activity, and they were genetically stable in bacterial cells. We also attempted to maximize suppressive activity by constructing a loop based on the sequence of micro-RNA and several other parameters (22). Furthermore, we constructed our own algorithm for the prediction of target sites of siRNA expression vectors (12). Our algorithm ensures a high probability of suppressive activity. Our final optimized siRNA expression vector, which included a hairpin-type sequence, had a U6 promoter, mutations in the sense strand, and an optimized loop sequence. Our vector system allowed the generation of genetically stable and very active siRNA expression libraries (12, 22–26). The vectors (the results are shown in Fig. 1*B*) were constructed according to this strategy with 50-bp stems in order to facilitate comparison between the interferon responses caused by two different dsRNAs (synthetic *versus* vector-driven). Other siRNA expression vectors used in this study had "regular" 21-bp stems.

Screening of Genes with the Library of siRNA Expression Vectors—To analyze dsRNA-induced apoptosis comprehensively by using our library of siRNA expression vectors, we induced apoptosis by using dsRNA (poly(I-C)). To optimize conditions for screening, we examined two parameters for the induction of apoptosis, the mode of exposure of cells to poly(I-C), and the method used for transfection. After optimization of transfection conditions, we were able to induce cell death effectively by using small amounts of poly(I-C). To confirm that cell death induced by poly(I-C) was apoptosis, we performed TUNEL assays (Fig. 1*C*). Fluorescence microscopy revealed aggregating nuclei (Fig. 1*C, arrowheads*) only in cells treated with poly(I-C), and poly(I-C)-treated cells were TUNEL-positive. The microscopic images of cells revealed features typical of apoptosis. In addition, the cleavage of PARP, as detected by Western blotting, indicated that poly(I-C) did actually induce apoptosis in HeLa S3 cells (Fig. 1*D*).

The activation of PKR is an important feature of dsRNA-induced apoptosis. Therefore, we selected PKR as a positive control. Fig. 2 shows stained cells, microscopic images, and the results of Western blotting analysis for siRNA expression vectors directed against two target sites in the gene for PKR. These observations confirmed that our siRNA expression vectors were able to suppress the expression of an endogenous gene and that our screening method should work in practice. For our screening analysis, we selected 241 specific genes, including genes for pro-apoptotic proteins, kinases, transcription factors, and various other proteins that appear to be related to apoptosis but whose functions are poorly understood. We defined at least two target sites in each gene to check the validity of our choice of targets.

Identification of Genes That Inhibit dsRNA-induced Apoptosis—We transfected HeLa S3 cells with our siRNA expression vectors (Fig. 3) by using one vector per well, and 36 h after transfection, we selected cells for resistance to puromycin by exposure to the drug for 24 h. After puromycin selection, cells were counted and divided equally into three wells of a 48-well plate. Apoptosis was induced by transfection with dsRNA (poly(I-C)). Twenty four hours after induction of apoptosis, we examined the effects of siRNA expression vectors, and we identified several siRNA expression vectors (Table I), which inhibited the dsRNA-induced apoptosis, such as *S3* in Fig. 3. We categorized the genes that inhibited the dsRNA-induced apoptosis to four groups, namely members of the caspase family, members of the Bcl-2 family, kinases, and others. Members of the Bcl-2 family and kinases other than PKR have not been implicated previously as a part of the network that resulted in dsRNA-induced apoptosis.

TABLE I
Genes identified using siRNA expression vectors that inhibited dsRNA-induced apoptosis

Group	Gene product	Accession no.
Caspase family	Caspase 8	NM_001228
	Caspase 3	NM_004346
	Caspase 9	NM_032996
	Caspase 10	NM_001230
Bcl-2 family	BAX	NM_138761
	Bid	NM_001196
Kinase family	PKR	NM_002759
	MAP3K1	XM_042066
	MAP3K2	NM_006609
	MAP3K6	NM_004672
	MAP2K1	NM_002755
	MAP2K2	NM_030662
	MAP2K4	NM_003010
	MAPK1	NM_002745
	MAPK9	NM_002752
	MAPK13	NM_002754
	MAPK14	NM_139013
	STK3	NM_006281
	MST2 (STK3)	NM_002737
	MLK4	NM_032435
	DAPK2	NM_014326
	RIPK2	NM_003821
	RIPK3	NM_006871
Others	CDC6	NM_001254
	DEDD	NM_004216
	VDAC	NM_003374
	Apaf-1	NM_001160
	Cytochrome *c*	NM_018947

Gil *et al.* (16) described how dsRNA activates PKR via the binding of the dsRNA to two dsRNA-binding motifs. They also showed that PKR-mediated apoptosis involves the FADD-caspase 8 pathway (16). In addition to the genes for the classical mediators of dsRNA-induced apoptosis, namely PKR, caspase 8, and caspase 3, we identified many interesting genes that have not been confirmed previously to play roles in dsRNA-induced apoptosis. The involvement of *Bid*, *BAX*, VDAC, cytochrome *c*, caspase 9, and *Apaf-1* suggests that the mitochondrial apoptotic pathway might play an important role in dsRNA-induced apoptosis. Moreover, identification of genes for *ERK2*, JNK/SAPK, p38 *MAPK*, *MAP2Ks*, and *MAP3Ks* suggests that MAPKs might be significant participants in dsRNA-induced apoptosis. Several factors, which we identified using our vector library, might work in concert with each other and represent various networks.

Mitochondrial Apoptotic Pathway and dsRNA-induced Apoptosis—The mitochondrial apoptotic pathway is one of the major pathways to apoptosis and is initiated by various apoptosis-inducing signals. When an appropriate apoptotic signal reached the mitochondria, Bcl-2 proteins mediated an increase in membrane permeability (27), and cytochrome *c* was released from the mitochondria to the cytoplasm. The released cytochrome *c* formed a complex with Apaf-1, and this complex activated caspase 9 and prolonged the apoptotic stimulus. The mediator between the apoptotic signal and the mitochondria was generally a Bcl homology 3 domain-containing pro-apoptotic member of the Bcl-2 family. Bid is also a member of the Bcl-2 family, and its expression is induced by caspase 8 (28). Only a few reports (29, 30) have noted a relationship between the Bcl-2 family and the IFN response on the one hand or between caspase 9 and PKR on the other hand. Moreover, there has been no detailed analysis of the association between the mitochondrial apoptotic pathway and dsRNA-induced apoptosis.

In our screening study, many siRNA expression vectors targeted to genes related to the mitochondrial pathway were able

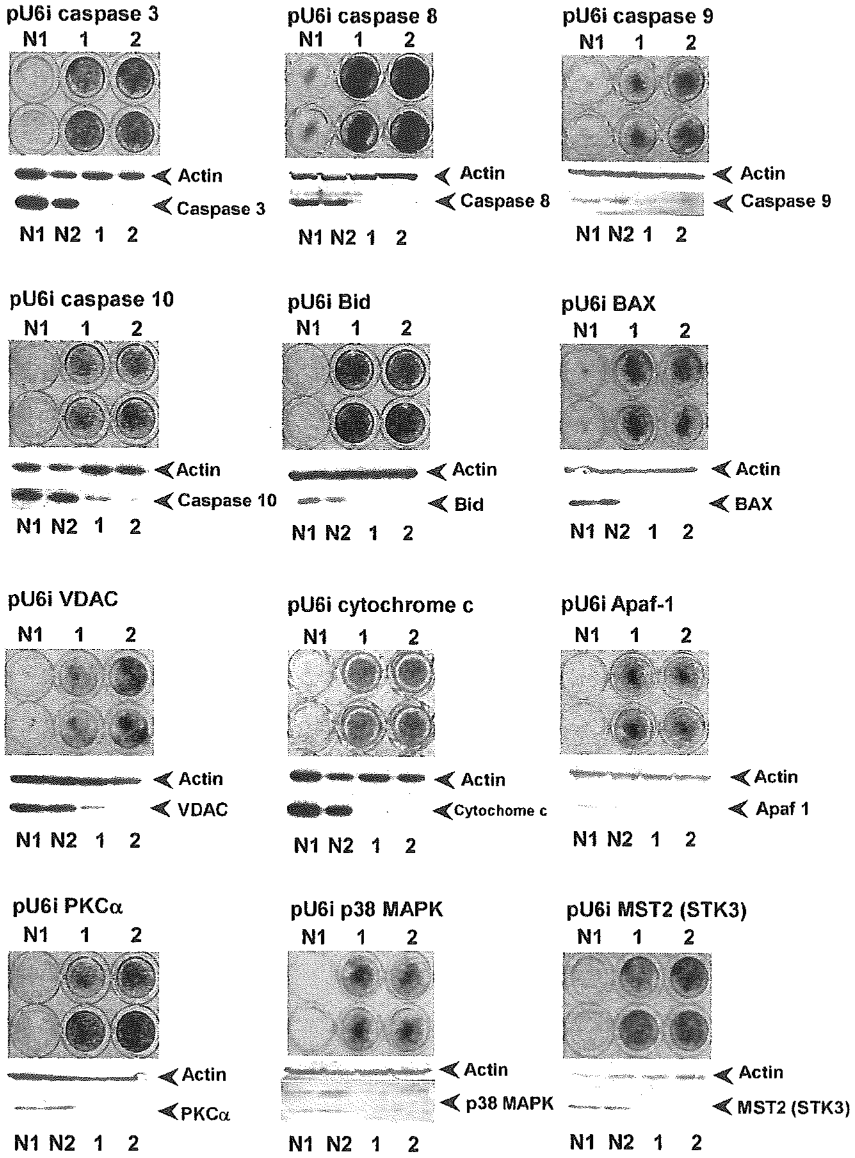

FIG. 4. **Cells in wells and Western blotting analysis of cells after transfection with specific siRNA expression vectors.** The target of each vector is indicated. The experiments were performed under the same conditions as those shown in Fig. 2. *N1,* negative control 1, siRNA expression vector targeted against *Renilla* luciferase; *N2,* negative control 2, siRNA expression vector targeted against HCV. *pU6*i indicates the empty siRNA expression vector.

to inhibit the dsRNA-induced apoptosis. As shown in Fig. 4, siRNA expression vectors against genes for *Bid*, *BAX*, VDAC, caspase 9, *Apaf-1,* and cytochrome *c* appeared to inhibit dsRNA-induced apoptosis. The suppressive activity for each vector was confirmed by Western blotting, as demonstrated also in Fig. 4. All our results together suggest a role for the mitochondrial pathway in dsRNA-induced apoptosis.

To verify that the mitochondrial apoptotic pathway is relevant to dsRNA-induced apoptosis, we performed Western blotting of cytoplasmic fractions isolated from HeLa S3 cells from 0 to 18 h after the induction of apoptosis (Fig. 5*A*). Western blotting indicated that cytochrome *c* was released from the mitochondria about 18 h after transfection with poly(I-C). This result demonstrated that the mitochondrial pathway was operative in dsRNA-induced apoptosis. Next, we examined whether the siRNA expression vectors directed against mitochondrion-related genes could block the release of cytochrome *c* in response to poly(I-C). As shown in Fig. 5*B*, when we "knocked down" genes for *Bid*, *BAX,* or VDAC, the release of cytochrome *c* from mitochondria was suppressed. These results demonstrated the participation of the mitochondrial apoptotic pathway in dsRNA-induced signaling. Moreover, we were surprised that "knock down" of the genes for PKR and caspase 8 did not affect the release of cytochrome *c* from mitochondria. Taken together, these results indicated that dsRNA-induced apoptosis required the mitochondrial pathway, which is independent of the pathway that includes PKR and caspase 8.

MAPK Superfamily and dsRNA-induced Apoptosis—MAPK cascades are ubiquitous signal-transduction modules that are involved in numerous intracellular phenomena (31). There are at least three different MAPK cascades, which include ERK1/2, JNK (also known as SAPK), and p38 MAPK. It is generally accepted that ERK1/2 signaling is involved in cell proliferation (32), whereas the JNK/SAPK and p38 MAPK pathways are activated preferentially by extracellular stresses (33), such as oxidative stresses, radiation, UV light, and tumor necrosis factor-α. These latter pathways play roles in the triggering of apoptotic signals (34, 35).

