Genome Editing by Programmable Nucleases and their applications in livestock species

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Abstract

Genetic modification in livestock species is almost 30 years old but many technical issues limited its use. The earlier methods - pronuclear injection and viral integration had several problems including random integration, silencing, low efficiency, inefficient gene expression regulation etc. With the advent of programmable nucleases such as Zinc Finger Nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs) and Clustered Regularly Interspaced Short Palindromic Repeats/Cas9 (CRISPR/Cas9), precision genome editing has now become possible. Genome editing by these nucleases is an efficient and powerful technique to alter the genome of organisms for site-specific modification and integration of endogenous and exogenous genes respectively. These techniques have already been used in species like Drosophila, zebrafish, mice, rats, monkeys, humans, pigs, cattle, sheep, goats and others. Generation of genome edited livestock has been proved feasible and valuable and they are useful for the understanding of complex physiology, generation of animals with improved traits and disease resistance, producing transgenic animals for the therapeutic proteins and large animal models for human diseases. Given the high efficiency of these techniques, it is expected that a large number of genome edited livestock will be produced in the future. In this review, we provide an overview of the programmable nucleases, their underlying mechanism, and usefulness of genome editing in livestock species.

Keywords- Nucleases; Genome editing; ZFN; TALEN; CRISPR/Cas9; Livestock

Introduction

Livestock species are important in agriculture, medical research, and medicine. Genetic modification of these species is important to improve animal welfare from feed conversion to disease resistance, improved nutrition and fit to the environment and also in the creation of humanized protein drugs and humanized transplant organs i.e. xenograft (Tait-Burkard et al., 2018). Large animal's genome modification is also important because of their similarity with humans in terms of physiology, anatomy and organ size where mouse model is not sufficient. For example, Cystic Fibrosis Transmembrane conductance Regulator (CFTR) KO pig model is similar to humans whereas mouse model of CFTR KO doesn't resemble at all (Keiser et al., 2011). The technique of livestock genome modification is more than three decades old. The use of integrating viruses for the genetic modification of animals is very old (Jaenisch et al., 1976) but in this case, the integration of gene is random. It also has smaller cargo size, limited promoter strength and specificity is worst (Hofmann et al., 2006). Additionally, the efficiency of transgene introduction into the genome of recipient animals is low. Pronuclear injection which was developed in the 1980s has been in use for a long period (Brinster et al., 1981) and somewhat better than the viral method. In this method, DNA having a desired gene construct is injected into a single cell fertilized embryo. The embryo is then implanted into the recipient mother and offspring are checked for the presence and expression of the transgene. But this method also has many drawbacks. First, the integration is random and that too takes place at multiple sites leading to the formation of concatamers which make the expression of gene difficult to control. Second, although the size of the construct is bigger than in viral method but still limited and hence the promoter should be small and genes are introduced in their spliced forms. The third problem is the fact that transgene introduction efficiency is very low. So considering the very low numbers of embryos in farm animals, it seems difficult. In mice, the use of embryonic stem (ES) cells is an alternative to produce transgenic animals (Doetschman et al., 1988) as these cells are immortal and available in extremely high numbers. In this method, ES cells are transfected with targeting vector having selection cassette (usually antibiotic resistance). These targeting vectors are exchanged by homologous recombination and the genetically modified ES cells are selected on the basis of antibiotic resistance and transferred into donor blastocyst which is implanted in recipient mice leading to the birth of chimera and then transgenic mice in next generation (Evans et al., 1985). However, with the exception of mouse and rat, germline competent ES cells have not been established yet in farm animals (Zafar et al., 2018) and this technique is not possible in these animals. In the absence of bonafide ES cells in livestock species, the only method left is Somatic Cell Nuclear Transfer (SCNT) developed in the late 1990s (Campbell et al., 1996). In SCNT, primary cells mostly fibroblasts are used for the manipulation of donor genome utilizing homologous recombination and the integration of a DNA sequence by this method is an extremely rare event (one in million cells). After a long period of clonal selection and propagation most of the cells die as these are finite or mortal cells and if survive then cells with correct integration are used to reconstitute an enucleated oocyte and the resulting offspring are clones. SCNT is still an inefficient method in which less than 10% of the embryos result in viable offspring (Gurdon et al., 2008; Wilmut et al., 2002; Yang et al., 2007; Petersen et al., 2008). Even some of the viable offspring have developmental anomalies due to poor reprogramming. Though SCNT is a feasible method to produce transgenic livestock, it is a very inefficient and costly procedure that requires enormous expertise available only in a few labs of the world. The idea of genome editing or modification is simple; base pairs at specific locations in the genome can be deleted, changed or added. These changes are permanent and if made in germ line cells, are heritable. Genome editing (GE) involves the modification of specific genomic sequences in living cells for the purpose of determining, changing, or expanding their function(s). With the discovery of programmable nucleases, targeted genome editing now seems practically feasible in a comparatively easy and cost-effective way with high efficiency. Almost all genome editors comprise of either customized protein sequence or small RNA and a nuclease. Nucleases are usually DNA cleaving enzymes which could be either exonucleases or endonucleases based on whether they cut at the end or the middle of DNA respectively.The nucleases in case of genome editors are invariably endonucleases. The customized protein sequence and RNA binds to the DNA at defined target site followed by cleavage by nuclease near or within the site. These genome editors (GEs) viz- ZFNs, TALENs, and CRISPR/Cas9 produce site-specific double-strand breaks (DSBs) which are repaired by cells own DNA repair mechanisms namely non-homologous end joining (NHEJ) and homology-directed repair (HDR). In the absence of a homology arm, NHEJ is functional which causes insertion and deletion of bases giving rise to indel leading to a frameshift mutation and hence gene knockout. HDR is a rare event and possible only in presence of a donor homologous template leading to knockout (KO) or knockin (KI). These nucleases have revolutionized the field of genome editing and can be used in all organisms including livestock. Here we give a brief introduction of these genome editors, their mechanism of action and how they have been and will be used in producing genetically modified livestock.

