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Transgenesis in farm animals-A review

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ABSTRACT

For a number of decades, attempts have been made to successfully produce transgenic animals which have numerous applications in the biotechnology industry with the foremost emphasis on production of monoclonal antibodies and recombinant proteins of human welfare. Different techniques are adopted in order to produce transgenic farm animals which could be further used as bioreactors. The most common traditional transgenesis technique employed is Somatic Cell Nuclear Transfer (SCNT) using genetically modified somatic cells or stem cells as nuclear donors. This review article summarizes the merits and demerits of the techniques currently used to produce transgenic livestock with major emphasis on somatic cell nuclear transfer. In the end, a brief discussion is done about the novel methods adopted to produce transgenic animals like Zinc Finger Nucleases (ZFN), Transcription Activator-like Effector Nuclease (TALEN) and Clustered regularly interspaced short palindromic repeats (CRISPR). It is expected that the new techniques developed would overcome the problems faced with existing traditional transgenesis methods.

Key words: Intra-cytoplasmic sperm injection, Pronuclear microinjection, Somatic cell nuclear transfer, Targeted genome editing, Transgenesis.

Background: Transgenic animal technology is one of the fastest growing areas in biotechnology. Transgenic animals are genetically modified animals that are produced by the introduction of a foreign gene/s of interest into their genome such that these genes are inherited and expressed by their offspring. The technology for generating transgenic animals exists for a number of vertebrate and invertebrate species. The generation of transgenic animals is essential for the analysis of gene function during different stages of the life cycle, such as development, organogenesis, ageing, etc. While the commercial applications include the preparation of recombinant proteins of therapeutic use, development of disease resistance animals and the introduction of new genetic characteristics into the herd (Miguel *et al*., 2010). Various methods with different efficiencies have been employed for the creation of transgenic animals. The systems include pronuclear injection (PI), embryonic stem (ES) cells – mediated, sperm- mediated gene transfer and somatic cell nuclear transfer (SCNT). Key factor for successful production of transgenic animal is transfection efficiency and precise expression level of the transgene. Various new technologies have been explored which are expected to be beneficial in improving targeted gene integration as well as enhancing the cloning efficiency. The new techniques could serve as a better platform for developing transgenic animals.

Methods of transgenic animal production: There are multiple methods available to produce transgenic animals. The merits and demerits of commonly used methods available for creating genetically modified animal are summarized below.

Pronuclear microinjection: The first transgenic animal produced was through microinjection of foreign DNA into the male pronucleus of zygotes (Gordon *et al*., 1980). The procedure simply involves the transfer of DNA containing the gene/s of interest under the control of appropriate promoter into the male pronucleus of zygotes followed by transfer of embryos into a surrogate mother. The transgene eventually integrates into the embryonic genome in a random fashion. This procedure has proved to be useful in species like goat (Freitas *et al*., 2007), rabbits, pigs and sheep (Hammer *et al*., 1985). But it shows poor results in case of certain species like cattle (Eyestone, 1999**)** due to difficulties faced in visualization of the male pronucleus (Salamone *et al*., 2012). The success rate of transgene integration ranges from 3% in rats, mice and rabbits to less than 1% in pigs, sheep and cows (Freitas *et al*., 2007; Hammer *et al*., 1985). However, this method results in a high percentage of mosaicism in which all the cells of the animal do not contain the transgene. Another major limitation is the determination of the transgene integration. The only way to detect transgene integration is to examine the transgenic animal thus produced and their offspring which may be problematic since in large animals - the reproductive (including the time period before attaining physiological maturity) is quite high such as 1.0-2.3 years in pigs, 0.9-2.3 years in goat and 2.3-4.5 years in cows (Maksimenko *et al.*, 2012). Also, transgenic animals

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produced by this method show great variability in transgene expression due to the high degree of mosaicism, variable efficiency of transgene integration and occurrence of position effect due to random transgene integration (Bosch *et al*., 2004). In order to create transgenic animals, testing of multiple lines of animals for proper transgene expression is required, which is both time consuming and costly way of producing such animals. These are the major drawbacks in case of large animal transgenesis through microinjection which makes it less suitable method of choice. But in case of small animals like mice, rats and rabbits, the methods are commonly employed for the creation of transgenic animals (Miguel *et al*., 2010).

