# Cell Mediated Transgenesis in Livestock

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# Declaration

I hereby declare that the work presented in this thesis is the product of my own efforts, and has not been submitted in any previous application for another degree. The work on which it is based is my own except where stated in the text or in the acknowledgement.

**Angelika Schnieke**

# Abstract

Production of transgenic livestock animals by DNA microinjection has become a routine procedure for research and commercial applications. However, this technique is inefficient and only capable of adding transgenes by random integration. A cell based system for the production of transgenic animals would offer significant advantages, allowing in vitro analysis of transgenes before the production of whole animals, precise placement of transgenes and the possibility of deletion, replacement or mutation of endogenous genes. In mice embryonic stem cells provide a tool for such precise genetic manipulation. However, despite considerable efforts, neither ES nor EG cells capable of contributing to the germline of any livestock species have been isolated.

This dissertation describes cell mediated transgenesis by in vitro transfection of cultured cells followed by nuclear transfer. Several different cell cultures were assessed for their suitability for nuclear transfer. Viable lambs were obtained from embryonic, fetal and adult somatic cells. This demonstrated for the first time that cellular totipotency is not irreversibly lost during differentiation. Some but not all of these cell types could support cell mediated transgenesis. A genomic Factor IX construct designed to express in the lactating mammary gland has been generated and used to demonstrate nuclear transfer as a means of transgenesis. Viable Factor IX transgenic female lambs were derived, and shown to express high concentration of the recombinant protein in their milk. These now provide the prospect of recombinant Factor IX as a safe alternative to human serum derived protein for the treatment of Haemophilia B.

Also reported are preliminary experiments to assess gene targeting in farm animals. An ovine HPRT targeting vector was constructed. Inactivation of the ovine HPRT locus was attempted using fibroblast cells capable of supporting development after nuclear transfer.

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## Abbreviations

| | |
|---|---|
| aa | amino acid(s) |
| Ala | Alanine |
| Arg | Arginine |
| APRT | Adenine phosphoribosyltransferase |
| ATP | Adenosine triphosphate |
| BLG | β-lactoglobulin |
| bp | Base pairs |
| BLWF | Black Welsh Mountain fibroblasts |
| °C | Degrees Celsius |
| cDNA | Complementary deoxyribonucleic acid |
| CFTR | Cystic fibrosis transmembrane conductance regulator gene |
| cfu | Colony forming unit |
| CHO | Chinese hamster ovary |
| CMV | Cytomegalovirus |
| CS | Cesarian section |
| DAPI | 4',6-diamidino-2-phenylindole |
| DMSO | Dimethylsulphoxide |
| DNA | Deoxyribonucleic acid |
| DTT | Dithiothreitol |
| E-coli | Escherichia coli |
| EDTA | Ethylenediaminetetraacetic acid |
| EG | Embryonic germ cells |
| EGF-1 | Epidermal growth factor 1 |
| EGTA | Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid |
| ELISA | Enzyme linked immunosorbent assay |
| EMBL | European molecular biology laboratory |
| ES | Embryonic stem cells |
| Fd | Farad |
| Fig. | Figure |
| FVIIa | Activated blood clotting factor VII |
| FVIII | Blood clotting factor VIII |
| FIX | Blood clotting factor IX |
| FIXa | Activated blood clotting factor IX |
| FX | Blood clotting factor X |
| FXIa | Activated blood clotting factor XIa |
| FGF 4 | Fibroblast growth factor 4 |
| Gla | Carboxy glutamate |
| HEPES | N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) |
| HPRT | Hypoxanthine Phosphoribosyltransferase |
| hr | Hours |
| I.D. | Identification |
| kb | Kilobase |
| kg | Kilogram |
| L | Litre |
| λ | Bacteriophage λ |
| LIF | Leukaemia inhibitory factor |
| mg | Milligram |
| ml | Millilitre |
| MOPS | 3-(N-morpholino)propanesulfonic acid |
| MPF | Meiosis/mitosis promoting factor |
| mRNA | Messenger ribonucleic acid |
| μg | Microgram |
| μJ | Microjoules |
| μl | Microlitre |
| n.a. | Not applicable |

| | |
|---|---|
| n.c. | Not counted |
| n.d. | Not done |
| neo | Neomycin |
| ng | Nanogram |
| No. | Number |
| n.t. | Not tested |
| OME | Ovine mammary epithelial |
| OP | Ovine preparation |
| PAGE | Polyacrylamide gel electrophoresis |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| PDFF | Poll Dorset fetal fibroblasts |
| pfu | Plaque forming units |
| PGCs | Primordial germ cells |
| PGK | Phosphoglycerate kinase |
| PIC | Polymorphic information content |
| pp | Post partum |
| RNA | Ribonucleic acid |
| rFIX | Recombinant blood clotting factor IX |
| rpm | Revolution per minute |
| r.t. | room temperature |
| SEC | Sheep embryo culture |
| SDS | Sodium dodecyl sulphate |
| SV40 | Simian virus 40 |
| ts | Temperature sensitive |
| TNT | Totipotent for nuclear transfer |
| 6-TG | 6-thioguanine |
| UV | Ultra violet |
| Val | Valine |
| V | Volts |
| X-CGD | X linked chronic granulomatous disease |
| X-gal | 5-bromo-4-chloro-3-indoyl-β-D-galactoside |

# Chapter 1
## Introduction

Genetic manipulation of domestic animals could bring numerous agricultural and medical benefits, including the introduction of desirable traits such as disease resistance or enhanced performance into food animals, the production of high volume biologically active human proteins for human therapy and donor tissues for human xenotransplantation.

Since the technology was established 13 years ago (Hammer *et al.*, 1985) the number of transgenic farm animals and the range of commercial applications have grown rapidly. These include sheep with improved wool production (Damak *et al.*, 1996), and pigs which carry the human regulatory genes for complement activation for use as xeno-transplantation organs (Byrne *et al.*, 1997; Cozzi *et al.*, 1997). Currently the greatest number of transgenic farm animals are produced for the production of pharmaceutical proteins in their milk. These include complex mammalian proteins which often require extensive post-translational modifications for their bioactivity. The latter requirement excludes both *E.coli* and yeast as a production system and often results in low levels of expression of functional protein in mammalian cell culture systems. Production of exogenous proteins in the milk of transgenic animals was established first in the mouse (Gordon *et al.*, 1987) and later in rabbits (Buhler *et al.*, 1990), sheep (Wright *et al.*, 1991), goat (Ebert and Schindler, 1991) cows (Krimpenfort *et al.*, 1991) and pigs (Wall *et al.*, 1991).

However, the production of transgenic domestic animals, such as sheep, pigs and cattle, by the current method of DNA microinjection into zygotes is inefficient (transgenic rate: 1-5%), time consuming and costly. DNA integration occurs at random and the quality and quantity of transgene expression as well as germline transmission is uncertain.

In mice, embryonic stem (ES) cells provide an alternative to pronuclear microinjection as a means of transferring exogenous DNA to the germ line of an animal and also allow precise genetic modifications by gene targeting (Brandon, Idzerda and McKnight, 1995). However, despite considerable efforts, there are still no ES cells capable of contributing to the germ line of any livestock species.

Recently, it has been shown that viable sheep can be produced by transfer of nuclei from *in vitro* cultured embryonic cells (Campbell *et al.*, 1996a). The aim of this work

was to demonstrate that nuclear transfer from stably transfected cells provides a means of cell mediated transgenesis in livestock. The major advantages of such an approach are:

1. All genetic manipulations and analysis are carried out *in vitro*.
2. All animals born are transgenic.
3. Germline transmission in 100% of animals.
4. Predetermination of the sex of the transgenic animal.
5. Reduction in the number of experimental animals.
6. Furthermore, if nuclear transfer competent cells support gene targeting by homologous recombination, then this would provide the opportunity for precise positioning of the transgene as well as deletion, replacement and mutation of an endogenous gene.

The main requirement for this approach was the identification and/or isolation of cells which can be genetically manipulated and still support development of fertile animals after nuclear transfer.

The following chapters give an overview on methods for the production of transgenic livestock, as well as production of nuclear transfer derived animals. The experimental sections were aimed at identifying somatic cell types suitable for cell mediated transgenesis. The work presented here established that cultured cells from different developmental stages, including adult, are suitable for nuclear transfer. The latter provided conclusive proof that differentiation is reversible.

Furthermore, the adult cell type, cultured ovine mammary epithelial cells, was chosen to establish an *in vitro* model for the prediction of transgene expression in the mammary gland of transgenic nuclear transfer derived animals (see Chapter 4).

The principle of cell mediated transgenesis in farm animals was exemplified by the production of sheep transgenic for the human clotting Factor IX gene. Recombinant Factor IX protein expressed in the mammary gland of these animals provides a safe alternative to human blood derived products for the treatment of haemophilia B.

The possibility of gene targeting in large animals using cultured differentiated cells as nuclear transfer donors is addressed in a review of somatic cell targeting and by an attempt to inactivate the ovine HPRT locus in nuclear transfer competent cells.

## 1.1. Approaches to transgenesis in farm animals

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). For the purpose of this account the definition: "transgenic animals" is taken to include animals in which endogenous genes have been mutated or deleted by homologous recombination in gene targeting experiments.

Most methods of gene transfer and gene manipulation were first established in the mouse and will therefore be discussed here. References to domestic livestock are given where applicable. Progress in genetic manipulation of livestock species is often driven by commercial aspects and publication of results is secondary to the protection of intellectual property. Consequently this field of research is often shrouded in rumours. This review will be restricted to data published in scientific journals.

### 1.1.1. Direct manipulation of zygotes and early embryos

#### 1.1.1.1 Pronuclear microinjection

This method was first described in 1980 (Gordon *et al.*, 1980), and since then has been used extensively in many species. In short, naked DNA is microinjected into the pronucleus of explanted zygotes which are then transferred to foster mothers to complete gestation. Transgene integration into the host genome is random and in mice typically occurs in 5-20% of offspring. This ratio can be increased to 40-56% by consecutive introduction of DNA into both pronuclei (Kupriyanov, Zeh and Baribault, 1998). Although injections are carried out at the zygote stage, delayed integration of exogenous DNA can lead to the production of mosaic founder animals with reduced germline transmission.

Pronuclear microinjection is by far the most widely used method of gene transfer in livestock (Ebert and Schindler, 1993). However, the proportion of offspring which are transgenic (1-5%) is often significantly lower than in mice and the costs considerably greater. It has been estimated that approximately 1200 microinjected bovine zygotes are required to produce a single transgenic calf (Eyestone, 1994). This would require approximately 300 super-ovulated cows as zygote donors and 600 cows as recipients for microinjected embryos.

Although methods have been developed to reduce these numbers, there is still a considerable incentive to use animals more efficiently. The use of donor animals for zygote production can be avoided by *in vitro* maturation and fertilisation (IVM/IVF) of oocytes obtained from slaughterhouse material. This technique was first developed in

cattle and has been used to produce transgenic calves (Krimpenfort *et al.*, 1991; Hill, Curry and De Mayo, 1992; Bowen *et al.*, 1994). Although zygotes produced *in vitro* are less viable then those derived *in vivo*, this is offset by the greater number available. Further refinements of IVM/IVF and culture conditions can be expected to improve zygote quality in the future.

Reduction in the numbers of recipients can be achieved by transgene analysis of embryo biopsies prior to embryo transfer. Although there are several reports in the literature (Bowen *et al.*, 1994) where embryo biopsies were analysed by PCR, no satisfactory method has yet evolved to distinguish between the integrated transgene and the residual amounts of non integrated DNA (Krisher *et al.*, 1994). Currently the most promising method is selective production of transgenic animals using green fluorescent protein (Ikawa *et al.*, 1995, Takada *et al.*, 1997). Alternatively, transgenic foetuses can be identified *in utero* from samples obtained by amnio- or allantocentesis.

Improvements in the rate of transgene integration could be achieved by optimal timing of zygote microinjection. Exogenous DNA is thought to integrate during DNA replication and thus microinjection should ideally be performed before or during early S-phase (Bishop and Smith, 1989). Attempts to synchronise bovine zygotes in S-phase before microinjection have had limited success (Gagné, Pothier and Sirard, 1995).

#### 1.1.1.2. Retrovirus mediated gene transfer

A retroviral vector is an infectious RNA virus that transduces a non-viral gene into mitotic cells *in vivo* or *in vitro* (Weiss *et al.*, 1985). Replication incompetent retrovirus vectors do not encode structural genes (*gag, pol,* and *env*) and therefore cannot produce viral particles, without the aid of a so called "packaging line". Such vectors can infect high numbers of cells, resulting in the integration of a single copy of vector DNA. (for Review on retroviral vectors see Miller 1992).

Transgenic mice have been produced by co-culture of early embryos with cells producing either replication competent (Jaenisch, Fan and Croker, 1975), or defective retroviruses (Soriano *et al.*, 1986, Stewart *et al.*, 1987). Retrovirus mediated gene transfer also facilitated the production of transgenic chickens, which because of the inaccessibility of the early avian embryos can not easily be achieved by other methods.

Although this approach is potentially more efficient than pronuclear injection, its use in the derivation of transgenic large animals has been limited (Kim, Leibfried-Rutledge

and First, 1993; Haskell and Bowen, 1995). Haskell and Bowen (1995) injected cells producing replication defective virus into the perivitelline space of bovine zygotes and achieved 7% mosaic transgenic foetuses at day 90. Retrovirus integration occurs in dividing cells and requires the breakdown of the nuclear envelope in mitosis. In the oocyte, during metaphase II of the second meiosis, the nuclear envelope is absent. Therefore infection of metaphase II oocytes should lead to a high integration rate and because integration occurs prior to fertilisation, a reduction in mosaicism. A recent publication by Chan *et al.* (1998) showed that 56% of infected bovine oocytes showed transgene expression at morula stage, while only 22% infected at zygote stage and 17% of microinjected zygotes were positive for the Lac-Z transgene. Furthermore, four live calves infected at oocyte stage were all transgenic and carried the transgene in their germline.

Retroviruses do, however, suffer several disadvantages which severely limit their usefulness. The size of DNA transduced is limited and effectively restricts the use of retroviral vectors to cDNAs, which are generally poorly expressed as transgenes. Delayed retroviral integration and the possibility of several different independent integrations leads to the frequent production of mosaic animals, which can fail to transmit the transgene through the germ line. Insertion of retroviral long terminal repeats (LTRs) into the host genome can also cause activation of adjacent genes with possible deleterious effects. Perhaps the most serious problem with transgenic animals carrying retroviral vectors is the risk of producing replication competent virus by recombination. Uncertainty regarding this possibility excludes at present, the use of animals containing retroviral transgenes for most human applications.

#### 1.1.1.3. Sperm-mediated DNA transfer

DNA uptake by spermatozoa of mice, pig, sheep, cattle, poultry, carp, blowfly and sea urchin has been demonstrated (Bachiller *et al.*, 1991; Castro *et al.*, 1990; Gavora *et al.*, 1991; Arezzo, 1989; Atkinson *et al.*, 1991). DNA internalisation into the sperm head has also been shown in mouse and cattle (Bachiller *et al.* , 1991; Atkinson *et al.*, 1991).

Lavitrano *et al.* (1989) have reported the production of transgenic mice by artificial insemination using spermatozoa exposed to exogenous DNA. This could provide a time and cost saving alternative for the production of transgenic animals and was initially received with great interest. However despite considerable efforts from many laboratories around the world, the results could not be repeated in mice (Brinster *et al.*, 1989). More optimistic results were reported for other species (Gandolfi, 1998).

Transgenic fish have been generated with good efficiency (>20%) (Khoo *et al.*, 1992; Tsai, Tseng and Liao, 1995). DNA uptake by fish sperm was further improved by the use of electroporation (Patil and Khoo 1996).

Using sperm-mediated DNA transfer Lavitrano *et al.* (1997) claimed to have achieved very high transgenic rate (54-60%) in pig. Although the structure of the integrated transgene was not analysed, its integrity was assumed as 9 out of 12 piglets expressed the transgene. This was in direct contrast with the reports by Schellander *et al.* (1995) and Sperandio *et al.* (1996) who produced transgenic calves and pigs by this method, but in both instances the transgenes were rearranged. Furthermore, recent experiments by Zoraqi and Spadafora (1997) showed that DNA internalised into the mouse sperm head became associated with the nuclear scaffold, was extensively rearranged and underwent recombination with sperm genomic DNA. It therefore seems that sperm mediated transgenesis may not be a realistic approach for livestock.

### 1.1.2. Cell mediated transgenesis

#### 1.1.2.1. Embryonic stem cells (ES)

Mouse embryonic stem cells (ES) are pluripotent cells derived from early embryos (Evans and Kaufmann, 1981; Martin 1981). These cells can be manipulated *in vitro* and when reintroduced into the preimplantation embryo will contribute to all cell types of a chimeric animal, including germ cells.

ES cells provided the first cell mediated method of transgenesis. DNA transfer into a cell intermediate, rather than directly into the embryo, has the advantage that genetic manipulation and analysis can be carried out *in vitro* before animals are produced. Thus, ES cells are a direct alternative to microinjection into zygotes for the production of transgenic animals and have been used where DNA microinjection is problematic, e.g. in the production of mice containing yeast artificial chromosomes (Pearson and Choi, 1993; Jakobovits *et al.*, 1993). However, the most powerful application of ES cell mediated transgenesis exploits the ability of ES cells to support homologous recombination between exogenous DNA and chromosomal sequences. Gene targeting by homologous recombination allows .precise modifications to be made at predetermined sites in the genome and has been used extensively to effect a wide variety of genetic manipulations (reviewed by Ramirez-Solis and Bradley, 1994 and by Brandon *et al.*, 1995).

Although great efforts have been made to derive ES lines from other species, definitive ES cell lines remain elusive, with the possible exception of human ES cells (Thomson

*et al.*, 1998), where the ultimate test, the ability to contribute to the germline, is not feasible due to ethical constrains. There are reports of ES or ES-like cell lines derived from hamster (Doetschman, Williams and Maeda, 1988), mink (Sukoyan *et al.*, 1992), sheep (Piedrahita, Anderson and BonDurant, 1990; Tsuchiya *et al.*, 1994), cattle (Sims and First, 1993; Stice *et al.*, 1994a; Stice *et al.*, 1996; Strelchenko and Stice, 1994), pig (Piedrahita, Anderson and BonDurant, 1990; Notarianni *et al.*, 1991; Talbot *et al.*, 1993, Gerfen and Wheeler, 1995) and rhesus monkey (Thomson *et al.*, 1995). In all of these cases, the limited definition of "cells which under the appropriate *in vitro* conditions, can differentiate along at least three different lineages" was used to underpin the "ES cell" claim. The production of pig (Wheeler, 1994) and rat chimeras (Iannaccone *et al.*, 1994) have also been reported, although in neither case has ES contribution to the germ line been demonstrated.The experiment in the rat was later proved to be erroneous (Iannaccone *et al.*, 1994). An improved method of ES cell derivation by drug selection may facilitate future ES cell derivation from other species (McWhir *et al.*, 1996). However, at present, ES cell technology remains restricted to the mouse.

If and when large animal embryonic stem cells do become available, their use in the production of chimeras in the same way as mouse ES cells will carry several disadvantages. Farm animals have a long generation interval and the production and breeding of chimeras can delay the analysis of transgene expression considerably. Chimera production can be avoided in mice by deriving animals entirely from ES cells e.g., by aggregation of ES cells with disadvantaged tetraploid embryos. However, this method is exquisitely sensitive to the particular ES cells used (Nagy *et al.*, 1993) and the usefulness of this method for large animal ES cells is unknown.

#### 1.1.2.2. Embryonic germ cells (EG)

Primordial germ cells (PGCs) are the progenitors of the gametes. Matsui, Zsebo and Hogan (1992) and Resnick *et al.* (1992) first identified a combination of growth factors (leukaemia inhibitory factor, steel factor and basic fibroblast growth factor) which promote long term growth of mouse PGCs and their conversion to a cell type termed embryonic germ (EG) cells. Morre recent experiments showed successful derivation of pluripotent stem cells from cultured human primordial germ cells (Shamblott *et al.*, 1998). EG cells closely resemble ES cells and are functionally equivalent for cell mediated transgenesis. EG cells can be manipulated *in vitro*, and then contribute to somatic and germ cell lineages of chimeric animals (Stewart, Gadi and Bhatt, 1994; Labosky, Barlow and Hogan, 1994).

In contrast to the small number of cells available from early embryos, PGCs can be isolated in relatively large numbers during their migration to the genital ridge. Thus, isolation of EG cells from farm animals might be a viable alternative to ES cell derivation.

Successful isolation and culture of chicken PG cells has led to the production of germline chimeric chickens (Vick, Lee and Simkiss, 1993; Naito *et al.*, 1994; Chang *et al..*, 1997; Naito, Sakurai and Kuwana, 1998). Several attempts have been made to isolate EG lines from PGCs in cattle (Cherny and Merei, 1994; Stokes, Cherny and Brandon, 1994), pig (Shim *et al.*, 1997; Piedrahita *et al.*, 1998) and rat (Mitani *et al.*, 1994). Blastocyst injection of cultured EG cells led to production of mid-gestation chimeric bovine embryos (Stokes, Cherny and Brandon, 1994). More recently chimeric male piglets have been produced from both genetically manipulated (Piedrahita *et al.*, 1998) and normal EG cells (Shim *et al.*, 1997). In both instances EG cell contribution to the testes was detected. Unfortunately germline transmission could not be established, as one of the animals was stillborn and the other failed to thrive and was sacrificed.

### 1.1.2.3. Spermatogonial stem cells

There has been a recent demonstration that transplanted spermatogonia can repopulate the testes of sterile, or sub-fertile recipients in mice (Brinster and Zimmermann, 1994; Brinster and Avarbock, 1994), and rat (Avarbock, Brinster and Brinster, 1996) and that rat spermatogonia transplanted into mice produce spermatozoa (Clouthier *et al.*, 1996). If a system can be developed which allows *in vitro* culture and genetic manipulation of spermatogonia or spermatogonial precursors, this could provide a future means of transgenesis (Lovell-Badge, 1996). Culture of immortalised spermatogenic cell lines have been reported previously (Hofmann, Abramian and Millan, 1995). More recently experiments have been published showing that primary spermatogonial stem cells can be cultured for up to four months and, following transplantation, produce spermatozoa (Nagano *et al.*, 1998; Brinster and Nagano, 1998).

### 1.1.3. Conclusions

Due to the observed transgene rearrangements sperm mediated transgenesis might never become a useful option for large animal transgenesis. The possible risk factors associated with retroviral vectors deems this method unsuitable for the transgenic production of proteins for human use.

Most transgenic farm animals are currently produced by pronuclear injection of zygotes. Multiple copies of the transgene are randomly inserted at a single locus. Transgene expression is generally unpredictable and the proportion of transgenic offspring is low. If integration occurs after the zygote stage, then animals may be mosaic and germline transmission reduced or absent (Figure 1.1).

Gene targeting in ES or EG cells not only allows the addition of transgenes at a defined chromosomal location but also the alteration of existing genetic information. Although progress is being made in establishing ES or EG cultures from large animals, their practical use so far is restricted to the mouse. Animals born are chimeric. Germline transmission has to be tested by breeding, which is cost and time consuming in large animals (Figure 1.1).

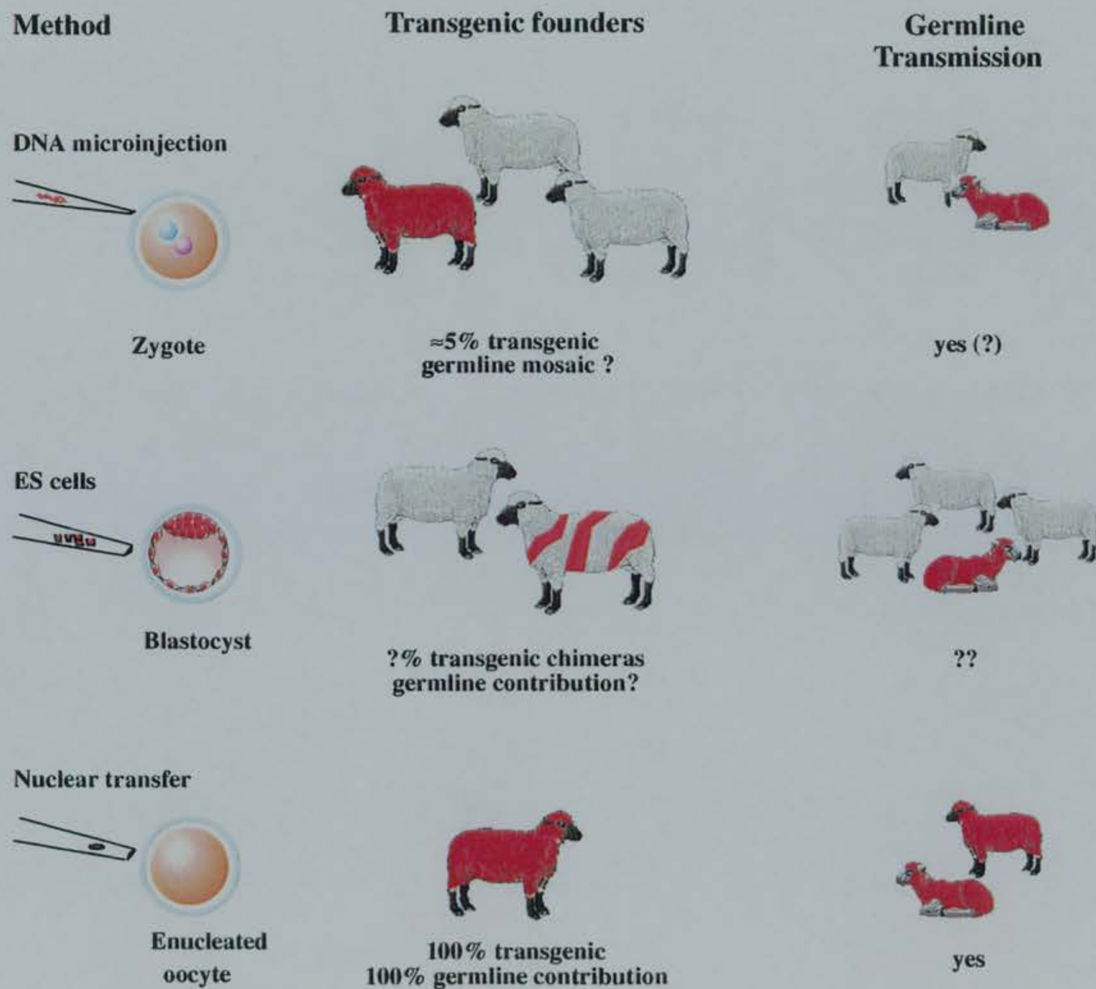



**Figure 1.1 Production of transgenic animals**
Schematic diagram of three alternative methods for the production of transgenic animals: DNA microinjection, ES cells, and nuclear transfer of genetically modified donor nuclei. Expected germline transmission is also indicated.

As will be discussed later, the lack of large animal ES cells may be circumvented by recent developments in nuclear transfer technology. Cells genetically manipulated *in vitro* could serve as nuclear transfer donors. All animals born would be transgenic and germline transmission would be ensured (Figure 1.1). To guarantee expression of transgenes, these could be inserted into a permissive locus by homologous recombination. Alternatively, if nuclear transfer competent cells express the transgene in a tissue specific manner, than quality and quantity of the recombinant protein could be assessed *in vitro* prior to nuclear transplantation.

## 1.2. Nuclear Transfer

### 1.2.1. Historical perspective

The replacement of the nucleus from a one-cell animal egg with that of another cell was first suggested by Spemann (1938) as a means of determining if nuclei of differentiated and undifferentiated cells have equivalent developmental potential. Pioneering work by Briggs and King (1952) showed that the nuclei from cells at the blastocyst stage, following transplantation to enucleated eggs, were able to direct normal development of the leopard frog *Rana pipiens* to feeding stage larvae, while nuclei from the mesoderm or endoderm of the late gastrulation stage were unable to do so (King and Briggs, 1955). This led to the conclusion that irreversible chromosomal changes occur during development and that nuclear totipotency is lost.

In later experiments with eggs of *Xenopus laevis*, using either early embryonic cells or larval intestinal cells as nuclear donors, not only normal larvae, but also some sexually mature frogs were obtained (Fischberg, Gurdon and Elsdale, 1958; Gurdon, 1962; Gurdon and Uehlinger, 1966; Di Berardino and Orr, 1992). The work was later extended to the use of terminally differentiated, keratinised skin cells of adult foot web as donor cells. Embryos derived did not survive much beyond metamorphosis (Gurdon, Laskey and Reeves, 1975). These results could either be interpreted as the loss of totipotency in somatic cells, or as failure of the technology employed to reveal the full developmental potential of these nuclei. Nevertheless it could be concluded that the developmental capacity of the transplanted nuclei decreased with age of the donor cell, and that this might be due to accumulated chromosomal abnormalities.

### 1.2.2. Nuclear transfer procedure

The recipient cell or cytoplast can be an enucleated zygote, or a metaphase II (MII) arrested oocyte. The latter is more commonly used for farm animals. Enucleation can

be achieved by a variety of methods, these include microsurgical removal (McGrath and Solter, 1983; Willadsen, 1986; Sun, Laurie and Moor, 1991), chemical enucleation (Fulka and Moor, 1993), or inactivation of the chromosomes by UV or laser treatment (Gurdon, 1986; Moor, Sun and Galli, 1992). Physical enucleation, e.g. by oocyte bisection, not only removes the chromosomes but also a proportion of the cytoplasm which may contain components essential for embryo development. Normal cytoplasmic volumes can be regained by fusing the donor nucleus with double cytoplasts (Peura, Lewis and Trounson, 1998).

Any diploid cell can be employed as a karyoplast, although in mammals the majority of studies have focused on the developmental capacity of early embryonic cells. Single blastomeres can be obtained by disaggregation of the embryos (Willadsen, 1986) or by aspiration (Prather *et al.*, 1987). Individual cells from later stages of development or from cells cultured *in vitro* are isolated by trypsinisation (Collas and Robl, 1990, Campbell *et al.*, 1996a).

The donor nucleus is introduced into the cytoplast either by microsurgical injection (Gurdon, 1986; Collas and Barnes, 1994; Ritchie and Campbell, 1995; Wakayama *et al.*, 1998) or by cell fusion. The latter can be achieved by polyethylene glycol treatment (Czolowska, Modlinski and Tarkowski, 1984), inactivated Sendai virus (McGrath and Solter, 1983) or electrofusion (McGrath and Solter, 1983; Willadsen, 1986; Campbell *et al.*, 1996a).

If the cytoplasts are MII oocytes, then the reconstituted embryo has to be activated to induce development. Although a number of methods have been developed (treatment with ionomycin, ethanol, electro-activation) (Loi *et al.*, 1998), none are capable of inducing the same range of activation responses as that initiated by sperm at fertilisation. Artificial means of activation induce only a single transient influx of $Ca^{2+}$ ions, while sperm entry induces multiple transients. In rabbits a series of electrical pulses can significantly increase the efficiency of embryo development (Ozil, 1990). Improvement in the development rate after ovine nuclear transfer has been reported after ionomycin induced activation followed by a three hour incubation with 6-dimethylaminopurine (Loi *et al.*, 1998).

### 1.2.3. Cell cycle and nuclear transfer

Experiments in amphibians highlighted the influence of the recipient cytoplasm on the cell cycle and transcriptional activity of the donor nucleus. These have later been confirmed in mammalian species .

Oocytes of most species pause twice during meiosis, before the first and then at the second meiotic division, at which stage the mature oocyte can be fertilised. Metaphase II oocytes have high levels of meiosis/mitosis promoting factor (MPF), which is a heterodimer of $p34^{cdc2}$ and cyclin. It is regulated by cytostatic factor (CSF/MAP kinase) activity. During sperm activation CSF is destroyed and MPF activity falls rapidly, due to proteolytic destruction of the cyclin subunit. The fertilised oocyte will exit meiosis, and enter S-phase of the first mitotic division giving rise to the two cell embryo (Whitaker, 1996; Figure 1.2).

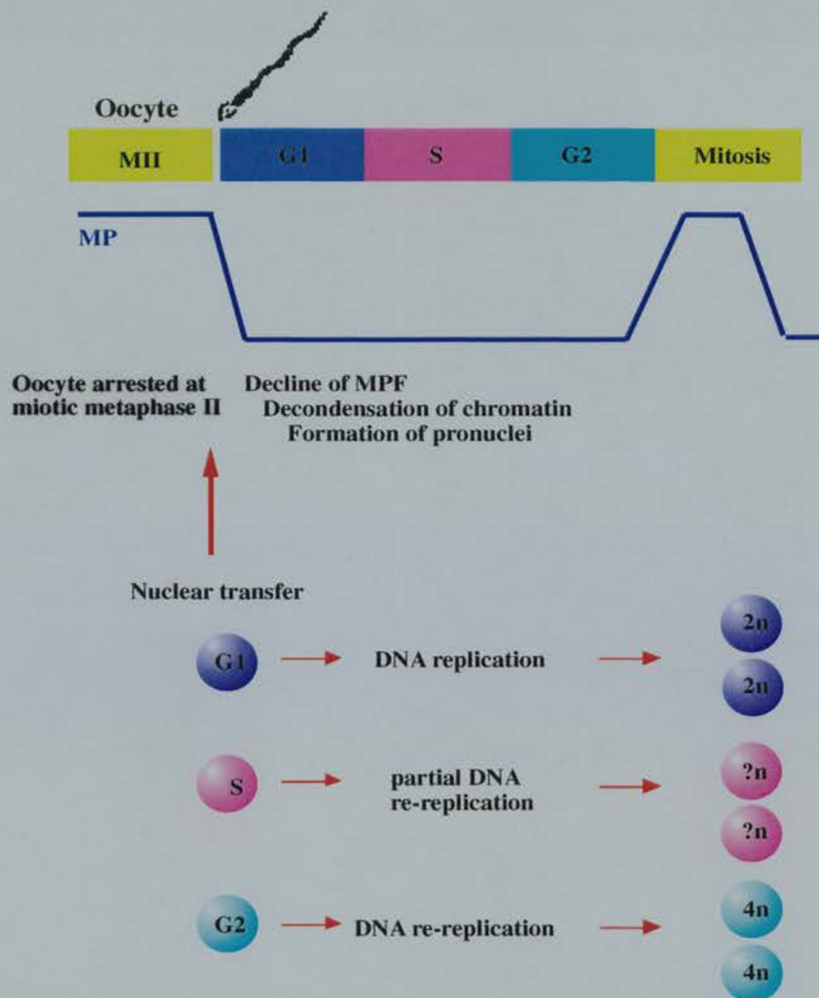



**Figure 1.2 Cell cycle and nuclear transfer**:Schematic representation of MPF levels in oocyte and zygote and its effect on nuclear transfer donor nuclei employed at different stages of the cell cycle and transferred to a metaphase II oocyte.

This has profound implications for nuclear transfer (Campbell *et al.*, 1996b). If a nucleus is transferred to an oocyte with high MPF levels, nuclear envelope breakdown and chromosome condensation occurs. This is followed by nuclear reformation and DNA replication regardless of the cell cycle stage of the donor nucleus. Previously

replicated DNA of the S and G2 phase nucleus will be re-replicated, which will lead to incorrect ploidy of the reconstituted embryo or to irreversible chromosomal damage (see Figure 1.2). Chromatin of S-phase nuclei show a typical pulverised appearance and high incidence of abnormalities. Only G1 donor nuclei can support normal development. In the mouse nuclear transfer efficiency and subsequent development to blastocyst could therefore be improved by synchronising donor nuclei in G1 (Otaegui *et al.*, 1994; Ouhibi *et al.*, 1994).

In contrast, if a donor nucleus is transferred to a fertilised oocyte/zygote in which MPF levels have declined, then nuclear envelope breakdown does not occur and the cell cycle of the incoming donor nucleus (G1, S-phase, G2) will be completed, resulting in normal development. As activation of the oocyte has the same effect as fertilisation (i.e. decline of MPF), nuclear transfer into preactivated oocytes can lead to live offspring from S-phase or G2 nuclei (Campbell *et al.*, 1994; Yong and Yuqiang, 1998).

### 1.2.4. Transcriptional activity in reconstituted embryos

The influence of the recipient cytoplasm on gene activity in amphibian embryos produced by nuclear transfer has been extensively studied by Gurdon (1986). His work established that the oocyte cytoplasm can reprogram differentiated nuclei. It will activate genes which are normally expressed in the oocyte or early embryo and inactivate inappropriate gene expression (Kono, 1997). These findings have later been confirmed for sheep (Sun, Laurie and Moor, 1991) and cow (Kanka *et al.*, 1991; Smith *et al.*, 1996). For example the bovine equivalent to the developmentally regulated mouse antigen TEC-3 is expressed on bovine embryos from the 8-cell stage onwards. Following nuclear transfer using TEC-3 positive blastomeres from the morula and blastocyst as donors, TEC-3 antigen expression disappeared from the early embryo and reappeared when embryos developed to morula or blastocyst stage (van Stekelenburg-Hamers *et al.*, 1994).

### 1.2.5. Nuclear transfer in livestock animals

Until recently, nuclear transplantation in livestock was only possible when using donor nuclei from cells obtained directly from early embryos, or subjected to very short periods in culture. Live animals have been produced by nuclear transfer from embryonic blastomeres into enucleated oocytes in rabbit (Stice and Robl, 1988), Rhesus monkeys (Meng *et al.*, 1997), pigs (Prather, Sims and First, 1989), cattle (Prather *et al.*, 1987; Bondioli, Westhusin and Looney, 1990; Keefer, Stice and Matthews, 1994; Sims and First, 1994), goat (Yong *et al.*, 1991; Yong and Yuqiang,

1998) and sheep (Willadsen, 1986; Smith and Wilmut, 1989). In spite of high rate of development to the blastocyst stage, the percentage of embryos that lead to successful pregnancy is generally low (20-25%; Westhusin, Pryor and Bondioli, 1991; Stice, Keefer and Matthews, 1994).

Nuclear transfer from later stage embryos up to blastocyst stage has been achieved in sheep (Smith and Wilmut, 1989) and cow (Collas and Barnes, 1994; Keefer, Stice and Matthews, 1994). However, attempts to employ other cell types such as primordial germ cells, somatic fibroblasts, or granulosa cells were less successful. Embryos developed to blastocyst stage, but pregnancy either failed, or was only sustained for the first trimester (Delhaise *et al.*, 1995; Moens *et al.*, 1996; Lavoir *et al.*, 1994; Moor, Sun and Galli, 1992; Collas and Barnes, 1994; Stice *et al.* 1994).

In spite of the progress achieved in livestock, nuclear transfer from late stage blastomeres into oocytes proved to be difficult in mice. Live offspring have been reported using 2-cell blastomeres (Robl *et al.*, 1986; Tsunoda *et al.*, 1989; Kono, Kwon and Nakahara, 1991), and more recently using synchronised 4-8-cell blastomere or synchronised cells of a compacted morula stage embryo in serial nuclear transfer (Kwon and Kono, 1996; Tsunoda and Kato, 1997). It was thought that the difficulties associated with mice may be related to the time at which the embryonic genome becomes transcriptionally active. Mouse embryos show a marked increase in transcription at the 2 cell stage, pigs at 4 cell, cows and sheep at the 8 cell stage, and rabbits at 16 cell stage (Prather, 1993; De Sousa *et al.*, 1998a; 1998b). It is conceivable that a delay before genome activation, as seen in the cow and sheep, allows the transplanted nucleus to be reprogrammed by its cytoplasmic environment more efficiently. In addition mouse oocytes are very fragile and techniques have only recently been developed, which achieve high survival rates of the manipulated oocyte (Wakayama *et al.*, 1998).

The small number of cells in the early embryo prohibits production of large numbers of cloned animals, but also the genetic manipulation of cells prior to nuclear transfer. This would require developmentally competent cells that proliferate in culture. Mid-gestation bovine foetuses have been produced by nuclear transfer from cultured bovine embryo explants (Stice *et al.*, 1994; Stice *et al.*, 1996). Live calves were obtained from cultured inner cell mass cells (Sims and First, 1994). The birth of sheep following nuclear transfer from cells cultured for long periods has been reported by Campbell (Campbell *et al.*, 1996a). Cells originally derived from day 9 ovine embryonic discs were passaged up to 13 times in culture and then used as nuclear donors. The authors

propose that the key to successful nuclear transfer is the induction of a quiescent state in the donor cell. In the quiescent state, termed $G_0$, cell cycle activity is absent, protein synthesis is reduced and changes occur to the chromatin (Whitfield *et al.*, 1985). It has been proposed that these alterations may render the nucleus more susceptible to modification by the oocyte cytoplasm (Campbell *et al.*, 1996a). Some cell types are naturally in $G_0$ (sertoli cells, neuronal cells, cumulus cells), but quiescence can also be induced in nuclear donor cells by culture in the presence of reduced amounts of serum, termed serum starvation.

### 1.2.6. Conclusions

Ovine and bovine nuclear transfer are well established. Most recent experiments successfully utilised quiescent karyoplasts derived from cultured differentiated embryonic cells (Campbell *et al.*, 1996a). If such nuclear transfer competent cell cultures can be genetically manipulated *in vitro*, then they provide a means for cell mediated transgenesis. If these cells support homologous recombination then they might also provide a viable alternative to large animal ES cells. Furthermore improvements in nuclear transfer should allow some of the fundamental questions of genomic totipotency of advanced embryonic and somatic nuclei to be answered (Sun and Moor, 1995).

## 1.3. Haemophilia B and Factor IX

The Factor IX gene was chosen to establish cell mediated transgenesis in livestock. Production of recombinant Factor IX is of medical and commercial importance.

