CRISPR-Cas9 genome editing in the livestock industry

A promising addition to current breeding programs

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3-11-2017
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Source image front page: https://cdn.technologyreview.com/i/images/gene.editingx392.jpg?sw=392 [Accessed 19 October 2017]

Abstract

Livestock plays an important role in the agriculture industry. Currently, selective breeding is used to produce offspring with specific trait, to increase profits and decrease production costs. Disadvantages of selective breeding are that it is unprecise, slow, costly and that animal welfare cannot be guaranteed. The aim of this essay is to investigate whether the genome editing method CRISPR-Cas9 can be an addition to current breeding programs. Clustered regularly interspaced short palindromic repeats (CRISPR) is a DNA sequence in bacteria and archaea. The sequence includes spacers, which come from invading viral genomic sequences. In combination with CRISPR associated (Cas) proteins, it functions as a prokaryotic antiviral mechanism. CRISPR-Cas9 is a site specific genome editing method, which is used for gene knock out, knock in or altering. CRISPR-Cas9 is currently used for several livestock applications. These include applications in animal welfare, disease resistance and performance. The advantages of CRISPR-Cas9 are the efficiency, simplicity, low costs and sensitivity, compared to selective breeding. Limitations of CRISPR-Cas9 are off target effects, formation of mosaic animals and ranging efficiencies. Currently transgenic animals are not approved for the market, but CRISPR-Cas9 is a promising method to increase food production, reduce pandemics and reduce pollution of livestock animals. To conclude, CRISPR-Cas9 genome editing has big potential to be used in combination with the current breeding programs in the livestock industry.

Introduction

Agriculture and livestock

According to the Oxford Dictionary, agriculture is 'The science or practice of farming, including cultivation of the soil for the growing of crops and the rearing of animals to provide food, wool, and other products'[1]. In the European Union, agricultural land covers 47% of its territory, which resembles 179 million hectares. In 2016, the output value of agriculture in the European Union was 400 billion euro. Over the last 15 years, output value of agriculture has been stable, while the input value increased from 200 to 220 billion euro. Main expenses are animal food, agricultural services and maintenance of materials and buildings[2].

Almost 40 percent the yearly output value comes from livestock animals. Livestock animals include cattle, goats, pigs and sheep, but also poultry, such as chicken, turkey, goose and duck. The majority of animal output value comes from meat production, especially from beef and pork (See figure 1). Milk and eggs are an important source as well. In addition to food production livestock is also used for fibers, clothes and drugs[3]. The manure of the animals can be used for fuel, building materials or fertilizers[4].

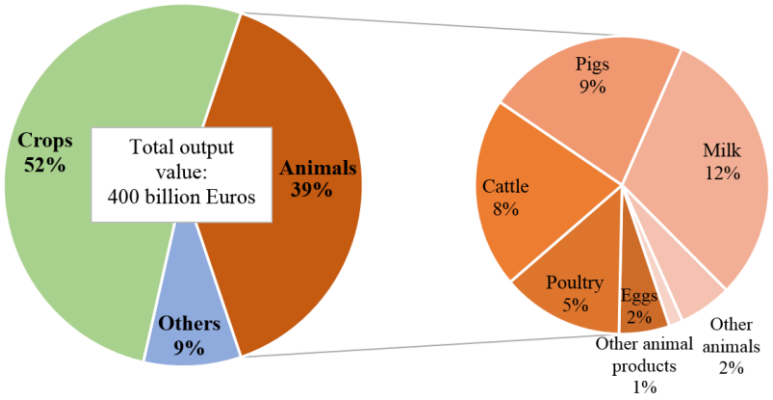

Figure 1: Yearly output of agricultural industry
39% of the total output value of the agricultural industry comes from animals. Animal output value comes mainly from milk and meat from pigs and cattle[2].

Selective breeding

In the livestock industry, selective breeding (artificial selection) plays an important role. Selective breeding is the mating process of a superior male and female to produce offspring with specific characteristics or traits. The aim of selective breeding is the reduction of production costs and increment of output values[3]. Currently, also impact on the environment plays a role in selective breeding. For example, traits that influence methane emission from manure[5]. When breeding programs started, in the 1950's, the aim was to select traits involved in growth and conformation (low fat percentage). Later also traits involved in feed efficiency were taken into account. In the nineties, reproduction, meat quality and health/behavioural traits were also of interest. Currently breeding programs also aim to select traits involved in longevity and infectious/metabolic diseases. Results from the selective breeding programs are increased carcass weight (chickens, cattle) and increased milk and egg production[7].

