Animal transgenesis technology: A review
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Abstract: Genetically modified organisms (GMO) are plants, animals, bacteria or viruses that have been altered through the transfer or deletion of genes into or from the organism under consideration. They have a variety of uses in biological and medical research, safety testing, pharming, agriculture, and xenotransplantation. The foreign gene of interest is prepared by different gene editing techniques like recombinant DNA technology, transcription activator-like effector nucleases (TALENs), Zinc-finger nucleases (ZFNs), and CRISPR/cas9 (clustered regularly interspaced short palindromic repeats). The prepared gene of interest is incorporated through different vectors like bacterial plasmids, cosmids and yeast artificial chromosomes. The gene with the vectors is inserted to the host cell through different gene insertion methods like heat shock, electroporation, viruses, gene gun, microinjection, and liposomes. Transgenesis may be done through the gonads, sperm, fertilized eggs and embryo by DNA microinjection, retroviruses, stem cells, and cloning. To date, the most ideal transgenic marker is green fluorescent protein. The success of transgene is checked by incorporation of antibiotic resistance gene, western and southern blot, PCR and ELISA. If the technology alleviates welfare and ethical issues, it becomes the most promising in the future.

1. Introduction
Life is a dynamic process with slower progress. This phenomenon was left unnoticed by humans until they invented agriculture and breeding. The ability to control plant and animal reproduction...
leads to empirical genetic selection and modification of genes. Through gene modification, it is possible to provide and fulfilled all the needs of humans (Houdebine, 2002).

An organism with deliberate modifications of its genome (either deletion or insertion) is called genetically modified organism (GMO) (Chrenek, Makarevic, Pivko, & Bulla, 2010; Isselmou, Wackernagel, Tabbara, & Wiart, 2008; Margawati, 2003). Transgenic animal technology is one of the fastest-growing biotechnologies, which integrate exogenous genome to be inherited and expressed by the offspring of GMO. The key limiting factors for the success of transgenesis are lower efficiency and precise control of gene expression while gene constructs have now been introduced into most species of food animals, including cattle, sheep, goats, pigs, rabbits, chickens and fish (Gordon, 1996; Miao, 2012).

Although farmers have manipulated the genetic constitution of cattle for centuries to increase productivity, it becomes possible after elucidation of the structure of DNA in 1953 by Watson and Crick in Cambridge and the development of recombinant DNA technology. This helps to expanding the range of options to answer many human problems, from the need for lifesaving drugs to the solution for potential world food shortages in the twenty-first century (Gordon, 1996; Rutovitz & Mayer, 2002).

In the mid-1970s, gene transfer with retroviruses, by early-1980s DNA microinjection and in 1982 production of foreign protein and growth hormone is possible. Later embryonic stem cell mediated gene transfer becomes perfected. For example, United Kingdom laboratories contain more than 575,000 transgenic mice in the year 2000. Technical and biological obstacles limit the success of transgenesis in livestock than rodents resulting availability of only handful of livestock while thousands of transgenic rodents are available currently (Blanchard & Kelly, 2005; Rutovitz & Mayer, 2002).

Advancements in Assisted reproductive technologies (ART) and molecular biological techniques helped the development of novel methods of gene delivery in mammals and accelerated the development of transgenic technology (Agca & Chan, 2007; Sirotkin et al., 2008). Therefore, the objective of this review is to highlight the major aspects of animal transgenesis technology.

2. Contributions of transgenic animals
Generally, animals are being genetically altered for different purposes (Rutovitz & Mayer, 2002; Whitelaw & Sang, 2005).

2.1. Research purpose
The research activity may be medical and/or biological with the concern of finding solutions for human disease and studying gene function for the betterment of human life (Bagle, Kunkulol, Baig, & More, 2012; Eenennaam, 2008; Isselmou et al., 2008; Margawati, 2003; Whitelaw & Sang, 2005). However, there is considerable controversy over the utility of transgenic animals to detect useful treatments for humans. To solve the real problems of humans it is better to use human cell culture with toxicity testing and human trials (Eenennaam, 2008; Rutovitz & Mayer, 2002).