### 1.3.1. Haemophilia

The two main forms of haemophilia (haemophilia A and B) are inherited X-linked, recessive bleeding disorders affecting mainly males. Haemophilia A affects one in 10,000 males and is caused by a mutation in the Factor VIII gene. Haemophilia B is less common, affecting one in 30,000 males and is caused by a defect in the Factor IX (FIX) gene. The severity of bleeding in both forms of haemophilia is linked to the amount of the relevant clotting factor in the blood. Haemophiliacs with >5% of normal level are classed as mild, 2-5% as moderate, and <2% as severe (Brownlee, 1987; 1995). Currently the treatment for haemophilia patients is human plasma derived FVIII or FIX which is limited in supply, may be contaminated with other plasma proteins or coagulation factors, and carries a risk of human infectious diseases. This has led to the search for alternative treatments either in form of recombinant protein or gene therapy.

### 1.3.2. Factor IX

Factor IX (FIX) is a vitamin K-dependent multi-domain glycoprotein and is synthesised in the liver as a precursor molecule of 461 amino acids. It has to undergo extensive post translational modifications to become biologically active (Tuddenham and Cooper, 1994; Reiner and Davie, 1994). For protein domains please see Figure 1.3. Cleavage of the signal sequence occurs during vectorial transport into the ER. The propeptide sequence is removed intracellularly by a furin protease. The propeptide and the gla-domain targets the vitamin K-dependent γ-glutamyl carboxylase present in the ER. γ-carboxylation of the first 12 glutamic acid residues ensures correct protein folding of FIX in the presence of calcium and allows binding to phospholipids. Other modifications include partial β-hydroxylation of aspartic acid residue 64, glycosylation of serine 53 in the EGF-1 domain and asparagine residues 157 and 167 in the activation peptide region.

The mature single chain protein (415aa, MW 56,000) circulates in the blood (5μg/ml). It is an essential component of the clotting cascade and can be activated by FVIIa/tissue factor or by FXIa. (see Figure 1.4). Activation occurs in two steps. First the Arg145-Ala146 bond is cleaved, resulting in a two-chain inactive intermediate (FIXα), containing the light chain (aa 1-145, Gla-domain and 2 EGF-domains) and a heavy chain (aa 146-415). Next the heavy chain of FIXα is cleaved at Arg180-Val181 to yield a 35-residue activation peptide and an active serine protease (Fig. 1.3). FIXa then activates FX to FXa leading to thrombin generation and fibrin clot formation (Fig.1.4).

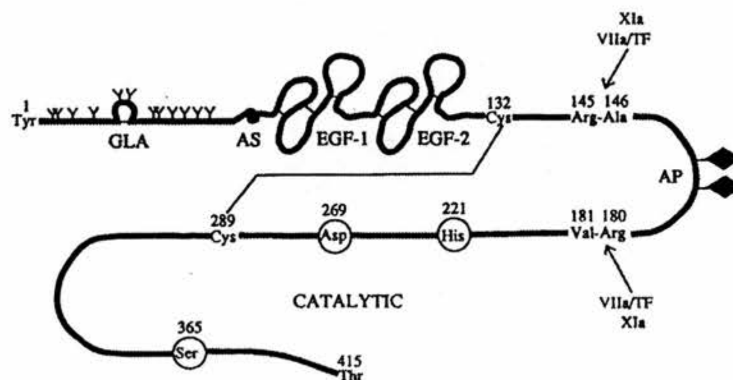



**Figure 1.3 FIX protein domains**
Schematic diagram of the FIX protein domains, also indicated are the amino acids involved FIX activation. Gla, γ-carboxyglutamic acid; AS, aromatic stack; EGF, epidermal growth factor-like domain; AP activation peptide, TF tissue factor. The diamond shapes represent potential N-linked carbohydrate side chains. Amino acids circled are members of the catalytic triad.

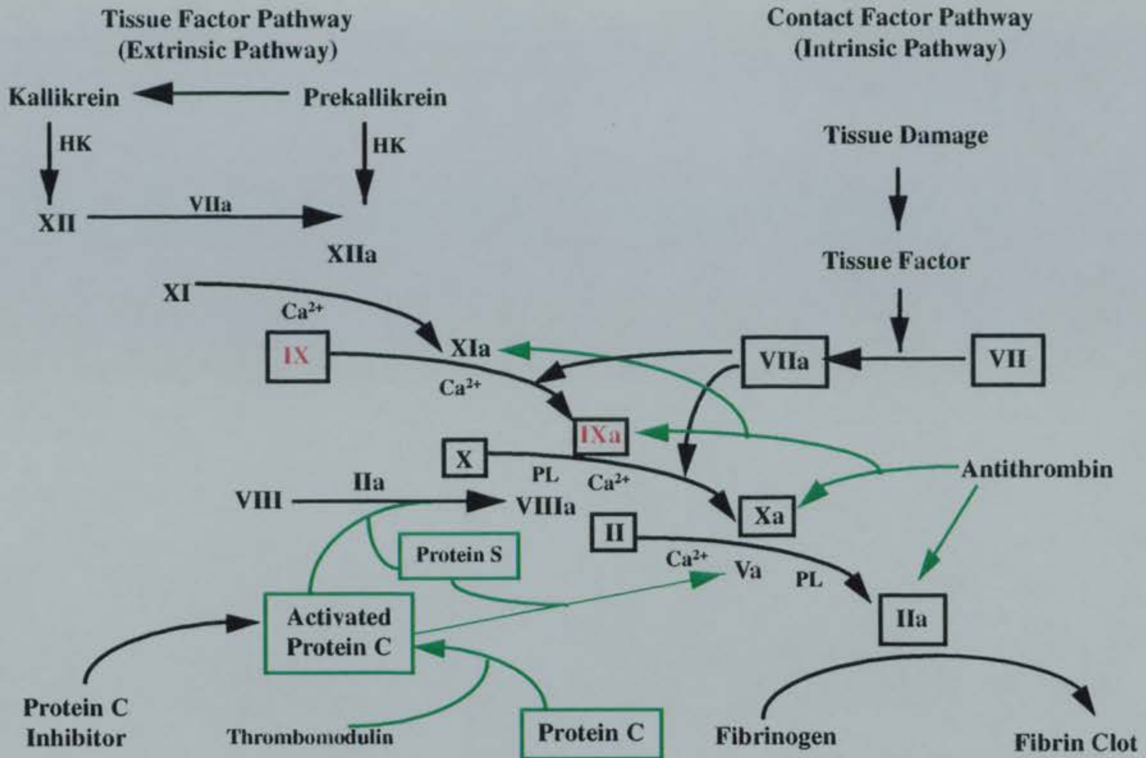



**Figure 1.4: Coagulation cascade**
Simplified version of the pathways of coagulation activation and inactivation (marked in green). FIX (marked in red) is activated by FVIIa/tissue factor or by FXIa. FIXa then activates FX to FXa leading to thrombin generation and fibrin clot formation. Abbreviations: HK, high molecular weight kininogen; PL, phospholipid.

The gene structure of the vitamin-K dependent clotting factors (FVII, FIX, FX, and protein C) is highly conserved (Furie and Furie, 1988). The human Factor IX gene is about 34kb long and consists of eight exons and seven introns (Yoshitake *et al.*, 1985). The exons roughly correspond to specific functional domains. Exon I encodes the majority of the signal peptide, exon II and III encode the propeptide and Gla domain. Exon IV and V encode EGF domains, exon VI the activation peptide, VII and VIII the catalytic domain. Mutations causing haemophilia B have been mapped. Most of these are point mutations or small deletions (Giannelli *et al.*, 1998).

The molecular cloning of the FIX coding sequence led to the development of recombinant protein and gene therapy for the treatment of haemophilia B.

### 1.3.3. Factor IX gene therapy

Gene therapy is based on the fact that the replacement gene needs to be expressed at only 5% of the normal FIX level to show a significant benefit.

In animal models the FIX gene was administered either by receptor mediated transfer (Perales *et al.*, 1994), by retrovirus (Dai *et al.*, 1992, Kay, Rothenberg and Landon, 1993), or by adenovirus vectors (Smith *et al.*, 1993; Walter *et al.*, 1996), in humans by autologous implant of cells transfected *ex vivo* with a functional FIX gene (Palmer , Thompson and Miller, 1989, Dai *et al.*, 1992). Common problems encountered with these methods included the insufficient or transient nature of the FIX gene expression and the host immune response to the vector. More encouraging results have been reported by Snyder *et al.* (1997), who describe stable expression of human FIX in mice transduced with adeno associated virus (AAV) vector encoding human FIX cDNA. Because the vector contained no viral genes, the treatment caused no detectable immune or inflammatory response. These experiments have now been extended to correction of haemophilia in a dog model (Snyder *et al.*, 1999; Herzog *et al.*, 1999). The AAV-FIX vector was either targeted to the liver or muscle, expression of rFIX (1-1.4% of normal FIX plasma level) was detected for up to 16 months and a partial correction of the coagulation defect was achieved. Several problems still have to be overcome before this technology can be applicable to humans. The main hurdle is the necessity to significantly increase the titer of the viral vector (Linden and Woo, 1999).

Loss of FIX function is often caused by single point mutations. Kren, Bandyopadhyay and Steer (1998) developed a novel method which allows efficient site-directed conversion of single nucleotides *in vivo*. The strategy is based on chimeric RNA/DNA molecules complexed with a protecting polycation and a ligand (lactose) for the hepatocyte asialoglycoprotein receptor. The complex was injected into the tail vein of rats. The chimeric RNA/DNA molecules then induced site directed mutagenesis of the FIX gene in the intact rat liver leading to a reduction in clotting activity. However, there are some surprising aspects to the work as reported. Even if 50% of the liver cells had been targeted, it is questionable whether this would lead to the reported reduction in clotting activity. Clotting activity is generally only reduced if FIX levels drop below 10% of normal. No other laboratory has been able to reproduce these results (Strauss, 1998).

### 1.3.4. Production of recombinant Factor IX

Due to the extent of post-translational modifications, production of functional recombinant human Factor IX (rFIX) is restricted to eukaryotic expression systems. rFIX has been expressed in a variety of mammalian cells including human embryonic kidney cells (Hamaguchi *et al.*, 1991), human hepatoma and fibroblast cells (De La Salle *et al.*, 1985; Palmer, Thompson and Miller, 1989), canine kidney cells (Rees *et*

*al.*, 1988), Chinese hamster ovary cells (Balland *et al.*, 1988), and baby hamster kidney cells (Busby *et al.*, 1985). These studies showed that an increase in expression led to a decrease in specific activity. Secretion of correctly processed material rarely exceeded 5μg/ml. Attempts to improve activity include, domain shuffling between FVII and FIX (Berkner *et al.*, 1987; Chang, J-Y. *et al.*, 1997), over-expression of the γ-carboxylase or the propeptide processing enzyme in CHO cells (Rehemtulla *et al.*, 1993). These resulted in no more then a 3 fold increase in activity.

Due to the size of the FIX gene, expression vectors were all based on the FIX cDNA. By producing a minigene containing a truncated first intron of the FIX gene expression levels improved 7-9 fold (Kurachi *et al.*, 1995).

To produce a large amount of biologically active recombinant human FIX, transgenic mice and sheep were generated which expressed the recombinant protein in their milk (Clark *et al.*, 1989; 1992). In these experiments expression of functional protein was low and was subsequently found to be a consequence of aberrant splicing of the cDNA construct (Yull *et al.*, 1995). Removal of the cryptic 3' splice site prevented the miss-splicing and led to production of relatively high levels (60.9μg/ml) of rFIX in the milk of transgenic mice (Yull *et al.*, 1995). Expression -although at low levels (13.7ng/ml)- of rFIX in goat milk was achieved by *in vivo* transfection directly into the lactating mammary gland (Zhang *et al.*, 1997). The highest levels of fully active rFIX protein (O.2mg/ml) were obtained in the milk of transgenic pigs (Lubon and Paleyanda, 1997). This proved that the mammary gland is capable of efficient post-translational modification of the human FIX gene and could provide a safe and cost effective alternative to human plasma derived FIX. Currently only a single recombinant FIX product (BeneFix™) produced in CHO cells has been marketed in the U.S.A. and is awaiting approval in Europe (White, Beebe and Nielsen, 1997).

### 1.4. Somatic cell targeting

Gene targeting is the use of homologous recombination to make defined alterations to the genome. It is well established in the mouse (Brandon, Idzerbda and McKnight, 1995, Torres, 1998), where it relies on the high frequency of homologous recombination between plasmids and cognate chromosomal sequences in murine ES cells. Frequent homologous recombination, however, does not seem to apply to a large number of other cell types. It is envisaged that first attempts towards gene targeting in livestock will be based on nuclear transfer of differentiated somatic cells. The literature reviewed will therefore be restricted to that describing gene targeting in somatic cells.

The driving force in this field is gene-therapy, the aim of which is the accurate correction of genetic defects. The main handicap lies in the inefficiency of gene targeting, which has been estimated to occur in 1 in $10^{-5}$ to $10^{-7}$ cells (Porter, 1989). Targeting efficiency -as with mouse ES cells- varies greatly depending on cell type, method by which DNA is introduced into the cell, targeted locus, targeting vector, and length of homology.

### 1.4.1. Cell type targeted

The first gene targeting experiment was accomplished in transformed somatic cells (bladder carcinoma cells), not in ES cells (Smithies *et al.*, 1985). Most of the early targeting constructs contained only small stretches of homology. More importantly, these homologous regions consisted of DNA sequences, which were not derived from the targeted cell line. The use of such non-isogenic DNA leads to mismatches between vector sequence and targeted locus, reducing the effective size of the homologous region. Most of the ES cell work now relies on use of isogenic DNA (te Riele, Maandag and Berns, 1992) and/or long stretches of homology to achieve high recombination efficiency. This led to the perception that ES cells are more proficient at homologous recombination than primary or immortalised somatic cells.

Very few experiments attempted a direct comparison of targeting efficiency in somatic cells versus ES cells. These include disruption of the N-myc gene in pre-B cell lines (Charron *et al.*, 1990), of the vincullin gene in F9 cells (Coll *et al.*, 1995), and inactivation of the interferon-gamma receptor in myoblasts (Arbones *et al.*, 1994). In all three instances absolute targeting efficiency in the ES or non-ES cells was comparable. Targeting efficiency in myoblasts even exceeded that in ES cells. Moreover the latter cells were primary cells, which supported homologous recombination without obvious alteration to phenotype, karyotype and growth characteristics.

Reproducibly high targeting efficiency was achieved using the chicken B cell tumour line DT40 (Buerstedde and Takeda, 1991), with homologous recombinants accounting for 6.5-100% of all transfectants (Buerstedde and Takeda, 1991; Takata *et al.*, 1994). As the absolute targeting frequency in these cells is not unusually high ($3.1\text{-}8.4 \times 10^{-6}$), it is presumed that transfectants obtained by non-homologous recombination are rare.

In most other mammalian cell lines the majority of stable transfectants are the result of random integration and the ratio of homologous to non-homologous integration is in

**Table 1.1: Targeting efficiency in somatic cells**

| Cell type | Targeted gene | No. of recombinants/ transfectants | Comment | Author |
|---|---|---|---|---|
| Transformed Cells | | | | |
| Bladder carcinoma | β-globin | 1:1000 | | Smithies *et al.*, 1985 |
| Erythroleukemia/ lymphoblast fusion cells | β-globin | 1:9700 | | Shesely *et al.*, 1991 |
| Mouse pre-B cell | N-MYC | Not reported | | Charron *et al.*, 1990 |
| T cell leukemia | CD4 | 1:900 | Promoter trap | Jasin *et al.*, 1990 |
| Chicken B cell line | | | | Buerstedde and Takeda, 1991 |
| Cervical carcinoma | Interferon inducable gene | 1:6550 | Promoter trap | Itzhaki and Porter, 1991 |
| Myeloid leukemia | X-CGD | 1:241 | | Zhen *et al.*, 1993 |
| Colon cancer | Ki-Ras | Not reported | Promoter trap | Shirasawa *et al.*, 1993 |
| Mouse embryo carcinoma | Vinculin | | | Coll *et al.*, 1995 |
| Colon cancer | p21 | Not reported | Promoter trap | Waldmann *et al.*, 1995 |
| Colon carcinoma | CFTR | Not reported | | Montrose-Rafizadeh *et al.*, 1997 |
| Fibrosarcoma | CDC2 | 1:50 to 1:230 | | Itzhaki *et al.*, 1997 |
| **Primary Cells** | | | | |
| Mouse myoblasts | Interferon-γ resecptor | 1:100 | Promoter trap | Arbones *et al.*, 1994 |
| Retinal epithelial | $\beta_2$ microglobulin | Not reported | | Williams *et al.*, 1994 |
| Foreskin keratinocytes | $\beta_2$ microglobulin | Not reported | | Williams *et al.*, 1994 |
| Embryonic lung fibroblasts | p21 | Not reported | Promoter trap | Brown *et al.*, 1997 |

All cells are of human origin unless otherwise indicated
HPRT Hypoxanthine Phosphoribosyltransferase
CFTR Cystic Fibrosis Transmembrane conductance regulator gene
X-CGD X linked Chronic granulomatous disease

the range of 1:30 to 1:40000 (Yanez and Porter, 1998). High numbers of random integrants necessitate screening of large numbers of cell clones to detect a single targeting event. Nevertheless targeting has been achieved in a wide variety of different cell lines (see Table 1.1), most importantly in several primary cell types, such as embryonic lung fibroblast (Brown, Wei, and Sedivy, 1997), retinal epithelial cells, and foreskin keratinocytes (Williams *et al.*, 1994). Targeting efficiency was between $4.3 \times 10^{-6}$ to $6 \times 10^{-8}$.

### 1.4.2. Targeting constructs

Gene targeting frequency can be improved by using isogenic DNA and increases exponentially with length of homology up to 14kb (Deng and Capecchi, 1992), see Table 1.2.

**Table 1.2: Length of homology influences targeting efficiency**

| Cell type | Targeted gene | No. of recombinants/ transfectants | Length of homology |
|---|---|---|---|
| CHO cells | APRT | 1:250000 | 0.896kb |
| CHO cells | APRT | 1:39000 | 1.4239kb |
| CHO cells | APRT | 1:12600 | 2.159kb |
| CHO cells | APRT | 1:2100 | 3.237kb |

Results shown in table 1.2 were taken from Scheerer *et al.* (1994)
CHO = Chinese Hamster ovary cells
APRT = Adenine Phosphoribosyltransferase

"Promoter trap" targeting vectors contain a promoterless selectable marker gene, which relies on the promoter at the targeted locus for activation. This strategy reduces the numbers of randomly integrated transfectants (see table 1.1). The same principle applies to "Poly A trap" vectors.

Modification of the DNA ends such as single strand overhangs (Selden, Heartlein and Treco, 1992) have been shown to improve targeting frequency, again by reducing the number of non-homologous recombinants.

Recent publications showed very high homologous recombination efficiency when using chimeric RNA/DNA oligonucleotides for targeting the β-globin gene in lymphoblastoid cells (Cole-Strauss *et al.*, 1996) and the Factor IX gene both *in vitro*

and *in vivo* (Kren, Bandyopadhyay and Steer, 1998). Unfortunately the technology does not seem to transfer easily and other researchers have failed to apply it to their gene of choice (Strauss, 1998).

Only a short stretch of homology (491bp) was sufficient, to correct a mutation in the cystic fibrosis transmembrane conductance regulator gene. The high targeting efficiency (1 in 100 cells) was achieved by denaturing the targeting DNA and combining it with *E.coli recA* protein (Kunzelmann *et al.*, 1996). The reason for the success is unclear. The authors claim that their studies do not indicate an enhancement of homologous replacement due to *recA*, and high targeting frequencies are not generally obtained when small targeting constructs are used either single stranded or double strand (Fujioka *et al.*, 1993), see also Table 1.2 .

Retroviral vectors have been employed for gene targeting with limited success (Ellis and Bernstein, 1989). More recently high targeting frequencies have been observed with both the adenoviral and adeno-associated viral (AAV) vectors. 1:5 to 1:14 transfected CHO cells had a correctly targeted adenine phosphoribosyltransferase gene (Wang and Taylor, 1993) and 11 out of 13 transfected primary human fibroblasts had a correctly targeted hypoxanthine phosphoribosyltransferase gene (Russell and Hirata, 1998). Targeting did not rely on extensive sequence homology, 2.7 to 3.8kb was sufficient. One critical factor is likely to be the delivery of large numbers of intact vector genomes into the cell nucleus which persist as single-stranded, episomal copies.

### 1.4.3. DNA transfer

The most commonly used DNA delivery method is electroporation, but other methods have been used. These include direct DNA microinjection (Zimmer and Gruss, 1989), calcium phosphate precipitation (Nairn *et al.*, 1993) and lipofection (Yanez and Porter, 1998). Many somatic cells have a significantly higher transfection efficiency when using calcium phosphate precipitation or lipofection but electroporation seems to give a higher overall targeting efficiency accompanied by fewer random integrations (Yanez and Porter, 1998). Other parameters to be considered are growth conditions, and cell cycle stage (Tempelton, Roberts and Safer, 1997).

### 1.4.4. Other factors influencing targeting efficiency

A variety of factors/conditions have been identified which influence targeting efficiency. These include transcription of the targeted locus (Nickoloff, 1990; Thyagarajan, Johnson and Campbell, 1995), introduction of double strand breaks at the targeted locus (Smith *et al.*, 1995), inactivation of the MSH2 gene (de Wind *et al.*,

1995), which is involved in DNA mismatch repair, and proteins involved in the homologous recombination pathway such as Rad 51 and Rad 54 (Shinohare and Ogawa, 1995). Greater understanding of these factors could lead to a reduction of undesirable random integration and an increase in homologous recombination.

### 1.4.5. Conclusion

Gene targeting can be performed in a variety of somatic cell lines. Overall targeting efficiency (targeted events per transfectant) at least for transformed cells, appears comparable to that in ES cells, but a much higher number of random integrants versus integration by homologous recombination was observed. A ratio as low as one correctly targeted event in a total of 10,000 transfectants is not uncommon. It therefore necessitates extensive analysis of transfectants, which is both laborious and time-consuming. Finally, gene targeting has been achieved in primary cells (Table 1.2), but the frequency of homologous recombination in primary cells is lower than in transformed cells (Thyagarajan *et al.*, 1996).

## 1.5. Model for somatic gene targeting: HPRT

The literature shows that gene targeting can be achieved in somatic cells. However, the ratio of random to targeted integrants is unfavourable. To shift this ratio many investigators have taken advantage of targeting vectors which rely on promoter trapping for the expression of the selectable marker gene (Table 1.1) or targeting of a selectable endogenous gene such as HPRT (Zheng *et al.*, 1991) or APRT (Scheerer and Adair, 1994). Alternatively an inactivated marker-gene construct can be introduced into cells and its function restored via homologous recombination (Russel and Hirata, 1998).

To assess gene targeting efficiency in somatic cells derived from livestock, in particular sheep, I carried out a model gene targeting experiment, to inactivate the ovine HPRT gene in cultured male cells.

### 1.5.1. HPRT function

The enzyme HPRT catalyses one of the first steps in the salvage pathway for the purine bases hypoxanthine and guanine, which are converted to dATP for DNA synthesis.

HPRT is located on the X-chromosome. It is ubiquitously expressed, with highest enzyme levels in the brain (Krenitsky, 1969; Howard, Kerson and Appel, 1970), particularly in the basal ganglia (Rosenbloom *et al.*, 1967). This area of the brain is

associated with low levels of *de novo* purine synthesis and is therefore sensitive to HPRT deficiency, leading to the neurological disorder, Lesch-Nyhan syndrome. Mouse models of the human disease were created by selection for spontaneous HPRT$^-$ ES cells (Williamson, Hooper and Melton, 1992), by gene targeting of the murine HPRT gene (Hwang *et al.*, 1996), and by retroviral insertion into the gene (Kuehn *et al.*, 1987).

HPRT expression is not required for cell growth in culture, which makes HPRT a useful marker gene, which can be selected both for and against (Stacey *et al.*, 1994). The toxicity of 6-thioguanine in cells expressing HPRT allows for isolation of spontaneous HPRT$^-$ mutants or for cells in which the HPRT locus was inactivated by gene targeting.

### 1.5.2. HPRT gene

The coding sequence and gene structure of the HPRT gene is highly conserved between human (Jolly *et al.*, 1983), rat (Jansen *et al.*, 1991), mouse (Konecki *et al.*, 1982), hamster (Konecki *et al.*, 1982), and pig (Mansfield, 1996). It is divided into 9 exons, covering 30-40kb. The gene structure for both the mouse (Melton, Brennand and Caskey, 1984) and human (Kim *et al.*, 1986) genes are known and the entire human HPRT gene has been sequenced (EMBL: HSHPRT8A).

Ovine and bovine HPRT genes have been mapped to the X-chromosome (Echard *et al.*, 1994) but have not been cloned. Isolation of isogenic ovine HPRT sequences is therefore a prerequisite prior to targeted disruption of the ovine HPRT locus.

# Chapter 2

## Construction and expression of a human Factor IX transgene in mice.

### 2.1. Background

This thesis describes the production of human FIX transgenic sheep by cell mediated transgenesis. Due to the large size of the FIX gene, all previously published experiments employed a FIX cDNA or mini-gene construct, which was expressed in some but not all transgenic animals. In an attempt to optimise expression and penetrance; e.g. increase the number of animals which express high levels of the recombinant protein, the transgene used was a full-length genomic FIX gene under the control of the ovine β-lactoglobulin promoter previously shown to provide high level transgene expression in ovine mammary glands (Carver *et al.*, 1993). This chapter describes the cloning of the FIX construct (pMIX1) and its expression in transgenic mice.

### 2.2. Transgene construct (pMIX1)

The nucleotide sequence of the human FIX gene (≈33kb) has been determined (Yoshitake, 1985). The gene is subdivided into 8 exons and 7 introns. It contains a variety of repetitive elements including ALU repeats and members of the KpnI (LINE) element family. The transcribed mRNA is 2083 bases of which 1245 bases are translated. 5' and 3' non-coding regions consists of 29 and 1390 bases respectively.

The pMIX1 construct comprises 31.35kb of the genomic human FIX gene linked to the ovine β-lactoglobulin (BLG) promoter and polyadenylation site.

**Cloning of pMIX1:** Human FIX DNA from a NheI site at position 2982 (12bp upstream of the AUG; positions refer to EMBL database file) to a BstBI site at position 33840 was derived from overlapping genomic plasmid and λ clones (kindly provided by Dr. G.G. Brownlee, University of Oxford).

A 3' fragment from the BstBI site to position 34346 (12bp downstream of the STOP signal) was derived by PCR amplification which also introduced EcoRI, XhoI and BamHI restriction sites. The amplified PCR product was digested with EcoRI/BamHI and inserted between the EcoRI/BamHI sites of Bluescript, giving rise to $FIX_{PCR}$ 3'. The integrity of the cloned fragment was confirmed by sequence analysis.

Figure 2.1 shows a schematic outline of the pMIX1 cloning strategy. Three subclones (pFIX 5', pFIX M, and pFIX 3') were derived from a number of overlapping λ and plasmid clones (Anson, 1984).

**pFIX 5'** consists of 4 fragments:
1. the 3.98kb NheI/BglII fragment derived from clone 9-3.
2. the 8.19kb BglII/PstI fragment derived from clone 8P-2.
3. the 2.07kb PstI/KpnI fragment derived from clone 7-4.
4. Bluescript KS- digested with XbaI and KpnI.

**pFIX M** (middle) consists of 4 fragments:
1. the 2.42kb XbaI/KpnI fragment derived from clone 7-4.
2. the 6.88kb KpnI/ApaI fragment derived from clone K-4.
3. the 5.81kb ApaI/EcoRI fragment derived from clone λHIX-1.
4. Bluescript KS- digested with XbaI and EcoRI.

**pFIX 3'** consists of 4 fragments:
1. the 5.36kb SmaI/EcoRI fragment derived from clone λHIX-1.
2. the 3.93kb EcoRI /BstBI fragment derived from clone λHIX-2.
3. the 0.49kb BstBI/XhoI fragment derived from $FIX_{PCR}$3'
4. Bluescript KS- digested with SmaI and XhoI.

**The complete genomic FIX gene** was assembled from the following four fragments (Fig. 2.1):
1. the 13.82kb NotI/NheI fragment derived from FIX5'.
2. the 11.89kb NheI fragment derived from FIX M.
3. the 5.64kb NheI/XhoI fragment derived from FIX3'.
4. Bluescript KS- digested with Not and XhoI

**pMIX1:** The 31.35kb NotI/XhoI fragment containing the entire FIX gene was inserted into the ovine BLG expression vector pMADS+ digested with NotI/XhoI (Fig.2.1).

**pMADS+** consists of 4.2kb of BLG promoter sequence, 30bp of 5' untranscribed region, multicloning sites (EcoRV, NotI, BglII, NheI, XhoI, ClaI), 170bp of 3' untranscribed region including a poly A addition site and 2kb of 3' flanking region cloned into pUC18 which has a modified polylinker to allow excision of the insert with either MluI or PvuI (PPL, unpublished). The NotI/XhoI fragment containing the

genomic FIX gene was inserted into the NotI/XhoI site of pMADS+. Figure 2.2 shows a schematic map of the pMIX 1 construct.

A decrease in the growth rate was observed in bacteria containing FIX 5' sequences, resulting in small colony size and reduced plasmid yield. The reason for this is unknown.

The final cloning step required the efficient isolation and purification of intact large DNA fragments, and the efficient transformation of bacteria with large plasmids. Isolation of DNA fragments from agarose gels using commercially available matrixes (Prep-A-Gene, Quiagen), led to some DNA degradation. After ligation and transformation bacterial colonies often contained small plasmids which were either the result of rearrangement or contamination with small DNA fragments/plasmids, which transformed preferentially.

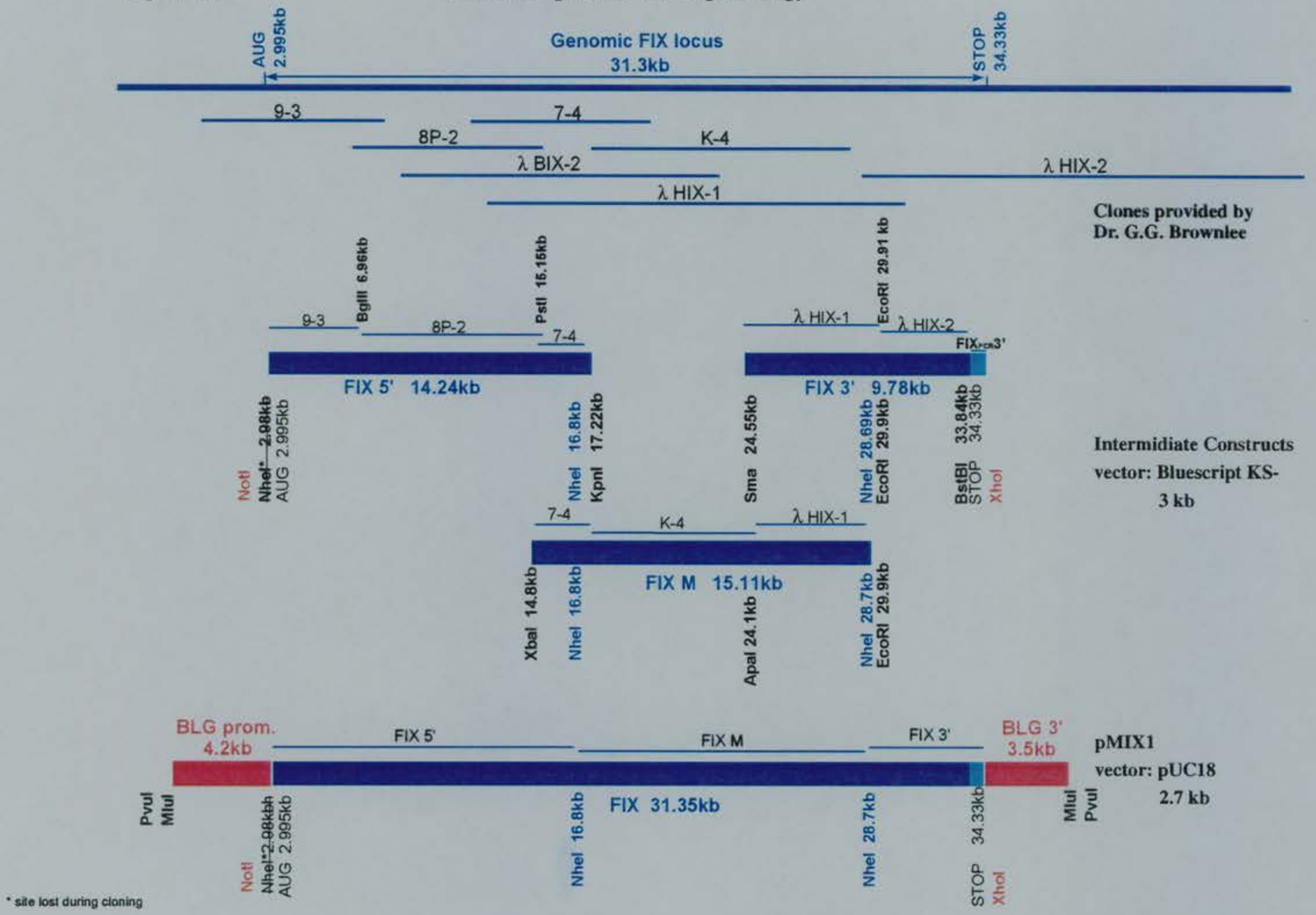
To avoid undesired recombination the bacterial substrain SURE was tested unsuccessfully. To increase efficiency highly competent DH5α or XL-1 bacteria commercially available (Stratagene) or prepared in house (see Material and Methods) were used either for chemically induced transformation or electroporation.

Cloning was attempted into two BLG expression vectors (pMADS+ and pMAD6), which differed in their 3' untranslated regions. pMAD6 contains parts of the untranslated exon 6 and the entire intron 6 of the BLG gene, which is absent in pMADS+. Cloning into pMAD6 proved repeatedly to be more difficult. The reason for this is unknown.

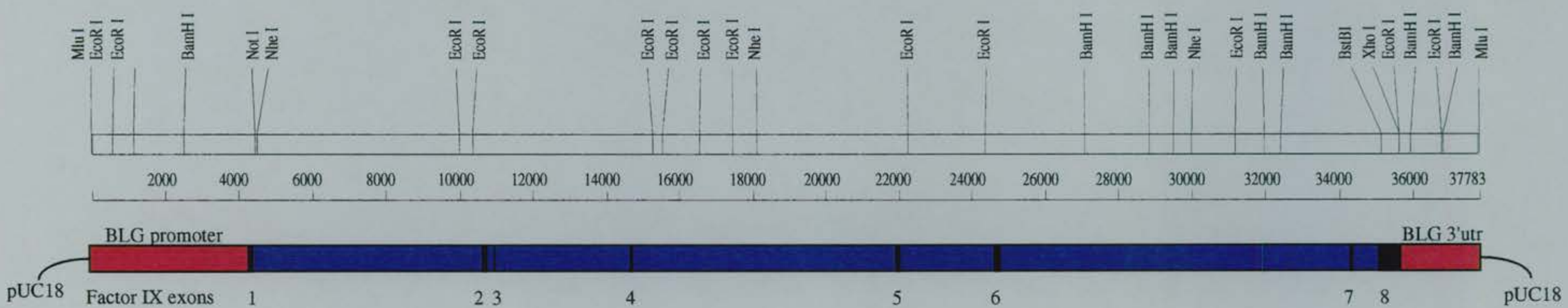
**Legend to Figure 2.1:** Schematic representation of pMIX1 cloning strategy. Factor IX sequences are indicated in blue, ovine ß-lactoglobulin (BLG) sequences in red, for simplicity the plasmid vector sequences have been omitted from the diagram. Overlapping plasmid and λ clones obtained from Prof. Dr G.G. Brownlee are indicated by single lines, intermediate constructs pFIX 5', pFIX M, and pFIX 3' and the final construct pMIX1 are indicated by solid boxes above which the individual fragments contributing to the constructs are shown. Restriction enzymes marked are those used during cloning, others have been omitted.

**Legend to Figure 2.2:** Schematic representation of construct pMIX1. pMIX1 comprises ~4.2kb of ovine ß-lactoglobulin (BLG) promoter and 5' untranslated sequence; ~31.35kb of the human Factor IX (FIX) gene from a NheI site 12bp upstream of the translational start site to an engineered XhoI site 12bp downstream of the translational stop signal; ~2.2kb of BLG 3' non-coding sequence, polyadenylation signal and 3' flanking sequence cloned into pUC18. Ovine BLG regions are indicated by red boxes, Factor IX exons are indicated by black bars. The pUC18 vector is indicated at either end of the construct.

Figure 2.1 Factor IX: pMIX1 cloning strategy



## Figure 2.2: pMIX1 map



The cloning succeeded when the FIX fragment was separated on a sucrose velocity gradient (see Material and Methods), large amounts of pure and intact fragment was isolated, which ligated efficiently. After conventional transformation a small number of bacterial colonies were obtained of which 75% contained the intact FIX gene cloned into pMADS+.

pMIX1 plasmid DNA preparations gave low yields, this was possibly the consequence of large size and some unknown sequence element in the 5' portion of the FIX gene.

For micro-injection MIX insert was purified away from bacterial vector sequence by digestion with MluI and purification by sucrose velocity gradient. For cell-mediated transgenesis either the isolated fragment or the linearised plasmid was used.

### 2.3. Factor IX transgenic mice

Transgenic mice were produced by microinjection of a 37.8kb pMIX1 MluI fragment into the male pronucleus of fertilised mouse oocytes. Milk was collected from transgenic founder females at day 10 post partum (This work was kindly carried out by Small Animal Unit, PPL).

FIX protein content in milk was assayed by ELISA using a polyclonal rabbit anti-human FIX antiserum (Dako) and quantified by comparison with purified human FIX standard (Diagnostica Stago). Two different sets of data were obtained. Assays carried out at time of milking, showed high levels of expression. Repeat experiments by a second operator showed lower levels. The reason for this discrepancy remains unresolved. Both results are given below (Table 3). (This work was kindly carried out by the Protein Chemistry Lab, PPL).

**Table 2.1: Expression of rFIX in the milk of transgenic mice.**

| | | Assay 1 | Assay 2 |
|---|---|---|---|
| **Mouse I.D.** | **Copy number** | **FIX protein μg/ml** | **FIX protein μg/ml** |
| 4 | 2 | 376-400 | 123.3 |
| 9 | 10 | 46-80 | 30 |
| 95 | 10 | 100 | 83.3 |
| 97 | 10 | 94-98 | 35 |
| 99 | 10 | 512-524 | 125 |
| 104 | 5 | n.t. | 2.2 |
| 105 | 5 | 1 | 1 |

n.t. not tested

## 2.4. Conclusions

This is the first report in which a full length genomic human FIX gene was cloned and expressed in transgenic animals. Due to the large size of the FIX gene all previous publications used either cDNA or minigene constructs.

Analysis of pMIX1 expression in transgenic mice showed that seven of seven female founders expressed FIX in their milk. Even taking into account the conservative expression level of 125µg/ml, both penetrance and expression levels exceeded any previous reports (Jallat *et al.*, 1990; Yull *et al.*, 1995), and indicated that pMIX1 was functional and suitable for introduction into sheep.

Efficient expression of the rFIX protein in the ovine mammary gland could lead to a reliable, cost effective and most of all save source of the protein for the treatment of the human disorder haemophilia B.

# Chapter 3
## Genetic Manipulation of TNT4 cells

### 3.1. Background

In 1994 lambs were produced by transfer of nuclei from early passages (passage 1-3) of cultured embryonic disc cells using preactivated recipient oocytes as reported by Campbell *et al.* (1996a). Out of 4 animals born one male lamb (S1024) survived for ≈6 months, before it died of kidney failure. This was the first evidence that live offspring can be obtained using cells maintained in culture through several passages. These ovine cells had been derived by Jim McWhir and were assigned the functional name TNT (**t**otipotent for **n**uclear **t**ransfer). The male lamb was derived from the primary cell population TNT4.

To investigate if TNT4 cells remained totipotent for nuclear transfer after extended culture, the experiment was repeated during the early part of the next breeding season (Oct.-Dec. 94) using cells from passage 6-11. No development to term was obtained from these later passages (Campbell *et al.*, 1996a).

Nuclear transfer experiments in the first quarter of 1995 tested serum starved ($G_0$) TNT4 cells (passage 6-13) using three different methods which varied in the timing between fusion of the karyoplast to the cytoplast and activation of the oocyte:
1. Nuclear fusion to preactivated cytoplasts.
2. Fusion of karyoplast and cytoplast at time of activation.
3. Fusion prior to activation.

The experiment led to the birth of two viable female nuclear transfer lambs (5LL2 "Megan" and 5LL5 "Morag", in summer of 1995) (Campbell *et al.*, 1996a). Both animals were healthy and fertile.

The authors interpretations were, that no significant difference was observed when comparing the 3 different fusion protocols, and that the success of the study was due to quiescent state of the donor nucleus ($G_0$). Not only did it provide a karyoplast at the right stage of the cell cycle, it also provided a nucleus which was possibly more amenable to reprogramming.

However the experiment as performed did not distinguish which factors were critical for successful development of nuclear transfer lambs, i.e. improvements in the experimental

manipulation, the type of cells used, the synchronisation of the cells in $G_0$ or a combination of method and cell type. It was therefore important to compare development to term using different cell types as nuclear transfer donors, either derived from an embryo or later stages of development (see Chapters 4 and 5).

Because TNT4 cells could be grown in culture for at least 13 passages without losing their nuclear transfer potential, it became evident that if they could be transfected with exogenous DNA, they could provide a novel method of producing transgenic animals. Furthermore, if TNT cells were shown to support homologous recombination between transfected DNA and chromosomal sequences, they might allow gene targeting in the same way as mouse ES cells. These issues will be addressed in the following chapters.

Work on this thesis started in March 1995. At that time the results of the nuclear transfer experiments using later passage TNT4 cells were still uncertain, birth of nuclear transfer lambs was expected 3-4 month later. Throughout, the timing of experiments were dictated by the sheep breeding season.

