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The Production of Transgenic Livestock and Its Applications

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ABSTRACT

During the last 20 years, transgenic animal technology has provided revolutionary new opportunities in many aspects of agriculture and biotechnology. Several gene delivery systems including pronuclear injection, retroviral vectors, sperm vectors, and somatic cell cloning have developed for making transgenic animals. In the future major improvements in transgenic animal generation will be mainly covered by somatic cell cloning technology. Many factors affecting integration frequency and expression of the transgenes should be overcome to facilitate the industrial applications of transgenic technology. Transgenic animal technology has settled down in some areas of the biotechnology, especially the mass production of valuable human proteins and xenotransplantation. In the 21st century animal biotechnology will further contribute to welfare of human being.

(Key words: Transgenic animals, Bioreactor, Xenotransplantation)

Ⅰ. INTRODUCTION

Transgenic animals have been defined as animals that have integrated foreign DNA into their germline as a consequence of experimental introduction of DNA (Palmiter and Brinster, 1985). Production of transgenic animals using various gene delivery systems has been demonstrated. The most commonly used methods include pronuclear injection (Hammer et al., 1985), retroviral vector (Kim et al., 1993), sperm vector (Lavitrano et al., 1989) and somatic cell nuclear transfer (Schnieke et al., 1997; Cibelli et al., 1998). Transgenic farm animals including goats (Ebert et al., 1991), sheep (Clark et al., 1989; Wright et al., 1991), pigs (Wall et al., 1991) and cattle (Krimpenfort et al., 1991) have been produced. However, the efficiency often varies between species and methodology, although numerous approaches have been used to improve transgenic efficiency. Nuclear transfer technology using mammalian somatic cells will greatly extend the efficiency of transgenic animals. In our laboratory a variety of transgenic animals including cattle and Korean Native goats have been generated, and nuclear transfer technology using transformed somatic cells is still under development. This paper describes the methods of gene transfer and the applications of transgenic animals.

Ⅱ. GENERATION OF TRANSGENIC LIVESTOCK

Several methods have been developed to introduce genetic materials into the developing mammalian embryo in such a way that it may become stably integrated into chromosomes of the animal.

1. Pronuclear Injection

Pronuclear injection is to introduce DNA into one of the pronuclei of a zygote by using a micromanipulator. Pronuclear injection is the predominant method for creation of transgenic animals, although it is labor intensive and requires a high level of technical skills and experience in embryo manipulation. The process of making transgenic farm animals by pronuclear injection is almost exactly the same as that used to produce transgenic mice. However, the major problem of pronuclear injection is the low transgenic rate in large animals as compared to mice (Wall, 1996). The efficiency in producing transgenic cattle is at least 40-fold lower than that of mice (Wall et al., 1997). Out of 74 calves that developed from DNA-injected bovine embryos in our laboratory, only 2 (2.7%) were identified as transgenic (Table 1). Another problem of pronuclear injection is mosaicism of transgenic founders. The number of mosaic transgenic mice represents approximately 15% of the total, but this may be an underestimate since many investigators have not reported frequencies of mosaicism among their transgenic mice. After microinjection of DNA, a high percentage of mosaic mouse embryos is detected in early cleavage stages (Burdon and Wall, 1992). Whitelaw et al. (1993) demonstrated that at least 62% of integrations following DNA microinjection resulted in mosaic embryos. Recently, Eyestone (1999) reported that 7 of 8 trangenic founder cattle passed their transgenes to the embryos at low transmission rates of less than 30%, showing varying degrees of mosaicism. When bovine oocytes matured *in vitro* were fertilized with sperm of one transgenic bull and the transgene for individual embryo was detected by PCR, overall transmission rate of the transgene was 20.9% (Table 2). This result suggests that our transgenic founder is germ-line mosaic. Thus, the mosaic frequency probably is high in transgenic cattle. Mosaic transgenesis is common due to late integration after the first round of zygotic DNA synthesis. In bovine zygotes, pronuclei do not become visible by Normarski optics 16 to 18 h post-insemination when DNA replication is already in progress (Wall, 1996). Therefore, most of the zygotes in the present work are