2.2. Pharmaceutical production “pharming” and safety testing
Transgenic animals used for pharmaceutical production are called Transpharmers which produces important human proteins in their milk and eggs after insertion of human genes (Whitelaw & Sang, 2005; Blanchard & Kelly, 2005; Chrenek et al., 2010; Murray, Mohamad-Fauzi, Cooper, & Maga, 2010; Bertolini, Bertolini, Murray, & Maga, 2014; Rutovitz & Mayer, 2002; Isselmou et al., 2008). Products so far from sheep milk include ATIII, α-antitrypsin and tPA through (Murray et al., 2010) while Blood, seminal plasma, urine, silk gland, and insect larvae haemolymph are other theoretically possible systems (Bagle et al., 2012).

Toxicity and carcinogenesis testing for newer chemicals and drugs undergone in animal experimentation which may take extended time to prove their safety while transgenics (mice) can do in shorter time (Eenennaam, 2008; Rutovitz & Mayer, 2002). Cell and tissue cultures may be non-animal
alternatives with greater accuracy in predicting human response. However, rush for transgenic development and the misapprehension that genetic modification will “fix” the problems inherent in using different species to assess human safety with animal testing will be lost (Giridharan, Kumar, & Muthusamy, 2000; Margawati, 2003). Examples: Enviro-pig, orb spider, and a sheep called “Tracy”.

2.3. Agriculture
Enhancing the quality of life for humans or the livestock being developed or significant resource conservation should be the goal of gene transfer (Isselmou et al., 2008; Murray et al., 2010). Enhancing metabolism, improving feed intake, better feed conversion efficiency, reducing pathogen load, creating novel or enhanced food and fiber products, permitting diversification of agricultural products, increasing diseases resistance, improving food quality and security, decreasing antibiotic use and enhancing resource conservation, improved carcass composition, enhancing lactation performance and wool production as well as reduced environmental impact are among the advantages of transgenesis in livestock and other agricultural commodities (Chrenek et al., 2010; Howard, Homan, & Bremel, 2001). For example, “Enviro-Pig” becomes pollution free by modifying to remove excretion of phosphorus (Rutovitz & Mayer, 2002), goats genetically modified to produce spider’s silk in their milk which is the strongest materials in the world and named as “Bio-Steel”. However, the possibility of Bio-Steel production in transgenic plants or in cell culture made the use of GM animals questionable for silk production (Bagle et al., 2012).

2.4. Foreign organ transplantation
The transfer of organ from one species to another is called Xenotransplantation. It becomes practical due to the organ gap between need and supply from the donors (Chrenek et al., 2010; Murray et al., 2010). The technology tries to produce humanized organs from pigs by controlling organ rejection due to physiological incompatibility in humans and pigs and transfer of gene-related diseases (Bagle et al., 2012; Einsiedel, 2005). Moreover, clinical and safety problems, welfare and ethical issues are among the challenges. Improving provision of National Health Services and encouraging donation and regeneration of tissues from stem cells may be alternatives to solve the problems of transgenic organs in the future (Einsiedel, 2005).

3. Transgenic vectors
A small piece of DNA containing foreign DNA with the ability to replicate itself for transferring or propagating in an organism is called cloning vector. Vectors increase the probability of gene expression (Giassetti, Maria, Assumpção, & Visintin, 2013).

3.1. Plasmid vectors
They are naturally occurring, small, self-replicating, circular and extrachromosomal pieces of DNA isolated from bacterial cells (Park, 2007). In a single cell, there may be 10–700 copies of plasmids. pUC18 is the most commonly used plasmid vector and they are restricted to accept DNA with ≤5000 base pairs (Wilmut, Schnieke, McWhir, Kind, & Campbell, 1997).

3.2. Specially developed bacteriophage
They have a characteristics future with one third of its genome is nonfunctional which made them suitable for gene transfer. Bacteriophage lambda has the ability to infect E.coli and can integrate up to 15–16 kilobases of the DNA segment (Nicholl, 2008; Food and Agriculture Organization of the United Nations (FAO), 2001).

3.3. Cosmids
They have both features of plasmids and bacteriophage chromosome without phage DNA so that they reproduce as plasmids and can integrate DNA segments less than 50 kilobases (Giassetti et al., 2013).

3.4. Yeast artificial chromosome (YAC)
It is a specially constructed linear yeast chromosome to incorporate less than 1 million base pairs of DNA strands (Nicholl, 2008).
4. Method and procedure of Foreign gene preparation

Preparing the transgene is the first step for foreign gene transfer technology. It is done by using conventional recombinant DNA techniques which functions by cutting and splice together pieces of DNA resulting in recombinant DNA. It is a DNA fragments that have been joined together in a laboratory which increases the possibility of exploring the regulation of gene expression and, cellular and physiological processes (Blanchard & Kelly, 2005; Chrenek et al., 2010; Eenennaam, 2008; Huldiner, 1996).

