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Advances in genome editing technologies for livestock improvement: A review of CRISPR-Cas9 and other genome editing tools

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Abstract
The emergence of genome editing technology has provided animal breeders with unprecedented alternatives for the efficient and precise modification of livestock genetics.
This article summarises the current state of genome editing technology, including CRISPR/Cas9, base editors, and prime editors, and how they have been applied to the enhancement of cattle. Genome editing has the ability to enhance cattle productivity, disease resistance, and welfare; however, there are also certain restrictions and difficulties that must be considered. Genetic engineering in cattle is also discussed, along with the public's views on the topic and the existing regulatory frameworks. In conclusion, we stress the need of continuing to study and advance genome editing technologies for the benefit of cattle.

Keywords: Genome editing, CRISPR/Cas9, Livestock improvement, Animal breeding, Genetic engineering, ZFNs, TALENs, Base Editors

Introduction
Genome editing is a form of genetic engineering that gives researchers the ability to make precisely targeted modifications to the DNA of an organism. This method gives researchers the ability to alter genes with a level of precision, efficiency, and adaptability that has never been seen before. Among genome editing technologies, the most widely used ones are:

1. **CRISPR-Cas9:** This is the most effective method for editing genomes by a wide margin. The CRISPR-Cas9 system use a short RNA molecule, referred to as guide RNA, to identify specific DNA sequences. The Cas9 enzyme is employed to cleave the DNA at a specific locus to modify the gene.
2. **ZFNs:** Zinc finger nucleases, which are tools for editing the genome, contain proteins that bind themselves to certain DNA sequences and then cut DNA at those specific locations.
3. **TALENs:** TALENs represent an additional tool applicable for genomic modification. They are efficacious as they utilise a protein to attach to a specific DNA sequence, subsequently cleaving the DNA at that precise location.
4. **Base Editors:** Researchers can use base editors, a kind of genome editing tool, to make exact modifications to specific DNA bases without causing a double-strand break in the genome.
5. **Prime Editors:** Prime editors are a new generation of genome editing tools that bring together the efficiency of homology-directed repair with the accuracy of CRISPR/Cas9.

CRISPR/Cas9
One of the most talked-about biological technologies right now is gene-editing technology that is related to CRISPR. The field studying CRISPR technology has seen tremendous growth since 2013, with thousands of studies published on the subject. "Developing a new approach to genome editing" was the rationale for the October 2020 Nobel Prize bestowed upon American Jennifer Doudna and French Emmanuelle Charpentier. Before the approach gained a lot of attention, experts had investigated it for about 30 years.

Steps and Procedure of CRISPR-CAS9

Fig 1: Steps of CRISPR-CAS9

Steps and stages of CRISPR-CAS9 gene editing

- **Selecting an organism:** It works well for plants, but we need to test it on model species before we can use it to treat genetic problems. To treat genetic problems using CRISPR-CAS9, choose a model organism with a genome that is very similar to the human genome.
- **Selecting a gene or target location:** The next step, following the choice of model organism, is to decide which gene or DNA sequence to examine, modify, or knockout.
- **Select a CRISPR-CAS9 system:** The CAS9 and CRISPR sequences that we select will be determined by the requirements necessary for our experiments. When it comes to DNA, nucleases, of which the CAS is a subtype, have the ability to break both single-and double-stranded patterns.
- **Selecting and Designing the sgRNA:** In addition to being a sort of guided RNA, the single-stranded RNA, also known as Single-guide RNA, possesses a sequence that is complementary to our target site. The complementary RNA, also known as crRNA, is composed of twenty nucleotides, whereas the tracrRNA, which is comprised of the loop that recognises the CAS9, comprises the remaining twenty nucleotides. Following the identification of the CAS, the tracrRNA component is responsible for directing the nucleus to the cleavage destination. For the design of the sgRNA, we are required to make use of computational approaches. It is common practice to determine the Single-guide RNA binding site upstream of the PAM, with the determination being contingent on the location of the PAM sequence.

