Transgenic Res (2009) 18:163–171
DOI 10.1007/s11248-008-9220-5

ORIGINAL PAPER

Production of *Prnp*$^{-/-}$ goats by gene targeting in adult fibroblasts

Caihong Zhu · Bei Li · Guohua Yu · Jianquan Chen · Huiqing Yu · Juan Chen · Xujun Xu · Youbing Wu · Aimin Zhang · Guoxiang Cheng

Received: 24 July 2008 / Accepted: 9 September 2008 / Published online: 24 September 2008

Abstract Homozygous mice devoid of functional *Prnp* are resistant to scrapie and prion propagation, but heterozygous mice for *Prnp* disruption still suffer from prion disease and prion deposition. We have previously generated heterozygous cloned goats with one allele of *Prnp* functional disruption. To obtain goats with both alleles of *Prnp* be disrupted which would be resistant to scrapie completely, a second-round gene targeting was applied to disrupt the wild type allele of *Prnp* in the heterozygous goats. By second-round gene targeting, we successfully disrupted the wild type allele of *Prnp* in primary *Prnp*$^{+/-}$ goat skin fibroblasts and obtained a *Prnp*$^{-/-}$ cell line without *Prnp* expression. This is the first report on successful targeting modification in primary adult somatic cells of animals. These cells were used as nuclear donors for somatic cell cloning to produce *Prnp*$^{-/-}$ goats. A total of 57 morulae or blastocytes developed from the reconstructed embryos were transferred to 31 recipients, which produced 7 pregnancies at day 35. At 73 days of gestation, we obtained one cloned fetus with *Prnp*$^{-/-}$ genotype. Our research not only indicated that multiple genetic modifications could be accomplished by multi-round gene targeting in primary somatic cells, but also provided strong evidence that gene targeting in adult cells other than fetal cells could be applied to introduce precise genetic modifications in animals without destroying the embryos.

Keywords Prion disease · *Prnp* · Second-round gene targeting · Adult fibroblasts

Abbreviations

DMSO	Dimethyl sulfoxide
Dpl	Doppel protein
FBS	Fetal bovine serum
GMEM	Glasgow minimal essential medium
neo	Neomycin phosphotransferase gene
Prnp	Prion protein gene
PrP	Prion protein
puro	Puromycin *N*-acetyl-transferase gene

Introduction

Prion diseases, such as scrapie in goats or sheep and bovine spongiform encephalopathy (BSE) in cattle,

C. Zhu · B. Li · A. Zhang · G. Cheng
School of Life Science and Technology, Tongji University, 1239 Si-Ping Road, Shanghai 200092, China

B. Li · G. Yu · J. Chen · H. Yu · J. Chen · X. Xu · Y. Wu · A. Zhang · G. Cheng
Shanghai Transgenic Research Center, 88 Cai-Lun Road, Shanghai 201210, China

G. Yu · J. Chen · H. Yu · J. Chen · X. Xu · Y. Wu · G. Cheng (✉)
Shanghai Genon Bio-Engineering Co. Ltd, 88 Cai-Lun Road, Shanghai 201210, China
e-mail: chenggx@cngenon.com

are a group of fatal and infectious neurodegenerative disorders of the central nervous system (CNS) (Prusiner 1998). The therapeutic research for prion diseases is intensive but they are still currently incurable (Stewart et al. 2008). There is considerable evidence that the PrP plays an essential role in prion diseases (Aguzzi et al. 2008). More interestingly, while the cellular PrP (PrP^C) is absolutely required for disease pathogenesis, it is dispensable for normal animal development. Mice devoid of functional *Prnp* ($Prnp^{-/-}$) do not present macroscopic developmental or anatomical alternations, and they are completely resistant to scrapie and do not propagate prions (Büeler et al. 1992; Büeler et al. 1993). Also, PrP-deficient cattle are clinically, physiologically, histopathologically, immunologically and reproductively normal, the brain tissue homogenates from $Prnp^{-/-}$ cattle are resistant to prion propagation in vitro as assessed by protein misfolding cyclic amplification (PMCA) (Richt et al. 2007). Based on these results, goat, a natural host of the prototype of prion diseases scrapie, with PrP genes disruption should also survive and reproduce normally.

Different from $Prnp^{-/-}$ mice, the mice heterozygous for disrupted *Prnp* ($Prnp^{+/-}$) still suffer from prion disease though with a prolonged incubation time, and the prion deposition and lesion distribution at terminal stage of disease are similar in heterozygous and wild type mice (Büeler et al. 1994; Manson et al. 1994). We have reported earlier on generation of five heterozygous cloned goats with one allele of *Prnp* be functionally disrupted (Yu et al. 2006). To obtain goats with both alleles of *Prnp* disruption which would be resistant to scrapie and prion propagation completely, the heterozygous goats could be bred to produce homozygosity. Alternatively, a second-round gene targeting could be carried out in $Prnp^{+/-}$ fibroblasts to disrupt the wile type allele of *Prnp* and followed with animal cloning.

