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Genome editing in animals - past, present and future: A review

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Abstract
Genome editing is a type of genetic engineering technology in which genetic elements (DNA) is inserted, deleted, modified or replaced in many organisms like plants, bacteria and animals. This technique can lead to changes in Physical traits like eye colour, coat colour and disease risk. Genome editing has its wide range of application in animals like human disease modelling, xenotransplantation, animal welfare aspects and production. Based on many literatures, different technologies of genome editing and it seems that CRISPR/Cas technologies are probably superseding ZFNs and TALENS. It aims in producing a better and healthier future generation of humans and animals. However, the CRIPSR/Cas methods are also being improvised, and newer additions have further enhanced its functional capabilities. In Genetic engineering field, better gene modification technologies are evolving and can one day replace even CRISPR/Cas, possibly shifting to synthetic genomics. At a same time, the unethical use of genome editing should be regularly monitored with proper rules and regulations.

Keywords: Genome editing, CRIPSR/Cas, ZFNs and TALENS

Introduction
Genome editing is a type of genetic engineering in which precise modifications like insertion, deletion or replacement of specific DNA sequence is achieved mainly using engineered nucleases or molecular scissors. In the present scenario, four different engineered nucleases are being utilised by the scientists for genome editing. The three basic steps in the process include the entry of genetic tool or scissors into the nucleus passing the cytoplasm, altering gene transcription and onward processing function and finally, the end output in the shape of a altered protein production. (Arai and Kagawa, 2014) [1].

History of genome editing
The discovery of restriction enzymes that normally protect bacteria against phages (Kelly *et al*., 1970) [12] was a turning point in the era of recombinant DNA technology. The ability to manipulate DNA in test tubes has revolutionized the field. Even though such efforts drove a number of discoveries genetics, the ability to precisely edit DNA in living eukaryotic cells came a few decades later.
An initial targeted gene disruption study in eukaryotic yeast cells was followed with breakthrough in mammalian cells (Smithies *et al*., 1984) [28]. Homologous recombination is the mechanism by which the cells incorporate exogenous DNA fragment to native DNA. This finding gave unprecedented power to characterize various genes in model organisms. However, the feasibility of this approach had several limitations. First and foremost is the low rate of spontaneous integration of an exogenous DNA copies (1 in 10^3–10^9 cells). Secondly, the influence of type of cell on rate of integration and finally the chance of faulty site incorporation of exogenous DNA at a higher frequency than the desired target site.
With constant efforts thereafter, researches realized that double strand breaks at target sites in the DNA can stimulate repair mechanisms that could increase the magnitude of targeted gene introgression (Rudin *et al*., 1989) [27]. To make this happen, researchers utilized rare cutting endonuclease enzymes, such as the 18-bp cutter I-SceI, to introduce specific DSBs in the mouse genome. Even then there were hurdles in the process. As a solution, alterations in the natural megaucleases were carried out to improve specificity of targeted editing. But only a very small fraction of genomes could be specifically targeted using meganucleases. To this end, eukaryotic zinc finger proteins were utilized for genome targeting and editing.
Zinc fingers are zinc ion-regulated small protein motifs that bind to DNA in a sequence-specific manner.

Each zinc finger motif recognizes a 3-bp DNA sequence (Klug *et al*., 1987) [16]. Therefore, unlike meganucleases, multiple zinc finger motifs could be assembled into a larger complex to achieve higher DNA binding specificity. Soon after the structure of zinc fingers was revealed, researchers started to create programmable nuclease proteins by fusing zinc finger proteins with the DNA cleavage domain of the Fok I endonuclease. While Zinc Finger Nucleases (ZFNs) generated substantial excitement as a genome-engineering tool, the discovery that transcription activator-like effector (TALE) proteins from Xanthomonas bacteria that can specifically recognize one single base instead of three bases has inspired further excitement about these proteins (Boch, 2009) [3].

The rise of CRISPR (Clustered Regularly Interspaced Short Palindromic Repeat) as the genome-editing technology

Although the discovery of artificially designed meganucleases followed by ZFNs and TALENs successively increased the genome-editing efficacy, targeting different sites in the genome required re-design or re-engineering of a new set of proteins. The difficulty in cloning and protein engineering ZFNs and TALENs, partially prevented these tools from being broadly adopted by the scientific community. In this respect, CRISPR has revolutionized the field because it is as robust as, if not more so than, the existing tools in terms of editing efficiency. More importantly, it is much simpler and more flexible to use.

