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Gene Targeting in Primary Fetal Fibroblasts from Sheep and Pig

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ABSTRACT

Nuclear transfer offers a new cell-based route for introducing precise genetic modifications in a range of animal species. However, significant challenges, such as establishment of somatic gene targeting techniques, must be overcome before the technology can be applied routinely. In this report, we describe targeted deletion at the *GGTA1* (α1,3-galactosyl transferase) and *PrP* (prion protein) loci in primary fibroblasts from livestock. We place particular emphasis on the growth characteristics of the primary cell cultures, since these are key to determining success.

INTRODUCTION

Pronuclear injection remains the mainstay for transgenic livestock production (Hammer et al., 1985) and indeed is routine in a variety of species. However, there has been little improvement in the efficiency of the technique over the years, with only 2–3% of injected eggs giving rise to transgenic offspring. In addition, the multicopy nature of most transgenic loci, coupled with the random nature of the site of integration, may give rise to unpredictable levels of expression (Clark et al., 1994). Most importantly, direct pronuclear injection provides no realistic means to modify endogenous genes in a targeted manner.

In contrast, gene targeting in the mouse using embryonic stem (ES) cells has been enormously successful, and, by exploiting the capacity of these cells to contribute to the germline and undergo homologous recombination with exogenous DNA, the introduction of precise, targeted changes into the mouse germline is now considered routine (reviewed by Hooper, 1992). The importance of this approach was understood at an early stage by those working with livestock, but ES cells capable of forming gonadal tissues in chimeras have not been isolated (Stice, 1998). This precluded the development of cell-based transgenesis and, consequently, gene targeting in livestock. The ability to clone animals by nuclear transfer (NT) from cultured somatic cells (Campbell et al., 1996; Wilmut et al., 1997), however, offers an alternative route to germline modification that could, in principle, be applicable to many species (Clark et al., 2000). In this approach, gene targeting is carried out in the primary somatic cell lines prior to NT. Targeting vectors are designed to disrupt the target locus by homologous recombination and enrichment of the targeting event is achieved by antibiotic selection; targeted cell lines are then used in NT procedures (McCreath et al., 2000; Denning et al., 2001).

Although gene targeting in mouse ES cells is now considered routine, targeting in somatic cells is a much more problematic exercise. There is little information on the frequency of homologous recombination in primary somatic cells and this

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is mainly restricted to human cells (Sedivy, 1999). In addition, the lifespan of primary cells in culture is a critical issue; unlike ES cells and transformed cell lines, primary somatic cells have a limited proliferative capacity. Genetic modification and subsequent preparation for NT must be accomplished before the cells senesce or enter crisis and transform. Cellular lifespan is determined by several parameters, including the culture conditions (Falanga and Kirsner, 1993; Saito et al., 1995), age of the donor animal (Kasinathan et al., 2001), and tissue and species of origin (Rubin, 1997).

This paper reports the characterization of primary cell cultures from sheep and pig fetuses and their use in gene targeting experiments. Data are presented on the growth characteristics of these cell populations in terms of the requirements for introducing and selecting targeted events, and expanding targeted cell populations. Promoterless targeting vectors were constructed to disrupt the ovine and porcine *GGTA1* (α1,3-galactosyl-transerase) genes and the ovine *PrP* (prion protein) gene. Using a high throughput screening protocol, targeting events were detected at all three loci but the limited proliferative capacity of the primary cells in culture was a significant impediment to overall success.

MATERIALS AND METHODS

Cell isolation and culture

Primary cultures of fibroblasts were recovered from 35-day-old fetuses derived from naturally mated sheep or pigs. Fetuses were eviscerated and decapitated for isolation of cells from the carcass of both species. In addition, for pigs, the gut, kidney, lung, and mesonephros were recovered to initiate cell lines. Tissues were dissociated manually, and then treated with trypsin/EDTA solution (Sigma, Dorset, U.K.). Primary cultures were grown on tissue culture plastic coated with 0.1% gelatin until the first passage, then on standard tissue culture plastic. The media (Sigma) was GMEM for ovine cells or DMEM for porcine cells, both supplemented with 10% fetal calf serum (Globe Farm, Gilford, Surrey, U.K.), 2 mM glutamine, 1 mM sodium pyruvate, and $1 \times$ nonessential amino acids (Life Technologies, Paisley, U.K.). Pig cultures were additionally supplemented with 2 ng/mL basic fibroblast growth

factor (Sigma). Cultures were exposed to 100 μg/mL gentamicin (Life Tech) for the first 24 h to prevent bacterial contamination. Growth conditions were at 37°C in an atmosphere composed of 90% N_2, 5% O_2, and 5% CO_2; or 75% N_2, 20% O_2, and 5% CO_2. At subconfluency, cells were cryopreserved at passage 1 in aliquots of ~1×10^6 per vial. Cultures were routinely passaged every 3–4 days by releasing with trypsin and plating at split ratios of 1:10 or 1:3 for sheep or pig cells, respectively.

