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Short Hairpin RNA Causes the Methylation of Transforming Growth Factor-β Receptor II Promoter and Silencing of the Target Gene in Rat Hepatic Stellate Cells

Jin-Wook Kim[1,2], **Yan-Hong Zhang**[1], **Mark A Zern**[1], **John J. Rossi**[3], and **Jian Wu**[1*]

1*Department of Internal Medicine, Transplant Research Program, University of California, Davis Medical Center, Sacramento, CA 95817*

2*Department of Internal Medicine, Seoul National University Bundang Hospital, Seongnam, South Korea*

3*Division of Molecular Biology, Beckman Research Institute of the City of Hope, Duarte 91010, CA*

Abstract

Small interfering RNA (siRNA) induces transcriptional gene silencing (TGS) in plant and animal cells. RNA dependent DNA methylation (RdDM) accounts for TGS in plants, but it is unclear whether siRNA induces RdDM in mammalian cells. To determine whether stable expression of short hairpin siRNA (shRNA) induces DNA methylation in mammalian cells, we transduced rat hepatic stellate SBC10 cells with lentiviral vectors which encode an U6 promoter-driven shRNA expression cassette homologous to the transforming growth factor-β receptor (TGFβRII) promoter region. Sequencing analysis of bisulfite-modified genomic DNA showed the methylation of cytosine residues both in CpG dinucleotides and non-CpG sites around the target region of the TGFβRII promoter in SBC10 cells transduced with the promoter-targeting lentiviral vector. In these cells, real-time RT-PCR showed a decrease in TGFβRII mRNA levels which were reversed by treatment with 5-aza-2-deoxycytidine. Our results demonstrate that recombinant lentivirus-mediated shRNA delivery resulted in the methylation of the homologous promoter area in mammalian cells, and this approach may be used as a tool for transcriptional gene silencing by epigenetic modification of mammalian cell promoters.

Keywords

DNA methylation; promoter; small interfering RNA; short hairpin RNA; transcriptional gene silencing; transforming growth factor-β receptor

INTRODUCTION

Double-stranded RNA-induced transcriptional gene silencing (TGS) was first reported in plants[1]. Methylation of a target gene promoter that is homologous to dsRNA was found to be involved in TGS[1,2]. Similarly, small interfering RNA (siRNA)-mediated TGS was associated with DNA methylation of the targeted sequence in mammalian cells[3], but

*To whom all correspondence should be addressed: University of California, Davis Medical Center, Transplant Research Institute, 4635 2nd Ave., Suite 1001, Sacramento, CA 95817, USA, Tel. +1-916-734-8044, Fax: +1-916-734-8097, E-mail: jdwu@ucdavis.edu.

subsequent reports failed to show sufficient DNA methylation as a major mechanism of TGS in these cells[4–6]. One possible explanation is that the action of siRNA did not last long enough to establish DNA methylation in a non-coding region[5], because synthetic siRNA is retained in cells for only a few days.

Fourth generation replication-defective/self-inactivating lentiviral vectors stably express a transgene or shRNA in mammalian cells for a prolonged period of time[7]. In this brief communication, we report that recombinant lentivirus-mediated delivery of short hairpin RNAs (shRNAs) against the transforming growth factor-β receptor II (TGFβII) promoter region led to the methylation of the target promoter sequences, and in turn suppressed the expression of TGFβII in rat hepatic stellate cells.

MATERIALS AND METHODS

Generation of lentiviral vectors producing shRNA against the TGFβII promoter and coding regions

