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Testing the putative effect of Dicer-substrate siRNAs in regulating gene expression at transcriptional level

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Abstract: RNA interference (RNAi) pathway is a gene silencing process during which small double-stranded RNA (dsRNA) molecules trigger the degradation of homologous RNA targets. Small interfering RNAs (siRNAs) are the mediators of the RNAi that can be induced in vitro and in vivo by direct application of chemically synthesized siRNAs. Recently, promoter-targeted small non-coding RNAs have been described to be capable of regulating gene expression at the transcriptional level. In the present study we tested the hypothesis that Dicer-substrate siRNAs (D-siRNAs), which trigger gene silencing through intrinsic RNAi pathway and are therefore more potent in gene knockdown than traditionally used 21-23mer siRNAs, may also display regulatory effects at the transcriptional level. Synthetic 27mer D-siRNAs targeting the promoter/early coding region of the *CDKN2A* gene were designed and transfected into HeLa cells, and 48 h post-transfection the *CDKN2A* expression was analyzed in quantitative real-time PCR using the housekeeping *G6PDH* gene as reference. In comparison to the mutant version, the *CDKN2A* gene was effectively knocked-down by the D-siRNA, but no evidence of transcriptional regulation was found. We conclude that the D-siRNA-induced suppression is likely to occur after transcription.

Key words: RNA interference, Dicer-substrate siRNAs, transcriptional silencing, *CDKN2A*

Dicer-substrat siRNA'ların gen ifadesini yazılım aşamasında düzenleme kabiliyetlerinin test edilmesi

Özet: RNA interferans (RNAi) yolağı, özdeş hedef mRNA moleküllerinin yıkımına yol açarak, gen ifadesinin yazılım sonrası aşamada düzenlenmesini içerir. Küçük müdahaleci RNA'lar (siRNA'lar), RNAi yolağının aracı molekülleridir ve RNAi, sentetik siRNA'lar kullanılarak in vitro ve in vivo olarak oluşturulabilir. Güncel çalışmalar, promotör bölgelerine hedeflendirilmiş küçük kodlamayan RNA'ların gen ifadesini yazılım aşamasında da düzenleyebilme özelliğinde olduğunu göstermektedir. Bu çalışmada, gen susturulmasını intrinsik RNAi yolağı ile gerçekleştiren ve bu nedenle geleneksel olarak kullanılan 21-23mer siRNA'lardan daha etkin olduğu gösterilen Dicer-substrate siRNA'ların (D-siRNA'lar) yazılım aşamasında düzenleyici işlevlere sahip olabileceği hipotezini test ettik. *CDKN2A* geninin promotör/erken kodlayan bölgesine özgül 27-mer D-siRNA ve bunun merkezi bölgesinde 4 adet baz değişimi içeren mutant versiyonu tasarladı. siRNA'lar HeLa hücrelerine transfekte edildi ve 48 saat sonra *CDKN2A* ifade düzeyi gerçek-zamanklı PCR'da yaşamsal gen *G6PDH* referans olarak kullanılarak değerlendirildi. Mutant versiyon ile karşılaştırıldığında, D-siRNA etkin bir şekilde *CDKN2A* geninin susturulmasına yol açtı, ancak baskılanmanın yazılım aşamasında gerçekleştiğine dair bir bulgu elde edilmedi. Bu bulgulardan yola çıkarak, D-siRNA'nın yol açtığı gen susturulmasının yazılım sonrası gerçekleştiğini kabul etmekteyiz.

Anahtar sözcükler: RNA interferans, Dicer-substrat siRNA'lar, gen susturulması, *CDKN2A*

Introduction

Post-transcriptional gene silencing (PTGS) is the first described feature of small interfering RNAs (siRNAs), the main players of the evolutionally conserved RNA interference (RNAi) pathway. In PTGS, homologous RNA targets are degraded in a sequence-specific manner through siRNAs, or initiation of the translation is inhibited. The RNase III-like nuclease Dicer plays a central role in RNAi cleaving long RNA molecules into 21-23 nt siRNAs with overhanging 3' ends (1).