There are two breeding types: inbreeding and outbreeding. Inbreeding is when animals with resembling genetics mate. This can be two siblings, sibling and parent or even between third degree related animals. Because of the low genetic diversity, inbreeding will result in homogeneity. Outbreeding is mating between non-related animals. This results in a genetic heterogenous group of animals. Bigger genetic variation can cause new characteristics and in the end even new breed. There are different types of outbreed systems, which differ in the relation between the two animals used. The most used outbreeding system is out-crossing, in which two animals from the same breed are mating and producing progeny[8]. Cross-breeding is when two animals from a different breed mate, resulting in a hybrid. Backcrossing is mating between a hybrid and its parent[8]. An advantage of inbreeding,

compared to outbreeding, is that it can result in advantageous recessive phenotypes. This is simultaneously a disadvantage, because also deleterious recessive alleles can be transmitted. Other disadvantages of inbreeding are increased chance of prenatal mortality, reduced fitness of offspring (inbreeding depression) and a reduced gene pool. A reduced gene pool leads to reduced adaptability[8,9].

In the past, breeding was done by traditional selection. This traditional selection was based on pedigree charts and phenotypes. A new development is genomic selection, which is used to predict breeding value of a genetic polymorphism. Genotyping of many animals from one species, will result in single nucleotide polymorphisms (SNPs). Genome wide association studies are then done to predict which phenotype or trait is associated with which SNP. Animals with a specific SNP for a phenotype can then be bred. The advantage of this is that animals can be selected early in life, which results in a bigger genetic change rate[7,10].

The current selective breeding programs have several disadvantages. The first disadvantage is that precise targeting is not possible. Selective breeding relies on the natural reproduction process, which makes precise selection of a target trait impossible. This can result in the passing on from adverse loci or mutations, which are located close to the target locus[11]. The second disadvantage is that selective breeding is a relative slow and expensive process. It can take years from mating of the selected male and female until the outcome of the breeding in the offspring can be seen. The third disadvantage of breeding is the possible harm to the animals. For example, selective breeding resulted in an increased milk production in cows. This causes discomfort for the animal, because of the weight of huge udders and frequent milking can cause painful inflammation. Another example is that selective breeding in chickens resulted in an increased egg production rate. To produce eggshells, high amounts of calcium are needed. When too little calcium is available, chickens will endure bone fractures[12]. Another example is the Belgian Blue cattle, which is a result of selective breeding (see figure 2). These cows have a mutation in their myostatin gene (MSTN), which results in extreme muscle production. Many cows have problems with calving, because the calf is too big. Causing that 80% of the calves are born via a caesarean section[12–14].

Figure 2: Full grown Belgian Blue bull
Belgian Blue bulls have an inactivated MSTN gene, which results in extreme muscle production[13].

Because of these disadvantages it is important to look for possible changes in the current breeding program or to find alternatives. A technique is genome editing, in which genes can selectively be turned on or off, or new genes can be introduced. In this essay, the genome editing method CRISPR-Cas9 and its applications in livestock will be described. The aim is to investigate whether CRISPR-Cas9 genome editing could be used as in the livestock industry, as alternative or addition for the current breeding procedure.

Literature overview

CRISPR

Clustered regularly interspaced short palindromic repeats (CRISPR) are a family of DNA sequences in bacteria and archaea. These sequences play a role in the antiviral defence mechanism, in which it sequence-specific targets foreign DNA. CRISPR was first discovered in 1987 in *E. coli*. At that time, short direct DNA repeats were observed, which had variable sequences at regular intervals[15]. In 2005, it was found that these variable sequences were homologous to viral DNA sequences. It was suggested that the whole CRISPR sequence, both repeats and variable sequences, played a role in the immune system of bacteria. In 2007 evidence for this was found by the observation that bacterial CRISPR had acquired exogenous DNA into it. From that moment, the idea of CRISPR in biotechnology was raised[15,16].

The CRISPR DNA sequence consists of three parts, one leader sequence followed by several repeats which are interrupted by spacers (see figure 3).

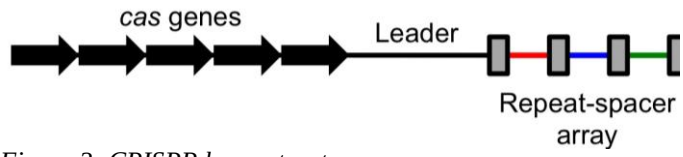

Figure 3: CRISPR locus structure
Several Cas genes are located upstream from the CRISPR sequence. The CRISPR sequence consists of a leader sequenced, followed by a repeat-spacer array. Spacers are showed in red, blue and green. Repeats in black-grey rectangles[17].

The leader sequence is a non-coding, AT-rich structure and is the transcription promotor of CRISPR. A spacer consists of a viral DNA sequence and has a length of 21 to 72 nucleotides. The repeats are preserved sequences, which have a length of 23 to 55 nucleotides. Because the repeats are often palindromic, they can form hairpin loops[18,19]. CRISPR needs CRISPR associated proteins (Cas proteins) to execute its antiviral defence function. Cas genes are located prior to the CRISPR sequence. Currently 35 Cas gene families are known with a total of 93 Cas genes. The Cas proteins play different roles in the CRISPR-Cas antiviral mechanism. They are among others involved in the recognition of foreign DNA (Cas1, Cas2) and the cleavage of DNA (Cas7, Cas10, Cas9)[16,20].

