CRISPR-Cas9-Based Genome Engineering in Animals

Subjects: Agriculture, Dairy & Animal Science

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Genetic engineering and transgenesis provide the opportunity for more significant gains and production in a short span of time. One of the best strategies is the genetic alteration of livestock to enhance the efficiency of food production (e.g., meat and milk), animal health, and welfare (animal population and disease). Moreover, genome engineering in the bovine is majorly focused on subjects such as disease resistance (e.g., tuberculosis), eradicate allergens (e.g., beta-lactoglobulin knock-out), products generation (e.g., meat from male and milk from female), male or female birth specifically (animal sexing), the introduction of valuable traits (e.g., stress tolerance and disease resistance) and their wellbeing (e.g., hornlessness).

genome editing ZFNs TALANs CRISPR-Cas9 guide RNA livestock precision specificity

1. Introduction

Genome editing is the captivating genetic engineering approach with enormous potential in the biomedical application of gene function manipulation. It ensures the ability to treat or anticipate various genetic disorders through deletion, addition, or base change at a specific location of the desired organismal genome's gene of interest (GOI). The ideal genome-editing approach needs to effectively alter a genomic sequence, showing higher DNA sequence specificity with less or no off-target effects. The strategy of genome engineering has to possible change genomic sequence, also should have higher DNA sequence specificity with fewer or no off-target effects. The idea of genome engineering begins with the enhancement of several specific molecular tools. They work as precise molecular scissor, known as Zinc Finger Nucleases (ZFN) [1], Translation Activator-Like Effector Nucleases (TALENs) [2], MegNs (Meganucleases), and CRISPR-Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated nuclease (Cas) 9) [3][4][5] (**Figure 1**).

