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• COLORECTAL CANCER •

Polymerase synthesis and potential interference of a small-interfering RNA targeting hPim-2

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Abstract

AIM: To synthesize three small-interference RNAs (siRNAs) by T7 RNA polymerase-catalyzed reaction, and to investigate their efficacy on modulating the expression of serine/threonine kinase Pim-2 in human colon cancer cell line.

METHODS: siRNA I, II and III were synthesized by T_7 RNA polymerase-directed *in vitro* transcription, then transfected into human colon cancer cells SW-480. After incubation for 6 h at 37 , 100 mL/L FBS in RPMI 1640 was substituted in each well. After the transfection was repeated twice to three times in each kind of siRNA, hPim-2 mRNA and protein expression were measured by RT-PCR and Western blotting, respectively.

RESULTS: Compared to the control group, after transfected for 48 h with hPim-2 siRNA I, II and III, the relative inhibition rates of hPim-2 mRNA expression in colon cancer cells were 65.4% (P<0.05), 46.2% (P<0.05) and 56.1% (P<0.05), respectively. The protein level of hPim-2 was decreased at 72 h compared to the untransfected cells. The relative inhibition percentages of hPim-2 protein by siRNA I, II, III were 61.6% (P<0.05), 45.8% (P<0.05) and 55.6% (P<0.05), respectively.

CONCLUSION: The *in vitro* transcribed siRNAs can be useful for silencing oncogene hPim-2 expression specifically and efficiently. This may open a new path toward the use of siRNAs as a gene-specific therapeutic tool.

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INTRODUCTION

RNAi is an evolutionarily conserved mechanism known to control insects, plants, and mammalian cells[1-4]. In this process, introduced double-stranded RNAs (ds-RNAs) silence gene was expressed through specific degradation of their cognate mRNAs[5,6]. Importantly, RNAi can be achieved in mammalian cells following transfection of synthetic 21- and 22-nucleotide (nt) small interfering (si) RNAs, indicating that RNAi may serve as a powerful tool to block the expression of target genes specifically[7-11].

Pim-2 is a member of a family of serine/threonine protein kinases that consists of two other members, Pim-1 and Pim-3, and it exists at high concentrations in many tumor cells[12,13]. Though it was identified 20 years ago, its function that maintains the cell size and its role in the survival of cancer cells have been just determined recently[14,15]. It is believed to be a cancer-causing gene, or oncogene. Here, we sought to use siRNA-targeting hPim-2 to determine whether this technique could be used to specifically inhibit hPim-2 expression.

MATERIALS AND METHODS

T_7 siRNA synthesis

siRNAs selection was based on the characterization of siRNA by Elbashir *et al*.[16]. Three hPim-2 siRNA sequences are given in Figure 1. For *in vitro* transcription, 40-nt DNA template oligonucleotides were designed to produce 21-nt siRNAs. siRNA sequences of the form $GN_{17}CN_2$ were selected for each target. Uridines in the last two nt form the 3' overhang of the siRNA duplex. The template and a 19-nt T_7 promoter (GGT AAT ACG ACT CAC TATA) were synthesized by Applied Biosystems 393 DNA synthesizer and purified by OPC (Perkin-Elmer, Foster city, CA). The oligonucleotide-directed mutagenesis of small siRNA transcription with T_7 polymerase is as follows: for each transcription reaction, 1 nmoL of each oligonucleotide was annealed in 50 μL of TE buffer (10 mmol/L Tris-HCl pH 8.0, and 1 mmol/L EDTA) by heating at 95 °C; after 5 min, the heating block was switched off and allowed to cool down slowly to obtain dsDNA. Transcription was performed in 50 μL of transcription mixture: 1×T_7 transcription buffer (40 mmol/L Tris-HCl pH 7.9, 6 mmol/L $MgCl_2$, 10 mmol/L DTT, 10 mmol/L NaCl and 2 mmol/L spermidine), 1 mmol/L rNTPs, 0.1 U yeast pyrophosphatase (Sigma), 40 U RNase (Life Technologies) and 100 U T_7 RNA polymerase (Fermentas) containing 200 pmoL of the dsDNA as template. After incubation at 37 °C for 3 h, 1 U RNase free-DNase (Promega) was added at 37 °C for 30 min. Sense and antisense 21-nt RNAs (single strand RNA, ssRNA) generated in separate reactions were annealed by mixing both crude transcription reactions, incubating at 37 °C overnight to obtain "T_7 RNA polymerase synthesized small interfering double-strand RNA (T_7 siRNA, dsRNA)". The mixture (100 μL) was then extracted with TE-saturated (pH 4.5) phenol:chloroform:isoamyl alcohol (25:24:1), purified with chloroform:isoamyl alcohol(24:1), isopropanol and 0.2 mol/L sodium acetate (pH5.2). The pellet was washed once with 750 mL/L ethanol, dried, and resuspended in 50 μL of water.

