ORIGINAL PAPER

Viral infection resistance conferred on mice by siRNA transgenesis

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Abstract RNA interference is an attractive strategy to fight against viral diseases by targeting the mRNA of viral genes. Most studies have reported the transient delivery of small interfering RNA or small hairpin (shRNA) expression constructs. Here, we present the production of transgenic mice stably expressing shRNA or miRNA targeting the IE180 mRNA (immediate early gene) of the pseudorabies virus (PRV) which infects mice and farm animals. We firstly designed non-retroviral shRNA or miRNA expression vectors. Secondly, we selected the most efficient shRNA construct that targeted either the 5′part or 3′UTR of the IE mRNA and was able to knockdown the target gene expression in cultured cells, by measuring systematically the shRNA content and comparing this with the interfering effects. We then produced four lines of transgenic mice expressing different amounts of shRNA or miRNA in the brain but without signs of stimulation of innate immunity. Lastly, we tested their resistance to PRV infection. In all transgenic lines, we observed a significant resistance to viral challenge, the best being achieved with the shRNA construct targeting the 3′UTR of the IE gene. Viral DNA levels in the brains of infected mice were always lower in transgenic mice, even in animals that did not survive. Finally, this work reports an effective strategy to generate transgenic animals producing shRNA from non-retroviral expression vectors. Moreover, these mice are the first transgenic animal models producing shRNA with a significant antiviral effect but without any apparent shRNA toxicity.

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Introduction

RNA interference (RNAi) is a natural cellular process mediated by small dsRNA that induces knockdown of gene expression through mRNA targeting. Since its discovery, numerous studies have implemented short hairpin RNA technology in order to achieve targeted gene silencing for therapeutic purposes [see review in (Davidson and McCray 2011)]. In terms of the fight against viral diseases, virus gene silencing using short interfering RNAs (siRNAs) or short hairpin RNAs (shRNAs) has become a valuable tool. Recently, a short-hairpin RNA was designed to function as a decoy thus interfering with the propagation of the avian influenza virus in ckicken farming (Lyall et al. 2011). Besides, strategies based upon the delivery of RNAi targeting viral RNA have been improved. Several types of human viruses have been targeted, such as hepatitis B or C viruses (Dimitrova et al. 2008; Chen et al. 2009; Keck et al. 2009; Pan et al. 2009), HIV-1 (Liu et al. 2007; Kumar et al. 2008), yellow fever virus (Pacca et al. 2009) or influenza A virus (Ge et al. 2004; Tompkins et al. 2004), and viruses affecting farm animals, such as porcine reproductive and respiratory syndrome virus (Li et al. 2009), porcine endogenous retrovirus (Jagdeece et al. 2009), porcine circovirus type 2 (Feng et al. 2008), Marek's disease virus in the chicken (Chen et al. 2009), or foot-and-mouth disease virus (Chen et al. 2006; Pengyan et al. 2008; Pengyan et al. 2010). Notably, in vivo experiments have demonstrated the efficacy of an RNAi strategy in diminishing the incidence of disease (Chen et al. 2009), reducing viral replication (Ge et al. 2004; Li et al. 2009; Pacca et al. 2009) and enabling viral clearance in the event of chronic infection (Uprichard et al. 2005).

During most in vivo studies, the siRNA was administered by injection in delivery vehicles. However, it is now widely acknowledged that systemic siRNA administration can induce high levels of inflammatory cytokines and Type I interferons in mammals (see (Robbins et al. 2009) for review). Activation of the host innate immune response may probably contribute to the efficacy of the siRNA, but could also interfere with it. Moreover, it has previously been shown that some siRNAs, which are potent inducers of interferon responses when transfected in a cell line and expressed transitorily, have not such effect when stably expressed by the same cell line (Robbins et al. 2006). This raises the possibility that in transgenic animals that permanently produce the siRNA, there is no stimulation of innate immunity by the siRNA. We thus decided to produce transgenic animals that stably express a shRNA-transgene to consistently deliver a siRNA targeting a viral mRNA. The present paper describes the production of transgenic strains and their ability to withstand a viral infection.

Pseudorabies virus (PRV), also known as Aujeszky's disease virus or suid herpesvirus 1, is an Alphaherpesvirus that infects many mammalian species including farm animals and particularly swine, which is its natural host [see review in (Pomeranz et al. 2005)]. Although vaccination has reduced its importance in many countries, it remains a serious health and economic problem in some regions.

The aim of the present work was to produce transgenic animals expressing permanently a siRNA targeting a viral gene and then to challenge these animals with the virus. The mouse was used as a model, since this species is highly sensitive to PRV. The immediate early gene (IE gene) was targeted because it is the first key gene expressed by the virus at the start of the infectious viral cycle. Indeed, the protein encoded by this gene is mandatory for the replication of the virus. Targeting this gene was therefore predicted to be sufficiently efficient to counteract the viral infection.

To attain this goal, we first performed an in vitro study to select efficient target sequences that would enable the inhibition of IE gene expression. We then generated transgenic mice expressing shRNAs or miRNAs and tested their resistance to PRV infection. Finally, we were able to report that transgenic mice permanently expressing efficient siRNA displayed greater resistance to a virus infection than wild type mice.

Materials and methods

Plasmid construction

The shRNA-producing genes were constructed from synthetic DNA fragments (Eurofins, Ebersberg, Germany) cloned downstream of the mouse U6 gene promoter (generous gift of Dr Shi, Harvard Medical School, Boston, MA, USA). The U6-shRNA genes were then inserted into the pM10 vector at the enzymatic BssHII restriction site. The mi-5′ gene

was constructed from a synthetic DNA fragment (Eurofins, Ebersberg, Germany) cloned into the BssHII site of the pM10 vector.

