Original Article

Establishment of Hamster- and Human-*PRNP* Transgenic Mice*

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Abstract

Objective To create transgenic mice expressing hamster- and human-*PRNP* as a model for understanding the physiological function and pathology of prion protein (PrP), as well as the mechanism of cross-species transmission of transmissible spongiform encephalopathies (TSEs).

Methods Hamster and human-*PRNP* transgenic mice were established by conventional methods. The copy number of integrated *PRNP* in various mouse lines was mapped by real-time PCR. PRNP mRNA and protein levels were determined by semi-quantitative RT-PCR, real-time RT-PCR, and western blot analysis. Histological analyses of transgenic mice were performed by hematoxylin and eosin (H & E) staining and immunohistochemical (IHC) methods.

Results Integrated *PRNP* copy number in various mouse lines was 53 (Tg-haPrP1), 18 (Tg-huPrP1), 3 (Tg-huPrP2), and 16 (Tg-huPrP5), respectively. Exogenous PrPs were expressed at both the transcriptional and translational level. Histological assays did not detect any abnormalities in brain or other organs.

Conclusion We have established one hamster-*PRNP* transgenic mouse line and three human-*PRNP* transgenic mouse lines. These four transgenic mouse lines provide ideal models for additional research.

Key words: PrP; *PRNP*; Transgenic mice; Copy number

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INTRODUCTION

Transmissible spongiform encephalopathies (TSEs), also known as prion diseases, are a group of lethal neurodegenerative diseases, including Creutzfeldt-Jakob disease (CJD), Kuru, Gerstmann-Straussler-Scheinker (GSS) syndrome, and fatal familial insomnia (FFI) in humans, bovine spongiform encephalopathy (BSE) in cattle, scrapie in sheep and goat, and chronic wasting disease (CWD) in deer and elk[1-2]. TSEs are caused by proteinaceous infectious particles, called prion (PrP^{Sc}), which consist mainly of a misfolded and aggregated protease-resistant isomer of a host-encoded

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glycoprotein (PrP^C)[3-4]. Prion diseases are readily transmitted within species, both experimentally and naturally, through various pathways, including intracerebral, intraocular, intraperitoneal, oral, intracardiac, and intramuscular mechanisms. In contrast, transmission of prion diseases between mammalian species is much less efficient, because of what is known as the "species barrier"[5], and is characterized by abortive transmission or transmission with an extended incubation time. This observation has prompted the development of transgenic rodent models that recapitulate most of the physiopathological features observed in natural prion disease[6-7].

Transgenic mice expressing the PrP of human[8-9], bovine[10-11], and ovine[12,7] have been produced. Those transgenic mice show a strongly reduced species barrier following inoculation by their respective species specific TSE agents. Transgenic mice expressing PrP^C from Syrian hamster in brain[13] appear to develop typical TSE 75 days after intracerebral inoculation of brain homogenates from scrapie-infected hamsters, whereas wild-type mice failed to show any clinical manifestations of TSE after 500 days. Recent studies have shown that native PrP^{Sc} can propagate efficiently by protein misfolding cycle amplification (PMCA) using brain homogenates expressing PrP[14] as the substrates. Transgenic animals will be critical for understanding the physiological functions of PrP and the pathological mechanism of TSEs.

In this study, one transgenic C57BL/6 mouse line containing full-length hamster-*PRNP* and three mouse lines containing full-length human-*PRNP* were established. The copy number of integrated *PRNP* was determined and their transcriptional and translational levels were evaluated. Moreover, the neurohistological characteristics of various transgenic mice were analyzed. Long-term observations of the transgenic mice confirmed no detectable abnormality during development or during passage.

MATERIALS AND METHODS

Plasmids

The recombinant plasmid pcDNA3.1-huPrP encoding full-length wild type human PrP (aa 1-253) and pcDNA3.1-haPrP encoding full-length hamster PrP (aa 1-253) were generated previously[15]. Plasmid DNAs were linearized by digestion with *Bgl* II that cuts upstream of the CMV promoter, to ensure the integrity of *PRNP* and maintain promoter function.

