al. (2013) showed that injection of RNA into the Drosophila embryo can induce highly efficient mutagenesis of desired target genes in up to 88% of injected flies.

Hai et al. (2014) made vWF-knockout pig models by coinjection with Cas9 and sgRNA mRNA in embryos, but the models turned out to be chimeras. Apart from this, other studies of Cas9 application in pig cells have been reported. Zhao et al. (2014) reported a modified CRISPR interference system (CRISPRi), by which inactive Cas9 can reversibly prevent the expression of both monocistronic miRNAs and polycistronic miRNA clusters. They demonstrated CRISPR/CRISPRi is capable of suppressing genes in murine and porcine cells (Zhao et al., 2014). Tan et al. (2013b) targeted 14 genes in pig, goat and cattle fibroblasts using TALEN-mediated HDR and edited the T1591C site of pig p65 using CRISPR/Cas gRNAs for comparison. Niu et al. (2014) applied the CRISPR/Cas9 system to target monkey genomes by co-injection of Cas9 mRNA and sgRNAs into one-cell-stage embryos, then successfully achieved precise gene targeting in cynomolgus monkeys, simultaneously disrupting two genes (Ppar-γ and Rag1) in one step, and no off-target mutagenesis was detected. Efficient targeting of a reporter transgene Tg(-5.1mnx1:egfp) and four endogenous loci (tyr, golden, mitfa, and ddx19) was achieved in zebrafish with custom guide RNAs and a zebrafish codon-optimized Cas9 protein (Niu et al. (2014); Liu et al. (2014)). Chen et al. showed that the CRISPR-associated system can be adapted for efficient and precise editing of the *C. elegans* genome (Chen et al. (2013)). Hou et al. (2013) demonstrated efficient targeting of an endogenous gene in three human pluripotent stem cell (hPSC) lines using HDR mediated by a distinct CRISPR-Cas system from *Neisseria meningitidis*. Their study could have a tremendous impact in regenerative medicine (Hou et al. (2013)). Nakayama et al. (2013) have assessed the efficacy of the CRISPR/Cas system in the amphibian *Xenopus tropicalis* by interrupting the six3 gene, which is required for proper eye and brain formation. Finally, Daimon et al. (2014) proved that CRISPR/Cas system works for the silkworm *Bombyx mori* by targeting the BmBLOS2 gene which is essential for the formation of urate granules in the larval epidermis.

Research and Development Progress of genomically modified pigs at China Agricultural University. Pigs are important economic animals and as model organisms, so there comes more and more research focused on improving porcine production characteristics such as body composition, disease resistance and reproduction. Moreover, pigs are physiologically closer to humans and have a longer lifespan than mice (Vodicka et al. (2005)), so pigs have become important tools for biomedical research, such as specific disease models. In our laboratory, we have generated several transgenic pigs which carry special characteristics.

For increasing lean meat percentage, we have produced myostatin (MSTN) knockout pigs. The significant role of myostatin (GDF-8) in inhibiting muscle development has been reported for many years, and in our research, we achieved knockout of myostatin in Landrace, Duroc and Large White pigs, which are commercial breeds highly selected for lean meat content. The myostatin knockout was targeted at the active C-terminal domain with a conventional homologous recombination method and the successful knockout event was shown by genotypic analysis. Phenotypic analysis of the resulting pigs reveal that with heterozygotes for the MSTN C-terminal domain KO (MSTN+/-) pigs, the lean meat proportion was increased remarkably ($P<0.01$), with concomitant decrease in backfat thickness ($P<0.01$) compared with that of MSTN+/+ pigs.

However, all of the homozygous KO (MSTN-/-) piglets had abnormal forelegs or hindlegs and cannot stand up and walk, so that they cannot hold themselves in a good position in the competition of suckling and died within 10 days after birth. We took muscle biopsies to detect myopathic features. The results showed hypertrophy and atrophy of the muscle fibers, and irregular distributed fiber types in MSTN-/- pigs. Using electron microscopy, MSTN knockout pigs demonstrate a disordered sarcomere with swelling and pathological mitochondria which may lead to the reduced muscle fiber endurance quality. In addition, the deletion of myostatin functional region in MSTN-/- pigs, down-regulates the genes important in tendon development such as tenomodulin ($P<0.05$), tenascin ($P<0.05$), and type I Collagen ($P<0.05$), which partly lead to abnormal legs in MSTN-/- pigs that die within a few days of birth. This research was the first report about MSTN knockout pigs. These results may explain why this mutation is not found in pigs but is somewhat common in other species such as cattle.