Cell culture

Human colon cancer cell line SW-480 was obtained from Chinese National Cancer Institute. The cells were grown in RPMI 1640 medium (Invitrogen) supplemented with 100 mL/L fetal bovine serum (GIBCO BRL, Grand Island, NY),100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C with 50 mL/L CO_2.

Transfection with siRNA oligonucleotides

Cells were seeded the day before the experiment in 6-well plates at a density of 1.5×10^5 per well to be 50% confluent on the day

hPim-2-I Target mRNA	5′	AAGUUUGCCCAGUUCCCUUCC	3′
siRNA	5′	GUUUGCCCAGUUCCCUUCCUU	3′ Sense strand
	3′	UUCAAACGGGUCAAGGGAAGG	5′ Antisense strand
hPim-2-II Target mRNA	5′	AAGACAUAAACCAAGUUUGCC	3′
siRNA	5′	GACAUAAACCAAGUUUGCCUU	3′ Sense strand
	3′	UUGUGUAUUUGGUUCAAACGG	5′ Antisense strand
hPim-2-III Target mRNA	5′	AAGUUGUUCCCAUUUUGAGCC	3′
siRNA	5′	GUUGUUCCCAUUUUGAGCCUU	3′ Sense strand
	3′	UUCAACAAGGGUAAAACUCGG	5′ Antisense strand

Figure 1 Sequences of 21-nt siRNA duplex that were used to target at hPim-2.

of the experiment. Transfection of the RNA oligonucleotides was performed using Lipofectamine 2000 (Invitrogen) as directed by the manufacturer to result in a final RNA concentration of 50 nmol/L. After transfection (incubation for 6 h at 37 °C), cells were washed with PBS and incubated in fresh culture medium until additional analyses.

Analysis of hPim-2 mRNA by RT-PCR

After transfection, total RNA was isolated using TRIZOL (Invitrogen) by a single-step phenol-extraction. Subsequent RT-PCR was performed (RT-PCR kit, Promega, Madison, WI.). Briefly, first strand cDNA was synthesized using an Oligo $(dT)_{15}$ primer at 42 °C for 30 min. PCR for hPim-2 and β-actin was performed in a single reaction of 20 μL volume. The latter served as a control following 28 cycles of denaturing at 95 °C for 45 s, annealing at 58 °C for 40 s, and extending at 72 °C for 40 s. Under this PCR condition, the amplification showed linearity as was determined experimentally (data not shown). PCR products were run on a 30 g/L agarose gel and visualized by ethidium bromide staining, and the intensities were then measured by scanning the gel with Gel Doc 1000 (Bio-Rad, Hercules, CA). Inhibition of hPim-2 mRNA was calculated according to the following formula:

$$\text{Inhibition percentage} = \frac{(1- A_{\text{sample}} \times A_{0\text{control}})}{A_{\text{control}} \times A_{0\text{sample}}} \times 100\%.$$

A_{sample}: the intensity of hPim-2 PCR product in cells transfected with siRNA and Lipofectamine; $A_{0\text{sample}}$: the intensity of hPim-2 PCR product in cells transfected with Lipofectamine alone; A_{control}: the intensity of β-actin product in cells transfected with siRNA and Lipofectamine; $A_{0\text{control}}$: the intensity of β-actin product in cells transfected with Lipofectamine alone.

