

*Minireview***TRANSGENIC FARM ANIMAL PRODUCTION AND APPLICATION**P. CHRENEK^{1,2*}, A. V. MAKAREVICH¹, J. PIVKO¹, J. BULLA²¹Animal Production Research Centre Nitra, Slovak Republic;²Slovak University of Agriculture in Nitra, Slovak Republic**ABSTRACT**

Transgenic farm animals are important in human medicine as a source of biologically active proteins, as donors in xenotransplantation and for a research in cell and gene therapy. Typical agricultural application include improved carcass composition, lactation performance and wool production, as well as enhanced disease resistance and reduced environmental impact. This minireview summarizes recent research based on the transgenic farm animal production and their potential applications.

Key words: farm animal; transgene; production; application

INTRODUCTION

Over the last three decades biotechnology has advanced to a level where it is generally feasible to make particular changes to the genome, and therefore to the expressed characteristics of living organisms. The product of such a change is called a transgenic or a genetically modified organism (GMO). GMOs are created and/or modified in the laboratory to amplify desired characteristics which are beneficial to mankind (Chrenek and Makarevich, 2008).

In the mid-1970s, several investigators infected mouse embryos with retroviruses and demonstrated that proviral DNA integrated into the genome and was passed to subsequent generations (germ-line transmission of foreign DNA). By the early-1980s the technology gained momentum with various laboratories producing transgenic mice by microinjecting genes into the pronucleus of a fertilized egg, which has been hailed as a seminal event in the development of animal biotechnology. The term “transgenic” describes animals carrying exogenous genes

(foreign DNA segments – gene construct) integrated into their genome. Since that time this definition has been extended to include animals that result from the molecular manipulation of endogenous genomic DNA, including all techniques from DNA microinjection to embryonic stem (ES) cell transfer and “knock-out” mouse production. In 1982, thanks to Palmiter and co-workers, a photograph of a giant transgenic mouse appeared on the cover of *Nature*, showing first visible phenotype in mice overexpressing rat growth hormone. In fact, early work of Richard Palmiter and Ralph Brinster (1986) related to growth performance and the dramatic phenotype of the growth hormone in transgenic mice subsequently influenced the emerging field in a most compelling manner for both basic and applied sciences. First reports of a transgenic rabbit obtained by pronuclear microinjection, which resulted in random insertional transgenesis, date back to 1985. And, by the 1985's sheep and pigs, goat (1991), cattle (1992) had joined the transgenic barn. Since then, the technology available has developed considerably with major turning points

*Correspondence: E-mail: chrenekp@yahoo.com
Peter Chrenek, Institute of Genetics and Animal Reproduction, Animal Production Research Centre Nitra, 951 41 Lužianky, Slovak Republic,
Tel.: +421 37 6546 285 Fax: +421 37 6546 189

Received: March 22, 2010

Accepted: March 24, 2010

continuing to accelerate at an incredible pace and more significantly the reach of the technology also extended to poultry and aquatic organisms such as Atlantic salmon and zebra fish (Houdebine, 1995).

Today, transgenic animals embody one of the most potent and exciting research tools in the biological sciences. Transgenic animals represent unique models that are custom tailored to address specific biological questions. Hence, the ability to introduce functional genes into animals provides a very powerful tool for dissecting complex biological processes and systems. This has made it possible to explore the regulation of gene expression as well as the regulation of cellular and physiological processes. Significant uses of live transgenic mammals are in the arenas of agricultural, biological, biotechnological and biomedical sciences including production of pharmaceuticals and in the field of organ transfer from transgenic animals to humans (Wall and Seidel, 1992). These require the ability to target gene expression and to control the timing and level of expression of specific genes. Experimental designs have taken advantage of the ability to direct specific expression (including cell types, tissue, organ type, and a multiplicity of internal targets) and ubiquitous, whole-body expression *in vivo*.

Gene transfer can be realized through:

a) Methods of transgenic animal creation through the gonads

The possibility of transfecting spermatogonia *in situ* via infusion of transgenes into seminiferous tubules or transfection of germ cell precursors *in vitro* followed by transplantation into host testis was tested. It was demonstrated that testis-derived cells transplanted into the testis of infertile males could populate the host testis, generate sperm, and produce offspring. Presumably the next step would be to transfect the testis-derived cells before transplantation.

