Gene targeting in livestock

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The development of nuclear transfer from tissue culture cells in livestock made it possible in principle to produce animals with subtle, directed genetic changes by in vitro modification of nuclear donor cells. In the short period since nuclear transfer was first performed, gene targeting in livestock has become a reality. Although gene targeting has immediate potential in biotechnology, it is unclear whether there are practical agricultural applications, at present. The first livestock targeting experiments have been directed at engineering animals either to render their organs immunologically compatible for human transplantation, or for improving the commercial production of recombinant proteins in the transgenic mammary gland. All successful examples of targeting have involved target loci that are expressed in the nuclear donor cell line. Two important barriers to the further development of this technology are adapting protocols for non-expressed genes and modifying procedures to enhance the lifespan of targeted cells in vitro. This review provides data that illustrate the difficulty in targeting non-expressed genes and discusses some of the practical issues associated with providing targeted nuclear donor cells that are competent for nuclear transfer.

Introduction

In 1982, Palmiter and colleagues reported the birth of transgenic mice that expressed human growth hormone and that grew to a mature size of up to twice that of their non-transgenic littersmates. This report was met with mixed reaction. Within the agricultural research community some researchers saw potential for improving the efficiency of animal production. However, when similar experiments were repeated in livestock, the resulting transgenic pigs showed minimal improvement in feed efficiency and suffered a variety of side effects with serious consequences for animal welfare (Hammer et al., 1985). A summary of the significant milestones in the development of transgenic technology is shown (Fig. 1). Arguably, there are still no good examples of transgenic approaches leading to agricultural improvement and it has really been the pharmaceutical/biotechnology potential of transgenic farm animals that has fuelled the further development of this technology. The production of pharmaceutical proteins in the mammary glands of transgenic sheep and cattle has developed into a new
industry and has underpinned the development of cloning of large animals by nuclear transfer from somatic cells. Although the announcement of the birth of Dolly in 1997 after nuclear transfer from an adult cell (Wilmut et al., 1997) was widely seen as a prelude to possible human cloning, Dolly was actually inspired by a very practical interest — development of...
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Cell-based transgenesis to facilitate gene targeting in livestock. The search for embryonic stem cells in livestock has also been motivated by this same interest in gene targeting. Why gene targeting? Why stem cells? Should we revisit the potential of these technologies in animal production?

A full review of the potential applications of gene targeting in livestock is beyond the scope of this paper. Indeed, a more appropriate source of such a review would be those scientists more directly involved in agricultural research of which there are several examples (Piedrahita, 2000; Di Berardino, 2001). The main objective of the present review is to describe the ‘state of the art’ of gene targeting in livestock and hopefully to stimulate some discussion of the potential for this technology in animal production and reproduction.

**Cell-based transgenesis**

The introduction of transgenes to mammalian embryos by pronuclear injection is an imprecise technique, which has changed little since its inception. Pronuclear injection is also inefficient as only 1–10% of injected transferred livestock embryos give rise to transgenic offspring. Perhaps the greatest deficiency of this technique is that there is no experimental control over the site of incorporation of the transgene or of transgene copy number. Hence the level and pattern of expression is highly variable. In practice, up to ten transgenic founders may be required to ensure that one animal has appropriate expression. The main difficulty with this technique is the unpredictable transgene expression, which arises as a direct consequence of random integration. It would be preferable to direct single copy transgenes to a specific site in the genome where the pattern and level of transgene expression can be predicted — or to make use of existing transcriptional machinery by targeting an open reading frame into an appropriate endogenous promoter. This is the experimental process of ‘gene targeting’ in which the inclusion in a transgenic construct of regions of homology to endogenous sequence mediates rare insertion events by homologous recombination.

The introduction of transgenes to tissue culture cells (cell-based transgenesis) rather than to embryos allows the use of mass methods of transfection, such as electroporation, in which thousands of transgenic clones are generated instantaneously. This procedure provides the opportunity to generate precise genetic change simply because it is adaptable to the development of high throughput rapid screening for events such as homologous recombination that occur at low frequency. The obvious disadvantage of cell-based transgenesis per se is that transgenic animals need to be generated from the genetically modified cell lines. In principle, there are now two ways in which this can be achieved: embryonic stem cells and nuclear transfer.

