Functional disruption of the prion protein gene in cloned goats

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The cellular prion protein (PrPC), a membrane glycoprotein anchored to the outer surface of neurons, lymphocytes and other cells, is associated directly with the pathogenesis of the transmissible spongiform encephalopathies (TSEs) occurring mainly in humans, cattle, sheep and goats. Although mice lacking PrPC develop and reproduce normally and are resistant to scrapie infection, large animals lacking PrPC, especially those species in which TSE occurs naturally, are currently not available. Here, five live PRNP+/− goats cloned by gene targeting are reported. Detailed RNA-transcription and protein-expression analysis of one PRNP+/− goat showed that one allele of the caprine PRNP gene had been disrupted functionally. No gross abnormal development or behaviour could be seen in these PRNP+/− goats up to at least 3 months of age. These heterozygous PRNP+/− goats are ready to be used in producing homozygous PRNP−/− goats in which no PrPC should be expressed.

INTRODUCTION

Prion diseases, also called transmissible spongiform encephalopathies (TSEs), are a group of lethal, infectious, neuro-degenerative disorders of the central nervous system. The primary characteristics of these diseases are brain vacuolation, neuronal apoptosis and astrogliosis that can lead to motor dysfunction, dementia and death. The most notable examples of these diseases are scrapie in sheep and goats, bovine spongiform encephalopathy (BSE) in cattle and Creutzfeldt–Jakob disease (CJD), Gerstmann–Straussler–Scheinker syndrome and fatal familial insomnia in human beings (Prusiner, 1998).

There is now considerable evidence that prions, the pathogens of these diseases, consist solely of an infectious, β-sheet-rich, protease-resistant conformational isoform (PrPSc) of the cellular prion protein (PrPC), a glycosylphosphatidylinositol-anchored membrane glycoprotein of uncertain function (Prusiner, 1982; Legname et al., 2004). The ‘protein-only’ hypothesis proposes that PrPSc, when introduced into animals, will cause the conversion of PrPC into a likeness of itself and that animals with no PrPC expression should be resistant to experimental scrapie infection, neither developing symptoms of prion diseases nor allowing propagation of the infectivity. This prediction was testified successfully by two independent lines of mice that are devoid of PrPC. Both lines of mice generated by homologous recombination in murine embryonic stem (ES) cells develop and reproduce normally and are resistant to scrapie (Büeler et al., 1992, 1993; Prusiner et al., 1993; Manson et al., 1994).

Due to the success of murine ES cells, gene targeting has been a routine tool for modifying the genome of mice precisely. In spite of considerable efforts, ES cells that can contribute to the germline of any livestock species are still not available, which limits the widespread use of this technology. With the development of cloning techniques, transgenic livestock can be generated by nuclear transfer from transfected fetal fibroblasts cultured in vitro (Schnieke et al., 1997) and the procedures are essentially the same as those required for gene targeting. This provides an alternative approach to circumvent the establishment of ES cells in livestock to modify their genome precisely, because fetal fibroblasts can be used to replace ES cells in gene targeting. Live gene-targeted sheep with a human ζ1-antitrypsin gene inserted into the ζ1 (I) procollagen locus have been produced successfully by gene targeting on ovine fetal fibroblasts (McCreath et al., 2000). One allele of the ζ(1,3)-galactosyltransferase gene in pigs and sheep has also been disrupted by similar procedures (Denning et al., 2001; Dai et al., 2002; Lai et al., 2002).

Scrapie, the prototype of prion diseases, is a naturally occurring disease of sheep and goats and was also the first prion disease to be transmitted to laboratory rodents.
(Chandler, 1961). Although targeted disruption of the murine prion protein gene does not have gross deleterious effects on mice carrying this mutation, there is no evidence that targeted disruption of this gene in sheep or goats will be analogous. In addition, there is evidence that goats are less susceptible than sheep to the scrapie agent (Billins et al., 2002). The availability of sheep and goats with reduced or no expression of PrPSc will be helpful in understanding the behaviour and adaptation of the TSE infectious agent in these models. Finally, four PRNP+/- cloned sheep were reported by Denning et al. (2001), but none of the animals survived for more than 12 days, leaving the question: what were the reasons for the death of these PRNP+/- sheep – cloning procedures or the genetic modification itself? In this report, we demonstrate the feasibility of producing live, healthy PRNP+/- cloned goats in which one allele of the PRNP gene has been disrupted functionally.

