Inhibition of PrP^{Sc} formation by lentiviral gene transfer of PrP containing dominant negative mutations

Carole Crozet[1], Yea-Lih Lin[2], Clément Mettling[2], Chantal Mourton-Gilles[3], Pierre Corbeau[2], Sylvain Lehmann[1,4] and Véronique Perrier[1,*]

[1]Laboratoire de Biologie des Encéphalopathies Spongiformes, and [2]Laboratoire Lentivirus et Transfert de Gènes – Institut de Génétique Humaine, CNRS UPR 1142, 141 rue de la Cardonille, 34396 Montpellier CEDEX 5, France
[3]BIO-RAD, Centre de Pharmacologie et Biotechnologies pour la Santé, CNRS UMR 5094, 15 avenue C. Flahault, 34060 Montpellier CEDEX 2, France
[4]Laboratoire de Biochimie. Hopital St Eloi, 80 avenue A. Fliche 34295 Montpellier CEDEX 5, France
*Author for correspondence (e-mail: vperrier@igh.cnrs.fr)

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Summary

Currently, there is no treatment to cure transmissible spongiform encephalopathies. By taking advantage of the 'prion-resistant' polymorphisms Q171R and E219K that naturally exist in sheep and humans, respectively, we have evaluated a therapeutic approach of lentiviral gene transfer. Here, we show that VSV-G (vesicular stomatitis virus G glycoprotein) pseudotyped FIV- (feline immunodeficiency virus) derived vectors carrying the mouse *Prnp* gene in which these mutations have been inserted, are able to inhibit prion replication in chronically prion-infected cells. Because lentiviral tools are able to transduce post-mitotic cells such as neurons or cells of the lymphoreticular system, this result might help the development of gene- or cell-therapy approaches to prion disease.

Key words: PrP, Prion, Dominant negative, Lentivirus, Therapy

Introduction

Transmissible spongiform encephalopathies (TSEs) are neurodegenerative disorders including Creutzfeldt-Jakob disease in humans, scrapie in sheep and goats, and bovine spongiform encephalopathy in cattle. These diseases are characterized by the accumulation of an abnormal 'scrapie' isoform of the prion protein called PrP^{Sc} in the brain (Prusiner et al., 1998). According to the prion hypothesis, the infectious isoform PrP^{Sc} can trigger the autocatalytic conversion of the normal 'cellular' prion protein PrP^{C} into PrP^{Sc} (Prusiner et al., 1990). However, certain cofactors, such as protein X or laminin-receptor precursor might also play a role in the conversion process (Kaneko et al., 1997; Leucht et al., 2003). Currently, no treatment is available to cure TSEs. A number of chemical molecules, targeting PrP^{Sc} itself or PrP^{Sc} replication, were discovered either serendipitously, such as branched polyamines (Supattapone et al., 1999), phtalocyanines and porphyrin derivatives (Caughey et al., 1998), or by using a more rational approach with the help of structure-based drug design, such as the identification of compound 60 (Perrier et al., 2000). These molecules were able to cure scrapie-infected cells of their infectivity, they were, however, poorly efficient on mice once the clinical symptoms had developed. Because PrP^{C} is a major cellular requirement for the propagation of infectivity (Bueler et al., 1993), it represents an attractive therapeutic target. Recent studies on conditional *Prnp* knockout mice, showing that the inhibition of the PrP^{C} production in neurons was able to prevent or stop the development of the disease, emphasized the importance of therapeutic strategies targeting PrP^{C} (Mallucci et al., 2003).

By taking advantage of 'prion-resistant' polymorphisms that naturally exist in sheep and humans, an alternative approach can be envisaged. Genotype analyses in sheep has shown that the polymorphism present at codon 171 of the ovine *Prnp* gene is correlated with the disease incidence and the modulation of its incubation time. Sheep carrying the polymorphisms Q/R or R/R at codon 171 of the PrP protein were resistant to different isolates of scrapie, whereas sheep harboring the Q/Q alleles developed prion disease (Belt et al., 1995; Bossers et al., 1996; Goldmann et al., 1994). Human genetic studies revealed that the gene encoding the prion protein has a large repertoire of polymorphisms and mutations. Studies among the Japanese population found that, 12% carried the polymorphism E/K at codon 219 of the PrP protein, whereas the remainder carried the E/E alleles. Because E/K-heterozygosity at codon 219 was not reported on the 85 autopsied cases of patients with sporadic CJD, this therefore suggests that the polymorphism E/K protects humans from prion diseases (Shibuya et al., 1998). Kaneko et al. introduced the Q171R sheep mutation and E219K human mutation in the mouse *Prnp* gene (MoPrPQ167R and MoPrPQ218K respectively) (Kaneko et al., 1997). These mutated PrPs could not be converted into the abnormal PrP^{Sc} isoform and, significantly, they also inhibited the formation of 3F4-tagged mouse PrP^{Sc} molecules during co-transfection experiments (Kaneko et al., 1997). The substitutions Q167R and Q218K in MoPrP were therefore described as dominant negative mutants because they block the replication of their wild-type counterparts (Kaneko et al., 1997). Recently, Perrier et al. analyzed the dominant negative inhibition effect in transgenic mice (Perrier et al., 2002).

