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THERAPY

Type I Interferons Impede Short Hairpin RNA-Mediated RNAi via Inhibition of Dicer-Mediated Processing to Small Interfering RNA

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RNAi by short hairpin RNA (shRNA) is a powerful tool not only for studying gene functions in various organisms, including mammals, but also for the treatment of severe disorders. However, shRNA-expressing vectors can induce type I interferon (IFN) expression by activation of innate immune responses, leading to off-target effects and unexpected side effects. Several strategies have been developed to prevent type I IFN induction. On the other hand, it has remained unclear whether type I IFNs have effects on shRNA-mediated RNAi. Here, we show that the type I IFNs significantly inhibit shRNA-mediated RNAi. Treatment with recombinant human IFN-α significantly inhibited shRNA-mediated knockdown of target genes, while it did not inhibit small interfering RNA (siRNA)-mediated knockdown. Following treatment with IFN-α, increased and decreased copy numbers of shRNA and its processed form, respectively, were found in the cells transfected with shRNA-expressing plasmids. Dicer protein levels were not altered by IFN-α. These results indicate that type I IFNs inhibit shRNA-mediated RNAi via inhibition of dicer-mediated processing of shRNA to siRNA. Our findings should provide important clues for efficient RNAi-mediated knockdown of target genes in both basic researches and clinical gene therapy.

INTRODUCTION

Chemically synthesized small interfering RNAs (siRNAs) or vectors expressing short hairpin RNA (shRNA) are widely used to induce RNAi in vitro and in vivo not only for gene-function analysis in basic research but also for the treatment of severe disorders due to their superior knockdown efficiencies.[1–3] siRNAs are transfected into cells using transfection reagents in the form of ∼21- to 23-bp double-stranded RNA (dsRNA), whereas shRNAs are delivered by non-viral or viral vectors encoding shRNA. Following the introduction of shRNA-expressing vectors into cells, shRNAs are transcribed mainly via the polymerase (pol) III promoter, exported from the nucleus to the cytoplasm by exportin 5, and processed by the endoribonuclease dicer to siRNAs. The generated siRNAs are then incorporated into the RNA-induced silencing complex (RISC) and guide the RISC to the target mRNA in an siRNA-sequence-specific manner, leading to knockdown of the target gene.[1–3] Among the RISC components, argonaute 2 (Ago2) cleaves target mRNA, while Ago1, Ago3, and Ago4 do not possess cleavage activities.[4]

Following introduction of non-viral and viral vectors into cells, innate immune responses, including type I interferon (IFN) responses, are triggered.[5,6] The induction of type I IFN responses results in upregulation of a large number of IFN-stimulated genes (ISGs), suggesting that expression levels of various non-target genes as well as target genes are altered by type I IFNs. The type I IFN responses might affect shRNA-mediated RNAi by positively or negatively regulating the RNAi pathway.

In this study, we demonstrate that the type I IFNs significantly inhibit shRNA-mediated RNAi via inhibition of dicer-mediated processing of shRNA to siRNA, although siRNA-mediated knockdown was not impeded by type I IFNs. These data suggest that care should be taken when using shRNA-expressing vectors in either basic research or clinical gene therapy, since they can induce type I IFN

A A549

B H1299

☐ mock
■ shLuc

C A549
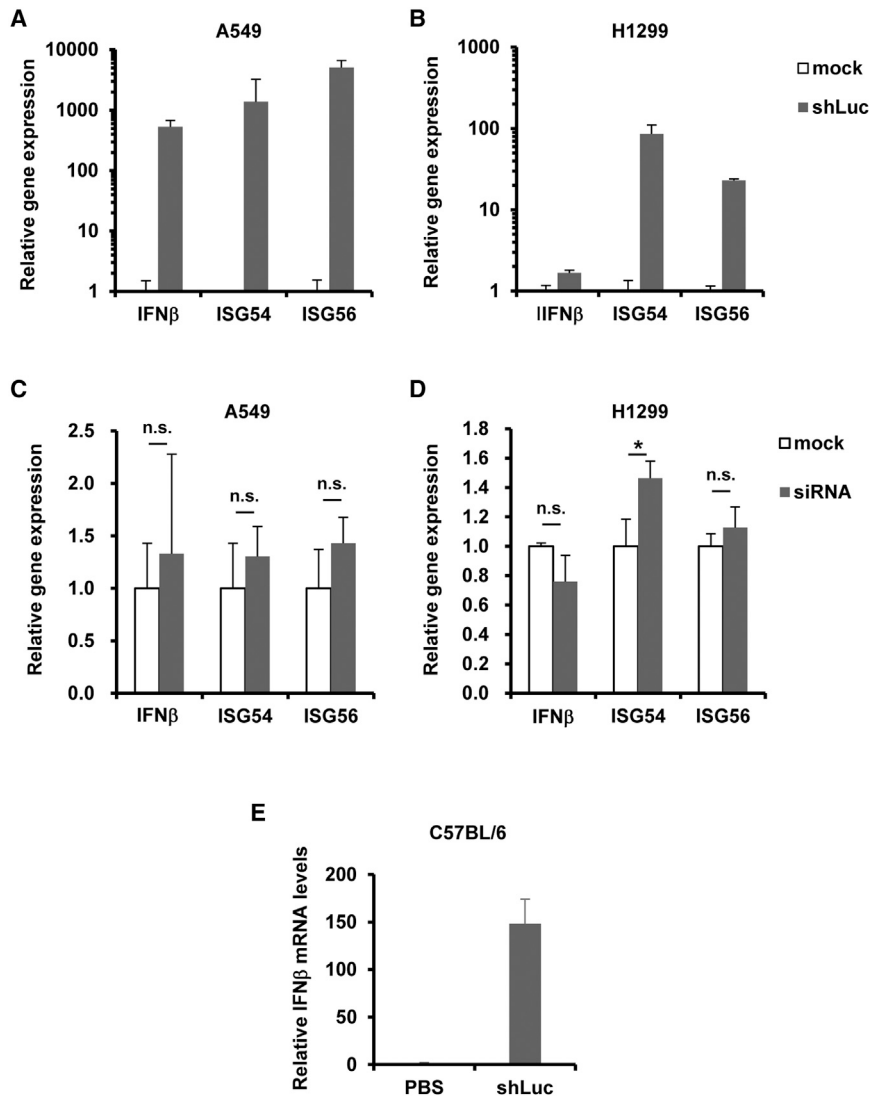
D H1299

☐ mock
■ siRNA

E C57BL/6

Figure 1. Type I IFN Responses Following Introduction of shRNA-Expressing Vectors

