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Full Length Research Paper

Disruption of the 37-kDa/67-kDa laminin receptor gene in bovine fetal fibroblasts

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The 37-kDa/67-kDa laminin receptor (LRP/LR), also known as ribosomal protein SA (RPSA), acts as a cell surface receptor for prions and plays an important role in internalization of cellular prion protein. In this study, we knocked out the part of prion binding sites (aa 161-205) by gene targeting in the bovine fetal fibroblasts (BFF). This is the first report about disrupting the gene encoding for the prion binding site in bovine fetal fibroblasts. The heterozygous BFF are ready to be used in producing homozygous cattle, which will be applied to study the interaction between prion and the 37-kDa/67-kDa LRP/LR.

Key words: Prion, PrP^{C}, PrP^{Sc}, 37-kDa/67-kDa laminin receptor, gene targeting.

INTRODUCTION

Prion diseases are a group of fatal, infectious, neuro-degenerative disorders, including Creutzfeldt-Jakob disease (CJD) in humans, scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle and wasting disease in deer (CWD) (Collinge, 2001). The principal hallmarks of these diseases are brain vacuolation, neuronal apoptosis and astrogliosis (Yu et al., 2006). Compelling evidences show that the infectious agent is prion, which is composed by a misfolded form of the prion protein (PrP^{Sc}) that propagates in the absence of nucleic acid (Prusiner, 1998). PrP^{C} is secreted in the cell surface and anchored there by glycosylphosphatidyl inositol (GPI) (Stahl et al., 1992). Studies show that PrP^{C} binds to its cell surface receptor, the 37-kDa/67-kDa laminin receptor (LRP/LR, "LRP" denotes the 37-kDa laminin receptor precursor, and "LR" denotes the 67-kDa high-affinity laminin receptor), which is important for the internalization of PrP^{C} (Gauczynski et al., 2001).

The 37-kDa/67-kDa laminin receptor (LRP/LR), also called as ribosomal protein SA (RPSA) is a non-integrin laminin binding protein. The different terminology of the 37-kDa LRP and 67-kDa LR can be explained that the mature 67-kDa LR is composed of 37-kDa LRP coupled with an unidentified factor (Castronovo et al., 1991; Butò et al., 1998). The function of 37-kDa/67-kDa LRP/LR is involved in many aspects. There is a relationship between 67-kDa LR and tumor invasion, expression of 67-kDa LR has been increased in various cancers (Zuber et al., 2008b; Mbazima et al., 2010; Omar et al., 2011); in addition, LRP/LR has been identified as a binding partner for cellular PrP (Rieger et al., 1997), as a receptor for PrP^{C} internalization (Gauczynski et al., 2001), and as a receptor for PrP^{Sc} (Prions) (Gauczynski et al., 2006) playing an important role for PrP^{Sc} propagation *in vitro* (Leucht et al., 2003).

The bovine 37-kDa/67-kDa LRP/LR gene is located on chromosome 22, and encodes a protein of 295 amino acids, there are two prion binding sites, direct binding site [amino acids (aa) 161-179] and another is indirect interaction site [amino acids (aa) 180-285]. The latter depends on the heparan sulfate proteoglycan (Hundt et al., 2001). The two binding sites can be blocked to bind with prion by antibodies specific for the 37-kDa/67-kDa LRP/LR, and antisense RNA and siRNA can also reduce the expression of the receptor (Leucht et al., 2003). The direct and part of the indirect (aa 180-205) prion binding sites are located in two adjacent exons. Therefore, in this study we sought to disrupt the two sites by gene targeting

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Both authors contributed equally to this work.

