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Generation and characterization of two strains of transgene mice expressing chimeric MiniSOG-MusPrP

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Highlights

- MiniSOG tag is an ideal marker not only for immunofluorescent assays, but also suitable for ultrastructural assays for prion morphology.
- Tg mice expressing miniSOG-PrP fusion proteins were generated and characterized.
- Those Tg mice can supply novel and useful experimental animals for further study on the potential morphological structure formation and deposits of prion in the brain tissues during prion infection.

Abstract

Background: Although the presences of scrapie associated fibril in the brain tissues is a ultrastructural hallmark for prion diseases, the exact morphological structure of prion during the progression of the disease is still unclear. The host prion protein (PrP) is encoded by PrP gene (*PRNP*) locating on the chromosome 20 in human and the chromosome 2 in mouse. Recently, a novel correlative light and electron microscopy with Mini Singlet Oxygen Generator (miniSOG) was generated. MiniSOG, a small protein of 106 amino acids, can absorb blue light and emit green fluorescence that is detectable under the fluorescence microscope. MiniSOG can also partially catalyze the polymerization of DAB to form black stained structures in the presence of osmium tetroxide, which is able to be observed under transmission electron microscope.

New methods: Two kinds of miniSOG-PrP expressing recombinant plasmids were generated. Correlative photooxidation and transmission electron microscope were used to detect these plasmids. The plasmids were microinjected into fertilized FVB/NJ eggs and Tg mice expressing miniSOG-PrP fusion proteins were selected after successive bred with *PRNP* KO Tg mice.

Results: Those two strains of Tg mice, Tg^{SOG23} and Tg^{231SOG}, developed normally and maintained healthy without detectable abnormality after one-year observation. Western blots and immunohistochemical assays with PrP- and miniSOG-specific antibodies confirmed that the chimeric miniSOG-PrP proteins were expressed in the brain tissues of Tg mice. Digital PCR assays proposed that the copy numbers of the inserted external gene in Tg^{SOG23} and Tg^{231SOG} were 2 and 12, respectively.

Comparison with Existing Method(s): Compared with GFP tag miniSOG is significantly smaller, which makes it easy be operated experimentally and possibly has less influence on the biological function of the labeled protein. Additionally, GFP tag is an ideal marker for immunofluorescent assays, but may not be suitable for ultrastructural assays for prion morphology.

Conclusion: Those Tg mice may supply novel and useful experimental animals for further study on the potential morphological structure formation and deposits of prion in the brain tissues during prion infection.

Key words: Prion diseases; miniSOG; transgenic mice; photooxidation

Introduction

Prion diseases are a series of lethal and transmissible neurodegenerative diseases in humans and animals, such as Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome (GSS) and fatal familial insomnia (FFI) in human, scrapie in goat and sheep, bovine spongiform encephalopathy in cattle [1, 2]. Scrapie infected rodents are widely used experimental models for prion study. The pathogen is an unique type of infectious, insoluble, limited protease digestion resistant scrapie prion protein (PrP^{Sc}), which is derived from the normal cellular prion protein (PrP^{C}) on the cell membrane, mostly enriching in the tissues of central nervous system (CNS) [2-4]. One of the hallmarks of prion diseases neuropathologically is the presences of amounts of scrapie associated fibril (SAF) in the brain tissues, which seems to be the only visible abnormal structure of prion [5-9]. Morphological observation of SAF mainly relies on electron microscopy, usually after negative stain with phosphotungstic acid. However, the morphology of SAF may vary greatly and the morphological association between SFA and the cells is impossible to be addressed due to the experimental processes of negative staining. The real morphological structure of prion during the progression of the disease is still unclear.

In the past years, a novel correlative light and electron microscopy (CLEM) with Mini Singlet Oxygen Generator (miniSOG) has been extensively used in biological studies. MiniSOG is a small protein consisting of 106 amino acids. It is a fluoroflavin protein modified from light-oxygen-voltage-sensing (LOV) domain of *Arabidopsis*

thaliana. Based on the non-covalent bonding with flavin mononucleotide (FMN), miniSOG can absorb blue light at the maximum intensity of 448 nm and emit green fluorescence [10-12], which can be monitored under the fluorescence microscope. Under the excitation of blue light, miniSOG can partially catalyze the polymerization of ¶ -diaminobenzidine (DAB) to form osmiophilic particles in the presence of osmium tetroxide, showing black stained structures under transmission electron microscope (TEM).