Recently, in addition to PTGS small non-coding RNAs have also been described to display regulatory effects at the transcriptional level. This regulation may act in both directions suppressing (2,3), and activating transcription (4,5), in human cells. Mechanistically, small RNA-directed transcriptional gene suppression functions by targeting of epigenetic modifications (e.g. histone methylations) to gene promoters (6,7). It has been shown that a low-copy promoter-associated RNA transcribed through RNA polymerase II is recognized by the antisense strand of the siRNA functioning as a recognition motif to attract epigenetic silencing complexes to the corresponding targeted promoters leading to transcriptional silencing (7). However, a more recent study has shown that promoter-targeting siRNAs, instead of directing silent epigenetic marks to promoters, interfered with the initiation of transcription blocking the assembly of the pre-initiation complex even if a noncoding promoter-associated RNA was involved in the process (8). On the other hand, deregulation of endogenous bidirectional transcription was postulated as the mechanism of transcriptional activation by small non-coding RNAs (9). The antisense transcript in the bidirectionally transcribed gene directs silent epigenetic marks to the sense gene promoter, and suppression of the antisense transcript results in gene activation.

Synthetic siRNAs used in in vitro studies are traditionally composed of 19-23 bp dsRNAs with 2-base 3' overhangs. When transfected into cells, they mimic the product of Dicer cleavage (10). It has been suggested that, in addition to cleaving longer dsRNAs, the Dicer endonuclease may direct loading the processed dsRNA into the RISC assembly. This hypothesis has led to the development of a new class of siRNAs, termed Dicer-substrate siRNAs (D-siRNAs). These are 25-30 nt in length and engage Dicer with high affinity and are passed from Dicer to RISC with strand-specific orientation (10,11). Of all sizes tested, the 27mer D-siRNA was found to be approximately 100-fold more potent in silencing the targeted gene than shorter (21-23mers) or longer (35-45mers) RNAs (10,12). It is assumed that cleavage by Dicer and loading to RISC contributes to the increased potency of D-siRNAs.

It is not known whether D-siRNAs are able to modulate gene regulation at transcriptional level. In the present study, we speculated that in addition to the PTGS D-siRNAs might have modulating activities at transcriptional level. We tested this hypothesis at the CpG island of the *CDKN2A* gene in which the promoter and early coding region overlap (13). The reason for choosing this sequence as the target is that we used non-validated D-siRNAs, and, in the case that these do not display regulating activities at the transcriptional level, gene suppression at the post-transcriptional level would favor the evidence of functionality of non-validated D-siRNAs.

Materials and methods

Cell culture

HeLa cell line was purchased from the German Resource Centre for Biological Materials (DSMZ) (DSMZ no: ACC 57). Cells were grown in the DMEM culture medium containing $NaHCO_3$ (3.7g/L), glucose (1 g/L), and stabile glutamine (Biochrom, Berlin, Germany) supplemented with 10% FCS (Biochrom) and antibiotics under standard conditions (37 °C and 5% CO_2 humidity). Cells were subcultivated at a confluency of 80%-90%. For siRNA transfection experiments, cells with a passage number < 10 were used.

Dicer-substrate siRNAs and transfection

A 27mer D-siRNA duplex that corresponds to the promoter/exon 1 region of the *CDKN2A* gene and its mutant version (mD-siRNA) containing 4 central mismatches (Table 1) were designed using the RNAi Design Tool of IDT Technologies (htpp://eu.idtdna.com/Scitools/applications/RNA/RNAi.aspx) and purchased from this company (IDT Technologies, Coralville, IA, USA). siRNA duplexes

Table 1. siRNA sequences for targeting the *CDKN2A* gene (NCBI Accession # NM_058197).

	Strand (5' to 3')	
D-siRNA	F	P-GCACCGAAUAGUUACGGUCGGAGgc
	R	GCCUCCGACCGUAACUAUUCGGUGCGU
mD-siRNA	F	P-GCACCGAAUCCCCACGGUCGGAGgc
	R	GCCUCCGACCGUGGGGAUUCGGUGCGU

Lower case base pairs indicate DNA; P: phosphorus; F: forward, R: reverse. Underlined bases represent mismatches

were transfected into HeLa cells via lipofection using the X-tremeGENE siRNA Transfection Reagent (Roche diagnostics, Mannheim, Germany) using the manufacturer's protocol. Briefly, 1 day prior to transfection, cells were seeded at a concentration of 1×10^5 cells/well in 12-well plates using 1 mL of DMEM medium to reach 30%-50% confluency. For transfection, 3 µL of transfection agent was mixed with 72 µL of serum-free medium, and 1 µL of siRNA was diluted in 74 µL of serum-free medium. Diluted siRNA was added immediately to the transfection agent and mixed and incubated at RT for 20 min. Finally, the transfection agent-siRNA complex was added to the cells in drops, and cells were incubated under standard growth conditions. Forty-eight hours after transfection, cells were washed, harvested and were kept at –80 °C until use for further analysis. To test the effect of the D-siRNA on the transcription, prior to harvesting transfected cells were incubated for additional 24 h in the presence (10 ng/mL) or absence of trichostatine A (TSA). Transfection experiments were performed 3 times, and 2 samples were included in each group. Mean values were calculated.