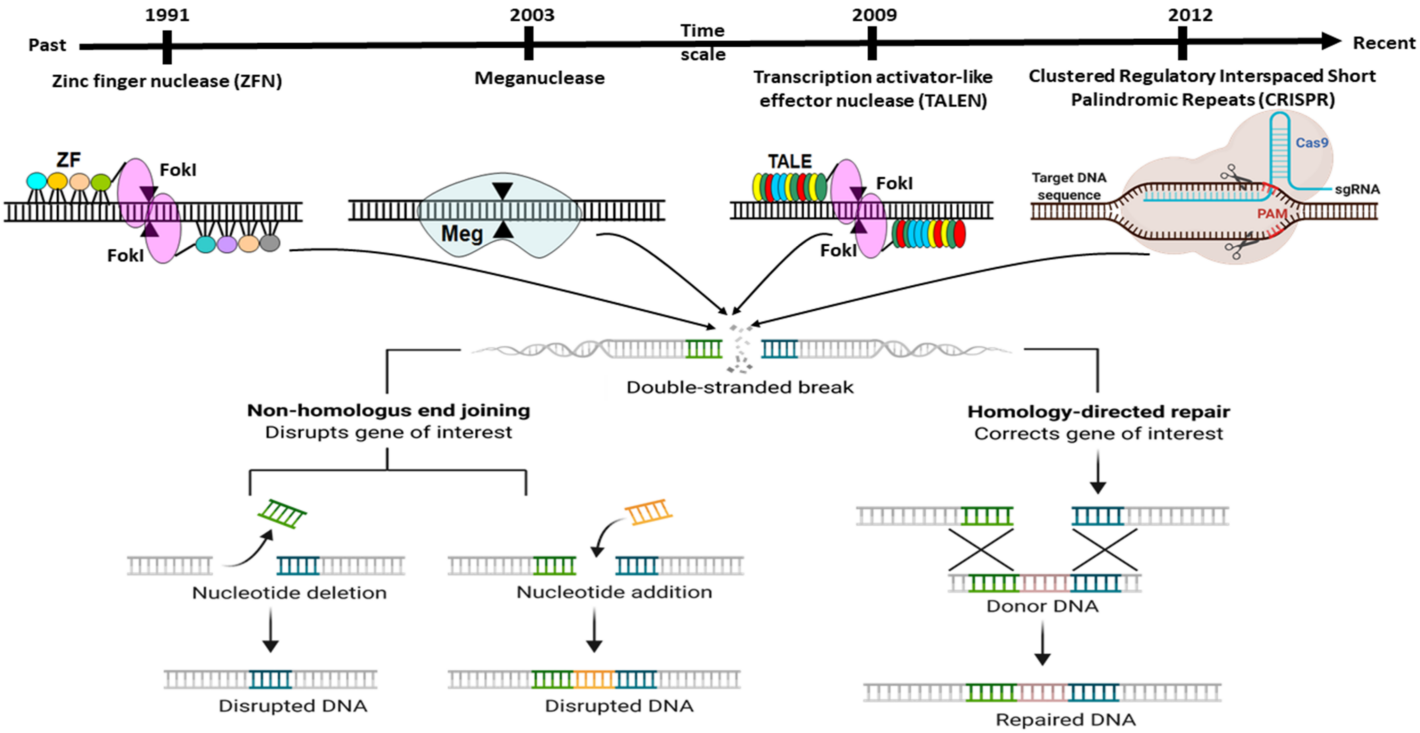

Figure 1. Diagrammatic illustration of various genome editing methodologies which is possible since the evolution of multiple precise molecular techniques. They work as exact molecular scissor, majorly, ZFNs (Zinc finger nucleases), TALENs (Translation activator-like effector nucleases), MegNs (Meganucleases), and CRISPR-Cas9 (Clustered regularly interspaced short palindromic repeats-CRISPR-associated system). Here, ZF: Zinc Finger, and PAM; protospacer adjacent motif.

CRISPR-Cas9, a bacterial antiviral framework, is the recently developed modern era of technology with gigantic potential capabilities. Shockingly, the thought of the strategy is motivated and adaptive from the single-celled microscopic organisms (bacteria) and archaea [6], where this life forms a utilisation endogenous CRISPR system as the versatile immune strategy. In essence, this is a defence mechanism against viruses or other pathogens' genetic sequences [7][8][9][10][11][12]. Moreover, these microbes are specialised in building up heritable memory of past assaulted phage or other pathogens through this strategy to cut up and devastate invader's DNA in peace and long-term prospects [13].

2. Adaptation of Adoptive Mechanism as CRISPR Editing System

CRISPR–Cas mediated immune response in microscopic organisms is noteworthy and comprises three mechanistic steps: spacer acquisition/adaptation, crRNA (CRISPR RNA) biogenesis/expression, and target interference. The molecular mechanism is specified all the way through each level; in the first stage, microbes capture a part of the hereditary/genetic material of viruses and integrate it as the primary spacer into the CRISPR cluster. In this way, it permits bacteria to remain immune against viruses or closely related ones in the future through making a genetic memory. During crRNA biogenesis, rehashed viruses' assault triggers the entire CRISPR

cluster expression to specific pre-crRNA (pre-CRISPR RNA), which assists into mature crRNA by ribonuclease RNase III and taken after binding with trans-activating crRNA (tracrRNA) through a direct repeat. Each crRNA contains a distinctive sequence for target interference; all crRNA and tracrRNA make a complex with Cas nuclease protein to form a ribonucleoprotein effector complex. The crRNA acts as a guide to superintend this effector complex to impair the viruses [8][14][15]. Few studies have shown 'how the prokaryotic CRISPR–Cas system can be utilised as a perfectional and exact molecular scissor after a couple of manipulations in crRNA', since single guide RNA (g-RNA) replaces the necessity of both the crRNA and tracrRNA. Therefore, effective gene editing through CRISPR employs two critical components: a g-RNA and the Cas9 protein [16][17].