As an important methodology, transgenic (Tg) mice are widely used in the researches of prion biology and prion disease. In this study, we inserted the sequences encoding miniSOG into the N-terminus (at the position of amino acid (aa) 23 after the signal peptide) and C-terminus (at the position of aa 231 in front of GPI anchor) of mouse *PRNP* gene, respectively, generating the recombinant plasmids expressing two kinds of miniSOG-PrP fusion proteins. After verified the expressions of those miniSOG-PrP fusion proteins in cultured cells, the plasmids were microinjected into fertilized FVB/NJ eggs and Tg mice expressing miniSOG-PrP fusion proteins were selected after successive bred with a PrP encoding gene knock-out (*PRNP* KO) Tg mice. Further assays confirmed that those two kinds of Tg mice were healthy after one-year observation and the miniSOG-PrP fusion proteins were detectable in the brain tissues. Those Tg mice may supply novel and useful experimental animals for further study on the potential morphological structure formation and deposits of prion in the brain tissues during prion infection.

Materials and Methods

Construction of eukaryotic expression plasmids

The full-length sequence (aa 1 to 253) of mouse *PRNP* gene (GenBank CT010345.1) was obtained by PCR using the DNA extracted from C57 mouse as the template. After verified by sequencing, the full-length *PRNP* fragment was inserted into vector pcDNA3.1(+) at the sites of EcoRI and XholI to generate pcDNA-MusPRNP, and into vector pDGF at the sites of MluI and HindIII to generate pDGF-MusPRNP. The sequence of miniSOG gene (GenBank JX999997.1) was obtained from plasmid pShuttle-CMV-IXSOGÄ pSh5-IXSOGÅ by PCR. To construct the recombinant plasmid expressing miniSOG-MusPrP fusion protein, the miniSOG sequence was inserted into the *PRNP* sequence in the plasmids pcDNA-MusPRNP and pDGF-MusPRNP at the position of aa. 23 (after the signal peptide) and aa. 231 (in front of GPI anchor) using overlap extension PCR technique, respectively, generating the plasmids pcDNA-MinoSOG-PrP23, pcDNA-PrP231-MiniSOG, pDGF-MinoSOG-PrP23, pDGF-PrP231-MiniSOG.

Cell culture and transfection

Cell lines SK-N-SH and 293T was FXOWXUHG LQ ¨XOEHFFR¶V PRGLILHG (DJOH¶V medium (Gibco, USA) supplemented with 10 % fetal bovine serum (FBS) (Gibco) in a humid atmosphere of 5 % CO_2 at 37 °C. To verify the expression of the recombinant proteins, the plasmids expressing pcDNA-MiniSOG-PrP23 and pcDNA-PrP231-MiniSOG were transfected into the cells with Lipofectamine 3000

[redacted] Green fluorescence was captured by Operetta (Perkinelmer) with Columbus software 24 and 48 h post-transfection.

Preparation of miniSOG polyclonal antibody

Recombinant fusion protein GST-miniSOG was expressed and purified from *E.coli*. according the protocol described elsewhere[13]. The purity of the purified product was determined by SDS-PAGE, which showed a single band, and its concentration was measured with BCA kit. 50 g of GST-miniSOG was mixed with the reagent of QuickAntibody-Mouse5W (Biodragon, KX0210041) and intramuscularly injected into the flexor digitorum profundus of the hind leg of the mice for three times, with the internal of two weeks. The sera from the immunized mice were collected, separated and stored at -80(.

