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ARTICLE

Non-specific Effects of siRNAs on Tumor Cells with Implications on Therapeutic Applicability Using RNA Interference

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Elimination of protein expression using RNA interference (RNAi) significantly improves the understanding of gene function and represents a promising technique for the treatment of diseases such as cancer and neurological disorders. Accumulating evidence suggests the so-called interferon-independent non-specific gene silencing of short interfering RNA (siRNA); however, its biological and functional cellular consequences are largely unidentified. We therefore analyzed the effects of different nonsense siRNAs on characteristic bio-parameters such as cell viability, proliferation, cell cycle distribution, apoptosis, and migration of tumor cells. All analyzed cellular aspects have been observed to be significantly affected by the presence of siRNA in an interferon-independent manner: viability, mitosis, and motility were significantly diminished and programmed cell death was significantly elevated. Moreover, all cell cycle stages (G0/G1-, G2/M-, and S-phase) were moderately shifted. Together, these results support the hypothesis that siRNA, due to sequence-specific cellular consequences, modulate bio-functionality independent of the target sequence. This phenomenon affects the design of siRNA experiments for future *in vitro* but also for *in vivo* tests as well as for potential therapeutic and preventive strategies. Moreover, monitoring interferon response after transfection of siRNAs is necessary but not sufficient to exclude potential off-target effects in non-diseased cells. (Pathology Oncology Research Vol 13, No 2, 84–90)

Key words: nonsense siRNA, RNA interference, RNAi, non-specific effects, functional aspects, cancer

Introduction

Gene-specific knockdown by RNA interference (RNAi) represents a potent tool for the examination of gene function and drug target identification in eukaryotic cells.[1] RNAi is characterized by the assembly of exogenous target-specific short interfering RNA (siRNA) into RISC (RNA-induced silencing complex), target detection, and subsequent target mRNA destruction. Moreover, increasing evidence also indicates that the transfection of mRNA-specific siRNA may display a powerful therapeutic strategy for the treatment of e.g. virus-mediated diseases,[2] neurological disorders[3] and cancer,[4] but also for cancer prevention.[5] Therefore, the exclusive target specificity of siRNA against disease-relevant mRNAs is an essential prerequisite for successful administration of this technology in humans. In this context, it has been observed that even single mismatches in siRNA molecules can abolish efficient degradation of target transcripts,[6] again emphasizing the specificity and applicability of this technique as a potential therapeutic approach.

Administration of longer (>30 bp) and shorter dsRNAs (<30 bp) are associated with the induction of immunomodulatory interferon (IFN)-responses, which have an evolutionary connection to anti-viral, anti-proliferative and pro-apoptotic activity.[7,8] To exclude these possible off-target effects in siRNA experiments, scientists frequently verify IFN-response deficiency by analyzing the expression of IFN target genes. Hence, nonsense/scrambled siRNA is used as a negative control in order to define the baseline in functional siRNA experiments. However, it

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has been shown that siRNA might also induce IFN-independent responses, which are associated with the regulation of many non-specific targeted mRNAs.[9] Although several investigators examined the biological impact of siRNA-mediated non-specific off-target effects on the molecular level using expression profiling approaches,[9-11] only few analyses of functional consequences (in most cases viability) in tumor cells have been performed yet.

In this study we analyzed whether exogenously introduced non-targeted siRNA may display IFN-independent functional off-target effects. Although no significant sequence homology with human mRNA/cDNA or genomic DNA has been identified, significant consequences on cell cycle distribution, cell viability, proliferation, apoptosis, and migration have been detected, which have a profound impact on siRNA design, experimental data interpretation, and potential therapeutic approaches.