## 3.2. Characterisation of TNT4 cells and their potential for cell mediated transgenesis.

The original aim of TNT4 cell isolation had been to establish an undifferentiated cell population of ovine embryonic stem cells. Groups of 4-6 ovine embryonic discs mechanically dissected from day 9 embryos were co-cultured on mitotically inactivated mouse STO feeder cells. Cells derived from these explants were cultured under ES cell conditions (Robertson, 1987; Magin, McWhir and Melton, 1992), TNT4 cells were routinely grown on gelatinised plates in the presence of inactivated mouse STO feeder cells (Ware and Axelrad, 1972) and ES cell culture medium supplemented with mouse leukemia inhibitory factor (LIF) (Smith *et al.*, 1988).

Little was known about the characteristics of this cell type. It was necessary to determine:

1. whether TNT4 cells represented ovine ES like cells.
2. if they were derived from single or multiple embryos.
3. if they had a limited lifespan or if, like mouse ES cells, they proliferated indefinitely.
4. which culture conditions were required for cell growth.
5. whether they were suitable for cell mediated transgenesis.

### 3.2.1. Do TNT4 cells resemble ES cells.

The morphology of TNT4 cells was quite different from mouse ES cells (Evans and Kaufmann, 1981). At passage 6 and later they appeared epithelioid (polygonal, cobblestone appearance). However, the morphology of ovine ES cells is unknown and it was therefore necessary to use available molecular criteria.

Initial characterisation by Campbell *et al.* (1996a) had shown that TNT4 cells expressed lamin A and C. Both markers are absent in ES cells, but present in all other cell types (Van Stekelenburg-Hamers *et al.*, 1995). A finding suggesting that TNT4 cells do not share characteristics with mouse ES cells.

There are a number of other markers characteristic for undifferentiated murine ES cells (Pedersen, 1994). The expression of two of these markers was examined. In the mouse alkaline phosphatase activity is a marker of undifferentiated cells of the embryo, ES cells and primordial germ cells, and is strongly down regulated upon differentiation. Unlike in the mouse expression in bovine fetuses is not strictly restricted to the inner cell mass (Van Stekelenburg-Hamers *et al.*, 1995). TNT4 cells were stained for alkaline phosphatase activity, in parallel with the mouse ES line HM-1 (Magin, McWhir and Melton, 1992). Strong staining for the developmental marker gene was observed in HM-1 cells, while TNT4 cells were negative (data not shown).

Mouse Oct3/4 is a transcription factor expressed in the embryonic epiblast, undifferentiated ES and EG cells and is strongly down regulated in differentiated cells (Yeom *et al.*, 1991). As the ovine equivalent to the mouse gene had not been characterised, expression of a murine Oct3/4 reporter gene (pOct-LacZ) was used as a convenient method to investigate if TNT4 cells expressed the gene. This reporter construct had been shown previously to function in mouse ES cells (McWhir *et al.*, 1996), and more recently in pig and bovine embryos (PPL, unpublished data).

TNT4 cells were transfected with the Oct3/4-LacZ reporter construct and control constructs (CMV-LacZ and PGK-LacZ). Details on constructs are outlined in Fig. 3.1, for details on LipofectAMINE transfection, please refer to Chapter 3.2.4.1. 36-48 hours after transfection cells were fixed, and incubated in the presence of X-gal, a histochemical substrate for β-galactosidase, which yields a blue precipitate upon hydrolysis. Promoter

activity was assessed by the number of stained cells per field of vision. Results are shown in Figure 3.2.

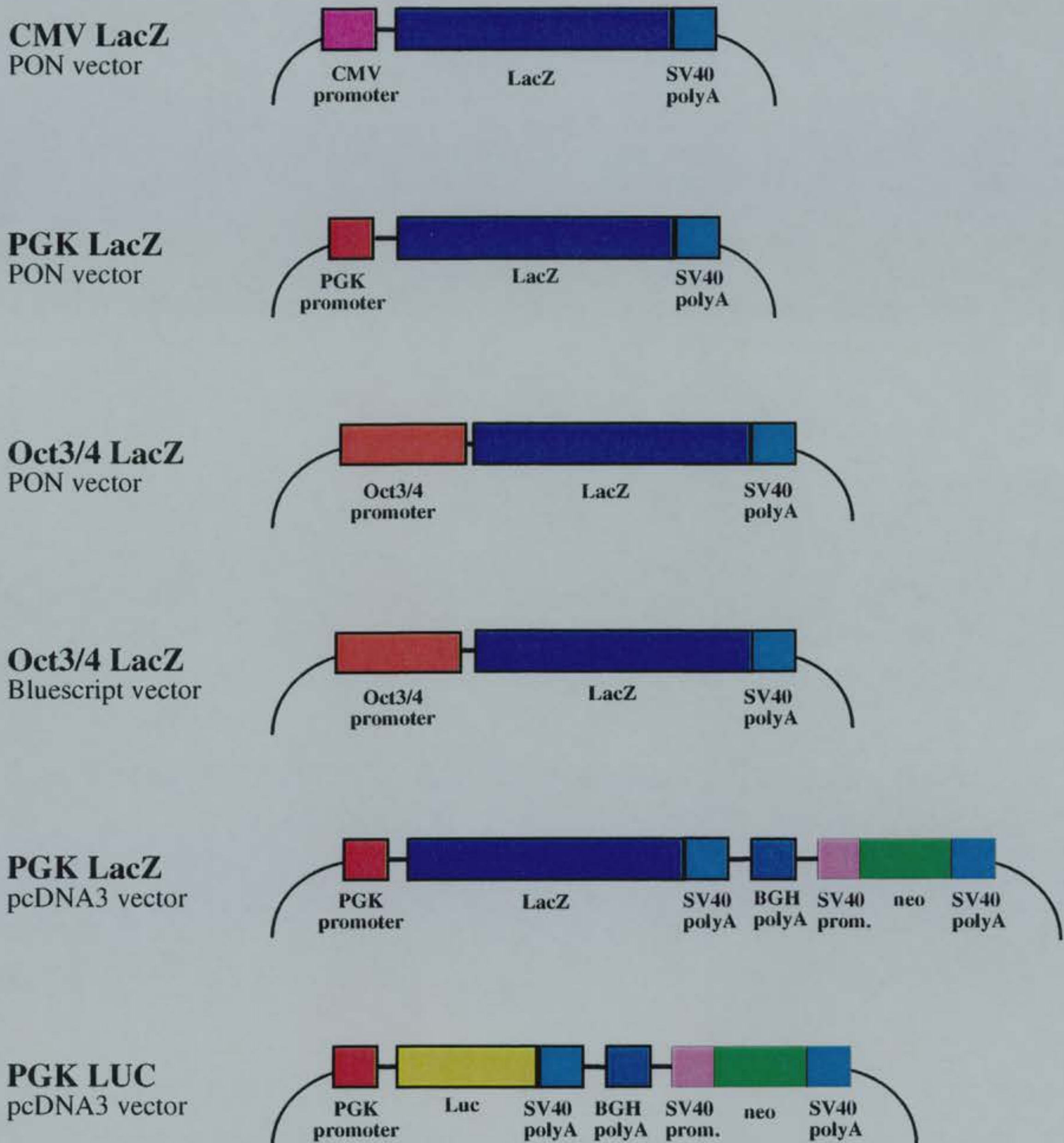



**Figure 3.1: Reporter gene constructs**
Schematic diagram of reporter gene constructs for investigating of either promoter activity in TNT4 cells or to establish transfection methods in cell types used for nuclear transfer. Solid boxes indicate the promoters, reporter gene, selectable marker gene and polyA signals, single lines indicate the plasmid vector sequences.

Expression derived from the Oct3/4 promoter could only be detected in 30 cells per $2x10^5$ cells transfected, more than a 100 fold lower compared to β-galactosidase expression directed by either the CMV or the mouse PGK promoter (see Figure 3.2).

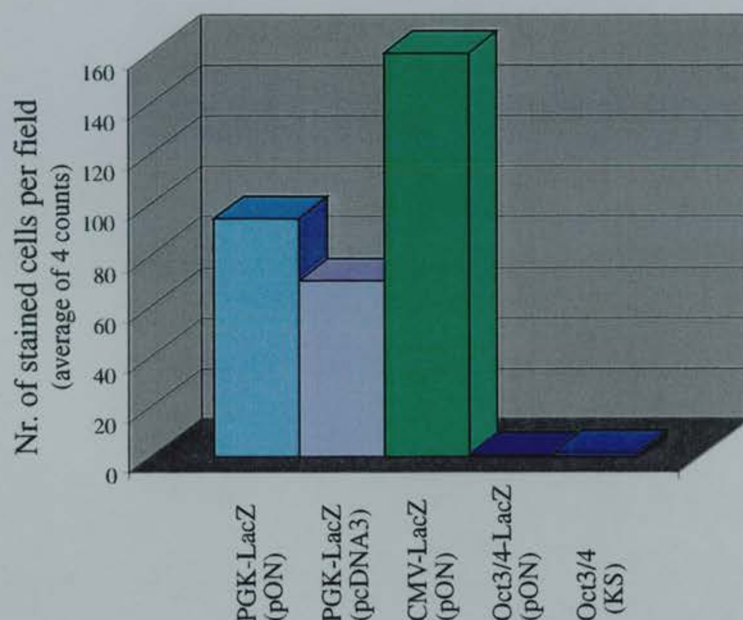


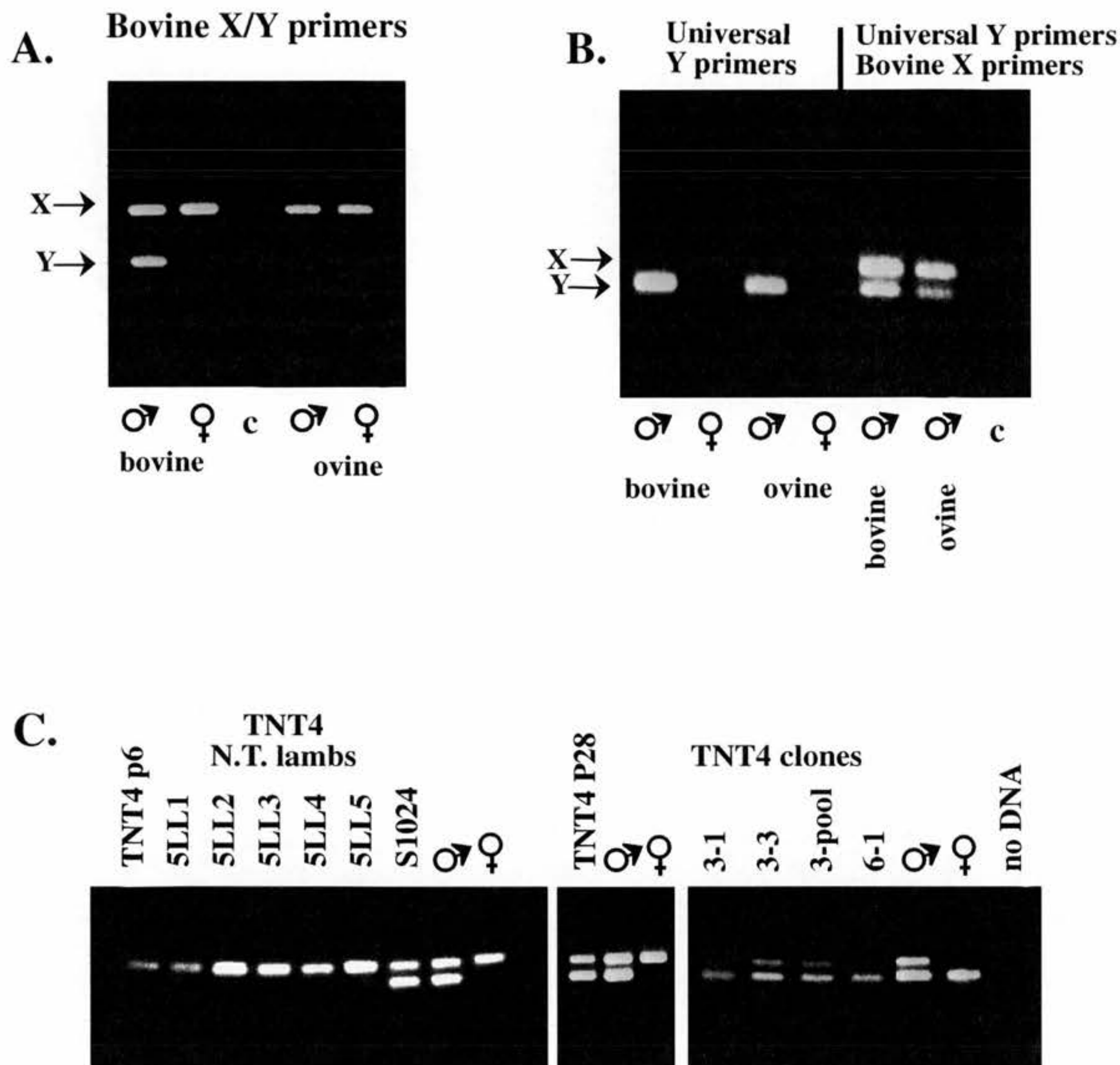

**Figure 3.2: Oct 3/4 directed expression in TNT4 cells**
Expression of the reporter gene was directed by either the PGK, CMV, or Oct3/4 promoter (for details on constructs please see Figure 3.1). Shown is a comparison of the number of cells staining positive for β-galactosidase.

The defining feature of ES cells is their ability to differentiate *in vivo* or *in vitro*. ES cells cultured in the absence of LIF and/or feeder cells will spontaneously differentiate into a variety of cell types. Although TNT4 cell cultures were originally established in the presence of feeder cells and mouse LIF, removal of these factors elicited no obvious change in morphology, while differentiation was observed for the control HM-1 mouse ES cells (data not shown).

### 3.2.2. Determining if TNT4 is of mixed origin

TNT cells were derived by co-culture of 4-6 ovine embryonic discs. When TNT4 cells were used at passage 3 for nuclear transfer both male and female lambs were born, indicating that at early passage the culture contained cells from at least two embryos. Because only the male lamb was viable, it seemed important at the time to determine if

both male and female populations were still present at a later passages and to be able to choose the sex of transfected cell clones for nuclear transfer experiments.




## Figure 3.3: Sex determination

PCR amplification of ovine X and Y sequences analysed on 2% agarose gels.

**A.** PCR primers employed were bovine zfx and zfy primers, which in combination successfully amplified both X and Y bovine sequences, but failed to amplify ovine Y sequences.

**B.** PCR primers employed were bovine zfx and the "universal" Y primers Rg4 and Rg7, which successfully amplified both X and Y ovine sequences.

**C.** Sex analysis of the lambs and abortuses produced by nuclear transfer during 1994 (S1024) and 1995 (5LL1 to 5LL5). Also included were samples from passage 6 and passage 28 TNT4 cells and TNT4 single cell clones.

C/no DNA, Control PCR reaction in the absence of DNA.

DNA was prepared from single cell clones at passage 12, and gender was determined by PCR using X and Y specific primers and DNA from male and female sheep as control. No protocol for gender determination by PCR in sheep was available at the time. The first set of primers tested were designed to amplify portions of the bovine zfx and zfy genes (Kirkpatrick and Monson, 1993). These bovine primers amplified ovine X but not Y sequences (Fig. 3.3A). As an alternative the "universal" Y primers Rg4 and Rg7 (Griffiths and Tiwari, 1993) were tested and successfully amplified ovine Y sequences. These primers also worked in the presence of the bovine X primers, allowing simultaneous detection of X and Y sequences in a single reaction (Fig. 3.3B).

Figure 3.3 C shows sex analysis of the lambs and abortuses produced by nuclear transfer during 1994 (S1024, male) and 1995 (5LL1 to 5LL5, females). Samples from passage 6 and passage 28 TNT4 cells showed that the amplified Y signal is weak at early passage and more prominent at later passage, indicating that the male population might have proliferated more readily. Of the three single cell clones analysed two were male (clone 3-1 and 3-3) and one female (clone 6-1), indicating that TNT4 was a mixed population at passage 12.

### 3.2.3. Growth characteristics of TNT4 cells

As the aim was to employ TNT4 cells for cell mediated transgenesis two important factors had to be established, whether the lifespan in culture was sufficiently long to allow for genetic manipulation and whether the cells could withstand single cell cloning.

To determine the TNT4 lifespan, cells were split 1:8 every 3-4 days. Cells were counted at every second or third passage. Doubling time of the cells up to passage 20 was 24-28hrs, and increased significantly after passage 20. At this point cells were split 1:2 to 1:4. Although some cells were cultured up to passage 30, many cells stopped dividing and a change in cell morphology was observed. Cells became large and flattened, an appearance consistent with replicative senescence (Goletz, Smith and Pereira-Smith, 1994). These results showed, that TNT4 cells were primary cells with a limited lifespan, which restricted the time available for genetic manipulation. Only a small number of cryopreserved aliquots were available at passage 6, the main stock of TNT4 cells was prepared at passage 9, which limited their usefulness.

To determine if clones of TNT4 cells could be derived from single cells, $10^2$ or $10^4$ cells at passage 9 were plated into 6 well dishes. The effect of different concentrations of serum and the requirements for both LIF and feeder cells for cell growth was determined (Table 3.1 ).

**Table 3.1: Serum, LIF and feeder cells requirements for TNT4 cell proliferation and single cell cloning.**

| LIF | Serum (FBS) | No. of colonies per $10^2$ cells plated | | $10^4$ cells plated. Cell count after 5 days | |
|---|---|---|---|---|---|
| | | - Feeders | + Feeders | - Feeders | + Feeders |
| Mouse | 5% | 0 | 5 | $4.7 \times 10^5$ | $8.6 \times 10^5$ |
| | 10% | 3 | ≈20 | $6.7 \times 10^5$ | $13.0 \times 10^5$ |
| | 20% | 5 | ≈20 | $6.7 \times 10^5$ | $12.7 \times 10^5$ |
| | | | | | |
| Human | 10% | 12 | >20 | $8.1 \times 10^5$ | $16.6 \times 10^5$ |
| | | | | | |
| None | 10% | n.t. | >20 | n.t. | $14.4 \times 10^5$ |
| | | | | | |

FBS Fetal Bovine Serum
n.t. not tested

Results summarised in Table 3.1 show that single cell colonies could be obtained with relatively high efficiency (1 colony per 50 cells plated). Cell proliferation and cloning efficiency was optimal when cells were plated onto a feeder layer and medium was supplemented with at least 10% FBS. Neither mouse nor human LIF was required for cell growth. LIF has been shown to be species specific in its action (Robinson *et al.*, 1994), and the effect of human or mouse LIF on ovine cells is unknown. Therefore the inclusion of mouse LIF during the derivation of TNT cultures might have been irrelevant.

### 3.2.4. Assessment of TNT4 cells for cell mediated transgenesis

Transfection and drug selection necessarily subject cells to potential metabolic insults and extended periods in culture. The usefulness of TNT cells in this respect depended on their ability to maintain totipotency throughout. A program of experiments to determine suitable transfection conditions was carried out using either the Luciferase or LacZ reporter genes under the control of constitutive promoters (e.g. PGK, CMV, SV40).

Once transfection and selection conditions were established, cells were co-transfected with a selectable marker gene and a FIX transgene. Stable integrants were obtained, their nuclear transfer suitability assessed by determination of their chromosome number and ability to survive serum starvation.

### 3.2.4.1. Transfection methods and optimisation

A wide variety of different methods to introduce DNA into mammalian cells are available. These include:

1. Electroporation, which uses an electric field to open pores in the cell, through which DNA diffuses into the cell (Neumann *et al.*, 1982).
2. Polycationic liposome-mediated transfection: The positively charged liposomes and the negatively charged DNA form a lipid/DNA complex, which is attracted by the negatively charged sialic acid residues on the cell surface (Felgner *et al.*, 1987). The precise mechanism by which the DNA enters the cell has not been determined.
3. Calcium phosphate transfection (Graham and van der Eb, 1973) produces a co-precipitate with the DNA. The particles adhere to the cell surface and enter the cytoplasm by phagocytosis (Loyter, Scangos and Ruddle, 1982).
4. Receptor mediated DNA endocytosis uses polycation-ligand conjugates, such as transferrin-polylysine (Wagner *et al.*, 1990; Zenke *et al.*, 1990). Ligand coated DNA particles bind to receptors on the cell surface, and upon endocytosis, the DNA is transported to the nucleus.

The method of choice based on previous experiments with other primary cell types was the polycationic reagent LipofectAMINE. Reporter constructs used to optimise transfection efficiency in TNT4 cells was the β-galactosidase gene under the control of the PGK promoter (Fig. 3.1).Transfection efficiency was estimated by the numbers of blue cells obtained (Fig. 3.4). The amount of DNA (1.5μg) and number of cells plated ($2x10^5$ per 6 well in the absence of feeder cells) was constant in all experiments. Preliminary experiment had indicated, that the optimum amount of the liposome was in the region of ≈7.5μl. Optimisation of the DNA/LipofectAMINE ratio and incubation time is shown in Table 3.2.

The lipid/DNA complex was added to the cells in serum free medium. At the end of the incubation time medium containing serum was added either directly to the cells or after the

transfection mixture had been removed by washing cells with PBS. The latter resulted in fewer transfectants. The presence of serum during transfection was investigated during a 16 hrs incubation and led to a 10 fold reduction in efficiency.

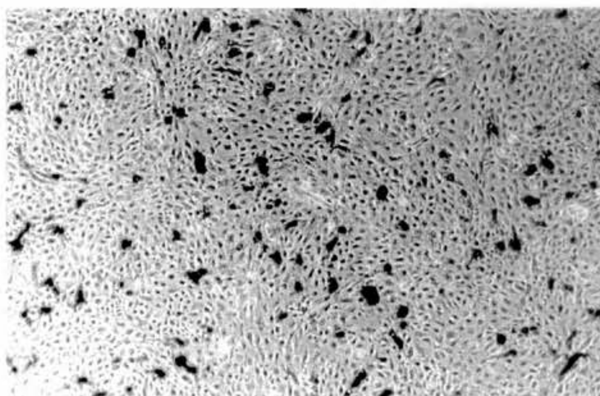


**Figure 3.4: TNT4 transfectants stained for β-galactosidase activity**
Phase-contrast photomicrograph of TNT4 transfectants stained for β-galactosidase activity. Magnification: 100X.

**Table 3.2: Optimisation of the DNA/LipofectAMINE ratio and incubation time**

| | No. of stained cells per field of vision* | | |
|---|---|---|---|
| Time | LipofectAMINE 5μl | LipofectAMINE 7.5μl | LipofectAMINE 10μl |
| 1 hr | 18-22 | 17 | 30 |
| 1 hr, PBS wash | 8 | 5 | 7 |
| 2 hrs | 40 | 70 | 65 |
| 2 hrs, PBS wash | 30 | 22 | 35 |
| 3 hrs | 45 | >100 | >150 |
| 3 hrs, PBS wash | 45 | 80 | 130 |
| 16 hrs | 120 | >200 | >250 |
| 16 hrs, serum | 18 | 23 | 25 |

*average of 2 counts

The highest transfection efficiency was obtained after overnight incubation with the lipid/DNA complex. An incubation time of 3 hrs was sufficient to obtain a reasonable

number of transfectants. To minimise insult to the cells all further experiments were carried out using 10µl of LipofectAMINE and a 3-5hrs incubation time.
LipofectAMINE transfection was also compared with transfection by calcium phosphate ($CaPO_4$) co-precipitation using the luciferase reporter gene. Conditions for an optimal $CaPO_4$/DNA co-precipitation had previously been established.

Firefly luciferase catalyses the oxidation of beetle luciferin with concomitant production of light. In nature the oxidation occurs from an enzyme intermediate, luciferyl-AMP which can be substituted in the *in vitro* assay by the substrate coenzyme A. Photon emission was measured in light units using a luminometer.

The addition of chloroquine enhances transfection efficiency in some cell types. Chloroquine is thought to increase the pH in the endosomal and lysosomal compartments and thus reducing degradation of transfected DNA. Its effect on TNT4 transfection efficiency was assessed (see Figure 3.5).

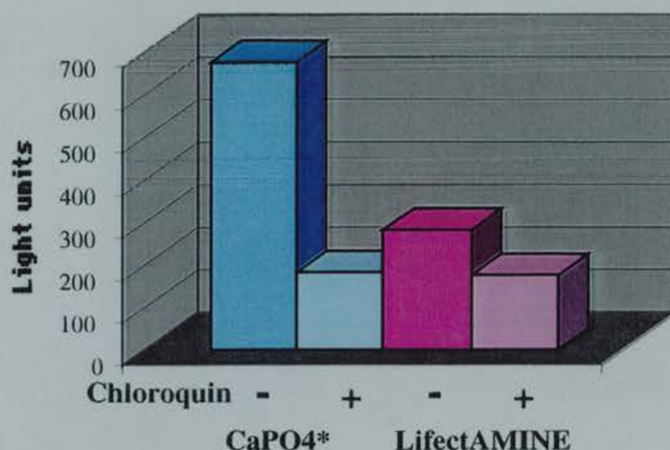



**Figure 3.5: Comparison of transfection methods**
Comparison of two transfection methods: LipofectAMINE and calcium phosphate co-precipitation. Transfection efficiency is measured indirectly (number of light units) using a luciferase reporter gene. Effect of chloroquine on transfection efficiency was also assessed. * Cells showed formation of syncytia (cell fusion)

Results in figure 3.5 show, that the greatest total number of transfectants was obtained by $CaPO_4$ co-precipitation. However this was accompanied by a high number of cell fusions. LipofectAMINE produced the greatest number of transfected cells per µg DNA without cell fusion. Chloroquine did not improve transfection efficiency.

Electroporation has been shown to be a convenient method for transfection of a variety of cell types. One drawback is the requirement for large amounts of DNA (80-200µg per experiment). A method had been published in which addition of DEAE-dextran to the electroporation medium allowed reduction in the amount of DNA used (Gauss and Lieber, 1992). Following this protocol electroporation conditions were optimised using the PGK-LacZ reporter gene and by varying the amount of DEAE-dextran (5-10µg/ml), the voltage (120-450V) and the capacitance (125-500µFd). Cell number ($1x10^6$), amount of DNA (1µg/ml) and electroporation medium (0.8ml serum free medium) was kept constant. Transfection efficiency was determined by the numbers of cells staining positive for β-galactosidase.

Results from two separate experiments are shown in Table 3.3. The highest number of transfected cells was obtained when using 10µg/ml DEAE-dextran, 180V and 500µFd. It has to be noted that 10µg/ml DEAE-dextran alone might have contributed to 32% of all transfectants (192/595, see Table 3.3).

**Table 3.3: Optimisation of electroporation conditions**
**Experiment 1**

| µFd | Volts | Cell death (% estimated) | No. of cells stained* |
|---|---|---|---|
| 5µg/ml DEAE-dextran | | | |
| 0 | 0 | 0% | 1 |
| 125 | 120 | 0% | 27 |
| 125 | 250 | 50-70% | 113 |
| 125 | 450 | 100% | 0 |
| 250 | 120 | 0% | 31 |
| 250 | 250 | 90% | 34 |
| 250 | 450 | 100% | 0 |
| 500 | 120 | 0% | 41 |
| 500 | 250 | 90-95% | 115 |
| 500 | 450 | 100% | 0 |

* stained cells per $25cm^2$ flask

**Table 3.3 (continued): Optimisation of electroporation conditions Experiment 2**

| μFd | Volts | Cell death (% estimated) | No. of cells stained* |
|---|---|---|---|
| 5μg/ml DEAE-dextran | | | |
| 500 | 140 | 0% | 45 |
| 500 | 160 | 0% | 120 |
| 500 | 180 | 5% | 147 |
| 500 | 200 | 20% | 345 |
| 500 | 220 | 50% | >350 |
| 10μg/ml DEAE-dextran | | | |
| 0 | 0 | 0% | 192 |
| 500 | 140 | 0% | 477 |
| 500 | 180 | 20% | 595 |
| 500 | 220 | 75% | 147 |

* stained cells per $25cm^2$ flask

Electroporation was considerably less efficient then lipofection (59 transfectants compared to >1000 per $2X10^5$ cells plated), therefore all further transfections were carried out using LipofectAMINE as described above.

### 3.2.4.2. Stable transfections of TNT4 cells with the FIX transgene.

In an attempt to use TNT4 cells for cell mediated transgenesis, cells were co-transfected with the selectable marker gene PGKneo (a kind gift of D. Melton, ICMB, University of Edinburgh) and the genomic FIX construct pMIX1 (see Chapter 2.2, Fig. 2.2).

At the time of this experiment the transgenic pMIX1 mouse study was still ongoing and the function of the pMIX1 construct unknown (see Chapter 2). TNT4 cells were therefore also co-transfected with PGKneo, the genomic BLG gene (pUCXSRV) and the FIX cDNA construct cFIXΔ3'SA (a kind gift of J. Clark, Roslin Inst.; Yull *et al.* 1995). The latter construct was known to express in transgenic mice when co-integrated next to an active BLG transgene.

TNT4 cells were transfected in the absence of feeder cells, followed by culture in the presence of feeder cells. Stable transfectants were selected in medium containing 0.2 or 0.4 mg/ml G418. Colonies were isolated and expanded for cryopreservation, DNA analysis, chromosome count, and assessment of viability after culture in medium with reduced serum (0.5% "serum starvation").

**Table 3.4: Transfection timeline**

| Manipulation | Day | Passage No. |
|---|---|---|
| Thaw | 0 | 9 |
| Expand culture | 3 | 10 |
| Transfection | 6 | 11 |
| Selection | 8 | 12 |
| Pick colonies (24 well dishes) | 21 | 13 |
| Expand colonies (6 well dishes) | 25-28 | 14 |
| Cryopreservation | 28-34 | 15 |
| Expand for DNA analysis | 31-43* | 16-17 |

* experiment was terminated at day 43. At this stage a large number of cell clones had stopped dividing.

During the course of the experiment (see Table 3.4) it was evident that the majority of isolated cell clones were reaching the end of their lifespan. Out of 70 clones isolated, 19 could be expanded sufficiently for cryopreservation, and 16 to allow Southern blot analysis (Table 3.5).

**Table 3.5: Single cell cloning of stably transfected TNT4 cells**

| **Amount of DNA transfected ($6x10^5$cells)** | **No. of colonies isolated** | **No. of clones cryopreserved** | **No. of clones analysed** |
|---|---|---|---|
| 3µg cFIXΔ3'SA<br>2µg BLG<br>0.5µg PGKneo | 22 | 10 | 8 |
| 4µg pMIX1<br>0.5µg PGKneo | 20 | 1 | 0 |
| 4µg pMIX1<br>1µg PGKneo | 28 | 8 | 8 |
| **Total number** | **70**<br>100% | **19**<br>27% | **16**<br>23% |

### 3.2.4.3. DNA analysis of stably transfected TNT4 cells

DNA isolated from individual cell clones was analysed by Southern blotting. The results are shown in Figure 3.6.

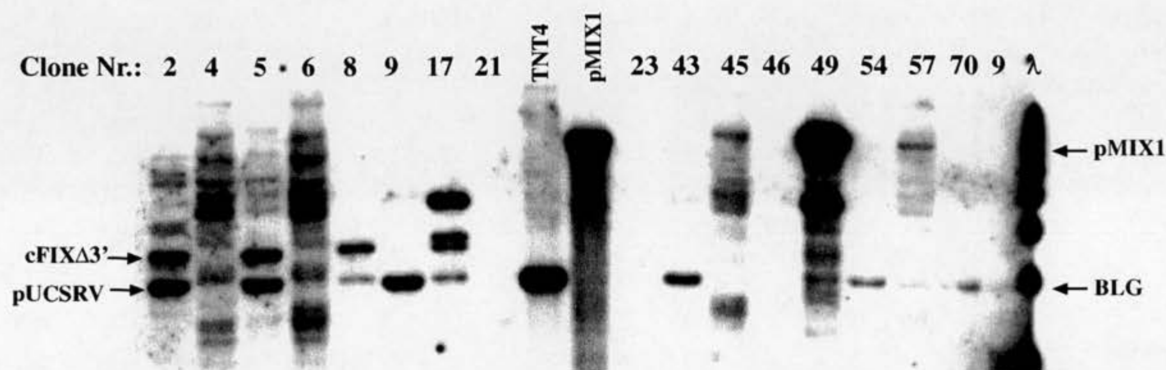



**Figure 3.6: Southern analysis of stably transfected TNT4 cells**
DNA was digested with BamHI and EcoRI and analysed on Southern blot using a 1.8kb probe derived from the ovine BLG promoter. Detected fragment size: cFIXΔ3'SA, 3.9kb; pUCXSRV, 2.9kb; pMIX1 7.4kb, endogenous BLG, 2.9kb. Please note that the amount loaded was not constant, because of variation in numbers of cells harvested. The presence of pUCSRV was determined by the intensity of the signal.

Four out of the eight clones transfected with the FIX cDNA contained both the BLG and cFIXΔ3'SA transgenes (Clones 2, 5, 8, and 17), one clone contained only the BLG transgene (Clone 9). The MIX1 transgene was detected in 3 out of nine clones (Clones 45, 49, and 57).

The gender of the clones was analysed by PCR. 14 out of the 16 clones were male, two were female. The latter 2 clones included cells of fibroblast morphology, indicating that they were contaminated with growing feeder cells.

### 3.2.4.4. Assessment of TNT4 transfectants for nuclear transfer

To determine suitability of transfected TNT4 cell clones for nuclear transfer their chromosome number and survival after serum starvation was determined. For the latter a known number of cells were plated into 6 well dishes, after 24hrs cells were washed and cultured for further 5 days with either 0.5% or 10% serum. At this point cells were counted. It was expected that cells grown in high serum would undergo several cell

doublings, while those in low serum would only replicate once or twice. Results are summarised in Table 3.6.

**Table 3.6: Serum starvation of stably transfected TNT4 cells**

| | | | No. of cells after 5 day culture | | | No. of cells after |
|---|---|---|---|---|---|---|
| Clone I.D. | Sex | No. of cells plated | 0.5% serum | 10% serum | Comment | addition of 10% serum |
| **cFIXΔ3'SA and BLG** | | | | | | |
| 2 | M | $2.8 \times 10^4$ | $2.5 \times 10^5$ | $3.8 \times 10^5$ | fib. morph. | n.t. |
| 5 | M | $1.25 \times 10^4$ | $4.12 \times 10^4$ | $15.2 \times 10^4$ | | n.t. |
| 8 | M | $1.05 \times 10^4$ | $0.87 \times 10^4$ | $1.6 \times 10^4$ | | $1.12 \times 10^4$ |
| 9 | M | $3.6 \times 10^4$ | $4.75 \times 10^4$ | $8.4 \times 10^4$ | | $7.5 \times 10^4$ |
| 17 | M | $1.5 \times 10^4$ | $1.37 \times 10^4$ | $2.5 \times 10^4$ | | n.t. |
| **pMIX1** | | | | | | |
| 45 | F | $3.88 \times 10^4$ | $1.66 \times 10^6$ | $1.68 \times 10^6$ | fib. morph. | n.t. |
| 49 | M | $2.0 \times 10^4$ | $3.2 \times 10^4$ | $5.87 \times 10^4$ | | n.t. |
| 57 | M | $2.7 \times 10^4$ | $0.87 \times 10^4$ | $2.12 \times 10^4$ | | $0.68 \times 10^4$ |

2 of the 9 cultures had lost their epithelial morphology. These fibroblast like cells were most likely escaped STO feeder cells, which showed continuous growth even in low serum. Clone 5 achieved 1.65 cell doublings in the 0.5% serum and 3.5 doublings in the 10% medium. The remaining 5 cultures underwent <1 cell doublings in low serum medium, and ≈1 in medium with 10% serum. Addition of full medium to serum starved cells resulted in limited cell proliferation (0-0.9 cell doublings). This indicated that all 6 epithelial cell clones had probably reached the end of their lifespan, with the possible exception of clone 5.

The chromosome number of stably transfected TNT4 cells was determined. To obtain metaphase spreads, cells were synchronised using colcemid, which inhibits polymerisation of spindle microtubules essential for chromosome movement during mitosis. Spreads were stained with DAPI and visualised under UV fluorescence. As the cells were dividing poorly, few metaphase spreads were obtained. None of the 69 spreads analysed showed the euploid chromosome number of 54. Chromosome counts varied between <40 to >100. These stably transfected TNT4 cells were unsuitable for nuclear transfer.

### 3.3. Conditional immortalisation of TNT4 cells

The above sections showed that TNT4 cells are differentiated, primary cells. Their limited lifespan rendered the main cell stock (available at passage 9) unsuitable for cell mediated transgenesis. One objective for further experiments was therefore to define other cell types, which can be genetically manipulated and support embryonic development following nuclear transfer (see Chapters 4 and 5). Should this fail, attempts could be made to increase the TNT4 lifespan by conditionally immortalising the cells using the thermolabile T-antigen mutant of SV40 (tsA58) (Jat *et al.*, 1991). Although steps were taken to minimise SV40 large T activity in future nuclear transfer derived animals by the addition of *loxP* sites (see below), it was not possible to rule out an accumulation of genetic aberrations during time spent in culture, which could lead to tumour formation. It was therefore the least favoured option.

#### 3.3.1. SV40 large T

SV40 is a small circular DNA virus whose natural host is primates. Its transforming ability is attributed to the early gene products small and large tumour antigen (T-antigen). Transforming ability of large T is mediated by its interaction with the tumour repressor proteins p53 and pRb (Schreier and Gruber, 1990). Both proteins are involved in cell cycle control and mutations in these genes are associated with cancer (Lane, 1992; Levine, 1990; Marshall, 1991; Hamel, Gallie and Philips, 1992; Hollingsworth, Hensey and Lee, 1993). Complexing of T-antigen with p53 and pRb renders the proteins non-functional, resulting in cells which progress unchecked through the cell cycle, ultimately leading to genetic instability and possibly tumour formation.

The temperature sensitive mutant of T-antigen tsA58 (substitution of alanine to valine at position 438) has the same properties as wild type at the permissive temperature (33°C) but at the restrictive temperature (39°C) T-antigen is inactive (Jat, 1991). It is not clear whether inactivation is due to a conformational change in the protein or to instability of the RNA (Deppert *et al.*, 1991, Tegtmeyer, 1975). Using the temperature sensitive T-antigen mutant for TNT4 immortalisation has the advantage that cells can be grown indefinitely in culture and the immortalising agent can be rendered inactive prior to nuclear transfer by culture at 39°C. Furthermore the immortalisation construct was flanked by *loxP* sites. Addition of *Cre* recombinase either prior or during nuclear transfer could excise the tsA58 transgene (Baubonis and Sauer, 1993, Sunaga *et al.,* 1997). Nuclear transfer derived lambs would be T-antigen free.

### 3.3.2. Generation of tsA58LOX construct

The SV40T construct "pUCtsA58" was obtained from C. Watson (Roslin Inst.). The cloning of PGKtsA58LOX was achieved in three steps which are outlined in Fig. 3.7.

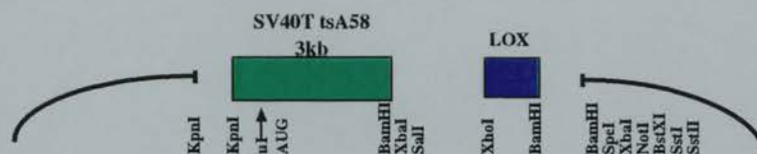
**1.** The 3kb KpnI/SalI fragment from pUC tsA58 and the XhoI/BamHI double stranded oligonucleotide comprising the *loxP* site were cloned into Bluescript digested with KpnI/BamHI (tsA58LOX).

**2.** The XhoI/BamHI double stranded oligonucleotide comprising the *loxP* site and the 0.5kb BamHI/HindIII fragment containing the PGK promoter were cloned into Bluescript digested with SalI/HindIII (PGK-LOX).

**3.** The StuI/NotI fragment from tsA58LOX was cloned into PGK-LOX digested with EcoRV and NotI.

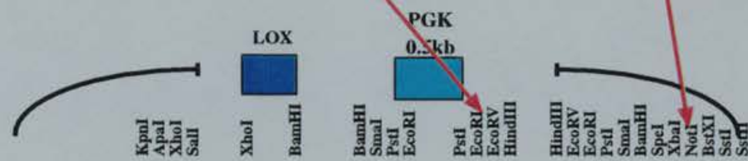
The presence of the *loxP* sites in the final construct was confirmed by restriction enzyme analysis and DNA sequencing.

**Cloning of SV40T ts A58 and LOX site**




StuI to NotI frag. inserted into EcoRV/NotI site

**Cloning of PGK promoter and LOX site**




**PGK-SV40T tsA58 flanked by LOX sites**

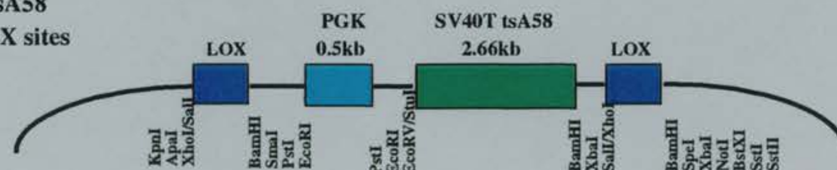



**Figure 3.7: PGKtsA58LOX** (Construct for conditional immortalisation)
Schematic diagram of cloning strategy. Indicated are the restriction enzyme recognition sites in the poly linker of the plasmid vectors and those used for cloning.

### 3.3.3. Immortalisation of TNT4 cells

TNT4 cells were transfected at passage seven with PGKtsA58LOX alone or co-transfected with PGKtsA58LOX and PGK-neo. Cells were cultured at 37°C, as it was known for rodent cells, that the tsA58 gene had some activity at this temperature, sufficient to immortalise cells but minimising phenotypic transformation (Gordon, 1999).

Cell clones obtained after single cell plating (plated at 1:5000) or G418 selection were analysed by PCR for the presence of the tsA58 gene and gender determination (see Fig. 3.8 and Table 3.7).