As shown in Table I, we identified many siRNA expression vectors, targeted against the MAPK superfamily in particular, against genes for *MAPK1*, *MAPK9*, *MAPK13*, and *MAPK14* and several types of *MKK* and *MEKK* that interfered with dsRNA-induced apoptosis. Thus, MAPK cascades appeared to be critical for dsRNA-induced apoptosis. There are several reports of the activation of p38 MAPK and JNK/SAPK by viral infection or dsRNA. Chu *et al.* (36) described two dsRNA-mediated pathways, one of which was the PKR-dependent NF-κB pathway, and the other was the PKR-independent JNK/SAPK pathway.

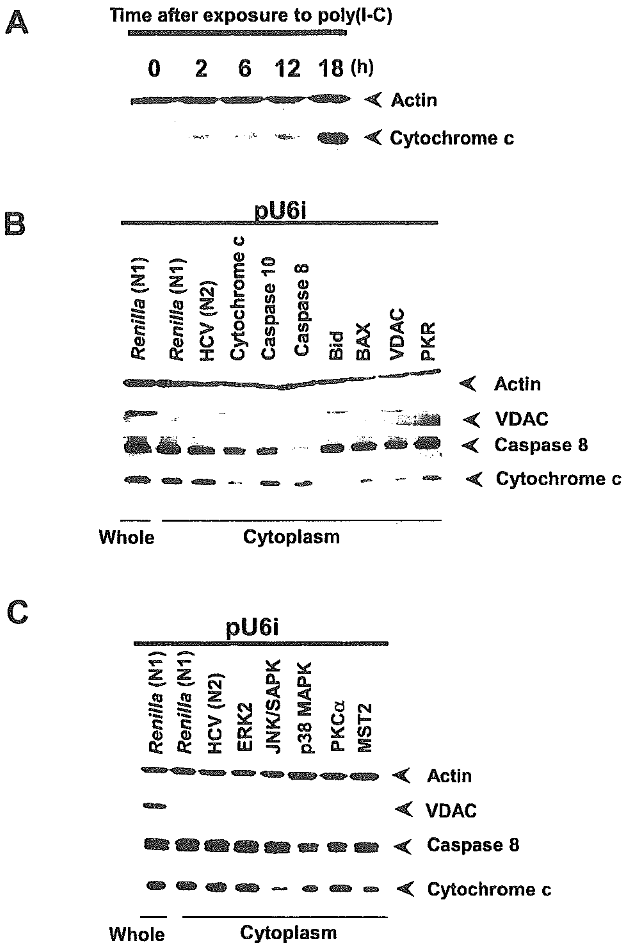

FIG. 5. *A*, time course of the release of cytochrome *c* after the transfection of cells with 0.5 μg of poly(I-C). *B*, analysis of the pathway upstream of the release of cytochrome *c*. HeLa S3 cells were transfected with specific siRNA expression vectors, with the indicated targets, which inhibited dsRNA-induced apoptosis. After selection with puromycin, cells were treated with poly(I-C). Eighteen hours after the induction of apoptosis, cells were harvested, and the release of cytochrome *c* was examined. *Whole* refers to nonfractionated whole-cell extracts. To verify the preparation of the cytoplasmic fraction, Western blotting was performed with VDAC-specific antibody. *N1*, negative control 1, siRNA expression vector targeted against *Renilla* luciferase; *N2*, negative control 2, siRNA expression vector targeted against HCV. *C*, similar analysis of the kinase pathway upstream of the release of cytochrome *c*.

Other researchers have noted that dsRNA signaling stimulates the activities of p38 MAPK and JNK/SAPK, with stimulation being dependent on PKR and RNase L (37). Goh *et al.* (38) reported that PKR is required for activation of p38 MAPK. However, to our knowledge, a role for ERK1/2 in dsRNA-induced apoptosis has not been described previously. It is generally accepted that the activation of ERK1/2 enhances cell survival (32). However, in the present study, the siRNA expression vector directed against *ERK2* strongly inhibited dsRNA-induced apoptosis. Therefore, we focused particular attention on the pro-apoptotic effects of ERK2 in dsRNA-induced apoptosis.

ERK2 Acts as a Pro-apoptotic Factor in dsRNA-induced Apoptosis—To confirm the role of ERK2 in dsRNA-induced apoptosis, we examined the activation, by phosphorylation, of ERK1/2 in cells after the introduction of dsRNA (poly(I-C); Fig. 6*A*). We examined the activation of JNK/SAPK over the same time period to validate our assay. Fig. 6*A* shows the biphasic increases in levels of phospho-ERK1/2 and phospho-JNK/SAPK with time. The mock-transfected control cells did not show any evidence of the activation of JNK/SAPK or of ERK1/2 (data not shown). Thus, it appeared that ERK1/2 was indeed activated by treatment of cells with dsRNA. Next, we confirmed the suppressive activity of the siRNA expression vectors against genes for JNK/SAPK and *ERK2* (Fig. 6*B*). Fig. 6*B* also shows the inhibition of dsRNA-induced apoptosis by siRNA expression vectors directed against *ERK2*. As shown in Fig. 6*C*, no phosphorylated ERK1/2 was detected in cells in the absence of treatment with poly(I-C). The suppression of the expression of ERK2 clearly decreased the level of phosphorylated ERK1/2 specifically, whereas suppression of the expression of JNK/SAPK and p38 MAPK did not affect the phosphorylation of ERK1/2. These results indicated the pro-apoptotic role of ERK2 in dsRNA-induced apoptosis.

To confirm this result, we performed the rescue experiment with an ERK2 expression vector (Fig. 6*D*), named pcEF9-ERK2-Rescue, which contains three point mutations, to avoid degradation by the ERK2-siRNA (pU6i ERK2), at the target sequence of siRNA expression vector without conversion of amino acids. We established the stable cell lines that overexpress pcEF9-ERK2-Rescue or pcEF9. These cell lines were transfected with the siRNA expression vector targeted against *Renilla* luciferase (negative control) or ERK2 (pU6i ERK2). Twenty four hours after transfection, cells were selected with puromycin for 24 h. We then induced apoptosis by transfection of poly(I-C). In the control cell line, which was transfected with the empty vector (pcEF9), depletion of ERK2 by pU6i ERK2 resulted in the blockage of apoptosis (Fig. 6*D, lower left*). In contrast, the ERK2-Rescue stable cell line, which is resistant to pU6i ERK2, rescued the phenotype of apoptosis. Concomitant with the rescue of apoptosis, the expression level of ERK2 was rescued in the ERK2-Rescue stable cell line (Fig. 6*D, lower right*). These results clearly demonstrate that ERK2 acts as a pro-apoptotic factor in dsRNA-induced apoptosis.

Identification of Genes That Function Upstream of ERK2—In our next experiments, we tried to identify factors located upstream of ERK2. After suppression of several selected genes that inhibited dsRNA-induced apoptosis, we induced apoptosis by transfection of cells with poly(I-C). As shown in Fig. 7, determination of levels of phospho-ERK1/2 by Western blotting demonstrated that phosphorylation of ERK1/2 was suppressed when expression of genes for *MST2* or PKCα was suppressed. To our surprise, activation of ERK1/2 was not inhibited by suppression of expression of PKR or of caspase 8. These results indicated that the ERK1/2 pro-apoptotic pathway was independent of the PKR-caspase 8 pathway and was instead mediated by MST2 and PKCα.

MST2 belongs to the family of SPS1/STE20-like kinases (39), and its expression is responsive, for example, to cell stress, various cytokines, and oxidative stress. It has been proposed that members of the MST subfamily might activate caspases via the JNK/SAPK pathway (40). Our results demonstrated that MST2 played a pro-apoptotic role by activating ERK1/2 in dsRNA-induced apoptosis. As for PKCα, it has been proposed that this enzyme is involved in the activation of ERK1/2, p38 MAPK, and JNK/SAPK (41). In view of these observations, it seems appropriate that suppression of the expression of PKCα inhibited the phosphorylation of ERK1/2. However, it has also been suggested that the activation of p38 MAPK and JNK/SAPK via PKCα might induce apoptosis, with ERK1/2 being involved in the inhibition of apoptosis. Our observations suggest a pro-apoptotic role for PKCα in dsRNA-induced apoptosis. Thus, both PKCα and MST2 appear to be involved, together with ERK2, in pro-apoptotic signal transmission.

Three Independent Pathways in dsRNA-induced Apoptosis—Here we identified several interesting enzymes that have not been associated previously with dsRNA-induced apoptosis. We also identified the connections among these enzymes and cat-

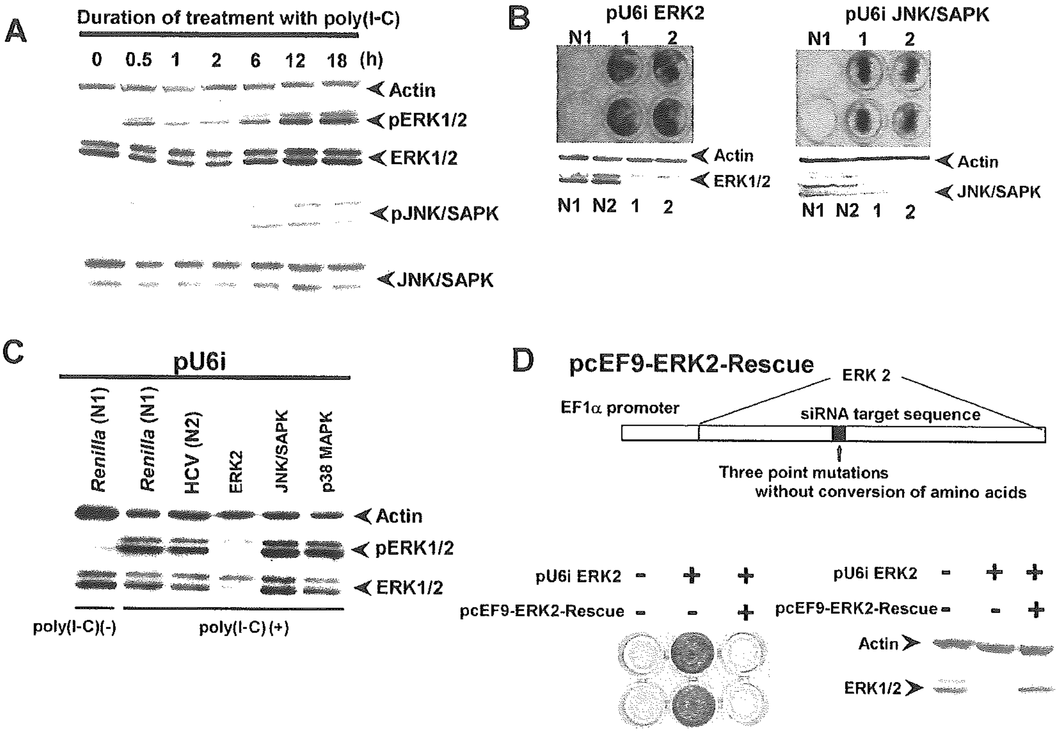

FIG. 6. *A*, time course of the phosphorylation of ERK1/2 and JNK/SAPK after treatment of cells with poly(I-C). *pERK1/2*, phosphorylated ERK1/2; *pJNK/SAPK*, phosphorylated JNK/SAPK. *B*, inhibition of dsRNA-induced apoptosis by siRNA expression vectors against genes for ERK1/2 and JNK/SAPK. *C*, the effects of ERK2, JNK/SAPK-, and p38 MAPK-targeted siRNA expression vectors on the activation of ERK1/2. *Poly(I-C)(−)* indicates the mock-transfected control in which no dsRNA (poly(I-C)) was introduced. *D*, the construct of ERK2 expression vector named pcEF9-ERK2-Rescue, in which three nucleotides were mutated at the target sequence of parental pU6i ERK2, without conversion of amino acids. The phenotypes after the induction of apoptosis were represented by the photographs (*lower left*). The anti-apoptotic phenotypes of pU6i ERK2-transfected cells were rescued by the introduction of the pcEF9-ERK2-Rescue vector. The Western blotting was performed to confirm that the transfection of pcEF9-ERK2-Rescue could rescue the expression of ERK2 from the degradation of endogenous ERK2 mRNA by the pU6i ERK2 (*lower left*). The *left lane* represents protein of the cells, which were transfected with negative control siRNA expression and pcEF9 vectors. The *middle lane* represents protein of the cells, which were transfected with pU6i ERK2 and pcEF9 vectors. The *right lane* represents protein of the cells, which were transfected with pU6i ERK2 and pcEF9-ERK2-Rescue.