Nuclease based genome editors (GEs) and their mode of function

***Zinc Finger Nucleases (ZFNs)* - F**irst zinc finger (ZF) domain having specific affinity to bind DNA was discovered as a member of the transcription factor IIIa in Xenopus oocytes (Miller et al., 1985). A typical ZF is comprised of Cys_2-His_2ZF DNA binding domain consisting of about 30 amino acids which contain two anti-parallel β-sheets and a α-helix and a zinc ion coordinated by two cysteine residues in the β-sheets and two histidine residues in the α-helix. Zinc finger domain is mainly involved in DNA binding as each finger recognizes a three nucleotide triplet through its α-helix called as recognition helix. So a three finger ZF domain binds with a 9 bp sequence. A ZFN consists of a site-specific zinc finger binding domain covalently linked to the non-specific DNA cleavage domain of the Fok1 restriction endonuclease (Kim et al., 1996, 1997). Chandrasegaran and his colleagues found that the DNA binding and non-specific DNA cleavage functions of Fok1 could be divided into different domains (Li et al 1992). They fused the Fok1 nuclease domain with ZFs and created artificial nucleases with novel specificities (Kim et al 1996). This represented the first demonstration of ZFNs. ZFNs basically cleave target DNA as a dimer and this doubles the number of specifically targeted nucleotides (Smith et al., 2000). So the target of three finger ZFNs will be 2x9=18 bp in length, which is considerably long enough to specify a single target site in the genome. The ZFN molecules bind to the target DNA separated by a 5-7 nucleotide spacer, with double-stranded breaks occurring in the spacer region (Figure-1).

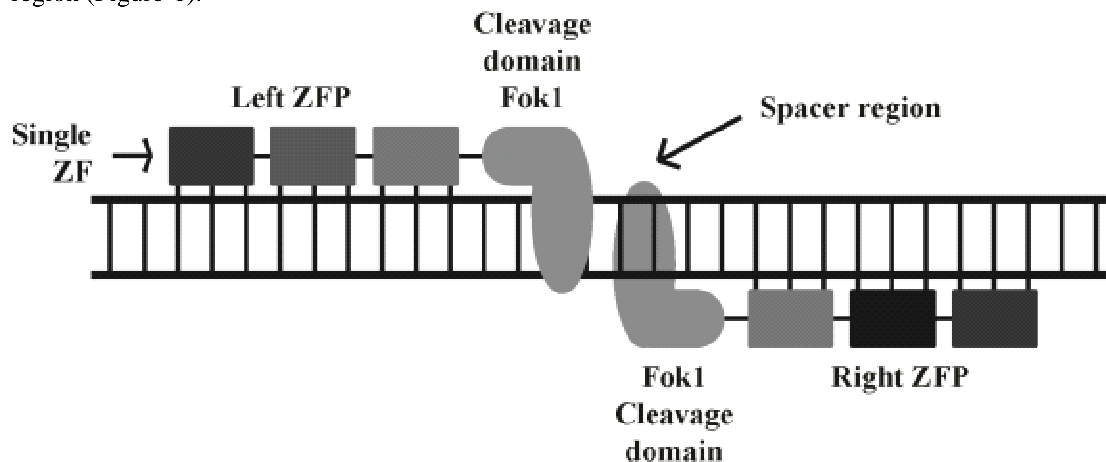

Fig 1:- Zinc Finger Nuclease (ZFN) bound to DNA- Shown here is three fingers containing Zinc finger domain fused with the cleavage domain of Fok1. Each zinc-finger consists of approximately 30 amino acids in a ββα arrangement and it binds to a three nucleotide triplet through its α-helix. ZFN target sites consist of two zinc-finger binding sites (left & right) separated by a 5–7-bp spacer sequence recognized by the FokI cleavage domain.

Transcription Activator-Like Effector Nucleases (TALENs)- TALE proteins first discovered by Bogdanove et al (2010) are derived from the plant pathogen Xanthomonas and related genera of bacteria. They are injected by type III secretion system of bacteria, bind to their host DNA, and act as transcription factors and activate the expression of plant genes that aid bacterial infection. They contain an N-terminal domain, a DNA binding domain and a C-terminal domain including an activator domain. The DNA binding domain consists of multiple nearly identical repeats (7-30), each of which is composed of approximately 34 amino acids. There are two key amino acids at position 12 and 13 which are involved in target recognition. These positions are highly variable among repeat units and called as repeat variable di-residues (RVDs). Each RVD recognizes one nucleotide (Boch et al., 2009; Moscou et al., 2009). The amino acids of RVD and the recognition nucleotides are well defined being NI for A, NG for T, HD for C, NN for G or A and NH for G only (Cong et al., 2012). The last repeat of the DNA binding domain is called half repeat as it contains RVD which is only conserved in the N-terminal half of the repeat. The target sequence for the TALE proteins always starts with thymine. TALE repeats can be used to engineer DNA binding motifs which can recognize specific sequences in the mammalian genome. The binding domain is linked with the non-specific cleavage domain of the type II restriction endonuclease Fok1 and the whole assembly is now called TALEN which can be used to introduce site-specific double strands breaks leading to stimulation of NHEJ and HDR (Cermak et al., 2011; Christian et al., 2010; Li, T. et al, 2011a, 2011b; Mahfouz et al., 2011; Miller et al., 2011).