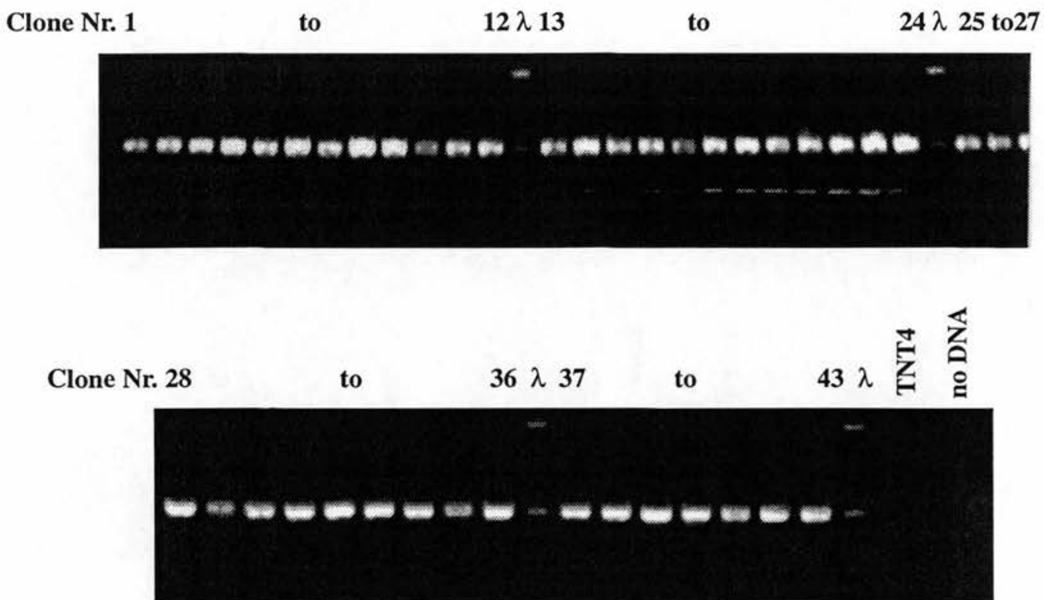



**Figure 3.8: PCR detection of SV40 T in TNT4 transfectants**
PCR amplification of SV40T sequences and analysis on agarose gel. λ, DNA size marker; No DNA, control PCR reaction lacking DNA.

Isolated cell clones had maintained epithelial morphology, but unlike the primary TNT4 cell clones, expanded rapidly even in the absence of feeder cells. 100% of picked cell clones could be expanded and frozen compared to 23-27% of primary clones transfected with a FIX transgene. Figure 3.8 shows that all clones contained the tsA58 gene, including those which had not been under drug selection. These results indicated that cells were conditionally immortalised.

**Table 3.7: Conditionally immortalised TNT4 cell clones:**

| Transfected DNA | Clones picked | Clones frozen | tsA58 positive | Sex | |
|---|---|---|---|---|---|
| | | | | F | M |
| PGKtsA58LOX | 15 | 15 | 15 | 15 | 0 |
| PGKtsA58LOX/ PGKneo | 28 | 28 | 28 | 26 | 2 |
| **Total** | **43** 100% | **43** 100% | **43** 100% | **41** 95.3% | **2** 4.7% |

The sex ratio was also reversed when compared to the primary cell clones derived from the FIX transfection. Only 2/40 clones were male. Whether this was due to the immortalisation or due to the fact that cells were used at an earlier passage is undetermined, although the latter is a more likely explanation.

## 3.4. Conclusions

The results in this chapter show that TNT4 cells do not share properties characteristic for ES or EG cells, e.g. lack of ES cell markers such as alkaline phosphatase, lack of expression of developmentally regulated genes such as Oct3/4. TNT4 cells represent a differentiated cell type of epithelial morphology with a limited lifespan in culture. Nevertheless, they are totipotent for nuclear transfer. At the time it still remained to be determined whether this was due to some specific, yet undefined characteristics of the cells or a result of improvements in the nuclear transfer technique.

A simple and reliable method for sexing of ovine cells has been established and applied to determine the gender of TNT cells. Results indicated that the cell population was derived from at least 2 individual embryos. Both male and female cells were present in the culture. At early passage the majority of cells were female, at later passage the male cells predominated, indicating a growth advantage of these cells. It should be noted that the only fertile animals obtained from TNT4 cells were female.

Culture and transfection conditions were optimised to allow production of genetically modified TNT4 cells. Clones which were stably transfected with either the cDNA or genomic FIX expression construct were obtained. These cell clones had an extended cell doubling time, or ceased to divide altogether. A change in morphology from epithelial phenotype to one reminiscent of senescence was also observed. Serum starved cells could not be induced to further cell divisions. All clones analysed were of male gender and had

an aberrant chromosome count. None of these clones were suitable as nuclear transfer donors. This led to the conclusion that the main cell stock (available at passage 9) was unsuitable for cell mediated transgenesis.

Attempts were made to increase the TNT4 lifespan by conditionally immortalising the cells using a temperature sensitive SV40T gene. As such a step could produce genetic aberrations, possibly leading to tumour formation in nuclear transfer derived animals, immortalised cells would have only been used if efforts to identify alternative cell types had failed. Furthermore the immortalisation construct contained *loxP* recognition sites to enable excision of the transgene prior to nuclear transfer.

Successful isolation of non-transformed nuclear transfer competent cell cultures suitable for cell mediated transgenesis is described in Chapters 4 and 5. Therefore work on TNT4 cells was abandoned. Conditionally immortalised TNT4 cells might still provide a resource to establish somatic gene targeting in ovine cells.

# Chapter 4

## Identification and isolation of nuclear transfer competent embryonic and adult cells

### 4.1. Background

The results in the previous chapter show that primary TNT4 cells were unsuitable in practice for cell mediated transgenesis. It was therefore essential to investigate the use of alternative cell cultures as donors for nuclear transfer. This would also establish if the nuclear transfer method was generally applicable, or if the success was based on special properties of the TNT4 cells.

If the nuclear transfer method was the enabling step (for example the use of quiescent nuclei), then nuclei from a wide variety of differentiated cells should support development after nuclear transfer, possibly even nuclei from adult cells. This therefore provided an opportunity to address a major question in mammalian biology, is genomic totipotency lost in somatic cells of the adult?

For the nuclear transfer season of winter '95/'96, I had the opportunity to test two different cell types. One was a conservative choice, newly derived ovine embryonic cells equivalent to TNT cells, and one was significantly more challenging, adult mammary cells from a 6 year old ewe. The use of adult cells for nuclear transfer has received widespread public attention and accounts of the experiment which ultimately gave rise to sheep 6LL3 "Dolly" have been published in popular books and magazines. I would like to make it clear that the choice and inclusion of adult mammary cells in the nuclear transfer experiments was entirely mine and that nuclear transfer procedures using these cells were carried out by the group of Ian Wilmut at my instigation.

Adult **o**vine **m**ammary **e**pithelial (OME) cells were chosen for their possible practical advantages for cell mediated transgenesis. An *in vitro* mammary culture may provide predictive data on transgene expression *in vivo* (Figure 4.1). At present, practical considerations make it necessary to use transgenic mice to assay constructs designed for expression in large animals. However, the shortcomings of the mouse as a predictor of the expression level in a large animal are widely recognised. For example, Carver (1993) found a markedly different range of expression of human $\alpha$-1 antitrypsin from the same construct in the milk of transgenic mice and sheep. Velander *et al.* (1992) obtained 1mg/ml

Ovine mammary epithelial culture
(OME)

Genetic manipulation
Co-transfection of OME cells with selectable marker gene and mammary specific transgene,
followed by selection and isolation of single cell clones.

Stable Transfectants

Addition of lactogenic hormones

RNA and/or protein analysis

Identification of cell clones expressing
high amounts of recombinant protein

Cryopreservation

Use of cryopreserved cells
for nuclear transfer

Transgenic lambs
from favourable cell clones

**Figure 4.1: Predictive model for *in vivo* transgene expression**
The schematic diagram shows the isolation and culture of ovine mammary epithelial cells. At first passage cells are transfected with selectable marker gene and a transgene under the control of a milk specific promoter. Single cell clones are isolated and expression of mammary specific genes is induced by addition of lactogenic hormones. Cell clones which show high expression of the recombinant protein are identified prior to use as nuclear transfer donors. Animals born are expected to express the transgene.

human Protein C from transgenic pigs using a construct which expressed poorly in transgenic mice. Similarly, post translational processing of a foreign protein by the mouse mammary gland is a poor guide to large mammals (Drohan *et al.*, 1994).

PPL in collaboration with Colin Wilde (Hannah Research Institute) tested the use of OME cells for milk specific transgene expression *in vitro*. Stably transfected OME cells, after addition of lactogenic hormones, were analysed for expression of recombinant protein. If the same cell clones could then be used as nuclear transfer donors, all animals born would be transgenic and should express the recombinant protein in their milk (Figure 4.1). This selection protocol would minimise the number of sheep which are normally required to produce one high expressing animal. It is envisaged that such an approach is not restricted to OME cells. Any nuclear transfer competent cell type, which mimics *in vivo* transgene expression, should be suitable.

## 4.2. Isolation of sheep embryonic cells

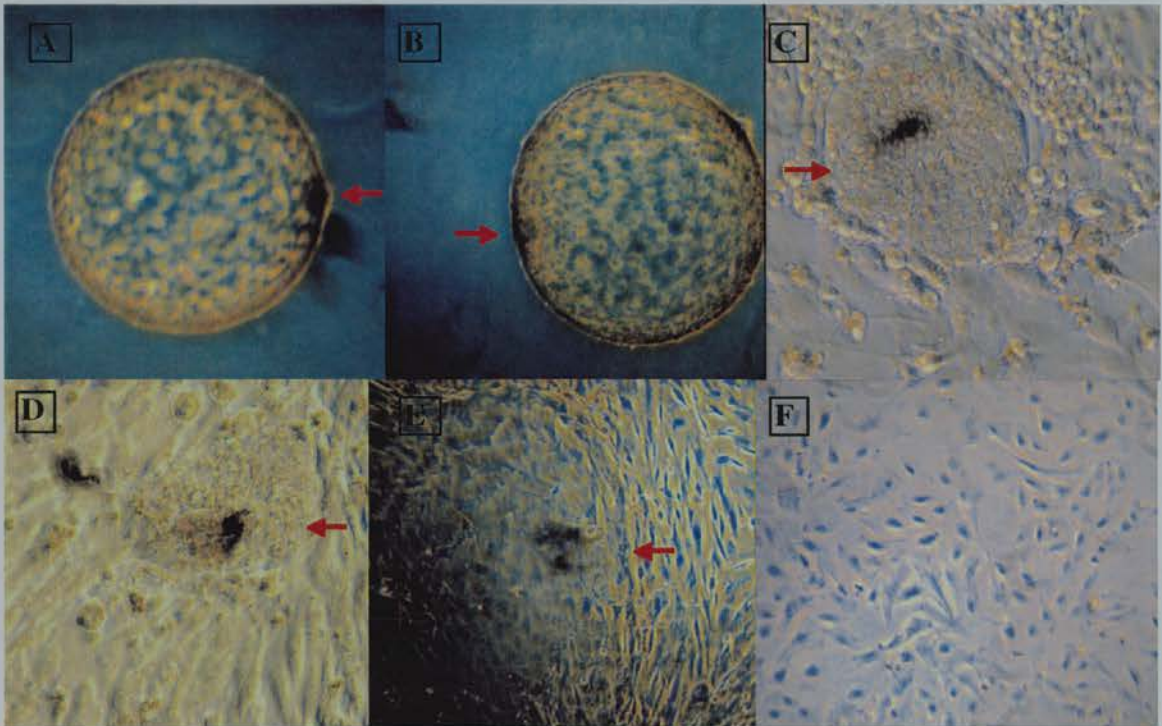



**Figure 4.2. Phase-contrast photomicrographs of ovine embryonic cultures** **A.** and **B.** day 10 hatched blastocysts (magnifications: A. 300X; B. 200X), the arrow indicates position of the embryonic disc; **C.** and **D.** disc explants at day 2-3 in culture, indicated by the arrow (magnification: 500X), also visible are STO feeder cells; **E.** disc explant after 9 days in culture (magnification: 200X); **F.** SEC1 embryonic cell culture at passage 5 (magnification:200X).

TNT4 cells had been isolated from cultures of several embryonic discs and were a mixed population which was undesirable. An attempt was therefore made to newly derive sheep embryo cultures (SEC) from single day 9-10 embryonic discs.

Day 9 to 10 ovine blastocyst stage embryos (Figure 4.2A and B) were derived from the specific pathogen free Poll Dorset herd at PPL on 4 separate days (kindly provided by the PPL Animal Husbandry Unit). The embryonic discs were mechanically dissected using fine forceps and placed individually in 24 well dishes on mitotically inactivated STO feeder cells (Figure 4.2 B). Culture conditions were essentially as for TNT and mouse ES cell derivation. Medium was supplemented with bovine rather than murine LIF. A bovine LIF expression vector had previously been cloned by the author and tested at PPL as part of a different project . Explant details and cell lines derived are shown in Table 4.1.

**Table 4.1: Derivation of SEC cells**

| Day isolated | Hatched embryos | Dissected embryos | Primary explant | Passage | | | Sex | I.D. |
|---|---|---|---|---|---|---|---|---|
| | | | | 1 | 4 | 5 | | |
| 24.10.95 (d9 emb.) | 43 | 22 | 4 | 2 | 1 | 1 | M | **SEC1** |
| 27.10.95 (d10 emb.) | 35 | 20 | 9* | | | | | |
| 06.12.95 (d10 emb.) | 31 | 12 | 5 | 3 | 2 | 2** | F<br>M | SEC2<br>SEC3 |
| 08.12.95 (d10 emb.) | 4 | 3 | 2 | 2 | 1 | 1** | M | SEC4 |
| **Total** | **113**<br>100% | **57**<br>50% | **20**<br>18% | **7**<br>9%+ | **4**<br>5%+ | **4**<br>5%+ | | |

* culture lost due to bacterial contamination
** culture lost due to incomplete inactivation of feeder cells
\+ % corrected for loss of 9 primary explants

Four explants (SEC 1, 2, 3, 4) derived from single embryonic discs were cultured to passage 4 and 5 and their gender determined by PCR (3 male and 1 female explant). Although SEC cells were co-cultured with feeder cells, they attached directly to the culture dish plastic. To test for feeder independence, SEC1 cells were grown in the absence of feeders from passage 2 onwards, while SEC2 to 4 were cultured in the presence of feeder cells. At passage 5 escaped STO feeder cells overgrew the embryonic cultures, which were then discarded. All subsequent experiments were carried out using SEC1 cells without feeders.

Although culture conditions were based on those used for mouse ES cell derivation, none of the SEC cultures resembled mouse ES cells, or TNT4 cells. When sub-confluent these were large very flattened cells (Fig. 4.3) which became closely packed at confluence. It is noteworthy that Gerfen and Wheeler (1995) describe the "ES like" pig cells used to produce chimeras as follows: "Initially, embryonic cells of interest were translucent in nature and difficult to locate". This was precisely the observation regarding SEC cells, they were almost invisible as early colonies.

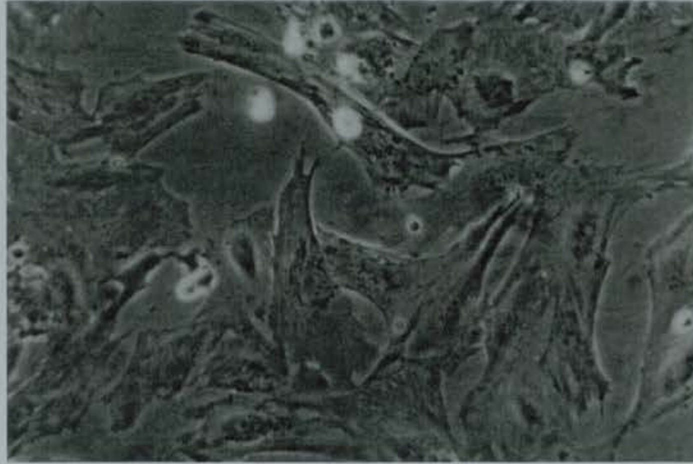


**Figure 4.3: Embryo derived SEC1 cells**
Phase-contrast photomicrograph of SEC-1 cells. Magnification: 750X.

### 4.2.1. Assessment of SEC1 cells for nuclear transfer

Prior to nuclear transfer, the chromosome number of SEC1 cells and their ability to survive serum starvation was assessed.

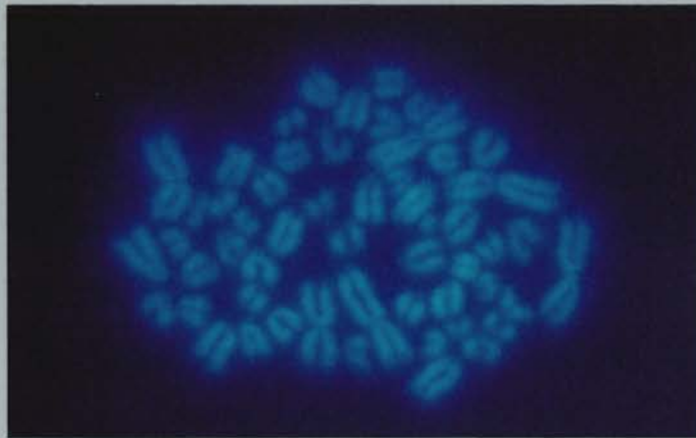


**Figure 4.4: Determination of SEC1 chromosome number**
Fluorescent micrograph of DAPI stained metaphase spread. Magnification 1,500X.

Metaphase spreads at passage 8 showed a modal chromosome number of 54 (Fig. 4.4). Cells cultured for >12 passages showed signs of senescence and few cells gave metaphase spreads, those which could be analysed were aneuploid.

Culture conditions necessary to arrest growth and the ability of cells to survive growth arrest were assessed by monitoring cell number (Table 4.2).

**Table 4.2: Serum starvation of SEC1 cells at passage 7**

| Day | 0.5% serum, cell count | 4 days after addition of 10% serum |
|---|---|---|
| 0 | $2.0 \times 10^4$ | n.t. |
| 2 | $2.1 \times 10^4$ | $7.1 \times 10^4$ |
| 3 | $1.9 \times 10^4$ | $6.5 \times 10^4$ |
| 6 | $1.6 \times 10^4$ | $4.8 \times 10^4$ |

Table 4.2 shows that cell growth was retarded in low serum after only 2 days. Addition of 10% serum allowed further cell growth even after 6 days of starvation

### 4.2.2. Nuclear Transfer using SEC1 donor nuclei

Cells which had undergone 5 days of serum starvation were provided for nuclear transfer, which was carried out in Ian Wilmut's laboratory at the Roslin Institute as described previously (Campbell *et al.*, 1996a). A schematic outline of the nuclear transfer procedure is shown in Figure 4.5. Animal care and veterinary procedures were carried out at the Roslin Institute's large animal unit. Results of the nuclear transfer experiment and details of veterinary examinations are included in this thesis for reasons of continuity and clarity.

**Figure 4.5 (next page):** Schematic outline of the nuclear transfer procedure. Oocytes derived from Scottish Blackface (symbolised as a yellow sheep) are enucleated. The donor cell derived from a different sheep breed (symbolised as a red sheep) is placed under the zona pelucida into the perivitelline space. The cell nucleus is introduced into the cytoplast by electrofusion, which also activates the oocyte. The reconstructed embryo is then either cultured *in vitro* up to blastocyst stage or is transferred into a pseudopregnant intermediate recipient ewe. At day 7 embryos are assessed for development. Late morulae and blastocysts are transferred into final recipients. Pregnancies resulting from nuclear transfer are determined by ultrasound scan at about 60 days after oestrus, and development is subsequently monitored at regular intervals.

**Figure 4.5: Nuclear transfer procedure**

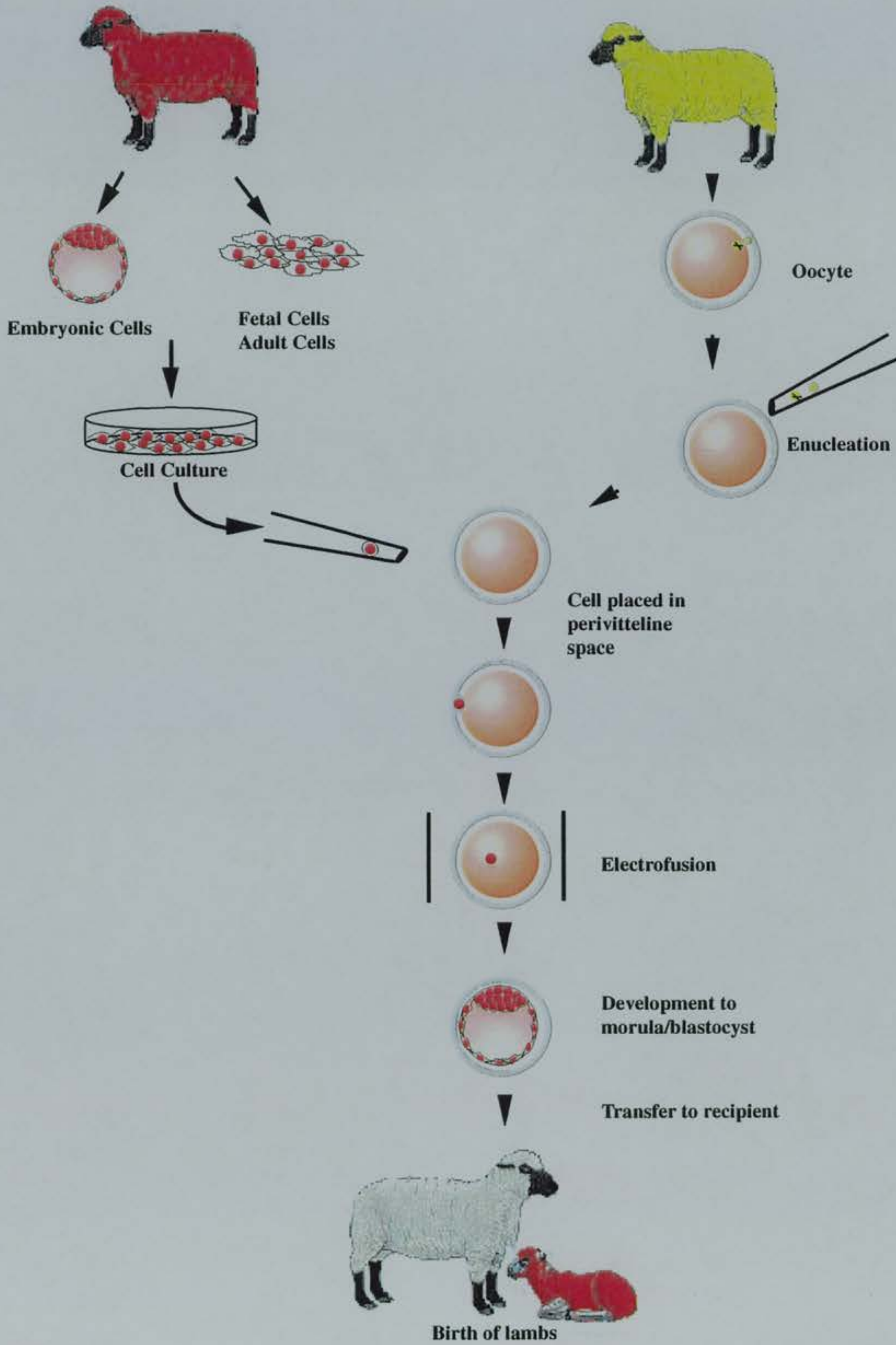


**Table 4.3: SEC1 nuclear transfer results**

| **Donor cell:** SEC-1<br>**Derived from:** day 9 embryo<br>**Breed:** Poll Dorset<br>**Passage:** 7-9<br>**Recipient oocyte:** Scottish Blackface | | |
|---|---|---|
| | *In vivo* culture | *In vitro* culture |
| No. of reconstituted embryos | 293 | 92 |
| ***In vivo*** **:**<br>No. recovered from oviduct | 231 | |
| ***In vitro*****:**<br>No. cultured | | 92 |
| No. of morulae and blastocysts | 90 | 36 |
| No. of embryos transferred | 72 | 15 |
| No. of pregnancies /<br>No. of recipients | 14/27 | 1/5 |
| No. of live lambs | 4 | 0 |
| Nuclear transfer efficiency<br>(% of live lambs from reconstructed embryos) | 1.36%<br>1.7%* | 0% |

* this percentage takes into account that only 80% of morula and blastocysts were transferred to final recipients.

The result of the SEC1 nuclear transfer experiment is summarised in Table 4.3. Out of 14 pregnancies 4 were lost prior to day 90. For details of the remaining 10 pregnancies please refer to table 4.4. The 4 liveborn nuclear transfer animals (6LL1, 6LL2, 6LL5, 6LL6), two of which were born by caesarean section, are shown in Figure 4.6. Birth weight varied from 4.2 to 6.5 kg (mean birth weight 5.55kg). Poll Dorset lambs in the PPL New-Zealand derived flock have a mean birth weight of 5.1kg for single birth. No live animals were obtained from the *in vitro* cultured embryos, indicating that further improvements to the culture conditions are required. Two of the four males have fathered healthy lambs, proving their fertility.

**Table 4.4: Summary of SEC1 pregnancies derived by nuclear transfer.**

| Ewe I.D. | Lamb I.D. | Duration of preg. (days) | Birth weight (kg) | Comments, Post Mortem results |
|---|---|---|---|---|
| 5E337 | | <118 | 0.4 | Mummified fetus |
| 5E205 | | <118 | 1.7 | Autolysis of organs, indications of liver abnormalities |
| 5E226 | | 117 | 4.53 | no gross abnormalities, kidney appeared shrunken |
| 1B44 | | 116 | 0.35 | Mummified fetus |
| 5E550 | twins | 130 | | One fetus died, pregnancy terminated |
| 5E1901 | **6LL1** | 149 | 6.5 | Vaginal prolapse, induced |
| 5E017 | **6LL2** | 152 | 6.0 | Caesarean section, breached position |
| 5E134 | 6LL4 | 149 | 5.8 | Caesarean section, born dead |
| 5E524 | **6LL5** | 148 | 4.2 | Normal birth |
| 9M399 | **6LL6** | 152 | | Caesarean section, temporary facial paralysis due to trapped nerve |

**Figure 4.6: SEC1 nuclear transfer sheep**
Photo of four Poll Dorset sheep derived from cultured embryonic donor cells by nuclear transfer.

### 4.3. Nuclear transfer competent adult cell culture

OME cell preparation was carried out in Colin Wilde's laboratory at the Hannah Research Institute as described by Finch *et al.* (1996). Ovine mammary tissue from a 6 year old Finn Dorset ewe was dissected post mortem in the third trimester of pregnancy, digested with collagenase and hyaluronidase, and fractionated by Percoll density gradient centrifugation. Fractions which were 98% positive for the epithelial marker cytokeratin were cryopreserved in multiple aliquots (OME (OP5)). Members of Wilde's group attempted to optimise culture conditions of OME (OP5) and analysed expression of endogenous milk genes. Frozen aliquots were shipped to PPL to investigate, if the cells could be genetically modified, e.g. addition of transgenes under the control of a lactation specific promoter.

Described here is the assessment of OME (OP5) as nuclear transfer donors. Figure 4.7 shows the morphology of these cells.

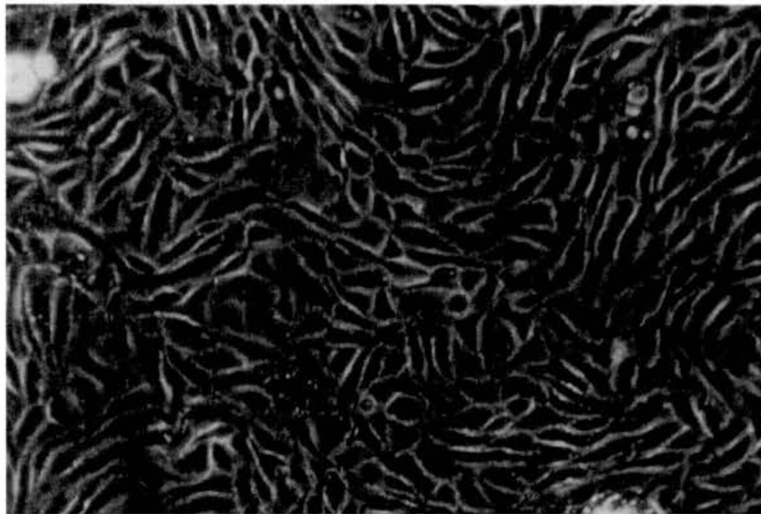


**Figure 4.7: Adult derived ovine mammary epithelium cells**
Phase-contrast photomicrograph of OME (OP5) cells. Magnification: 300X.

#### 4.3.1. Assessment of OME (OP5) cells for nuclear transfer

Both chromosome number and the ability to survive serum starvation was assessed prior to nuclear transfer as described for TNT4 and SEC1 cells.

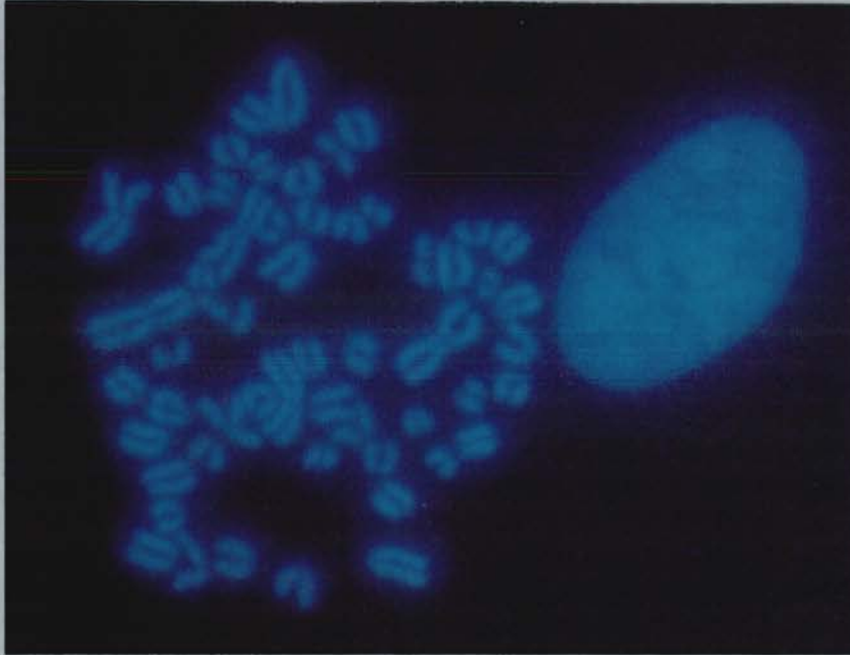


**Figure 4.8: Determination of OME (OP5) chromosome number**
Fluorescent micrograph of DAPI stained metaphase spread, also shown is an interphase nucleus. Magnification 1,500X.

OME (OP5) metaphase spreads at passage 3 showed a modal chromosome number of 54 (Fig. 4.8). Serum conditions necessary to arrest growth and the ability of cells to survive growth arrest were assessed by monitoring cell number (Table 4.5). Cell growth was retarded in low serum after 3-4 days. After addition of 10% serum further cell growth was observed. 5 days of serum starvation was used to prepare cells for nuclear transfer.

**Table 4.5: Serum starvation of OME (OP5) cells at passage 3**

| Day | 0.5% serum, cell count | 4 days after addition of 10% serum |
|---|---|---|
| **0** | $1.0x10^4$ | n.t. |
| **3** | $7.6x10^4$ | $55.5x10^4$ |
| **4** | $21.3x10^4$ | $64.4x10^4$ |
| **5** | $11.6x10^4$ | $73.0x10^4$ |

### 4.3.2. Nuclear Transfer using OME donor nuclei

Cells which had undergone 5 days of serum starvation were provided to Ian Wilmut's group at the Roslin Institute. Nuclear transfer was carried out as described in chapter 4.2.2. with the exception that reconstituted embryos were only cultured *in vivo*. The results are summarised in Table 4.6.

**Table 4.6: OME nuclear transfer results**

| **Donor cell:** OME (OP5)<br>**Derived from:** 6 year old adult<br>**Breed:** Finn Dorset<br>**Passage:** 3<br>**Recipient oocyte:** Scottish Blackface | |
|---|---|
| | *In vivo* culture |
| No. of reconstituted embryos | 277 |
| *In vivo* :<br>No. recovered from oviduct | 247 |
| No. of morulae and blastocysts | 29 |
| No. of embryos transferred | 29 |
| No. of pregnancies /<br>No. of recipients | 1/13 |
| No. of live lambs | 1 |
| Nuclear transfer efficiency<br>(% of live lambs from reconstructed embryos) | 0.36% |

Twenty nine embryos were transferred to final recipients, a single pregnancy was established, and developed to term. Duration of pregnancy was 148 days and birth weight 6.6kg. The adult cell derived nuclear transfer sheep 6LL3 (better known as Dolly) is shown in figure 4.9 together with her lamb 8LL5 (Bonny), which was produced by natural mating and was born in Spring 1998. Nuclear transfer efficiency for the adult cells was lower than for the embryonic cells (0.36 live lambs per 100 reconstituted embryos compared to 1.36).

**Figure 4.9: OME (OP5) derived nuclear transfer sheep**
Photo of Dolly (6LL3) at 2 years of age together with her female offspring (Bonny, 8LL5), which was derived by natural mating.

## 4.4. Microsatellite analysis of nuclear transfer derived lambs.

Sections 4.2 and 4.3 showed that healthy and fertile lambs were derived from both embryonic and adult cells. In a separate experiment Keith Campbell and Patricia Ferrier in Ian Wilmut's Group had isolated fetal fibroblasts from a day 25 Black Welsh Mountain fetus (BLWF1). These too were nuclear transfer competent, and resulted in the birth of two male lambs (Animal I.D. 6LL7, 6LL8) (Wilmut, 1997).

All nuclear transfer lambs displayed the morphological characteristics of the breed from which the donor cell was derived (SEC-1, Poll Dorset; BLWF1, Black Welsh Mountain; and OME, Finn Dorset). In addition, microsatellite analysis was carried out to confirm genomic identity of donor cell and nuclear transfer derived sheep.

Microsatellites are repetitive elements containing simple sequence motifs, usually dimers or trimers, e.g. (dG-dT)•( dC-dA) dimers. Generally they are less then 100bp and are

found embedded in unique DNA sequences (Tautz, 1989), allowing specific PCR amplification of individual microsatellite loci. (Litt and Luty, 1989; Weber, 1990). Differences in the size of alleles are due to variation in the number of repeats, which is detected by gel electrophoresis.

Buchanan *et al.* (1993) have described a number of highly polymorphic ovine microsatellite markers. Five of these (Table 4.7) were used to compare DNA from nuclear transfer lambs, donor cells and recipient ewes.

**Table 4.7: Microsatellite Markers**

| Name | Allele sizes (bp) | No. of alleles | PIC* |
|---|---|---|---|
| MAF33 | 121-141 | 9 | 0.70 |
| MAF209 | 109-135 | 8 | 0.79 |
| OarFCB11 | 121-143 | 9 | 0.79 |
| OarFCB128 | 99-131 | 8 | 0.72 |
| OarFCB304 | 150-188 | 9 | 0.54 |

* PIC = polymorphic information content

Figure 4.10 shows the results obtained with four of these markers, as well as the gender analysis of both cells and lambs using PCR. In each case donor cells and the lambs derived from those cells had identical banding patterns, while the banding pattern for individual recipient ewes varied.

Figure 4.11 shows that the microsatellite markers used are indeed polymorphic and can distinguish between individual animals from the same breed. Four random Finn Dorset sheep were compared with duplicate DNA samples from 6LL3 (Dolly) and the adult derived donor cells OME (OP5). Each animal has its individual microsatellite banding pattern (Fig. 4.11, sample 1-4). Again the nuclear transfer derived lamb and the donor cell culture have an identical pattern (Fig. 4.11, OME and 5LL3). The latter samples were analysed in duplicate.

These results confirm that nuclear transfer lambs were derived from their respective donor cells.

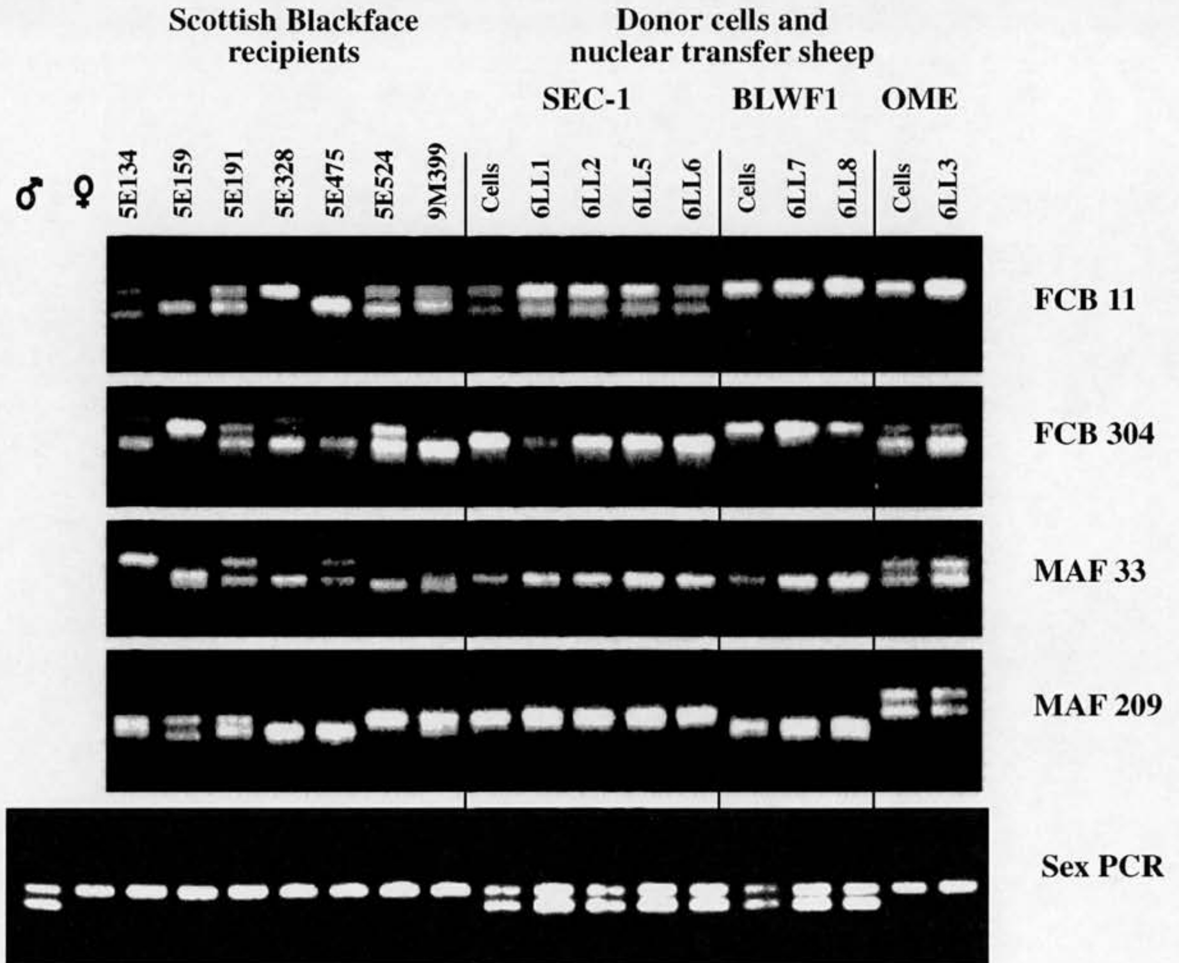



**Figure 4.9: Microsatellite analysis of nuclear transfer sheep**
Microsatellite analysis of recipient ewes, nuclear donor cells and lambs using four polymorphic ovine markers. Cell populations are embryo derived (SEC1), fetal derived (BLWF1) and adult derived (OME). Bottom panel shows gender determination by PCR.

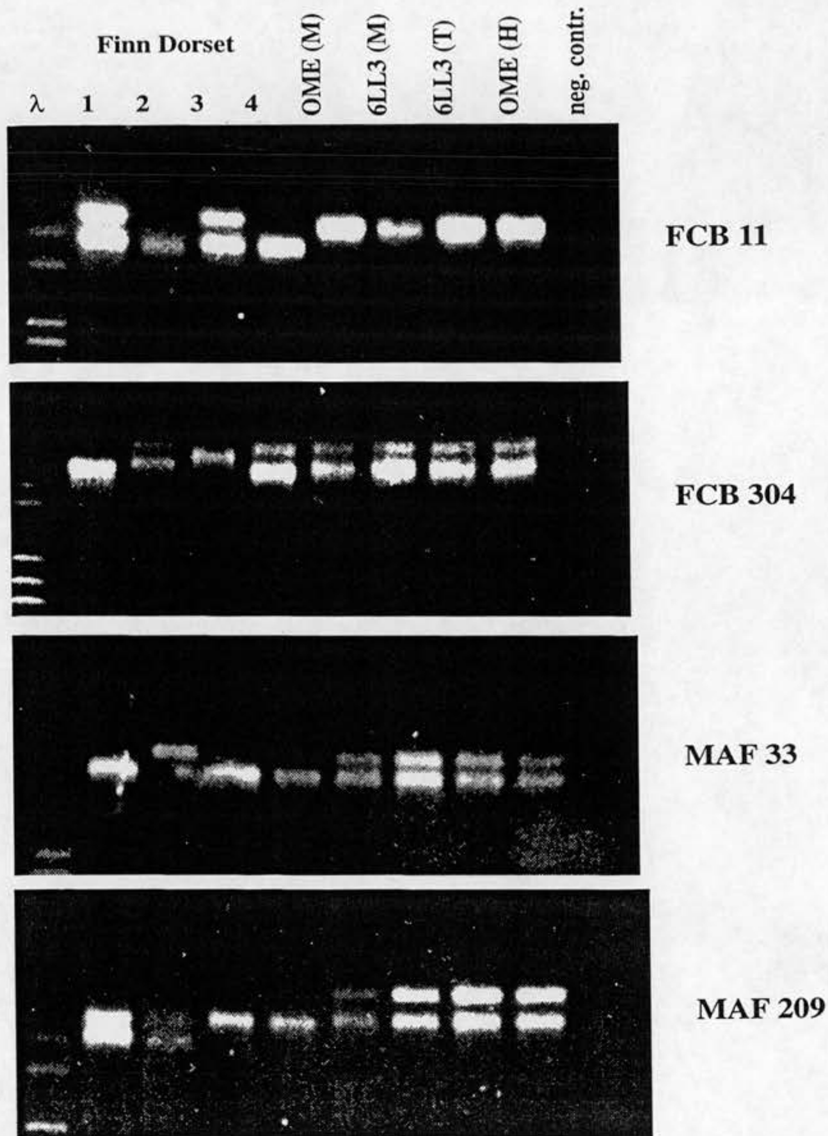



**Figure 4.10: Microsatellite analysis of 6LL3 (Dolly), the adult derived donor cells 0ME (OP5) and random Finn Dorset sheep**
Microsatellite analysis of four random Finn Dorset sheep (1 to 4), and two samples each of the mammary cell derived OME culture and nuclear transfer derived animal 6LL3 (Dolly). The duplicated DNA sample had been prepared by independent investigators. M, DNA prepared for microsatellite analysis; T, DNA prepared for telomere analysis by Paul Shiels; H, DNA prepared at the Hannah Institute.

