Development of cattle with a modified genotype: Embryological techniques needed for the efficient production of transgenics

J.G.E. THOMPSON, A.J. PETERSON AND H.R. TERVIT

MAFTech, Ruakura Agricultural Centre, Hamilton

ABSTRACT

The expansion of molecular biology across most biological fields has meant an increasing application of this technology to agriculture. One of the most exciting new areas to emerge has been that of transgenic animal production. Genes, from a variety of sources, can be incorporated into the early embryo and therefore effectively alter the genotype, and even the physiology of an animal.

Most of the work has been conducted using the laboratory mouse. Translation of the technology to the domestic animal has not met with great success, partly because of the genes and promoters chosen and partly because of the difficulty and expense in obtaining large numbers of suitable early embryos for gene incorporation. An efficient gene incorporation system in cow embryos ideally requires the development of a number of novel embryological techniques. They include *in-vitro* maturation and fertilisation of bovine oocytes from abattoir-sourced ovaries, *in-vitro* culture to allow the development of fertilised embryos to readily tranferable stages, satisfactory techniques for incorporating genes into the embryo and techniques such as embryonic cloning to exploit the transgenic cattle produced. The various techniques, their problems and successes are described in depth in this paper.

A research programme has been initiated to link embryology , dairy science and molecular biology tschniques with the aim of producing transgenic cattle with modified milk composition.

Keywords Embryological techniques; gene injection; cattle; transgenic cattle

INTRODUCTION

Recently there has been a gradual but rapidly expanding input of recombinant DNA technology into animal production. Although most of this work has been conducted overseas, the value of this technology has been recognised in New Zealand. Already the New Zealand Society of Animal Production has been addressed on the application of restriction fragment length polymorphisms (RFLPs) in animal breeding to identify markers for single genes which are important in animal production (Montgomery *et al.*, 1988).

One of the most exciting applications of recombinant DNA technology is that of the production of transgenic animals. Broadly speaking, a transgenic is derived when the genotype of an animal has been altered by the incorporation of exogenous DNA. Therefore, the technique allows the addition of new genes into an existing gene pool, or possibly to inhibit expression of existing genes. This, in turn, can dramatically change physiological characteristics of specific organs or the whole animal. Such techniques have an obvious application in animal production.

The Embryology group at Ruakura has embarked on a programme developing embryological techniques necessary for the incorporation of genes into domestic animal embryos, with particular emphasis on a joint programme with the Dairy Science and Molecular Biology groups to modify the milk composition of dairy cattle. L'Huillier *et al.* (1989) have dealt with the molecular biology approach to be undertaken. In this paper we will outline our programme from an embryological viewpoint.

Gene Transfer in Domestic Animals

There are three techniques which have been successfully used to produce transgenic animals. All have been developed in the laboratory mouse. The most striking demonstration of the potential for transgenics was the incorporation of the human growth hormone gene into mice, which

resulted in increased growth rates and body weight (Palmiter *et al.*, 1983). More recently, the mouse has also been used as a model to demonstrate the ability of gene transfer to alter milk composition (Gordon *et al.*, 1987; Simons *et al.*, 1987). Both the alteration of growth and milk composition have now been applied to domestic animal transgenics, but with differing levels of success. This demonstrates that although the mouse is a powerful model, there is no simple transferrence of the technology to domestic animals.

The three techniques most widely used are: 1) Direct gene injection into a pronucleus of an embryo; 2) Retroviral mediated transfer into embryos; 3) Incorporation (physical or proviral) into embryonic stem cells, followed by transfer of transformed stem cells to a host blastocyst. Details of these techniques and their relative success rates can be found in a variety of reviews (Gordon and Ruddle, 1986; Palmiter and Brinster; 1985; Renard and Babinet, 1987; Ward *et al.*, 1985). Of the techniques, direct gene injection has proven to be the most successful and popular approach (Renard and Babinet, 1987). However, the field is moving rapidly and new methods, or adaptions of existing techniques, are continuously emerging. For instance, the recent development of gene targetting (inactivation of expression in a selectively targetted gene) in embryonic stem cells to produce mice with specific homozygous deletions, is a good example (Mansour *et al.*, 1988).