(A–D) A549 (A and C) and H1299 (B and D) cells were transfected with pHMU6-shLuc (A and B) or siControl (indicated as siRNA) (C and D). After 24-hr incubation, IFNβ, ISG54, and ISG56 mRNA levels in the cells were determined by qRT-PCR. The data are expressed as the means ± SD (n = 3). (E) 100 μg pHMU6-shLuc was intramuscularly administered to C57BL/6 mice. 3 hr after administration, the IFN-β mRNA levels in the mouse muscle were determined by qRT-PCR. Data are expressed as the means ± SE (n = 3–4). *p < 0.05.

with the siControl (Figures 1C and 1D). Next, we examined whether an shRNA-expressing plasmid would induce type I IFN responses in vivo. Intramuscular administration of the shLuc-expressing plasmid induced high levels of IFNβ expression (more than 100-fold compared to the PBS-administered group) (Figure 1E). These results indicate that introduction of shRNA-expressing plasmids, but not siRNAs, induces type I IFN responses, leading to elevation in ISG expression levels.

IFN-α Stimulation Inhibited shRNA-Mediated Gene Knockdown

In order to examine whether type I IFNs affect shRNA-mediated knockdown efficiencies, H1299 and A549 cells were transfected with shRNA-expressing plasmids, followed by treatment with recombinant human IFN-α. The cell viabilities (Figure S2) and transfection efficiencies, which were assessed by the percentages of GFP-positive cells following transfection with a GFP-expressing plasmid (Figure S3), were not significantly altered after 43-hr exposure to 10^4 U/mL IFN-α. In H1299 cells, transfection with an shRNA against c-*myc* (shmyc)-expressing plasmid (pHMU6-shmyc) led to significant knockdown of the c-*myc* gene in the absence of IFN-α stimulation (Figures 2A and 2B). Surprisingly, c-*myc*-knockdown efficiencies were reduced by ~35% when cells were treated with IFN-α (Figure 2A). In agreement with the shRNA-mediated alteration of c-*myc* mRNA levels, knockdown of c-*myc* at the protein level was also inhibited by IFN-α stimulation (Figure 2B). mRNA and protein levels of c-*myc* were not significantly altered after treatment with 10^4 U/mL IFN-α (Figures 2A and 2B). The reduction in the knockdown efficiencies of pHMU6-shmyc by IFN-α was dependent on the doses of IFN-α (Figure 2C). IFN-α inhibited target gene knockdown in A549 cells when mediated not only by shmyc but also by shRNA against p53 (shp53) (Figures 2D and 2E). Treatment with recombinant human IFN-β, which is another type I IFN, also inhibited shmyc-mediated knockdown in H1299 cells (Figure 3A). On the

production that may in turn inhibit the knockdown efficiencies of shRNAs.

RESULTS

Type I IFN Responses following Introduction of shRNA-Expressing Vectors

In order to examine whether type I IFN expression was induced following introduction of shRNA-expressing plasmids and siRNAs, A549 and H1299 cells were transfected with a plasmid expressing shRNA against luciferase (shLuc) (pHMU6-shLuc) or a control siRNA (siControl). Treatment of both cells with recombinant type I IFN (IFN-α) significantly induced ISG expression (Figure S1). qRT-PCR analysis showed that transfection with the shLuc-expressing plasmid significantly induced high levels of IFN-β and ISG expression (Figures 1A and 1B), whereas significant levels of upregulation of IFN-β and ISG expression were not found following transfection
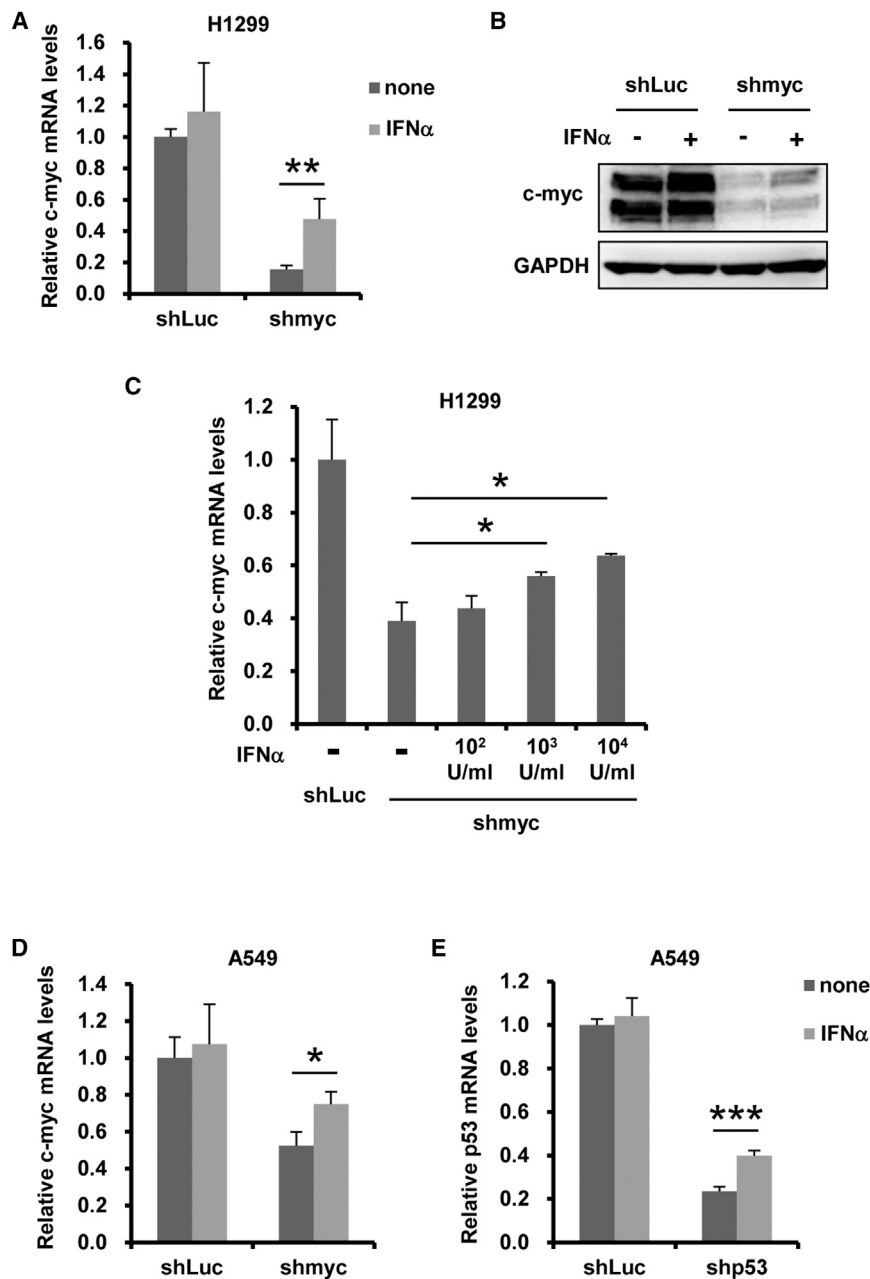
Figure 2. Inhibition of shRNA-Mediated Knockdown by IFN-α Stimulation

