

Review

## Specific genetic modifications of domestic animals by gene targeting and animal cloning

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### Abstract

The technology of gene targeting through homologous recombination has been extremely useful for elucidating gene functions in mice. The application of this technology was thought impossible in the large livestock species until the successful creation of the first mammalian clone "Dolly" the sheep. The combination of the technologies for gene targeting of somatic cells with those of animal cloning made it possible to introduce specific genetic mutations into domestic animals. In this review, the principles of gene targeting in somatic cells and the challenges of nuclear transfer using gene-targeted cells are discussed. The relevance of gene targeting in domestic animals for applications in bio-medicine and agriculture are also examined.

### Introduction

Discovery of the functions of genes is a remarkably important aspect of biological research. Precise genetic modifications of animals are a powerful methodology to study physiological mechanisms at the molecular level. Gene knock-in and knock-out through gene targeting mediated by DNA homologous recombination paved the way towards the era of mammalian functional genomics. Following the first report of mouse embryonic stem (ES) cells in 1981 [1,2] and the successful alteration of the hypoxanthine phosphoribosyl transferase (HPRT) gene locus through homologous recombination in mouse ES cells in 1989 [3,4], numerous mouse mutants generated through gene targeting have been reported. By the year 2000, an estimated 5000 genes had been inactivated in the mouse [5]. It has become routine in many laboratories around the world to produce mice with specific genetic modifications including gene disruption, gene replacement, and even engineered chromosomal translocation. However, it has been extremely difficult to alter genes in mammals,

other than the mouse, by homologous recombination. Although several ES cell lines have been established, primarily from the 129/SvJ mouse strain, and are now commercially available, cloning of ES cells from other mammalian species has made only limited progress. Embryo-derived pluripotent cell lines have been reported in pigs [6] and cattle [7], but successful germ-line chimeric offspring have not yet been reported. The failure to development of ES cell lines has hampered many applications of gene targeting technologies in domestic animals.

The breakthrough in animal cloning using somatic cells [8] implies that targeted genetic manipulations of domestic animals could be achieved by combining gene targeting in somatic cells and cloning. In this review, the strategy and potential challenges of gene targeting manipulation in the domestic animal are discussed. Some of the applications of gene targeting in domestic animals for bio-medicine and agriculture are also addressed.

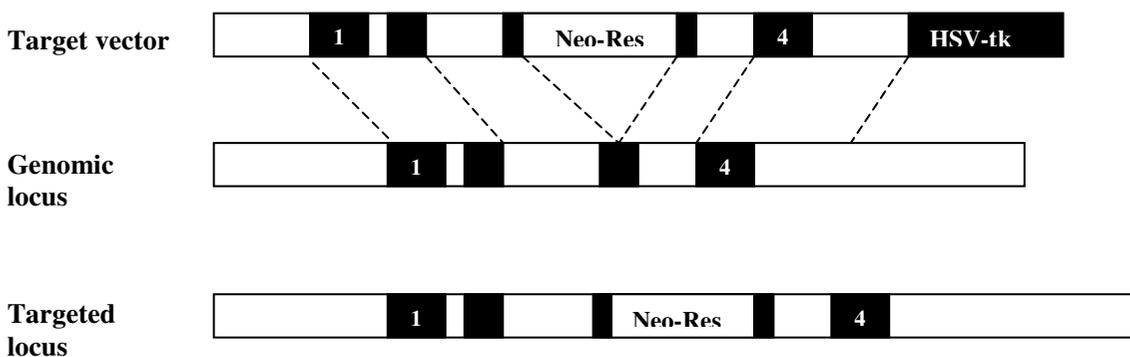
**Homologous recombination and gene targeting**