Transgenic mice carrying PrP with Q167R or Q218K substitutions on a *Prnp*$^{-/-}$ background, were unable to replicate and propagate prion diseases. In transgenic mice, whose wild-type (wt) *Prnp* gene was re-inserted, the conversion of wt PrP^{C} into PrP^{Sc} was dramatically slowed down and correlated with an increase in the incubation time in these animals. This dominant negative effect seemed to depend on a particular ratio of the mutated PrP molecules produced by the transgene and the endogenous wt PrP molecules.

These transgenic experiments demonstrate the relevance of the dominant negative mutants but because they are not directly applicable in gene therapy, it appeared necessary to develop vectors for their in vivo delivery. The lentiviral gene transfer system is one of the most appropriate systems that could be employed. We used the vesicular stomatitis virus G glycoprotein (VSV-G) pseudotyped feline immunodeficiency virus (FIV) lentiviral vectors to evaluate a gene transfer therapeutic strategy. Here, we show that FIV vectors carrying the MoPrPQ167R or MoPrPQ218K dominant negative mutants are able to abolish endogenous wt PrP^{Sc} replication in chronically prion-infected N2a58/22L cells. In addition, injections of virion preparations into mice, using the intracerebral (ic) or intravenous (iv) routes, demonstrate that our lentiviral vectors can transduce cells from brain and spleen tissues. Therefore, these results present an advance in the development of gene or cell-therapy approaches to combat prion disease.

Materials and Methods

Nomenclature

Residue 171 in ovine PrP corresponds to codon 167 of the mouse prion protein (MoPrP). Residue 219 in human PrP (HuPrP) corresponds to codon 218 in MoPrP.

Cell lines description

The *Prnp*$^{-/-}$ cerebellar cells are derived from *Prnp* knockout mice and were kindly provided by T. Onodera (Kuwahara et al., 1999). *Prnp*$^{-/-}$ cells were cultured in Dulbecco's modified Eagle's medium (DMEM/F12) containing 10% fetal calf serum (FCS), 10 U/ml penicillin and 10 mg/ml streptomycin at 37°C in 5% CO_2.

The mouse neuroblastoma (N2a) cell line was purchased from the American Type Culture Collection (ATCC CCL 131). N2a cells were stably transfected with wild-type mouse *Prnp* cDNA. N2a58 subclone, overexpressing MoPrP proteins, was chronically infected with the mouse-adapted scrapie strain 22L as described by Nishida et al., (Nishida et al., 2000) resulting in N2a58/22L cells. Cells were cultivated in MEM supplemented with 10% FCS, 300 μg/ml geneticin, 10 U/ml penicillin and 10 mg/ml streptomycin, at 37°C in 5% CO_2.

Construction of lentiviral-derived vector carrying the PrP mutants Q167R and Q218K

Mutagenesis was performed on the open reading frame of the MoPrP using the QuikChange site directed mutagenesis kit (Stratagene) with primers containing the mutations for Q167R (5′-GTACTACAGG-CCAGTGGATCGGTACAGCAACCAGAACAACT-3′ and 5′-GTT-GTTCTGGTTGCTGTACCGATCCACTGGCCTGTAGTAC-3′), and Q218K (5′-GATGTGCGTCACCCAGTACAAAAAGGAGTCCCA-GGCCTATTA-3′ and 5′-TAATAGGCCTGGGACTCCTTTTTGTA-CTGGGTGACGCACATC-3′). The resulting cDNAs (MoPrPQ167R and MoPrPQ218K) were subcloned in the gene-transfer vector used for the virion production. This transfer vector was derived from the prototypic FIV-14-Petaluma genome (NIH AIDS Research and Reference Reagent Program, http://www.aidsreagent.org/), by replacing the U3′ region of the 5′ LTR with a cytomegalovirus (CMV) promoter supporting the expression of the vector in the cells (Lin et al., 2004). Moreover, the *gag* gene was truncated at position 1243, and a frameshift was introduced at position 926. The *pol*, *vif*, *orfA*, *env*, and *rev* genes were replaced by a cloning cassette containing the mutated PrP sequences driven by the phosphoglycerate kinase promoter. The PrP sequence was followed by an internal ribosome entry sequence (IRES) and the enhanced green fluorescent (GFP) protein marker gene (Fig. 1).

