Wendel. Genes duplicated by polyploidy show unequal contributions to the transcriptome and silencing that are reciprocal and organ-specific. Proc. Natl. Acad. Sci. USA (In Press.)

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RNA Interference by Production of Short Hairpin dsRNA in ES Cells, Their Differentiated Derivatives, and in Somatic Cell Lines

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ABSTRACT

dsRNA of several hundred nucleotides in length is effective at interfering with gene expression in mouse oocytes, pre-implantation embryos, and embryonic stem (ES) cells but is not as efficient in differentiated cell lines. Here we describe a method to achieve RNA interference in totipotent and differentiated ES cells together with a wide range of other mammalian cell types that is both simple and efficient. It utilizes a linearized plasmid that directs the expression of a hairpin RNA with a 22-nucleotide-paired region. This molecule has a 13-nucleotide 5′ overhang that would be subject to capping on its 5′ phosphoryl group and thus differs from the ideal structure suggested for effective small interfering RNAs. Thus, it appears either that the structure of small inhibitory RNA molecules may not need to be as precise as previously thought or that such a transcript is efficiently processed to a form that is effective in interfering with gene expression.

INTRODUCTION

The specific blocking gene expression by double-stranded RNA (dsRNA) of up to several hundred nucleotides in length has been documented in a variety of organisms including *Caenorhabditis elegans* (1), *Drosophila melanogaster* (2), trypanosomes (3), planaria (4), *paramecium* (5), hydra (6), zebrafish (7,8), and mouse (9,10). The use of dsRNA of such molecular weight for silencing gene expression has not been as efficient in cultured mammalian somatic cell lines where it appears to also inhibit gene expression nonspecifically (11). This reflects the ability of dsRNA to trigger the interferon response in mammalian cells, lead-

ing to the activation of the RNA-dependent protein kinase PKR and 2′,5′-oligoadenylate synthetase (2′,5′-AS). Activated PKR stalls translation by phosphorylation of the initiation factor eIF2, and activated 2′,5′-AS causes mRNA degradation by 2′,5′-oligoadenylate-activated ribonuclease L (12). These responses to introduction of any dsRNA lead to an overall block of translation and cell death. However, three reports have now suggested such longer dsRNAs can mediate RNA interference (RNAi) without accompanying interferon responses (13–15).

Longer dsRNA appears to mediate specific RNAi efficiently in mouse oocytes and embryos (9,10,16). Similarly, embryonic stem (ES) cells, which are derived from the mouse embryo, seem to show a specific response to RNAi with dsRNA of this size (17,18). These findings suggest that either embryonic cells have a more effective RNAi response or that the interferon response to dsRNA is absent or not yet fully developed.

dsRNA with an average length of 500 bp has been typically delivered by microinjection into embryos or syncytial tissues or by transfecting cultured cells with dsRNA. The efficiency of this approach has recently been improved by an electroporation approach that can introduce dsRNA into multiple mouse oocytes or embryos (16). In *C. elegans*, RNAi is sufficiently robust that the effects can be achieved by feeding the worms an *E. coli* strain that is expressing the specific dsRNA (19). Interference with specific gene expression can also be achieved in stable transgenic lines of *D. melanogaster* and *C. elegans* by the expression of a construct directing the production of a hairpin loop of RNA (20,21). Such constructs can be placed under the control of regulatable promoters to interfere with specific gene expression in a spatiotemporal manner (21,22).

An important clue to understanding the mechanism of RNAi came from the discovery that 21–23 nucleotide pieces of dsRNA are intermediates that are generated from the cleavage of longer dsRNA (23). Such small dsRNAs induce efficient sequence-specific mRNA degradation in lysates prepared from *Drosophila* embryos (23,24). Moreover, when transfected into cultured mammalian cell lines, small interfering RNAs (siRNAs) of 23 nucleotides appear to evade the interferon response to elicit specific gene silencing (24,25). Recently, plasmids that direct the synthesis of a small hairpin RNA from an RNA polymerase III promoter have been described (26–28). The products are analogous to synthetic siRNAs because they have 3′ overhanging T or U nucleotides but are folded back on themselves to give a 19- to 20-nucleotide base paired region and a 4- to 9-nucleotide loop.

