Functional disruption of the prion protein gene in cloned goats

Guohua Yu,[1,2] Jianquan Chen,[2,3] Huiqing Yu,[2,3] Siguo Liu,[2,3] Juan Chen,[2,3] Xujun Xu,[2,3] Hongying Sha,[2,3] Xufeng Zhang,[2,3] Guoxiang Wu,[2,3] Shaofu Xu[2,3] and Guoxiang Cheng[2,3]

Correspondence
Guoxiang Cheng
chenggx@cngenon.com

[1]Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Graduate School of Chinese Academy of Sciences, 320 Yue-Yang Road, Shanghai, 200031, China

[2,3]Shanghai Transgenic Research Center[2] and Shanghai Genon Bio-Engineering Co. Ltd[3], 88 Cai-Lun Road, Shanghai 201203, China

Received 27 July 2005
Accepted 14 December 2005

The cellular prion protein (PrP^C), 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 PrP^C develop and reproduce normally and are resistant to scrapie infection, large animals lacking PrP^C, 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 PrP^C should be expressed.

INTRODUCTION

Prion diseases, also called transmissible spongiform encephalopathies (TSEs), are a group of lethal, infectious, neurodegenerative 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–Sträussler–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 (PrP^{Sc}) of the cellular prion protein (PrP^C, a glycosylphosphatidylinositol-anchored membrane glycoprotein of uncertain function) (Prusiner, 1982; Legname *et al.*, 2004). The 'protein-only' hypothesis proposes that PrP^{Sc}, when introduced into animals, will cause the conversion of PrP^C into a likeness of itself and that animals with no PrP^C 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 PrP^C. 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 α1-antitrypsin gene inserted into the α1 (I) procollagen locus have been produced successfully by gene targeting on ovine fetal fibroblasts (McCreath *et al.*, 2000). One allele of the α(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 (Billinis *et al.*, 2002). The availability of sheep and goats with reduced or no expression of PrP^C 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× non-essential amino acids (Sigma), 2 ng 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′-CAATCGTATGCTTCTGCTATGTTC-3′) and SRY654r (5′-CAATGTTACCCTATCGTGGCC-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 from purified genomic DNA of GFF88 fetal fibroblasts by using primers [PrPLF: 5′-GAATCCGCGGTGTCGACTCTCTAGTCCATGATCGTTCCTC-3′, with two artificial restriction-enzyme sites (underlined) at its 5′ end for molecular cloning (*Sac*II) or vector linearization (*Sal*I), and PrPLR: 5′-TGTTCAATGGCCGATCCCAT**GATGACTTCTCTGCAAAATAAAG**-3′, 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′-**CTTTATTTTGCAGAGAAGTCATC**ATGGGATCGGCCATTGAACA-3′, with a 5′ tail (23 bp, in bold) within the *PRNP* locus and complementary to the left arm, and neoR: 5′-GAATGCGGCCGCAGTACTCCCCAGCTGGTTCTTTCCG-3′, with two sites for molecular cloning (*Not*I) or genomic DNA analysis (*Sca*I)]. These two fragments were used to prime from each other to give a 3·0 kb product. This product, digested with *Sac*II and *Not*I, was ligated to a 4·5 kb right arm also amplified from purified genomic DNA of GFF88 fetal fibroblasts by using primers [PrPRF: 5′-ATAAGCGGCCGCGGATCCAGACTATGAGGACCGTTACTATCGTG-3′, with two sites for molecular cloning (*Not*I) or genomic DNA analysis (*Bam*HI), and PrPRR: 5′-CCGCTCGAGGTCGACGTATCATTCACTTCGGCTCTGTAAA-3′, with two sites for molecular cloning (*Xho*I) or vector linearization (*Sal*I)] to complete the GTPrP vector. The GTPrP targeting vector was linearized with *Sal*I before electroporation.

