Gene Therapy (2009) 16, 142–147

www.nature.com/gt

SHORT COMMUNICATION

Prevention of interferon-stimulated gene expression using microRNA-designed hairpins

M Bauer[1,2,3], N Kinkl[1,2], A Meixner[1,2], E Kremmer[4], M Riemenschneider[5], H Förstl[5], T Gasser[3] and M Ueffing[1,2]

[1]Helmholtz Center München-German Research Center for Environmental Health, Institute of Human Genetics, München-Neuherberg, Germany; [2]Institute of Human Genetics, Klinikum rechts der Isar, TU München, München, Germany; [3]Department of Neurology, Hertie Institute for Clinical Brain Research, University of Tuebingen, Tübingen, Germany; [4]Helmholtz Center München-German Research Center for Environmental Health, Institute of Molecular Immunology, München, Germany and [5]Psychiatrische Klinik, Klinikum rechts der Isar, TU München, München, Germany

RNA interference allows selective gene silencing, and is widely used for functional analysis of individual genes in vertebrate cells and represents an attractive therapeutic option for treating central nervous system diseases. However, growing evidence exists that the expression of short hairpin RNAs (shRNAs) can trigger cellular immune response resulting in unspecific cellular phenotypes and severe side effects. We found that lentiviral vector (LV)-mediated expression of shRNAs in primary cortical cultures resulted in strong expression of the interferon-stimulated gene oligoadenylate synthetase 1 (Oas1), which was accompanied by accelerated apoptosis and substantial net neuron loss. Modification of the shRNA construct by implementing features of the naturally occurring microRNA-30 (miR-30) precursor avoided Oas1 induction in transduced primary cultures, whereby modification of the passenger strand seems to be a crucial feature to circumvent interferon-stimulated gene expression. This work represents the first experimental study showing that an miR-30-based shRNA construct prevents Oas1 pathway associated off-target effects, which we consider as an essential prerequisite for shRNA use in future gene therapeutic approaches.
Gene Therapy (2009) **16,** 142–147; doi:10.1038/gt.2008.123;

Keywords: *shRNA; microRNA; Lrrk2; lentiviral vectors; cortical neurons; Parkinson's disease*

Introduction

RNA interference is an evolutionarily conserved mechanism of sequence-specific double-stranded RNA (dsRNA)-mediated gene silencing that might become a promising therapeutic tool for treating central nervous system diseases, which are associated with gain- or change-of-function mutations in specific genes. For example, silencing of mutated gene expression in a mouse model of amyotrophic lateral sclerosis decelerated disease progression and future gene therapeutic approaches decreasing *leucine-rich repeat kinase-2 (LRRK2)* gene expression could provide a causal therapy for Parkinson's disease patients harboring *LRRK2* gain-of-function mutations.[1,2] Although an increasing number of *in vitro* and *in vivo* studies have shown efficient and specific silencing of gene expression, growing evidence exists that vector-driven expression of first generation short hairpin RNAs (shRNAs) can trigger cellular immune response and can lead to deleterious off-target effects.[3] For example, activation of innate cellular immune response by shRNA expression in primary hippocampal neurons resulted in a retraction of synapses and dendritic spines and shRNA expression in zygotes caused early embryonic lethality, which was associated with increased expression of an interferon-induced gene.[4,5]

In general, the introduction of dsRNA into a cell may provoke diverse antiviral effects mediated by several intracellular receptors. Studies have shown that dsRNA located and processed in the endosome may activate Toll-like receptor 3 or 7 leading to the induction of type 1 interferons.[6,7] In addition, cytoplasmic sensors including the RNA-dependent protein kinase can mediate dsRNA-triggered interferon response and in polymerase III driven shRNA expression systems, specific sequences around the transcription start site have been identified, which can also lead to interferon (IFN) induction.[8,9] IFN then binds to cell surface receptors in an auto- or paracrine fashion and confers a more global antiviral state by inducing a complex array of IFN-stimulated genes (ISGs), including RNA-dependent protein kinase and a family of oligo adenylate synthetase (Oas) enzymes.[10]

More recently, second generation shRNA constructs have been designed, in which the stem of the endogenously expressed primary microRNA-30 (miRNA-30) was replaced with gene-specific duplexes for different target genes.[11,12] It has been shown that second generation