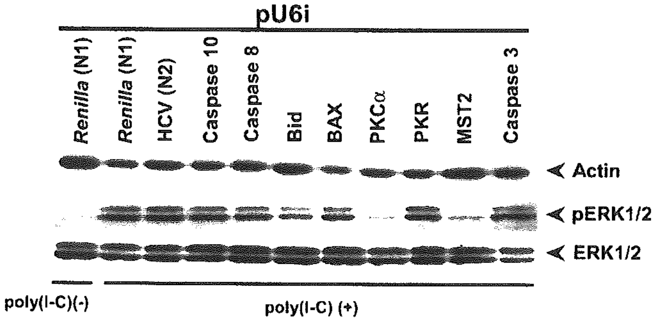

FIG. 7. **Analysis of the pathway upstream of the activation of ERK1/2.** *Poly(I-C)(−)* indicates the mock-transfected control in which no dsRNA (poly(I-C)) was introduced. The siRNA expression vectors targeted against PKCα and MST2 inhibited the activation of phosphorylated ERK1/2 by the introduction of dsRNA (poly(I-C)).

egorized them to two groups: enzymes in the mitochondrion-related apoptotic pathway, and enzymes in the MAPK pathway. As shown in Fig. 5*C*, we examined the connection between the mitochondrial pathway and the MAPK pathway. After suppression of the expression of ERK2, p38 MAPK, and JNK/SAPK, we monitored the releasing of cytochrome *c* from mitochondria by Western blotting.

Only JNK/SAPK-suppressed cells failed to release cytochrome *c* from their mitochondria, whereas suppression of the expression of ERK2 and p38 MAPK had no effect on the releasing of cytochrome *c*. These results demonstrated that JNK/SAPK acted upstream of the mitochondrial pathway. In addition, as shown in Fig. 5*B*, suppression of the expression of PKR and caspase 8 did not influence the release of cytochrome *c*. These results indicated that the pathway from JNK/SAPK to mitochondria is independent of the classical PKR-caspase 8 pathway.

As described above, the ERK2-dependent pro-apoptotic pathway was mediated by MST2 and PKCα and was unrelated to the PKR and JNK/SAPK pathways. We can summarize our data as depicted by the model of the mechanism of dsRNA-induced apoptosis that is shown in Fig. 8. There appear to be at least three independent pathways, all of which appear to be necessary for the induction of apoptosis by dsRNA.

DISCUSSION

By using our library of siRNA expression vectors, we have identified many genes that are associated with dsRNA-induced apoptosis. We selected at least two target sites in each target mRNA by using our original algorithm, and each of the two target sites was often sufficient for suppression of the target mRNA. The average relative efficiency of suppression of endogenous genes was more than 80%, as a result of the use of optimized vectors.

Our screening revealed the unexpected pro-apoptotic role of ERK2 in dsRNA-induced apoptosis. It was proposed previously that ERK1/2 delivers a survival signal, which counteracts the pro-apoptotic effects of the activation of JNK/SAPK and p38 MAPK. Thus we were surprised that ERK2 was implicated in the dsRNA-induced apoptosis, because ERK1/2 has typically been associated with cell survival (42). However, Murray *et al.* (43) reported that ERK1/2 had pro-apoptotic effects in hippocampal neurons, and there are several reports suggesting a

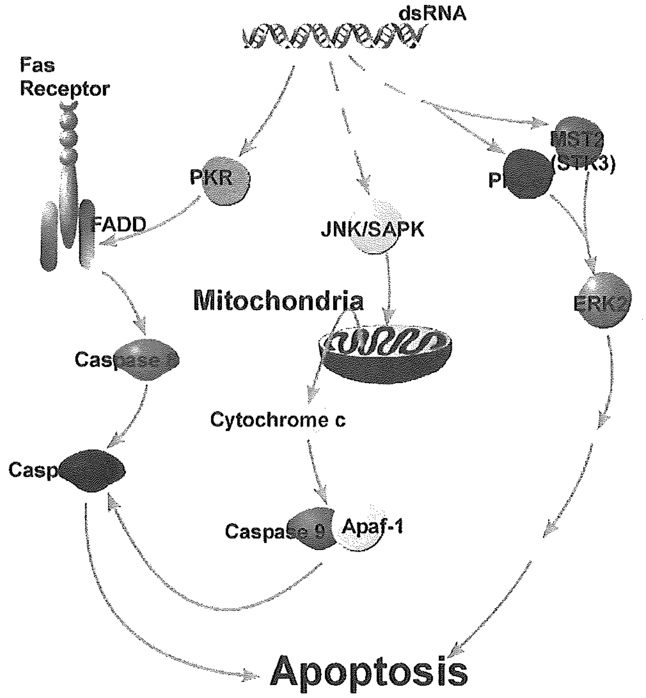

FIG. 8. **Schematic representation of the mechanism of apoptotic signaling that occurs in response to dsRNA.**

requirement for ERK1/2 in cisplatin-induced apoptosis of human cervical carcinoma cells and ovarian cell lines (44). Moreover, Lee *et al.* (45) reported that oxidative stress-induced apoptosis is mediated by the phosphorylation of ERK1/2. Our result was thus in tune with conclusions obtained in studies of other apoptotic pathways. The pro-apoptotic effect of ERK1/2 might be a general phenomenon. We found that MST2 and PKCα mediated the activation of ERK1/2. The MST2- and PKCα-mediated pro-apoptotic effects of ERK2 provide new clues to the mechanism of dsRNA-induced apoptosis, and these effects are completely independent of the established PKR-dependent pathway.

The participation of the JNK/MAPK pathway in dsRNA-induced apoptosis has been reported by several groups. However, the events that occur upstream and downstream of JNK/MAPK remain poorly defined (36–38). To summarize earlier results, it appears that there are p38 MAPK and JNK/SAPK pathways; the p38 MAPK pathway is dependent on PKR, whereas the JNK/SAPK pathway is dependent on PKR and RNase L. In higher vertebrates, IFN mediates the intrinsic antiviral response, in part through the action of RNase L. RNase L, a uniquely regulated enzyme, is activated by 2′,5′-oligoadenylate, which is produced by IFN-inducible and dsRNA-dependent synthetases. Li *et al.* (46) described how RNA damage caused by RNase L can lead to the activation of JNK/SAPK and apoptosis. However, to our knowledge no events downstream of the activation of JNK/SAPK in dsRNA-induced apoptosis have been reported previously. It has only been reported that JNK/SAPK causes mitochondrial death signaling in UV-induced apoptosis and the neuron growth factor-reduction pathway (47, 48). Our results demonstrate directly and for the first time the existence of a JNK/SAPK-mediated mitochondrial pathway in dsRNA-induced apoptosis.

CONCLUSION

In the present work, we have succeeded in developing an original vector library that has high suppressive activity and specificity and that allows us to avoid the confounding effects of the interferon response of dsRNA. We screened ~700 clones that were targeted against different genes. We propose that there are at least three independent pathways in the dsRNA-induced apoptotic network as follows: the PKR-caspase 8-dependent classical pathway, the JNK/SAPK-mediated mitochondrial pathway, and the ERK2-related pathway. Apoptosis appears to require the operation of all three pathways simultaneously, because suppression of the expression of only one mediator was able to inhibit the induction of apoptosis by poly(I-C).

In the present experiment, we demonstrate the strength of our vector-based RNAi screening technology, which is free from interference by the interferon response, and we show how it can be used for comprehensive analysis of dsRNA-induced apoptotic pathways. Our large scale screening technology is applicable to the entire human genome and should help in the discovery of the functions of vast numbers of functional genes.

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Minireview

Genome-wide application of RNAi to the discovery of potential drug targets

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Abstract Progress is being made in the development of RNA interference-based (RNAi-based) strategies for the control of gene expression. It has been demonstrated that small interfering RNAs (siRNAs) can silence the expression of target genes in a sequence-specific manner in mammalian cells. Various groups, including our own, have developed systems for vector-mediated specific RNAi. Vector-based siRNA- (or shRNA) expression libraries directed against the entire human genome and siRNA libraries based on chemically synthesized oligonucleotides now allow the rapid identification of functional genes and potential drug targets. Use of such libraries will enhance our understanding of numerous biological phenomena and contribute to the rational design of drugs against heritable, infectious and malignant diseases.

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Keywords: RNA interference; small interfering RNA; Vector-based siRNA

1. Introduction

The sequence of human genome has been determined but the functions of many genes remain unknown. Methods for bridging the gap between sequence and function are obviously necessary. Various methods have been used in attempts to suppress gene expression, exploiting, for example, antisense oligonucleotides and ribozymes. Recently, RNA interference-based (RNAi-based) strategies have been developed and show considerable promise. RNAi is an evolutionarily conserved biological phenomenon in plants and animals whereby double-stranded RNA (dsRNA) induces the sequence-specific degradation of cognate RNA [1]. This process is recognized as a mechanism in defense of the genome against molecular parasites such as viruses and transposable genetic elements (transposons) [2]. It is now well established that some gene regulation is mediated by small RNAs of 19–28 nucleotides (nt) in length, such as microRNAs (miRNAs) [3] and small interfering RNAs (siRNAs) [4], generated from precursor dsRNAs by ribonuclease III type enzyme Dicer. By accumulation of many researches, RNA-mediated gene silencing has now been developed from only biological phenomenon into very useful experimental tool to suppress gene expression by miRNAs and/or more popular dsRNAs such as expressed dsRNAs and synthetic siRNAs. This review summarizes progress to date in optimizing RNAi and exploiting its potential.

In RNAi, a long dsRNA is processed intracellularly by Dicer to yield siRNAs. Human Dicer has been cloned and its ribonucleolytic activity and dsRNA-binding properties have been characterized. Recombinant Dicer generates 21- to 23-nt products from dsRNA [5,6]. The experimental silencing of specific genes by RNAi in mammalian systems was hampered initially by the non-specific dsRNA-dependent inhibition of protein synthesis via the protein kinase R (PKR) pathway and the non-specific degradation of RNA that occurs upon activation of RNase L. However, it was demonstrated that synthetic 21- or 22-nt RNAs with 2-nt 3′-overhangs (siRNAs) can silence the expression of target genes without any non-specific inhibition of gene expression in cultured mammalian cells [7]. The synthetic siRNA duplex is similar to a naturally processed siRNA product from long dsRNAs both in terms of length and in terms of structure. The dominant products of processing by Dicer are duplexes of 21- and 22-nt RNAs with 2-nt 3′ overhangs, which are very effective mediators of the degradation of mRNA [7,8], although slightly longer siRNAs of approximately 27 nts have been reported to be more effective [9,10]. It has proved possible to use siRNAs to control the expression of both exogenous and endogenous genes in mammalian cells [7–10].