Mechanism of Genome Editing

Genome editing technologies works on the principle that the endogenous repair mechanism gets ignited when there is a double strand break in the DNA (DSB). These breaks are typically repaired through two major pathways- non-homologous end joining (NHEJ) and homology-dependent repair (HDR). NHEJ is template independent and works by direct relegation of the cleaved ends. This repair pathway is error-prone and can cause insertion or deletion of unintended bases at the break points that may lead to frame shift mutations. Whereas HDR is a template dependent pathway in which the broken end will invade into a homologous sequence and subsequently repaired by synthesis (Szostak *et al*., 1983) [29]. So, the targeted sequence can be converted to a sequence of interest by introducing a homologous donor DNA sequence along with the nucleases.

Genome Editing Technologies

Genome editing methods include chemical methods, protein based nuclease and RNA-protein based system. But the old techniques are out of the scene as newer technologies are more promising. So currently there are four major engineered nucleases that do the job. Furthermore, these technologies are variable in terms of their specificity and sensitivity, off-target effects, finances and technique expertise.

Chemical Modalities of Genome Editing

This method follows a non-restriction enzyme protocol including a pseudo-complementary peptide nucleic acid (pc-PNA), to specify the cleavage the site in the chromosome, along with cerium and EDTA mixture, to cleave the target site and DNA ligase to glue the site with desirable DNA. The methodology was termed as artificial restriction DNA cutter (Komiyama, 2014) [18]. Upon initial introduction, the technique looked quite appealing to the clinical market; however, later issues like increased turn-around time and specific manufacturing of site-specific pcPNA became huge hurdles.

Protein-Based Nuclease Systems

There are different techniques under the system which includes meganucleases, Zinc Finger Nucleases (ZFN) and Transcription Activator like Effector Nucleases (TALEN).

Meganucleases or Homing Endonucleases

Homing endonucleases (HE) are endodeoxyribonucleases characterized by large recognition site of about 12-40 bp and hence are considered the most specific naturally occurring restriction enzymes. The large endonucleases are transported into the cell with the help of recombinant Adeno-Associated Viruses (rAAV) (Yoon *et al.*, 2018) [40]. But there were many off-target effects for this method which includes reduced site specificity, less DNA integration and possible host genome mutations. This was overcome by redesigning the structure of endonucleases to more site-specific and less toxic chimeric endonucleases like DmoCre and E-Drel.

Still, the use of HE has been limited because of two major reasons. Firstly, despite the presence of hundreds of naturally found meganucleases, each of them has a unique recognition sequence. Thus, for an endonuclease to act the specific recognition sequence must be present in the target DNA. Secondly, and more critically, the majority of induced DSBs are repaired through the error-prone non-homologous end joining (NHEJ) DNA repair mechanism. Thereby, not only may the exogenously introduced DNA template not incorporate at the DSBs, but also the NHEJ repair mechanism may randomly insert or delete DNA pieces at the break sites (Jeggo, 1998) [13].

Zinc Finger Nucleases (ZFN)

The term zinc finger protein was coined to describe the finger like appearance of the protein motifs isolated from *Xenopuslaevis*. Zinc finger protein is characterized by the coordination of one or more zinc ions that stabilize the protein motif. The most common ZF protein in humans is the Cys_2 His_2 (C_2H_2) zinc-finger domain. The C_2H_2 zinc finger domain is a peptide comprised of ~30 amino acids (aa). It contains two β-strands and one α-helix. It has been well known that amino acids (aa) beside α-helix (−1 to +6 aa from the start aa of α-helix) recognize a 3-nucleotide sequence. Therefore, the α-helix is referred to as the recognition helix (Klug, 2010) [17]. Since, each zinc finger recognize a 3-bp DNA code, combinatorial assembly of 6–7 zinc fingers out of the unique 64-finger pool could uniquely target any 18–21 bp genomic sequences.