Transfection and selection of the caprine fetal fibroblasts. After being linearized with *Sal*I, the GTPrP targeting vector was introduced into passage 3 GFF88 fetal fibroblasts by electroporation. About $1·0 \times 10^7$ 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 subconfluence, a small number of cells was isolated and transferred to 48-well plates for PCR analysis and the remaining cells were expanded by passaging 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 the

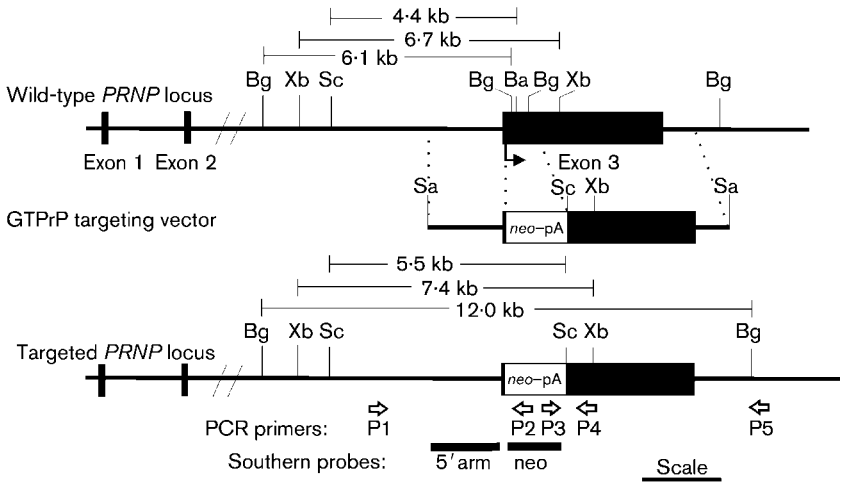

Fig. 1. Diagrams of the wild-type caprine *PRNP* locus, GTPrP targeting vector and targeted *PRNP* locus. The wild-type *PRNP* locus shows the three exons represented by black boxes. The arrow at the third exon indicates the translation-initiation site. The open box represents the *neo*-pA cassette. 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 *Bgl*I, *Xba*I or *Sca*I/*Bam*HI for both the wild-type *PRNP* locus and the targeted *PRNP* locus are also shown. Restriction-enzyme sites: Bg, *Bgl*I; Xb, *Xba*I; Sc, *Sca*I; Ba, *Bam*HI, Sa, *Sal*I. 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′-CACAGCCAGGCATTCAGAAAC-3′; P2, 5′-CCACCATGATATTCGGCAAG-3′; P3, 5′-CGCCTTCTTGACGAGTTCTTC-3′; P4, 5′-CACGATAGTAACGGTCCTCATAGTC-3′; P5, 5′-GTATGATGCAGGGAAACCAAAG-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 *Bgl*I, *Xba*I or *Sca*I/*Bam*HI 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 $PRNP^{+/-}$ goat and one wild-type goat at similar age or from wild-type GFF88 cells by using TRIzol reagent (Gibco-BRL) following the manufacturer's instructions. RNA samples (20 μg) were separated on 1·0 % agarose/formaldehyde denaturing gels, transferred to a nylon membrane and hybridized with four different probes: a 2·9 kb ex3 fragment located in the third exon of the *PRNP* gene and produced by PCR with a forward primer (5′-GACTATGAGGACCGTTACTATCGTG-3′) and a reverse primer (5′-CCAATCCCACCATACACACATC-3′), a 1·1 kb *neo* fragment, a 143 bp ex1 + 2 probe containing the sequences of the first two exons of the *PRNP* gene and produced by RT-PCR with the forward primer (5′-TGCCAGTCGCTGACAGCC-3′) and reverse primer (5′-GTGATTCAGCTCAAGTTGGATCTG-3′), and a 470 bp glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene fragment produced by RT-PCR using forward primer (5′-GCAAGTTCCACGGCACAG-3′) and reverse primer (5′-CGCCAGTAGAAGCAGGGAT-3′).

RT-PCR was performed by using an RT-PCR kit (TaKaRa) following the manufacturer's instructions. First-strand cDNA was synthesized with 2 μg total RNA by using reverse transcriptase in a volume of 20 μl. Subsequent PCR was carried out by using 2 μl cDNA sample as template. The primer sequences were: P6, 5′-CAACCAAGCTGAAGCATCTGTC-3′; P7, 5′-AAGAACTCGTCAAGAAGGCGATAGAAGGCG-3′. P2 and P4 have the same sequences as were used in the DNA analysis.