A protospacer is a spacer that is still incorporated in the viral DNA, see figure 4.A protospacer adjacent motif (PAM) is a short DNA sequence of 3 to 5 nucleotides adjacent of the spacer sequence. It plays a role in specific targeting and discriminating between endogenous and exogenous DNA sequences[15,21].

Prokaryotic antiviral mechanism

There are three systems (type I – III) of the antiviral mechanism of CRISPR-Cas. Multiple Cas genes are involved in these systems. The three steps of the mechanism are acquisition, crRNA processing and interference (see figure 4). During acquisition (or adaption) spacers are formed from exogenous nucleic acids, and inserted into the CRISPR sequence. This step is the same in all three systems. During acquisition, the invading DNA is recognized by Cas1 and Cas2. The protospacer is cleaved by nuclease activity of Cas1. The protospacer is then ligated to the repeat which is located next to the leader sequence of the CRISPR sequence. This results in a single strand part in the double stranded CRISPR sequence. Single strand extension will make the protospacer into a double stranded spacer sequence[22].

The second step of the antiviral mechanism is crRNA processing (or biogenesis). During CRISPR RNA (crRNA) processing the primary CRISPR transcript is cleaved by Cas proteins, which result in crRNA. The cleavage step of crRNA processing is different in all three systems. In system type I, the cleavage is done by Cas6. A hairpin loop is formed in a direct repeat, which results in partially double stranded RNA. Cas6 cleaves after the hairpin, where double stranded RNA goes into single stranded RNA. In system type II the crRNA is cleaved by Cas9 and RNase III. Double stranded RNA is formed by trans-activating crRNA (tracrRNA). At the double stranded part, the RNA is cleaved. In system type III a Cas6 homolog is involved, which can cleave single stranded crRNA. After cleavage, trimming is needed in system type II and III. This is done to produce mature crRNA. In system type II this is done at the 5' end, while in system type III this is done at the 3' end[22,23].

The third step of the CRISPR mechanism is interference. The mature crRNA will associate with Cas proteins to form interference complexes. This complex can recognize viral DNA which has the same sequence as the acquired spacer sequence. In system type I and type II the complex will interact with PAM sequences on exogenous DNA. In system type III the crRNA will base pair with exogenous nucleic acids. When the exogenous DNA or mRNA is recognized, it will be cleaved by Cas nuclease activity[22].