The ZFN is custom-designed C_2H_2 zinc finger protein fused with the cleavage domain of FokI restriction endonuclease. The binding domain in the recognition helix of protein motif identifies the desirable splice site, which is then cut at a specific codon by FokI of ZFN. The choice of the Fok I restriction enzyme was a well considered, deliberate choice for couple of reasons. Firstly, unlike many other restriction enzymes, FokI has distinct DNA recognition and DNA cleavage domain. Knowing this, researchers removed the DNA sequence recognition domain of Fok I and fused only the DNA cleavage domain to zinc finger protein motifs. Another critical consideration is that Fok I requires homodimerization at the target site to cleave DNA. Therefore,

designing two separate zinc finger motifs, each monomer with specific recognition sequence of 18 bp targeting two proximal sites next to each other allows Fok I to homodimerize and result in DNA strand breaks at the target sites. The double strand DNA breaks created by ZFNs are repaired by either non-homologous end joining (NHEJ) or homology directed repair (HDR).

The improved efficiency in the design of ZFNs like fusing the recognition helix of many ZFP in tandem to create polydactyl ZFP that can recognise larger segment of the DNA has tremendously enhanced the capabilities to edit genomes of living cells and opened doors for therapeutic applications of such genome-editing tools. The technique in recent years has gained widespread popularity due to its simplicity and specificity, and it is being employed in clinical usage for certain diseases.

Transcription Activator like Effector Nuclease (TALEN)

TALE proteins are bacterial effectors produced by *Xanthomonas* bacteria that can cleave the DNA at specific sites. Like ZFNs, TALENs consists of amino terminal TALE DNA-binding domain fused to a carboxy terminal FokI cleaveage domain (Miller *et al*., 2011) [22]. The binding domain is a repeat domain of ~34 residues in length and a highly variable 12th and 13th residues called repeat variable diresidue (RVD). Amino acid composition other than RVD among repeats is basically the same, but some non-RVD variations on particular positions such as 4th and 32nd residues have been determined to be important for TALEN activity (Hatada, 2017) [10]. TALENS almost resemble ZFNs in terms of manufacturing and mode of action with only difference being in TALENs more specific with 3 nucleotide target whereas ZFNs can only address 1 nucelotide thus making TALENs slightly more accurate with fewer off-target effects. . Both the number of amino acid residues between the TALE DNA binding domain and the FokI cleavage domain and the number of bases between the two individual TALEN binding sites appear to be important parameters for achieving high levels of activity. To overcome the limitations of the above mentioned methods, chimeric proteins, by fusing meganucleases, ZFs, and TALEs (eg."megaTALs") have been engineered to generate novel monomeric enzymes that take advantage of the binding affinity of ZFs and TALEs and the cleavage specificity of meganucleases (Boissel *et al.*, 2014) [4].

RNA Protein based systems

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) is a defence mechanism present in bacteria to provide protection from invading viruses and plasmids via RNA-guided DNA cleavage by CRISPR-associated (Cas) proteins genes. Short segments of foreign DNA are integrated within the CRISPR locus called as spacer and transcribed into CRISPR RNA (crRNA) which then anneal to trans-activating crRNA (tracrRNA) to direct sequence specific degradation of pathogenic DNA by Cas9 protein (Brouns *et al*., 2008) [5].

The crucial work, which arguably marked the beginning of CRISPR as a biotechnology tool, has been the demonstration that Cas9 enzymes can be reprogrammed to target a desired DNA sequence. The CRISPR gene-editing technology, as we know it today, is developed by combining an endonuclease protein whose DNA-targeting specificity and cutting activity can be programmed by a short guide RNA (sgRNA), which consists of both crRNA and tracrRNA (Barrangou, 2007) [2]. These studies were immediately followed by groundbreaking publications showing that CRISPR can be adapted for in vivo genome editing in eukaryotic cells. For the design of gRNA, the only restriction is that the locus needs to contain the "NGG" protospacer adjacent motif (PAM) downstream of the target sequence. Therefore, the user only needs to design the gRNA and construct the gRNA expression vector. After transfection of cells with the gRNA and Cas9 expression vectors, the Cas9/gRNA complex then binds to the target genomic locus and induces double-stranded breaks (DSBs). Cas9-induced DSBs can be repaired by either non-homologous end joining (NHEJ) or homology-directed repair (HDR). CRISPR systems are of three different types based on Cas gene as type I, II and III. Type I and III contains multiple cas proteins whereas type II contains only one cas protein which is Cas9 complexed with crRNA. The classification is based on the respective signature Cas genes (Cas3 in type I, Cas9 in type II and Cas10 in type III) and the typical organization of the respective loci.