Because livestock like goats, sheep, pigs, and cows have a long generation interval, the time required for production of homozygous livestock by sequential gene targeting could be greatly reduced compared to the traditional breeding strategy (Kuroiwa et al. 2004). In this research, while making efforts to produce homozygous goats by breeding traditionally, we also applied a second-round gene targeting in $Prnp^{+/-}$ fibroblasts to disrupt the wild type allele of *Prnp*, and then prepared cloned goats through nuclear transfer. Different from traditional somatic cell gene targeting in fetal fibroblasts (McCreath et al. 2000; Denning et al. 2001; Dai et al. 2002; Lai et al. 2002; Kuroiwa et al. 2004; Yu et al. 2006; Rogers et al. 2008), we chose the skin fibroblasts derived from an adult $Prnp^{+/-}$ goat ear biopsy as target cells for second-round gene targeting because $Prnp^{+/-}$ fetal is rare and difficult to obtain and the cloning efficiency of goat using fetal fibroblast or adult fibroblasts are almost the same in our laboratory.

In this report, we successfully targeted the wild type allele of *Prnp* in primary $Prnp^{+/-}$ skin fibroblasts and obtained a $Prnp^{-/-}$ cell line without *Prnp* expression. To our knowledge, it is the first report on successful targeting modification of a gene in primary adult somatic cells of animals. Using these targeted skin fibroblasts as nuclear donor in nuclear transfer, we finally obtained a cloned fetus with both alleles of *Prnp* be disrupted at day 73 of gestation.

Methods

Isolation and culture of $Prnp^{+/-}$ goat skin fibroblasts

The primary goat skin fibroblasts GSF3-1 were isolated from an ear skin biopsy of a 1.2-year-old male $Prnp^{+/-}$ Saanen dairy goat as described by Kubota et al. (2000). Briefly, skin biopsy was cut into small pieces after washing in PBS containing penicillin and streptomycin for 3 times and transferred into 100 mm tissue culture dishes containing 4 ml GMEM (Gibco) supplemented with 2 mM L-glutamine (Gibco), 1 mM sodium pyruvate (Gibco), 1× non-essential amino acids (Gibco), 10% FBS (Gibco), 100 U penicillin ml^{-1} and 100 μg streptomycin ml^{-1} (Gibco). When the expanding cells became confluent, they were disaggregated by 0.25% trypsin-EDTA (Gibco) and passaged to new dishes. The cells at different passages were cryopreserved in 10% DMSO (Sigma) for further manipulation.

Construction of the second-round targeting vector

Two fragments including the homologous arms around the exon 3 of *Prnp* in the targeted allele (Allele A) and the wild type allele (Allele B) of GSF3-1 were

amplified, respectively. A 1.9 kb 5′ homologous arm and a 6.1 kb 3′ homologous arm from the two fragments of allele B were used to construct a promoter-less targeting vector GTPrP*puro* with *puro*-pA sequence directly adjacent to the endogenous gene start codon. The 1.9 kb 5′ homologous arm was amplified by using primers PrP5f: 5′-GAACGTCGAC TCTCCAGTCCATGGTCGTTCCTC-3′ with an artificial restriction-enzyme sites (underlined) at its 5′ end for molecular cloning and vector linearization (*Sal* I), and PrP5r: 5′-GTGGGCTTGTACTCGGT**CATGA TGACTTCTCTGCAAAAT**-3′; with a 3′ tail (22 bp; in bold) within the *Prnp* locus and a 5′ tail complementary to the start of *puro* coding sequences (17 bp). The 0.9 kb *puro*-pA fragment was amplified by using primers puroF: 5′-**ATTTTGCAGAGAAGT CATCATG**ACCGAGTACAAGCCCAC-3′, with a 5′ tail (22 bp, in bold) within the *Prnp* locus and complementary to the 5′ homologous arm, and puroR: 5′-CGCGGATCCGCGCCCCAGCTGGTTCTTTCC-3′, with a sites (underlined) at its 5′ end for molecular cloning (*Bam* H I). These two fragments were used to prime from each other to give a 2.8 kb product which was ligated to a 6.1 kb right arm amplified by using primers PrP3f: 5′-CGCGGATCCGGATCCTGGTTC TCTTTGTGG-3′ with a site for molecular cloning (*Bam H* I) and PrP3r: 5′-CCGCTCGAGGTCGACAT GCTGGAGAGGATGTGGAGA-3′, with two sites for molecular cloning (*Xho* I) or vector linearization (*Sal* I) to complete the targeting vector.

The targeting vector GTPrP*puro* was linearized with *Sal* I before electroporation.

Transfection and selection of the GSF3-1

The GTPrP*puro* targeting vector was linearized with *Sal* I and introduced into passage 4 GSF3-1 by electroporation. About 1.0×10^7 exponentially growing GSF3-1 cells were disaggregated and washed in PBS twice, then mixed with 10 μg linearized and purified GTPrP*puro* and subjected to a pulse of 400 V/250 μF in a 0.4 cm Gene Pulser Cuvette (Bio-Rad). The transfected cells were plated into two 10 cm-dishes in GMEM without selection. After 48 h, all cells were trypsinized and reseeded in selective cell-culture medium with 0.8 μg/ml Puromycin (Sigma). After 9–10 days selection, healthy and well-separated colonies were isolated with cloning rings and transferred to 48-well cell-culture plates. At subconfluence, half of cells were isolated for PCR analysis and the remaining cells were expanded by passaging until sufficient cells were obtained for cryopreservation and nuclear transfer.