**Embryonic stem cells**

In 1981, Evans and Kaufman showed that permanent undifferentiated cell lines could be derived from early mouse embryos. These so called embryonic stem (ES) cells were characterized by a remarkable set of properties: (i) they could be cultured indefinitely without differentiation and without the accumulation of karyotypic abnormalities, and (ii) when reintroduced to the preimplantation embryo, they would resume their normal differentiation programme and participate in normal development, including contribution to functional germ cells. Germ line competence is referred to as totipotentiality. Hence, totipotential ES cells offered an alternative, cell-based, route to whole animal (germline) transgenesis. The murine ES system has now become a routine laboratory tool for the generation of ‘knockout’ mice in which gene function can be investigated by studying the developmental consequences of disrupting the target
Table 1. Comparison of gene targeting efficiency in fibroblasts of sheep fetuses

| Targeted locus                        | McCreath et al. (2000) | Denning et al. (2001a) | M. M. Marques, A. J. Thomson and J. McWhir (unpublished) |
|---------------------------------------|------------------------|------------------------|-----------------------------------------------------------|
|                                       | α1-procollagen         | α(1,3)-galactosyl transferase (GGTA1) | α1-procollagen                                              |
|                                       |                        | prion protein (PrP)     |                                                            |
| Promoter trap                         | Yes                    | Yes                    | Yes                                                       |
| Transfection method                   | Lipofection            | Electroporation         | Electroporation                                             |
| Number of cells                       | $5 \times 10^5$        | $5 \times 10^6$         | $1 \times 10^7$                                            |
| Amount of DNA                         | 6 μg                   | 10 μg                  | 50 μg                                                     |
| Dilution plating in wells             | No                     |                        | Yes                                                       |
| 96-wells (2.5 $\times 10^3$ cells per well) |                        |                        | 24-wells (7 $\times 10^3$ cells per well)*                  |
| Selection                             | G418 (800 μg ml$^{-1}$) | G418 (400 μg ml$^{-1}$) | G418 (600 μg ml$^{-1}$)                                     |
| 48 h after transfection               | 24 h after transfection |                        | 48 h after transfection                                     |
| Ring cloning                          | Yes                    | No                     | No                                                        |
| Resistant colonies                    | PDFF2 (COLT1) $\sim$ 200 | BW6F2 (PrP) 533         |                                                            |
|                                      | PDFF5 (COLT1) $\sim$ 200 | BW6F2 (GGTA1) 877      |                                                            |
|                                      | PDFF2 (COLT2) $\sim$ 200 | G65F4 (GGTA1) 568      |                                                            |
| Targeted events (PCR)                 | 5 out of 36 (14%)      | 55 (10.3%)             | 36 out of 43 (83.7%)                                       |
|                                      | 4 out of 56 (7%)       | 10 (1.1%)              | 28 (3.8%)                                                 |
|                                      | 46 out of 70 (66%)     | 35 (6.2%)              |                                                            |

*Only half of the electroporation was plated out in 24-well plates.
gene. Attempts to develop a similar system in livestock species have invariably floundered at the germ line transmission step. Although there are numerous reports of the development of ES-like lines with apparent multipotentiality in vitro, no putative ES lines have given rise to functional ES-derived germ cells (germline transmission) in chimaeric animals from any species other than the mouse. Until the development of nuclear transfer this represented an absolute barrier to gene targeting in species other than mouse.

**Nuclear transfer and gene targeting**

Transgenic sheep (Schnecke et al., 1997), calves (Cibelli et al., 1998; Brink et al., 2000) and goats (Keefe et al., 2001) have all been generated by nuclear transfer from transgenic fetal fibroblasts, modified by the addition of transgenes. This technique involves the fusion of a genetically modified nuclear donor cell with an enucleated oocyte to generate a 'reconstructed embryo', which is then transferred to a recipient female after a period of in vitro culture. More recently several groups have shown the successful generation of gene targeted nuclear donor cells (Tables 1 and 2) with insertions directed to the ovine α1(I) procollagen (Col1A1) locus (McCreath et al., 2000), the ovine α1,3 galactosyltransferase (GGTA1) and prion protein (PrP) genes (Denning et al., 2001a), and the porcine GGTA1 gene (Dai et al., 2002; Harrison et al., 2002). Crucially, only Col1A1 targeted ovine cells gave rise to viable animals that subsequently survived to adulthood. Slightly more encouraging are the porcine data (Dai et al., 2002) in which five of seven liveborn piglets survived to adulthood; however, it is perhaps premature to conclude that this represents a true species difference.

A particular difficulty in the development of targeting procedures for fetal fibroblasts is premature senescence, which frequently makes it impossible to confirm the targeted event by Southern blot analysis before nuclear transfer. GGTA and PrP targeting in sheep, for example, and GGTA in pigs (Denning et al., 2001a,b; Harrison et al., 2002) could not be confirmed by Southern blot analysis. In the former example, targeting was confirmed only post hoc by analysis of fetal tissue, but crucially this depended upon prior nuclear transfer in the absence of that confirmation.