For a long time, transgenesis in mammalian cells and especially embryos contains hurdles, mainly for large animals such as livestock. Since the discovery of the engineered nucleases adopted allows us, by adding a site-specific double-stranded break (DSB), to make precise the genetic manipulation of specific genes or sequences by means of HR (Homologous Recombination) and NHEJ (Non-Homologous End Joining) repair pathways [18]. However, site-specific Cas9 generated DSB effectively stimulated the HR pathway approximately 10,000-fold in the lower organism [19][20]. In contrast, the competitive NHEJ route for DSB repair, is routinely favoured and leads, as much as possible, to minor insertions or deletions (indels) in mammals [21][22]. For the development of biomedical models, therapeutic trials, and joint breeding, site-specific genome manipulation is a critical method. Although preliminary research on the use of engineered nucleases for precise genetic engineering of food animal species focused on ZFN [23][24][25][26][27], meganucleases [28], and TALENs [29][30][31][32]. Later, CRISPR/Cas9 gradually emerged as the tool of choice due to its easy architecture and implementation [33][34].

To begin, gene editing technology was developed in the late twentieth century and is still evolving. Despite this, the whole subject has garnered considerable attention since its discovery. The first gene editing technique that established a foundation in the area of recombinant DNA technology was ZFN, launched in 1991 and widely utilized for many decades [35]. Additionally, another gene editing technique known as TALENs was developed in 2009 in response to the discovery of the genome-targeting capacity of TAL effectors (TALE) [36]. Later that year, another interesting genome modification technique, termed CRISPR, was discovered (**Figure 1**). It synthesizes a combination of short directed RNAs (guide RNA) and Cas-9 nuclease and is forced to build a tailored endonuclease for each target, a need that TALEN and ZFN cannot meet (**Figure 2**). Since this discovery, the entrance barrier to genome editing has been substantially reduced, allowing for more user participation and creativity [36]. The CRISPR/Cas9 protein complex (tracrRNA) requires two RNA transcripts: the crRNA and the trans-acting CRISPR RNA [37][38]. When this dual RNA restriction is reconfigured as a single-guide RNA (sgRNA) of 19–24 bp, Cas9 is functional and effective in generating DSB into the target gene's DNA sequence [37].