2. Design of effective siRNAs

Schwarz et al. [11] showed that the two strands of an siRNA duplex are not equally eligible for assembly into the RNA-induced silencing complex (RISC) and siRNA duplexes can be functionally asymmetric, with only one of two strands having the ability to trigger RNAi. Therefore, it might be preferable to design an siRNA duplex with an antisense strand that can enter RISC. It has been shown statistically that siRNAs with functional duplexes have a lower internal stability at the

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5′-antisense end than that of non-functional duplexes [12]. Reynolds et al. [13] identified some characteristics for the rational design of siRNAs for RNAi, such as a bias towards low internal stability at the 3′ terminus of the sense strand. Ui-Tei et al. reported results that were basically consistent with these findings and the following rules appear to apply to the induction of effective gene silencing in mammalian cells: (i) there should be A or U at the 5′ end of the antisense strand; (ii) there should be at least five A or U residues in the 5′-terminal one-third of the antisense strand and (iii) there should be no GC stretch of more than 9 nt in length [14]. We also showed that U at the 10th position in the sense strand (the middle nucleotide of the target) tends to be associated with strong activity [15]. Strategies for the design of effective siRNAs are becoming clearer [11–18].

Although an siRNA duplex with a bulge in its sense strand retained most of its RNAi activity, bulges in the antisense strand, when this strand formed a duplex with its target, completely abolished the ability of the duplex to induce RNAi. These observations suggested that siRNAs should be designed to be perfectly complementary to their targets [19], although our recent data suggest that some predetermined bulges within expressed short hairpin RNAs (shRNAs; Fig. 1) in the sense or antisense strand do not disturb RNAi [20]. Miller et al. [21] demonstrated the allele-specific silencing of dominant genes associated with two unrelated diseases and concluded that siRNA can be engineered to silence the expression of disease-related alleles that differ from wild-type alleles by as little as a single nucleotide. However, it has been demonstrated that siRNAs can also cross-react with targets with limited sequence similarity [22]. Sledz et al. [23] reported that, in addition to the specific gene-silencing effects of RNAi, global interferon-stimulated enhancement of gene expression can be detected in response to the intracellular stimulation by siRNAs. Kim et al. reported that short single-stranded RNAs (ssRNAs) and siRNAs synthesized by phage polymerase also induced

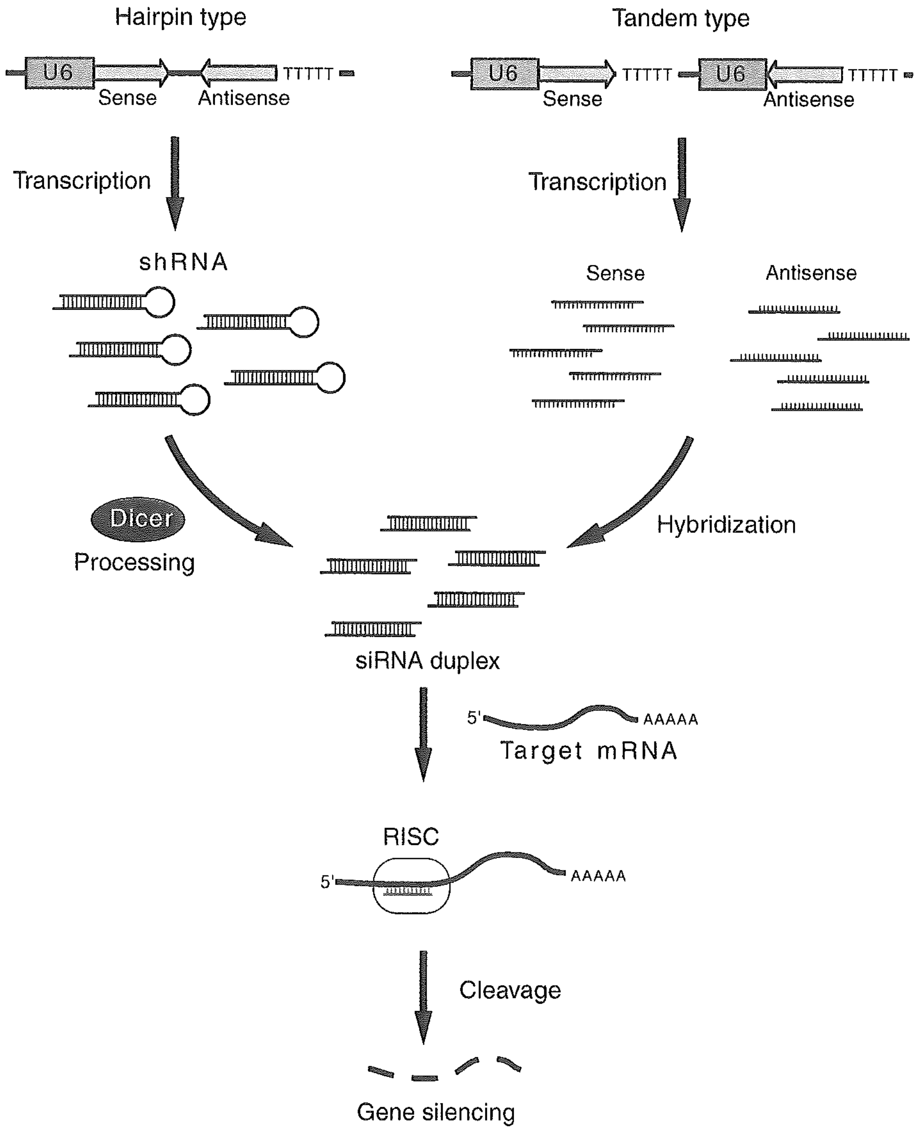

Fig. 1. Schematic representation of gene silencing by an shRNA-expression vector. The shRNA is processed by Dicer. The processed siRNA enters the RNA-induced silencing complex (RISC), where it targets mRNA for degradation.

the interferon response and that removal of the 5′ triphosphate of the transcripts prevented activation of the interferon response. Thus, their findings suggested that the interferon response could be circumvented if the 5′ triphosphate was removed from the transcribed RNA [24]. It has been reported that the interferon response can be induced by both a substantial number of shRNA vectors [25] and synthetic siRNAs with certain sequences [26,27]. Thus, use of the lowest effective dose of an shRNA vector or siRNA was recommended in order to limit the risk of the interferon response [25].

We demonstrated recently that the introduction of G:U mismatches within transcribed shRNAs significantly reduced the extent of induction of the interferon response [28,29], without any reduction in the extent of RNAi [30].

3. Systems for the expression of shRNA and genome-wide RNAi libraries

Because transfected synthetic siRNAs in cells can be degraded within a short time, inhibition of target genes does not continue for very long. To overcome this problem, various groups, including our own, have developed systems for vector-mediated specific RNAi in mammalian cells [31–39] (Fig. 1). Each of these systems exploits a polymerase III (pol III) promoter, such as a U6 or H1 promoter or the promoter of the gene for a tRNA. The various systems can be divided into two groups depending on whether the expressed RNA is of the tandem type or the hairpin type (Fig. 1). In the expression system for the tandem type, both of the sense and the antisense strand are driven separately by their own respective promoters. In the expression system for the hairpin type, a sequence that contains the sense strand of the siRNA of interest, followed by a loop sequence and the antisense strand, is driven by a single promoter. The H1 and U6 promoters have been used to drive the expression of shRNAs because they produce small RNA transcripts that lack a polyadenosine tail and have a well-defined site for initiation of transcription and a termination signal that consists of more than four T residues in a row (>T4), which results in 3′ overhanging U residues. Since it dose not contain any additional nucleotides at the 3′ end, the shRNA that is transcribed from such a vector system can function effectively. The structure of the transcribed and processed shRNA closely resembles that of synthetic double-stranded siRNA [40]. This shRNA system has been used to inhibit gene expression in mammalian cells very effectively and to an extent equal to that achieved with synthetic siRNA [41].

The genome-wide and comprehensive analysis of gene expression in various organisms should be facilitated by exploitation of the above-described phenomenon. For example, systematic functional analysis by RNAi of a number of genes in *Caenorhabditis elegans* [42–47] and *Drosophila* cells [48–52] has allowed the identification of the functions of many genes. In the near future, a similar approach using a genome-wide RNAi library should be feasible in mammalian cells because libraries of siRNA oligonucleotide and siRNA-expression vectors are becoming available. Randomized ribozyme libraries have already been used for the identification of novel genes [53–68] and several groups have begun to generate siRNA libraries directed against the entire human genome [69–79].

4. Approaches to the construction of RNAi libraries

The successes achieved by the exploitation of RNAi for the analysis of the functions of individual genes and by use of ribozyme libraries have led inevitably to efforts to apply these approaches on a large scale to reverse genetic analysis. In the case of construction of an RNAi library, the library can consist of either synthetic siRNAs or siRNA-expression vectors. Plasmids and viral vectors are exploitable expression systems for the siRNA-expression library. Each library has the advantages and disadvantages in its delivery strategies. For example, although viral vectors can be imported efficiently into various cell lines by infection, siRNAs and plasmid vectors cannot be imported efficiently into some cell lines, such as primary, neural and hemopoietic cells, by transfection. Transfection of siRNAs and plasmid vectors can be performed easily in ordinary laboratories, but handlings of viral vectors require more experiences and special equipments. It is necessary to choose the delivery strategy that is optimal under a fixed experimental condition.

Genome-wide libraries of siRNAs can be constructed in several fundamentally different ways, which include chemical synthesis and the enzymatic digestion of long dsRNAs. Because the effectiveness of siRNAs is strongly dependent on their target sites in their target RNAs, several groups developed their own algorithm that allows one to predict favorable target sites for generation of a high-quality library [11–18,80–90]. Some of these algorithms are open to the public on the web. In our case, in order to optimize various parameters, we decided to produce a large number of siRNA pairs and measure sequence–activity relationships by walking along an entire gene and shifting one base at a time [41]. Unfortunately, in the year 2001, the price of one siRNA pair in Japan was close to $1000! However, Suzuki's group has since developed a transcription system that can be used to produce siRNAs at a reasonable price [91] (Fig. 2). The advantage of their strategy is that only completely base-paired siRNAs are collected from the gel and, thus, the concentration of oligonucleotides reflects that of duplexed siRNAs. Examination of the sequences of approximately 1000 siRNA pairs directed against the mRNA for EGFP and their activities enabled us to develop an original and reliable algorithm [16,92,93].