Western blots

10% cell lysates or brain homogenates were separated by 12% SDS-PAGE and electroblotted onto nylon membranes. Membranes were blocked in TBST containing 5% skimmed milk at room temperature (RT) for 2 h and incubated with anti-PrP primary antibody (6D11, 1:1000, Sc-58581, Santa Cruz Biotechnology, USA), anti- -actin antibody (1:5,000, Huaxingbio, HX1827), at 4°C overnight. After washing with TBS containing 0.1% Tween 20 (TBST) three times (10 min each time), the membranes were incubated with HRP conjugated secondary antibodies (1:5,000,

Jackson ImmunoResearch Labs, 115-035-003) at RT for 1 h. The blots were developed using enhanced chemiluminescence system (ECL, PerkinElmer, NEL103E001EA). Images were captured by Clinx ChemiCapture System with ChemiScope 6000 (Qinxiang, China).

Photooxidation and transmission electron microscope (TEM)

48 h post-transfection, the expressions of the MiniSOG fusion proteins in the cells were screened with fluorescent microscope. The target regions were fixed with 2.5% glutaraldehyde (SPI, USA) in 0.1 M sodium cacodylate buffer, pH 7.4 (SPI, USA) on the ice for 1 h, rinsed five times in chilled 0.1 M sodium cacodylate buffer, and incubated in blocking buffer (20 mM aminotriazole, 50 mM glycine, and 10 mM KCN) at RT for 30 min, in order to block the photooxidation activity of endogenous flavin proteins. After adding ice-cold freshly-prepared oxygen saturated diaminobenzidine (DAB) (1mg/mL, pH 7.4, Amresco) [14], the cell wells were immediately transferred to an inverted fluorescence microscope (Nikon, Eclipse Ti-U) and exposed to the blue light irradiation for 15-20 min using 40-fold objective lens. The light source of the fluorescence microscope was short arc mercury lamp (100w, OSRAM GmbH). In order to maintain low temperature of the DAB solution and sufficient oxygen concentration, the DAB solution was replaced for 2 to 3 times during the irradiation. Illumination was stopped as soon as a very light brown reaction product began to appear in place of the green fluorescence as monitored by transmitted light (usually 2 to 10 min, depending on the initial fluorescence intensity,

the brightness of the illumination and the optics used). After rinsed with chilled 0.1 M sodium cacodylate buffer for five times, the cells were post-fixed with 1% osmium tetroxide (OsO_4) on the ice for 30 min and stained with 2% aqueous uranyl acetate (SPI, USA) at 4 for 1 h. The cells were dehydrated in an ice-cold graded ethanol series (20, 50, 70, 90 and 100%) for 2 min each. After treated with anhydrous ethanol and acetone for 10 min at RT, cells were infiltrated in Epon-812 resin (SPI, USA) using 1:1 acetone at RT for 20 min, followed with 3:1 for 1h and with fresh Epon-812 resin for 2h. Finally, the products were polymerized in a vacuum oven at 40, 60 and 80(for 5h, respectively. Ultrathin slices (80 nm) were prepared with the ultramicrotome (Leica EM UC7). The slices were screened with the transmission electron microscope (Tecnai 12, FEI, USA) and images were captured with Erlangshen Model 785, Gantan software after stained with Uranium acetate and lead citrate.

Immunofluorescent assay (IFA)

48 h post transfection, cells receiving the plasmids expressing MiniSOG-PrP23 and PrP231-MiniSOG were subjected into various IFA assays. To label endoplasmic reticulum (ER), the cells were incubated with 0 prewarmed ER-7UDFNHUTM Red dye (Thermo Fisher, E34250) DW & for approximately 15-30 min. To label cell membrane, the cells were incubated with 5.0 μg/ml wheat germ agglutinin (WGA), Texas Red®-X conjugate (Thermo Fisher, W32466) at 37°C for 10 minutes. To label Golgi body, the cells with 1:20000 diluted Golgi-Tracker Red (Beyotime, C1043) at

4°C for 30 minutes. After washing, cells were incubated with 1:200 diluted PrP specific monoclonal antibody (mAb) 6D11 at 4 °C overnight and subsequently incubated with Alexa Fluor 488-conjugated donkey anti-mouse antibody (1:200, Thermos) at RT for 1 h. The cell nucleus was stained with 1 g/mL 4¶6-diamidino-2-phenylindole (DAPI, Beyotime, China) for 15 min. Images were captured with Olympus FV1000 confocal microscopy with FV10-ASW software.