Gene targeting is the terminology used for genetic manipulations of animal genomes using homologous recombination for the altering of gene activity in any purposeful manner. Homologous recombination is the exchange of homologous segments of two DNA molecules anywhere along their length. The technology of homologous recombination allows the precise modification (replacement or deletion) of certain alleles in the genome. In mammals, homologous recombination naturally occurs during the meiosis cleavage of gamete cells. Two homologous chromosomes undergo a crossing-over process at recombinant hot areas, resulting in the exchange of chromosomal fragments. A primary step in homologous recombination is DNA exchange, which involves pairing of a DNA duplex with at least one DNA strand containing a complementary sequence to form an intermediate recombinant structure. When two complementary DNA strands pair with a DNA duplex, a classical Holliday recombination-joint may form [9]. Once formed, a hetero-duplex structure may be resolved by strand breakage and exchange, so that all or a portion of an invading DNA strand is spliced into a recipient DNA duplex, adding or replacing a segment of the recipient DNA.

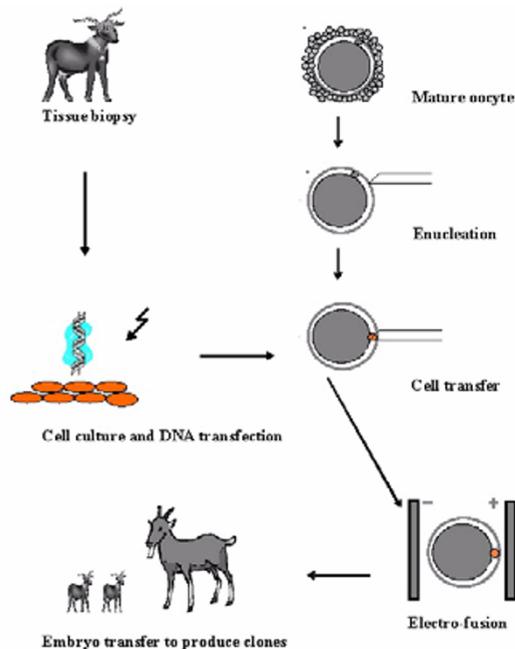
The majority of foreign DNA integration events in yeast are homologous recombination [10] as opposed to random illegitimate recombination in mammalian cells [11]. The research on DNA transformation in the 1970s in yeast led to the development of the basic principles of gene dis-

ruption via homologous recombination in mammalian cells. The insertion of DNA sequences into a human chromosome by homologous recombination was first demonstrated in 1985 by Smithies et al. [12] for the human  $\beta$ -globulin locus. Later, Capacchi's work in 1989 [13] laid the foundation for the development of the strategies of gene targeting using mouse ES cells.

The most commonly used targeting vector in mouse ES cells is the replacement vector (Fig. 1), which contains a positive selection marker for selection of transformed cells. Additionally, a negative selection marker is added outside of the region of the homology to counter-select the random integration following positive selection. During homologous recombination, the negative selectable gene is lost because it is located at the distal region of homology between a vector and a target. Therefore, the cells containing negative selection markers are resulted from random DNA integrations. Usually, the simplex virus-thymidine kinase (HSV-tk) gene is used for negative selection. The cells with the HSV-tk gene are sensitive to the nucleotide analogs gancyclovir or 1-(2-deoxy-2-fluoro-beta-D-arabino-furanosyl)-5-iodouracil (FIAU). As a result, only the cells transformed through homologous recombination are isolated following negative selection [13]. This procedure is termed positive-negative selection (PNS). The ES cells with targeted mutant genes can then be injected into host blastocysts to derive chimeric mice. Eventually, some of the chimeric mice could transmit the genotype of the ES cells to their progeny.



**Figure 1**  
**The replacement vector for gene targeting.** The third exon of a target gene is disrupted by neomycin resistance marker (Neo-Res) after homologous recombination. The HSV thymidine kinase gene (HSV-tk) is removed following homologous recombination.



