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REVIEW

Gene engineering in swine for agriculture

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Abstract

Domestic pigs are the second most important source of meat world-wide, and the genetic improvement of economic traits, such as meat production, growth, and disease resistance, is a critical point for efficient production in pigs. Through conventional breeding and selection programs in pigs, which are painstakingly slow processes, some economic traits, such as growth and backfat, have been greatly improved over the past several decades. However, the improvement of many polygenetic traits is still very slow and challenging to be improved by conventional breeding strategies. The development of reproductive knowledge and a variety of techniques, including foreign gene transfer strategies, somatic cell nuclear transfer (SCNT) and particularly, recently developed nuclease-mediated genome editing tools, has provided efficient ways to produce genetically modified (GM) pigs for the dramatic improvement of economic traits. In this review, we briefly discuss the progress of genomic markers used in pig breeding program, trace the history of genetic engineering, mainly focusing on the progress of recently developed genome editing tools, and summarize the GM pigs which have been generated to aim at the agricultural purposes. We also discuss the specific challenges facing application of gene engineering in pig breeding, and future prospects.

Keywords: gene engineering, genome editing, pig, agricultural application

1. Introduction

Humans have a long history of investigating the genetic makeup of beneficial traits to optimize pig production to meet increasing global demands for high-quality pork, thereby contributing to human consumption habits and food security. In conventional selection and crossbreeding systems, to obtain genetic improvements in the pure lines that contribute to market production, multiple nucleus populations must be built and maintained with extensive selection, including phenotype recording, genetic evaluation, selection of parents, etc. The processes are painstakingly slow, however, through this strategy of genetic improvement, some economic traits, such as growth rate and backfat, have been improved rapidly (Chen *et al*. 2002).

Since the 1980s, genetic markers have been developed and applied in livestock improvement programs, which have shown great potential for overcoming the above limitations during selection. The earliest and most successful story is the Halothane gene genetic test in selection for meat quality (Fujii *et al*. 1991). Since then, and until the end of the last century, many scientists have attempted to

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identify genetic markers from microsatellites to single nucleotide polymorphisms (SNPs) that are associated with economically important traits *via* candidate gene approaches and quantitative trait loci (QTL) mapping. Some well-known economically important genes, including *ESR*, *RN*, *MC4R*, etc., have been identified (Van Eenennaam *et al*. 2014). The marker-assisted-selection (MAS) approach significantly improves the accuracy of breeding value estimations for monogenic traits. However, this is not the case for quantitative or polygenic traits with low heritability, such as traits of reproductive and meat quality.

At the beginning of this century, a dense set of genetic markers that are evenly spread throughout the genome was predicted to be able to overcome many limitations that were previously identified in traditional strategies and evaluate the genetic merit of individuals (Meuwissen *et al*. 2001). The 60K SNP panel for pigs was released in 2009, which allows for the genetic merit evaluation and selection in candidate breeding animals with more accuracy through genome-wide association analysis (GWAS), especially for polygenic traits (Ramos *et al*. 2009). By GWAS, the genomic markers controlling genetic variation in economically important pig phenotypes, including causative genes and QTLs, have been successfully identified (Ernst and Steibel 2013). However, regarding to the genome selection for pig breeding, the high cost of DNA isolation, genotyping and phenotypic data collection greatly limits its application. To reduce the cost without affecting the selection accuracy, trait-line-specific low-density panels were developed to genotype on dams and have been combined with high-density panels to genotype breeding males (Hickey *et al*. 2011, 2012). This strategy has been reported to be effective and has been applied in some large-scale pig breeding companies to select for specific traits (Van Eenennaam *et al*. 2014). In addition, N-ethyl-N-nitrosourea (ENU)-mediated artificial random mutagenesis in pigs has been reported very recently, which provided powerful tool to efficiently generate the reservoir of mutants at the genome levels and screen the mutants with desired alleles for agricultural and biomedical research (Hai *et al*. 2017). We summarized the timeline for the historical use of DNA markers in pig breeding programs, as well as the ENU-mediated mutagenesis at the genome levels in pigs (Fig. 1-A). No doubt, the conventional or 'artificial' selection program in pig is largely uncontroversial, however, due to the limitations we described above, the innovations in breeding strategies are expected to improve the pig production efficiently.

2. Techniques for genetic engineering

Over the past three decades, with the increasing ability to read and interpret pig genomes and with the development of modern biotechnologies, especially recently developed and optimized genome editing tools, desirable alleles can