Generation and characterization of the transgenic mice (Tg MiniSOG-PrP23 and Tg PrP231-MiniSOG)

The generations of the transgenic mouse expressing MiniSOG-PrP23 or PrP231-MiniSOG were conducted in Institute of Experimental Animal Medicine, Chinese Academy of Medical Sciences. Briefly, the plasmids pDGF-MiniSOG-PrP23 and pDGF-PrP231-MiniSOG were microinjected into fertilized FVB/NJ eggs, respectively, and further planted into the oviducts of pseudopregnant FVB mice. Founder mice were screened by pDGF-specific PCR of tail DNA with the primers 1 (Forward ¶ -GAGATGTCCTTCCTCCTCTGTTC-3¶ Reverse: ¶ -CACCCTGCTGTTTACTATCTCCC¶-3). All founder mice carrying the introduced DNAs were bred with FVB WT firstly, and then positive offspring mice were subsequently bred with FVB Tg $PRNP^{0/0}$ mice with PrP-null background[15, 16]. The mice were further screened by pDGF-specific PCR using primers 1 and primers 2 (Forward: 5¶-ACTATCAGTCATCATGGCGAACCTT-3¶, Reverse: 5¶ -GAAGGCCTCCCTCATCCCACGATCA-3¶), to select Tg^{SOG23} expressing

MiniSOG-PrP23 and Tg^{231SOG} expressing PrP231-MiniSOG. The expressions of MiniSOG-PrP in in the brains of these two strains of Tg mice were examined by Western blots and IHC assays.

Preparation of brain homogenates

The whole brains of the tested mice were removed surgically and dissected immediately. Brain homogenates, 10% (w/v), were prepared based on the protocol described previously [17, 18]. Briefly, brain tissues were homogenized in lysis buffer (100 mM NaCl, 10 mM EDTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 10 mM Tris, pH 7.5) containing protease inhibitor cocktail set III (1%, v/v, Merck, 535140). The tissue debris was removed with low speed centrifugation at 2000 g for 10 min and the supernatants were collected for further study.

Immunohistochemical (IHC) Assay

Brain tissue was fixed in 10% buffered formalin solution and paraffin sections (5 P LQ WKLFNQHVV ZHUH SUHSDUHG URXWLQHO\6HFWLRQV ZHUH TXHQFKHG IRU HQGR peroxidases in 3% H_2O_2 in methanol for 10 min before blocked with 5% BSA for 15 min at RT. The sections were incubated with 6D11 (1:100) and anti-MiniSOG (1:100) at 4 °C overnight. Subsequently, the sections were incubated with HRP-conjugated goat anti-mouse antibody (Zsbio, PV-6002) at 37°C for 1 h and visualized by incubation with 3,3-diaminobenzidine tetrahydrochloride (DAB) (Boster, AR1000). The slices were counterstained with hematoxylin (Boster, AR 0005), dehydrated, and

mounted in permount.

Digital PCR

Genomic DNA were extracted from two individual mice of each Tg mouse. The gene Fabpi was used as internal control [19]. Digital PCR reactions were performed based on the Naica System for Crystal Digital™ PCR (Stilla Technologies, France), using a qPCR mix supplied by manufacturer. 10 O of qPCR mix, 1 ORI Alexa 647 reference Fluorescent dye, 1 O of HDFKSULPHU0 , 0.5 OR f genomic DNA, 1.9 ORI Evagreen Fluorescent dye and 9.6 ORI1XFOHDVH-free H_2O were mixed in a total volume of 25 O PCR was performed under following conditions: 95 °C for 30s, 60 °C for 15s and 72°C for 30s, totally 45 cycles. The data were captured by the software of Crystal Reader in digital PCR data captured equipment Naica Prism3 (Stilla Technologies, France). All PCR reactions were performed in triplicate. The copy numbers of the inserted external gene were evaluated by the ratio of positive spots of *PRNP*/Fabpi.