Analysis of hPim-2 protein

The expression levels of hPim-2 protein in cells transfected with siRNAs were measured by scanning the density of bands on Western blotting. The expression level of hPim-2 mRNA was analyzed by the method described above. After 72 h of transfection, cells were lysed in RIPA buffer [10 mmol/L Tris-HCl (pH 7.4), 10 g/L deoxycholate, 10 g/LNP40, 150 mmol/L NaCl, 1 g/L SDS, 0.2 mmol/L phenylmethyl sulfonyl fluoride, 1 μg/mL aprotinin and 1 μg/mL leupeptin] for 30 min on ice. The lysates were centrifuged at 15 000 r/min for 15 min to remove debris. Equal amounts (30 μg) of proteins were separated by 120 g/L SDS-PAGE and transferred onto PVDF membrane (Hybond-polyvinylidene difluoride membranes, Amersham Biosciences). The transferred membrane was incubated with anti-hPim-2 goat polyclonal or anti-β actin rabbit polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and followed with peroxidase-linked secondary antibody. Finally, the immunoreactive proteins were detected by an ECL-plus detection kit (Amersham Biosciences) and scanned by Gel Doc 1000 (Bio-Rad), and the inhibition percentage (%) was calculated according to the following formula: inhibition percentage = $(1-A_{\text{sample}}/A_{\text{control}})\times 100$.

Statistics

The data were expressed as mean±standard deviation (mean±SD). Statistical analysis was performed by Student's-*t*-test (two tailed). All data represented at least two independent experiments.

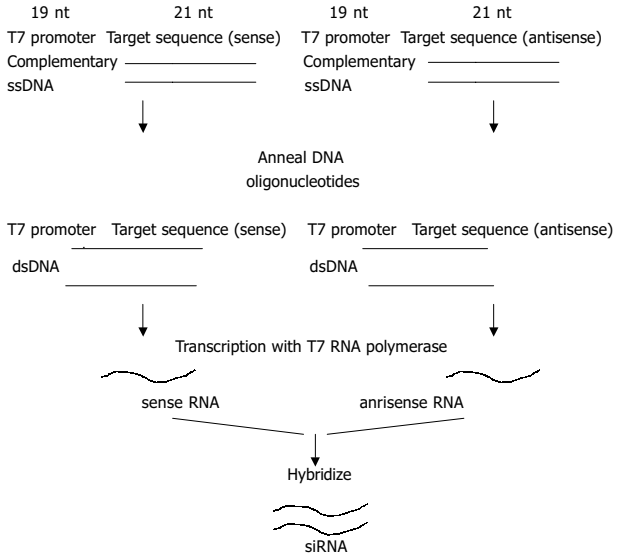

Figure 2 Strategy to generate T7 siRNA.

RESULTS

Synthesis of siRNA by in vitro transcription

To generate siRNAs by *in vitro* transcription, we designed the strategy presented in Figure 2. Target sequences for siRNA were identified by scanning the length of the hPim-2 gene with AA sequences. The AA and downstream 19 nucleotides were recorded and compared to an appropriate genome database to eliminate any sequences with significant homology to other genes. Those sequences that appear to be specific are the potential siRNA target sites. Besides, it is noteworthy that T_7 RNA polymerase can transcribe a template efficiently if only the first nucleotide of the RNA transcript is G. Thus, the design of T_7 siRNAs requires that the sequence starts with a G and has a C at position 19 ($GN_{17}CN_2$) to allow annealing with the complementary RNA, which also starts with a G[17,18]. The T_7 promoter oligonucleotide is invariant and common to any target gene. A 40 mer DNA oligonucleotide template was synthesized

by a 21 mer oligonucleotide encompassing the T_7 promoter with complementary sequence preceded by two additional nucleotides (reading the sequence 5'----3'). Following transcription reactions, sense and antisense transcriptions were annealed, ethanol precipitated and yielded what we refer to as T_7siRNAs. The integrity of the transcriptions was checked on a 30 g/L agarose gel (Figure 3).

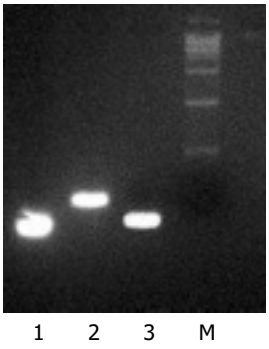

Figure 3 Lane 1: T7 *in vitro* transcribed single-strand RNA. Lane 2: annealed double-strand DNA template. Lane 3: hybridized double-strand small interference RNA.

Effect of siRNAs on hPim-2 expression

The mRNA level of hPim-2 was determined by semi-quantitative RT-PCR. A 237-bp DNA fragment of hpim-2 gene and a 317-bp DNA fragment of β-actin gene were amplified by RT-PCR with specific primers, respectively. As shown in Figure 4A, mRNA expression level of hPim-2 was decreased when compared to the uninduced cells, while the mRNA level of β-actin as the control was almost unchanged. As shown in Figure 4B, after transfection with hPim-2 siRNA I, II and III and compared with the levels of β-actin, the relative inhibition rates of hPim-2 mRNA expression were 65.4% (P<0.05), 46.2% (P<0.05) and 56.1% (P<0.05) in colon cancer cells, respectively.