Materials and Methods

Cell culture and transfection

Hep3B, HuH-7, HepG2 and HaCaT cells were maintained in DMEM, MEM or RPMI (PAA, Cölbe, Germany) supplemented with 10% FCS and 1% Penicillin/Streptomycin, at 37°C in a 5% CO_2 atmosphere. All transfection assays were done using Oligofectamine (Invitrogen) according to a modified protocol. In brief, for FACS analysis, $1.2x10^5$ cells (48 h) or $8x10^4$ cells (72 h) were seeded in a 6-well dish one day prior transfection in 2 ml DMEM without antibiotics. Solution I (200 µl OptiMEM (Gibco/Invitrogen, Karlsruhe, Germany) +10 µl siRNA (20 µM stock)) and solution II (55 µl OptiMEM + 5 µl Oligofectamine) were incubated for 10 min at room temperature and carefully mixed. After 20 min, the solution was added to the cells, carefully mixed by shaking, and incubated at 37°C. After 4 h the transfection mixture was replaced by fresh medium (10% FCS with antibiotics).

mRNA preparation, RT-PCR, siRNA, and primer sequences

Total RNA was isolated using the RNA Nucleospin RNAII kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. The total RNA concentration was assessed using a Nano-Drop photometer (Peqlab Biotechnologie, Erlangen, Germany). For cDNA synthesis 1 µg of total RNA was used for reverse transcription (random primer) using RevertAid™ H Minus MuLV Polymerase (1 U/µl), according to the manufacturer's instructions (MBI Fermentas, St. Leon-Rot, Germany).

The sequences for nonsense siRNA #1 (sense: 5'-UUC UCC GAA CGU GUC ACG U dtdt-3') and nonsense siRNA #2 (sense: 5'-CCG AUA UGA GUC AAC AGA U dtdt-3') were blasted using the Ensembl blast algorithm. No significant homologies with cDNA or genomic sequences have been detected. siRNAs were chemically synthesized by Eurogentec (Seraing, Belgium). The following primers were used for PCR analysis: OAS-2 (forward: 5'-TCA AGG ATT TAC CCT CGC TG-3'; reverse: 5'-CTG GAT CCA AGA TTA CTG GC-3'), 18S-rRNA (forward: 5'-AAA CGG CTA CCA CAT CCA AG-3'; reverse: 5'-CCT CCA ATG GAT CCT CGT TA-3'); program: 30 cycles of 94°C for 30 seconds, 60°C for 40 seconds, 72°C for 60 seconds.

Cell viability-, proliferation-, and migration assay

Cell viability was analyzed using the MTT assay. MTT stock solution (5 mg/ml in PBS; Sigma-Aldrich, Taufkirchen, Germany) was diluted in DMEM medium (1:10) and applied on cells growing in 48-well plates. After incubation (3 h), the medium was aspirated, 200 µl DMSO/ethanol (1:1) were added and the extinction was measured at 570 nm in an ELISA-reader.

Cell proliferation was examined using a BrdU-ELISA (Cell proliferation ELISA Biotrak, GE Healthcare/Amersham, Freiburg, Germany) according to the manufacturer's instructions.

For determining migratory activity, 3 days after siRNA transfection HaCaT, HuH-7, and Hep3B cells were treated with mitomycin c (Sigma-Aldrich; 5 µg/ml) for 3 h to repress proliferation (determined by BrdU-ELISA). The cell monolayer was damaged with a 'scratch' using a pipette tip. Cells were treated with HGF (10 ng/ml) and incubated for 18 further hours. Scratches were digitally documented and relative migratory activity was ascertained by calculating the cell-free areas.

Western blot analysis

Cell extract isolation, quantification, separation and blotting were performed as previously described.[12] Anti-phospho-STAT1 antibody (1:1000; Cell Signaling) and anti-actin antibody (1:5000; MP-Biomedicals, Aurora, Ohio, USA) were diluted in TBST (5% milk powder) and incubated overnight at 4°C. The appropriate secondary antibody was applied for 1 h (1:2000; HRP-anti-mouse and HRP-anti-rabbit) at room temperature. The immunoblots were visualized by enhanced chemiluminescence (Amersham).

