Precision genetic modification of the Mammalian Genome

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Abstract

The potential of transgenesis to achieve rational and efficient animal improvement has been recognised for some time. Commercial application of this technology has been restricted by inherent limitations of existing methodologies, and a shortage of candidate genes for transfer. Recent advances, notably in the areas of mammalian reproductive technologies and whole genome sequencing, provide, for the first time, realistic prospects for directed genetic modification of the mammalian genome in a commercially relevant manner. Augmentation of traditional breeding objectives with transgenic technologies can be expected to improve the efficiency of existing breeding programs, while the opportunity to introduce novel genes and gene combinations, and modify endogenous genes with precision, raises the possibility of developing animals with novel properties, specifically designed for niche markets of enormous value.

Modification of the genome by transgenesis is therefore likely to become an important component of livestock industries. While Australia has access to the relevant technologies, the pace of international progress has not been matched locally, and this could compromise the competitiveness of local industries. Animal transgenesis provides an example of how strategic investment in key emergent technologies at a local level can be critical for both the establishment and maintenance of industries of international importance.

Transgenesis and animal improvement

The production of heritable, economically valuable genetic alterations in commercially important livestock has long been a goal of animal breeders. The last few decades have seen an explosion in our understanding of the molecular basis for inheritance, recognition of the importance of genes in the animal phenotype, and the identification and functional analysis of many genes associated with important production characteristics. Arising from this basic information grew recognition that direct molecular intervention to achieve genetic modification, or transgenesis, might provide an improved route to animal breeding. In particular, the ability to achieve predetermined genetic alteration might improve the efficiency of traditional breeding programs, and lead to the development of new breeds with production characteristics of enormous agricultural or medical value. For example:

1. One-step introduction of genes which improve livestock properties such as yield, quality and disease resistance into superior breeding stock would provide an efficient mechanism for incremental improvement of existing breeds, without a requirement for extensive selection trials and backcrossing programs.

2. Introduction of genetic variability by transgenesis is not restricted by the species barrier, allowing the introduction of genes from diverse sources, and generation of animals with altered characteristics of agricultural or medical relevance.

In general, the perceived promise of animal transgenesis injection (Palmiter and Brinster 1986) has not been matched by outcomes of applied relevance with only a few exceptions. It has become apparent that deficiencies in our current knowledge and inherent limitations in the existing transgenic technology underlie this failure. Nonetheless, recent technical innovations provide, for the first time, realistic prospects for precise genetic modification of the animal genome in accordance with desire, thereby opening the way to achievements of commercial relevance.
Transgenesis by pronuclear injection

Traditionally, animal transgenesis has been carried out by pronuclear injection (Figure 1). Developed in mice (Gordon and Ruddle 1981), this technique entails direct microinjection of DNA fragments into the pronucleus of a fertilised oocyte. In a percentage of microinjected embryos, injected DNA integrates in tandem arrays at apparently random positions in the genome, and is inherited stably by each daughter cell resulting from embryonic division. Return of the embryo to a recipient female allows development to term, and birth, in around 5-10% of microinjected embryos, of transgenic offspring. Each cell of the transgenic liveborn, described as a founder, contains copies of the injected transgene which are passed on to any offspring (Niemann and Kues 2000).

Expression of protein encoded by the injected DNA can alter the phenotype of the transgenic individuals. For example, transfer of growth hormone genes has been shown to increase animal size and growth rate in several species including mouse, rabbit, sheep, pig and cattle (Palmiter et al. 1982; Hammer et al. 1985; Bondioli et al. 1991). The injected DNA is designed to achieve the desired phenotypic alteration. In general it comprises two components: a gene that encodes a biologically active protein, and a linked promoter which directs expression of the gene to the appropriate cell types and stages of development. For example, use of the actin promoter has been used to direct ubiquitous expression of transgenes, while the casein promoter can be used to direct expression of transgenes specifically to the mammary gland, thereby allowing secretion of specific proteins into milk (Ziomek 1998).

Figure 1: Diagrammatic representation of animal transgenesis by pronuclear injection. Random integration of injected DNA into the genome precludes effective control of transgene expression.
Control over transgene expression is a critical feature of transgenic animal design. For example, ubiquitous expression of growth hormone in the embryonic and adult pig has been associated with arthritic symptoms which are largely overcome by expression from regulated promoters, expressed specifically in response to dietary changes such as heavy metals (metallothionein promoter; Palmiter et al. 1982) or antibiotics (Tet promoter).

**Deficiencies of transgenesis by pronuclear injection**

Accurate regulation of transgene expression has proven difficult to achieve in practise. Some limitations are inherent in our knowledge of promoter biology. Few promoters with tightly regulated expression patterns have been identified, and in many cases the relatively small promoter fragments that are used in transgene constructs do not recapitulate precisely the expression pattern of the endogenous gene. While some progress has been made using larger promoter fragments and DNA fragments, these can reduce the efficiency of transgenesis.

