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New basic approach to treat non-small cell lung cancer based on RNA-interference

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Abstract

Background: To date the therapy for non-small cell lung cancer (NSCLC) is associated with severe side effects, frustrating outcomes, and does not consider different tumor characteristics. The RNA-interference (RNAi) pathway represents a potential new approach to treat NSCLC. With small interfering ribonucleic acids (siRNAs), it is possible to reduce the expression of proliferation-dependent proteins in tumor cells, leading to their apoptosis. We propose that siRNAs could be adapted to the tumor type and may cause fewer side effects than current therapy.

Methods: Four NSCLC cell lines were cultured under standard conditions and transfected with three different concentrations of siRNAs targeted against the hypoxia-inducible factors 1 α and 2 α (HIF1 α and HIF2 α) and signal transducer and activator of transcription 3 (STAT3). The expression was observed by quantitative real-time polymerase chain reaction and western blots. For the analysis of cell growth three days after transfection, the cell number was detected using a CASY cell counter system.

Results: The results of the silencing of the analyzed factors differ in each cell line. Cell growth was significantly reduced in all cell lines after transfection with HIF1 α - and STAT3-siRNA. The silencing of HIF2 α resulted in a significant effect on cell growth in squamous, and large-cell lung cancer.

Conclusions: This study shows that the knockdown and viability to siRNA transfection differ in each tumor type according to the used siRNA. This implies that the tumor types differ among themselves and should be treated differently. Therefore, the authors suggest a possible approach to a more personalized treatment of NSCLC.

Introduction

In most countries, non-small cell lung cancer (NSCLC) is still one of the most common diseases. Its tremendous malignancy is demonstrated by the low five-year survival rates, especially in advanced stages of the disease. The current standard treatment is the combined use of surgery, chemotherapy, and radiotherapy, adapted to the stage of the disease. With its various and partial severe side effects, for example, the toxicity of chemotherapy and the still very frustrating therapeutic outcome, it is crucial that new targets and therapeutic strategies are found. To date, only some of the patients with a mutation in the epidermal growth factor receptor (EGFR) benefit from treatment with gefitinib.¹ For all other cases, standard therapy is the only available treatment, where neither the histological type nor the expressions of certain

factors in the tumor play important roles. However, the expression or overexpression of some factors can have an effect on the overall survival of patients, and this represents a promising possibility for new treatment options.^{2,3}

The RNA-interference pathway that was discovered by Fire and Mello in 1998⁴ offers one possible method to reduce overexpression of certain factors in tumors and, therefore, reduce their growth. This pathway is a natural defense mechanism in cells and can be stimulated with synthetic short double stranded small interfering RNAs (siRNA). Thereby, the sequence of the siRNA determines the degradation of a certain mRNA and their reduced translation into a protein. The discovery and understanding of this pathway represents potential for the treatment of diseases like tumors. There have been clinical trials between phase I-III, the most well-known study being the inhibition of the macula degeneration with an

siRNA targeted against vascular endothelial growth factor (VEGF).⁵ There are further trials in phase I-II, working with siRNAs targeted against vascular endothelial growth factor receptor-1 (VEGFR-1), RTP801 or ribonucleoside reductase M2 (RRM2).^{6–8} There are very important prerequisites that have to be fulfilled for an efficient and safe therapeutic application of siRNAs in order to reduce or eliminate side-effects, for example: effective siRNA design and synthesis to avoid immune stimulation and off-target effects; improved biological stability of the siRNA; formulation of a delivery system with the most favorable pharmacokinetics and biodistribution; target to the organ/cell type; ensure siRNA uptake into the cell; siRNA must escape from endosome and/or lysosome avoiding errant siRNA compartmentalization; bioavailability of the siRNA at the site of action; and siRNA must exert gene-silencing activity.⁹ We propose that siRNA application is safe and efficient when these conditions are achieved.