Virion production

VSV-G pseudotyped FIV lentiviral vectors were produced by transient transfections of HEK293T cells with the following three plasmids: the gene transfer vector, the packaging vector and the pMD.G vector (Lin et al., 2004). The packaging vector was also derived from the FIV-14-Petaluma genome whose 5′ LTR had been replaced by a CMV promoter and the 3′ LTR had been replaced by a poly-A signal. The third vector, pMD.G, used for pseudotyping contains the VSV-G envelope gene, which encodes the envelope G protein of the vesicular stomatitis virus (Fig. 1).

HEK293T cells, plated at a density of 3.10^6 in 100 mm plates, were transfected by using the calcium phosphate method (Sambrook et al., 1989) with 9 μg of the gene-transfer plasmid harboring the PrP mutant, 15 μg of the FIV-derived packaging plasmid which codes for virion structural protein and 6 μg of the VSV-G envelope plasmid for pseudotyping. After 18 hours, the medium was replaced with 10 ml of DMEM containing 125 mM Hepes/plate. Virions were collected at day 2 and 3. The virion suspension was cleared by filtration through a 0.45 μM filter and concentrated by two successive ultra-centrifugations at 52,112 ***g*** for 90 minutes (SW28, Beckman). Virions were resuspended in PBS. The viral titer was determined by FACS analysis of the GFP in HEK293T cells transduced with virion preparations according to the protocol described by Curran et al., (Curran et al., 2000), and by measuring the p24 levels in the virions with the PetChek Idexx kit (IDEXX Laboratories, Westbrook, Maine, USA). As an indication, 20 μl of virion suspension with a titer of 10^8-10^9 transducing unit (TU)/ml can be obtained from a 100 mm plate of HEK293T cells.

In vitro lentivirus transduction

Cells were transduced with a multiplicity of infection (MOI) of 1 and 10 (1 to 10 TUs per cell) in a medium supplemented with polybrene (8 μg/ml), using a 12-well plate containing 10^5 cells/well. The plates were then centrifuged (1200 ***g***) for 90 minutes at 30°C. At the next day, medium was replaced with fresh one.

Immunoblotting of PrP^{C} and PrP^{Sc}

Six days after transduction, *Prnp*$^{-/-}$ cells were lysed in lysis buffer (10 mM Tris HCl pH 8.0, 100 mM NaCl, 0.5% NP-40 and 0.5% sodium deoxycholate) and samples were analyzed by immunoblotting (Perrier et al., 2004). PrP^{C} was detected with SAF32 monoclonal antibody directed against the 59-89 epitope of the human PrP.

To detect PrP^{Sc} in N2a58/22L cells, cellular lysates were collected at different times after transduction and were proteinase K (PK) treated (Perrier et al., 2004). Immunoblotting was performed with SAF mix as described elsewhere (Perrier et al., 2004).

In situ detection of GFP, PrP^{C} and PrP^{Sc}

Fluorescence of the GFP protein was observed by indirect microscopy (Leica) after fixation with formaline (3.7%). Immunocytofluorescence