Typical transgenes contain nucleotide sequences of the gene of interest with all components essential for efficient expression especially the promoter or regulatory region such as that of b-actin or simian virus 40T antigens and tissue-specific promoters (Acquaah, 2004). The transgene can be expressed in many tissues of the transgenic animal by using a promoter from a ubiquitously expressed gene; Alternatively, with tissue-specific promoters like adipocyte P2 promoter (fat cells), myosin light-chain promoter (muscle), amylase promoter (acinar-pancreas) and insulin promoter (islet beta cells) (Huldiner, 1996). The major method for preparation of foreign gene of interest is recombinant DNA technology which consists of the following three steps.

4.1. Isolating the gene of interest

It is done by using recombinant DNA technology by using restriction enzymes (Table 1) (Acquaah, 2004). For example, Eco RI recognizes the DNA sequence GAATTC and cleaves it between G and A (G↓A) (Giasetti et al., 2013).

4.2. Cloning of target gene

Cloning is the process of introducing a foreign gene (called insert) into a vector (plasmid). The vector containing a cloned gene is referred as chimera, which is introduced to the host cell to live in different ways. The host cell containing the vector is also called transformed cell (Blanchard & Kelly, 2005).

Vector and target DNA sticky ends should be produced by the same enzyme to be complementary to insert the cut DNA in to the vector and joined by DNA ligase enzyme which covalently joins the sugar-phosphate backbone of bases together. It can make joining of sticky or blunt ends, but it is more efficient at closing sticky ends (Giasetti et al., 2013; Wilmut et al., 1997).

4.3. Incorporation of cloned gene into a host cell

The prepared foreign DNA should be incorporated to the host cell through different methods rather it cannot be readily sent across the membrane (Acquaah, 2004).

4.3.1. Heat shock

Sudden heating of a solution containing cold calcium chloride with chimera plasmids and normal host bacteria at 42°C for 2–5 min increases the permeability of the host bacterial membranes for plasmid chimeras to be incorporated in the host cell (Wilmut et al., 1997).

4.3.2. Electroporation

Applying high voltage pulse temporarily disrupts the host cell membrane, which allows passage of the vector in to the cell (Acquaah, 2004).

| Restriction Enzyme | Enzyme Source         | Target sequence |
|--------------------|-----------------------|-----------------|
| BamHI              | Bacillus amyloliquefaciens | G↓GATCC         |
| EcoRI              | Escherichia coli       | G↓AATTC         |
| HindIII            | Haemophilus influenza  | A↓AGCTT         |
| TaqI               | Thermus aquatics      | T↓CGA           |
4.3.3. Viruses
The ability of viruses to infect susceptible cell and replicate themselves made possible the incorporation of desired DNA sequence into the target host cells (Miao, 2012).

4.3.4. Gene gun
It functions by firing golden particles coated with foreign DNA segments into the host cell (Whitelaw & Sang, 2005).

4.3.5. Microinjection
By using a fine needle foreign DNA is injected directly into the nucleus by holding a cell place with a pipette under a microscope (Houdebine, 2002).

4.3.6. Liposome
A small membrane-bound vesicle (liposome) can enclose vectors and transfer foreign DNA while fusing with a cell or nuclear membrane of the host cell (Whitelaw & Sang, 2005).

5. Methods of transgenesis
Efficient methods for incorporation of foreign DNA should exclude chemical or physical mutagenesis. Hence, it should be in a relatively stable form. Genetically modified animals can be totally altered or partially altered (www.hse.gov.uk/biosafety/gmo/acgm/acgmcomp). The methods can be transferring genes through the gonads, microinjection, stem cells, sperm vectors, somatic cells and retroviral methods (Miao, 2012).

5.1. Transgenesis through the gonads
It works by transfecting the spermatogonia in situ through infusion of transgenes into seminiferous tubules or transfection of germ cell precursors in vitro followed by transplantation into host testis. Testis-derived cells transplanted in to the testis of infertile males populate the host testis, generate sperm, and produce offspring (Chrenek et al., 2010).

