Antiprion Prophylaxis by Gene Transfer of a Soluble Prion Antagonist

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Prion diseases are untreatable neurodegenerative disorders characterized by accumulation of PrP^{Sc}, an aggregated isoform of the normal prion protein PrP^C. Here, we delivered the soluble prion antagonist PrP-Fc₂ to the brains of mice by lentiviral gene transfer. Although naïve mice developed scrapie at 175 ± 5 days postintracerebral prion inoculation (dpi), gene transfer before inoculation delayed disease onset by 72 ± 4 days. At 170 days postintracerebral prion inoculation, PrP^{Sc} accumulation and prion infectivity in PrP-Fc₂-treated brains were reduced by 3.6 and 4.2 logs, respectively. When PrP-Fc₂ was delivered 30 days after prion inoculation, survival of the treated animals was extended by 25 days. We then used tissue-specific recombination to express PrP-Fc₂ in the entire central nervous system, in only astrocytes, or in only oligodendrocytes. Oligodendrocyte-restricted PrP-Fc₂ expression impaired PrP^{Sc} deposition and delayed disease even though oligodendrocytes are completely resistant to prion infection, suggesting that PrP-Fc₂ affords protection via noncell autonomous mechanisms. These results suggest that somatic gene transfer of prion antagonists may be effective for postexposure prophylaxis of prion diseases. (*Am J Pathol* 2008, 172:1287–1296; DOI: 10.2353/ajpath.2008.070836)

To date, no efficacious treatments for prion diseases are available in clinical settings.^{1,2} Anti-PrP antibodies can delay scrapie after peripheral challenge of prions.^{3,4} However, this strategy does not target PrP^{Sc} replication in the brain, and intracerebral delivery of anti-PrP^C antibodies may be neurotoxic.⁵ Active immunization strategies are meeting with little success most likely because endogenous PrP^C induces robust immune tolerance.^{6,7} Transgenic expression of a soluble dimeric PrP, termed PrP-Fc₂, affects prion propagation and scrapie patho-

genesis by interfering with the buildup of PrP^{Sc}.⁸ The high solubility and stability of PrP-Fc₂ prompted us to explore its therapeutic potential. We therefore used lentiviral vectors⁹ to investigate in a scrapie mouse model whether sustained intracerebral expression of PrP-Fc₂ inhibits prion replication.

Materials and Methods

Mice

Mouse lines *tg550Stop*, carrying a loxP-flanked transcription-termination cassette upstream of the open reading frame of PrP-Fc, and *tg588^{CMV}*, in which the transcription-termination cassette was eliminated, were described previously.⁸ Mice carrying the Cre recombinase under the transcriptional control of the Nestin, glial fibrillary acidic protein (GFAP), or proteolipid protein (PLP) regulatory elements have been described elsewhere.^{10–12} *tg550Stop* × PLP/Cre-ERT2, *tg550Stop* (but Cre-negative), and PLP/Cre-ERT2 (but *tg550Stop*-negative) littermates were treated with tamoxifen dissolved in a sunflower oil/ethanol (10:1) mixture at 10 mg/ml. Twice 1 mg of tamoxifen per day for 5 consecutive days was injected intraperitoneally, as described.¹²

Vector Production and Injection

PrP-Fc cDNA was cloned into the lentiviral vector pRRL.sin.cPPT.hCMV.GFP.Wpre⁹ in place of GFP. Vectors stocks were generated as described⁹ by co-trans-

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Table 1. Sequences of PCR Primers and PrP^{Sc} Open Reading Frame