Western blot analysis. For brain tissue, 10 % (w/v) homogenates were made in lysis buffer [10 mM Tris/HCl (pH 7·4), 100 mM NaCl, 10 mM EDTA, 0·5 % Nonidet P-40, 0·5 % sodium deoxycholate] at 4 °C and were centrifuged at 1000 ***g*** for 5 min. Then, the supernatant was used for Western blot. Equal amounts of protein were separated by SDS-PAGE (12 % gels) and transferred to PVDF membranes by semi-dry electroblotting (Bio-Rad). After preincubation for 1 h in blocking buffer [50 mM Tris/HCl (pH 7·5), 150 mM NaCl, 10 % non-fat dry milk], the membranes were incubated overnight at 4 °C in the same buffer containing a 1 : 4000 dilution of a mouse anti-prion protein monoclonal antibody (mAb) (4C6, National BSE Reference Laboratory, Tsingtao, China) or a 1 : 1000 dilution of a mouse anti-actin mAb (Sigma, catalogue no. A4700). After washing, the membranes were incubated for 2 h in the blocking buffer containing a 1 : 1000 dilution of the peroxidase-conjugated goat anti-mouse IgG antibody. Then, the membranes were visualized by using SuperSignal West Pico chemiluminescent substrate (Pierce).

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

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

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

*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.

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 (GTPrP74 and GTPrP78) 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 GTPrP74 and GTPrP78 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 GTPrP78 colony could not be expanded to yield enough cells for genomic DNA extraction for Southern blot. As a result, only the GTPrP74 colony was analysed by Southern blot analysis. Genomic DNA of wild-type GFF88 cells or of GTPrP74 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 *Bgl*I sites within the 0·4 kb coding region would be deleted and result in a new 12·0 kb *Bgl*I fragment from the targeted locus, in addition to the 6·1 kb endogenous *Bgl*I 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 GTPrP74 was digested with *Xba*I or *Sca*I/*Bam*HI, there was a new 7·4 kb *Xba*I fragment in addition to the 6·7 kb endogenous *Xba*I fragment, or a new 5·5 kb *Sca*I/*Bam*HI fragment in addition to the 4·4 kb endogenous *Sca*I/*Bam*HI 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.

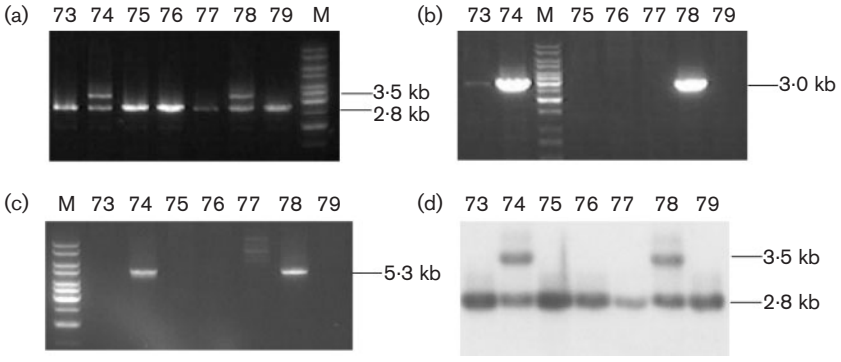

Fig. 2. PCR analysis of G418-resistant colonies. Colonies are indicated above each lane. M, 1 kb DNA marker. The PCR primers used are P1/P4 (a), P1/P2 (b) and P3/P5 (c). The positions of the primers are indicated in Fig. 1. The bands amplified by PCR with different sets of primers are also indicated. (d) Southern blot analysis of the PCR products amplified with P1/P4 primers. After being transferred to a nylon membrane, the PCR products were hybridized with a 1·9 kb 5′-arm probe.

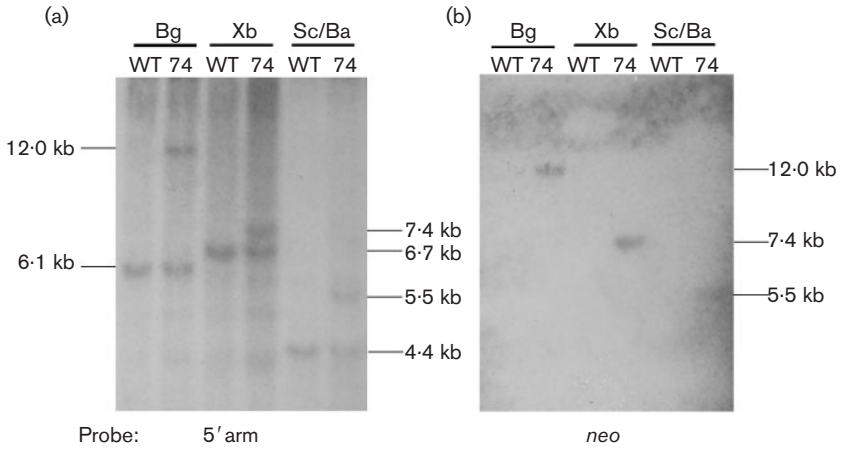

Fig. 3. Southern blot analysis of the PCR-positive GTPrP74 colony. Genomic DNA purified from wild-type GFF88 cells or GTPrP74 cells was digested with *Bgl*I, *Xba*I or *Sca*I/*Bam*HI and was hybridized with a 1·9 kb 5′-arm probe (a) or a 1·1 kb *neo* probe (b). Restriction-enzyme sites: Bg, *Bgl*I; Xb, *Xba*I; Sc, *Sca*I; Ba, *Bam*HI.