**METHODS**

Isolation and culture of primary caprine fetal fibroblasts. The GFF88, B38A and 1126B caprine fetal fibroblast cell lines were derived from 30–40-day fetuses of Saanen dairy goats and cultured in Glasgow minimal essential medium (GMEM; Sigma) supplemented with 2 mM glutamine (Gibco-BRL), 1 mM sodium pyruvate (Sigma), 1 x non-essential amino acids (Sigma), 2 mg basic fibroblast growth factor ml⁻¹ (Sigma), 10% fetal calf serum (Hyclone), 100 U penicillin ml⁻¹ and 100 µg streptomycin ml⁻¹ (Sigma). Sex of the fetal fibroblast cell lines was determined according to the presence or absence of the SRY gene by PCR amplification using the following primers: SRY16f (5'-CAATCTGTAGCTCTGTATGTTTC-3') and SRY654r (5'-CAATGTACCTATCGTGGC-3').

Gene-targeting vector construction. The promoterless gene-targeting vector GTPrP was constructed by placing the neomycin phosphotransferase gene followed by a polyadenylation signal (neo-pA) directly adjacent to the endogenous initiation codon of the PRNP locus. The 1-9 kb left arm was amplified by PCR using primers [PrPLF: 5'-GAATTCCGGCCGATGATTATGTTCTTGATGC-3'] and two artificial restriction-enzyme sites (underlined) at its 5' end for molecular cloning (SacII) or vector linearization (SalI), and PrPLR: 5'-TGTGTAATGGGCGATCCCAATGATGACTTCTCTGAAAATAG3', with a 3' tail (23 bp, in bold) within the PRNP locus and a 5' tail complementary to the start of neo coding sequences). The 1-1 kb neo-pA fragment was amplified by using primers [neoF: 5'-CTTATATTTGCAGAAGTCACTGATGGATTGGCCATTAGACAATG3', with a 5' tail (23 bp, in bold) within the PRNP locus and complementary to the left arm, and neoR: 5'-GAATGTGGGCACTACTCCACGGTGTTCTTGTCG-3'], with two sites for molecular cloning (NotI) or genomic DNA analysis (SacII). These two fragments were used to prime from each other to give a 3-0 kb product. This product, digested with SacII and NotI, was ligated to a 4-5 kb right arm also amplified from purified genomic DNA of GFF88 fetal fibroblasts by using primers [PrPFR: 5'-ATAAACCCGGCGGATCTGACTATTGGGACCGTTCACTACGTCG-3'], with two sites for molecular cloning (NotI) or genomic DNA analysis (BamHI), and PrPRR: 5'-CGCGCTGGATCTGGATCTGATTACCTCGTTCTCCTAGAAA3', with two sites for molecular cloning (XhoI) or vector linearization (SacII) to complete the GTPrP vector. The GTPrP targeting vector was linearized with SacII before electro-poration.

Transfection and selection of the caprine fetal fibroblasts. After being linearized with SacII, the GTPrP targeting vector was introduced into passage 3 GFF88 fetal fibroblasts by electroporation. About 1-0 x 10⁷ cells were harvested at 60–70% confluence, mixed with 20 µg linearized and purified GTPrP vector, transferred into a 0-4 cm cuvette (Bio-Rad) and subjected to a pulse of 220 V, 950 µF, delivered by a Gene Pulser II (Bio-Rad). The transfected cells were plated into 10 cm dishes in GMEM without selection. After 48 h, all cells were trypsinized and reseeded in selective cell-culture medium with 250 µg G418 ml⁻¹ (Gibco-BRL). After 8–10 days selection, healthy and well-separated colonies were isolated with cloning rings and transferred to 96-well cell-culture plates. At subculture, a small number of cells was isolated and transferred to 48-well plates for PCR analysis and the remaining cells were expanded by passing until sufficient cells were obtained for cryopreservation and DNA extraction for Southern blot analysis.