Prior to the construction of the siRNA-expression library, we also optimized our siRNA-expression system. We constructed three types of siRNA vector system, namely, a tandem type, a dual-promoter type, and a hairpin type, and compared the activities of the resultant siRNAs. We determined that the hairpin-type system had the highest suppressive activity at the low concentrations of plasmids. However, we observed a high rate of mutation in the stem-loop region when we used plasmids to transform *Escherichia coli*. These mutations presented a serious problem with respect to the construction of a reliable library of siRNA vectors. However, we found that introduction of multiple C to T (or A to G) mutations into the sense strand rendered the plasmids genetically stable and did not affect silencing activity (Fig. 3). Moreover, we optimized the loop sequence that connected the sense and antisense sequences and other parameters that might be expected to affect the activity of the siRNA. Together, the optimization of the siRNA-expression system and the development of our algorithm enabled us to construct a large-scale and high-quality library of siRNA vectors. Now, using our algorithm and our

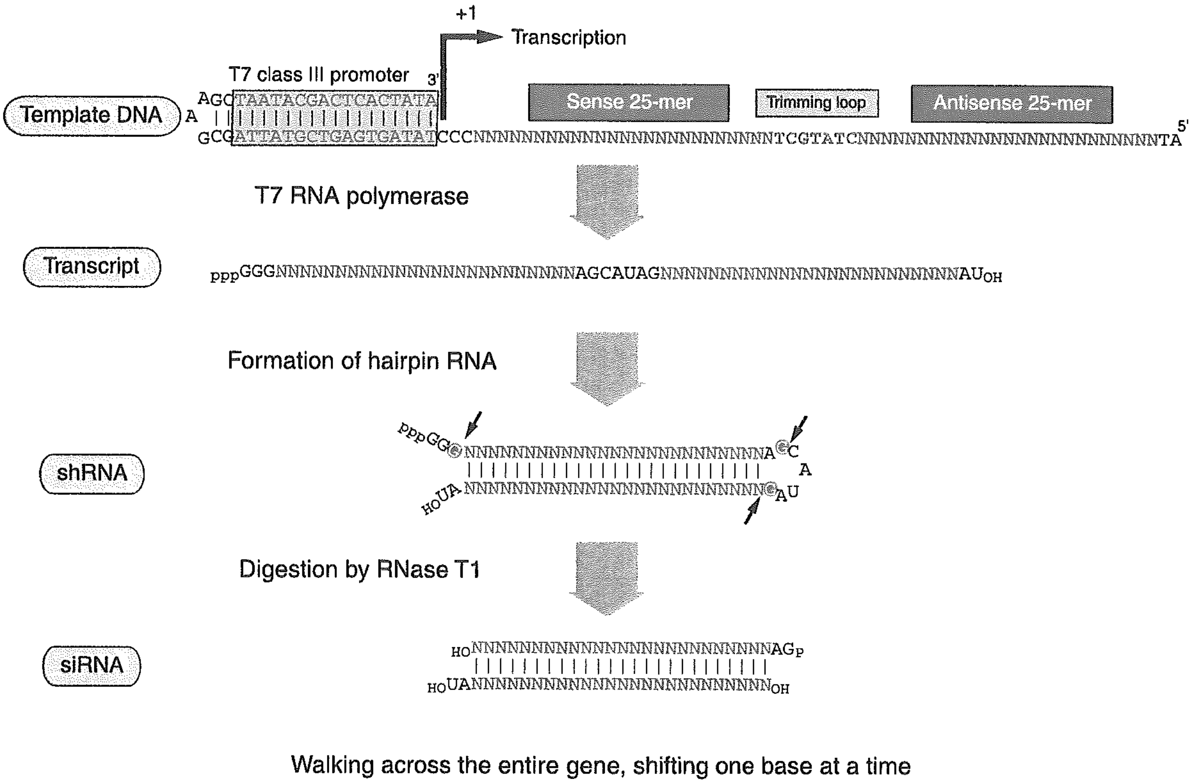

Fig. 2. Schematic representation of a transcription system for production of siRNA.

optimized siRNA-expression system, we are constructing an siRNA-expression library that will encompass the complete array of transcripts from the human genome (Fig. 3). To confirm that the same phenotypic changes occur when siRNAs are targeted to multiple independent sites in an mRNA, our library contains at least two target sites per gene [79].

Although our libraries are based on the optimized algorithm for selection of the best two target sites, it might also be possible and more economical to use enzymatically fragmented cDNAs for the construction of libraries. Indeed, several groups have reported methods for generating libraries of random shRNAs using fragments of enzymatically digested cDNA [94–96]. Sen et al. described an enzyme-mediated method for generating numerous functional siRNA constructs from any gene of interest or any pool of genes. The restriction enzyme-generated siRNA (REGS) system produced, on average, 34 unique siRNAs per kilobase of sequence. REGS enabled Sen et al. to create a complex siRNA library from double-stranded cDNA that encompassed known and unknown genes, with 96% of the clones containing inserts of the appropriate size [94].

Shirane et al. produced RNAi libraries from cDNAs using another system that they called EPRIL (enzymatic production of RNAi library). EPRIL involves several enzymatic treatments for the production of a library of shRNA-expression vectors from cDNAs of interest and should contribute significantly to the elucidation of the gene function at the whole-genome level [95].

Luo et al. described a system for "siRNA production by enzymatic engineering of DNA (SPEED)". Theirs is a simple, effective, and inexpensive strategy for construction of genome-wide siRNA-expression libraries from populations of double-stranded cDNAs. The method dispenses with the need for expensive chemical synthesis of oligonucleotides and facilitates the generation of siRNA libraries that encompass all expressed genes, including those of unknown structure and function, from diverse sources of mRNA in many species. In addition to cDNA libraries, other DNAs can be converted into an siRNA library by SPEED, for example, the HIV genome. Screening of such a library for siRNAs that strongly inhibit viral replication or other virus-encoded functions should help to identify potential siRNA-based therapeutic agents [96].

One potential drawback associated with this kind of cloning of enzymatically fragmented short DNAs is the possible production of fragments of palindromic DNA (which lead to formation of completely matched hairpin RNAs) that tend to mutate during amplification in *E. coli*, as described above. The maintenance of reliable libraries requires special care. Thus, we shall have to wait to assess the utility of such libraries for the successful identification of functional genes.

5. Application of RNAi libraries

We have used our library to screen for genes involved in the endoplasmic reticulum stress-dependent apoptosis that is induced by thapsigargin, a plant-derived sesquiterpene lactone [77]. Thapsigargin triggers endoplasmic reticulum stress, with subsequent apoptosis, but the molecular mechanisms underlying this process are incompletely understood. Using the library, we were able to identify some unexpected and novel pathways in thapsigargin-induced apoptosis, and our results provide evidence for the efficacy and utility of the comprehensive analysis of signaling networks and pathways with a library

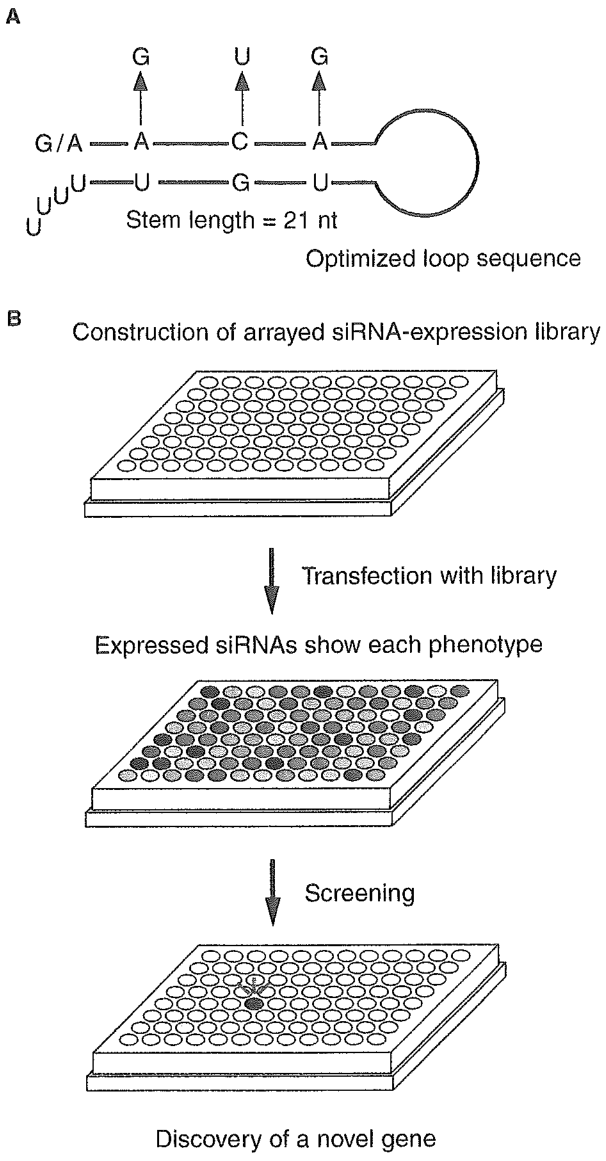

Fig. 3. (A) Schematic representation of the proposed siRNA-expression system. Three or four C to U or A to G mutations are introduced into the sense strand. (B) Schematic representation of the discovery of a novel gene using an siRNA library.

of siRNA-expression vectors. The most significant advantages of our siRNA-expression library, as compared with the widely used library of synthetic siRNAs are as follows. The interferon response in transfected cells can be avoided [28,29], and the RNAi effect is sustained for a longer period of time than are the inhibitory effects of synthetic siRNAs, in particular, in proliferating cells. Using the siRNA-expression library, we demonstrated the straightforward identification of novel pathways that had not previously been identified by other strategies [66,67,75,77,79]. We have already constructed siRNA libraries directed against the genes for all human kinases and phosphatases and against the genes for all human and mouse transcriptional factors and nuclear factors, as well as against other human genes (~15000 clones). Further analyses using such large libraries of siRNA-expression vectors should provide more precise information about various signaling pathways and enhance our understanding of numerous physiological phenomena. As a consequence, they should allow us to identify specific disease-related target genes.

Many groups have used either libraries of synthetic siRNAs or of siRNA-expression vectors that had been produced by the selection of specific cleavage sites within target mRNAs [69–74,78]. Some groups have reported the application of libraries of synthetic siRNAs directed against certain families of genes [70,72,78]. Aza-Blanc et al. screened HeLa cells using an siRNA library directed against 510 genes, including the genes for most human kinases, to identify genes that impact TRAIL-induced apoptosis. TRAIL is a member of the tumor necrosis factor (TNF) superfamily that induces the selective killing of tumor cells when bound to its cognate receptor. Using this approach, Aza-Blanc et al. [70] identified a variety of known and previously uncharacterized genes that modulate TRIAL activity.

Mackeigan et al. systematically screened the kinase- and phosphatase-encoding components of the human genome and identified new regulators of apoptosis and chemoresistance using a custom-made set of libraries of siRNAs that were designed to include two siRNA duplexes for each gene target. In total, their kinase library was directed against 650 target genes and their phosphatase library was directed against 222 genes. The development of inhibitors that target these kinases or phosphatases may lead to new anti-cancer strategies [78].

Zheng et al. reported the development of a dual-promoter siRNA-expression system. In this system, a gene-specific siRNA sequence is inserted between two different opposing pol III promoters, the mouse U6 and the human H1 promoters. They used their system to construct a library of siRNA-expression cassettes that targets 8000 human genes with two designed sequences per gene [71]. They were able to identifiy both known and unique regulators of NF-κB signaling using this library.

Brummelkamp et al. reported the production of an shRNA library directed against the family of de-ubiquinating enzymes [69]. Protein modification via the conjugation of ubiquitin moieties, known as ubiquitination, plays a major role in many biological processes, including the cell cycle and apoptosis. The enzymes that mediate ubiquitination have been well studied, but much less is known about the ubiquitin-specific proteases that mediate de-ubiquitination of cellular substrates. To study this family of proteins, they designed shRNA vectors for suppression of the expression of 50 human de-ubiquitinating enzymes, and used these vectors to identify de-ubiquitinating enzymes in cancer-relevant pathways. Using the library, they identified the tumor suppressor CYLD (encoded by the familial cylindromatosis-susceptibility gene) as a suppressor of NF-κB activity. CYLD binds to the NEMO (also known as IKKg) component of the IkB kinase (IKK) complex and appears to regulate its activity through de-ubiquitination of TRAF2, since the ubiquitination of TRAF2 can be modulated by CYLD. Inhibition of CYLD increases resistance to apoptosis, suggesting a mechanism through which loss of CYLD might contribute to oncogenesis. This effect can be relieved by aspirin derivatives that inhibit NF-κB activity, an observation that suggests a therapeutic intervention for restoration of growth control in patients with familial cylindromatosis. These results led to proposals for treating cylindromatosis with existing drugs and provided powerful confirmation that unbiased, genetic approaches can lead not only to new insights in biology but also to practical advances in the treatment of disease.