b) Methods of transgenic animal creation through the germ cells

In germline gene transfer, the parents' egg and sperm cells are changed with the goal of passing on the changes to their offspring. Germline gene transfer is not being actively investigated, at least in larger animals and humans, although a great deal of discussion is being conducted about its value and desirability. Since 1989, a new method for the production of transgenic animals has been available, namely sperm-mediated gene transfer (SMGT), based on the intrinsic ability of sperm cells to bind and internalise exogenous DNA molecules and to transfer them into the oocyte at fertilisation. The major benefits of the SMGT technique were found to be its

high efficiency, low cost and ease of use compared with other methods. Furthermore, SMGT does not require embryo handling or expensive equipment. Sperm-mediated gene transfer could also be used to generate multigene transgenic pigs that would be of benefit as large animal models for medical research, for agricultural and pharmaceutical applications and, in particular, for xenotransplantation, which requires extensive genetic manipulation of donor pigs to make them suitable for grafting to humans. Three studies that combine sperm-mediated gene transfer with the other methodology are a case in point:

- SMGT + REMI (restriction enzyme-mediated integration);
- SMGT + electroporation (electroporation of DNA into sperm before performing SMGT);
- SMGT + mAb (antibody to associate transgene with sperm before surgical oviduct insemination).

c) Methods of transgenic animal creation through fertilized eggs or embryos

For practical reasons, i.e., their small size and low cost of housing in comparison to that for larger vertebrates, their short generation time, and their fairly well defined genetics, mice have become the main species used in the field of transgenics. There are several principal methods used for the creation of transgenic animals.

1) DNA single microinjection

This method involves the direct microinjection of a chosen gene construct (a single gene or a combination of genes) from another member of the same species or from a different species, into the pronucleus of a fertilized ovum. It is one of the first methods that proved to be effective in mammals. Microinjection in mice and rabbit eggs is relatively simple, thanks to better visualization of pronucleus. However, this does not hold good for embryos of rats and other higher species of animals, where it is much more complex. Total amount of microinjected DNA is about 1-2 pL, which at concentration of 1-2 ng/ μ L represents around 200-400 copies of DNA, depending on the type of gene construct though. Eggs are fixed on one side with the help of holder pipette and are microinjected from the other end with the help of microinjection syringe of several micrometers in size automatically or using manual microinjector. Microinjection process causes a visible increase in the volume of the injected pronucleus. After microinjection followed by short time or long time embryo culture under *in vitro* conditions, embryos are transferred to the oviduct or uterus of the hormonally treated female or using vasectomized males. Most of the gene constructs were prepared using cDNA for the studied gene. Under ideal circumstances and thanks to promoter,

targeted expression of the gene in the desired tissue at sufficient level in proper time was achieved and thereby the ability of transgenic individual to carry the integrated gene to offspring confirmed. Animal accounts for a model organism for the purpose of transgenesis into which it is possible to introduce and integrate gene constructs of different sizes ranging from 10-20 kb using plasmids to about 250-300 kb using YAC, BAC and MAC vectors. The insertion of DNA is, however, a random process, and there is a high probability that the introduced gene will not insert itself into a site on the host DNA that will permit its expression. A major advantage of this method is its applicability to a wide variety of species.

2) DNA double microinjection

On the same principle as the microinjection of a foreign DNA into the pronucleus lies the method which facilitates the transfer of a foreign gene into both pronuclei of fertilized egg, which was successfully used for production of transgenic mice (Kupriyanov *et al.*, 1998). This approach was extrapolated by Chrenek and his co-workers (2005, 2007) in a larger species thereby increasing the transgene integration efficiency upon microinjection of DNA into both pronuclei of rabbit embryos. In the case of double microinjection, degradation of embryos (lysis, fragmentation, irregular division) takes place based on the information as above. Obtained results also showed that double microinjection doesn't cause significantly higher percentage of embryo degeneration. Success of the integration of a foreign gene through this method was at least 30% more than that obtained by the single microinjection method.

3) Cytoplasmic injection

However, using the established method of DNA-ligand-polylysine conjugate to transfect mammalian cells/DNA-transferrin-polylysine transfecting avian cells it was suggested that this might provide a mean for cytoplasmic injection to be successful. The reported success of cytoplasmic injection of DNA/polylysine mixtures in producing transgenic animals also examined the effects of a number of DNA concentrations and lysine-to-phosphate ratios on transgenic efficiency.

Thus, cytoplasmic injection of DNA is possible by this method, although there is a loss of efficiency over the standard pronuclear injection procedure. This method allows the injection of the gene into the zygote even if the pronuclei are not visible (sheep, pigs, cattle). There is also the unexplored avenue that injection of DNA/polylysine mixtures anywhere in the zygote (including the pronucleus) may yield transgenic animals, thereby allowing trainee injectors a chance to be productive while acquiring the skills necessary to complete pronuclear injection proper.