Table 1. Production of transgenic calves carrying human lactoferrin gene

Injected embryos	Cultured embryos	Blastocysts (%)	Transferred embryos	Pregnant / recipients(%)	Transgenic / calves(%)
10252	5391	722 (13.4)	579	78/395 (19.7)	2/74 (2.7)

Table 2. Identification of the transgene and sex of bovine embryos fertilized *in vitro* with sperm of transgenic bull by PCR

Group	Replica					Positive /Total (%)
	1	2	3	4	5	
Transgene(+)	3/9	5/32	10/30	5/30	5/33	28/134 (20.9)
Sex(+)*	6/9	22/32	20/30	11/30	24/33	83/134 (61.9)

(+)* is positive for male

injected during DNA synthesis, increasing the probability that the resulting transgenic offspring will be mosaic and transmit their transgenes only to 25% of their offspring. Despite these limitations, pronuclear injection has several advantages over other gene delivery systems. It is considered a safe and reliable method for gene transfer. Transgenic animals have been consistently produced by pronuclear injection in a variety of species, although there is a great variation in the transgenesis frequency between species. Schedl et al. (1993) reported the production of transgenic mice carrying a yeast artificial chromosome (YAC) ranging in size 350 to 460 kb. Thus, there is no size limitation of DNA fragment being injected (Brem et al., 1996). Another advantage is that the integration efficiency has no apparent correlation with DNA length (Brinster et al., 1985).

2. Retroviral Vectors

Retroviruses have a single-stranded RNA genome, which after infection of the host cell is reverse-transcribed into DNA and integrated into a host chromosome in a singe copy. Retroviruses can be genetically modified to act as vectors allowing the infection of embryos with exogenous DNA (Jaenisch et al., 1975). Superficially, this is an attractive option as the process is relatively efficient. The major physical limitation of retroviral vectors is their size. A maximum of 10 kb of retroviral sequences can be efficiently reverse- transcribed and encapsidated to form an infectious particle (Temin, 1987). Another drawback is that transgenic mice produced by retroviral infection are almost mosaic because infection occurs after several cleavages. Kim et al. (1993) demonstrated successful infection of bovine preimplantation embryos by replacing the endogenous MoMLV envelope glycoprotein with that of the gibbon ape leukemia virus. When retroviral vectors were infected into bovine oocytes arrested at metaphase Ⅱ (MⅡ) of the second meiotic division before fertilization, all the offspring born from infected oocytes were transgenic (Chan et al., 1998). These transgenic animals showed germline transmission and transgene expression. Thus, retroviral vector systems provide a very promising method for producing genetically modified animals.

3. Sperm Vectors

The use of spermatozoa as noninvasive delivery vesicles to transfer exogenous DNA into oocytes during *in vitro* fertilization has provided an ideal alternative in generation of transgenic animals (Lavitrano et al., 1989; Spadafora, 1998). Due to its relative simplicity compared to pronuclear injection, many researchers have attempted to create transgenic animals by sperm vector. So far, offspring have been produced in several species including mice (Lavitrano et al., 1989; Maione et al., 1998), pigs (Sperandio et al., 1996), fish (Khoo et al., 1992) and cattle (Schellander et al., 1995) by using this method. The success of sperm transformation depends on the binding efficiency of DNA and its subsequent internalization into sperm nuclei. To improve the binding and internalization of DNA, different methods including the use of liposome (Bachiller et al., 1991), the direct injection of DNA complex into seminiferous tubules or vas deferens (Kim et al., 1997; Huguet and Esponda, 1998), and the electroporation of spermatozoa (Gagne et al., 1991). Interestingly, Perry et al. (1999) reported an adaptable method of transgenesis that exogenous DNA could reproducibly be delivered into an oocyte by microinjected spermatozoa. Although only limited success has been demonstrated, sperm vectors still hold great promise for future transgenic technology.