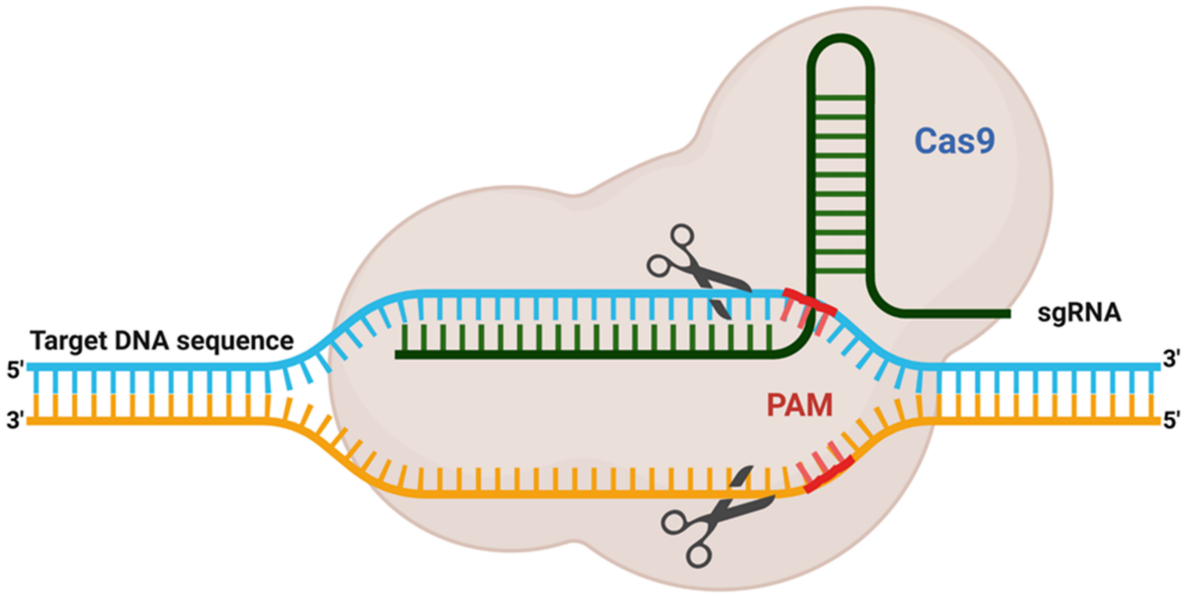

Figure 2. This picture is depicting the adopted functional CRISPR complex containing single guide RNA and Cas9 protein. It is the reconfiguration of natural dual RNA (tracrRNA and crRNA) system to a single-guide RNA (sgRNA) of 19–24 bp, which is good enough to program Cas9 to introduce DSB in target DNAs in vivo. PAM; protospacer adjacent motif.

3. Bioinformatics Tool Used to Design sgRNA for Gene Editing

As per previous studies, CRISPR/Cas9 protein recognises PAM sequence, sgRNA act to help to identify target loci followed by activation of endonuclease activity to cleave at a specific site. Cas9 enzyme cleavage activity varies significantly among different locations and cell types, owing to several factors that can affect the linking and cleavage potential of the sgRNA–Cas9 system. Therefore, various investigations have revealed that all included guide RNA characteristic (like composition, position and GC content), physical attributes (like melting temperature, and secondary structure formation) and chromatin remodelling for differential gene expression, together affecting the sgRNA efficiency. Various characterising tools were created to design highly efficient guide RNAs (**Figure 3**).

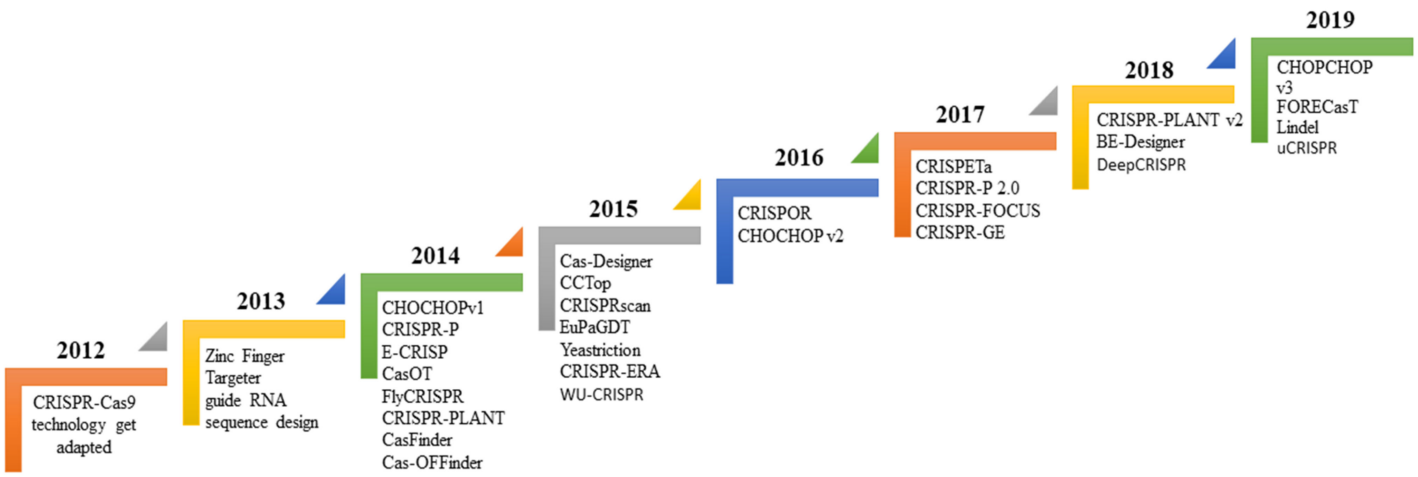

Figure 3. Time scale-based evolutionary representation of various Web-based sgRNA design tools from the past year to present.