Two independent groups have recently reported the usefulness of viral libraries with bar code detection [73,74]. Berns et al. reported the construction of a set of retroviral vectors that encode 23742 distinct shRNAs and target 7914 different human genes [73]. They used their library in human cells to identify one known and five previously unknown modulators of arrest of the p53-dependent proliferation. Suppression of these genes conferred resistance to the arrest of both the p53-dependent and the p19ARF-dependent proliferation of cells, and abolished the DNA damage-induced arrest of the cell cycle at G1 [73]. Paddison et al. reported the construction and application of an shRNA-expression library that targeted 9610 human and 5563 mouse genes. Their library consists of close to 28000 sequence-verified shRNA-expression cassettes contained within multifunctional vectors, which permit the shRNA cassettes to be packaged in retroviruses, tracked in mixed populations of cells by means of DNA 'bar codes', and shuttled to customized vectors by bacterial mating. In order to validate the library, Paddison et al. used a genetic screen designed to identify defects in human proteasome function. Their results suggest that large-scale RNAi libraries can be used in specific, genetic applications in mammals and will become valuable resources for gene analysis and the discovery of potential drug targets [74,97].

Genome-wide RNAi screenings can contribute to the rational design of drugs against heritable, infectious and malignant diseases. For example, Eggert et al. performed genome wide RNAi screening combined with small molecule screening [98]. Their parallel chemical genetic and genome wide RNAi screens identified cytokinesis inhibitors and targets and showed that the parallel RNA interference with small molecule screening is useful approach to discover leads for therapeutic drugs. In addition, Agaisse et al. performed genome-wide RNAi screen for host factors required for intracellular bacterial infection [99].

Now, a number of libraries are commercially available, for example the MISSION shRNA library from Sigma Aldrich, the Hannon-Elledge shRNA library from Open Bio Systems, the *s*mart & *i*ntelligent *si*RNA library from iGENE therapeutics, etc. These libraries should be useful for discovery of potential drug targets.

6. Conclusion

The available methods for construction of RNAi libraries each has various advantages and defects. Random libraries are relatively inexpensive to produce and can cover an individual gene with many different shRNAs. However, they do not necessarily cover all genes and the vector might not be stable. By contrast, libraries produced from chemically synthesized siRNAs are easy to handle and transfection efficiencies are generally high, but they are expensive and the duration of their efficacy is limited. Structurally optimized effective shRNAs, transcribed from vectors in cells, can now be generated by applying an optimized algorithm and they can be maintained with ease. In future, siRNA libraries will be useful for wide-range screening for drug development. There is no question that these libraries and large-scale RNAi libraries will contribute to our understanding of numerous biological phenomena and to the design of entire classes of novel therapeutic agents.

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RESEARCH ARTICLE

Gene therapy for human small-cell lung carcinoma by inactivation of Skp-2 with virally mediated RNA interference

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Increase of Skp-2, which is involved in the degradation of cell cycle regulators including $p27^{Kip1}$, p21 and c-myc, is one of the important mechanisms for dysregulation of cell cycles in various cancers. We applied RNA interference (RNAi) for Skp-2 by using HIV-lentiviral or adenoviral vectors for a human small-cell lung carcinoma cell line with increased Skp-2 to evaluate RNAi strategy for cancer gene therapy. HIV-lentivirus-mediated RNAi for Skp-2 resulted in efficient inhibition of the in vitro cell growth of cancer cells with increased Skp-2 through the increase of $p27^{Kip1}$ and p21, but no significant effect on the growth of cells without high Skp-2 expression. Furthermore, intratumoral administration of adenovirus siRNA vector for Skp-2 efficiently inhibited growth of established subcutaneous tumor on NOD/SCID mice. These results indicate that the Skp-2 RNAi may be a useful strategy for gene therapy of cancers with high Skp-2 expression.

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Introduction

Dysregulation of cell cycle is one of the important mechanisms for uncontrolled growth in most cancers. $p27^{Kip1}$, a cyclin-dependent kinase (cdk) inhibitor, inhibits the transition from G1 to S phase by suppressing the activity of a cyclin E/cdk2 complex in the late G1 to S phase.[1] In many cancers, including gastric, breast and colorectal cancers, low expression of $p27^{Kip1}$ was reported to be associated with poor prognosis and highly aggressive nature of the tumors.[2] Since the level of the $p27^{Kip1}$ protein is mainly controlled by ubiquitin–proteosomal proteolysis, enhanced degradation of $p27^{Kip1}$ appeared to be an important mechanism for the reduction of $p27^{Kip1}$ in cancers.[3] Skp-2, a member of the F-box protein family, is a specific substrate-recognition subunit of an SCF ubiquitin–protein ligase complex and is involved in the $p27^{Kip1}$ degradation.[4] Increased expression of Skp-2, accompanied by inverse decrease of $p27^{Kip1}$, was reported in many cancers, including small-cell lung carcinoma (SCLC),[5] oral squamous cell carcinoma,[6] lymphoma[7] or gastric carcinoma,[2] indicating that Skp-2 may be involved in the tumorigenesis of some cancers with the reduced $p27^{Kip1}$. The Skp-2 was overexpressed in 44% of primary SCLC with the reduced expression of $p27^{Kip1}$ through the gene amplification in 5p11–13.[5] Yokoi *et al*[5,8] have previously shown that downregulation of Skp-2 in an SCLC cell line with antisense oligonucleotides resulted in the inhibition of *in vitro* cell growth. However, the precise mechanism of growth inhibition by the Skp-2 inactivation remains to be investigated. In this study, we have analyzed the mechanism for the inhibition of tumor cell growth using newly developed HIV and adenoviral vectors expressing the Skp-2 siRNA, which may be useful for the future gene therapy.

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Results

HIV vector-mediated RNA interference (RNAi) for Skp-2 resulted in the inhibition of in vitro cell growth of SCLC cell line with elevated Skp-2 expression

We constructed several HIV vectors expressing siRNAs targeting at the Skp-2 mRNA and analyzed their RNAi effects by determining the Skp-2 protein by Western blot analysis after infecting an SCLC cell line, ACC-LC-172, which has the gene amplification and increased expression of Skp-2. Of these siRNA HIV vectors, we selected two HIV vectors, S2 and S5, which mediated efficient reduction of the Skp-2 protein. We next evaluated effects of the RNAi for Skp-2 on *in vitro* growth of the ACC-LC-172 SCLC cells by infection of these Skp-2 siRNA HIV vectors and a control siRNA HIV vector for firefly lucifrase (GL3B) whose infection did not affect *in vitro* growth, cell cycle status and amounts of Skp-2 protein of ACC-LC-172 compared to uninfected ACC-LC-172 (data not shown). *In vitro* cell growth of ACC-LC-172 was significantly inhibited when infected with the S5 siRNA HIV vector compared to the control GL3B siRNA HIV vector ($P<0.0001$), although transduction efficiency monitored by GFP-expressing cells was comparable among the infected cells (98.7–99.9%). (Figure 1a). The similar cell growth inhibition was observed in a melanoma cell line, A375mel, with the overly expressed Skp2 (data not shown). *In vitro* growth of ACC-LC-172 infected with the S2 siRNA HIV vector was less inhibited than that with S5 ($P=0.0005$) (Figure 1a), associated with the weaker suppression of Skp-2 and less induction of $p27^{Kip1}$ and p21 compared to S5 (Figure 1b). The decrease of the Skp-2 protein accompanied by the reduction of $p27^{Kip1}$ protein, shown by Western blot analysis of cell lysates at day 9 after the infection, was positively correlated with the inhibition of *in vitro* cell growth (Figure 1a and b). Other cdk inhibitor p21 was slightly elevated in the cells infected with the S2 and S5 siRNA HIV vectors similarly with $p27^{Kip1}$ (Figure 1b). The $p57^{Kip2}$ protein was under the detectable limit in this cell line (data not shown). The Rb protein was not changed after the infection. Cell cycle analysis performed on day 9 after the infection demonstrated decrease of the population in S and G2/M phases in the cells infected with the S5 siRNA HIV vector (44.6%) compared to those infected with the GL3B siRNA HIV vector (57.1%) (Table 1). No significant apoptosis was observed in the infected cells by flow cytometry analysis and DNA fragmentation assays (data not shown). These results indicated that the Skp-2 RNAi inhibited cellular growth of ACC-LC-172 through the increase of both cdk inhibitors $p27^{Kip1}$ and p21. In contrast, *in vitro* growth of 293T cells without the Skp-2 overexpression was less sensitive to the inhibitory effect of the Skp-2 RNAi ($P=0.1835$) (Figure 2a), although similar pattern of changes was observed in the Skp-2 and $p27^{Kip1}$ proteins (Figure 2b). The constitutively low-level expression of the Skp-2 in 293T cells may explain the reason for the 293T resistance to the Skp-2 RNAi (Figure 2c). Similarly, *in vitro* cell growth of SBC-1, an SCLC cell line that lacks detectable Skp-2 expression, was not inhibited by the Skp-2 siRNAs (Figure 2d). Thus, virally mediated Skip2 RNAi does not appear to affect cells with low Skp2 expression.

Less involvement of myc in the inhibition of the ACC-LC-172 cell growth by the Skp-2 RNAi

Since Skp-2 was also reported to act as a transcriptional cofactor for c-myc,[9,10] which is involved in cell cycle progression by mediating the activation of cyclin E–cdk2 and cyclin D–cdk4, promoting the G1/S transition,[11] we evaluated the role of myc in the Skp-2 RNAi-mediated cell growth inhibition of the ACC-LC-172 SCLC cells. Increased myc expression was previously reported in most SCLC[11] and mild myc increase (2.03-fold) was detected in ACC-LC-172 (data not shown).

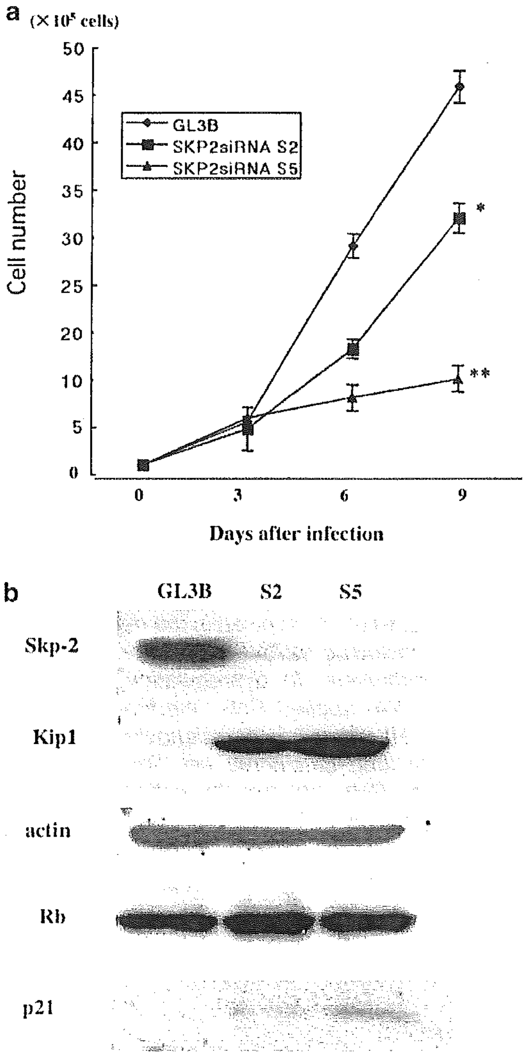

Figure 1 *Inhibition of in vitro growth of an SCLC cell line along with decrease of Skp-2 and increase of $p27^{Kip1}$ and p21 by infection of the HIV vectors expressing siRNA for Skp-2: (a) inhibition of in vitro tumor cell growth by virally medicated RNAi for Skp-2. SCLC cell line ACC-LC-172 (100 000) were infected with siRNA HIV vectors for control firefly luciferase (GL3B) or Skp-2 mRNA (S2 and S5) at 100 MOI on day 0, then the cell numbers were determined by trypan blue dye exclusion method on days 3, 6 and 9. The vertical bars indicate the s.d. of the triplicate assays (*$P=0.0005$; **$P<0.0001$). This is one representative result of three independent experiments with similar results. (b) Decrease of Skp-2 protein and increase of $p27^{Kip1}$ and p21 proteins by virally medicated RNAi for Skp-2. Cell lysates were prepared from the cells infected with siRNA HIV vectors in (a) on day 9 after the infection. The Skp-2 protein was significantly decreased in cells infected with S2 and S5 compared to GL3B, and $p27^{Kip1}$ and p21 proteins were reciprocally increased in cells infected with S2 and S5. The degrees of the increase of the $p27^{Kip1}$ and p21 proteins were correlated with the degrees of the decrease of the Skp-2 protein. The transduction efficiency determined by GFP expression by flow cytometry was equivalent among the three groups (98.7–99.9%) at the harvest.*

Table 1 Cell cycle status of ACC-LC-172 cells transduced with siRNA HIV vectors for firefly luciferase or Skp-2 mRNAs

siRNA	*% G0/G1*	*% S*	*% G2/M*
GL3B	42.92	45.40	11.68
S2	53.36	31.41	15.23
S5	55.39	35.38	9.23

When a firefly luciferase expression plasmid, whose expression is under the control of human telomerase reverse transcriptase (hTERT) gene promoter containing two myc-binding E-box (CACGTG) motifs[12] (pGL3-hTERT), was transiently transfected to ACC-LC-172, the firefly luciferase activity normalized by *Renilla luciferase* activity was only minimally increased (1.1- to 2.7-fold) compared to that with a pGL3-Basic plasmid (Figure 3), suggesting that constitutive myc activity was relatively low in the ACC-LC-172 cells. Transfection of the same plasmid into the ACC-LC-172 cells stably transduced with the HIV Skp-2 siRNA vector (siRNA S5) did not change the luciferase activity. These results indicated that enhancement of myc activity was not the mechanism for the cell growth inhibition of ACC-LC-172 by the Skp-2 RNAi.