4. Somatic Cell Cloning

The successful production of offspring derived from nuclear-transferred (NT) embryos using somatic cells in mammals has important implications not only for multiplication of valuable domestic animals, but also for elucidation of genomic totipotency of donor nuclei. Fetal and adult somatic cells have been used to produce animals after nuclear transfer using electrical fusion or intracytoplasmic injection of nuclei into oocytes. Consequently, cloned several species animals including sheep (Wilmut et al., 1997; Schnieke et al., 1997), goat (Baguisi et al., 1999), mouse (Wakayama et al., 1998) and cattle (Cibelli et al., 1998; Kato et al., 1998; Wells et al., 1999; Zakhartchenko et al., 1999) have been successfully produced by nuclear transfer. The major obstacle for the application of somatic cell cloning remains its low efficiency. Intensive studies on remodeling/reprogramming, oocyte synchronization, nuclei synchronization of donor cells, low pregnancy, and telomere restoration will improve the cloning success. Nonetheless, cloning from transformed cells has other advantages for making transgenic farm animals (Anderson and Seidel, 1998). The production of transgenic animals by nuclear transfer method using transformed somatic cells showed 100% efficiency (Schnieke et al., 1997; Cibelli et al., 1998). These results suggest the future place of cloning technology for the production of transgenic animals. Thus, the enhanced transgenic efficiency must be achieved by cloning technology using somatic cells in the 21 century.

Ⅲ. FACTORS AFFECTING TRANSGENESIS EFFICIENCY AND EXPRESSION OF TRANSGENES

Complex techniques such as *in vitro* maturation (IVM), fertilization (IVF) and culture (IVC), DNA injection and embryo transfer are essential to produce transgenic animals. The production of many transferable embryos after DNA injection is required for making transgenic animals. Transfer of expanded blastocysts that developed from DNA-injected bovine embryos had a higher pregnancy rate as compared with early and mid blastocysts (Han et al., 1996). As shown in Table 3, co-culture of DNA-injected bovine embryos with mouse embryonic fibroblasts (MEF) showed a higher *in vitro* development than without MEF (Park et al., 1999). Linear DNA is integrated much more efficiently than supercoiled circular molecules (Brinster et al., 1985), suggesting that free DNA ends are important for integration. Generation of transgenic livestock by pronuclear injection is inefficient, showing low integration frequency at less than 1% of injected embryos (Wall, 1996). Some techniques for selection of transgenic embryos (Bowen et al., 1994; Hyttinen et al., 1994) have been tried to enhance the production efficiency of transgenic animals. Integration frequency is a

Table 3. Comparison of the *in vitro* development of DNA-injected embryos co-cultured with or without MEF

Group	Injected embryos	Survived embryos(%)	Cultured embryos(%)	Blastocysts (%)
CR1aa	2931	2505(85.5)	1804(72.0)	131(7.2)[a]
MEF	3370	2516(74.6)	1805(71.7)	302(16.7)[b]

a vs b; $P<0.05$

major factor in transgenic animals. The use of repetitive DNA sequences can be considered to increase transgenic frequency in transgenic animals. The repetitive sequences such as the short interspersed repeated DNA elements (SINEs) are present in high copy numbers, located in euchromatic regions and known to have recombinational activity (Weiner et al., 1986). Enhanced integration frequency has been shown in the embryos and fetuses after DNA injection when the SINE sequences were connected to the expression vector (Kang et al., 1999).

The level of transgene expression usually differs between lines of transgenic mice that carry the same transgene (Palmiter and Brinster, 1986). The differences in expression must reflect variation in the structure of the integration site and could be due to differences in number of integrated copies, the precise arrangement of the integrated DNA, mutation or modification of the transgene. DNA sequence elements such as β-globin locus control region (LCR) which overcome the effects of position or arrangement (Grosveld et al., 1987). Matrix attachment region (MAR) sequences may function as dominant boundaries and partition chromosomes into independently regulated units (Gasser and Laemmli, 1986). All transgenic mice carrying MAR sequences expressed the transgenes (McKnight et al., 1992; Lee et al., 1998). Another factor influencing the expression of transgenes is the presence of introns. The level of expression in transgenic mice was consistently higher when introns were present (Brinster et al., 1988). Our data showed that the genomic sequences induced a higher level expression of the transgenes as compared to cDNA (Kim et al., 1999).