Genomic PCR analysis of drug resistant cell colonies

Drug-resistant colonies were screened for targeting events by three different sets of PCR amplification across the 5′-homologous arm or the 3′-homologous arm. Approximately 5,000 cells in 48-well plates were lysed in 40 μl embryo lysis buffer (ELB) (40 mM Tris/HCl, pH 8.9, 0.9% Triton X-100, 0.9% Nonidet P-40, 0.4 mg/ml proteinase K) at 65°C for 15 min and heated to 95°C for 10 min to inactivate the proteinase K. PCR amplification was performed in a 20 μl reaction volume using the TaKaRa LA system with GC buffer with 3 μl cell lysate as DNA template. The positions of primers are indicated in Fig. 1. The primer sequences were: P1, 5′-CACAGCCAGGCATTCAGAAAC-3′; P2, 5′-AGTTGCCAGCCATCTGTTGTT-3′; P3, 5′-AACAACAGATGGCTGGCAACT-3′; P4, 5′-CACG ATAGTAACGGTCCTCATAGTC-3′; P5, 5′-GCA G AGGACCCAAACAGACAT-3′; The thermal cycling conditions were: 5 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 62°C and 3.5 min (P1/P3 and P1/P4) or 6.5 min (P2/P5) at 72°C; followed by 10 min at 72°C.The PCR products of positive colonies were sent to sequencing to further confirm the targeting events.

RT-PCR analysis

Analysis of *Prnp* expression was performed in skin fibroblasts. Total RNA was extracted from $Prnp^{+/+}$, $Prnp^{+/-}$ and $Prnp^{-/-}$ skin fibroblasts by using TRIzol reagent (Invitrogen), the first strand cDNA was synthesized with 2 μg RNA using RT-PCR kit (TaKaRa) following the manufacturer's instructions. Subsequent PCR was carried out using primers P6 and P7 in 30 cycles of 94°C, 30 s, 58°C, 30 s, 72°C, 45 s. To detect expression of goat β-actin mRNA as control, primers GBAF and GBAR were used in the same PCR conditions. To exclude the possibility of genomic DNA contamination, another RT-PCR was carried out without reverse transcriptase. The positions of primers P6 and P7 are indicated in Fig. 1. The primers sequences were: P6, 5′-GAGTGCTGAA GAGTTGATGC-3′; P7, 5′-CTTACCAGTCCAA

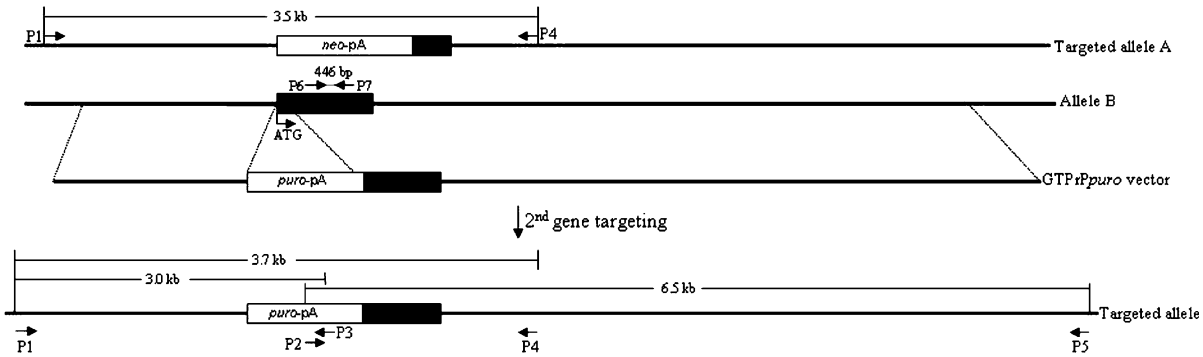

Fig. 1 Diagrams of the targeted allele A, allele B, second-round targeting vector GTPrP*puro* and targeted allele B. The black box represents the coding sequence (CDS) of *Prnp*, the open boxes represent *neo*-pA and *puro*-pA cassettes. PCR primers are indicated in the targeted *Prnp* locus and the RT-PCR primers are indicted in wild type *Prnp* locus. The expected size of PCR and RT-PCR products are also shown in the maps

CACTAGCA-3′; GBAF, 5′-TGGCACCACACC TTCTACAA-3′; GBAR, 5′-TCCTTGATGTCACGG ACGAT-3′.