Preparation of Hamster-PRNP and Human-PRNP Transgenic Mice

Hamster- and human-*PRNP* transgenic mice were developed by the professional staff at the Institute of Laboratory Animal Sciences, Chinese Academy of Medical Sciences & Peking Union Medical College (Peking, China). All animal experiments were approved by the Institute's Committee of Animal Welfare and Administration, Chinese Academy of Medical Sciences. Briefly, 1 μL of 5 ng/μL linearized pcDNA3.1-haPrP and pcDNA3.1-huPrP were microinjected into fertilized eggs of C57BL/6 mice. The implantation of the fertilized eggs and feeding of mice were carried out as described previously[16].

Extractions of Tissue DNA and RNA

Animal DNA from tail and lower limb muscles was extracted using the QIAamp DNA Kit (Qiagen, Hilden, Germany). RNA from brain, heart, liver, spleen, kidney, intestine, and muscle were obtained with RNeasy Lipid Tissue Mini Kit (Qiagen) and RNeasy Mini Kit (Qiagen).

Preparation of Tissue Homogenates

Tissues (10% w/v) from transgenic and wild-type C57BL/6 mice were homogenized in lysis buffer (100 mmol/L NaCl, 10 mmol/L ethylenediaminetetraacetic acid, 0.5% Nonidet P-40, 0.5% sodium deoxycholate in 10 mmol/L Tris-HCl, pH 7.4). Homogenates were centrifuged at 20 000× g for 30 min, and the supernatants were stored for further use.

PCR of Hamster-PRNP and Human-PRNP transcripts in the Transgenic Mice

PCR protocols specific for targeting hamster-*PRNP* and human-*PRNP* were developed. Primers for hamster-*PRNP* (forward 5'-TGCAAGAAG-CGGCCAAAGCC-3' and reverse 5'-GGTTCTTTCCGCCT-CAGAAGCC-3') and human-*PRNP* (forward 5'-GGCA-AACCTTGGATGCTGG-3' and reverse 5'-TGGCACTTCC-CAGCATGTAGCC-3') were synthesized. The specificity and sensitivity of each primer pair was evaluated by PCR, using recombinant plasmids containing full-length human, mouse or hamster *PRNP*, as well as tissue DNAs extracted from human, mouse or hamster. 0.2 μg extracted mouse DNA was mixed with 1 μL (10 μmol/L) of each primer pair and 12.5 μL 2×Taq PCR StarMix (GenStar) in a final volume of 25 μL. The cycling conditions for PCR (30 cycles) were 94 °C for 5 min, 94 °C for 30 s, 60 °C for 30 s, 72 °C for

30 s. PCR products were analyzed using 1.2% agarose gel electrophoresis. GAPDH was used as an internal control.

Real-time PCR for Mapping of Hamster and Human-PRNP Copy Number in Transgenic Mice

We adapted real-time PCR methodology for mapping copy number of hamster- and human-*PRNP* in transgenic mice. To prepare reference samples, hamster- and human-*PRNP* fragments of the same length were amplified by PCR and cloned into vector pMD18-T. Copy number was calculated as follows: 6.02×10^{23} (copy number/mol/L) × concentration (g/mL)/MW [g/(mol/L)] = copies/mL, in which MW = base pair (bp)×660 (daltons/bp). The reference solutions were adjusted to 10^3, 10^4, 10^5, 10^6, 10^7, and 10^8 copies/μL. Standards consisted of 1 μL of the reference solution mixed with solution containing 0.2 μg DNA of wild type C57BL/6 DNA (3.1×10^4 copies of genome).

Real-time PCR was conducted on an ABI Prism 7500 sequence detector (Applied Biosystems, Foster City, CA, USA) in a 96-well microamp optical reaction plate format. PCR was performed in 25 μL, containing 0.2 μg DNA of transgenic or reference sample, 1 μL (1 μmol/L) primers for hamster or human *PRNP* and 12.5 μL of Power SYBR Green PCR Master Mix. Each PCR amplification was performed in triplicate using the following profile: 5 min at 50 °C and 10 min at 95 °C, followed by 40 three-temperature cycles (15 s at 95 °C, 30 s at 56 °C for hamster-*PRNP*, 30 s at 60 °C for human-*PRNP,* with a final extension for 30 s at 72 °C). The standard curve was made according to average Ct values of reference solutions. The copy number of hamster or human *PRNP* in individual transgenic mice was calculated as the average Ct relative to the standard curve, in which the weight of the mouse diploid genome was taken as 6.42×10^{-12} g[17]. β-actin served as an internal control.