Target sequences for siRNA were selected by using the Ambion's web-based program (siRNA Target Finder: http://www.ambion.com/techlib/misc/siRNA_finder.html). Two target sequences were chosen from the CpG islands in the promoter region of rat TGFβII (Accession No. AF474028): 5′-AAGGCGAGGGCTGGAAGCTGG-3′ (−412 to −392) and 5′-AAGCTGGAGGAGAGGACCGGA-3′ (−398 to −378). The CpG islands were defined as more than 200-bp stretches of DNA with a C+G content of 50% or greater and observed CpG/expected CpG in excess of 0.60[8], and were identified by using EMBOSS CpGPlot (http://www.ebi.ac.uk/emboss/cpgplot/)[9]. One siRNA sequence against rat TGFβRII mRNA (519–539, Accession No. NM_031132), which was identified as the most potent siRNA sequence in other experiments, was selected for this pilot study: 5′-AAGAACGACAAGAACATTACT-3′. For each of these target sequences, a short hairpin RNA (shRNA) expression cassette was assembled by PCR as reported[10–12]. Briefly, the pTZ U6+1 plasmid with the human U6 promoter was amplified using a 5′ U6 universal primer (5′-ATAAGAATGCGGCCGCCCCGGGGATCCAAGGTCGGG-3′) and 3′ primers that contain shRNA target sequences (Table 1). Amplified shRNA expression cassettes were subcloned into a lentiviral vector backbone (pHIV7-GFP PL) between the *NotI* and *XbaI* restriction enzyme sites. The orientation of the insert was confirmed by sequencing analysis. The recombinant lentiviral backbone plasmid was cotransfected with three other plasmids (core packaging plasmids: pMDLg/pRRE and pRSV-Rev, and envelope plasmid: pMD2.VSVG) of the 4^{th} generation vector into a packaging 293T cell line, by calcium phosphate precipitation as described[13]. Recombinant lentiviral particles were harvested 5 days after transfection by ultracentrifugation at 50,000g for 2hrs at 4°C, and resuspended in PBS containing 0.5% BSA. The lentiviral titer was determined by measuring HIV-1 p24 content with an ELISA assay kit (Beckman Coulter, Haleah, FL, USA) according to the manufacturer's instructions. Double-stranded siRNAs against these target regions (designated as siRNA-p-412 against −412 to −392 and siRNA-TGFβRII519 against 519–539, respectively) and control scrambled RNA (5′-AATTCTCCGAACGTGTCACGT-3′) were synthesized by Qiagen Inc (Valencia, CA).

Transduction of lentiviral vectors in SBC10 cells

SBC10 cells, a rat hepatic stellate cell line, were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS). On the day of transduction, cells were replated at 6×10^4 cells/well in 6-well plates, along with recombinant lentivirus encoding for shRNA against the TGFβRII promoter region at an MOI of 50 in the presence of polybrene at a final concentration of 4μg/ml. Cells were passaged every 2–3 days when confluent, and maintained in DMEM with 0.4% FBS from day 4 after the lentiviral transduction. Total RNA was extracted on day 12 for real-time RT-PCR analysis as described below. For inhibition of DNA

methylation, 5-aza-2-deoxycytidine (5-aza, Sigma-Aldrich Chemical Co. St. Louis, MI) was replenished daily for 48hrs at a final concentration of 5μM[14] before extracting total RNA. For transfection with dsRNAs, siRNA(s) at a final concentration of 50 nM were transfected into SBC10 cells with TransIT-TKO® transfection reagent (Mirus, Madison, MI) at a concentration of 8 μl/1.25 ml according to the manufacturer's instructions, and total RNA was extracted 2 days after transfection for real-time RT-PCR analysis.

Bisulfite sequencing PCR analysis of TGFβRII promoter DNA

Seven days after lentiviral transduction, genomic DNA was extracted from SBC10 cells using QIAamp DNA Blood Mini Kit (Qiagen) according to the manufacturer's protocol. Seven-hundred nanograms of DNA were used for bisulfite modification as described[15]. Briefly, isolated DNA was digested with *BamHI* for 2 hrs and denatured in 0.3M NaOH for 20 min at 37°C. This reaction was mixed with 2 volume of 2% low-melting point agarose dissolved in H_2O. Ten μl aliquots of agarose/DNA mixtures were pipetted into chilled mineral oil to form agarose beads, then 200 μl of 2.5 M sodium metabisulfite (Sigma Chemical Co.) in 125 mM hydroquinone was added. The reaction mixture was incubated for 3.5 hrs at 50–55°C in the dark, followed by de-sulphonation in 0.3M NaOH and neutralization in 1/5 volume of 1M HCl. After 3 washes with Tris-EDTA buffer (pH 8.0), the beads were directly used for PCR reactions. Bisulfite-treated genomic DNA was amplified by PCR primers which were designed using MethPrimers, a web based program for bisulfite sequencing PCR[16] (http://www.urogene.org/methprimer/index1.html). Two sets of primer pairs were designed for each of the shRNA target sites: for the site −543~−334, forward 5′-TTATTTTTGAGTTTGAATTTGGAGT-3′ and reverse 5′-CCAACAACTAAACAAAACCTCTC-3′; for the site −486 ~ −312, forward 5′-GTTGGGAGTGGGTAATTTAAAGTT-3′, reverse 5′-AAAAAAAACAAAAAACTCCTC-3′.