The IE-5′ region was a BamHI-SmaI fragment of the IE-cDNA encompassing 217 nucleotides upstream of the AUG site and 167 downstream. The IE3′-UTR region obtained by PCR from the viral genome (5′-P-GGACTCTGACTCTGACTCT-3′OH as forward primer and 5′-P-CTCTTTCTCTCTTTC-3′OH as reverse primer) encompassed 943 nucleotides downstream of the STOP codon. These fragments were cloned, respectively upstream and downstream of the luciferase gene to generate the plm25-IE5-Luc-IE3 plasmid (Fig. 1).

Generation of transgenic mice

The inserts to be used for microinjections were released from plasmids by NotI digestion, separated on 1 % agarose gel in 1× TBE, purified using the Qiaquick gel extraction kit (Qiagen, Courtaboeuf, France) and then EluTipD (Schleicher & Schuell, Mantes la Ville, France). The resulting DNA preparations were microinjected into FVB/NRj mouse embryo pronuclei at a concentration of 2 ng/µl. The transgenic mice were identified using PCR performed on tail tip DNA extracts. Three sets of primers were designed to cover the integrity of the integrated construct: the 5′HS4 region (5′HS4F 5′-TCAAATCA TGAAGGCTGGAA-3′, 5′HS4R 5′-GAGTTGGATG AGAGATAAT-3′), the E2 region (SUR123S 5′-GG CCACAATTCGCCGGCG-3′, SUR123AS 5′-GCGG CAGAACGCGACTCA-3′), and the hGH transcription terminator (hGH5′ 5′-AAGTTCGACACAAACT CACA-3′, hGH3′ 5′-AGCAATTTGGGAGGCCAAG G-3′). The copy number of the transgene was determined by qPCR using 5′-GCCCCTCTGCTAAC CATGTTC-3′ and 5′-TTGCCAAAATGATGAGAC AGCAC-3′ as specific primers normalized by the level of the endogenous mouse β2-microglobulin gene in
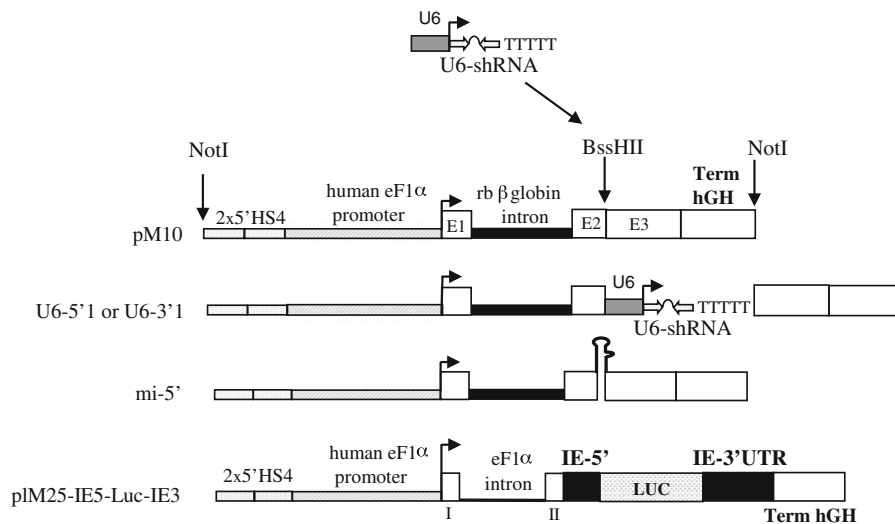

Fig. 1 Structure of siRNA-producing constructs. The pM10 construct encompassed a tandem of the chicken β-globin gene fragment 5′HS4 as insulator, the promoter of the human eF-1α gene (−1,430, +2), the rabbit (rb) β-globin second intron and several enhancers (E1, E2 and E3). The U6-shRNA gene encompassed the mouse U6 promoter (grey box), two complementary copies of the targeted sequence separated by a loop structure, and a 5-T sequence as transcription termination signal. It was introduced at one of the BsaBI, BssHII or AatII sites on the pM10 construct to generate the U6-5′1 or U6-3′1 constructs targeting the 5′ part or 3′UTR of the IE mRNA, respectively. The mi-5′ construct was a miRNA expressing gene. The endogenous sequence of the human miR30a gene was replaced by the IE targeted sequence. This gene was inserted in the BssHII site of the pM10 vector. The plm25-IE5-Luc-IE3 target construct encompassed the luciferase gene linked to IE-5′ and IE-3′ mRNA sequences. The IE-5′ mRNA region (black box, left) surrounding the initiation of IE gene translation (a 384 bp long fragment) was inserted downstream of the human eF-1α promoter and the first intron [−1,430 to +984, respectively to the tsp, encompassing the first exon (I) and the beginning of the second exon (II)]. The IE 3′-UTR region (black box, right) encompassing a 943 bp long fragment (+23 to +966, respectively to the STOP codon) was linked downstream of the luciferase gene

each DNA extract using the following sets of primers: (5′-GTGACGACCTCCGGATCTGA-3′ as forward primer and 5′-GCCGAGTAGCAGCCACTGAA-3′ as reverse primer). All sets of primers were selected to insure an even efficiency in amplifying their respective target sequences. The number of transgene copies was thus given by the formula $2^{(Ctb2mglo-Cttransgene)}$, where Ct represents the cycle threshold number for β2-microglobulin and the transgene, respectively.