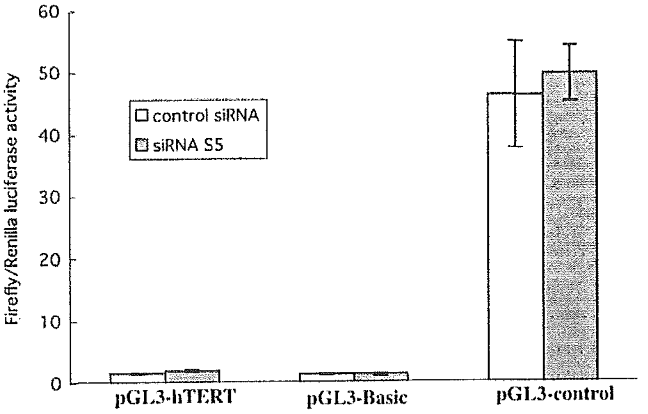

Figure 3 *No involvement of myc in the Skp-2 RNAi-mediated cell growth inhibition in the ACC-LC-172 SCLC cell line: ACC-LC-172 cells stably expressing siRNA for Skp-2 (siRNA S5) or control siRNA were transfected with 1 μg of pRL-SV40 (Renilla luciferase expressing plasmid) and 1 μg of pGL3-hTERT, pGL3-Basic or pGL3-control (firefly luciferase expressing plasmids driven by different promoters) by using lipofectamine. At 48 h after the transfection, the cells were harvested and the both Renilla and firefly luciferase activities were determined. Each firefly luciferase activity normalized by the Renilla luciferase activity was calculated. The normalized firefly luciferase activity with pGL3-hTERT was minimally elevated by 1.6-fold compared to with pGL3-Basic without any inhibition in the presence of siRNA for Skp-2. Each bar represents the mean value of triplicate assays and error bars represent the s.d. This is one representative result of three independent experiments with similar results.*

In vivo therapeutic activity of intratumoral administration of adenovirus siRNA for Skp-2

To evaluate therapeutic ability of the RNAi for Skp-2, we constructed an adenovirus vector expressing siRNA for

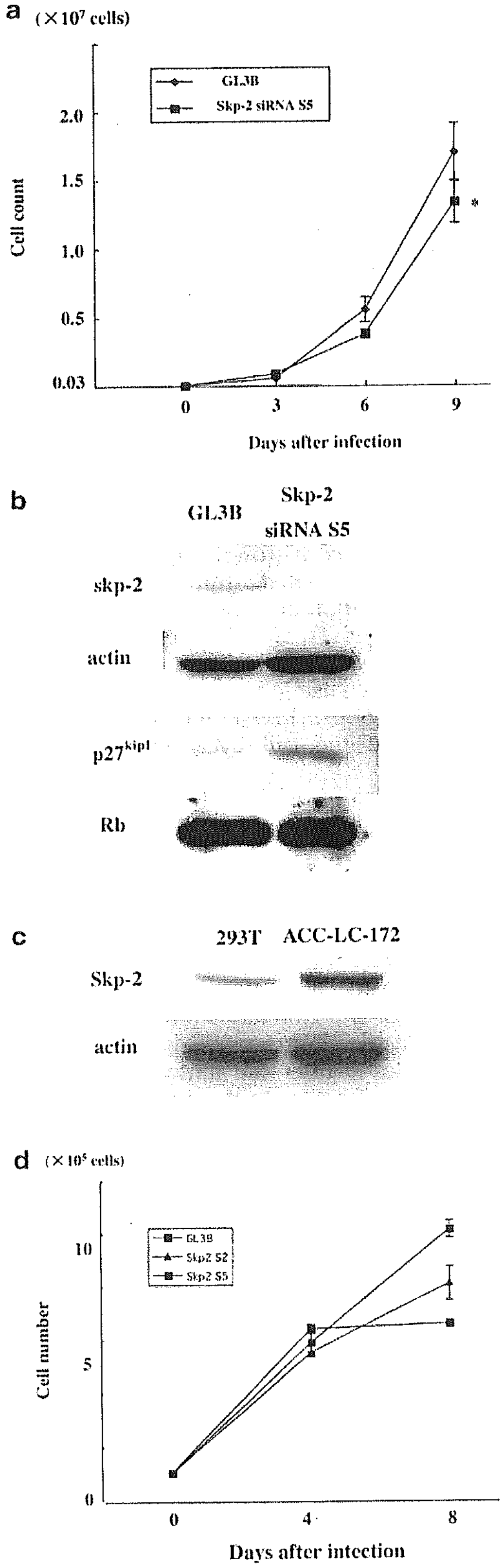

Figure 2 *Minimal inhibition of in vitro growth of 293T cells without increase of Skp-2 expression by infection of the Skp-2 siRNA HIV vectors: minimal inhibition of in vitro growth of 293T cells by the Skp-2 siRNA HIV vector infection. The 293T cells (30 000) were infected with siRNA HIV vectors for control GL3B or Skp-2 mRNA (S5) at 100 MOI on day 0, then the cell numbers were determined as Figure 1a. The vertical bars indicate the s.d. of the triplicate assays (*P = 0.1835). This is one representative result of three independent experiments with similar results. (b) Decrease of the Skp-2 protein and increase of the p27^{Kip1} protein by the Skp-2 siRNA HIV vector infection. Cell lysates were prepared as in Figure 1b. Relatively low expression Skp-2 protein was decreased in cells infected with S5 compared to GL3B, and the p27^{Kip1} protein was reciprocally increased in cells infected with S5, although the changes were less prominent than observed in ACC-LC172 cells. The transduction efficiency determined by GFP expression by flow cytometry was equivalent between the two groups (GL3B: 95.4%; S5: 100.0%) at the harvest. (c) Low expression of Skp-2 in 293T cells compared to ACC-LC-172 SCLC cell line. The amount of the Skp-2 protein was much lower in 293T cells than in ACC-LC-172 cells. The percentage of S+G2/M populations in 293T cells and ACC-LC-172 cells was 74.0 and 58.4%, respectively, at the harvest. (d) SBC-1 SCLC cells lacking detectable Skp-2 were refractory to Skp-2 RNAi. The in vitro cell growth of SBC-1, an SCLC cell line without detectable Skp-2 protein, was not inhibited with Skp-2 siRNA vectors.*

Skp-2, AdF35-Skp-2 siRNA S5, since adenovirus vector is more effective for *in vivo* gene transfer with more efficient production of high-titer viruses. The constructed adenovirus reduced the Skp-2 protein very efficiently in the ACC-LC-172 SCLC cells at only 5 multiplicity of infection (MOI) (Figure 4a), and inhibited the cell growth compared with the control AdF35-GL3B (data not shown). When these adenoviruses were intratumorally injected three times every 2 days to subcutaneously grown ACC-LC-172 SCLC tumors with the largest diameter of 3–4 mm on immunodeficient NOD/SCID mice, growth of tumors injected with the AdF35-Skp-2 siRNA (S5) was significantly reduced compared to that with the control AdF35-GL3B ($P < 0.05$) (Figure 4b). These results indicated that Skp-2 is an excellent target for the treatment of cancers with the increased Skp-2, and gene therapy by virally mediated RNAi may be applicable for these cancers.

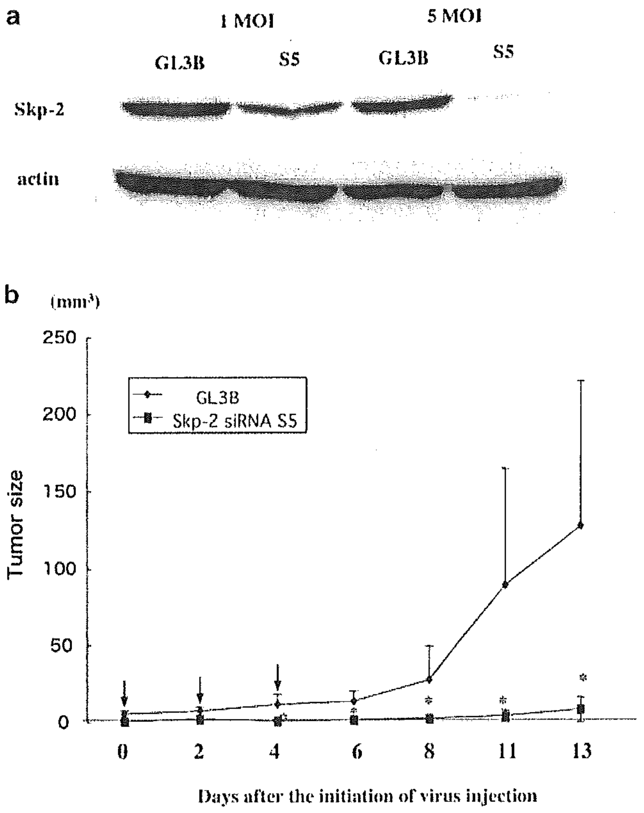

Figure 4 *Inhibition of in vivo tumor growth by intratumoral administration of adenoviral vector expressing siRNA for Skp-2. (a) Efficient downregulation of Skp-2 protein by infection of the Skp-2 siRNA adenoviral vector. Cell lysates from ACC-LC-172 cells infected with AdF35-Skp-2 siRNA S5 or AdF35-GL3B at either 1 or 5 MOI were prepared and analyzed for Skp-2 protein by Western blot analysis. (b) Inhibition of in vivo tumor growth by intratumoral injection of the Skp-2 siRNA. A total of* 1×10^8 *IFU of AdF35-Skp-2 siRNA S5 (*$n = 5$*) or AdF35-GL3B (control) (*$n = 4$*) were intratumorally injected three times every 2 days as shown in the figure (arrow) to subcutaneously established ACC-LC-172 on NOD/SCID mice. The largest diameter of the tumor reached 3–4 mm when injected with adenoviral vectors. The tumor size was compared between the two groups. **$P < 0.05$*, the vertical bars indicate s.d.*

Discussion

The cdk inhibitor, $p27^{Kip1}$, controls progression of cell cycles in response to mitogenic stimuli, and is a dosage-dependent tumor suppressor protein.[3] Reduced expression of $p27^{Kip1}$ is not usually caused by genetic change,[3] but often caused by enhanced proteolysis in cancers. The targeted disruption of an ubiquitin–protein ligase Skp-2 resulted in cell cycle arrest in G1 with the accumulation of $p27^{Kip1}$,[13] indicating that Skp-2 regulate cell cycle progression through proteolysis of $p27^{Kip1}$. Patients with cancers with the reduced $p27^{Kip1}$ by increased expression of Skp-2 were reported to have relatively poor prognosis in clinical studies.[6–8]

In this study, we attempted virally mediated RNAi for Skp-2 on the SCLC cell line to analyze the role of Skp-2 in the cell growth as well as to develop possible gene therapy for the Skp-2 overexpressing cancers. The observation that cell growth was inhibited by the virally mediated Skp-2 RNAi, which was accompanied by downregulation of Skp-2 and upregulation of both $p27^{Kip1}$ and p21, indicated that the increased Skp-2 expression is a primary event causing cell cycle progression of the SCLC cell line through the enhanced proteolytic degradation of $p27^{Kip1}$. Interestingly, the degree of the Skp-2 suppression with different siRNAs was correlated with the degree of the elevation of $p27^{Kip1}$ and p21 as well as *in vitro* growth suppression. Although p21 is mainly regulated transcriptionally, Skp-2-mediated ubiquitination and the subsequent proteolysis appear to be involved in the regulation of p21.[14] Although the increase of p21 was not as prominent as that of $p27^{Kip1}$, we also observed significant increase of p21 after the Skp-2 RNAi, indicating that it may also contribute to the cell cycle progression in the SCLC cell line.