Correspondence: Dr M Bauer, Helmholtz Center München-German Research Center for Environmental Health Institute of Human Genetics, Ingolstaedter Landstraße 1, D-85764 München-Neuherberg, Germany.
E-mail: matthias.bauer@helmholtz-muenchen.de
Received 21 May 2008; revised 30 June 2008; accepted 2 July 2008; published online 14 August 2008

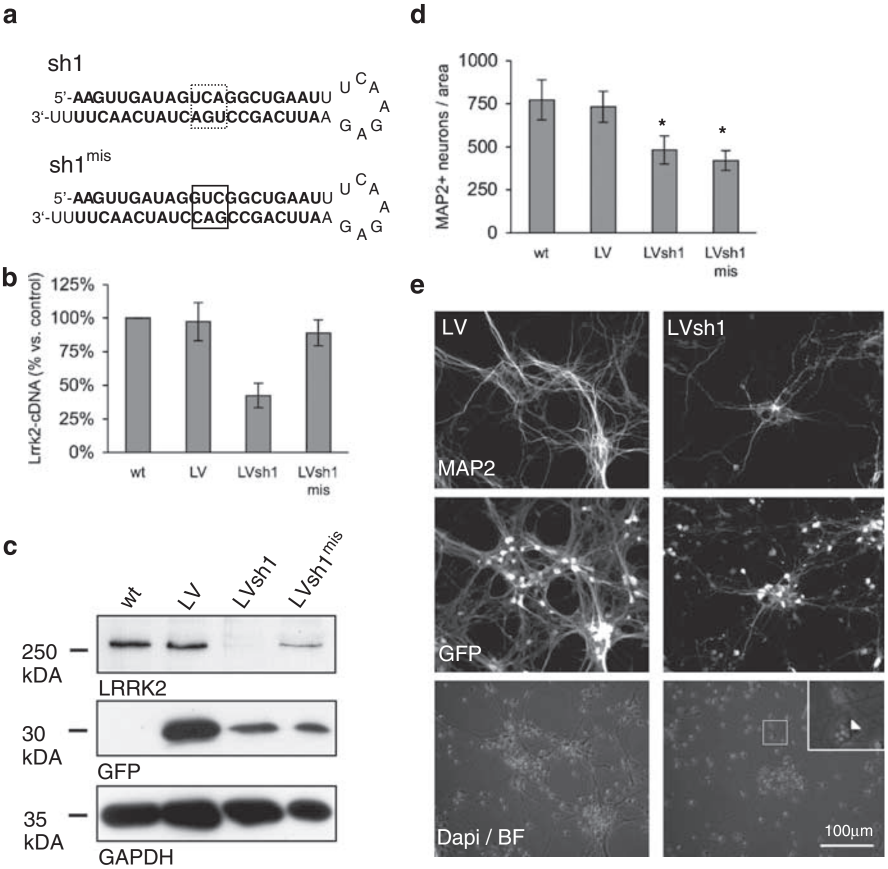

Figure 1 Cytotoxic effects of *Lrrk2* targeting and mismatch control shRNAs in primary cortical neurons. (**a**) Primary cortical neurons derived from E15.5 mouse embryos were cultivated on pDLysine-coated (Sigma-Aldrich, Taufkirchen, Germany) glass slides as described previously and transduced (100 kU^{NIH3t3}, MOI1) with lentiviral vectors (LVs) encoding *Lrrk2* targeting (sh1, *Lrrk2* target sequence in bold) and mismatch control shRNAs (sh1^{mis}, mismatch sequence hemmed) 1 day after plating (day one *in vitro*, DIV1) and analyzed at DIV7. *Lrrk2*-target sequences were identified using Ambion-web-based oligo-search software, one sequence (AAGTTGATAGTCAGGCTGAAT, AY792512: 58–79) was selected, synthesized and subcloned into pBC KS+(ClaI)-H1. The H1sh cassette was then cloned into the pLV-EFα1-GFP transfer vector as described previously.[16] LVsh1^{mis}-transduced cultures were included as controls for unspecific dsRNA-mediated off-target effects and nontransduced (wt) as well as GFP-encoding LV-transduced cultures served as negative controls for transduction-associated toxicity. (**b**) Total RNA was obtained from DIV7 cortical cultures (RNeasy Mini Kit, Qiagen, Hilden, Germany) and RNA (2 μg per 20 μl reaction) was reversely transcribed with the Omniscript RT Kit (Qiagen). *Actin* (forward primer: 5′-GCTGAGAGGGAAATCGTGCG-3′, reverse primer: 5′-TCCA GGGAGGAAGAGGATGC-3′), and *Lrrk2* (forward primer: GCTATCTTGCATTTCGTTGTGC; reverse primer: CCCAGGATTCC-CAATGAACC) was amplified by real-time PCR (ABI Prism 7700 detection system, Applied Biosystems, Foster City, CA, USA) with the QuantiTect SybrGreen PCR Kit (Qiagen): 95 °C for 15 min, followed by 35–40 cycles at 95 °C for 15 s, 54 °C for 30 s and 72 °C for 30 s. Absolute expression levels were normalized to *Actin* expression within the same sample, calculated as 'mean normalized expression' and presented as relative to control expression changes.[23] Each assay for a particular gene was repeated at least three times in triplets. *Lrrk2* mRNA in LVsh1-transduced cultures was reduced to 38% compared with nontransduced controls ($n=3$; C_t values were calculated as mean normalized expression levels and presented as relative changes compared with wt controls). (**c**) LRRK2 protein content in LVsh1-transduced cortical neurons was reduced and GFP expression in both, LVsh1 and LVsh1^{mis}-transduced cultures was decreased compared with LV controls. Quantification of protein bands and loading controls (GAPDH) was carried out with the image analysis software ImageJ (Rasband, WS, US National Institutes of Health, Bethesda, MD, USA; http://rsb.info.nih.gov/ij/) and data are presented as previously published.