Forward primer recognizing PrP-moiety of PrP ^{Sc}
5'-AACGACTGGGAGGACCGCTA-3'
Reverse primer recognizing Fc-moiety of PrP ^{Sc}
5'-CCTTGCCATTTCAGCCAGTCC-3'
Open reading frame of PrP ^{Sc}
5'-ATGGCGAACCTTGGCTACTGGCTGCTGGCCCTCTTTGTGACTATGTGGACTGATGTGGCCTCTGCAAAAAGCGGCCAAAGCCTGGAGGGTGGAA- CACCGGTGGAAGCCCGGTATCCCGGGCAGGGAAGCCCTGGAGGCAACCGTTACCCACCTCAGGGTGGCACCTGGGGGCAGCCCCACGGTGGTGGC- TGGGGACAACCCCATGGGGGCAGCTGGGGACAACCTCATGGTGGTAGTTGGGGTCAGCCCCATGGCGGTGGATGGGGCCAAGGAGGGGGTACC- CATAATCAGTGAACAAGCCAGCAAAACCAAAACCAACCTCAAGCATGTGGCAGGGGCTGCGGCAGCTGGGGCAGTAGTGGGGGGCCTTGGTGG- CTACATGCTGGGGAGCGCCATGAGCAGGCCCATGATCCATTTTGGCAACGACTGGGAGGACCGCTACTACCGTGAAAACATGTACCGCTACCCTA- ACCAAGTGTACTACAGGCCAGTGGATCAGTACAGCAACCAAGCAAACTTCGTGCACGACTGCGTCAATATCACCATCAAGCAGCACACGGTCACC- ACCACCACCAAGGGGAGAACTTCACCGAGACCGATGTGAAGATGATGGAGCGCGTGGTGGAGCAGATGTGCGTCACCCAGTACCAGAAGGAGT- CCGAGGCCATATACGACGGGAGAAATCCGGGGGCTCGACAAACTCACACATGCCCAACCGTGCCCGAGCACCTGAAGCCGAGGGGGCACCG- TCAGTCTTCCTCTTCCCCCAAAACCAAGGACACCCCTCATGATCTCCCGACCCCTGAGGTCACATGCGTGGTGGTGGACGTGAGCCACGAA- GACCCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCAATAATGCGCAAGCAAGCCGCGGAGGAGCAGTACAACAGCACGTACCG- TGTGGTCAGCGTCCCTCACCGTCTGCACCAAGGACTGGCTGAATGGCAAGGAGTACAAGTGAAGGTCTCCAACAAAGCCCTCCAGCCTCCA- TCGAGAAAACCATCTCCAAAGCCAAAGGGCAGCCCGAGAACACAGGTGTACACCTTGCCCCCATCCCGGGATGAGCTGACCAAGAACCAGGT- CAGCCTGACCTGCCTGGTCAAAGGCTTCTATCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGAGAGAACACTACAAGACCAC- GCCTCCGCTGTTGGATCCGACGGCTCCTTCTCTCAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGGAGGGAACGTCTTCTCA- TGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTGTCTCCGGGTAAA-3'

Target sequences of the primers are underlined.

fection of 293T with pCMV-ΔR8.91,¹³ pMD.G,¹³ and pRRL.sin.cPPT.hCMV.PrP^{Sc} vectors. 293T-conditioned media were filtered and ultracentrifuged to concentrate the vector.⁹ Particle content was measured by HIV-1 p24 antigen immunocapture.⁹ HIV-1 p24 concentration was between 500 to 600 μg/ml (corresponding to 100,000 infectious units per ng of p24). C57BL/6 mice were slowly injected with 5 μl of the lentiviral preparations or phosphate-buffered saline (PBS) stereotactically into the hippocampus or 30 μl intracerebrally into the right telencephalic hemisphere. Integration of the transgene was detected by polymerase chain reaction (PCR) with standard conditions using primers amplifying the PrP and Fc regions (Table 1).

Western Blot Analysis and NaPTA Precipitation

Sodium phosphotungstate precipitation and quantitative Western blot were performed as described.⁸ Brain and spleen samples were digested for 30 minutes at 37°C with 50 μg/ml and 20 μg/ml of proteinase K, respectively. Antibody incubations were done in 1% Top-Block (Sigma-Aldrich Chemia GmbH, Buchs, Switzerland) in Tris-buffered saline Tween 20 with ICSM18³ or POM1¹⁴ for PrP and PrP-Fc₂ for 1 hour at room temperature or overnight at 4°C. The samples were analyzed with a VersaDoc digital imager (model 5000; Bio-Rad, Hercules, CA).

Bone Marrow (BM) Chimeric Mice

BM cells (5 × 10⁶) were isolated from tibiae and femurs, and injected into tail veins of 8- to 10-week-old recipients conditioned by whole-body irradiation (1100 rad). Several paradigms were generated: wt BM was injected into wt or tg588-irradiated mice, and tg588 BM was injected into wt or tg588-irradiated mice. The tg588^{CMV} line (for brevity tg588) was used in the BM reconstitution experiments because of the sixfold higher expression of PrP-Fc₂ in spleen in tg588 compared to tg550.⁸ Eight weeks

after grafting, reconstitution was assessed by enzyme-linked immunosorbent assay (ELISA) analysis of peripheral blood taken from the retro-orbital plexus of ether-anesthetized mice.

ELISA Analysis

ELISA to detect PrP-Fc₂ was performed as described.^{3,4} Briefly, plates (Maxisorp; Nunc, Roskilde, Denmark) were coated with monoclonal anti-PrP antibody ICSM 18, washed with PBS containing 0.1% (v/v) Tween 20 (PBST), and blocked with 5% bovine serum albumin (BSA). After washing, plates were incubated with 30 μl of twofold serially diluted serum (1:20 prediluted) in PBST containing 1% BSA and then probed with horseradish peroxidase-conjugated goat anti-human IgG-Fc horseradish peroxidase (1:1000 dilution; Rockland Immunochemicals, Gilbertsville, PA). Plates were developed with 2,2'-azino-diethyl-benzothiazolinsulfonate, and optical density was measured at 405 nm. Titer was defined as the highest dilution showing an OD more than two times the technical background, which was calculated as the average of uncoated wells and wells incubated omitting serum. For detection of anti-human Fc and anti-GFP antibodies in serum of lentivirus-treated mice, the plates were coated with purified human Fc and GFP-His,¹⁵ respectively. The antibodies from the serum were detected in the plates by using rabbit anti-mouse IgG + A + M (H + L)-horseradish peroxidase conjugate (Zymed/Invitrogen AG, Basel, Switzerland).