In this study, we describe a simple and efficient method for the inhibition of gene expression in mouse ES cells, their differentiated derivatives, and in somatic cell lines, by producing siRNAs in vivo as short hairpin structures transcribed from an RNA polymerase II promoter. These RNAs are most likely not polyadenylated, so they may be an effective trigger for the RNAi response. The effectiveness of these transcripts suggests that either the precise structure of siRNA molecules may not be as critical as previously thought (25) or that the structure of the transcript per se facilitates its conversion into effective siRNA.

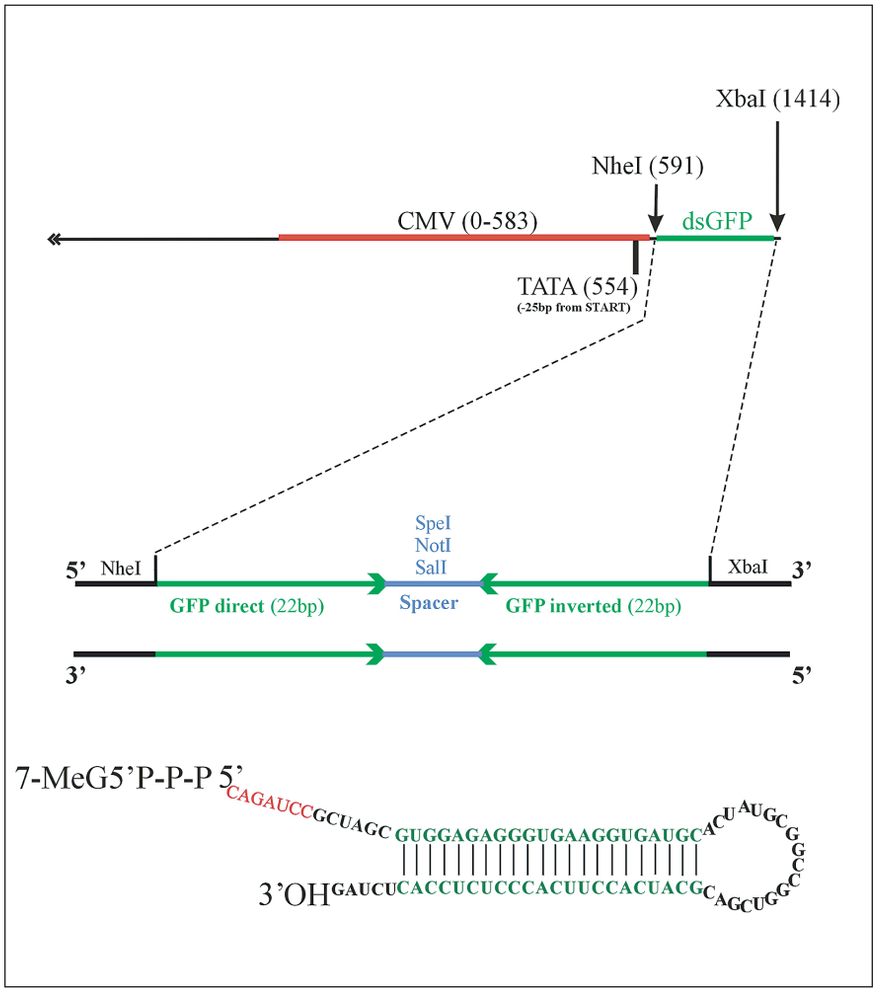

Figure 1. Schematic view of the construct encoding for short hairpin GFP RNA. (A) Linearized pds-GFP-N1 plasmid. Double-stranded synthetic DNA fragment was inserted between *Nhe*I and *Xba*I sites of the pGFPesm-N1 plasmid, downstream of the CMV promoter. (B) The inserted synthetic DNA fragment consisted of indirect repeats of a 22-nucleotide-long GFP sequence that were separated by an 18-nucleotide-long spacer of three restriction sites. Flanking restriction sites were added to the insert to produce a *Nhe*I site at its 5′ end and a *Xba*I site at its 3′ end. (C) Predicted structure of the short hairpin RNA: a 7-MeG cap is likely to be added to the 5′ ends of a large proportion of the transcripts. The 5′-transcribed sequence preceding the *Nhe*I restriction site is marked in red and the target GFP sequence in green.