Western blot analysis

Total protein was extracted from $Prnp^{+/+}$, $Prnp^{+/-}$, and $Prnp^{-/-}$ skin fibroblasts with RIPA lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1.0% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate) at 4°C. Equal amounts of protein sample were run on a 12% SDS-PAGE gel and transferred to PVDF membranes by semi-dry electroblotting (Bio-Rad). After preincubation for 1 h in blocking buffer (25 mM Tris, pH 8.0, 140 mM NaCl, 3 mM KCl, 0.05% Tween-20, 5% non-fat dry milk), the membrane were incubated for 1 h in the same buffer containing a 1:2,000 dilution of a mouse anti-PrP monoclonal antibody 4C6 (National BSE Reference Laboratory, Qingdao, China) or a 1:1,000 dilution of a mouse anti-actin monoclonal antibody (Sigma, catalog no. A4700). After washing, the membranes were incubated for 1 h in the blocking buffer containing a 1:1,000 dilution of the horseradish peroxidase-conjugated goat anti-mouse IgG antibody. Then the membranes were washed again and visualized with diaminobenzidine (DAB).

Chromosome analysis

Targeted cells ($Prnp^{-/-}$) were cultured in 100 mm dishes to about 80% confluence and arrested at metaphase (M) by adding colecmid (Sigma) to the culture at a final concentration of 0.5 μg/ml. After 1 h, the cells were collected and treated with hypotonic KCl (0.075 M) for 15 min at 37°C. The cells were then fixed in acetic methanol (vol/vol = 1:3), and drops of cell suspension were spread on clean microscopic slides. The chromosomes were stained with 5% Giemsa for 10 min. The numbers of well spread chromosomes within a clear cell boundary were counted under a light microscope at 1,000× magnification under oil.

Embryonic cloning

The cloned goat embryos were produced by nuclear transfer as described previously (Zou et al. 2002) with slight modification. In brief, the healthy targeted cells ($Prnp^{-/-}$) with normal karyotypes were treated with starved medium and then introduced into the perivitelline of enucleated oocytes with a beveled pipette, subsequently, the reconstructed oocytes were electrically activated and cultured in oviducts of temporary recipients. The morulae and blastocytes from reconstructed embryos cultured in vivo for 5 days were surgically transferred into uteri of the synchronized final recipients. At day 35, the surrogates were scanned with a B-ultra-sound scanner to identify pregnant goats which were singled out and observed closely until they gave birth.

All animal work was done following a protocol approved by Shanghai Municipal Experimental Animal Committee.

Genomic PCR analysis of cloned embryo

Genomic DNA was extracted from the tissue of cloned goat embryo using FlexiGene DNA kit (QIAGEN) following the manufacturer's instructions. PCR analysis was carried out using primers P1 and P4 to detect the genotypes of cloned embryo.

Results

Construction of the promoter-less targeting vector GTPrP*puro*

The *Prnp* is expressed in goat skin fibroblasts, it is also possible to enrich for homologous recombination events using a promoter-trap strategy as in fetal fibroblasts.

In attempting to target the allele B, there is the possibility that the second targeting vector will undergo homologous recombination with the first integrated targeting vector GTPrP (Yu et al. 2006), resulting in replacement of the knockout vector in allele A rather than disruption of the allele B. Two fragments including the 5′ and 3′ homologous arm of the first targeting vector GTPrP were amplified from *Prnp* allele A of GSF3-1, the analogous fragments were also amplified from allele B. These fragments were analyzed and compared. The results indicated that the allele A and allele B of *Prnp* have 2% discrepancies in the homologous arms. To targeting the allele B as we expected, the targeting vector GTPrP*puro* in which the homologous arms were isogenic to allele B was constructed by inserting the *puro*-pA directly adjacent to the initiation codon of *Prnp*. If homologous recombination occurs between GTPrP*puro* vector and allele B, a 23 bp coding region followed the initiation codon will be deleted and replaced by the 0.9 kb *puro*-pA cassette (Fig. 1). But if the recombination occurs in allele A, the fist targeting vector GTPrP will be replaced by the second-round targeting vector GTPrP*puro*. The same as the first vector GTPrP, the GtPrP*puro* also retained the splice acceptor site of exon 3 to avoid causing severe ataxia and Purkinje cell loss in aged animal (Moore et al. 1999; Rossi et al. 2001).

Targeting of the allele B of *Prnp* with GTPrP*puro* vector

Linearized GTPrP*puro* vector was delivered into passage 4 GSF3-1 fibroblasts by electroporation. After Puromycin selection for 9–10 days, the drug-resistant colonies were isolated using cloning ring. Puromycin-resistant colonies were first screened by PCR using P1/P4 to detect targeted events. Of 204 colonies analyzed by PCR using a forward primer, P1, that is located upstream of the 5′ homologous arm and a reverse primer, P4, that is located within the 3′ homologous arm, five colonies (2.5%) were found to have targeted allele B to produce cells with both alleles of *Prnp* be disrupted ($Prnp^{-/-}$), as determined by the presence of two bands of the expected sizes: a 3.5 kb band from the first targeted allele A and a 3.7 kb band from the second targeted allele B (Fig. 2a). Other five colonies (2.5%) were found to have replaced the first targeting vector in allele A as determined by the presence of two bands: a 2.8 kb band from the normal *Prnp* locus in allele B and a 3.7 kb band from the replaced allele A (Fig. 2a; Table 1). These results indicated that the vector GTPrP*puro* had no bias on targeting efficiency for allele A and allele B of *Prnp* though it was isogenic to allele B.