**Figure 2**  
Procedure of producing gene-targeted animals (goats) by cloning.

### Gene targeting in somatic cells and animal cloning

#### The general strategy of gene targeting using animal cloning

The first animal cloned via nuclear transfer of an adult somatic cell, Dolly the sheep [8], sparked vast interest in nuclear reprogramming of somatic cells and animal cloning. Nuclear transfer technology is now widely used by numerous laboratories to produce animals from the somatic cells of animal fetuses and adults. In the last several years, the procedures of nuclear transfer have been successfully applied to various mammalian species and have resulted in live births of mice [14], cattle [15], goats [16,17], pigs [18], rabbits [19] and cats [20]. Furthermore, transgenic animals have been produced by cloning gene-transfected fetal somatic donor cells in sheep [21], cattle [22] and goats [17]. A procedure for producing cloned animals through nuclear transfer of gene-targeted somatic cells is shown in Fig. 2. Somatic cells (usually fetal fibroblastic cells) are transfected in vitro by liposomes or electroporation, screened for gene targeting, and transferred into enucleated oocytes. The couplets are electrically pulsed to induce fusion and then activated for further development in vitro. The embryos derived from nuclear transfer are

transferred into recipients which give birth to offspring carrying mutations from the gene-targeted somatic cells. Since cloning of pluripotent ES cells in domestic animals has not been fully demonstrated, to date, it has not been possible to knock-in or knock-out genes in domestic animals by the homologous recombination in ES cells and the production of animal chimeras. Alternatively, the technology of animal cloning might provide a basis for gene targeting in farm animals. In fact, gene-targeted animal clones have been successfully produced in sheep [23] and pigs [24] by homologous recombination in fetal fibroblast cells and nuclear transfer.

#### Gene targeting in somatic cells

A number of parameters have been shown to influence the frequency of homologous recombination in mouse ES cells: (1) the frequency increases when longer homologous sequences are included in the targeting constructs [25]; (2) homologous recombination with isogenic DNA sequences is more efficient than with non-isogenic sequences [26]; (3) absolute frequency of homologous recombination appears to be locus dependant [27]. The absolute targeting frequency in mouse ES cells varies from  $1 \times 10^{-5}$  to  $1 \times 10^{-6}$  per electroporated cell [28]. However, accumulated evidence shows that gene targeting in somatic cells is more difficult than in mouse ES cells, and the frequency in somatic cells is about two orders of magnitude lower than in mouse ES cells [29], for example, ranging from  $2.8 \times 10^{-7}$  to  $27.5 \times 10^{-7}$  per cell in sheep [30]. Apparently, mouse ES cells possess higher recombination frequency than somatic cells. Therefore, a more efficient strategy for promoting homologous recombination is required for gene targeting in somatic cells.

Although PNS for mouse ES cells can yield an enrichment of about 2000 fold [13], PNS in somatic cells can only achieve a 2–3 fold enrichment [12]. A powerful promoterless selection has been employed for increasing enrichment of gene targeting events in somatic cells. In a promoterless vector, the gene of the positive selection marker lacks a promoter, it can only be expressed from the internal promoter of the target gene following homologous recombination. In some very rare cases, non-homologous recombination events could still be recovered if the targeting vectors integrate into the sites of chromosomes close to an active promoter. Hanson et al. [12] reported that an enrichment ratio of 5,000- to 10,000-fold with a promoterless vector in a rat fibroblastic cell line for the c-myc gene had been achieved. In a report of generating  $\alpha$  (1,3) galactosyltransferase knock-out porcine fetal fibroblasts, it was also demonstrated that the promoterless targeting vector provided a significant enhancement in gene targeting efficiency by eliminating a larger proportion of random integration events in comparison to the PNS strategy [31].

The somatic cell lines selected for gene targeting and nuclear transfer must have high potency for population expansion and a high stability of cytogenetic normalcy. Theoretically, a total of ~45 population doublings are required to generate gene-targeted cells for nuclear transfer [32]. An obvious choice among the somatic cell types is the fetal fibroblast cell due to its proved ability to produce cloned animals and the ease of population expansion. The life span of the fetal fibroblast is about 30–50 population doublings in pigs and cattle [33] and 40–120 population doublings in sheep [30]. Therefore, it is possible to carry out gene targeting procedures with these cells. Furthermore, it is feasible to introduce targeted mutations into animals through nuclear transfer, since evidence shows that populations of bovine fibroblasts, after more than 45 cell doublings [34], or even in senescence [35], retained their totipotency for supporting full development of cloned embryos. Up to now, several reports of gene-targeted modifications in sheep [23,36] and pigs [24,37] using promoterless targeting vectors and fetal fibroblastic cell lines have been published.