[16] In general, cells were lysed in PBS-containing 0.5% NP-40 and a protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany) and protein samples (20 μg) were separated on 8% (Figures 1c, 3c and f, upper panel) or 12% (Figures 2a and 3f, lower panel) SDS-polyarcylamide gels and transferred onto Hybond-P PVDF membranes (GE Healthcare, Munich, Germany). After blocking with 5% nonfat dry milk in TBST (Tris-buffered saline, 0.1% Tween-20) membranes were incubated with primary antibodies in blocking buffer (anti-LRRK2, clone 1E11, rat monoclonal, cell culture supernatant 1:10), washed with TBST and incubated with horseradish peroxidase (HRP)-coupled secondary antibodies. Membranes were washed and antibody–antigen complexes were visualized using the ECL+ chemiluminescence detection system (GE Healthcare) on Hyperfilms (GE Healthcare). The membranes were then stripped with 100 mM β-mercaptoethanol and 2% SDS in 62.5 mM Tris-HCl (pH6.8) for 30 min at 56 °C, washed with TBST and subsequently reprobed with anti-GFP (rabbit polyclonal, 1:10.000, Invitrogen, Karlsruhe, Germany) and anti-GAPDH (mouse monoclonal, 1:1000, Chemicon) antibodies. Western blots are representative of three independent experiments that gave similar results. (**d**) Cortical neurons were prepared for immunocytochemistry as published previously and incubated with antimicrotuble-associated protein 2 (MAP2) antibodies overnight (1:1000, mouse; Sigma).[16] For fluorescent labeling, secondary antibodies conjugated to Cy2 and Cy3 were incubated for 3 h at room temperature (1:200, donkey, Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Dapi (1:10000, Sigma-Aldrich) was added to the secondary antibodies. Cultures were analyzed by fluorescence microscopy (Axioscope, Zeiss, Jena, Germany). A significant decline of MAP2+ neurons was evident in *Lrrk2* shRNA and mismatch shRNA expressing cortical cultures at DIV7 ($n=4$–8, $^*P<0.05$, ANOVA, Tukey's Test; MAP2+ neurons were counted in 10 randomly assigned 40 × eye fields comprising 1 mm^2). (**e**) Rarefaction of neurites (right, upper panel) and numerous cells with fragmented (right lower panel, high magnification inset, arrowhead) and condensed nuclei were present in sh1 expressing cultures. Cy, cyanine; GFP, green fluorescent protein; PBS, phosphate-buffered saline; shRNA, short hairpin RNA; wt, wild type.