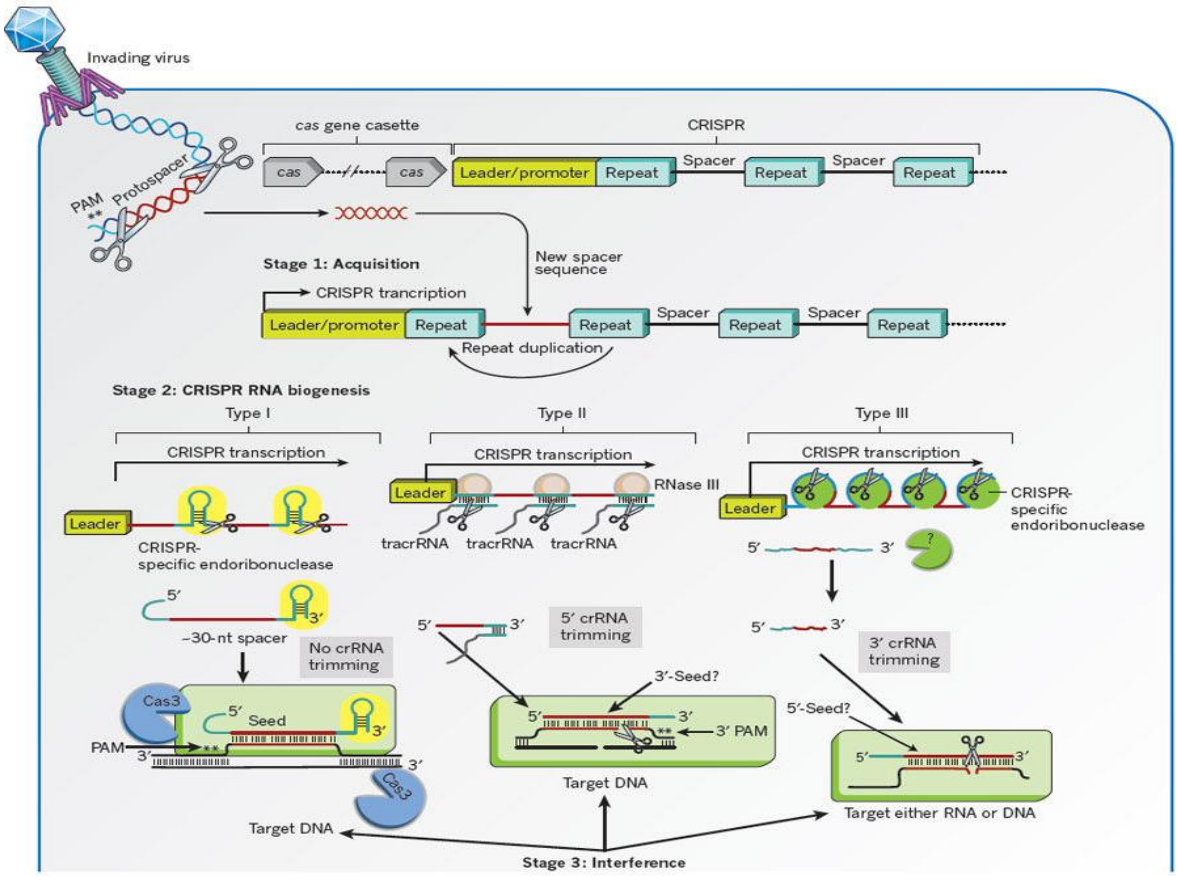

Figure 4: The antiviral mechanism of CRISPR-Cas.
Step 1: During acquisition spacers from viral DNA are inserted into the CRISPR sequence. Step 2: During crRNA progressing the primary CRISPR transcript is cleaved by Cas proteins into crRNA. In type I a hairpin loop is formed and the crRNA is cleaved by Cas6. In type II the tracer RNA forms double stranded RNA, which is cleaved by Cas9 and RNase III. In type III a Cas6 homolog cleaves the crRNA. Trimming is needed in type II (5' end) and III (3'end) to produce mature crRNA. Step 3: Interference complexes are formed by crRNA and Cas proteins. In type I and type II the complex interacts with PAM sequences of exogenous nucleic acids. In type III the complex directly base pairs with either exogenous nucleic acids. After base pairing the exogenous sequence is cleaved by Cas nuclease activity[22,23].

Genome editing

The idea of genome editing started after the discovery of the double helix structure of DNA in 1953. Many studies about possible gene editing showed that base pairing is essential for site specific genome editing. The first transgenic organism was created *in vitro* in 1979, when a gene was replaced in yeast, making use of homologous recombination[24].

Embryotic stem cells were identified for the first time in the early seventies. These were mouse embryotic stem cells from blastocysts. At that time gene transfer was also studied. It was observed that gene copies from injected viral DNA are integrated in the host genome by homologous recombination. Combination of this knowledge led in 1989 to the birth of the first ever genetically modified animal, namely a knockout mouse. In 2007 the Nobel prize in physiology or medicine was awarded for the 'discoveries of principles for introducing specific gene modifications in mice by the use of embryonic stem cells'[25].

CRISPR-Cas9 gene editing

CRISPR is used to edit genome, making use of antiviral mechanism system II. For this system, crRNA, tracrRNA and Cas9 are needed. A two-component system was developed, in which crRNA and tracrRNA were combined into one single synthetic guide RNA (sgRNA). The sgRNA consists of the target sequence of 20 nucleotides, which is specific for the gene of interest[26]. CRISPR-Cas9 can be used in two ways. First for gene disruption, which results in gene knockout or site-specific gene mutations. Second for introduction of new genes in the genome, which results in knock-in animals[27].

CRISPR-Cas9 genome editing consists of several steps. The first step is designing the sgRNA. It is important that efficacy is high for the target, and that there is no off-target cleavage. The second step is the construction of a plasmid. The sgRNA sequence is made into a vector, which also includes Cas9 RNA[21].

The third step is the introduction of the plasmid into the cells of the organism. When the plasmid is introduced, the sequence of the plasmid is translated such that an active Cas9 protein, which includes the gRNA, is formed. Cas9 can than site specifically cleave the host DNA[21]. The plasmid can be introduced by either micro-injection (MI) or by somatic cell nuclear transfer (SCNT)[28]. During cytoplasmic MI DNA is injected into an oocyte. The DNA is injected in the pronuclear phase of fertilization. During this phase, the genetic material from the sperm and egg cell have not fused yet[5,21]. The oocytes are after MI placed in the oviduct of a pseudo-pregnant animal. This method has an efficiency of 10 to 40%. During SCNT an embryo is created by an oocyte and a somatic cell. The CRISPR-Cas9 plasmid is introduced to cultured somatic cells. Transgenic somatic cells are selected for nuclear transfer. The nucleus from the oocyte is removed and implanted with the nucleus of the somatic cell[9,21]. The oocyte is cultured and transferred into the oviduct of a pseudo-pregnant animal. This method has an efficiency of 100%, because only transgenic somatic cells are introduced into the oocyte[29].