The PCR reaction of 20 μl includes: 1 unit AmpliTaq Gold with 1× buffer, 2.5 mM $MgCl_2$, 200 μM dNTP, 1 μM primers, and 100 ng of modified DNA. The reactions were incubated at 94°C for 10 min, followed by 45 cycles of 94°C for 20 s, 57°C for 20 s, and 72°C for 30 s, and a final extension at 72°C for 7 min. The PCR products were cloned using pCR8/GW/TOPO TA Cloning□ Kit (Invitrogen Inc., Carlsbad, CA) according to the manufacturer's instructions, and sequenced using GW1 primer: 5□-GTTGCAACAAATTGATGAGCAATGC-3□.

Real-time RT-PCR for determination of TGFβII mRNA levels

Total RNA was isolated from SBC10 cells using Trizol® Reagent (Invitrogen Inc.) according to the manufacturer's instructions. Isolated RNA (2 μg) was converted into cDNA using the Thermoscript RT system with random hexamers and DNAse treatment. Primer pairs for rat TGFβII and β-actin were designed with Primer Express software (version 2.0, Applied Biosystems, Foster city, CA). Final concentrations of each primer are shown below in parenthesis:

TGFβII-F: 5′-GGAGGACGACCGCTCTGA-3 (900 nM)

TGFβII-R: 5′-GGGCAGCAGTTCCGTATTGT-3 (900 nM)

β-actin-F: 5′-TGACCCAGATCATGTTTGAGACC-3 (300 nM)

β-actin-R: 5′-CAGTGGTACGACCAGAGGCA-3′(300 nM)

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Real-time PCR was performed using a standard TaqMan PCR kit protocol in an Applied Biosystems Prism 7700 Sequence Detection System. The 12 μl PCR mixture includes 1× SYBR®□ Green PCR Master Mix (Applied Biosystems), primer pair, and 100 ng template.

Semi-log amplification curves were evaluated by the comparative quantification method (ΔΔCT) and the gene expression levels were normalized to rat β-actin as described previously [17,18].

RESULTS AND DISCUSSION

CpG methylation of the TGFβIIR promoter by lentiviral vector mediated-shRNA delivery

We generated two lentiviral vectors which produce shRNA against the TGFβRII promoter region (designated as shRNA-p-412 against −412 to −392 and shRNA-p-398 against −398 to −378, respectively), one against the TGFβRII coding region (shRNA-TGFβRII 519 against 519–539) and one with a scrambled RNA sequence (shRNA-scrambled). The transfection efficiency of lentiviral vectors in SBC10 cells was excellent (94.5% on day 13 by FACS analysis) and specific shRNA production was detected by real-time RT-PCR using a loop-stem method [19] (data not shown). Sequencing analysis of bisulfite-modified genomic DNA revealed the methylation status of four CpG dinucleotides within or adjacent to the target sequences of the shRNAs in the CpG island which begins at −414 downwards (Fig. 1A,B). Two clones from a control group (without lentiviral transduction) showed the absence of methylation at all four CpG dinucleotides, indicating that wild-type CpG dinucleotides in the target area of our shRNAs are maintained in the non-methylated state. Clones from cells transduced with lentiviral scrambled shRNA vector displayed no methylation in these target areas. In contrast, the transduction with shRNA-TGFβRII promoter-412 in SBC10 cells led to methylation at all of the four CpG dinucleotides. The level of methylation is 100% in the homologous target area and 10bp downstream CpG, and 50% in the further downstream CpGs. All non-CpG cytosine residues were also methylated between methylated CpG dinucleotides in the CpG island, a typical methylation pattern of RNA-directed DNA methylation observed in plants[20–23]. Transduction with lentiviral shRNA-p-398 vector induced less methylation (25–50%). The methylations tended to spread: when methylation was present, every CpG and non-CpG cytosine residues were methylated in the target CpG island. Three cytosine residues located upstream of the target CpG island showed varying degrees of methylation in shRNA-p-412 and shRNA-p-398-transduced cells, and also in controls as well as in those transduced with the lentiviral scrambled shRNA vector.

