New frontiers in gene targeting and cloning: success, application and challenges in domestic animals and human embryonic stem cells

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Until recently, precise modification of the animal genome by gene targeting was restricted to the mouse because germline competent embryonic stem cells are not available in any other mammalian species. Nuclear transfer (NT) technology now provides an alternative route for cell-based transgenesis in domestic species, offering new opportunities in genetic modification. Livestock that produce human therapeutic proteins in their milk, have organs suitable for xenotransplantation, or that could provide resistance to diseases such as spongiform encephalopathies have been produced by NT from engineered, cultured somatic cells. However, improvements in the efficiency of somatic cell gene targeting and a greater understanding of the reprogramming events that occur during NT are required for the routine application of what is currently an inefficient process. The ability to reprogramme and genetically manipulate cells will also be crucial for full exploitation of human embryonic stem (hES) cells, which offer unparalleled opportunities in human health and biotechnology. Particularly pertinent are directed differentiation of hES lines to specific cell lineages, production of cells that evade the patient's immune system and ensuring the safety of ensuing transplants. This review will discuss some of the successes, applications and challenges facing gene targeting in livestock and hES cells.

One of the fundamental dogmas in biology is that through the processes of transcription and translation, DNA templates can be decoded to produce peptides. This two-step amplification process means that one gene can produce numerous RNA transcripts, each in turn leading to production of many peptides. Consequently, studying gene function by attempting to completely eliminate specific RNAs or proteins is challenging because of the high turnover and numbers of these molecules. By contrast, gene disruption at the DNA level by gene targeting (targeted integration by homologous recombination) is absolute and unambiguous (Capecchi, 1989). However, the precision of gene targeting is not limited simply to gene disruption. Directed insertion or substitution of genetic sequence is also possible. More subtle changes can also be made, for example promoter sequences can be altered to modulate expression or coding sequences can be mutated to study function

of individual domains. Thus, gene targeting potentially enables the genetics of virtually any organism to be altered in a precise manner (Capecchi, 1989).

Gene targeting of an endogenous gene was first reported in mice by Smithies *et al.* (1985). This technology has since provided tremendous insight into a plethora of biological questions in functional genomics, allowed modelling of diseases including cancer and is the major basis of the desire to extend targeting to other species. This review begins by giving a brief account of how targeted modification is achieved in mice. However, pluripotent embryonic stem (ES) cells, which have been central to success in mice, are not available in any domestic species, despite considerable efforts to isolate them. This precluded extension of the technology to livestock until the development of an alternative route for cell-based transgenesis that involved cloning by nuclear transfer (NT; Campbell *et al.*, 1996). This issue will form the second part of the review. There are now several reports describing gene targeting in livestock (McCreath *et al.*, 2000; Denning *et al.*, 2001a; Dai *et al.*, 2002; Lai *et al.*, 2002). However, these successes mask tremendous inefficiencies. Increasing the efficiency of isolation of gene-targeted primary somatic cells and deepening the understanding of the underlying

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reprogramming events that occur during NT will be crucial for routine use of this technology. The final part of the review will consider the newly emerging field of human embryonic stem (hES) cells. The authors will speculate on the feasibility of using NT in 'therapeutic cloning' to produce blastocysts to permit isolation of patient specific hES cells. Finally, it will be proposed that whether hES cell lines are derived from blastocysts produced by NT or IVF, or by alternative methods involving direct reprogramming of somatic cells, gene targeting will play a central role in unlocking the full potential of hES cell lines for uses such as tissue transplantation and directed differentiation.

Development of gene targeting in mammals

Until ES cells were derived from mouse blastocysts, direct 'pro-nuclear injection' into the male pro-nucleus of the zygote was the only method available to modify mammals genetically. Although pro-nuclear injection is a robust technique, the random nature of integration is inefficient, often gives rise to unpredictable transgene expression and suffers from mosaicism such that the transgene is not expressed in all cells of the animal (Clark *et al.*, 1994). The most serious limitation is that pro-nuclear injection provides no means to modify endogenous genes.

Isolation of pluripotent murine ES (mES) cells that can contribute to all tissues of the fetus (Evans and Kaufman, 1981; Martin, 1981) provides a route for precise modification of the mouse genome by gene targeting (Capecchi, 1989). In this approach, the desired modification is introduced into a cloned copy of the chosen gene by standard recombinant DNA technology. The modification is then transferred to the genome of mES cells by means of homologous recombination between the exogenous modified DNA and the cognate DNA sequence in the ES-cell chromosome. Screening procedures such as PCR or Southern blot analysis are used to identify the rare ES cells in which the correct targeting event has occurred. The appropriate cell is then clonally expanded and maintained as a pure population, whereupon these cells are microinjected into the blastocoel of the preimplantation mouse embryo (Fig. 1). This blastocyst is surgically transferred to the uterus of a foster mother, where development is allowed to progress to term. The requirement is to produce a 'germline chimaera', that is, the donor ES cells contribute to a range of tissues, specifically including the germ cells. Breeding from the chimaera produces heterozygous offspring that can be interbred to produce a pure line of homozygous animals with the engineered modification (Capecchi, 1989).