Histopathology

Brains were fixed in 4% paraformaldehyde, paraffin-embedded, and cut into 2-μm sections. Sections were stained with hematoxylin and eosin (H&E) and with anti-GFAP.¹⁶ Histoblots were performed according to published protocols.¹⁷ For immunohistochemistry, cryosections were fixed 5 minutes in paraformaldehyde, 2

minutes in 50% acetone, 2 minutes in 100% acetone, 2 minutes in 50% acetone, PBS-washed, and blocked for 30 minutes in 5% donkey serum. Rabbit anti-human Fc γ (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted 1:1000 in 0.5% BSA was used as primary antibody and biotinylated donkey anti-rabbit (Jackson ImmunoResearch Laboratories) was used as a secondary antibody diluted 1:100 in 0.5% BSA. In our experience, histoimmunochemical detection of PrP-Fc₂ in tissue sections proved difficult because PrP-Fc₂ is a molecule secreted from cells.

Prion Inoculations

Mice were inoculated intracerebrally or intraperitoneally with 3×10^2 or 10^3 LD₅₀ infectious units of Rocky Mountain Laboratory strain (RML, passage 5) scrapie prions prepared as described.⁸ Mice were sacrificed on the day of onset of terminal clinical signs of scrapie. Significance *P* values were derived by comparing mean survival (Student's *t*-test; two-tailed distribution, two-sample unequal variance). Survival data were plot by using GraphPad Prism 4.

Infectivity Bioassays with tga20 Mice

Assays were performed on 1% homogenates of brain or of spleen tissues as described.¹⁸ Tissues were homogenized in 0.32 mol/L sucrose with a homogenizer and diluted 1:10 in 5% BSA in PBS. Supernatants (30 μ l) were inoculated intracerebrally into groups of three to four tga20 mice.¹⁹ Infectivity titers were calculated as described.²⁰

Scrapie Cell Assay in Endpoint Format (SCEPA)

For SCEPA, highly RML prion-susceptible neuroblastoma cells (subclone N2aPK1²¹) were exposed to prion samples for 3 days in 96-well plates, and split three times 1:3 every 2 days, and three times 1:10 every 3 days. After reaching confluence, 25,000 cells from each well were filtered onto the membrane of white Immobilon P plate (Millipore, Billerica, MA) treated with PK, denatured, and individual infected (PrP^{Sc}-positive) cells were detected by ELISA using antibody POM-1 to PrP. After reaching confluence, 25,000 cells from each well were processed as above. Wells were counted positive if the spot number was clearly exceeding background. From the proportion of negative to total wells the number of infectious tissue culture (TCI) units per aliquot was calculated by the Poisson equation as described previously.²¹ The potency of the SCEPA is based on the finding that the proportion of infected cells, and with it the signal-to-background ratio, increases on average ~25% per day during culturing. The sensitivity of the assay can be further enhanced by increasing the number of replicate samples and the number of 1:10 splits.

Preparation of Cerebellar Granule Cells, Oligodendrocytes, and Astrocytes

Cerebellar granule neurons were prepared from 7- to 8-day-old mice as previously described.²² Contamination with glial cells was <5%. Mixed glial cell cultures containing oligodendrocytes and astrocytes were produced from 1-day-old neonatal mice as described.^{23,24} Cultures were prepared with high-glucose Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and replenished on day 4 and every 3 to 4 days thereafter for 10 days, with Dulbecco's modified Eagle medium plus 10% heat-inactivated horse serum. Oligodendrocytes were purified from mixed glial cultures by differential detachment and negative selection of microglia by adherence to hydrophobic plastic. Purified oligodendrocytes were then plated onto glass or plastic culture chambers coated, respectively, with 100 μ g/ml or 10 μ g/ml poly-L-lysine, whereas astrocytes were kept in the same dish. Oligodendrocyte precursors were expanded with platelet-derived growth factor- and fibroblast growth factor-supplemented SATO medium for 2 days and subsequently differentiated with 1% horse serum-supplemented SATO medium for 3 days.

Immunofluorescence

Cells were fixed with 4% paraformaldehyde in PBS for 15 minutes at room temperature. The cultures were permeabilized and blocked in PBS supplemented with 0.1% Triton X-100 and 10% fetal calf serum. Cells were then incubated with antibodies against neuronal-specific nuclear protein (NeuN, 1:50), glial fibrillary acidic protein (GFAP, 1:100; DAKO, Glostrup, Denmark), and myelin-associated glycoprotein (MAG; 1:100; Chemicon, Temecula, CA) diluted in 1% BSA in PBS at 4°C overnight. After washing, cells were incubated with goat anti-mouse secondary antibodies conjugated with Alexa 546 (1:200; Molecular Probes, Eugene, OR) or with donkey anti-rabbit secondary antibodies conjugated with fluorescein isothiocyanate (1:50; Jackson Laboratory, Bar Harbor, ME). Nuclear staining was performed with DAPI.