(A and B) H1299 cells were transfected with pHMU6-shLuc or -shmyc, followed by treatment with recombinant IFN-α at 10^4 U/mL. After 48-hr incubation, c-*myc* mRNA (A) and protein (B) levels in the cells were determined by qRT-PCR and western blotting analysis, respectively. (C) H1299 cells were transfected with pHMU6-shLuc or -shmyc, followed by treatment with recombinant IFN-α at 10^2, 10^3, or 10^4 U/mL. After 48-hr incubation, c-*myc* mRNA levels in the cells were similarly determined. (D and E) A549 cells were transfected with pHMU6-shLuc, -shmyc (D), or -shp53 (E), followed by treatment with recombinant IFN-α at 10^4 U/mL. After 48-hr incubation, c-*myc* (D) and p53 (E) mRNA levels in the cells were similarly determined. These experiments were repeated at least three times, and representative data are shown. All data are expressed as the means ± SD (n = 4). *p < 0.05, **p < 0.01, ***p < 0.001.

gene following transfection with pHMH1-shmyc (Figures 4A and 4B), suggesting that type I IFNs inhibit knockdown by an shRNA driven by any type of pol III promoter. These results suggest that type I IFNs inhibit shRNA-mediated knockdown, at least within 43 hr after type I IFN treatment.

Knockdown by Chemically Synthesized siRNAs Was Not Inhibited by IFN-α Stimulation

Following transfection with shRNA-expressing plasmids, an shRNA is transcribed and processed to siRNA by dicer, leading to the knockdown of target genes. We next examined the effects of type I IFNs on chemically synthesized siRNA-mediated knockdown. When A549 cells were transfected with an siRNA against p53 (sip53), followed by incubation in the presence of recombinant human IFN-α, p53 was significantly knocked down at comparable levels in the presence or absence of IFN-α (Figure 5A). Similar results were found in an siRNA against c-*myc* (simyc)-transfected cells (Figure 5B). These results suggest that type I IFNs do not inhibit siRNA-mediated knockdown and that steps before the incorporation of siRNA into RISC are impaired by type I IFNs.

other hand, a type II IFN (IFN-γ) did not alter the knockdown efficiencies of an shmyc-expressing plasmid, although a slight but statistically significant inhibition of shmyc-mediated knockdown was found following treatment with tumor necrosis factor α (TNF-α), which is a representative inflammatory cytokine (Figure 3B). Next, in order to examine whether the results described above were found only when the U6 promoter was used as an shRNA-expressing promoter, we tested a plasmid containing an H1-promoter-driven shmyc-expression cassette (pHMH1-shmyc). Treatment with IFN-α significantly inhibited shmyc-mediated knockdown of the c-*myc*

IFN-α Stimulation Inhibited Dicer-Mediated Processing of shRNA to siRNA

Next, in order to examine the copy numbers of shRNA and the processing product, siRNA, northern blotting analysis was performed following transfection with shRNA-expressing plasmids. Copy numbers of shp53 and shmyc were increased by IFN-α stimulation in H1299 cells transfected with shp53- and shmyc-expressing
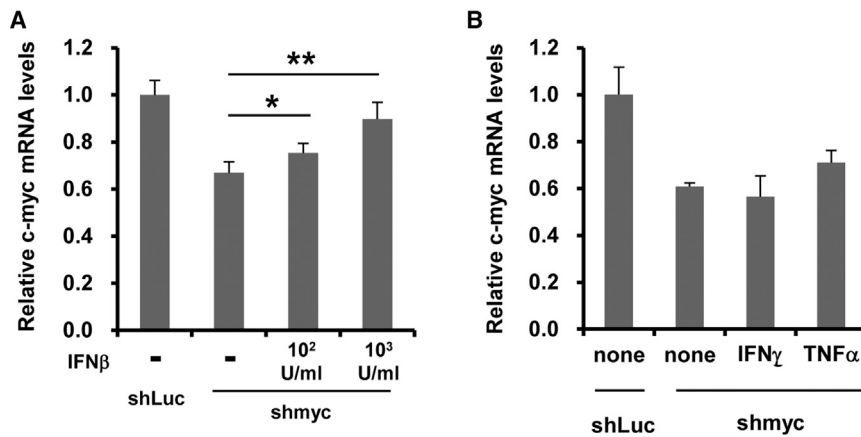
Figure 3. Efficiencies of shRNA-Mediated Knockdown following Exposure to IFN-β, IFN-γ, and TNF-α