As this strategy allows defined genes to be modified in a precise manner, gene targeting theoretically provides the opportunity to inactivate or alter gene activity at will. This approach has now been used to engineer

mice so that a range of biological processes within the setting of the whole animal can be systematically analysed. For example, knockout mice have been used to establish potential links between female reproduction and energy homeostasis. Mice lacking insulin receptor substrate 2 (IRS-2^{-/-}) are overtly diabetic, displaying a similar pathophysiology to that of human type II diabetes. IRS-2^{-/-} mice also have small, anovulatory ovaries with reduced numbers of follicles and as a consequence are infertile (Burks *et al.*, 2000).

Clearly targeted genetic manipulation of mice has played, and continues to play, an essential role in the understanding of functional genomics. Extension of gene targeting to other mammalian species would have a significant impact on biotechnology and also the understanding of human disease and development.

The need for nuclear transfer

In mice, gene targeting is a relatively routine procedure as ES cells can be maintained in culture for long periods and gene-targeted clones that can contribute to the germ line of chimaeras can be isolated at high efficiency (for example, see Okkenhaug *et al.*, 2002). Given these qualities, isolation of similar pluripotential cells from livestock is likely to permit similar studies to be carried out in these species. However, despite years of effort, stable and reliable ES-cell lines that can contribute to the germ line, a step that is critical for generating engineered animals by this route, have not been isolated from any domesticated animals. For example, Piedrahita *et al.* (1998) isolated pluripotent primordial germ cells from pig fetuses at day 25–27 of gestation. These cells were transfected with a DNA construct and transgenic cells were isolated. Transgenic chimaeras were produced but germline transmission was not reported when injected into recipient blastocysts. Cibelli *et al.* (1998) adopted an alternative approach. By using bovine fetal fibroblasts as donors in nuclear transfer (see below), cloned blastocysts were produced which were used to isolate cultured ES-like cells. In the chimaeras produced from these cells, contribution was seen in several tissues but these did not include the germ cells.

The lack of suitable ES cells represented a considerable barrier to gene targeting in livestock so the discovery that animals could be cloned from somatic cells in culture by NT was met with significant excitement. Although NT had been possible for many years in sheep and cattle using embryonic blastomeres (Willasden *et al.*, 1986; Prather *et al.*, 1987), it was not until the landmark paper by Campbell *et al.* (1996) that this technique could have a significant impact on gene targeting in livestock. Campbell and co-workers demonstrated that NT could reset the developmental programme; viable offspring could be produced from cells that had been maintained in culture and expressed markers of the differentiated state. In this approach, a primary somatic cell was taken

Fig. 1. Comparison of successful gene targeting strategies in mice and livestock. Steps that have been successfully reported for mice (solid arrows) or livestock (broken arrows) are indicated. Routes of transgenesis shown: left, blastocyst injection; right, nuclear transfer (NT). See text for details of these methods. Although gene targeting in mouse embryonic stem (ES) cells has been achieved, there are no similar reports for livestock. The opposite is true for somatic cells, thus generating a need for NT. Gene-targeted heterozygous mice have been produced by both blastocyst injection and NT, whereas heterozygous pigs and sheep have been produced only via NT. Although to date animals have been bred to homozygosity using only the blastocyst injection route, clones are usually fertile and so producing homozygous animals via breeding should also be possible. Alternatively, homozygosity can be achieved by isolating cells from a gene-targeted cloned animal, disrupting the second allele and then using these cells as donors for NT (for example, see Phelps *et al.*, 2003; see text for details).

from a culture dish and injected under the zona pellucida of an enucleated metaphase II oocyte. Through electrical stimulation, the membranes of the oocyte and somatic cell were fused, while also activating the developmental programme. After culturing the embryo to the blastocyst stage, it was transferred to a surrogate mother and allowed to progress to term. Similar techniques have

been successfully used to clone sheep, cows, pigs, mice, goats, rabbits and cats using a range of primary types of cell, including fibroblast, cumulus, Sertoli, gonad, neural, oviduct, uterine, granulosa, mammary, muscle, liver, testis and germ cells, which have been isolated from embryonic, fetal or adult stages of development (see Wilmut and Peterson, 2002).