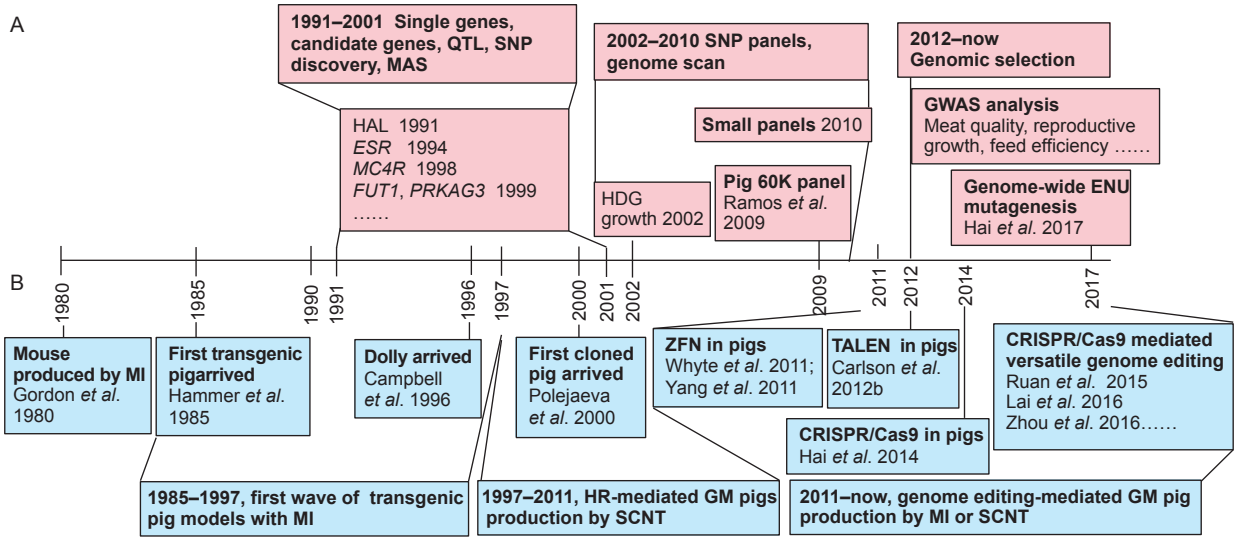

Fig. 1 A, timeline for progress of genomic markers used in conventional pig breeding programs. B, specific milestones of genetic engineering, genome editing tools and the generated genetically modified pigs over the past 35 years. QTL, quantitative trait loci; SNP, single nucleotide polymorphisms; MAS, marker assisted selection; HAL, halothane; ESR, estrogen receptor; MC4R, melanocortin 4 receptor; FUT1, fucosyltransferase 1; PRKAG3, protein kinase AMP-activated non-catalytic subunit gamma 3; GWA, genome-wide association study; ENU, N-ethyl-N-nitrosourea; MI, pronuclear microinjection; HR, homologous recombination; GM, genetically modification; SCNT, somatic cell nuclear transfer; ZFN, zinc finger nuclease; TALEN, transcription activator-like effector nuclease; CRISPR/Cas9, clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated (Cas) protein 9 system.

be almost instantly introgressed into the host genome, which offers the precedent potential to accelerate pig breeding for agricultural applications. Fig. 1-B highlighted the specific milestones of genetic engineering, genome editing tools and the genome-modified pigs over the past 35 years.

2.1. Random integration of foreign DNA

In 1980, the generation of transgenic mice *via* the direct injection of exogenous DNA into single-cell embryos was reported (Gordon *et al*. 1980), and this technique was quickly applied to large animals. For many years, before other alternative strategies, pronuclear microinjection (MI) was the only method for creating transgenic large animals (Hammer *et al*. 1985). The main limitation of MI strategies is the random integration and variable expression of the transgene, as well as a considerable proportion of mosaicism. Additionally, this procedure has low efficiency (1–4% transgenic offspring) and is extremely time-consuming and costly. Few transgenic pigs were generated with MI that aimed at improving pig growth rates (Table 1). Except for MI, several strategies have been developed successfully for foreign DNA transfer in pigs, including sperm-mediated gene transfer (SMGT) (Lavitrano *et al*. 1997; Chang *et al*. 2002), virus-mediated gene transfer (Cabot *et al*. 2001; Hofmann *et al*. 2003), intracytoplasmic sperm injection (ICSI)-mediated transgenesis (Pereyra-Bonnet *et al*. 2008).

2.2. Cell-mediated transgene

The remarkable step-change in mouse gene targeting was the discovery of murine embryonic stem cells (ESCs). The characteristics of these pluripotent cells, such as indefinite growth *in vitro*, high homologous recombination (HR) efficiencies, and the ability to differentiate into all types of cells, provided a powerful cell-mediated transgenesis strategy for generating gene-modified mice (Capecchi 2005). However, this system is still not available for genetic modifications in pigs because no characterized porcine ESCs have so far been isolated. The landmark establishment of cell-mediated transgenesis methods in large animals was the arrival in 1996 of the cloned sheep Dolly, which was the first mammal produced by somatic cell nuclear transfer (SCNT) (Campbell *et al*. 1996). Cloned piglets from a cultured adult somatic cell population using a nuclear transfer procedure were generated in 2000 (Polejaeva *et al*. 2000). Compared with MI, SCNT possesses many beneficial advantages, such as fewer animals required and the fact that a wide range of gene modifications can be applied; thus, SCNT quickly became the preferred method for producing gene-modified pigs. However, the SCNT-mediated gene engineering in