When somatic cells nearing senescence are used, chromosomal abnormality is the critical factor related to poor efficiency of animal cloning. In sheep, telomeres degrade at a rate of ~0.59 kb per year in vivo, but much faster in culture [38]. When telomeres fall below a critical length, chromosomal abnormalities occur more frequently. Somatic cells can be induced to prolong their longevity and retain cytogenetic normalcy after a large number of population doublings. Previous reports indicate that human diploid fibroblasts can be immortalized by transfection with the telomerase catalytic component. With this treatment, the human fibroblasts could be cultured beyond 300 passages without phenotypic or chromosomal abnormalities [39–41]. Therefore, the expression of telomerase in targeted somatic cells could be an effective treatment for improving the efficiency of animal cloning.

## Applications of gene targeting in domestic animals

### Xenotransplantation

Organ transplantation is the only known treatment for many diseases involving terminal organ failure. Unfortunately, the lack of availability of human organs has greatly limited the number of patients who can receive this life-saving treatment. Because of the serious shortage of human organs, tremendous effort has been made worldwide to the study for the use of genetically engineered animal organs as replacement organs for human transplantation (Xenotransplantation).

Pigs are considered to be the most suitable animal for supplying organs because their organs are physiologically and anatomically compatible with those of humans [42], and

they are already a production animal slaughtered for food. Furthermore, pigs can be easily bred, they are very prolific, and can be housed in pathogen-free facilities to prevent the transmission of infectious diseases [43]. One of the major constraints to using pig organs for xenotransplantation is complement-dependant hyper-acute rejection (HAR), which occurs within a few minutes following tissue transplantation between discordant species of animals. Hyper-acute rejection results in lyses of the endothelial cells in the vessels of the transplanted organs and ultimately in graft failure [43]. Human and old world monkeys have natural antibodies against  $\alpha$  (1, 3)-galactosyl epitopes on pig cells. Therefore, the best way to circumvent HAR is to knock-out the function of the  $\alpha$ -1, 3-galactosyltransferase gene in the pigs. Mice with an  $\alpha$ -1, 3-galactosyltransferase gene inactivated through homologous recombination are viable, and when human serum bound to cells and tissues of these mice they showed substantially less xeno-antibody production than normal mice [44]. One allele of the  $\alpha$ -1, 3-galactosyltransferase locus in the pig was successfully knocked out through homologous recombination in somatic cells and subsequent nuclear transfer [24,37,45]. More recently, the second allele of  $\alpha$ -1, 3-galactosyltransferase gene has also been inactivated by selection of a point mutation at the second base of exon 9, which resulted in inactivation of the gene. Four healthy piglets with double knock-out of  $\alpha$ -1, 3-galactosyltransferase gene were produced by nuclear transfer [46]. Further analysis of the tissues and organs from these double knock-out pigs in nonhuman primate models will provide crucial information regarding the mechanisms of HAR.

### Knock-out of Prnp gene

Prion diseases have had a devastating impact on the production of livestock, bovine spongiform encephalopathy (BSE, one type of prion disease in bovine) is expected to cause about \$50 billion loss in UK. Now, it has been ascertained that one variant of deadly Creutzfeldt Jacob disease (CJD) in humans can be caused by consumption of animal products contaminated with the BSE pathogen [47]. Control of the BSE epidemic has become top priority worldwide.