The success of the NT technique enabled precise engineering of the genome to be extended to animals other than mice (Fig. 1), providing new opportunities in biotechnology and disease modelling. Genetically engineered mice have been fundamental to assigning function to genes and there are now over 2000 published murine gene knockouts (see <http://research.bmn.com/mkmd>). However, these knockouts provide few realistic opportunities to develop clinical treatments, monitor development of the fetus or the developing reproductive system in the fetus and adult, or study the long-term effects on health caused by the engineered mutations. Thus, the major restrictions to modelling human pathology in mice are differences in size, lifespan, physiology and the nature of the reproductive system, including the number of progeny produced.

Sheep have already been used to investigate the effects of maternal nutrition on the resulting offspring. The long gestation period in this species has allowed exploration of the existence of discrete 'windows of sensitivity' to the programming of fetal and juvenile physiological control mechanisms following maternal undernutrition (Heasman *et al.*, 1998). There is also evidence that sheep are superior to mice for modelling certain human diseases. For example, numerous strains of mice have been engineered with a mutation in the *CFTR* gene and so would ideally display the cystic fibrosis (CF) phenotype (reviewed in Harris, 1997). In humans this is primarily characterized by recurrent lung infection and pancreatic destruction. However, CF mice do not present the disease; they have normal pancreatic function and little airway disease in a limited lifespan. Instead, the majority of strains die from intestinal obstruction. By contrast, it is anticipated that sheep would provide a better model. Tissue-specific and developmental patterns of expression of the *CFTR* gene in sheep are very similar to those seen in humans. Furthermore, there are significant anatomical, functional and electrophysiological similarities between the lung in sheep and humans (Harris, 1997).

Modifications to livestock could also be made to influence traits such as growth rate and feed efficiency in agricultural livestock breeding. From a biotechnological standpoint, genes in livestock could be replaced with their human counterparts in order to use them as a source of the protein (reviewed in Clark *et al.*, 2000). Models need not be restricted to farm animals; gene targeting in rabbits will be of importance to the pharmaceutical industry when, for example, cannulation of specific blood vessels is required, a technique that is technically challenging in mice (Fan *et al.*, 1999).

Alternatively, human therapeutic proteins could be produced in the milk of domestic species by directing expression to mammary tissue. Using this approach, Schnieke *et al.* (1997) transfected sheep fibroblasts with a DNA construct encoding human blood-clotting factor IX and selected those with the highest expression for NT. Although the strategy was not designed specifically to

target the transgene to a specific locus, it provided the first evidence that cells could be transfected and selected, while still remaining competent for NT, thus setting the stage for gene targeting in livestock.

Gene targeting in livestock

A major driving force in the development of gene targeting in livestock has been the biotechnology sector. Consequently, gene targeting has so far been directed towards commercial or medical applications. Human alpha-1-antitrypsin (hAAT) is a plasma protein that inhibits elastase, a key enzyme in the inflammatory response that can lead to excessive tissue destruction. Therefore, hAAT is a potential candidate for the treatment of CF lung disease and other conditions in which connective tissue is broken down irreversibly. McCreath *et al.* (2000) adopted a gene-targeting strategy to insert a transgene encoding hAAT into the 3' untranslated region of the $\alpha 1(I)$ procollagen (*COL1A1*) locus. The design of the construct enabled high expression of hAAT in the milk of the transgenic sheep.

This gene-targeting strategy involved specific insertion of a transgene rather than deletion of endogenous coding sequence and hence gene function, which was the aim of the current authors' strategy. The production of sheep fetuses and lambs that carried targeted disruptions in one allele of either the *PrP* gene, which encodes prion protein, or the *GGTA1* gene, which encodes $\alpha(1,3)$ -galactosyl transferase, was reported (Denning *et al.*, 2001a). Prions are a novel form of infectious agent that cause spongiform encephalopathies in humans and animals (Bueler *et al.*, 1992). As sheep and particularly cattle have functional *PrP* genes and are used to produce biomedical products such as gelatin, collagen and, increasingly, human proteins after genetic modification, it may be appropriate to produce prion-resistant populations. Galactosyl transferase is responsible for the presentation of galactose- $\alpha(1,3)$ -galactose (the gal epitope) on the extracellular surface of cells of all organisms, with the exception of catarrhines (Old World monkeys, apes and humans), which have lost the function of the *GGTA1* gene (Galili *et al.*, 1985). The difference in cell-surface epitopes is believed to be a major cause of hyperacute rejection of livestock organs xenografted to humans, and so eliminating the gal epitope could alleviate some aspects of the rejection stimulus.