(A) H1299 cells were transfected with pHMU6-shLuc or -shmyc, followed by treatment with recombinant IFN-β at 10^2 or 10^3 U/mL. After 48-hr incubation, c-*myc* mRNA levels in the cells were determined by qRT-PCR. (B) H1299 cells were similarly transfected, followed by treatment with recombinant IFN-γ and TNF-α at 5×10^3 U/mL and 100 ng/mL, respectively. After 48-hr incubation, c-*myc* mRNA levels in the cells were similarly determined. All data are expressed as the means ± SD (n = 4). *p < 0.05, **p < 0.01.

plasmids, respectively (Figure 6A). In contrast, IFN-α stimulation reduced the copy numbers of the sip53 and simyc, which were produced from shp53 and shmyc, respectively, by dicer-mediated processing (Figure 6A). qRT-PCR analysis also demonstrated that IFN-α treatment led to significant decreases in the copy numbers of simyc in H1299 cells following transfection with an shmyc-expressing plasmid (Figure 6b). Significant reductions in the copy numbers of si-myc and sip53 were also found in IFN-α-stimulated A549 cells following transfection with shmyc- and shp53-expressing plasmids, respectively (Figure S4). These results suggest that type I IFNs inhibit the processing of shRNA to siRNA.

Similarly to the processing of shRNA to siRNA, microRNA (miRNA) is also processed from precursor miRNA (pre-miRNA) to miRNA by dicer.[7] In order to examine whether type I IFNs also inhibit the processing of pre-miRNA to miRNA, we examined the copy numbers of the representative miRNAs let-7a, miR-27a, and miR-17 following IFN-α stimulation by northern blotting and qRT-PCR analyses and found that IFN-α stimulation hardly reduced the copy numbers of these miRNAs (Figure S5).

IFN-α Stimulation Did Not Alter the Expression Levels of Dicer, Ago2, or TRBP

Processing of shRNA is mediated by a complex composed of dicer, Ago2, and other proteins, including the dsRNA-binding protein TRBP.[7,8] In order to examine whether the expression levels of dicer, Ago2, and TRBP were altered after IFN-α stimulation, the expression levels of dicer, Ago2, and TRBP in IFN-α-stimulated cells were determined by qRT-PCR and western blotting analyses. Neither dicer nor Ago2 expression was altered by IFN-α stimulation at the mRNA or protein level (Figures 7A and 7B). TRBP protein levels were also not altered by IFN-α stimulation (Figure 7C). Recently, it was reported that Ago2 is modified by poly-ADP-ribose after viral infection, leading to a decrease of the RNAi activity;[9,10] however, IFN-α stimulation did not appear to elevate the levels of poly-ADP-ribosylated Ago2 in this study (Figure S6A). A previous study reported that phosphorylation of Ago2 inhibited RNAi activity.[11] Similar levels of mobility shift of Ago2 were observed in the presence and absence

of IFN-α by western blotting analysis using Phos-tag gels, indicating that the phosphorylation levels of Ago2 were comparable between the IFN-α-treated group and vehicle-treated group (Figure S6B). These results suggest that the expression levels of dicer, Ago2, and TRBP, which are representative components of the RNAi pathway, are not significantly affected by IFN-α stimulation.

PKR Had No Effect on Type I IFN-Mediated Inhibition of the Processing of an shRNA

dsRNA-dependent protein kinase (PKR) is rapidly upregulated by type I IFNs at the mRNA and protein levels (Figures S1A and S7A). TRBP, which supports dicer function, binds to PKR and functions as an inhibitor of PKR,[8] raising the possibility that PKR might capture TRBP and competitively impair its ability to process an shRNA following type-I-IFN-mediated upregulation of PKR. We evaluated the processing efficiencies of an shRNA to an siRNA in PKR-knockdown cells in order to examine the involvement of PKR in type I IFN-mediated inhibition of the processing of an shRNA. Comparable levels of sip53 were produced from shp53 in PKR-knockdown cells with or without IFN-α treatment (Figure S7B), indicating that siRNA-mediated PKR knockdown did not cancel the IFN-α-mediated inhibition of the processing of an shRNA. These results suggest that PKR has no effect on type I IFN-mediated inhibition of the processing of an shRNA.

IFN-α Stimulation Also Inhibited the Knockdown Efficiencies of an shRNA-Expressing Adenovirus Vector

The adenovirus (Ad) vector is a powerful framework for shRNA-mediated knockdown due to its superior transduction efficiencies.[12–14] We examined whether an shRNA-expressing Ad vector would induce type I IFN responses. Intravenous administration of an Ad vector expressing shLuc (Ad-shLuc) induced ~500-fold higher levels of expression of IFN-α in the spleen compared with administration of PBS (Figure 8A). In order to examine whether type I IFNs inhibit the knockdown efficiencies of Ad vectors expressing shRNA, A549 cells were transduced with an Ad vector expressing shp53 (Ad-shp53), followed by treatment with recombinant human IFN-α. The transduction efficiencies of the Ad vector were not
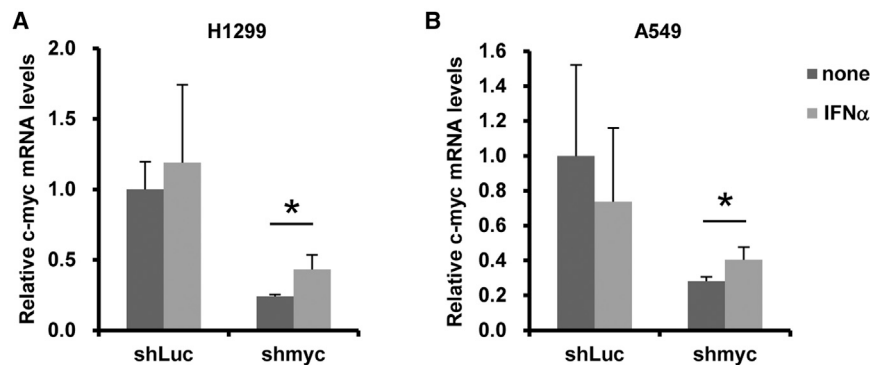
Figure 4. IFNα-Mediated Inhibition of Knockdown following H1 Promoter-Driven Expression of shRNA