Karyotypic analysis

Confluent cultures of cells were split at a ratio of 1:2 to fresh flasks. After 24 h, pig cells were treated with colcemid at a final concentration of 100 ng/mL (KaryoMax colcemid solution, Gibco) for 2 h, and then prepared for analysis. Sheep cells were grown for 26 h without additional treatment before preparation for analysis. Briefly, cells were trypsinized and pelleted by centrifugation. Cells were resuspended in a hypotonic solution of 0.4% trisodium citrate for 30 min to induce swelling. Samples were pelleted by centrifugation then fixed in a 3:1 mix of methanol/acetic acid. This step was repeated a further three times, before finally resuspending in 0.5 ml fixative.

Fixed cells were dropped to cleaned (100% ethanol, 5% HCl solution) slides to ensure successful chromosome spreads. After drying, slides were stained with Gurr's Geimsa stain diluted 1:20 in phosphate-buffered water, pH 6.8, for 8 min. Chromosome number was determined from a minimum of 10 separate spreads.

Targeting vector construction

To facilitate construction of targeting vectors, ovine and porcine genomic libraries were made in λDASHII (Stratagene). Partially digested Sau3A genomic DNA from Black Welsh sheep (BWF1) or Large White pig (PFIF17) cells was ligated to compatible BamHI phage vector arms. The ligation products were packaged to produce recombinant phage, which were propagated on *spi* selective XL1-Blue-MRA(P2) bacterial cells. Probes corresponding to the coding region of the *GGTA1* and *PrP* genes were used to isolate the corresponding phage. For isolation of the ovine and porcine *GGTA1* genes, RT-PCR, using sense (5'-GAGAAAATAA TGAATGTCAA AGGA-3' and antisense (5'-TGATAATCCCAGCAGTAT-TC-3') primers, was used to amplify coding se-

quence from exon 4 to 9. For the ovine *PrP* gene, coding sequence corresponding to nucleotides 22278 to 23048 of accession U67922 (Entrez) was used.

Promoterless vectors, with *neo-pA* sequence (Stratagene) adjacent to the endogenous gene start codon, were used to target the *GGTA1* and *PrP* loci. The ovine *GGTA1* vector was constructed by amplifying a truncated left arm (300 bp; using primers 199001, 5′-ACGTGGCTCC AA-GAATTCTC CAGGCAAGAG TACTGG-3′ and 199006, 5′-CATCTTGTTC AATGGCCGAT CC-CATTATTT TCTCCTGGGA AAAGAAAAG-3′, with tail complementary to the start of *neo* coding sequence), and *neo-pA* sequence (using primers 199005, 5′-CTTTTCTTTT CCCAGGA-GAA AATAATGGGA TCGGCCATTG AACAA-GATG-3′, with tail complementary to left arm, and 199004, 5′-CAGGTCGACG GATCCGAACA AAC-3′). These fragments were used to prime from each other to give a 1.2-kb fusion product. This was ligated to intron 3 sequence (1kb EcoRV-EcoRI fragment), to extend the left arm, and to ~9kb (EcoRV partial digest—NotI) of 3′ sequence to create the right arm.

The ovine *PrP* vector was constructed by amplifying the left arm (2.4 kb; using primers prp6F, 5′-CCGAGCTCGC CAATTTCATG GCTGCAG-TCACC-3′ and prp7R, 5′-CGATCCC ATGATGA-CTTCTC TGCAAAATAA AG-3′, with tail complementary to the start of *neo* coding sequence) and *neo-pA* sequence (using primers prp10F, 5′-GAGAAGTCAT CATGGGATCG GCCATTGA-ACA-3′, with tail complementary to left arm; and prp8R, 5′-TGCAGGTCGA CGGATCCGAA-3′). These fragments were used to prime from each other to give a 3.3-kb fusion product, which was ligated to a 3-kb KpnI fragment to complete the vector.

The porcine *GGTA1* vector was constructed by amplifying a truncated left arm (200 bp; using primers PAGTI3A2, 5′-ACCAGGGT GAGAGTT-CCCAT-3′ and NEOPAGT, 5′-CGATCCCATT ATTTTCTCCT GGGAAAAGAA AA-3′, with tail complementary to the start of *neo* coding sequence) and *neo-pA* sequence (using primers PAGTNEO, 5′-CAGGAGAAAA TAATGGGATC GGCCATTGAA CA-3′, with tail complementary to left arm, and PRP8R, 5′-TGCAGGTCGA CG-GATCCGAA-3′). These fragments were used to prime from each other to give a 1.1-kb fusion product. This was ligated to a 3-kb EcoRI to StuI fragment and a 7-kb EcoRI fragment to extend the left arm and create the right arm, respectively. The ovine *GGTA1* and *PrP*, and porcine *GGTA1* targeting vectors were linearized at unique NotI, SacI and BsaBI sites, respectively.

Northern blot analysis

Polyadenylated RNA was isolated from approximately 1×10^7 Black Welsh (BWF1) or Large White (PFIF17) cells using an mRNA isolation kit (Roche, East Sussex, U.K.). The total sample (8 or 4 μg for sheep and pig, respectively) was separated by formaldehyde gel electrophoresis. The RNA was transferred to nylon membrane using standard procedures. Ovine cDNA probes were used for *GGTA1* and *PrP*, while a murine cDNA probe was used for the GAPDH control. Hybridization and washes to a stringency of $2 \times$ SSC/0.1% SDS were carried out at 65°C.