The only method successfully applied to the production of transgenic domestic animals is that of pronuclear injection. In general, lower success rates of gene incorporation and expression occur in domestic animals compared with mice. This can be attributed to: 1) The comparative difficulty and cost in obtaining and transferring viable pronuclear embryos; 2) The increased difficulty in manipulating domestic animal embryos (eg. poor visualisation of pronuclei); 3) Lack of understanding concerning gene incorporation; 4) Lack of understanding concerning the nature of hybrid gene transcription and expression. Given the difficulty in obtaining large numbers of embryos and the resources required to handle them, it is not surprising that there are few transgenic livestock. Furthermore, most work was prompted by the earlier success in mice and focussed on altering growth and leaness (Lamming, 1988). In hindsight, this was a poor choice for two reasons: Firstly, there is little understanding of the response to high circulating levels of exogenous growth hormone (GH) during pre- and post-natal development. Secondly, because the gene has been linked to 'leaky' non-specific promotors such as metallothionein, GH has been overexpressed from a number of tissues. The resulting GH transgenics have usually shown altered growth characteristics but have died at an early age as a result of a whole list of complications. Even so, the "growth hormone lesson" has itself been an important one to learn (Lamming, 1988), and the production of transgenic sheep with altered milk composition has given clear evidence that functionally useful transgenic livestock can be achieved when gene expression is specific to a tissue such as the mammary gland (Simons *et al.*, 1988).

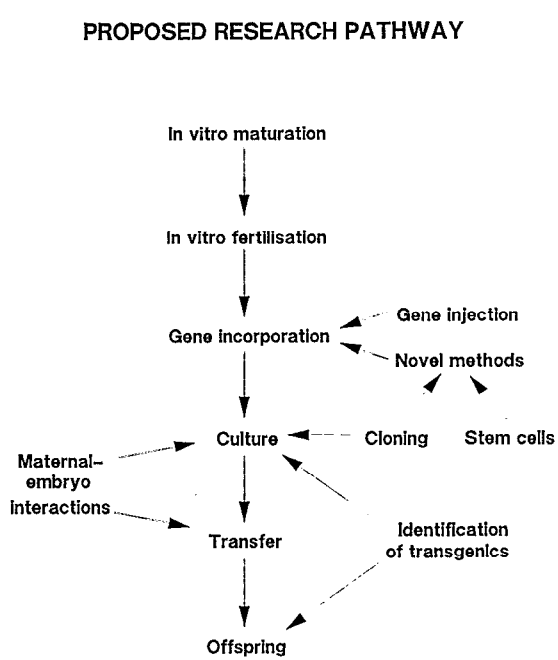

FIG. 1 Schematic representation of the proposed research progamme.

Production of Transgenic Cattle

Our proposed programme to develop transgenic cattle is schematically described in Figure 1. The programme has a number of components within it, such as *in-vitro* maturation and fertilisation of bovine oocytes, gene incorporation and subsequent embryo culture. These components are necessary to ensure the success of the programme.

***In-vitro* Maturation and Fertilisation of Bovine Oocytes**

The development of an *in-vitro* maturation and fertilisation system (IVM/F) for bovine oocytes is the only practical solution to the problem of embryo collection for gene transfer in cattle. Although superovulation and subsequent non-surgical embryo recovery is widely used, it is limited in its application here, primarily because of the late developmental stages of embryos obtained. Surgical collection could be used, but is cost prohibitive. The main advantage of the IVM/F technique is that abattoir material can be utilised. Fortunately there are now a number of reports from overseas laboratories outlining successful IVM/F techniques.

In-vitro maturation deals with the isolation of unfertilised oocytes from the ovary and incubating them to a stage where they are readily fertilisable. In mammals, follicle-bound oocytes are arrested by ovarian factors at a particular developmental stage, known as the germinal vesicle stage. Selection of a follicle to grow (under the influence of FSH) and then ovulate (due to the ovulatory LH surge) removes the inhibitory factors and stimulates further oocyte development. Development proceeds to a point where, for continuation, fertilisation by a single, normal sperm cell is required. Development up to fertilisation is known as oocyte maturation and involves changes to both the nucleus and cytoplasm. An alternative to *in-vivo* maturation can be achieved by removing the oocyte from the follicle, as this is also effective in removing the inhibitory influences (Edwards, 1965); such is the process of *in-vitro* maturation.