Flow cytometric analysis

Cell cycle analysis was performed using a PAS II flow cytometer (Partec, Münster, Germany) according to a modified protocol.[13] In brief, samples were treated with 2.1% (w/v) citric acid with 0.5% Tween 20 followed by slight shaking at room temperature for 20 min. The 500 µl cell suspensions were carefully mixed with 3.5 ml 0.4 M

sodium hydrogen-phosphate buffer (pH 8.0; containing 0.5 ml of DAPI stock solution: 10 mg/28.5 ml distilled water). Each histogram depicting the DNA index and cell cycle of one sample represents 40,000 to 80,000 cells. Human lymphocyte nuclei from healthy donors were used as an internal standard. The mean coefficient of variation (CV) of diploid lymphocytes was 0.8-1.0.

For the detection of apoptotic cells and viability, FACS-Calibur flow cytometer (Becton Dickinson, Heidelberg, Germany) was used with filter combinations for propidium iodide (PI). Each histogram and dot plot represented 10,000 cells. For determination of cellular viability both adherent and detached cells in the supernatant were harvested and collected by centrifugation (800 rpm, 5 min). Cells were carefully suspended in 1 ml Cell wash solution (Becton Dickinson) and incubated with 10 µl of propidium iodide (2 mg/ml).[14] Subsequently, 0.5 ml of the Nicoletti buffer (0.1% sodium citrate, pH 7.4; 0.1% Triton X-100; 50 µg/ml propidium iodide) per sample were added and the number of apoptotic cells was determined.[15] Measurements were aquired in Fl-2 in logarithmic mode and calculated by setting gate over the first three decades to detect apoptotic cells. Depicted FACS analyses (cell cycle and apoptosis) were performed three times in independent experiments (each performed in triplicate).

Software

For DNA and cell cycle histogram analysis, and calculation of apoptotic cell fractions the Multicycle program (Phoenix Flow Systems, San Diego, CA, USA) and Cell Quest software (Becton Dickinson) were used. The data were submitted to statistical analysis using SPSS (SPSS GmbH Software, Munich, Germany). Area determination was performed using an analysis soft imaging system (Olympus, Hamburg, Germany).

Results

Transfection of nonsense siRNAs is not associated with an induction of interferon in tumor cells

Since the cytokine interferon (IFN) itself is a potent modulator of several cellular effects (e.g. mitosis and apoptosis) in multiple cell types, we first tested whether transfection of so-called nonsense siRNAs (nonsense #1, nonsense #2) induces an IFN response in our *in vitro* model system (HuH-7, *Fig. 1*). No phosphorylation of typical IFN downstream effectors such as the signal-transducer and activator of transcription (STAT)-1 after 48 h or 72 h (*Fig. 1a*), or activation of the IFN target gene oligoadenylate synthetase (OAS)-2 (*Fig. 1b*) were observed using the standard transfection protocol. These experiments were also performed with additional tumor cell lines showing similar results (e.g. Hep3B, data not shown). Thus, potential secondary IFN-mediated biological effects such as secretion of cytokines which may influence cellular bio-functionality can be excluded in this experimental setup.

Nonsense siRNAs reduce tumor cell viability and tumor cell proliferation

Because most studies concentrate on the analysis and verification of expression profiles after siRNA transfection (gene-specific and nonsense siRNA), only limited data regarding potential functional consequences are currently available. To clarify this issue we tested two commonly used nonsense sequences with regard to their capability to regulate tumor cell viability (HuH-7, *Fig. 2a*). The number of viable cells was significantly reduced after transfection of both siRNAs as compared to cells treated with cationic carriers or to untreated cells. Moreover, both tested siRNAs also significantly reduced proliferation as compared

