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Review

Recent advances in genome editing and creation of genetically modified pigs

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HIGHLIGHTS

- Relying on homologous recombination, the creation of gene-targeted pigs was originally slow and tedious.
- 2000, somatic cell nuclear transfer (SCNT) was successfully used to clone pigs.
- 2013, ZFN were used to create a biallelic knockout of both the GGTA1 and CMAH genes in vitro prior to SCNT.
- 2013, clustered randomly interspaced short palindromic repeats and the associated protein 9 (CRISPR/Cas9) were introduced.
- 2015 CRISPR/Cas9 system has been used to simultaneously silence 3 genes.

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ABSTRACT

The field of xenotransplantation is benefiting greatly from recent advances in genetic engineering. The efficiency and pace with which new model animals are being created has dramatically sped progress towards clinical relevance. Endonuclease-driven genome editing now allows for the efficient generation of targeted genetic alterations. Herein we review the available methods of genetic engineering that have been successfully employed to create genetically modified pigs.

Contents

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1. Introduction

Gene-knockout pigs hold great promise for creating donor animals with a negative crossmatch for clinical xenotransplantation. Capecchi, Smithies, and Evans developed methods for creating targeted alterations in the genomes of mice more than 20 years ago [1]. The creation of gene-targeted pigs has been slow and tedious because of the lack of identifiable embryonic stem cells (ES) in the pig, the reliance upon homologous recombination to create targeted gene deletions, and the need for lengthy breeding programs to create homozygous knockout pigs. Despite these limitations, the field has overcome many barriers in the past decade.

In 2002 the first GTKO pigs were created using homologous recombination to delete a single copy of GGTA1, followed by backbreeding to homozygosis, a process that took three years to complete. In 2010 Zinc Finger Nucleases (ZFN) were introduced, making it possible to create homozygous knockout cells in a single reaction so that true knockout pigs could be created in 5 months instead of 36 months. In 2012 ZFN were used to create a biallelic knockout of two xenoantigens simultaneously [2]. In 2013, the CRISPR/Cas9 system was introduced and accelerated the creation of multiple concurrent homozygous gene deletions. Recently CRISPR/Cas9 system has been used to simultaneously silence 3 genes; the majority of people now exhibit very low levels of antibody binding to cells from this triple knockout pig [3]. The process of creating genetically modified pigs has progressed rapidly over the past decade.

2. Random integration

Initially, due to the absence of pig ES, creating a true genetic knockout pig was not feasible. In this era, attention focused on transgenic methods to regulate αGal expression. The process of 'competitive glycosylation' involved inserting a gene for a fucosyltransferase or sialyltransferase [4]; by depleting the common acceptor substrate N-acetyllactosamine, αGal was competitively replaced with less immunoactive oligosaccharides. At the genomic level, one such method placed a human cDNA fucosyltransferase -under CMV and H2K-b promoters-into the pig genome by embryo microinjection [5]. Despite variable and mosaic expression patterns, this method was successful in *reducing* αGal expression.

3. Somatic cell nuclear transfer

One of the early barriers to creating genetically modified pigs was the biologic tenant that cellular differentiation is unidirectional. This dogma was rewritten in 1997 when a mammary epithelial cell was used to create an embryonic cell [6]. Fused with an enucleated egg, a somatic sheep cell was redesigned to become an embryonic cell capable of producing a cloned sheep. This process of somatic cell nuclear transfer (SCNT) has now become of central importance to the field of xenotransplantation. In the absence of pig embryonic stem cells, genetically modified pigs are created directly from somatic cell modification; by 2000, SCNT was successfully used to clone pigs [7,8].

4. Homologous recombination

With new ability to create clonal animals from somatic cell nuclear donors, the field shifted focus from microinjection of randomly integrating transgenes towards site-directed mutagenesis. The first of these gene-targeting strategies was homologous recombination (Fig. 1B). In 2002, Lai et al. used a gene trap-targeting vector, pGalGT, for homologous replacement of the endogenous GGTA1 allele [7]; after antibiotic selection and SCNT, 4 single-allele knockout pigs were created. Later that year, Phelps et al. accelerated the production of a double αGal knockout pig by employing three consecutive rounds of cloning instead of backcross breeding [9]. In this process, heterozygous fetal fibroblasts were isolated from a pregnancy and subjected to a second ATG-targeting knockout vector with antibiotic selection. After enrichment for αGal − cells by *C. difficile* toxin A, the second allele was found to be serendipitously disrupted by a rare spontaneous mutation. Aside for this one spontaneous mutation, antibiotic selection markers complicated homologous recombination strategies; the obligatory insertion of antibiotic resistance genes limited clinical application of these animals.

5. Nuclease editing

Site-specific nuclease technology shifted attention away from homology-driven recombination knockout. Table 1 compares the 4 nuclease classes. Broadly speaking, these strategies involve creating a double-strand break (DSB) by targeting a bacterial nuclease to a specific genomic sequence. Nuclease-driven DSBs are subsequently repaired by the error-prone process of non-homologous end joining (NHEJ). The product often includes small deletions or insertions that yield missense or nonsense mutations and a null phenotype. (Fig. 1C–E) Alternatively, homology-directed repair (HDR) offers the opportunity to control the mutation event [10]. By co-transfecting a site-specific nuclease with oligonucleotides or donor sequences containing locus-specific homology to the DSB site, a specific insertion may be created [11]. Regardless of repair pathway, the introduction of DSBs can increase the random recombination frequency from 1 event per 10^-6 cells to 1 in 5 cells [12].

6. Zinc finger and TALEN nuclease

In 2010, Zinc Finger Nucleases (ZFN) offered the first of these site-directed approaches [13]. ZFNs are fusions of a nonspecific DNA cleavage motif with a sequence-specific zinc finger protein. (Fig. 1) A_4 The nuclease activity is a derivative of the FokI bacterial restriction endonuclease, capable of creating a single strand break. ZFNs operate in concert by dimerizing two DNA-binding domains with two FokI enzymes to produce DSBs with 18 bp specificity. The subsequent damage response pathway of NHEJ afford ZFNs their mutagenic potential. (Fig. 1C) In 2013 ZFN were used to create a biallelic knockout of both the aGal and CMAH genes in vitro prior to SCNT; this set an important precedent of using endonuclease technology to remove 2 xenoantigens from scratch within 7 months [2].

Transcription activator-like effector nucleases (TALENs) were introduced in 2011, and function like ZFNs to create DSBs by