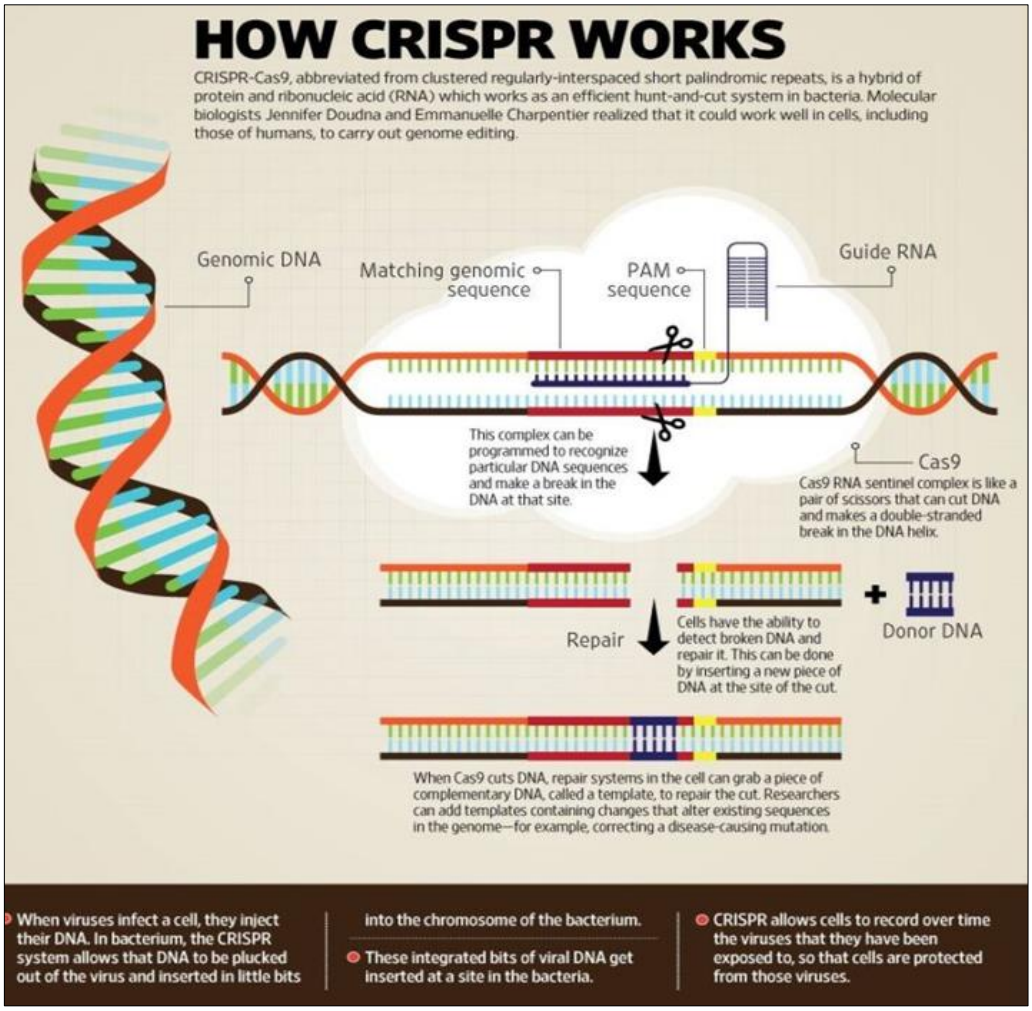

Fig 2: Mechanism of CRISPR-CAS9 system

Credit to microbiologynotes.org

- **Synthesizing and cloning of sgRNA:** We need to make a duplicate or stock of it. The process involves making several copies of the chosen plasmid, inserting the gRNA gene into it, and then achieving the desired result. Proceed by removing the plasmid-expressed gRNA. In addition, sgRNA synthesis can be aided by *in vitro* transcription. After the synthesis of our gRNA, CAS9 will be prepared to modify the target gene.
- **Delivering the sgRNA and CAS9:** The CAS9 and sgRNA are commonly introduced into the target cell by the electroporation method. In this case, the nuclease and sgRNA are able to enter the cell through holes that are formed in the cell by means of the present current. Another option for creating CAS9 in a host cell is to insert a gene or mRNA specific to it, rather than CAS. Unlike bigger molecules, inserting a CAS is extremely challenging. Adenovirus, Lentivirus, Retrovirus, and Adeno-associated virus are some examples of viral vectors that can carry out this same activity. The viral vector-mediated transfection, however, requires CAS-specific messenger RNA. The viral nuclease protein is itself produced. Microinjection, gene gun, sonication, and chemical alterations are further CRISPR-CAS9 delivery techniques. Our cargo-CAS and sgRNA-has arrived at the cell of interest.
- **Validating the experiment:** Validating a CRISPR-Cas9 experiment entails confirming that the target gene or sequence has undergone the desired modification.
- **Culture the modified cells:** A genetically engineered cell line that is representative of the altered cell has been obtained by us at this time**.** Maintain sterile conditions and use the proper culture medium to cultivate the cell line. Once we have enough cell lines, we can introduce them into the creature we've chosen to hold the tests**.**
- **Gene expression studies:** One way to verify gene expression is using quantitative PCR, often known as real-time RT-PCR. Here, the messenger RNA (mRNA) is extracted from the cell line, converted to complementary DNA (cDNA), and then measured using polymerase chain reaction (PCR).
- **Analyzing results:** Computational and physical analysis of results are also possible. Computational tools are useful for checking the accuracy of gene sequences, gene expression profiles, and other similar datasets**.**

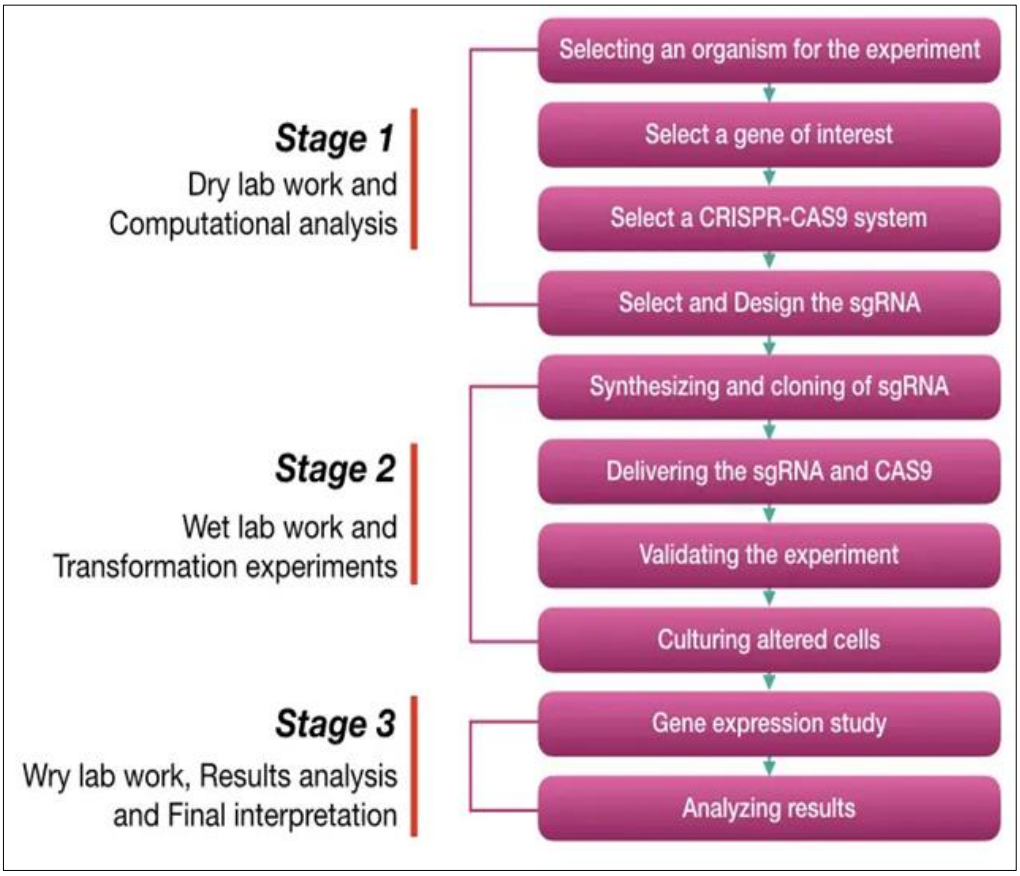

Fig 3: Phases and procedures of CRISPR-Cas9 gene modification

Applications of CRISPR-CAS9

Effective, site-specific genome editing in both single cells and entire animals is made possible by programmable DNA cleavage utilising CRISPR-Cas9. The research community has made extensive Utilisation of CRISPR-mediated genome editing for a wide range of purposes, including chromosome imaging, genome-wide screening, transcription control, and epigenome modification. The CRISPR technique is already helping animals with genetic problems, and it may soon be utilised in clinics to treat blood and eye ailments in humans. Both China and the US have authorised two CRISPR-Cas9 targeted cancer therapy clinical trials. These methods are finding new uses outside of biomedicine, including as speeding up the breeding process for crops and cattle, creating novel antibiotics, and using gene drives to manage insects that carry diseases.