Although Yokoi *et al* reported that antisense oligonucleotide-mediated inactivation of Skp-2 resulted in the induction of apoptosis characterized by an increase of the sub-G1 population, fragmentation of nuclei and activation of caspase-3 when evaluated on days 2–4,[8] we did not observe significant increase of the sub-G1 population or DNA fragmentation in the ACC-LC-172 cells with the siRNA HIV vectors in spite of very efficient downregulation of Skp-2 on day 9 (data not shown). This discrepancy may result from the different time point. Remaining on day 9 after the lentiviral infection cells might have been refractory to the apoptotis.

In contrast to the previous reports using $Skp\text{-}2^{-/-}$ mice[13] or forced expression of $p27^{Kip1}$,[1] Skp-2 RNAi did not lead to complete G1 arrest. Although almost complete downregulation of the Skp-2 protein accompanied by the increase of the $p27^{Kip1}$ protein was observed with the siRNA S5 (Figure 1b), these infected cells continued to grow slowly (Figure 1a), possibly due to relative resistance of this cell line to cell cycle inhibition by $p27^{Kip1}$.

In terms of possible use of this strategy for treatment of cancer patients, along with the effective tumor growth inhibition, it should be noted that growth of cells without the increased Skp-2, including 293T cells and primary fibroblasts (data not shown) was hardly affected by Skp-2 RNAi, suggesting that inactivation of Skp-2 is a relatively safe targeting therapy for cancers with high Skp-2 expression. In this study, we also demonstrated that intratumoral injection of adenovirus vector expressing the Skp-2 siRNA efficiently inhibited *in vivo* growth of the established tumor subcutaneously implanted in NOD/SCID mice, suggesting that viral-mediated Skp-2 RNAi may be applicable for gene therapy. These results altogether indicated that Skp-2 is a good target for gene therapy or other molecular target therapy for patients with cancers expressing high level of Skp-2.

Materials and methods

Cell lines

An ACC-LC-172 cell line (a kind gift from Dr Takahashi, Aichi Cancer Center, Research Institute, Japan) and SBC-1 (purchased from Japanese Collection of Research Bioresources, Japan) established from Japanese patients with SCLC were maintained in RPMI-1640 (Sigma, Japan) supplemented with 10% (v/v) fetal bovine serum, penicillin and streptomycin. The 293T cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in DMEM (Sigma, Japan) supplemented with 10% (v/v) fetal bovine serum, penicillin and streptomycin.

HIV vectors

HIV vectors for siRNA expression were constructed from an HIV-U6i-GFP plasmid, which was described previously.[15,16] Briefly, HIV-U6i-GFP has two expression units: one was an siRNA expression cassette, from which a short hairpin RNA was transcribed from human U6 promoter, and the second was a GFP expression cassette, from which GFP gene was transcribed from the CMV promoter. For siRNA expression, *in vitro* annealed complementary oligonucleotides for target sequences were inserted into the two *Bsp*MI sites downstream of the human U6 promoter. Two siRNA target sequences were selected for the Skp-2 RNAi: (S2) ATCA GATCTCTCTACTTTA and (S5) AGGTCTCTGGTGTTT GTAA. Two complementary oligonucleotides, cacc-(target sense)-TTCAAGAGA-(target antisense)-TTTTT and gcatAAAAA-(target sense)-TCTCTTGAA-(target antisense) were synthesized for each target sequence and annealed *in vitro*. The annealed double-stranded (ds) oligonucleotides with 5′-protruding ends complementary to the two *Bsp*MI sites in the HIV-U6i-GFP plasmid were then subcloned into the HIV-U6i-GFP. Control GL3B siRNA (anti-firefly luciferase siRNA) HIV vector was also constructed with the target sequence GTGCGCTGCTGGTGCCAAC. A mutation-specific anti-BRAF siRNA HIV vector (target: GCTACAGA GAAATCTCGATGG) was used for control in a reporter assay. These HIV vectors produce a short hairpin RNA with the linker sequence (TTCAAGAGA) forming a loop structure, then the linker is processed by Dicer, forming a dsRNA that act as an siRNA. The third-generation HIV vectors were produced by transfecting 293T cells with HIV plasmid vectors, pMD.G (VSV-G env expression plasmid), pMDLg/p.RRE (the third-generation packaging plasmid) and pRSV Rev (Rev expression plasmid) (the latter two plasmids were provided by Cell Genesys, USA) by calcium phosphate transfection. The culture supernatants were collected and used as virus stocks after concentration. The viral titer was measured by counting GFP-positive cells after infection on 293T cells.

In vitro growth inhibition assay

ACC-LC-172 cells (100 000) were infected with the siRNA HIV vectors for Skp-2 (S2 or S5) or firefly luciferase (GL3B) at 100 MOI at day 0. Cell numbers were counted every 3 days by trypan blue dye exclusion method until day 9. The 293T cells (30 000) were infected with siRNA HIV vectors for control GL3B or Skp-2 (S5) at 100 MOI at day 0, then the cell numbers were determined every 3 days until day 9. SBC-1 cells (100 000) were infected with the siRNA HIV vectors, S2, S5 or GL3B at 100 MOI. Cell numbers were counted every 4 days until day 8.

Western blot analysis

Cell lysates were prepared in the lysis buffer (20 mM Tris-HCl (pH 7.5), 12.5 mM β-glycerophosphate, 2 mM EGTA, 10 mM NaF, 1 mM benzamide, 1% NP-40, protease inhibition cocktail (complete, EDTA-free (Roche, Germany)) and 1 mM Na_3VO_4) from the infected cells used in *in vitro* growth inhibition assay on day 9 after confirmation of equivalent GFP expression among the groups by flow cytometry. The protein concentration was determined by DC protein assay kit (Bio-Rad, USA). Anti-p45^{Skp-2} (Zymed Laboratories Inc., CA, USA), anti-actin (Sigma, USA), anti-p27^{Kip1} (BD Transduction, USA), anti-Rb (Cell Signaling, USA) or anti-p21 (Santa Cruz, USA) Abs was used for the first antibody. An HRP-conjugated anti-IgG antibody was used for the second antibody, and the reaction was detected by chemiluminescence with SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, USA).

Cell cycle analysis

The cells used in the *in vitro* growth inhibition assays were harvested on day 9 and stained with propidium iodide (PI) by using CycleTEST PLUS DNA Reagent Kit (Becton Dickinson, San Jose, CA, USA) according to the manufacturer's instruction. After the stained cells were analyzed by FACSCalibur (Becton Dickinson), the cell cycle status was analyzed with ModFit software (Becton Dickinson).

hTERT reporter construction

A 0.4 kb hTERT promoter sequence was amplified by genomic PCR with the forward primer: CGCTGG GGCCCTCGC TGGCGTCCCT (nts −324 to −300, numbered relative to the translation initiation site); and the reverse primer: CAGCGGCAGCACCTCGCGGTAGTGG (nts +48 to +72). After denaturation for 4 min at 95°C, 27 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 70°C and extension for 1 min at 72°C were performed and followed by completion for 7 min at 72°C. The PCR product was subcloned into a pCRII vector of TA Cloning kit (Invitrogen, San Diego, CA). After the confirmation of the correct sequence, the translation initiation codon was mutated from ATG to TTG by using QuikChange site-directed mutagenesis kit (STRATAGENE, La Jolla, CA, USA). Then, the hTERT promoter was subcloned into a pGL3-Basic vector (Promega, Madison, WI, USA). The resultant construct, pGL3-hTERT, transcribes the firefly luciferase gene under the control of the 0.4 kb hTERT promoter.

Reporter assay

ACC-LC-172 cells (500 000) stably expressing siRNA for Skp-2 (S5) or for BRAF (V599E) (control siRNA specific for mutated BRAF (V599E) after the infection with HIV vectors) were transfected with 1 μg of *Renilla luciferase* expression plasmid, pRL-SV40 (Promega) and 1 μg of one of the following firefly luciferase expression plasmids: pGL3-hTERT, pGL3-Basic or pGL3-control (Promega) by using Lipofectamine (Invitrogen). At 48 h after the transfection, the cells were harvested and the luciferase activity was analyzed using Dual-Glo Luciferase Assay System (Promega) and a Berthold luminometer. Each firefly luciferase activity was normalized to *Renilla luciferase* activity.

Adenovirus vectors for siRNA

The adenovirus vectors containing Ad5/35 chimeric fiber protein[17] were used in this study. The vector plasmids pAdF35 and the shuttle vector plasmid pHMCMV-GFP1 were described previously.[18] pHMCMV-GFP1 contains the CMV promoter, the GFP gene derived from pEGFP-N1 (Clontech, Palo Alto, CA, USA) and the bovine growth hormone (BGH) poly(A) signal. The siRNA expression unit containing human U6 promoter and two *Bsp*MI cloning sites were excised from the HIV-U6i-GFP plasmid by *Eco*RI digestion, then subcloned into the *Eco*RI site in pHMCMV-GFP1, which was located downstream of the BGH poly(A) signal. This vector was designated as pHMCMV-GFP-U6i. The ds oligonucleotides for the short hairpin RNA can be directly subcloned into the two *Bsp*MI sites of the pHMCMV-GFP-U6i as in HIV-U6i-GFP. Accordingly, the shuttle vector plasmids containing ds oligonucleotides for Skp-2 (S5) or GL3B were constructed. The adenovirus vectors, AdF35-Skp-2 siRNA S5 and AdF35-GL3B, were constructed by an improved *in vitro* ligation method as described.[19] Both adenovirus vectors were propagated in 293 cells and the viral titers were determined using Adeno-X Rpaid Titer Kit (Clontech) according to the manufacturer's instructions.

Animal experiments

Male NOD/SCID mice (6 weeks old) (Japan Clea, Japan) were subcutaneously implanted with 5×10^6 ACC-LC-172 cells. About 1 week after the implantation, when the largest tumor diameter reached about 3–4 mm, we injected 1×10^8 IFU of AdF35-Skp-2 siRNA S5 or AdF35-GL3B into the tumor (day 0). The adenovirus injection was repeated twice every 2 days. The tumor volume (the largest diameter × the perpendicular diameter × the height) was measured every 2 or 3 days until day 13. The animal experimental protocol was approved by the Laboratory Animal Care and Use Committee at Keio University School of Medicine. Mice were treated according to the Guidelines for the Care and Use of Laboratory Animals of Keio University School of Medicine.

Statistical analysis

All statistical analyses were performed according to unpaired Student's *t*-test.

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