For knock-in experiments, another donor plasmid (repair template) is made, which includes the sequence of the gene which is inserted. This sequence should be flanked by sequences of the target site[21]. Important for gene insertion is the target site. The gene must be inserted somewhere in the genome where expression is stable. Also (toxic) side effects must be minimal. These sites are called safe harbour sites or loci. Examples of these are the AAVS1 gene on chromosome 19 in humans and the pH11 locus on chromosome 14 in pigs[27].

Livestock CRISPR-Cas9 applications

Genome editing for livestock applications started more than 30 years ago. In 2013, CRISPR-Cas9 genome editing was used for the first time in cells from livestock animals. Tan et al., from the university of Minnesota, aimed to produce horn free bulls. Naturally horn free Agnus bulls have the Celtic polled (Pc) allele on chromosome 1 in their genome. This allele of 212 base pairs confers polledness, associated with a polled phenotype. A plasmid was constructed with a homologous directed repair template and the Pc allele. This plasmid was introduced in fibroblasts from a horned breed. But due to lack of efficiency at this time, only genetically modified fibroblasts were produced and no transgenic bulls were produced yet[30]. Currently there are many applications of CRISPR-Cas9 in livestock. These include applications for disease resistance and increased performance. In addition, there are several biomedical applications of CRISPR-Cas9 gene editing. A few examples of CRISR-Cas9 genome editing are described below.

Species	Gene	Effect
Animal welfare		
Cattle	Pc allele	Polledness
Disease resistance		
Pigs	*CD163*	Reproductive and respiratory disease
Cattle	*NRAMP1*	Tuberculosis
Cattle	*PRNP*	Bovine spongiform encephalopathy
Performance		
Pigs, goats	*MSNT*	Increased muscle mass
Pigs	*NANOS2*	Infertility
Goats	*FGF5*	Increased hair growth
Biomedical applications		
Disease models		
Pigs	*TPH2*	Serotonin deficiency disorders
Pigs	*ApoE*	Cardiovascular disease
Pigs	*LDL-R*	Cardiovascular disease
Pigs	*RUNX3*	Cancer progression
Pigs	*C3*	Complement system
Pigs	*vWF*	Coagulation disorders
Xenotransplantation		
Cattle	*PERV*	Retroviral infection prevention
Cattle	*GGTA1*	Acute immune rejection prevention
Human protein production		
Cattle	*FGF2*	Fibroblast growth factor 2 production
Cattle	*ALB*	Albumin production

Table 1: Overview of applications of CRISPR-Cas9 genome editing.
In livestock CRISPR-Cas9 genome editing is used for disease resistance, increased performance or animal welfare. CRISPR-Cas9 is also frequently used in biomedical applications, to create disease models, to increase success rate in xenotransplantation or for human protein production.

Disease resistance

Reproductive and respiratory syndrome

The reproductive and respiratory syndrome virus in pigs, costs more than 1 billion euro per year in Europe. Infection with this virus in mature pigs causes reproductive failure and low sperm quality. In growing pigs, it results in respiratory disease and reduced body size. CD163 on macrophages makes entry into cell possible for the virus. Domain 5 of CD163 enables entry and thus infectivity. Exon 7 of the CD163 gene encodes for this domain. CRISPR-Cas9 was used to knockout this gene. A sgRNA that targeted exon 7 of CD163 was designed. The sgRNA and Cas9 mRNA were injected into a zygote, 18 hours after fertilization. Blastocyst stage embryos were transferred into the oviduct of a female pg. In 50% of the born piglets the DNA sequence was completely removed. The CD163 knockout pigs were phenotypically normal. CD163 knockout pigs showed resistance to the reproductive and respiratory syndrome virus[31,32].

Tuberculosis in cattle

The natural resistance-associated macrophage protein-1 (NRAMP1) is associated with an innate resistance to pathogens like salmonella and mycobacterium. It is also associated with resistance to *mycobacterium bovis (m. bovis)* which can lead to tuberculosis in cattle. This protein is present in macrophages and phagocytes, where it is involved in reactive oxygen species and macrophage reprogramming[33,34]. CRISPR-Cas9 was used to introduce the NRAMP1 gene into the cattle genome. The NRAMP1 gene was inserted on chromosome 25. A plasmid with the NRAMP1 sequence was constructed, including the flanking region of the gene, and the Cas9 mRNA. The plasmid was inserted in bovine fetal fibroblast cells (BFFs). Cell colonies with the right (transgenic) genotype were used for SCNT. In vitro reconstructed embryos were transferred into oviducts of cows, in the blastocyst stage. The transgenic calves showed increased resistance to tuberculosis. NRAMP1 knockout animals which were infected with *m. bovis* had lower growth rate of *m. bovis*, lower necrosis level of challenged macrophages, and higher apoptosis level, compared to control animals. Potential off-target sites were predicted. The level of off target modifications was affected by the amount of plasmid used. Optimal plasmid concentration was therefore determined. To reduce off-target effects instead of Cas9 RNA, Cas9 nickase RNA was used CRISPR[33].