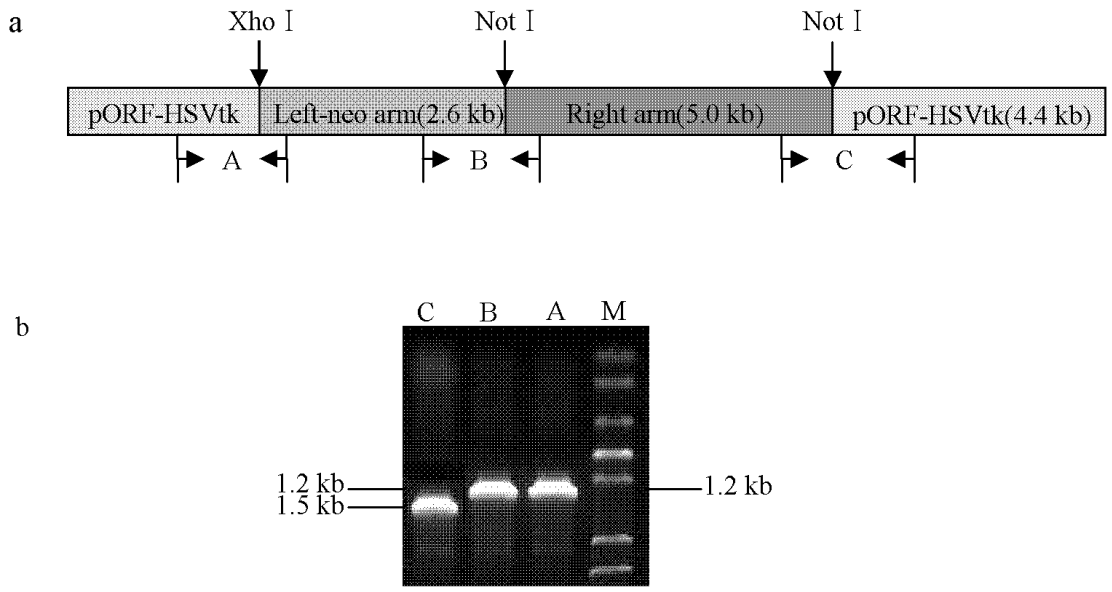

Figure 1. Diagram of the targeting PNS-GLR vector. The PNS-GLR vector was about 12 kb, XhoI and NotI were restriction-enzyme sites between pORF-HSVtk and L-neo, L-neo and right arm, right arm and pORF-HSVtk. A, B, C, represented three products of PCR amplification, which were 1258, 1235 and 1564 bp, respectively. M: Trans 2k plus DNA marker.

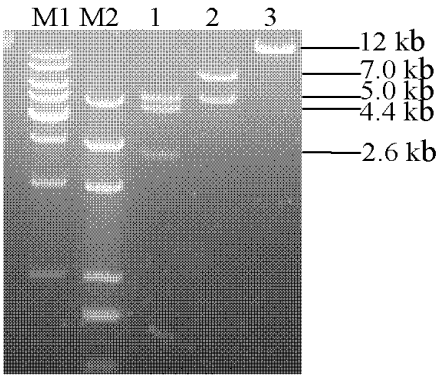

Figure 2. Restriction-enzyme digestion analysis of the PNS-GLR vector. Lane M1: 1kb DNA marker; lane M2: Trans 2k plus DNA marker; lane 1: digestion with XhoI and NotI; lane 2: digestion with NotI; lane 3: digestion with XhoI. The positions of the restriction-enzyme sites were also shown.

in the bovine fetal fibroblasts (BFF) by using the vector of positive-negative selection. The results showed that the 37-kDa/67-kDa LRP/LR gene was successfully disrupted.

