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Transcriptional targeting of small interfering RNAs into cancer cells

Trang Huynh [a], Sébastien Wälchli [b], Mouldy Sioud [a,*]

[a] Department of Immunology, Molecular Medicine Group, University Department, University of Oslo, The Norwegian Radium Hospital, Montebello, N-310 Oslo, Norway

[b] Department of Biochemistry, University Department, University of Oslo, The Norwegian Radium Hospital, Montebello, N-310 Oslo, Norway

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Abstract

Small interfering RNAs (siRNAs) are widely used for analyzing gene function and have the potential to be developed into human therapeutics. However, persistent siRNA expression in normal cells may cause toxic side effects. Therefore, the therapeutic applications of RNAi in cancer require either the specific delivery of synthetic siRNAs into cancer cells or the control of siRNA expression. Accordingly, we have developed a cancer-specific vector that expresses siRNAs from the human survivin promoter. A plasmid vector expressing siRNAs under this promoter enabled efficient gene silencing of gene expression in different cancer cell lines. The levels of inhibition were comparable to that obtained with the constitutively active U6 promoter. By contrast to U6 promoter, no significant gene silencing was obtained with the Survivin promoter in normal mammary epithelial cells. Collectively, these data indicate that the survivin promoter is suitable for directing siRNA expression in cancer cells, but not normal cells.

Keywords: RNA interference; Hairpin RNAs; Transcriptional regulation; Gene therapy; Survivin promoter; Tissue-specific promoter; Small interfering RNAs

Although initial experiments with RNAi in human cells have been unsuccessful because of the interferon pathway, the demonstration that small interfering RNAs (siRNAs) can bypass the potent ds RNA IFN response and induce gene silencing in a sequence-specific manner has paved the way for functional genomics and drug target validation in human cells [1–5]. However, one of the drawbacks of synthetic siRNAs is the transient nature of the inhibition [6]. To circumvent this problem, several investigators have shown that short hairpin siRNA (shRNA) can be produced from both plasmid and viral vectors [7–11]. shRNAs mimic the pre-miRNA hairpin that result from the processing of endogenous primary (pri-miRNA) transcripts by the RNase III enzyme Drosha. The most common approach relies on the transcription by RNA polymerase III of shRNAs. In such strategy, an RNA polymerase III promoter (e.g., U6, H1, and tRNA) is used to transcribe a short stretch of inverted DNA sequence. Upon transport to the cytoplasm by Exportin 5, the double-stranded short hairpin RNAs (shRNAs) are recognized by Dicer to generate functional siRNAs. In addition to plasmid DNA, viral-based shRNA allows long-term gene silencing in a variety of cell types, including normal [12–18]. Despite the success obtained with Pol III promoters, they do not allow tissue/cell-specific gene inactivation. A complementary strategy has been developed in which the siRNAs are transcribed from a pol II promoter and have been shown to mediate efficient gene silencing in cultured mammalian cells and animals [9,19]. Compared to Pol III promoters, Pol II promoter-directed expression of shRNAs offers several advantages, including tissue-specific promoters and inducible transcription [20]. Therefore, these promoters may be used to direct cell- or tissue-specific gene silencing. However, in most of the reported examples a constitutively active Pol II promoter was used (e.g., CMV promoter) [9]. For cancer cell targeting, a potential strategy would be the use of a tissue- or cancer-specific promoter. In this respect, several

* Corresponding author. Fax: +47 22 500 730.
E-mail address: mosioud@ulrik.uio.no (M. Sioud).

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genes have shown to be expressed exclusively or predominantly in cancer cells as compared to normal cells [21–23]. In addition, several promoters have already been evaluated for transcriptional targeting in cancer gene therapy, such as the α-promoter for hepatic carcinoma [24], the tyrosinase gene promoter for melanoma [25], the prostate-specific antigen promoter for prostate cancer, and the cyclooxygenase-2 (Cox-2) promoter for different cancers [26–29].

Survivin is a member of the inhibitors of apoptosis (IAP) gene family, which play crucial roles in the survival of cancer cells and the progression of malignancies. It is expressed in nearly all-human cancers, but not or poorly in normal adult human tissues [30]. Moreover, its expression is transcriptionally regulated, suggesting that survivin promoter may be useful in controlling siRNA expression in cancer cells. Here, we report on the development of a broadly applicable minimal survivin promoter that contains all the naturally regulatory elements capable for evoking gene silencing in cancer cells, but not normal cells.

Materials and methods

Reagents. DNA oligonucleotides and monoclonal antibodies were purchased from Invitrogen and Santa Cruz, respectively.