Chang and Fang (pers. comm.) generated another gene modification in pig with the human follistatin gene that can antagonise the function of myostatin (Amthor et al. (2004)), which promoted muscle hypertrophy and decreased adipose accumulation without such negative effects.

Another priority is the development of transgenic porcine models aimed to improve disease resistance. Hu et al. (2012) showed that bovine transgenic milk containing recombinant human lactoferrin (rhLF) can modulate intestinal flora in the neonatal pig as an animal model for the human infant. Li and others (L Li. pers. comm.) generated transgenic pigs which were resistant to porcine reproductive and respiratory syndrome virus (PRRSV) infection based on RNAi technology. While Lu and others have generated LF-HLZ BAC gene modified pigs which secrete porcine milk containing human lysozyme, to improve the diarrhea-resistant ability of piglets (D. Lu. Pers comm.). Twenty-nine cloned female pigs were born, the concentration of recombinant human lysozyme was up to 2.5g/L. Another transgenic model produced by this latter team is TPBC1-HLZ BAC transgene pig. In this study, they genetically added recombinant human lysozyme (rhLZ) to sow milk and investigated whether the presence of recombinant human lysozyme can influence intestinal microbiota and morphology in sucking pigs. The average concentration of rhLZ was 116.34 ± 24.46 mg/L in the milk of F1 sows, which was 1500-fold higher than that of the native pig lysozyme, and the results of the feeding experiments demonstrated that rhLZ-enhanced milk can inhibit the growth of *E. coli* in the duodenum and positively influence intestinal morphology without adversely affecting weight gain or piglet growth. Song Z et al (unpublished) have generated transgenic pigs that overexpressed HDAC6 and examined their antiviral ability in both *in vitro* and *in vivo* challenge experiments using standard protocols. Our results demonstrated that the HDAC6 transgenic pigs exhibited enhanced resistance to PRRSV infection both *in vitro* and *in vivo*. And the transgenic pigs showed the germline transmission of the overexpression of HDAC6 proteins to the next generation.

We have also investigated ways of building disease models using pigs. Atherosclerosis is a potentially serious disease in humans. An ApoE knock-out mouse model showed atherosclerosis development quickly. Li et al. (2014) knocked out the ApoE gene in mini pigs by gene targeting to build the disease model. Laron syndrome is an autosomal recessive genetic disorder that is characterized by normal or high levels of growth hormone and low levels of insulin-like growth factor I in the circulation, resulting from defects in the GH receptor (GHR) gene. To unravel the underlying mechanism of this disease, Cui and others (D. Cui unpublished results) disrupted the GHR gene in miniature pigs using engineered zinc-finger nucleases and it could serve as a desirable human disease model compared with the existing mouse model. He and colleagues (He J. pers. comm) constructed a PKD1 single allele disruption miniature pig model by ZFN. In this research they generated PKD1TGCT ins/+ and PKD1T ins/+ pigs. These pigs had obvious vesicles in their kidneys at 5 months old, and such vesicles were increasing in number and size at 11 months of age. The next histological and physiological inspection implied that these pigs were suffering diminished renal function. The results mentioned above indicate that it could be a good research model for studying pathogenesis of autosomal dominant polycystic kidney disease (ADPKD). When Ye J. et al. (unpublished) generated c-Myc transgenic pigs, overexpression of c-Myc gene in the kidney does not really lead to polycystic kidney disease. Since eleveted c-Myc exression accelerates cell proliferation, it implies that the cysts formed in ADPKD could not only be attributed to abnormal

proliferation. So that these other models may be more useful. Meanwhile, TALEN and CRISPR-Cas9 technology have been exploited to knock out the PKD2 gene in minipig. Song Z. et al. have succeeded in building obesity mini pig models through the efficient knockout of the Leptin gene by ZFNs technology. The leptin mutant pigs display obesity, high blood glucose, high CHOL and high insulin. And the same phenotype was observed in the next generation.

Conclusion

Directly manipulating the genotype of animals establishes a powerful approach to investigate gene function in biological research and can directionally alter productive characteristics in farm animals as a means of molecular breeding. For more complex exogenous gene expression, BAC and YAC are increasingly used to carry genes of interest in pigs and cattle, and moreover, site-specific insertion of such large constructions are required sometimes. With gene editing technology such as newly developing tools of ZFN, TALEN and CRISPR/Cas9, we can even modify the epigenome of a locus of interest to alter regulatory pathways, which lead eventually to dramatic changes of the phenotype of animal. What we can expect to see in the near future is genome editing combining with many other modern genetic and reproductive tools to bring us to a new era of farm animal breeding and medicine.