Suppression of TGFβII mRNA by shRNA against the promoter region

We measured the relative TGFβRII mRNA levels by real-time RT-PCR to determine whether the delivery of shRNA against the promoter region with recombinant lentiviral vector reduced the TGFβRII gene expression in SBC10 cells. Transduction of lentiviral shRNA-p-412 vector significantly suppressed the TGFβRII mRNA level in SBC10 cells compared to the control cells or those transduced with lentiviral scrambled shRNA vector (Fig. 2A). This suppressive effect was not observed before day 7 (data not shown), indicating that the methylation of the target promoter sequence precedes silencing of the TGFβRII gene. shRNA-p-398 had only a modest effect in the gene silencing and this observation may be related to the lesser degree of methylation of the CpG island, especially the CpG dinucleotide at −360 *cis*-acting Sp1 binding site which drives basal TGFβRII gene promoter activity[24].

Next, we transfected siRNA against the same promoter and coding regions in SBC10 cells and assessed the TGFβRII gene expression (Fig. 2B). siRNA against the TGFβRII coding region (siRNA-TGFβRII519) significantly reduced the mRNA level, whereas siRNA against the promoter region (siRNA-p-412) did not. This finding suggests that siRNA-p-412 does not induce post-transcriptional silencing of the TGFβRII gene in the same way as siRNA-TGFβRII519 does.

Finally, we sought to determine whether the inhibition of methylation could reverse the suppressive effect of shRNA against the TGFβRII promoter region (Fig. 2C). After 48 hrs of treatment with 5-aza, cells transduced with lentiviral shRNA-p-412 vector did not show any inhibition in TGFβRII gene expression, whereas shRNA against the TGFβRII coding region (shRNA-TGFbRII519) maintained its silencing activity. These results strongly suggest that the gene silencing seen in SBC10 cells transduced with lentiviral shRNA vector against the TGFβRII promote region was indeed associated with the methylation of cytosine residues in the promoter region.

To our knowledge, this is the first report showing that lentivirus-based shRNA delivery caused the methylation at the homologous promoter sequence, leading to silencing of gene expression in mammalian cells. Recent reports regarding RNA-directed TGS showed contradictory results with respect to the consequence of the promoter methylation[3–5]. Nuclear transport of siRNA was shown to be a crucial factor for promoter-targeted silencing[3]; this fact may explain the negative[4] or minimal (<10%) level of the promoter methylation[5] introduced by transfection of siRNAs/shRNAs without MPG (a nuclear import-mediating peptide) or lentivirus-mediated nuclear transport[25]. The high levels of cytosine residue methylation in our study might be due to augmented nuclear delivery of shRNA by lentiviral vectors for an extended period of time, because levels of shRNA produced by the recombinant lentiviral vector were very high in our study. However, we cannot exclude the possibility that the CpG island in the TGFβRII promoter region is more susceptible to DNA methylation than other target genes employed in the previous studies[4,5]. Further studies of targeting different promoters by lentiviral shRNA vectors may help confirm this issue.

In the present study, we found that the extent of methylation was quite dense, occurring at every available CpG and non-CpG cytosine residue in the CpG island, quite similar to RNA-directed DNA methylation in plants. This methylation pattern is not observed in control cells or those transduced with lentiviral scrambled shRNA vector, in which isolated one- or two-cytosine residues were methylated in the upstream area of the CpG island. This may be due to either low level methylation in the wild state, incomplete sulfonation during bisulfite sequencing, or sequence-nonspecific methylation by scrambled shRNA. In fact, there was 12.2% similarity in sequences between the TGFβRII promoter and scrambled shRNA as analyzed by the ClustalW method (data not shown). Thus, we can not role out the possibility that DNA methylation in the promoter region after the lentiviral scrambled shRNA vector transduction may be partially due to the sequence similarity. However, as described above, the pattern of methylation does not support this hypothesis.