Table 1 GM pigs created for potential agricultural applications

Trait/Goal	Modification target	Technology[1)]	References
Growth	*GH*	Microinjection	Hammer *et al*. (1985); Pursel *et al*. (1990); Pursel and Rexroad (1993)
	GRF	Microinjection	Pursel *et al*. (1990)
	IGF-1	Microinjection	Pursel *et al*. (1990)
	GHR	TALEN and handmade cloning	Li *et al*. (2014)
Meat fatty acids composition	*Fat-1*	SCNT	Lai *et al*. (2006); Pan *et al*. (2010)
	FAD2	Microinjection	Saeki *et al*. (2004)
Meat production	*MSTN*	ZFN and SCNT	Qian *et al*. (2015)
	MSTN	CRISPR/Cas9 and SCNT	Wang K *et al*. (2015); Bi *et al*. (2016)
	MSTN	CRISPR/Cas9 and zygotes injection	Tanihara *et al*. (2016)
Disease resistance	*mIgA*	Microinjection	Lo *et al*. (1991); Weidle *et al*. (1991)
	RELA	TALEN or ZFN and zygotes injection	Lillico *et al*. (2013)
	SIGLEC1	HR and SCNT	Prather *et al*. (2013b)
	CD163	CRISPR/Cas9 and SCNT	Whitworth *et al*. (2014); Wells *et al*. (2017)
	PBD-2	SCNT	Yang *et al*. (2015)
	CD1d	CRISPR/Cas9 and SCNT	Whitworth *et al*. (2014)
	Mx1	Microinjection	Muller *et al*. (1992)
	Mx1	SCNT	Yan *et al*. (2014)
	FMDV shRNA	SCNT	Hu *et al*. (2015)
	HDAC6	SCNT	Lu *et al*. (2017)
Reproduction	*FSHα/β*	SCNT	Xu *et al*. (2016); Jiang *et al*. (2017)
Lactation performance	*rHLA*	SCNT	Ma *et al*. (2016)
Environmental friendly	*Phytase*	Microinjection	Golovan *et al*. (2001)

[1)] TALEN, transcription activator-like effector nuclease; SCNT, somatic cell nuclear transfer; ZFN, zinc finger nuclease; CRISPR/Cas9, clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated (Cas) protein 9 system; HR, homologous recombination.

pigs had been impeded since the low cloning efficiency and difficulties of establishing cell lines with the desired genetic modification due to a lack of available germ line-competent pluripotent stem cells (Brevini *et al*. 2007; Keefer *et al*. 2007). Accumulating evidence suggests that defective epigenetic reprogramming of DNA and histones are likely associated with the low overall success rate related to cloning (Dean *et al*. 2001; Kang *et al*. 2001; Santos *et al*. 2003).

Before the emergence of effective genome editing tools, DNA HR, which was followed by SCNT, was the primary tool for generating knockout pigs. However, only a few successful examples have been created by this strategy due to the extremely low frequency of HR in somatic cells (less than 10^{-6}) and only one allele can be targeted during one transfection. The low efficiency of gene targeting in cultured somatic cells became the bottleneck of GM large animal generation. Therefore, it is understandable why this inefficient gene-targeting strategy was abandoned once powerful genome editing tools emerged.

2.3. Nuclease-mediated genome editing

The emergence of advanced meganucleases-mediated gene-editing tools introduces a new era for gene targeting, especially in large animals, and provides the powerful approaches to improve the efficiency of GM animal production. There are many meganucleases, including designed endonucleases and engineered meganucleases, have been reported to be used for gene editing. Petersen and Niemann (2015) reviewed the each meganuclease and their application in farm animals. Here, we will briefly discuss three most commonly used meganucleases, including zinc finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN) and the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated (Cas) protein 9 system. Compared with HR-mediated gene targeting, these nucleases are used to generate a double-strand break (DSB) at a desired genomic locus and enable site-directed genome engineering, while also possessing many advantages, including having a high efficiency and being inexpensive, easy and user-friendly. Moreover, creating bi-allelic knockout pigs by HR is a time-consuming process that requires 3–5 years of serial cloning and breeding. By contrast, homozygous knockout animals can be achieved in one step by ZFNs (Geurts *et al*. 2009; Whyte *et al*. 2011; Yu *et al*. 2011), TALENs (Song *et al*. 2013; Huang *et al*. 2014; Liu *et al*. 2014) and CRISPR/Cas9s (Hai *et al*. 2014; Wang *et al*. 2015, 2016), which remarkably reduces the time required to generate homozygous mutant offspring in pigs. Most importantly, all three nuclease-mediated gene editing tools have been reported to be somatic cell reprogramming-free strategies, which avoid prenatal or postnatal death caused by incomplete epigenetic reprogramming (Yao *et al*. 2016).

The nuclease-mediated gene-targeting protein, ZFNs (Kim *et al*. 1996) and TALENs (Christian *et al*. 2010), are modular proteins containing two domains: a DNA recognition domain and a *Fok*I nuclease domain. In ZFNs, each individual zinc finger binds to three DNA base pairs, while in TALENs, each repeat binds a single base. This characteristic makes any DNA sequence a theoretical TALEN target. ZFNs have greatly improved gene-targeting efficiencies (from 10^{-6} to 10%), but the drawbacks associated with off-target cleavage, cytotoxicity, and limited target sites have narrowed their applications (Cornu *et al*. 2008). TALENs exhibit comparable advantages, including an even higher efficiency, lower cytotoxicity, and relative ease of generating homozygote mutants. Although many ZFN-mediated genetically modified pigs have been generated since the first example of the use of this strategy was reported in 2011 (Whyte *et al*. 2011; Yang *et al*. 2011; Yao *et al*. 2016), the ZFN strategy was quickly replaced by TALEN due to the advantages described above.