MATERIALS AND METHODS

Construction of positive-negative selection targeting vector

Several studies were performed to confirm that the fragments were ligated correctly or not when L-neo fragment and right arm were inserted into the gene targeting vector pORF-HSVtk. The restriction-enzyme sites of the PNS-GLR vector are shown in Figure 1a. The total length of the PNS-GLR vector was 11996 bp; the fragment should be about 12 kb (pORF-HSVtk vector) when digested by Xho I. Two bands were obtained when digested by Not I, one was about 5.0 kb (right arm) and another was 7.0 kb (L-neo fragment and pORF-HSVtk vector). The L-neo arm (2.6 kb) and right arm (5.0 kb) and pORF-HSVtk vector (4.4 kb) were obtained (Figure 2) when the PNS-GLR vector was digested by Xho I and Not I. In addition, we designed three pairs primers across the L-neo fragment, the right arm and the pORF-HSVtk sequence, the

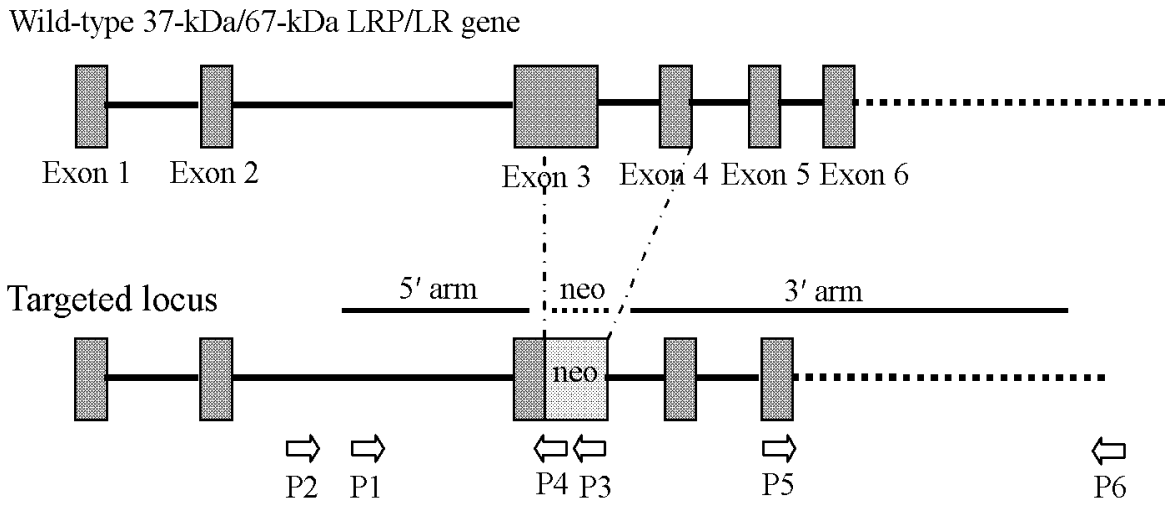

Figure 3. Diagram of the gene targeting LRP/LR locus. P1/P3, P2/P4, P5/P6 represent the three pairs of PCR primers; the direct prion binding site and part of the indirect binding site were replaced by neo fragment.

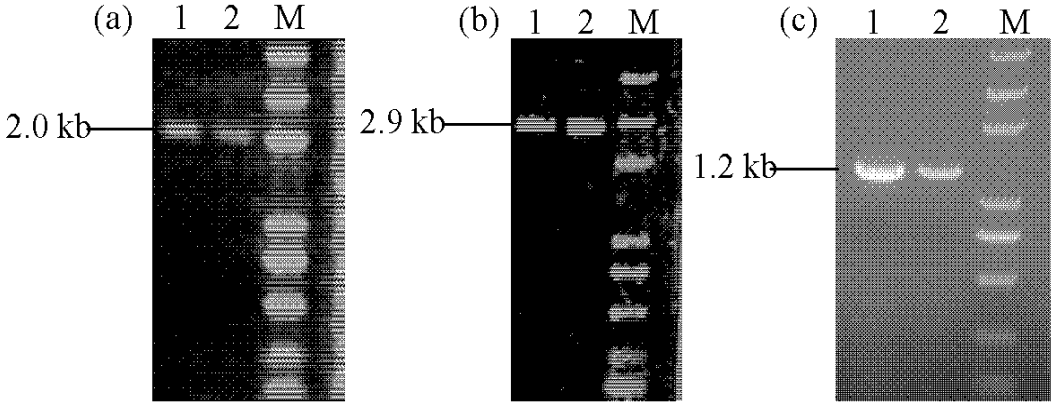

Figure 4. PCR analysis of G418-resistant and GANC-resistant colonies. Colonies are indicated above each lane. Lane M: Trans 2k plus DNA marker. The locations of the PCR primers are shown in Figure 1. The PCR primers are P1/P3 (a), P2/P4 (b) and P5/P6 (c).