Earlier genome editing systems, such as zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), require complicated processes for construction of DNA-binding proteins, making these methods time-consuming and expensive. Thus, CRISPR/ Cas9 is the most widely used genome editing technology.

Newer Technologies

Scientists are in constant effort to overcome the hurdles in genome editing using CRISPR-Cas system, which is supposed to be the most efficient method, by developing newer strategies. Two such methods of genome editing are base editing and prime editing.

Base Editing

Base editing is a method used to convert a single nucleotide directly into another nucleotide without breaking both DNA strands. This method can be used to change T to a A or G to a C and hence, can be employed in treating genetic disorders governed by a single base mutations like sickle cell anaemia or beta thalassemia.

There are two types of base editing; one is adenine base editing (ABE) and other is cytosine base editing (CBE). Like cytosine and adenine contains an exocyclic amine that can deaminated to alter its base pairing preferences, it was considered for the editing. When adenosine is deaminated, inosine will be formed which has base pairing preference as that of guanosine, as a result of which cytosine will be added to the complementary strand giving an end product with a change of AT to GC base in the target DNA (Ledford, 2019) [20].

Similarly deamination of cytosine yields uracil to which adenine is added in the complementary strand later repaired with thymine forming DNA with single nucleotide conversion

Prime Editing

Even though the CRISPR technology has gained wide spread popularity it is prone to off-target effects. Alternate method called prime editing can help to reduce this off-target effects that makes it safer to use in gene therapies. In prime editing the nick is made only in one strand of the DNA by the Cas9 enzyme. A second enzyme called reverse transcriptase guided by a strand of RNA makes the edit at the site of cut. The prime editing guide RNA (pegRNA) guides the Cas9-enzyme complex to the target site, where Cas9 forms a nick in one

strand of the DNA. The pegRNA has two sections; one with the sequence having DNA bases (binding sequence) and other with RNA bases that encode the desired edit (edited site). After attachment of the binding site to the target sequence, reverse transcriptase will add new bases to the target DNA complementary to the edited sequence of pegRNA. Later, the old strand will be excised by endonucleases and new strand will be added to the DNA. Now, one strand of the DNA double helix will be edited and other strand will be non-edited making a mis-match there. To rectify the mis-match the guide RNA directs prime editor to make nick on the second strand. This nick will be repaired with edited strand taken as template, forming a completely edited DNA (Ledford, 2019) [20].

Advantages of Prime Editing Over Conventional CRISPR-Cas System

Prime editing is more precise with minimum off-target effects and versatile than conventional CRISPR-Cas systems. The older methods may create uncontrollable mixture of edits that vary between the cells called as mosaicism, which has been overcome by these newer methods. In certain cases it is difficult or rather impossible for researchers to overwrite one piece of DNA with sequence of their interest using CRISPR-Cas system because the endogenous cell repair systems are far more likely to make random insertions or deletions rather than to add a specific sequence to the DNA. Another important factor is that in the prime editing method only single strand DNA is nicked that makes it easier and accurate than other methods.

Genetic Engineering in Large Animals for Disease Models

Non human primates (NHP), pigs and dogs are generally accepted by researchers to be useful animals for human disease modelling and have been widely used in biomedical research.

Non-Human Primates (NHP)

NHPs, such as rhesus and cynomolgus monkeys, resemble humans in evolution, anatomy, physiology and pathology more than any other animal, which make them the most attractive species for human disease models. The first NHPs disease model was created in 2008 using lentivirus-mediated expression of the polyglutamine-expanded huntingtin gene (HTT) in rhesus macaque. With the most abundant NHPs resources in the world, Chinese scientists are leading the field of genome editing in NHP models.