Measurement of IE mRNA knockdown in CHO transfected cells

In order to select shRNAs with highly specific activity, an in vitro system was developed using the transfection of shRNA or miRNA producing genes and their respective target genes cloned in the plm25-IE5-Luc-IE3 plasmid. The plm25-IE5-Luc-IE3 construct (Fig. 1) encompassed a transcription unit composed of IE gene fragments corresponding to the 5′ part (IE5′) and the 3′-UTR (IE-3′UTR) of the IE viral gene, linked, respectively upstream and downstream to the luciferase coding sequence. Degradation of the IE region in IE5-Luc-IE3 mRNA by siRNAs was expected to prevent translation of the luciferase cistron. Thus, a quantification of siRNA-induced knockdown could be achieved by measuring luciferase activity in the plm25-IE5-Luc-IE3 transfected cells. The reliability of this method was previously established (Hung et al. 2006), showing that it is possible to fuse short target sequences (such as the IE5′ or IE3′ sequences) in the UTR of a reporter gene in order to establish a quantitative reporter-based siRNA validation system.

The transfections were carried out in CHO.K1 cells (ATCC number CCL-61) using ExGen500 (Euromedex, Souffelweyersheim, France), according to the manufacturer's protocol. Plasmid pCH110 was cotransfected as a source for β-galactosidase in all experiments to monitor transfection efficiency. Luciferase activity was assayed and normalized to β-galactosidase activity.

Evaluation of the siRNA concentration

The concentration of siRNA produced by the shRNA or miRNA constructs in transfected cells and transgenic mouse tissues was estimated by RT-qPCR (Shi and Chiang 2005). Briefly, 5 µg of total RNA,

prepared as previously described (Chomczynski and Sacchi 1987), were polyadenylated according to Ambion's protocol (PolyA Polymerase, Ambion, Applied Biosystems, France). The polyadenylated RNAs were reverse transcribed with a polyT adapter (High Capacity cDNA Archive kit, Applied Biosystems). The poly-T adapter was made up of a polyT (12 T residues) sequence and a universal oligonucleotide (5′-GCGAGCACAGAATTAATACGACTCACTAT AGGTTTTTTTTTTTT(AGC)(AGCT)-3′). Quantification was achieved by SYBR Green quantitative PCR (Applied Biosystems) using a set of primers composed of the universal primer corresponding to the 5′ end of the polyT-adapter (5′-GCGAGCACAGAAT-TAATACGACTCACTATA-3′) and a primer specific to the siRNA sequence, resulting in the amplification of a 65–70 bp long fragment. The sequences of the specific primers were as follows: 5′-TGAAGTCAA-GAGATCGTCGGCC-3′ to assay the siRNA targeting the 5′ part of the IE mRNA; 5′-TTTTCTCACCCGA TGGGAG-3′ to assay the siRNA targeting the 3′UTR of the IE mRNA.

The concentration of siRNA in transfected CHO cells was normalized to the concentration of β-galactosidase mRNA in order to take account of transfection efficiency. The expression of β-galactosidase was measured by SYBR green qPCR (forward primer: 5′-CGCGAATTGAATTATGGCCC-3′; reverse primer: 5′-AGATGGCGATGGCTGGTTTC-3′). The normalized concentration was given by the formula $2^{(Ct\beta-Gal-CtsiRNA)}$ where Ct represents the cycle threshold number for siRNA and β-galactosidase PCR.

The concentration of siRNA in tissue samples was estimated after normalization by the concentration of Let7c miRNA in each sample. It was thus given by the formula $2^{(CtLet7c-CtsiRNA)}$.

Quantification of the innate immune response

The expression of IFN-inducible IFIT1 mRNA (Geiss et al. 2001) was measured by SYBR green qPCR (forward primer: 5′-GAACAGCTACCACCTTTAC AGCAA-3′; reverse primer: 5′- AGCTTCCATGTGA AGTGACATCTC-3′) using the RT reaction obtained for siRNA measurements. The concentration of IFIT1 mRNA was normalized to that of GAPDH as given by the formula $2^{(CtLet7c-CtsiRNA)}$ (forward GAPDH primer: 5′-TCCTGCACCACCAACTGCTT-3′; reverse GAPDH primer: 5′-GTGGCAGTGATGGCATGGAC-3′).

Viral challenge of mice

Viral challenges were performed using the Bartha strain of PRV. A viral stock was prepared by growing the virus on the PK15 cell line as previously described (Flori et al. 2008), and was then stored in aliquots at −80 °C. The viral stock titre was determined by plaque assay on PK15 cells (Riteau et al. 2006). Viral inoculates were prepared just before use by performing appropriate dilutions of the viral stock in complete Eagle's minimum essential medium (EMEM, Lonza, Verviers, Belgium) with no serum or antibiotics. Anesthetized mice maintained in a disease-secure isolated facility were inoculated by the instillation of 30 µl of inoculate via the nostrils. The viral challenge was performed by inoculating groups (15–26 animals) of 10-week-old FVB/NRj mice with 2,500 plaque forming units per animal, a viral dose giving rise to 20–25 % survival in wild-type animals. Mice were provided with food and water ad libitum, to insure day-by-day food accessibility even in infected and weak animals. Mice were weighed and monitored daily for signs of illness, with special care to fur appearance and behaviour, especially apathy or loss of appetite. Within each group, animals were sacrificed when signs of illness became evident. Around 10 animals were killed 8 days after infection (except for line SH30), and their brains were removed and frozen immediately at −80 °C. Finally, all surviving animals were sacrificed at the end of the experiment 23 days after infection. Brains were then collected.