RT-PCR and Real-time PCR Detection of Hamster- and Human-PRNP Transcriptions in Transgenic Mice

Hamster- and human-*PRNP* expression in transgenic mice was measured using two-step RT-PCR and real-time PCR. Briefly, 2 μg extracted RNA were incubated with 15 U AMV reverse transcriptase at 42 °C for 15 min, followed by reverse transcriptase inactivation at 95 °C for 5 min. Reactions without reverse transcriptase were used as negative controls. Human- or hamster-*PRNP* expression levels were evaluated by real-time RT-PCR using 1 μL cDNA. β-actin served as the internal control.

Western Blot Analysis

Tissue homogenates were separated by 15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred electronically to PVDF membranes (Immobilon-P, Millipore Billerica, MA). After blocking with 5% non-fat milk powder in PBST, membranes were incubated with diluted PrP-specific monoclonal antibody (mAb) 3F4 (1:2000) (Dako, Denmark) or diluted 1E4 (1:1000) (Sanquin, Holand) at 4 °C overnight. After washing twice with PBST, the membrane was incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Boehringer, Germany) (diluted 1:8000 in PBST) at room temperature for 1 h. Signal was detected with an enhanced chemiluminescence detection kit (Amersham-Pharmacia Biotech, Piscataway, NJ, USA).

Histological and Immunohistochemical (IHC) Assays

Transgenic brain was processed using standard formalin fixation and paraffin embedding. Tissue slices were subjected to conventional staining with hematoxylin and eosin (H & E) or immunostaining of PrP and GFAP. Briefly, for PrP staining, tissue slices were exposed to microwave irradiation (Galanz, China) in distilled water for 30 min and 3% hydrogen peroxide-methanol for 10 min. Slices were blocked with normal goat serum for 15 min, incubated with PrP mAb 3F4 (1:250) or mAb 6D11 (1:100) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4 °C overnight, followed by horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibody. (Boster, China) at 37 °C for 1 h. DAB (ZSGB-Bio, Beijing, China) was used to visualize immunostaining and slices counterstained with hematoxylin. For GFAP staining, the procedure was the same as for PrP, wherein the first antibody, GFAP pAb (Boster, China), was diluted 1:200, and the secondary antibody was anti-rabbit IgG coupled with HRP (Boster, China).

Statistical Analysis

Quantitative assessment of total PrP levels in transgenic and WT mice was evaluated using digital immunoblot images obtained with Image Total Tech software (Pharmacia, Piscataway, NJ, USA). Briefly, immunoblot images were scanned with Typhoon (Pharmacia) digitalized, and saved in TIFF format. All data values are presented as the mean±SD. Statistical analysis was performed using the *t*-test. A $P<0.05$ was considered statistically significant.

RESULTS

Establishment of Hamster-PRNP and Human-PRNP Transgenic Mice

DNA from F1 tail biopsies was extracted and screened for the presence of exogenous DNA by PCR using primers specific for the T7 promoter (forward primer) and *PRNP* sequence (reverse primer). Three mice microinjected with hamster-*PRNP* and five mice injected with human-*PRNP* yielded PCR products at the expected position (data not shown). After mating with wild type C57BL/6, the offspring (F2) were collected to determine the integration site of hamster-*PRNP* or human-*PRNP*. *PRNP* species-specific PCR produced bands in one hamster-*PRNP* transgenic mouse (designated as Tg-HaPrP1) and three human-*PRNP* transgenic mice (designated as Tg-HuPrP1, Tg-HuPrP2, and Tg-HuPrP5) (Figure 1). Further mating of these transgenic mice showed that the offspring developed normally, without any obvious behavioral abnormalities, including trembling or ataxia. The body weight increase in each group of the founder transgenic mouse was similar to that of wild type C57BL/6. The breeding of transgenic mice was normal.