4) Embryonic stem cell-mediated gene transfer

This method involves prior insertion of the desired DNA sequence by homologous recombination into an in vitro culture of embryonic stem (ES) cells using electroporation or liposomes. ES cells are isolated in culture from blastocyst-stage embryos, and embryonic germline (EG) cells are isolated from cultured primordial germ cell (PGC). Development of a stem cell line for a species that could be used as a recipient of the desired DNA and subsequent nuclear transfer of the stem cell nuclei or the incorporation of the stem cells into an embryo with the resulting birth of a chimeric animal are potential solutions to the inefficient incorporation of the injected DNA. ES cell-mediated gene transfer is the method of choice for gene inactivation, the so-called knock-out method. ES cells first were isolated from mouse embryos and since have been used as a model for mammalian embryogenesis and more recently for genetic manipulations. Due to their ability to integrate foreign DNA and thereafter differentiate into any and all tissues of a normal individual, ES cells are effective vehicles for genetic engineering and for creating laboratory animal models of human diseases. Despite considerable efforts, progress towards isolation of ES cells from agricultural species has been slow. Some of the more promising results have been achieved with pigs. To date, production of embryonic stem cell lines has been much more difficult in livestock species than in mice.

5) Retrovirus (Adenovirus)-mediated gene transfer

To increase the probability of an expression, the gene transfer is mediated by means of a carrier or vector, generally a virus or a plasmid. Retroviruses are commonly used as vectors to transfer genetic material into the cell, taking advantage of their ability to infect host cells in this way. Offspring derived from this method are chimeric, i.e., not all cells carry the retrovirus. Transmission of the transgene is possible only if the retrovirus integrates into some of the germ cells. For any of these techniques the success rate in terms of live birth of animals containing the transgene is extremely low. Providing that the genetic manipulation does not lead to abortion, the result is a first generation (F1) of animals that need to be tested for the expression of the transgene. Depending on the technique used, the F1 generation may result in chimeras. When the transgene has integrated into the germ cells, the so-called germ line chimeras are then inbred for 10 to 20 generations until homozygous transgenic animals are obtained and the transgene is present in every cell. At this stage embryos carrying the transgene can be frozen and stored for subsequent implantation.

6) Nuclear Transfer

Nuclear transfer, which involves the transfer of each nucleus (genetically modified) from a multicellular embryo into an enucleated metaphase II oocyte, has been developed in several species. Nuclear transfer has become an active field of research over the last decade, culminating in reports over the past two years of live offspring from differentiated cells of sheep, cattle, and mice. Dozens of laboratories are producing calves and lambs whose genetic parents are transgenic somatic cells, often derived from adults, transfected via electroporation. In spite of the fact that research orients towards modification of somatic cell genome through homologous recombination with the aim of producing so called "knock-out" (targeted gene is blocked) or "knock-in" (which refers to the integration of the transgene to specific site in the cell). After selecting the positive cells ("knock-out" or "knock-in"), these may serve as a donor of nucleus mainly for the purpose of cloning. As the production of cloned (or transgenic) animals is demanding, combination of the techniques of transgenesis, cloning allows achieving up to 30% better results. The principle depends on the utilization of transgenic somatic cells (or nucleus) for the purpose of cloning. Blastomeres isolated from cloned together with early transgenic embryos are subsequently used for the purpose of chimera. In this manner, chimeric cloned transgenic animals can be obtained.

d) Methods of transgenic animal creation through somatic cells

In somatic gene transfer the recipient's genome is changed, but the change is not passed on to the next generation. Matsumoto and his colleagues (2001) developed a non-viral and plasmid-based method for arterial gene transfer by *in vivo* electronic pulse, using a newly designed T-shaped electrode. Using rabbit carotid arteries, they first optimized gene transfer efficiency, and firefly luciferase gene transfer via electronic pulse under 20 V voltage (the pulse length: P_{on} time 20 mins, the pulse interval: P_{off} time 80 mins, number of pulse: 10 times) showed the highest gene expression. Electroporation-mediated gene transfer of *E. coli lacZ* with nuclear localizing signal revealed successful gene transfer to luminal endothelial cells and to medial cells. They concluded that *in vivo* electroporation-mediated arterial gene transfer is readily facilitated, safe and may prove to be an alternative form of gene transfer to the vasculature (Wall, 1996).

The use of transgenic animals

A representative, but non-inclusive, list of purposes for which transgenic animals have been used indicates the wide ranging application of this technology:

- a) in medical research, transgenic animals are used to identify the functions of specific factors in complex homeostatic systems through over- or under-expression of a modified gene (the inserted transgene);
- b) in toxicology: as responsive test animals (detection of toxicants);
- c) in mammalian developmental genetics;
- d) in molecular biology, the analysis of the regulation of gene expression makes use of the evaluation of a specific genetic change at the level of the whole animal;
- e) in the pharmaceutical industry, targeted production of pharmaceutical proteins, drug production and product efficacy testing;
- f) in biotechnology: as producers of specific proteins;
- g) genetically engineered hormones to increase milk yield, meat production; genetic engineering of livestock and in aquaculture affecting modification of animal physiology and/or anatomy; cloning procedures to reproduce specific blood lines; and
- h) developing animals specially created for use in xenografting.