Results

We generated a recombinant lentiviral vector transducing PrP-Fc₂ (henceforth termed ψ PrPFc; Figure 1a). In a first series of experiments we investigated whether ψ PrPFc, when precisely targeted to a defined group of cells, would afford local protection. We therefore injected a relatively small amount of ψ PrPFc (3×10^8 infectious units in 5 μ l) into a group of five mice intrahippocampally under stereotaxic guidance. Controls were injected with PBS (*n* = 5). One mouse from each group was sacrificed 6 months after virus injection to provide a first indication about disease progression. PrP-Fc₂ was detected by immunohistochemistry in the injected hippocampus, but not in the contralateral hemisphere (Figure 1b, top row). Accordingly, lentiviral integration in the brain was demonstrated by PCR analysis (Figure 1c). No adverse reac-

Figure 1. Intrahippocampal lentiviral transfer of PrP-FC₂. **a:** Schematic representation of the transfer vector used in this study. **b:** Hippocampal PrP-FC₂ expression in the injected hippocampus (**top row**) coincided with suppressed prion pathogenesis in ψ PrPFC-treated mice sacrificed at 170 dpi (**middle and bottom rows**), as assessed by immunohistochemistry using an anti-human Fcy antibody. **c:** PCR using primers indicated in **a** of various tissues and genotypes showing brain-specific lentiviral integration. Genomic actin amplification served for normalization of the PrPFC signal (concentration of genomic DNA in crude extract from the tissue samples was not controlled in this experiment). **d:** No pathology in the brain was associated with the lentiviral injection as determined by H&E staining and by various markers (GFAP, CD11b, Iba-1; data not shown) in nonprion inoculated mice. **e:** Histoblots showed a broad area of decreased PrP^{Sc} deposition extending well beyond the site of ψ PrPFC injection (arrow). In contrast, PrP^{Sc} signals were saturated in the contralateral hemisphere and in a PBS-treated brain. **f:** Survival plots displaying the incubation time until development of terminal scrapie in PBS- and ψ PrPFC-treated mice after intracerebral challenge. Median disease onset was delayed by 36 days in intrahippocampally ψ PrPFC-treated mice. LTR, long terminal repeats; CMV, cytomegalovirus; SD, splice donor; SA, splice acceptor; Ψ , packaging sequence; GA, 5' portion of *gag* gene with truncated reading frame including extended packaging signal; RRE, rev response element (RRE); cPPT, central polypurine tract; WPRE, posttranscriptional regulatory element from the genome of the woodchuck hepatitis virus.

tions were detected by conventional histology (Figure 1d), nor by immunohistochemistry with various astroglial, microglial, and inflammatory markers (GFAP, CD11b, Iba-1; data not shown).

Twenty days after virus injection, four ψ PrPFC-treated mice and four PBS controls were inoculated intracerebrally with 3×10^2 LD₅₀ RML prions. All PBS-injected control animals succumbed to disease [177 $\pm$ 8 days postintracerebral prion inoculation (dpi)], whereas none of the ψ PrPFC-treated animals showed clinical signs at

177 dpi (Figure 1f). One clinically unaffected ψ PrPFC-treated mouse was sacrificed at 170 dpi and analyzed for the presence of PrP^{Sc} by histoblotting. Whereas PBS-treated animals showed massive PrP^{Sc} accumulation, the ψ PrPFC-injected mouse showed markedly reduced accumulation of PrP^{Sc} in the injected hippocampus. Importantly, ipsilateral reduction in PrP^{Sc} signal intensity extended well beyond the track of the stereotaxic needle, suggesting that ψ PrPFC exerts long-range antiprion effects. In the contralateral hemisphere, however, PrP^{Sc}

deposition was indistinguishable from that of PBS-treated controls (Figure 1e). Histological analysis failed to reveal any vacuolation and astrogliosis in the ipsilateral hippocampus (Figure 1b, middle and bottom rows). The contralateral side presented with rather strong vacuolation and astrogliosis, although the corresponding region of a terminally sick PBS-treated animal may have been

more strongly affected (Figure 1b, middle and bottom rows). ψ PrPFc-injected mice succumbed to disease at 213 ± 6 dpi (Figure 1f), which is remarkable, considering substantial vacuolation and PrP^{Sc} deposits detected in the contralateral side of the ψ PrPFc-injected mice sacrificed for analysis at 170 dpi (see above; Figure 1b, middle row; and Figure 1e, bottom row). Therefore, a single