Genomic, PCR, and construction of siRNA expression pGSU-ST2 vector. Genomic DNA was isolated by proteinase K digestion and sequential phenol extraction from RAMOS cancer cell line. Initially, 1.680 kb DNA fragment containing the survivin promoter was amplified from genomic DNA of human RAMOS cells using the following PCR primers: Forward primer 5′-GGGTAAGAGAGGGAGAGGAGGAGA-3′ and reverse primer 5′-TCTTGAATGTAGAGATGCGGTG-3′. Subsequently, a 600 bp DNA containing all the regulatory sequences (34) was PCR amplified using the following primers: Forward primer 5′GGGTAA GAGAGGGAGAGGAGGA-3′, 5′-AAGCTCTCCTGTCTGACT-3′ and reverse primer 5′-AGAGATGCGGTGGTCCTTGA-3′. The 600 bp PCR product was first cloned into pGEM-T Easy Vector (Promega) and confirmed by sequencing. A second PCR was performed, adding *Bam*H1 and *Hin*dIII cloning sites for cloning siRNAs. These sites were added immediately downstream the second potential transcription start site [33]. The PCR products were recloned into pGEM-T Easy Vector. The resulting vector was named pGSU-ST2, sequenced, and used to express shRNAs. The sequence of the reverse primer used for second PCR was: 5′-GTAAGCTTATGGATCCGCCGCGGGGCATGTCGGGA-3′.

Cloning of siRNAs. To clone siRNAs against the targeted genes, for each gene two overlapping oligonucleotides were designed.

Equal amounts of sense and antisense oligonucleotides were annealed and then subcloned into *Hin*dIII and *Bam*HI-cleaved pGSU-ST2 vector. Positive clones were selected and confirmed by DNA sequencing.

Cell lines and cell culture. Human breast cancer cell lines MCF7, HEK293, HT29, and Hela were obtained from American type culture collection (ATCC). The cells were cultured in RPMI supplemented with 10% FCS and antibiotics. The human normal mammalian epithelial cells (HMEC) were obtained from (Clonetics/BioWittaker) and cultured in MEBM (Clonetics, CAMBREX) according to the manufacturers' instructions. Cells were maintained at 37 °C in a humidified 5% CO_2 incubator.

Transfection. Cells were transfected at 60–70% confluence in 6-well plates with indicated amounts of shRNA plasmid constructs and pEGFP-N3 plasmid DNA using lipofectAMINE according to the manufacterers' instructions. After 48 h of transfection, the cells were analyzed by flow cytometry in order to check for GPF expression.

Western blot analysis. For Western blot analysis, the cells were washed with PBS, lysed in lysis buffer containing 1% NP-40 and 1% proteinase inhibitors (Sigma–Aldrich), and then incubated in ice for 30 min. Subsequently, cell lysates were centrifuged at 15,000 rpm for 10 min at 4 °C. Supernantant was collected and protein concentrations were determined. Around 15 μg of protein was loaded per lane and separated by 10% SDS (sodium dodecyl sulfate)–polyacrylamide gel electrophoresis. Subsequently, proteins were transferred to nitro-cellulose membranes and then membranes were blocked with 5% milk/Tween in (PBS). Membranes were incubated with an anti-GFP polyclonal antibody followed by incubation with peroxidase-conjugated goat anti-rabbit second antibody and immunoreactive proteins were detected by the ECL method. To check for protein loading, the blots were also incubated with an anti β-actin polyclonal antibody.

Results and discussion

Design of the surviving promoter

Although Poll III promoters are widely used for the expression of siRNAs, high level expression of shRNA increases the induction of bystander effects, including off-target silencing, interferon response, and cellular toxicity. Moreover, constitutive expression of shRNAs could compete with endogenous miRNA processing and functionality, leading to cell death [31,32]. Therefore, the therapeutic applications of RNAi require either the specific delivery of synthetic siRNAs into cancer cells or the control of siRNA expression. To develop a cancer specific siRNA-based gene therapy, we used the survivin promoter because surviving is expressed in nearly all-human cancers. Also, recent studies demonstrated the utility of survivin promoter to drive transgene expression in a cancer-specific manner, and survivin expression correlates with clinical progression and resistance to chemo- and radiotherapy [33]. To construct human survivin promoter-driven shRNA vector, we first PCR amplified a 1.680 kb genomic DNA of

GFP

BamH1 Loop sequence Poly A signal Hind III

5′-GATCCGCAAGCTGACCCTGAAGTTCTTCAAGAGAGAACTTCAGGGTCAGCTTGCAATAA-3′
3′ GCGTTCGACTGGGACTTCAAGAAGTTCTCTCTTGAAGTCCCAGTCGAACGTTATTTCGA-5′

CSPP

5`-GATCCGAAGATTTGCGCAGTGGACTTCAAGAGAGTCCACTGCGCAAATCCTTTCAATAA-3`
5′GCTTCTAAACGCGTCACCTGAAGTTCTCTCAGGTGACGCGTTTAGGAAAGTTATTTCGA-3′