DNA analysis. Drug-resistant colonies were first screened for targeting events by three different sets of PCR amplification across the 5'-homologous arm or the 3'-homologous arm. The positions of PCR primers are shown in Fig. 1. About 5000 cells in 48 wells were lysed in 40 µl lysis buffer [40 mM Tris/HCl (pH 8.0), 0.9% Triton X-100, 0.9% Nonidet P-40, 0.4 mg proteinase K ml⁻¹] at 65°C for 30 min and then heated to 95°C for 10 min to inactivate

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**Fig. 1.** Diagrams of the wild-type caprine PRNP locus, GTPrP targeting vector and targeted PRNP locus. The wild-type PRNP locus shows the three exons represented by black boxes. The arrow at the third exon indicates the translation-initiation site. The open box represents the neo-pA cassette. PCR primers and Southern blot probes are indicated in the map of the targeted PRNP locus. The predicted sizes of Southern blot fragments digested with BglI, XbaI or Scal/BamHI for both the wild-type PRNP locus and the targeted PRNP locus are also shown. Restriction-enzyme sites: Bg, BglI; Xb, XbaI; Sc, Scal; Ba, BamHI, Sa, SalI, Bar, 2 kb.
proteinase K. PCR amplification was performed in a 20 μl reaction volume using the Takara LA system with 2 μl cell lysate as DNA template. The primer sequences were: P1, 5′-CACAGCCAGGAGTTCAGAACAAC-3′; P2, 5′-CTCCCATGATATTGCAGCAGAC-3′; P3, 5′-CGCCCTTCTTGAGGATTCTTCC-3′; P4, 5′-CACAGATAGACGGTCCTCATAAGTC-3′; P5, 5′-TGGATGTCGAGGAAACCAAG-3′. The thermal-cycling conditions were: 2 min at 94 °C; 30 cycles of 30 s at 94 °C, 30 s at 62 °C and 3-5 min (P1/P2 and P1/P4) or 5 min (P3/P5) at 72 °C; followed by 10 min at 72 °C. PCR-amplification conditions for purified DNA from tissue or blood were the same as those for cell lysate, except that 1 μl purified DNA was used as template.

For Southern blot analysis, genomic DNA was prepared from cells, tissue or blood of goats. Samples were lysed in lysis buffer [10 mM Tris/HCl (pH 8.0), 50 mM EDTA, 10 mM NaCl, 0.5% SDS, 0.4 mg proteinase K ml⁻¹]. Then, DNA was extracted by standard methods. About 5–10 μg DNA was digested with BglI, XbaI or Scal/BamHI and separated by electrophoresis on a 0.7% agarose gel. After being transferred to a nylon membrane, the DNA was hybridized with two different probes (shown in Fig. 1): a 1–9 kb fragment corresponding to the 5′-homologous arm of GTPrP or a 1–1 kb fragment corresponding to the neomycin cassette.

Nuclear transfer. Saanen dairy goats were used as oocyte donors, temporary recipients and final recipients. These animals were purchased from other farms and were maintained in the Shanghai Nanhui Transgenic Experimental Animal Base of Shanghai Transgenic Research Center for several months before they were used in nuclear transfer. All of the animal work was done following a protocol approved by Shanghai Municipal Experimental Animal Committee. The procedures were carried out essentially as described previously (Zou et al., 2002).

Northern and RT-PCR analysis. Total RNA was isolated from brain tissue of one naturally dead PRNP region of the ovine PRNP gene sequence has not been reported, the ovine PRNP gene has previously been cloned and well characterized. It has three exons spanning 21 kb genomic DNA, with the 770 bp coding region contained entirely within the final exon (Lee et al., 1998). Two homologous arms, a 1–9 kb fragment and a 4–5 kb fragment, of the GTPrP targeting vector (Fig. 1) were generated by PCR from purified DNA of GFF88 cells by using primers that were designed according to reported sequences of the ovine PRNP gene (GenBank accession no. U67922). In the constructed GTPrP vector, a 1–1 kb neo-pA cassette was inserted directly adjacent to and in frame with the endogenous initiation codon of the PRNP gene. If homologous recombination occurs between the endogenous PRNP locus and the GTPrP vector, a 436 bp coding region following the initiation codon will be deleted and replaced by the 1–1 kb neo-pA cassette.