Transfection and identification of targeted clones by high throughput polymerase chain reaction screening

Early passage ovine or porcine cells were transfected with linearized targeting vector. Cells were plated to 96-well plates (0.5–5 $\times 10^3$ cells/well). G418 selection was applied after 24 h at 600 μg/mL for sheep cells or 72 h at 100 μg/mL for pig cells. At subconfluence, drug resistant colonies were replica plated to two 96-well plates for DNA analysis and cryopreservation.

Drug-resistant colonies were screened for targeting events by polymerase chain reaction (PCR). DNA was isolated in 96-well plates by overnight lysis (50 mM Tris, pH 8, 20 mM EDTA, 100 mM NaCl, 0.3% SDS, 10 mg/mL protinease K) then isopropanol precipitation. Pellets were resuspended in 50 μL of TE (10 mM Tris-HC1, pH 8, 1 mM EDTA). Amplification was performed using Roche Expand HiFi kit, with 1 μl of DNA template. Primer locations and fragment sizes are indicated in Figure 4 below.

- Ovine PCR: G1 (5′-CAGCTGTGTGGGTATG-GGAG GG-3′), G2 (5-′CTGAACTGAA TGTT-TATCCA GGCCATC-3′), G3 / P3 / PG3 (5′-AGCCGATTGT CTGTTGTGCC CAGTCAT-3′), P1 (5′-TTCAGTCGCT CTGTTGTGTC CCA-3′), P2 (5′-AGCATCCCTC CTGCCTTCAGTT-CTTC-3′)
- Porcine PCR: PG1 (5′-AGCATCCTCC TCTTC-CTCTT TCTC-3′), PG2 (5′-GCCCAATAGC AGCCAGTCCC TTC-3′)

Cycling conditions for ovine *GGTA1* were 94°C, 2 min; 94°C, 30 sec/65°C, 30 sec/68°C, 2.5 min—10 cycles; 94°C, 30 sec/65°C, 30 sec/68°C, 2.5 min + 5 sec per cycle—20 cycles; 68°C, 7 min. For ovine *PrP* and porcine *GGTA1*, the elongation phase was increased to 4 min. PCR products from porcine cells were digested with StuI restriction enzyme prior to analysis to release a common 3-kb fragment and enhance resolution of the 1.3- and 1.6-kb fragments that correspond to nontargeted and targeted alleles, respectively. Products were analyzed by agarose gel electrophoresis.

RESULTS

Growth characteristics of primary fetal cells

Successful nuclear transfer from cultured cells has only been achieved in livestock using primary somatic cells, a fundamental characteristic of which is their limited lifespan. Previously, we have estimated that achieving targeted genetic modification requires approximately 45 population doublings to proceed from isolation of primary cells from fetal tissue, through the targeting process to preparing targeted cells for NT (Clark et al., 2000). Therefore, we set out to characterize the growth properties of fetal cells derived from different breeds of sheep and pigs, and determine whether their proliferative capacity could be improved by modifying the culture conditions.

Fetuses were isolated from Black Welsh, Finn Dorset, and Shetland breeds of sheep that had

been naturally mated. Following disaggregation of the carcass, individual cells and clumps of cells attached to the gelatin-coated tissue culture plastic and most cultures grew to confluency after 3–7 days. Most cells from each culture were cryopreserved for use in future gene targeting experiments. The remaining cells were used to determine the maximum proliferative capacity by serial passage on standard tissue culture plastic, in an atmosphere composed of 75% N_2, 20% O_2, and 5% CO_2 (Fig. 1A). Based on morphological appearance, fibroblastic cells predominated after about the third passage (~12 doublings; Fig. 2A). A surprising observation was the large variation seen both between cultures derived from the same breed and between different breeds. For example, BW6F2 divided ~110 times at intervals of ~20 h, but BWF1 senesced after 50 populations doublings (PD), each taking ~40 h to complete. We have now investigated the growth characteristics of many primary sheep cultures (data not shown). All have shown a proliferative capacity of between 40 and 120 divisions, suggesting that these values may represent the lower and upper extents of longevity of bulk ovine cultures.

In the same way as described for ovine cultures, the characteristics of porcine cells, derived from the Large White breed, were determined (Fig. 1B). Again, fibroblastic like cells predominated after two to three passages (Fig. 2B). There were, however, several notable differences between the two species. The porcine cells were much larger than sheep cells, as judged by microscopic analysis and by the presence of higher
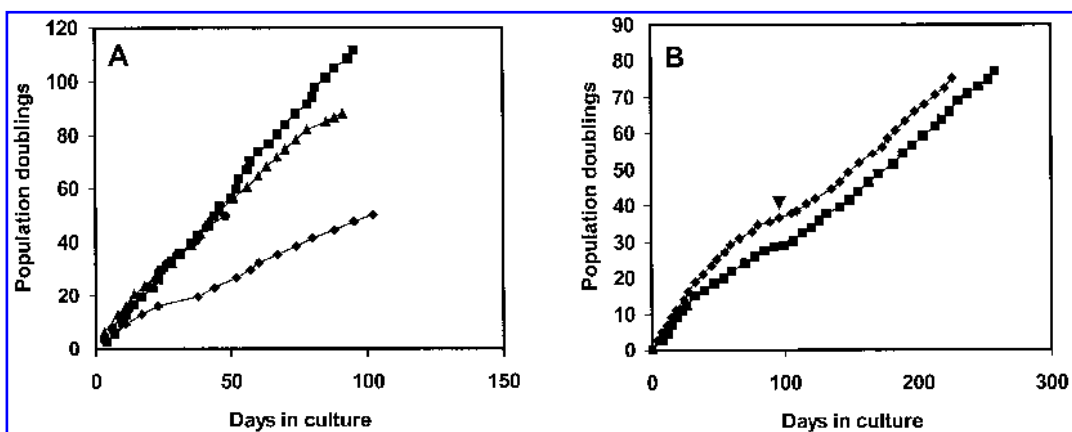