4. Guide RNA Sequence Features

Target sequence nucleotide constitution is one of the concerning factors of sgRNA efficiency and specificity for the genome editing activities by Cas9 [39]. The broad-scale screening of CRISPR-based editing in mammals demonstrated that cytosine is more favourable at the cleavage position (-3 position proximal to PAM [40]. Similarly, guanine is most advantageous at site 1 and 2 ahead of the PAM sequence, whereas GC content of the downstream sequence of the PAM region, especially 4–13 bases, come up with sgRNA efficiency. Contrarily, thymine is not likely preferred at +/−4 nucleotides which neighbours the PAM [41].

However, sequence upstream to PAMs sequence may not influence sgRNA efficiency. The downstream line, on the other hand, is expected to have a major impact on efficiency [42]. Based on this valuable information, various efficiency models have been generated. The energetics related to the emergence of the guide RNA, DNA, and Cas protein complex are customary and might elucidate to eliminate biases between distinctive models, because a few energetics approaches may better outline the Cas nuclease editing effectiveness [42][43][44]. Furthermore, other factors, such as genetic and epigenetic properties, including gene position, chromatin accessibility, and expression, are also essential constraints that influence Cas nuclease activity and sgRNA binding [42]. However, various studies have investigated that nucleosomes negatively affect Cas9 target cleavage activity; on the other hand, DNase I hypersensitivity and epigenome markers affect guide RNA efficacy [45][46]. Keeping all property mentioned above and their effects on efficiency, numerous computational tools for evaluating guide RNA efficiency and prediction of its specificity have been created so far (**Table 1**).

Table 1. Computational tools are available for the design of sgRNAs to evaluation of guide RNA efficiency and prediction of its specificity. All the links were accessed on 7 June 2021.

Evaluation Guide RNA Efficiency	Link to Access the Algorithms
E-CRISP (Cas9)	http://www.e-crisp.org/E-CRISP/
CRISPRscan (Cas9, Cpf1)	https://www.crisprscan.org/gene/
evaluateCrispr (Cas9)	https://eu.idtdna.com/site/order/designtool/index/CRISPR_SEQUENCE

Evaluation Guide RNA Efficiency	Link to Access the Algorithms
sgRNAScorer (Cas9, Cpf1)	https://sgrnascorer.cancer.gov/
SSC (Cas9)	http://cistrome.org/SSC/
WU-CRISPR (Cas9)	http://crisprdb.org/wu-crispr/
Azimuth (Cas9)	https://github.com/MicrosoftResearch/Azimuth
CRISPRater (Cas9)	http://www.leukemia-research.de/resources/crisprater/
CRISPRpred (Cas9)	https://bmcbioinformatics.biomedcentral.com/articles/10.1186/s12859-020-3531-9
CASPER (Cas9, Cpf1)	https://pubmed.ncbi.nlm.nih.gov/28968798/
DeepCpf1 (Cpf1)	http://deepcrispr.info/
TSAM (Cas9)	https://pubmed.ncbi.nlm.nih.gov/29672669/
TUSCAN (Cas9)	https://github.com/BauerLab/TUSCAN
uCRISPR (Cas9)	https://github.com/Vfold-RNA/uCRISPR
Predict guide RNA specificity	
CasOT (Cas9)	http://eendb.zfgenetics.org/casot/
Cas-OFFinder (custom)	http://www.rgenome.net/cas-offinder