It is tempting to speculate that the methylation of cytosine residue dinucleotides in the promoter area played a major role in transcriptional silencing of the TGFβRII gene in our study because: 1) the silencing effect can be reversed by 5-aza, a DNA demethylating agent, and 2) shRNA-TGFβRII promoter-412, which causes more methylation, also had a more potent silencing activity than did the shRNA-TGFβRII promoter-398. However, the promoter methylation may not be an absolute prerequisite for siRNA-induced TGS because TGS could be induced in cells devoid of DNA methyltransferase activity[4]. Recent reports show that siRNA induces dimethylation at Lys9 of histone H3 (H3K9me2), an epigenetic marker of heterochromatin and silent genes[4,6,26]. Increasing evidence points to a close interplay between DNA methylation, histone methylation and TGS in mammalian cells[26–28]. We believe that our data, along with the evidence of siRNA-induced histone methylation, strongly supports the unifying model for siRNA-mediated TGS where DNA methylation and histone modification work together, leading to silencing of a target gene or chromatin[29]. Inhibition of transcriptional initiation by antigene RNAs (agRNAs) is also an intriguing possibility [30]; however, the transcription start site of the TGFβRII gene is about 100 bps downstream from our shRNA target site[24].

In conclusion, our results demonstrate that lentiviral vector delivery of shRNA targeting the TGFβRII promoter region caused the methylation of cytosine residues in the region, and that this approach may be applicable in various scenarios of transcriptional silencing of mammalian genes.

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Abbreviations used in this paper

5-aza, 5-aza-2-deoxycytidine; CpG island, a region with increased density of CpG dinucleotides; IFN-α, interferon-α; siRNA, small interfering RNA; shRNA, small hairpin RNA; RdDM, RNA dependent DNA methylation; RT-PCR, reverse transcriptase polymerase chain reaction; TGFβRII, transforming growth factor-β receptor II; TGS, transcriptional gene silencing..

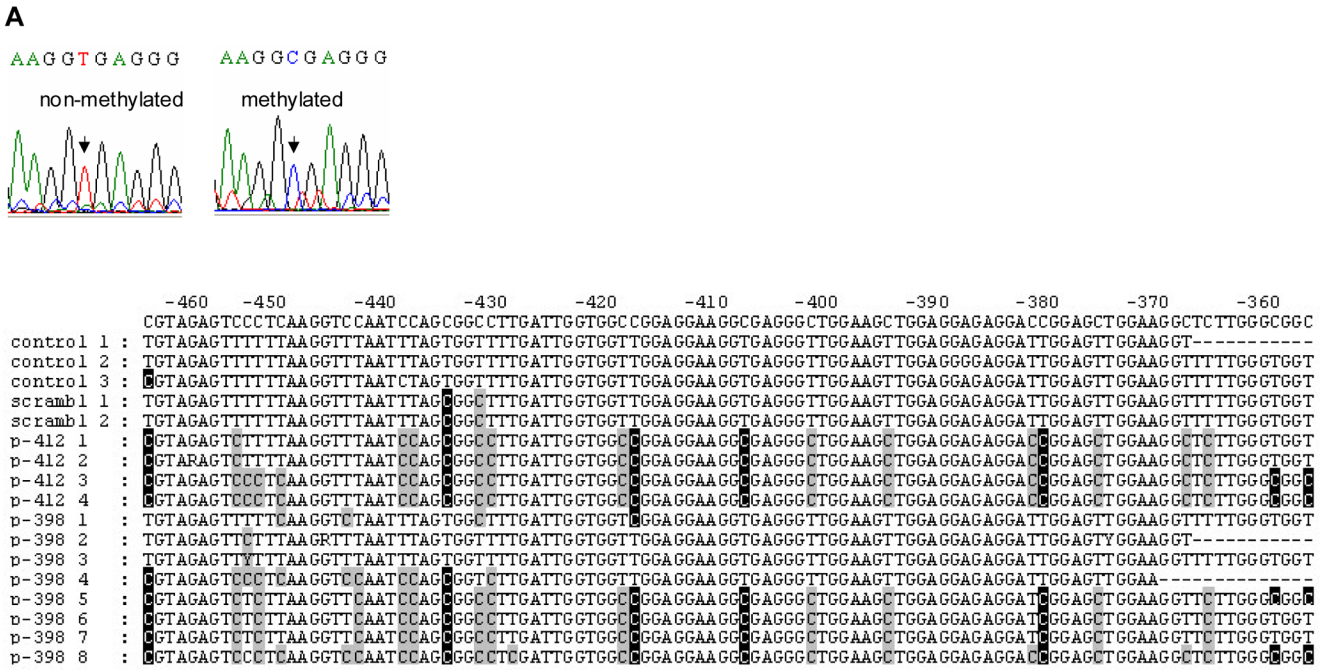
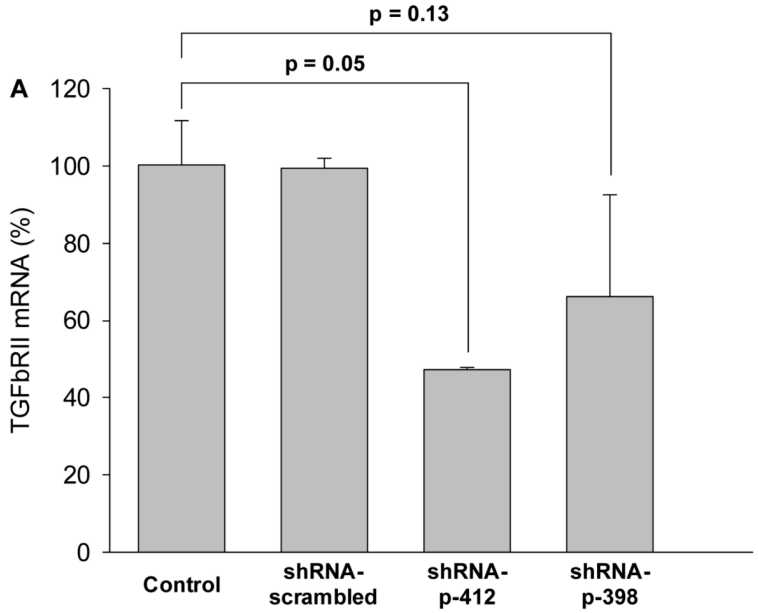
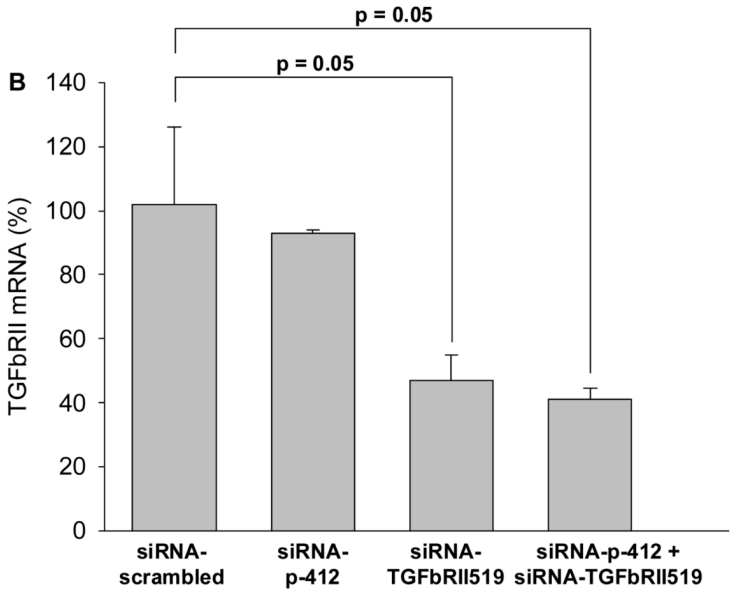