Although certain tissues in a gal-knockout sheep would be useful for xenotransplantation and for assessing the extent to which this epitope is responsible for rejection, considerable efforts have been made to create similar genetic disruptions in pigs due to their close similarity in organ size and physiology to humans, and large litter size. Two independent groups (Dai *et al.*, 2002; Lai *et al.*, 2002) reported production of live pigs in which one allele of the *GGTA1* gene had been disrupted. Importantly, Lai *et al.* (2002) sought

Table 1. Efficiency of gene targeting in primary livestock fibroblasts

Target gene	Number of cells transfected	G418 resistant colonies ^a	Colonies confirmed as gene targeted ^b	Absolute targeting frequency ^c	Reference
Sheep					
<i>COL1A1</i>	5×10^5	70	46 (66%)	9×10^{-5}	McCreath <i>et al.</i> , 2000
<i>PrP</i>	5×10^6	533	5 (1%)	1×10^{-6}	Denning <i>et al.</i> , 2001a
<i>GGTA1</i>	1×10^7	1445	25 (1.7%)	2.5×10^{-6}	Denning <i>et al.</i> , 2001a
Pigs					
<i>GGTA1</i>	4.9×10^7	159	8 (5%)	1.6×10^{-7}	Lai <i>et al.</i> , 2002
<i>GGTA1</i>	8×10^6	1105	17 (1.5%)	2.1×10^{-6}	Dai <i>et al.</i> , 2002

^aG418 drug selection allows isolation of cells that have been stably transfected with the targeting construct and are expressing the neomycin phosphotransferase drug resistance gene. Most colonies will result from random integration of the targeting vector; only some will be correctly targeted.

^bParentheses indicate the ratio of confirmed gene-targeted colonies to G418 resistant colonies.

^cAbsolute targeting frequency expresses number of targeting events on a per cell basis, demonstrating the low frequency of the targeting process.

to achieve this in a line of pigs that had long been used for xenotransplantation studies and that consistently tested negative for transmission of porcine endogenous retrovirus to human cells *in vitro*. Ultimately, these studies paved the way for the production of gal-knockout pigs. Phelps *et al.* (2003) attempted to target the disruption of the second *GGTA1* allele by gene targeting and selecting for gal-negative cells with toxin A from *Clostridium difficile*, which has a cytotoxic effect on gal-positive cells. However, inactivation of the second allele did not arise by gene targeting; rather it occurred by a spontaneous T > G transversion that was selected for by toxin A. These gal-knockout pigs should now permit a full assessment of any benefit on hyperacute rejection and the subsequent delayed rejection mechanisms.

However, these successes mask the tremendous inefficiencies of producing gene-targeted livestock. Significant demands are placed on primary somatic cells during gene targeting. The efficiency of homologous recombination in these cells compares with the lower end of the range reported for mES cells (Templeton *et al.*, 1997). As a result, it is virtually a necessity to use promoterless-type gene-targeting strategies (Clark *et al.*, 2000; Harrison *et al.*, 2002), which enrich for correct targeting events by 100- to 500-fold, because drug-resistant colonies are only produced when construct integration occurs in frame and downstream of an active promoter. Although this approach has enabled gene-targeted colonies to be detected at frequencies of 1–66% (Table 1), primary cells have a limited lifespan that is further compromised by growth from clonal density. This means few have sufficient vigour to be successfully prepared for NT. Indeed, of 25 targeted colonies identified in experiments targeting the *GGTA1* gene in sheep (Table 1), 23 senesced before they could be sufficiently expanded for use in NT (Denning *et al.*, 2001a), thus reducing the effective targeting efficiency

by 10-fold (Denning *et al.*, 2001b). Lai *et al.* (2002) attempted to circumvent this issue in pigs by reducing the overall time in culture and using targeted donor cells immediately on thawing from cryopreservation.

Extended culture and clonal selection of genetically modified cells may also impact on the efficiency of production of viable animals by NT. 'Normal' NT from unmodified donor cells typically has a success rate of between 0 and 4% of reconstructed embryos developing to become surviving young (Wilmut *et al.*, 2002). The figure is 0–0.5% in reports using gene-targeted cells (Table 2). In a similar way to unmodified clones that fail to survive, those carrying targeted modifications display a range of anomalies, including defects in the cardiovascular system, liver, kidneys or placenta (De Sousa *et al.*, 2001; Wilmut *et al.*, 2002).