Figure 2. Intracerebral lentiviral vector administration. **a:** Western blot analysis of frontal brain of transgenic PrP-Fc₂ mice,⁸ and PBS-treated and ψ PrPFc-treated animals. Using anti-PrP antibody,³ sustained PrP-Fc₂ expression was detected 6 months after virus injection in the brain of ψ PrPFc-treated animals, but not in the spleen. **b:** Severe spongiosis and astrogliosis in terminally sick PBS-treated animals, but no sign of prion-associated pathology in a ψ PrPFc-treated animal sacrificed 170 dpi, similarly to age-match uninoculated mice. Sections were stained with H&E and immunostained with GFAP for detection of astrogliosis. **c:** Whereas PrP^{Sc} signals were visible in wt brains and spinal cords at terminal stage, no or weak signals were present in the brain and spinal cord of a ψ PrPFc-treated animal sacrificed at the same time point. However, spleens of treated and controls animals exhibited similar quantities of PrP^{Sc}. **d:** NaPTA-enhanced quantitative Western analysis showed that PrP^{Sc} accumulation in the tested ψ PrPFc-treated brain was 0.03% of the tested PBS-treated brain. To reach a comparable intensity level for Western blot quantification, control samples were diluted linearly up to 32 times, whereas for ψ PrPFc-treated brains, PrP^{Sc} was concentrated by NaPTA precipitation from 125- or 250-fold more starting material than was used directly for Western blot in the case of PBS-treated mouse, **lane 1**. **e:** Prion infectivity, determined in the brain of one ψ PrPFc-treated animal by the scrapie cell assay in endpoint format (SCEPA), was reduced by 4.2 log TCID₅₀/g tissue compared to PBS-treated animals. **f:** Survival of scrapie-infected mice treated with PBS or ψ PrPFc. Whereas PBS-treated mice ($n = 4$) succumbed to disease at 175 ± 5 dpi, ψ PrPFc-treated animals ($n = 3$) had an extended survival of 72 days (247 ± 8 dpi).

unilateral hippocampal injection sufficed to delay onset of disease by 36 days ($P < 0.01$) as compared to PBS-injected controls.

The above results, along with the observation that the antiprion action of PrP-Fc₂ is dose-dependent in transgenic mice,^{8,25} encouraged us to explore whether non-stereotaxic delivery of a larger number of ψ PrPFc particles would enhance protection. We injected 30 μ l of ψ PrPFc (1.5×10^9 infectious units) intracerebrally into the right telencephalic hemisphere of mice ($n = 4$), and inoculated them with 3×10^2 LD₅₀ RML prions intracerebrally 20 days later. Six months after virus injection, immunoblot analysis revealed sustained PrP-Fc₂ expression in brains of ψ PrPFc-treated mice (Figure 2a).

We sacrificed one presymptomatic ψ PrPFc-treated mouse and two terminally sick PBS-treated mice at 171 dpi to estimate prion pathogenesis at this experimental paradigm. Profound histopathological changes were identified in PBS-treated brains (Figure 2b). In contrast, the ψ PrPFc-treated brain displayed no spongiform changes ipsi- or contralaterally. Astrogliosis, estimated by GFAP immunoreactivity, was similar to age-matched uninfected mice (Figure 2b).

Brain, spinal cord, and spleen homogenates were subjected to proteinase K (PK) digestion and analyzed for accumulation of PrP^{Sc} by Western blot analysis. Surprisingly, whereas wild-type (wt) animals showed extensive buildup of PrP^{Sc}, no PrP^{Sc} was detectable in the brain of the ψ PrPFc-treated animal and only traces thereof in the spinal cord (Figure 2c). Conversely, spleens from all animals showed similar accumulation of PrP^{Sc}, suggesting that protection was restricted to the central nervous system (CNS) (Figure 2c). To increase the sensitivity of PrP^{Sc} detection, we concentrated PrP^{Sc} by differential precipitation with sodium phosphotungstic acid (NaPTA). Quantitative Western blot analysis⁴ showed that PrP^{Sc} accumulation in ψ PrPFc-treated brain was 0.03% of PBS-treated brain. To reach a comparable intensity level for Western blot quantification, control samples were diluted linearly up to 32 times, whereas for ψ PrPFc-treated brains, PrP^{Sc} was concentrated by NaPTA precipitation from 125- or 250-fold more starting material than is was used directly for Western blot in the case of PBS-treated mouse, lane 1 (Figure 2d). Prion infectivity titers determined by end-point scrapie cell assay²¹ were similar in spleens of PBS- and ψ PrPFc-treated mice [4.1 and 4.6 log infectious tissue culture units (TCI U)/g tissue, respectively, Figure 2e]. In contrast, prion titers in control brains ($n = 2$) reached 7.6 log TCI U/g tissue, but only 3.4 log TCI U/g tissue in the treated animal (Figure 2e). Typical progressive signs of scrapie, including ataxia and kyphosis, led to terminal disease in all PBS-treated mice by 175 ± 5 dpi ($n = 4$). In contrast, ψ PrPFc-treated animals succumbed to prion disease at 247 ± 8 dpi ($n = 3$), suggesting remarkable resistance to the disease (72 days) and extending their lifespan by more than 40% (Figure 2f). Therefore, single-dose gene transfer of PrPFc provides very potent protection against prion disease, and suppresses prion replication by $>10,000$ -fold.