Analysis of the CDKN2A gene expression

RNA was extracted from siRNA-treated cells using the SV Total RNA Isolation System (Promega, Madison, WI, USA) and converted to cDNA using the Revert Aid FirstStrand cDNA Synthesis kit (Fermantas, Vilnius, Lithuania). Five microliters of cDNA were used in quantitative PCR in which the qPCR results were standardized to *G6PDH*. Primers (IDT, Coralville, IA, USA) and hybridization probes (TIB Mol Biol, Berlin, Germany) shown in Table 2 were designed using the LightCycler Probe Design Software (Roche Diagnostics). Three independent cell-culture experiments were used to calculate the average value of gene expression.

DNA methylation analysis

For methylation analysis on the promoter region of the *CDKN2A* gene, genomic DNA was isolated from siRNA-treated cells using the standard method and 1 µg was digested with the methylation-sensitive restriction enzyme *Hpa*II to eliminate non-methylated DNA sequences. Subsequently, DNA was exposed to bisulfite modification using the EZ DNA Methylation Gold kit (Zymo-Research, Orange, CA, USA) according to the manufacturer's instructions; 100 ng of modified DNA was used for methylation analysis. The methylation assay used consisted of a 2-step approach performed as described previously (14) and included a positive control that consisted of in vitro methylated DNA of HeLa cells.

Table 2. Primer and probe sequences to analyze the *CDKN2A* gene expression.

	Strand (5' to 3')	
Primers	F	CACCGAATAGTTACGGTCGG
	R	GGCGGGGTCGGCGAGTTGG
Probes		GATGGGCAGCGCCCGGATGCG-FLU
		LC640-GAGCTGCTGCTCCACGGCG-P

The quenching probe is labeled with flourescein (FL), and the reporter probe with LC640 at its 5' end.

Chromatin Immunoprecipitation (ChIP) assay

Formaldehyde was added to the cells to a final concentration of 1.42% 48 h post-transfection and incubated for 15 min at RT. To quench the formaldehyde, glycine (125 mM) was added and incubated for 5 min at RT. Cells were harvested by scraping and collected by centrifugation at 2000 ×*g* for 5 min at 4 °C, washed twice with PBS. For lysis of cells, pellets were resuspended in 200 µL of IP buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.5, 5 mM EDTA, 0.5% NP-40 (V/V), 1% Triton x-100 (V/V) and protease inhibitors) and homogenized by pipetting up and down several times. To remove insoluble material, lysates were centrifugated at 12,000 ×*g* for 1 min at 4 °C. Following washing, the nuclear pellet was sonicated in 200 µL of IP buffer, and the lysate was cleared by centrifuging at 12,000 ×*g* for 10 min at 4 °C. The supernatant was divided into 2 aliquots; one was used as DNA input. The supernatant of the 2nd aliquot was added to protein A agarose beads that had been blocked with 2% BSA and pre-incubated with ChIP-grade antibody against H3K27me3 for 4 h and incubated overnight. The beads were washed for 20 min each with high salt, low salt, LiCl, and TE buffers, and DNA/histone complexes were eluted twice by adding 150 µL of elution buffer (100 mM $NaHCO_3$ and 1% SDS) and incubating for 15 min at 65 °C. Following Proteinase K treatment, DNA was purified using an appropriate purification kit, suspended in 65 µL of water and stored at –20 °C. Then 3 µL of ChIP-associated DNA was amplified by real-time PCR using SYBR Green as fluorescence molecule and primers specific to the *CDKN2A* promoter region, and quantitation of *CDKN2A*-specific sequences in immunoprecipitated DNA was performed as reported previously (15).

Statistical analyses

In order to test the effectiveness of the D-siRNA and mDsiRNA molecules on the gene expression or epigenetic parameters, the Mann-Whitney U test was used to compare the results of transfection experiments and DNA and histon methylation analyses.