RESULTS

Construction of promoterless targeting vector

PrP(C) is encoded by a single-copy gene that comprises three exons, with the entire coding region contained in the third exon (Basler et al., 1986). Although the whole caprine PRNP gene sequence has not been reported, the ovine PRNP gene has previously been cloned and well characterized. It has three exons spanning 21 kb genomic DNA, with the 770 bp coding region contained entirely within the final exon (Lee et al., 1998). Two homologous arms, a 1–9 kb fragment and a 4–5 kb fragment, of the GTPrP targeting vector (Fig. 1) were generated by PCR from purified DNA of GFF88 cells by using primers that were designed according to reported sequences of the ovine PRNP gene (GenBank accession no. U67922). In the constructed GTPrP vector, a 1–1 kb neo-pA cassette was inserted directly adjacent to and in frame with the endogenous initiation codon of the PRNP gene. If homologous recombination occurs between the endogenous PRNP locus and the GTPrP vector, a 436 bp coding region following the initiation codon will be deleted and replaced by the 1–1 kb neo-pA cassette.

Disruption of the PRNP gene with the GTPrP vector

Linearized GTPrP was transfected into early passage fetal fibroblasts by electroporation. A total of four rounds of independent transfection, G418 selection and colony isolation were carried out in three different cell lines (Table 1). We first transfected linearized GTPrP into female GFF88 cells, from which two homologous arms were generated. After 8–10 days drug selection, 163 colonies were isolated. G418-resistant colonies were initially screened by three independent PCRs to detect targeted events. Of 112 colonies analysed by PCR using a forward primer, P1, that is located 5′ of the left homologous arm and a reverse primer, P4, that is located within the right homologous arm, ten were found to have undergone the desired recombination event as determined by the presence of two bands of the expected sizes: a 2.8 kb band from the normal PRNP locus and a 3.5 kb band from the targeted PRNP locus. When homologous recombination occurred between the endogenous PRNP locus and the GTPrP vector, about 0.4 kb coding region of the PRNP locus was replaced by the 1–1 kb neo-pA cassette, resulting in a new 3.5 kb band when analysed by PCR using primers P1 and P4. Fig. 2(a) shows the results of
several representative colonies analysed by PCR using primers P1 and P4. However, eight of the ten P1/P4 PCR-positive colonies were found to be mixed colonies, as determined by the lower intensity of the 3-5 kb band compared with that of the 2-8 kb band.

To confirm further successful targeting at the PRNP locus, an additional two independent PCRs were carried out. Two P1/P4 PCR-positive colonies (GTPPrP74 and GTPPrP78) were also confirmed by these two PCRs (Fig. 2b, c). In addition, the PCR products amplified with primers P1 and P4 were transferred to a nylon membrane and hybridized with a 1-9 kb 5'-arm probe (Fig. 2d), which demonstrated that the 3-5 kb bands generated with primers P1 and P4 were not non-specific bands. Finally, the 3-0 kb PCR products generated with P1/P2 and the 5-3 kb PCR products generated with P3/P5 from GTPPrP74 and GTPPrP78 were sequenced and the results were consistent with gene targeting at the PRNP locus (data not shown).

Due to the possibility of false positives produced by PCR, Southern blot analysis of the PCR-positive colonies was also employed to confirm successful targeting. The GTPPrP78 colony could not be expanded to yield enough cells for genomic DNA extraction for Southern blot. As a result, only the GTPPrP74 colony was analysed by Southern blot analysis. Genomic DNA of wild-type GFF88 cells or of GTPPrP74 was digested with three different sets of restriction enzymes and hybridized with two different probes (Fig. 3). If the PRNP gene had been targeted successfully, two BglI sites within the 0-4 kb coding region would be deleted and result in a new 12-0 kb BglI fragment from the targeted locus, in addition to the 6-1 kb endogenous BglI fragment from the non-targeted locus, when the digested DNA samples were hybridized with a 1-9 kb 5'-arm probe (Figs 1 and 3a). Similarly, when genomic DNA of GTPPrP74 was digested with XbaI or Scal/BamHI, there was a new 7-4 kb XbaI fragment in addition to the 6-7 kb endogenous XbaI fragment, or a new 5-5 kb Scal/BamHI fragment in addition to the 4-4 kb endogenous Scal/BamHI fragment, respectively (Fig. 3a). When the same membrane was hybridized with a 1-1 kb neo probe, only the newly generated fragments of the expected size from the targeted locus were visible (Fig. 3b), which was consistent with successful targeting at this locus and also indicated that only a single copy of the targeting vector had been integrated into the caprine genome.