A real technological breakthrough came in 2013 with the discovery of CRISPR/Cas9, which promises even greater efficiency, flexibility, simplicity and a lower cost and has sparked a new revolution in many research areas (Mussolino and Cathomen 2013). This latest tool is derived from bacterial immune systems and uses a small RNA to guide a universal monomeric nuclease (Cas9) to the specific target DNA site, where it induces a DNA DSB. The most dominantly used variant is the Cas9 protein, which is 1 368 residues long and from *Streptococcus pyogenes* (SpCas9) (Haft *et al*. 2005). Current Cas9 nucleases can use a single-guide RNA (sgRNA) to form a functional guide RNA:Cas9 complex that achieves RNA-programmed DNA cleavage and simplifies the use of the CRISPR/Cas9 system (Jinek *et al*. 2012). The guide RNA sequence is complementary to the specific target site, and if more guide RNAs are added, which can target multiple different genes, Cas9 nuclease activity can be directed to multiple different target sites in the genome. This ability of modifying all alleles of multiple genes precisely and simultaneously has been reported in mice firstly (Wang *et al*. 2013), soon after that, GM pigs with multiple gene targets were generated (Whitworth *et al*. 2014; Li *et al*. 2015; Zhou *et al*. 2016). Furthermore, CRISPR/Cas9-mediated versatile genome editing in pigs, such as swapping of the entire exon (Whitworth *et al*. 2014), site-specific knock-ins (Peng *et al*. 2015; Ruan *et al*. 2015; Lai *et al*. 2016) and single amino acid substitutions independent of SCNT (Zhou *et al*. 2016), have been recently reported. Due to the very short target recognition sequence and tolerance for mismatches, this relatively simple system might have off-target effects that can result in the introduction of unintended

mutations elsewhere in the genome. However, it has been demonstrated that off-target mutations are rare events in mice (Iyer *et al*. 2015) and human cells (Kim *et al*. 2015). Additionally, significant effort has been undertaken and a multitude of strategies have been evaluated to reduce the number of off-target effects. For example, some scientists have mutated FokI and Cas9, giving rise to nickases that can cut single-stranded rather than double-stranded DNA to create nicks rather than DSBs; thus, subsequent repair by the break excision repair pathway leaves no marks on the genome (Ren *et al*. 2014; Frock *et al*. 2015).

For the purpose of versatile genome editing and fewer off-target effects, several other natural CRISPR nucleases aside from SpCas9 have been developed and used for genome editing. The *Staphylococcus aureus* (Sa) Cas9 analog (SaCas9) is smaller (1 053 residues) than the SpCas9 and facilitates some applications (Komor *et al*. 2017). More than 10 types of Cas9 have been identified with different features (Komor *et al*. 2017). Recently, additional nucleases have been identified as being capable of RNA-guided sequence-specific DNA cleavage; for example, Cpf1 from *Acidaminococcus* sp. (AsCpf1) and *Lachnospiraceae bacterium* Cpf1 (LbCpf1) have been used for mammalian cell genome editing (Zetsche *et al*. 2015). These two enzymes are different in size, protospacer adjacent motif (PAM) requirement and location of the introduced DSB within the protospacer, in addition, the cleavage of the two DNA strands is staggered, rather than blunt-ended, as compared with SpCas9. Although these endonucleases already provide us with a variety of genome-editing options, the increasing popularity of genome editing coupled with the development of precise genome-editing techniques implies the importance of discovering new programmable DNA-binding or DNA-cleaving enzymes.

3. Genetic modified pigs for potential agricultural applications

The history of genetic engineering in pigs can be traced to the born of first transgenic pig *via* the MI method in 1985 (Hammer *et al*. 1985). Over past three decades, as shown in Fig. 2, the genetic modification in pigs progressed from the random integration of foreign DNA through a variety of techniques, to manipulation endogenous genes *via* HR in somatic cells followed by SCNT. In the last few years, the remarkable development of nuclease-mediated gene editing technologies is set to revolutionize the field, as large animal genomes can be modified efficiently and sophisticatedly. It is not surprising that many more GM pig lines have been produced in the last few years compared with the previous three decades. Much of data and publications described GM pigs for biomedical researches, including the fields of xenotransplantation, regenerative medicine, and tumor biology, etc. (Prather *et al*. 2013a; Butler *et al*. 2015; Redel and Prather 2016; Yao *et al*. 2016), these animals are beyond the scope of review. Despite the recent several reviews described the gene manipulation application in food animals for agricultural purpose (Laible *et al*. 2015; Petersen and Niemann 2015; Tan *et al*. 2016; Van Eenennaam 2017), quite few specifically focuses on pigs. Very recently, Wells and Prather (2017) reviewed the genome-editing technologies to improve research, reproduction and production in pigs. Here, we summarized the GM pigs used for potential agricultural applications. As shown in Table 1, to date, about 20-gene GM pigs have been produced which are aimed at potential agricultural applications. Clearly, *MSTN*, *CD163*, *RELA*, *GHR*, *CD1d* and *SIGLEC1* gene-modified pig were generated by editing endogenous genes rather than introducing exogenous genes. In the following section, we will introduce these GM pigs, including their generation, detailed mechanisms and relative efficiencies.