In this study, we evaluated the expression of three different factors after non-viral transfection with siRNAs and analyzed the influence of the knockdown on the cell proliferation for the different NSCLC lines. The factors that were used in this study were chosen because of their roles and importance in the viability of cancer cells and, thereby, connected expression or overexpression with its observed effects on other malignancies.^{10,11} HIF1 α and HIF2 α are important transcription factors in cells for the handling of a lack of oxygen. Both help the cells with inducing angiogenesis and differentiation in the case of hypoxia.^{12–14} Whereas STAT3, with its two isoforms STAT3 α and STAT3 β , which are results of an alternative splice acceptor side in an exon, is a transcription factor and also plays an important role in tumour angiogenesis, cell proliferation, and in the inhibition of apoptosis.¹⁵ It also plays a role in interacting with the acute-phase-response of the immune system and in embryogenesis where the absence of STAT3 is lethal.¹⁶ As the different types of NSCLC are often not distinguished between in terms of treatment, this study was created to test the three different types of NSCLC (squamous cell lung carcinoma, adenocarcinoma, and large cell lung carcinoma), for the effects of specific siRNA transfection on cell growth. In a previous study, the authors have already observed the effects on NSCLC cell lines for the factors serum-response-factor (SRF), E2-promoting binding factor (E2F1), and Survivin.¹⁷ This new study has been conducted to attempt to find a new approach in the creation of a more effective and more specialized treatment for each type of NSCLC.

Methods

Cell culture

The four used NSCLC cell lines (SK-MES squamous cell lung carcinoma; LXF-289 adenocarcinoma; A549 adenocarci-

noma; and NCI-H-460 large cell lung carcinoma [DSMZ-German Collection of Micro-organisms and Cell Cultures, Braunschweig, Germany]) were cultured under standard conditions at 37°C and 5% CO₂.

All cell culture media and supplements were purchased from PAA (Cölbe, Germany). All cell line media were supplemented with 100 U penicillin and 20 μ g streptomycin per ml and with 2 mM L-glutamine. Additionally, the medium was supplemented with 10% fetal calf serum (FCS) (PAA, Cölbe, Germany). A549 and SK-MES were cultured in high-glucose Dulbecco's modified eagle medium (DMEM), NCI-H-460 in PRMI 1640 medium, and the LXF-289 cell line in Ham's F10 medium.

Transfection of small interfering (si)RNAs

One day prior to siRNA transfection, cells were seeded in 12-well cell culture plates. For quantitative real time-polymerase chain reaction (qRT-PCR) 150 000 and for CASY analysis, 10 000 cells were seeded into one well of a 12-well plate. The next day, cells were transfected with different siRNA concentrations: 25 nM, or 100 nM for qRT-PCR and 50 nM for CASY and western blot analysis. The amount of siRNA was calculated for one well of a 12-well plate and diluted in 300 μ L basal medium (DMEM high-glucose medium without supplements). Then, 1.1 μ L or 1.75 μ L of INTERFERin, purchased from Polyplus (Illkirch, France), were pipetted into a siRNA concentration of 25 nM or of more than 50 nM, respectively. For CASY analysis of the mixture HIF1 α plus HIF2 α -siRNA, 25 nM of each siRNA was diluted in 300 μ L basal medium and the appropriate amount of INTERFERin was added.

To form transfection complexes, mixtures were incubated for 20 minutes at room temperature. Subsequently, the medium of the cells was aspirated and cells were washed with 500 μ L phosphate buffered saline (PBS) derived from PAA (Cölbe, Germany). For siRNA transfection, 300 μ L of the transfection complexes were added to each well and incubated for two hours at 37°. The complexes were then removed and fresh medium, supplemented with FCS, antibiotics, and L-glutamine was added to the cells.

siRNA sequences

Scrambled-siRNA (SCR-siRNA) was purchased from Qiagen (Hilden, Germany), however, they do not provide the sequence of their non-silencing siRNA. They ensure that it has no homology to any known mammalian gene. To ensure minimal nonspecific effects on gene expression and phenotype, their non-silencing siRNA was validated using Affymetrix Gene Chip arrays and a variety of cell-based assays. The used siRNAs have the following sequences: -HIF1 α sense- 5'-AGAGGUGGAUAUGUGUGGG-3';

antisense 5'-CCCACACAUAUCCACCUCU-3'; HIF2 α sense 5'-AGA UUC CUC GUU AUU GUU G-3'; antisense 5'-CAACA AUAACGAGGAAUCU-3'; STAT3 sense 5'-GCCUCUCU GCAGAAUCAA-3' antisense 5'-UUGAAUUCUGCAGA GAGGC-3'.