FIG. 1. Growth characteristics of ovine and porcine fetal fibroblasts in culture. Cells were isolated from 35-day-old carcasses and passaged continually in an atmosphere of 75% N_2, 20% O_2, and 5% CO_2. (**A**) Sheep cultures from Black Welsh (BW6F2, ■; BWF1, ◆), Finn Dorset (7G65F4, ▲) or Shetland (2327F2, ●) breeds wee grown to senescence. (**B**) Cultures from Large White pig carcasses (PF6C, ◆; PF1C, ■) showed karyotypic instability after approximately 40 population doublings and transformed (arrow), giving rise to a sigmoidal growth curve.
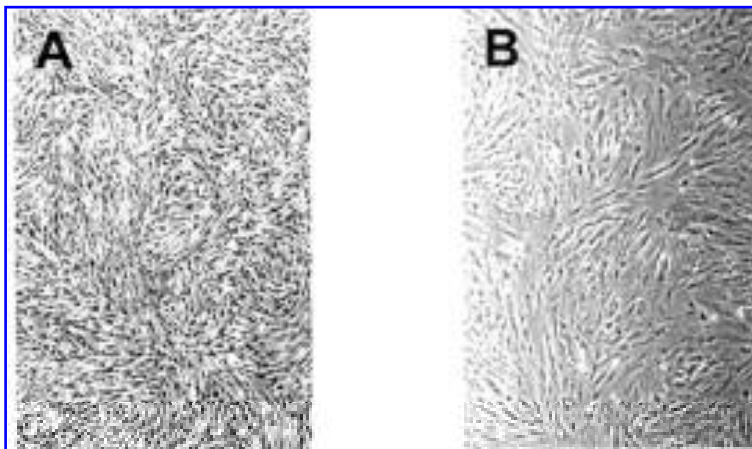
FIG. 2. Fibroblastic morphology of a confluent monolayer of cells at passage 4–6 cultured from a 35-day-old sheep (**A**) or pig fetus (**B**). Original magnification, $\times$ 10.

numbers of sheep cells at confluency, per unit area. The porcine cells grew more slowly than the majority of sheep cell cultures and had a doubling time of 48–60 h. Furthermore, many of the porcine cell cultures appeared to go through crisis after ~40 population doublings and continued growth was associated with an aneuploid genotype (Fig. 1B and Table 1). This was in contrast to the growth of the sheep cells in culture, which retained a stable karyotype and did not go through crisis (Fig. 1A and Table 1).

Several studies have suggested that growth of cells in an atmosphere with reduced oxygen content may increase the rate and extent of proliferation (Falanga and Kirsner, 1993; Saito et al., 1995). We compared the characteristics of two sheep fetal lines (BW6F2 and BWF1) grown in 5% or 20% O_2 but failed to detect any growth advantage of bulk cultures in the low oxygen atmosphere (Fig. 3A). Similar observations were made with pig cultures (data not shown). Furthermore, growth characteristics were not improved by addition of epidermal growth factor (EGF) at concentrations of 3–100 ng/mL to sheep and pig cells, or by growing pig cultures on murine feeder layers (data not shown).

The experiments so far examined the growth characteristics of cells derived from bulk fetal carcasses. Cultures of pig cells derived in this manner showed a substantially reduced proliferative capacity as compared to sheep cells. To determine whether porcine cells with greater proliferative capacity could be isolated from other tissues, we initiated cultures from fetal gut, kidney, lung and mesonephros, and compared them to the growth of cells derived from the carcass (Fig. 3B). We found, however, these cultures senesced or entered crisis after even less doublings than those from the carcass (Fig. 3B).

Construction of targeting vectors and optimization of transfection

To facilitate targeting vector construction, we used *GGTA1* or *PrP* DNA probes to screen genomic libraries prepared from Black Welsh sheep cells (BWF1) or Large White (PFIF17) pig cells. The genomic organization of the genes is shown in Figure 4. The coding exons of the ovine and

TABLE 1. KARYOTYPIC ANALYSIS OF OVINE AND PORCINE CARCASS FIBROBLASTS DURING CULTURE

Breed	Population doublings (percent karyotypic normality[a])		
	1–21	*22–50*	*51+*
Sheep[b]			
Black Welsh	ND	96%	97%
Finn Dorsett	100%	94%	96%
Shetland	98%	93%	Senesced
Pig[b]			
Large White	92%	75%[c]	15%[c]

[a]Karyotypic normality was determined by counting the number of chromosomes present in a minimum of 10 separate spreads.