Bovine spongiform encephalopathy

The cow prion protein gene (PRNP) encodes for the cellular prion protein (PrP^c). Misfolding of the protein results in the PRPBSE isomer. Accumulation of this isomer in the brain causes bovine spongiform encephalopathy (mad cow disease). Exon 3 of the PRNP gene was targeted by CRISPR-Cas9. A plasmid was developed with a sgRNA that both made indels and removed an 875 bp part of exon 3. Cultured bovine fetal fibroblast were transfected with the plasmid. After 2 days, cells were lysed and analyzed. Bovine oocytes were *in vitro* fertilized with bull semen, and injected with sgRNA and Cas9 mRNA. The zygote was cultured for 7 days. In the fibroblasts target efficiency was 67%, in blastocysts this was 46%[35].

Performance

Increased muscle mass

In pigs, the myostatin gene (MSTN) was targeted with CRISPR-Cas9. As previously mentioned, MSTN negatively regulates skeletal muscle growth. A plasmid was made with a sgRNA that targeted exon 3 of the MSTN gene. Embryonic fibroblasts were isolated and cultured. The plasmid was added to the fibroblast. Oocytes were collected from pig ovaries. They were maturated and the polar body was aspirated. Then the nucleus of the fibroblast was fused with donor cells. Embryos were transferred into the oviduct of a pig. MSNT knockout pigs had increases muscle growth and reduced fat thickness[36].

Infertility in pigs

Park et al. studied infertility in male pigs. Infertile pigs can become surrogates for sperm stem cells transplantation. These stem cells will proliferate, and can generate large amounts of sperm cells. Stem cells from superior male pigs can then be used for transplantation, so these pigs have high amount of progeny. NANOS genes are RNA binding proteins which are involved in germline development. A sgRNA, containing a sequence that targeted exon 1 of the NANOS2 gene, was microinjected into fertilized porcine embryos. These embryos were cultured and transferred into oviducts of female pigs. Knockout male pigs were infertile, due to apoptosis of sperm progenitor cells, but had no morphological abnormalities. Because female knockout pigs and heterozygous male knockout pigs are fertile, these animals can be bred to create homozygous knockout males[37].

Increased cashmere production

Several CRISPR-Cas9 studies are done in Cashmere goats. Cashmere goats are bred for their meat and fur. CRISPR-Cas9 was used to knockout a gene involved in hair growth. Fibroblast growth factor 5 (FGF5) is a hair growth inhibitor. FGF5 mutations are associated with hair length in several animals. A sgRNA was designed that specifically targeted FGF5. The sgRNA was injected into goat embryos, and transferred into pseudo pregnant goats. Efficiency of gene knockout was 21%. Off-target site prediction, resulted in 13 potential sites. Several knock-out animals showed off-target modifications[38]. FGF5 knockout animals had significant increased amount of cashmere producing follicles and an increased hair fibre length, compared to control goats. In addition, it was shown that FGF5 knockout animals had normal germ line transmission[39].

Biomedical applications

In addition to the livestock applications, CRISPR-Cas9 is also used in biomedicine. It is mainly used to generate animal models, but also to improve xenotransplantation and for the production of human proteins.

Animal models are used to gain knowledge about biological mechanisms in healthy and disease processes. Pigs are often used as animal models, because of their physiological similarity to humans. In the past couple of years several disease models were generated by CRISPR-Cas9. First an animal model for mood disorders and behavioural abnormalities was developed. Various mood disorders, like depression, are characterised by serotonin deficiency. Also, behavioural abnormalities can be characterised by serotonin deficiency. A gene involved in serotonin synthesis is the tryptophan hydroxylase 2 (TPH2) gene. Knockout of this gene results in serotonin deficiency. TPH2 knockout pigs were made to study mood disorders which are characterized by serotonin deficiency[40]. Second an animal model for cardiovascular disease. Cardiovascular disease is the main cause of death worldwide. Current cardiovascular animal models have a slow and laborious production process. Therefor CRISPR-Cas9 was used for the development of an animal models is important. The ApoE and LDL-R genes were knocked out in pigs, which resulted in elevated cholesterol and apolipoprotein E levels. Elevated levels of these lipoproteins are associated with an increased risk for cardiovascular disease. ApoE and LDL-R knockout pigs can thus be used as animal model for the development and progression of cardiovascular disease. Other examples are knockout of the tumour suppression gene RUNX3 to study the progression of cancer, knockout of the complement protein 3 gene to study the complement system and knockout of the von Willebrand Factor (vWF) gene to study coagulation disorders[41–43].