Other inherent deficiencies in the pronuclear injection process contribute to failure of transgene regulation (Nieman and Kues 2000). A major weakness derives from our inability to control integration of the transgene into the genome. The number of inserted copies can vary widely, between one and several hundred copies, with consequent impact on RNA and protein expression levels. Further problems are associated with inability to control the site of transgene integration. Integration at specific sites in the genome, often associated with heterochromatin, can lead to rapid shutdown or irreversible silencing of transgene expression. Integration adjacent to powerful enhancers can lead to inappropriate expression of the transgene irrespective of the transgene promoter, while other sites of genome integration may be associated with mutation of endogenous genes.

Transgenesis by pronuclear injection is also restricted by the nature of the genetic modifications that can be made. At present, the identity and function of only a small number of mammalian genes has been established. Of greater long-term impact is the limitation of pronuclear injection technology to addition of genes, when many of the more valuable genetic alterations incorporate gene deletion or modification. Accordingly, commercially important outcomes of pronuclear injection technology have been restricted to gene insertion, for example to ruminants modified genetically to express therapeutic human proteins in milk (Ziomek 1998).

In summary, commercial application of transgenesis by pronuclear injection has been restricted by three major deficiencies:

1. Inability to control the site of transgene integration or copy number prevents adequate control of gene expression. The associated unpredictability of genetic modification generates a requirement for extensive and costly screening of multiple transgenic founder animals, and breeding programs to ascertain heritability of transgenic alterations.

2. Pronuclear injection allows only the insertion of additional genes into the animal, and not commercially valuable modifications such as gene deletion or modification.

3. Few mammalian genes of demonstrated commercial significance have been identified.

**Reproductive technologies and precision modification of the mammalian genome**

It has been recognised for some time that many deficiencies associated with pronuclear injection could be overcome by the development of techniques which allow specific modification of the genome in predetermined fashion. Problems associated with variable transgene copy number could be overcome by
specific integration of a single transgene at a predetermined genomic location. Tight regulation of transgene expression could be achieved by directed integration of transgenes adjacent to defined endogenous promoters, thereby exploiting the properties of tightly regulated promoters in situ. Precision modification of the genome in this manner is therefore expected to reduce markedly the variability of the transgenic procedure, with consequent advantages in reduced screening requirements. Further, the ability to modify endogenous genes with precision opens the possibility of new kinds of genetic modification including mutation, deletion and correction of endogenous genes. This genetic flexibility would dramatically increase the potential applications of animal transgenesis.

The prospects for animal transgenesis have been revolutionised by the development and validation of reproductive techniques which, in conjunction with molecular biology and tissue culture-based selection systems, appear to make precision genomic modification a realistic possibility. These include the use of animal embryonic stem (ES) cells, and the application of animal cloning technology.

Embryonic Stem (ES) cells: Embryonic stem cells are isolated from the pluripotent cells of the Inner Cell Mass (ICM) of the mammalian embryo, the founder stem cell population for the entire embryo. ES cells can be maintained in the undifferentiated state in vitro for extended periods of time. Upon reintroduction into the mammalian embryo (Figure 2), usually by microinjection into the blastocoelic cavity, ES cells integrate into the ICM and participate normally in embryogenesis, contributing differentiated derivatives to all tissues in chimaeric offspring. In a proportion of cases the germline of chimaeric offspring is also derived from the injected ES cells, and breeding of the animal results in transmission of ES cell genes to further generations (Robertson et al. 1986).

Application of ES cell technology has been used for the generation of mice bearing a wide variety of genetic alterations, including mutations responsible for human genetic disease (Brandon et al. 1995a; 1995b; 1995c). Unfortunately, the techniques developed for isolation of mouse ES cells have not extended easily to other mammalian species, and despite some promising observations, useful ES cell lines from non-rodent species have not been reported.

Animal cloning: The demonstration that mammals can be generated by cloning, or nuclear transfer (NT) from adult somatic cells, has been a major scientific advance of the late 20th century (Wilmut et al. 1997; Figure 2). Removal of the nucleus from an oocyte is followed by transplantation of the nucleus from another cell into the enucleated oocyte. Activation of the reconstructed NT embryo by electrical or chemical stimulation, followed by development in vitro and transfer to a recipient female, results in embryogenesis programmed by the donor nucleus. The entire nuclear genome of the resulting offspring is derived from the transplanted nucleus, with contribution from the oocyte only to cytoplasmic components.