Like ZFNs, TALEN also binds to the target site as a dimer with nuclease domain of Fok1 sitting on 13-33 bp spacer sequence where cleavage occurs (Figure-2). TALENs have been used to produce knock out in mice, rats and zebrafish (Huang et al., 2011; Sander et al., 2011; Tesson et al., 2011) and in cattle, sheep and pigs, thus showing that TALENs are effective in inducing genetic modification in a broad range of different species (Carlson et al., 2012; Proudfoot et al., 2015). TALENs are superior to ZFNs in terms of simplicity, time, cost and straightforwardness in design and assembly strategies. They also have higher genome editing activity and low off-target effects than ZFNs. These properties of TALENs are attractive for many researchers who are interested in genome editing.

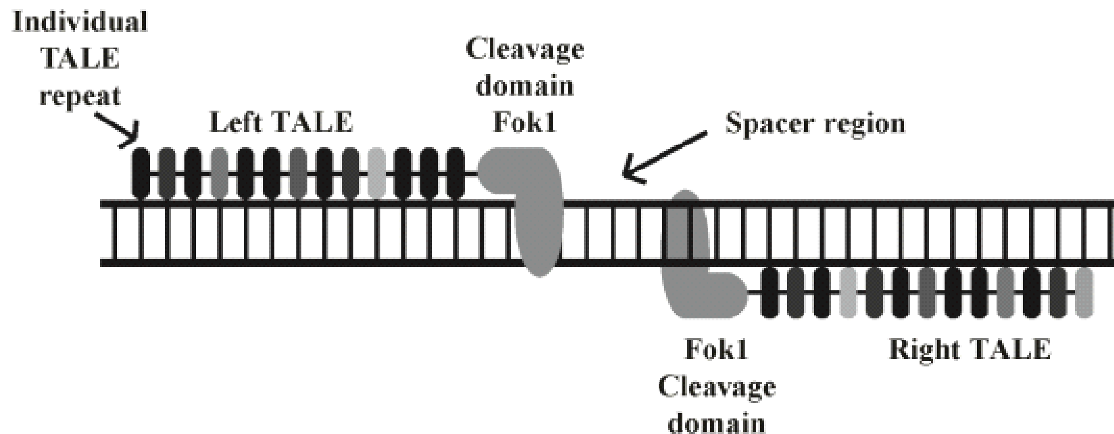

Fig 2:- TALEN dimer bound to DNA- Individual TALE repeats consist of approximately 34 amino acids that bind to a single nucleotide via repeat-variable di-residues (RVD). TALEN target sites consist of two TALE binding sites (left & right) separated by a spacer sequence of varying length (12–20 bp).

CRISPR/Cas9 (RNA-guided genome editing) **-** An RNA guided endonuclease (RGEN), known as CRISPR/Cas9 has revolutionized the field of genome engineering. This system is much easier to introduce than ZFNs and TALENs because of its simple construction of customized vectors targeting specific loci. The CRISPR locus was first discovered by Ishino and his colleagues in the genome of E.coli and later on by different groups in other eubacteria and archaeabacteria (Ishino et al., 1987; Mojica et al., 2000; Jansen et al., 2002). It contains tandem repeats of the same sequence separated by spacers having different sequences derived from foreign DNA (Mojica et al., 2005; Pourcel et al., 2005). The CRISPR locus works with CRISPR-associated (Cas) proteins as an adaptive immune system against invading viruses and foreign DNA (Wiedenheft et al., 2012; Westra et al., 2014). In type II CRISPR system, the sequence from the invading DNA is incorporated between repeat sequences encoded as an array within the bacterial genome. CRISPR repeat arrays are transcribed as CRISPR RNAs(crRNAs) each having a variable sequence from the invading DNA (Protospacer) and part of the CRISPR repeat. Each crRNA binds with transactivating CRISPR RNA (tracrRNA) and these two RNAs form a complex with the Cas9 nuclease. The protospacer-encoded part of crRNA guides Cas9 to cleave complementary target DNA sequence if they are adjacent to protospacer adjacent motifs (PAMs). Protospacers in the CRISPR locus are not cleaved because PAM sequence is not present next to them (Bhaya et al., 2011; Reeks et al., 2013; Barrangou et al., 2014). The canonical PAM sequence in most of the cases is 5’-NGG but rarely 5’NAG or others. When introducing a DSB, two nuclease domains HNH and RuvC in Cas9 independently induce a nick at the Watson and Crick strands resulting in a linear DSB between the bases 3- and 4-bp upstream of the PAM sequence (Jinek et al., 2012; Nishimasu et al., 2014). Type II CRISPR system from *Streptococcus pyogenes* has been adapted for genome editing. The process needs only two components- a chimeric guide RNA (gRNA) resembling the crRNA-tracrRNA complex and Cas9 protein with nuclease activity (Jinek et al., 2012; Cong et al., 2013; Mali et al., 2013). Twenty nucleotides at the 5’ end of the gRNA (the protospacer part of crRNA) direct cas9 to a specific target DNA site adjacent to PAM using standard RNA-DNA complementarity base-pairing rules. So Cas9 nuclease activity can be directed to any DNA sequence of the form N_{20}- NGG simply by altering the first 20 nt of the gRNA to correspond to the target DNA sequence (Figure-3). The CRISPR/Cas9 system has proven to be a simple and versatile system for targeted genome editing in a large number of organisms. Furthermore, CRISPR/Cas9 allows simultaneous targeting of multiple genomic loci (multiplex genome editing).