Xenotransplantation is the transplantations of tissue or cells from one specie to another. A lot of research is done in the possible application of xenotransplantation to humans. Challenges are occurrence of an immune response due to non-human molecules after transplantation, or that an infectious disease can be transmitted from animal to human. To prevent these issues, CRISPR-Cas9 can be used. For example, the bovine PERV gene can cause retroviral infection in humans. Knock-out of the PERV gene prevents this[44]. Another example is knock out of the bovine GGTA1 gene. This glycoprotein can cause an acute immune rejection in humans. This can be prevented by GGTA1 gene knockout[45].

CRISPR-Cas9 can also be used to produce human proteins, by introducing a human gene into the genome of the animal. This is for example done in cattle. The human FGF2 gene was introduced in the cattle genome. This gene encodes for fibroblast growth factor 2 protein, which could be a potential therapeutic substance, because of its wound healing and tissue repairing properties[46]. Another example is the introduction of the human albumin (ALB) gene into the pig genome. Albumin is the major protein in blood serum. It is used therapeutically for patients who are in shock suffer from severe burn injuries or patients who have low protein levels[47].

Conclusion and discussion

CRISR-Ca9 has several advantages. The main advantage of CRISPR-Cas9 is its efficiency. CRISPR-Cas9 gene targeting has an efficiency of over 80%[48]. Another advantage is the simplicity of the procedure. The simplicity also makes it a fast and cheap process. In comparison to another genome editing method (with Zinc finger nucleases), the costs of CRISPR-Cas9 are 150 times less[49]. Third, the sensitivity is high, because CRISPR-Cas9 has sequence specific gene targeting[20]. The last advantage is that multiplexed genome editing is possible. With multiple sgRNA's several genes can be targeted at once[48].

Disadvantages of the current selective breeding programs are that precise targeting is impossible, it is a slow and expensive process and that animal welfare cannot be guaranteed. With CRISPR-Cas9, all these three factors can be addressed. CRISPR-Cas9 could thus be used in combination with current breeding program to overcome these factors. This will result in increased production of animal products and increase profits. But before that is possible, some challenges must be addressed. The current CRISPR-Cas9 genome editing procedure has several limitations, transgenic animals are not yet approved on the market and the general public is sceptic about production and consumption of transgenic animal products[50,51].

Limitations of CRISPR-Cas9

Currently there are several limitations for the CRISPR-Cas9 genome editing method. The main challenge is the specificity of CRISPR-Cas9. The sgRNA is made to cleave one particular sequence in the genome. But it is possible that there are partially homologous sequences, which are also cleaved by CRISPR-Cas9. Normally the sgRNA contains 20 nucleotides. Off-target effects can occur, because DNA binding can still occur when maximal 5 nucleotides differ from the target sequence[19]. This off-site targeting will result in side effects. There are currently three strategies to increase specificity. First, Cas9 protein with double nickase activity, instead of nuclease activity. This will result in higher specificity because both complementary strands must both be recognized and cleaved, before a double strand break will occur[15,33]. Second, usage of a protein which combines a mutant of catalytically inactive Cas9 and Fok1 endonuclease. Two sgRNAs are needed for specific targeting. Fok1 dimerization is needed for double strand cleavage. They can only dimerize when they are 30 base pairs apart[52]. Third, usage of truncated sgRNAs. Truncated sgRNAs have a length of 17 or 18 nucleotides. These shorter sgRNAs show higher specificity and thus lower off-target effects[26].

Another limitation of CRISPR-Cas9 is the formation of mosaic animals. A mosaic animal has cells with two or more genotypes. This is due to a delayed mRNA expression of CRISPR-Cas9 during MI in zygotes. Only one of the two alleles is then edited[32]. Mosaic animals can fail in transmission of the desirable trait[53]. A third limitation of CRISPR-Cas9 is the variability in efficiency. Even though the procedure of CRISPR-Cas9 is simple, highly skilled personnel is still needed. Efficiencies can differ greatly between different batches. Increased automatization and standardization could reduce the ranging efficiency. This will also contribute to lowered numbers of mosaic animals[32].

Market approval

Currently transgenic animals are not yet approved for the market. This is due to two factors, namely the guidelines for transgenic animals and the attitude of the general public.

Guidelines

There are strict guidelines for the use and consumption of genetically modified animals. First there is the international Cartagena protocol on Biosafety. This protocol aims to protect the movement, handling and use of living modified organisms that may have negative effects on biological diversity and human health. In the Cartagena protocol, a living modified organisms is defined as a 'living organism that possesses a novel combination of genetic material obtained through the use of modern biotechnology'. The Cartagena protocol currently contains 171 parties from all over the world. The Cartagena protocol includes transgenic animals for food, feed or processing. For the use and handling of these living modified organisms, there are two requirements. First the parties involved need to be informed and second there must be sufficient scientific information about the safety and adverse effects[54]. In addition to the Cartagena protocol, there are guidelines for placement on the market. This are the guidelines of the Food and Drug Administration (FDA). Currently no transgenic animals are authorized for the production and consumption of food by the FDA[50].