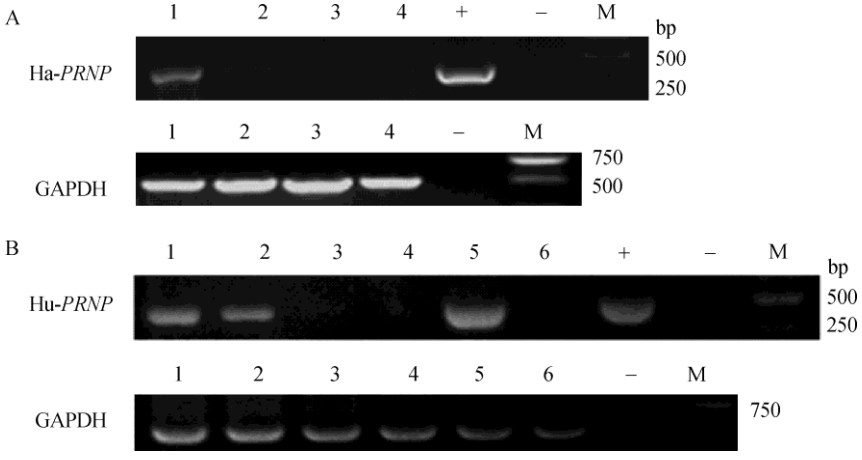


Figure 1. Determination of the location of integrated hamster or human-*PRNP* fragments in transgenic mice by species-specific PCR. Mouse tissue DNA was extracted from brain tissue. A. Hamster-*PRNP* transgenic mice. Lane 1-4: individual mice. "+": positive control for hamster DNA. "-": negative control of wild-type C57BL/6 mouse DNA. B. Human-*PRNP* transgenic mice. Lanes 1-6: individual mice. "+": positive control for human DNA. M: DNA markers. Amplification of the segment of GAPDH was used as an internal control.

Mapping Hamster-PRNP and Human-PRNP Copy Number

To determine the copy number of hamster- or human-*PRNP* in the transgenic mice, two reference standards consisting of various copy numbers of hamster- or human-*PRNP* segments, diluted into wild-type C57BL/6 mouse DNA, were prepared. Both hamster- and human-*PRNP* specific RT-PCR identified specific DNA amplification from 15 to 30 cycles, using reference solutions of 10^3, 10^4, 10^5, 10^6, 10^7, and 10^8 copies/μL (data not shown). Standard curves prepared using those standards demonstrated a high degree of correlation between *Ct* values and copy number (for hamster-*PRNP*, R^2=0.996, and for human-*PRNP,* R^2=0.991). The copy number of hamster or human-*PRNP* in founder transgenic mice was calculated using this data. Based on these *Ct* values, the single-gene copy number of hamster-*PRNP* transgenic mouse Tg-haPrP1 was 53, and that of human-*PRNP* transgenic mice Tg-huPrP1, Tg-huPrP2, and Tg-huPrP5 were 18, 3, and 16, respectively (Table 1).

Table 1. Determination of the Copy Numbers of Exogenous *PRNPs* in Transgenic Mice by Species-specific Real-time PCR

	Ct Value	Copies Number	Quality of Mouse Genome	Single-gene Copy Number
Tg-HaPrP1	23.5	996471.9	6.42×10^{-12}	53
Tg-HuPrP1	22.1	563887.6	6.42×10^{-12}	18
Tg-HuPrP2	25.5	91633.2	6.42×10^{-12}	3
Tg-HuPrP5	22.2	512025.4	6.42×10^{-12}	16

Determination of Hamster- and Human-PRNP Transcriptional Levels in Transgenic Mice

To evaluate the transcriptional integrity of the exogenous *PRNP* in the brains of transgenic mice, total cellular RNA was extracted and the relative amounts of two species-specific *PRNP* mRNAs were evaluated by semiquantitative RT-PCR. *PRNP* specific bands were detected in the brain (Figure 2A). The same assays were performed on heart, liver, spleen, kidney, intestine, and muscle. Specific bands were detected in all tested tissues, although the relative expression levels varied (Figure 2B).