*Colonies were scored as mixed when the 2-8 kb band from the non-targeted locus was more intense than the 3-5 kb band from the targeted locus in the PCR analysis with P1 and P4.
†Colonies were scored as senesced when cell numbers could not be seen to increase after 7 days.
‡ND, Not done.

### Table 1. Summary of PCR and Southern blot analysis results of G418-resistant colonies

<table>
<thead>
<tr>
<th>Fetal fibroblast cell line</th>
<th>GFF88</th>
<th>B38A</th>
<th>B38A</th>
<th>1126B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>Female</td>
<td>Male</td>
<td>Male</td>
<td>Male</td>
</tr>
<tr>
<td>No. G418-resistant colonies isolated</td>
<td>163</td>
<td>198</td>
<td>238</td>
<td>450</td>
</tr>
<tr>
<td>No. G418-resistant colonies analysed by PCR</td>
<td>112</td>
<td>170</td>
<td>167</td>
<td>261</td>
</tr>
<tr>
<td>No. PCR-positive colonies analysed by P1/P4</td>
<td>10</td>
<td>1</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>No. mixed colonies*</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>No. P1/P4 PCR-positive colonies confirmed by P3/P5</td>
<td>2</td>
<td>1</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>No. senesced colonies†</td>
<td>1</td>
<td>1</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>No. PCR-positive colonies confirmed by Southern blot</td>
<td>1</td>
<td>ND†</td>
<td>ND†</td>
<td>0</td>
</tr>
<tr>
<td>No. targeted colonies suitable for nuclear transfer</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Colonies were scored as mixed when the 2-8 kb band from the non-targeted locus was more intense than the 3-5 kb band from the targeted locus in the PCR analysis with P1 and P4.
†Colonies were scored as senesced when cell numbers could not be seen to increase after 7 days.
‡ND, Not done.

![Fig. 2. PCR analysis of G418-resistant colonies.](image-url)
In order to shorten the interval between the production of heterozygous \( PRNP^{+/-} \) goats and the production of homozygous \( PRNP^{-/-} \) goats by animal breeding, it was important to produce several male and female \( PRNP^{+/-} \) goats at the same time. In an effort to produce several male \( PRNP \) gene-targeted colonies, we transfected linearized GTPrP vector into two lines of male fetal fibroblasts in three independent experiments (Table 1). Although a total of six colonies isolated from transfected B38A cells were found to be positive by PCR analysis, all of them died before sufficient cells could be recovered for nuclear transfer or DNA extraction for Southern blot analysis. In addition, 450 colonies were isolated from transfected 1126B cells and 261 colonies were analysed by PCR, but no colonies were found to be positive.

**Production of \( PRNP^{+/-} \) goats by nuclear transfer**

The healthy and targeted GTPrP74 colony was used as karyoplast donor for reconstructing embryos with nucleated oocytes. Two independent groups of oocyte-collection and embryo-transfer procedures were carried out within an interval of about 1 month (Table 2). At the first group of nuclear transfers, a total of 66 morulae or blastocysts were transferred to 30 final recipients, which produced 10 pregnancies at day 35. Two pregnancies subsequently aborted, but no tissue of fetuses could be recovered for DNA analysis. Five pregnancies were maintained to term, resulting in five live births and one stillbirth. Three kids perished soon after birth and the remaining two kids remained alive and healthy for up to at least 4 months. Five live kids were delivered in the second group of nuclear transfers. Two of them also died within 48 h of birth and the other three kids also remained healthy for up to at least 3 months. The major characteristics of the 11 cloned goats were summarized in Table 3.