products were 1258 bp (A), 1235 bp (B) and 1564 bp (C), respectively (Figure 1b). Restriction-enzyme digestion and gene sequencing analysis indicated that the PNS-GLR vector was constructed successfully.

Transfection and selection of the bovine fetal fibroblasts

The bovine fetal fibroblasts cell lines were from 35-day fetuses of Herstan cow. The PNS-GLR vector was linearized by Xho I and transfected into bovine fetal fibroblasts by an electroporation apparatus. When homologous recombination occurred between the endogenous LRP/LR and the PNS-GLR vector, the targeted cells can survive from G418 and GANC selection. In order to examine whether the homologous recombination occurred between PNS-GLR vector and endogenous LRP/LR locus, we designed three pairs of primers, P1/P3, P2/P4 and P5/P6 (Figure 3). The results of gene sequencing confirmed that the homologous recombination occurred correctly (Figure 4).

Detection of 37-kDa/67-kDa LRP/LR mRNA expression levels in targeted cells

To examine the 37-kDa/67-kDa LRP/LR mRNA expression levels in targeted cells, the reverse transcription PCR and real-time quantitative PCR analysis were performed, and the results are shown in Figure 5a and b, respectively. The levels of 37-kDa /67-kDa LRP/LR gene expression in targeted cells (colony 1 and colony 2) were significantly lower than that of in the control cells; all samples were tested in triplicate. Differences in mRNA expression levels between targeted and control groups were statistically significant ($p < 0.05$, Figure 5b), which indicated that the 37-kDa/67-kDa laminin receptor gene in bovine fetal fibroblasts were disrupted successfully.