HIF1 α , HIF2 α and STAT3 siRNAs were synthesized by Eurofins MWG Operon (Ebersberg, Germany).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Twenty-four hours after siRNA transfection, the total RNA was purified from cells using the Aurum total RNA mini-kit supplied by Bio-Rad (Hercules, CA, USA). Bio-Rad's iScript cDNA Synthesis Kit was used according to the manufacturer's instructions to consecutively reverse-transcribe 200 ng of each RNA sample. Primer design was conducted using Primer 3 and Primer Premier 5 software (PREMIER Biosoft International, Palo Alto, CA, USA). The following primer sequences were used: GAPDH forward 5'-TCAACAGCGACACCC ACTCC-3'; reverse 5'-TGAGGTCCACCACC-CTGTTG-3'; HIF-1 α forward 5'-TGCAGAATGCTCAGAGAA-3'; reverse 5'-GCTGCATGATCGTCTGGCTGCT-3'; HIF-2 α forward 5'-TGTCAGGCATGGCAAGCCGG-3'; reverse 5'-GCACG GGCACGTTACCTCA-3'; STAT3 forward 5'-CGGAGAA ACAGTTGGGACCCCT-3'; reverse 5'-GAGCTGCTCCAG GTACCGTGT-3'. All primers were synthesized by Eurofins MWG Operon (Ebersberg, Germany). All PCR reactions contained IQ SYBR Green Supermix from Bio-Rad, 400 nM forward and reverse primer and 2 ng of cDNA in a total volume of 15 μ L. All PCR reactions were performed in triplicates. Normalized gene expression was calculated by the Δ Ct method using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a reference.

Cell growth analysis by CASY

Three days after siRNA transfection, the cell number was determined for the analysis of cell growth using a CASY cell counter system (Schärfe System, Reutlingen, Germany). Because of the lower resistance of dead cells in an electronic pulse area analysis, they were distinguishable from living cells. Cells were detached by using trypsin and neutralized with trypsin neutralizing solution (TNS) before they were counted by CASY. For the purpose of further analysis, only the numbers of living cells per ml were used and the number of non-transfected cells was set to 100%.

Protein expression by western blot

Twenty-four hours after siRNA transfection, the total protein was isolated from cells with the use of a radio-immunoprecipitation assay (RIPA) lysis buffer from Thermo

Fisher Scientific (Bonn, Germany). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the isolated proteins was then performed and blotted on nitrocellulose membrane, which was blocked overnight in 5% bovine serum albumen (BSA). Subsequently, the membrane was incubated with a 1:500 dilution of anti-STAT3 and anti-actin antibody, both from Santa Cruz (Heidelberg, Germany), for two hours at room temperature. After washing, the second antibody from Sigma Aldrich (Seelze, Germany) with conjugated alkaline-phosphatase was applied to the membrane for two hours in a 1:5000 dilution. Visualization was carried out with 5-bromo-4-chloro-3-indolyl-phosphate-nitro blue tetrazolium (BCIP/NBT) substrate from Sigma Aldrich (Seelze, Germany). For calculation of the relative expression of the protein level, Image J was used. Actin expression was, therefore, used as reference.

Statistics

The mean $\pm$ standard deviation (SD) is used for the expression of the results. The results represent the average of $n = 1$ to $n = 4$ independent experiments (performed in duplicates for qRT-PCR and in quadruplicates for CASY). To confirm that there was a significant knockdown the t-test for independent samples was performed with $n = 3$.