## 4.5. Conclusions

The above results show that healthy and fertile nuclear transfer lambs were derived from both cell cultures, embryonic SEC1 cells and, more significantly, from the fully differentiated adult OME cells. From this it can be concluded that using the current nuclear transfer method live animals can be derived from a variety of differentiated cell types and that it is the practical aspects of the procedure, such as use of quiescent nuclei which lead to successful birth of animals, rather than the characteristics of the euploid cell.

The nuclear transfer animal Dolly (6LL3) is the first animal cloned from an adult cell. It provides ultimate proof that the process of differentiation at the level of the nucleus is not irreversible. These findings are consistent with the view that differentiation is achieved by systematic, sequential changes in gene expression and that the effects of epigenetic changes are reversible. That the animal was indeed derived from the adult cells was proven by microsatellite analysis using highly polymorphic ovine markers.

Nuclear transfer efficiency (% of live lambs per reconstructed oocyte) for the embryonic cell line SEC1 (1.7%) was slightly higher than that obtained with TNT4 cells (1.07%), while that for OME cells was lower (0.36%). The main difference in efficiency resulted from the number of reconstituted embryos that developed to blastocyst (11.74% for OME compared to 38.96% for SEC1). The percentage of transferred blastocysts that developed to birth was only slightly lower (3.45% compared to 5.55%). The reduction of the developmental capacity of the adult nucleus might reflect an accumulation of chromosomal damage during ageing, such as telomere shortening and oxidative damage.

A major drawback of production of transgenic livestock by microinjection is the random integration of the transgene, often resulting in wildly varying expression levels. This requires the production of multiple transgenic animals which all have to be assessed for the quality and quantity of the recombinant protein. This can both be cost and time consuming, especially if the founder animal is male and the transgene is expressed in milk. The use of mammary epithelial cells for nuclear transfer opens up the possibility to predict expression of milk specific transgenes *in vitro* prior to the production of animals (Figure 4.1), therefore reducing time, cost and number of experimental animals. It is envisaged that such an approach is not restricted to OME cells. Any nuclear transfer competent cell type, which mimics in vivo transgene expression, should be suitable.

The success of the experiments described here provide new research opportunities for evaluation of the molecular mechanisms underlying differentiation, reprogramming, and genomic imprinting. Production of animals without germline involvement may also provide a new tool to investigate the mechanisms of ageing. Further beneficial aspects of cloning from somatic cells could include easy preservation of genetically important strains of laboratory and farm animals as well as endangered species (Wells *et al.*, 1999). It might also prove to be useful to humans as a means of providing autologous cells for regenerative transplants (Smith, 1998; Trounson and Pera, 1998).

# Chapter 5

## Human Factor IX transgenic sheep produced by transfer of nuclei from transfected cells

### 5.1. Background

The proceeding chapters showed that normal sheep can be produced by transfer of nuclei from adult mammary and embryonic cells cultured *in vitro*. In addition Wilmut *et al.* (1997) described successful nuclear transfer from fetal fibroblast cells. The aim of work described in this chapter is to determine if nuclear transfer from stably transfected somatic cells could provide a means of producing transgenic livestock. Gene transfer into cultured cells offers several important advantages over DNA microinjection into embryos, the most significant of which is the ability to identify and analyse genetically manipulated nuclear donor cells before producing whole animals. It also allows the sex of the animals to be predetermined, which leads to a more efficient use of experimental animals.

The first objective was to identify a cell type most amenable for cell mediated transgenesis. These cells would then be transfected with pMIX1, a vector designed to express human rFIX protein in the milk of transgenic animals and which had been shown to be expressed in the mouse mammary gland (see Chapter 2).

### 5.2. Assessment of cell types for cell mediated transgenesis

Embryonic, fetal and adult cell culture were each assessed for their potential use in cell mediated transgenesis. The results are summarised in Table 5.1.

**Table 5.1: Assessment of embryonic, fetal, and adult cells for cell mediated transgenesis**

| | TNT4 | SEC1 | BLWF1 | OME |
|---|---|---|---|---|
| **Source** | Day 9 embryo | Day 9 embryo | Day 25 fetus | Adult 6 years |
| **Nuclear transfer efficiency** | 1.07% | 1.70% | 1.35% | 0.36% |
| **Transfection efficiency** | 1-5% | n.d. | n.d. | 0.5% |
| **Single cell cloning** | yes | no | n.d. | yes |
| **Euploidy after cloning** | **no** | **no*** | n.d. | **yes** |

n.d. not done

* Cells aneuploid at passage >12

In contrast, both fetal and adult cells can be isolated in essentially unlimited numbers with no requirement for expansion. Large numbers of cells can be cryopreserved for convenience and are available for genetic manipulation at first passage. Cell clones from the adult OME (OP5) culture had been previously isolated (by A. Scott, Cell Biology, PPL) as part of the aforementioned collaboration with Colin Wilde.

The euploidy of both cell clones and oligoclonal pools was tested as a basic requirement for nuclear transfer. Table 5.2 shows the results of the chromosome counts, no adverse effect on gross karyotype is indicated.

**Table 5.2: Modal chromosome number of OME (OP5) transfectants**

| Transfectants | Passage | No. of spreads | Modal number | Comments |
|---|---|---|---|---|
| Pool 1 | 5 | 15 | 54 | |
| Pool 2 | 5 | 18 | 53 | |
| Clone 3 | 6 | 18 | 54 | |
| Clone 4 | 6 | 3 | 54 ? | poor spreads |
| Clone 5 | 6 | 18 | 54 | |
| **Total** | **5-6** | **99** | **54** | |

No experiments were carried out with the fetal fibroblast cells BLWF1 as neither pathogen status of the cell culture nor the animal, from which cells were derived, was known. Previous experience by the author had shown that ovine fibroblast cells can be efficiently transfected (data not shown) and, as shown for the adult cells,- the relatively short time in culture should guarantee euploidy. The main advantage of fetal fibroblasts compared to OME (OP5) cells, was the higher nuclear transfer efficiency and therefore the greater likelihood of obtaining transgenic animals. For this reason it was decided to isolate both male and female fetal fibroblast from PPL's specific pathogen free flock and assess those for cell mediated transgenesis.

## 5.3. Isolation and manipulation of ovine fetal fibroblasts

Primary strains of ovine cells, termed PDFF1-7 (**P**oll **D**orset **F**etal **F**ibroblast), were derived from seven day 35 fetuses kindly provided by PPL's Animal Husbandry Unit. Sex analysis of each cell line by PCR revealed cell line PDFF5 to be male and the other six female (Figure 5.1). As the immediate aim was to express recombinant FIX in the ovine

mammary gland, a female cell line PDFF2 was chosen for transfection. Male PDFF5 cells were also tested for their ability to support nuclear transfer because future experiments such as gene targeting may benefit from the production of male animals (see Chapter 6).

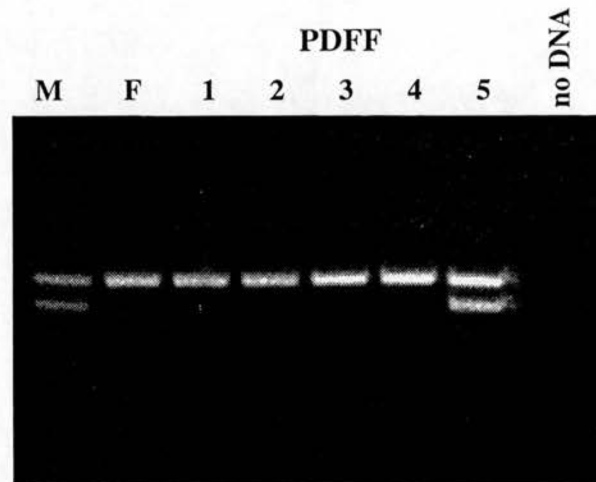



**Figure 5.1: Sex determination of Poll Dorset fetal fibroblasts**
PCR amplification of ovine X and Y sequences analysed on 2% agarose gels.
PCR primers employed were bovine zfx 5' and 3' and the "universal" Y primers Rg4 and Rg7, which successfully amplified both X and Y ovine sequences. M, male control DNA; F, female control DNA.

Trial transfections indicated that PDFF1, PDFF2 (both female) and PDFF5 (male) cells could be readily transfected with a LacZ reporter gene using the cationic lipid reagent LipofectAMINE (see also Chapter 3). pMIX1 DNA and the selectable marker construct PGKneo were cotransfected into PDFF2 cells at passage 1, after 3 days in culture, and stable transfectants selected with G418. Because the possible effects of drug selection and growth as single cell clones on the ability of cells to support nuclear transfer were unknown, cells were treated in two ways after transfection. One group was grown at high density under G418 selection, then cryopreserved as a pool for nuclear transfer. The other group was plated at low density and cloned transfectants grown from isolated colonies. Twenty four clones were isolated, of which 21 were expanded for analysis of genomic DNA. Southern analysis similar to that in figure 5.4 showed that 10 clones contained pMIX1 DNA.

### 5.3.1. Assessment of PDFF2 transfectants for nuclear transfer

The chromosome number of untransfected PDFF2 and 5 cells cultured up to passage 19 over a period of 80 days, was determined and showed a modal chromosome number of 54, the euploid ovine chromosomal complement. The chromosome number of pMIX1 transfected PDFF2 clones was determined at passage 6-7, after an average 40 days in culture and the uncloned PDFF2 pool at passage 5, after only 19 days in culture.

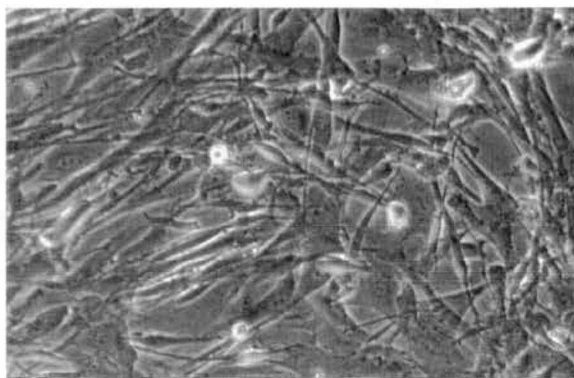
**Table 5.3: Euploidy of transfected and untransfected PDFF cells.**

| Cells | Passage | No. of spreads counted | Euploid |
|---|---|---|---|
| | Non-transfected | | |
| PDFF2 | 7 | 7 | 71.4% |
| PDFF2 | 19 | 29 | 65.5% |
| **PDFF5** | 4 | 32 | 83.3 |
| | Transfected | | |
| **PDFF2 pool** | 5 | 37 | 75.7 |
| PDFF2-7 | - | n/c | - |
| **PDFF2-12** | 6-7 | 26 | 92.3 |
| **PDFF2-13** | 6-7 | 33 | 75.8 |
| PDFF2-14 | - | n/c | - |
| PDFF2-15 | - | n/c | - |
| PDFF2-31 | 6-7 | 46 | 70% |
| PDFF2-32 | - | n/c | - |
| PDFF2-37 | - | n/c | - |
| PDFF2-38 | 6-7 | 48 | 50% |
| PDFF2-42 | - | n/c | - |

n/c not counted, poor cell proliferation, few spreads.

Not all single cell clones could be readily expanded. The 4 clones which grew best in culture showed a modal chromosome number of 54 indicating no gross chromosomal instability after long term culture and drug selection (see Table 5.3). Clone PDFF2-12 and PDFF2-13 were chosen for nuclear transplantation, their morphology is shown in Figure 5.2.

**PDFF2-12** **PDFF2-13**



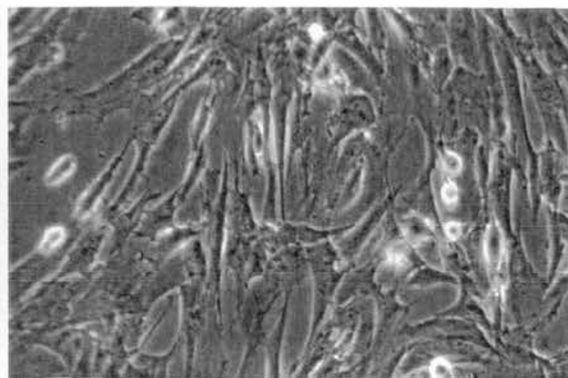


**Figure 5.2: Morphology of PDFF2 transfected cell clones**
Phase-contrast photomicrograph of the two stably transfected PDFF2 cell clones (PDFF2-12 and PDFF2-13). Magnification: 250X.

The ability of PDFF2 cells to undergo and survive serum starvation was tested. After 5 days culture in medium containing reduced serum (0.5%), restoration of serum content to 10% reversed the effect and cell growth resumed (Table 5.4).

**Table 5.4: Serum starvation of PDFF2 cells**

| **Serum** | **0.5%** | **0.5%** | **0.5%** | **10%** | **10%** | **10%** |
|---|---|---|---|---|---|---|
| **Day** | 0 | 3 | 5 | 6 | 7 | 8 |
| **PDFF2 non-transfected** | | | | | | |
| Cell number ($x10^4$) | 1 | 2.8 | 3.4 | 8.6 | 22.2 | n.t. |
| Cell number ($x10^4$) | 2 | 4.6 | 10.4 | 26.4 | 81.0 | n.t. |
| **PDFF2 transfected pool** | | | | | | |
| Cell number ($x10^4$) | 1 | n.t. | 2.2 | n.t. | n.t. | 52 |

For nuclear transfer experiments cells were provided to Ian Wilmut (Roslin Institute) after a 5 day culture in low serum.

## 5.4. Nuclear transfer of transfected and non-transfected PDFF cells

Four cell types were used as nuclear donors: untransfected PDFF5, pooled PDFF2 transfectants, and two transfected clones, PDFF2-12 and PDFF2-13 confirmed as containing >10 and ~5 copies of the pMIX1 transgene respectively (see Fig. 5.4). Transfer of nuclei from each cell type into enucleated oocytes derived from Scottish Blackface ewes was carried out by members of Ian Wilmut's group at the Roslin Institute as previously described (Campbell *et al.*, 1996a, Chapter 4). Table 5.5 shows the results of nuclear transfer.

**Table 5.5: Summary of the results of nuclear transfer**

| | **PDFF5 non-transfected** | **PDFF2 pool** | **PDFF2-12** | **PDFF2-13** |
|---|---|---|---|---|
| No. of reconstructed embryos | 82 | 224 | 89 | 112 |
| No. developed to morulae or blastocysts | 5 (6.1%) | 22 (9.8%) | 19 (21.4%) | 23 (20.5%) |
| No. of embryos transferred | 5 | 22 | 19 | 21 |
| No. of recipients | 2 | 9 | 7 | 6 |
| No. of pregnancies at day 60 | 2 | 4 | 4 | 1 |
| No. of fetuses at day 60 (% of embs. transf.) | 3 (60%) | 4 (18.2%) | 6 (31.6%) | 1 (4.8%) |
| No. of liveborn lambs * (% of embs. transf.) | 1 (20%) | 3 (13.6%) | 2 (10.5%) | 1 (4.8%) |
| **N.T. efficiency** (% live lambs from reconstructed embryos) | **1.22%** | **1.34%** | **2.25%** | **0.89%** |

* Liveborn lambs were defined as those with a heart beat and able to breathe unassisted at birth.

Live lambs were obtained from all four cell types. As expected, animals derived from PDFF5 were male and those from PDFF2 were female. Figure 5.3 shows the four transgenic lambs 7LL8, 7LL12, 7LL13 (Polly), and 7LL15 (Molly). All animals from

PDFF2 cells had a slightly undershot jaw which did not interfere with the animal's well-being. This is a genetic trait known to occur sporadically in the Poll Dorset breed and is considered to be unrelated to nuclear transfer. The PDFF5 and SEC1 lambs (also Poll Dorset) did not show this feature.

**Figure 5.3: Transgenic nuclear transfer derived lambs.**
Lambs 7LL8, 7LL12, derived from PDFF2 pool, 7LL13 (Polly, derived from PDFF2-13), and 7LL15 (Molly, derived from PDFF2-12).

Table 5.6 provides details of all 11 pregnancies established in surrogate mothers. Of the original 14 fetuses, one was lost prior to day 80, 3 were lost at late stage pregnancy (130 days), 4 were lost perinatally and 7 were liveborn, as defined by heart beat and unassisted breathing. One (7LL9) died shortly after birth due to meconium in the lung, and a second (7LL16) was diagnosed with a heart defect and euthanised at 14 days old for animal welfare reasons. Post mortem examination of abortuses and dead lambs did not indicate any common factor as a cause of death.

**Table 5.6: Summary of pregnancies derived by nuclear transfer.**

| Preg. No. | N.T. donor cell type | Lamb I.D. | Gest. length | Birth weight | Neo | FIX | Sex | Comments |
|---|---|---|---|---|---|---|---|---|
| 1 | PDFF5 | 7LL5 | 147d | 3.8kg | n/a | n/a | m | Unassisted birth |
| 2 twins | PDFF5 | 7LL6† 7LL7† | 150d 150d | 3.4kg 3.7kg | n/a | n/a | m m | Still birth, 1 fetus dead for 1 week or less. |
| 3 | PDFF2 pool | | <80d | | | | | Regressed |
| 4 | PDFF2 pool | 7LL8 | 155d | 7.6kg | yes | no | f | Assisted birth due to position of lamb |
| 5 | PDFF2 pool | 7LL9† | 161d | 6.3kg | yes | no | f | Induced, CS 52hrs later, died 90 min pp, meconium in lung |
| 6 | PDFF2 pool | 7LL12 | 155d | 8.7kg | yes | no | f | Induced, CS 52hrs later |
| 7 | PDFF2-12 | 7LL3 | 130d | | n/d | n/d | f | Spontaneous abortion |
| 8 twins | PDFF2-12 | 7LL10† 7LL11† | 132d 132d | 3.6kg 4.5kg | yes yes | yes yes | f f | Loss of fetal heart beat, induced, CS, stillbirth, 1 fetus abnormal |
| 9 | PDFF2-12 | 7LL14† | 148d | 3.6kg | yes | yes | f | Induced, CS 24hrs later, heart beat, no breathing |
| 10 twins | PDFF2-12 | 7LL15 7LL16† | 155d 155d | 4.6kg 3.0kg | yes yes | yes yes | f f | Induced, unassisted birth, 7LL16 euthanised at 14 d, heart defect |
| 11 | PDFF2-13 | 7LL13 | 155d | 5.5kg | yes | yes | f | Induced, unassisted birth |

†: Animals died or were euthanised for animal welfare reasons.
n/d: Not done
n/a: Not applicable
CS: Cesarian section
pp: post partum

All animals derived from PDFF cells exhibited a prolonged gestation (breed average =145 days) and with the exception of animals 7LL5-8, labour was induced artificially. Delayed parturition was almost certainly the cause of death of lamb 7LL9. Subsequent to this, all surrogate ewes were induced at day 153, and if necessary Cesarian section was carried out. Three of 11 pregnancies were twin pregnancies. In two instances of twin pregnancies (7LL6, 7LL7 and 7LL10, 7LL11) the death of one fetus in late pregnancy probably resulted in the death of the sibling.

The birth weight of nuclear transfer derived lambs whose gestation exceeded 145 days ranged from 3.0 to 8.7kg, with a mean value of 3.7kg for twin and 5.9kg for single pregnancies. This is within the normal range for Poll Dorset lambs in PPL's New Zealand derived flock (range 3 - 9kg for single pregnancies, mean weights 3.75kg for twins and 5.1kg for singles). It is not certain whether the apparently slightly higher mean birth weight is significant given the small number of nuclear transfer lambs and increased gestation time.

### 5.5. DNA analysis of nuclear transfer lambs

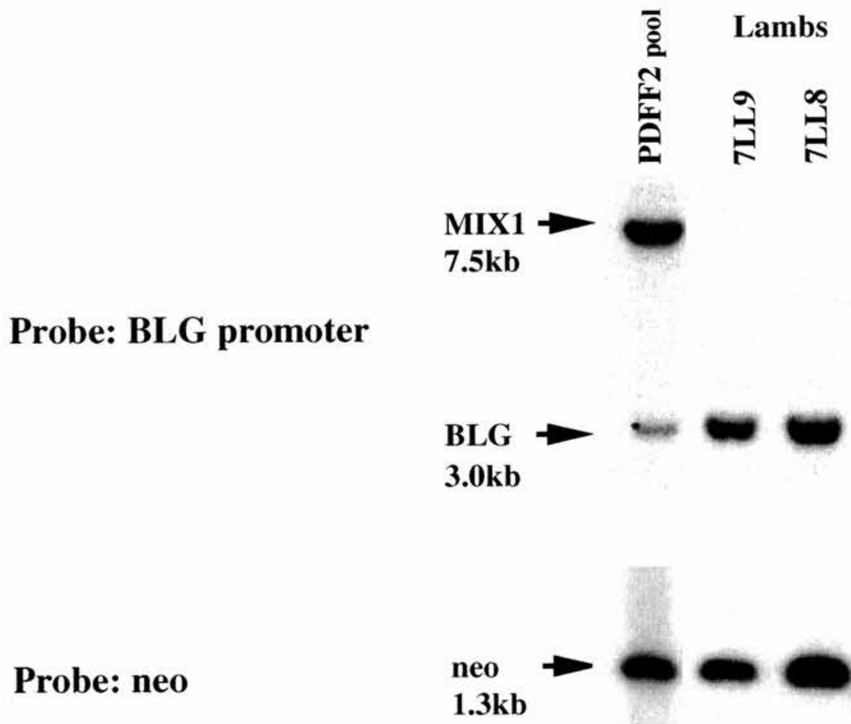
DNA was prepared from blood samples from live lambs, or tongue biopsies of dead animals and was analysed for the presence of the pMIX1 and PGKneo transgenes. The results are shown in Figure 5.4. A, B and C.

All fetuses and animals derived from the transfected PDFF2 cells were transgenic (Table 5.5). The three animals derived from the PDFF2 pool (7LL8, 9, 12) contained the selectable marker gene PGKneo (Figure 5.4 A), but lacked the FIX transgene (Figure 5.4 B and C). This result was not unexpected as the transfection was carried out with a 3 fold molar excess of PGK-neo, a consequence of the large size of the FIX transgene. Analysis of single cell clones showed ≈50% of clones contained both transgenes.

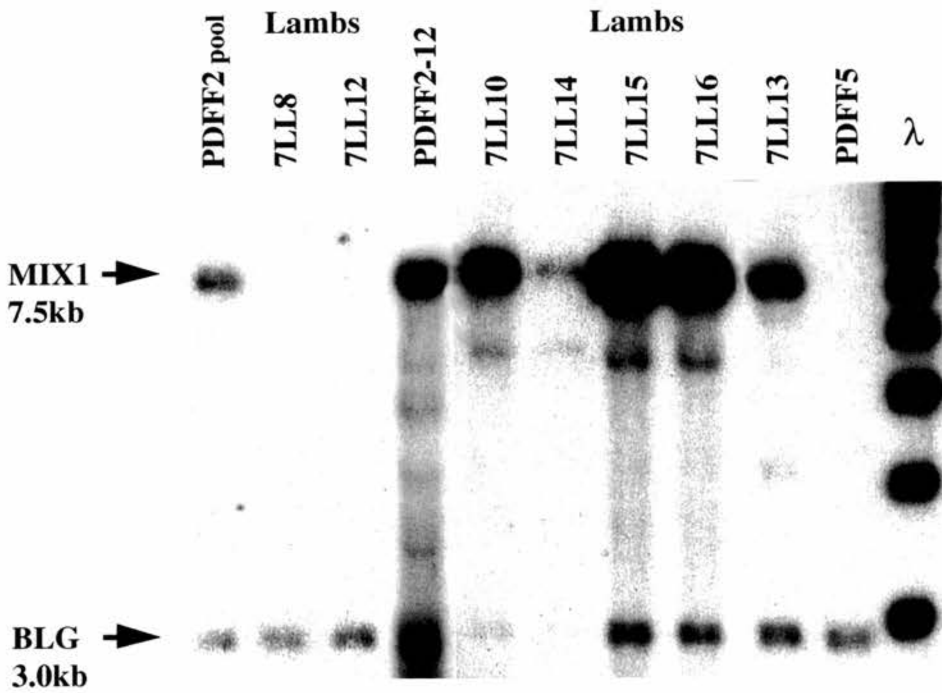
Southern blots hybridised with a 1.8kb probe derived from the BLG promoter showed that fetuses and lambs derived from the cell clones PDFF2-12 (7LL10, 14, 15, 16) and PDFF2-13 (7LL13) contained the FIX transgene (Figure 5.4 B, 7.5kb). The additional bands in animals derived from either PDFF2-12 (≈5.5kb) or PDFF2-13 (≈4.2kb) could indicate an incomplete copy of the transgene. Also visible is a ≈3kb band which represents the endogenous BLG gene.

**Figure 5.4: Southern analysis of nuclear transfer derived sheep**

**A.**




**B.**



C.

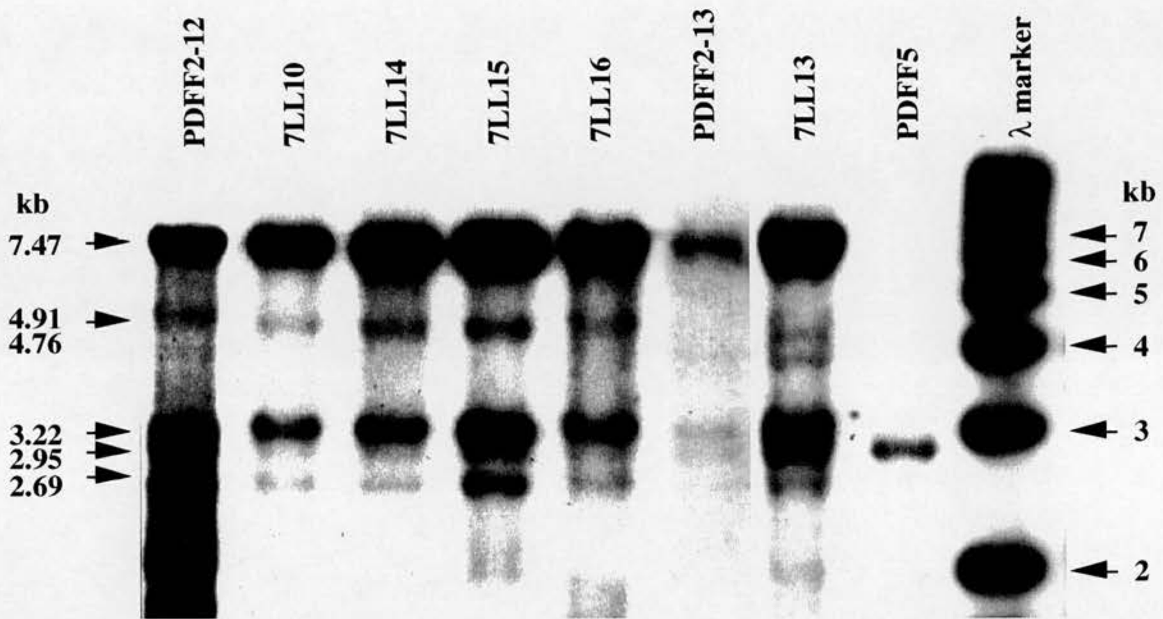



**Figure 5.4: Southern analysis of nuclear transfer derived sheep**

**A**. Southern analysis of the uncloned pool of cells (PDFF2 pool) and two lambs derived from them (7LL8, and 7LL9) assayed for the presence of pMIX1 and PGKneo transgenes by hybridisation with BLG promoter and neo probes.

**B**. Assay for the presence of the pMIX1 transgene in lambs derived from the pool (PDFF2 pool, lambs 7LL8, 7LL12) and the transfected clone (PDFF2-12, lambs 7LL10, 7LL14 to 7LL16. Lamb 7LL13 was derived from clone PDFF2-13. PDFF5 cells were non transfected. The positions of the transgenes and the endogenous BLG gene are indicated.

**C.** Southern analysis to determine the integrity of the pMIX1 transgene in lambs derived from the transfected clones (PDFF2-12, lambs 7LL10, 7LL14 to 7LL16 and PDFF2-13, lamb 7LL13) by hybridisation with BLG/FIX cDNA probe. Sizes of fragments containing the 8 coding exons are indicated (see also Table 5.7). DNA was digested with BamHI and EcoRI (for number and sizes of fragments please see Figure 5.5).

The integrity of the FIX transgene (pMIX1) was assessed after hybridisation with a FIX cDNA probe, which detected all eight coding exons (Figure 5.4 C). The intensity of the hybridisation signal is determined by the extend of homology, i.e. the size of the exonic sequence contained within the fragment (see Table 5.7). Figure 5.5 shows a schematic map of pMIX1, indicated are the locations of the eight exons and the BamHI and EcoRI restriction enzyme recognition sites used for the Southern analysis.

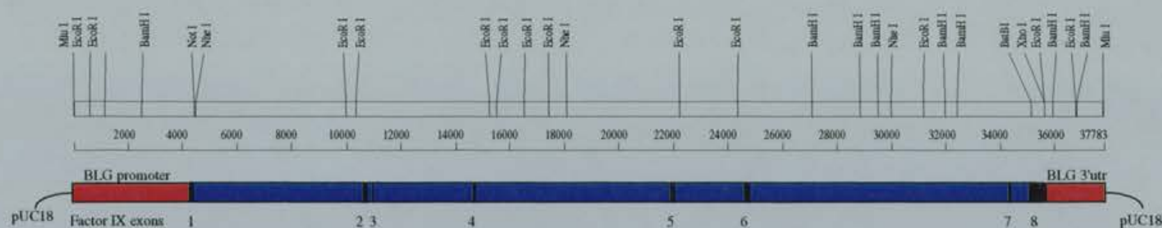



**Figure 5.5: Schematic map of pMIX1**

Schematic representation of construct pMIX1. pMIX1 comprises ~4.2kb of ovine ß-lactoglobulin (BLG) promoter and 5' untranslated sequence; ~31.4kb of the human Factor IX (FIX) gene from a NheI site 12bp upstream of the translational start site to an engineered XhoI site 12bp downstream of the translational stop signal; ~2.2kb of BLG 3' non-coding sequence, polyadenylation signal and 3' flanking sequence cloned into pUC18. Ovine BLG regions are indicated by red boxes, Factor IX exons are indicated by black bars. The pUC18 vector is indicated at either end of the construct.

**Table 5.7: pMIX1 fragment sizes after digestion with BamHI and EcoRI and detection with a BLG/FIX cDNA probe.**

| Fragment size (kb) | Gene region | Extent of homology to probe (kb) |
|---|---|---|
| 7.47 | Promoter and exon 1 | 1.9 |
| 4.91 | Exons 2, 3, 4 | 0.3 |
| 4.76 | Exon 5 | 0.13 |
| 3.22 | Exons 7, 8 | 0.45 |
| 2.69 | Exon 6 | 0.2 |
| | | |
| 2.95 | Endogenous BLG | 1.8 |

## 5.6. Recombinant Factor IX expression in nuclear transfer sheep.

Transgenic nuclear transfer derived sheep could not be bred in winter 1997/98 as they were still immature. rFIX expression was therefore analysed in milk obtained from induced lactation of 7LL13 and 7LL15 at 11 months of age. Twenty one days after application of lactogenic hormones, the two transgenic FIX animals were milked twice daily for a period of 2 weeks. This work was carried out by Tim King at the Large Animal Husbandry Unit, Roslin Institute.

7LL13 yielded 3-10ml per milking over a period of 12 days. Milk from 7LL15 was only obtained on day one (1-2ml). The concentration of rFIX in milk samples was estimated by ELISA using human plasma derived FIX as a standard. Fig. 5.6 shows the standard curve for human plasma derived FIX standard and table 5.8 shows the results for 7LL13 (milk from day 1-3) and 7LL15 (day 1 only). The amount of rFIX in milk from 7LL15 (5mg/ml) at day one of lactation was more then twice that of 7LL13 (2mg/ml), possibly reflecting the difference in transgene copy number.

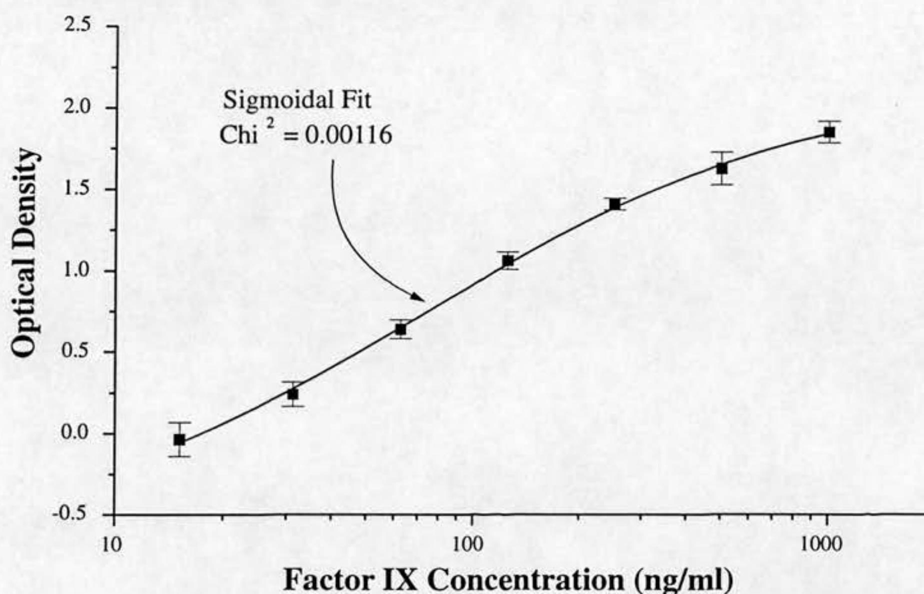



**Figure 5.6: ELISA: Standard curve**
The human FIX standard (ERL) was diluted in milk. Dilutions ranging from 1000 to 15.625μg/ml were assayed in triplicates using a Dynex microtiter plate reader. Results were analysed using ORIGIN computer program.

**Table 5.8: Concentration of rFIX in induced lactation milk**

| Sheep I.D. | Copy Nr. | Day of sample | Mean μg/ml |
|---|---|---|---|
| 7LL13 (Polly) | ≈5 | Day 1 | 2442.93 |
| 7LL13 (Polly) | ≈5 | Day 2 | 1463.58 |
| 7LL13 (Polly) | ≈5 | Day 3 | 2123.52 |
| 7LL15 (Molly) | >10 | Day 1 | 5043.72 |

The high concentration of rFIX obtained by ELISA assay was confirmed by Western blot analysis (Fig. 5.7). and comparison with purified human standard. It is noteworthy that expression levels obtained on the first day of induced lactation can be an overestimate and generally drop slightly during the first week of lactation.

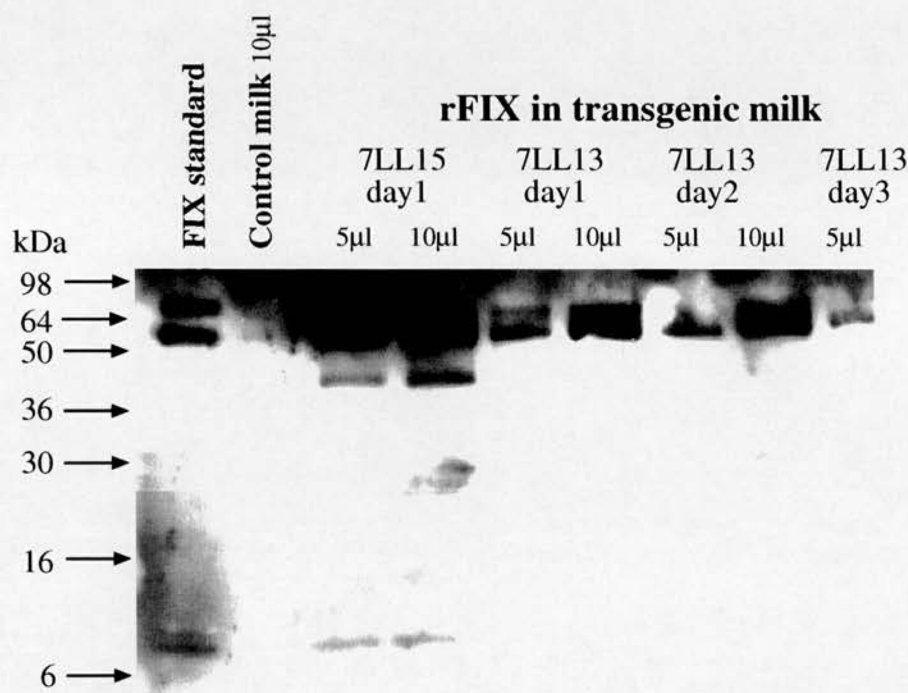



**Figure 5.7: Western analysis of induced lactation milk from FIX transgenic sheep derived by nuclear transfer**
Samples were analysed on a reducing SDS-PAGE gel (18% Tris/glycine gel). Milk samples had been diluted 1:500, amounts loaded are indicated above the lanes. Amount of human FIX standard (ERL) loaded: 45ng.

Figure 5.7 shows that rFIX migrates slightly faster then the human standard, possibly due to a difference in glycosylation. The same migration pattern was also observed in transgenic mice and in transgenic sheep obtained by microinjection of the FIX cDNA construct cFIXΔ3'SA (PPL, unpublished data). FIX is secreted as a single chain protein of about 56kDa, however it migrates slower under SDS-PAGE gel electrophoresis than would be predicted on the basis of its molecular weight because it has a high net negative charge. Both the human standard and the recombinant protein show the presence of lower molecular species which represent activated FIX molecules.

Although only two nuclear transfer derived MIX1 transgenic animals were obtained, both expressed high levels of rFIX protein, compared to only two out of nine transgenic sheep obtained by microinjection of the FIX cDNA construct cFIXΔ3'SA (maximum expression level 1.2mg/ml ; PPL, unpublished data).

## 5.7. Production of transgenic animals by nuclear transfer compared with pronuclear microinjection

It has long been recognised that the gestation of large numbers of non transgenic embryos represents a major source of inefficiency in large animal transgenesis (Bowen *et al.*, 1994). Cell mediated transgenesis has the advantage that transgene analysis is carried out *in vitro*. All animals born are transgenic. The number of animals required for the production of a single transgenic founder animal should therefore be reduced.

Since 1989, PPL has carried out a number of studies to generate transgenic sheep by pronuclear microinjection. Data collected from this work provided a realistic measure of the overall efficiency of pronuclear microinjection and allowed comparison with cell mediated transgenesis (see Table 5.9).

**Table 5.9: Comparison of transgenesis by nuclear transfer and pronuclear microinjection.**

| | Pronuclear microinjection 1989-1996 | Nuclear transfer of PDFF2 transfectants |
|---|---|---|
| Oocyte donors | 982 | 68 |
| Intermediate recipients | Not applicable | 14 |
| Final recipients | 1895 | 22 |
| Total no. of sheep used | 2877 | 104 |
| Established pregnancies in final recipients | 912 (48%) | 9 (41%) |
| Total no. of lambs born | 1286 | 6 |
| Total no. of viable transgenic lambs born[a] | 56 | 5 |
| Percentage of offspring transgenic | 4.35% | 100% |
| No. of sheep required for production of 1 transgenic lamb | 51.4 | 20.8 |

a: Viable lambs were defined as those alive at 1 week of age

Table 5.9 shows that a total of 51.4 animals were necessary to produce one transgenic lamb by pronuclear microinjection compared to 20.8 animals using nuclear transfer, a factor of approximately 2.5. The most significant difference being in the number of final recipients used. The embryo transfer efficiency from both methods is similar.

Because nuclear transfer allows the sex of transgenic animals to be predetermined, cell mediated transgenesis offers a further two fold increase in efficiency over pronuclear microinjection when the sex of the transgenic founder animal is critical. The primary interest of PPL is the expression of human proteins in milk, which requires transgene analysis in female animals.

## 5.8. Conclusions

Cell mediated transgenesis is based on successful *in vitro* manipulation of cells without diminishing their developmental potential after nuclear transplantation. Therefore a careful assessment of all cell types previously used in nuclear transfer was carried out. The choice of fetal fibroblasts for the production of transgenic animals was based on the following criteria:

1. Large numbers of cells were available at early passage for genetic manipulation.
2. Cells were readily transfectable.
3. Cells remained euploid over prolonged time in culture.
4. Acceptable nuclear transfer efficiency.

Because no ovine fetal fibroblast cells were available from animals with the appropriate pathogen free status, it was essential to establish new cultures. One of these (PDFF2) was then co-transfected with PGK-neo and the human FIX construct pMIX1. Two cloned transfectants and a population of neomycin resistant cells were used as donors for nuclear transfer to enucleated oocytes. Six transgenic animals were live born. Three produced from cloned cells contained the FIX and neo transgenes, whereas three produced from the uncloned population contained the marker gene only. Animals transgenic for the FIX transgene produced high concentrations of the recombinant protein in their milk (5mg/ml), exceeding any previously reported expression data.