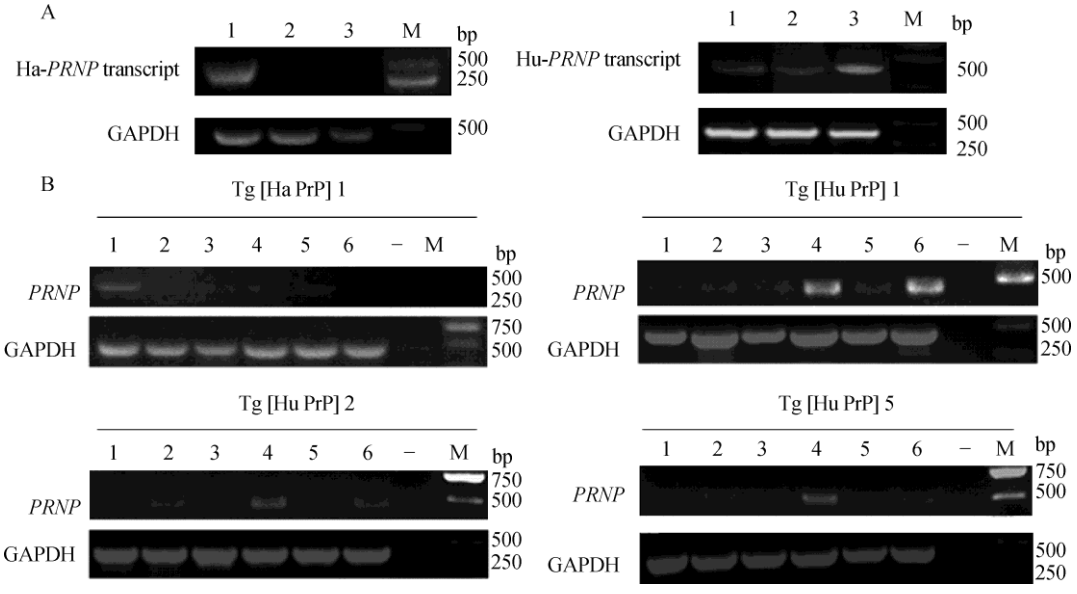


Figure 2. Evaluation of transcripts derived from exogenous hamster- or human-*PRNP* in transgenic mice by species-specific *PRNP* RT-PCR. A. Exogenous PrP mRNAs in brain. Lanes 1-3: individual mice. B. Exogenous PrP mRNAs is expressed in peripheral organs. Lane 1: heart; lane 2: liver; lane 3: spleen; lane 4: kidney; lane 5: intestine; lane 6: skeletal muscle. Transgenic genotypes are indicated above the gel images. M: DNA markers. GAPDH from the extracted tissues was used as the internal control.

To confirm the transcriptional levels of hamster- and human-*PRNP* mRNAs, species-specific real-time RT-PCR assays were performed. Human or hamster specific *PRNP* mRNAs were clearly amplified from brain and other peripheral organs of transgenic mice, whereas no signal was detected in samples prepared from wild-type mice. The average *Ct* values from different tissues of the transgenic mice ranged from 22.670 to 27.663 (Table 2). Further analysis of the *Ct* values from three human-*PRNP* transgenic mice revealed relative lower average *Ct* values in brain (24.535), muscle (skeletal muscle: 24.206) and heart (muscle: 25.044) (Figure 3). These data, together with the results of traditional RT-PCR, suggests that the exogenous *PRNPs* are integrated and are transcriptionally active in brain as well as peripheral organs. The transcriptional activity of integrated *PRNPs* seems to vary among tissues, with more active transcription in the CNS and muscle.

Table 2. The *Ct* Values of Transcripts Derived from Exogenous *PRNP* in Various Transgenic Tissues

	Brain	Heart	Liver	Spleen	Kidney	Intestine	Muscle
Tg-HuPrP1	22.951	23.061	26.390	25.490	25.165	27.015	25.247
Tg-HuPrP2	25.643	26.288	26.365	26.697	28.196	24.444	22.670
Tg-HuPrP5	25.013	25.783	27.036	27.663	25.136	27.706	24.701
Average	24.53567	25.044	26.597	26.61667	26.16567	26.38833	24.206

Evaluation of Hamster- and Human-PrP Expression in Transgenic Mice

To assess expression of hamster- or human-derived PrP proteins in transgenic mice, 10% (w/v) brain homogenates were subjected to western blotting with PrP-specific mAb 3F4, while brain homogenates of wild type mice were loaded as control. PrP specific

signals were detected in brain from both wild type and transgenic mice, but the signal of the transgenic samples, whether the transgene was hamster-*PRNP or* human-*PRNP*, were much stronger than that of wild type (Figure 4A). Analyses of the intensities of each PrP signal after equilibration with that of individual β-actin revealed significantly higher levels of total PrP in transgenic brain relative to wild type (P<0.05, Figure 4B).