CRISPR/Cas9 has similar specificity as ZFNs and TALENs (Gaj et al., 2013), but due to the RNA guided recognition site, it has the advantage of being very simple to generate, easy to handle, efficient and cost-effective. To improve specificity and reduce off-target cleavage many modifications have been done. Cas9 with nickase activity (HNH or RuvC domain inactive) to avoid off-target events (Shen et al., 2014) or Cas9 in which both the nuclease domain were made inactive, called dead Cas9 (dCas9) connected to Fok1 nuclease, which has to dimerize before cleavage and so increases the specificity (Gullinger et al., 2014; Tsai et al., 2014) are already available. The specificity can be further enhanced by using truncated gRNA (Fu et al., 2014) or by modification of Cas9 with no off-target cleavage (Kleinstiver et al., 2016; Slaymaker et al., 2016). dCas9 has also been used in single base editing without introducing DSBs (Komor et al., 2016).

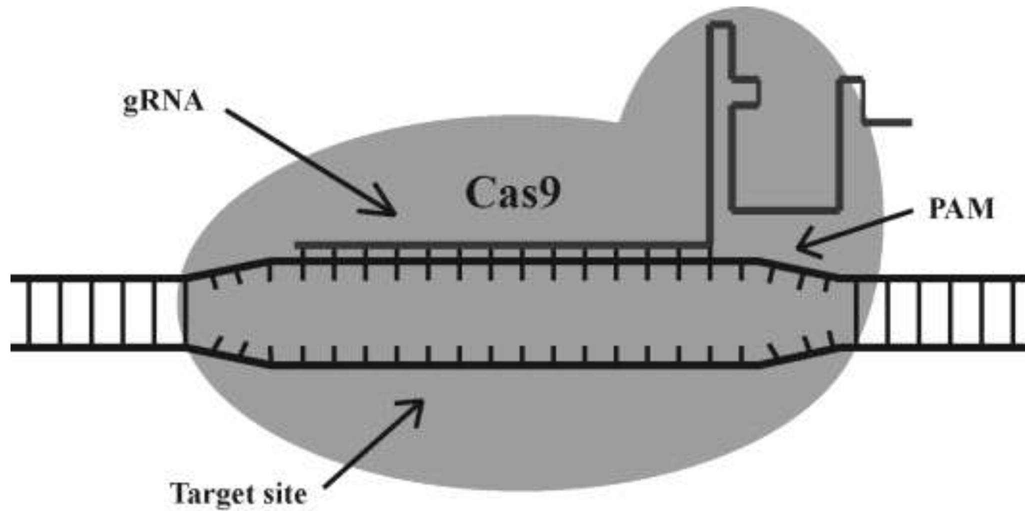

Fig 3:- CRISPR/Cas9- A chimeric RNA equivalent to crRNA-tracrRNA hybrid binds with Cas9 nuclease making a surveillance complex which searches the target site and binds to it. After 20-bp hybridization, the target DNA is cleaved by Cas9 nuclease, resulting in a blunt end at the 3-bp upstream of the PAM site.

All the three genome editing techniques mentioned above generate double-strand break (DSB) in the target DNA which is then repaired by Non-homologous end joining (NHEJ) or homologous recombination (HDR) (Figure 4).

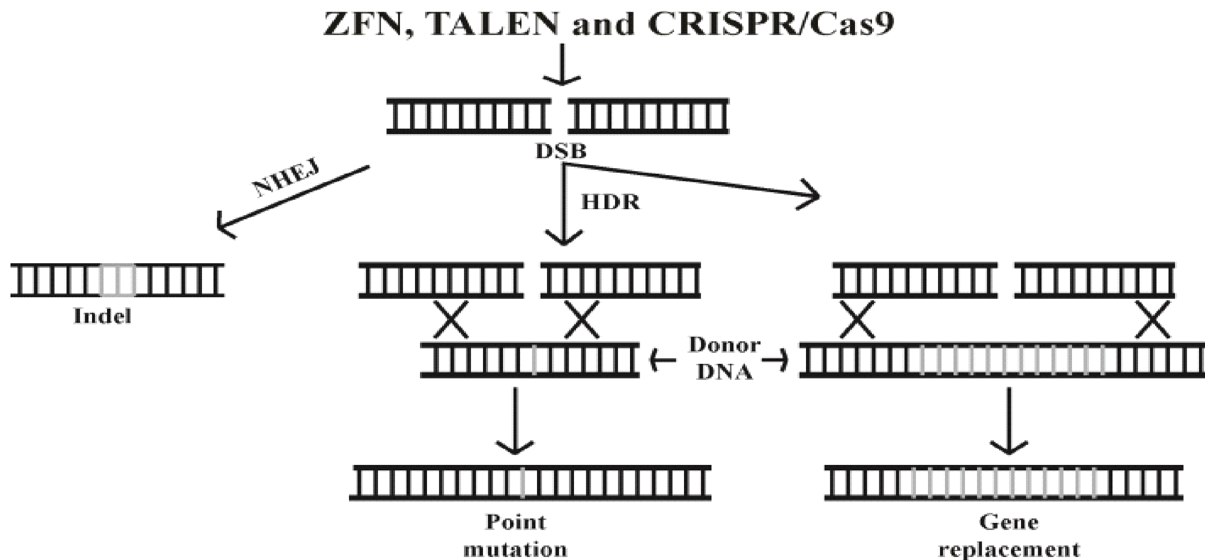

Fig 4:- Double Strand Break (DSB) repair by Non- homologous end joining (NHEJ) and Homology-directed repair (HDR). NHEJ gives rise to Indel leading to disruption of gene whereas HDR will cause substitution through homologous recombination with donor DNA.

