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TRANSGENIC AND TRANSCHROMOSOMIC LIVESTOCK - METHODS AND APPLICATIONS

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ABSTRACT

Transgenesis is the process by which gene modification is achieved through the introduction of gene(s) of interest into a cell or an animal embryo to develop transgenic cell lines or transgenic animals, respectively. A variety of methods such as lipofection, electroporation, magnetofection, sonoporation, biolistic particle delivery and viruses have been employed to introduce DNA into *in vitro* cultured cells. Viral transfection and microinjection are the two main methods used to introduce DNA into preimplantation embryos to generate transgenic animals. Transgenesis occur through random integration of introduced genes into the host genome. The advent of animal cloning allowed modification of donor fibroblasts to generate stable transgenic cells *in vitro* which can then be used to create transgenic animals through cloning via somatic cell nuclear transfer (SCNT). This approach allows creating multiple clones that would express the same phenotype, thereby expanding the scope of creating herds of animals with the desired phenotype. While viral and the microinjection methods produced transgenic animals with random integration, recent advances in gene targeting/editing technologies such as Zinc Finger Nuclease (ZFN), Transcription Activator-like Effector Nuclease (TALEN) and CRISPR/Cas9 promise precise gene targeting for better outcomes. The methods, and pros and cons of various approaches used to produce transgenic animals and the utility of transgenic animals are discussed in this review.

INTRODUCTION

The possibility of genetic manipulation of farm animals carrying foreign DNA from any source captivated the imagination of animal scientists because it opened the door for advancing genetic progress for traits that are controlled by single copy genes. Generation of transgenic

animals has two purposes (a) to improve production traits for agricultural purposes and (b) for medical and pharmaceutical purposes. The former aims to improve physiological characteristics such as growth rate, fertility, fecundity, feed utilization, carcass quality, milk production and/or composition, increased disease resistance etc. In the latter, the aim is to use transgenic animals as bioreactors to produce large amounts of recombinant proteins (food additives, biotherapeutics, antibodies) or for organ pharming for xenotransplantation.

The report of the first transgenic mouse in 1974 by Jaenisch and Mintz heralded a new era in transgenic animal research. Even though enormous strides have been made in generating transgenic farm animals, the means to manipulate quantitative trait loci (QTL) in livestock species remains a distant dream. This is in large part due our inability to control variables such as copy number and the location(s) on the host chromosomes where foreign DNA gets integrated; as well as difficulties in maintaining robust tissue- and stage-specific transgene expression. Targeting the recombinant protein to the target organ (for e.g., mammary glands) has been challenging. More importantly, to make transgenic venture a commercial success, transmission of the transgene through the germline is critical in generating herds of animals that express the transgene as efficiently and faithfully as the parent animal.

A transgenic animal produced using viral or pronuclear injection method can be said to be a one-of-a-kind animal that cannot be reproduced again because of the randomness of gene integration. Progeny from these transgenic animals, produced by traditional breeding, may not exhibit the same production characteristics as the parent animal. One way to overcome this difficulty is to create clones of founder transgenics so that the gene expression patterns are conserved. Recent advances in gene targeting technologies promises to overcome these challenges. Regardless of the approach used, in the case of farm animals, high R&D expense, high cost of breeding and maintenance of transgenic animals and the long time it takes to evaluate and prove the value of transgenics are major factors that have slowed the progress in livestock transgenesis (Wall, 1996).

METHODS OF PRODUCING TRANSGENIC LIVESTOCK

When discussing the production of transgenic livestock, it is important to discuss the methods used before and after the advent of somatic cell nuclear

transfer (SCNT) (Campbell *et al.*, 1996; Wilmut *et al.*, 1997; Cibelli *et al.*, 1998). Before SCNT, two general methods were used to produce transgenic animals - viral method and pronuclear injection. Viral method, adapted in the very early stages of transgenic research, used either integrating viruses or non-integrating viruses (for more information on viruses, see review by Lundstrom, 2018). Jaenisch and Mintz (1974) reported the development of the first transgenic mice by injecting simian virus 40 (SV40) viral DNA into mouse pre-implantation blastocysts. This resulted in generating mosaic animals and no germline transmission of the transgene was observed. Earliest reports of transgenic mice using pronuclear microinjection were that of Gordon and Ruddle, (1981), Brinster *et al.*, (1981) and Hammer *et al.*, (1985). Pioneering experiments in mice by Palmiter *et al.* (1985) gave the much-needed impetus to livestock transgenesis. Transgenic mice were produced through pronuclear injection of human growth hormone gene. These animals exhibited enhanced growth rate. This demonstrated the feasibility of using similar approaches in farm animals.