Results

Determinations of the expression of fusion proteins of MiniSOG-PrP23 and PrP231-MiniSOG in cultured cells

To evaluate the expressed fusion proteins of MiniSOG-PrP23 and PrP231-MiniSOG, plasmids pcDNA-MiniSOG-PrP23 and pcDNA-PrP231-MiniSOG were transfected into the cell line 293T that was confirmed without detectable endogenous PrP, as well as the cell line SK-N-SH that contained endogenous PrP [20-22]. 48 h after transfection, cells were subjected into the observation by Operetta (Perkinelmer) with Columbus software. Numerous green fluorescent signals were observed in both 293T and SK-N-SH cells receiving plasmids pcDNA-MiniSOG-PrP23 and pcDNA-PrP231-MiniSOG, but not in the cells receiving the plasmid pcDNA-MusPrP (Fig 1A). It indicates that the inserted miniSOG is able to emit green fluorescence under our experimental condition.

The lysates of the cells transfected with plasmids pcDNA-MiniSOG-PrP23 and pcDNA-PrP231-MiniSOG, as well as the cells receiving plasmid pcDNA-MusPrP (as PrP-Ctrl) and blank vector pcDNA3.1 (as vector-Ctrl), were separately prepared and subjected into PrP-specific Western blots. In the preparation of 293 cells, multiple PrP-specific bands ranging from 30 to 45 kDa were detected in the cells transfected with pcDNA-MinoSOG-PrP23 and pcDNA-PrP231-MiniSOG, while several bands ranging from 20 to 35 kDa were also observed in the cells receiving pcDNA-MusPrP (PrP-Ctrl) and no positive signals in the cells of pcDNA3.1 (vector-Ctrl) (Fig 1B). In

SK-N-SH cells, multiple PrP-specific bands ranging from 30 to 45 kDa were detected in the cells transiently expressing MiniSOG-PrP23 and PrP231-MiniSOG, while many bands ranging from 20 to 35 kDa were also observed in the cells receiving pcDNA-MusPrP (PrP-Ctrl) and pcDNA3.1 (Vector-Ctrl) (Fig 1C). The PrP signals in the cells of PrP-Ctrl were much stronger than that of Vector-Ctrl. Those data indicate that the recombinant proteins MiniSOG-PrP23 and PrP231-MiniSOG are efficiently expressed with the expected molecular weights.

Determination of the subcellular localizations of MiniSOG-PrP23 and PrP231-MiniSOG in SK-N-SH cells

To see the distributions of the expressed MiniSOG-PrP23 and PrP231-MiniSOG subcellularly, SK-N-SH cells growing on the slices were double-stained with commercial membrane, ER or Golgi labelling kits (red) and PrP specific mAb 6D11 (green). Confocal microscopy revealed that the expressed PrP231-MiniSOG colocalized with the signals of membrane, whereas MiniSOG-PrP23 seemed to distribute inside of membrane (Fig 2A). Both MiniSOG-PrP23 and PrP231-MiniSOG colocalized with the signals of Golgi body (Fig 2B), whereas did not colocalized with ER signals (Fig 2C).

To test the ultrastructure of the cells expressing MiniSOG-PrP23 and PrP231-MiniSOG, the regions of the 293T cells transfected with the relevant plasmids that revealed green fluorescence under the fluorescent microscopy were collected and ultrathin slices were prepared. After treated with photooxidation and exposed to DAB,

the slices were screened by TEM. A mass of black fine filar stainings were observed in cytoplasm and on the membranes of nucleus (Fig 3). It highlights that the fusion miniSOG-PrP proteins can be detected by EM after photooxidation.

Determination of the expressions of MiniSOG-PrPs in the brains of two strains of Tg mice.