Embryonic Stem (ES) cells-mediated trangenesis: Embryonic stem (ES) cells are pluripotent stem cells obtained from the inner cell mass of the blastocyst and are largely used for creating transgenic mice. Under optimum culture conditions, these cells show great potential to divide endlessly**.** Due to this characteristic feature, ES cells are rapidly propagated and can be manipulated easily by the insertion of DNA construct containing gene/s of interest. The method involves isolation and *in vitro* culturing of ES cells and subsequent insertion of the transgene. The transgenic ES cells are then selected from non-transgenic cells and allowed to propagate further in order to form transgenic ES cell colonies. The transgenic ES cells obtained are picked, inserted into blastocysts and then transferred to the surrogate mother. The chimeric animals, thus produced are tested for germline transmission and through various breeding strategies, pure transgenic animals are created. Major advantage of using ES cells is the prior selection of transgene integration through use of selectable markers which was absent in microinjection where transgene integration could be detected in transgenic animal or their progeny (Hodges and Stice, 2003). Also, targeted integration via homologous recombination is also possible, which solves the problem of position effect as seen in the case of random integration and also facilitates the production of gene knockout mice useful for genetic studies (Capecchi, 1989). Earlier use of ES cells as a method of transgenesis was seen in mouse only, but attempts have been made to isolate ES cells in the case of farm animals (Iannaccone *et al*., 1994; Notarianni *et al*, 1991; Wells *et al*, 1999). But in these species, this method is not much successful due to difficult culture conditions, requirements for maintenance of culture in undifferentiated form and moreover, tricky genetic manipulation is needed (Cibelli *et al*., 1998). Unfortunately, even though livestock ES cells were compared to mouse ES cells, the generation time and high cost of maintenance of multiple chimeric animals is a hurdle to test for germline transmission.

Sperm- mediated transgenesis: There are plenty of reports available regarding the use of sperm cells as vector carrying transgene as an alternative technique to conventional pronuclear microinjection. In 1971, it was explored that the sperm cells have the potential to carry foreign DNA into the oocyte during the process of fertilization (Brackett *et al*, 1971). Since its discovery, this potential of sperm cells is being exploited to produce transgenic animals. This technique involves initial co- incubation of sperms with transgene such that the sperm cells take up the foreign DNA containing gene/s of interest. DNA binds to sperm cell's plasma membrane mediated by specific DNA- binding protein present in sperm (Farace and Spadafora, 1989). About 15-20% of the total sperm bound DNA is internalized by the process mediated by CD4 molecules (Spadafora, 1998). In order to deliver sperms carrying gene of interest into oocytes, various different mechanisms are adopted with varying efficiencies (Fernández-Martín and Salamone, 2010). The different mechanisms comprise of *in vitro* fertilization (IVF) (Lavitrano *et al.*, 2002), laproscopic insemination (LI) (Lavitrano *et al.*, 2002) and intra-cytoplasmic sperm injection (ICSI) (Spadafora, 1998; Perry *et al.*, 1989). Though IVF and LI show better results in terms of the embryo production rate compared to ICSI but it has been reported that ICSI is a better technique for transgenic animal production (Brinster *et al.*, 1989). A study showed that despite of obtaining high rates of morulae and blastocysts through IVF and LI, none of the embryos expressed the transgene (Perry *et al.*,1999). On the other hand, various reports indicate that ICSI- mediated gene transfer is an effective and repeatable method to produce transgenic mice **(**Perry *et al.*, 1999**)** and pigs (Kurome *et al*., 2007). In 2011, by using ICSI, 91.6% GFP expressing embryos were obtained (Pereyra-Bonnet *et al.,* 2011). ICSI- mediated gene transfer was also reported in cattle, but efficiency was quite low (Perry *et al.*, 1989)**.** Various studies were done in order to improve the efficiency of the embryo production rate by ICSI. Attempt was made in 2012, by a group of scientists, for improving efficiency of embryo production via ICSI in which ICSI was done in five different mammalian species, including cat, pig, sheep, cattle and horse and it was reported that after injection of sperm carrying transgene into metaphase-II (MII) oocytes, the oocytes need to be chemically activated (Salamone *et al.,* 2012; Pereyra-Bonnet *et al.,* 2008). After using different chemical activation treatments, it resulted in production of > 80% EGFP expressing blastocysts (Bevacqua *et al.,* 2010). The most appealing feature of this technique is its simplicity since no embryo manipulation is needed and possibility of mass production of transgenic animals via *in vivo* or *in vitro* insemination of numerous oocytes. But this method suffers from certain drawbacks such as similar to pronuclear microinjection, in sperm- mediated gene transfer targeted integration cannot be achieved resulting in random integration of foreign gene and also high rate of mosaicism is present (Salamone *et al.,* 2012)(Fig.1).