Improving gene targeting in livestock

As the issues highlighted above hamper the efficient production of gene-targeted livestock, methods are required either that achieve more efficient gene disruption so less cell population doublings are needed and less proliferative capacity is used, or that allow the somatic nucleus to be more efficiently reprogrammed. For successful NT, the donor nucleus must be returned to an embryonic-like state after brief exposure to the oocyte cytoplasm. This remodelling process involves a loss of about 85% of protein from the donor nuclei concomitant with substantial uptake of proteins from the egg cytoplasm (Gurdon *et al.*, 1979). As a result, the somatic nucleus experiences major changes in epigenetic status, which includes modification of chromatin structure and patterns of methylation. Consequently, major efforts to characterize the remodelling process are underway. In *Xenopus* egg extract, nucleosomal adenosine triphosphatase (ATPase) ISWI, a member of

Table 2. Rates of developmental progression from gene-targeted primary somatic fibroblasts after cloning by nuclear transfer

Target gene	Reconstructed embryos	Blastocyst	Fetuses at day 60	Lambs at birth	Lambs > 6 months of age	Reference
Sheep						
<i>COL1A1</i>	417	80 (19.2%)	20 (4.8%)	14 (3.4%)	2 (0.5%)	McCreath <i>et al.</i> , 2000
<i>PrP</i>	454	47 (10.3%)	8 (1.8%)	3 (0.7%)	0 (0%)	Denning <i>et al.</i> , 2001a
<i>GGTA1</i>	265	55 (20.7%)	7 (2.6%)	0 (0%)	0 (0%)	Denning <i>et al.</i> , 2001a
Target gene	Reconstructed embryos	Pregnancies at day 25	Pregnancies at day 45	Pigs at term	Pigs surviving	Reference
Pig						
<i>GGTA1</i>	3104	28 (0.9%)	9 (0.3%)	7 (0.2%)	4 (0.1%)	Lai <i>et al.</i> , 2002
<i>GGTA1</i>	2513	11 (0.4%)	ND	5 (0.2%)	5 (0.2%)	Dai <i>et al.</i> , 2002

All percentages in parentheses relate to numbers of reconstructed embryos.

Animal survival is 0–0.5% using targeted cells as donors for nuclear transfer; using unmodified cells the figure is 0–4%.

ND: not determined.

the SWI2/SNF2 superfamily, was identified as a key molecule in chromatin remodelling of *Xenopus* epithelial cells (Kikyo *et al.*, 2000). Furthermore, Tada *et al.* (2001) demonstrated reprogramming of the thymocyte genome. Oct 4 is a protein produced during early development. It is not produced in thymocytes, yet a transgene encoding Oct 4–green fluorescent protein engineered into the thymocyte genome was reactivated upon fusion with mES cells. In addition, fusion also caused the inactive X chromosome in the thymocyte to adopt some characteristics of an active X chromosome. Håkelién *et al.* (2002) analysed the characteristics of human 293T fibroblasts that had been exposed to nuclear and cytoplasmic extract from human T cells. There was evidence of nuclear uptake and assembly of transcription factors, induction of activity of a chromatin remodelling complex, histone acetylation, and activation of lymphoid cell-specific genes. These reports are encouraging in that they indicate that it may be possible to pre-treat somatic cells with reprogramming factors before NT; however, it is likely to be some time before they translate into real improvements in the current low efficiency of viable cloned animals.

One of the significant challenges to be overcome in gene targeting in livestock is the limited lifespan of primary cells in culture. It has been estimated that a total of about 45 population doublings are required to generate targeted cells from fetal tissue using a combination of electroporation, drug selection and high-throughput screening (Clark *et al.*, 2000). Although this appears well within the lifespan of bulk populations of sheep fibroblasts, which can divide up to about 100 times, it is a somewhat artificial comparison because of the heterogeneous nature of the primary culture, and in reality only represents the longevity of the best cells. In fact, from studies in human cells, it has been estimated that cells decline in replicative capacity from the time of explantation and do so in a stochastic manner with

a half-life of only about eight doublings (Rubin, 2002). Therefore, as gene targeting protocols in livestock cells use early passage primary cultures, it is not surprising that most colonies fail to proliferate sufficiently to be used in NT and those that do are close to senescence. Increasing the lifespan of the cultured cells would expand the window of opportunity available for gene targeting. For example, primary human fetal foreskin or lung cells can divide an additional ten times if treated with L-carnosine (McFarland and Holliday, 1999). The authors' own research indicates that the superoxide dismutase mimetic, MnTMPyP, can increase longevity in sheep fetal fibroblasts (C. Denning, unpublished). The use of low oxygen tensions (2%) rather than standard ambient (20%) conditions has been reported to promote the proliferation of pig fetal fibroblasts from single cells, perhaps by reducing the effect of oxidative damage to DNA that may contribute to replicative senescence (Harrison *et al.*, 2002). Finally, when telomeres fall below a critical length, chromosome abnormalities occur more frequently and replicative senescence occurs (Counter *et al.*, 1992). The restoration of telomeres and proliferative capacity in late passage cells to a longer 'juvenile' state by NT (Lanza *et al.*, 2000) has enabled knockout of both *GGTA1* alleles in pigs (Phelps *et al.*, 2003). Furthermore, the finding that ectopic expression of human telomerase can maintain telomere length and extend the lifespan of many primary human cells without transformation (Bodnar *et al.*, 1998) may be important for somatic-cell manipulation.