To further confirm the successful targeting events, two additional independent PCRs were carried out in the targeted colonies. The results were as expected (Fig. 2b, c). In addition, the 3.0 kb PCR products generated with P1/P3 and the 6.5 kb PCR products generated with P2/P5 from positive colonies were sequenced and the results were also consistent with our expectation (Fig. 3). These results firmly indicated the targeting events by homologous recombination had been occurred between the targeting vector GTPrP*puro* and *Prnp* locus in GSF3-1. We successfully targeted a gene in adult somatic cells and obtained a cell line with both alleles of *Prnp* disruption.

Functional disruption of *Prnp* expression in skin fibroblasts

To evaluate the functional disruption of *Prnp* gene in fibroblasts, RT-PCR was carried out to detect the mRNA level of *Prnp* in $Prnp^{+/+}$, $Prnp^{+/-}$, and $Prnp^{-/-}$ goat skin fibroblasts. Primers P6 and P7 are

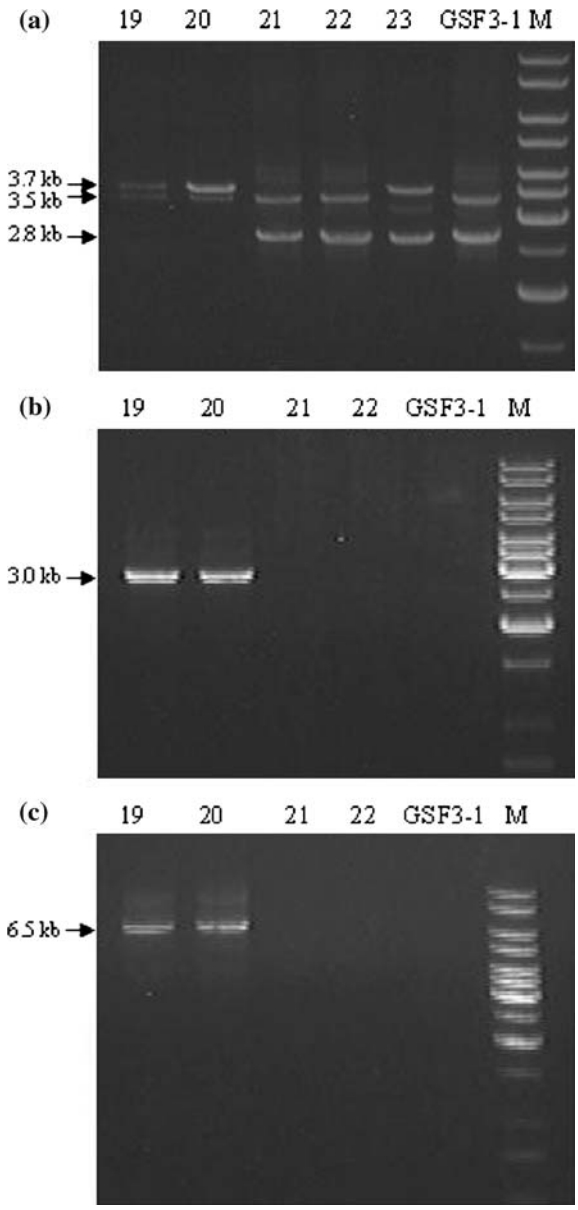

Fig. 2 PCR analysis of Puromycin-resistant colonies. Colony numbers are indicated above each lane. The PCR primers are (**a**) P1/P4, (**b**) P1/P3, (**c**) P2/P5. The position of primers are indicated in Fig. 1. No. 19 and No. 20 colonies were $Prnp^{-/-}$ colonies as the presence of a 3.5 kb and a 3.7 kb bands amplified by P1/P4, a 3.0 kb band amplified by P1/P3, and a 6.5 kb band amplified by P2/P5. No. 21, No. 22 colonies were untargeted colonies. No. 23 colony was replaced colony as the presence of a 2.8 kb and a 3.7 kb bands amplified by P1/P4. GSF3-1 was untransfected goat skin fibroblast. M: 1 kb DNA ladder

located in the exon 3 of *Prnp*. Amplification with P6 and P7 generated a 446 bp fragment indicated the expression of the wild type allele of *Prnp* in $Prnp^{+/+}$ and $Prnp^{+/-}$ fibroblasts. No fragment was generated in $Prnp^{-/-}$ fibroblasts in which both alleles of *Prnp* had been disrupted by insertion of *neo*-pA and *puro*-pA cassette (Fig. 4). These results indicted that the transcription of *Prnp* had been functionally disrupted in skin fibroblasts.

Western blot was also performed on $Prnp^{+/+}$, $Prnp^{+/-}$, and $Prnp^{-/-}$ goat skin fibroblasts using an anti-PrP monoclonal antibody 4C6 to confirm the functional inactivation of *Prnp*. PrP-specific bands (approximately 35 kDa) were detected from both $Prnp^{+/+}$ and $Prnp^{+/-}$ skin fibroblasts, but no band was observed in $Prnp^{-/-}$ skin fibroblasts (Fig. 5). These results clearly demonstrated that the expression of *Prnp* was functionally disrupted in goat skin fibroblasts.