[b]Normal diploid karyotype for sheep and pig cells is 54 and 38 chromosomes, respectively.

[c]Crisis occurred after ~40 doublings; at this stage, most cells in the pig cultures had a low mitotic index and the chromosomes were elongated. The transformed cells that emerged were mainly triploid or tetraploid.
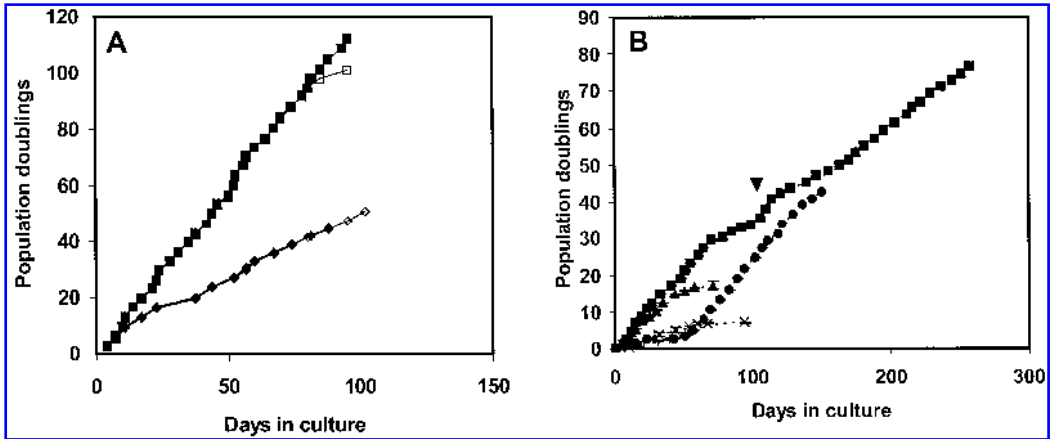
ND, not done.

FIG. 3. Effect of low oxygen and cell origin on growth characteristics. (**A**) Black Welsh sheep cultures were grown in an atmosphere of 75% N_2, 20% O_2, and 5% CO_2 (BW6F2, ■; BWF1, ◆) or 90% N_2, 5% O_2, and 5% CO_2 (BW6F2, ■; BWF1, ◆) and showed no significant difference in growth pattern. (**B**) Cells were isolated from various tissues of 35-day-old Large White pig fetuses and passaged continually in an atmosphere of 75% N_2, 20% O_2, and 5% CO_2. Senescence or transformation occurred in cells from gut (+), kidney (×), lung (▲), and mesenpheros (●) occurred after less doublings than did transformation of cells from the carcass (■), which occurred at approximately 40 doublings (arrow).

porcine *GGTA1* genes were designated 4–9 because translation initiates in exon 4 of the well-characterized mouse gene (Joziasse et al., 1992).

It has become increasingly clear that efficient gene targeting in somatic cells requires powerful strategies to select for the targeted cells (Sedivy, 1999; Sedivy and Dutriaux, 1999). The promoterless method has been reported to increase the ratio of targeted to nontargeted (nonhomologous recombination) clones by 100–500-fold because
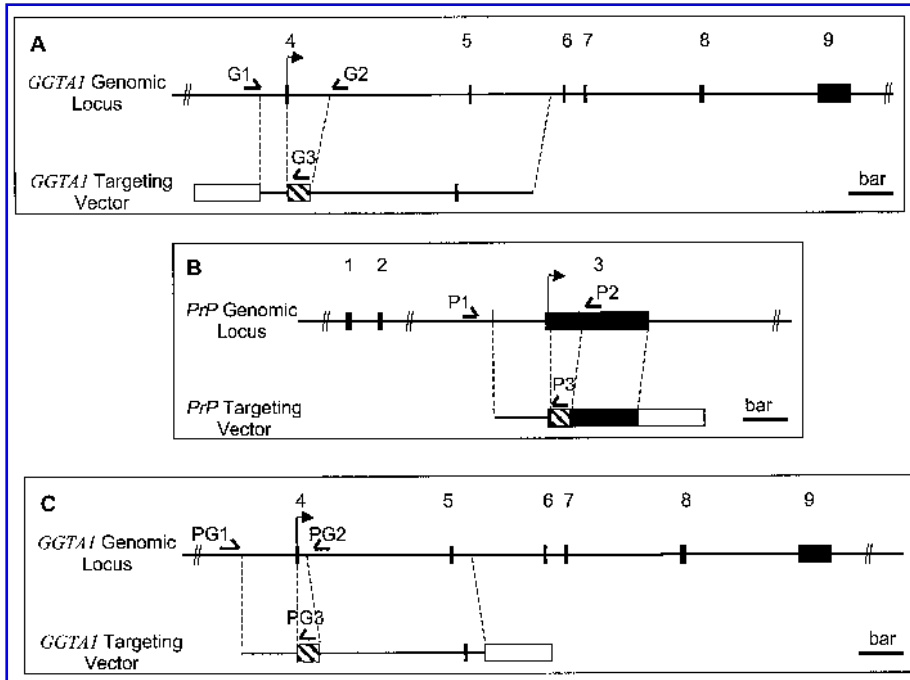