Next, we tested whether PrP-Fc₂ might afford postexposure prophylaxis. Mice were inoculated with 3×10^2

Figure 3. Intracerebral lentiviral vector transfer during prion disease progression. **a:** Survival plots displaying the incubation time until development of terminal scrapie in ψ GFPFc and ψ PrPFc ($n = 7$) mice treated 30 days after RML prion inoculation. Median disease onset was delayed by 25 days in ψ PrPFc-treated mice. **b:** When prion-inoculated mice were injected with ψ PrPFc 121 dpi, they succumbed at the same time as ψ GFPFc-treated mice (respectively, 197 ± 17 dpi and 197 ± 9 dpi).

LD₅₀ RML prions intracerebrally and, 30 days later, intracerebrally treated with ψ PrPFc (1.5×10^9 IU; $n = 7$). Control animals injected with ψ GFPFc (0.8×10^9 IU; $n = 6$) succumbed to scrapie at 163 ± 4 dpi, whereas ψ PrPFc-treated animals survived 188 ± 11 dpi ($P < 0.01$) (Figure 3a). Terminally sick mice from both groups showed similar PrP^{Sc} accumulation in their brains (data not shown). We then administered ψ PrPFc at 121 dpi, when scrapie-induced brain damage is already evident by histology (A.A., unpublished data). ψ GFPFc ($n = 7$)- and ψ PrPFc ($n = 8$)-treated animals succumbed with similar incubation times (197 ± 17 dpi and 197 ± 9 dpi, respectively), suggesting that treatment at this stage is ineffective (Figure 3b). Importantly, we detected by ELISA anti-GFP antibodies in serum of the ψ GFPFc-treated mice and anti-human Fc antibodies in serum of the ψ PrPFc-treated mice (data not shown). This indicates that secreted GFP-Fc₂ and PrP-Fc₂, both fused to human Fc, crossed the blood-brain-barrier, and were processed by the murine immune system as immunogens. Induction of the transgene-specific antibodies may affect prion propagation. It has been reported that chronic activation of the immune system in the periphery facilitates prion pathogenesis.^{26–28} This may also explain faster onset of disease seen for the groups treated with the same lentivirus at 30 dpi compared to 121 dpi. (Figure 3, a and b).

Figure 4. Effects of hematopoietically expressed PrP-Fc₂. **a:** PrP-Fc₂ titers (relative units) in blood of chimeric mice. *tg588*→wt reconstituted mice showed weaker PrP-Fc₂ expression than *tg588* mice. **b:** Fifty days after RML prion inoculation, spleens from *tg588*→wt and wt→*tg588* showed decreased accumulation of PrP^{Sc} compared to wt→wt spleens. **c:** Infectivity titer of wt→*tg588* ($n = 1$) was below detectability and those of *tg588*→wt ($n = 2$) were 1.2 log lower than wt→wt ($n = 2$, average is represented) controls. **d:** wt→wt and *tg588*→wt reconstituted mice succumbed to scrapie at the same stage, whereas wt→*tg588* and *tg588*→*tg588* mice were retarded.

We then sought to determine whether peripherally expressed PrP-Fc₂ contributes to the antiprion effect. We used transplantation of genetically modified BM progenitors, because this technique may represent a gene therapy vehicle. BM of PrP-Fc₂-expressing mice (*tg588*^{CMV})⁸ or of wt mice was transferred to wt or to *tg588*^{CMV} mice. Eight weeks after BM grafting, the concentration of PrP-Fc₂ was assessed in peripheral blood by ELISA (Figure 4a). Blood PrP-Fc₂ concentrations of *tg588*^{CMV} mice that had received wt BM approached those of *tg588*^{CMV}→*tg588*^{CMV} mice and were four to six times higher than those of wt mice reconstituted with *tg588*^{CMV} BM, indicating that circulating PrP-Fc₂ is mainly contributed by nonhematopoietic radioresistant cells (Figure 4a).

Twelve weeks after reconstitution, chimeric mice were challenged intraperitoneally with 10³ LD₅₀ RML prions. At 50 dpi, immunoblot analysis showed that PrP^{Sc} accumulation was reduced in spleens of *tg588*^{CMV}→wt ($n = 2$) and wt→*tg588*^{CMV} ($n = 2$) compared to wt→wt mice (Figure 4b). Accordingly, splenic prion titers in *tg588*^{CMV}→wt ($n = 2$) were 1.2 log lower than wt→wt ($n = 2$) (Figure 4c) and wt→*tg588* spleen ($n = 1$) showed no detectable infectivity (Figure 4c). Wt→wt mice ($n = 3$) and *tg588*^{CMV}→wt mice ($n = 3$) developed scrapie with identical latency (232 ± 7 dpi and 232 ± 6 dpi, respec-

tively), whereas wt→*tg588*^{CMV} ($n = 4$) and *tg588*^{CMV}→*tg588*^{CMV} ($n = 2$) were affected later (300 ± 21 dpi and 337 ± 25 dpi, respectively) (Figure 4d). These results indicate that PrP-Fc₂-expressing hematopoietic cells antagonized PrP^{Sc} accumulation and prion replication, yet failed to modify the clinical course of prion disease.