3.1. Growth

The growth hormone (GH) pathway is well-recognized for being critical for regulating linear growth, carbohydrate homeostasis, and fat metabolism. With the MI method, *GH*, *GH*-releasing factor (*GRF*) and insulin-like growth factor-I (*IGF-1*) transgenic pigs were created by successfully integrating chimeric genes, which included a regulatory sequence (either metallothionein, albumin, viral long terminal repeat (LTR), prolactin, or transferring, etc.) fused to the structured sequences encoding bovine *GH*, human *GH*, porcine *GH* or human *GRF* and human *IGF-1*, into pig genomes (Hammer *et al*. 1985; Pursel *et al*. 1990; Pursel and Rexroad 1993). Some of these *GH* transgenic pigs displayed significantly elevated plasma *GH* levels and gained weight faster; however, many adverse health effects were also observed in these pigs, including joint pathologies, gastric ulcers and infertility (Pursel and Rexroad 1993). In 1999, an Australian group reported transgenic pigs bearing a modified porcine *GH* under the promoter of human metallothionein that could be regulated by zinc feeding; these pigs showed significant improvements in growth rate, feed conversion and body fat muscle ratio without any health or reproductive side effects due to the transgenes (Nottle *et al*. 1999). However, due to the safety issues associated with the extra hormones in the meat, overexpression of *GH* in pigs for promoting growth may not be a good choice. Recently, combined with TALENs and hand-made cloning technology, growth hormone receptor (GHR) knockout pigs had been created and these $GHR^{-/-}$ pigs were 50% smaller than that of the controls at the age of 20 weeks (Li *et al*. 2014).