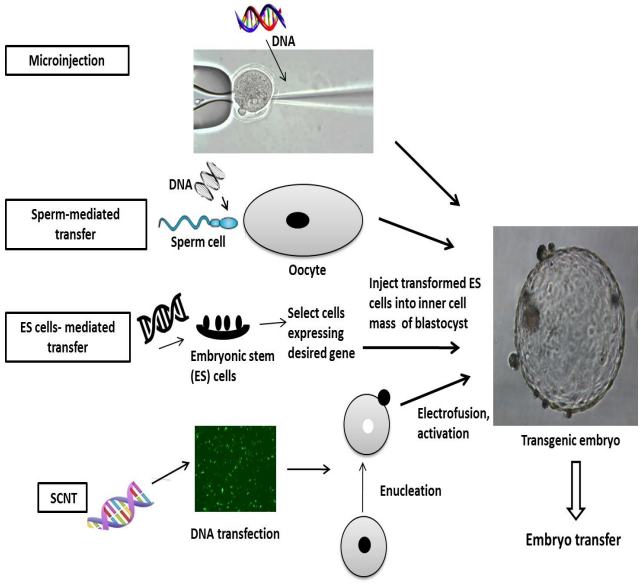

Fig.1: Methods for creation of transgenic animals.

Somatic Cell Nuclear Transfer (SCNT): Somatic cell nuclear transfer (SCNT) involves transfer of the nucleus of a somatic cell into cytoplasm of enucleated metaphase-II (MII) oocytes for the creation of a genetically identical copy of nuclear donor. Cloning through SCNT has led to the production of elite animals with useful traits and the creation of transgenic animals of agricultural and biomedical use. SCNT has been successfully used for the production of cloned (Baguisi *et al.,* 1999; Kato *et al.,* 1998; Polejaeva *et al.,* 2000) and transgenic cloned (George *et al.,* 2011; Lipinski *et al.,* 2007; Liu *et al.,* 2013) animals. The use of SCNT in producing transgenic livestock offers many advantages over other methods as discussed above. One of the important advantages of using SCNT is that offspring produced is entirely transgenic compared to PI, which accounts for large amount of *mosaicism* in transgenic animals. In addition, SCNT allows predetermination of the sex and phenotype of transgenic animals to be produced and thus, offers a further increase in efficacy when sex and phenotype of the transgenic founder animals is critical, e.g., for the mass production of recombinant proteins into milk, transgenic animal must be a female with good phenotype and genotype. The success rate of SCNT ranges from 1-3% in most of the animals, including cattle. On the other hand, SCNT faces major problems of low efficiency and high mortality rates of fetuses and embryos (Kishigami *et al.,* 2008). There are a number of factors that influence the efficiency of SCNT which includes the donor cell type (Oback and Wells, 2007; Salamone, 2006), enucleation protocol (Moro et al., 2010), electrofusion and activation steps (Canel *et al.,* 2010; Vichera *et al.,* 2009). The technique involves numerous critical steps which drastically affects the cloning efficiency, which is briefly discussed below.