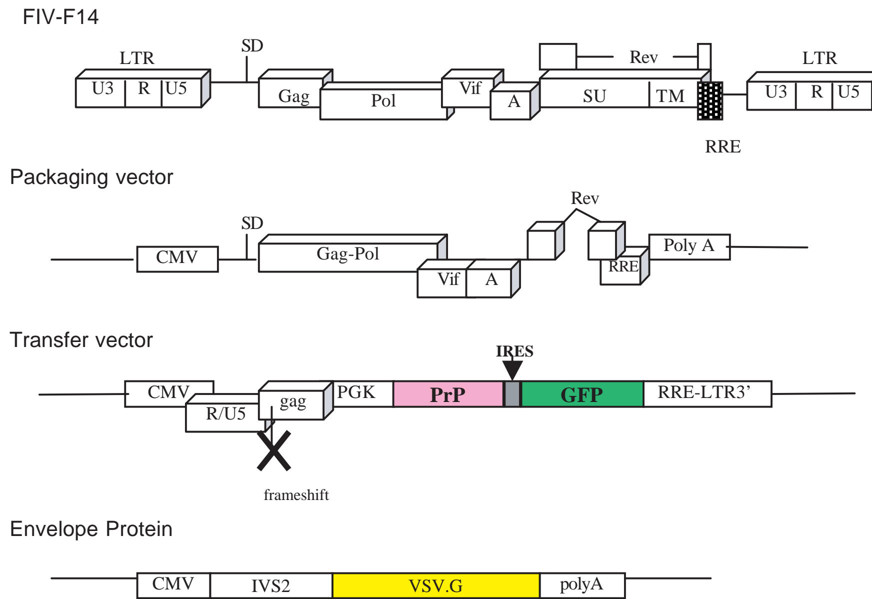

Fig. 1. Characteristics of the lentiviral vectors. The packaging vector, was derived from the prototypic FIV-14-Petaluma genome (AIDS Research and Reference Reagent Program), in which the 5′ LTR is replaced by a CMV promoter and the 3′ LTR by a poly-A signal. The transfer vector was also obtained from FIV-14-Petaluma by replacing the U3 region of the 5′ LTR with a CMV promoter in order to enable the expression of the vector in cells. Moreover, the *gag* gene was truncated at position 1243 and a frameshift introduced at position 926. The *pol*, *vif*, *orfA*, *env* and *rev* genes were replaced by a cassette containing the mutated PrP sequences driven by the phosphoglycerate kinase promoter. The PrP sequence is followed by an IRES sequence and the enhanced GFP marker gene. The third vector pMD.G, used for pseudotyping, contains the VSV-G envelope gene, which encodes the envelope G protein of the vesicular stomatitis virus. PGK, phosphoglycerate kinase promoter; CTE, constitutive transport element; cPPT CTS, central DNA flap sequence; SD, splice donor site.

of PrP^C and PrP^{Sc} was performed as previously described (Mange et al., 2004). The SAF61 antibody was directed against the144-156 epitope of the HuPrP.

Animal models

C57Bl/6 mice used for the experiments were from Charles Rivers/IFFA CREDO L'Arbresle, France. They were housed in an independent, filtered and pressurised enclosure in an A3 facility according to French Ethical Committee guidelines (Decree 87-848) and the European Community Directive 86/609/EEC.

Injection of the lentivirus in mice

Mice were deeply anaesthetized with a mixture of 100 μg/g of ketamine and 15 μg/g of xylazine. For the ic injection of the lentivirus, we used a 10 μl Hamilton syringe with a 26 gauge (G) needle fixed on a stereotaxic frame and connected to a microinjection pump (David Kopf Instruments, Tujunga, CA). Five microliters of the lentiviral preparation expressing the β-galactosidase gene (10^8 TU/ml) were injected at a rate of 0.5 μl/minute, either into the ventricle (anteroposterior 0, lateral –1, dorsoventral –2,5; see Fig. 4C,D) or into the thalamus (–1.58, 0.2, –3.2; see Fig. 4A,B) and (0.34, 0.5, –2.2; see Fig. 4E). One to three weeks after the injections, the brains were removed and fixed in 2% paraformaldehyde (PFA). For iv injections, 100 μl of the lentiviral preparation expressing the β-galactosidase gene (2×10^8 TU/ml) were injected with a 26G needle into each mouse. Five weeks after the injections, the spleens were removed and fixed in 2% PFA.

Detection of β-galactosidase expression in C57Bl/6 mice

After a short (30 minutes) fixation period in 2% PFA, β-galactosidase staining was carried out by incubating the entire brain overnight in a X-Gal-solution at 37°C.

For immunohistochemical analysis, fixation of spleen tissue or brains was performed overnight in 0.1 M PBS (pH 7.4) with 2% PFA at 4°C. The brains were subsequently dehydrated with graded concentrations of alcohol and embedded in paraffin. Sections of 5 μm were collected onto pre-treated glass slides (Polylysin or StarFrost, Fisher Scientific). Slides were then dewaxed and treated with proteinase K (20 μg/ml) (Roche-Boehringer) for 10 minutes at 37°C. All these steps were carried out at room temperature (22°C-24°C). Endogenous peroxidase activity was inhibited with 2% H_2O_2 (Merck) in 0.1 M PBS for 5 minutes. Non-specific antigenic sites were blocked by a 30-minute incubation in 4% normal goat serum. An anti-β-galactosidase monoclonal antibody (Chemicon) was then applied overnight. Brain sections were rinsed before detection of the primary antibody using the ABC system (Vector). These steps were followed by rinsing the sections in 0.1 M PBS, peroxidase was finally revealed by incubating the sections in 0.1 M PBS containing aminoethylcarbazole (AEC) (Dako) to give red deposits. The slides were weakly counterstained with aqueous hematoxylin before mounting (GelMount). Images were collected under a Zeiss Axiophot microscope, using a Nikon DXM1200 camera.