For many years the viability of oocytes matured *in vitro* from the domestic species, such as cattle and sheep, was very poor. Media composition and the role of granulosa cells and gonadotrophins in signalling development have only been realised in this decade (Warnes *et al.*, 1977; Moor and Osborn, 1983; Staigmiller and Moor, 1984; Eppig and Schroeder, 1986).

In-vitro fertilisation is dependent on two components; the availability of mature oocytes from either *in-vitro* or *in-vivo* sources, and the requirement for sperm cells to have undergone their final step, known as capacitation. Capacitation is a series of biochemical events in sperm cells which alter membrane properties and sperm motility, so that fusion and penetration of an oocyte can occur. Capacitation is inhibited until sperm are in the female reproductive tract. Once sperm have capacitated they may undergo the acrosome reaction, a morphological event which releases enzymes that aid penetration of the egg vestments. Unlike the vast quantity of literature available on capactation of rodent sperm, little is understood of the requirements for capacitation of bull and ram sperm. Only recently have successful capacitation systems for the domestic species been developed.

The first successful IVF studies in cattle occurred earlier this decade in Brackett's laboratory (Brackett *et al.*, 1980; Brackett *et al.*, 1982). Oocytes matured in- vivo were inseminated with sperm which had been osmotically shocked, hence induced to capacitate. Although successful, fertilisation rates and developmental capacity of resulting embryos were poor. First's laboratory at the University of Wisconsin have published a number of reports dealing with both IVM and IVF of bovine oocytes. They recognised the need for oocytes to be incubated with cumulus and granulosa cells for competent maturation (Ball *et al.*, 1984; Liebfried-Rutledge *et al.*, 1987), an observation also made by Japanese workers (Fukui and Sakuma, 1980). Furthermore, the Wisconsin group outlined factors involved in sperm capacitation, such as the beneficial effects of epinephrine and the amino acid taurine (Ball *et al.*, 1983). In particular, they observed the beneficial effects of proteoglycans on capacitation

(Lenz *et al.*, 1982) and found that the sulphated glycosaminoglycans were responsible (Lenz *et al.*, 1983).

In the past few years, a number of laboratories have published successful results. Fertilisation rates of between 70-80% can be expected from *in-vitro* matured oocytes. Such results are encouraging in terms of our programme, which presently is at the stage of developing a successful IVM procedure.

Embryo Culture

For reasons of basic efficiency, successful embryo culture of *in-vitro* fertilised embryos is a major developmental step in our programme. The alternative to culturing embryos from the 1-cell stage to blastocyst is to transfer them immediately to recipients. This represents an enormous input in resources; surgical transfers would be required and the transfer of many non-viable embryos would occur.

In-vitro culture of very early developmental stage cattle and sheep embryos to late preimplantation stages has had little success. Normal development in vitro does occur up to the 8-cell stage, but at this stage, a developmental "block" occurs, and only a small percentage continue on to blastocysts (Camous *et al.*, 1984). Tervit *et al.*, (1972) appeared to overcome this problem by incubating in atmospheres of low oxygen content. Since then, many conflicting reports have emerged concerning the function of O_2 tension in media. The most successful method for preimplantation development to date is the transfer to a surrogate oviduct (ie. rabbit or sheep) (Lawson *et al.*, 1972a,b; Eyestone *et al.*, 1987). This is particularly true of *in-vitro* fertilised embryos (Sirard *et al.*, 1985; Lambert *et al.*, 1986; Fukui *et al.*, 1987; Greve *et al.*, 1987; Lu *et al.*, 1987). Few offspring have been reported from IVF cattle embryos cultured in vitro and transferred beyond the 2-cell stage. In recent times, early embryos co-cultured with trophoblastic vesicles (Camous *et al.*, 1984) or an oviduct cell suspension (Gandolfi and Moor, 1987) have led to encouraging results. Our culture studies are currently investigating factors affecting embryo development, such as media components, incubation systems and pO_2 levels. Future studies will examine more closely the relationship between the embryo and its environment, by examining metabolic events of the early embryo and the response of embryos to secretions from the oviduct under *in-vivo* or *in-vitro* conditions.