Cloning of mammals from somatic cells was originally demonstrated in the sheep, but has since been shown to be transferrable to other mammals including mice, goats and cattle (Wakayama et al. 1998; Baguisi et al. 1999; Cibelli et al. 1998). A variety of different cell types appear competent to act as sources of nuclear material. While low cloning efficiencies demonstrate a need for technical refinement of the procedure, and there is some controversy over the importance of specific aspects of the process such as cell cycle synchrony between nucleus and cytoplast, ongoing modification of experimental procedures is already improving the efficiency of this process.
Figure 2: The use of ES cell- and cloning-based strategies for precision genetic modification. Specific genetic alterations can be introduced into cultured ES or somatic cells, which can be used to transmit the required alteration into the genome of liveborn animal by blastocyst injection and NT respectively.
In the context of animal transgenesis, the key feature of both ES cell and cloning technologies is that the
starting material is a single cultured cell which is used either after expansion *in vitro* for chimaera
formation, or as a source of nuclei for oocyte transplantation (Figure 2). Application of both technologies
leads to the generation of animals in which the gametes are derived from the cultured cell. Genetic
modifications introduced into this cell during culture *in vitro* can thus be passed to future generations.
The genomes of mammalian cells cultured *in vitro* can be modified with exquisite precision by powerful
genetic modification technologies first developed in yeast and bacteria (Shearwin-Whyatt *et al*. 1999).
The desired genetic modification, which can take any form, is created *in vitro* using recombinant DNA.
Introduction of this DNA into a cultured cell population is followed, in rare instances, by incorporation
of the exogenous DNA into the genome by homologous recombination (HR), thereby resulting in
replacement of endogenous sequences by the modified sequence. Rare cultured cells in which integration
by HR has occurred, often at a frequency of 1/1,000,000, can be identified using standard molecular
biological techniques *in vitro*, and then propagated to provide a useable population of cells for use in
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Extensive experience has demonstrated that ES cells are ideally suited to modification by homologous
recombination, and can be used as vectors for transmission of the resulting gene alterations into animals.
The creation of genetically modified mice has proven of immense value in deciphering gene function,
While there was initial uncertainty as to whether HR would be supported by the cell lines suited to
nuclear transplantation, recent evidence indicates that this is feasible, and that genetic modifications
introduced into cultured cells *in vitro* can be transmitted to animals using cloning technologies
(McCreath *et al*. 2000). Combination of these techniques can be expected to yield precisely modified
livestock animals in the near future.

**Genome projects: gene identification and function**

A major scientific activity over the last decade has been the sequencing of entire genomes, and this can
be expected to have considerable impact on animal transgenesis. The genomes of some simpler
organisms, including eukaryotes, have already been reported. Significantly, the human genome project is
now well advanced (Pennisi 2000). The next few decades can expect to be dominated by research
activity directed to understanding the function of all the genes that make up an animal, and in particular a
mammal. While much of this work will focus on gene function in the human for obvious medical
reasons, and gene function in experimentally accessible organisms such as the mouse, it is already
apparent that there is very considerable conservation of gene identity, organisation and function within
different mammals. Accordingly, data obtained from basic genetic investigation in any mammalian species will be applicable to livestock species.

The projected impact of the genome projects is in the short term, knowledge/identification of the repertoire of genes that make up a mammalian genome, and in the foreseeable future, significant understanding of the role that these genes play in mammalian development, physiology, homeostasis and disease. Coupled with the genetic alteration and reproductive technologies described above, this can be expected to increase vastly the potential of animal transgenesis for agricultural, medical and commercial application.

**The commercial impact of precision animal transgenesis**

The pivotal technological advances discussed above provide for the first time realistic prospects for directed genetic alteration of the livestock genome. While the key molecular and reproductive techniques require expertise, the skills are not prohibitively specialised and widespread adoption of transgenic approaches can be anticipated. In many cases, transgenesis can be expected to augment breeding programs with traditional objectives, allowing rapid and predictable introduction of genetic modifications affecting size, growth rates, food conversion efficiencies, product quality and waste management into animals of high value. Compared with introduction of genetic modifications by interbreeding, this approach would reduce the requirement for phenotypic screening, and for backcrossing to remove the effects of unwanted genes, thereby improving the predictability and efficiency of the breeding process. The range of traditional characteristics amenable to modification by transgenesis can be expected to increase as knowledge of mammalian gene function is advanced.

Further developments can be expected to include novel agricultural and medical uses for livestock animals, with the development of high value animals for specific applications. For example, transgenic sheep and goats that express high value proteins for use as human pharmaceuticals have been developed, while research programs in Australia and elsewhere seek the development of pig organs genetically modified for use in xenotransplantation into humans (Collignon 1999). Applications of widespread and currently unforeseen utility can be predicted, and in many cases these will be of considerable value. Transgenesis is ideally suited to these purposes since it provides unparalleled advantages for the movement of genes between species, thereby expanding the spectrum of potential applications. Further, techniques for the introduction of subtle and defined mutations to endogenous genes will be obligatory for certain applications, such as xenotransplantation, which require subtle humanisation of endogenous genes in a manner that cannot be achieved by accessing existing genetic variation.

Regarding the key technologies, Australian scientists have historically been significant contributors to biological research and can be expected to play an equivalent role in the identification of gene organisation and function that will be the major achievement of the genome projects. Australia has had strong, longstanding research programs in the reproductive technologies, and significant local contributions to both cloning and ES cell technologies have been reported. Given the likely importance of these technologies described above, the delay between the first reports of animal cloning from somatic cells in 1997 and equivalent Australian achievements in cattle and sheep in 2000 must therefore be regarded with concern. Realisation of the benefits that are expected to accompany application of transgenic technologies requires both participation in the development of core technologies accompanied by generation of Intellectual Property, and application of these techniques to animal improvement for local conditions and international markets. In the absence of IP ownership, Australian scientists may be restricted in their ability to transfer genes of value to Australian breeds for ongoing incremental improvement, and may be precluded from the development of animals suited to local conditions, with novel and valuable production characteristics.
References