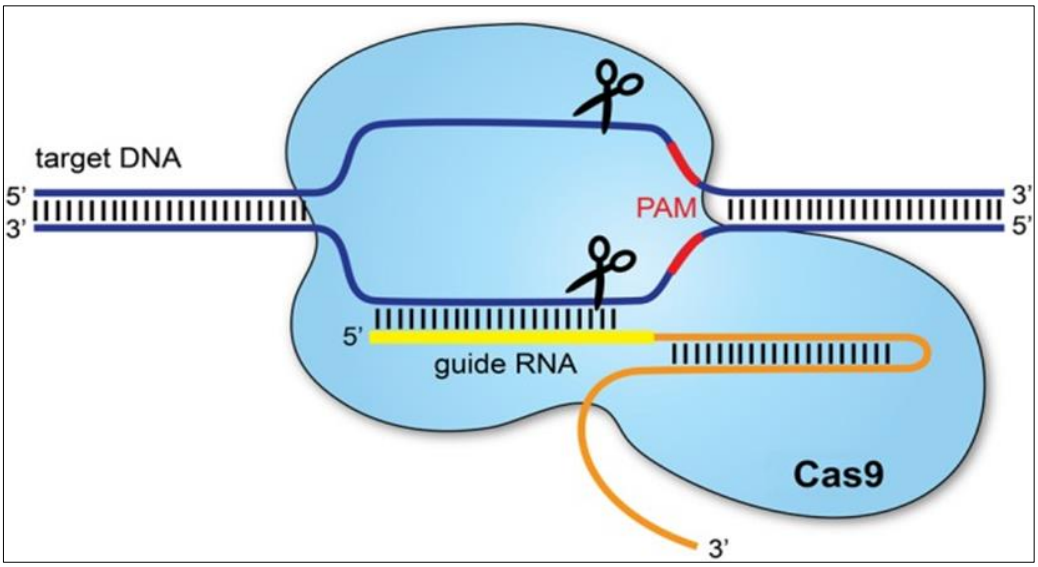

Fig 4: Workflow of CRISPR-Cas9

Livestock Improvement

1. Pigs: Improvements in growth rates and disease resistance are just two examples of the beneficial qualities introduced into pigs using CRISPR-Cas9. A potential alternative animal model, the pig has a number of noteworthy characteristics that set it apart from other animals (Qian *et al*., 2015) [57]. Model pigs are comparable to humans in many ways (Miller *et al*., 2011) [44]. The biochemical metabolism, anatomy, and physiology of humans and pigs are quite comparable (Ruan *et al*., 2015) [60]. The reproductive cycle of pork is relatively brief, it produces a large number of offspring with each litter, and it reaches sexual maturity at a young age. Pork offers several advantages because of these characteristics. In addition, genome editing and SCNT technologies have recently advanced to the point that large animals can be efficiently genetically changed (Yang *et al*., 2018) [89]. In 1985, the first genome-edited pigs were produced via pronuclear DNA microinjection in zygotes (Hammer *et al*., 1985) [21]. The generation of genome-edited pig models has been accelerated due to the advent of CRISPR Cas9 technology.

As previously demonstrated that genome-modified pigs may be efficiently produced in a single step by microinjecting zygotes with the CRISPR/Cas9 system. The mutations can then be efficiently passed down to the next generation (Hai *et al.*, 2014) [20]. After that, other researchers found that by combining the CRISPR/Cas9 system with SCNT, it is possible to successfully produce pigs with one or two genes targeted, without causing mosaic mutations or noticeable off-target consequences (Whyte *et al*., 2011; Zhu *et al*., 2022) [27, 104]. Additionally, it was possible to create pigs with modified genes targeting three different genes at once (Wang *et al*., 2015) [69].

Besides its application in developing knock-in models that replicate human diseases, CRISPR/Cas technology is utilised to generate knock-out pigs. Researcher employed CRISPR-Cas9 to insert a substantial CAG repeat of 150 CAGs into the endogenous pig HTT gene within fibroblast cells (Yang *et al.*, 2014) [86-88] This was done in the same way that HD patients were. This was followed by the creation of an HD KI pig model through the utilisation of SCNT, which expressed endogenous full-length mutant HTT. A Significant and selective neurodegeneration was observed within the medium spiny neurones of the brains of these animals as a result of the experiment. The use of huge fragment knock-in in pigs has also been observed in other circumstances (Song *et al*., 2022; Li *et al*., 2019) [64, 32, 36]. These occurrences involve genes or models.

The CRISPR-Cas9 technology has been shown to be effective in many experiments for generating knockout or knock-in mice. As a result, several pig models that mimic human diseases have been developed. These models include 5-hydroxytryptamine (5-HT) deficiency, cardiovascular disease, cancer, type II collagenopathy, HD, and complement protein deficiency.

Pigs, on the other hand, are among the most vital animals to the agricultural sector. Possibilities for unique traits in genetically engineered pigs include enhanced resistance to the disease and larger muscle mass. Researchers have focused on improving viral resistance and augmenting muscle mass by employing the CRISPR-Cas9 technology to target genes that inhibit muscle hypertrophy (Wang *et al*., 2017; Wang *et al.*, 2018; Zou *et al*., 2018; Liu *et al*., 2013; Li *et al*., 2017; Yan *et al*., 2020) [70, 71, 105, 39, 35, 84].

In addition, with the increasing demand for xenotransplantation, pigs are seen as a valuable supply of organs for transplantation. Immunological compatibility between pigs and humans is a major concern following xenotransplantation. To eradicate the antigens that cause immunological rejection in humans, several genome editing pigs have been developed (Porrett *et al*., 2022; Chuang *et al*., 2017; Gao *et al.*, 2017; Wu *et al*., 2016; Kalds *et al*., 2019) [55, 11, 16, 81, 25]. As an additional concern, pig endogenous retroviruses (PERVs) provide a threat of transmission between oth er species. As a result, scientists used a SCNT and CRISPR/Cas9 system to inactivate all Pig endogenous retroviruses in a porcine primary cell line and generate pigs devoid of PERVs (Yu *et al*., 2011; Niu *et al.*, 2017; Zhang *et al*., 2018) [92, 47-48, 95, 98-99]. A more secure use of pig organs in therapeutic settings was the intended goal of these initiatives. A major step forward in the field of clinical application has been the publication of a number of groundbreaking studies that demonstrate the viability of transplanting organs, such as kidneys and hearts, from transgenic pigs into people (Prather

et al., 2003) [56].

2. Cattle: In an effort to enhance their resilience to diseases and growth rates, scientists have utilised CRISPR-Cas9 to modify the genomes of cattle.

3. Goats and sheep, with their manageable size and brief gestation period, have also emerged as valuable model animals in the field of biomedical research. When it comes to the agricultural and pharmaceutical industries, the dairy, meat, fibre, and additional by-products that come from sheep and goats are just as valuable as those that come from pigs. When (Han *et al*., 2014) [22] reported the effective one-step generation of gene knockout sheep by a single zygote injection of the CRISPR/Cas9 system, they were the first to demonstrate that it is possible to precisely target genes in sheep by utilising the CRISPR/Cas9 system. The investigators focused their attention on the myostatin (MSTN) gene. Genome editing facilitated by CRISPR/Cas9 was proved to be an effective method for modifying goat genomes in the same year (Niu *et al*., 2017) [47-48]. Additionally, Live-born goats with biallelic mutations were successfully produced using single-gene knockout fibroblasts for SCNT.