Results

After siRNA transfection of A549 cells with HIF1 α -, HIF2 α -, and STAT3-siRNA, the gene expression of HIF1 α was unchanged, but HIF2 α targeted siRNA led to a 58% reduction of HIF2 α gene expression with 25 nM siRNA, and a 53% reduction after transfection with 100 nM siRNA HIF2 α -siRNA. The transfection with 25 nM STAT3 siRNA resulted in a 68% reduction, while the transfection with 100 nM STAT3 siRNA resulted in a 61% reduction of STAT3, analyzed by qRT-PCR (Fig 1a–c). SCR-siRNA transfection did not result in a decrease of HIF1 α , HIF2 α or STAT3 gene expression. The transfection with 50 nM HIF1 α - and STAT3-siRNA resulted in a significant reduction of cell growth of more than 40%; HIF2 α -siRNA and the mixture of HIF1 α - plus HIF2 α siRNA did not result in a significant reduction in cell growth (Fig. 1d and Table 1).

The examination of the gene expression in LXF-289 cells after HIF1 α -targeted siRNA transfection with concentrations of 100 nM siRNA showed a 50% reduction of HIF1 α after transfection with HIF1 α siRNA, but no visible knockdown with 25 nM siRNA concentration (Fig. 2a). The transfection with different concentrations of HIF2 α -siRNA did not result in a knockdown (Fig. 2b). The transfection of LXF-289 cells with STAT3-siRNA led to a reduction of 10% of the protein level (Fig. 2c,e). The cell growth was reduced significantly to 50%, 30%, and 40%, compared to

Figure 1 Relative (a) HIF1 α , (b) HIF2 α and (c) STAT3 expression of A549 cells 24 hours after transfection with corresponding siRNAs. The x-axis describes the different siRNAs and their used concentrations, while the y-axis shows the relative expression of the mRNA silenced gene, with untransfected cells set to one. (d) Cell numbers in % three days after transfection with siRNA concentrations of 50 nM. Untransfected cells were set to 100%. (e) Relative protein level of STAT3 α and STAT3 β 24 hours after transfection with 50 nM STAT3-siRNA, compared to untransfected cells. Actin expression was measured to calculate a relative expression. (f) Western blot of STAT3 expression after transfection with 50 nM STAT3 siRNA ($n = 2$). Each chart shows the mean $\pm$ standard deviation (SD) of (a), (b), (c) $n = 1$, (d) $n = 3$. * indicates statistical significance at a level of $P < 0.05$, compared with untransfected cells.

non-transfected cells after transfection with HIF1 α -, STAT3-, and HIF1 α - plus HIF2 α -siRNA as a mixture, respectively (Table 1). HIF2 α -siRNA transfection did not result in a decrease of cell growth (Fig. 2d).

The analysis of gene expression after transfection of the NCI-H-460 cell line with HIF1 α -, HIF2 α -, and STAT3-

siRNA showed a 46% decrease of the HIF1 α expression after transfection with HIF1 α -siRNA, a 23% reduction of the HIF2 α expression after transfection with 25 nM HIF2 α -siRNA, and a reduction of 53% after transfection with 100 nM HIF2 α -siRNA (Fig. 3a,b). Furthermore, the expression of STAT3 is reduced 70% after transfection with 25 nM STAT3-siRNA, and 75% at a 100 nM siRNA concentration (Fig. 3c). After transfection with 50 nM HIF1 α -, HIF2 α -, and STAT3-siRNA the cell growth is significantly reduced by the transfection of HIF1 α -siRNA with 80%, followed by a significant reduction of 40% after transfection with HIF2 α - and STAT3-siRNA (Table 1). The mixture of HIF1 α -, plus HIF2 α -siRNA also revealed a significant 80% decrease in cell growth. However, the SCR-siRNA transfection seems to be responsible for a low, but significant, decrease in cell growth of 27% (Fig. 3d). Furthermore, SCR-siRNA led to an increase

Table 1 Reduction of cell growth after siRNA transfection

Tumor type	HIF1 α	HIF2 α	STAT3	HIF1 α +HIF2 α
A549	44%*	8%	47%*	20%
LXF	48%*	No reduction	30%*	40%*
NCIH	78%*	60%*	60%*	80%*
SK-MES	20%*	20%*	27%*	44%*

p* indicates statistical significance at a level of $P < 0.05$ compared with untransfected cells.