TALENs were used to mutate the X-linked, *methyl-CpG binding protein 2 (MECP2)* gene in rhesus and cynomolgus monkeys to model Rett Syndrome (RTT). *Ppar-γ* (*peroxisome proliferator- activated receptor gamma*) and *Rag1* (*recombination activating gene 1*) double-mutant cynomolgus monkeys were first created without detecting off-targeting mutagenesis (Niu *et al*., 2014) [24]. Later, optimized CRISPR/Cas9 system was injected into monkey zygotes, ultimately producing a p53 biallelic (homozygous) mutant monkey that offered a model to study tumorigenesis (Wan *et al*., 2015) [30]. Another example is the Duchenne muscular dystrophy (DMD) monkey model, successful created by CRISPR/Cas9 mediated deletion of the *dystrophin* gene (Chen *et al*., 2015) [6]. The cynomolgus monkeys *DAX1 (dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1)* knock out and the *DAX1*-deficient monkey displayed adrenal gland development defect, abnormal testis architecture, and unaffected Sertoli cell formation. One year later using TALENs, bi-allelic *microcephalin 1* (*MCPH1*) mutant cynomolgus monkey was created that recapitulated most of the clinical characteristics observed in individuals with microcephaly: smaller head circumference, hypoplastic corpus callosum, premature chromosome condensation, and behavioral abnormalities (Ke *et al*., 2016) [14]. The first successful somatic cell nuclear transfer was made in two cynomolgus monkeys. By incorporating this delicate yet difficult procedure, researchers could circumvent some of the variability problems and accelerate the production of genetically uniform monkey models for human diseases.

Pigs

Pigs have been widely used in biomedical research for the past several decades because of the breeding and handling advantages and fewer ethical concerns when compared with NHPs. ZFNs were first applied to pigs with the knockout of the transgenic *enhanced green fluorescent protein* (*eGFP*) and *PPARγ* gene, providing the feasibility of nuclease-mediated gene editing in pigs. TALENs were used to generate *low density lipoprotein receptor (LDLR)* mono-allelic and bi-allelic mutant Ossabaw pigs as models of familial hypercholesterolemia. Later, CRISPR/Cas9 system was first applied to pigs and generated vWF (von Willebrand Factor) bi-allelic mutants, which exhibited significantly reduced coagulation factor FVIII activity and a severe bleeding phenotype, consistent with von Willebrand disease (vWD). In Chinese Bama miniature pigs, zygote co-injection of Cas9 mRNA and sgRNA (single guide RNA) was used to delete *Npc1l1* gene *(Niemann-Pick C1-Like 1)*, efficiently producing bi-allelic mutant pigs to study how Npc1l1 influences cardiovascular and metabolic diseases (Wang *et al*., 2015) [32]. Recently, to study hypertrophic cardiomyopathy (HCM), HCM-point mutation (R723G) was introduced into the porcine *MYH7* gene using TALENs (Montag *et al*., 2018) [23].

Cancer Research Models

Cancers in mice are biologically different from humans and findings based on murine models often do not translate into clinic. Thus, animal models that are more representative of the human cancer spectrum are in great demand. The first nuclease-mediated porcine cancer model was created using TALENs to introduce the *adenomatous polyposis coli (APC)* heterozygous mutation, providing a colon cancer model. Later, created *PKD1* (polycystic kidney disease 1) mono-allelic knockout pigs using ZFNs, the resultant pigs exhibited renal cysts at 6 months that progressively grew, providing a good model for studying renal cystogenesis (He *et al*., 2015) [11]. A pROSA26-iCas9 pig line was established with Cre-inducible Cas9 expression by TALENs, which allowed *ex vivo* and *in vivo* genome modifications. By delivering Cre recombinase and sgRNAs targeting multiple cancer gene loci to the lungs, F1 pRosa26-iCas9 pigs grew large primary lung tumors and showed lung cancer symptoms. These pigs with primary tumors will provide a new platform to develop models of human cancer, possibly facilitating new diagnostic and therapeutic technologies (Wang *et al*., 2017) [33].

Neurodegenerative Disease Models

Because the brain structure of a pig is more similar to that of a human than a rodent, a number of pig models have been generated to study Neurodegenerative diseases. Three pig

lines that model Parkinson disease were created using TALENs and CRISPR/Cas9: DJ1 knockout, PARK2/PINK1 double knockout, or Parkin/DJ-1/PINK1 triple knockout. These pigs served as models for Parkinson disease pathology studies and therapeutic intervention development (Yao *et al*., 2014; Zhou *et al*., 2015; Wang *et al*., 2016) [39, 42, 31].

Recently, a Huntingtin (HTT) knock-in pig model of Huntington's Disease (HD) was created using CRISPR/Cas9, and the resultant pigs exhibited movement, behavioural abnormalities, and selective degeneration of striatal medium spiny neurons at early stages, which recapitulated the selective neurodegeneration of individuals with HD perfectly (Yan *et al*., 2018) [38].