After successive bred with FVB WT and Tg PRNP$^{0/0}$ mice and genetically typed with PCR techniques, two strains of heterozygous Tg mice, Tg$^{SOG23/WT}$ expressing MiniSOGPrP23 and WT-PrP and Tg$^{231SOG/WT}$ expressing PrP231-MiniSOG and WT-PrP, as well as two strains of homozygous Tg mice, Tg^{SOG23} and Tg^{231SOG} were selected. All types of Tg mice appeared healthy during the observation period (more than 10 months old). The increases of body weights of the Tg mice in the first three months were similar as the WT ones (data not shown). Routine neuropathological assays of HE stainings for the brain slices of two strains of homozygous Tg mice (10 months old) did not find any obvious abnormality in different brain regions, including cortex, cerebellum, hippocampus and pons (Supp Fig 1).

To assess the expressions of the fusion MiniSOG-PrP in the brains of different strains of Tg mice, the brain homogenates of Tg mice (3 to 5 mice each strain) were prepared and employed to Western blots. In the preparations of heterozygous Tg mice, Tg$^{SOG23/WT}$ and Tg$^{231SOG/WT}$, both native PrP bands (ranging from 20 to 35 kDa) and large molecular weight PrP signals (from 40 to 55 kDa) were detected in the reaction

with mAb 6D11 (Fig 4A). In the reaction of anti-MiniSOG, large amounts of positive blots from 40 to 50 kDa were seen, while no clear blot was detected in the position from 25-35 kDa (Fig 4B). Meanwhile, in the preparations of brain lysates from WT FVB mice, only native PrP signals were detected in the blot with mAb 6D11 and no positive signal was observed in that of anti-MiniSOG (Fig 4A and B). In the preparations of homozygous Tg mice, Tg^{SOG23} and Tg^{231SOG}, almost no native PrP signal was observable in the blots with mAb 6D11, but clear positive bands from 40 to 50 kDa were detected in the blots with anti-MiniSOG (Fig 4C). We also noticed that the PrP reactivity in #78 was weak compared with others. The exact reason is unknown, possibly due to individual difference. The technique problem could not be excluded. Nevertheless, this mouse (#78) has been already scarified, which is not used for further breeding and infectious experiment. As expected, positive bands from 40 to 50 kDa were also detected in the reaction with anti-MiniSOG (Fig 4D). Those data indicate that both native PrP and the fusion miniSOG-PrP proteins are expressed in the brain tissues of the heterozygous Tg mice, and only the fusion PrP-miniSOG is expressed in that of the homozygous Tg mice.

The potential PK resistances of the *de novo* expressed miniSOG-PrP in the brain tissues of homozygous Tg mice were evaluated by PK-digested Western blots. At the same amount of 10% brain homogenate, no PrP signal was observed in the preparation of WT mice, however, PrP signals were clearly detectable in the brain homogenates of two Tg mice under the exposure to 5 g/ml PK and turned to weak

but still visible in the reactions of 10 g/ml PK (Fig 5). It seems that the miniSOG-PrP expressed in the brains of two types of Tg mice possess weak PK resistance.

The brain slices of those Tg mice were prepared and IHC assays with mAb 6D11 and anti-miniSOG were conducted. In the preparations of mAb 6D11, large amounts of brown stains were detected in the brains of two heterozygous Tg mice, which were mostly outside the cells, whereas much fewer brown stains were in the brains of two homozygous Tg mice, which restricted surrounding the cell nuclei (Fig 6A). On the contrary, in the reactions of anti-miniSOG, both heterozygous and homozygous Tg mice showed the same reactive profiles, in which deeply brown signals were seen in the cytoplasm (Fig 6B and Fig 6C). No morphological difference in the IHC assays were figured out between Tg^{SOG23} and Tg^{231SOG}.

To assess the copy numbers of the inserted external gene in the brain tissues of Tg mice, the genomic DNA of two individual animals each type of Tg mouse were extracted and subjected into digital PCR assays using gene Fabpi as the internal control. With the primers for mouse *PRNP*, specifically positive amplified spots were identified in all tested samples (Fig 7). Calculation the ratios of the positive spots of miniSOG-PrP and Fabpi proposed that the copy numbers of the inserted external gene in Tg^{SOG23} and Tg^{231SOG} were 2 and 12, respectively.