Production of $Prnp^{-/-}$ embryo by nuclear transfer

The healthy No. 19 targeted skin fibroblasts ($Prnp^{-/-}$) with normal karyotype (data not shown) were used as nuclear donor for reconstructing embryos with enucleated oocytes. After nuclear transfer, a total of 57 (40.1%) morulae or blastocytes were transferred to 31 recipients, which produced 7 (22.6%) pregnancies at day 35. But unfortunately, all pregnancies aborted subsequently with only 1 of the fetuses recovered at day 73 of gestation (Table 2). Genomic DNA analysis by PCR using primers P1 and P4 was carried out to detect the genotype of this cloned embryo, the results show that this aborted fetus was $Prnp^{-/-}$, which was consistent with the donor cell No. 19 colony (Fig. 6).

Discussion

By second-round gene targeting, we successfully targeted the wild type allele of *Prnp* in primary $Prnp^{+/-}$ skin fibroblasts using a promoter-less vector GTPrP*puro*. The targeting modification in adult somatic cells may have wider application, because the adult skin fibroblasts are more convenient than fetal fibroblasts to obtain and have no ethical issues. In contrast to the gene targeting in adult rhesus macaque fibroblasts (Meehan et al. 2008), the primary adult goat fibroblasts without transfection of hTERT expression cassette could be cultured in vitro long enough to complete gene targeting

Table 1 Efficiency of gene targeting of *Prnp* in goat skin fibroblast GSF3-1

Total fibroblasts	Puromycin resistant colonies	Total targeting events in allele A	Total targeting events in allele B	Mixed colonies[a]	Senesced colonies[b]	Targeted colonies suitable for NT[c]
1×10^7	204	5 (2.5%)	5 (2.5%)	5	2	1

[a] Colonies were scored as mixed when all 3 bands (2.8 kb, 3.5 kb, 3.7 kb) had been amplified by PCR using P1/P4

[b] Colonies were scored as senesced when the cell numbers could not be seen to increase after 7 days

[c] The other two pure and unsenesced colonies were targeted in allele A

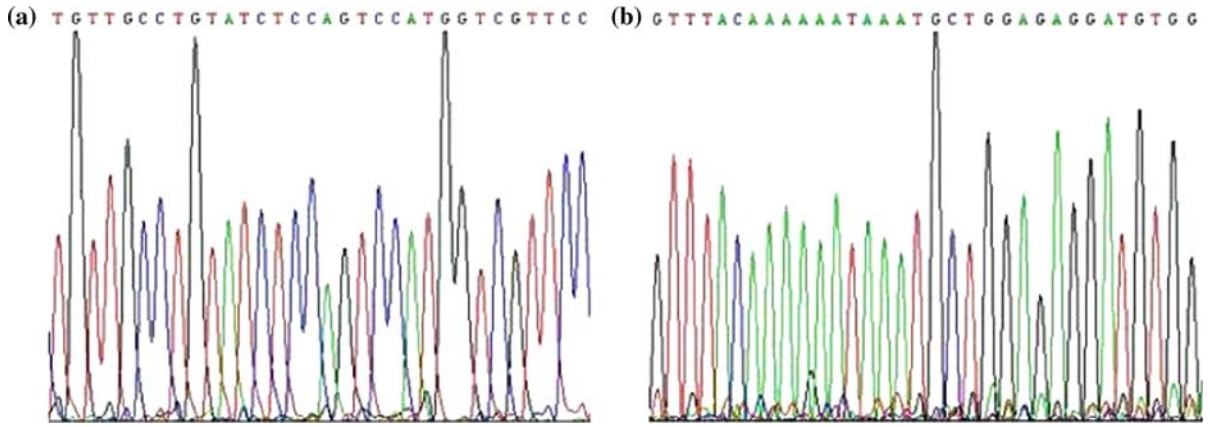

Fig. 3 Sequencing analysis of the targeted allele. (**a**) The sequences of the connection between the 5′ homologous arm of GTPrP*puro* and *Prnp* locus. (**b**) The sequences of the connection between the 3′ homologous arm of GTPrP*puro* and *Prnp* locus. The results indicated successful homologous recombination between the targeting vector GTPrP*puro* and *Prnp* locus

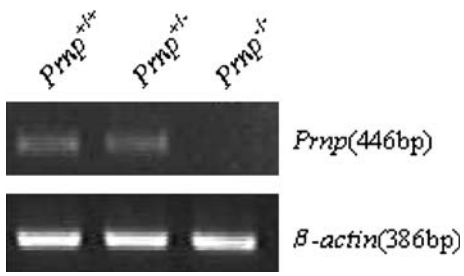

Fig. 4 RT-PCR analysis of $Prnp^{+/+}$, $Prnp^{+/-}$, and $Prnp^{-/-}$ goat skin fibroblasts. By the presence or absence of expected size of RT-PCR products, *Prnp* transcription were observed in $Prnp^{+/+}$ and $Prnp^{+/-}$ fibroblasts but not in $Prnp^{-/-}$ goat skin fibroblast with both alleles be disrupted. β-actin was used for control to monitor template amounts