Evaluation Guide RNA Efficiency	Link to Access the Algorithms
sgRNAcas9 (Cas9)	http://www.biootools.com/
FlashFry (custom)	https://aaronmck.github.io/FlashFry/
Crisflash (Cas9)	https://github.com/crisflash/crisflash
MIT (Cas9)	https://crispr.mit.edu
CCTop (Cas9, Cpf1)	https://cctop.cos.uni-heidelberg.de:8043/
CFD (Cas9)	https://www.genscript.com/gRNA-detail/mouse/11537/Cas9/Cfd-CRISPR-guide-RNA.html
CRISPRoff (Cas9)	https://www.genscript.com/gRNA-detail/mouse/11537/Cas9/Cfd-CRISPR-guide-RNA.html
uCRISPR (Cas9)	https://github.com/Vfold-RNA/uCRISPR
CRISTA (Cas9)	https://crista.tau.ac.il/
Elevation (Cas9)	https://github.com/microsoft/Elevation
DeepCRISPR (Cas9)	http://deepcrispr.info/DeepSpCas9/

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5. A Different Mechanism for the Transport CRISPR System

The delivery of Cas9 in cells is an imperative thought in gene editing. Adopted CRISPR–Cas can be utilised in different ways or formats, for instance, m-RNA (direct transfection of sgRNA and Cas9 RNA), DNA (vector-based strategy), and in the form of RNP (ribonuclease protein complex). For detailed information around the CRISPR-Cas transport systems, it would be ideal to follow the recent review article by Lino et al. [47][48] (**Figure 4**A).