Figure 2 Relative (a) HIF1α and (b) HIF2α expression of LXF cells 24 hours after transfection with corresponding siRNAs. The x-axis describes the different siRNAs and their used concentrations, while the y-axis shows the relative expression of the mRNA, with untransfected cells set to one. (c) Relative protein level of STAT3α and STAT3β 24 hours after transfection with 50 nM STAT3-siRNA, compared to untransfected cells. Actin expression was measured to calculate a relative expression. (d) Cell numbers in % three days after transfections with the concentrations of 50 nM. Untransfected cells were set to 100%. (e) Western blot of STAT3 expression after transfection with 50 nM STAT3 siRNA ($n = 2$). Each chart shows the mean $\pm$ standard deviation (SD) of (a), (b), $n = 2$ and (d) $n = 3$. p^* indicates statistical significance at a level of $P < 0.05$, compared with untransfected cells.

of STAT3 expression in qRT-PCR (Fig. 3d), but mediated no increased protein level analyzed by western blot (Fig. 3e,f). The transfection of STAT3-siRNA resulted in a 5% reduction of the STAT3 protein level.

The results of the transfection of SK-MES cells with HIF1α-, HIF2α-, and STAT3-siRNAs revealed no reduction of HIF1α expression, analyzed by qRT-PCR (Fig. 4). The expression of HIF2α was reduced 60% at a 25 nM siRNA concentration (Fig. 4b). SK-MES cells transfected with STAT3-siRNA showed a 40% and 60% knockdown of STAT3 expression after transfection with 25 nM and 100 nM siRNA, respectively (Fig. 4). Cell growth was significantly reduced 20%, 20%, 27%, and 44% after transfection with HIF1α-, HIF2α-, STAT3-, and the mixture of HIF1α-, plus HIF2α-siRNA, respectively (Fig. 4d and Table 1).

The transfection of the adenocarcinoma cell lines (A549 and LXF-289) with SCR-siRNA resulted in a visible increase of the cell viability, which was insignificant (Figs 1,2d).

SK-MES and NCI-H-460 cells showed a decrease, however, only the decrease in growth of the NCI-H-460 cell line is significant (Figs 2d,4d).

The analysis of the protein level after transfection with STAT3-siRNA reveals a decrease of the protein level of STAT3α and STAT3β for three of the four cell lines (except SK-MES). STAT3 as a transcription factor is produced all the time. If siRNA is introduced into these rapid proliferating cancer cells, the effect of the RNAi is limited by dilution of the siRNA by the dividing cells. From our results we see a knockdown of the STAT3 mRNA level after 24 hours, but the half-life of the protein is much longer and the effect of the protein knockdown is not as high as desired. In a therapeutic setup one would have to apply the siRNA every two to three days to also significantly decrease the protein levels of STAT3.

The transfection with SCR-siRNA did not alter the protein level of STAT3α and STAT3β in the cell lines A549, LXF-289 and SK-MES compared to untransfected cells (Figs 1–4e).

Figure 3 Relative (a) HIF1 α , (b) HIF2 α and (c) STAT3 expression of NCI-H460 cells 24 hours after transfection with corresponding siRNAs. The x-axis describes the different siRNAs and their used concentrations, while the y-axis shows the relative expression of the mRNA, with untransfected cells set to one. (d) Cell numbers in % three days after transfection with siRNA concentrations of 50 nM. Untransfected cells were set to 100%. (e) Relative protein level of STAT3 α and STAT3 β 24 hours after transfection with 50 nM STAT3-siRNA, compared to untransfected cells. Actin expression was measured to calculate a relative expression. (f) Western blot of STAT3 expression after transfection with 50 nM STAT3 siRNA ($n = 2$). Each chart shows the mean $\pm$ standard deviation (SD) of (a), (b), (c) $n = 1$ and (d) $n = 3$. p^* indicates statistical significance at a level of $P < 0.05$, compared with untransfected cells.