MATERIALS AND METHODS

Plasmid for dsRNA Production

Two oligonucleotides were synthesized corresponding to an inverted sequence repeat of a 22-nucleotide fragment of GFP carried in the pQBIgfp vector (Qantum/Qbiogene, Montreal, QC, Canada), 5′-GTGGAGAGGGTGAAGGTGATGC-3′, which extended from nucleotides 92 to 113, relative to the first nucleotide of the start codon. The inverted repeats were separated by three unique restriction sites (*Spe*I-*Not*I-*Sal*I), and additional unique restriction sites were added to both ends. The two complementary oligonucleotides were incubated at 80°C and left until the temperature decreased to 30°C. After annealing, the resulting dsDNA fragment possessed an *Nhe*I restriction site at the 5′ end and a *Xba*I restriction site at the 3′ end. The annealed DNA was cloned between the *Nhe*I and *Xba*I sites of the plasmid pGFPemdN1 such that the inverted repeat was located downstream from the cytomegalovirus (CMV) promoter and upstream of the *Xba*I restriction site in this construct (Figure 1).

Cell Culture

Wild-type (CBB) ES cells (kindly provided by Azim Surani, University of Cambridge) and transgenic (β-actin) ES cells expressing *Mus musculis* GFP (MmGFP) under the β-actin promoter (Zernicka-Goetz, unpublished data) were grown on gelatinized plates in DMEM/nut mixture F12 (Invitrogen, Carlsbad, CA, USA) supplemented with 12% FBS (Invitrogen), 100 IU/mL leukemia inhibitory factor (LIF) (CHEMICON, Temecula, CA, USA), L-glutamine, sodium bicarbonate, β-mercaptoethanol, sodium pyruvate, and nonessential amino acids (Invitrogen). ES cells used for experiments were kept in culture between 15 and 25 passages from the isolation of ES cells from the inner cell mass of the blastocyst. To induce differentiation, LIF was removed from the medium. The cells were transfected 10 days after the induction of differentiation.

STO cells were grown in modified DMEM (Invitrogen) supplemented with 10% FBS (Sigma, St. Louis, MO, USA). NIH-3T3 cells were grown in DMEM supplemented with 10% FBS (both from Sigma).

Transfection

Prior to transfection, the pdsGFP-N1 plasmid was linearized with *Xba*I. The pCMV-nls LacZ plasmid was kindly provided by Dr. Aaron Zorn (University of Cambridge). Synthetic 21-bp siRNAs [kindly provided by J. Pearson

(University of Cambridge)] corresponding to nucleotides 469–489 of QBIgfp and to nucleotides 489–509 of MmGFP (relative to the first nucleotide of the start codon) were chemically synthesized and annealed (Dharmacon Research, Lafayette, CO, USA).

The cells were trypsinized 24 h before transfection at 80% confluency and transferred to gelatinized 4-well dishes (4×10^5 cells/well). In all experiments, a constant amount of 3 µg DNA/well was maintained by the addition of Bluescript® plasmid when required. DNA was transfected to adherent cells by lipofection using the Transfast Reagent™ (Promega, Madison, WI, USA), according to the manufacturer's instructions. We used 1 µg each of pQBIgfp, pCMV-nls LacZ, and pdsGFP-N1 or 60 pmol siRNA per well, with the exception of those in which the amount of the pQBIgfp plasmid was reduced to 0.5 or 0.1 µg (see Results). For each 1 µg DNA, we used 2 µL Transfast Reagent in 200 µL serum-free medium. After 1 h of incubation at 37°C in 5% CO_2, 600 µL complete cell culture medium were added, and the cells were cultured for 24 h or longer, as specified in the Results section.

Gene-Silencing Evaluation

To estimate changes in GFP fluorescence, the cells were observed under a 1024 scanning head (Bio-Rad Laboratories, Hercules, CA, USA) attached to an Eclipse 800™ microscope (Nikon, Melville, NY, USA) using 10% [for GFP from Quantum pQBIgfp (QGFP)] or 3% (for MmGFP) of laser power.

To detect β-galactosidase activity histochemically, the cells were fixed in 1% formaldehyde, 0.2% glutaraldehyde for 10 min, washed with PBS, and incubated with X-gal (Sigma).

To quantify β-galactosidase activity using a Dynex MLX luminometer (Dynex Technologies, Chantilly, VA, USA), the cells were trypsinized, washed twice with ice-cold PBS, and resuspended in lysate buffer (K_2HPO_4, KH_2PO_4, dithiothreitol). The cells were then subjected to the two cycles of freezing in dry ice and thawing at 37°C and centrifuged at 17 000× *g* for 5 min at 4°C. The supernatant was stored at 70°C until use. For each lysate, the same amount of protein was used for the luminescent measurements. β-galactosidase expression was monitored with the Luminescent β-Gal kit (BD Biosciences Clontech, Palo Alto, CA, USA).