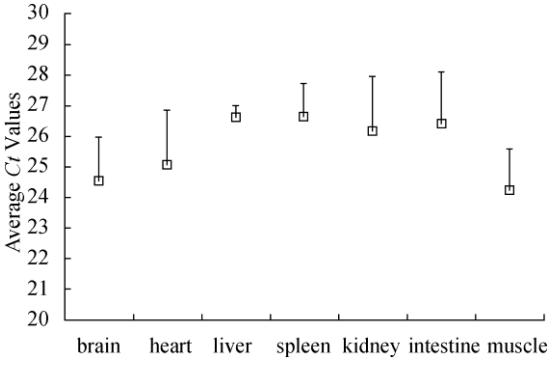
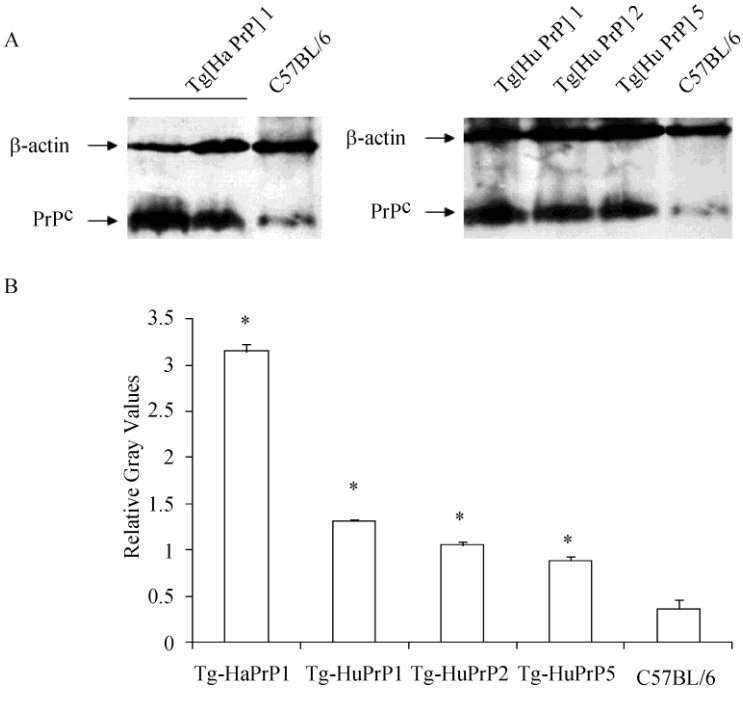


Figure 3. Comparison of average *Ct* values of human *PRNP* transcripts in three transgenic mice by species-specific real-time RT-PCR. *Ct* values are plotted along the Y-axis and the different tissues are plotted along the X-axis. The averaged data are presented as mean±SD.

To examine PrP expression in peripheral tissues, 10% (w/v) homogenates of heart, liver, spleen, kidney, intestine, and muscle from transgenic mice were subjected to western blot analysis with mAb 3F4. PrP specific signals were observed in all transgenic and wild type tissues tested, but transgenic PrP signals were remarkably stronger than that of wild type, especially in heart, kidney, liver, and muscle (Figure 4C). Quantitative analyses of the grey values of each PrP and equilibration with the corresponding grey values of β-actin demonstrated that total PrP levels in transgenic tissues were higher than that in wild-type (P<0.05, Figure 4D). Total PrP in the liver of Tg-huPrP2 and Tg-huPrP5, muscle of Tg-haPrP1, Tg-huPrP1 and Tg-huPrP5, spleen of Tg-huPrP2 and Tg-huPrP5, kidney of Tg-huPrP1, Tg-huPrP2 and Tg-huPrP5, intestine of Tg-haPrP1, Tg-huPrP1, Tg-huPrP2 and Tg-huPrP5, and heart of Tg-huPrP1, Tg-huPrP2, and Tg-huPrP5 were significantly higher than that in WT (P<0.01). These data suggest that the integrated hamster- and human-*PRNP* construct induces PrP expression in transgenic mice. Additionally, the immunoreactive profiles of PrPs in transgenic brain were tested by western blotting with PrP-specific mAbs 1E4 and 6D11, which are able to recognize endogenous PrP. No obvious difference in PrP profiles was observed between transgenic and wild type mice, regardless of the signal intensity, glycosylation pattern, and migration position (data not shown).