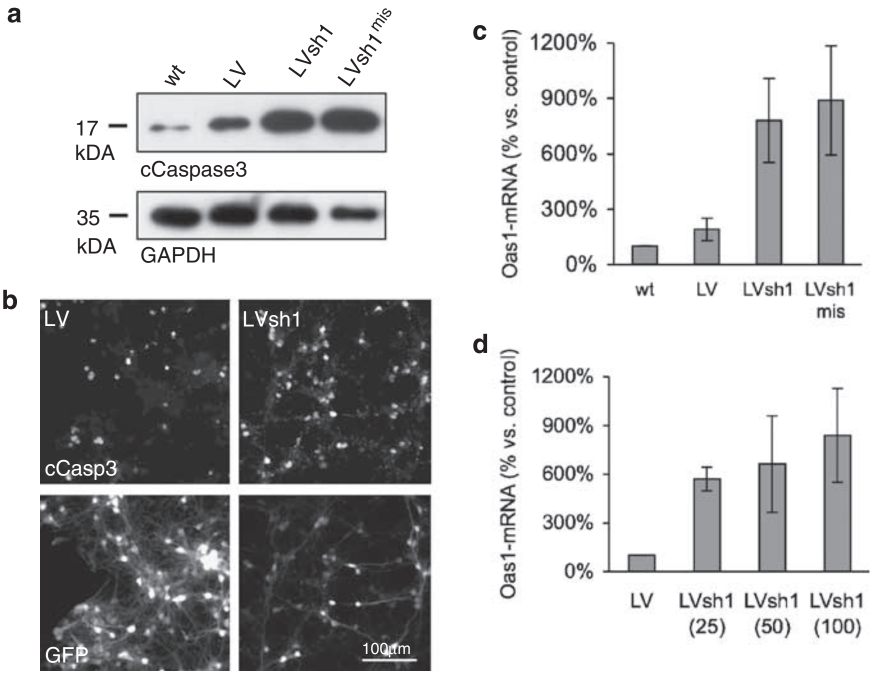

Figure 2 Acceleration of apoptosis and induction of *Oas1* gene expression in primary cortical cultures after sh1 and sh1$^{\text{mis}}$ expression. (**a**) Lentiviral vector (LV)-mediated expression (DIV1, 100kU$^{\text{NIH3t3}}$, MOI1) of *Lrrk2* targeting (LVsh1) as well as mismatch control shRNAs (LVsh1$^{\text{mis}}$) resulted in elevated amounts of cleaved caspase 3 protein (anti-cleaved caspase 3, rabbit polyclonal, 1:1000, Cell Signaling Technology, Danvers, MA, USA), compared with nontransduced (wt) and LV vector-transduced cultures. Western blots are representative of three independent experiments that gave similar results. (**b**) Increased numbers of cleaved caspase 3 immunoreactive cells (cCasp3, 1:50) were detected at DIV7 in LVsh1-transduced cortical cultures. (**c**) In LVsh1 and LVsh1$^{\text{mis}}$-transduced cultures, *Oas1* mRNA expression (forward primer: 5′-CCATCCTCAAGTGGACAAGAACTG-3′, reverse primer: 5′-TTGGGCTTTGGGCACCTTC-3′) was increased by eight- to ninefold ($n=3$). (**d**) Titering down the amount of infectious lentiviral particles from 100 kU$^{\text{NIH3t3}}$ to 25 kU$^{\text{NIH3t3}}$ ($n=3$–5) could not prevent *Oas1* induction in primary cortical cultures. Oas1, oligoadenylate synthetase 1; wt, wild type.

shRNA constructs (also denominated as shRNA$^{\text{mir}}$) are more efficient in gene silencing and it has been speculated that due to its similarity to miRNAs, shRNA$^{\text{mir}}$ constructs are less likely to induce immune response.[11,13]

We show here that the induction of the interferon-response gene Oas1 by expression of first generation shRNA can be abolished by the introduction of the targeting sequence into a miR-30 backbone, whereby the modification of the passenger strand seems to be a crucial feature to avoid innate cellular immune response.

Results and Discussion

Mutations in the *LRRK2* gene have been first described in a familial Parkinsonian syndrome and have been subsequently recognized to be present in up to 2% of all patients with Parkinson's disease.[2] Biochemical analysis performed in our laboratory disclosed that *LRRK2* mutations are functionally characterized by an increase in LRRK2 kinase activity and attempts have been initiated aiming at experimentally downregulating *Lrrk2* gene expression in primary neurons.[14]