Basic tools and steps for genome editing

The main steps in any genome editing process include- construction or designing of genome editors, their delivery into cells, selection of the transfected cells, single cell cloning, genomic DNA isolation, PCR based amplification of target site and eventually sequencing of the PCR product to confirm the editing or modification of the gene of interest. These steps are briefly discussed below.

***Designing of GEs*-** Construction of ZFNs and TALENs is a tedious process whereas the method of engineering of CRISPR component – gRNA and Cas9 is comparatively easy.

Five basic methods for designing ZFNs-
a) Sequential selection of ZFs with novel specificity using a randomized library and a phage display
b) Bipartite library and two-finger archive
c) Modular assembly
d) Bacterial-based selection of ZFs from a randomized library
e) Context-dependent assembly (CoDA)

Similarly, there are 4 ways by which TALENs can be constructed-
a) Golden gate cloning-based assembly
b) Golden gate PCR-based assembly
c) Restriction enzyme and ligation (REAL) assembly
d) Fast ligation-based automatable solid-phase high-throughput (FLASH) system

For CRISPR/Cas9, there are many web-based software for designing gRNA. Plasmids for constructing custom gRNA and Cas9 expressing vectors are available commercially. The construction process only involves insertion of annealed oligonucleotides into the vectors which is much simpler than the procedures of ZFNs and TALENs. The gRNA and Cas9 can be expressed using either separate vectors or a single combined vector. The details of the designing of the GEs are beyond the scope of this review.

***Delivery of GEs into cells*-** Introducing plasmids encoding nucleases into target cells by electroporation or transfection based on liposome or cationic polymer are most common (Porteus and Baltimore, 2003; Urnov et al; 2005). Methods based on delivering nuclease directly as proteins or as in vitro transcribed RNA have also been used (Shi et al; 2014; Kuhn et al; 2012). Transfection reagent free strategies based on direct nuclease delivery can be used on Protein Transduction Domains (PTD). Genetic fusion or chemical conjugation of nucleases to these positively charged moieties favour their uptake by cells (Shi et al; 2014). Protein transfection procedures for example electroporation of Cas9-gRNA ribonucleoprotein has been achieved in human embryonic stem cells (Kim et al; 2014). Chemical transfection agents such as cationic lipid are also being used as these agents protect protein cargos from serum inhibition or protease mediated degradation and help in endosomal escape. Although viral vectors are generally less straightforward to produce than most non-viral systems as they constitute instrumental gene, and in some cases protein and mRNA transfer tools (Cai et al; 2014; Mock et al; 2014) and are highly efficient in entering into the cells. Because the ideal mode of operation for GE tools is via “hit-and-run”, episomal viral vectors are preferable over their chromosomally integrating counterparts. Thus Integrase defective viral vectors (IDLVs, Baculoviral vectors (BVs), Adenoviral vectors (AdVs) and recombinant Adeno-associated viral vectors (rAAVs) are all being used for transducing nucleases or donor DNA into mammalian cells.

***Selection of the transfected cells*-** Usually the delivery vector has either antibiotic resistance cassette or Green Fluorescent Protein (GFP) gene to select the transfectant from a mixed population. In case of antibiotic selection, after 24 hours of transfection cells are grown in presence of respective antibiotic which leads to death of all the non-transfected cells and only transfected cells proliferate. When there is a GFP gene in vector, all the transfected cells are checked for the expression of green protein after 24 hours and GFP+ cells are sorted with the help of Fluorescent Activated Cell Sorter (FACS).

Single cell cloning- Using serial dilution method single transfected cells are transferred in each of the wells of 96 well plate for expansion.

***Genomic DNA extraction*-** Scaling up of cells derived from single clone is done from 96 to 24 and then 6 well plate. At this stage genomic DNA is extracted from the individual clones using either phenol-chloroform method or commercially available kits.

***PCR of target site* -** Using primers designed against the target site flanking region, the amplification is done for all the expanded clones.

***Sequencing* -** The PCR products of individual clones are used for sequencing to check if there is any mutation in the target site. Any modification in the nucleotide sequence is an indication that gene editing has taken place. These gene edited positive clones are further expanded for downstream process.

Application of genome editors in livestock species

Genome editing has completely transformed the field of transgenic livestock production. In earlier days generation of knockout or knockin in livestock species has been extremely difficult due to the absence of germline-competent embryonic stem cells in these animals but with the discovery of customized nucleases, it has now been possible to produce animals with desired genotype using either cytoplasmic microinjection or SCNT. These genome editors offer an easy and user-friendly way to modify the genome of any organism including farm animals of economic importance. In the last two decades, genome editing has been done in a large number of livestock species such as cattle, pig, sheep, and goat using ZFNs, TALENs and CRISPR/Cas9 (Ruan et al., 2017). Many of the traits are controlled by multiple genes in higher animals and with CRISPR/Cas9 technique it is now possible to do multiplexed genome editing at a given time (Van Eenennaam et al., 2018). In recent years, many published reports of genome editing appeared in livestock and we are mentioning here only important ones mainly in cattle, pig, sheep and goat.