Before SCNT, pronuclear microinjection was the method of choice for generating transgenics in livestock species. In this method, about 1 to 2 pl of a solution containing 2 to 3 ng/µl of plasmid DNA vectors carrying the gene of interest is injected into the pronuclei of embryos. If one assumes an average size of the plasmid construct to be about 5 to 10 kb, the number of copies of plasmid DNA injected per embryo amounts to hundreds of thousands of copies of the gene. Inside the cells, the DNA vector tends to recombine to produce long tandemly arranged concatemerized DNA. As the embryo undergoes rapid division, the transgene integrates randomly into the embryonic genome either as single copy or as concatemers (Rulicke and Hubscher, 2000). Depending on the stage of the embryo at which genome integration occurs, transgenic progeny may exhibit 100% transgenesis or various degrees of mosaicism (Wilkie *et al.*, 1986). In order for the transgene to be transmitted through germline, integration must occur before germ cell differentiation. During mammalian embryogenesis, segregation of somatic and germline lineage occurs at about the time of gastrulation. Primordial germ cells (PGCs), the precursors of future gametes, migrate and populate the developing gonads where they undergo meiosis to produce oocytes or spermatocytes (Nikolic *et al.*, 2016). Therefore, it is critical for gene integration to occur in the precursor cells of PGCs, the epiblasts, to result in germline transmission of the transgene.

With the advent of SCNT, creating

transgenic animals with the desired phenotype became more practical. It became feasible to isolate fibroblasts from donor animals, generate transgenic cells *in vitro*, and select cells harboring the right transgene configuration. These cells could be used as nuclear donors to generate cloned animals through SCNT. Since transfections are performed in cultured cells, a variety of commonly used transfection procedures such as electroporation (Wong and Neumann, 1982), lipofection (Holmen, 1995), or lentiviral transfection (Wolkowicz *et al*, 2004) can be used. Over the years, several gene targeting technologies have been developed. These approaches include the Zinc Finger Nuclease (ZFN), Transcription Activator-like Effector Nuclease (TALEN) and Clustered Regularly Interspaced Short Palindromic Repeats/Cas9 (CRISPR/Cas9) technology (Kim *et al.*, 1996; Joung and Sander, 2013; Jinek *et al.*, 2012; Ran *et al.*, 2013).

TRANSGENIC LIVESTOCK.

Pursel *et al.* (1989) was the first to report the production of large transgenic livestock. They reported transgenic pigs created by pronuclear microinjection of human growth hormone gene. They reported that transgenic pigs showed significant improvements in daily weight gain, feed efficiency and reduction in subcutaneous fat. However, long-term uncontrolled expression of the growth hormone resulted in animals developing gastric ulcers, arthritis, cardiomegaly, dermatitis, and renal diseases. Attention soon turned to the idea of utilizing livestock animals as bioreactors to produce large quantities of pharmaceutically important biomolecules. Pioneering research in this area include the expression of human anti-hemophilic factor IX and human alpha-1-antitrypsin in sheep milk (Clarke *et al.*, 1989; Wright *et al.*, 1991); human tissue-type plasminogen activator and human antithrombin (Atyrn) in goat milk (Denman *et al.*, 1991; Edmunds *et al.*, 1998); and human protein C in milk of transgenic pigs (Velander *et al.*,1992). Later developments include the expression of human α-lactalbumin in goat milk (Yuan *et al.*, 2014), human lactoferrin in cow milk (Wang *et al.*, 2017), and human plasminogen activator in goat milk (He *et al.*, 2018). Earlier, Archer *et al.* (1994) had created transgenic goats secreting human growth hormone in milk. This was made possible through retrovirus mediate transfection procedure. Replication-deficient retroviruses carrying human growth hormone gene were infused through the teat canal of goats during a period of hormone-induced mammogenesis. The transgene was expressed under the control of a mammary gland-specific promoter to restrict the expression to mammary gland epithelial cells. A drawback of this

approach is that since only somatic cells are transfected, germline transmission is not possible.