Skin disease models

The structure of pig skin, including thickness, the dermal-epidermal interface, hair follicle content, pigmentation, collagen and lipid composition, and dermal blood, is very similar to the structure of human skin.

When tyrosinase (TYR) was bi-allelically mutated with CRISPR/Cas9, typical albinism was observed in the mutant pigs, including pigment loss in skin, hair and eyes. Another pig line that shows albinism was developed. These *MITF* bi-allelic mutant pigs have a white coat color phenotype and clinical manifestations and underlying causal genetics of human Waardenburg syndrome (Wang *et al*., 2015) [34]. Pigs are also been used to model ectodermal dysplasia-9 (ED-9) by deleting *Hoxc13* with CRISPR/Cas9.

Dogs

The first *MSTN* (myostatin) bi-allelic knockout dogs developed with zygote injection of Cas9 mRNA and sgRNA combined with autologous embryo transfer exhibited a double-muscle phenotype of the thighs at four months, which demonstrated the feasibility of generating dog models for biomedical research (Zou *et al*., 2015) [43]. Another dog model created with genome editing technology was an atherosclerotic cardiovascular disease model incorporating an apolipoprotein E (ApoE) bi-allelic mutation (Feng *et al*., 2018) [7]. In a DMD dog model, the expression of the dystrophin gene was successfully restored by systemic delivery of CRISPR gene-editing components. The dogs showed improved muscle histology, demonstrating the potential application of gene editing approaches in the treatment of individuals with DMD.

Genome Editing for Xenotransplantation

Organ transplantation provides a very promising solution for patients suffering from end-stage organ failure. However, the severe shortage of human organs has led to a major transplantation crisis.

Genetically engineered pigs

During early 2000's, pigs were produced with heterozygous and homozygous inactivated GGTA1 gene (Lai *et al*., 2002) [19]. Among the patients with hearts from GGTA1-knockout (GTKO) pigs one survived for six months. It was seen that transgenic expression of two genes; human heme oxygenase 1 (HO-1) gene and human zinc finger protein A20 gene conferred potential protection against xenograft rejection (Petersen *et al*., 2011) [26].To further decrease the xenoreactive process in hyperacute rejection, pigs lacking CMAH (encoding cytidine monophosphate-N-acetylneuraminic acid hydroxylase) and β4GalNT2 (encoding β1,4-N-acetylgalactosaminyltransferase) were created and these double knockout pigs showed a decreased humoral barrier to xenotransplantation compared with pigs lacking only GGTA1 (Lutz *et al*., 2013) [21].

Another vital factor leading to hyperacute rejection is complement activation, and several human complement regulatory proteins (e.g. CD46, CD55, CD59) have been identified as promising targets to reduce complement activity in xenotransplantation. In fact, organs from transgenic pigs with human hCD59 have been shown to be protected from complement attack. In 2005, transgenic pigs were produced that expressed all three human complement factors, hCD46, hCD55 and hCD59.

Additionally, physiological incompatibilities during xenotransplantation can activate a blood-mediated inflammatory reaction (IBMIR), giving rise to coagulative disorders. pig models expressing human CD39 (platelet aggregation genes), TFPI (tissue factor pathway inhibitor), and thrombomodulin (an inhibitor of factors Va and VIIIa,) have demonstrated coagulation inhibition (Iwase *et al*., 2014) [12].

Genome Editing in Livestock Breeding

Darwin clearly pointed out that both nature and artificial selection have shaped animal and plant breeds. "The key is man's power of cumulative selection: nature gives successive variations; man adds them up in certain directions useful to himself" he says. In the last 50 years, many sophisticated breeding procedures have been developed in quantitative genetics to select animals with outstanding breeding values.

Milk Modification

β-Lactoglobulin (BLG) is a major whey protein that is the dominant allergen in milk from goats, cows and other ruminants (normally absent in human milk) and can cause allergy symptoms ranging from mild to life threatening. Genetic modification could be a more direct approach to reduce BLG levels in ruminants' milk, and both ZFNs and TALENs have been used to mutate BLG in cattle (Wei *et al*., 2018) [35]. The knockout of BLG gene and knock in of human lactoferrin (hLF) gene using TALENs was done in goats to obtain goats with high expression of hLF and absence of BLG. Similar strategy was followed in cows to produce animals with high expression levels of human serum albumin in the milk.