Animal prion diseases include scrapie of sheep and goats, transmissible mink encephalopathy, chronic wasting disease of mule deer and elk, feline spongiform encephalopathy and BSE. The characteristics of these diseases are infectious and vacuolar degeneration of the gray matter neuropil. It was discovered by Prusiner in 1982 [48] that the infectious agent was a protease-resistant protein, which was termed a prion (PrP). A prion has a molecular weight of 27–30 kDa and is encoded by a single copy gene, Prnp in mammals. Prions exist in two major isoforms: the nonpathogenic or cellular form, designated

PrP<sup>C</sup>, and the pathogenic or scrapie-inducing form, designated PrP<sup>Sc</sup>. Prion diseases are caused by conversion of PrP<sup>C</sup> to PrP<sup>Sc</sup> through infection or genetic mutation. Once a cell contains PrP<sup>Sc</sup>, it appears to act as a conformational template by which PrP<sup>C</sup> is converted to a new molecule of PrP<sup>Sc</sup> through protein-protein interactions [49].

Some evidence shows that normal prions play a role in synaptic function [50] and copper binding [51]. However, no developmental or behavioral abnormalities were found in prion knock-out mice. Furthermore, in the prion knock-out mice, homozygous null mice (Prnp<sup>0/0</sup>) fail to develop the characteristic clinical and neuropathological symptoms of scrapie after inoculation with mouse prions, and they do not propagate prion infectivity [52,53]. Therefore, it is expected that removal of the Prnp gene from cattle and sheep will result in these animals being resistant to BSE and scrapie. Fetal fibroblast cell lines with the deletion of one allele of the PrP gene have been established in sheep by homologous recombination [30]. Four cloned lambs were produced from nuclear transfer of the PrP<sup>+/-</sup> somatic cells [36]. More investigations are needed to evaluate the survivability of PrP<sup>0/0</sup> domestic animals and their resistance to prion diseases.

#### **Inactivation of animal Ig genes**

Human antibodies have numerous diagnostic and therapeutic applications. Current antibody production platforms used in industry are not expected to meet future demand for production capacity. Some of the strategies for mass production of human antibodies involve: (1) introducing the human immunoglobulin gene into mice or large domestic animals (e.g. cattle) with their endogenous immunoglobulin gene inactivated, (2) producing human monoclonal antibodies from mouse hybridoma or polyclonal antibodies from large animal blood immunized against the human antigen. To inactivate the endogenous immunoglobulin gene, gene disruption by homologous recombination in mouse ES cells, or somatic cells of large domestic animals, must be carried out in order to introduce the mutation into the animals through cloning.

Each antibody molecule consists of two classes of polypeptide chains, light (L) chains ( $\kappa$  L-chain or  $\lambda$  L-chain) and heavy (H) chains. A single antibody molecule has two identical copies of the L chain and two of the H chain. The loci of antibodies are very large and located on different chromosomes (chromosome 14 (H-chain locus), 22 ( $\lambda$  L-chain locus) and 2 ( $\kappa$  L-chain) in humans). In their germ line configuration, these loci consist of diverse segments encoding the variable (V), diversity (D) and joining (J) genes that comprise the variable domains along with the segments that encode the constant (C) domains [54].

Mouse antibody is immunogenic in humans. The major factors contributing to this immunogenicity are thought to be the sequences of the murine C- and V-regions of an antibody molecule [55]. Immunogenicity of therapeutic antibodies is a significant problem and severely limits their widespread and repeated applications to treat many diseases. A process of antibody humanization has been employed to structure the mouse antibodies within the framework of the human antibody molecule, while retaining the mouse antigen-binding complementary determining regions [56]. Although partially humanized antibodies have demonstrated significantly reduced immunogenicity, the most desirable antibodies for therapeutic applications in humans would be fully humanized antibodies [57]. Transgenic mice have been constructed which have had their own immunoglobulin genes functionally replaced with human immunoglobulin genes so that they produce human antibodies upon immunization [58-60]. Elimination of mouse antibody production was achieved by inactivation of mouse Ig genes in the ES cells by using gene-targeting technology to delete crucial *cis*-acting sequences involved in the process of mouse Ig gene rearrangement and expression. B cell development in these mutant mice could be restored by the introduction of megabase-sized YACs containing a human germline-configuration H- and  $\kappa$  L-chain minilocus transgene. The expression of fully human antibody in these transgenic mice was predominant, at a level of several 100  $\mu$ g/l of blood [60]. This level of expression is several hundred-fold higher than that detected in wild-type mice expressing the human Ig gene [60], indicating the importance of inactivating the endogenous mouse Ig G genes in order to enhance human antibody production by mice. More recently, a human artificial chromosome (HAC) vector containing the entire unarranged sequences of the human Ig H-chain and  $\kappa$  L-chain was successfully introduced into cows (TC cows) with the technology of microcell-mediated chromosome transfer and nuclear transfer of bovine fetal fibroblast cells [61]. The HAC was retained in calves at a rate of 78–100% of cells. Human immunoglobulin proteins were detected in the blood of new born calves at a level of 13–258  $\mu$ g/l. This report suggests that production of a specific human polyclonal antibody could be easily scaled up by direct immunization of TC cows. Nevertheless, greater challenges remain for increasing expression levels of the human antibodies by inactivation of the endogenous bovine Ig genes. With further improvements to gene targeting technology in somatic cells and nuclear transfer, immunologically deficient cattle or other large domestic animals will be produced in order to provide a platform for expressing various human Ig genes.