Discussion

In the last few years, new approaches in the therapy of NSCLC have been established. Traditional procedures including surgery, chemotherapy, and radiotherapy lead to a frustrating outcome in most patients, even at early diagnosis. Now there are new treatment options, especially for patients in advanced stages. For example, preoperative chemotherapy or the tyrosine-kinase inhibitor erlotinib can be used in some patients to prolong survival.^{18–20}

However, as early symptoms are rather unspecific, the disease is often discovered in advanced stages with already contralateral lymphatic, or, even worse, metastatic lesions. In this case the prognosis is limited and therapy might consist of the traditional treatments to achieve a better outcome for the patient. In the event of an inoperable tumor, a palliative approach is taken to increase the patients' quality of life.

The purpose of this study was to investigate the application of RNA as a possible alternative therapeutic application for NSCLC patients.

HIF1 α and HIF2 α and their adapted expression to hypoxia in cancer cells play key roles as transcription factors in angiogenesis, cell proliferation, and survival. HIF2 α is also known as endothelial PAS domain-containing protein (EPAS1).²¹ HIFs promote the activation of genes involved in cancer initiation, progression, and metastasis. Hypoxia has been shown to enhance the invasiveness and metastatic potential of tumor cells by regulating the genes involved in the breakdown of the extracellular matrix (ECM), as well as genes that control motility and adhesion of tumor cells.²²

STAT3 and its two isoforms α (93 kDa) and β (84 kDa), play a role, not only in cell proliferation and angiogenesis, but also interact with many different factors, including Interleukin-6 (IL-6).²³ Constitutive STAT3 activation is

Figure 4 Relative (a) HIF1 α , (b) HIF2 α and (c) STAT3 expression of SK-MES cells 24 hours after transfection with corresponding siRNAs. The x-axis describes the different siRNAs and their used concentrations, while the y-axis shows the relative expression of the mRNA, with untransfected cells set to one. (d) Cell numbers in % three days after transfection with siRNA concentrations of 50 nM. Untransfected cells were set to 100%. (e) Relative protein level of STAT3 α and STAT3 β 24 hours after transfection with 50 nM STAT3-siRNA compared to untransfected cells. Actin expression was measured to calculate a relative expression. (f) Western blot of STAT3 expression after transfection with 50 nM STAT3 siRNA ($n = 2$). Each bar shows the mean $\pm$ standard deviation (SD) of (a), (b), (c) and (d) $n = 3$. p^* indicates statistical significance at a level of $P < 0.05$ compared with untransfected cells.

associated with various human cancers and commonly suggests a poor prognosis.^{24–27}

The three main subtypes of NSCLC are adenocarcinoma, squamous-cell lung carcinoma, and large-cell lung carcinoma. Our results remarkably reveal that every histological subtype of NSCLC showed different knockdown and viability results. Even among the adenocarcinoma cell lines A549 and LXF-289, some differences in the knockdown and cell growth were found. The knockdown of the mRNA level by siRNA transfection also differs among the other cell lines SK-MES (squamous cell lung carcinoma) and NCI-H-460 (large cell lung carcinoma).

The transfection with HIF1 α -siRNA led to a knockdown of HIF1 α mRNA in LXF-289 and NCI-H-460 cells, but not in A549 and SK-MES cells (Figs 1–4a). However, the cell viability is significantly decreased in all of the four analyzed cell lines after transfection of 50 nM HIF1 α -siRNA. That implies that a HIF1 α -siRNA mediated knockdown has an impact on

cell proliferation in NCI-H460 and LXF-289 cells, but this cannot be confirmed in A549 and SK-MES cells. The transfection procedure seems not to have an impact on the expression of HIF1 α , as SCR-siRNA transfection did not result in an altered HIF1 α expression.

The transfection with HIF2 α -siRNA led to a silencing in large cell carcinoma (NCI-H-460), adenocarcinoma (A549), and in squamous cell carcinoma (SK-MES). However, transfection with HIF2 α -siRNA resulted in a statistically significant reduction of cell growth in NCI-H-460 and SK-MES cells, whereas the A549 and LXF-289 cells showed no significant decrease.