Further characterisation of the rFIX protein will be carried out on natural lactation milk and will include an *in vitro* clotting assay to determine bioactivity. Other vitamin K dependent proteins have been successfully expressed in the mammary gland of transgenic animals,

including mice (FVII and Protein C; PPL unpublished data), sheep (FVII and Protein C PPL unpublished data), and pigs (Protein C, Velander *et al.*, 1992). High concentration of recombinant protein can result in incomplete γ-carboxylation and reduced bioactivity. Recombinant FIX expressed at concentrations of up to 5g/l may exhaust the γ-carboxylation capacity of the mammary gland, therefore the rFIX protein expressed in Polly and Molly might not be fully bioactive. Natural lactation milk is expected in late spring 1999.

The efficiency of nuclear transfer, expressed as the number of liveborn lambs obtained per 100 reconstructed embryos, varied from 0.89% (PDFF2-13) to 2.25% (PDFF2-12) for transfected cell clones. This rate is similar to that for non-manipulated embryonic cells (1.70%, SEC1) and fetal fibroblasts (1.35%, BLWF1) (see Chapter 4). Although the small sample size does not allow firm conclusions to be drawn about the relative efficiency of each treatment, these data provide evidence that fetal cells which have undergone transfection, drug selection and single cell cloning are capable of supporting nuclear transfer.

The number of live lambs was reduced by the high mortality rate (46%) observed, which was almost certainly exacerbated by two instances of twin pregnancies in which the death of one lamb in late gestation led to loss of both. The mortality rate for non twin pregnancies was 28.6%, higher than that occurring after normal breeding (~8%), but similar to that observed after nuclear transfer from embryo blastomeres (5-40%) (Kruip and den Daas, 1997). The data do not therefore suggest any correlation between lamb mortality and extended culture or genetic manipulation of the donor cells.

These results represent the first example of cell mediated transgenesis in a mammalian species other than mouse, and provide an unequivocal demonstration that differentiated cells can undergo genetic manipulation in culture and produce viable animals by nuclear transfer. Furthermore, comparison of the present data and that obtained from previous microinjection studies at PPL indicates that production of a single transgenic founder by cell mediated transgenesis requires approximately 2.5 fold fewer animals than pronuclear microinjection, largely by reducing the size of the recipient flock.

# Chapter 6

## Cloning and targeting of the HPRT gene in ovine fetal fibroblasts

### 6.1. Background

The success of gene targeting in mice relies on the high frequency of homologous recombination between exogenous DNA and cognate chromosomal sequences in ES cells. Current literature would indicate that the ratio of homologous to non-homologous integration events in most somatic cells is lower than in ES cells. However, compared to ES cells, far less work has been done using somatic cells lines and even less using primary cells. It was therefore essential to establish the frequency of homologous recombination in primary fetal fibroblasts which were nuclear transfer competent. Similar to early experiments which established targeting in mouse ES cells (Thomas and Capecchi, 1987), a model gene targeting experiment was designed to inactivate the selectable endogenous HPRT gene. The aim was not to produce HPRT$^-$ sheep, because of possible deleterious effects. HPRT deficiency in human causes Lesch-Nyhan syndrome, although HPRT negative mice show no such symptoms (Kuehn *et al.*, 1987; Williamson, Hooper and Melton, 1992).

### 6.2. Cloning of ovine HPRT sequences

The strategy for ovine HPRT targeting was to delete important exonic sequences and replace these with a selectable marker gene. Details of the design of the targeting vector are shown in Fig. 6.1. Although the ovine HPRT gene has been localised to the X-chromosome (Echard *et al.*, 1994), neither the gene nor cDNA had been cloned. The first step therefore was to identify and clone portions of the ovine HPRT gene from isogenic DNA derived from the male fibroblast cells PDFF5. This would provide 5' and 3' homologous regions for the production of a replacement targeting vector.

Cloning of the HPRT gene could be achieved by generating a library of genomic fragments in phage λ, then isolating ovine HPRT sequences using mouse or human cDNA probes. However, it was considered that this approach could be complicated by the presence of HPRT pseudogenes which have been demonstrated in mouse and human. An alternative was long range PCR (Randolph *et al.*, 1996) using primers based on sequences conserved between HPRT genes from different species. This strategy is supported by the fact that the gene structure of the human and mouse HPRT genes are almost identical and that the coding sequence from mouse (Konecki *et al.*, 1982), rat (Jansen *et al.*, 1991), pig (Mansfield, 1996), hamster (Konecki *et al.*, 1982), and human (Jolly *et al.*, 1983) are known to be highly conserved.

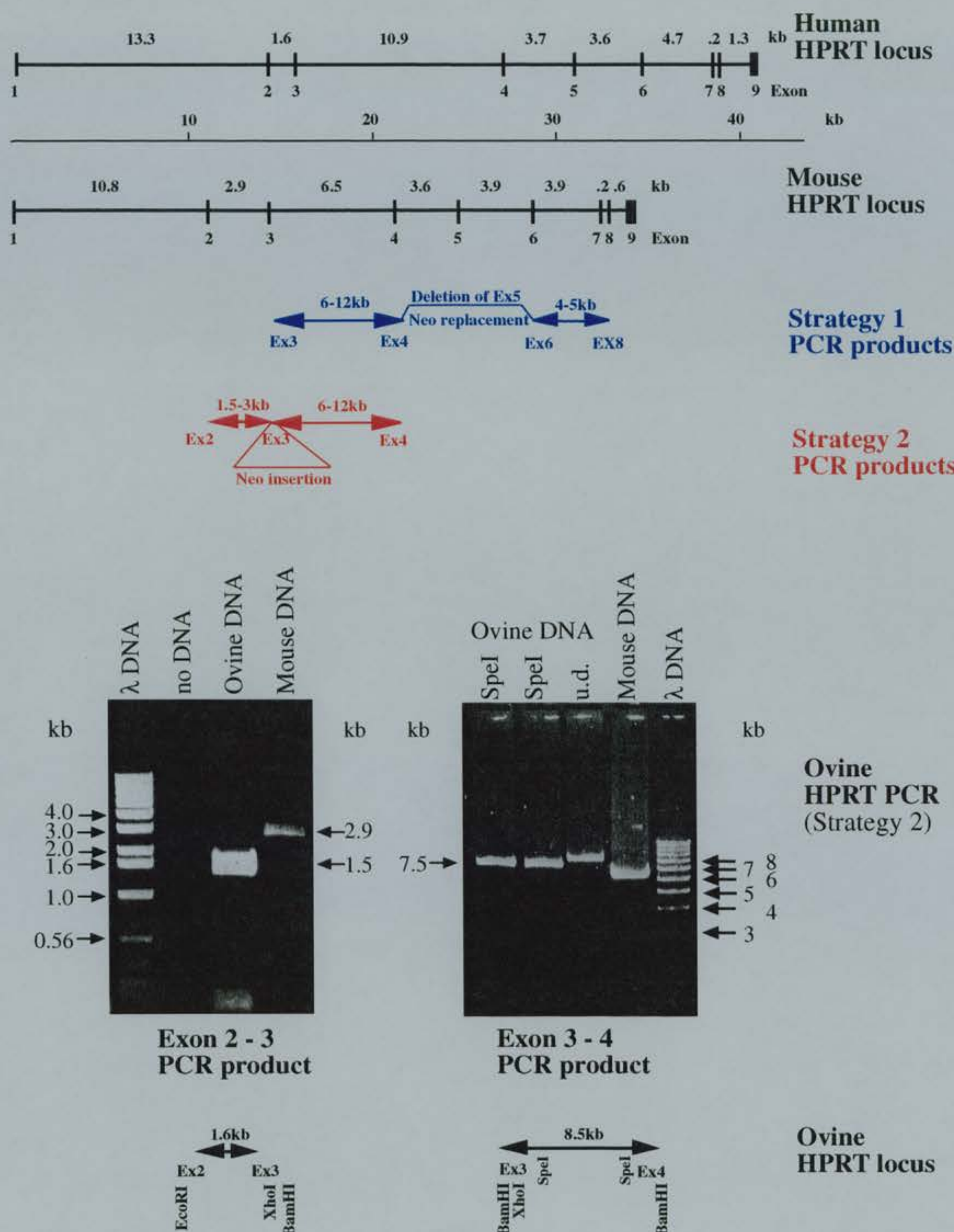



**Figure 6.1: HPRT cloning strategy and PCR amplification of ovine sequences**

Schematic diagram of the human and mouse HPRT gene, indicated are the positions of exons and sizes of introns. The two cloning strategies for the ovine HPRT gene are outlined in correlation to the mouse HPRT gene. Also shown are the PCR amplification products obtained from either mouse or ovine DNA and analysed on 1% agarose gel. Primer sequences are given in Material and Methods.

u.d., undigested DNA; SpeI, DNA digested with SpeI.

The original PCR and cloning strategy is shown in Fig. 6.1 (Strategy 1). The two ovine PCR products were: an estimated 6-12kb fragment spanning from exon 3 to exon 4, and a 4-5kb fragment spanning from exon 6 to exon 8. It was intended that these two fragments should provide the 5' and 3' homologous regions for a targeting vector. The function of the targeted gene would be interrupted by the deletion of the 5th and parts of the 4th and 6th exons and their replacement with a selectable neo marker.

The sequence of the primer pairs used for amplification of ovine sequences was deduced from regions of the HPRT coding sequence conserved between human, rat, mouse and hamster. Long range PCR was performed on DNA from mouse embryos, and DNA from the ovine fetus from which the PDFF5 culture was established. Using mouse DNA as a template, the 6.5kb exon 3 to 4 fragment and the 4.3kb exon 6 to 8 fragment both amplified efficiently. Using sheep DNA exon 3/4 primers repeatedly amplified a weak 8.5kb fragment (Figure 6.1, bottom right). Exon 6/8 primers failed to amplify ovine HPRT sequences. A new set of primers was therefore designed to amplify a region spanning from exon 2 to exon 3, which would then be used as the 5' homologous region of the targeting vector while exon 3 to 4 sequences would provide 3' homology (Figure 6.1, Strategy 2). Figure 6.1 (bottom left) shows successful amplification of a $\approx$1.6kb fragment in sheep and 2.9kb in control mouse DNA comprising of exon 2 to 3 sequences.

**Cloning exon 2 to 3:** The PCR primers contained EcoRI and BamHI restriction sites. The amplified 1.6kb PCR fragment contained an additional internal EcoRI site (see Fig. 6.4). After partial digestion with EcoRI and complete digestion with BamHI it was cloned into the EcoRI and BamHI sites of Bluescript. Sequence analysis confirmed that it was HPRT (Fig. 6.3 and Table 6.1).

**Cloning exon 3 to 4:** The PCR primers contained BamHI restriction sites. The attempt to clone the 8.5kb PCR fragment either as a BamHI fragment or directly into the PCR cloning vector pGEM-T failed repeatedly. Restriction enzyme mapping of the PCR product indicated that this fragment contained two internal SpeI sites releasing a 7.5kb fragment which was cloned into the SpeI site of Bluescript (see Fig. 6.2). Orientation of the SpeI insert was determined by restriction enzyme analysis and DNA sequencing.

Although the subcloned fragment contained no exon, sequence analysis showed a stretch of homology (91.8%) with sequences from the 3rd intron of the human HPRT

gene located about 900bp 5' of exon 4. Search of the entire EMBL data base produced only a single match, again intron 3 of the human HPRT gene. This ruled out the possibility that sequence homology was due to a repetitive element. To confirm this result, PCR primer pairs were designed to amplify sequences spanning from exon 3 into intron 3 and from intron 3 into exon 4 (Fig. 6.2). Sequence analysis of the subcloned PCR fragments, (designated as Exon 3 and Exon 4), confirmed that sequences were derived from exon 3 and 4 of the HPRT gene (Fig. 6.3 and Table 6.1).

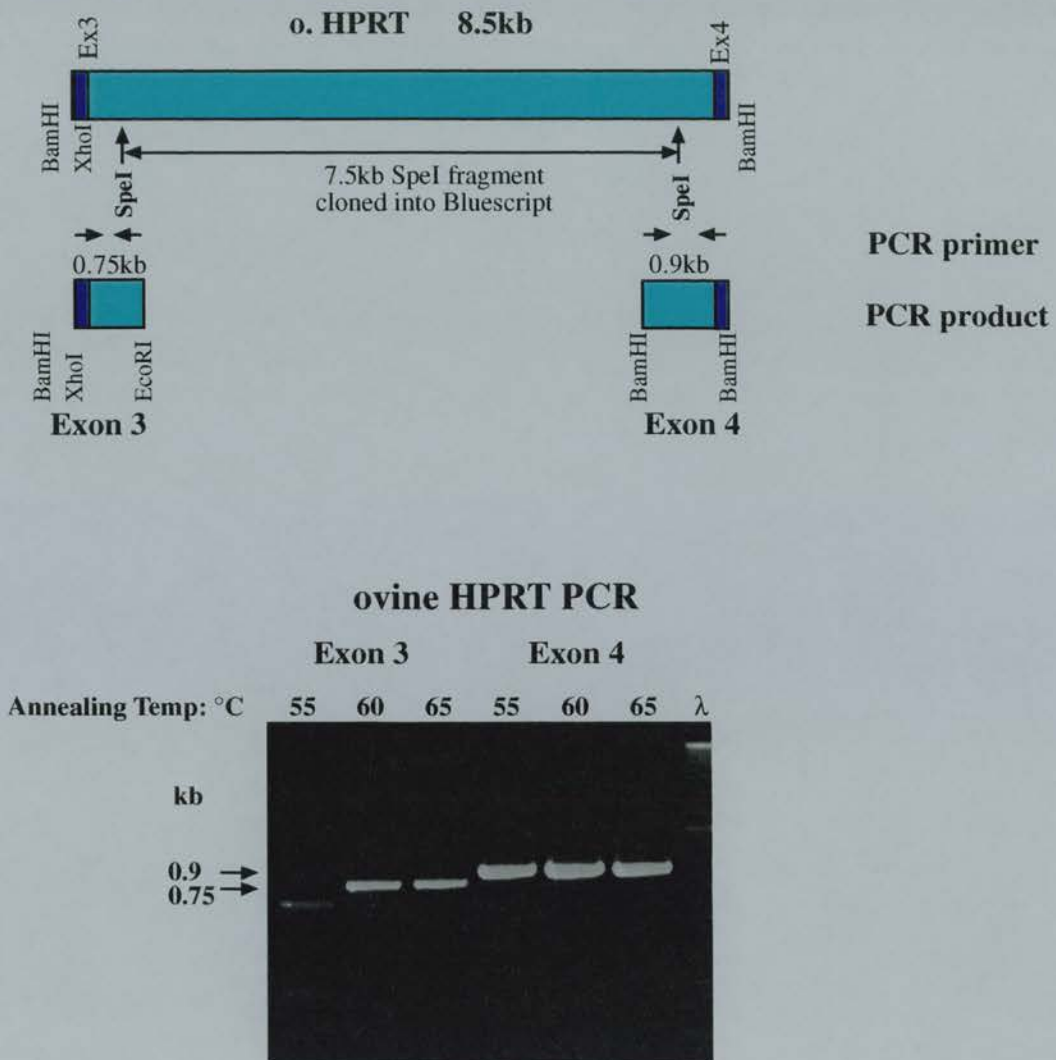



**Figure 6.2: Ovine HPRT exon 3 to 4**
Shown is the amplified PCR product spanning from exon 3 to exon 4 of the ovine HPRT gene. Because efforts to clone the 8.5kb BamHI fragment failed, the 7.5kb SpeI fragment was subcloned into the plasmid vector Bluescript and partial DNA sequence was obtained using "universal" and "reverse" primers. From this sequence PCR primers were deduced for amplification of the "exon 3" and "exon 4" fragments. Their analysis on a 1% agarose gel is also shown. Both fragments were subcloned into the plasmid vector Bluescript and partial DNA sequence was obtained.

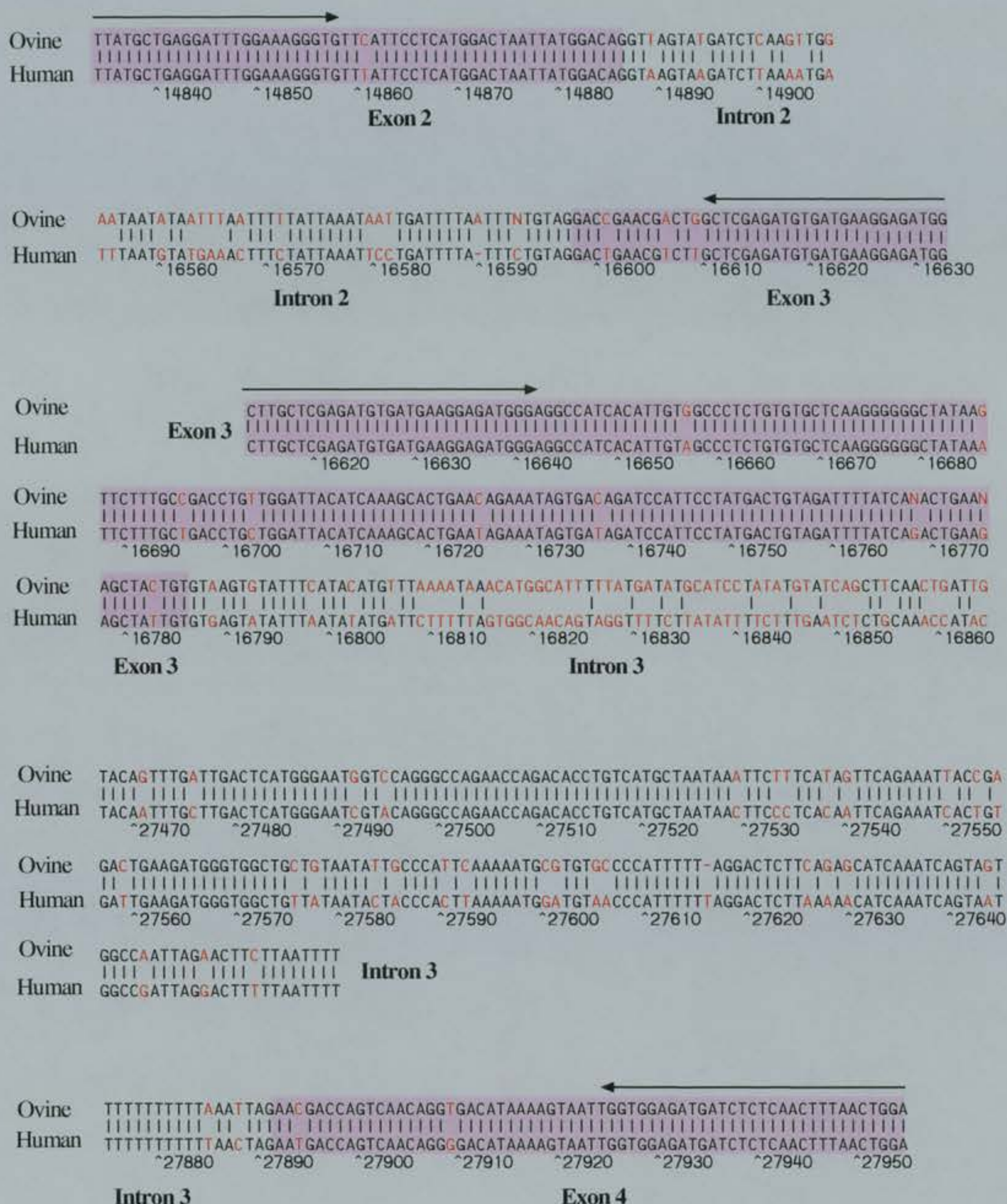



**Figure 6.3: Sequence comparison of ovine and human HPRT**
Comparison of ovine and human HPRT sequences was carried out using the DNASTAR Align program. Bases in red indicate mismatches, shadowed areas the exon sequences, and the arrows indicate positions of primers used for PCR amplification of the ovine HPRT gene.

**Table 6.1: Sequence comparison of ovine and human HPRT**

| Region of homology to human HPRT | | Homology % | Number of gaps |
|---|---|---|---|
| Location | Size | | |
| 14822-14888 | 66 | 92.5 | 0 |
| 16575-16640 | 65 | 86.8 | 1 |
| 16613-16788 | 175 | 94.9 | 0 |
| 27467-27670 | 203 | 84.4 | 1 |
| 27910-27954 | 44 | 100 | 0 |

Position of the relevant human HPRT exons are:

Exon 2: 14780-14886

Exon 3: 16603-16786

Exon 4: 27892-27957

## 6.3. Construction of ovine HPRT targeting vector

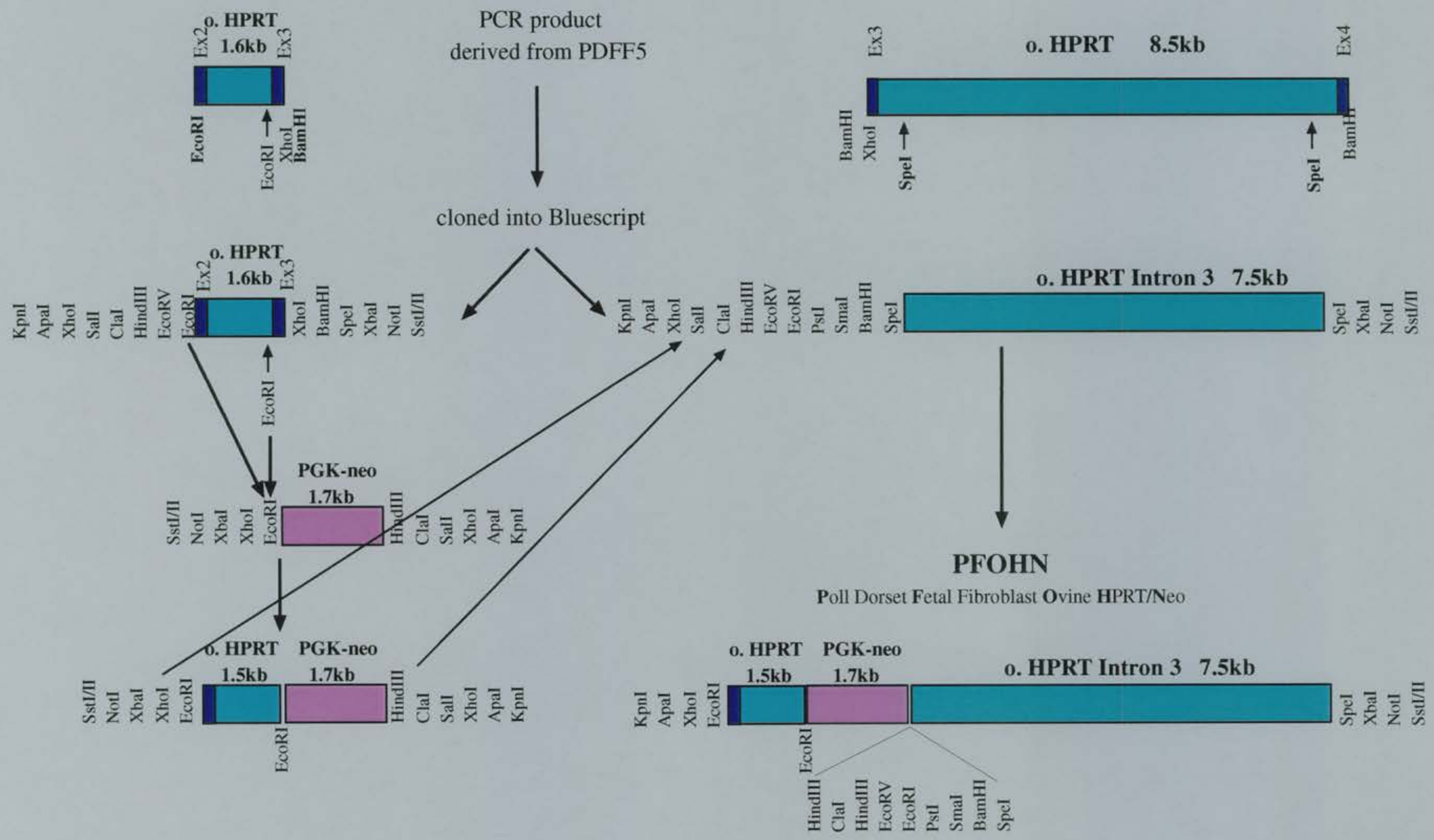
The cloning strategy is outlined in Figure 6.4, the individual cloning steps are outlined below.

**1.** The 1.5kb EcoRI fragment containing parts of the ovine HPRT exon 2 and intron 2 was cloned into the EcoRI site of PGK-neo (Bluescript). Orientation of the insert was determined by restriction enzyme analysis.

**2.** The 3.2kb XhoI/ClaI fragment containing the ovine HPRT exon 2/intron 2 sequence and PGK-neo gene was then cloned into the XhoI/ClaI site of o.HPRTint.3 giving rise to **PFOHN** (**P**oll Dorset **f**ibroblast, **o**vine **H**PRT, **n**eo). This targeting vector was designed to inactivate the ovine HPRT gene by deleting exon 3 and replacing it with PGK-neo.

**Figure 6.4 (next page):** Schematic diagram of the construction of the ovine HPRT targeting vector PFOHN. Restriction sites indicated are those used for cloning or present in the polylinker of the plasmid vectors. Ovine HPRT sequences are marked in blue: exon sequences in dark blue; PGKneo in pink.

Figure 6.4: Cloning strategy for ovine HPRT targeting vector



## 6.4. Targeting of the endogenous HPRT gene in PDFF5 cells.

The male PDFF5 cells have been shown to be nuclear transfer competent (Chapter 5). The frequency of homologous recombination in these primary fetal fibroblasts was to be determined by targeted inactivation of the HPRT gene. Cells transfected with the PFOHN targeting vector were first selected for PGK-neo expression for 5-7 days to allow elimination of non transfected cells and for the HPRT protein in correctly targeted cells to decay. Loss of HPRT was then determined by 6-TG selection.

Because little is known about the influence the DNA delivery method has on gene targeting efficiency both electroporation and LipofectAMINE were used. For no clearly defined reason, most gene targeting experiments in mouse ES cells employ electroporation as a method of DNA transfer, but LipofectAMINE gives higher transfection efficiencies in somatic cells. In a preliminary experiment the optimal transfection conditions for both methods were determined.

A range of electroporation parameters for transfection of PDFF5 cells, similar to those applied for determination of electroporation efficiency of TNT4 cells (Chapter 3), were examined by transient transfections with the SV40 LacZ reporter gene. It was established that PDFF5 cells are readily transfectable ( 5%) at 350V, 500μF, 150μg DNA/$10^7$ cells.

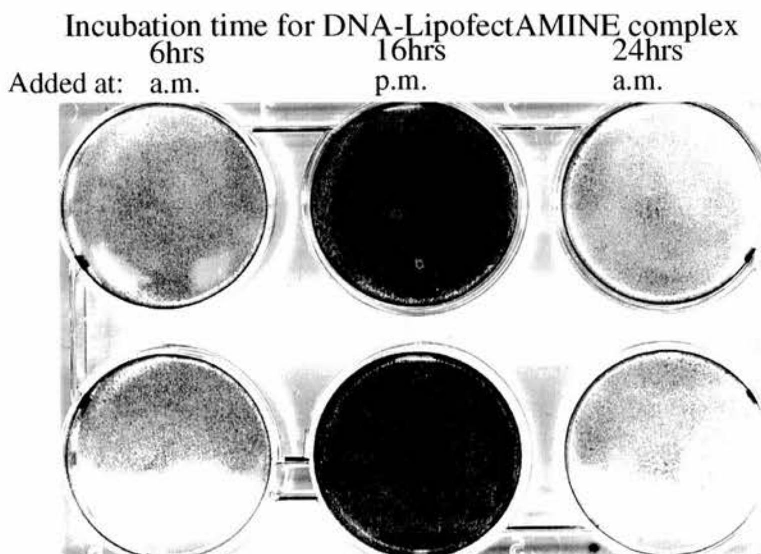



**Figure 6.5: β-galactosidase staining of PDFF5 transfectants**
$2x10^5$ cells per well were transfected with 1.5μg of CMV-LacZ using 10μl LipofectAMINE and incubated for the times indicated above the wells. After 48hrs cells were stained for β-galactosidase activity.

LipofectAMINE transfection had so far been the most efficient method to introduce DNA into both embryonic and fetal ovine cells. Under optimised conditions >75% of PDFF5 cells could be transfected. This is visualised in Figure 6.5 (middle panel) in a transient experiment using the CMV-LacZ reporter gene. During this experiment the exposure time of cells to the DNA-LipofectAMINE complex varied from 6 to 24 hrs. Transfection efficiency was measured by the number of stained cells. The 16hr exposure gave by far the greatest number of positive cells (>75%). It is not assumed that the result is purely a function of exposure time, as repeat experiments gave lower transfection rates. It is more likely that the cell-cycle stage was optimal for efficient uptake of DNA. A more systematic analysis is part of future experiments.

Targeting was attempted both by introduction of DNA via electroporation and by lipofection. $2x10^7$ PDFF5 cells were electroporated with 150μg of linearised PFOHN DNA. After selection in 0.6 mg/ml of G418 for 5 to 7 days selection was switched to 35μg/ml 6-TG for 2 weeks. No 6-TG resistant colonies were obtained from a total of 1000 transfected $neo^r$ clones. The delay of 5-7 days prior to 6-TG selection was to allown degradation of the ovine HPRT protein, based on the assumption that its half life was similar to the mouse HPRT protein.

Using LipofectAMINE no 6-TG resistant colonies were obtained from a total of 3500 transfected $neo^r$ clones.

### 6.5. Conclusions

4500 $neo^r$ transfectants were analysed for inactivation of the endogenous HPRT gene using 6-TG selection. No resistant colonies were obtained. These results may indicate that homologous recombination events in ovine primary fibroblasts, in particular in PDFF5 cells, are rare and hence gene targeting events, especially for non-selectable loci might be difficult to identify. The ratio of homologous to non-homologous integration for other somatic cell types range from 1 in 50 to 1 in 10,000 (see Table 1, chapter 1). It is therefore possible that the number of transfectants analysed was not sufficient to detect a targeting event, and further experiments are required.

Other formal possibilities for the low targeting efficiency are:

1. By far the largest number of colonies screened were obtained by lipofection, and it is unknown what influence the DNA delivery method has on targeting efficiency.
2. The regions of homology present in the targeting vector were obtained by PCR amplification and could therefore contain single base differences to the target locus, thus reducing targeting efficiency.

3. Different loci have markedly different targeting efficiencies and it is therefore possible that homologous recombination frequency at the ovine HPRT locus is low.

Most significant though were results obtained from David Melton's group (ICMB, Edinburgh University), which questioned the suitability of HPRT as a model for gene targeting in sheep. His aim was to isolate spontaneous HPRT mutants from the ovine PDFF5 cells. $1.09 \times 10^8$ cells were plated in 6-TG and no colonies were obtained. This was a unique result, as all previously tested cell types showed spontaneous HPRT deficiency in about 1 in $10^6$ cells (David Melton, personal communication). His findings indicated uncertainty regarding the purine metabolism/HPRT pathway in sheep. As 6-TG has never been used for selection of ovine cells it was also conceivable that it has a non specific toxic effect in sheep fibroblast. Until these questions are resolved, targeting of the HPRT locus was discontinued.

Ongoing and future experiments include production of an isogenic λ library, restoring activity of a mutated selectable marker gene (Gfp-neo fusion gene), which was stably integrated into ovine fetal fibroblast cells and targeting of endogenous genes including knockout of ovine PrP and transgene placement into milk gene loci.

# Chapter 7
## Discussion

Work presented here showed that nuclear transfer animals can be derived from a number of differentiated cell types, including embryonic, fetal and adult cells and that some, but not all of these cells can be employed for cell mediated transgenesis.

The lamb 6LL3 (Dolly) is the first mammal to be derived from an adult cell. Not only did it prove that chromosomal changes occurring during differentiation are reversible, it also offers a unique possibility to study the possible persistence of epigenetic changes, in particular ageing related changes such as oxidative damage and telomere shortening. Analysis of telomere length has recently been carried out (Shiels *et al.*, 1999).

As Dolly was the only example of a cloned animal derived from an adult cell, questions were raised both about the significance and veracity of the result. That these doubts were unfounded, was proven both by extensive microsatellite (Ashworth *et al.*, 1998) and DNA fingerprint analysis (Signer *et al.*, 1998). More significantly a report by Wakayama *et al.* (1998) has now shown, that fertile mice can be obtained by nuclear transplantation using cumulus donor nuclei derived from adult animals. The importance of this report also lies in the improvement of the nuclear transfer technique, overcoming for the first time the difficulties associated with cloning in the mouse, for example the fragility of the oocyte. Mice -having a short generation time- provide an ideal model system to investigate factors influencing nuclear transfer efficiency. The experiments in mice and sheep have now also been repeated in the cow, where cloned calves were obtained from oviduct or cumulus cells (Kato *et al.*, 1998), from granulosa cells (Wells *et al.*, 1999), from ear cells (Vignon *et al.*, 1999), and mammary gland cells (Zakhartchenko *et al.*, 1999). Furthermore birth of nuclear transfer calves is expected for April 1999 derived from mammary cells present in early bovine milk (Nature Biotechnology 1998, Business and Regulatory News Brief).

Nuclear transfer technology is clearly still in the early stages of development and several problems remain to be addressed. In particular the incidence of perinatal mortality and the lack of spontaneous parturition (Chapter 5; Wells *et al.*, 1997; Wells, Misica and Tervit, 1998; Kato *et al.*, 1998). Many types of manipulation of preimplantation embryos other than nuclear transfer have been reported to increase fetal morbidity and mortality e.g., *in vitro* culture, IVM/IVF, asynchronous embryo transfer and progesterone treatment of the mother (Kruip and en Dass, 1997; Walker,

Hartwich and Seamark, 1996). An increased understanding of the interaction between the transplanted nucleus and the host cytoplasm, the relationship between the early embryo and the maternal environment and improved culture systems will increase the success of embryo production and manipulation *in vitro*. Factors already known to affect late fetal development include the presence of serum in embryo culture medium (Kruip, and en Dass, 1997; Walker, Hartwich and Seamark, 1996), and imprinting of the early embryonic genome (Reik *et al.*, 1993; Jaenisch, 1997; Römer *et al.*, 1997).

This report is also the first example of cell mediated transgenesis in a mammalian species other than mouse, and provides an unequivocal demonstration that differentiated cells can undergo genetic manipulation in culture and produce viable animals following nuclear transfer. This work has now been repeated by Cibelli *et al.* (1998), producing 3 cloned transgenic calves from non-quiescent fetal fibroblasts, while the work reported here and by others (Wakayama *et al.*, 1998; Kato *et al.*, 1998; Wells *et al.*, 1999; Vignon *et al.*, 1999; Zakhartchenko *et al.*, 1999) employed nuclei in $G_0$. Although first indications confirm that nuclei at the $G_0$ stage produce nuclear transfer embryos that are more developmentaly competent (Galat *et al.*, 1999; Hill *et al.*, 1999), further experiments are required to determine the importance of quiescence to the success of nuclear transfer.

Although still in its infancy, the use of somatic cell donors for nuclear transfer in livestock offers many advantages over pronuclear microinjection as a means of generating transgenic animals. Perhaps the most significant of these is a large reduction in the number of experimental animals required, which is desirable for both ethical and commercial reasons.

It has long been recognised that the gestation of large numbers of non transgenic embryos represents a major source of inefficiency in large animal transgenesis (Wall, 1992). Several schemes have been devised to address this problem by identifying transgenic embryos before embryo transfer, either by detection of the transgene in embryo biopsies by PCR (Bowen *et al.*, 1994), or by coexpression of a marker gene (Takada *et al.*, 1997; Thompson *et al.*, 1995). However these methods, with the possible exception of that of Takada *et al.* (1997), are restricted by the persistence of unintegrated DNA during the short time embryos can be cultured *in vitro* before embryo transfer. Cell mediated transgenesis circumvents this problem altogether. Cells transfected *in vitro* can be analysed extensively in the laboratory before any effort is devoted to large animals. This will be especially useful when producing transgenic

animals with very large transgenes such as YACs, where microinjection often fails to produce animals with a complete transgene.

Delayed integration of microinjected DNA into the embryo genome often results in mosaic founder animals. The reduced rate of transgene transmission resulting from germ line mosaicism can hinder or prevent the establishment of transgenic lines from potentially valuable founder animals. In contrast, animals produced by nuclear transfer from an *in vitro* manipulated donor cell are entirely transgenic.

Because nuclear transfer allows the sex of transgenic animals to be predetermined, cell mediated transgenesis offers a further two fold increase in efficiency over pronuclear microinjection when the sex of the transgenic founder animal is critical, for example if the primary interest is the expression of human proteins in milk, which requires transgene analysis in female animals. However expansion of a flock is far easier from males. These conflicting requirements result in a delay in flock expansion where the founder is female, and breeding of animals in which transgene expression is unknown where the founder is male. Transgene expression *in vitro*, in nuclear transfer competent cells, might even allow prediction of transgene expression *in vivo* and remove a further uncertainty in transgenic production.

In principle, cell mediated transgenesis allows the process of founder analysis and flock expansion to be significantly streamlined. The founder generation can be entirely female. Sheep carrying different random integrations of the transgene can be produced by nuclear transfer from independently transfected cell clones and milk analysed at an early stage. Once a suitable clone has been identified, the corresponding stock of cells can be used to generate an "instant flock" by further nuclear transfer. Animals within such a flock could be superior to those produced by conventional breeding as a source of proteins for human therapy, because genetic identity would contribute to the consistency of the medicinal product. Although only two animals were generated from two independent clones, these serve to illustrate the scheme. Animals 7LL13 and 7LL15 were induced to lactate at ~11 months of age, at which time the FIX levels in milk were determined. Both animals expressed rFIX at levels up to 100 fold higher than any previously reported (Yule *et al.*, 1995; Kurachi *et al.*, 1995) with a total amount of recombinant protein of up to 5g/l. It is presumed that this result is a consequence of using a large genomic FIX construct, while all previous investigators employed cDNA based vectors.

It is anticipated that cell mediated transgenesis will be widely applicable. The procedures of transfection, drug selection and growth from single cell clones are essentially the same as those required for gene targeting. The results presented here, coupled with previous demonstrations of homologous recombination in several somatic cell types (please see Chapter 1, Table 1), now provide the first realistic prospect of targeted genetic manipulation in a livestock species. The power of gene targeting has been amply demonstrated in mice. Extension of this technique to large animals will open a huge range of new applications and research possibilities.

# Chapter 8
## Methods

### 8.1. Nucleic acid methods

### 8.1.1. Isolation of DNA

#### 8.1.1.1. From blood

DNA was extracted using the Nucleon biosciences kit as recommended by the supplier.

1. 4.5ml of 10mM Tris pH 8, 0.32M sucrose, 5mM $MgCl_2$, 1% Triton X-100 was added to 1.5ml of blood collected in an EDTA coated vial, and centrifuged for 5 min, at 3,000rpm.
2. Cell pellet was resuspended in 350μl 0.4M Tris pH 8, 60mM EDTA, 150mM NaCl, 1% SDS and incubated at $65^0$C for 30 min, after which a 100μl of sodium perchlorate was added for deproteinisation.
3. The sample was extracted with 600μl of ice cold chloroform, 150μl of "Nucleon" silica resin was added, followed by centrifugation for 5min. at 4000rpm.
4. Supernatant was collected, DNA was ethanol precipitated and resuspended in 50μl of TE.

#### 8.1.1.2. From tissue culture cells or tissue samples

1. Cells or small pieces of tissue samples were incubated in 10mM NaCl, 25mM EDTA pH 8, 1% SDS, 100-200μg/ml of proteinase K, at $65^0$C for 1-16hrs (depending on tissue type and sample size).
2. After 2 phenol extractions DNA was ethanol precipitated and resuspended in TE.

#### 8.1.1.3. From small numbers of cells for use in PCR reactions

1. Cells were collected by centrifugation, resuspended in 50-100μl of 50mM KCl, 1.5mM $MgCl_2$, 10mM Tris pH 8.0, 0.5% NP40, 0.5% Tween, 100μg/ml proteinase K.
2. Incubated for 20-60 min. at $65^0$C.
3. Proteinase K was heat inactivated by incubation for 10 min, at $95^0$C.
4. Typically 4-10μl was used for PCR reactions.

### 8.1.1.4. From bacteria: mini prep

1. 1-2ml of Luria broth or 2YT (50-100μg/ml ampicillin) was inoculated with bacteria and incubated overnight at 37$^0$C.
2. After centrifugation the pellet was resuspended in 100μl 50mM glucose, 25mM Tris PH 8, 10mM EDTA, 5mg/ml lysozyme, incubated for 5min, at r.t..
3. 150μl 1% SDS, 0.2M NaOH was added.
4. After 5 min of incubation at r.t. 200μl of cold 3M potassium acetate pH 4.9 (pH adjusted with acetic acid) was added, incubated for 5 min on ice and centrifuged for 5 min, 16,000rpm.
5. Supernatant was extracted once with phenol, DNA was ethanol precipitated and resuspended in 20-100μl TE.

### 8.1.1.5. From bacteria transformed with pMIX1

This method was employed for isolation of pMIX1 DNA. Due to the large size of the plasmid >40kb plasmid yields were generally low and multiple small cultures gave generally the best yields.

1. 6x100ml of terrific broth, 200μg/ml ampicillin was inoculated with a single bacterial colony and grown for 16-24hrs, at 37$^0$C.
2. After centrifugation each pellet was resuspended in 2ml 50mM glucose, 25mM Tris PH 8, 10mM EDTA, 5mg/ml lysozyme, and incubated for 5min, r.t..
3. 4ml 1% SDS, 0.2M NaOH was added.
4. After 5min of incubation 3ml of cold 3M potassium acetate pH 4.9 (pH adjusted with acetic acid) was added, incubated for 5min on ice and centrifuged for 20 min, 3,500rpm.
5. Supernatant was extracted once with phenol, DNA was ethanol precipitated and resuspended in 500μl TE.
6. Quantity and quality of the six plasmid preparations were analysed by restriction enzyme digestion (see Figure 8.1). Due to the large size of the pMIX1 construct (41.5kb) plasmid yields were often low. Only samples which contained a reasonable amount of DNA were pooled for further purification (see Fig. 8.1, sample 3, 4, 5).
7. 1.7g CsCl and 120μl EtBr was added to 1.5ml of DNA solution and transferred into 11x32mm quick-seal tubes (TLA-100.2 Beckmann rotor)
8. Centrifuged for 16hrs, 80,000rpm, at r.t..
9. Plasmid band was withdrawn with the help of a syringe.