(A and B) H1299 and A549 cells were transfected with pHMH1-shLuc or -shmyc, followed by treatment with recombinant IFN-α at 10^4 U/mL. After 48-hr incubation, c-*myc* mRNA levels in the cells were determined by qRT-PCR. All data are expressed as the means ± SD (n = 4). *p < 0.05.

altered by treatment with IFN-α (Figure S8). The copy numbers of the siRNA produced by processing from shp53 were significantly reduced following IFN-α stimulation in the cells following transduction with Ad-shp53 (Figure 8B). An ∼15% decrease in the knockdown efficiencies of Ad-shp53 was observed following treatment with IFN-α (Figure 8C). Ad-shp53-mediated knockdown of p53 protein was also inhibited by IFN-α stimulation (Figure 8D). These results suggest that type I IFNs also inhibit Ad-vector-expressing shRNA-mediated knockdown via inhibition of the processing of shRNA to siRNA.

DISCUSSION

The aim of this study was to investigate the effects of type I IFNs on shRNA-mediated RNAi and to provide insights that could lead to more efficient knockdown by shRNA. The results showed that type I IFNs inhibit the processing of shRNA to siRNA (Figure 4), leading to a reduction in the knockdown efficiencies of shRNA-expressing plasmids (Figures 1 and 2) and shRNA-expressing Ad vectors (Figure 6). On the other hand, synthetic siRNA-mediated knockdown of target genes was not altered by IFN-α stimulation (Figure 3).

This study demonstrated that IFN-α treatment leads to apparent inhibition of the processing of shRNA to siRNA (Figure 4). Several previous studies also suggested that the RNAi pathway was negatively regulated by innate immune responses. Wiesen et al. reported that treatment with type I IFN or transfection with poly(I:C), a synthetic analog of double-stranded RNA that strongly induces type I IFN production, suppressed dicer protein levels in the trophoblast cells; however, they did not examine whether the dicer expression levels were suppressed by type I IFNs in other types of mammalian cells.[15] We demonstrated that mRNA and protein levels of dicer were not significantly reduced by IFN-α stimulation in H1299 and A549 cells (Figures 5A and 5B). Type-I-IFN-mediated suppression of dicer expression might occur only in limited types of cells. Seo et al. demonstrated that infection with Sendai virus and herpes simplex virus leads to a reduction in mammalian RNAi activity via modification of Ago2 by poly-ADP-ribose and that this effect is mediated by RIG-I/ mitochondrial anti-viral signaling adaptor (MAVS)-dependent signaling.[10] The RIG-I-MAVS pathway is involved in type I IFN production following virus infection;[16] however, we demonstrated that

IFN-α stimulation did not significantly induce the ribosylation of Ago2 by poly-ADP (Figure 5C). This suggests that the modification of Ago2 by poly-ADP-ribose is triggered not by the type I IFN production pathway but by other downstream pathways via RIG-I-MAVS signaling.

Several studies have demonstrated that type I IFN responses are induced following introduction of siRNA and shRNA.[17,18] However, transfection with an siRNA did not also induce detectable levels of IFN responses in the cultured cells in this study (Figures 1C and 1D). Previous studies also demonstrated that introduction of siRNA alone did not induce type I IFN expression.[19,20] siRNA-induced type I IFN responses would be caused under restricted situations. On the other hand, introduction of an shRNA-expressing plasmid significantly induced type I IFN responses (Figures 1A, 1B, and 1E). The type I IFN responses induced by the shRNA-expressing vector might affect the knockdown efficiencies of shRNAs.

As shown in Figure S5A, processing of pre-miRNAs was hardly affected by IFN-α treatment. This might have been due to the high processing efficiency of pre-miRNAs to mature miRNAs. As shown in Figure S5A, pre-let-7a, -miR-27a, and -miR-17 were not significantly detected by Northern blotting analysis, whereas detectable levels of mature let-7a, miR-27a, and miR-17 were found. These results suggest that IFN-α did not sufficiently inhibit pre-miRNA processing under this condition and that the IFN-α-mediated inhibition of the processing of shRNA was different from that of pre-miRNA.

Viral and non-viral vectors expressing an shRNA are a promising agent for severe disorders. Various pre-clinical studies using shRNA-expressing vectors have been reported.[1–3] For example, several studies demonstrated that shRNAs against mRNAs of hepatitis B virus (HBV) and hepatitis C virus (HCV) efficiently inhibited the virus replication in culture cells and in mice.[21–23] On the other hand, treatment with pegylated IFN-α (PEG-IFN) is often chosen for therapy against persistent infection with HBV and HCV.[24–26] The combination of therapies with PEG-IFN and shRNA against HBV and HCV should be performed with caution, because PEG-IFN inhibits the therapeutic efficacy of shRNA against HBV and HCV. Viral and non-viral vectors expressing an shRNA have also been widely tested as potential anti-cancer agents.[27] On the other hand, IFN therapy has been demonstrated to mediate significant tumor regression and is now in clinical use.[28] The drug packaging insert for PEG-IFN
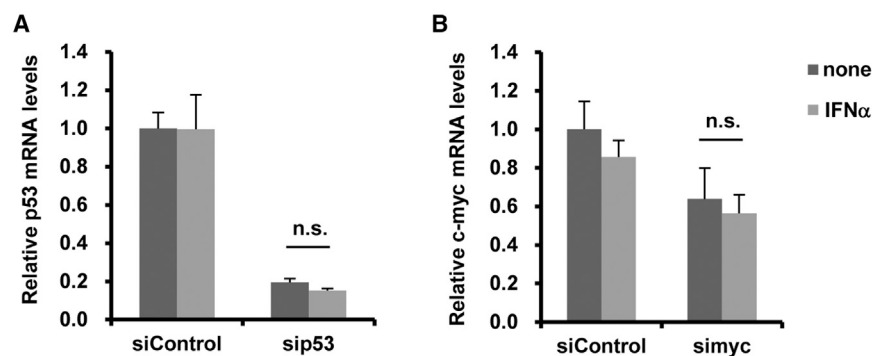
Figure 5. Knockdown Efficiencies of Chemically Synthetic siRNAs following IFN-α Stimulation