Donor cell type and *in vitro* culture conditions of donor cells: Numerous cell types including fetal and adult fibroblasts, embryonic stem cells, brain cells, sperm cells, testicular cells, urine cells, cumulus cells, sertoli cells, macrophages, blood leukocytes, embryonic stem cells, etc have been used as nuclear donors (George *et al.,* 2011; Arat *et al.,* 2002; Cibelli *et al.,* 1998; Huang *et al.,* 2010; Jyotsana *et al.,* 2015; Madheshiya *et al.,*2015). But till now, it is not clear which cell type is best suited for cloning (Kato *et al.,* 1998; Goto *et al.,* 2013; Tani *et al.,* 2001). The culture conditions also have an impact on cloning efficiency. The donor cells before being used as nuclear donors are cultured *in vitro* (Wells *et al.,* 1999; Cho *et al.,* 2004; Kato *et al.,* 2010). In 2014, comparative analysis of the developmental potential of bovine SCNT embryos using cumulus cells cultured under different culture conditions such as fresh cells (non-cultured), 20h culture, cycling culture and serum- starved culture was done. The blastocyst rate of Nuclear Transfer (NT) embryos derived from cultured cells (cycling and serum starved culture) was significantly higher than the embryos derived from fresh cells (Akagi *et al.,* 2003; Akagi *et al.,* 2014**).** A similar study was done in other species such as rabbit (Cervera and García-Ximénez, 2003) and goat (Akshey *et al*., 2010). Therefore, a number of studies conducted showed that nuclei of short term, long term cultured cells and also cells near to senescence have the ability to undergo reprogramming and have potential to generate healthy clones after NT (Lanza *et al.,* 2000).

Stage of cell cycle and recipient oocytes: The co-ordination of cell cycle of donor cells and recipient oocytes is very important in order to prevent DNA damage and also to maintain the appropriate ploidy number (Campbell and Alberio, 2003). Oocytes arrested at metaphase II (MII) have been majorly used as recipient cytoplasts (Oback and Wells, 2003). The cell cycle stage of somatic cells also affects the developmental potential of embryos. The cells in G0 and G1 phase of the cell cycle have been reported in almost every successful study (Kato and Tsunoda, 2010). Reports are also present, showing that the metaphase cells can be reprogrammed successfully (Tani *et al.,* 2001). Several studies were conducted in which the developmental potential of NT blastocysts derived from fibroblast cells in G0 and G1 phases were compared (Goto *et al*., 2013; Ideta *et al*., 2001; Ideta *et al.,* 2010; Kasinathan *et al.,* 2001). The results indicated that no significant difference was found in *in vitro* development potential of embryos constructed from G0 and G1 stage of the cell cycle. However, *in vivo* developmental competence of NT embryos was found to be higher when G1 phase fibroblast cells were used as nuclear donors (Goto *et al*., 2013; Ideta *et al*., 2001; Ideta *et al.,* 2010; Kasinathan *et al.,* 2001).

Oocyte activation: Unlike IVF, SCNT lacks natural activation, therefore NT embryos must be manually activated for further development of embryos. Different activation methods are employed such as electrical activation and chemical activation (calcium ionophore A23187, ethanol, 3-phosphatidylinositol (IP3) and chlorine strontium). It was found that these methods can increase the intracellular calcium concentration in the reconstructed embryos (Miao, 2012).