Concerns about the potential for this genomic engineering approach utilising HR to generate targeted point mutations are similar to those around the frame shifting insertion-deletion mutation (indel) strategy, which relies on aberrant DNA repair. Afterwards, a G→A point mutation in the GDF9 gene was identified and confirmed by (Pan *et al*., 2021) [52]. This mutation considerably affects the yearly birth rate of cashmere goats. Furthermore, the inaugural CRISPR/Cas9 gene knock-in sheep program was launched by (Xie *et al*., 2019) [82]. Highly efficiently, they integrated an external tGFP (turboGFP) gene into specific genes within the frame. The thymosin beta 4 (Tβ4) gene was introduced into the goat's CCR5 locus in an independent study. Research on knock-in goats can be modelled after this study (Li *et al*., 2014) [37].

Various biomedical studies have made use of sheep and goats as fascinating models. The size and architecture of sheep and goats make them a better choice than experimental rats for simulating human diseases. As with humans, it has been possible to use the CRISPR-Cas9 method to alter the genome of sheep and goats in a way that makes them sick. CRISPR-Cas9 is used to alter the CFTR gene in sheep, they created the first animal model of (CF) in humans (Fan *et al*., 2018) [15]. Severe disease phenotypes, which are identical to those found in humans, were observed in newborn CFTR−/-sheep. Consistent with human cystic fibrosis liver illness, these phenotypes included pancreatic fibrosis, intestinal blockage, and severe liver and gallbladder disease. In another study it is found that using sheep that have had their otoferlin (OTOF) genes disrupted can help researchers better understand and create new medicines for genetically-related deafness in humans. This allows for improved understanding and development of these new therapies (Meurens *et al*., 2012) [42]. An additional fascinating model of human hypophosphatasia (HPP) is presented which is a, a rare metabolic bone illness, in the research on sheep that utilised CRISPR-Cas9. Hypophosphatasia is a rare disease that affects the bones. This work involved the use of genetic engineering to insert a single point mutation into the tissue-nonspecific alkaline phosphatase (ALPL) gene. The fact that the gene-edited lambs that were developed phenocopied human HPP is one of the reasons why this large-animal model of unusual human bone ailments is useful (Wu *et al*., 2017) [80]. These findings provide credence to the notion that the CRISPR/Cas9 technology shows enormous promise for creating GM animals with traits similar to human diseases (Komor *et al*., 2017) [28].

Just like sheep, pigs and goats are highly valued in the livestock business. As a result, there is a growing push from scientists to modify their features through genome editing in various ways, taking into account their physical demands. Donor organs for xenotransplantation could be sourced from sheep and goats, which can develop human organs. In order to accomplish this, scientists came up with the concept of the PDX1−/− foetus, which is an acronym that stands for pancreatic and duodenal homeobox protein 1. This paves the road for the construction of gene-edited sheep that may be used as a vehicle for the growth of organs from diverse species (Wang *et al*., 2015) [69]. When it comes to the heritable regulation of sheep's reproductive efficiency, the BMPR-IB, is one of the most promising choices. Researchers used CRISPR/Cas9 technology to make sheep BMPR-IB genes functionally useless by inserting mutations. As per the research this caused ovulation rates to rise, which boosted litter sizes (Zhang *et al*., 2018) [95, 98-99]. A mammary gland bioreactor built utilising the CRISPR/Cas9 system was used to produce milk enhanced with melatonin in sheep and goats (Menchaca *et al*., 2020; Zhou *et al*., 2015; Vilarino *et al*., 2017) [41, 102, 68]. This bioreactor was an AANAT/ASMT transgenic animal model. By inhibiting the activity of FGF5 using CRISPR/Cas9, additional studies demonstrated that wool growth could be enhanced, resulting in longer and more abundant wool in sheep or goats (Li *et al*., 2019; Hu *et al*., 2017; Wang *et al*., 2016) [32, 36, 72-73]. It was also demonstrated that comparable studies were conducted to alter the coat colour of these animals (Zhang *et al*., 2017) [96-97].

In an effort to keep up with the increasing demand for sheep and goat meat, numerous studies have sought to enhance the quantity and quality of this meat by modifying the genes responsible for producing muscles in sheep or goats, such as MSTN. A significant number of these research have concentrated on either sheep or goats (Crispo *et al*., 2015; Niu *et al*., 2018; Zhou *et al*., 2019; Wang *et al*., 2020; Guo *et al*., 2016; Zhang *et al*., 2017; He *et al*., 2018) [12, 49, 100, 75, 18, 96-97, 23]. The pig's approach also included this tactic.

Disease Modeling and Treatment

1. Studying disease mechanisms and developing novel treatments has been made possible by using CRISPR-Cas9 to produce mice models of human diseases.
2. Gene treatment, Scientists have investigated the possibility of utilising CRISPR-Cas9 for gene therapy in rat models of hereditary diseases in an effort to eradicate the mutated DNA that causes these conditions (Wang *et al*., 2016) [73-74].

Conservation Biology

1. **The conservation of endangered species:** By using CRISPR-Cas9, researchers are looking into the prospect of modifying the genomes of endangered species in order to make them more resistant to or adaptive to changes in their environment (Carlson *et al*., 2012) [9-10].
2. **Invading Species:** CRISPR-Cas9 is being studied by scientists as a potential tool to manage invading species, which pose a threat to native ecosystems and biodiversity. Eight (Niu *et al*., 2018) [49].

3. These novel uses for CRISPR-Cas9 in animal genetics show how far the technology has come, from increasing crop yields to better understanding disease causes and creating effective treatments.

ZFNs (Zinc Finger Nucleases)

Another type of proteins with motifs that can attach to particular DNA sequences are zinc finger (ZF) nucleases. These nucleases share 30 amino acid long ZF motifs that create one alpha-helix and two antiparallel beta sheets (Pabo *et al*., 2001) [50]. They were initially identified as a fragment of the transcription factor IIIa in clawed frog oocytes (Miller *et al.*, 1985) [43]. Two histidine and two cysteine amino acid residues attached to Zn2+ stabilise the domains of zinc finger nucleases, resulting in a structurally compact domain. Through the residues of the α-helix, the ZF motif attaches itself to the main groove of the DNA double helix (Pavletich *et al.*, 1991) [53], A more specialised DNA recognition domain could be formed by a collection of zinc fingers (Kim *et al.*, 1996) [28]. ZFNs have a non-specific cleavage domain of the Fok1endonuclease in addition to a particular DNA binding domain. In most cases, certain chromosomal alterations require a few ZFN motifs. There are more specific targeted sequences when two ZFNs are important (Smith *et al*., 2000) [63]. The type II restriction endonuclease FokI can dimerise and cleave the targeted dsDNA at the insertion site by binding to the opposing DNA sequences in an antiparallel manner, separated by 5-7 bp. Utilising a pre-selected library of zinc-finger modules produced either by rational design or by selecting vast combinatorial libraries is known as the "modular assembly" approach (Beerli *et al.*, 2002; Segal *et al*., 1999) [4, 62]. Pre-selected zinc-finger modules can be joined in tandem to target DNA sequences that contain a sequence of these DNA triplets since zinc-finger domains have been constructed that recognise almost all of the 64 potential nucleotide triplets (Beerli *et al.*, 2002; Beerli *et al*., 1998; Bhakta *et al*., 2013; Kim *et al*., 2011; Gonzalez *et al*., 2010; Beerli *et al*., 2000) [4, 6, 7, 27, 17, 5]. As an alternative, fresh zinc-finger arrays can be chosen from randomised libraries using selection-based techniques like OPEN (Oligomerized Pool Engineering), which account for context-dependent interactions between nearby fingers. (Sander *et al.*, 2011) [61]. Additionally, strategies that combine the previously mentioned techniques have been devised, employing zinc-finger modules that have been pre-selected for context-dependency in order to modularly create longer arrays (Sander *et al.*, 2011; Gupta *et al.*, 2012) [61, 19]. For a long time, the only method for producing unique site-specific DNA-binding proteins and enzymes was zinc-finger protein technology.