In accordance with previously published studies, a preselected shRNA construct (sh1) and a mismatch control construct (sh1$^{\text{mis}}$, central, three base-pair mismatched; Figure 1a) under the control of the human H1 promoter was cloned into a lentiviral vector (LV).[1,15,16] Viral supernatants were obtained and primary cortical cultures derived from E15.5 mouse embryos were transduced with 100kU$^{\text{NIH3t3}}$ (multiplicity of infection, MOI1) 1 day after plating (1 day *in vitro*, DIV1). Transduction of cultures with LVsh1 but not with LVsh1$^{\text{mis}}$ resulted in an ~40% reduction of *Lrrk2* mRNA at DIV7 (Figure 1b). Compared with LV controls, semiquantitative analysis of western blot bands revealed decreased LRRK2 protein levels down to 64 ± 33% ($n=3$) in LVsh1-transduced cultures (LVsh1$^{\text{mis}}$: 91 ± 31%, $n=3$). Green fluorescent protein (GFP) expression in both, LVsh1 (72 ± 28%, $n=3$) and LVsh1$^{\text{mis}}$-transduced cultures (59 ± 22%, $n=3$) seemed to be impaired compared with LV-transduced controls, although equal amounts of GFP expressing LV particles were applied (Figure 1c). Cell counts in shRNA-expressing cortical cultures revealed a significant reduction of MAP2+ neurons in LVsh1 as well as in LVsh1$^{\text{mis}}$-transduced cultures (Figure 1d). Further immunocytochemical analysis of sh1 (Figure 1e, right panel) and sh1$^{\text{mis}}$ (data not shown) expressing cultures showed rarefaction of neurites and increased numbers of condensed and fragmented nuclei suggestive for apoptosis (right panel, inset, the lowest picture). In accordance to the latter finding, expression of sh1 and mismatch controls led to increased amounts of cleaved caspase 3 (LVsh1: 320 ± 114%; LVsh1$^{\text{mis}}$: 321 ± 110% vs LV, $n=3$ each, Figures 2a and b). The phenotype observed in sh1-silenced cultures stands in contrast to a previously published report showing that *Lrrk2* silencing in primary cortical neurons leads to increased neurite length and branching rather than to increased neuron cell death.[17] In