5.2. Sperm-mediated gene transfer
It is based on the intrinsic ability of sperm cells to bind and internalize exogenous DNA molecules and to transfer them into the oocyte at fertilization used as alternative for microinjection (Houdebine, 2002; Miao, 2012; Rutovitz & Mayer, 2002). Higher efficiency, low cost, and ease of use, absence of embryo handling or the use of expensive equipment are among the advantages over other methods while it results in extremely variable result in different species of animals (Chrenek et al., 2010).

5.3. Transgenesis through fertilized eggs or embryos

5.3.1. DNA microinjection
It is a direct and microsurgical procedure conducted on a single cell to introduce foreign DNA into either cytoplasm or nucleus. It uses glass needle (i.e., a fine, glass microcapillary pipette), a precision positioning device (a micromanipulator) to control the movement of the micropipette, and a micro-injector. Most of the time male pronuclei of embryos are used because of its large size (Chrenek et al., 2010; Houdebine, 2002). It is the only successful method for producing GM livestock until recently with only 3% to 5% injected embryos get the transgene (Markkula & Huhtaniemi, 1996; Miao, 2012; Whitelaw & Sang, 2005). In a single embryo, 200 to 500 copies of the gene construct can be injected (Chrenek et al., 2010). It results in random incorporation of desired genome in the injected embryos (Blanchard & Kelly, 2005; Markkula & Huhtaniemi, 1996; Rutovitz & Mayer., 2002). The resultant embryos containing the transgene should be cultured in vitro for 24 h implanted into a “pseudo pregnant” surrogate mother (Chrenek et al., 2010). It could not work for poultry because of extreme difficulty to gain access to the fertilized egg when it is still at the single-cell stage (Rutovitz & Mayer., 2002).
5.3.2. A retrovirus (adenovirus) mediated gene transfer

They are RNA viruses having reverse-transcriptase enzyme to make DNA from RNA (Blanchard & Kelly, 2005). They have the ability to integrate into the host DNA and copied when the cell divides (Chrenek et al., 2010; Rutovitz & Mayer, 2002; Whitelaw & Sang, 2005). It results in the production of chimeras because not all cells can get the transgene (Blanchard & Kelly, 2005; Miao, 2012). The pure homozygous transgenic animals may result after 10 to 20 generations through inbreeding and can be stored for subsequent implantation (Chrenek et al., 2010).

It is suitable to make transgene in poultry and other species of animals (Houdebine, 2002). Examples of viruses used for this purpose include Moloney Leukaemia Virus (causes lymphoid leukemia in mice, rats, and hamsters); Rous Sarcoma Virus (cancer formation in humans) and Avian Leukosis Virus (poultry flocks) (Rutovitz & Mayer, 2002).

Size limit on the amount of DNA inserted, unable to replicate in early embryonic cells, lower efficiency than the natural coupled with the danger of forming new pathogens are among the drawbacks of this method (Rutovitz & Mayer, 2002).

5.3.3. Stem cell-mediated gene transfer

According to Gordon (1996), Markkula and Huhtaniemi (1996) and Miao (2012), it involves insertion of the desired gene into totipotent stem cells and the stem cells containing the gene of interest are incorporated to the host embryos resulting in chimeric animals. It does not need a live transgenic animal to test the presence of a desired transgene and it allows testing at the cell stage (Evans & Kaufman, 1981; Margawati, 2003). It allows gene targeting by allowing directed modification of endogenous genes (Miao, 2012; Robertson, 1991).

5.4. Somatic cell nuclear transfer

The technique involves transfer of somatic cell nucleus to the cytoplasm of enucleated egg to be reprogrammed by egg cytoplasmic factors to form a zygote (BallP & Peters, 2004; Campbell, McWhir, Ritchie, & Wilmut, 1996; Wilmut & Whitelaw, 1994; Camara et al., 2008). In mammals, the zygote must be artificially placed into the uterus of a surrogate mother (Denning et al., 2001; Heyman et al., 1998).

It starts during the mid-1980s, after 30 years of initial successful experiments with frogs and now it is practicable in different species of animals except humans (Heyman et al., 1998; Kuroiwa et al., 2002; McCreath et al., 2000). DOLLY is the best example of somatic cell nuclear transfer technique (Eenennaam, 2008).

6. Transgenic markers and screening of transgenesis

To test whether the cells incorporate the transgene or not, we should also incorporate transgenic markers which should be screened in different ways. This increases efficiency of transgenesis by identifying true transgenes (Blanchard & Kelly, 2005).