RESULTS

dsRNA of 21–22 nucleotides mediates efficient sequence-specific silencing in several mammalian cell lines and has been termed siRNA (23). We sought to test whether the interfering response could be achieved by expressing a 22-nucleotide short hairpin GFP RNA from a CMV promoter carried on a plamid vector. To avoid the synthesis of unnecessarily long RNA, we linearized the plasmid by cleavage at an *Xba*I site distal to the inverted sequence repeat before transfecting it into different cell types derived from the mouse embryo. This could have the advantage of directing the synthesis of non-polyadenylated RNA that itself might facilitate the triggering of an RNAi response.

We first tested the effects of introducing the pdsGFP-N1 plasmid into wild-type ES cells that expressed GFP transiently from an exogenous plasmid, pQBIgfp. Co-transfection of ES cells with pQBIgfp and the plasmid encoding for short hairpin RNA, pdsGFP-N1, resulted in the almost complete inhibition of GFP expression within 24 h (Figure 2A). This was in contrast to cells transfected only with the pQBIgfp plasmid (Figure 2A). This inhibition of GFP expression was similar in extent to that observed following the co-transfection of cells with pQBIgfp plasmid and cognate synthetic siRNA (data not shown). In experimental groups transfected with plasmid directing the expression of the short hairpin or with siRNA, GFP fluorescence did not reappear following up to five days of culture. In each of these experiments, we included a plasmid pCMV-nls LacZ, which directs the expression of β-galactosidase from a CMV promoter, and assayed for the activity of this enzyme at intervals after trasfection. Although both the plasmid that encoded short hairpin GFP RNA and GFP siRNA resulted in a diminution of GFP expression, we were unable to detect any de-

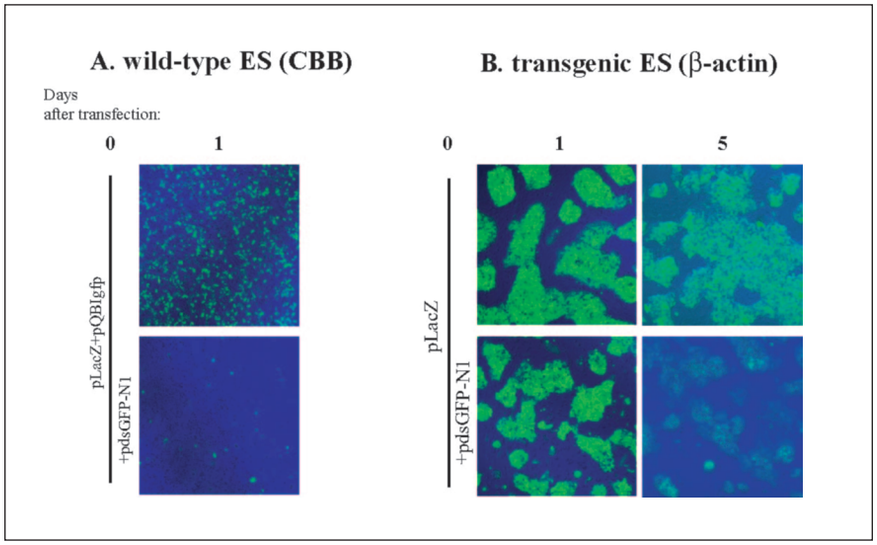

Figure 2. RNAi in ES cells. (A) Change in GFP fluorescence after RNAi in wild-type ES (CBB) cells. The cells were transfected with 1 µg each of GFP plasmid and LacZ plasmid. Experimental groups were additionally transfected with 1 µg pdsGFP plasmid (encoding short hairpin RNA) or siRNA for GFP (data not shown). The upper panel shows the control transfected cells. The lower panel shows the cells transfected with the plasmid encoding for short hairpin RNA. GFP silencing was already evident 24 h after transfection. (B) Changes in GFP fluorescence after RNAi in transgenic ES cells expressing GFP from the β-actin promoter (β-actin). The cells were transfected with 1 µg LacZ plasmid. The experimental group was additionally transfected with 1 µg pdsGFP plasmid (encoding short hairpin RNA) or siRNA for GFP. The upper panels show the control transfected cells, and the lower panels show the cells transfected with plasmid encoding for short hairpin RNA. GFP silencing resulted in a decrease of MmGFP fluorescence intensity and became evident after five days of culture.

crease in β-galactosidase activity (data not shown). These results demonstrate that short hairpin RNA produced in vivo is as potent as siRNA in inhibiting gene expression in ES cells directed from exogenous plasmids.