Fig. 1.

Methylation of the TGFβRII promoter region in SBC10 cells transduced with shRNA-producing lentiviral vector. **A**. Wild type non-methylated ‘C’ in CpG dinucleotide (CpG2) is converted to ‘U (read as T)’ by sodium bisulfite, whereas methylated CpG2 is resistant to bisulfite treatment. **B**. Sequences of bisulfite-modified TGFβRII promoter region. The upstream boundary of the CpG island begins at −414. The target sequences of shRNA were −412 to −392 for shRNA-p-412, and −398 to −378 for shRNA-p-398. The wild sequence at the top row represents the TGFβRII promoter sequence before bisulfite modification. The shaded C’s represent methylated cytosine residues. A variable degree of methylation was present in all 4 CpG sites (black shade) and 6 non-CpG cytosines (grey shade) around the target sequences in the CpG island region of shRNA-p-412- and shRNA-p-398-treated SBC10 cells. Cytosine residues were also methylated upstream of the CpG island. In control or lentiviral scrambled shRNA-transduced cells, cytosine methylation was not found in the CpG island region, whereas several cytosine residues were methylated upstream of the CpG islands.





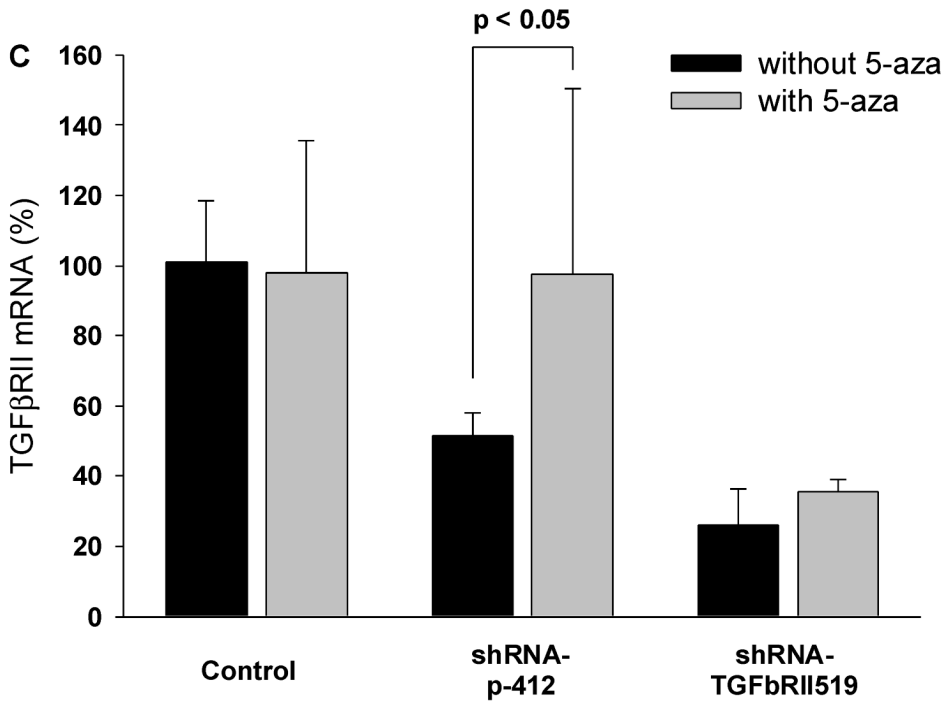


Fig. 2.

Suppression of TGFβRII mRNA expression after transduction of lentiviral shRNA vector against the TGFβRII promoter regions. **A**. Relative levels of TGFβRII mRNA in SBC10 cells 12 days after transduction with lentiviral shRNA vector against the promoter region. Lentiviral vectors against −412 to − 392 of the TGFβRII promoter (shRNA-p-412) significantly suppressed TGFβRII gene expression. **B**. Relative levels of TGFβRII mRNA in SBC10 cells 2 days after transfection with siRNA against the same region as shRNA-p-412 (siRNA-p-412), siRNA against the coding region of TGFβRII (siRNA-TGFβRII519), and a combination of the two siRNA. siRNA-p-412 did not show significant silencing of the TGFβRII gene, whereas siRNA-TGFβRII519 and the combination of the two siRNAs did. **C**. Effects of 5-aza on TGFβRII mRNA levels in SBC10 cells transduced with lentiviral shRNA vectors against the promoter region. 5-Aza reversed the silencing effect of shRNA-p-412, but had no effect on shRNA-TGFβRII519-treated cells. Values are means ± SD (n=3).

Table 1

Primer sequences for the generation of shRNA-expressing cassettes

shRNA name	Sequence
shRNA-scrambled	5′-p-GGAAGATCTAGAAAA*AATTCTCCGAACGTGTCACGT*CTACACAAA*ACGTGACACGTTCGGAGAA*CGGTGTTTCG TCCTTTCCACAAG-3′
shRNA-TGFβRII 519	5′-p-GGAAGATCTAGAAAAA*AGAACGACAAGAACATTACT*TCTCTTGAAA*GTAATGTTCTTGTCGTTC*CGGTGTTTCGTCCTTTCCACAAG-3′
shRNA-p-412	5′-p-GGAAGATCTAGAAAAA*AGGCGAGGGCTGGAAGCTGG*CTACACAAA*CCAGCTTCCAGCCCTCGCC*CGGTGTTTCGTCCTTTCCACAAG-3′
shRNA-p-398	5′-p-GGAAGATCTAGAAAAA*AGCTGGAGGAGAGGACCGGA*CTACACAAA*TCCGGTCCTCTCCTCCAGC*CGGTGTTTCGTCCTTTCCACAAG-3′

Italicized sequences denote siRNA target sites (sense/antisense) separated by a loop sequence.