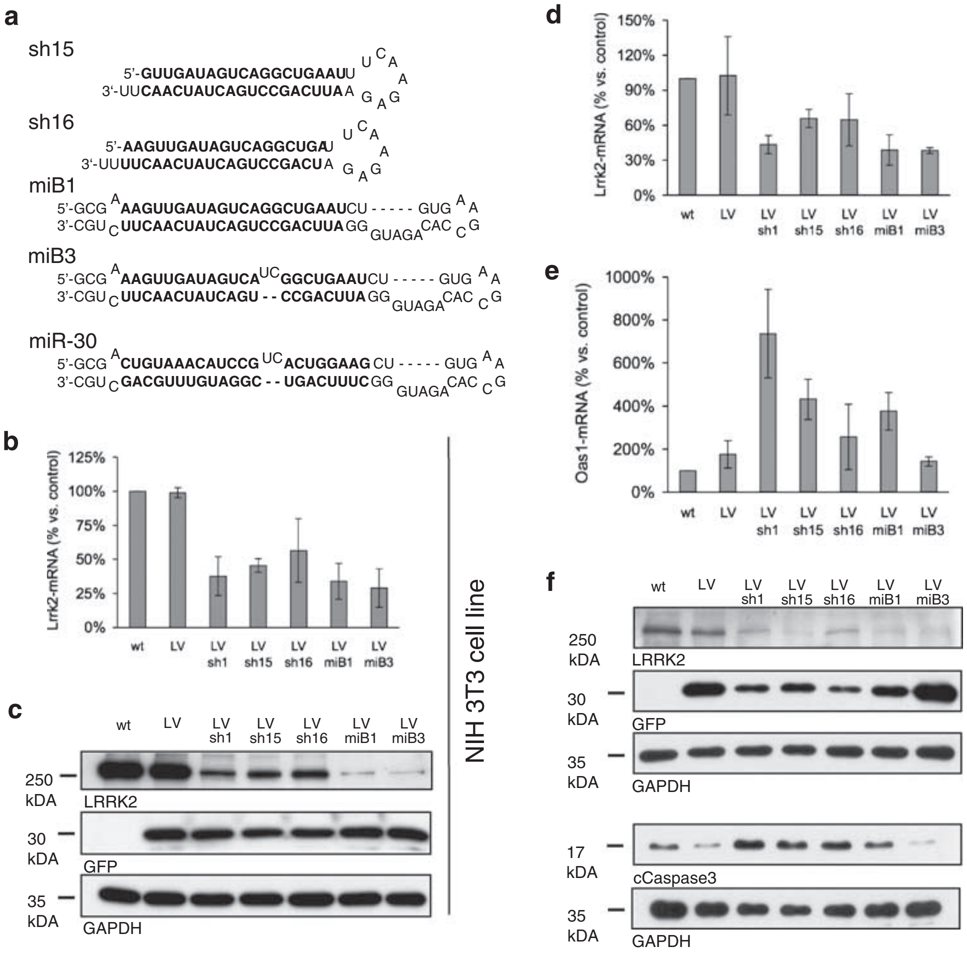

Figure 3 Implementation of microRNA-30 (miR-30) design features in *Lrrk2* targeting shRNA constructs prevents *Oas1* induction and apoptosis in primary cortical cultures. (**a**) *Lrrk2* specific 21-nt target stem duplexes (bold) in the sh1 construct were shortened to 19 nucleotide duplexes by either deletion of the 5′ (sh15) or the 3′ (sh16) ends. Second generation shRNA^{mir} constructs were obtained by replacing the primary miR-30 transcript (miR-30) with the *Lrrk2* specific 21-nt stem duplex (miB1); a di-nucleotide bulge was introduced in the passenger strand of the miB3 construct. H1-shRNA and shRNA^{mir} constructs were cloned into the pLV transfer vector and endogenously *Lrrk2* expressing NIH3t3 cells were transduced (MOI5) with viral supernatants to evaluate silencing efficiencies. *Lrrk2* mRNA levels and protein content was quantified 5 days post-transduction by (**b**) qPCR ($n = 3$) and (**c**) western blot analysis (GFP and GAPDH immunoblot served as controls for transduction efficiency and protein load, respectively. Primary cortical cultures were transduced (100kU^{NIH3t3}, MOI1) 1 day after plating (DIV1) and analyzed at DIV7: mRNA was obtained for *Lrrk2* mRNA quantification (**d**, $n = 3$) and expression analysis of IFN-inducible gene *Oas1* (**e**, $n = 3$–5); (**f**) LRRK2 and cleaved caspase 3 protein amounts were measured by western blot analysis. Nontransduced (wt) and LV-transduced (LV) cultures served as controls. Expression of miB1 and miB3 in cortical cultures resulted in *Lrrk2* mRNA reduction down to 40% (**d**) and was accompanied by a marked reduction of LRRK2 protein levels (**f**, upper panel). Only in LVmiB3-transduced cultures, efficient *Lrrk2* silencing was not accompanied by increased cleaved caspase 3 protein levels (**f**, lower panel). Western blots are representative of three independent experiments that gave similar results. LRRK2, leucine-rich repeat kinase-2; shRNA, short hairpin RNA; Oas1, oligoadenylate synthetase 1; qPCR, quantitative PCR.

addition, the expression of a control shRNA construct (sh1^{mis}) resulted in exactly the same phenotype seen in cultures expressing the specific silencing construct, suggesting an unspecific effect in LVsh1-transduced cultures. We, therefore, further investigated the expression of 2′5′-Oas1, which has been used as surrogate marker for shRNA-triggered interferon response and found that in LVsh1 as well as in LVsh1^{mis}-transduced cortical cultures *Oas1*-mRNA levels were increased approximately ninefold compared with control cultures (Figure 2c).[9,18] Lowering the viral titers by a quarter did not essentially prevent *Oas1* interferon response gene expression (Figure 2d). It has been shown previously that the presence of ectopically introduced dsRNA can lead to the activation of the cellular antiviral defense machinery including the expression of IFNα and IFNβ resulting in the expression of ISGs.[8] dsRNAs then further activate ISGs including Oas1 and RNA-dependent protein kinase and lead to translational inhibition, RNA degradation and apoptosis.[19] The observed increase of apoptotic cells (Figures 1e and 2b), the reduction of vector-driven GFP expression (Figure 1c) in combination with markedly increased *Oas1* expression (Figure 2c) strongly indicates an shRNA-induced cellular immune response rather than a specific *Lrrk2* silencing effect.