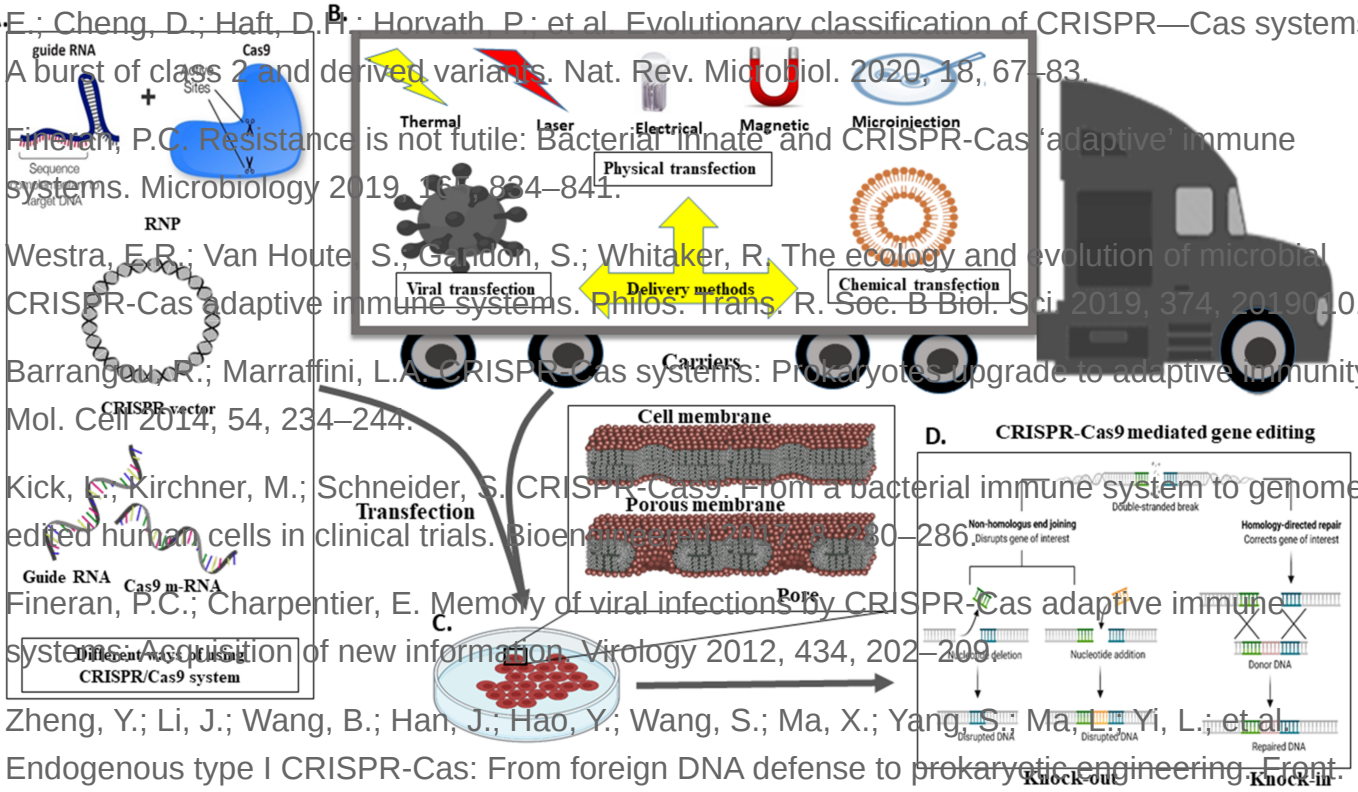

Figure 4. Different Methods of Delivering CRISPR/Cas9 into Cells. Schematic demonstration of in vivo CRISPR/Cas delivery modes and vehicles in numerous biological frameworks. Frameworks utilised to deliver CRISPR/Cas components can be separated into two major categories, CRISPR/Cas delivery mode and delivery carrier. (**A**) Three CRISPR/Cas delivery models, including protein (Cas protein with guide RNA as a ribonucleoprotein complex, RNP), DNA (plasmid encoding both the Cas protein and the gRNA), and RNA (mRNA for Cas protein translation and a separate gRNA), (**B**) Can be delivered into mammalians, aquacultures or plants by means of bacterial or viral vectors, chemical and physically directed delivery method, (**C**) To facilitate the delivery of the CRISPR system in the cell, transfection is accomplished by creating a membrane pore, and (**D**) Through the CRISPR framework, indel creation (knock out) or knock-in of a gene of interest in a targeted cell is possible.

The delivery method for CRISPR is very much similar to the standard transfection method for nucleic acid. CRISPR/Cas9 system delivery inside the cell usually conducts through either viral or chemical processes. Generally, physical processes are taken on electrical or mechanical forces to form transient pores in the membrane of cells, facilitating the update of CRISPR molecules. Moreover, recently, due to nanotechnology and microtechnologies, the physical method for transfection is in higher demand. For instance, nanostructure-mediated electroporation permits miniaturisation or shortened the physical transfection method to enhance transfection

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efficiency and precision [47]. It has the advantage that it homogeneously treats cells with improved viability and less damage to cells than bulk electroporation. Usually, CRISPR/Cas9 protein complexes have to be delivered in the cytoplasm of the transfected cells. To achieve efficient gene editing, CRISPR/Cas9 protein complexes must cross both the cell membrane and the nuclear membrane. As a result, the nuclear localisation sequence (NLS) directs the CRISPR/Cas9 system to the nucleus-encoded by the plasmid vector or the Cas9 protein. In the absence of an NLS sequence or signal, the CRISPR/Cas9 complex only enters the nucleus at the time of cell division when the membrane is disrupted [47].

6. Genome Editing in Ruminants Such as Cattle and Buffalos

It has been anticipated that in the population of 7.6 billion humans globally, every ninth individual (821 million people) does not have sufficient food to cover an active life [49]. Despite the lack of food, the human population is expected to rise to 8.5 billion in 2030, 9.7 billion in 2050, and 11.2 billion in 2100 [50]. As a result, the United Nations' Food and Agriculture Organization (FAO) predicts that total agricultural yield (crop yield and animal-based products) should rise to 60% to fulfil global demand. More specifically, this percentage is further contributed to by animal protein, such as meat production by 76%, and milk productivity will need to increase by 63%. In order to achieve this ultimate goal, a precise and practical approach should be used [51]. Meanwhile, genomic targets for genome engineering are possible by screening the differential expression using high throughput proteomics or genomics techniques [52][53][54][55]. In this regard, the generation of collective knowledge across the globe allows one to share and build the more efficient farm animals breeds [56].