To assess the efficiency of these two approaches on the expression of an endogenous chromosomally located gene, we used a line of ES cells that stably express MmGFP from the β-actin promoter. The transfection of these cells with the pdsGFP-N1 resulted in a decrease in GFP fluorescence 4–5 days following transfection (Figure 2B). Similar results were obtained after the transfection of GFP siRNA. The persistence of MmGFP expression until four days after transfection may largely reflect the levels of this stable form of protein in cells at the onset of the experiment. There was no decrease in the levels of expression of β-galactosidase from the pCMV-nls LacZ co-transfected into these cells with either plasmid directing the expression of the short hairpin or siRNA.

It has recently been shown that long dsRNAs trigger the nonspecific inhibition of gene expression in differentiated cells derived from mouse ES cells and in several cell lines of embryonic origin (13,21). Thus, we sought to examine whether short hairpin RNA could elicit specific RNAi in differentiated cells derived from ES cells. For this purpose, we induced the differentiation of wild-type and GFP-transgenic ES cells by removing LIF from the culture and then carried out transfections 10 days after removal of LIF. We found that short hairpin RNA produced from the pdsGFP-N1 plasmid to be effective in silencing GFP from a co-transfected plasmid (Figure 3A) and from a chromosomally inserted GFP transgene (Figure 3B). This effect was maintained for at least five days of culture following transfection. Once again, we could detect no depression of β-galactosidase expression from co-transfected pCMV-nls LacZ.

We also carried out a similar experiment in which ES cells were co-transfected with GFP-expressing plasmid together with the plasmid encoding for short hairpin RNA or with synthetic siRNA and then induced to differenti-

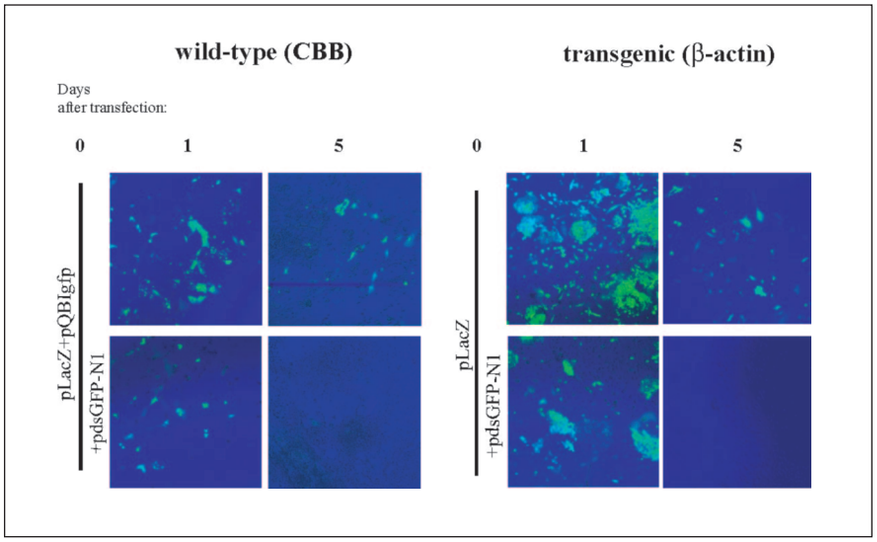

Figure 3. RNAi in differentiated derivatives of ES cells. (A) Changes in GFP fluorescence after RNAi in differentiated wild type ES cells (CBB). Cells were transfected with 1 μg each of GFP plasmid and LacZ plasmid. The experimental group was additionally transfected with 1 μg pdsGFP plasmid (encoding for short hairpin RNA) or siRNA for GFP (data not shown). GFP silencing was already evident 24 h after transfection by the decrease in the number of fluorescent cells and was visible as a total loss of GFP fluorescence after five days of culture. (B) Changes in GFP fluorescence after RNAi in differentiated transgenic ES cells (β-actin). The cells were transfected by using 1 μg LacZ plasmid. The experimental group was additionally transfected with 1 μg pdsGFP plasmid (encoding for short hairpin RNA) or siRNA for GFP. GFP silencing appeared 24 h after transfection as a decrease in intensity of GFP fluorescence and was visible as loss of GFP fluorescence after five days of culture. (A and B) The upper panels show the control transfected cells, and the bottom panels show the cells transfected with plasmid encoding for short hairpin RNA.