Quantification of viral DNA in the brain

The brains were collected rapidly after death. The right and left hemispheres were frozen separately at −80 °C until further use. The amount of viral DNA was measured by SYBR Green quantitative PCR (Applied Biosystems) in the DNA extract from one hemisphere. Specific sets of primers targeting the IE gene were used (5′-GCCTCGCTCAGGCAGAAAG-3′ as forward primer and 5′-CAGGAGCTGGCTGAAGTTGC3′ as reverse primer). Values were normalized to the level of the endogenous mouse β2-microglobulin gene in each DNA extract. The relative amount of viral DNA was thus given by the formula $2^{(Ctb2mglo-CtIE)}$, where Ct represents the cycle threshold number for β2-microglobulin and IE, respectively.

Statistical analysis

Concentrations of siRNA and percentages of initial body weights were compared using Student's T test. Comparisons of survival were performed using Fisher's exact test.

Results

In vitro selection of efficient siRNA constructs to induce significant expression knockdown of the targeted construct

The aim was to select in vitro efficient target sequences on IE mRNA to construct shRNA expressing genes and generate siRNA expressing transgenic mice. We designed a set of 19–22 nucleotide-long siRNAs by complying with empirical rules (Li et al. 2007) and also all possible secondary structures that could influence target site accessibility (Lu and Mathews 2008). The targeted sequences and their complementary sequences separated by a loop were introduced downstream of the mouse U6 gene promoter to generate U6-shRNA genes. To prevent transgene silencing, these U6-shRNA genes were inserted into the pM10 vector (Fig. 1) designed in our laboratory; this contains regulatory elements to ensure reliable transgene expression (Attal et al. 2000; Rival-Gervier et al. 2003). The pM10 vector thus acted as a carrier for each U6-shRNA gene.

To analyse the efficiency of the shRNA expressing constructs, co-transfections of CHO cells were carried out with a target construct (plm25-IE5-Luc-IE3) and the different shRNA genes. Among the ten shRNA expressing constructs studied, three induced more than 70 % inhibition of luciferase gene expression (Table 1; Fig. 2a). The inhibition was obtained with the U6-5′1 and U6-3′1 constructs targeting the 5′ part and 3′UTR of the IE mRNA, respectively. Interestingly, an additive inhibitory effect was observed after the simultaneous transfection of U6-3′1 and U6-5′1 constructs (Fig. 2b), raising the possibility to rise the level of inhibition of IE expression by simultaneously targeting the 5′- and the 3′-UTR of the IE gene.

The U6-5′1 sequence was also introduced into a miRNA construct (named mi-5′). This mi-5′ gene construct was a modified miR30a gene where the endogenous specific sequence of the human miR30a

Table 1 Comparison of in vitro knockdown (KD) activities and the siRNA production of a set of U6-shRNA constructs

	Luciferase activity (% of control cells)	siRNA level (arbitrary units)	Targeted sequence
U6-5′1[*]	10	150	GCCGACGATCTCTTTGACTTCA
U6-5′2	30	100	GGCGCGGACTCTGAAGA
U6-5′3	100	ND	GGCTCTCCGGCGGCTATCA
U6-5′4	86	ND	GGCCGCCTTCCTCCTTCTTCT
U6-5′5	72	ND	GCCTTCCTCCTTCTTCT
U6-5′6	74	ND	GGCCTCGCTCAGGCAGAAA
U6-3′1[*]	15	15	CTCCCATCGGGTGAGAAAA
U6-3′2	94	ND	CCGGTCCCCTTCTCCTCCTT
U6-3′3	94	ND	GATCGTCCCGGTCCCCTTCT
U6-3′4	78	ND	GCCCTCCTCCTCTCCTTCT

Six IE-5′ and 4 IE-3′UTR targeting constructs were assayed by transfecting CHO cells (0.75 µg/P35 dish) with the pLM25-IE5-Luc-IE3 target construct (0.75 µg/P35 dish) as described in "Materials and Methods". The U6-5′1 targeted sequence was located just after the AUG initiation codon, and the U6-3′1 sequence in the 3′UTR region of the IE gene. The residual luciferase activity is given as the percentage of luciferase activity in cells transfected by plm25-IE5-Luc-IE3 and the empty pM10 vector. siRNA levels were assayed by RT-qPCR as described in "Materials and Methods" section when luciferase activity was less than 30 %. *ND* non determined.

* The sequences that have been used to produce transgenic mice

gene was replaced by the IE-5′1 sequence and introduced into the pM10 vector (Fig. 1). This miRNA construct caused a significant knockdown of the target construct (around 50 % inhibition, Fig. 2a). Thus, several siRNAs targeting the 5′ part or 3′ UTR of IE mRNA were able to knockdown in vitro the expression of the target construct, but with variable efficiency.

The production of siRNA in transfected CHO cells was measured in order to compare the ability of the various constructs to express siRNAs. The highest level was achieved by the U6-5′1 and U6-5′2 constructs (Fig. 2c; Table 1). Both the mi-5′ and U6-3′1 constructs produced small quantities of siRNA. We also compared the efficiency of the siRNA sequences produced by the U6-5′1 and U6-3′1 constructs to knockdown target gene expression. A similar knockdown of luciferase gene expression was achieved with a smaller quantity of U6-3′1 siRNA than U6-5′1 siRNA (Fig. 2d). Thus, the siRNA targeting the 3′UTR of the IE gene was more efficient than that targeting the 5′ region.

Generation of transgenic mice harbouring shRNA or miRNA constructs

Given the diversity of production level and efficiency of the constructs, we decided to use three of them to generate transgenic mice by embryo microinjections. Two of these constructs targeted the 5′ part of the IE mRNA (the mi-5′ and U6-5′1 constructs) and one targeted the 3′UTR (the U6-3′1 construct).