Steps and stages of ZFN

- **Designing the Zinc Finger Proteins:** Creating zinc finger proteins with the ability to identify and attach to particular DNA sequences. This entails choosing zinc finger motifs that are able to identify particular DNA base triplets.
- **Construction of ZFNs:** ZFNs are created by attaching a nuclease domain, usually the FokI endonuclease, to the zinc finger proteins that have been developed. By doing this, a chimeric enzyme is produced that is able to identify and cleave particular DNA sequences.
- **Delivery of ZFNs to Cells:** Use electroporation, transfection, or other techniques to deliver the ZFNs to the target cells.
- **Recognition and Binding of Target DNA:** The nuclease domain is positioned for cleavage by the ZFNs' recognition and binding to the target DNA sequence.
- **Cleavage of Target DNA:** The target DNA is cleaved by the nuclease domain, resulting in a double-strand break.
- **Activation of DNA Repair Mechanisms:** The cell's DNA repair processes, including as homologous recombination (HR) and non-homologous end joining (NHEJ), are triggered.
- **Editing the Genome:** Researchers can make precise changes to the genome, including insertions, deletions, or replacements, by offering a template for repair.
- **Verification of Genome Editing:** Confirming the genome editing event by a variety of techniques, including Southern blotting, PCR, and sequencing.

Applications of ZFNs

Due to the fact that milk is one of the most consumed products from cattle, there has been a significant amount of discussion over the possibility of employing genome editing technology to enhance the nutritional integrity of milk. Research has previously shown that ZFNs can be used to eliminate the main β-lactoglobulin gene, which codes for a milk protein that is highly allergic in cattle (Yuan *et al.*, 2020) [93]. The first pigs with an eGFP transgene were produced by experts using a ZFN-mediated knockout approach (Williams *et al*., 2018) [78]. Following the introduction of the ZFN plasmids into fibroblasts derived from pigs, the selection of eGFP knockout cells, which accounted for approximately 0.1% of the sorted cells, was carried out through the utilisation of fluorescence-activated cell sorting. Next, the mutant cells that had been mediated by ZFN were utilised in the process of somatic cell nuclear transfer as well as embryo transfer. During the course of the research, a number of pigs were created that displayed KO eGFP fluorescence. Pigs that were deficient in the ZFN-mediated MSTN gene exhibited faster muscle growth, less body fat, and more muscle mass (Rao *et al*., 2016) [58]. Novel animal models for the purpose of studying cardiovascular sickness were ZFN-mediated endogenous peroxisome proliferator-activated receptor-γ KO-pigs (Yang *et al.*, 2014) [86-88]. With the development of α-1,3-galactosyl-transferase gene biallelic KO pigs by ZFN (Bao *et al.*, 2014) [2], the possibility of xenotransplantation became conceivable. In spite of the fact that KO pigs are not yet prepared for large long-term xenotransplantation, we thought this was a reasonable starting point for xenotransplantation with pig organs. The lysostaphin coding vector was introduced into the endogenous β-casein locus in bovine foetal fibroblasts by Liu *et al*. using ZFNs. As a result of gene editing, these cows were able to ameliorate the symptoms of mastitis by producing milk that included lysostaphin (Ma *et al*., 2017) [40]. Because of the many restrictions and regulations that exist, the commercial availability of these creatures that have had their genes altered is restricted to a select few locations.