The production of transgenic animals by SCNT is a multistep procedure, involving a number of technical and biological factors affecting the developmental potential of NT embryos. Various improvements have been incorporated in the cloning procedure for improving developmental potential of NT embryos (Niemann and Lucas-Hahn, 2012) but unfortunately, the cloning efficiency in terms of live offspring born still remains low. The main reason for poor results in cloning efficiency is believed to be a failure to reprogram nuclear genome of donor cells (Rodriguez-Osorio *et al.,* 2012)(Fig 2).

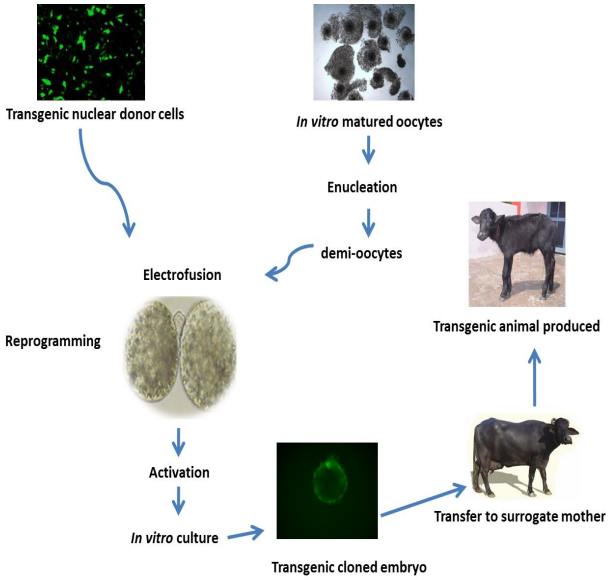

Fig. 2: Production of cloned transgenic animal using SCNT.

New approaches of genome editing: So far, the results presented indicate that there are number of techniques capable of transferring the transgenes to the cytoplasm and nuclei of the cells/ oocytes/ zygotes. But merely the presence of the transgene in the nucleus does not imply its integration into the host genome which is a key factor for transgenic animal production. Therefore, development of such techniques that allows the integration of transgene is essential. In addition, the new techniques developed also allow for the site- directed integration of the transgene. Zinc finger nucleases (ZFN), Clustered regularly interspaced short palindromic repeat (CRISPR) and Transcription Activator-like Effector Nucleases (TALEN) are the new technologies that employs nuclease, recombinases and integrases that are derived from lower organisms and function actively in mammalian cells and allows site- specific transgene integration. These strategies are briefly discussed below.

Zinc Finger Nucleases (ZFN): Zinc finger nuclease (ZFN) consists of one DNA binding domain and one non-specific endonuclease domain. It recognizes and binds DNA at specific site with the help of DNA binding domain and produce cut thus causing double stranded break (DSB) in DNA at a specific location due to endonuclease domain present followed by transferring of external DNA by induction of homology directed repair or non-homology terminal joining (Miao, 2012). ZFN-mediated genome editing has shown good results in mice, rats, human and recently, in 2014, mastitis resistant transgenic calves have been produced using ZFN (Liu *et al.,* 2014). Even though ZFNs have been used for targeted genome editing in various organisms, the major limitations faced with ZFN are limited modularity due to context dependent DNA-binding effects, making difficult for ZFNs to target any desired DNA sequence (Ramirez *et al.,* 2018). Moreover, lack of specificity of some ZF domains can generate off-target cleavage, leading to undesirable mutations and chromosomal aberrations (Pattanayak *et al.,*2011; Radecke *et al.,* 2010).