RESULTS AND DISCUSSION

In the process of the prion disease, the 37-kDa/67-kDa

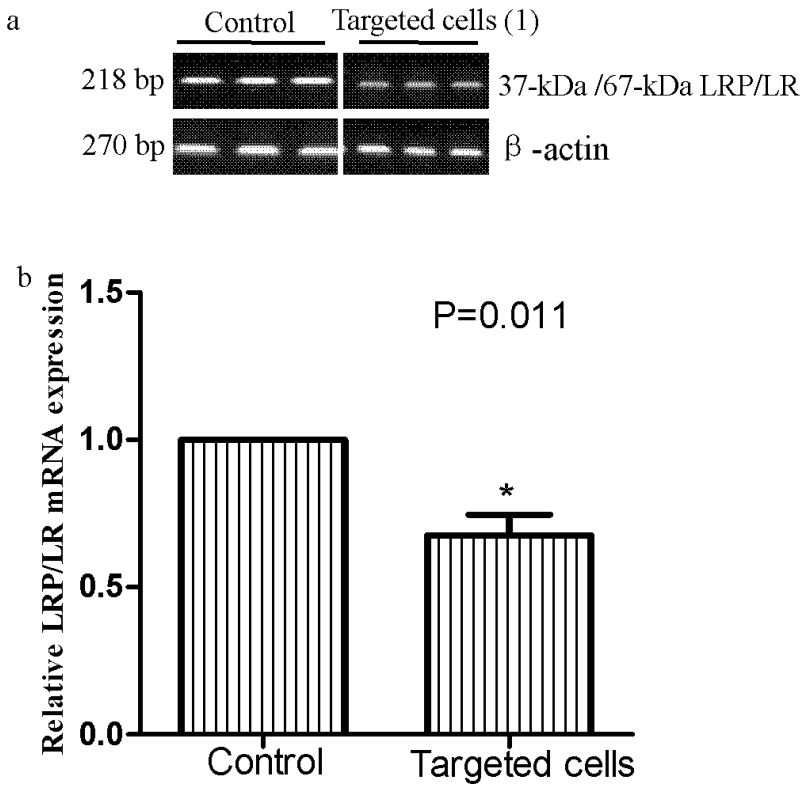

Figure 5. RT-PCR and real-time quantitative PCR analysis of expression levels of 37-kDa/67-kDa LRP/LR in control and targeted cells (1). (a) 1% agarose gel electrophoresis of RT-PCR products of LRP/LR gene. As a control, RT-PCR products were standardized by analysis of constitutive expression of β-actin gene. (b) Relative LRP/LR mRNA expression, all values were normalized to β-actin mRNA expression levels and are expressed as means ± SD (n = 3). A significant difference is indicated by * ($p<0.05$).

LRP/LR plays an important role in internalization of PrP^C and propagation of PrP^{Sc}. The 37-kDa/67-kDa LRP/LR is a multifunctional protein and is expressed within the cytoplasm, the nucleus and the plasma membrane, respectively (Vana et al., 2007, 2009; Mbazima et al., 2010; Omar et al., 2011), and the 37-kDa/67-kDa LRP/LR was identified as the cell surface receptor for PrP^C (Gauczynski et al., 2001; Hundt et al., 2001) and for infectious prions (Gauczynski et al., 2006), bovine prions are endocytosed in an LRP/LR-dependent manner by human enterocytes (Morel et al., 2005; Kolodziejczak et al., 2010), which confirmed the importance of the 37-kDa/67-kDa LRP/LR in the prion infection. Therefore, several therapeutic approaches targeting the 37-kDa/67-kDa LRP/LR have been developed including (a) LRP/LR decoy mutant (Pflanz et al., 2009b), (b) small interfering RNAs directed against the LRP/LR mRNA (Leucht et al., 2003; Pflanz et al., 2009a), (c) anti-LRP/LR antibodies (Zuber et al., 2007, 2008a, c), and (d) polysulfated glycans (Gauczynski et al., 2006), and all of these approaches can prevent PrP^{Sc} propagation efficiently.

With the development of cloning techniques, transgenic animals 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 (Capecchi, 1989; Wang et al., 2003). In this study, in order to further understand the relationship between PrP^C and PrP^{Sc}, we knocked out one allele of the direct and part of the indirect binding sites gene so that the bovine fetal fibroblasts with reduced LRP/LR expression will be useful in prion research. Moreover, to avoid the possible influence of ribosomal protein SA (RPSA) pseudogenes on the RT-qPCR measurements, precautions were taken to rule out this influence such as identifying the sequence of the genes amplified during the experiment to make sure there are no pseudogenes. Additionally, it should be noted that up to now, no detailed analysis of the RPSA gene family has been performed in bovine; hence it is therefore difficult to know if no pseudogenes with (nearly) identical sequence exist.

After gene targeting, DNA analyses indicate that one allele of the 37-kDa/67-kDa LRP/LR gene has been disrupted successfully. In addition, real-time quantitative PCR analyses showed that the expression level of 37-kDa/67-kDa LRP/LR gene is significantly decreased. The

targeted cells had the normal abilities of cell division; however, more details about whether the two binding sites are essential for normal animal development will be conducted in future work. We are aware of the existence of RPSA pseudogenes and their possible influence on the RT-qPCR measurements. Precautions were taken to rule out this influence, such as identifying the sequence of the genes amplified during the experiment to make sure there are no pseudogenes

To date, this is the first report about disrupting the binding sites of the 37-kDa/67-kDa LRP/LR gene in bovine fetal fibroblasts. In the future, we hope to conduct nuclear transfer to produce heterozygous cattle. We expect to achieve the homozygous bovine devoid of the 37-kDa/67-kDa LRP/LR gene after finishing the second-round gene targeting, and eventually, we will test if bovine devoid of the LRP/LR gene are resistant to prion infection.

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