Meat production, composition and quality

Myostatin (MSTN) is a protein secreted in muscle tissues and its primary function is negatively regulating muscle growth. The natural mutation of MSTN leads to a double muscle trait first reported in cattle and then in sheep, dogs, and humans, making *MSTN* an attractive target for genome editing to increase lean meat in livestock. A ZFNs-mediated MSTN-mutation in Chinese Meishan pigs led to developmentally normal animals that exhibited an increase in muscle mass by 100% and a decrease in fat accumulation compared with wild-type animals. Enhanced body weight and larger muscle fibre size were also observed in goats with disrupted MSTN. In goats, the MSTN locus was also used to insert the *fat-1* gene, which converts n-6 polyunsaturated fatty acid (PUFA) to n-3 PUFA, which has been reported to be a risk factor for many life-threatening diseases (Zhang *et al*., 2018) [41].

Pigs lack functional uncoupling protein (UCP1), which makes them cold intolerant and prone to fat deposition, causing

neonatal death and lower production efficiency. In pigs, CRISPR/Cas9 was used to insert mouse adiponectin-UCP1 into the endogenous UCP1 locus, and the UCP1-knockin pigs showed an improved ability to maintain body temperature during acute cold exposure with normal physical activity. The UCP1-knockin pigs showed increased lean meat and decreased fat deposition compared with control pigs, making them a valuable resource for the pig industry.

Disease Resistance

Porcine reproductive and respiratory syndrome (PRRS) is the most economically devastating disease affecting industrial swine worldwide. The cellular receptor for the PRRSV has been identified as CD163, a cellular protein in the scavenger receptor cysteine-rich (SRCR) superfamily, making the receptor a potential target to block PRRSV infection. With the aid of the CRISPR-Cas9 system, CD163-null pigs were quickly generated. In the CD163-nullpigs that were exposed to PRRSV or infected penmates, no viremia or clinical signs were observed (Whitworth *et al*., 2016) [36], providing proof-of-concept that a single gene deletion establishes PRRSV-resistant pig breeds.

Bovine tuberculosis, which is caused by *Mycobacterium bovis*, is becoming a serious threat to the agricultural economy and global public health. One gene of interestis the SP110 gene found in mice. It has emerged as a promising candidate to control infection by *Mycobacterium bovis* by limiting its growth in macrophages and inducing apoptosis in infected cells. With TALEN-mediated insertion of the mouse SP110 gene into the cattle genome, transgenic cattle were capable of controlling the growth of *Mycobacterium* and limiting transmission of tuberculosis in penmates (Wu *et al*., 2015) [37].

Animal Welfare

Physical dehorning of cattle is used to protect animals and producers from accidental injury but is costly and painful for the animals. Genetic analyses have identified variants that are associated with cattle hornlessness (i.e. 'polled'), a trait that is common in beef but rare in dairy breeds. Thus, a candidate "polled" allele was introgressed into dairy cattle using TALEN-mediated genome modification and reproductive cloning. Hornless dairy cattle were obtained, providing evidence for genetic causation and a means to introduce "polled" into livestock with the potential to improve the welfare of millions of cattle without crossing.

Bioreactor

Livestock have also been used as bioreactors to produce human biological products. Blood-derived human serum albumin (HSA) is prescribed for a number of severe diseases such as liver failure and traumatic shock and is in high demand. Due to the shortage of human blood supplies and the infection risks associated with human blood, alternative ways to produce HSA has long been sought. Success was found when CRISPR/Cas9 was used to knock in human albumin cDNA to the pig endogenous albumin locus, leading to transgenic piglets with human albumin in their blood (Peng *et al*., 2015) [25].

Challenges and Future Prospects

Even though genome editing is a potential technology with immense applications in the field of biomedicine, agriculture and animal husbandry, challenges still remain with integrating genome editing to these areas. It is obvious that the effectiveness and specificity of genome editing with current tools still need improvement, and the safety and ethical concerns of using genetic modified tissues, organs and animals remain a focus of considerable debate.

One of the technical challenges is the delivery of such tools into living cells and organisms. Researchers commonly use viral vectors to deliver genes of interest *in-vivo* or *in-vitro*. Due to their low immunogenicity, AAV vectors are particularly attractive therapeutic delivery vehicles for *in-vivo* settings. However, the large size of current Cas proteins creates a major challenge in their packaging into AAV vectors. Therefore, future advancements in reducing the size of existing Cas proteins or the discovery of smaller Cas9 proteins is highly needed.