Results and Discussion

Lentivirus-mediated gene transfer provides a potentially useful tool for therapeutic strategies because it can transduce both dividing and non-dividing cells, such as neurons, in a stable and long term fashion (Blomer et al., 1997; Blomer et al., 1996; Marr et al., 2003; Naldini et al., 1996). This system also allows the transfer of genes without any selection process, preventing an eventual undesirable clonal effect. We therefore developed FIV-derived vectors to evaluate a gene transfer therapeutic strategy by using the inhibitory properties of PrP dominant negative mutants against prion replication. We prepared VSV-G and pseudotyped FIV vectors containing the mouse *Prnp* gene in which the Q167R or Q218K mutations were generated by PCR mutagenesis. To identify the transduced cells and measure the efficiency of transduction, the gene of interest in these vectors is followed by an IRES sequence and a reporter gene coding for GFP. Virion particles were produced by transfecting HEK293T cells with a three-plasmid expression-system (Fig. 1): the gene transfer vector carrying the mutated *Prnp* genes, the FIV-derived packaging plasmid coding for structural proteins of virions and the VSV-G envelope plasmid that determines the cellular pseudotyping. Virions produced in the culture medium were filtrated and concentrated by successive ultra-centrifugations (Curran et al., 2000). The viral

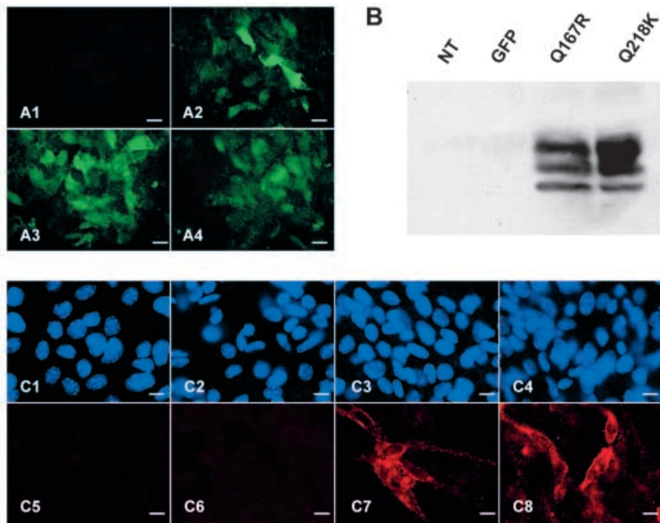

Fig. 2. Transduction of *Prnp*$^{-/-}$ cells with lentivirus carrying the PrP dominant negative mutants MoPrPQ167R and MoPrPQ218K. (A) Transduction efficacy of *Prnp*$^{-/-}$ cells with lentiviral vectors assessed by GFP fluorescence. Images A1, A2, A3 and A4 correspond to non-transduced, GFP, MoPrPQ167R and MoPrPQ218K transduced cells, respectively. (B) Detection of PrP^C by immunoblotting using SAF32 anti-PrP antibody (C) SAF32 immunocytofluorescence of the PrP^C in *Prnp*$^{-/-}$ cells: no staining is visible in (C5) non-transduced and (C6) GFP lentiviral transduced cells, whereas cells transduced with the (C7) MoPrPQ167R, or (C8) MoPrPQ218K lentivirus exhibit a strong rhodamine fluorescence. C1, C2, C3, C4 correspond to the Hoechst nuclear staining of the cells represented in figure C5, C6, C7, C8 respectively. Scale bars, 8 μm.

titers were determined by counting the number of GFP positive fluorescent cells using FACS analysis after transduction of HEK293T cells with different dilutions of the virions. These titers were about 3.5×10^8 to 8.8×10^8 TU/ml.

To evaluate whether viral particles were able to transduce our cellular models and efficiently express the mutated genes, *Prnp*$^{-/-}$ cerebellar cells derived from *Prnp* knockout mice (Kuwahara et al., 1999) were transduced with virion preparations with a MOI of 10 (10 TU/cell). The success of the gene transfer was assessed by the fluorescence properties of the GFP (Fig. 2A). The transduced *Prnp*$^{-/-}$ cells expressed the MoPrPQ167R and MoPrPQ218K proteins as demonstrated by immunoblotting analysis using the SAF32 antibody (Fig. 2B). The similar levels of PrP^C detected agree with the identical viral titers used for the transduction. Furthermore, immunofluorescence of these cells also showed that the mutated proteins were correctly exported at the cell surface, which suggests that dominant negative mutations do not affect the cellular trafficking of the PrP protein (Fig. 2C).

To investigate the inhibitory potential of lentivirus vectors carrying the dominant negative PrP mutants, the prion infected neuroblastoma cells (N2a58/22L) were transduced. This N2a58/22L cell line provides a model for prion replication because it constitutively produces high levels of PrP^{Sc} proteins (Nishida et al., 2000). The cells were transduced with the different preparations of virions using a MOI of 1. By counting the number of GFP-positive cells we were then able to determine an efficacy of transduction of 80% (Fig. 3A), without any toxicity to the cells.