Discussion

In this study, we have generated two strains of Tg mice, Tg^{SOG23} and Tg^{231SOG}, expressing fusion protein miniSOG-MusPrP, one inserted at the N-terminus of PrP after the signal peptide and the other at its C-terminus in front of the GPI anchor, respectively. Digital PCR reveals that the copy numbers of the transgenic recombinant gene are 2 in Tg^{SOG23} and 12 in Tg^{231SOG}. Those two strains of Tg mice are healthy without observable abnormality after one-year observation. Further assays have confirmed that the miniSOG-PrP fusion proteins were efficiently expressed in the brain tissues of the Tg mice.

In the past decades, there are huge improvements for understanding prion biology, structure, infectivity and pathogenesis. However, the prion morphology seems to progress slowly. We still identify the concept that PrP^{Sc} can exist in different degree of aggregation in the brain, forming fibrous or rod-like structures with smaller oligomers and more molecules, and then forming amyloid structures with large molecular weight. Some studies propose that oligomer PrP^{Sc} is the most infectious [23, 24]. So far, SAFs and prion rods are considered to be the main morphological structures of prion under the observation of electron microscopy, which share the same protein composition and antigenicity [25]. However, these prion morphologies are usually purified by superionization and observed by electron microscopy with negative staining [26, 27]. In addition, immunoelectron microscopy for prion morphological assay has also obviously drawbacks. As PrP^{Sc} is an aggregation of multiple monomers, which is insoluble in water and surfactant, treatment with strong denaturants is often required

in order to expose partial epitopes for antibody recognition and binding. Moreover, PrP^{Sc} contains the same primary sequences as PrP^{C} and the routinely used antibodies could not distinguish PrP^{Sc} and PrP^{C}, which needs prior treatment with protease or formic acid to remove endogenous PrP^{C}. Treatment with strong denaturants or exposure to proteases will definitely destroy many components and native structures of the cells or tissue, meanwhile result in the loss of many structural details of PrP^{Sc}.

To overcome the difficulty of PrP^{Sc} immunoassay *in vivo*, many different attempt have been tried, including adding fluorescent labels to PrP. It is reported that GFP label has been inserted into PrP^{C} at the position of aa 223 to generate Tg(PrP-EGFP)/$PRNP^{0/0}$ mice. However, further bioassays have revealed that this type of Tg mice are tolerant to the infection of prions [28-30]. Another strain of Tg mouse contains GFP tag at N-terminus after the signal peptide. Such type of Tg mouse can form PrP^{Sc}, but the ability of formation is weak [31]. It might be due to the influence of the external tag on the unknown spatial structure of PrP, which is essential for the conversion from PrP^{C} to PrP^{Sc}.

In recent years, the technology of protein marker recognized by electron microscope has developed greatly. MiniSOG is one of the ideal labels, which is useful in the studies both *in vitro* and *in vivo*[32]. The most valuable feature of miniSOG is its double usages in both fluorescent and ultrastructural assays via photooxidation. The tested tissues or cells can be fixed with routine methods and the components (O_2, DAB, and OsO_4) for subsequent photooxidation are small molecules that easily permeate tissue, which ensures good preservation of ultrastructure without concern

for retention of antigenicity [10]. Compared with GFP tag (235 aa-long) miniSOG is significantly smaller (110 aa-long), which makes it easy be operated experimentally and possibly has less influence on the biological function of the labeled protein. Additionally, GFP tag is an ideal marker for immunofluorescent assays, but may not be suitable for ultrastructural assays for prion morphology. Those features of miniSOG may supply an ideal tool for exploring the formation, change and distribution of prion agents morphologically in the brain or other peripheral tissues during prion infection.

The expressing level of the inserted *PRNP* gene in the transgenic mice may influence the susceptibility to prion infection[33-35]. Based upon the blots in Western blot, the average gray values of PrP in the brain tissues of those two strains of transgenic mice were analyzed after normalized with the data of the corresponding actin. The expression levels of PrP-miniSOG in both transgenic mice are estimated about 0.5 time of that of PrP in wild type mouse, respectively. We have to confess that such estimation is not accurate as the molecules of PrP-miniSOG and wild-type PrP are different that may affect on the reactivities in Western blot. Further tests of limit dilution of brain homogenates in PrP-specific Western blot will be helpful for evaluation of the expressing level of the recombinant PrP in transgenic mouse.