Specifically, a strategy to generate large founder animals with a desired allele in one step, without a prolonged period of breeding, is in high demand. But mosaic mutations, which are commonly observed in zygote injection-based genome editing, is a potential challenge in the editing of large animals. These issues could potentially be solved by tagging Cas9 with ubiquitin-proteasomal degradation signals and introducing editing components in an appropriate format (i.e. Cas9 protein/sgRNA complex) into very early-stage embryos. The investigators largely eliminated chimerism by formulating CRISPR-Cas9 as a short-lived synthetic protein-RNA complex and co-injecting it into the oocyte at the same time as the sperm.

In spite of the substantial potential of genome editing in clinical and agricultural application, safety and ethical issues cannot be ignored. Xenotransplantation provides hope to patients living with organ failure and waiting for a donor, yet the use of animal organs and tissues in humans is still not fully accepted due to safety and ethical concerns. Further confirmation on the efficacy and safety of xenotransplantation is urgently needed for the procedure to gain acceptance. On the other hand, with the advent of interspecies chimeras using blastocyst complementation, human organs may one day be produced in large animals.

As CRISPR technologies grow in scope and power, social and ethical concerns over their use are also rising, and applications of these powerful tools deserve greater considerations. One such CRISPR application with a long-lasting outcome is the so-called "gene drive" that can potentially target an entire population or a species (Gantz *et al*., 2015) [8]. In this powerful CRISPR application, researchers have demonstrated that a gene allele that provides parasite-resistant phenotype in mosquitos can quickly spread through the population in a non-Mendelian fashion (Hammond, 2016) [9] such applications may greatly empower us in the war against malaria-type diseases. However, due to the global effect of such applications, safety backups should be carefully designed and additional regulatory procedures should be considered and implemented in advance.

Another one major obstacle is the potential immunogenicity to CRISPR-Cas9 proteins. The most widely used Cas9 proteins are from *S. aureus* and *S. pyogenes*. Notably, since these bacteria cause infectious disease in humans at high frequencies, a recent report documented that more than half of humans may already have pre-existing humoral and cell-mediated adaptive immune responses to Cas9 proteins. Therefore, as the CRISPR-Cas9 system moves forward into clinical trials, this factor must be taken into account. Studying and understanding such challenges will enable us to better

determine the scope of their limitations and ways to overcome them. To this end, one proposed solution to the immunogenicity problem could be to identify and utilize orthogonal CRISPR-Cas9 proteins to which we as humans have not been introduced before. It is likely that many more naturally occurring CRISPR systems will be discovered and that they will be harnessed for additional genome-targeting platforms. Therefore, in parallel to the current advancements, additional studies are needed to address the safety and specificity of such tools. Furthermore, sufficient considerations need to be devoted to the social and ethical implications of such technologies so that they will be accessible to all layers of society and benefit all humankind.
Unlike transgene technology, which introduces an exogenous gene into the host genome randomly, genome editing only changes the endogenous gene in an efficient and accurate way. FDA has determined that animals with intentionally altered genomes should be subjected to regulations under the provisions of new animal drug. Unlike FDA, the U.S. Department of Agriculture (USDA) stated that the USDA will not regulate genetically modified plants produced by the new genome editing techniques, which will definitely accelerate the commercialization of genome edited organisms.

Conclusion

Genome-editing technologies offer the opportunity for larger gains over a shorter time-period and can be utilised in manipulating the genome of animal for a better future. Resilience to ASFV is a potential example whereby an allele, only present in the wild warthog population, which has co-evolved with the pathogen for many thousands of years, has been introduced into domesticated pigs by genome editing. We have gone through different technologies of genome editing and it seems that CRISPR/Cas technologies are probably superseding ZFNs and TALENS. Genome editing has its wide range of application in animals like human disease modelling, xenotransplantation, animal welfare aspects and production. It aims in producing a better and healthier future generation of humans and animals. However, the CRIPSR/Cas methods are also being improvised, and newer additions have further enhanced its functional capabilities with reduced off-target effects. Furthermore, with the process of engineering, better gene modification technologies is evolving and can one day replace even CRISPR/ Cas, possibly shifting to synthetic genomics. But the unethical use of genome editing should be regularly monitored with proper rules and regulations.

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