(A and B) A549 cells were transfected with siControl, sip53 (A), or simyc (B), followed by treatment with recombinant IFNα at 10^4 U/mL. After 48-hr incubation, c-*myc* and p53 mRNA levels in the cells were determined by qRT-PCR. All data are expressed as the means ± SD (n = 4).

(PEGINTRON, MSD) reported that ~10^3 U/mL of IFN-α was detected in the serum following repetitive administration of PEG-IFN in patients with HBV, HCV, or melanoma (http://database.japic.or.jp/pdf/newPINS/00050436.pdf). Pepinsky et al. also reported that following intravenous administration of PEG-IFN to rhesus monkeys, 10^3–10^4 U/mL PEG-IFN was observed by 24 hr post-administration.[29] In this study, the knockdown efficiencies of the shRNAs were reduced when cells were treated with IFN-α at more than 10^3 U/mL (Figure 2C). We should exercise care when applying shRNA-expressing vectors to patients undergoing an IFN therapy or other therapies associated with the production of type I IFNs.

We and other groups have demonstrated that Ad vectors have numerous advantages as a delivery vehicle of shRNA.[12–14] For example, Ad vectors can efficiently transduce dividing and non-dividing cells, and high titers of Ad vectors are easily obtained. However, in spite of their superior transduction profiles, especially in vivo, the knockdown efficiencies of shRNA-expressing Ad vectors are not higher than expected. One of the reasons for this fact is that VA-RNAs, which are small RNAs transcribed from the Ad genome, inhibit shRNA-mediated knockdown.[14,30] Our present investigations reveal another reason: the type-I-IFN-mediated inhibition of shRNA processing. A number of previous reports demonstrated that the administration of Ad vectors can trigger severe innate immune responses associated with high levels of production of type I IFNs.[5,31] Type I IFN production following treatment with an Ad vector leads to the inhibition of Ad-vector-mediated RNAi. In order to avoid the potential inhibition of Ad-vector-mediated RNAi efficacy by type I IFNs, a promising approach would be to deliver shRNAs using Ad vectors that were engineered to reduce Ad-vector-induced innate immune responses. Thus, a helper-dependent Ad (HD-Ad) vector, in which all the Ad genes are deleted, would be promising as an shRNA delivery vector, since an HD-Ad vector has been shown to attenuate innate and adaptive immune responses.[32]

In this study, IFN-α-mediated inhibition of the knockdown efficiencies of shmyc was statistically significant, but not larger than expected based on the level of IFN-α-mediated reduction in simyc copy numbers (Figure 2A). More than 50% reduction in simyc copy

numbers was observed after IFN-α treatment (Figure 6B). Even if the simyc copy numbers were in fact reduced to this degree, sufficient levels of simyc for knockdown of c-*myc* were produced in IFN-α-stimulated cells. In addition, the effects of type I IFNs on shRNA-mediated RNAi differ between cell types, target genes, and delivery vectors.

In summary, we have demonstrated that type I IFNs inhibit shRNA-mediated RNAi via inhibition of the processing of shRNA to siRNA. This study suggests that it is important to avoid IFN responses for the efficient knockdown of target genes not only in basic research but also in medical studies.

MATERIALS AND METHODS

Cells and Mice

HEK293 (a transformed embryonic kidney cell line) and A549 (a human lung adenocarcinoma epithelial cell line) cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), streptomycin (100 μg/mL), and penicillin (100 U/mL). H1299 (a non-small cell lung carcinoma cell line) cells were cultured in RPMI 1640 supplemented with 10% FBS, streptomycin (100 μg/mL), and penicillin (100 U/mL). Female C57BL/6 mice aged 6 weeks were obtained from Nippon SLC. The mouse experimental procedures used in this study were approved by the Animal Experimentation Committee of Osaka University and performed in accordance with the institutional guidelines for animal experiments at Osaka University.

Reagents

Recombinant human IFN-α, IFN-β, and IFN-γ were purchased from PBL Interferon Source. Recombinant human TNF-α was purchased from Invivogen.

Plasmids

The plasmids expressing shRNA against luciferase (shLuc), p53 (shp53), and c-*myc* (shmyc) were previously constructed.[12,33] Briefly, in order to insert the sequences that encode shLuc, shp53, and shmyc, the corresponding oligonucleotides were synthesized, annealed, and cloned under the human U6 and H1 promoter sequences in the two types of plasmids, pHM5-U6 and -H1,[12,33] resulting in pHM-U6-shLuc, -U6-shp53, -U6-shmyc, pHM-H1-shLuc, -H1-shp53, and -H1-shmyc. pHMCMV-GFP, a plasmid expressing GFP, was also previously constructed.[34]
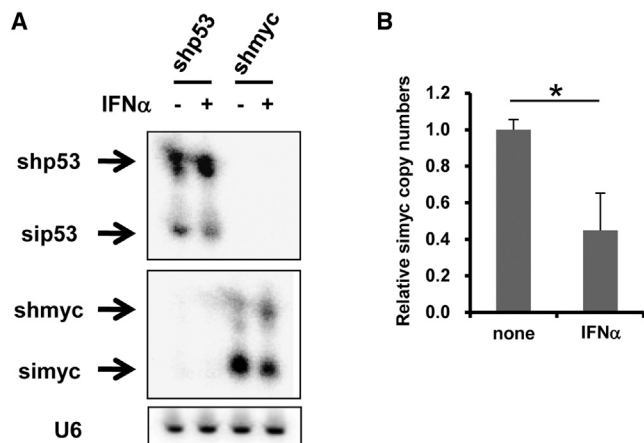
A