Autopsies were performed on all of the dead kids. We could not see any direct relationship between the death of these kids and the disruption of their \( PRNP \) gene. The most common abnormality was atelectasis at birth. The lungs of kid 7A did not expand completely and it died several hours after birth. The whole lungs of kid 16A did not expand at all and it died at birth. Kid 18A had an apparently abnormal birth weight (6·2 kg) that was about two times the birth weight of other kids and showed serious respiratory distress. Kid 7B showed some cysts on its left kidney and some coprostasis. During its 3 day life, it defecated only once. We found that its rectum was clogged with several plaques after autopsy. All of these defects have been observed or described in our previous nuclear transfer with non-transfected cells (data not shown) or in nuclear transfer with targeted cells reported by others (McCreath et al., 2000; Denning et al., 2001). In addition, no gross abnormalities in the brains of these dead kids, such as vacuolation, neuronal apoptosis or astrogliosis, were evident as judged by microscopic examination of haematoxylin/eosin-stained sections (data not shown).

We performed PCR and Southern blot to analyse these cloned goats. PCR analysis showed that five of the six kids delivered in the first group of nuclear transfer and all five kids delivered in the second group of nuclear transfer had one targeted and one normal \( PRNP \) allele (Table 3). Fig. 4(a) shows only the PCR results of the first seven

**Table 2. Summary of nuclear-transfer results**

<table>
<thead>
<tr>
<th>Group*</th>
</tr>
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<tbody>
<tr>
<td>1</td>
</tr>
<tr>
<td>--------</td>
</tr>
<tr>
<td>No. embryos transferred to temporary recipients</td>
</tr>
<tr>
<td>No. embryos recovered from temporary recipients</td>
</tr>
<tr>
<td>No. embryos developed to morula or blastocyst</td>
</tr>
<tr>
<td>No. embryos transferred to final recipients</td>
</tr>
<tr>
<td>No. final recipients</td>
</tr>
<tr>
<td>No. fetuses at day 35</td>
</tr>
<tr>
<td>No. kids at birth: live (dead)</td>
</tr>
<tr>
<td>No. kids alive over 1 week</td>
</tr>
</tbody>
</table>

*There was an interval of about 1 month between the two groups of nuclear transfers.
delivered kids. Surprisingly, one non-targeted kid (16A) was also generated, which indicated that the GTPPr74 colony still contained some non-targeted cells in spite of being scored as a non-mixed colony by the P1/P4 PCR analysis. Southern blot results (Fig. 4b) also revealed fragments that were consistent with the presence of one targeted and one normal PRNP allele in three live kids (8A, 14A and 45B).

All of the five live PRNP+/2 cloned goats showed normal development up to at least 3 (45B, 50A and 50B) or 4 (8A, 14A) months of age (Fig. 4c; Table 3), as judged by their size and body weight (data not shown). We also did not see any abnormal behaviour, such as ataxia or dementia, in these PRNP+/2 goats.

Expression analysis of the PRNP+/− goats

To further confirm that the replacement of 0.4 kb coding region of the PRNP gene by the 1.1 kb neo-pA cassette had disrupted the expression of this gene functionally, we carried out Northern blot, RT-PCR and Western blot analysis of the brain tissue of the PRNP+/2 46A goat, which was delivered after only 131 days pregnancy by the final recipient, PrP46, in the second group of nuclear transfer and died at the second day after birth (Table 3).

Northern blot analysis is shown in Fig. 5(b). Hybridization with the 2-9 kb ex3 probe containing the exon 3 sequences of the PRNP gene detected a 4.2 kb mRNA band in the brain tissue of both the wild-type goat and the 46A goat and in the GFF88 fetal fibroblast cells, consistent with the expression of a normal PRNP allele. When the RNA samples were hybridized with a 143 bp ex1 + ex2 probe containing the exon 1 and exon 2 sequences of the PRNP gene, the sample of the wild-type goat only showed the 4.2 kb band, but the sample of the 46A goat showed another new 1.2 kb mRNA band, which was consistent with a PrP−neo fused mRNA in the targeted PRNP allele (Fig. 5a, b). However, the 1.2 kb fused mRNA from the targeted allele was apparently less abundant than the 4.2 kb endogenous mRNA from the normal allele (Fig. 5b). Whether this reflects different mRNA stability or transcription activity has yet to be determined. Hybridization of the same samples with a 1.1 kb neo probe detected