Only one animal was ever approved on the market. This was the AquaAdvantage® salmon in 1989 by the FDA. It was approved because the cDNA that was introduced met the definition of a drug. The growth rate of salmon was induced by insertion of the growth hormone (GH) gene. Body weight of knockout salmon was four times larger than control fish and the final body weight was reached earlier, which resulted in lower production costs and higher profits. Since then the guidelines have changed, so that animals with an inserted transgene also falls under a living modified organism[55].

Attitude of the general public

The general view of the public towards genetical modified (GM) animals is negative. The attitude of people towards use of GM animals depends on several factors, such as age, sex and religion[56]. The perception of biotechnology and GM animals is monitored by the European Commission in a so-called Eurobarometer, which surveys thousand participants per member country. In 2010, more than 80% of the participants had heard of GM foods. Their opinion was that GM foods are unsafe for their health, harmful, fundamentally unnatural, and unsafe for the environment. In addition, their opinion was that development of GM foods should not be encouraged and that it is not good for the economy[51].

There are several arguments opponent of GM foods use. This are mainly safety, animal welfare and usefulness. People are afraid for both health and environmental risks, especially on long term[57]. People think that there is a lack of knowledge and scientific uncertainty about the risks of consumption of products from GM animals. This idea of lack of knowledge is strengthened by disease outbreaks, such as mad cow disease. The risk for the environment of GM foods comes from the risk that transgenic animals can spread unwanted genes to their wild counterparts. Also, there is a fear that the unnaturalness of GM animals can circumvent the constitutional survival mechanisms of nature, which can have unknown consequences[56,57]. The second argument of opponents is animal welfare. Animal welfare is a general issue in animal research. There are some ethical issues with the production of transgenic animals. This are that the procedures to create a transgenic animal are invasive, many animals are needed and off-target effects which lead to side effects[28]. The third argument is the usefulness of GM animals. The general public does not see the added value of the use of GM animals. This includes economic usefulness (technical and financial aspects), societal usefulness (disease limitation, third world poverty reduction) and individual usefulness. In addition to these three arguments, the general public also fears possible applications in humans. It is feared that modifying animals is the starting point toward an inevitable use on humans[57].

Future livestock applications of CRISPR-Cas9

There are several challenges in the livestock industry. The first is the growing world population. It is expected that the global population will grow with 40% in the coming 34 years. This will result in an increase of 70% of animal product need[58]. Increased amount of livestock animals will cause an increased risk for pandemics, which influences both livestock animals and humans. A third challenge is climate change. Livestock animals produce immense amounts of greenhouse gases. So, need for animals with reduced methane and phosphorus excretion are needed. In addition, climate change has an influence on the efficiency of animal production in the livestock industry. Climate change results in changed temperatures, which can influence the production of animal products. For example, milk production is maximal at certain temperature. The last challenge is the balance between economics and animal welfare. On one hand, high profits and high food production are needed, while on the other hand animal welfare is very important[59]. These four challenges, increasing world population, increased pandemic risk, climate change and animal welfare can all be addressed by CRISPR-Cas9 gene editing.

A solution for increased need of food and climate change, is the production of animals which can live under specific circumstances. For example, animals that are resistant to food or water scarcity, for the same meat production. These animals can then be used in places with low water and food availability for the animals. In addition, resistance to heat or cold can be addressed[60]. Increased production of animal products can also be achieved by targeting the reproduction of animals. For female animals, genes involved in litter size can be targeted, while for male animals, they can be made sterile, to be used for germ cell transplantation of superior animal sperm cells[37,61]. In addition, CRISPR-Cas9 can be used to produce milk with less allergens or an increased amount of nutrients. This will also contribute to the increasing need for food[21].

CRISPR-Cas9 can also be used to target disease resistance, which reduces the chance of pandemics. For the livestock industry, the main pollutants are methane and phosphorus. CRISPR-Cas9 can therefore, be used to produce transgenic animals which have a reduced methane/phosphorus excretion[58].

To summarize, livestock is an important factor for worldwide food supply. Currently selective breeding programs are used to increase production rate, production quantity and quality of animal products. Disadvantages of the current breeding programs are that it is a non-precise, slow and expensive method, while animal welfare cannot be guaranteed. CRISPR-Cas9 genome editing can be added to the selective breeding programs, to overcome these disadvantages. CRISPR-Cas9 can be used in livestock animals to increase production of animal products, increased disease resistance or improve animal welfare. Transgenic animal for consumption are though not yet approved on the market. Prior to market approval and use by consumers, the guidelines of the government and FDA must be reassessed and the sceptic attitude of the general population towards genetically editing food, must be changed. To conclude, CRISPR-Cas9 has great potential to be used within current breeding programs to increase the quality and quantity of food production.

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