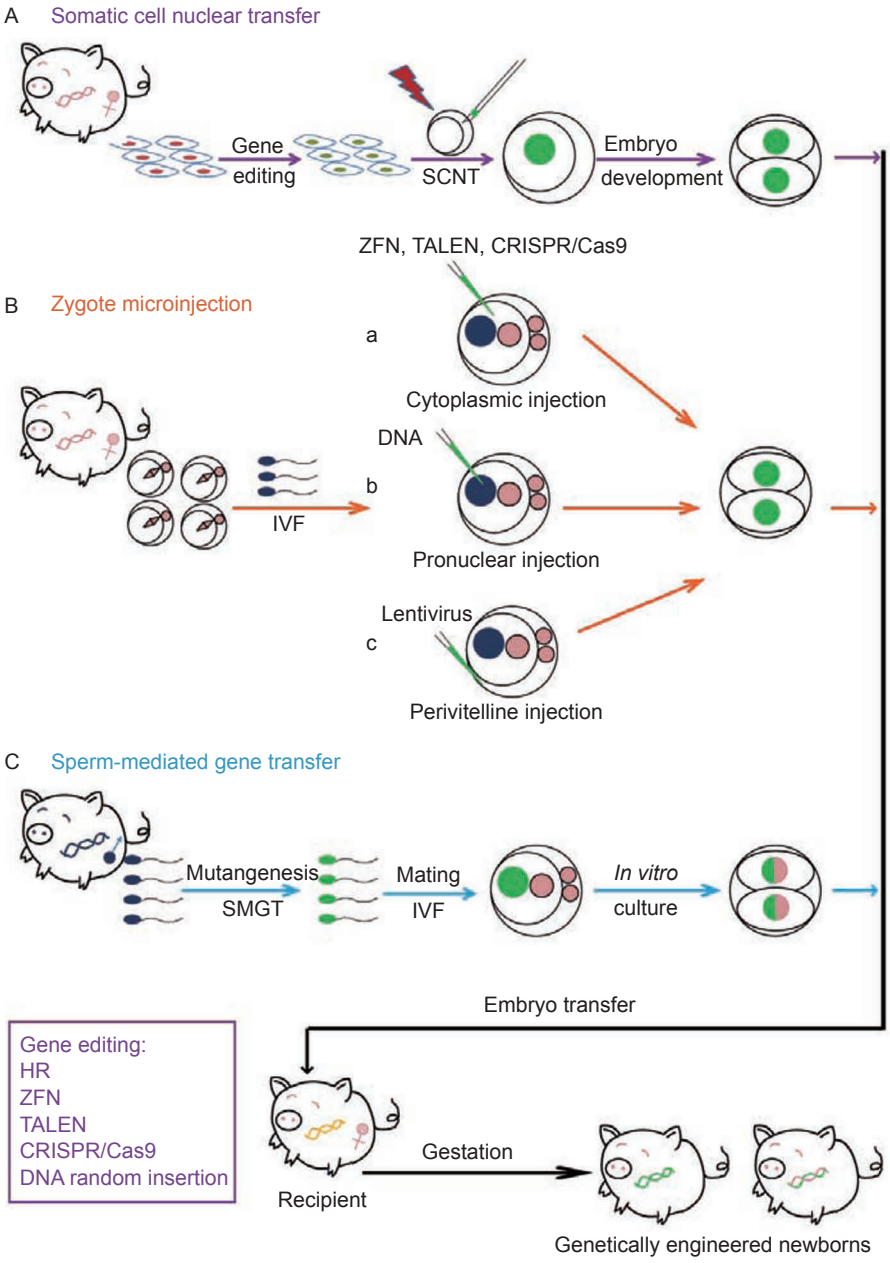

Fig. 2 Schematic diagram of dominant approaches for generation of genetically modified (GM) founders in pigs. MI or MII oocytes were obtained from flushing or slaughterhouse ovaries. GM pigs can be generated by various strategies: A, SCNT-mediated GM. Fibroblasts were obtained from day 35 pig fetuses and used for making genetic modifications. GM cells were used as donors and injected into recipient cytoplasm for SCNT. B, microinjection mediated GM. a, pronuclear microinjection of DNA fragments and mRNA or protein of endonucleases (ZFNs, TALENs and CRISPR/Cas system). b, cytoplasmic microinjection of mRNA or protein of endonucleases (ZFNs, TALENs and CRISPR/Cas9 system). c, perivitelline injection of lentiviral vectors. C, sperm-mediated GM. Sperm may be incubated with DNA before insemination or DNA injected to the testes or mutation in sperm resulted from N-ethyl-N-nitrosourea (ENU) mutagenesis. GM founders maybe produced by *in vitro* fertilization (IVF), or intracytoplasmic sperm injection (ICSI) or nature mating. SCNT, somatic cell nuclear transfer; ZFN, zinc finger nuclease; TALEN, transcription activator-like effector nuclease; CRISPR/Cas9, clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated (Cas) protein 9 system.

3.2. Meat production, composition and quality

Increasing global demands for high-quality pork have pushed scientists to continually look for ways to maximize muscle growth in pigs without genetically compromising meat product palatability. Meat production and quality are complex traits both at phenotypic and genotypic level. Despite some candidate genes, QTLs or indirect markers

associated in linkage disequilibrium with candidate genes for meat quality have been extensively studied over the past many years (Pena *et al*. 2016), the causality of some important markers still need to be proved further. Below, we will describe GM pigs created to potentially improve the meat production and quality.

The myostatin (*MSTN*) gene was a well-known ideal target for increasing muscle growth and improving meat production through genetic manipulation in livestock. *MSTN* is a dominant inhibitor of skeletal muscle development and growth, and its mutation leads to muscular hypertrophy, or a double-muscled (DM) phenotype, in cattle (Grobet *et al*. 1997; Kambadur *et al*. 1997; Marchitelli *et al*. 2003), sheep (Clop *et al*. 2006; Kijas *et al*. 2007), mice (McPherron *et al*. 1997; Lin *et al*. 2002), dogs (Mosher *et al*. 2007) and humans (Schuelke *et al*. 2004; Schafer *et al*. 2016). Although no natural *MSTN* mutations have been reported in pigs, *MSTN*-mutant Meishan pigs without any select marker genes were generated by combining ZFNs and SCNT methods. The resulting pigs showed a normal grow rate but increased muscle mass and decreased fat accumulation compared with wild-type pigs (Qian *et al*. 2015). Additionally, *MSTN*$^{-/-}$ pigs exhibited obvious intermuscular grooves and enlarged tongues, which are characteristic of the DM phenotype, at 8 months due to myofiber hyperplasia. Interestingly, 20% of the *MSTN*$^{-/-}$ pigs had one extra thoracic vertebra (Qian *et al*. 2015). At almost the same time, *MSTN*-mutant pigs were also successfully generated by a combination of CRISPR/Cas9 and SCNT methods and these pigs exhibited the DM phenotype as expected (Wang K *et al*. 2015). In 2016, Bi *et al*. (2016) reported a selectable marker-free MSTN knockout cloned pigs generated by the combined use of CRISPR/Cas9 and Cre/LoxP. Although the SCNT-mediated genome editing approach could produce GM pigs with the desirable and biallelic mutations in one step, the required complex micromanipulation techniques and the low production efficiencies of the viable cloned offspring due to a high incidence of developmental abnormalities increased the risks of both prenatal and postnatal death by faulty epigenetic reprogramming of donor somatic cell nuclei. In 2016, a Japanese group generated *MSTN* gene knockout pigs by injecting Cas9 protein and a single-guide RNA into *in vitro* fertilized zygotes, and the biallelic mutant piglet showed increased muscle mass (Tanihara *et al*. 2016). These *MSTN*$^{-/-}$ pigs demonstrate the possibility of quick genetic improvements for lean meat from fat-type indigenous pig breeds.