FIG. 4. Organization of the genomic loci of ovine *GGTA1* (**a**), ovine *PrP* (**b**), or porcine *GGTA1* (**c**) genes and the promoterless targeting vectors used for disruption. Numbering of the exons in *GGTA1* is based on the mouse; translation initiates in exon 4 and terminates in exon 9. The coding sequence of *PrP* is entirely within exon 3. Location of PCR primers is shown. The size of the PCR product in correctly targeted alleles changes from 2.8–2.2 kb, primers G1 and G2 (**A**); 4.6–3.9 kb, primers P1 and P2 (**B**); and 4.3–4.6 kb, primers PG1 and PG2 (**C**). Fragments of 1.5 kb, primers G1 and G3 (**A**); 3 kb, primers P1 and P3 (**B**); and 3.5 kb, primers PG1 and PG3 (**C**) are produced only when correctly targeted cells are present. Arrows indicate the translation initiation sites. Black boxes represent exons, hatched boxes represent *neo-pA* sequence, and open box represents pBlueScript sequence. Bar = 2 kb.
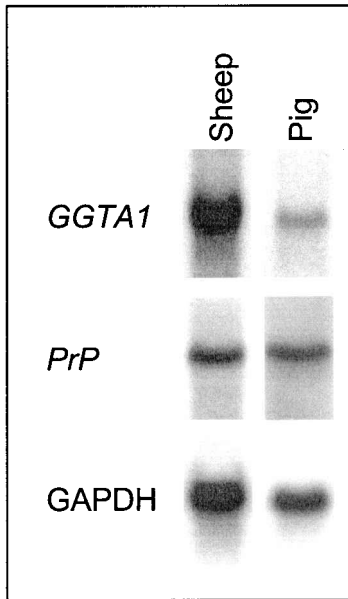
FIG. 5. *GGTA1* and *PrP* genes are expressed in sheep and pig fetal fibroblasts. Ovine coding sequence probes were used for *GGTA1* and *PrP*; thus, poor cross-species hybridization was likely to account for low signal intensity in porcine *GGTA1*. A murine coding sequence probe was used for the GAPDH control. While for sheep and pig, exposure times were the same, they were different between the three genes; thus, the transcript abundance shown is qualitative.

the selectable marker will only be expressed if integration occurs in frame and downstream of a transcriptionally active gene (Sedivy and Dutriaux, 1999). Using northern analysis, we were able to easily detect transcripts generated from the *GGTA1* and *PrP* loci (Fig. 5). In the replacement vectors we constructed, the neomycin phosphotransferase (*neo*) gene was placed directly adjacent to the initiation codon of the targeted genes and a section of the downstream sequence was deleted (Fig. 4). In correctly targeted clones, this strategy places the *neo* gene under the control of the endogenous promoter of either GGTA or PrP genes (Fig. 4).

From the results of the previous section, we elected to perform the targeting experiments using cells derived from pig or sheep fetal carcasses grown in an atmosphere of 20% O_2. Electroporation is the most commonly used method of transfection in gene targeting experiments (Sedivy, 1999). We first optimized conditions by counting the number of blue cells after transient transfection with the β-galactosidase marker plasmid pCMV-Sport-βgal and staining with the chromogenic agent x-gal. Electroporation of Black Welsh, Finn Dorset, Shetland cells using a pulse

of 250 μF : 400 V; 125 μF : 350 V; and 250 μF : 250 V, respectively, resulted in ~50% cell survival, of which ~10% stained blue. However, electroporation of Large White porcine cells using these conditions produced less than 1% blue cells and this prompted us to investigate lipofection. Using a 3:1 ration of lipofectamine to DNA, we were able to achieve a transient transfection efficiency of ~5–10%, similar to that seen using electroporation in sheep cells.

Gene targeting in primary somatic ovine and porcine cell cultures

Since primary cultures have a finite lifespan, we transfected the GGTA1 and PrP targeting constructs into early passage ovine or porcine cells. Furthermore, we expected the targeting efficiency to be low, so used a high throughput strategy to detect correctly targeted colonies. Following transfection, cells were seeded into a 96-well plate format and G418 selection was applied. Drug-resistant colonies were derived at an efficiency on a per cell basis of ~1 in 10^{-4} to 10^{-5}. These were grown to subconfluence and then replica plated for cryopreservation and DNA analysis. We used two independent PCR reactions to detect targeting events for each construct (Figs. 4 and 6). One reaction amplified from genomic DNA external to the left arm of homology into neo coding sequence and indicated whether targeted cells were present. In contrast, the second reaction was designed to detect both alleles, wild-type and targeted, resulting in fragments of different sizes (Figs. 4 and 6). Moreover, since targeted and nontargeted alleles should be present in equimolar ratios, so producing bands of similar intensity, it was possible to determine with a reasonable degree of confidence whether a colony contained only targeted cells or was contaminated by additional non-targeted cells (Table 2 and Fig. 6). The determination of the purity of colonies by S. blotting was not attempted because it was not possible to isolate sufficient quantities of DNA from individual colonies on a routine basis.