β-galactosidase, firefly luciferase, secreted placental alkaline phosphatase and green fluorescent protein (GFP) are currently available transgenic markers but GFP is the most ideal marker which allows selection of transgenic embryos soon after gene transfer or prior to embryo transfer (Ikawa et al., 1995). Enzymes that inactivate aminoglycoside antibiotics such as neomycin or kanamycin are common markers used in selecting transgenic cells, which are mostly important in molecular biology when the efficiency of transferring gene constructs is poor and a pool of many cells is targeted for transfection (Howard et al., 2001).

Southern blot assay is the most widely used for testing the presence of transgene in the host animals. It involves digestion of DNA with restriction enzymes and analysis is made on agarose gel electrophoresis, which uses electric current. The fragments of DNA moved towards the positive pole from the negative one and settled in the gel according to their size with the bigger fragments
on the top while the smaller fragments move faster to the gel and settled on the positive pole. Then, the DNA is denatured by strong base or acid and blotted on to the membrane with hybridization of a probe with DNA of the gene of interest. If the gene of interest is present the blotted membrane picks the probe and illuminates the gene (Blanchard & Kelly, 2005).

The other essay is western blot, which is used to detect the transgenic protein produced by animals. SDS-polyacrylamide gel is used for electrophoresis. If the protein is small it moves towards the positive pole and blotted with nitrocellulose membrane. Then, it is incubated with a primary antibody, which sticks to a transgenic protein to form protein-antibody complex and visualization is made by hybridizing with the secondary antibody that forms a color. The presence of a transgenic protein makes a dark band in the film (Khalsa, Jewett, Duffy, & Czeisler, 2000; Sridhar, 2006).

Enzyme-Linked Immuno-absorbent Assay is also used to identify the presence of transgenic proteins by measuring the number proteins in serum, blood, and urine. It works by using plastic tray having wells, which are coated with specific antigen. If the sample contains transgenic protein it reacts with the antigen and color is produced (Blanchard & Kelly, 2005). DNA hybridization and PCR have also the ability to detect transgenes (Sridhar, 2006).

7. Application of CRISPR-Cas systems in animal transgenesis
CRISPR-Cas adaptive immune system is formed by conjugation of Clustered regularly interspaced short palindromic repeats (CRISPR) and associated sequences (Cas genes and Cas proteins). It is present in 80% of archaea and 48% of bacteria mostly on chromosomes and in some plasmids. It consists cassettes of regularly alternating repeats and spacers, a leader sequence and a set of Cas genes, which participate in different stages of the CRISPR-Cas pathway to generate CRISPR-RNAs (crRNAs) and Cas proteins. Defense against foreign genetic elements, regulation of lysogeny and regulation of biofilm formation are some of the roles of CRISPR-Cas systems (Barrangou & van der Oost, 2013).

8. Animal welfare and ethical concerns
The genetic modification of animals faced active opposition from animal welfare and ethics point of views. The researchers working in this area should ensure the absence of welfare interference in using recombinant DNA technology and should applaud the merits of gene modification for protecting welfare of animals like increasing disease resistance and others. Breaching the species barrier or playing God which may result in unwanted characters (altered behavior like enhanced aggression) and unknown hazards for humans is the major area of opposition from religious peoples and animal right and welfare groups. For example, if an animal is modified to contain a receptor for a human virus, it may act as a novel reservoir for human disease (Blanchard & Kelly, 2005; Eenennaam, 2008; Gordon, 1996, 2004; Isselmou et al., 2008; Rutovitz & Mayer., 2002).

9. Conclusions
Animal transgenesis technology is promising in the future to replace conventional use of drugs through the creation of disease-resistant animals and other methods of improving production potential of animals to avoid global food insecurity through diversification and improvement of agricultural products. It also used to improve human health by filling organ gap and production of important pharmaceutical products to treat human diseases. Animal Welfare and Ethics are the major issues which make the acceptance of the technology controversial and the efficiency of transgenesis is low. The efficiency of transgenesis should be enhanced by the innovation of other efficient technique, the researchers of the technology should be equipped with a high level of ethical values and keeping the welfare of animals they manipulate, innovation of new technologies should be invented for use in the field condition and awareness should be created to avoid strong opposition of the technology.
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