In order to verify the decrease in mRNA expression, which corresponded to the decreases at protein levels, Western blotting was performed. Figure 5A shows that the protein level of hPim-2 was decreased at 72 h compared to the uninduced cells. The relative inhibition percentages of hPim-2 protein by siRNA I, II and III were 61.6% (P<0.05), 45.8% (P<0.05) and 55.6% (P<0.05), respectively (Figure 5B).

DISCUSSION

Oncogene overexpression has been implicated in the development and progression of a variety of human cancers and, therefore, provides a potential target for cancer gene therapy[19-22]. For years, research has been focused on effective tools to specifically down-regulate oncogene overexpression such as antisense oligonucleotide strategy. However, there has been only limited success because of the lack of specificity and potency for this method. For example, screening of more than 20 oligomers is usually required before identifying one antisense that functions effectively, and the dose required for inhibiting gene expression is often not much different from the doses that lead to nonselective toxicity[23-25].

Recent progress of RNAi techniques has demonstrated the potential to overcome those limitations. The selection of targeting sequences of RNAi is less restricted, once the site is identified, sense and antisense oligonucleotides with 3'-UU overhangs can be designed, so the success rates of producing effective duplexes are higher. Just like in this experiment, siRNAs were designed complimentary to three different regions of the corresponding Pim-2 mRNA, and each of them has different level of inhibition efficacy, the suppression of hPim-2

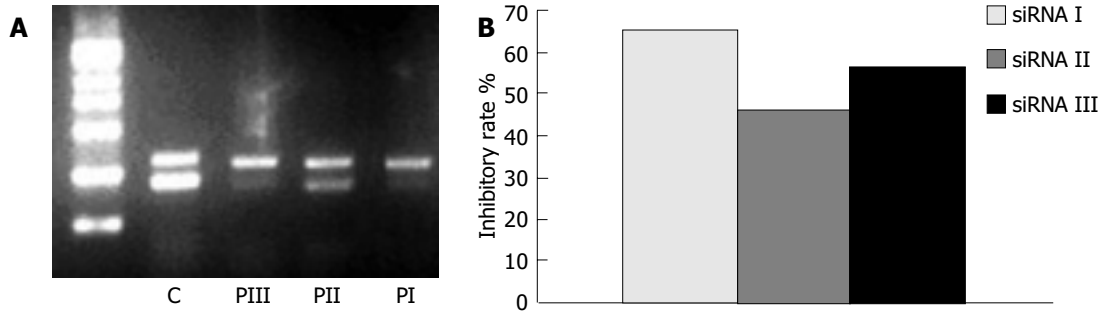

Figure 4 Inhibitory effects of siRNA on mRNA level of hPim-2. A: Electrophoresis of RT-PCR products of hPim-2 gene and β-actin gene in colon cancer cells transfected with siRNA I,II,III. B: Quantitation of inhibitory percentage of hPim-2 mRNA in transfected cells.Each level of PCR product of hPim-2 gene was. quantitated and normalized to the level of β-actin. Inhibitory rate was calculated by comparing to the control cells.The results were expressed as means±SD from independent experiments. P<0.05 *vs* the cells transfected with lipofectamine alone.