#### **Other applications of gene targeting in domestic animals**

Gene targeting in domestic animals has other potential applications in both agriculture, and as human disease

models. In animal production, the candidate genes for targeting modifications may include: (1) Myostatin gene, which is involved in the expression of the 'double muscle' phenotype of some breeds of cattle [62], it has been suggested that knocking-out this gene would increase skeletal muscle growth in animals [32]; (2) Growth hormone gene, early studies of pigs transgenic for an exogenous growth hormone gene showed an increased growth rate, however poor control of its expression caused many deleterious side effects. Knock-in with extra copies of the growth hormone gene should result in an elevated level of growth hormone with a physiological pattern of release, which may lead to an enhancement of the animal growth rate without the negative effects. Gene targeting has been extensively used in mice to create disease models for studying the pathologies of and therapies for human diseases. However, in some disease models, knock-out mice may exhibit the biochemical pathways of the pathology, but often do not produce clinical symptoms [63]. Genetic engineering of livestock through gene targeting could create better models for human diseases due to greater similarities in anatomy, physiology and organ size. For example, the mouse model of human cystic fibrosis was generated by disruption of the cystic fibrosis transmembrane conductance regulator (CFTR) gene, however, the utility of the CFTR-knock-out mice in studying the pathogenesis of cystic fibrosis has been limited because of their failure, despite the presence of severe intestinal disease, to develop lung disease [64,65] due to differences in lung physiology. There are substantial similarities between humans and sheep in lung physiology, anatomy, DNA sequence of the CFTR gene, and tissue specific expression patterns of the CFTR gene [66]. Therefore, a knock-out of the CFTR gene in sheep could produce a better model for human cystic fibrosis.

## Conclusions

The combined technologies of gene targeting in somatic cells and animal cloning permit the introduction of specific genetic modifications into the genome of domestic animals. Although gene targeting in mouse ES cells has become a routine procedure in many laboratories worldwide, the efficiency of gene targeting in somatic cells is still very low. For some genes, it is even impossible to implement the strategy of gene targeting using promoterless targeting vectors, due to the lack of active promoters in fibroblasts and other types of somatic cells. Nevertheless, continuous research efforts aimed at cloning stem cells of domestic animals might eventually provide a platform for efficient gene targeting using the technology similar to that for mouse ES cells. The application of gene targeting technologies in domestic animals for xenotransplantation has made significant progress in recent years. Generation of homozygous knock-out pigs for the  $\alpha$ -1, 3-galactosyltransferase gene will eventually elucidate the

mechanism(s), and lead to the prevention of hyper-acute immuno-rejection of transplanted tissues and organs. In the foreseeable future, gene targeting will likely play a major role in the manufacture of pharmaceuticals, and comprise much of the research examining physiological mechanisms as well as analyzing gene functions in domestic animals.

## Additional material

### Additional File 1

Biographies

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