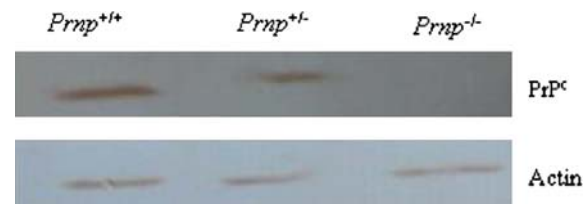

Fig. 5 Western blot analysis of $Prnp^{+/+}$, $Prnp^{+/-}$, and $Prnp^{-/-}$ goat skin fibroblasts. The absence of PrP-specific band in $Prnp^{-/-}$ goat skin fibroblasts indicated the functional disruption of *Prnp* expression in goat skin fibroblasts. The anti-actin antibody was used to ensure that each lane contained an equal amount of total protein

procedures, and the homologous recombination could be successfully achieved without S-phase synchronization or adding SV 40 enhancer element to the targeting vector in our experiments. Our research also indicated that multiple genetic modifications could be accomplished by multi-round gene targeting in primary adult somatic cells.

To specifically target the allele B of *Prnp*, we constructed a second targeting vector in which the homologous arms were isogenic to allele B, which has 2% discrepancies to allele A in this location. But the results indicated that there were no bias on the

Table 2 Summary of nuclear transfer results

Stage of nuclear transfer	Number
Embryos transferred into temporary recipients	166
Embryos recovered from temporary recipients	142
Morula or blastocyst developed in vivo	57 (40.1%)
Embryos transferred to final recipients	57
Final recipients	31
Fetuses at day 35	7 (22.6%)
Lambs at birth	0

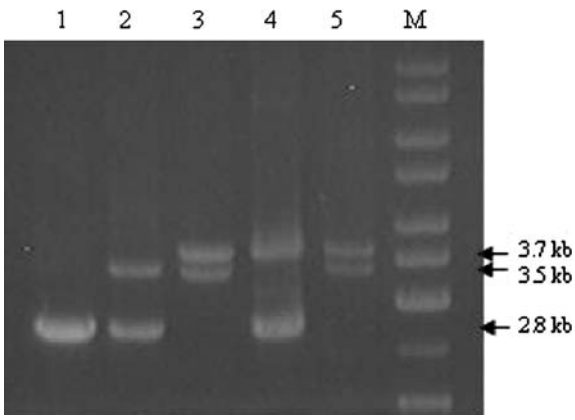

Fig. 6 Genomic PCR analysis of the aborted cloned fetus. 1: Wild type skin fibroblasts ($Prnp^{+/+}$); 2: Heterozygous skin fibroblasts GSF3-1 ($Prnp^{+/-}$); 3: No.19 skin fibroblasts colony with allele B targeted ($Prnp^{-/-}$); 4: No.23 skin fibroblasts colony with allele A replaced ($Prnp^{+/-}$); 5: Aborted cloned fetus. M: 1 kb DNA ladder

targeting efficiency between the allele A and allele B of *Prnp* (1:1) using this vector. Our data, together with others (Sedivy et al. 1999; Kuroiwa et al. 2004), supported a postulation that the isogenicity does not significantly influence the efficiency of homologous recombination in some gene locus in goat skin fibroblast.

Although we have not obtained live-born $Prnp^{-/-}$ goats by second-round gene targeting and nuclear transfer, we did not expect this failure as a direct result of the gene disruption of *Prnp* because three $Prnp^{-/-}$ goats obtained through breeding strategy remained healthy up to 5 months of age (data not shown). The aborted cloned fetus had similar abnormalities as other nuclear transfer experiments with untransfected cells in our laboratory. And the development rates at different stages of the animal cloning procedure were also similar with other experiments (Yu et al. 2006). So we attributed this result to the inherently low efficiency of somatic cell cloning technology (Wilmut et al. 1997).

All the $Prnp^{-/-}$ goats obtained from breeding strategy have remained healthy for at least 5 months without showing obvious abnormalities, this indicated that "loss of function" of goat PrP^c is unlikely in itself to be significant in the pathogenesis of scrapie and that ablation of the normal cellular prion protein PrP^c function does not adversely affect the normal goat development. Therefore, our research supplied more evidence supporting a general hypothesis that PrP^c function is not vital for normal animal development (Tremblay et al. 1998; Mallucci et al. 2002). However, detailed characterization of these goats should be carried out to investigate the physiological function of PrP^c in goats.

The $Prnp^{-/-}$ goats could be a more relevant model than PrP-deficient mice for elucidating the basic mechanisms of prion diseases and PrP^c functions. They also could be a better source of a variety of goat-derived products that have been extensively used in biotechnology, and be useful for production of prion-free therapeutic recombinant human protein, tissue and organs in transgenic goats for biomedical application.

Acknowledgments We gratefully thank Hongying sha, Laixiang Ge, Dawei Hu, Fazhan Xie and Lijun Ding for their technical assistance to this work. This work was supported by grants from Shanghai Rising-Star Program (07QB14022) and China Postdoctoral Science Foundation (20060400174).