When comparing the protein and mRNA expression of STAT3 after transfection with SCR-siRNA, the results of the qRT-PCR showed an increase of mRNA expression in all cell lines. However, in the results of the protein level, only the LXF-289 cell line (Fig. 2c) exhibited this behavior. From latter results in our laboratory we found increased or decreased

expression of the analyzed genes after transfection with SCRsiRNA.^{17,28,29} In most cases, these changes were not significant and we suggest that the expression changes are derived from the transfection itself and not from the SCRsiRNA. In one of our studies we compared different transfection reagents and our results showed fewer and less toxic side effects with Interferin compared to Lipofectamine²⁰⁰⁰ (Invitrogen).³⁰ However, as cationic liposomal transfection is based on endocytosis, every efficient transfection is accompanied by cell stress. This cell stress can then lead to a higher expression of STAT3 and increased viability, particularly in invasive cells, such as cancer cells.

The decrease of STAT3 after STAT3-siRNA transfection is clearly visible on the mRNA and protein level, as well as the significant reduction of cell growth in all cell lines. This implies that STAT3-siRNA shows a specific knockdown and the silencing of STAT3 mediates an antiproliferative or apoptotic effect to the cells. STAT3 as a transcription factor is constitutively activated in cancer cells. If siRNA is introduced into these rapid proliferating cancer cells, the effect of the RNAi is limited by dilution of the siRNA by the dividing cells. Our results reveal a knockdown of the STAT3 mRNA level after 24 hours, but the half-life of the protein is much longer and the effect of the protein knockdown is not as high as desired. In a therapeutic setup one would have to apply the siRNA every two to three days to also significantly decrease the protein levels of STAT3. Interestingly, the mixture of HIF1 α and HIF2 α -siRNA shows a better reduction of cell growth in only one cell line (SK-MES) compared to HIF1 α - or HIF2 α -siRNA alone. We suggest that the antiproliferative effects of the siRNAs do not add themselves when used in a 25 nM concentration compared to the viability of cells transfected with one siRNA of 50nM. Furthermore, HIF1 α - and HIF2 α -siRNA did not reduce cell growth in all of the analyzed cell lines.

The transfection of SCR-siRNA showed an increase of STAT3 mRNA expression in all used cell lines. In squamous cell carcinoma SCR-siRNA transfection resulted in an increase or decrease of the analyzed factors. In NCI-H-460 cells SCR-siRNA transfection also showed a significant decrease in cell growth. The used SCR-siRNA or the transfection seems to cause stress to the cells which either leads to proliferative or antiproliferative effects. There are several other studies with similar observations using the same SCR-siRNA.^{17,28}

Our results suggest that these double stranded RNA molecules offer high potential for further therapies, as, in vitro, the cell lines showed a decreased cell viability, depending on the histological type of NSCLC and the used factor. This implies that choosing the right factor in the appropriate concentration could present a possible new method of therapy.

The authors' actual aim, in the near future, is to further improve the ways to transfect cells, not only in vitro, but in vivo, to establish a new approach in the treatment of NSCLC.

Conclusion

The therapeutic possibilities to treat advanced stages of NSCLC are restricted to surgery, radiotherapy, and chemotherapy with low five-year survival rates. Therefore, it is necessary to investigate new therapeutic approaches. We analyzed the knockdown and cell proliferation of four NSCLC cell lines after transfection with siRNAs targeted against HIF1 α , HIF2 α , and STAT3. We found that the cell proliferation was mostly reduced after transfection in all cell lines, but the three histological cell types reacted differently to siRNA silencing. Therefore, new therapeutic application needs to be personalized and specialized to the different tumor cell types. A clinical application of siRNAs would achieve these prerequisites.

Disclosure

No authors report any conflict of interest.