10. DNA was dialysed against TE for 2hrs, at $4^0C$, then phenol extracted, ethanol precipitated and resuspended in TE.

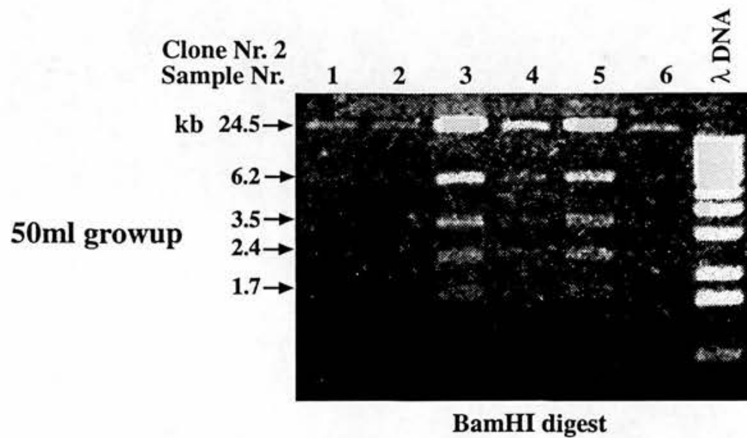



**Figure 8.1: Isolation of pMIX1 plasmid DNA**
BamHI digest of pMIX1 DNA. DNA was isolated as described above and a 100th of each sample was analysed on a 1% agarose gel.

### 8.1.1.6. From bacteria: Maxi prep

1. Bacterial colony was transferred to 200-500ml of terrific broth, 100µg/ml ampicillin and grown for 16-24hrs, at $37^0C$.
2. After centrifugation cell pellet was resuspended in 3ml 15% sucrose, 50mM EDTA, 50mM Tris pH 8.0, 5mg/ml lysozyme, and incubated for 15min on ice.
3. 6ml 62.5mM EDTA, 50mM Tris pH 8.0, 0.1% Trition was added, mixed gently and incubated on ice for 30min.
4. Centrifuged 45min, 18000rpm.
5. Supernatant was decanted, its volume adjusted to 10ml. 9.5g CsCl and 1ml EtBr was added and transferred to quickseal tube (NVT 65 rotor, Beckmann)
6. Centrifuged at 55,000rpm for 16hrs, at r.t..
7. Plasmid band was withdrawn using a syringe.
8. DNA was dialysed against TE for 2hrs, at $4^0C$, then phenol extracted, ethanol precipitated and resuspended in TE.

### 8.1.1.7. From phage lambda.

1. Single phage plaques were isolated and incubated in phage buffer (10mM Tris pH 7.4, 20mM NaCl, 20mM $MgCl_2$), resulting in $\approx 1\times10^9$ pfu/ml.
2. For the plate lysate ≈10,000pfu were absorbed to 1ml of LE392 bacteria (these had been resuspended in 10mM magnesium sulphate, $O.D._{600} = 0.5$) for 20min, $37^0C$.
3. 7ml 0.8% agarose in L broth, 1mM $MgCl_2$, 10mM $MgSO_4$ was added and spread onto fresh L agarose plates, and incubated overnight.
4. Plates were cooled, 12-15ml of phage buffer and 1-2 drops of chloroform was added, followed by incubation for 2-16hrs, $4^0C$.
5. Phage buffer was collected, 1μg/ml of RNase and DNase was added and incubated for 30min, at $37^0C$.
6. Centrifuged for 25min, 7000rpm.
7. Supernatant was collected and centrifuged at 30,000rpm for 2hrs, at $4^0C$ in a swing-out rotor (SW41, Beckmann).
8. Pellet was resuspended in 0.5ml of phage buffer, insoluble particles removed by centrifugation and DNA extracted by addition of EDTA (final concentration 20mM), SDS (final concentration 0.5%), proteinase K (final concentration 50μg/ml) and incubation for 1hr, at $65^0C$, followed by phenol extraction.
9. DNA was then dialysed against TE, for 16-24hrs, at $4^0C$.
10. Collected phage DNA could either be used directly or was concentrated following ethanol precipitation.

### 8.1.2. Preparation and transformation of competent E-coli (DH5α, XL1)

The methods listed below achieved transformation efficiencies of $>10^7$cfu/μg of input plasmid DNA.

### 8.1.2.1. Chemical method

1. Overnight culture was diluted 1:100 in L broth, and grown to $O.D._{600} = 0.4$-$0.6$.
2. Chilled, centrifuged and resuspended in 1/4 volume 100mM RbCl, 50mM $MnCl_2$, 30mM K Acetate, 10mM $CaCl_2$, 15% Glycerol, and incubated for 1-2hrs on ice.
3. Centrifuged and resuspended in 1/40 of original volume 10mM MOPS pH 7.O, 10mM RbCl, 75mM $CaCl_2$, 15% Glycerol.
4. 200μl aliquots were frozen in liquid nitrogen and then stored at $-70^0C$.

5. For bacterial transformation 20-100μl of competent and 1-5μl of DNA or ligation mix was added.
6. After incubation for 30min on ice, cells were heat shocked 45sec, at $42^0$C, and then placed on ice for 2min.
7. 1-2ml of L Broth was added and cells were incubated for 20min, at $37^0$C before plating on LB plates containing 100μg/ml ampicillin and if required X-Gal and IPTG (see buffers below).

#### 8.1.2.2. Electroporation method

The method used is essentialy as described by Wu, 1993.

1. 500ml of 2YT medium was inoculated with 2.5ml of an overnight culture of DH5α bacteria. These were grown to $O.D._{600}$ of 0.6, and then cooled on ice for 10-15min.
2. Cells were pelleted and resuspended in 250ml of ice cold 1M HEPES pH 7 and harvested by centrifugation.
3. Cells were resuspended in 20ml of ice cold 10% (V/V) glycerol and harvested again by centrifugation.
4. Cells were resuspended in an equal volume of ice cold 10% (V/V) glycerol and 100μl aliquots were stored frozen.
5. Cells were thawed on ice, DNA added to 50μl of competent cells and transferred to a chilled cuvette (0.1cm).
6. Cuvette was placed into the chilled electroporation chamber and pulsed once (2000V, 200Ω, 25μF).
7. 1ml of SOC medium was added, cells were transferred to 15ml tube and incubated for 30min-1hr, $37^0$C before plating on LB plates containing 100μg/ml ampicillin and if required X-Gal and IPTG (see buffers below).

### 8.1.3. Recombinant DNA manipulation

Enzymes were supplied by Böhringer or New England Biolabs and used as recommended by the supplier. Bacterial vectors had previously been purchased from Promega (pGEM-T), Stratagene (Bluescript) and New England Biolabs (pUC18).

#### 8.1.3.1. Polymerase chain reaction (PCR)

Primers were designed using the OLIGO 4 computer program. PCR reactions were carried out essentially as recommended by the supplier of the thermostable Taq and Expand™ DNA polymerases (Böhringer). The latter was used for long range PCR and is composed

of both thermostable Taq and Pwo DNA polymerases. Reaction conditions were optimised by varying either annealing temperature, buffer composition (e.g. $MgCl_2$ concentration) or cycle conditions. Genomic DNA was diluted to ≈50ng/μl either in $H_2O$ or in 50mM KCl, 1.5mM $MgCl_2$, 10mM Tris pH 8.0, 0.5% NP40, 0.5% Tween, the latter often facilitated a more efficient PCR reaction. Primer sequences and optimised PCR conditions are given in the relevant sections (see below).

#### 8.1.3.1.1. PCR based method for ovine sex determination

Primers employed had previously been described for amplification of SRY and bovine zfx sequences (Griffiths, 1993; Kirkpatrick, 1993) and are shown below. PCR conditions were optimised for the use of these primers in combination with ovine DNA.

| | |
|---|---|
| Primer zfx5' | GACAGCTGAACAAGTGTTACTG |
| zfx3' | AATGTCACACTTGAATCGCATC |
| | |
| Rg4 | GGTCAAGCGACCCATGAA(C/T)GCNTT |
| Rg7 | GGTCGATACTTATAGTTCGGGTA(C/T)TT |

1. PCR reactions were carried out in a total volume of 50μl, containing 5μl 10x PCR reaction buffer (Böhringer, 1.5mM $MgCl_2$), 2.5μl of each primer Rg4 and 7 (Primer stock: 20μMolar), 0.8μl of each primer zfx5' and 3' (Primer stock: 20μMolar) and ≈0.1μg of DNA or 5-10μl of cell lysate (see isolation of DNA from small numbers of cells for use in PCR reactions).
2. After incubation at $95^0$C, for 5min, reaction was cooled to $80^0$C and 8μl dNTP (final concentration 0.2mM), 0.5μl Taq DNA polymerase (Böhringer) were added, followed by 35 cycles of $94^0$C, 30sec; $55^0$C, 30sec and $72^0$C, 45sec, extension was completed by final incubation at $72^0$C, 5min.
3. A 5μl aliquot of the PCR reaction was analysed on a 2% agarose gel (1:1 mix of agarose and Nusieve agarose).

#### 8.1.3.1.2. PCR conditions for microsatellite analysis

Microsatellite analysis was carried out as described by Buchanan *et al.* (1993). The primers and PCR conditions used are listed below.

| | |
|---|---|
| Primer FCB 11 (5') | GCA AGC AGG TTC TTT ACC ACT AGC ACC |
| Primer FCB 11 (3') | GGC CTG AAC TCA CAA GTT GAT ATA TCT ATC AC |
| | |
| Primer FCB 128 (5') | ATT AAA GCA TCT TCT CTT TAT TTC CTC GC |
| Primer FCB 128 (3') | CAG CTG AGC AAC TAA GAC ATA CAT GCG |

| | |
|---|---|
| Primer FCB 304 (5') | CCC TAG GAG CTT TCA ATA AAG AAT CGGP |
| Primer FCB 304 (3') | CGC TGC TGT CAA CTG GGT CAG GG |
| Primer MAF 33 (5') | GAT CTT TGT TTC AAT CTA TTC CAA TTT C |
| Primer MAF 33 (3') | GAT CAT CTG AGT GTG AGT ATA TAC AG |
| Primer MAF 209 (5') | TCA TGC ACT TAA GTA TGT AGG ATG CTG |
| Primer MAF 209 (3') | GAT CAC AAA AAG TTG GAT ACA ACC GTG G |

PCR reactions were carried out in a total volume of 20μl, containing 2μl 10xPCR reaction buffer (100mM Tris pH 8.3, 15mM $MgCl_2$, 500mM KCl), 2μl of both 5' and 3' primer (Primer stock: 3.33μMolar) and ≈0.1μg of DNA. After incubation at $95^0C$, 5min, reaction was cooled to $80^0C$ and 2μl dNTP (final concentration 0.125mM), 0.25μl Taq (Böhringer) were added, followed by 10 cycles of $94^0C$, 30sec; $63^0C$, 45sec and 23 cycles of $90^0C$, 30sec; 63°c, 45sec. For primer pair MAF33 the annealing/extension temperature was lowered from $63^0C$ to $59^0C$. The PCR products were analysed on 4% Metaphor agarose gels.

### 8.1.3.1.3. PCR detection of SV40T in TNT4 transfectants

Primers designed to amplify a 516bp fragment of the SV40T are shown below.

| | |
|---|---|
| Primer SV40T 5' | GGGGAGTCCAGAGTTGCCTTCA |
| SV40T 3' | CTCCCCACCATCTTCATTTTTATC |

1. PCR reactions were carried out in a total volume of 50μl, containing 5μl 10x PCR reaction buffer (100mM Tris pH 8.3, 15mM $MgCl_2$, 500mM KCl), 2.5μl of each primer (primer stock: 20μMolar) and 5-10μl of cell lysate (see isolation of DNA from small numbers of cells for use in PCR reactions).
2. After incubation at $95^0C$, 5min, reaction was cooled to $80^0C$ and 8μl dNTP (final concentration 0.2mM), 0.5μl Taq DNA polymerase (Böhringer) were added, followed by 35 cycles of $94^0C$, 30sec; $58^0C$, 30sec and $72^0C$, 60sec, extension was completed by final incubation at $72^0C$, 10min.
3. 5-10μl aliquot of the PCR reaction was analysed on a 1.5% agarose gel.

### 8.1.3.1.4. Isolation of ovine HPRT sequences by long range PCR

Cloning of portions of the ovine HPRT gene by long range PCR is described in Chapter 6. Listed below are the PCR primer pairs and conditions employed for the amplification of

ovine HPRT sequences. Cloning of the amplified product was either attempted directly (see cloning of FIX3'$_{PCR}$; Chapter 8.1.4) or after DNA separation on a 1% agarose gel.

**Primers**

Exon 2-5' CGGAATTCTTATGCTGAGGATTTGGAAA(A/G)GGTG
EcoRI
Exon 3-3' GCGGATCCATCTCCTTCAT(C/G)ACATCTCGAGC
BamHI

Exon 3-5' GCGGATCCTTGCTCGAGATGTGATGAAGGAGATGGGA
BamHI XhoI
Exon 4-3' GCGGATCCAGTTAAAGTTGAGAGATCATCTCCACCA
BamHI

Exon 6-5' GGACTAGTTAATTAATTGACACTGGTAAAACAATGC
SpeI PacI
Exon 8-3' CGCTCGAGATCGATCATTATAGTCAAGGGCATATCC
XhoI ClaI

Exon 3-5' GCGGATCCTTGCTCGAGATGTGATGAAGGAGATGGGA
BamHI
Intron 3-P1 CCGAATTCAGCGCCAGAGAGGATATCCGCCACCAG
EcoRI

Intron 3-P2 CCGGATCCGTGTATGCTATGAGGTAGGGATCATAC
BamHI
Exon 4-3' GCGGATCCAGTTAAAGTTGAGAGATCATCTCCACCA
BamHI

**PCR conditions**

**Amplification of ovine sequences spanning from exon 2 to 3 and 6 to 8**

1. PCR reactions were carried out in a total volume of 50µl, containing 5µl 10x PCR reaction buffer (22.5mM $MgCl_2$, 500mM Tris pH 9.2, 160mM $(NH_4)_2SO_4$, Böhringer), 1µl of each primer (Primer stock: 20µMolar) and 0.1µg of genomic DNA.
2. After incubation at 95°C, 5min, reaction was cooled to 80°C and 14µl dNTP (final concentration 0.35mM), 0.75µl Expand™ DNA polymerase (Böhringer) were added, followed by:
   1 cycle: 94°C, 30sec; 55°C, 45sec and 68°C, 2min;
   10 cycles: 94°C, 30sec; 60°C, 45sec and 68°C, 2min;
   25 cycles: 94°C, 30sec; 65°C, 45sec and 68°C, 2min plus 20sec extension per cycle
   1 cycle: 68°C, 15min

**Amplification of ovine sequences spanning from exon 3 to 4**

PCR reaction was carried out as described for amplification of sequences spanning from exon 2-3, with the following modifications to the cycle conditions:

1 cycle: $94^0C$, 30sec; $60^0C$, 45sec and $68^0C$, 8min;

10 cycles: $94^0C$, 30sec; $66^0C$, 45sec and $68^0C$, 8min;

25 cycles: $94^0C$, 30sec; $65^0C$, 45sec and $68^0C$, 8min plus 20sec extension per cycle

1 cycle: $68^0C$, 25min

**Amplification of ovine sequences spanning from exon 3 to intron 3 and from intron 3 to exon 4**

PCR reaction was carried out as described for amplification of SV40T sequences, with the following modifications to the cycle conditions: 35 cycles of $94^0C$, 30sec; $65^0C$, 30sec and $72^0C$, 50sec, final extension $72^0C$, 10min. The PCR reactions contained 0.1μg of ovine genomic DNA.

### 8.1.3.2. Restriction digest

Restriction digests were carried out as recommended by the supplier of the restriction enzyme. Reactions were stopped by addition of 1/10 volume of gel loading buffer. DNA was analysed on 0.8-1.0% agarose, 0.5μg/ml EtBr gels in TBE unless otherwise stated.

### 8.1.3.3. Purification of DNA fragments

#### 8.1.3.3.1. Fragments up to ≈20kb: "Prep-A-Gene" (Bio-Rad) method

1. DNA band was excised from the agarose gel and incubated with 3 volumes of Prep-A-gene binding buffer (50mM Tris pH 7.4, 1mM EDTA, 6M $NaClO_4$) for 5min, at $55^0C$.
2. 5μl/μg of DNA of Prep-A-gene matrix was added and incubated for 5-10min at room temperature.
3. Spun in Eppendorf centrifuge for 30sec, pellet was washed 3 times in 50x volume of binding buffer, followed by three washes in 20mM Tris pH 7.4, 2mM EDTA, 0.4M NaCl, 50% Ethanol.
4. After a final spin, DNA was eluted from the matrix in either $H_2O$ or TE, 5min, at $55^0C$.

#### 8.1.3.3.2. Fragments over 20kb: Sucrose density gradient

This method was used for fragment isolation during pMIX1 cloning and for purification of the MIX1 micro-injection fragment.

1. For a 20-40% sucrose gradient 0.5ml of 40% sucrose was overlaid first with 0.5ml of 30% then 20% sucrose and left to equilibrate at $4^0$C for 2hrs.
2. This was then overlaid with 100µl of restriction enzyme digested DNA and centrifuged for 20hrs, at $15^0$C, 26,000rpm (TLS 55 rotor, Beckmann ultracentrifuge TL-100).
3. After centrifugation fractions were collected in 96 well microtiter plates, a 3-10µl aliquot of each fraction was analysed on a 1% agarose gel.
4. Fractions containing the purified DNA fragment were pooled, ethanol precipitated and resuspended in TE

### 8.1.3.4. Ligation

Ligations were carried out as recommended by the supplier of the T4 DNA ligase (Böhringer). Amounts of DNA in a typical 20µl reaction would vary depending on the number of fragments to be ligated (2, 3, or 4 part ligations) and on the size of vector and insert DNA (e.g. 10-50ng of plasmid vector DNA, 50-250ng of insert DNA). Reactions were stopped by heat inactivation at $70^0$C for 10min.

### 8.1.3.5. DNA sequence analysis

Double stranded DNA sequencing was carried out using the "ABI PRISM™" dye terminator cycle sequencing ready reaction kit with AmpliTaq® as recommended by the supplier (Perkin Elmer).

1. Each 20µl reaction contained 0.4µg of DNA template, 8µl Terminator Ready Reaction Mix, 4pmole primer.
2. The PCR reaction was incubated for 1 min at $94^0$C followed by 25 cycles: $95^0$C, 30sec; $55^0$C, 15sec; $60^0$C, 2min.
3. The sample was transferred to 1.5ml microcentrifuge tube and 2µl of 3M sodium acetate pH 5.2 and 50µl 95% ethanol was added, incubated on ice for 10min and centrifuged for 20min.
4. Pellet was washed with 70% ethanol, pellet was dried and then resuspended in 5µl formamide/25mM EDTA pH 8, 50mg/ml blue dextran (5:1 ration), heated at $90^0$C for 2min to denature, and placed on ice until loading.

5. 2.5µl was loaded onto a 4.75% acrylamide/bis acrylamide (19:1), 6M urea gel in 1x TBE. Sequencing gel was run and analysed with the help of ABI PRISM™ 377 automated sequencer. DNA sequences were analysed using either Factura or DNASTAR computer programs.

| Sequencing primer: | Reverse | AACAGCTATGACCATG |
| --- | --- | --- |
| | Forward | GTAAAACGACGGCCAGT |

### 8.1.3.6. Southern Analysis

**Southern blot:** After electrophoresis the agarose gel was exposed to U.V. light to nick the DNA, which facilitates transfer of larger fragments. After incubation in 0.5M NaOH to denature the DNA the gel was neutralised in 0.5M Tris, pH 7.2, 1.5M NaCl). DNA was transferred to Duralon UV membrane by capillary action (transfer buffer: 20x SSPE) and DNA was crosslinked to the membrane using an ultra violet "Stratalink" machine (Stratagene) at 1200µJ.

**$^{32}$P DNA labelling:** The "Random Primed DNA Labelling Kit" from Böhringer was employed for incorporation of $^{32}$P into the randomly primed, and newly synthesised complementary DNA strand. The reaction was carried out as recommended by the supplier. A typical 20µl reaction contained 25-100ng of DNA, 1µl of each dATP, dCTP, dGTP, and dTTP (Stock: 0.5mM), 5µl $^{32}$P-dCTP, 1µl Klenow. After incubation for 30-60min at 37$^0$C, 80µl of TE was added to the reaction and the unincorporated $^{32}$P nucleotides were separated from the DNA using a Sephadex G-50 column according to the manufactures recommendations (Nick Column, Pharmacia).

### Southern blot hybridisation

1. Filter membrane was wetted in 2x SSPE and placed in hybridisation bottle.
2. After addition of 30ml 0.5M $NaPO_4$ pH 7.2, 7% SDS, 1mM EDTA the filter was pre-hybridised for 1-4hrs at 65$^0$C.
3. The pre-hybridisation solution was discarded and replaced with 20ml 0.5M NaPO4 pH7.2, 7% SDS, 1mM EDTA, $10^6$-$10^7$cpm/ml of denatured $^{32}$P labelled DNA probe and hybridised over night at 65$^0$C.

4. Filter was rinsed in 2x SSPE and then washed three times in 40mM $NaPO_4$ pH7.2, 1% SDS, 1mM EDTA at $60^0C$ for 15min.
5. Signal was detected by exposure to X-OMAT AR5 film.

### 8.1.4. Construction of the FIX expression vector pMIX1

Construction of pMIX1 was carried out as described in the Chapter 2 using standard methods as described above. All fragments required for cloning were isolated from mini plasmid or λ prep. DNA, using gel electrophoresis and Prep-A-Gene purification. The final cloning step required manipulation of DNA fragments >30kb, these were separated and purified on sucrose gradients (see 8.1.3.3.2.).

Cloning of $FIX3'_{PCR}$ required amplification of the 3' end of the human FIX gene by PCR. The PCR primers used are listed below, also indicated are the restriction enzyme recognition sites contained within the primer sequence.

Primer FIX1: 5' GC GAATTC AGATCT TTA ACA TTG CCA ATT AGG 3'
EcoRI BglII

Primer FIX2: 5' GC GGATCCTCGAG ATC CAT CTT TCA **TTA** AGT GAG C 3'
BamHI XhoI STOP

1. PCR reactions were carried out in a total volume of 50μl, containing 5μl 10x PCR reaction buffer (100mM Tris pH 8.3, 15mM $MgCl_2$, 500mM KCl ), 2.5μl of both primer FIX1 and 2 (primer stock: 20μMolar) and ≈10ng of λ HIX2 DNA.
2. After incubation at $95^0C$, 5min, reaction was cooled to $80^0C$ and 8μl dNTP (primer stock 1.25mM; final concentration 0.2mM), 0.5μl TAQ DNA polymerase (Böhringer) were added, followed by 35 cycles of $94^0C$, 30sec; $65^0C$, 30sec and $72^0C$, 45sec, extension was completed by final incubation at $72^0C$, 5min.
3. An aliquot of the PCR reaction was analysed on a 1% agarose gel.
4. The PCR product was purified away from unincorporated nucleotides, primers and the thermostable polymerase using the Böhringer "PCR Clean Up Kit" as recommended by the supplier.
5. After digestion with the restriction enzymes EcoRI and BamHI the PCR product was cloned into the EcoRI/BamHI site of the plasmid vector Bluescript.
6. The integrity of the cloned PCR fragment was confirmed by DNA sequence analysis.

### 8.1.5. Construction of the immortalisation vector PGKtsA58LOX

Construction of PGKtsA58LOX was carried out as described in the Chapter 3 using standard methods as described above.

For the *loxP* site two complementary oligonucleotides were synthesised (see sequence below), the 5' and 3' overhangs were designed to facilitate cloning to vector DNA digested with XhoI and BamHI.

*loxP*: **TCGAG**ATAACTTCGTATAGCATACATTATACGAAGTTAT**G**
**C**TATTGAAGCATATCGTATGTAATATGCTTCAATA**CCTAG**
XhoI BamHI

1. The two complementary DNA strands were annealed at a concentration of 1μg/μl.
2. Four μg of annealed oligonucleotides were then phosphorylated using 4μl of T4 Polynucleotide Kinase in 20μl, 76mM Tris pH 7.6, 10mM $MgCl_2$, 1mM ATP, 15mM DTT, 1mM spermidine.
3. After incubation at $37^0$C for 30min the reaction product was stored frozen at $-20^0$C until required.

## 8.2. Protein Analysis

### 8.2.1. Western Blot analysis (Factor IX)

Reducing SDS PAGE

1. Samples were loaded in 5-15μl 0.5M Tris pH 6.8, 2% Glycerol. 10% SDS, 0.1% bromophenol blue, 5% β-mercaptoethanol onto a 18% 10 well Tris-glycine gel (Novex).
2. After electrophoresis the Tris-glycine gel was washed once in transfer buffer (25mM Tris, 129mM glycine), 15min at room temperature.
3. The nitrocellulose membrane (Immulon P, Millipore)was cut to size, rinsed first in methanol then in transfer buffer.
4. The protein was transferred to the nitrocellulose membrane using an Electroblotter (100mA, 25Volts, 30min).
5. The membrane was then incubated in 50ml blocking buffer (PBS, 2% BSA, 0.1% Tween 20) for 1hr.
6. Followed by a 1hr incubation in 50ml of 1:2000 rabbit anti-FIX antibody (Dako P0300) in blocking buffer.

7. Followed by a 1hr incubation in 50ml of 1:5000 anti-rabbit horseradish peroxidase antibody (Sigma) in blocking buffer.
8. Followed by 4 washes in PBS, 10min. each.
9. 2.5ml of the two developing chemicals (Supersignal chemiluminescent substrate peroxide and Luminol/enhancer solution; Pierce) was added for ≈1min, then rinsed in PBS.
10. The signal was visualised using Hyber film.

### 8.2.2. ELISA (Factor IX)

1. 96 well microtiter plates were coated with 1:1200 dilution of anti-FIX polyclonal antibody (Dako P0300) in PBS pH 7.2, 0.02% Na azide, 100µl per well, and incubated over night at room temperature.
2. Plates were washed 3 times with wash buffer (PBS pH 7.2, 0.5M NaCl, 0.2% Tween)
3. 130µl of Blocking buffer (PBS pH 7.2, 3% w/v gelatin) was added per well and incubated for 1hr at r.t., followed by 3 rinses with wash buffer.
4. Milk samples were used in a dilution series from 1: 500 to 1:16000 and the human FIX standard (ERL) was used in a dilution series from 1000µg/ml to 15.625µg/ml. Both were diluted in PBS pH 7.2, 0.5M NaCl, 0.2% Tween.
5. 50µl of each dilution was loaded per well in triplicates and incubated for 1hr at r.t., followed by 3 rinses with wash buffer.
6. The anti-FIX polyclonal antibody horseradish peroxidase conjugate (Dako) was diluted 1:1000 in PBS pH 7.2, 0.5M NaCl, 0.2% Tween, 50µl was added to each well and incubated for 1hr at r.t., followed by 3 rinses with wash buffer.
7. 100µl of TMB Colour Reagent (KPL single component peroxidase substrate) was added to each well and incubated for ≈15min.
8. The reaction was stopped by addition of 100µl 0.2M $H_2SO_4$ and signal analysed using a Dynex automated microtiter plate reader.

## 8.3. Cell culture

Cells were grown on standard tissue culture plastic pre-coated with 0.1% gelatin, and incubated at $37^0$C in a 5% $CO_2$ humidified atmosphere. For routine passage cells were washed twice with PBS, disaggregated enzymatically using trypsin, which was inactivated by addition of medium containing serum. Cells were then pelleted, resuspended in growth

medium and transferred at appropriate dilution to culture dishes/flask. Isolation of single cell colonies was performed with the help of cloning rings.

### 8.3.1. Calcium phosphate transfection

Transfection was carried out as described by Chen and Okayama (1987).

1. $2x10^5$ cells were seeded per 3.5cm diameter well on the day before transfection.
2. Day 1: 5µg DNA was added to 0.25ml of 50mM HEPES, 280mM NaCl, 1.5mM $Na_2HPO_4$ pH 7.0, mixed, and dropwise 0.25ml of 250mM $CaCl_2$ was added.
3. After 30 min at r.t. the precipitate was added to the medium and incubated overnight
4. Day 2: Cells were washed with PBS, and medium replaced.
5. Day 3: Cells were lysed for Luciferase assay.

### 8.3.2. Polycationic liposome-mediated transfection

LipofectAMINE transfection was carried out as recommended by the supplier (GIBCO BRL)

1. $2x10^5$ cells were seeded per 3.5cm diameter well on the day before transfection.
2. Day 1: 1.5-2µg DNA was added to 0.1ml OptiMEM, mixed, and dropwise 0.1ml of OptiMEM containing 10µl of LipofectAMINE was added.
3. After 30-45 min at r.t. 0.8ml OptiMEM was added to the precipitate, and this was then added to the cells which had been washed twice with OptiMEM.
4. After 1-24hrs 2ml of culture medium plus 10% serum was added.
5. Day 3: Cells were either fixed for β–galactosidase assay, lysed for Luciferase assay, or plated at a 1:10 dilution into medium containing G418 for selection of single cell colonies.

### 8.3.3. Electroporation

The method used is described below.

1. 0.8ml of serum free medium containing $1x10^6$ cells, 1µg/ml DNA, and 5-10µg/ml DEAE-dextran was transferred to an electroporation cuvette.
2. Electroporation parameters varied for the voltage from 120-450V and for the capacitance from 125-500µFd.
3. After the cells were subjected to the electroporation they remained for a further 10min, room temperature in the cuvette and were then transferred in 5ml of culture medium plus 10% serum to a $25cm^2$ flask and incubated at $37^0C$ for 48hrs.

4. Cells were either fixed for β–galactosidase assay or plated at a 1:10 dilution into medium containing G418 for selection of single cell colonies

### 8.3.4. Luciferase assay

The assay was carried out essentially as described by the supplier of the reagents (Promega).

1. Cells were rinsed twice with PBS.
2. Cell lysis buffer was added and incubated for 10-15min at room temperature.
3. Cell lysate was transferred to Eppendorf microcentrifuge tubes and centrifuged for 5-10sec to pellet debris. At this point cell lysate was frozen for future analysis.
4. Cell lysate was diluted 1:100 in lysis buffer.
5. 20μl of cell lysate was mixed with 100μl of Luciferase assay reagent and reaction was placed in luminometer to measure light emission.

**Lysis buffer:** 25mM Tris pH 7.8
2mM DTT
2mM 1,2 diaminocyclohexane-N, N, N', N'-tetraacetic acid
10% glycerol
1% Triton X-100

**Luciferase assay reagent:** 20mM Tricine
1.07mM $(MgCO_3)_4Mg(OH)_2 \cdot 5H_2O$
2.67mM MgSO4
0.1mM EDTA
33.3mM DTT
270μM coenzyme A
470μM Luciferin
530μM ATP

### 8.3.5. ß-galactosidase assay (Histochemical reaction)

1. The medium was aspirated and cells were washed with PBS.
2. Cells were fixed with 2% Formaldehyde, 0.2% Glutaraldehyde in PBS for 5 min at room temperature.
3. Fixative was aspirated off, and cells washed with PBS.
4. Overlay solution including 1mg/ml X-Gal was added and incubated for 30 min-4hrs at 37$^0$C. Reaction was stopped by washing with PBS.
5. Cells were dried with methanol for 10 min before assessment.

**Overlay solution:** 5mM $K_4Fe(CN)_6$
5mM $K_3Fe(CN)_6$
5mM $MgCl_2$

### 8.3.6. Alkaline phosphatase staining

Alkaline phosphatase staining was carried out as described by Wassarman (1993) with some minor modifications.

1. Cells were fixed with 4% (w/v) paraformaldehyde for 20min.
2. Washed 3x in Tris-maleate buffer pH 9, for 10min.
3. Staining solution (Tris-maleate pH 9, 0.4mM $MgCl_2$, 0.4mg/ml Naphthol- AS-MX phosphate, 1mg/ml Fast Red TR salt) was added and staining reaction was allowed to develop for 15-20min (control cells became bright pink).
4. Reaction was stopped by addition of PBS.

### 8.3.7. Freezing of cells

1. Cells were trypsinised, spun, and resuspended in 0.5 ml of medium.
2. One volume freezing medium (growth medium +20% DMSO +20% serum) was added.
3. Cells were frozen at $-80^0$C and after 24hrs transferred to liquid nitrogen.

### 8.3.8. Production of mitotically inactivated feeder cells

Medium containing 10μg/ml mitomycin C was added to STO cells and cells were incubated for 2-4 hours, after which cells could be used directly or were stored in liquid nitrogen until required.

### 8.3.9. Determination of chromosome number (metaphase spreads)

1. Typically cells (25cm$^2$ flask)were grown until about 75% confluent, 0.2μg/ml colcemid was added to the culture medium and cells were incubated for 30min-6hr.
2. Cells were trypsinised, pelleted and resuspended in 4ml 75mM KCl, incubated for 8-10min at room temperature before centrifugation.
3. Pellet was resuspended in 5ml methanol/acetic acid (3:1) added dropwise while vortexing and incubated for 1hr at room temperature.
4. Step 3 was repeated twice and then stored at $-20^0$C.
5. Metaphase spreads were prepared by dropping fixed cell nuclei onto cold slides. These were allowed to dry for a few minutes at room temperature.
6. 40μl of mounting solution, 10μg/ml DAPI was added to the slide, viewed under fluorescence microscope using appropriate filter.
7. Photographs were taken and chromosome number determined.

### 8.3.10. Serum starvation

1-2x10$^4$ cells were plated into 12 well dishes. After 18-24hrs cells were washed twice with either PBS or serum free medium, medium containing 0.5% serum was added and cells were incubated at 37$^0$C for 5 day. Cells were disaggregated enzymatically 1-2hrs prior to nuclear transfer.

### 8.3.11. Derivation of ovine embryonic cell culture

Embryos were collected at day 9-10 and rinsed 3 times in ES isolation medium, 2x pen/strep (see 8.4 for medium and buffers). The embryonic disc was dissected with the help of watchmaker forceps #5. Once ≈60-90% of the trophectoderm had been removed, the individual discs were rinsed 6 times in ES isolation medium, 2x pen/strep, transferred to gelatinised 24 well dishes and cultured in the presence of STO feeder cells, ES isolation medium and bovine LIF. Embryonic cultures were observed at 1-2 day intervals, if required growing trophectoderm cells were removed mechanically. After 8-14 days the explant was trypsinised and transferred onto fresh feeder cells. Cell growth after the first trypsinisation was a good indication that embryonic cultures had been established, these where then passed regularly at 1:4 to 1:10 dilutions.

### 8.3.12. Fetal fibroblast derivation, culture and transfection with pMIX1.

Fetuses were collected at day 35 and rinsed 3x in PBS. Head, hooves, heart, liver and kidneys were dissected. The remaining fetus was finely chopped with a scalpel and then transferred to a 125ml flasks containing 15ml trypsin, incubated with magnetic stirrer for 20-40min at 37$^0$C. 35ml fibroblast medium, 2x pen/strep was added, cells were pelleted, washed twice in fibroblast medium, 2x pen/strep and then plated onto gelatinised plates.

At first passage, 2x10$^5$ PDFF2 cells were plated into a 3.5cm diameter well and cotransfected with 0.5µg PGKneo (kindly provided by David Melton, University of Edinburgh), 1.5µg MluI digested pMIX1 DNA using "LipofectAMINE" (GIBCO) under conditions recommended by the supplier. 48hrs after transfection cells were split 1:10 and G418 added to 0.6mg/ml. Transfected PDFF2 cells reached subconfluence after 6 days selection. At third passage a portion of the cells were split 1:10, subjected to a further 5 days selection and cryopreserved as an uncloned population. Other portions were split 1:1000 and 1:5000 and subjected to G418 selection for a further 7 days. Individual colonies were isolated, expanded for cryopreservation at passage 5 and a portion of each clone grown further for chromosome counting and Southern analysis.

## 8.4. Buffers, medium and stock solutions

### 8.4.1. Stock solutions:

**20xSSPE** 3M NaCl
0.2M $NaH_2PO_4$
20mM EDTA, pH 7.4

**10xTBE** 0.9M Tris pH8.3
0.9M boric acid
20mM EDTA

**10x running buffer:** for 1Litre
(protein gels) 29g Tris Base
144g glycine
10g SDS

**Ethidium Bromide** 10mg/ml

**Gel loading buffer:** 50% glycerol
0.2M EDTA pH 8.2
0.1% dye (bromophenol blue, bromocresol green)

**10xTBE** 0.9M Tris pH8.3
0.9M boric acid
20mM EDTA

**TE** 10mM Tris, pH7.4
1mM EDTA

**Xgal** 20mg/ml in dimethylformamide

### 8.4.2. Bacterial media

| | |
|---|---|
| **L Broth** | 1Litre<br>10g NaCl<br>5g yeast extract (Difco)<br>10g Tryptone |
| **L Agar** | 1Litre<br>10g NaCl<br>5g Bacto yeast extract<br>10g Bacto Tryptone<br>11g agar |
| **SOC Medium** | 1Litre<br>0.5NaCl<br>5g Bacto yeast extract<br>20g Bacto Tryptone<br>10ml 250mM KCL<br>Autoclave, then add 5ml 2M $MgCl_2$, 20ml 1M glucose |
| **Terrific broth** | 1Litre<br>24g Bacto yeast extract<br>12g Bacto Tryptone<br>4ml glycerol<br>Autoclave, then add 100ml 0.17M $KH_2PO_4$, 0.72M $K_2HPO_4$ |
| **2YT** | 1Litre<br>5g NaCl<br>10g yeast extract (Difco)<br>16g Tryptone |
| **X Gal plates** | 50µl 20mg/ml X-Gal<br>5µl 0.2M IPTG spread on 10cm plate |

### 8.4.3. Tissue culture media and solutions

**100 X pen/strep stock**
5000 units penicillin (Glaxo)
50mg streptomycin (Glaxo) in 100ml PBS.

**Gelatin** 0.1% w/v in $H_2O$

**G418** 100 mg (active component)/ml in PBS.

**PBS**
137mM NaCl
2.7mM KCl
4.3mM $Na_2HPO_4$
1.4mM $KH_2PO_4$

**Trypsin EGTA** (1 litre)

| | |
|---|---|
| 2.5g | Trypsin (porcine Difco) |
| 0.4g | EGTA |
| 7.0g | NaCl |
| 0.3g | $Na_2HPO_4 \cdot 12H_20$ |
| 0.24g | $K_2HPO_4$ |
| 0.37g | KCl |
| 1.0g | D-Glucose |
| 3.0g | Tris pH 7.6 |
| 1.0ml | Phenol red (Difco) |
| 0.1ml | Polyvinyl alcohol |

**ES cell medium**

| | |
|---|---|
| Glasgow MEM (BHK-21) | 500ml |
| Fetal calf serum | 56ml |
| 100mM Sodium pyruvate | 5.6ml |
| 100x Non-Essential Amino Acids | 5.6ml |
| 500mM β-mercaptoethanol | 1.1ml |
| 200mM L-Glutamine | 5.6ml |

**ES isolation medium**: As ES cell medium,
5% New born calf serum
1x pen/strep

**OME cell meduim**

| | |
|---|---|
| F12:DMEM | 500ml |
| Fetal calf serum | 50ml |
| EGF | 0.1ml |
| Insulin | 5.0ml |
| Linoleic acid | 1.0ml |
| L-glutamine | 5.0ml |

| | | |
|---|---|---|
| **Fibroblast medium** | Glasgow MEM (BHK-21) | 500ml |
| | Fetal calf serum | 56ml |
| | 100mM Sodium pyruvate | 5.6ml |
| | Non-Essential Amino Acids | 5.6ml |
| | 200mM L-Glutamine | 5.6ml |
| **STO medium** | F12:DMEM | 500ml |
| | Fetal calf serum | 50ml |
| | L-glutamine | 5.0ml |

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# Appendices

# Appendix 1

## Paper published in the journal Nature

# Viable offspring derived from fetal and adult mammalian cells

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**Fertilization of mammalian eggs is followed by successive cell divisions and progressive differentiation, first into the early embryo and subsequently into all of the cell types that make up the adult animal. Transfer of a single nucleus at a specific stage of development, to an enucleated unfertilized egg, provided an opportunity to investigate whether cellular differentiation to that stage involved irreversible genetic modification. The first offspring to develop from a differentiated cell were born after nuclear transfer from an embryo-derived cell line that had been induced to become quiescent[1]. Using the same procedure, we now report the birth of live lambs from three new cell populations established from adult mammary gland, fetus and embryo. The fact that a lamb was derived from an adult cell confirms that differentiation of that cell did not involve the irreversible modification of genetic material required for development to term. The birth of lambs from differentiated fetal and adult cells also reinforces previous speculation[1,2] that by inducing donor cells to become quiescent it will be possible to obtain normal development from a wide variety of differentiated cells.**

It has long been known that in amphibians, nuclei transferred from adult keratinocytes established in culture support development to the juvenile, tadpole stage[3]. Although this involves differentiation into complex tissues and organs, no development to the adult stage was reported, leaving open the question of whether a differentiated adult nucleus can be fully reprogrammed. Previously we reported the birth of live lambs after nuclear transfer from cultured embryonic cells that had been induced into quiescence. We suggested that inducing the donor cell to exit the growth phase

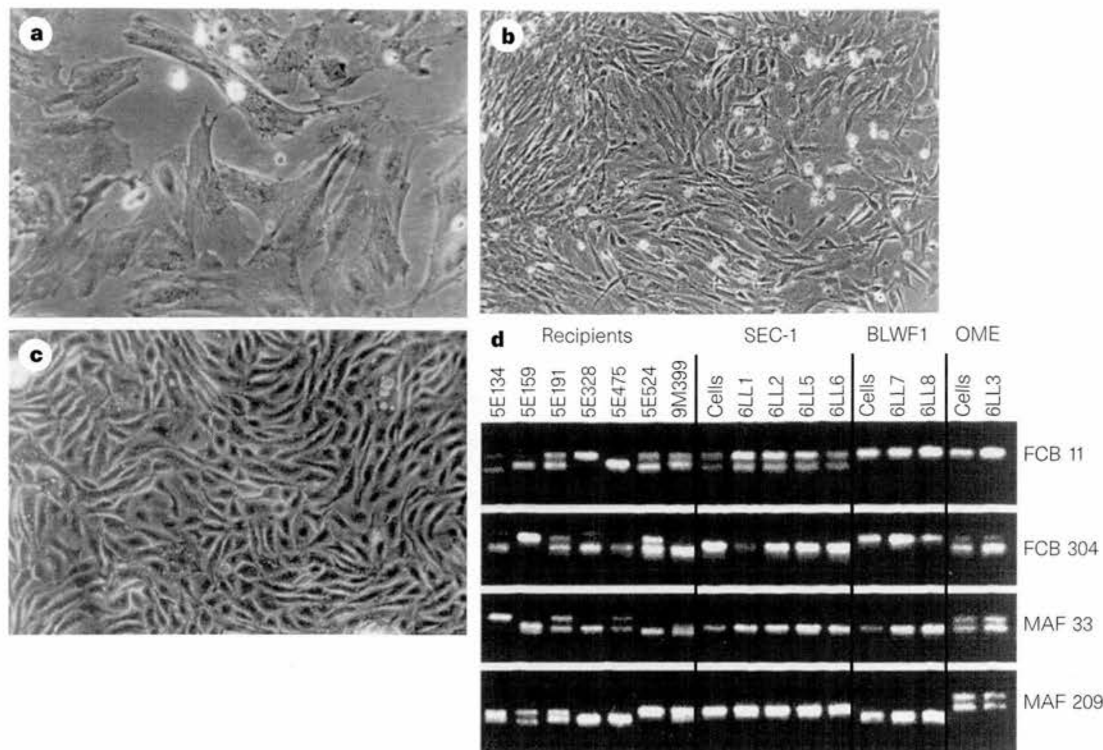


**Figure 1** Phase-contrast photomicrograph of donor-cell populations: **a**, Embryo-derived cells (SEC1); **b**, fetal fibroblasts (BLWF1); **c**, mammary-derived cells (OME). **d**, Microsatellite analysis of recipient ewes, nuclear donor cells and lambs using four polymorphic ovine markers[22]. The ewes are arranged from left to right in the same order as the lambs. Cell populations are embryo-derived (SEC1), fetal-derived (BLW1), and mammary-derived (OME), respectively. Lambs have the same genotype as the donor cells and differ from their recipient mothers.

causes changes in chromatin structure that facilitate reprogramming of gene expression and that development would be normal if nuclei are used from a variety of differentiated donor cells in similar regimes. Here we investigate whether normal development to term is possible when donor cells derived from fetal or adult tissue are induced to exit the growth cycle and enter the G0 phase of the cell cycle before nuclear transfer.