Using the U6-5′1 construct, the proportion of transgenic founders was abnormally low. Among 153 newborns, no founders harbouring the full-length U6-shRNA gene were characterized. This suggests that for some reason, this construct was deleterious and induced the death of embryos or foetuses.

By contrast, using the miRNA-expressing construct (mi-5′) targeting the same 5′ sequence, seven transgenic mice were obtained from 70 newborns. It is probable that this construct enabled the production of transgenic mice because it expresses siRNA at a lower level than the U6-5′1 construct. Two lines of transgenic mice were established, as the others did not transmit their transgene, and one line (L15) was studied further.

Using the U6-3′1 construct, 11 transgenic founders were obtained from 32 newborns. Five transgenic mice transmitted the transgene to their descendants. Three of these lines were further studied (lines SH03, SH05 and SH30).

The copy number of the transgene and the production of siRNA were measured in each transgenic mouse line. Measurements were performed in the brain because this organ is the major site for viral multiplication following nasal instillation (Brittle
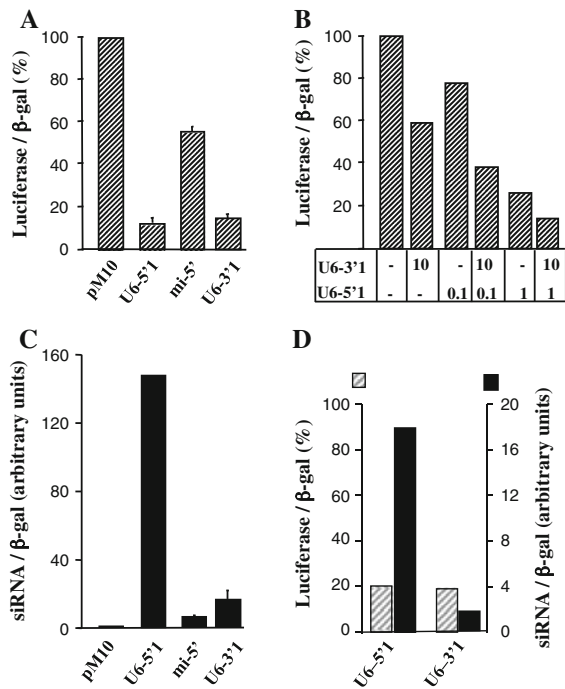
Fig. 2 In vitro measurement of the knockdown efficiency of siRNA constructs. **A** and **B** Inhibition of target mRNA. The different siRNA-expressing constructs U6-5′1, U6-3′1, mi-5′, or the control empty vector pM10, were transfected into CHO cells with the reporter luciferase construct (plm25-IE5-luc-IE3; 0.75 μg/P35 dish) and the β-galactosidase vector pCH110 (Pharmacia, 1 μg/P35 dish). The same amount of each siRNA-expressing construct (0.75 μg/P35 dish) was used. Luciferase and β-galactosidase activities were measured 48 h after transfection. The results are given as percentages of luciferase activity in cells transfected by plm25-IE5-Luc-IE3 and the empty pM10 vector. All luciferase values were normalized to β-galactosidase activities. Values are mean ± SEM of values obtained in three separate experiments. **C** and **D** siRNA expression in transfected CHO cells. **C** Cells were transfected as in **A**. The amounts of siRNA normalised to the relative level of β-galactosidase mRNA determined in each sample are given in arbitrary units. Each *bar* is the mean ± SEM of three separate experiments. **D** The amount of transfected constructs was adapted to ensure 80 % inhibition of luciferase expression. Therefore CHO cells were transfected by 10 ng of U6-5′1 or 100 ng of U6-3′1 construct. The pM10 empty vector was added as carrier to reach an equal amount of DNA in the transfected mixtures. The amount of siRNA is given as in **C**. This figure is representative of three separate experiments

Fig. 3 Expression of siRNA in the brains of transgenic mice. **A** Qualitative analysis of fragments amplified by qPCR. siRNA amplification by qPCR was performed as described in "Materials and Methods". Electrophoresis was performed on a 2 % agarose gel to analyse qualitatively the amplified material. A specific amplified fragment was observed with the expected size (65 bp, *black arrow*) in PCR samples obtained from reverse transcriptase-treated RNA (+). The non-specific amplification or dimers of primers were detected in control animals (WT) and non-reverse transcribed RNA (−). Let7c miRNA detected in all RT+ samples was used to normalize siRNA production. **B** siRNA levels in the brain. The expression of siRNA or miRNA, measured by quantitative PCR, was normalized to the Let7c miRNA expression in each tissue sample and is shown as arbitrary units. *Numbers in brackets* indicate the copy number of the integrated transgene. Note that the level of expression of shRNA or miRNA was apparently correlated to the number of integrated transgene copies. Each value was determined on 3–5 animals from each line