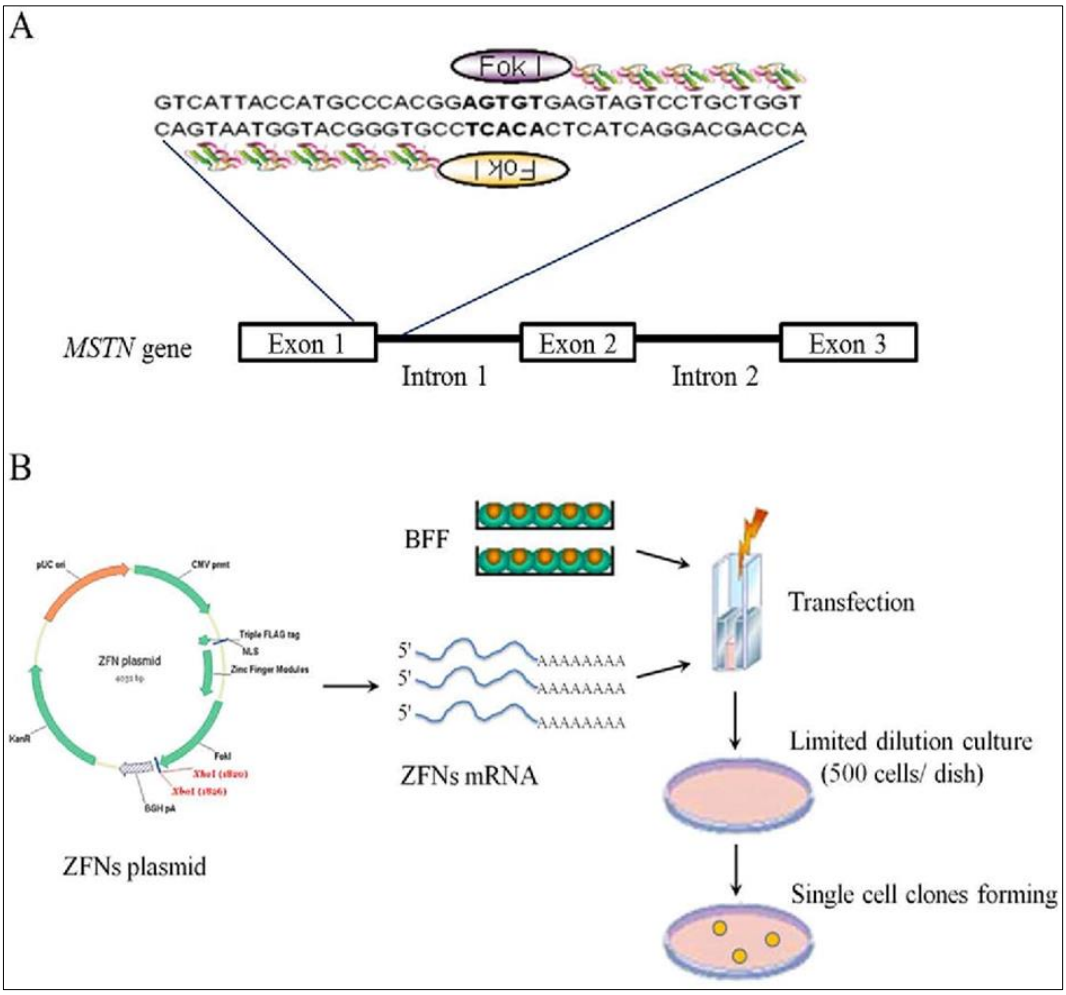

Fig 5: Different steps of ZFNs

Talens

The structure of TALENs is comparable to that of ZFNs. Additionally, they have a cleavage domain and a DNA-binding domain (Rao *et al.*, 2016) [58]. To generate DSBs, two TALENs are necessary (Moscou *et al.*, 2009) [45]. Xanthomonas effector proteins were the first to have the DNA-binding domain of TALENs, which is known as TALE (Bogdanove *et al.*, 2010) [8]. One pair of bases is recognised by each of the thirty tandem repeats that make up the DNA-binding domain (Deng *et al.*, 2012) [14]. These tandem repeats range from thirty-three to thirty-five amino acids in length with each repeat domain. TALE may possibly target any DNA sequence (Ni *et al.*, 2014) [46]. By linking an artificial TALE to the single-strand DNA-cleaving domain of FokI, researchers successfully created TALEN, a potent tool that integrates the features of both TALE and FokI. In comparison to Zinc-finger nucleases, the TALEN technology is not only simpler to operate but also more affordable. Through the use of the RVDs NN, NW, and HD, the letters G, A, T, and C can be distinguished (Ni *et al.*, 2014) [46]. In a manner analogous to that of ZFNs, talens have the ability to modify specific DNA sites through the creation of DSB-mediated NHEJ and HDR. TALEN's more straightforward structure and more precise recognition of DNA sequence contribute to improvements in gene editing efficiency, toxicity, cost, and off-target risk. These improvements are all made possible by TALEN. Through a substantial body of research, it has been proven that TALEN pairs have the potential to efficiently induce knockout (KO) of target genes in a vast variety of cattle, with knockout efficiencies ranging from 20-60% (Carlson *et al.*, 2012; Yang *et al.*, 2015) [9-10, 90]. TALEN-mediated gene knock-in efficiencies of more than thirty percent have been seen in certain loci (Yang *et al.*, 2015; Tian *et al.*, 2018; Liu *et al.*, 2019) [90, 38], which is quite surprising.

Steps and Stages of TALEN

It includes the following steps

Designing TALENs, Construction of TALENs, Delivery of TALENs to Cells, Recognition and Binding of Target DNA, Cleavage of Target DNA, Activation of DNA Repair Mechanisms, Editing the Genome, Verification of Genome Editing.

The intricate design, high cost, and lack of viable targets are limitations of the ZFN approach, despite the fact that it has been an effective tool for modifying cattle genomes (Carlson *et al.*, 2012) [9-10]. The benefits of TALEN technology led many research organisations to swiftly apply it to the alteration and enhancement of cattle genomes. Scientists demonstrated a promising application of TALEN by using it to modify the genomes of cattle. This application was demonstrated by the following (Carlson *et al.*, 2012) [9-10]. Primary pig cells demonstrated high activity for 64% of the TALENs tested. When TALEN mRNA was directly injected into the zygotes of cattle, 75% of the embryos had their target genes knocked off. This number was 29% in pigs and 43% to 75% in cows. The use of TALENs has been found to be

effective in producing MSTN-KO pigs, cattle, and lambs (Roura *et al.*, 2016; Yang *et al.*, 2011; Li *et al.*, 2020) [59, 87, 31, 34]. Significant changes occurred in the phenotypic of the muscles, and the TALEN showed at least a 10% effectiveness rate in cattle. Additionally, TALEN has been successfully utilised in the generation of porcine models of cardiovascular disorders featuring biallelic mutations in the LDLR expression gene (Carlson *et al*., 2012) [9-10]. In a curious study, Cui and colleagues conducted an investigation into the impact of TALENs on goats. They began by removing the β-Lactoglobulin gene, and then proceeded to knock in the human lactoferrin gene (Cui *et al.*, 2015) [13]. These findings demonstrate the potential of TALEN-mediated HDR for gene editing, which could lead to genetically modified cattle that could be utilised as mammary gland bioreactors for the efficient production of targeted products. One of the diseases that plague cattle and is seriously harming the livestock business is tuberculosis, also referred to as consumption. TALENs were used in bovine somatic cells to introduce the resistance gene SP110 into a particular spot on chromosome 28 in order to create calves resistant to the disease. 23 calves were born after 147 surrogate moms received SCNT embryo transplants. Due to real infection testing on this newborn calf, a Chinese study team concluded in 2015 that the calves were resistant to tuberculosis (Wu *et al*., 2015) [79]. A study that created PARK2 gene editing pigs using TALENs in 2014 or concurrently dropped Parkin, DJ-1, and PINK1 genes in 2016 for neurological disease research was one instance of hAPP, hTAU, and hPS1 gene overexpression in pig somatic cells in 2017. The creation of loxP-engineered chromosomes and the potential for conditional gene activation in this model organism have been made possible by the targeted integration of TALENs in zebrafish, which was accomplished by micro-injecting single-cell embryos with TALEN mRNA and single stranded DNA oligonucleotides (Bedell *et al.*, 2012) [3] or donor plasmid with extended (>800 bp) homology arms. A single set of injections was also conducted in porcine zygotes using TALENs targeted to the porcine *RELA* gene (*p65*) for which a tolerance allele for African swine fever has been proposed.