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 enucleated 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

	Group*	
	1	2
No. embryos transferred to temporary recipients	230	132
No. embryos recovered from temporary recipients	151	127
No. embryos developed to morula or blastocyst	66	59
No. embryos transferred to final recipients	66	59
No. final recipients	30	25
No. fetuses at day 35	10	9
No. kids at birth: live (dead)	5 (1)	5
No. kids alive over 1 week	2	3

*There was an interval of about 1 month between the two groups of nuclear transfers.

Table 3. Major characteristics of the cloned goats

Characteristic	Goats in group 1						Goats in group 2				
	7A	7B	8A	14A	16A	18A	45A	45B	46A	50A	50B
Final recipients*	PrP7	PrP7	PrP8	PrP14	PrP16	PrP18	PrP45	PrP45	PrP46	PrP50	PrP50
Gestation (days)	149	149	153	154	150	148	148	148	131	145	145
Birth weight (kg)	2·6	2·0	3·6	3·0	3·0	6·2	3·1	4·8	1·8	2·2	2·8
Status at birth	Live	Live	Live	Live	Dead	Live	Live	Live	Live	Live	Live
Targeted	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes
No. days alive	1	3	>120	>120	0	1	2	>90	1	>90	>90

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

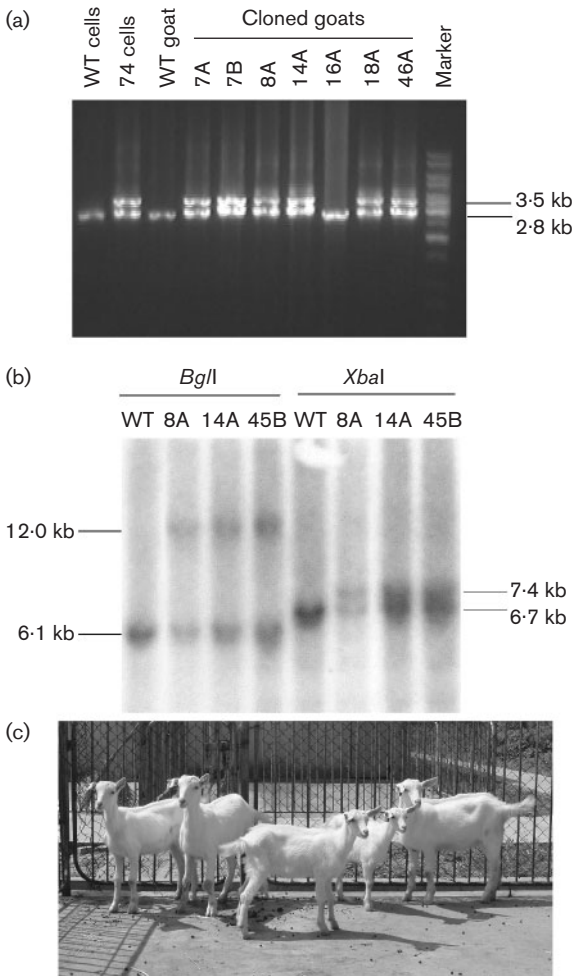

Fig. 4. DNA analysis of cloned goats. (a) PCR analysis. The PCR primers used were P1 and P4. The positions of the primers are indicated in Fig. 1. (b) Southern blot analysis. Genomic DNA of goats was digested with *Bgl*I or *Xba*I and was hybridized with a 1·9 kb 5′-arm probe. (c) Five $PRNP^{+/-}$ cloned goats at about 3 (45B, 50A and 50B) or 4 (8A, 14A) months of age. WT, Wild-type.

delivered kids. Surprisingly, one non-targeted kid (16A) was also generated, which indicated that the GTPrP74 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 $PRNP^{+/-}$ 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 $PRNP^{+/-}$ 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 $PRNP^{+/-}$ 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 + 2 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