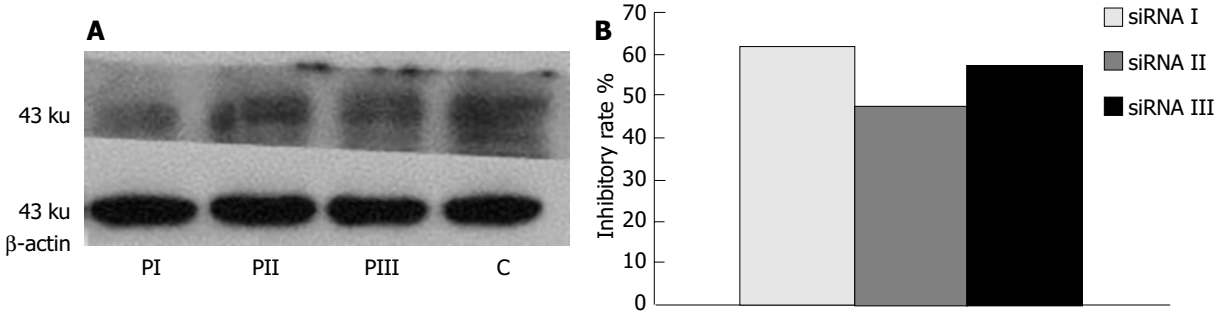

Figure 5 Inhibitory effects of siRNA on protein level of hPim-2. A: Western blot analysis of hPim-2 protein in colon cancer cells transfected with siRNA I, II, III. B: inhibitory percentage of hPim-2 protein in transfected cells compared to the control cells.Each level of hPim-2 protein was quantitated.Inhibitory rate was calculated by comparing to the control cells. The results were expressed as mean±SD from independent experiments. P<0.05 *vs* the cells transfected with lipofectamine alone.

gene expression by these siRNAs directed at different sites varied from 45-65%. This indicates that screening potential target of RNAi is much more easy.

Besides, our results demonstrate that *in vitro* transcribed siRNA can effectively down-regulate oncogene expression with great efficiency. It has been suggested that siRNA may inhibit gene expression through diverse effects, inhibition of mRNA can occur through the formation of a nuclease complex called RISC (RNA-induced silencing complex) that targets and cleaves mRNA which is complementary to the siRNA. The damaged mRNA may deteriorate through the action of the RNA-dependent RNA polymerase (RdRP), producing new siRNAs to target other mRNA. This incessant waterfall-like amplification can produce RNA interference effect at a very small dose, and inhibit the protein translation quickly and efficiently[26-30]. In our experiment, the dose required for inhibiting Pim-2 gene expression was 50 nmol/L, far below the dose required for the antisense oligonucleotide[31], indicating that siRNA synthesized by the *in vitro* transcription strategy can suppress the hPim-2 gene expression sensitively.

Here, we used the *in vitro* transcription method for the synthesis of siRNAs by T_7 RNA polymerase and transferred them into cells. The main advantage of this technique is its simplicity. It provides a reproducible and highly efficient means to inhibit the target gene expression. Human Pim-2 gene, a regulated transcriptional apoptotic inhibitor, has a novel role in promoting cell autonomous survival. Over-expression of Pim-2 allows the tumour cells to ignore or become insensitive to boosters of the immune system[14]. Application of Pim-2-directed siRNA can significantly reduce Pim-2 mRNA and protein levels efficiently. Our next step is to try to manipulate the action of Pim-2 with siRNA, so that we can interfere with the survival of cancer cells.