Table 3. Major characteristics of the cloned goats

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Goats in group 1</th>
<th>Goats in group 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7A</td>
<td>7B</td>
</tr>
<tr>
<td>Final recipients*</td>
<td>PrP7</td>
<td>PrP7</td>
</tr>
<tr>
<td>Gestation (days)</td>
<td>149</td>
<td>149</td>
</tr>
<tr>
<td>Birth weight (kg)</td>
<td>2.6</td>
<td>2.0</td>
</tr>
<tr>
<td>Status at birth</td>
<td>Live</td>
<td>Live</td>
</tr>
<tr>
<td>Targeted</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>No. days alive</td>
<td>1</td>
<td>3</td>
</tr>
</tbody>
</table>

*Three final recipients (PrP7, PrP45 and PrP50) gave birth to two kids.

Fig. 4. DNA analysis of cloned goats. (a) PCR analysis. The PCR primers used were P1 and P4. The positions of the primers are indicated in Fig. 1. (b) Southern blot analysis. Genomic DNA of goats was digested with BglI or XbaI and was hybridized with a 1.9 kb 5′-arm probe. (c) Five PRNP+/− cloned goats at about 3 (45B, 50A and 50B) or 4 (8A, 14A) months of age. WT, Wild-type.
a 1.2 kb mRNA band only in the sample of the 46A goat, again consistent with a PrP–neo fused mRNA.

RT-PCR was also performed with the RNA samples of the brain tissue of the wild-type goat and the dead 46A goat (Fig. 5c). A forward primer (P6) located in the second exon and three reverse primers (P2, P7 and P4) located in the neo-pA cassette or in the third exon were used. Amplification with P6/P4 primers revealed a 536 bp band in both samples, which indicated the expression of the normal PRNP allele. As expected, amplification with P6/P2 or P6/P7 revealed a 670 bp band or an 865 bp band only in the sample of the 46A goat, which further confirmed the presence of a PrP–neo fused mRNA in the targeted PRNP allele.

In order to confirm functional disruption of the PRNP gene at the protein-expression level, Western blot analysis of the brain tissue of the 46A goat was carried out to detect PrPC expression in the brain tissue of both the wild-type goat and the 46A goat (Fig. 5d), but the PrPC expression level in the brain of the 46A goat was apparently reduced, which suggested that the expression of the targeted PRNP allele had been disrupted.

**DISCUSSION**

Prion diseases have caused great concern because of the BSE epidemic in cattle and the recent appearance of a new and highly lethal variant of CJD (vCJD) in humans that has caused more than 100 deaths in the UK and a few cases in other countries (Aguzzi & Polymenidou, 2004). Scrapie, a naturally occurring disease of sheep and goats, is used as the prototype of prion diseases, so sheep or goats with reduced or no PrPC expression will be useful in research on prion diseases.

Due to the low efficiency of homologous recombination in somatic cells, it is essential to use a powerful selection strategy to enrich targeted events. Two fundamentally different enrichment methods have been developed: positive–negative selection (PNS) and promoterless selection.
Generally, PNS vectors can achieve enrichments of only two- to fivefold, but promoterless vectors can achieve enrichments of 100–1000-fold (Sedivy & Dutriaux, 1999). Such powerful selection of promoterless vectors, whilst not necessary in murine ES cells because of the intrinsically high recombination efficiency, seems essential for efficient gene targeting in somatic cells. Indeed, of the 23 disruptions made in human somatic dells (Sedivy et al., 1999) and of the three genes targeted in livestock (McCreath et al., 2000; Denning et al., 2001; Dai et al., 2002; Lai et al., 2002), all involved a promoterless strategy. Although a milestone was achieved by Kuroiwa et al. (2004), who succeeded in disrupting one transcriptionally silent gene and one transcriptionally active gene in one bovine cell line by sequential targeting with PNS vectors, we chose to use a promoterless vector in our targeting experiment to ensure successful disruption of the caprine PRNP gene.