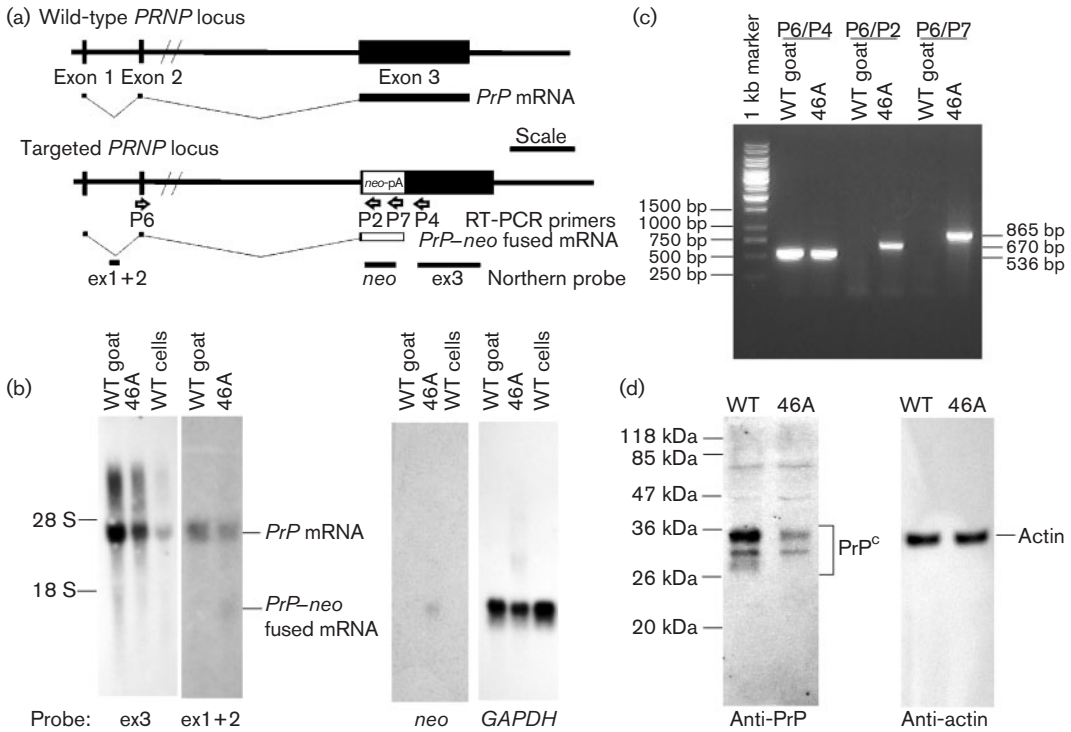

Fig. 5. Expression analysis of brain tissue of the 46A goat. (a) Diagrams of RNA transcription and cleavage of the normal *PRNP* locus and the targeted *PRNP* locus. Primers used in RT-PCR and probes used in Northern blot analysis are indicated. Bar, 2 kb. (b) Northern blot analysis. Three different probes (ex3, ex1 + 2 and *neo*) were used to analyse the RNA transcription and cleavage of the *PRNP* gene. A 470 bp glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) probe was also used as an indication of the amount of total RNA loaded. The positions of the 18S and 28S rRNA bands are indicated. (c) RT-PCR analysis. Three different sets of primers were used. (d) Western blot analysis of brain tissue of the 46A goat. Brain homogenates from the wild-type goat and the 46A goat were treated with a mouse anti-prion protein mAb to detect PrP^C expression or a mouse anti-actin mAb to ensure that each lane contained an equal amount of total protein. 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 PrP^C expression in the brain. Brain tissue isolated from the same region of the wild-type goat and the 46A goat was homogenized and an anti-prion protein mAb was used for detection of PrP^C. A typical profile of PrP^C with an apparent molecular mass of approximately 28–36 kDa was detected in the brain tissue of both the wild-type goat and the 46A goat (Fig. 5d), but the PrP^C 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 PrP^C 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 RNA 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.

ACKNOWLEDGEMENTS

We gratefully thank Aimin Zhang, Jianyan Zhao, Junlan Geng, Youbing Wu, Min Zhu, Yanhua Tang and Yinfeng Cai for their contributions to this work. We also thank Dr Jiake Zuo for his helpful suggestions on this manuscript. This work was supported by grants from the Shanghai Municipal Commission of Science and Technology (04ZR14134 and 004319001) and a grant from the '863 project' (2002AA2066052).

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