ate by removing LIF from the culture medium 24 h after transfection. Such differentiating ES cells ceased to express GFP 48 h after LIF removal, and this drop in GFP expression was maintained up to six days after LIF removal (data not shown). Thus, the silencing of GFP expression induced by in vivo transcription of short hairpin RNA is maintained after the induction of ES cell differentiation.

Finally, we decided to test whether the short GFP hairpin RNA would interfere with GFP expression in somatic cell lines. To this end, we co-transfected mouse fibroblasts (NIH-3T3) and fetal fibroblasts (STO) with GFP-expressing plasmid and plasmid directing the synthesis of short hairpin RNA. We found that STO cells showed little or no expression of GFP 24 h after transfection compared to the control cells. Similar results were observed after the transfection of cells with cognate siRNA (Figure 4A). In contrast, we found that equivalent amounts of siRNA oligonucleotides or plasmid-expressing hairpin RNA were less effective at interfering with the extremely efficient expression of GFP from the pQBIgfp plasmid transfected into NIH-3T3 cells (Figure 4B). However, when we increased the ratio of plasmid encoding for short hairpin RNA to pQBIgfp plasmid using 0.5 or 0.1 µg pQBIgfp plasmid instead of 1 µg, we began to see an interfering effect on GFP expression. Transfection of NIH-3T3 cells with pGFPds-N1 plasmid caused a significant decrease in the GFP fluorescence in a large number of cells, which was especially visible when a 10:1 ratio of pdsGFP-N1 and pQBIgfp plasmids was used (Figure 4B). A similar dose response was seen with siRNA. There was no decrease in the expression from the co-transfected *lacZ* gene in these experiments.

DISCUSSION

Several reports have shown that it is possible to inhibit gene expression in cultured mammalian cells by the introduction of long dsRNA. It seems particularly effective in totipotent and pluripotent cells. This is perhaps not surprising because sequence-specific RNAi by long dsRNA was demonstrated to be effective in the cells of the mouse embryo (9,10,14). Recently, long dsRNA has been shown to produce an efficient specific response in undifferentiated ES and embryonic carcinoma (EC) cells but not when differentiation is induced (13,18). Results with long dsRNA have been more varied, leading to an unspecific response in several mammalian cell types, including mouse embryonic fibroblasts (STO), and several pluripotent cell lines, including N-Tera-1 and F9 cells (13,18).