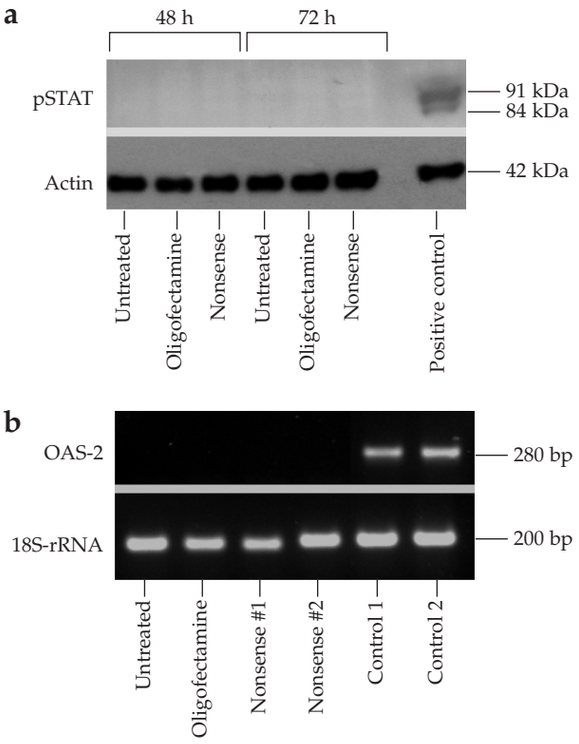


Figure 1. *Lack of an IFN-response after administration of nonsense siRNAs.* ***(a)*** *No phosphorylation/activation of STAT-1 (STAT-1α, 91 kDa; STAT-1β, 84 kDa) was observed 48 h or 72 h after transfection of nonsense siRNA. Protein extracts of IFNγ-treated HuH-7 cells served as positive controls (600 U for 16 h). Actin was used as a loading control.* ***(b)*** *No induction of OAS-2 expression was detected after administration of nonsense siRNAs. 18S-rRNA served as a loading control. RNAs (cDNA) isolated from different primary human liver tumors served as positive controls.*

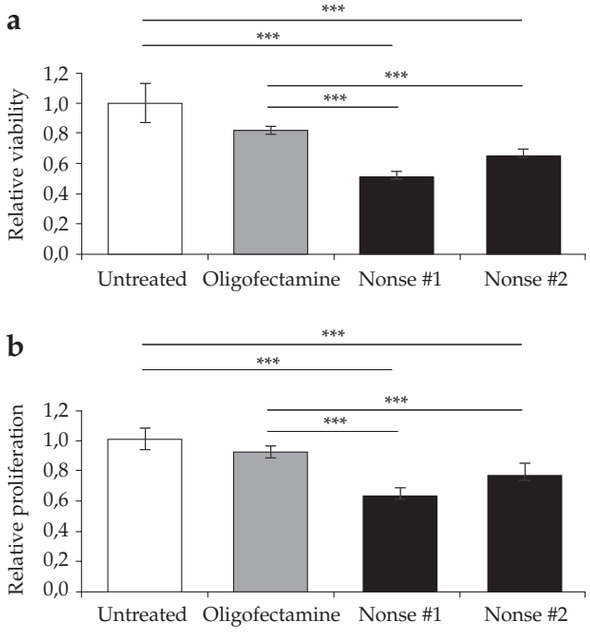


Figure 2. *Nonsense siRNAs reduce cell viability and proliferation.* ***(a)*** *Transfection of different siRNAs (nonsense #1, nonsense #2; 100 nM) was associated with reduced cell viability as compared to cells processed with the transfection agent and untreated cells. MTT-assay was performed 4 days after siRNA administration. Results were verified in independent experiments.* ***(b)*** *Application of nonsense siRNAs (nonsense #1, nonsense #2; 100 nM) led to reduced proliferation as compared to controls. BrdU-ELISA assay was performed 3 days after siRNA administration. Results were independently reproduced. P-values were determined by Student's two-sided t-test; ***$p<0.001$*

to controls (HuH-7, *Fig. 2b*), which might be in part the reason for the decreased number of viable cells after transfection. These results were verified using further tumor cell lines showing equal results (Hep3B and HepG2, data not shown).