Replication-defective lentiviral vectors are not expected to spread throughout the brain after a single unilocal injection.²⁹ PrP-Fc₂ may therefore owe its potent antiprion efficacy to secretion and extracellular spread. This scenario assumes that PrP-Fc₂ would be capable of antagonizing PrP^{Sc} deposition and prion replication in a noncell autonomous manner. We tested this hypothesis in *tg550Stop* mice, which bear a PrP-Fc₂ transgene preceded by a loxP-flanked transcriptional stop cassette.⁸ *tg550Stop* mice⁸ were bred to nestin/*Cre*¹⁰ (*tg550*^{Nestin}), GFAP/*Cre*¹¹ (*tg550*^{GFAP}), or PLP/*Cre*-ERT2¹² (*tg550*^{PLP}) recombinator mice, activating expression of PrP-Fc₂ in the entire CNS, in astrocytes, or in oligodendrocytes, respectively. Before prion inoculation, *tg550Stop* × PLP/*Cre*-ERT2, *tg550Stop* (but *Cre*-negative), and PLP/*Cre*-ERT2 (but *tg550Stop*-negative) littermates were treated daily with 2 mg of tamoxifen intraperitoneally for 5 days to activate *Cre* recombination.¹²

Restriction of transgene expression to the specified cell populations was tested on primary cell cultures of cerebellar granule neurons, astrocytes, and oligodendrocytes from *tg550^{Nestin}*, *tg550^{GFAP}*, and *tg550^{PLP}* mice. Purity in these primary cultures was typically >95%, as determined by specific cellular marker stains (see Supplemental Figure S1 at <http://ajp.amjpathol.org>). Cells were lysed and subjected to Western blot analysis for PrP-Fc₂ (see Supplemental Figure S2 at <http://ajp.amjpathol.org>). Expression of PrP-Fc₂ was relatively weak in *tg550^{PLP}* oligodendrocytes (maybe because the *Pmp* promoter is weakly active in oligodendrocytes^{30,31}), but undetectable in neurons and astrocytes (see Supplemental Figure S2 at <http://ajp.amjpathol.org>). *Tg550^{Nestin}* showed sustained generalized PrP-Fc₂ expression in the CNS, whereas PrP-Fc₂ was mainly secreted by *tg550^{GFAP}* astrocytes (see Supplemental Figure S2 at <http://ajp.amjpathol.org>).

Tg550^{Nestin}, *tg550^{GFAP}*, and littermate control mice were inoculated with 3×10^2 LD₅₀ RML prions intracerebrally or 10^3 LD₅₀ RML prions intraperitoneally. Typical signs of scrapie, including ataxia and kyphosis, occurred in all inoculated mice. However, *tg550^{Nestin}* and *tg550^{GFAP}* mice showed delayed disease after intracerebral inoculation (151 and 65 days, respectively; $P < 0.01$) (Figure 5, a and c) and after intraperitoneal inoculation (90 and 65 days, respectively; $P < 0.01$) (Figure 5, b and d). Astrocytes have been shown to be competent for

prion replication,³² although the toxicity of astrocytic PrP^{Sc} to neurons is somewhat controversial.^{33,34}

To determine whether PrP-Fc₂ exerts its antiprion effect when produced by cells resistant to prion infection, we inoculated *tg550^{PLP}*, which expressed PrP-Fc₂ selectively in oligodendrocytes (see Supplemental Figure S2 at <http://ajp.amjpathol.org>) and Schwann cells.¹² Transgenic mice expressing PrP under the control of the myelin basic protein-promoter are resistant to prion infection, suggesting that oligodendrocytes and Schwann cells do not support cell-autonomous prion replication and neural spread of prions.³¹

We found that *tg550^{PLP}* mice ($n = 8$), which expressed low amounts of PrP-Fc₂, showed delayed disease over tamoxifen-treated *tg550Stop* and PLP/Cre-ERT2 mice. The delay was 35 days ($P < 0.01$) after intracerebral inoculation (Figure 5e) and 50 days ($P < 0.01$) after intraperitoneal inoculation (Figure 5f). Terminal illness was unambiguously accompanied by clinical scrapie signs. Spleens from *tg550^{Nestin}*, *tg550^{GFAP}*, *tg550^{PLP}*, and controls did not show any PrP-Fc₂ expression (see Supplemental Figure S3a at <http://ajp.amjpathol.org>) nor any difference in their accumulation of PrP^{Sc} (see Supplemental Figure S3b at <http://ajp.amjpathol.org>).