Ⅳ. APPLICATIONS OF TRANSGENIC TECHNOLOGY IN LIVESTOCK

Transgenic technology has the possibility for diverse applications of research and industry. It has been used for studying the mechanisms of developmental regulation of gene expression (Rossant and Joyner, 1989), the identification of new genes (Jaenisch, 1988), the production of animal models of human disease (Hooper, 1990), the production of large amount of specific proteins through mammary gland (Clark et al., 1987), the modification of milk compositions (Wilmut et al., 1990), the enhanced growth performance (Pursel et al., 1989), the development of transgenic animals for xenotransplantation (Hammer et al., 1998) and so on. In this paper we would like to focus on transgenic animals to produce human pharmaceutical proteins and modify genetically pig organs for xenotransplantation.

1. Aniaml Bioreactors

It was proposed that mammary specific expression of human genes might be exploited for the production of recombinant proteins in the milk of transgenic livestock (Clark et al., 1987). To direct tissue specific gene expression, structural genes should be connected to mammary specific promoter sequences such as caseins, whey acid protein (WAP) and lactoglobulin. Transgenic animals named as animal bioreactors have many benefits as compared to conventional production systems through microorganisms or animal cells (Janne et al., 1992; Bremel, 1996). The advantages include high productivity, low operating costs, appropriate post-translational modification of proteins, and that most transgenic animals can give rise to their transgenic progeny. Since the feasibility of genetically engineering farm animals to produce human proteins in milk has been established, the generation of transgenic farm animals expressing human prot-

eins have been reported (Wall et al., 1991; Wright et al., 1991; Krimpenfort et al., 1991). In 1996 and 1998, we produced transgenic cattle with human lactoferrin gene and transgenic goats carrying human granulocyte-colony stimulating factor (G-CSF) gene, respectively. However, a limiting phenomenon in attaining high expressing transgenic lines for commercial production is the so-called position effect. The level of transgene expression will be largely governed by its integration site into the genome. Unfortunately, there is no control over the site of integration of a injected gene. Therefore, the generation of a high expressing line is basically a numbers game. Recently, products from trangenic milk have made considerable progress towards the market. It has been known that PPL Therapeutics have recombinant α-1-antitrypsin (AAT) in phase Ⅲ clinical trials for the treatment of cystic fibrosis and Genzyme Transgenic Transgenic Corporation have human antithrombin Ⅲ (ATⅢ) in phase Ⅲ trials.

2. Transgenic Animals for Xenotransplantation

The grafting of living organs, tissues or cells between members of different species, whether genetically modified or not, is called xenotransplantation (Auchincloss, H.J., 1988). Such transfer of animal organs to humans appears on the horizon as a last resort to solve the severe shortage of transplants of human origin for patients suffering from end-stage disease. The remaining alternative is to use an animal similar in size to human, readily available, without the danger of infectious diseases. The domestic pig is found to possess several advantages except immunological and physiological compatibility. Porcine and human organs have approximately the same size and a similar efficiency. The most serious huddle to xenotransplantation is immediate destruction of xenografts by hyperacute rejection (HAR). The histology of this type of rejection is marked by extensive intravascular thrombosis and extravascular hemorrhage. The general principles of genetic modification to promote xenotransplantation are to reduce the expression of the additional antigens and to correct the molecular incompatibilities responsible for dysregulation of the immune response. An efficient approach to mitigate HAR is to express human complement regulator proteins such as human decay-accelerating factor (h-DAF) or membrane cofactor protein (MCP) on the surface of the xenogenic donor cells (Rosengard et al., 1995). Orthotopic transplantation of h-DAF transgenic pig hearts to juvenile baboons ended at a maximal time of 10 days (White, 1996). With vigorous immunosuppression, transgenic pig organs have survived in primates for up to 60 days without evidence of rejection (Waterworth et al., 1997). Another approach to alleviate HAR has been to eliminate the galactosyl transferase gene by homologous recombination (Sandrin et al., 1994). Although homologous recombination is not available in the pig, it is possible to insert transgenes into the pig genome. Sandrin et al. (1995) suggested the idea of competing with the α-galactosyl transferase in pigs by expressing the α-1,3 fucosyl transferase (H-transferase) gene that humans use to form the blood group O antigen. Recently, it has been reported that α-galactosyl transferase gene can be knocked-out in pig fibroblast cells. Thus, the knocked-out pigs will be produced in near future by nuclear transfer using transformed somatic cells.

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