Results and discussion

We transfected HeLa cells with 50 nM 27mer D-siRNA. This has been reported to suppress gene expression effectively (12). Forty-eight hours later, the expression level of the *CDKN2A* was determined relative to *G6PDH*. Results of this experiment showed that D-siRNA is highly effective in suppressing the *CDKN2A* gene. As depicted in Figure 1a, expression level of the *CDKN2A* gene in the cells transfected with D-siRNA was reduced by average 94% when compared with cells transfected with its mutant version (mD-siRNA), showing the functionality of the non-validated D-siRNA. The extent of suppression through the D-siRNA is higher than the rates reported previously (10,12). However, extent of knockdown of a given gene may be affected by several factors including the characteristics of the sequence of interest, localization of the sequence relative to regulatory sequences, etc. We also showed

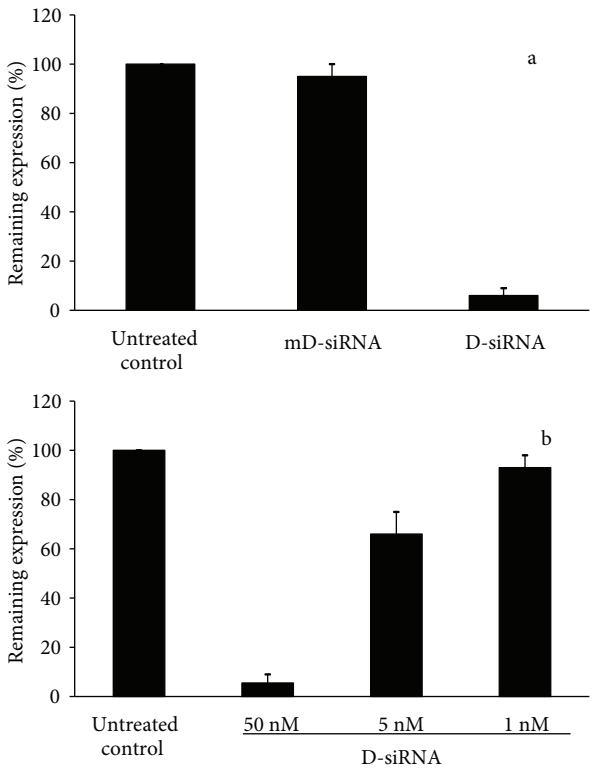

Figure 1. (a) Knock-down of the *CDKN2A* gene by the D-siRNA; 50 nM of the D-siRNA and its mutant version was transfected into HeLa cells, and 48 h post-transfection the expression of the *CDKN2A* was analyzed relative to *G6PDH* in qPCR. Shown is also untreated control. (b) Dose-dependent gene suppression by the D-siRNA. HeLa cells were transfected with indicated concentrations of the D-siRNA, and the *CDKN2A* expression was analyzed as described above.

that with decreasing concentrations of the D-siRNA the degree of silencing was also reduced (Figure 1b), suggesting that suppression is dose-dependent.

As our target sequence compromises the promoter and the early coding region, the observed effect may result from suppression at the transcriptional and/or post-transcriptional level. To find out whether the knockdown of the *CDKN2A* is associated with repressive chromatin changes we used the histone deacetylase inhibitor trichostatin A (TSA). The cells transfected with the D-siRNA (50 nM) were treated with 10 ng/mL of TSA for an additional 24 h, and the expression level of the *CDKN2A* was analyzed. The test revealed that suppression of the *CDKN2A* through D-siRNA was not affected by TSA treatment (Figure 2a), indicating that chromatin changes are not involved in the D-RNA-induced suppression.

To confirm the above finding that the D-siRNA effect was not affected by TSA, we further investigated DNA methylation and histone methylation in the promoter of the *CDKN2A* gene. In HeLa cells no or very low levels of *CDKN2A* promoter methylation have been reported (16). Concordantly, very low levels of basal methylation were also detected in these cells. Upon D-siRNA treatment, no significant change in the basal rate methylation was found, excluding DNA methylation as a repressive epigenetic mark (Figure 2b). The trimethylation of the histone H3 lysine 27 (H3K27) is another epigenetic mark associated with gene silencing (17). Similar to DNA methylation, following D-siRNA treatment no significant change was found in the amount of trimethylated H3K27 (Figure 2c). These findings suggest that the D-siRNA directed *CDKN2A* suppression occurs not at