et al. 2004). In transgenic animals only, an amplification product specific to the expected siRNA was detected (Fig. 3a) and quantified by real time PCR (Fig. 3b). SiRNA production was norma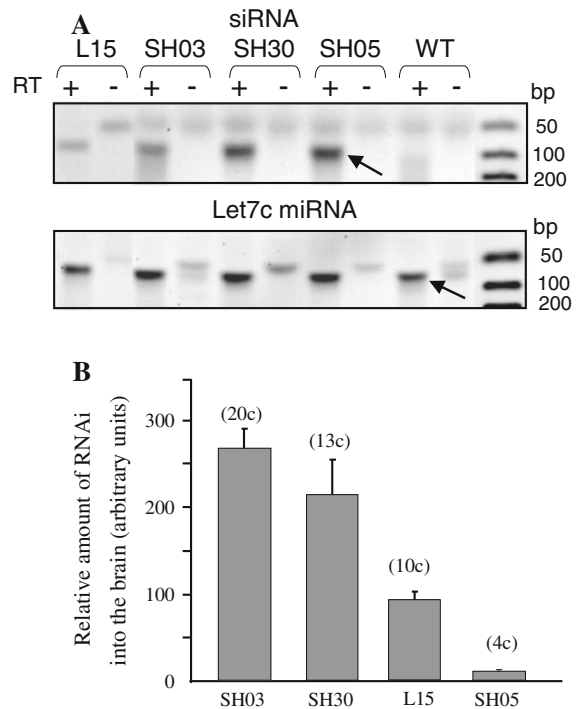lized to the amount of Let7c miRNA, which expression is abundant and stable in mouse brain. The lowest level of expression was found in line SH05; intermediate levels were found in line L15 and the highest expression was found in lines SH03 and SH30. In addition, siRNA levels were similar in all the mice tested within each line, and stably transmitted over at least five generations.

We therefore decided to study these four lines in greater detail in vivo in order to compare the ability of

shRNA- or miRNA-producing constructs to induce resistance against a viral infection.

Transgenic mice resistance to PRV infection

Viral challenges were performed by the intranasal inoculation of an attenuated strain of PRV, the Bartha strain. The dose of instilled virus was low in order to ensure the death of 75–80 % of wild-type animals. We chose to use this attenuated strain under these conditions because it causes the death of the animals after a long clinical phase, thus providing a better opportunity to study the course of the symptoms, which is not the case for the highly virulent Kaplan or NIA-3 strains that kill animals within 2–3 days post inoculation.

The resistance of pools of mice from each of the four selected transgenic lines (SH05, SH03, SH30 and L15) to PRV infection was tested and compared with that of wild-type mice. Table 2 reports the number of infected mice in each group. On day 0 of the experiment, pools of animals received a unique and identical dose of virus via the nostrils. The percentage of surviving mice was monitored for 3 weeks postinfection (PI). Two experiments were performed using line L15 and control mice only (not reported here), and one using all four lines simultaneously (Fig. 4).

During all experiments, the percentage of surviving mice was higher in transgenic lines than in control mice. This difference was statistically significant in lines SH03 and SH30 (Fig. 4a). Thus transgenic animals were better able to resist viral infection. Furthermore, the first death occurred later in transgenic mice (day 10 or even day 11) than in the control group (day 9) (Fig. 4a).

It has been previously observed that the first sign of viral infection with the Bartha strain was a loss of weight; accordingly all infected mice were weighed every day. We quantified the severity of the viral infection by measuring the degree of weight loss and counting the days between the first detection of weight loss and death. As shown in Fig. 4b, the number of days with weight loss before death was higher in transgenic lines than in controls, and this difference was significant in lines L15 and SH03. Thus, transgenic animals with clinical signs of viral attack survive longer than control mice.

In most cases, mice displaying major weight loss died before the end of the experiment. Interestingly, in transgenic lines only, some of the mice presenting a major weight loss recovered a few days later, and subsequently returned to their initial weight (Fig. 4c). These animals finally survived the viral infection. This was never observed in wild-type animals.

The course of infection in the brains of infected animals was estimated by quantifying viral DNA by qPCR in brain DNA extracts (Fig. 5) since it has already been published that in several viral species, viral DNA quantified by qPCR is correlated to the viral titre (Gallina et al. 2006). Viral DNA was detected in all infected animals, both transgenic and wild-type. A first set of animals was sacrificed on day 8 PI (day of the first death in control mice) to monitor the progress of viral replication. The highest levels of viral DNA were detected in wild-type animals and the lowest in SH03 mice (Fig. 5a). Transgenic animals in the SH03 line were thus able to counteract viral replication within 8 days. As expected, the lowest levels of viral DNA were detected in mice that survived the viral infection and were killed 23 days PI (Fig. 5b), while extremely high and variable levels were found in the mice that were sacrificed during the experiment (Fig. 5c). However, in this latter group, the mean amounts of viral DNA were lowest in the SH03 and SH30 lines. Interestingly, the proportion of mice with extremely low levels was greater in the SH30 line (2/5), that expressed the highest amounts of siRNA,

Table 2 Distribution of mice after PRV infection in transgenic lines and in controls

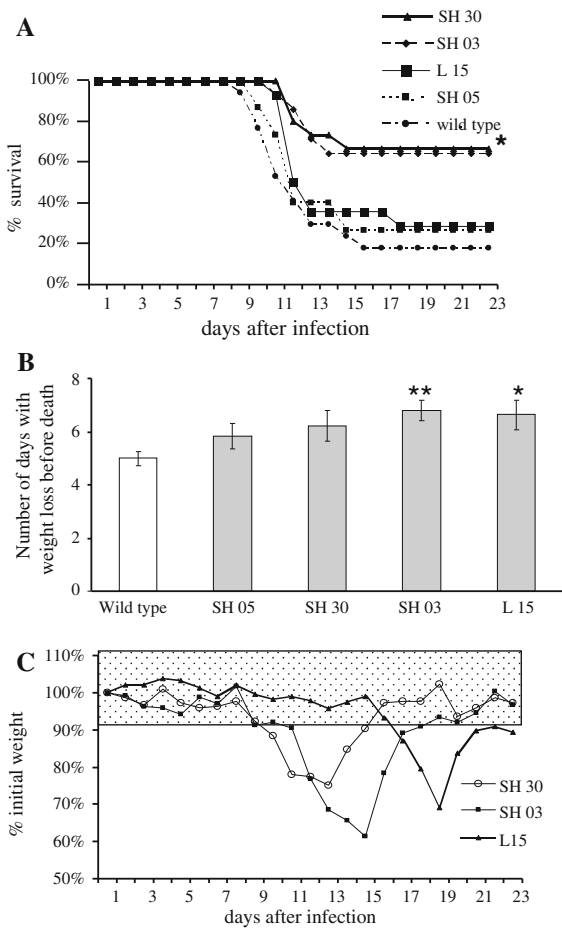| Lines	Total number of mice	Mice dead after anaesthesia	Mice sacrificed 8 days PI	Naturally dead mice during infection	Surviving mice at the end of experiment
Control	27	0	10	14	3
L 15	24	1	9	10	4
SH 03	25	3	8	5	9
SH 05	26	1	10	11	4
SH 30	15	0	0	5	10