**Practical aspects of gene targeting in livestock**

**Association of developmental abnormality with the modification of nuclear donor cells**

Nuclear transfer is associated with developmental abnormalities, such as increased birth weight, longer gestation period, high perinatal mortality, congenital malformations and increased abortion rate (Van Reenen et al., 2001). Collectively, these effects are referred to as large offspring syndrome (LOS). This phenomenon is usually attributed to incomplete reprogramming, but may also arise as a result of accumulation of subkaryotypic abnormalities in cultured nuclear donor cells, to the loss of imprinting normally laid down during gametogenesis, or to both. Although there are few data, the incidence of LOS may increase with nuclear donor cells that are genetically modified. Of 14 liveborn lambs reported by McCreath et al. (2000) from targeted cells, only three survived beyond 6 months. Denning et al. (2001) reported that of seven targeted lambs born, the only survivor was killed for animal welfare reasons at 1 week of age. The same cell line used in these targeting experiments had previously given rise to clones that survived to adulthood. If single-cell cloning does impose an additional developmental burden, this can be explained in at least two ways. If only a proportion of nuclear donor cells are capable of being reprogrammed then that proportion may be closely
Table 2. Comparison of gene targeting efficiency in fibroblasts of pig fetuses

| Targeted locus          | Dai et al. (2002) | Lai et al. (2002) | Harrison et al. (2002) |
|-------------------------|-------------------|-------------------|------------------------|
| Promoter trap           | Yes               | Yes               | No                     |
| Transfection method     | Electroporation   | Electroporation   | Lipofection            |
| Number of cells         | 2.0 x 10^6        | 2.0 x 10^7        | 2.5 x 10^5             |
| Amount of DNA           | 10 µg             | 0.4 pmol          | Not known              |
| Dilution plating in wells| Yes              | Yes              | No                     |
| Selection               | G418 (250 µg ml⁻¹) | G418 (100 µg ml⁻¹) | G418 (0.15 µg ml⁻¹) |
| 48 h after transfection |                   | 48 h after transfection | 24 h after transfer to 100 mm plates |
| Ring cloning            | No                | No                | Yes                    |
| Resistant colonies      | SLA1-10 (pPL654) 127 | 159 analysed      | 499                    |
|                         | PCFF4-4 (pPL657) 179 |                  | 1400                   |
|                         | PCFF4-3 (pPL657) 200 |                  |                        |
|                         | PCFF4-6 (pPL657) 599 |                  |                        |
| Targeted events (PCR)   | 0                 | 8 (5%)            | 9 out 395 (2.3%) |
|                         | 11 (6%)           |                   | 1 (0.07%)              |
|                         | 1 (0.5%)          |                   |                        |
|                         | 18 (3%)           |                   |                        |
correlated with development to term and beyond. If that proportion is low, then there is a correspondingly high likelihood that any one subclone will be developmentally compromised. Alternatively, or in addition, the stress of subcloning may introduce new modifications, such as loss of imprint, that lead to developmental failure. In either event it is important to develop targeting protocols that reduce the period from transfection to identification of targeted cells and reduce the stress imposed by single-cell cloning during this period. The ideal targeting protocol would eliminate the subcloning step completely.

**Senescence and somatic cells**

Fetal fibroblasts from livestock species cease division (senesce) within 90–100 population doublings. Although this finding would appear to give a very large window for targeting experiments, it probably masks a situation in which senescence is ongoing even at early passage but is largely unnoticed as proliferating cells overtake the senescing population. However, such a dynamic becomes rapidly apparent after single-cell cloning in which typically almost 50% of the chosen colonies senesce within 1 week of isolation. A high but variable proportion of the remaining colonies subsequently senesce, and do not provide sufficient DNA for Southern blot analysis. Representative senescent and non-senescent single-cell colonies are shown (Fig. 2). Although the evidence is still anecdotal, we believe this problem arises as the cumulative effect of clonal variability in proliferative potential and the additional stress due to growth of cells at low density.