Cattle- Various genome-editing techniques have been used to date in cattle for specific gene modifications (Ericksson et al, 2017; Yum et al., 2018).Using CRISPR/Cas9 system NRAMP1 (natural resistance to infection with intracellular pathogens) gene was introduced in cattle giving resistance against *Mycobacterium bovis* which causes tuberculosis in these animals (Gao et al., 2017). SP110 gene has been introduced using TALEN in the intergenic region of macrophage of bovine making it resistant to M.bovis (Wu et al., 2015). Myostatin (MSTN) is a negative regulator of growth hormones and causes less muscle tissue production in Animals. Naturally occurring Belgian bull in which this gene is mutated shows a high level of muscle production and increased body mass. By knocking out the MSTN gene using TALEN mRNA, cattle with increased muscle mass was produced (Proudfoot et al., 2015). In another report, this phenotype was achieved using ZFN followed by SCNT (Luo et al., 2014). β-lactoglobulin (BLG) is a major allergen in cow milk. Using ZFN, BLG knockout cow after SCNT was generated (Yu et al., 2011). It was also produced by ZFN and TALEN microinjection in the embryo (Wei et al., 2015). Human lysozyme (Hlyz) gene was inserted into the cattle β-casein locus using ZFN (Liu et al., 2014). The human lysozyme was secreted in the milk making it resistant to bacteria causing mastitis. Polled locus is responsible for the growth of horn in cattle and using TALEN this locus was knocked out in Holstein cattle to produce hornless animal (Carlson et al., 2016). Efficient edition of the bovine PRNP prion gene in somatic cells and IVF embryos were done using TALEN (Choi et al., 2015) and CRISPR/Cas9 system (Bevacqua et al., 2016). This strategy can be further used to produce prion-free cattle. Lysostaphin is an antimicrobial agent against Staphylococcus aureus. ZFN nickase mediated insertion of the lysostaphin gene into the β-casein locus of cloned cows has been done (Liu et al., 2013).

Pigs: - The genome editing techniques have been maximally used in pigs (Ryu et al., 2018; Yang and Wu, 2018). Pig resistant against infection with Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) was produced via genetic knockout of the CD163 receptor using cytoplasmic microinjection mediated delivery of CRISPR/Cas9 construct in embryos (Whitworth et al., 2016). The same goal was achieved by CRISPR/Cas9 mediated knockdown of SCRC5 domain from exon 7 of CD163 gene (Burkard et al., 2017). Using CRISPR/Cas9, Bi et al (2016) knocked out the MSTN gene for excess muscle production in the pig. Such pigs were also produced by ZFN (Qian et al., 2015) and TALEN (Rao et al., 2016). Rosa26 locus was used to knockin EGFP gene using CRISPR/Cas9 and Cre-recombinase (Li et al., 2014). EGFP transgene was also knocked out using ZFN (Whyte et al., 2011). The very same locus was also used to integrate Fat1 gene using ZFN which gives higher unsaturated fatty acid content and healthier meat (Tao et al., 2016). The uncoupling protein1 (UCP1) knockin using CRISPR/Cas9 in the white adipose tissue of pigs decreases fat deposition and thermogenic capacity (Zheng et al., 2017). RELA KO pigs having loss of expression of transcription factor p65 were produced using TALEN and ZFN (Lillico et al., 2013 and 2016). Ruan et al (2015) generated knockin pig using CRISPR/Cas9 by integration into H11 genomic safe harbor. This was the first safe harbor knockin pig. Swine induced pluripotent stem cells (iPSCs) were also made using CRISPR/Cas9 (Feng et al., 2015). NANOS2 locus is responsible for germ cells formation. Using CRISPR/Cas9, NANOS2 KO pigs were

produced which can be used as a host to transfer the germ cells from genetically superior male to produce gametes (Park et al., 2017).