Transfection of nonsense siRNAs modulate cell cycle distribution in tumor cells

These results strongly suggest siRNAs mediated time-dependent off-target effects on the cell cycle. To visualize these non-specific biological effects of siRNA we analyzed the distribution of different cell cycle phases (G_0/G_1-, G_2/M-, and S-phase) in three independent experiments. A weak but distinct increase in the number of G_0/G_1-phase cells was observed after 48 h, which became more prominent 72 h after transfection (*Fig. 3a*). Simultaneously, a slightly but significantly reduced number of cells accumulated in the G_2/M-phase after 72 h (*Fig. 3b*). Consequently, a slight bidirectional global cell cycle shift for nonsense #1 siRNA was detected, which lowered the number of S-phase cells (*Fig. 3c*) and proliferating cells after 72 h (*Fig. 3d*; proliferation index (PI) containing S- and G_2/M-phase cells). Similarly, a significant shift in the cell cycle distribution after transfection of the other nonsense siRNA (nonsense #2) was detected (data not shown). The molecular reason for this shift might be a dysregulation of cell cycle components such as cyclins, cyclin-dependent kinases (cdks), or cdk-inhibitors. Using semiquantitative real-time PCR we observed a moderate induction of $p27^{kip1}$ (up to 1.42-fold), cyclin B1 (up to 1.84-fold), cyclin D1 (up to 1.65-fold), and cyclin A2 (up to 1.49-fold) after transfection of both nonsense siRNAs as compared to controls. Other proteins such as cdk-1 and $p21^{cip1}$ were not regulated at all (data not shown).

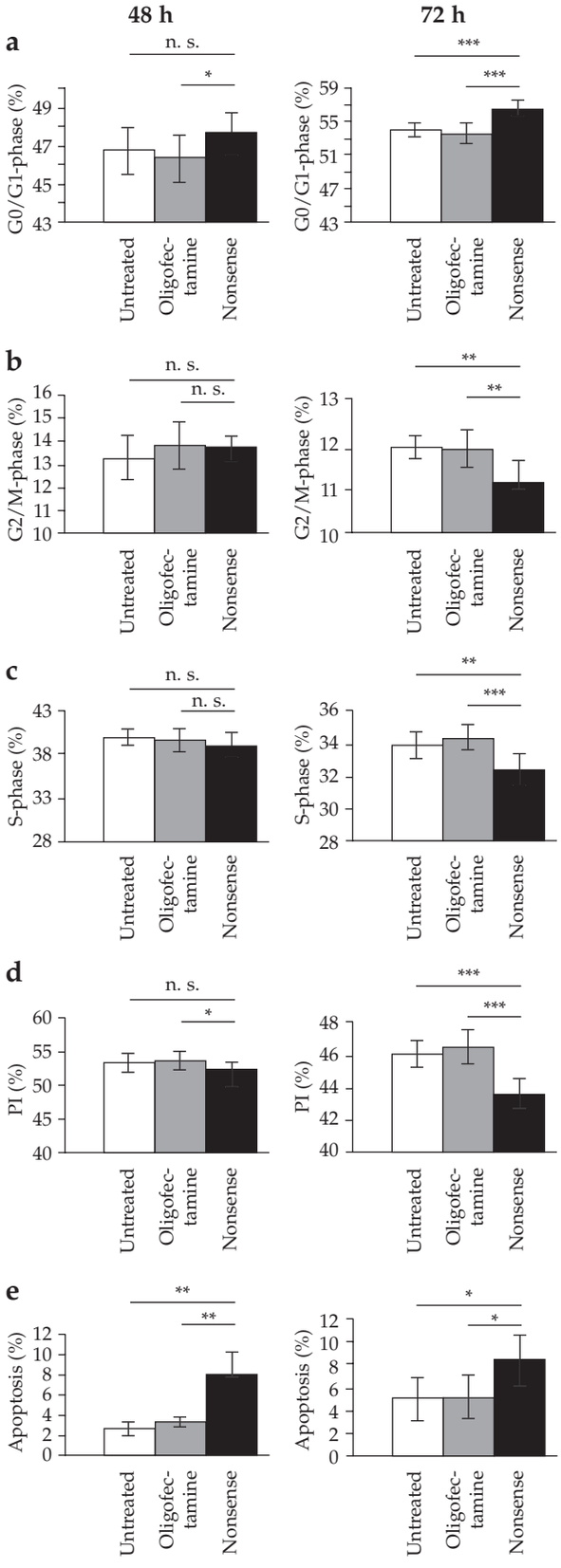
Since programmed cell death also affects the number of viable cells, we further analyzed the number of apoptotic cells after transfection of nonsense siRNAs. After 48 h and 72 h a mild but significant induction of apoptosis was observed (*Fig. 3d*). Together these data clearly show that both reduced proliferation (probably based on moderate cell cycle shifting) and elevated apoptosis participate in the modulation of cell viability after transfection of nonsense siRNAs.