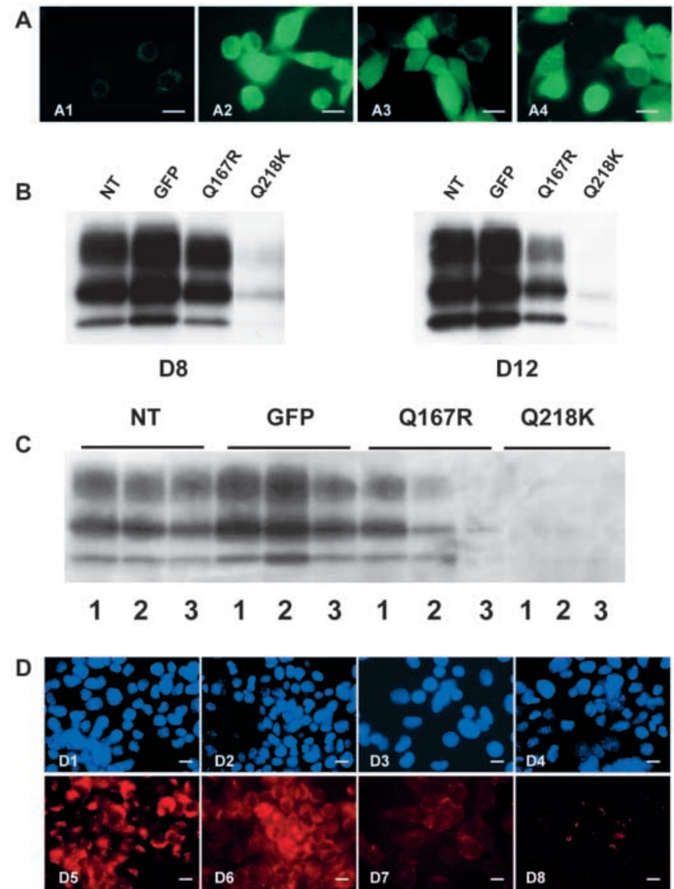

Fig. 3. Transduction of N2a58/22L cells with the MoPrPQ167R and MoPrPQ218K virions. (A) GFP analysis after transduction of N2a58/22L cells. (A1) non-transduced cells, (A2) cells transduced with GFP vector, (A3) MoPrPQ167R or (A4) MoPrPQ218K PrP mutants. (B) Inhibition of endogenous $MoPrP^{Sc}$ levels by dominant negative mutants in N2a58/22L cells. Proteinase K digested cell lysates were analyzed by immunoblotting and the wild-type $MoPrP^{Sc}$ levels revealed with SAF mix. N2a58/22L cells expressing the MoPrPQ218K mutant showed a strong inhibition of the PrP^{Sc} level at day 8 (D8) and day 12 (D12) after transduction, whereas cells expressing MoPrPQ167R mutants showed a slight diminution. (C) Kinetic analysis of PrP^{Sc} inhibition in N2a58/22L cells by western blot analysis, at 8, 12 and 15 days (lanes 1, 2 and 3, respectively) after transduction. (NT) non-transduced cells, (GFP) cells transduced with virions carrying GFP only, or virions carrying (Q167R) MoPrPQ167R or (Q218K) MoPrPQ218K. These results are representative for several independent experiments. (D) Immunofluorescence of the PrP^{Sc} accumulation using SAF61 anti-PrP antibody 20 days after transduction. D5 non-transduced, D6: GFP, D7: MoPrPQ167R, D8: MoPrPQ218K. D1, D2, D3, D4 correspond to the Hoechst nuclear staining of the cells presented in figure D5, D6, D7, D8 respectively. Scale bars, 8 μm.

Transduced N2a58/22L cells were serially split in duplicates and cultivated for several weeks. Every four days, the cellular lysates were collected and proteinase-K-treated to analyse the inhibition-kinetic of the endogenous $MoPrP^{Sc}$ levels by immunoblotting. After 8 and 12 days, the N2a58/22L cells expressing the dominant negative MoPrPQ167R and MoPrPQ218K mutants showed a strong reduction in endogenous wt $MoPrP^{Sc}$ levels (Fig. 3B). After 15 days of