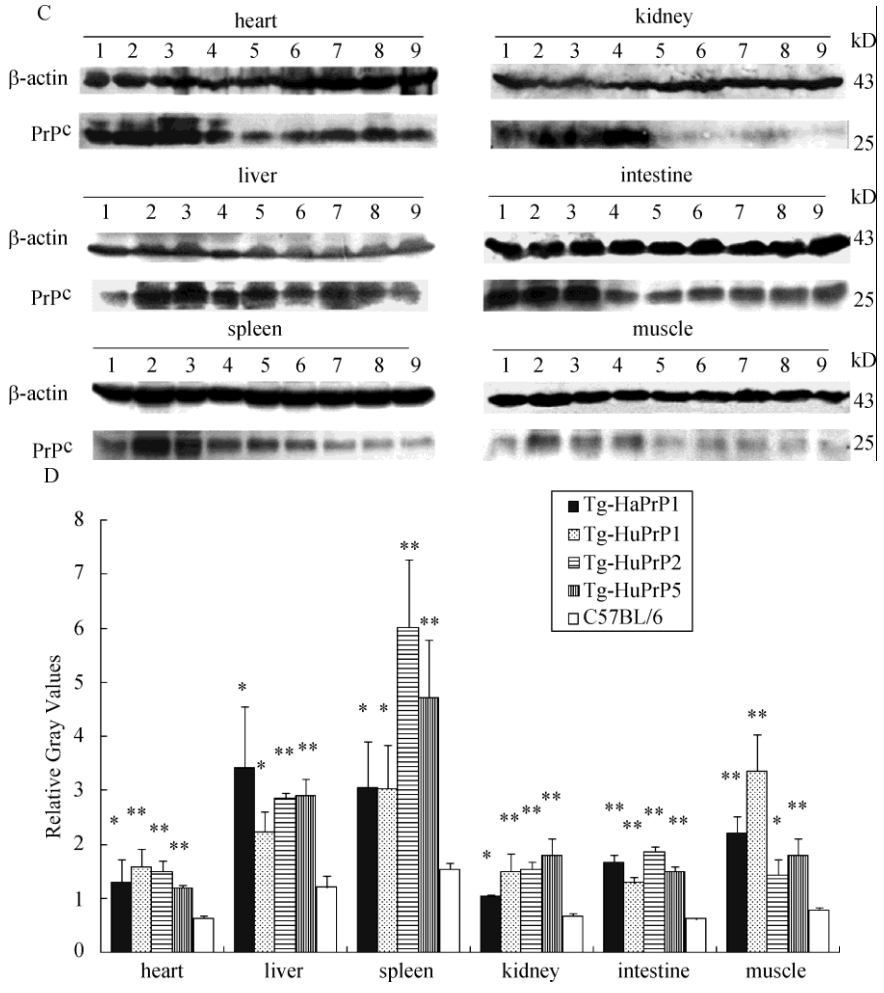


Figure 4. Western blot analyses of PrP in transgenic mice by mAb 3F4. A. Western blot of brain. Transgenic and wild type (C57BL/6) genotypes are indicated above images. PrP and β-actin signals are denoted by the arrows on the left. B. Quantitative analyses of each gray value of PrP *vs.* that of β-actin. The average relative gray value is calculated from three independent blots and presented as mean±SD. *:$P<0.05$. C. Western blots of other organs. Lane 1: Tg-HaPrP1; lane 2: Tg-HuPrP1; lane 3: Tg-huPrP2; lane 4: Tg-HuPrP5; lanes 5-9: wild-type C57BL/6 mice. Different organs are denoted above the images. D. Quantitative analyses of each gray value of PrP *vs.* that of β-actin. The average relative gray value is calculated from three independent blots and presented as mean±SD. *:$P<0.05$; **:$P<0.01$.

Histological Characteristics of Transgenic Brain

To determine if expression of transgenic PrPs in mouse brain caused TSE-like pathology, adult brain was subjected to histological analyses. H & E staining assays did not identify any abnormalities in transgenic brain, including spongiform changes or neuron loss (data not shown). GFAP specific IHC tests confirmed that the number of GFAP-positive cells in the transgenic mice were generally similar to that of wild-type's. The positive cells were mainly small, and distributed predominantly in white matter (Figure 5). These data indicate that exogenous PrPs in *PRNP*-transgenic mice do not cause neuropathological abnormality, at least as determined by the methods employed here.

DISCUSSION

We have established one hamster-*PRNP* transgenic mouse line and three human-*PRNP* transgenic mouse lines. We were not able to detect any obvious abnormality during development or during breeding, following long-term observation. Histological assays also did not detect any abnormality in brain or other organs. The copy number of integrated *PRNP* in various mouse lines have been determined, and are quite stable among different tissues and during passage.