Nonsense siRNAs affect tumor cell migration

We further analyzed the impact of nonsense siRNAs on other complex and strictly regulated cellular processes such as cell migration. We utilized epithelial derived cells (HaCaT); in which migration can be strongly induced by the application of soluble factors such as hepatocyte growth factor (HGF). Using a 'scratch'-assay we demonstrated that 'wound-closure' is nearly completed 18 h after initial scratching and HGF-stimulation in both controls (untreated and oligofectamine-treated cells), while application of both nonsense siRNAs significantly reduced cell migration and subsequent area recovery *(Figs. 4a* and *4b*). Similar results were observed for tumor cells such as HuH-7 and Hep3B; however, to a lower extent (data not shown).

Discussion

Until now, various studies (with multiple siRNA sequences and transfection protocols) have demonstrated that application of siRNA non-specifically modulates expression profiles of various cancer cell types.[9,11,16] We further substantiate and expand this hypothesis by presenting the first comparative functional data (cell cycle distribution, viability, proliferation, apoptosis, and migration), which prove the presence and functional significance of IFN-independent off-target effects of siRNA lacking significant homology on the RNA/cDNA- or the genomic



DNA level in eukaryotic cells. Because these biological effects are normally negligible as compared to those observed after inhibition of e.g. tumor suppressor genes or oncogenes, these functional consequences could have easily been missed. However, the effects are of central relevance for scientists working with *in vitro* models for different diseases (e.g. cancer, neurological disorders) since these results affect data interpretation and prospective therapeutic/preventive approaches.

However, the underlying molecular mechanisms that induce non-specific gene regulation are not completely understood and have been discussed intensely in the literature. (I) Partial sequence identity of siRNA with unintended transcripts might facilitate the reduction of numerous genes.[11] (II) Partially complementary siRNA have been shown to induce non-specific translational repression through micro-RNA (miRNA) mechanisms.[16,17] (III) Cellular stress is mediated through siRNA sequence/motif-dependent activation of sub-cellular mechanisms.[18] These hypotheses argue that off-target effects are sequence-specific and therefore 'theoretically' predictable. In contrast, expression analysis with alternative sequences imply more global biological effects, since completely different siRNAs give rise to identical gene regulation.[9] The latter argument is supported by our results and by published data[18] showing that transfection of completely different nonsense siRNAs is associated with nearly identical functional consequences in regard to a reduction in viability, proliferation, migration and induction of apoptosis; contrary effects after transfection of non-specific siRNA have hardly ever been observed. Thus, we reason that the administration of siRNAs modulate the expression of target structures in a sequence-specific manner (e.g. via siRNA- and miRNA-effects) with its unique biological effects and additionally induce a more global (but IFN-independent) 'dysfunction', which is associated with a reduced cellular turnover, limited functionality, and even elevated apoptotic rate.

It is tempting to speculate that this IFN-independent dysfunction (reduced viability, migration etc.) is not simply a pure experimental artifact but rather a passive mechanism to protect organisms from cells invaded by

Figure 3. *Nonsense siRNA mediates a cell cycle shift and induces apoptosis after 48 h and 72 h. A weak increase in the number of G_0/G_1-phase cells* ***(a)*** *and a slight decrease in the number of G_2/M-phase cells* ***(b)*** *were observed after transfection of nonsense siRNA (nonsense #1). The number of S-phase cells* ***(c)*** *and the proliferation index (****d;*** *PI) are significantly diminished. Moreover, apoptosis was induced* ***(e)****. All values represent the mean of three independent experiments (each performed in triplicate). P-values were determined by Student's two-sided t-test; *p<0.05; **p<0.01; ***p<0.001; n.s., not significant*