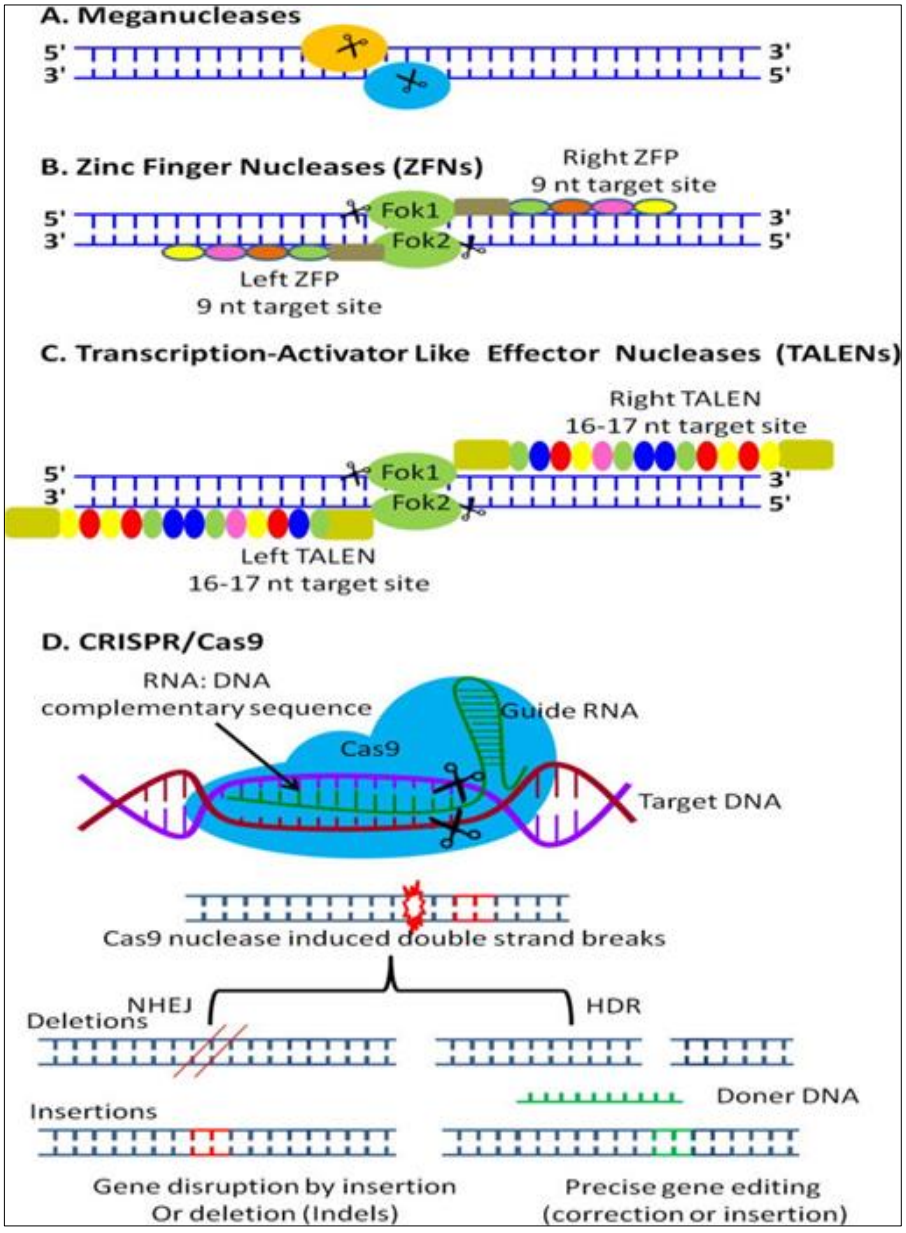

Fig 6: Comparison between ZFNs, CRISPR-CAS9 & TALENs

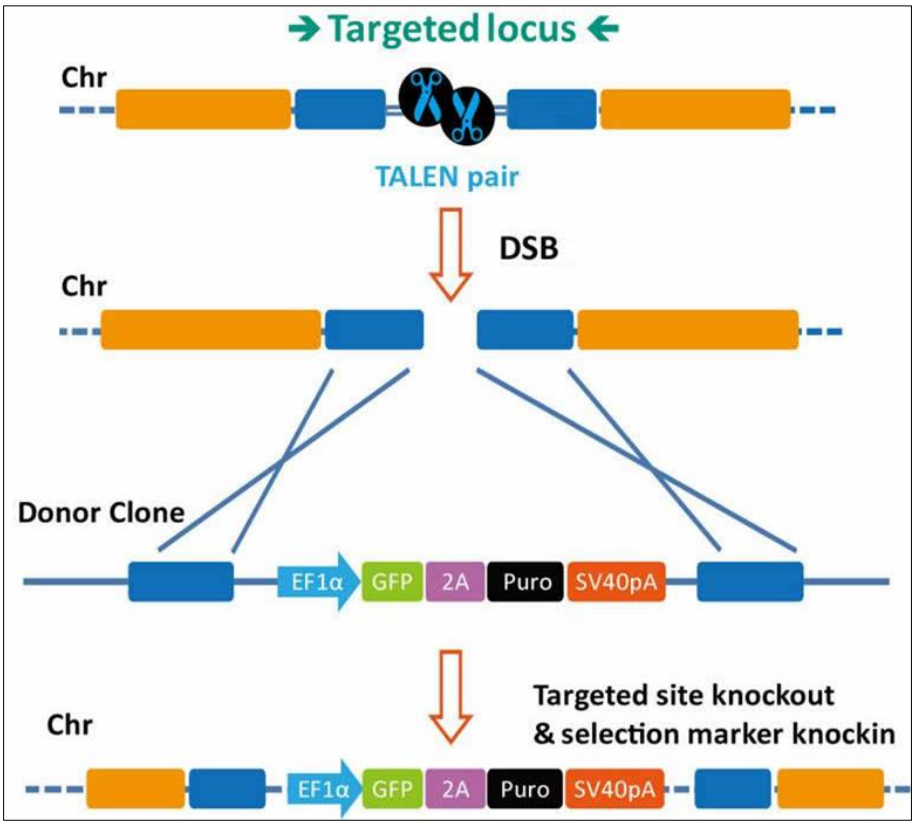

Fig 7: Different Steps of TALENs

Base Editors

The majority of human diseases are caused by single nucleotide variations (SNVs), which also play a significant role in the genetic foundation of phenotypic variation in livestock (Li *et al*., 2020) [31, 34]. So, it's critical and pressing to create a tool that can accurately and efficiently replace single bases. In this context, researchers developed a novel tool for editing target genes that they named the base editing system. This implement was founded on the CRISPR-Cas system. Base-editing procedures, in contrast to CRISPR/Cas, include cutting a single strand of DNA at the target location and inserting new bases with the use of nucleotide deaminases and modified Cas proteins. CBE and ABE are the two main enzymes in the base editing system; CBE can convert G to T-A, while ABE can convert A to G-C, according to several base modification enzymes (Landrum *et al*., 2016) [29]. One potent technique for precise genome editing has emerged with the introduction of the BE system, and it offers numerous advantages. First of all, double-strand breaks are not necessary for BE-mediated gene editing. The NHEJ repair mechanism initiated by DSBs is not well-defined and has the potential to produce unnecessary indels. On top of that, cytotoxicity can be achieved by utilising DSBs in excess (Tan *et al*., 2013) [66]. Additionally, screening for extremely active sgRNA and Cas nucleases is unnecessary for researchers. Second, unlike the HDR repair route, which relies on donor DNA, the BE system can function independently of it. Practical issues include designing donor DNA that animals can efficiently use and efficiently transferring donor DNA into livestock cells (Zhou *et al*., 2019) [100].