PI post infection

Fig. 4 Survival rates and weight loss in mice after intranasal PRV inoculation. Groups of animals from transgenic lines (26, 25 and 24 animals from lines SH05, SH03, and L15; 15 animals from line SH30) and 27 wild-type mice (controls) received one dose of the virus as described in "Materials and Methods" (day 0): 14–15 animals from lines SH05, SH03, SH 30, L15 and 17 controls were then monitored for 23 days PI, see Table 2. The animals were weighed daily. **A** Percentage survival from animals monitored for 23 days. **B** Number of days with weight loss before death. Values are mean ± SEM for 5–10 animals depending of mice survival. The number of stars indicates the significance of the difference from the control group (*$p < 0.05$; **$p < 0.01$). **C** Pattern of weight loss in transgenic mice showing major weight loss followed by recovery. The characteristic patterns of weight changes in three animals are given. The *dotted area* represents the normal variation of mouse weight (±10 %). Such a pattern was observed in 4 animals from line SH30, 2 from line SH03 and only one from line L15

than in other transgenic lines and control mice (2/14). The number of viral DNA copies detected in the brain correlated with the outcome of the infection. Transgenic mice that died from the viral infection were able

to overcome viral replication, at least to some extent. This effect was more visible in mice lines expressing high levels of siRNA.

Taken together, these data indicate that transgenic mice were able to partly counteract the PRV infection according to several criteria such as reduced mortality, delayed or reduced weight loss and a reduction in the amounts of viral DNA in the brain.

The innate immunity of transgenic mice is similar to that of control mice

In order to determine whether the antiviral effect could be mediated by non-specific innate immune mechanisms induced by siRNA expression, we checked the innate immunity of transgenic mice by measuring the level of IFN-inducible IFIT1 mRNA in the brains of transgenic and wild-type animals (Geiss et al. 2001). As shown in Fig. 6, the relative amount of IFIT1 mRNA was similar in the brain extracts from non-infected transgenic and control animals, and more than 140 times lower in uninfected animals than in those infected. Therefore, non-infected transgenic animals did not display a constitutive state of innate immune protection. It is thus reasonable to assume that the antiviral effects of the siRNA were not mediated by non-specific innate immune mechanisms.

Discussion

As previously reported in the case of the avian influenza infection in chicken (Lyall et al. 2011), this study is a new demonstration that genetic modification can be used for counteracting viral infection. It reports for the first time on the extent to which transgenic animals expressing siRNA that targets a viral RNA are resistant to a viral challenge. A number of previous papers had reported the efficacy of exogenous siRNAs in reducing viral infection or eradicating the chronic presence of the virus [see review in (Haasnoot et al. 2007) and (Davidson and McCray 2011)]. However, most of these studies were based on the simultaneous injection of both the virus and the siRNA targeting the virus (delivered as 19–21 mer siRNA or through the injection of an adenovirus producing the siRNA). These papers showed that the viral infection could be efficiently targeted by transiently produced siRNA, thus offering the possibility of a therapeutic approach.
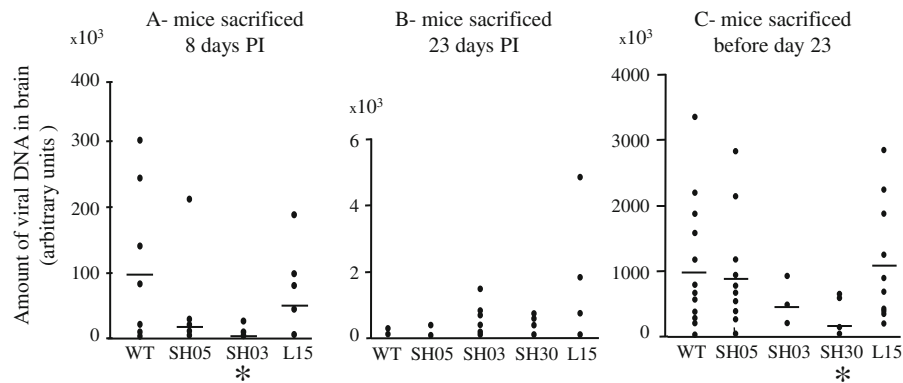
Fig. 5 Quantification of viral DNA in infected mice. The amount of viral DNA was quantified by qPCR determination of the amount of IE gene in brain extracts in mice killed 8 days PI (**A**), in mice killed 23 days PI (**B**) and in sacrificed mice before the end of the experiment (**C**). In this later group, the time of sacrifice was the day at which mice elicited signs of suffering. Values were normalized to the level of the endogenous mouse β2-microglobulin gene in each DNA extract and are shown in arbitrary units. Each *point* represents one mouse. *Horizontal lines* represent the mean considering all mice in one group. Note the change of scale between the three sets of determinations. Note also that the viral DNA was detected in all mice, proving that nasal incubation was efficient. No mice from line SH30 were sacrificed on day 8 PI, because we could not obtain sufficient mice from this line. The *star* indicates the significance of the comparison between wild-type and transgenic lines ($p < 0.05$)