Ethics statement

Generation of transgenic mice expressing MiniSOG-PrP23 or PrP231-MiniSOG, the usage of animal specimens in this study was approved by the Ethical Committee of

the National Institute for Viral Disease Control and Prevention (Beijing, China) under the protocol 2011ZX10004-101. Animal housing and experimental protocols were in accordance with the Chinese Regulations for the Administration of Affairs Concerning Experimental Animals. All methods were performed in accordance with the relevant guidelines and regulations.

Conflict of Interest Statement

The authors declare that they have no conflict of interest.

Author Contributions

L.P.G. contributed to study design, performed assays and data analysis, and prepared the manuscript. K.X. and Y.Z.W. assisted with the assays of Western Blot analysis. X.H.Y. and D.D.C. assisted with the animal tests. Q.S. and X.P.D., corresponding authors, contributed to design, study concept, and manuscript preparation.

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Informed consent

Not applicable.

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Figure legends

Figure 1. Determination of the expressions of the chimeric miniSOG-PrP proteins in the cells transiently transfected with the plasmids pcDNA-MiniSOG-PrP23 and pcDNA-PrP231-MiniSOG. Cells (293T and SK-N-SH) were harvested 48 h post-transfection. Cells transfected with the plasmid pcDNA-MusPrP (PrP-Ctrl) and pcDNA3.1 (Vector-Ctrl) were used as controls. **A.** Fluorescent assays. **B.** Western blot with PrP specific antibody for the 293T cells transfected with different plasmids. **C.** Western blot with PrP specific antibody for the SK-N-SH cells transfected with different plasmids.

Figure 2. Morphological localization of the expressed chimeric miniSOG-PrP inside of the transfected 293T cells. **A.** Representative images of double stained IFA with PrP antibody and anti-membrane. **B.** Representative images of double stained IFA with PrP antibody and anti-Golgi. **C.** Representative images of double stained IFA with PrP antibody and anti-ER.

Figure 3. Ultrastructural morphological localization of the expressed chimeric miniSOG-PrP in the transfected cells. Cells were undergone into photooxidation process and analyzed with TEM after stained with DAB. Arrows indicate the positive stained chimeric miniSOG-PrP. The magnifications of the images are indicated at the bottom.

Figure 4. Determinations of the expressions of chimeric miniSOG-PrP in the brain tissues of different Tg mice by Western blots. The brain homogenates of various Tg mice in Western blots were prepared from cortex regions. **A.** $Tg^{SOG23/WT}$ and $Tg^{231SOG/WT}$ with PrP mAb 6D11. **B.** $Tg^{SOG23/WT}$ and $Tg^{231SOG/WT}$ with anti-MiniSOG. **C.** Tg^{SOG23} with PrP mAb 6D11 and anti-MiniSOG. **D.** Tg^{231SOG} with PrP mAb 6D11 and anti-MiniSOG.

Figure 5. Evaluations of PK-resistances of the expressed chimeric miniSOG-PrP in the brain tissues of different Tg mice. Same amounts of brain lysates of Tg^{SOG23} (left), Tg^{231SOG} (middle) and wild-type (WT, right) mice were exposed to different concentrated PK (0, 5, 10, 50 and 100 μg/ml) prior to Western blots with PrP mAb 6D11.

Figure 6. Determinations of the expressions of chimeric miniSOG-PrP in the brain tissues (cortex region) of different Tg mice by IHC assays. **A.** PrP mAb 6D11. **B.** anti-miniSOG-PrP mAb. **C.** anti-miniSOG-PrP pAb. Various Tg mice are indicated on the left and the magnifications are showed on the bottom.

Figure 7. Evaluations of the transcriptions of chimeric miniSOG-PrP in the brain tissues (cortex region) of different Tg mice by digital PCR assays. **A.** Tg^{SOG23}. **B.** Tg^{231SOG}. The ratios of the numbers of the positive spots of miniSOG-PrP/Fabpi in the tested mouse were showed in the panels.

Fig.1

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