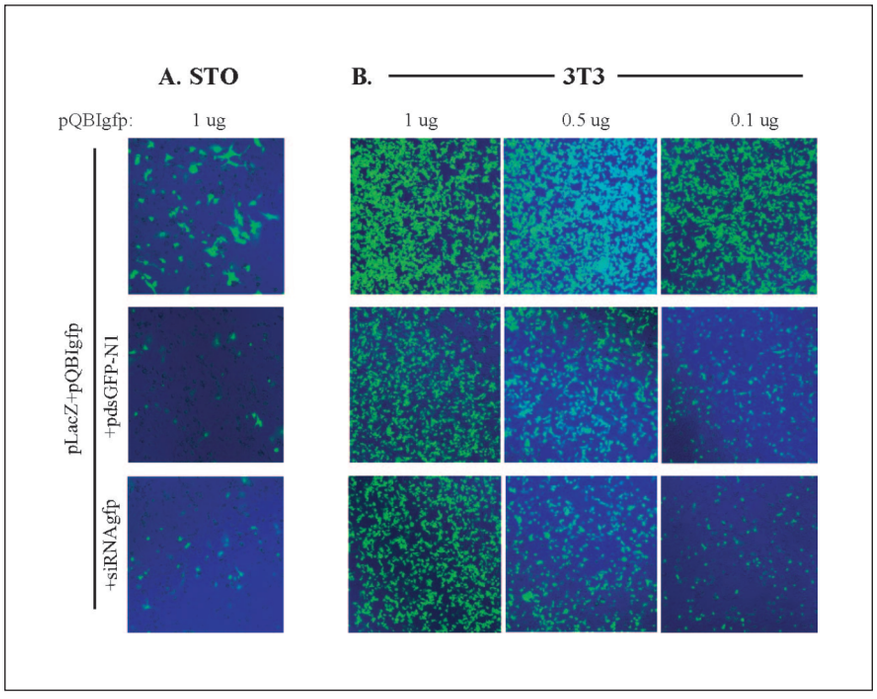

Figure 4. RNAi in somatic murine cells 24 h after transfection. (A) Changes in GFP fluorescence in STO cells. Cells were transfected with 1 µg each of GFP plasmid and LacZ plasmid. The experimental group was additionally transfected using 1 µg pdsGFP plasmid (encoding for short hairpin RNA) or siRNA for GFP. The upper panel shows the control transfected cells; the middle panel shows the cells transfected with plasmid encoding for short hairpin RNA; and the lower panel shows the cells transfected with siRNA for GFP. GFP silencing is evident from the reduced number of fluorescent cells. (B) Changes in GFP fluorescence in NIH-3T3 cells. The cells were transfected with 1 (first column), 0.5 (second column) or 0.1 µg (third column) GFP plasmid, respectively, and 1 µg LacZ plasmid. The experimental group was additionally transfected with 1 µg pdsGFP plasmid (encoding for short hairpin RNA) or siRNA for GFP. The upper panels show the control transfected cells; the middle panels show the cells transfected with plasmid encoding short hairpin RNA; and the bottom panels show the cells transfected with siRNA for GFP. GFP silencing is evident from the loss in the number of fluorescent cells, the most evident in the group was transfected with 0.1 µg GFP-expressing plasmid (third column).

However, three groups have reported specific effects with long dsRNA (13–15). On the other hand, siRNAs produced in vitro induce a robust specific response in several cell lines including human embryonic kidney (293), HeLa cells, and NIH-3T3 cells (25,29). This confirmed that the mechanism for RNAi exists in mammalian cells and suggests that it can be evoked most efficiently if other effects mediated by longer dsRNA can be bypassed. Here we have shown that short hairpin RNA produced in vivo leads to the efficient and specific inhibition of gene expression not only in ES cells but also in their differentiating or differentiated derivatives and in mouse fibroblasts (STO and NIH-3T3). In each case, the effect was as potent as the effect of siRNA. Thus, this approach offers a simple alternative method for the inhibition of gene expression in many cell types.

It has been shown previously that siRNAs mediate the efficient sequence-specific mRNA degradation in lysates prepared from *Drosophila* embryos and in mammalian cells (23–25). These reports suggested that the presence of overhanging 3′ ends in synthetic siRNAs are necessary to induce RNAi in examined cells. Moreover, Harborth and colleagues (29) found that a 5′ phosphate on the target complementary strand of the siRNA duplex was required for siRNA function. An alternative system for expressing small siRNA-like structures from a plasmid has recently been described (26,27, 30–32). It attempts to emulate the synthetic siRNAs described by Elbashir and colleagues (25). Importantly, this uses the RNA polymerase III promoter and thus produces a transcript that is neither capped nor polyadenylated. The constructs that were effective in eliciting dsRNAi and a 19-nucleotide paired region had two 3′ overhanging T or U residues, as specified by Elbashir et al. (24). However, in our case, short hairpin RNA produced in cells from linearized plasmid should have five unpaired residues at its 3′ end. In fact, these molecules also have 13-nucleotide 5′ overhangs and are of a length such that a high proportion of them are highly likely to have 7MeG 5′ caps typical of RNA polymerase II transcripts. Despite these significant differences in the structure of siRNA and short hairpin RNA, our results show that both are equally effective at interfering with gene expression. We speculate that these hairpin structures can function as siRNAs or are efficiently processed into siRNA molecules. They might also be particularly efficient since being produced intracellularly with a 5′ cap, they might evade the interferon response. Furthermore, it is possible that they are not recognized as "foreign" RNA molecules because they resemble the stem-loop structures of small temporal RNAs (stRNAs) (33,34) or microRNAs (miRNAs) (35,36) that have been proposed to have regulatory roles for eukaryotic gene expression. Indeed, some features of the miRNAs were recently incorporated into a system for carrying out RNAi in cultured human cells (37). In conclusion, the technique we described presents a very effective, simple, and low-cost method to knock down gene expression in all mammalian cell types.

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