REFERENCES

1 **McCaffey AP**, Meuse L, Pham TT, Conklin DS, Hannon GJ, Kay MA. RNA interference in adult mice. *Nature* 2002; **418**: 38-39
2 **Tiscornia G,** Singer O, Ikawa M, Verma IM. A general method for gene knockdown in mice by using lentiviral vectors expressing small interfering RNA. *Proc Natl Acad Sci U S A* 2003; **100**: 1844-1848
3 **Brummelkamp TR**, Bernards R, Agami R. A system for state expressing short RNAs in mammalian cells. *Science* 2002; **296**: 550-553
4 **Elbashir SM**, Lendeckel W, Tuschl T. RNA interference is mediated by 21-and 22-nucleotide RNAs. *Genes Dev* 2001; **15**: 188-200
5 **Wang Q**C, Nie QH, Feng ZH. RNA interference: Antiviral weapon and beyond. *Word J Gastroenterol* 2003; **9**: 1657-1661
6 **Fire A,** Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interfernce by double-stranded RNA in Caenorhabditis elegans. *Nature* 1998: **391**: 806-811
7 **Hammond SM**, Bernstein E, Beach D, Hannon GJ. An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosphila cells. *Nature* 2000; **404**: 293-296
8 **McManus MT**, Petersen CP, Haines BB, Chen J, Sharp PA. Gene silencing using micro-RNA designed hairpins. *RNA* 2002; **8**: 842-850
9 **Novina CD**, Murray MF, Dykxhoorn DM, Beresford PJ, Riess J, Lee SK, Collman RG, Lieberman J, Shankar P, Sharp PA. siRNA-directed inhibition of HIV-1 infection. *Nat Med* 2002; **8**: 681-686
10 **Paddison PJ**, Caudy AA, Bernstein E, Hannon GJ, Conklin DS. Short hairpin RNAs induce sequence-specific silencing in mammalian cells. *Genes Dev* 2002; **16**: 948-958
11 **Yu JY**, DeRuiter SL, Turner DL. RNA interference by expression of short-interfering RNAs and hairpin RNAs in mammalian cells. *Proc Natl Acad Sci U S A* 2002; **99**: 6047-6052
12 **Datta SR**, Ranger AM, Lin MZ, Sturgill JF, Ma YC, Cowan CW, Dikkes P, Korsmeyer SJ, Greenberg ME. Survival factor-mediated BAD phosphorylation raises the mitochondrial threshold for apoptosis. *Dev Cell* 2002; **3**: 631-643
13 **Plas DR,** Thompson CB. Cell metabolism in the regulation of programmed cell death. *Trends Endo Met* 2002; **13**: 74-78
14 **Fox CJ**, Hammerman PS, Cinalli RM, Master SR, Chodosh LA, Thompson CB. The serine/threonine kinase Pim-2 is a transcriptionally regulated apopototic inhibitor. *Genes Dev* 2003; **17**: 1841-1854
15 **Allen J,** Verhoeven E, Domen J, van der Valk M, Berns A. Pim-2 transgene induces lymphoid tumors, exhibiting potent synergy with *c-myc*. *Oncogene* 1997; **15**: 1133-1141
16 **Elbashir SM**, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 2002; **411**: 494-498
17 **Milligan JF**, Uhlenbeck OC. Synthesis of small RNAs using T7 RNA polymerase. *Methods Enzymol* 1989; **180**: 51-62
18 **Konarska MM**, Sharp PA. Structure of RNAs replicated by the DNA-dependent T7 RNA polymerase. *Cell* 1990; **63**: 609-618
19 **Gottlieb E,** Thompson CB. Targeting the mitochondria to enhance tumor suppression. *Methods Mol Biol* 2003; **223**: 543-554
20 **Watanabe RN**. Oncogene and tumor suppressor gene. *Rinsho Byori* 2002; **123**: 131-136
21 **Gerdes AM**. Cancer genetics. A review of oncological molecular biology seen in relation to the human genome. *Ugeskr Laeger* 2002; **164**: 2865-2871
22 **Willians JL**. Malignancy: an evolving definition of a cancer cell. *Clin Lab Sci* 2002; **15**: 37-43
23 **Miyagishi M,** Hayashi M, Taira K. Comparison of the suppressive effects of antisense oligonucleotides and siRNAs directed against the same targets in mammalian cells. *Antisense Nucleic Acid Drug Dev* 2003; **13**: 1-7
24 **Kretschmer-Kazemi Far R**, Sczakiel G. The activity of siRNA in mammalian cells is related to structural target: accessibility comparition with antisense oligonucleotides. *Nucleic Acid Res* 2002; **31**: 4417-4424
25 **Aoki Y**, Cioca DP, Oidaira H, Kamiya J, Kiyosawa K. RNA interference may be more potent than antisense RNA in human cancer cell lines. *Clin Exp Pharmacol Physicol* 2003; **30**: 96-102
26 **Chiu YL**, Rana TM. RNAi in human cells: basic structural and functional featuresof small interfering RNA. *Mol Cell* 2002; **10**: 549-561
27 **Scherr M**, Morgan MA, Eder M. Gene silencing mediated by small interfering RNAs in mammalian cells. *Curr Med Chem* 2003; **10**: 245-256
28 **Carthew RW**. Gene silencing by double-stranded RNA. *Curr opin Cell Biol* 2001; **13**: 244-248
29 **Smith NA**, Singh SP, Wang MB, Stoutjesdijk PA, Green AG, Waterhouse PM. Total silencing by intron-spliced hairpin RNAs. *Nature* 2000; **407**: 319-320
30 **Doi N**, Zenno S, Ueda R, Ohki-Hamazaki H, Ui-Tei K, Saigo K. Short-intrfering-RNA-mediated gene silencing in mammalian cells requires Dicer and eIF2C translation initiation factors. *Curr Biol* 2003; **13**: 41-46
31 **Bertrand JR**, Pottier M, Vekris A, Opolon P, Maksimenko A, Malvy C. Comparison of antisense oligonucleotide and siRNAs in cell culture and *in vivo*. *Biochem Biophys Res Commun* 2002; **296**: 1000-1004

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