Using the PCR method, we were able to demonstrate targeting events at the *GGTA1* locus in both sheep and pig cells, and at the *PrP* locus in sheep cells (Figs. 4 and 6, and Table 2). The overall frequency ranged from 0.8% to 9% (targeted to nontargeted cell ratio) and corresponded to an absolute targeting frequency of 2.8×10^{-7} to 27.5×10^{-7} (per cell basis). There was a large amount of variation both between the targeting
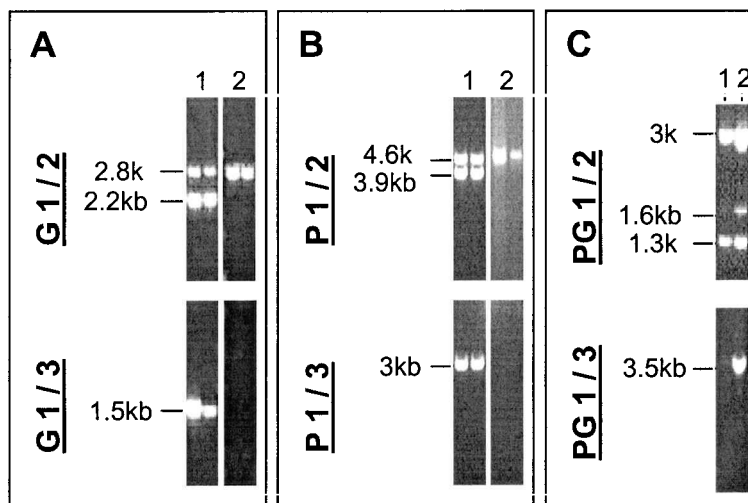
FIG. 6. Gene targeting in primary somatic cultures. DNA was isolated from colonies of transfected sheep or pig cells and analyzed by PCR. See Figure 4 for location of primers. Asterisk indicates the bands that show correct targeting. (**A**) Ovine *GGTA1* PCR (lanes in duplicate): panel 1 shows targeted cells; panel 2 shows nontargeted parental cells. (**B**) Ovine *PrP* PCR (lanes in duplicate): panel 1 shows targeted cells; panel 2 shows nontargeted parental cells. (**C**) Porcine *GGTA1* PCR: lane 1 shows nontargeted parental cells; lane 2 shows targeted cells. Following PCR using primers PG1 and PG2, products were cleaved with StuI digestion to release a common 3-kb fragment, and 1.3-kb or 1.6-kb fragments representing nontargeted or targeted alleles, respectively.

efficiencies in the different cell lines (Table 2) and between experimental groups using the same line (data not shown).

Although many of the sheep colonies were targeted, a high proportion were mixed—that is, they contained targeted and nontargeted cells. Therefore, the data shown here represents the upper estimates of targeting efficiency. Nevertheless, on the basis of PCR, we did detect a substantial number of clones that contained only targeted cells. However, many of these pure clones senesced before they could be expanded

sufficiently for NT. Hence the effective targeting frequency, in terms of generating useful targeted cells, was 10–30-fold lower than our estimate of actual targeting frequency—that is, the effective frequency was in the order of 1 in 10^7.

In the pig cells these problems were even more acute. Although we were able to detect targeting events in 20 colonies, none of these appeared to comprise pure targeted clones (Fig. 6 and Table 2). Furthermore, all clones senesced before they could be analyzed further and we failed to generate any targeted populations suitable for NT.

TABLE 2. TARGETING EFFICIENCIES OVINE AND PORCINE CARCASS FIBROBLASTS

Breed	Locus	Drug-resistant colonies	Detected targeting events[a]	Targeting frequency[b]	Absolute targeting frequency[c]
Sheep					
Black Welsh	*PrP*	856	55 (50)	6.4%	27.5×10^{-7}
Finn Dorsett	*PrP*	917	14 (12)	1.5%	23×10^{-7}
Shetland	*PrP*	1241	19 (ND)	1.5%	6.3×10^{-7}
Black Welsh	*GGTA1*	1467	11 (3)	0.8%	2.8×10^{-7}
Finn Dorsett	*GGTA1*	1485	45 (25)	3.0%	11.3×10^{-7}
Pig					
Large White	*GGTA1*	215	20 (20)	9%	14.3×10^{-7}

[a]The total number of detected targeting events is indicated, and the proportion of these that contained targeted and nontargeted cells (mixed colonies) is shown in parentheses.
[b]Targeting frequency describes the ratio between detected targeting events and drug-resistant colonies.
[c]Absolute targeting frequency is targeting efficiency per cell basis.
ND, not done.

DISCUSSION

The development of cloning technology in which viable animals can be generated by NT from cultured somatic cells has enabled gene targeting in livestock to be developed. The fact that successful cloning has now been achieved in sheep (Campbell et al., 1996), cattle (Wells et al., 1999), mice (Wakayama et al., 1998), goats (Baguisi et al., 1999), and pigs (Polejaeva et al., 2000) means that, in principle, it should be possible to knockout or otherwise modify endogenous genes in these species.