Gene Transfer

As described above, the most successful method of gene transfer into embryos is by pronuclear injection (Palmiter and Brinster, 1985). Although our programme will include this method of gene transfer, other methods will be investigated, and may become more efficient once the technology is developed.

Gene transfer by pronuclear injection (more simply known as gene injection) involves the injection of linearised DNA into one of the pronuclei of a single cell embryo. The DNA is derived from either a bacterial plasmid or bacteriophage source into which structural genes encoding for particular proteins (or enzymes) have been spliced, so that they exist within the plasmid or phage DNA. Normally, some promotor/regulator region, derived from a tissue-specific protein gene, is also added to control and regulate the structural gene.

After sperm incorporation into the oocyte has occurred, both the male and female haploid nuclei form pronuclei, which normally fuse together ("syngamy") and the embryo begins to cleave. For gene injection, a finely drawn glass pipette is inserted into one of the pronuclei and picolitre quantities of solubilised DNA is injected. Injection is performed at this point of embryonic development, as it appears to be the only time that the introduction of new genes will lead to incorporation (Walton *et al.*, 1987). The reasons why cytoplasmic injection of DNA into embryos, or injection into latter stage nuclei fails to produce transgenic offspring are unknown.

There have been few reports of successful gene transfer to domestic livestock. The difficulty in obtaining large numbers of embryos and the low gene incorporation rates have been cited as reasons. There is also a problem of visualising the

pronuclei of domestic, and in particular, cattle embryos. The only effective method currently used is high speed centrifugation. This causes the organelles to aggregate, thus "clearing" the cytoplasm.

Inner cell mass (ICM) cells, isolated from blastocysts, can be cultured and remain undifferentiated. Such cultured cell lines are known as stem cells. Stem cells have the remarkable property of being able to recolonise blastocysts and contribute to foetal cell lines, producing chimeric animals. Although nearly all work has been performed using the mouse, our aim is to develop and maintain cell lines derived from ICM cells from bovine blastocysts. Stem cells will be maintained in a co-culture system using an inactive cell monolayer to act as "feeder cells". Such stem cell lines have been developed using pig ICM cells (Piedrahita *et al.*, 1988). If successful, these cell lines can be transfected with exogenous DNA. Cells identified as transfected would then be injected into blastocysts to produce chimeric individuals which can be bred to homozygosity, or alternatively, blastocysts devoid of ICM cells could be injected to produce a complete transgenic offspring.

Embryonic Cloning

Although not directly related to the production of transgenic cattle, embryonic cloning (using the techniques of nuclear transplantation and electrofusion) has great potential for the production of identical offspring, and for dramatically increasing the number of offspring produced from one embryo.

The technique involves separating the cells from an early embryo and transplanting each cell so that it is positioned next to an unfertilised oocyte, from which the chromosomes have been removed (enucleation). Placing the two cells in an electrofusion chamber and applying a period of very short pulses of low voltage causes the two cells to fuse. This technique has been successfully applied to both sheep (Willadsen, 1986) and cattle embryos (Prather *et al.*, 1987), and experimentation will shortly begin at Ruakura.

SUMMARY

In conclusion, the Ruakura embryology group has embarked on a research programme involving new and powerful technologies. It will involve the development of IVM and IVF techniques in cattle and detailed basic studies on the culture of these embryos. The studies will provide a cheap and practical source of pronuclear cow embryos to be injected with foreign genes to produce transgenic animals. In collaboration with other groups at Ruakura, the aim will be to produce transgenic dairy cattle with altered milk composition and to multiply transgenics using embryo transfer and cloning technology. Perhaps more importantly, these studies should increase our understanding of factors involved in early embryonic development and improve the survival of embryos in embryo transfer and other manipulative procedures.

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