References

Aguzzi A, Sigurdson C, Heikenwaelder M (2008) Molecular mechanisms of prion pathogenesis. Annu Rev Pathol 3:11–40. doi:10.1146/annurev.pathmechdis.3.121806.154326

Büeler H, Fischer M, Lang Y et al (1992) Normal development and behaviour of mice lacking the neuronal cell-surface PrP protein. Nature 356(6370):577–582. doi:10.1038/356577a0

Büeler H, Aguzzi A, Sailer A et al (1993) Mice devoid of PrP are resistant to scrapie. Cell 73(7):1339–1347. doi:10.1016/0092-8674(93)90360-3

Büeler H, Raeber A, Sailer A et al (1994) High prion and PrPSc levels but delayed onset of disease in scrapie-inoculated mice heterozygous for a disrupted PrP gene. Mol Med 1(1):19–30

Dai Y, Vaught TD, Boone J et al (2002) Targeted disruption of the a1,3-galactosyltransferase gene in cloned pigs. Nat Biotechnol 20(3):251–255. doi:10.1038/nbt0302-251

Denning C, Burl S, Ainslie A et al (2001) Deletion of the a(1,3)galactosyl transferase (GGTA1) gene and the prion protein (PrP) gene in sheep. Nat Biotechnol 19(6):559–562. doi:10.1038/89313

Kubota C, Yamakuchi H, Todoroki J et al (2000) Six cloned calves produced from adult fibroblast cells after long-term culture. Proc Natl Acad Sci USA 97(3):990–995. doi:10.1073/pnas.97.3.990

Kuroiwa Y, Kasinathan P, Matsushita H et al (2004) Sequential targeting of the genes encoding immunoglobulin-mu and prion protein in cattle. Nat Genet 36(7):775–780. doi:10.1038/ng1373

Lai L, Kolber-Simonds D, Park KW et al (2002) Production of a-1,3-galactosyltransferase knockout pigs by nuclear transfer cloning. Science 295(5557):1089–1092. doi:10.1126/science.1068228

Mallucci GR, Ratté S, Asante EA et al (2002) Post-natal knockout of prion protein alters hippocampal CA1 properties, but does not result in neurodegeneration. EMBO J 21(3):202–210. doi:10.1093/emboj/21.3.202

Manson JC, Clarke AR, McBride PA et al (1994) PrP gene dosage determines the timing but not the final intensity or distribution of lesions in scrapie pathology. Neurodegeneration 3(4):331–340

McCreath KJ, Howcroft J, Campbell KH et al (2000) Production of gene-targeted sheep by nuclear transfer from cultured somatic cells. Nature 405(6790):1066–1069. doi:10.1038/35016604

Meehan DT, Zink MA, Mahlen M et al (2008) Gene targeting in adult rhesus macaque fibroblasts. BMC Biotechnol 8:31. doi:10.1186/1472-6750-8-31

Moore RC, Lee IY, Silverman GL et al (1999) Ataxia in prion protein (PrP)-deficient mice is associated with upregulation of the novel PrP-like protein doppel. J Mol Biol 292(4):797–817. doi:10.1006/jmbi.1999.3108

Prusiner SB (1998) Prions. Proc Natl Acad Sci USA 95(23):13363–13383. doi:10.1073/pnas.95.23.13363

Richt JA, Kasinathan P, Hamir AN et al (2007) Production of cattle lacking prion protein. Nat Biotechnol 25(1):132–138. doi:10.1038/nbt1271

Rogers CS, Hao Y, Rokhlina T et al (2008) Production of CFTR-null and CFTR-DeltaF508 heterozygous pigs by adeno-associated virus-mediated gene targeting and somatic cell nuclear transfer. J Clin Invest 118(4):1571–1577. doi:10.1172/JCI34773

Rossi D, Cozzio A, Flechsig E et al (2001) Onset of ataxia and Purkinje cell loss in PrP null mice inversely correlated with Dpl level in brain. EMBO J 20(4):694–702. doi:10.1093/emboj/20.4.694

Sedivy JM, Vogelstein B, Liber HL et al (1999) Gene targeting in human cells without isogenic DNA. Science 283(1):9. doi:10.1126/science.283.5398.9a

Stewart LA, Rydzewska LH, Keogh GF et al (2008) Systematic review of therapeutic interventions in human prion disease. Neurology 70(15):1272–1281. doi:10.1212/01.wnl.0000308955.25760.c2

Tremblay P, Meiner Z, Galou M et al (1998) Doxycycline control of prion protein transgene expression modulates prion disease in mice. Proc Natl Acad Sci USA 95(21):12580–12585. doi:10.1073/pnas.95.21.12580

Wilmut I, Schnieke AE, McWhir J et al (1997) Viable offspring derived from fetal and adult mammalian cells. Nature 385(6619):810–813. doi:10.1038/385810a0

Yu G, Chen J, Yu H et al (2006) Functional disruption of the prion protein gene in cloned goats. J Gen Virol 87(Pt4):1019–1027. doi:10.1099/vir.0.81384-0

Zou X, Wang Y, Cheng Y et al (2002) Generation of cloned goats (*Capra hircus*) from transfected foetal fibroblast cells, the effect of donor cell cycle. Mol Reprod Dev 61(2):164–172. doi:10.1002/mrd.1143