Cibelli *et al.* (1998) reported the generation of chimeric transgenic animals using embryonic stem like (ES-like) cells derived from transgenic bovine fetal fibroblasts. In this approach, transgenic fibroblasts were fused with enucleated oocytes to produce cloned embryos and cultured *in vitro* up to the blastocyst stage. These blastocysts were plated on fibroblast feeder layers to generate transgenic ES-like cells. About 8-10 ES-like cells were injected into day 3 embryos, cultured *in vitro* until day 7.5 and transferred into synchronized recipients to produce chimeric progeny harboring cells from the recipient embryo and the injected transgenic ES-like cells. DNA analysis of various organs from 5-month-old progeny indicated the presence of transgene in at least one organ, with one animal showing transgene in the oocytes. McCreath *et al.* (2000) reported gene targeting at the ovine α1(I) procollagen (COL1A1) locus in cultured fibroblasts and cloned to produce transgenic lambs. Improvement of meat quality was attempted by Saeki *et al.* (2004) by expressing spinach Δ12 fatty acid desaturase (FAD2) cDNA in transgenic pigs. The gene was expressed under the control of an adipocyte P2 (aP2) promoter to restrict transgene expression in white and brown adipocytes. The purpose of this study was to create value added animals wherein meat from these animals could help to reduce the incidence of lifestyle-related diseases such as coronary heart disease and thrombotic disease. Lai *et al.* (2006) reported fat-1 transgenic pigs that produced high levels of n-3 fatty acids in their tissues and organs. Brophy *et al.* (2003) reported the creation of transgenic goats expressing rat stearoyl-CoA desaturase (SCD) cDNA in the mammary gland to improve the nutritional value of milk by converting specific medium- and long-chain saturated fatty acids to the more healthy monounsaturated form.

Gene targeting methods have become the method of choice for generating transgenic animals. Many research groups reported the use of homologous recombination as a means to perform gene targeting in livestock (Dai *et al.*, 2002; Denning *et al.*, 2001a, 2001b; Kuroiwa *et al.*, 2004; Lai *et al.*, 2002; Laible and Alonso-Gonzalez, 2009; Marques *et al.*, 2006; Thomson *et al.*, 2003). However, the efficiency of homologous recombination is extremely low. Next generation gene targeting approaches used ZFN, TALEN and the CRISPR/Cas9 systems. These methods exploit the ability of endonucleases to induce targeted Double Stranded DNA