There is also concern that senescence may be more than simply a logistical problem, but may also be associated causally with developmental failure. Time in culture is known to be associated with the accumulation of both imprinting errors (Rideout et al., 2001) and genetic abnormalities (Eggan et al., 2002). Cloning efficiency has been negatively correlated with passage number (Wilmut et al., 1997; McCreath et al., 2000; Denning et al., 2001a) although there are also reports to the converse (Kubota et al., 2000, Lanza et al., 2000). Research into the use of alternative cell populations, telomerization of fetal fibroblasts to extend doubling time or alternative methods of identifying targeted cells are all high priorities.
Strategies to overcome senescence

Novel strategies are under investigation for the analysis of pooled cells in which single-cell cloning may be unnecessary or in which the underlying frequency of homologous recombination may be increased. This work is in its infancy and is not discussed here in detail. Other studies are investigating the potential for telomerization to improve the viability of clonally derived fetal fibroblast cells. One simple modification that markedly improves the proportion of colonies that continue to proliferate is to use a dilution plating targeting protocol (outlined in Fig. 3) to establish an average of 1-3 clones per well of a series of 24 well plates. This procedure has the disadvantage that most of the resulting clones are mixed and candidate targeted clones may also contain non-targeted cells. However, this technique has the advantage that clones remain vigorous and can provide sufficient DNA for Southern blot analysis.

An alternative solution may be the isolation of livestock-derived cells that do not senesce—ES cells. In mice, ES cells are ideally suited to genetic manipulation. Unlike fetal fibroblasts, ES cells proliferate indefinitely, and retain a normal euploid karyotype. Recently it has been shown that in mice ES cells may also be the preferred nuclear donor (Wakayama et al., 1999; Rideout et al., 2000; Eggan et al., 2001; Humpherys et al., 2001). This preference is probably because ES cells retain a chromatin structure similar to that of cells in the early embryo and require less reprogramming. These features of murine ES cells have renewed interest in isolating ES cells from farm animal species as a potential solution to senescence of nuclear donor cells. It would also overcome the difficulty in obtaining confirmation of targeted status by Southern blot analysis and possibly the developmental abnormalities associated with incorrect reprogramming.

Targeting expressed genes in livestock

We sought first to establish whether an expressed ovine gene (Col1A1) could be targeted with both promoter trap and non-promoter trap strategies. PPL Therapeutics had previously targeted Col1A1 at high frequency in ovine fetal fibroblasts (McCreath et al., 2000). The Col1A1 targeting construct was modified so that it contained an additional selectable marker (blasticidin) driven by the SV40 promoter (Fig. 4a). In this way the same cells could be compared, transfected with the same DNA, for promoter trap (G418 selection) and non-promoter trap (blasticidin selection). A representative Giemsa stained plate in which approximately 50% of wells give rise to colonies that comprise predominantly single-cell clones is shown (Fig. 4b). Targeted colonies were obtained under both forms of selection and therefore there is no reason to believe that a promoter trap per se is essential to successful targeting in ovine fetal fibroblast cells. However, because the non-promoter trap will always be associated with a high background of non-targeted clones, it is also inherently prone to an increase in false positives obtained by PCR analysis.

PCR analysis was performed using one primer within the neo cassette and a second primer within the upstream Col1A1 sequence (but outside the sequence included in the targeting construct) to identify targeted clones. Southern blot analysis of 15 clones that amplified the targeting-specific band (Fig. 4c) uses an internal probe within the 5' homology and gives a targeting-specific band of 5 kb and a band of random size in non-targeted clones. Hence, clone 53 is confirmed as a random integrant by Southern blot analysis (Fig. 4c), but was originally diagnosed as a targeted clone by PCR analysis. This is most likely attributable to the presence of a minority of targeted cells within mixed clones, although it may also be due to PCR artefacts. These data demonstrate the danger of relying upon a PCR screen alone.
When a similar analysis was performed from non-promoter trap clones, the proportion of false positives was higher, reflecting the inherent ability of the promoter trap to enrich for targeted clones and illustrating that the screening of non-promoter trap clones is complicated by a high incidence of artefacts.
Targeting unexpressed genes in livestock has not yet been accomplished

To address the related problems of targeting unexpressed genes and of overcoming senescence, we previously attempted to target two unexpressed genes in sheep fetal fibroblasts and obtained no candidate targeted clones at either the cystic fibrosis transmembrane conductance regulator (CFTR) locus (S. Pells, unpublished) or the β-casein locus (A. J. Thomson and M. M. Marques, unpublished). There are presently no reports in the literature of targeted fetal fibroblasts in farm animal species at non-expressed genes.

In principle, the difficulty in targeting unexpressed genes can be accounted for in two ways. Targeting an expressed gene enables the use of a promoter trap vector in which expression of a selectable marker is dependent upon homologous recombination to provide transcriptional activation. This is only an option with an expressed target gene because the selectable marker
uses the promoter of the target gene. The promoter trap provides very high enrichment for the targeted event because very few random integrants are capable of expressing the selectable marker. Even if the underlying frequency of targeting is low, such a strategy may mean that most of the surviving clones are actually targeted. For example at the Col1A1 locus > 60% of the clones analysed were targeted (McCreath et al., 2000). Alternatively (or additionally) there may be features of the chromatin structure at certain unexpressed genes that render the target unavailable for homologous recombination. In the latter event, targeting frequency may be very low or even non-existent.