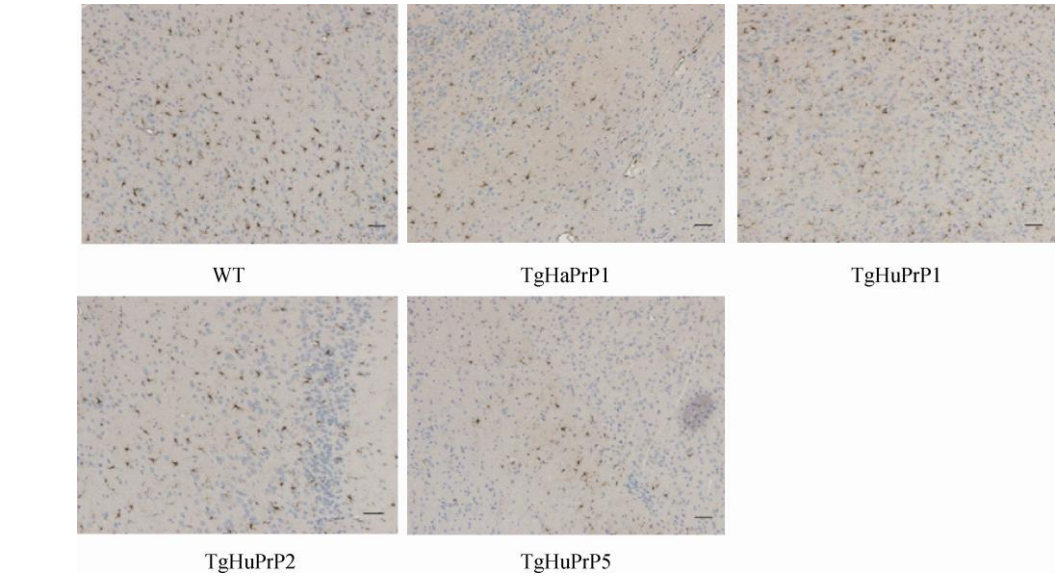


Figure 5. Immunohistochemical assay of glial fibrillary acidic protein (GFAP) in the cortex of transgenic brain. WT: wild-type mouse. Transgenic genotype is denoted under the pictures.

Active transcription of exogenous PrP mRNAs was observed in brain harvested from individual transgenic lines. Although the copy number of the inserted *PRNP* varied widely, the level of human PrP mRNA in brain among three human-*PRNP* transgenic mice, determined by both real-time PCR and conventional RT-PCR, were quite comparable. In addition, there are specific transcripts of exogenous *PRNP* in peripheral tissues, including heart, liver, spleen, kidney, intestine, and muscle. The results demonstrate that the integrated *PRNP* transcribed efficiently. This is consistent with many observations described previously, in which the copy number of exogenous genes often does not directly correlate to levels of transcription[18].

To assess the presence of exogenous PrP in various tissues, the PrP-specific mAb 3F4, specific for human- and hamster-PrP but not mouse-PrP, was used in western blot analysis. However, under our experimental conditions, mAb 3F4 cross-reacted with native mouse PrP, although the signal was weak. Stronger PrP signals are detected not only in brain but also in peripheral tissues of each transgenic mouse line, suggesting efficient expression of exogenous hamster or human-PrP. The PrP signal intensity observed in tissues of human-*PRNP* transgenic mice did not correspond to the copy number of integrated *PRNP*. We did note, however, that in transgenic brain, the mRNA and protein levels of exogenous PrPs were higher when their copy numbers were very high, but were not proportional when the copy numbers were low. The site of integration and copy number of exogenous genes are the two major factors affecting expression levels in transgenic animals, and are critically dependent upon the characteristics of target gene[17]. It seems that copy number variation of human-*PRNP* (from 3 to 18) under our experimental condition do not significantly influence the mRNA and protein levels of exogenous *PRNP* in transgenic brain. The slight differences in mRNA and protein levels in peripheral tissues among transgenic mice may result from the different integration sites of the target genes.

Since endogenous *PRNP* is 85 and 89% identical to hamster- and human-*PRNP,* respectively*,* at the nucleotide level, detection requires species-specific PCR to determine copy number and expression level of the integrated *PRNP* in transgenic mice. The protocols developed here should prove to be useful tools for distinguishing between endogenous and exogenous *PRNPs* transcripts in both cell and animal models.

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