We further investigated whether modifications of the hairpin stem length, short-hairpin backbone structure and/or passenger strand modifications may influence the emergence of cellular immune response. Two additional first generation shRNAs were designed: one lacking the AA-dinucleotide at the transcription initiation site (Figure 3a, sh15) and one lacking the AU-dinucleotide at the 5′ region of the guide strand (Figure 3a, sh16). shRNAs were cloned into a LV and silencing efficiencies were first monitored in NIH3t3 cells (Figures 3b and c), which endogenously express *Lrrk2* and did not show induction of *Oas1* after transduction with LVsh1 and LVsh1^{mis} at an MOI of up to 10 (data not shown). *Lrrk2* mRNA in LVsh15 and LVsh16-transduced NIH3t3 (MOI5) was decreased by $\sim$50–60% (Figure 3b) resulting in a slightly less LRRK2 protein reduction compared with LVsh1-transduced cells (Figure 3c). In primary cortical cultures, *Lrrk2* mRNA was reduced by both constructs (Figure 3d); however, sh15 expression resulted in a marked induction of *Oas1* mRNA (Figure 3e). The latter finding was rather unexpected since a previous study has shown that the deletion of an AA-dinucleotide at the transcription initiation site prevents *Oas1*-induction.[9] Thus, our finding may indicate that U6 promoter driven shRNA expression differs from H1-driven constructs with respect to stimulate dsRNA-triggered immune response. More recently, second generation shRNA^{mir} constructs based on endogenously expressed miR-30 primary transcripts have been shown to be more efficient in gene silencing than first generation shRNA constructs and it was speculated that shRNA^{mir} might be more favorable to avoid interferon response.[11,13] To experimentally test this hypothesis, we designed two second generation shRNA^{mir}s: the miB1 construct was adapted from Boden *et al*.[11] and an additional two nucleotide bulge was integrated in the passenger strand of miB3 (Figure 3a). In accordance with the findings from others, lentiviral-mediated expression of miB1 and miB3 constructs in NIH3t3 cells resulted in a more efficient LRRK2 protein decline compared with the first generation sh1 expressing cells (Figure 3c).[11] However, miB1 expression in cortical cultures resulted in an approximately fourfold induction of *Oas1* (Figure 3e) and was accompanied by elevated cleaved caspase 3 levels (Figure 3f). In contrast and compared with LV-transduced controls, no increase of *Oas1* expression was detected in LVmiB3-transduced cortical cultures (Figure 3e). As an additional indication for viable cultures, cleaved caspase 3 protein levels were comparable with those of wild-type (wt) and LV-transduced cultures (Figure 3f) and MAP2+ neuron cell counts were equal to controls (data not shown). Endogenous miRNAs are evolutionary optimized to preclude the induction of dsRNA-triggered cellular immune response. In consequence and in accordance with this selection, the introduction of bulges, which are invariably present in miRNA stems, are instrumental to prevent the activation of dsRNA-dependent ISGs.[20,21]

Interestingly, it has been shown very recently that shRNA-associated neurotoxic effects in a mouse model of Huntington's disease could be mitigated by placing the target sequence into an artificial miR-30-based miRNA expression system. However, the authors of the study have not investigated the expression of ISGs as a possible cause for the observed toxic effects.[22]

To our knowledge, we provide here the first study showing that an exact implementation of all design features of naturally occurring miR-30 precursor is capable to (i) efficiently silence a given target gene and (ii) avoids induction of the interferon-response gene Oas1, which otherwise occurs when first generation designed shRNA constructs are used. This finding has implications regarding vector-based shRNA construct design in experimental gene function studies as well as in gene therapeutic approaches.

Acknowledgements

We thank Stephanie Schöffmann for excellent technical assistance and Dr Thomas Meitinger for helpful discussions. This work was supported by the NGFN ('SMP-Human Brain', FKZ-01GR0449 to MU). and by a KKF grant from the Technical University Munich (KKF-Project 8725151 to MB).

References

1 Raoul C, Abbas-Terki T, Bensadoun JC, Guillot S, Haase G, Szulc J *et al*. Lentiviral-mediated silencing of SOD1 through RNA interference retards disease onset and progression in a mouse model of ALS. *Nat Med* 2005; **11**: 423–428.

2 Zimprich A, Biskup S, Leitner P, Farrer M, Lincoln S, Kachergus J *et al*. Mutations in LRRK2 cause autosomal-dominant parkinsonism with pleomorphic pathology. *Neuron* 2004; **44**: 601–607.

3 Brummelkamp TR, Bernards R, Agami R. A system for stable expression of short interfering RNAs in mammalian cells. *Science* 2002; **296**: 550–553.