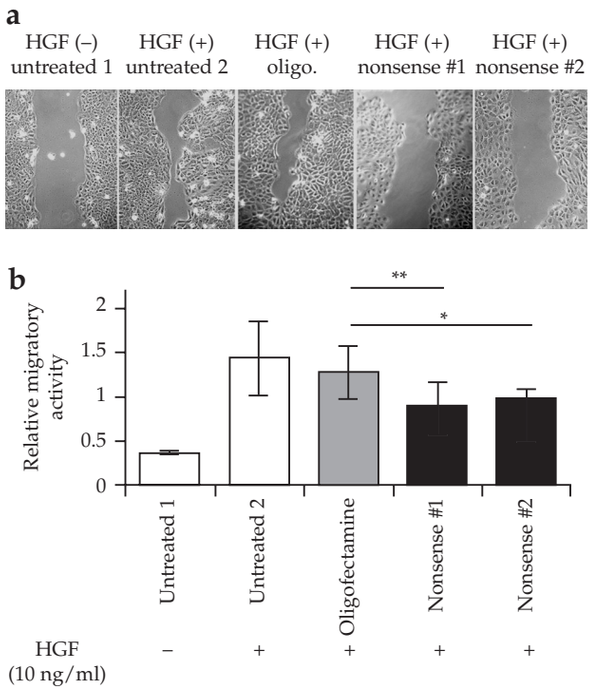


Figure 4. *Nonsense siRNAs reduce cell migration.* ***(a)*** *Three days after siRNA administration (100 nM) a 'scratch'-assay with HGF-induced adherent HaCaT cells was performed (HGF: 10 ng/ml). Epithelial cells were treated with mitomycin C prior wounding to prevent mitosis-dependent dish recovery. Reduced proliferation (<15% residual proliferation) was checked using a BrdU-ELISA kit. Uncovered areas were documented and analyzed 18 h after 'wounding'; magnification: 50x.* ***(b)*** *Side-by-side comparison of reduced relative migratory activity after transfection of two different nonsense siRNAs. Results were verified in an independent experiment. P-values were determined by Student's two-sided t-test; *p<0.05; **p<0.01*

foreign nucleic acid (e.g. viruses). The repeating synchronous behavior of nonsense siRNA-transfected cells (reduced viability) and unidirectional regulation of gene expression provide further evidence for this hypothesis.[9] Thus, siRNA-transfected cells are possibly subjected to an alternative anti-viral mechanism that retract rather than kill infected cells from multi-cellular microenvironments without inducing a global IFN-response. This hypothesis is supported by the fact that RNAi is present in several ancient organisms (e.g. *Trypanosoma brucei* and *Entamoeba histolytica*).[19] In conclusion, IFN-independent off-target effects after administration of siRNA may possibly represent a basic ancient molecular mechanism to protect multi-cellular and mono-cellular organisms from foreign nucleic acid.

Certainly, an important question for scientists working with siRNA is: how can off-target problems be dealt with, especially when minor specific effects of target-specific RNAi may be obscured by its off-target effects? Firstly, multiple nonsense siRNAs should be used for each experimental setup, because they may modulate biofunctionality to a different extent.[18] Secondly, siRNAs with preferably little homology in the so-called 'seed'-region should be analyzed because these sequences play a central role in the recognition of miRNA.[20] Thirdly, intrinsic sequences responsible for the induction of toxicity should be avoided.[18] Fourthly, chemical modifications of siRNA (e.g. 2'-*O*-methyl ribosyl substitution) significantly reduced silencing of off-target transcripts without affecting target gene recognition.[21] Fifthly, optimization of siRNA concentrations is required.[22] Irrespective of number, design, and chemical modification, the observed biological effects after specific inhibition of biologically relevant target genes using RNAi should always be statistically analyzed compared to appropriate nonsense siRNA controls. Only this procedure guarantees that monitored functional consequences after RNAi treatment reflect authentic and target-specific biological effects.

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