The promoterless gene-targeting strategy is the only method that has been reliably used in primary somatic livestock cells. Although this strategy provides a method to enrich for targeting events, it limits the approach to those genes that are actively transcribed in fibroblasts, currently the only validated type of cell with sufficient longevity to allow selection of gene-targeted cells for NT (Denning *et al.*, 2001b). However, primordial germ

cells have been isolated from the bovine genital ridge and have displayed a proliferative capacity of up to 85 population doublings (Forsberg *et al.*, 2002). If these cells can support gene-targeting events, they would significantly expand the repertoire of genes open to precise modification. The use of recombinant adeno-associated virus (AAV) has been reported to result in high efficiency (up to 1% of the cells in culture) targeted insertion of a functional transgene into the hypoxanthine phosphoribosyl transferase (*HPRT*) and type I collagen (*COL1A1*) genes in normal human fibroblasts without drug selection or enrichment (Hirata *et al.*, 2002). Although the AAV vectors used in this study were tested only in human cells and were restricted to carrying a maximum of 4 kb of insert, this approach appears sufficiently promising to warrant further testing in livestock cells.

In specific instances, such as developing ovine models of CF, it may be preferable to reduce the extent of gene function rather than eliminate it *per se*. In mammalian systems, short regions of double stranded RNA less than 30 bp in length are recruited by the RNA-induced silencing complex, leading to specific cleavage of homologous RNAs in a process known as RNA interference (RNAi) (Hammond *et al.*, 2001). Until recently this approach was confined to short-term, transient assays. However, several groups have developed plasmid-based systems using the RNA polymerase type III promoters U6 and H1 to drive expression of short RNAs (shRNAs; Brummelkamp *et al.*, 2002; Lee *et al.*, 2002). Importantly, plasmid and lentiviral vectors expressing shRNAs are effective at long-term silencing of gene expression in mES cells and transgenic mice (Kunath *et al.*, 2003; Robinson *et al.*, 2003). Although RNAi is yet to be tested in livestock, the approach may be extended to gene knockdown in these species.

A solution that could potentially address all of the issues raised above would be isolation of ES cells from livestock species. If these cells could be isolated and were to display characteristics similar to mES cells, such as long lifespan, high efficiencies of homologous recombination and contribution to the germ line in chimaeras, then gene targeting in livestock could be achieved by using ES cells in either the blastocyst injection route or NT, both of which are possible in mice (Fig. 1; Smithies *et al.*, 1985; Rideout *et al.*, 2002). Unfortunately, isolation of livestock ES cells remains a challenging goal.

Human ES cells

In contrast to livestock, isolation of hES cells has been achieved. Although stem cells have been isolated from other sources, such as cord blood, bone marrow and other adult tissues, this review will focus on hES cells. First derived from the human blastocyst in 1998 (Thomson *et al.*, 1998), hES cells are a unique

population (Fig. 2) that meets several key requirements. First, the cells can be propagated indefinitely *in vitro* in the primitive undifferentiated state. Second, even after long periods of cultivation *in vitro* the cells can differentiate spontaneously into multiple types of cell, including representatives of all three embryonic germ layers, both *in vivo* in teratomas after grafting in severe combined immunodeficient (SCID) mice or *in vitro* under appropriate conditions (Fig. 2). Third, they are stably diploid and karyotypically normal *in vitro*.

Potentially, human cells with these characteristics offer many opportunities: new approaches to the study of human embryonic development, including birth defects and embryonal tumours; access to human embryonic cells on an unparalleled scale for use in gene expression and genomics; creation of *in vitro* human disease models for basic research, drug discovery and toxicology; and new delivery systems for gene therapy. A major initiative for hES cells is to direct their differentiation to specific lineages for transplantation, and hence address the current chronic shortage of tissue available for treatment of degenerative diseases. A critical issue, as with most transplants, is graft rejection.