These advantages have prompted certain organisations to employ BE systems for the purpose of conducting research on animal genetic improvement. Before this, (Xie *et al*., 2020) [83] utilised the CBE system, which consisted of BE3 and hA3A-BE3, in order to effectively induce single base alterations at several loci in pigs simultaneously, including the cellular, embryonic, and individual phases. In the beginning, the CBE system was utilised to set up larger animal models by producing base editing pigs. This was the initial application of the method. As a consequence of this, various groups have employed CBE and ABE in order to eradicate specific genes in pigs. These genes include GGTA, MSTN, CD163, GHR, and IGF2, and their elimination has been done in an effort to improve pig characteristics (Yue *et al*., 2021; Whitworth *et al*., 2014; Petersen *et al*., 2016; Zou *et al*., 2018; Symington *et al*., 2011). Sheep and goats mutated in Socs2, GFG5, and BMPRIB were produced at Northwest A&F University using BE3 and ABEmax (Li *et al*., 2016; Zhou *et al*., 2020; Zhou *et al*., 2017) [33, 101-102]. When it comes to precise control, BE-mediated mutation is superior to DSB-mediated mutation. Based on these findings, it appears that BE systems are a great way to boost reproductive, milk-making, wool-producing, and livestock output. Finally, a base editing system can greatly enhance the precision and efficiency of cattle breeding, in comparison to earlier gene editing methods. This, in turn, is anticipated to increase genetic improvement in large animals.

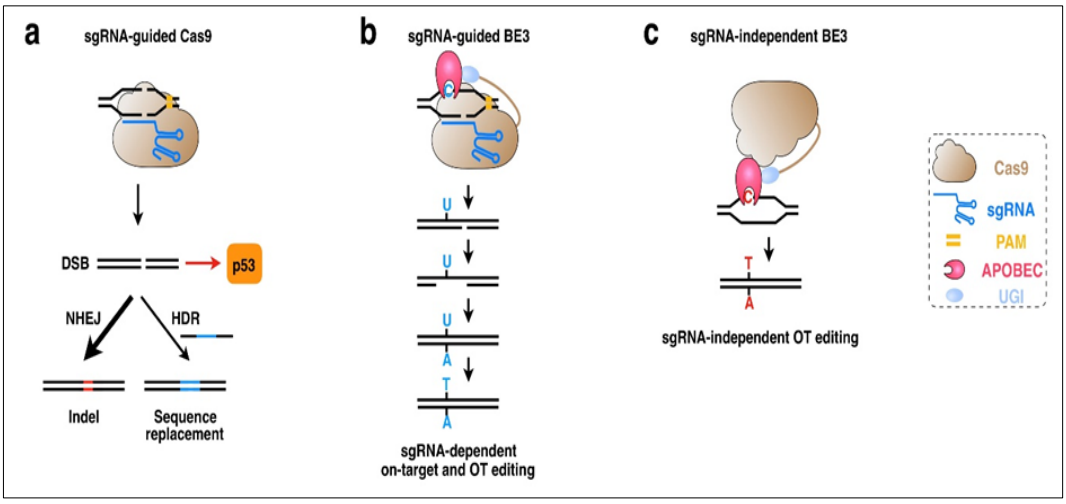

Fig 8: Different steps of Base Editors

Prime Editors

According to scientist, prime editor (PE), a new gene editing tool, has the ability to repair human genetic inherited mutations and is one of the most accurate methods for introducing point mutations (Anzalone *et al.*, 2019) [1]. This novel approach to prime editing eliminates the need for a donor DNA template or DSB in the target sequence, allowing for the insertion of point mutations. This gene editing tool, PE, is able to be transfected with a pegRNA thanks to a fusion of RT-nCas9 with a catalytically impaired nCas9 (H840A). Prime editing works molecularly by consistently locating the DNA target using a 5′ end of pegRNA that contains 20 nucleotides and a long 3′ end that extends to engage with the target sequence on the opposite strand. Through its RuvC nuclease domain, RT-nCas9 is able to disrupt single-strand DNA. After that, the fragmented DNA strand is positioned at the 3′ end of the pegRNA, which has a PBS attached to it. It had been stated that RT-nCas9 is capable of synthesising a new sequence by making use of the pegRNA template, which is located upstream of the PBS and contains the modification site. It is certain that the primary editing method developed by Dr. David Liu's group may successfully introduce targeted insertions and deletions into cells without the need for a double-strand break (Anzalone *et al.*, 2019) [1]. Using human HEK293T cells, they carried out 175 alterations, with an indels rate of less than 10% and an editing efficiency of 20 to 50%. Prime editing remains in its nascent stages, necessitating further research to fully harness its capability (Yan *et al.*, 2018) [85]. Despite the fact that prime editing shows significant potential for gene editing, the approach is yet in its early stages.

A long-standing goal of modern medicine is *in vivo* gene editing as a treatment for people with hereditary disorders. Prime editing has been tested for the treatment of several genetic illnesses and has been quickly implemented *in vivo* because to its many advantages over other genome-editing approaches mediated by CRISPR-Cas9 (Zhang *et al.*, 2018) [95, 98-99].

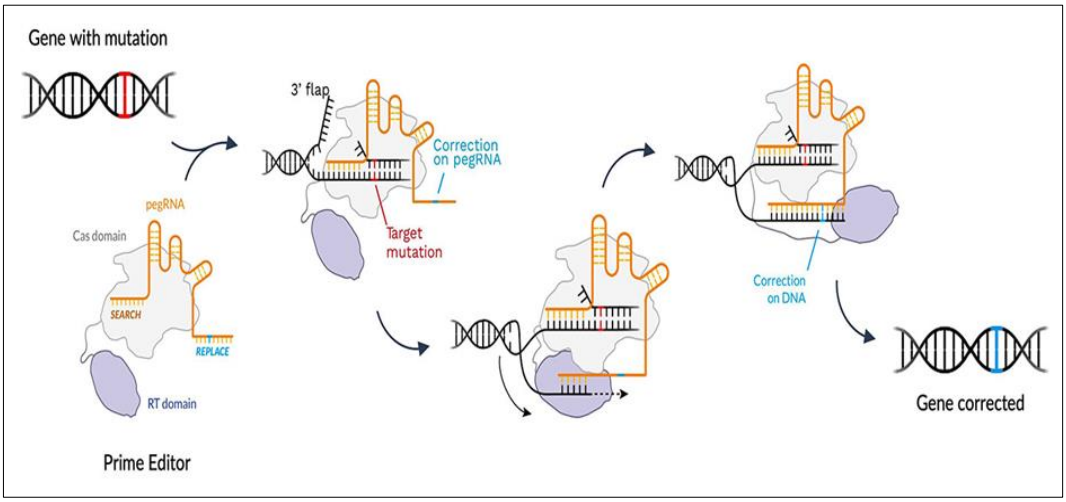

Fig 9: Different steps of Prime Editors

Conclusion

Finally, CRISPR-Cas9 and other genome editing technologies have completely altered the landscape of animal breeding. Improved production, disease resistance, and animal welfare can be achieved by the introduction of desired traits into cattle populations with the use of these techniques, which enable unparalleled accuracy, efficiency, and flexibility. Although TALENs and ZFNs are additional genome editing techniques that have demonstrated promise, CRISPR-Cas9 is now the most popular and flexible platform. Significant advances in cattle development are anticipated as a result of ongoing research into these technologies, which will contribute to

sustainable agriculture and global food security.

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