Three new populations of cells were derived from (1) a day-9 embryo, (2) a day-26 fetus and (3) mammary gland of a 6-year-old ewe in the last trimester of pregnancy. Morphology of the embryo-derived cells (Fig. 1) is unlike both mouse embryonic stem (ES) cells and the embryo-derived cells used in our previous study. Nuclear transfer was carried out according to one of our established protocols[1] and reconstructed embryos transferred into recipient ewes. Ultrasound scanning detected 21 single fetuses on day 50–60 after oestrus (Table 1). On subsequent scanning at ~14-day intervals, fewer fetuses were observed, suggesting either mis-diagnosis or fetal loss. In total, 62% of fetuses were lost, a significantly greater proportion than the estimate of 6% after natural mating[4]. Increased prenatal loss has been reported after embryo manipulation or culture of unreconstructed embryos[5]. At about day 110 of pregnancy, four fetuses were dead, all from embryo-derived cells, and post-mortem analysis was possible after killing the ewes. Two fetuses had abnormal liver development, but no other abnormalities were detected and there was no evidence of infection.

Eight ewes gave birth to live lambs (Table 1, Fig. 2). All three cell populations were represented. One weak lamb, derived from the fetal fibroblasts, weighed 3.1 kg and died within a few minutes of birth, although post-mortem analysis failed to find any abnormality or infection. At 12.5%, perinatal loss was not dissimilar to that occurring in a large study of commercial sheep, when 8% of lambs died within 24 h of birth[6]. In all cases the lambs displayed the morphological characteristics of the breed used to derive the nucleus donors and not that of the oocyte donor (Table 2). This

**Table 1 Development of embryos reconstructed with three different cell types**

| Cell type | No. of fused couplets (%)* | No. recovered from oviduct (%) | No. cultured | No. of morula/blastocyst (%) | No. of morula or blastocysts transferred† | No. of pregnancies/no. of recipients (%) | No. of live lambs (%)‡ |
|---|---|---|---|---|---|---|---|
| Mammary epithelium | 277 (63.8)[a] | 247 (89.2) | - | 29 (11.7)[a] | 29 | 1/13 (7.7) | 1 (3.4%) |
| Fetal fibroblast | 172 (84.7)[b] | 124 (86.7) | - | 34 (27.4)[b] | 34 | 4/10 (40.0) | 2 (5.9%) |
| | | | 24 | 13 (54.2)[b] | 6 | 1/6 (16.6) | 1 (16.6%)§ |
| Embryo-derived | 385 (82.8)[b] | 231 (85.3) | - | 90 (39.0)[b] | 72 | 14/27 (51.8) | 4 (5.6%) |
| | | | 92 | 36 (39.0)[b] | 15 | 1/5 (20.0) | 0 |

* As assessed 1 h after fusion by examination on a dissecting microscope. Superscripts a or b within a column indicate a significant difference between donor cell types in the efficiency of fusion ($P < 0.001$) or the proportion of embryos that developed to morula or blastocyst ($P < 0.001$).
† It was not practicable to transfer all morulae/blastocysts.
‡ As a proportion of morulae or blastocysts transferred. Not all recipients were perfectly synchronized.
§ This lamb died within a few minutes of birth.

**Figure 2** Lamb number 6LL3 derived from the mammary gland of a Finn Dorset ewe with the Scottish Blackface ewe which was the recipient.

**Table 2 Delivery of lambs developing from embryos derived by nuclear transfer from three different donor cells types, showing gestation length and birth weight**

| Cell type | Breed of lamb | Lamb identity | Duration of pregnancy (days)* | Birth weight (kg) |
|---|---|---|---|---|
| Mammary epithelium | Finn Dorset | 6LL3 | 148 | 6.6 |
| Fetal fibroblast | Black Welsh | 6LL7 | 152 | 5.6 |
| | Black Welsh | 6LL8 | 149 | 2.8 |
| | Black Welsh | 6LL9† | 156 | 3.1 |
| Embryo-derived | Poll Dorset | 6LL1 | 149 | 6.5 |
| | Poll Dorset | 6LL2‡ | 152 | 6.2 |
| | Poll Dorset | 6LL5 | 148 | 4.2 |
| | Poll Dorset | 6LL6‡ | 152 | 5.3 |

* Breed averages are 143, 147 and 145 days, respectively for the three genotypes Finn Dorset, Black Welsh Mountain and Poll Dorset.
† This lamb died within a few minutes of birth.
‡ These lambs were delivered by caesarian section. Overall the nature of the assistance provided by the veterinary surgeon was similar to that expected in a commercial flock.

alone indicates that the lambs could not have been born after inadvertent mating of either the oocyte donor or recipient ewes. In addition, DNA microsatellite analysis of the cell populations and the lambs at four polymorphic loci confirmed that each lamb was derived from the cell population used as nuclear donor (Fig. 1). Duration of gestation is determined by fetal genotype[7], and in all cases gestation was longer than the breed mean (Table 2). By contrast, birth weight is influenced by both maternal and fetal genotype[8]. The birth weight of all lambs was within the range for single lambs born to Blackface ewes on our farm (up to 6.6 kg) and in most cases was within the range for the breed of the nuclear donor. There are no strict control observations for birth weight after embryo transfer between breeds, but the range in weight of lambs born to their own breed on our farms is 1.2–5.0 kg, 2–4.9 kg and 3–9 kg for the Finn Dorset, Welsh Mountain and Poll Dorset genotypes, respectively. The attainment of sexual maturity in the lambs is being monitored.

Development of embryos produced by nuclear transfer depends upon the maintenance of normal ploidy and creating the conditions for developmental regulation of gene expression. These responses are both influenced by the cell-cycle stage of donor and recipient cells and the interaction between them (reviewed in ref. 9). A comparison of development of mouse and cattle embryos produced by nuclear transfer to oocytes[10,11] or enucleated zygotes[12,13] suggests that a greater proportion develop if the recipient is an oocyte. This may be because factors that bring about reprogramming of gene expression in a transferred nucleus are required for early development and are taken up by the pronuclei during development of the zygote.

If the recipient cytoplasm is prepared by enucleation of an oocyte at metaphase II, it is only possible to avoid chromosomal damage and maintain normal ploidy by transfer of diploid nuclei[14,15], but further experiments are required to define the optimum cell-cycle stage. Our studies with cultured cells suggest that there is an advantage if cells are quiescent (ref. 1, and this work). In earlier studies, donor cells were embryonic blastomeres that had not been induced into quiescence. Comparisons of the phases of the growth cycle showed that development was greater if donor cells were in mitosis[16] or in the G1 (ref. 10) phase of the cycle, rather than in S or G2 phases. Increased development using donor cells in G0, G1 or mitosis may reflect greater access for reprogramming factors present in the oocyte cycoplasm, but a direct comparison of these phases in the same cell population is required for a clearer understanding of the underlying mechanisms.

Together these results indicate that nuclei from a wide range of cell types should prove to be totipotent after enhancing opportunities for reprogramming by using appropriate combinations of these cell-cycle stages. In turn, the dissemination of the genetic improvement obtained within elite selection herds will be enhanced by limited replication of animals with proven performance by nuclear transfer from cells derived from adult animals. In addition, gene targeting in livestock should now be feasible by nuclear transfer from modified cell populations and will offer new opportunities in biotechnology. The techniques described also offer an opportunity to study the possible persistence and impact of epigenetic changes, such as imprinting and telomere shortening, which are known to occur in somatic cells during development and senescence, respectively.

The lamb born after nuclear transfer from a mammary gland cell is, to our knowledge, the first mammal to develop from a cell derived from an adult tissue. The phenotype of the donor cell is unknown. The primary culture contains mainly mammary epithelial (over 90%) as well as other differentiated cell types, including myoepithelial cells and fibroblasts. We cannot exclude the possibility that there is a small proportion of relatively undifferentiated stem cells able to support regeneration of the mammary gland during pregnancy. Birth of the lamb shows that during the development of that mammary cell there was no irreversible modification of genetic information required for development to term. This is consistent with the generally accepted view that mammalian differentiation is almost all achieved by systematic, sequential changes in gene expression brought about by interactions between the nucleus and the changing cytoplasmic environment[17]. □

## Methods

Embryo-derived cells were obtained from embryonic disc of a day-9 embryo from a Poll Dorset ewe cultured as described[1], with the following modifications. Stem-cell medium was supplemented with bovine DIA/LIF. After 8 days, the explanted disc was disaggregated by enzymatic digestion and cells replated onto fresh feeders. After a further 7 days, a single colony of large flattened cells was isolated and grown further in the absence of feeder cells. At passage 8, the modal chromosome number was 54. These cells were used as nuclear donors at passages 7–9. Fetal-derived cells were obtained from an eviscerated Black Welsh Mountain fetus recovered at autopsy on day 26 of pregnancy. The head was removed before tissues were cut into small pieces and the cells dispersed by exposure to trypsin. Culture was in BHK 21 (Glasgow MEM; Gibco Life Sciences) supplemented with L-glutamine (2 mM), sodium pyruvate (1 mM) and 10% fetal calf serum. At 90% confluency, the cells were passaged with a 1 : 2

division. At passage 4, these fibroblast-like cells (Fig. 1) had modal chromosome number of 54. Fetal cells were used as nuclear donors at passages 4–6. Cells from mammary gland were obtained from a 6-year-old Finn Dorset ewe in the last trimester of pregnancy[18]. At passages 3 and 6, the modal chromosome number was 54 and these cells were used as nuclear donors at passage numbers 3–6.

Nuclear transfer was done according to a previous protocol[1]. Oocytes were recovered from Scottish Blackface ewes between 28 and 33 h after injection of gonadotropin-releasing hormone (GnRH), and enucleated as soon as possible. They were recovered in calcium- and magnesium-free PBS containing 1% FCS and transferred to calcium-free M2 medium[19] containing 10% FCS at 37 °C. Quiescent, diploid donor cells were produced by reducing the concentration of serum in the medium from 10 to 0.5% for 5 days, causing the cells to exit the growth cycle and arrest in G0. Confirmation that cells had left the cycle was obtained by staining with antiPCNA/cyclin antibody (Immuno Concepts), revealed by a second antibody conjugated with rhodamine (Dakopatts).

Fusion of the donor cell to the enucleated oocyte and activation of the oocyte were induced by the same electrical pulses, between 34 and 36 h after GnRH injection to donor ewes. The majority of reconstructed embryos were cultured in ligated oviducts of sheep as before, but some embryos produced by transfer from embryo-derived cells or fetal fibroblasts were cultured in a chemically defined medium[20]. Most embryos that developed to morula or blastocyst after 6 days of culture were transferred to recipients and allowed to develop to term (Table 1). One, two or three embryos were transferred to each ewe depending upon the availability of embryos. The effect of cell type upon fusion and development to morula or blastocyst was analysed using the marginal model of Breslow and Clayton[21]. No comparison was possible of development to term as it was not practicable to transfer all embryos developing to a suitable stage for transfer. When too many embryos were available, those having better morphology were selected.

Ultrasound scan was used for pregnancy diagnosis at around day 60 after oestrus and to monitor fetal development thereafter at 2-week intervals. Pregnant recipient ewes were monitored for nutritional status, body condition and signs of EAE, Q fever, border disease, louping ill and toxoplasmosis. As lambing approached, they were under constant observation and a veterinary surgeon called at the onset of parturition. Microsatellite analysis was carried out on DNA from the lambs and recipient ewes using four polymorphic ovine markers[22].

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# Appendix 2

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SCIENCE

# Human Factor IX Transgenic Sheep Produced by Transfer of Nuclei from Transfected Fetal Fibroblasts

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# The Lamb That Roared

Elizabeth Pennisi

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# Human Factor IX Transgenic Sheep Produced by Transfer of Nuclei from Transfected Fetal Fibroblasts

Angelika E. Schnieke,* Alexander J. Kind, William A. Ritchie, Karen Mycock, Angela R. Scott, Marjorie Ritchie, Ian Wilmut, Alan Colman, Keith H. S. Campbell†

Ovine primary fetal fibroblasts were cotransfected with a neomycin resistance marker gene (*neo*) and a human coagulation factor IX genomic construct designed for expression of the encoded protein in sheep milk. Two cloned transfectants and a population of neomycin (G418)–resistant cells were used as donors for nuclear transfer to enucleated oocytes. Six transgenic lambs were liveborn: Three produced from cloned cells contained factor IX and *neo* transgenes, whereas three produced from the uncloned population contained the marker gene only. Somatic cells can therefore be subjected to genetic manipulation in vitro and produce viable animals by nuclear transfer. Production of transgenic sheep by nuclear transfer requires fewer than half the animals needed for pronuclear microinjection.

Microinjection of DNA into the pronuclei of fertilized oocytes has been the only practical means of producing transgenic livestock since the method was established in 1985 (*1*). However, only a small proportion (~5%) of animals integrate the transgene DNA into their genome (*2, 3*). In addition, because the timing and site of integration are random, many transgenic lines do not provide sufficiently high levels of transgene expression or germline transmission. The consequent inefficient use of animals and associated high costs are a major drawback to pronuclear microinjection.

In mice, embryonic stem cells provide an alternative to pronuclear microinjection as a means of transferring exogenous DNA to the germline of an animal and allow precise genetic modifications by gene targeting (*4, 5*). However, despite considerable efforts, embryonic stem cells capable of contributing to the germline of any livestock species have not been isolated (*6–11*).

Recently, viable sheep have been produced by transfer of nuclei from a variety of somatic cell types cultured in vitro (*12–14*). We now demonstrate that nuclear transfer from stably transfected somatic cells provides a cell-mediated method for producing transgenic livestock.

We have used a transgene designed to express human clotting factor IX (FIX) protein in the milk of sheep. FIX plays an essential role in blood coagulation, and its deficiency results in hemophilia B (*15*). This disease is currently treated with FIX derived mainly from human plasma. Recombinant FIX produced in milk would provide an alternative source at lower cost and free of the potential infectious risks associated with products derived from human blood.

The transgene construct, pMIX1 (*16*), comprises the human FIX gene, containing the entire coding region (*17*), linked to the ovine β-lactoglobulin (BLG) gene promoter, which has been previously shown to provide a high level of transgene expression in ovine mammary glands (*18*). Analysis of pMIX1 expression in transgenic mice showed that seven of seven female founders expressed FIX in their milk (*19*). The level of expression in two animals (125 μg/ml) exceeded that achieved in previous studies (*20, 21*), indicating that pMIX1 is functional and suitable for introduction into sheep.

Primary strains of ovine cells, termed PDFF (Poll Dorset fetal fibroblast) 1 to 7, were derived from seven day-35 fetuses from the specific pathogen-free flock at PPL Therapeutics (*22*). Sex analysis of each cell strain by the polymerase chain reaction (PCR) (*23*) revealed PDFF5 to be male and

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**Table 1.** Results of nuclear transfer. Nuclear transfer was performed as described previously (*12, 13*). All cells were exposed to a reduced serum concentration (0.5%) for 5 days before use as nuclear donors. PDFF5 cells were used for nuclear transfer at passage 2 or 3, PDFF2 transfected pools at passage 5 to 7, and transfected clones PDFF2-12 and PDFF-13 at passage 7 to 9. Liveborn lambs were defined as those with a heartbeat and able to breathe unassisted at birth.

| Measurement | PDFF5 (non-transfected) | PDFF2 pool | PDFF2-12 | PDFF2-13 |
|---|---|---|---|---|
| Reconstructed embryos | 82 | 224 | 89 | 112 |
| No. developed to morulae or blastocysts | 5 (6.1%) | 22 (9.8%) | 19 (21.4%) | 23 (20.5%) |
| Embryos transferred | 5 | 22 | 19 | 21 |
| Recipients | 2 | 9 | 7 | 6 |
| Pregnancies at day 60 | 2 | 4 | 4 | 1 |
| Fetuses at day 60 (% of embryos transferred) | 3 (60%) | 4 (18.2%) | 6 (31.6%) | 1 (4.8%) |
| Liveborn lambs (% of embryos transferred) | 1 (20%) | 3 (13.6%) | 2 (10.5%) | 1 (4.8%) |
| Nuclear transfer efficiency (% live lambs from reconstructed embryos) | 1.22% | 1.34% | 2.25% | 0.89% |

the other six to be female.

Trial experiments indicated that both PDFF2 and PDFF5 cells could be readily transfected with a *lacZ* reporter gene with the use of the cationic lipid reagent Lipofectamine. PDFF2 cells at passage 1, after 3 days in culture, were cotransfected with pMIX1 DNA and the selectable marker construct PGKneo, and stable transfectants were selected with G418. Because the effects of drug selection and growth as single-cell clones on the ability of cells to support nuclear transfer were unknown, cells were then treated in two ways: One group was grown at high density under G418 selection and then cryopreserved as a pool for nuclear transfer. The other group was plated at low density under G418 selection, and cloned transfectants were grown from isolated colonies (*22*). A total of 24 clones was isolated, of which 21 were expanded for analysis of genomic DNA. Ten clones were found to contain pMIX1 by DNA hybridization analysis (*24*).

Untransfected PDFF2 cells cultured to passage 19 over a period of 80 days exhibited a modal chromosome number of 54, the euploid ovine chromosomal complement. The chromosome number of the four most rapidly growing pMIX1-transfected clones (PDFF2-12, -13, -31, -38) was determined at passage 6 or 7, after an average of 40 days in culture, and that of the uncloned PDFF2 pool was determined at passage 5, after 19 days in culture. Each clone and the pool showed a modal chromosome number of 54, indicating the absence of gross chromosomal instability during culture and drug selection.

We have proposed that induction of quiescence in nuclear donor cells by serum deprivation is necessary for successful nuclear transfer (*12*). After 5 days of culture in medium with a reduced serum content (0.5%), immunofluorescence detection of proliferating-cell nuclear antigen (PCNA), which is an indicator of active DNA replication, showed that none of the cells analyzed was in S phase, consistent with cell cycle arrest (*25*). Restoration of serum content to 10% reversed this effect and cell growth resumed.

Four cell types were used as nuclear donors: untransfected male PDFF5 cells, pooled female PDFF2 transfectants, and two transfected clones, PDFF2-12 and PDFF2-13, which contained $>10$ and $\sim 5$ copies of the pMIX1 transgene, respectively. Transfer of nuclei from each cell type into enucleated oocytes derived from Scottish Blackface ewes was performed as previously described (*12, 13*).

Live lambs were obtained from all four cell types (Table 1). As expected, animals derived from PDFF5 cells were male and those from PDFF2 cells were female. The efficiency of nuclear transfer, expressed as the number of liveborn lambs obtained per 100 reconstructed embryos, varied from 0.89% for PDFF2-13 to 2.25% for PDFF2-12. This efficiency is similar to the value (1.35%) that we obtained previously for nonmanipulated fetal fibroblasts from another breed of sheep (BLWF1) (*13*).

Pregnancies resulting from embryo trans-

**Table 2.** Characteristics of nuclear transfer–derived lambs. Outcomes of 11 pregnancies resulting from nuclear transfer of PDFF donor cells. When judged necessary, labor was induced by injection of dexamethasone at day 153 of gestation; when required, cesarean section (CS) was performed 24 to 52 hours later. The average duration of gestation for the Poll Dorset flock at PPL Therapeutics is 145 days.

| Pregnancy no. | Nuclear transfer donor cell type | Lamb | Gestation (days) | Birth weight (kg) | *neo* | FIX | Sex | Comments |
|---|---|---|---|---|---|---|---|---|
| 1 | PDFF5 | 7LL5 | 147 | 3.8 | | | M | Unassisted birth |
| 2 (twins) | PDFF5 | 7LL6* | 150 | 3.4 | | | M | Stillbirth, one fetus dead for $\leq 1$ week |
| | | 7LL7* | 150 | 3.7 | | | M | |
| 3 | PDFF2 pool | | <80 | | | | | Regressed |
| 4 | PDFF2 pool | 7LL8 | 155 | 7.6 | (+) | (−) | F | Assisted birth because of position of lamb |
| 5 | PDFF2 pool | 7LL9* | 161 | 6.3 | (+) | (−) | F | Induced, CS 52 hours later, died 90 min postpartum, meconium in lung |
| 6 | PDFF2 pool | 7LL12 | 155 | 8.7 | (+) | (−) | F | Induced, CS 52 hours later |
| 7 | PDFF2-12 | 7LL3* | 130 | | | | F | Spontaneous abortion |
| 8 (twins) | PDFF2-12 | 7LL10* | 132 | 3.6 | (+) | (+) | F | Loss of fetal heartbeat, induced, CS, |
| | | 7LL11* | 132 | 4.5 | (+) | (+) | F | stillbirth, one fetus abnormal |
| 9 | PDFF2-12 | 7LL14* | 148 | 3.6 | (+) | (+) | F | Induced, CS 24 hours later, heartbeat, no breathing |
| 10 (twins) | PDFF2-12 | 7LL15 | 155 | 4.6 | (+) | (+) | F | Induced, unassisted birth, 7LL16 |
| | | 7LL16* | 155 | 3.0 | (+) | (+) | F | euthanized at 14 days, heart defect |
| 11 | PDFF2-13 | 7LL13 | 155 | 5.5 | (+) | (+) | F | Induced, unassisted birth |

*Lamb died or was euthanized for animal welfare reasons.

fer were determined by ultrasound scan at about 60 days after estrus, and development was subsequently monitored at regular intervals. Of the original 14 fetuses, 7 were liveborn, as defined by heartbeat and unassisted breathing (Table 2). Postmortem examination of aborted fetuses and dead lambs did not reveal any common factor as a cause of death.

All animals derived from PDFF cells exhibited a prolonged gestation, and, with the exception of animals 7LL5 to 7LL8, labor was induced artificially. Delayed delivery was likely the cause of death of lamb 7LL9. Subsequently, all surrogate ewes were induced at day 153, and, if necessary, cesarean section was performed. Three of 11 pregnancies were twin pregnancies. In two instances (7LL6 and 7LL7 and 7LL10 and 7LL11), the death of one fetus in late pregnancy probably resulted in the death of the sibling.

The birth weight of nuclear transfer–derived lambs whose gestation exceeded 145 days ranged from 3.0 to 8.7 kg, with a mean of 3.7 kg for twins and 5.9 kg for single pregnancies. Poll Dorset lambs in the PPL Therapeutics New Zealand–derived flock have mean weights of 3.75 kg for twins and 5.1 kg for single pregnancies. However, comparison is complicated by the fact that nuclear transfer–derived lambs were gestated in Scottish Blackface surrogate mothers. All animals from PDFF2 cells had an undershot lower jaw that did not interfere with their well-being. This characteristic is a genetic trait that occurs sporadically in the Poll Dorset breed and is considered to be unrelated to nuclear transfer. The PDFF5 lambs did not show this feature.

DNA from nuclear transfer–derived lambs was analyzed for the presence of pMIX1 and PGKneo transgenes (Fig. 1). All fetuses and animals derived from the transfected PDFF2 cells were transgenic. The three animals derived from the PDFF2 pool (7LL8, -9, -12) contained the selectable marker gene but lacked the FIX transgene (Fig. 1, A and B). Fetuses and lambs derived from the cell clones PDFF2-12 (7LL10, -14, -15, -16) and PDFF2-13 (7LL13) contained both the FIX transgene (Fig. 1B) and PGKneo.

Our approach has shown that cell-mediated transgenesis is possible in a mammal other than the mouse. The technique is still in the early stages of development and problems remain to be addressed—in particular, the lack of spontaneous parturition and the incidence of perinatal mortality. However, the mortality rate we observed (46%) was exacerbated by two twin pregnancies in which the death of one lamb in late gestation may have resulted in the loss of the other. The mortality rate for nontwin pregnancies was 28.6%, higher than that occurring after normal breeding (~8%) but similar to that observed after nuclear transfer with embryonic blastomeres (5 to 40%) (*26*). Our data therefore do not suggest any correlation between lamb mortality and extended culture or genetic manipulation of the donor cell. Many types of manipulation of preimplantation embryos—for example, in vitro oocyte maturation and fertilization, in vitro culture, asynchronous embryo transfer, and progesterone treatment of the mother—have been shown to increase fetal morbidity and mortality (*26, 27*). An increased understanding of the interaction between the transplanted nucleus and the host cytoplasm and the relation between the early embryo and the maternal environment, together with improved culture systems, should increase the success of embryo production and manipulation in vitro.

The use of somatic cell donors for nuclear transfer in livestock offers many advantages over pronuclear microinjection. Since 1989, PPL Therapeutics has generated a substantial number of transgenic sheep by pronuclear microinjection. A total of 51.4 animals are required to produce one transgenic lamb by pronuclear microinjection, compared with 20.8 animals in the present study by nuclear transfer, values that differ by a factor of ~2.5 (Table 3). The most important difference is that no recipients are wasted gestating nontransgenic lambs in the nuclear transfer technique.

Gestation of large numbers of nontransgenic embryos represents a major source of inefficiency (*28*). Several schemes have been devised to identify transgenic embryos before embryo transfer, either by detection of the transgene in embryo biopsies by PCR (*29*) or by co-expression of a marker gene (*30, 31*). However, these methods, with the possible exception of that of Takada *et al.* (*30*), are restricted by the persistence of

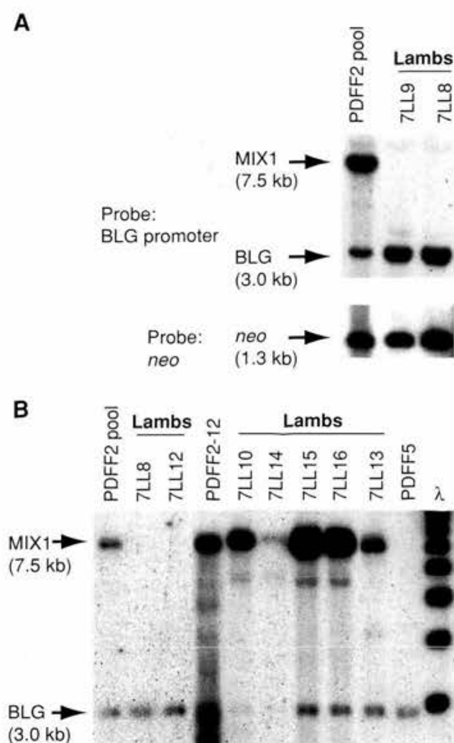


**Fig. 1.** DNA analysis of transfected clones and transgenic sheep. Genomic DNA was isolated from the blood of live animals or tongue samples from dead animals, digested with Bam HI and Eco RI, and subjected to Southern hybridization with either a 1.8-kb fragment of the BLG promoter or the *neo* gene. (**A**) Southern analysis of the uncloned pool of cells (PDFF2 pool), and two lambs (7LL8 and 7LL9) derived from them, for the presence of pMIX1 and PGKneo. (**B**) Assay for the presence of the pMIX1 transgene in lambs derived from the PDFF2 pool (7LL8 and 7LL12) and from the transfected clones PDFF2-12 (7LL10, 7LL14 to 7LL16) and PDFF2-13 (7LL13). PDFF5 cells were not transfected. The positions and sizes of fragments corresponding to the transgenes and the endogenous BLG gene are indicated. The lane marked λ is a 1-kb ladder of phage λ fragments from 3 to 12 kb.

**Table 3.** Comparison of the production of transgenic sheep by nuclear transfer or pronuclear microinjection.

| Parameter | Pronuclear microinjection (1989–1996) | Nuclear transfer of PDFF2 transfectants |
|---|---|---|
| Oocyte donors | 982 | 68 |
| Intermediate recipients* | Not applicable | 14 |
| Final recipients | 1895 | 22 |
| Total number of sheep used | 2877 | 104 |
| Established pregnancies (% of final recipients) | 912 (48%) | 9 (41%) |
| Lambs born | 1286 | 6 |
| Viable transgenic lambs born† | 56 | 5 |
| Percentage of offspring transgenic | 4.35% | 100% |
| Sheep required for production of one transgenic lamb | 51.4 | 20.8 |

*After nuclear transfer, intermediate recipients are used to allow development of reconstructed embryos to blastocyst stage. †Defined as those alive at 1 week of age.

unintegrated DNA during the short time that embryos can be cultured before embryo transfer. In contrast, cells transfected in vitro can be analyzed extensively before effort is devoted to large animals. This advantage will be particularly important in instances in which microinjection is inefficient; for example, with large constructs such as yeast artificial chromosomes.

Delayed integration of microinjected DNA into the embryo genome often results in mosaic founder animals. The reduced rate of transgene transmission resulting from germline mosaicism can hinder or prevent the establishment of transgenic lines from potentially valuable founder animals. In contrast, animals produced by nuclear transfer are entirely transgenic.

Nuclear transfer allows the sex of transgenic animals to be predetermined and thus offers a further twofold increase in efficiency relative to pronuclear microinjection when the sex of the transgenic founder animal is critical. If, for example, the primary interest is the expression of human proteins in milk, the founder generation can be all females. Sheep with different random integrations of the transgene can be produced by nuclear transfer from independent cell clones and the milk analyzed. After a suitable clone has been identified, the corresponding stock of cells can be used to generate an "instant flock" by further nuclear transfer. Such a flock could be superior to those produced by conventional breeding as a source of proteins for human therapy because genetic identity would contribute to the consistency of the medicinal product.

The procedures of transfection, drug selection, and growth from single-cell clones described here are essentially the same as those required for gene targeting. The realistic prospect of targeted genetic manipulation in a livestock species should open a vast range of new applications and research possibilities.

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16. For construction of pMIX1, human FIX DNA from an Nhe I site at position 2982 to a Bst BI site at position 33,840 (positions refer to EMBL database file HSFIXG) was derived from overlapping genomic λ clones. A 3′ fragment from the Bst BI site to position 34,346 was derived by PCR amplification and a Xho I site introduced. The 31.36-kb fragment containing the FIX coding region was assembled in Bluescript KS− vector (Stratagene) that had been digested with Xba I and Xho I. The entire FIX gene was then excised as a Not I–Xho I fragment and inserted into the ovine BLG expression vector pMADS+ that had been digested with Not I and Xho I. The pMADS+ vector consists of 4.2 kb of BLG promoter sequence, 30 bp of 5′ untranscribed region, a multicloning site (Eco RV, Not I, Bgl II, Nhe I, Xho I, Cla I), 170 bp of 3′ untranscribed region including a polyadenylate addition site, and 2 kb of 3′ flanking region cloned into pUC18, containing a modified polylinker to allow excision of the insert with either Mlu I or Pvu I.
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22. PDFF cell strains were isolated by disaggregation of day-35 fetuses and cultured as described previously (*13*). At first passage, $2 \times 10^5$ PDFF2 cells were plated into a 3.5-cm-diameter well and cotransfected with 0.5 μg of PGKneo and 1.5 μg of Mlu I–digested pMIX1 DNA with the use of Lipofectamine (Gibco). Forty-eight hours after transfection, cells were split 1:10 and G418 added to a final concentration of 0.6 mg/ml. Transfected PDFF2 cells achieved subconfluence after selection for 6 days. At the third passage, one portion of the cells was split 1:10 and then subjected to selection for an additional 5 days before cryopreservation as an uncloned population. Other portions were split 1:1000 or 1:5000 and subjected to G418 selection for an additional 7 days. Individual colonies were isolated and expanded for cryopreservation at passage 5, and a portion of each clone was grown further for chromosome counting and Southern (DNA) blot analysis.
23. The sex of strains PDFF1 to PDFF7 was determined by PCR, essentially as described [R. Griffiths and B. Tiwari, *Mol. Ecol.* **2**, 405 (1993); B. W. Kirkpatrick and R. L. Monson, *J. Reprod. Fert.* **98**, 335 (1993)].
24. Genomic DNA isolated from cloned pMIX1 transfectants was digested with Bam HI and Eco RI and subjected to Southern blot analysis with a 1.8-kb fragment of the BLG promoter.
25. PCNA analysis of serum-deprived cells was performed essentially as described [I. R. Kill *et al.*, *J. Cell Sci.* **100**, 869 (1991)].
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32. We thank J. Bracken and M. Malcolm-Smith for technical assistance in large animal work, H. Bowran and D. McGavin for care of the animals, D. Cottom for providing data on microinjection studies, the Animal Sciences Team at PPL Therapeutics for help in deriving the PDFF cells, Y. Gibson and the Small Animal Unit at PPL Therapeutics for generating FIX transgenic mice, S. Bruce for FIX protein analysis, G. G. Brownlee for human FIX genomic λ clones, D. Melton for PGKneo, A. R. Scott for technical assistance with molecular biology, and I. Garner for constructive discussions during the course of the project. The experiments were conducted under the Animals (Scientific Procedures) Act 1986 and with the approval of the Roslin Institute Animal Welfare and Experiments Committee.

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# The Lamb That Roared

A lamb cloned from a single cell of an adult sheep demonstrated the power of cloning technology, surprising both researchers and the public, and igniting a fierce debate about ethics

A year ago, few researchers would have guessed that science's most stunning achievement in 1997 would come from a barn. But in late February, a bleating, white-nosed lamb swept into the public eye: 7-month-old Dolly, the first animal cloned from an adult cell. She electrified both the research community and the general public, for although animals had been cloned before, creating a sheep from a single cell of a 6-year-old ewe was a stunning technological feat that many had thought impossible.

Cloning is of practical import, as it can be used to quickly create herds of identical animals that churn out medically useful proteins; the first such animals—a handful of transgenic sheep clones—are described on page 2130 of this issue. But the implications of cloning technology go much further, opening up new avenues of research in cancer, development, and even aging. Indeed, Dolly forces a reexamination of what it means to grow old, for although she is now 18 months old, her DNA, taken from the donor cell, may be almost 8 years old.

According to conventional wisdom, adult cells cannot give rise to new, mature organisms. So after Dolly's debut, researchers scrambled to understand how she was created. Scientific societies convened their own impromptu meetings to discuss both the scientific and ethical implications of the work, and companies specializing in transgenic animals saw their stock value jump overnight.

But despite Dolly's soft brown eyes, some feared that she was a wolf in sheep's clothing, come to steal humankind's individuality and autonomy. She sparked calls for a ban on human cloning in the United States, Switzerland, China, and other nations, and to some, she raised the sci-fi specter of cookie-cutter clones grown for spare parts. But whether welcomed or feared, cloning in 1997 forced scientists and the public alike to rethink their basic ideas about life, and to confront the implications of our growing ability to manipulate life's blueprint.

As is true for many breakthroughs, cloning represents the convergence of advances in several disciplines over several decades. Painstaking progress in sheep reproductive biology, genetic manipulation, and cell culture all paved the way for Dolly. But the critical technique is nuclear transfer, in which the intact nucleus of one cell is absorbed into an egg whose own nucleus has been removed.

Researchers seeking to unlock the secrets of embryonic development had been working to perfect this technique for 40 years, starting with experiments in frogs in 1952. They transferred the nuclei of embryonic or tadpole cells into frog eggs and succeeded in raising cloned tadpoles and even adult frogs. But the older the frog cell donating the DNA, the less likely was the resulting clone to develop normally. When donor cells from an adult were used, no frog clone ever developed beyond the tadpole stage. And in mice, the typical mammalian model organism, results were even more discouraging. At the time, researchers couldn't get viable young from anything but nuclei taken from very early embryos—the two- to four-cell stage. So most biologists came to accept that mature cells could not give rise to entire organisms, especially in mice. Only an egg cell possessed that mysterious power, called totipotency.

But those working with cows and sheep were not quite persuaded. A team of researchers at the Roslin Institute outside Edinburgh, Scotland, for example, suspected that previous failures were caused by donor DNA that was in a different stage of the cell cycle than the recipient egg cell. They used nuclear transfer to clone sheep from embryonic cells, and in 1996 announced the birth of two cloned lambs. Next, they cloned sheep from fetal fibroblast cells. And in partnership with a local biotechnology company, they attempted what everyone had said was impossible: to clone a sheep from adult cells.

To do this, the team used cultured udder cells, taken from a 6-year-old ewe, and then starved them, forcing most of their genes to enter an inactive phase that the researchers hoped would match the cell-cycle stage of the recipient eggs. Once the udder-cell nuclei were transferred into the eggs, still-unknown factors coaxed that "inactivated" 6-year-old DNA to go back in time, so to speak, and apparently become totipotent once more, directing the eggs to develop into lambs. Out of 277 such eggs, only one produced a healthy living animal: Dolly.

To a startled public, Dolly made the horrors of science fiction clones seem all too possible. If she could be cloned from an udder cell, people wondered, then why not a dictator from his nose, as was attempted in the movie *Sleeper*, or a spare self as a reservoir of replacement body parts? Such things are safely in the realm of fiction, of course, but many people, scientists included, became concerned that cloning people would dehumanize our species and spoke out against it.

Yet upon reflection it's clear that just as identical twins grow up to be individuals, clones would never be truly identical. Even Dolly is not an exact replica of the ewe used to clone her, because she did not develop in that ewe's uterus nor receive its genes in the cellular organelles called mitochondria.

For now, Dolly stands alone. No one, not even the Roslin team, has made a second animal from an adult cell. Of course, most biomedical researchers work with mice—and mouse nuclear transfer results are still dismal. So attention is focused on the handful of labs worldwide working on cloning in livestock. Most are starting with fetal cells, whose DNA can more easily be made totipotent. So far, several firms say they too have cloned either sheep or cows from fetal cells, and one group has cloned monkeys from embryonic cells.

Nuclear transfer experiments are under way in other species too, ranging from zebrafish to rabbits. Among basic researchers, the Scottish group's success has inspired new experiments looking at how DNA changes as a cell matures. Clarifying the nature of totipotency may spark insight into what makes cells and organisms age, and how cell growth can go awry, as in cancer. Researchers are watching Dolly closely, for although so far she seems the 18-month-old she's supposed to be, her DNA may make her age prematurely.

Cloning experts point out that the true identity of Dolly's progenitor cell is not known for sure—it's possible that it was a stem cell, known to be able to develop into several

kinds of tissues. Even if that's true, the ability to restore totipotency to easily harvested adult cells would offer a potentially simple method to replace lost or damaged cells.

Meanwhile, the Roslin team has taken the next step toward making cloning economically useful by cloning sheep carrying foreign genes. Three sheep carry a marker gene, and two have both the marker gene and the gene for the human factor IX protein, which some hemophiliacs take to aid blood clotting. These sheep were cloned from transgenic fetal fibroblast cells, not adult cells, so they are most remarkable not as clones, but because they developed successfully despite having undergone genetic manipulation.

On the drawing board are flocks of sheep that make factor IX and other useful proteins in their milk. Other scientists are developing nuclear transfer techniques to create other types of genetically tailored livestock, opening the door to better animal models of genetic diseases, animals as organ donors, and possibly leaner, faster growing livestock.

Indeed, as with all breakthroughs, it's not possible yet to foretell exactly where cloning will lead. Although initial reactions were universally against all human cloning, there have been whispers that such cloning may one day have a place in giving infertile couples genetic offspring. Whatever direction the research takes, however, the public is likely to demand a say in how cloning is applied. Biologists, ethicists, and others will be wrestling with the implications of this birth in a barn for years to come.

**–Elizabeth Pennisi**