Transcription Activator-like Effector Nucleases (TALEN): Transcription activator-like effector (TALE) proteins having simpler DNA recognition code than ZFN have emerged recently (Gay *et al*., 2013; Joung and Sander, 2013). TALE proteins are found naturally in plant pathogenic bacteria 'Xanthomonas' and regulate the level of gene transcription. Similar to ZFN, TALEN consists of one DNA binding domain and the other domain having endonuclease activity. The DNA binding domain composed of set of 33-35 amino acid repeats in a way that each repeat recognizes a single target nucleotide. TALEN specificity is conferred by two hypervariable amino acids present at position 12 and 13 which are known as repeat-variable di-residues (RVD) (Boch *et al*., 2009; Moscou and Bogdanove, 2009). Like ZFN, Tale repeats are linked together in order to recognize specific DNA sequences. TALEN has been rapidly and widely used to perform precise genome editing in a wide range of cell types and organisms, including rats, mice (Panda *et al.*, 2013), human and livestock (Moghaddassi *et al.*, 2014).

Unlike the context-dependent DNA binding of ZFNs, TALENs can be rapidly and easily constructed to target almost any DNA sequence due to the simple protein-DNA code. In addition, TALEN exhibits significantly reduced off-target effects and lower cytotoxicities, ease of construction and high efficiency compared to ZFNs, proving to be an efficient genome editing tool (Ding *et al.*, 2013; Mussolino *et al.*, 2011). Recently, a group of scientists has successfully made use of TALEN to edit myostatin (MSTN)

gene in case of sheep and cattle (Proudfoot *et al.*, 2015). Also, the custom designed TALEN are available in the market through the companies such as Life Technologies (Grand Island; New York), Transposagen Biopharmaceuticals (Lexington; Kentucky) and Cellectis Bioresearch (Paris; France).

Clustered regularly interspaced short palindromic repeats (CRISPR): CRISPR/Cas 9 is the latest and most effective alternative to TALEN and ZFN available for targeted genome editing that is derived from a defense system present in bacteria and archaea that wards off invading phage and viral attack (Gay *et al.,* 2013). These organisms capture small DNA fragments (~20bp) from the foreign DNA of invading phages or plasmids and insert these DNA sequences, termed as proto-spacers, into their own genome to form a CRISPR. This was the case of type I CRISPR system while in type II CRISPR system, the CRISPR regions are transcribed as pre-CRISPR RNA (pre-crRNA) and processed to generate target-specific CRISPR RNA (crRNA). This system has been exploited from *Streptococcus pyrogenes* in which, a short RNA molecule known as a single guide RNA (sgRNA) molecule is bound by a conditional DNA nuclease called Cas9 (Jinek, 2012). The 20 nucleotides at the 5' end of the sgRNA guides the Cas9/sgRNA (protein/RNA) complex along the DNA strands until a match is made between the 'guide' RNA and the 'target' DNA sequence. Once a match is made and if an NGG trinucleotide (PAM) sequence is present immediately downstream of the target site, the Cas9 molecule immediately undergoes a conformational change that activates two separate nuclease domains earlier present in an inactive form in Cas9 molecule (Jinek, 2014).This results in cleavage of both strands of the target DNA, which are three nucleotides upstream of the PAM site (Jinek, 2012). The CRISPR/Cas system can be used a number of times to target any DNA sequence just by redesigning of the crRNA. This characteristic feature of CRISPR/Cas9 system was demonstrated through a study conducted in 2013, in which targeting of specific genes in mammalian cells using the CRISPR/Cas9 system was shown and also the ability to target more than one DNA sequence in the same cell by using multiple guide RNAs was illustrated (Cong, 2013; Mal, 2013). New CRISPR plasmids are easily prepared by cloning 20 bp guide DNA sequences in a vector that encodes sgRNA. A crucial advantage of CRISPRs over ZFNs and TALENs is their simple design and preparation, its high specificity and low cytotoxicity.

CONCLUSION

This review shows a summarized comparison of the available technologies for the transgenic animal production. Number of new alternatives available for site specific gene transfer is also discussed. The proper combination of available technologies will give a chance to various research groups to have better accessibility to animal transgenesis. Transgenic research involves exploration and creation of simple and reliable method which will contribute to increased efficiency of generation of transgenic animals and therefore, the related products which will improve our wellbeing and livelihood.

Competing interests: The authors declare that they have no competing interests.

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