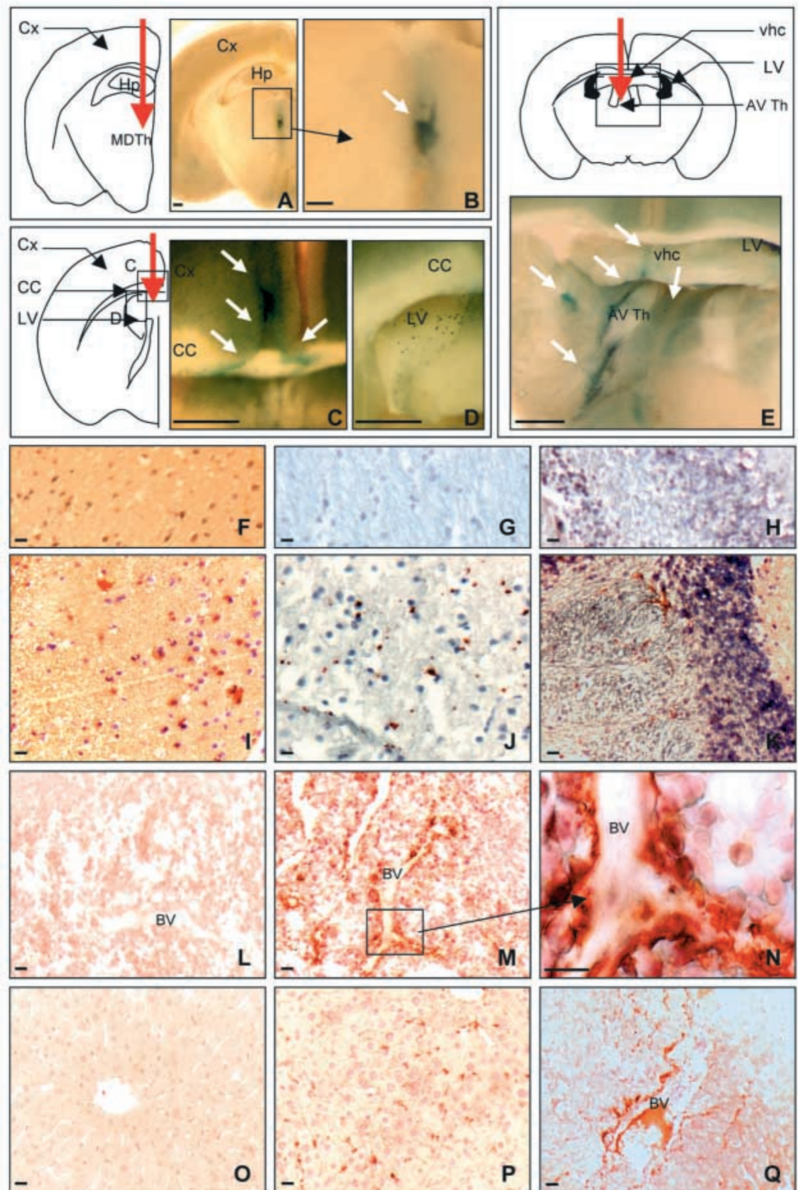

Fig. 4. In vivo delivery of FIV lentiviral vectors. (A-E) Brain slices of mice intracerebrally injected with FIV lentiviral vectors carrying the β-galactosidase gene after X-Gal staining (blue coloration) 1 week after injection. (A, B) According to the site of injection (red arrow), the blue coloration was observed in the mediodorsal thalamic nucleus. (C-E) X-Gal staining was also observed along the needle passage: (C) the cortex as well as in the fibers of the corpus callosum, (D) the wall of the ventricules and (E) the anteroventral thalamic nucleus. (F-K) After intraventricular injection, close immunohistochemical examination using anti-β-galactosidase antibody, allowed the detection of the β-galactosidase expression (red staining) 3 weeks after injection in (I) the cortex, (J) the striatum, and (K) the cerebellum. No staining was observed in non-injected mice used as control. F, cortex, G, striatum, H, cerebellum). (L-Q) Sections of spleen and liver from intravenously injected mice were analysed by immunocytochemistry. Positive cells were detected in the vicinity of the blood vessels, in (M-N) the spleen, (Q) the liver and (P) some isolated cells in the liver. No staining was observed in non-injected mice (L, spleen; O, Liver). (Cx, cortex; Hp, hippocampus; MDTh, mediodorsal thalamic nucleus; CC, corpus callosum; LV, lateral ventricule; vhc, ventral hippocampal commissure; AV Th, anteroventral thalamic nucleus; BV, blood vessel. Scale bars in A-E, 1 mm; in F-N, 10 μm.