Therapeutic cloning is often suggested as a route to reducing the extent of rejection (Colman and Kind, 2000). In this approach, the patient's own healthy cells, for example from the skin, would be used as donors in NT (Fig. 2). The sole purpose of the blastocysts created from this procedure would be to derive immune compatible hES cells that could be differentiated *in vitro* to replace the diseased tissue in the patient, for example, dopaminergic neurones in the case of Parkinson's disease. Rideout *et al.* (2002) demonstrated not only the utility of this approach in mice, but also extended it to include correction of a genetic defect. Somatic nuclei from the tail tip of mice carrying a homozygous mutation in the *Rag2* gene, which results in complete absence of B and T cells in the lymphoid organs, were used as donors in NT. After development to the blastocyst stage, ES cells (NT-ES) were isolated and propagated *in vitro*. Genetic correction of the damaged *Rag2* gene was then achieved by gene targeting in the NT-ES cells. These cells were differentiated to a haematopoietic stem cell (HSC) lineage, which restored immune function when transplanted into adult *Rag2* mutant mice.

Although this approach represents a major advance, there are a number of issues that remain unresolved. For example, the authors note that the extent of engraftment of the *in vitro*-derived HSC was low because of rejection by natural killer immune cells, and they speculate that even genetically matched cells derived from therapeutic cloning may face barriers to effective transplantation (Rideout *et al.*, 2002). Furthermore, NT produces only a genomic copy of the donor cell, so peptides derived from mitochondria contributed by the oocyte may also stimulate rejection, although preliminary studies have questioned the validity of

Fig. 2. Isolation and potential uses of human embryonic stem (hES) cells. There are three routes by which hES could be produced: (1) from blastocysts produced by IVF; (2) from blastocysts produced by 'therapeutic cloning' by nuclear transfer (NT) from somatic cells in culture; and (3) by directly reprogramming somatic cells using exogenous factors. All of the approximately 70 hES cell lines established worldwide have been produced from IVF blastocysts; (2) and (3) above have not yet been achieved. The inner cell mass (ICM) is removed from the blastocyst and placed into culture conditions that favour propagation of these embryonic cells. The hES cultures produced can be differentiated *in vivo* in severe combined immunodeficient mice or *in vitro* to produce representatives of all three embryonic germ layers. With hES this includes tissues of the fetus and placenta, unlike murine ES cells, which usually contribute to tissues of fetal origin only. There are many potential uses for differentiated derivatives of hES cells, including direct applications in human health, reduction of the number of animals used in biomedical research and as research tools in development and functional genomics.

this concern (Lanza *et al.*, 2002). However, there are significantly more formidable barriers: overcoming the technical challenge of performing the inefficient process of NT with limited supplies of human oocytes, ethical concerns and the financial implications of providing totally characterized, individualized hES cell-based patient therapy and achieving this on a timescale that will benefit a critically ill patient. Whether NT, and hence some of the above issues, can be bypassed by using cellular reprogramming with exogenous factors (see Fig. 2 and previous discussion) is a matter of intense scientific interest. However, even if this approach does become feasible, safety, financial and timescale issues would still remain, raising the question of whether patient-specific therapy is a realistic goal.

Other alternatives must therefore be considered concurrently. A representative bank of hES cells corresponding to a large range of human leukocyte antigen (HLA) types would allow close rather than exact transplant matching. In association with the National Institute for Biological Standards and Control, the Medical Research Council and Biotechnology and Biological Sciences Research Council have established the first international stem-cell bank, which should be in operation

by mid-2003 (<http://www.nibsc.ac.uk/divisions/cbi/stemcell.html>). Although the size of stem-cell bank required to provide a wide enough range of tissue types to supply the population is not precisely known, extrapolation from a different source, the US National Marrow Donor Program, Minneapolis, may give an indication. In 1995 this programme had access to over 1.9 million HLA-typed donors (Beatty *et al.*, 1995), which matched 70% of patients, but under-representation of racial minorities meant only 34% of Afro-American patients were matched. By any standards, generating comparable numbers of hES lines would be challenging. Thus, an alternative suggestion is to recruit selected HLA-typed individuals to act as somatic-cell donors for NT to supply specific HLA-typed embryos and reduce the size requirement for a stem-cell bank (Bradley *et al.*, 2002).

Selecting strategies that allow differentiated derivatives of hES cells to evade the immune system may further condense banking initiatives. Derivation of hES lines from blood group O embryos and inducing donor-specific tolerance via a chimaeric haematopoietic system may alleviate graft rejection. Bradley *et al.* (2002) reviewed these topics recently and readers are directed

to this resource. Alternatively, it may be necessary to 'neutralize' HLA antigens by genetic modification to prevent expression of major histocompatibility class (MHC) I and II molecules (Bradley *et al.*, 2002). This may go some way to providing a universal donor cell line suited to a large number of patients, although it may be associated with its own dangers.