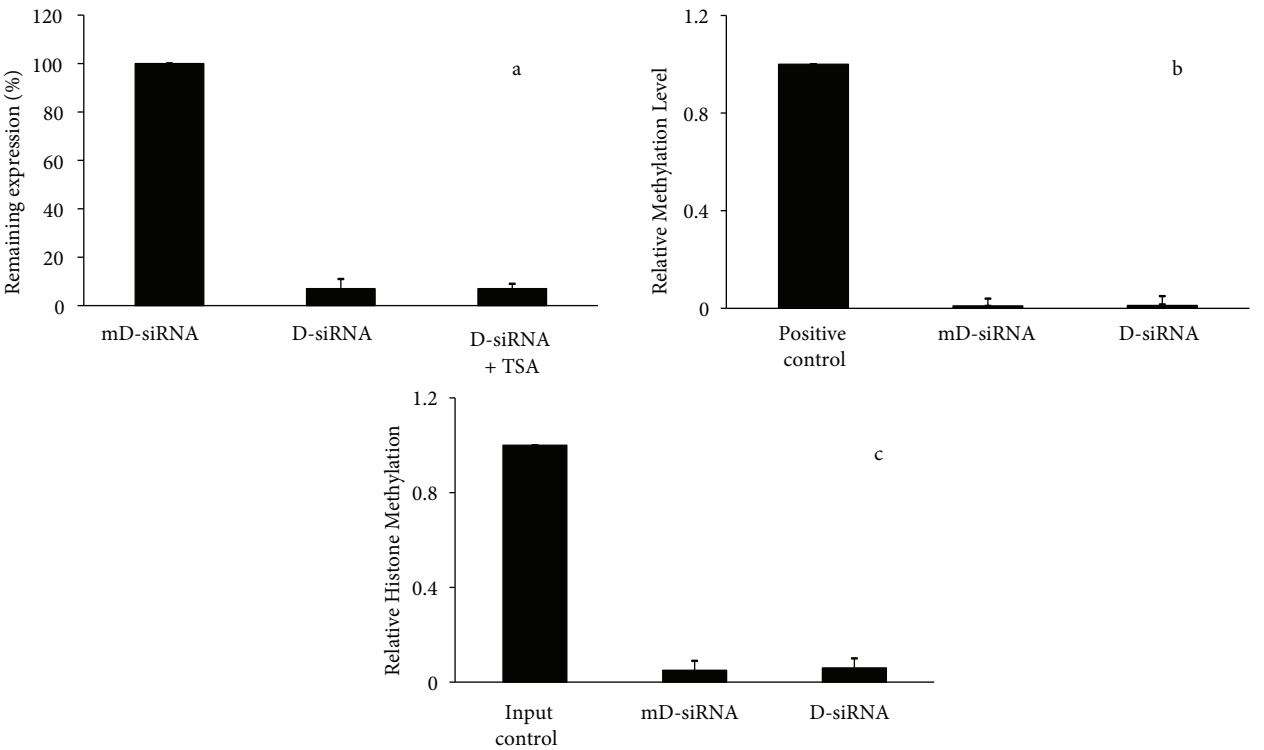

Figure 2. (a) Effect of TSA on the D-siRNA-induced suppression. Transfected cells were incubated in the presence (10 ng/mL) or absence of TSA for additional 24 h, and the *CDKN2A* expression was analyzed. Note that TSA had no effect on the D-siRNA-induced suppression. (b) Role of DNA methylation in the D-siRNA effect. Following incubation, DNA methylation was analyzed by a 2-step approach and relative methylation levels were calculated using a positive control. In the D-siRNA-induced suppression no change in the basal methylation levels was detected. (c) Involvement of H3K27me3 in the D-siRNA-related suppression. siRNA-treated cells were exposed to ChIP assay using the antibody against the H3K27me3, and *CDKN2A* promoter sequences were amplified from the ChIP-associated DNA by real-time PCR. Error bars indicate standard deviations.

transcriptional level, but likely at post-transcriptional level.

Our finding that the D-siRNA mediated suppression is not associated with transcriptional events led us to speculate that following the cleavage duplexes are introduced into the RISC assembly preventing them from acting on transcription. Another speculation would be that, in a sequence like our target region in which regulatory and coding sequences overlap, PTGS might be the dominant mechanism of gene silencing.

This is the first study investigating the ability of D-siRNAs to modulate gene expression at transcriptional level. Our experiments provided no signs of an action of the *CDKN2A*-targeting D-siRNA at the transcriptional level. We conclude that the highly efficient suppression of the *CDKN2A* we observed is likely to occur at post-transcriptional level.

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