The splice-acceptor site of the third exon of the PRNP gene was not deleted in these PRNP+/−/− goats, because there was considerable evidence that deleting the splice-acceptor site of exon 3 of the murine prion protein gene (Prnp) could cause severe ataxia and Purkinje cell loss in later life of Prnp−/− mice (Sakaguchi et al., 1996). This abnormal phenotype was due to the ectopic expression of another gene (Prnd), which shows some sequence similarity to the Prnp gene and is located 16 kb downstream of the Prnp gene. Deleting the splice-acceptor site of the third exon of the murine Prnp gene causes abnormal exon skipping and formation of chimeric transcripts that place Prnd transcription under the control of the Prnp promoter, resulting in abnormal expression of the Prnd gene in the brain of Prnp−/− mice (Moore et al., 1999; Rossi et al., 2001). Disruption of the Prnd gene can prevent the appearance of this abnormal phenotype in Prnp−/− mice (Genoud et al., 2004). Our RNA analysis shows that the RNA of the targeted caprine PRNP allele is transcribed and cleaved normally (Fig. 5), excluding the possible ectopic expression of the caprine Prnd gene in the brain of these PRNP+/− or prospective PRNP−/− goats. In addition, in contrast to the prion protein gene targeting in mice reported by Büeler et al. (1992), who reported that RNA transcription of the targeted murine Prnp allele was not terminated at the neo polyadenylation site, but instead terminated at the downstream endogenous Prnp polyadenylation site, the RNA transcription of the targeted caprine PRNP allele reported here was terminated effectively at the neo polyadenylation site, resulting in a 1.2 kb, rather than a 4.9 kb, PrP−neo fused mRNA.

One minor defect in our DNA analysis was that no external probe outside the homologous arms was used in the Southern blot analysis of the GTPrP74 colony or the cloned goats. We have tried several external probes outside the 5’-homologous arm, but in all of the lanes, including the wild-type samples and the targeted samples, no specific band could be seen (data not shown). One possible explanation for this is the presence of many DNA sequence repeats in this region (Lee et al., 1998). Although external probes play some roles in excluding possible false positives resulting from random integration, we believe that the data shown in this report support the demonstration of successful gene targeting.

Regardless of the fact that six (one non-targeted and five targeted) of the 11 cloned goats perished after birth, we did not see any abnormal development or behaviour in the remaining five live PRNP+/− cloned goats up to at least 3 months of age (Fig. 4c; Table 3). This was consistent with our expectations, because only one allele at the PRNP locus was disrupted. Indeed, mice with both alleles of the Prnp gene disrupted showed no gross abnormalities (Büeler et al., 1992; Manson et al., 1994); however, in contrast, none of the PRNP+/− cloned sheep reported by Denning et al. (2001) survived for longer than 2 weeks after birth, because of various abnormalities. Our results suggest that the incidence of mortality reported here or by Denning et al. (2001) was not a consequence of the disrupted PRNP gene per se, but was due to the nuclear-transfer procedures and/or the prolonged culture or drug selection of the cells used in nuclear transfer. We think that the following aspects need to be considered carefully to produce viable animals by nuclear transfer. First, the cell line to be used in nuclear transfer needs to be selected carefully. Fetal fibroblasts isolated from fetuses of different genetic backgrounds or isolated at a different time have differing abilities of cell division. Second, the concentration of G418 used should be optimized to kill non-transfected cells in about 7 days. Increased concentrations will kill other cells over a shorter time and result in transfected cells at a low density, which will cause the cells to senesce too quickly. Third, the reconstructed embryos should be cultured in vivo to develop to morulae or blastocysts before they are transferred to final recipients.

We have shown the feasibility of modifying the genome of goats precisely by gene targeting of fetal fibroblasts, and the PRNP gene is also the first gene of goats that has been targeted successfully. We have also presented strong evidence at the DNA and protein-expression levels that demonstrates the functional disruption of one allele of the caprine PRNP gene. These heterozygous PRNP+/− goats are now ready to be used in producing homozygous PRNP−/− goats in which no PrP C should be expressed.

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REFERENCES

Prion protein gene knockout goats