In developing this technology, however, there are several key issues that must be addressed. The first relates to the efficiency whereby endogenous genes can be targeted by homologous recombination in somatic cells in culture. In mouse ES cells, isogenic DNA is important for efficient gene targeting (Deng and Capecchi, 1992; te Riele et al., 1992). Furthermore, large variations in frequency are seen when targeting different loci (Hasty et al., 1994). Data relating to homologous recombination frequencies are sparse in somatic cells, especially primary cell lines, although the general consensus is that these are lower than reported for mouse ES cells (Arbones et al., 1994; Thyagarajan et al., 1996). It was therefore important to establish whether it was feasible to target loci in primary somatic cells from livestock. The limited proliferative capacity of these cells also has major implications for successfully introducing targeted changes. We have previously estimated that ~45 doublings are required to transfect, select, expand, and prepare cells for NT (Clark et al., 2000). Therefore, the longevity of donor cells in culture is a key parameter in the development of the capability to carry out gene targeting.

With the above considerations in mind, we set out to establish these parameters in primary somatic cells, isolated from sheep and pig fetuses. Cultures of sheep primary fibroblasts grew over a range of time periods with many completing more than 80 population doublings before senescing. Ostensibly this should be well over our estimates of 45 doublings for the targeting window. Nevertheless, it should be born in mind that this represents the lifespan of the longest-lived cells in the culture and a high proportion of the starting population senesce earlier. This is clearly evident from the high proportion of targeted cells that failed to survive in between their identification and final expansion.

The proliferative capacity of the sheep cells was similar to those reported previously for human fibroblasts (Gupta, 1980; Wertz et al., 1981). By contrast, the pig cells divided more slowly, and after about 40 population doublings either senesced or entered crisis, resulting in the generation of aneuploid cells (Table 1). This behavior was evident in a range of cultures isolated from different sources. Therefore, it is perhaps not surprising that, although we were able to identify targeting events in the pig cultures, we failed to expand the targeted colonies. In both pig and sheep cells, we tried a variety of culture conditions to improve proliferative capacity, including low O_2 and the provision of growth factors with no obvious benefits.

Targeting vectors were constructed using genomic libraries produced from Black Welsh sheep cells or Large White pig cells. These followed conventional designs for promoterless *neo* constructs, which are generally thought to be the most efficient form of targeting vector (Sedivy, 1999). We detected targeting at both the *GGTA1* and *PrP* loci in sheep and the *GGTA1* locus in pigs at moderate frequencies (3×10^6 to 10^7), consistent with frequencies reported in human somatic cells (Sedivy, 1999), but towards the lower end of the range reported for mouse ES cells (Templeton et al., 1997). As far as we could tell, the targeting frequency was not dependent on the breed of cells used in sheep. We do not know the polymorphic variation of the DNA across the segments used to construct the targeting vectors. Therefore, it is difficult to conclude whether this truly reflects a lack of requirement for isogenicity, as has been suggested for human somatic cells (Sedivy, 1999), or relates to lack of polymorphism between breeds at these loci.

Despite our capability in targeting the two sheep genes by homologous recombination in primary cells, we experienced significant problems in isolating populations that could be used for NT. One reason for this was the high incidence of colonies that contained mixed populations of targeted and nontargeted cells. This occurrence could be explained in several ways. Although the plating density may have been too high in some experiments, we were surprised that mixed colonies were still abundant even when the plating density was subsequently reduced by up to 10-fold. It is possible the establishment and growth characteristics of a colony that arose from two or more cells was superior to those from individual cells, thus preferentially selecting for

mixed colonies. Alternatively, simultaneous targeted and random integrations into different chromosomes of the same cell could give rise to two drug-resistant daughter cells, one carrying the targeted event and one with a random event.

The failure to sustain any neo[R] pig cells after selection, whether targeted or not, correlates with the more limited proliferative capacity of these cells in culture. The maximum lifespan of these cells in culture is ~40 PDs, less than the 45 PDs we have estimated to be required to target and isolate clonal populations of cells for NT. Therefore, it is difficult to imagine how it will be possible to isolate such lines from normal pig fibroblasts such as these with the frequencies of gene targeting that we report, unless culture conditions can be established which substantially extend *in vitro* lifespan.

We have recently reported the use of some of the targeted sheep cell lines described above in NT experiments (Denning et al., 2001). We generated both fetuses (*GGTA1* and *PrP*) and live born lambs (*PrP*) from these cells (Denning et al., 2001). However, we experienced a high incidence of pre- and perinatal losses during these experiments, and no animals survived beyond 2 weeks. In the only other report to describe gene targeting by NT in livestock, McCreath et al. (2000) reported the birth and survival of two sheep carrying a specific gene insertion. Again, in this study there was a high incidence of pre- and perinatal loss compared to experiments using unmodified early passage fetal fibroblasts (Campbell et al., 1996). Thus, it is possible that the overall efficiency of NT may be reduced through a combination of the stringent selection and extended culture required to isolate targeted cells.

The parameters of cell growth and targeting efficiency reported in this study indicate that it is just about feasible to generate targeted sheep using a combination of gene targeting and NT with current approaches (McCreath et al., 2000; Denning et al., 2001). For pig, the lower proliferative capacity indicates that the likelihood of success with these approaches is marginal, at least with the cells and culture conditions we have used. There is clearly a need to optimize as well as develop new approaches in both species. Possibilities include methods to improve the frequency of gene targeting, which would reduce the number of total doublings required to achieve a targeted clone. Alternatively, methods to enhance the proliferative capacity of primary cells in culture, such

as expressing the telomerase gene (Bodnar et al., 1998), could be used, although this would have to be without comprising the viability of the cells for NT.

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