The different animal systems to produce recombinant proteins

- 1) **Milk:** Milk is currently the best available bioreactor. Extensive studies have shown that it can be the source of a variety of recombinant proteins, some of them being rather complex molecules. On the other hand, a certain number of failures occurred for various reasons. Some of them are purely technical such as the generation of transgenic animals. Other, such as protein maturation or secretion, is more fundamental.
- 2) **Blood:** Serum, which collects secretion from many tissues, may be the source of recombinant proteins. Although serum from transgenic animals is an abundant by-product of slaughterhouses it does not appear presently as a potential source for many recombinant proteins. Indeed, many proteins are poorly stable in serum and some of them may hamper the health of the animals.
- 3) **Urine:** Urine is an abundant biological fluid already used to prepare proteins such as gonadotropins for pharmaceutical use. The system allowed expression in urothelium may be useful if it happens that the foreign protein is matured in a more appropriate manner in urothelium than in the mammary gland or if the side-effects of the protein are less deleterious for the animals.
- 4) **Seminal plasma:** Seminal plasma is relatively abundant biological fluid in some species and it can be easily collected. This system, as urine, may have specific advantages. In both cases, it is not known how complex proteins are matured and secreted.

- 5) **Egg white:** Egg white, as milk, is an abundant fluid containing huge amount of proteins and is being secreted out of the body. It is thus expected to be an excellent system to produce recombinant proteins. Yet, it has not been implemented so far, essentially for technical reasons.
- 6) **Silk worm cocoon:** The cocoon of insects contains large amount of a few proteins which form silk. From this point of view, this system shares a certain number of properties with milk and egg white. Silk worm can be easily reproduced into large number of individuals. It is therefore, a good candidate to be a living fermentor.

The mammary gland is probably the most promising target tissue because it produces large amounts of protein in a temperature-regulated fluid that may be collected daily in a non-invasive fashion. Transgenic animals are not only cost-effective bioreactors but, with the complex secretory cell types and organs of the mammalian organism, can perform much more complicated protein modifications than simply cultured cells (Houdebine, 2000).

REFERENCES

- CHRENEK, P. – VASICEK, D. – MAKAREVICH, A. V. – JURCIK, R. – SUVEGOVA, K. – PARKANYI, V. – BAUER, M. – RAFAY, J. – BATOROVA, A. – PALEYANDA, R. K. 2005. Increased transgene integration efficiency upon microinjection of DNA into both pronuclei of rabbit embryos. *Transgenic Res.*, vol. 14, 2005, p. 417-428.
- CHRENEK, P. – RYBAN, L. – VETR, H. – MAKAREVICH, A. V. – UHRIN, P. – PALEYANDA, R. K. – BINDER, B. R. 2007. Expression of recombinant human factor VIII in milk of several generations of transgenic rabbits. *Transgenic Res.*, vol. 16, 2007, p.353-361.
- CHRENEK, P. – MAKAREVICH, A. V. 2008. Transgenic rabbits – production and application. *Slovak J. Anim. Sci.*, vol. 41, 2008, no. 3, p. 113-120.
- HOUEBINE, L. M. 1995. Transgenic animals. Generation and use. Published in The Netherlands by Harwood Academic publishers. 1995, ISBN 90-5702-068-8.
- HOUEBINE, L. M. 2000. Transgenic animal bioreactors. *Transgenic Res.*, vol. 9, 2000, p. 305-320.
- KUPRIYANOV, S. – ZEH, K. – BARIBAUT, H. 1998. Double pronuclei injection of DNA into zygotes increase yields of transgenic mouse lines. *Transgenic Res.*, vol. 7, 1998, p. 223-226.
- MATSUMOTO, T. – KOMORI, K. – SHOJI, T. – KUMA, S. – KUME, M. – YAMAOKA, T. – MORI, E. – FURUYAMA, T. – YONEMITSU, Y. – SUGIMACHI K. 2001. Successful and optimized *in vivo* gene transfer to rabbit carotid artery mediated by electronic pulse. *Gene Ther.*, 2001 Aug;8(15), p. 1174-9.
- PALMITER, R. D. – BRINSTER, R. L. 1986. Germ-line transformation of mice. *Annu Rev Genet*, 1986, vol. 20, p. 465-499. *Review*.
- WALL, R. J. – SEIDEL G. E. Jr. 1992. Transgenic farm animals - A critical analysis. *Theriogenology*, vol. 38, 1992, no. 2, p. 337-357.
- WALL, R. J. 1996. Transgenic livestock: progress and prospects for the future. *Theriogenology*, vol. 45, 1996, p. 57-68.