Breaks (DSB) on host chromosomes that stimulates cellular DNA repair mechanisms by nonhomologous end joining (NHEJ) and homology directed repair (HDR) (Wyman and Kanaar, 2006). Hauschild *et al.* (2011) reported using ZFN for biallelic knockdown of GGTA1 (α1, 3-galactosyltransferase) in primary porcine fibroblasts. Modified cells were used to produce transgenic pigs through SCNT. Liu *et al.* (2014) reported the creation of mastitis resistant cattle by targeted integration of human lysozyme gene to the bovine β-casein locus in bovine fetal fibroblast using ZFN. Milk from transgenic clones were able to kill *S. aureus* in *in vitro* assays. Carlson *et al.*, (2012) and Proudfoot *et al.*, (2015) reported the use of TALEN to generate transgenic livestock. CRISPR/Cas9 has shown great promise for gene targeting in livestock. Because of its precision, specificity, and simplicity, CRISPR/Cas9 has seen extensive use across various species. Unlike ZFN and TALEN, CRISPR/Cas9 uses an RNA guided method to target gene(s) of interest. Hai *et al.* (2014) reported the generation of biallelic knockout pigs by direct injection of Cas9 mRNA and small guide RNA (sgRNA) into zygotes to target exon 5 of the Von Willebrand factor gene. This was quickly followed by Han *et al.* (2014) who reported successful targeting of sheep myostatin gene (MSTN). They targeted the third exon of MSTN in sheep fibroblasts to produce out-of-frame indels (insertions or deletions) to abolish normal MSTN function. Crispo *et al.* (2015) were able to successfully target MSTN in sheep by microinjecting Cas9 mRNA and sgRNA into ovine embryos to create monoallelic and biallelic knockouts. MSTN is a negative regulator of muscle growth. Founder transgenics in the MSTN knockouts exhibited heavier body weight than their wild type counterparts, indicating successful disruption of endogenous MSTN gene. Between 2014 and 2020, more gene targeting studies in livestock mediated by CRISPR/Cas9 have been reported (Ikeda *et al.*, 2017; Chuang *et al.*, 2017; Niu *et al.,* 2018; de Oliveira *et al.*, 2019; Zhang *et al.*, 2019; Menchaca *et al.* 2020).

It is evident from gene targeting publications to date that a large percentage of gene targeting/editing studies attempted in livestock species is performed by pronuclear injection. In this approach, even though, proper targeting/editing of genes can occur, the chances of off-target effects are very high. Off-targeting is irreversible, and its effects and target vary with each construct. Therefore, outcomes become unpredictable. There are conflicting reports about the degree of off-targeting when CRISPR/Cas9 system is used. Zhang *et al.* (2019) reported low frequency of Cas9 off-targeting compared to TALEN. However, analysis by Nerys-Junior *et al* (2018) and

Zheng *et al.* (2020) indicate that off-target effects of CRISPR/Cas9 can be as much as 50 percent. They concluded that while CRISPR/Cas9 is superior to TALEN in targeting efficiency, TALEN provides low off-target modifications.

TRANSCHROMOSOMIC ANIMALS

Vrious DNA vectors in various configurations carrying gene(s) of interest, under the control of promoters and regulatory elements have been used in generating transgenic cell lines and transgenic animals. The most common vector types are the plasmid vectors with a capacity of 5 to 25 kilobases of DNA. Vectors capable of carrying larger DNA fragments have been developed. These include phage vectors (35 to 45 kb), P1 phage vectors (70 to 100 kb), P1-derived artificial chromosomes, Bacterial Artificial Chromosomes (PACs and BACs, up to 300 kb), and Yeast Artificial Chromosomes (YACs, 20 to 2000 kb). These vectors can intergrate as single copy or as concatamers, and potentially disrupt normal endogenous gene(s). Transgenes may also undergo gene silencing due to positional effect if they integrate into inactive regions of the chromsomes. To overcome these difficulties, Human Artificial Chromosmes (HACs) capable of carrying payloads greater than 2000 kb, with no upper size limit, were developed. Their large capacity allowed the incorporation of all the necessary gene regulatory elements, including centromere, into HACs for proper functioning and control of gene expression. The presence of centromere allows HACs to replicate and migrate independently, permitting faithful transmission to daughter cells (Bajpai, 2013). Three methods have been used to introduce HACs into host cells. The first transchromosomic mice reported by Tomizuka and colleagues used a technique termed Microcell Mediated Chromosome Transfer (MMTC) (Tomizuka *et al.*, 1997). Lee *et al.* (2014) and Macdonald *et al.* (2014) used a technique called Recombinase Mediate Gene Transfer (RMGT). A third method uses flow sorting of artificial chromosomes followed by pronuclear microinjection (Co *et al.*, 2000). Transgenic animals carrying HACs are termed "humanized animals" since they are able to recapitulate human expression profiles in the transgenic host. Such animals allow evaluation of drugs and the production of human therapeutic antibodies. Transchromosomic mice have been generated using human chromosome fragments (hCF) of native human chromosomes 2, 14, 21, 22, IgH, Igκ, and Igλ (Tomizuka *et al.*, 1997; Tomizuka *et al.*, 2000; Shinohara *et al.*, 2001; O'Doherty *et al.* 2005). Suzuki *et al.* (2006) used *de novo* synthesized HACs to generate transchromosomic mice. Kuroiwa