Genome editors have also been used to produce genetically modified pigs which would be extremely useful in the field of biomedicine. TALEN was used for porcine recombination activating gene 2 (RAG 2) knockouts to produce immunodeficient pigs useful in medical studies (Lee et al., 2014). Using direct injection of the CRISPR/Cas9 system into developing embryos, Lei et al (2016) generated RAG2/IL2RG deficient pigs presenting SCID (Severe Combined Immuno Deficiency) phenotype. The α 1,3- galactosyltransferase (α 1,3-GT) encoded by the GGTA-1 gene produces a sugar epitope on the surface of porcine cells and play a major role in xenotransplantation. So organs from α 1, 3-GT knockout pigs can be accepted by the human without a major immune rejection. α 1, 3-GT KO pigs were produced using ZFN followed by SCNT (Hauschild et al., 2011), by TALEN (Cheng et al., 2016; Kang et al., 2016), by cytoplasmic microinjection mediated delivery of CRISPR/ Cas9 (Petersen et al., 2016), CRISPR/Cas9 and SCNT (Butler et al., 2016) and α 1, 3-GT, PARKIN & DJ-1 knockouts without selection marker using TALEN (Yao et al., 2014). Knockouts of peroxisome proliferator-activated receptor gamma (PPAR-Ɣ) using ZFN (Yang et al., 2011) and low-density lipoprotein receptor (LDLR) using TALEN (Carlson et al., 2012) to produce a large animal model for cardiovascular disease. Dystrophin KO pigs using TALEN (Carlson et al., 2012) are a good model of Duchenne muscular dystrophy (DMD). Adenomatous-polyposis-coli protein (APC) KO using TALEN mRNA & CRISPR/Cas9 (Tan et al., 2013) is a model for certain types of intestinal cancer. Knockout of von Willebrand factor (vWF) using CRISPR/Cas9 (Hai et al., 2014) is a good model for a clotting disorder. SLA-1, 2, 3 and B2M (MHC family genes) knockout pigs were produced respectively using CRISPR/Cas9 (Reyes et al., 2014) and TALEN (Wang, Y et al., 2016). These knockouts are a universal organ donor for xenotransplantation. The active porcine endogenous retrovirus (PERV) sequence is a risk in xenotransplantation. PERV sequence knockout pig was produced using CRISPR/Cas9 (Yang et al., 2015). MITF protein is a master regulator of melanocyte development and an important oncogene in melanoma. Using CRISPR Cas9 MITF gene was mutated in pig giving Melanoma model (Wang, X et al., 2015b). CRISPR/Cas9 was used for the Knockin of human albumin cDNA into swine albumin locus in the zygotes for the production of human albumin in pigs (Peng et al., 2015). By co-injection of Cas9 mRNA and multiplexing single guide RNAs (sgRNAs) targeting parkin, DJ-1, and PINK1 genes, respectively, into in vivo derived pronuclear embryos, pig model of Parkinson's disease was generated (Wang, X et al., 2016). Tryptophan hydroxylase 2 (TPH 2) is the rate-limiting enzyme of serotonin synthesis in the brain. By knocking out this gene using CRISPR/Cas9 pig model of depression has been generated (Li et al., 2017). Generating B cell-deficient mutant is the first step to produce human antibody repertoires in a large animal model. Using CRISPR/Cas9, JH region of the pig IgM heavy chain gene which is crucial for B cell development and differentiation was targeted (Chen et al., 2015).

Goats and sheep- MSTN knockout goats with increased muscle mass were produced using CRISPR/Cas9 (Ni et al., 2014; Guo et al., 2016) and by TALEN (Yu et al., 2016). Additionally MSTN and FGF5 KO goats were also generated by CRISPR/Cas9 (Wang, X et al., 2015a). FGF5 inhibits the growth of hair, so knocking out this gene will lead to long hair in cashmere goat. Goats with β-lactoglobulin knockout (BLG KO) were produced using CRISPR/Cas9 (Ni et al., 2014; Zhou et al., 2017), ZFN (Xiong et al., 2013; Song et al., 2015) and TALEN (Cui et al., 2015). Growth differentiation factor 9 has a profound effect on litter size and by knocking out this gene in cashmere goat using CRISPR/Cas9, litter size was increased (Niu et al., 2017b). Sheep with MSTN KO was also produced by injecting TALEN mRNA and CRISPR/Cas9 mRNA (Proudfoot et al., 2015; Crispo et al., 2015). Niu et al (2017a) used CRISPR/Cas9 to increase yellow fat in sheep meat by removing β-carotene oxygenase 2 (BCO2) gene. Melatonin in sheep milk was produced using CRISPR/Cas9 (Ma et al., 2017).

Future prospects and challenges

Genome editors (GEs) have emerged as valuable molecular tools which have revolutionized the field of biological science including basic science, clinical and agricultural research. The eruption of GEs is meant to cause an exponential rise in the appearance of genetically engineered livestock aimed to improve production rates, the product itself or animal and human health (Toranzo et al., 2017). Their importance in livestock production and improvement is already clear from a large number of genetic modifications done in these species. Genome editing technology can rapidly edit key genes that affect target traits and obtain the required genotype through a single generation of editing, thereby greatly reducing breeding time (Ruan et al., 2017). We can create genetic diversity by editing the gene with defects while keeping the superior traits untouched (Bastiaansen et al., 2018) They may be used for introducing traits that do not naturally exist in a particular species which is impossible by traditional breeding such as environmentally friendly gene edited animals having low phosphorous discharge in the excreta, transfer of African swine fever allele from warthog to pig using ZFN (Lillico et al., 2016). Using conventional

genetic engineering method transgenic sheep with TLR4 receptor gene was produced which may increase anti-brucellosis ability (Li et al., 2016) and lysozyme secretion was increased by anti-mastitis TLR2 transgenic sheep which effectively removed pathogen from milk (Deng et al., 2012). These modifications can be achieved rapidly and efficiently in a much convenient way by genome editing. Improved wool production in sheep, increased omega-3-fatty acids content in meat, production of humanized milk in dairy animals expressing protein from human milk such as Lactoferrin, α- lactalbumin, high yield of nutraceuticals such as lysozyme, β- and ß- casein and pharmaceutical proteins such as α-1-antitrypsin, C1 esterase inhibitor in milk are possible by genome editing technology. Many other proteins of therapeutic value in milk, blood, urine or seminal fluid are currently on clinical and pre-clinical trials (Bertolini et al., 2016). Models of a few human diseases have been made in pigs by genome editing and future efforts will be promising in producing many models of metabolic and other diseases. Many genes in the pigs can be edited to produce tailored organs for xenotransplantation. Human organs in pigs can be developed by interspecies chimerism (Wu et al., 2017). The gene responsible for a specific organ development is ablated in the pig embryo by using either ZFN or TALEN or CRISPR/Cas9 and chimera are produced by injecting human induced pluripotent cells (hiPSCs) in blastocysts followed by implantation in the recipient female. The offspring will have the organ of interest developed from the hiPSCs similar to a human organ.