Genetic modification as a tool for providing clinically relevant, functional and safe types of cell will also be important in realising other opportunities for hES cells as therapeutic agents. hES cells have been induced to form embryoid bodies which can differentiate and form types of cell of all three embryonic germ layers (Itskovitz-Eldor *et al.*, 2000). Differentiation to specific types of cell of developmental (trophoblast; Xu *et al.*, 2002a) and clinical interest has been demonstrated, with the formation of cardiomyocytes (Mummery *et al.*, 2002; Xu *et al.*, 2002b), insulin-producing cells (Assady *et al.*, 2001), haematopoietic cells (Kaufman *et al.*, 2001; Chadwick *et al.*, 2003), endothelial cells (Levenberg *et al.*, 2002) and cells of neuronal origin (Carpenter *et al.*, 2001; Schuldiner *et al.*, 2001; Zhang *et al.*, 2001). However, most differentiation protocols result in a non-pure, heterogeneous population of cells, containing not only the desired type of cell but also a wide range of other cells, potentially including undifferentiated ES cells. As undifferentiated hES cells form teratomas upon injection into SCID mice (Thomson *et al.*, 1998), there are serious safety concerns about transplantation where there is risk of contamination by undifferentiated cells. Additionally, to ensure a pure and substantial source of the desired types of cell before transplantation, it is likely that the desired differentiated types of cell will need to be purified from the heterogeneous population.

Ectopic expression of transgenes to direct differentiation has been successful in other systems. For example, expression of human bone morphogenetic protein-2 (BMP-2) cDNA in a multipotential murine embryonic mesenchymal cell line is highly effective in directing cells towards a chondrocyte phenotype (Carlberg *et al.*, 2001), whereas expression of HoxB4 in mouse ES cells induces differentiation to haematopoietic stem cells (Kyba *et al.*, 2002). Once the desired types of cell are present in a differentiated culture, they can be purified by expressing selectable genes from a tissue-specific promoter, a technique already applied experimentally to mES cells to isolate cardiomyocytes at 97% purity (Muller *et al.*, 2000). Conversely, markers that lead to cell ablation can be used under the control of promoters specific to undifferentiated hES cells in order to address the safety concerns about these potentially tumorigenic cells.

Gene targeting will certainly be required in many of these applications, for example disruption of specific genes involved in MHC expression. For others, random integration of a transgene may be sufficient. However, as random addition often suffers from mosaicism and

unpredictable gene expression (Clark *et al.*, 1994), gene targeting to a known non-essential locus, not subject to repressive influences, would be preferable.

Development of techniques for transgenesis and gene targeting in human ES cells is rapidly progressing. Exogenous DNA has been introduced into hES cells using electroporation (Eiges *et al.*, 2001; Zwaka and Thomson, 2003) and lipofection (Eiges *et al.*, 2001), or viral transduction with lentiviral (Pfeifer *et al.*, 2002) and adenoviral (Smith-Arica *et al.*, in press) vectors. Gene targeting has now been reported in human ES cells (Zwaka and Thomson, 2003). Electroporation of targeting constructs directed to selectable (*HPRT1*) and non-selectable (Oct 4 or *POU5F1*) loci resulted in homologous recombination at both, although lipofection-mediated transfection did not. Importantly, targeting frequencies were similar to those associated with mouse ES cells. Targeted cells had a normal, undifferentiated hES morphology and continued to express Oct 4. Although these data are very encouraging, further reports investigating the pluripotentiality and karyotypic stability of gene-targeted hES cells are required. Additionally, both loci targeted by Zwaka and Thomson (2003) are expressed genes and although non-expressed genes are routinely targeted in mouse ES cells (Johnson *et al.*, 1989), this goal has remained elusive in somatic cells so requires investigation in hES cells.

Conclusions

Targeted genetic modification in mice revolutionized functional genomics in the 1980s. With the development of NT from cultured somatic cells in 1996 and the isolation of hES cells from human blastocysts in 1998, we may be on the verge of another scientific revolution. Both these new fields are currently technically challenging. However, the first insights into the underlying events that occur during NT are emerging. With further development, this will not only be significant to reprogramming of somatic cells from domesticated animals but also in the dedifferentiation of human somatic cells to hES cells, which in turn will be directed to specific differentiated lineages. The role of targeted genetic modification is clear too; it will play a major role in most areas of hES-cell research, from immune compatibility and safety of transplants to directed differentiation and *in vitro* modelling of human diseases. With sufficient resources, these technologies will make the imagination the limit.

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