These transgenic techniques also opened up the possibilities for modifying the fatty acid composition of pork to make it healthier. Linoleic acid (18:2n-6) and alpha-linolenic acid (18:3n-3) are polyunsaturated fatty acids that are essential for mammalian nutrition, as mammals lack the desaturases required for the synthesis of Δ12 (n-6) and n-3 fatty acids. Pigs that carry the fatty acid desaturation 2 gene for a Δ12 fatty acid desaturase from spinach were generated and showed increased linoleic acid (18:2n-6) content in white adipose tissues compared with wild-type pigs (Saeki *et al*. 2004). A high ratio of omega-6 to omega-3 fatty acids has been reported to be a risk factor for many life-threatening diseases, such as cardiovascular diseases, diabetes and cancer. Two omega-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are increasingly being recognized as important modulators for health and are recommended as human dietary supplements. Marine products are a major source of omega-3 fatty acids for both food and animal feeds. In 2006, n-3 fatty acid desaturase gene (*fat-1*) transgenic pigs were generated *via* SCNT, which produced high levels of n-3 fatty acids from n-6 analogs. The tissues of these pigs have a significantly reduced ratio of n-6:n-3 fatty acids compared with wild-type pigs (Lai *et al*. 2006; Pan *et al*. 2010). These transgenic pigs provide a land-based source of omega-3 fatty acids and have a valuable impact not only on public health but also on the sustainable development of marine resources.

3.3. Disease resistance

Another major area of interest is the improvement of resistance/tolerance to pathogens by genome editing with the aim of increasing pig robustness, decreasing the use of antibiotics and drugs and further enhancing the health benefits of pork as a human food. Over the past several decades, traditional genetic selection for disease resistance was not as successful as the selection for other economic traits, such as growth, carcass leanness, meat production, etc. The main reason for this is that disease resistance is a complex and polygenic trait, and vaccination strategies and the use of antibiotics slowed the progress of disease resistance selection programming to a certain extent. However, with continually progressing knowledge regarding gene function, as well as the fast development of genome editing tools, the objective of selecting for disease resistance is once again a focus of scientists, and several disease-resistant GM pigs have been successfully generated.

As early as 1991, light and heavy chain of mouse monoclonal antibodies (mAbs) had been introduced into pigs by MI with titers of up to 1 000 μg mAb mL^{-1} being detected in the sera of the transgenic pigs (Lo *et al*. 1991; Weidle *et al*. 1991). The elevated serum antibodies could help to improve the immunity of these pigs and protect them against some diseases.

As the most economically important disease plaguing swine world-wide, porcine reproductive and respiratory syndrome (PRRS) is a particular concern to the swine

industry. Vaccines have been developed against this virus, but the industry has been unable to control the disease due to the genetic diversity of the virus. In 2010, virologists proposed the PRRSV infection model, in which SIGLEC1 (*CD169*) was identified as a necessary surface receptor for PRRSV entry into porcine cells. The virus was then uncoated by *CD163* in the endosome and the viral genome was released into the cytoplasm (Van Breedam *et al*. 2010). Based on the hypothesis that a host with a defective receptor would be immune to PRRSV, geneticists moved towards preventing PRRSV infection from the host side. SIGLEC1 knockout pigs were generated *via* a HR and SCNT strategy. However, when these knockout pigs were infected with PRRSV, no differences were observed in viral replication compared with wild-type pigs (Prather *et al*. 2013b). Next, geneticists turned their attentions to the *CD163* gene, and with the aid of the CRISPR/Cas9 system, *CD163*-null pigs were quickly generated (Whitworth *et al*. 2014). Compared with the obvious clinical signs consistent with PRRS in wild-type piglets, no viremia or clinical signs were observed in the *CD163* knockout pigs even though they were infected and exposed to infected pen mates (Whitworth *et al*. 2016), which suggested that *CD163* is required for PRRSV infection. Of note, these pigs possess a complete knockout of *CD163*, which is a member of the scavenger receptor cysteine-rich (*SRCR*) superfamily and consists of 10 *SRCR* domains. It has been demonstrated that domain 5 from exon 7 is responsible for virus binding. To explore the function of domain 5 in PRRSV infection, genetically modified pigs with a normal SRCR domain 5 or the SRCR domain 5 replaced with a synthesized exon encoding a homolog of the *hCD163L1* SRCR domain 8 (domain swap) were generated by CRISPR/Cas9 technology. Infection studies with different PRRSVs revealed that CD163 is likely to be the receptor for all PRRS viruses and that domain 5 knockout pigs are resistant to these viruses (Wells *et al*. 2016). Refining the modification of *CD163* provides the opportunity to breed pigs that are resistant to PRRS while retaining the important biological functions associated with *CD163*. Very recently, *CD163*-null pigs were shown to have no resistance following infection with the African swine fever virus (ASFV) isolate, Georgia 2007/1, which rules out a significant role for *CD163* in ASFV infection (Popescu *et al*. 2017). In addition, except for *CD163*, histone deacetylase 6 (*HDAC6*) gene has been demonstrated to be critical for PRRS infection. *HDAC6* transgenic pigs were produced by SCNT and they exhibited enhanced resistance to PRRS infection (Lu *et al*. 2017) .