B

Figure 6. Processing of shRNA to siRNA in IFN-α-Treated Cells

(A and B) H1299 cells were transfected with pHMU6-shp53 or -shmyc, followed by treatment with recombinant IFN-α at 10^4 U/mL. After 48-hr incubation, shRNA and siRNA copy numbers in the cells were determined by northern blotting analysis (A) and qRT-PCR (B). Data are expressed as the means ± SD (n = 4). *p < 0.05.

Viruses

The Ad vectors expressing shLuc and shp53 under the human U6 promoter (Ad-shLuc and -shp53, respectively) were previously prepared by an in vitro improved ligation method.[14,35,36] The Ad vector expressing GFP (Ad-GFP) was also previously prepared.[34] These Ad vectors were amplified in HEK293 cells and purified by two rounds of cesium-chloride-gradient ultracentrifugation, dialyzed, and stored at −80°C.[35] The virus particles (VPs) were determined by a spectrophotometric

method.[35] Determination of infectious units (IFU) was accomplished using an Adeno-X Rapid Titer Kit (Clontech Laboratories).

Transfection with siRNAs and shRNA-Expressing Plasmids

Control siRNA (siControl) was purchased from QIAGEN (Allstars Negative Control siRNA, QIAGEN). sip53, simyc, and siRNA against PKR (siPKR) were obtained from Gene Design. The target sequences of sip53, simyc, and siPKR were 5′-ctacttcctgaaaacaacg-3′, 5′-gatgaggaagaaatcgatg-3′, and 5′-ggtgaaggtagatcaaaga-3′, respectively. Efficient knockdown of PKR after transfection with siPKR was previously demonstrated.[37]

Cells were transfected with siRNAs at 50 nM or shRNA-expressing plasmids at doses identical to those of the control plasmids using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions. After 5-hr incubation, cells were treated with recombinant cytokines, including IFN-α, and incubated for a total of 48 hr. Expression levels of target genes were evaluated as described below.

qRT-PCR Analysis

Cells were transfected with siRNAs or shRNA-expressing plasmids or were transduced with Ad vectors at the indicated titers. After 5-hr incubation, the cells were treated with recombinant IFN-α and incubated for a total of 48 hr. Total RNA was isolated from the cells using ISOGEN (Nippon Gene). cDNA was synthesized using 500 ng total RNA with a Superscript VILO cDNA synthesis kit (Life Technologies). Real-time RT-PCR analysis was performed using Fast SYBR Green Master Mix (Life Technologies) and a StepOnePlus real-time
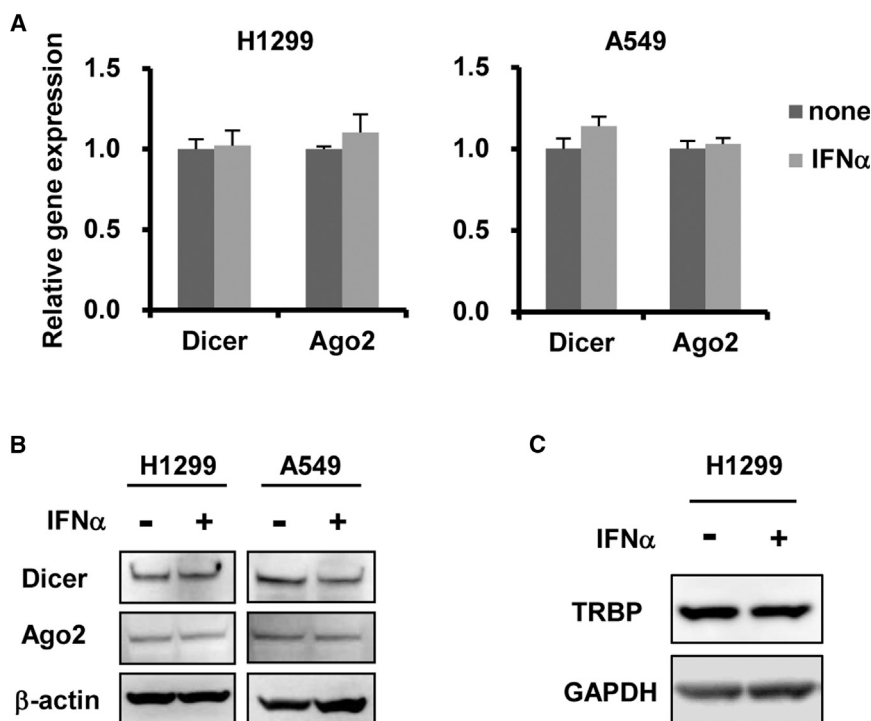
A

B

C

Figure 7. Dicer and Ago2 Expression Levels following IFN-α Stimulation

H1299 and A549 cells were treated with recombinant IFN-α at 10^4 U/mL. After 48-hr incubation, dicer and Ago2 mRNA (A) and protein (B) levels in the cells were determined by qRT-PCR and western blotting analysis, respectively. (C) TRBP protein levels in the cells were determined by western blotting analysis. The qRT-PCR data are expressed as the means ± SD (n = 4).