et al. (2002) and Robl *et al.*, (2003) were the first to report the generation of transchromosomic cattle. HAC vectors containing the entire unrearranged sequences of the human immunoglobulin heavy-chain and lambda light-chain were introduced into primary bovine fetal fibroblasts using MMCT. Transgenic cells were partially selected in the presence of low concentrations of a selectable antibiotic marker (G418) to avoid loss of HACs during initial selection and expansion of transgenic cells. Selected transgenic cells were used to produce transgenic fetuses via SCNT. Fibroblasts isolated from these fetuses were subjected to stricter antibiotic selection, analyzed, and further used to create healthy and phenotypically normal cloned offspring carrying stable HACs. They determined that 78 to 100 percent of analyzed transfected fibroblasts retained intact HAC. Kuroiwa *et al.* (2009) and Matsushita *et al.* (2014) demonstrated that HAC carrying transgenics were able to express human polyclonal antibodies following hyperimmunization. Wu *et al.* (2019) reported the generation of transchromosomic goats carrying HACs containing the entire human immunoglobulin gene in the germline configuration. However, one issue they encountered is the dominance of the intact endogenous goat immunoglobulin genes, which negatively affected the quantity and quality of immunoglobulins produced in these animals. Competition between endogenous goat and transgenic human immunoglobulin genes suppressed the expression of human IgGs. A small percentage of the antibodies produced by the transgenic animals were chimeric in nature. They speculated that this was formed by the fusion of goat derived light chain and human derived heavy chain or vice versa. One approach to avoid the production of chimeric antigen is to inactivate endogenous immunoglobulin genes. A sequential gene targeting strategy can be employed to produce homozygous knockouts (Kuroiwa *et al.*, 2004; Sano *et al.* 2013; Matsushita *et al.*, 2014). A process termed "rejuvenation cloning" can be used to overcome poor cloning efficiencies of fibroblasts. In this approach, gene targeting is performed in fibroblasts cultured *in vitro*. Properly targeted fibroblasts are selected and used as nuclear donors to produce cloned fetuses using SCNT. Fibroblasts isolated from these fetuses are used for additional rounds of gene targeting and cloning. This process is continued until the desired homozygous knockout is accomplished. Transchromosomic cattle have shown promises in delivering personalized antimicrobial therapy (Silver *et al.* 2018), treating lethal models of Hantavirus pulmonary syndrome (Hooper *et al.*, 2014), antibody production against

Venezuelan Equine Encephalitis Virus (Gardner *et al.*, 2017), treat Ebola infection in non-human primates (Luke *et al.*, 2018), against lethal Zaire Ebola virus challenge in rhesus macaques (Rosenke *et al.*, 2018), and neutralizing activity against Hantavirus (Perle *et al.*, 2020).

CONCLUSION

Transgenic technology is a powerful platform that allows researchers to study gene function, dissect developmental pathways, or use animals as bioreactors to produce pharmacologically important biotherapeutics. Currently, CRISPR/Cas9 seems to be the most promising technology to generate animals with targeted gene correction, gene addition or gene deletion. It allows researchers to create transgenic cell lines from donor animals, analyze and choose correctly targeted fibroblasts to produce transgenic animals via SCNT. This is particularly relevant where multiple rounds of targeting are necessary to produce homozygous targeting. While considering commercial applications, one must be mindful of advances made in manufacturing of biologics using mammalian cell culture systems. Cell manufacturing systems have made enormous strides in scaling up and adapting to the changing needs in the field of recombinant protein production. Scaling up a transgenic animal project is difficult, time consuming and expensive. Therefore, if one is contemplating commercial transgenic animal operation, the suitability of transgenic animal as the right model for the target product, the time and expense involved in generating transgenic animals and the time it takes to build up a transgenic herd should be key considerations.

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