There is no doubt that genome editing by these nucleases is the best way to get the livestock and other species of the desired genotype but this technology has certain limitations and drawbacks which need to be resolved before using it in a full-fledged way. A high degree of specificity is the main challenge and would be a critical prerequisite for employing these technologies in human patients or for the generation of livestock species (Petersen, 2017). The current genome editing technologies (ZFNs, TALENs, and CRISPR/Cas9) all have the potential to induce off-target mutations in the genome. Although these mutations may not have any impact on the health of individual animals, they still carry a potential risk and can create obstacles for the future promotion of genome editing (Ruan et al., 2017). Modification through NHEJ is hard to predict because the outcome of the modifications is random. Mosaic genotypes generated through direct injection of engineered endonuclease into zygotes could lead to founders with unexpected phenotypes (Ryu et al., 2018). A number of strategies have been suggested to overcome these shortcomings. Use of Cas9 nickase, modified to introduce only single-stranded breaks, was proposed to minimize complications associated with DSBs (Cong et al., 2013). Development of dead Cas9 (dCas9) conjugated to Fok1 endonuclease which requires dimer formation has higher affinity to the target site and hence increased specificity (Guilinger et al., 2014; Tsai et al., 2014). Application of Cpf1, a single RNA-guided endonuclease of a class 2 CRISPR-Cas system, can provide higher diversity to target sequences to overcome limitations of designing effective sgRNAs for the CRISPR/Cas9 system (Zetsche et al., 2015). Next-generation genome editing tools such as HF-Cas or eCas (Kleinstiver et al., 2016; Slaymaker et al., 2016) seem to be more promising in high fidelity genome editing. A recently discovered nuclease of the CRISPR-Cas system called CasX has been found more efficient and specific for genome editing (Liu et al; 2019).However, to exploit the full potential of these new technologies, important questions and challenges must be addressed before making genome editing a full proof technology.

Safety and Regulatory concerns

Though genome editing can be helpful in solving a plethora of problems associated with conventional breeding and genetic engineering it is mandatory to take precautionary measures before applying it in the livestock industry. Seeing its present use, it is expected that hundreds of genome edited animals will be available in the future. There are strict guidelines for the use of genetically modified animals (GMAs). The Cartagena protocol on biosafety and the food and drug administration (FDA) of US say that there must be sufficient scientific information about the safety and adverse effects of GMAs and their products and it should not have negative effects on biological diversity and human health. The regulation of GMA includes safety assessment with respect to their possible impact on human/animal health and environment (Pauwels et al., 2014). The points that come under the regulatory network are- information on the genomic locus/loci of editing and effect on the function of the endogenous gene after disruption and generation of new reading frames and what adverse effects the altered or truncated protein would have on the health of edited organisms and their consumers. Genome editing generates indel mutation (by NHEJ) or precise point mutation (by HDR) resulting in an animal that is similar to those obtained through natural or conventional (physical/chemical) mutagenesis. So such animals have been excluded from the European Union (EU) regulatory policy whereas animals in which foreign DNA has been incorporated in the genome come under this regulation. Methods that do not deliver nucleic acids into the recipient cells and that rely on direct delivery of nuclease proteins without the use of repair templates excluded from EU regulations, whereas the vector-mediated delivery of nuclease encoding sequence, with or without repair templates might produce GMA that are regulated (Pauwels et al., 2014). However, the FDA regulations are stricter and don't allow to use GMA derived food for

human consumption. For GMA derived food to be approved, it must be determined that genetic modification should not alter the physiology and composition of edible tissue and the main purpose of regulation is to avoid potential risk on consumers. Currently, no GMA has been approved for food except AquaAdvantage Salmon which is yet to be commercialized. One of the reasons behind the lack of approval or commercialization of genetically modified organisms is the concerns of the opponents regarding safety, animal welfare, and usefulness. In most cases the non-approval of GMA is not supported by scientific evidences rather it reflects the negative opinion of people towards them who think that the GM foods are unsafe and harmful for health and environment because of being unnatural (Ishii ., 2017). There is a lack of knowledge and scientific uncertainty involved with the use of genome edited animals. They can have an environmental risk if transgenic animals can spread unwanted genes in wild animals. The unnaturalness of the organisms may lead to unknown consequences and off-target cleavage may have side effects which is difficult to predict. Therefore before using the genome edited animals, it is necessary for the scientists, industries, and government to educate people about the risks and benefits of such animals and their products to develop regulatory laws and policies which will help in gaining positive public perception.

Conclusion- Programmable nucleases are considered as the most efficient and precise tools of the 21st century for the genome editing of organisms including livestock. Genome editors can be used in livestock animals to increase production of animal products, increased disease resistance, improve animal welfare and to produce gene edited animals for biomedical research. All the above-mentioned genome editors have been used to get the desired genotype in livestock for the different traits and utility. A large number of gene-edited livestock species have been generated till date and many more in the pipeline. The beauty of this technology lies in the precise editing of any specific site in the genome of the organism which could be even footprint-free. As compared to conventional genetic engineering, genome editing is safer and has less undesirable effects. The off-target cleavage associated with this technology is a matter of concern but with the improvement and modifications of the system, it is expected that this problem could be resolved soon. However, the regulatory policies related to safety concerns and conservative public opinion are the biggest hurdles in using genome edited animals and the food derived from them. There is no gene edited farm animal which has been approved as food till now by FDA. We need to scientifically assess the safety-related issues before making laws and regulations for the use of genome edited animals. Additionally, positive efforts should be made by researchers, company stakeholders and government to educate common people about the benefits which would be helpful in erasing their doubts and negative opinion about the food derived from such animals.

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