Genome-editing and transgenic innovations offer the chance for more significant gains over a shorter time. Until now, genome editing investigation in cattle has centred fundamentally on enhancing the efficiency of food productivity (e.g., meat and milk), animal health, and welfare (animal production, such as cow hornlessness and disease), generate all-male offspring, eradication of allergens from products (e.g., beta-lactoglobulin knock-out). On the other hand, genome editing might be utilised to precisely knock-in valuable alleles (such as heat tolerance, illness resistance), as well as haplotypes into our native locally well-adapted cattle breeds genome, subsequently to improve their productivity [57]. We recently used the buffalo mammary epithelial cells to understand lactogenic signalling [58][59].

Early research was majorly focused on animal growth. Skeletal muscle gives meat for human utilisation or consumption, consisting of muscle fibres, intramuscular adipose tissues, and connective tissues [60]. The importance of growth hormone (GH) and insulin-like growth factor I (IGF-I) in regulating body size in developing animals has long been recognised. GH and IGF-I play an essential role in muscle growth, both before and after birth [61]. The GH–IGF axis (growth hormone-insulin-like growth factor axis), regulated by the pituitary gland and liver, is responsible for muscle growth and body mass [62]. GH, on the other hand, induces the development of IGF-I in almost all tissues. The liver is the only organ that can primarily produce serum IGF-I. The pituitary gland produces GH, which stimulates the development of IGF-I in other tissues (liver and muscle). Even though some cIGF-I is released from other tissues, such as muscle, the liver is the most common source of circulating IGF-I (cIGF-I). cIGF-I is a component of the negative feedback loop that controls GH secretion [61]. IGF-1 derived from

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both muscle and liver plays a crucial role in myogenesis [63]. At the same time, a mutation in the IGF-2 gene's regulatory function has been linked to increased muscle growth in pigs [64]. In recent years, effective microinjection of the GH and IGF-1 genes into pig zygotes has been reported. Later, two lines of GH-expressing transgene pigs gained 11.1 and 13.7 percent more mass than control pigs [65][66][67]. When transgenic technology is combined with recent genome editing technology, it creates a new age or property for animal protein that could affect animal welfare, while meeting human diet demands. The cloned pig, for example, that expresses the fat-1 gene from the nematode C. elegans, has a lower ratio of n–6 to n–3 fatty acids. A higher ratio of n–6 to n–3 fatty acids has been linked to poor bone health in humans. A lower ratio is related to healthier bone properties; thus, reducing both fatty acids can have nutritional health benefits in a diet [68].

Furthermore, related modifications have been observed in pigs containing the C. elegans n–3 fatty acid desaturase gene (encoded by the fat-1 gene) [69][70][71]. Similar findings were obtained when CRISPR/Cas9 was used to insert the fat-1 gene into the pig in the rosa 26 locus [72]. This is in proximity with gene alteration (genetic manipulation), which depends on the internalisation of the artificial gene (transgenes) to improve characteristic traits in animals. The genome/gene editing method allows us to make precise and error-free modifications to a livestock animal's genome, to increase productivity, production, and infection resistance. In the genome editing region, targeted gene editing of the myostatin gene is a popular goal for increasing growth and muscle production. They were first noticed in heavily muscled sheep and cattle like Piedmontese and Belgian Blue cattle and the Texel sheep breed. Additionally, it was discovered that decreased expression of the myostatin gene (also known as GDF8, or growth differentiation factor 8) results in increased muscle growth. Single-nucleotide polymorphisms in the myostatin gene trigger a fundamental genetic change. The Piedmontese and Belgian Blue have a single-nucleotide polymorphism in the myostatin gene and an 11-bp deletion in the myostatin gene [72][73][74].

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