Determination of prion titers¹⁹ 50 days after intracerebral inoculation demonstrated that all spleens from *tg550^{Nestin}*, *tg550^{GFAP}*, and *tg550^{PLP}* and their littermate controls contained approximately the same infectivity,

Figure 5. Protection against scrapie can be mediated both by neuronally and glially restricted expression of PrP-Fc₂. **a–f**: Survival plots of scrapie-infected transgenic mice expressing PrP-Fc₂ in the entire CNS (**a, b**), in astrocytes (**c, d**), or in oligodendrocytes (**e, f**) after intracerebral (**a, c, e**) or intraperitoneal RML prion inoculation (**b, d, f**). Expression of PrP-Fc₂ in oligodendrocytes, which are inherently resistant to scrapie, delayed prion disease, suggesting a noncell autonomous antiprion effect. **g**: Immunoblot analysis revealed reduced levels of PrP^{Sc} in brains of transgenic mice expressing PrP-Fc₂ cells specifically. **h**: GFAP staining indicated diminished pathological changes, in agreement with delayed disease progression.

confirming the similar prion replication rate in spleens of each genotype (see Supplemental Table S1 at <http://ajp.amjpathol.org>). Although brains of wt mice contained prion titers between 2.7 to 4.5 log LD₅₀/g tissue after 50 dpi intracerebrally, *tg550^{Nestin}*, *tg550^{GFAP}*, and *tg550^{PLP}* brain titers were below detectability. At 100 dpi, *tg550^{Nestin}* brain titers were still reduced compared to wt controls (see Supplemental Table S1 at <http://ajp.amjpathol.org>).

Immunoblot analysis of PrP^{Sc} in *tg550^{Nestin}*, *tg550^{GFAP}*, *tg550^{PLP}*, and wt littermate brains revealed similar PrP^{Sc} deposition at terminal disease (Figure 5g). In contrast, *tg550^{Nestin}*, *tg550^{GFAP}*, and *tg550^{PLP}* sacrificed at the time at which wt controls mice had developed terminal scrapie, showed markedly reduced PrP^{Sc} accumulation (Figure 5g). With the caveat that Western blot analyses are only semiquantitative, this suggests that PrP^{Sc} deposition is counteracted even when PrP-Fc₂ is expressed by CNS cells unable to replicate prions.

Next, we analyzed neuropathological changes during the course of the disease. At terminal stage (181/183/224 dpi), all control animals showed pathological evidence of neuroinvasive CNS scrapie infection to a similar extent (Figure 5h and data not shown). At these time points, *tg550^{Nestin}* mice showed only weak astrogliosis, vacuolation, and PrP^{Sc} deposition (Figure 5h and data not shown), whereas in *tg550^{GFAP}* and *tg550^{PLP}* mice, neuropathological changes were more advanced, as reflected by astrogliosis, PrP^{Sc} deposition, and spongiform degeneration (Figure 5h and data not shown), but were still reduced in comparison to wt sick mice. Therefore, PrP-Fc₂ not only delays PrP^{Sc} accumulation in the brain, but also retards histopathological changes. The entirety of the above results strongly suggests that PrP-Fc₂ delays PrP^{Sc} accumulation and prion disease in a noncell autonomous manner.

Discussion

We have explored *in vivo* gene therapy in a mouse model of prion disease. Our approach resulted in sustained expression of a soluble prion antagonist in the brain, impaired replication of disease-associated PrP^{Sc}, effective neuropathological protection, and delayed disease progression. Even though lentiviral transduction is limited to the site of injection,²⁹ PrP^{Sc} accumulation was decreased in most of the brain. The most plausible interpretation is that secreted PrP-Fc₂ was released into the cerebrospinal fluid, where it antagonized PrP^{Sc} replication noncell autonomously, as suggested in a previous report.⁸

Why was ψ PrP-Fc less efficient to delay prion pathogenesis when delivered during disease progression? Transgene expression may take 4 to 6 weeks before reaching its maximum.³⁵ Therefore, after injection at 30 dpi, high expression of PrP-Fc₂ was achieved in the brain ~60 to 75 dpi, when prion infectivity and PrP^{Sc} have already colonized the brain. Indeed, we detected PrP^{Sc} in the brain by Western blot as early as 58 dpi (data not shown). Because there is a competitive interaction between PrP-Fc₂ and PrP^C for PrP^{Sc},⁸ the presence of substoichiometric PrP-Fc₂ may not

be sufficient to efficiently prevent PrP^{Sc} formation and may only weakly slow down its accumulation.

Direct gene delivery of a soluble prion antagonist into the CNS by lentiviral vectors may be an effective strategy against prion diseases. In contrast, almost all previous therapeutic interventions only showed benefits after extracerebral injection, probably reflecting the difficulty of most of the drugs to cross the blood-brain barrier.^{3,36–38} Because variant Creutzfeldt-Jakob disease can be diagnosed before death on lymphoid tissues³⁹ and patients may display extraneural PrP^{Sc} long before neuroinvasion,⁴⁰ gene delivery of prion antagonists directly in the brain may be sufficient to block PrP^{Sc} before it has already colonized the CNS. In this context, our findings may provide a promising new approach to prion therapeutics.

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