The authors would like to acknowledge the help of Graham Plastow in preparing the manuscript.

References

Amthor, H., Nicholas, G., McKinnell, I., et al. (2004) *Dev Biol* 270, 19-30.
Bassett, A.R., Tibbit, C., Ponting, C.P., and Liu, J.L. (2013) *Cell Rep* 4, 220-228.
Carlson, D.F., Tan, W., Lillico, S.G., et al. (2012) *Proc Natl Acad Sci USA* 109, 17382-17387.
Chan, A.W., Chong, K.Y., Martinovich, C., et al. (2001) *Science* 291, 309-312.
Chan, A.W., Homan, E.J., Ballou, et al. (1998) *Proc Natl Acad Sci USA* 95, 14028-14033.
Chen, C., Fenk, L.A., and de Bono, M. (2013) *Nucleic Acids Res* 41, e193.
Dai, Y., Vaught, T.D., Boone, J., *et al.* (2002) *Nat Biotechnol* 20, 251-255.
Daimon, T., Kiuchi, T., and Takasu, Y. (2014) *Dev Growth Differ* 56, 14-25.
Gordon, J.W., Scangos, G.A., Plotkin, D.J., et al. (1980) *Proc Natl Acad Sci USA* 77, 7380-7384.
Hai, T., Teng, F., Guo, R., et al. (2014) *Cell Res* 24, 372-375.
Hauschild, J., Petersen, B., Santiago, Y., et al. (2011) *Proc Natl Acad Sci USA* 108, 12013-12017.
Hofmann, A., Kessler, B., Ewerling, S., et al. (2003) *EMBO Rep* 4, 1054-1060.
Hou, Z., Zhang, Y., Propson, et al. (2013). *Proc Natl Acad Sci USA* 110, 15644-15649.
Hu, W., Zhao, J., Wang, J., et al. (2012) *Biochem Cell Biol* 90, 485-496.
Jaenisch, R. (1976) *Proc Natl Acad Sci USA* 73, 1260-1264.
Li, X., Yang, Y., Bu, L., et al. (2014) *Cell Res.in press*
Lillico, S., Vasey, D., King, T., and Whitelaw, B. (2011) *Transgenic Res* 20, 441-442.
Liu, D., Wang, Z., Xiao, A., et al. (2014) *J Genet Genomics* 41, 43-46.
Liu, X., Wang, Y., Guo, W., et al. (2013) *Nat Commun* 4, 2565.
Ma, Y., Shen, B., Zhang, X., et al. (2014) *PLoS One* 9, e89413.
McCreath, K.J., Howcroft, J., Campbell, K.H., et al. (2000) *Nature* 405, 1066-1069.
Nakayama, T., Fish, M.B., Fisher, M., et al. (2013) *Genesis* 51, 835-843.
Niu, Y., Shen, B., Cui, Y., *et al.* (2014) *Cell* 156, 836-843.
Platt, J.L., and Lin, S.S. (1998) *Ann N Y Acad Sci* 862, 5-18.
Richt, J.A., Kasinathan, P., Hamir, A.N., et al. (2007) *Nat Biotechnol* 25, 132-138.
Tan, W., Carlson, D.F., Lancto, C.A., et al. (2013a) *Proc Natl Acad Sci USA* 110, 16526-16531.
Tan, W., Carlson, D.F., Lancto, C.A., et al. (2013b) *Proc Natl Acad Sci USA* 110, 16526-16531.
Thomas, K.R., and Capecchi, M.R. (1987) *Cell* 51, 503-512.
Vodicka, P., Smetana, K.J., Dvorankova, B., et al. (2005) *Ann N Y Acad Sci* 1049, 161-171.
Wall, R.J. (1996) *Theriogenology* 45, 57-68.
Watanabe, M., Nakano, K., Matsunari, H., et al. (2013) *PLoS One* 8, e76478.
Xin, J., Yang, H., Fan, N., et al. (2013) *PLoS One* 8, e84250.
Yang, D., Yang, H., Li, W., et al. (2011) *Cell Res* 21, 979-982.
Yu, S., Luo, J., Song, Z., et al. (2011) *Cell Res* 21, 1638-1640.
Zhao, Y., Dai, Z., Liang, Y., et al. (2014) *Sci Rep* 4, 3943.
Zhou, J., Wang, J., Shen, B., et al. (2014) *FEBS J. in press*