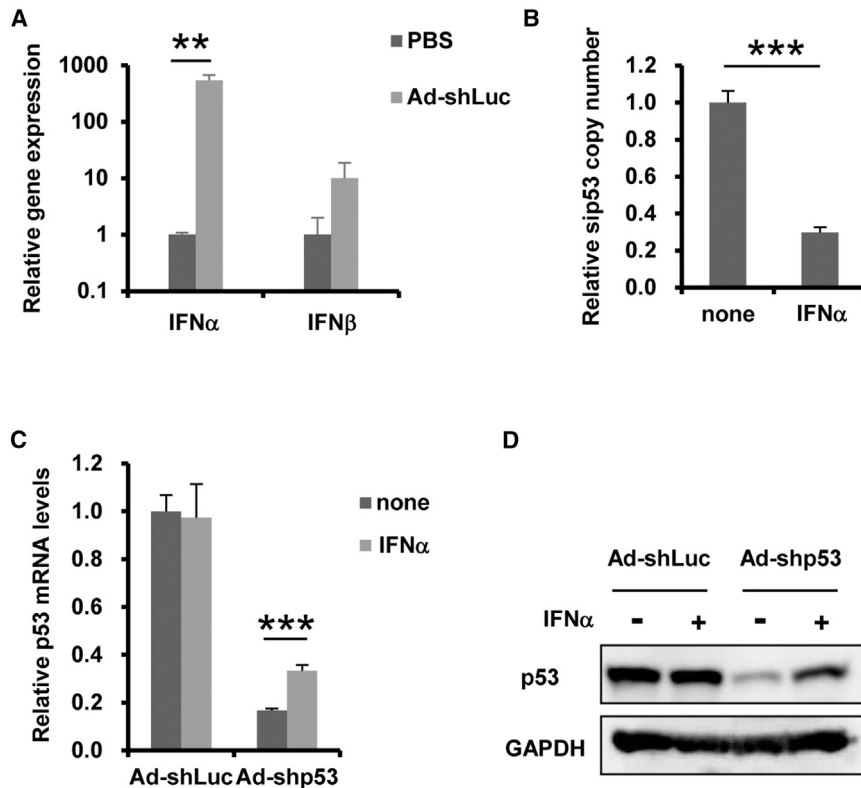**Figure 8. Inhibition of Ad Vector-Expressing shRNA-Mediated Knockdown by IFN-α Stimulation**

(A) 4×10^8 IFU Ad-shLuc was intravenously administered to C57BL/6 mice. 3 hr after the administration, IFN-β mRNA levels in the spleen were determined by qRT-PCR. Data are expressed as the means ± SE (n = 3–4). (B–D) A549 cells were transduced with Ad-shp53, followed by treatment with recombinant IFN-α at 10^4 U/mL. After 48-hr incubation, the copy numbers of sip53 in the cells were determined by qRT-PCR (B). After 48-hr incubation, p53 mRNA (C) and protein (D) levels in the cells were determined by qRT-PCR and western blotting analysis, respectively. Data are expressed as the means ± SD (n = 4). **p < 0.01, ***p < 0.001.

PCR system (Life Technologies) as previously described.[38] The data were normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels. Sequences of the primers used in this study are described in Table S1.

siRNA copy numbers were determined by qRT-PCR using Mir-X miRNA First-Strand Synthesis and SYBR qRT-PCR (Clontech-Takara) and primers specific for the sip53 and simyc. Sequences of the primers used in this study are described in Table S1.

Determination of Type I IFN Expression Levels In Vivo

100 μg shLuc-expressing plasmid (pHMU6-shLuc) and 4×10^8 IFU Ad-shLuc were intramuscularly and intravenously administered to mice, respectively. Total RNA was extracted from the muscles and spleens 3 hr after intramuscular and intravenous administration, respectively. Type I IFN mRNA levels were determined by qRT-PCR analysis.

Western Blotting Analysis

Western blotting analysis was performed as previously described.[14] Briefly, whole-cell extracts were prepared and electrophoresed on 10% SDS-polyacrylamide gels under reducing conditions, followed by electrotransfer to polyvinylidene fluoride (PVDF) membranes (Millipore). After blocking with 5% skim milk prepared in TBS-T (Tween-20, 0.1%), the membrane was incubated with primary antibodies (Table S2), followed by incubation in the presence of horseradish peroxidase (HRP)-labeled anti-rabbit or anti-mouse immunoglobulin G (IgG)

antibody (Cell Signaling Technology). Phosphorylation levels of Ago2 were determined by western blotting analysis using Phos-tag polyacrylamide gels (Wako), as previously described.[39]

Northern Blotting Analysis

Total RNA was extracted from the cells with ISOGEN (Nippon Gene). 10 μg total RNA per lane was loaded onto 15% polyacrylamide denaturing gel. After electrophoresis, bands of RNA were transferred to Hybond-N+ membranes (Roche). The membranes were then probed with ^{32}P-labeled synthetic oligonucleotides that were complementary to the sequence of sip53, simyc, let-7a, or human U6 small nuclear RNA (sip53: 5′-gactccagtggtaatctac-3′; simyc: 5′-gatgaggaagaaatcgatg-3′; let-7a: aactatacaacctactacctca; U6: 5′-tgctaatcttctctgtatcgt-3′).

Determination of Poly-ADP-Ribosylation Levels of Ago2

Whole-cell extracts were prepared from IFN-α-stimulated cells, and Ago2 was immunoprecipitated using a mouse anti-Ago2 antibody (Table S2) and a microRNA Isolation Kit (human Ago2) (Wako). After immunoprecipitation, poly-ADP-ribosylation levels of the immunoprecipitated Ago2 were determined by western blotting analysis using a mouse anti-pADPr antibody (Table S2).

Statistical Analysis

Statistical significance was determined using the Student's t test. Data are presented as the means ± SD or SE.

SUPPLEMENTAL INFORMATION

Supplemental Information includes eight figures and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.omtn.2016.12.007.

AUTHOR CONTRIBUTIONS

M.M. designed and performed the experiments, analyzed data, and wrote the manuscript; F.S. designed and supervised the projects, analyzed data, and wrote the manuscript; K.W. and K.T. supported

the experiments; M.T. analyzed data; and H.M. supervised the projects, interpreted data, and wrote the manuscript.

CONFLICTS OF INTEREST
The authors declare no competing financial interests.

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