4 Alvarez VA, Ridenour DA, Sabatini BL. Retraction of synapses and dendritic spines induced by off-target effects of RNA interference. *J Neurosci* 2006; **26**: 7820–7825.

5 Cao W, Hunter R, Strnatka D, Mcqueen CA, Erickson RP. DNA constructs designed to produce short hairpin, interfering RNAs in transgenic mice sometimes show early lethality and an interferon response. *J Appl Genet* 2005; **46**: 217–225.

6 Kariko K, Bhuyan P, Capodici J, Weissman D. Small interfering RNAs mediate sequence-independent gene suppression and induce immune activation by signaling through Toll-like receptor 3. *J Immunol* 2004; **172**: 6545–6549.

7 Hornung V, Guenthner-Biller M, Bourquin C, Ablasser A, Schlee M, Uematsu S *et al*. Sequence-specific potent induction of IFN-alpha by short interfering RNA in plasmacytoid dendritic cells through TLR7. *Nat Med* 2005; **11**: 263–270.

8 Sledz CA, Holko M, De Veer MJ, Silverman RH, Williams BR. Activation of the interferon system by short-interfering RNAs. *Nat Cell Biol* 2003; **5**: 834–839.

9 Pebernard S, Iggo RD. Determinants of interferon-stimulated gene induction by RNAi vectors. *Differentiation* 2004; **72**: 103–111.

10 Garcia-Sastre A, Biron CA. Type 1 Interferons and the virus–host relationship: a lesson in détente. *Science* 2006; **312**: 879–882.

11 Boden D, Pusch O, Silbermann R, Lee F, Tucker L, Ramratnam B. Enhanced gene silencing of HIV-1 specific siRNA using microRNA designed hairpins. *Nucleic Acids Res* 2004; **32**: 1154–1158.

12 Zeng Y, Wagner EJ, Cullen BR. Both natural and designed microRNAs can inhibit the expression of cognate mRNAs when expressed in human cells. *Mol Cell* 2002; **9**: 1327–1333.

13 Cullen BR. Induction of stable RNA interference in mammalian cells. *Gene Therapy* 2006; **13**: 503–508.

14 Gloeckner CJ, Kinkl N, Schumacher A, Braun RJ, O'Neill E, Meitinger T *et al*. The Parkinson disease causing LRRK2 mutation I2020T is associated with increased kinase activity. *Hum Mol Genet* 2006; **15**: 223–232.

15 Wiznerowicz M, Trono D. Conditional suppression of cellular genes: lentivirus vector-mediated drug-inducible RNA interference. *J Virol* 2003; **77**: 8957–8961.

16 Bauer M, Szulc J, Meyer M, Jensen CH, Abbas Terki T, Meixner A *et al*. Delta-like 1 participates in the specification of ventral midbrain progenitor-derived dopaminergic neurons. *J Neurochem* 2008; **104**: 1101–1115.

17 MacLeod D, Dowman J, Hammond R, Leete T, Inoue K, Abeliovich A. The familial Parkinsonism gene LRRK2 regulates neurite process morphology. *Neuron* 2006; **52**: 587–593.

18 Bridge AJ, Pebernard S, Ducraux A, Nicoulaz AL, Iggo R. Induction of an interferon response by RNAi vectors in mammalian cells. *Nat Genet* 2003; **34**: 263–264.

19 Karpala AJ, Doran TJ, Bean AG. Immune responses to dsRNA: implications for gene silencing technologies. *Immunol Cell Biol* 2005; **83**: 211–216.

20 Lagos-Quintana M, Rauhut R, Lendeckel W, Tuschl T. Identification of novel genes coding for small expressed RNAs. *Science* 2001; **294**: 853–858.

21 Manche L, Green SR, Schmedt C, Mathews MB. Interactions between double-stranded RNA regulators and the protein kinase DAI. *Mol Cell Biol* 1992; **12**: 5238–5248.

22 McBride JL, Boudreau RL, Harper SQ, Staber PD, Monteys AM, Martins I *et al*. Artificial miRNAs mitigate shRNA-mediated toxicity in the brain: implications for the therapeutic development of RNAi. *Proc Natl Acad Sci USA* 2008; **105**: 5868–5873.

23 Simon P. Q-Gene: processing quantitative real-time RT-PCR data. *Bioinformatics* 2003; **19**: 1439–1440.