Foot-and-mouth disease virus (FMDV) is also an economically devastating viral disease facing the swine industry world-wide. This disease is highly contagious and spreads very quickly and easily. Vaccination programs aim to prevent the spread of this disease; however, once a breakout occurs, it may be too late for vaccines to stop it spreading. The generation of genetically engineered pigs that are resistant to infection will provide a novel alternative strategy for preventing outbreaks and spending on FMDV disease. Transgenic (TG) pigs that constitutively express FMDV-specific short interfering RNAs (siRNAs) derived from small hairpin RNAs (shRNAs) were generated and challenged by intramuscular injection with virus. These TG pigs displayed no clinical signs of viral infection while the wild-type control pigs exhibited high fever, severe clinical signs of foot-and-mouth disease and the typical histopathological changes (Hu *et al*. 2015)

African swine fever virus (ASFV) is a highly infectious disease affecting both domestic and wild pigs. ASFV-infected pigs exhibit an acute, rapidly fatal hemorrhagic fever, and ASFV infection is considered one of the most infectious animal pathogens. ASFV infections in domestic pig populations have serious socioeconomic impacts world-wide, especially in Africa. Functional studies have revealed that the polymorphic sequence variation S531P in RELA proto-oncogene, NF-kB subunit (RELA), which is a major component of the NF-κB transcription factor, promotes the majority of the distinct host response to ASFV in warthogs and domestic pigs (Palgrave *et al*. 2011). Subsequently, TALENs or ZFNs, designed for the porcine RELA gene, were delivered to the pig zygote and resulted in the birth of bi-allelic edited animals (4% of those born) (Lillico *et al*. 2013). Generation of RELA-modified pigs paves the way for the development of pig lines with tolerance against ASFV.

Furthermore, beta-defensin 2 (*PBD-2*) transgenic pigs which were produced by SCNT have showed the enhanced resistance to *Actinobacillus pleuropneumoniae* infection (Yang *et al*. 2015). Myxovirus resistance gene (*Mx1*) has a broad spectrum of antiviral activities, *Mx1* transgenic pigs were produced by MI method (Muller *et al*. 1992) or SCNT (Yan *et al*. 2014). Cells from the transgenic pigs showed a profound decrease of influenza A proliferation and resistant to classical swine fever virus (CSFV) (Yan *et al*. 2014).

Taken together, the generation of GM pigs that are resistant to specific pathogens will allow for new breeding approaches that maximize disease resistance, and provides the potential solution to the inefficiencies of current breeding systems.

3.4. Lactation performance

Lactation performance of sows is very important for piglet survival and growth. It has been demonstrated that human α-lactalbumin (HLA) plays the critical role in maintaining the neonatal nutritional and physiological functions. The two lines of transgenic pigs that over-expressed recombinant

HLA were produced *via* SCNT. The contents of r*HLA* in milk from the two lines of transgenic cloned sows were around 2.5 mg mL^{-1}. Furthermore, better performance in body weight gain and intestine growth was observed in the piglets nursed by recombinant *HLA* transgenic sows, compared to the control piglets reared by non-transgenic sows. This pig model provides an alternative way for pig breeders to improve lactation performance (Ma *et al*. 2016).

3.5. Reproduction

Follicle-stimulating hormone (FSH) is a critical hormone regulating reproduction in mammals. *FSHα/β* transgenic pigs were generated by SCNT and the effect of *FSHα/β* on reproduction was evaluated. Data showed that overexpression of *FSH* could improve spermatogenesis ability of Large White boars (Xu *et al*. 2016), while a decreased fecundity was observed in transgenic Large White female pigs (Jiang *et al*. 2017). These findings will provide insight into the possibility of improving reproduction traits in pigs by genetic engineering strategies.

3.6. Environmentally friendly

Gene engineering also provides another solution for the sustainable development of agricultural environments. Phytate phosphorus, the most abundant source of phosphorus in the pig diet, can not be digested by pigs due to a lack of phytase; thus, it has been passed into manure and led to environmental pollution. Phytase transgenic pigs were created to solve the pollution problem caused by the manure and to mitigate the adverse environmental effects from intensive farming systems (Golovan *et al*. 2001). The saliva of these pigs contains the phytase that digests the dietary phytate phosphorus, relieves the requirement for inorganic phosphate supplements, and reduces fecal phosphorus output by up to 75%. This study demonstrated that gene modification technology could offer sufficient scope to prevent or reduce the environmental pollution from intensive pig farming activities.

4. Challenges and perspectives

As described above, GM pigs can be produced through various technologies with the reduced cost and a shorter time frame, which holds much promise to accelerate genetic improvements in pigs. Especially, the quantitative traits that are determined by multi loci could also be modified by genome editing. However, challenges present here are that to what extent the gene engineering will be applied in pig breeding and how it intersects with conventional breeding. It has been reported that combining conventional breeding program with gene engineering strategy would improve the response to selection (Jenko *et al*. 2015). It is likely that gene engineering will complement, rather than replace or disrupt the conventional breeding program completely.

Another challenge we are facing is that few agriculturally useful genes have been identified, and discovering causative variants that significantly impact important agricultural traits is a much more difficult obstacle to overcome. However, with the increasing ability to sequence, read and interpret pig genomes, especially with the release of swine genome sequence (Groenen *et al*. 2012), it is expected that, more genomic markers controlling genetic variation in economically important pig phenotypes will be revealed. On the other hand, the development of genome editing technology will facilitate the identification of causative genes in pigs by establishing the pig mutants with precision genotypes. No doubt, the substantial progress in genetic modification will be made in coming years. Despite these biotechnologies are more frequent applicability for scientific aims so far, we hope that the application of these innovative technologies in agriculture will benefit of animals, mankind and the environment.

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