Other studies have described the generation of transgenic animals expressing RNAi molecules that target different regions of viral genomes (Dieckhoff et al. 2008; Wise et al. 2008; Pengyan et al. 2010). However, in vivo resistance was not confirmed by these studies. Indeed, the number of animals surviving a viral challenge has rarely been documented; instead, the animals were killed a few days after the start of the challenge in order to compare the course of the virus in infected and control animals. By contrast, the data presented here concern a viral challenge of transgenic mice stably expressing shRNA or miRNA targeting sequences of a viral RNA. Finally, this work highlights two siRNAs that are able to block the PRV infection to varying degrees without causing any visible toxicity.

The problems encountered when trying to obtain siRNA-producing transgenic animals are now well documented (Jackson et al. 2006; Sioud 2010); these arise either from the overproduction of siRNA that interferes with endogenous miRNA pathways, or from an interaction between the siRNA and a non-targeted RNA, resulting in deleterious gene silencing. It is not easy to identify the possible siRNA sequences responsible for this unwanted interaction, because partial sequence homology between the siRNA and the target may be sufficient to induce an interfering effect. In the present paper, the siRNA content was systematically
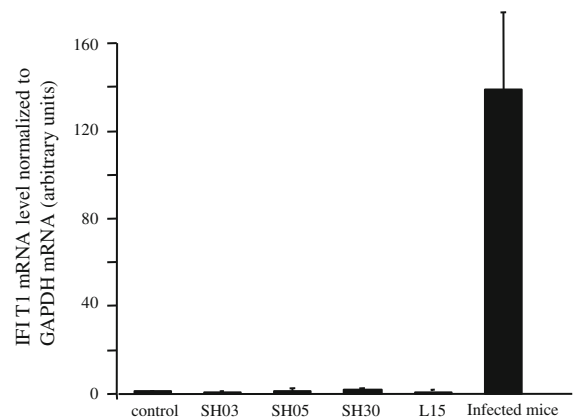

Fig. 6 IFIT1 mRNA levels in the brains of transgenic and control mice. IFIT1 mRNA levels were determined by RT-qPCR from brain extracts as described in "Materials and Methods". At least three animals were studied from each line. The *bar* for infected mice represents the mean ± SEM of values assayed in transgenic animals that died naturally 12–14 days after infection

measured and compared with the interfering effects in vitro before the transgenic animals were generated, which allowed us to identify the best shRNAs capable of inhibiting their target RNA with optimum efficiency at a low concentration so as to avoid any side effects. Finally, this strategy was effective since we obtained transgenic lines with reduced susceptibility

to PRV infection using the U6-3′1 construct that only produced small quantities of the most efficient shRNA.

The antiviral effects observed in transgenic siRNA-expressing mice did not result from an insertion-point mutation effect because they were seen in four separate lines. In addition, they were correlated to the expression level of the siRNA genes. The efficiency of constructs to produce siRNAs is dependent on both the potency of the promoter and the further maturation process by cellular enzymes. This could explain why the U6-5′1 construct was much more efficient than the mi-5′ gene. Indeed, the former was transcribed by the strong U6 gene promoter and the latter by the less potent eF1-α gene promoter. Moreover, the difference in maturation processes between shRNA and miRNA might also have modulated the final concentration of siRNAs. Surprisingly, the U6-3′1 construct led to a low production of siRNA. A likely explanation is that the 3′end of the siRNA sequence contains four A residues, which is the natural termination signal for genes transcribed by RNA polymerase III. This could have induced an unwanted premature termination of transcription.

The antiviral effect is also dependent on the sequence being targeted, since transgenic animals from lines SH30 and SH03 targeting the 3′UTR of the IE gene displayed better resistance against the viral infection than transgenic animals in line L15 targeting the 5′ part of the IE gene. In particular, the best effect was achieved with transgenic lines expressing moderate levels of siRNA. Thus, a judicious selection of highly efficient shRNA allowed us to delay and even sometimes to stably block the viral infection in the absence of phenotypically detectable toxicity. Because we observed a significant additive effect induced by the simultaneous transfection in CHO cells of plasmids expressing siRNA that targeted the 5′ and 3′-UTR of the IE gene, it would be useful to obtain mice lines expressing simultaneously siRNAs that target the 5′part and 3′UTR of the IE gene by breeding animals from the L15 and SH03 or SH30 lines. We expect that double-transgenic mice would display better resistance to the virus because of the additive effect of the two siRNAs. It is also possible that the production of two different siRNAs might better counteract the evolution of escape variants of the virus.

Several studies have reported the success of transient siRNA treatment against acute viral infection (Haasnoot et al. 2007). This was particularly effective in reducing the peak viral load in the case of respiratory viruses. However, it was not systematically determined whether the antiviral activity of the siRNA resulted, at least in part, from a systemic innate immune response induced by the siRNA duplexes. In the present study, transgenic animals stably expressing siRNAs did not show any signs of constitutive innate immune protection in the absence of any infection, as indicated by the very low level of IFN-inducible IFIT1 mRNA in brain extracts from non-infected animals. The absence of siRNA-induced innate immunity prior to infection and the significant reduction of the viral titre after infection are suggestive of safe and efficient RNAi-induced antiviral effect. Even if we are aware that siRNA induced a moderate resistance in our model, siRNA transgene technology thus appears to be promising in ensuring resistance against PRV infection.

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