culture, the majority of PrP^{Sc} was cleared (Fig. 3C). To confirm the elimination of PrP^{Sc}, we performed a SAF61 immunocytofluorescence of the PrP^{Sc} after 20 days. We used a standard guanidinium thiocyanate protocol that allows PrP^{Sc} staining by promoting antigen retrieval of the PrP^{Sc} and elimination of PrP^{C} staining (Mange et al., 2004). This method also showed a large decrease of PrP^{Sc} in cells containing the dominant negative construct (Fig. 3D); the inhibitory effect was reproduced in three independent experiments (data not shown). Generally, these results show that the virions harboring the PrP dominant negative mutants are functional and that they are efficient to inhibit the endogenous wt PrP^{Sc}-conversion. These results are in accordance with those obtained in previous studies (Kaneko et al., 1997; Perrier et al., 2002) who showed that inhibition of wt $MoPrP^{Sc}$-formation can occur with MoPrPQ167R and MoPrPQ218K mutants. In addition, the lentiviral gene-transfer method allowed us to amplify the inhibitory effect of these dominant negative mutants in scrapie-infected cells. The transient transfections of prion-infected cells performed by Kaneko et al. (Kaneko et al., 1997), resulted in only a minority of cells expressing the PrP mutants, which had an inhibitory effect only on the conversion of 3F4-tagged $MoPrP^{C}$ but not on the conversion of endogenous wild-type PrP^{C} into PrP^{Sc}. In our case, and because the majority of the cells expressed the PrP mutants, we were able to obtain a strong inhibition of the conversion of the endogenous wt PrP^{C} into PrP^{Sc}. Our results show that the delivery of dominant negative PrP mutants through lentiviral vectors, can not only provide a strong expression of the transgene, but also transdominantly inhibit the accumulation of the endogenous wt $MoPrP^{Sc}$ in chronically scrapie-infected N2a58/22L cells.

Lentiviral vectors possess the ability to transduce post-mitotic cells such as differentiated neurons as well as the cells of the lymphoreticular system in a prolonged and stable manner (Aguzzi et al., 2003; Blomer et al., 1997; Blomer et al., 1996; Glatzel et al., 2004; Mabbott and Bruce, 2003; Mangeot et al., 2002; Naldini et al., 1996). To analyse whether our lentiviral vectors had the potential to deliver the gene to the brain or the peripheric organs, we performed ic and iv injections of lentivirus expressing the β-galactosidase gene in mice. For ic delivery, we used a stereotaxic frame to inject the lentiviral preparation in the right lateral ventricle or in the thalamus of adult C57BL/6 mice. β-galactosidase expression was assessed by direct X-Gal staining of the entire brain. Following the intrathalamic injection, we observed a β-galactosidase activity mainly in the injection site (Fig. 4A,B,E), which demonstrated the capacity of our lentivirus to transduce neural cells. Following intraventricular injection, X-Gal-blue coloration was limited all along the syringe trajectory and around the lateral ventricle (Fig. 4C,D). This

was probably owing to the diffusion of the lentiviral particle in the whole ventricle. However, close examination of the brain by immunohistochemical analysis allowed us to detect β-galactosidase staining in the area of striatum, the cortex and in some more distant sites as well as the area of the cerebellum (Fig. 4I-K). After systemic lentiviral injections, β-galactosidase was observed around the blood vessels in the spleen of the mice and in some dispersed liver cells, probably due to the iv injection (Fig. 4L-Q). The spleen localisation in the vicinity of the vessels is somehow very pertinent because these structures are innervated by the noradrenergic peripheric autonomous nervous system implicated in the neuroinvasion process (Aguzzi et al., 2003; Bencsik et al., 2001; Haik et al., 2004; Prinz et al., 2003). These data showed that our lentiviral vectors can be used to transduce cells in the brain and in the spleen. Because neurons are the main cells targeted in prion disease, the use of lentiviral vectors carrying dominant negative PrP mutants could slow or even prevent prion replication, at least in the brain area transduced with the virion particles. In addition, the first stages of prion diseases are characterized by an increase of the PrP^{Sc} levels in the lymphoreticular system and in the spleen (Lasmezas et al., 1996; Maignien et al., 1999). Thus, lentiviral vectors could be useful effectors for the development of a gene therapy through repeated peripheral injections, with the aim of slowing down or preventing prion replication during the very early stages of disease, at least before neuroinvasion. Moreover, a gene therapy strategy was recently evaluated by Marr et al., as an alternative therapeutic approach for the treatment of Alzheimer's disease (Marr et al., 2003). Lentiviral vectors expressing neprilysin, a major Aβ-degrading enzyme identified in the human brain and implicated in the clearance of Aβ amyloids, was tested in transgenic mouse models of amyloidosis. A single lentiviral injection decreased the number and size of amyloid plaques by about 45%. Altogether, these data have encouraged us to further evaluate therapeutic strategies based on injections of the lentivirus carrying dominant negative PrP mutants, by both ic and peripheral routes. Moreover, the recent and promising results of the neuroprotective cell-therapy treatment have prompted us to consider an approach combining both cell therapy and gene therapy using these dominant negative PrP mutants (Bachoud-Levi et al., 2000; Brown et al., 2001; Brustle and McKay, 1996; Lindvall, 2003; Lindvall and McKay, 2003). Indeed, neural precursors obtained after in vitro differentiation from embryonic stem cells could thus be transduced with the dominant negative PrP virion preparations before injection in the prion-infected brain. The recent description of soluble dimeric PrP (Meier et al., 2003) or RNAi interferences (Daude et al., 2003) that, antagonize prion replication may also be engineered in lentiviral vectors to extend the inhibitory effect.

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