Targeting β-casein in livestock

We are interested in conducting gene targeting in ovine fetal fibroblasts to place transgenes at the β-casein locus, to provide high level, mammary-specific transgene expression. As β-casein expression is highly tissue- and stage-specific, the gene is not transcribed in fetal fibroblasts. Hence, the targeting construct (Fig. 5a) is necessarily a non-promoter trap in which selection is for the expression of a constitutively expressed blasticidin cassette that is expected to be active in both targeted and non-targeted cells. A large scale targeting experiment at the ovine β-casein locus was carried out using the dilution plating procedure. A PCR pre-screen identified 28 candidate-targeted clones out of 720 clones analysed. A representative panel of PCR-positive clones is shown (Fig. 5b). It was then confirmed by digestion with enzymes that cleave at known sites within the fragment that the targeting-specific band amplified from these samples was the expected β-casein fragment (Fig. 5c). This finding would seem to provide convincing evidence of successful targeting at an unexpressed locus. However, the possibility could not be excluded of so-called ‘jump’ PCR in which a fragment polymerized non-geometrically from the blasticidin primer in a random site hybridizes with a fragment polymerized from the upstream primer but generated at the unmodified β-casein locus. Were such a hybrid to form then it would generate a product capable of geometric amplification and identical to the targeting-specific band. Although jump PCR is a formal explanation, it is unlikely that this actually occurs because multiple PCR reactions on the same clones invariably give rise to the same diagnostic fragment. In addition, this fragment cannot be generated artefactually by mixing the targeting construct with wild-type genomic DNA (A. J. Thomson and M. M. Marques, unpublished). Another possibility is that false positives result from mispriming due to fortuitous similarity between the sequence near the integration site and the upstream β-casein PCR primer.

Preliminary data after Southern blot analysis indicate that the candidate targeted clones (Fig. 5b) are not actually targeted (A. J. Thomson and M. M. Marques, unpublished). The provisional explanation of this result is that these clones contain a mixture of targeted and non-targeted cells and that the targeted cells are preferentially lost with culture. Although it is not clear why targeted clones should be at a growth disadvantage, the most likely explanation is that the chromatin structure at the inactive β-casein gene locus prevents long-term expression of the blasticidin resistance gene resulting in the senescence or death of the targeted cells. This finding highlights the importance of experimental detail. For example, if we had lower levels of blasticidin selection perhaps the targeted cells would have survived. Once again, this is a salutary lesson in the danger of over-reliance on PCR data alone – particularly with a non-promoter trap strategy. It appears that the dilution plating technique, although very successful in addressing the problem of senescence, may have led to predominantly mixed clones and eventual loss of targeted cells. This problem is being addressed by further optimization of the dilution plating procedure.
Fig. 5. β-Casein gene targeting studies. (a) Structure of the target locus, targeting construct and targeted locus after homologous recombination with the vector. (b) PCR reactions from 11 candidate clones after electrophoresis. (+) is positive control DNA; samples 105 and 712 are negative for β-casein. Candidate clones show the expected band and an additional non-specific band of unknown origin. (c) PCR reactions were digested with restriction enzymes to determine whether the targeting-specific band contains the expected restriction sites (Kpn I and Xba I).
Conclusions

Gene targeting in livestock will provide opportunities for improving the repeatability and regulation of transgene expression. The main impact of the development of this technology is likely to be within the biotechnology industry rather than agricultural industries, and will alter the economics of the production of pharmaceutical proteins in the mammary gland of transgenic sheep, cattle and goats. In social terms this means that gene targeting in livestock is likely to make it possible for the biopharming industry to develop new products of lower economic value and hence, to expand the proportion of society that stands to benefit. The practical limitations of targeting in livestock include the relative inefficiency of the nuclear transfer procedure and the apparent association of developmental abnormality with the period of time that the nuclear donor cell is maintained in culture. There is also a fundamental problem associated with gene targeting at loci that are silent in the nuclear donor cell; the absolute targeting frequency at silent loci appears to be much lower than at active loci. In addition, the inapplicability of promoter traps at silent loci is associated with a high background of non-targeted clones and greatly complicates the verification of targeted clones. In both expressed and non-expressed genes there is a serious problem associated with senescence of the nuclear donor cell. We believe that these are challenges of high priority not only for applications in livestock, but also for somatic targeting in human adult stem cells with potential therapeutic application in human medicine.

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