References

- 1 Azzoli CG, Baker S Jr, Temin S *et al.* American Society of Clinical Oncology Clinical Practice Guideline update on chemotherapy for stage IV non-small-cell lung cancer. *J Clin Oncol* 2009; **27**: 6251–66.
- 2 Andersen S, Donnem T, Stenvold H *et al.* Overexpression of the HIF hydroxylases PHD1, PHD2, PHD3 and FIH are individually and collectively unfavorable prognosticators for NSCLC survival. *PLoS ONE* 2011; **6** (8): e23847.
- 3 Stenvold H, Donnem T, Andersen S *et al.* Overexpression of matrix metalloproteinase-7 and -9 in NSCLC tumor and stromal cells: correlation with a favorable clinical outcome. *Lung Cancer* 2012; **75**: 235–41.
- 4 Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 1998; **391** (6669): 806–11.
- 5 Mousa SA, Mousa SS. Current status of vascular endothelial growth factor inhibition in age-related macular degeneration. *BioDrugs* 2010; **24**: 183–94.
- 6 Alderton GK. Therapy: A big step for targeting RNAs. *Nat Rev Cancer* 2010; **10**: 313.
- 7 Davis ME, Zuckerman JE, Choi CH *et al.* Evidence of RNAi in humans from systemically administered siRNA via targeted nanoparticles. *Nature* 2010; **464** (7291): 1067–70.
- 8 Tiemann K, Rossi JJ. RNAi-based therapeutics-current status, challenges and prospects. *EMBO Mol Med* 2009; **1**: 142–51.
- 9 Akhtar S, Benter IF. Nonviral delivery of synthetic siRNAs in vivo. *J Clin Invest* 2007; **117**: 3623–32.

- 10 Kim WY, Perera S, Zhou B *et al.* HIF2alpha cooperates with RAS to promote lung tumorigenesis in mice. *J Clin Invest* 2009; **119**: 2160–70.
- 11 Schoppmann SF, Jesch B, Friedrich J, Jomrich G, Maroske F, Birner P. Phosphorylation of signal transducer and activator of transcription 3 (STAT3) correlates with Her-2 status, carbonic anhydrase 9 expression and prognosis in esophageal cancer. *Clin Exp Metastasis* 2012; **29**: 615–24.
- 12 Chiavarina B, Whitaker-Menezes D, Migneco G *et al.* HIF1-alpha functions as a tumor promoter in cancer-associated fibroblasts, and as a tumor suppressor in breast cancer cells: autophagy drives compartment-specific oncogenesis. *Cell Cycle* 2010; **9**: 3534–51.
- 13 Kamlah F, Eul BG, Li S *et al.* Intravenous injection of siRNA directed against hypoxia-inducible factors prolongs survival in a Lewis lung carcinoma cancer model. *Cancer Gene Ther* 2009; **16**: 195–205.
- 14 Pawlus MR, Wang L, Ware K, Hu CJ. Upstream stimulatory factor 2 and hypoxia-inducible factor 2alpha (HIF2alpha) cooperatively activate HIF2 target genes during hypoxia. *Mol Cell Biol* 2012; **32**: 4595–610.
- 15 Onishi A, Chen Q, Humtsoe JO, Kramer RH. STAT3 signaling is induced by intercellular adhesion in squamous cell carcinoma cells. *Exp Cell Res* 2008; **314**: 377–86.
- 16 Takeda K, Noguchi K, Shi W *et al.* Targeted disruption of the mouse Stat3 gene leads to early embryonic lethality. *Proc Natl Acad Sci U S A* 1997; **94**: 3801–4.
- 17 Walker T, Nolte A, Steger V *et al.* Small interfering RNA-mediated suppression of serum response factor, E2-promotor binding factor and survivin in non-small cell lung cancer cell lines by non-viral transfection. *Eur J Cardiothorac Surg* 2013; **43**: 628–34.
- 18 Bozcuk H, Abali H, Coskun S. The correlates of benefit from neoadjuvant chemotherapy before surgery in non-small-cell lung cancer: a metaregression analysis. *World J Surg Oncol* 2012; **10**: 161.
- 19 Liao WY, Chen JH, Wu M *et al.* Neoadjuvant chemotherapy with docetaxel-cisplatin in patients with stage III N2 non-small-cell lung cancer. *Clin Lung Cancer* 2013; **14**: 418–24.
- 20 Lyseng-Williamson KA. Erlotinib: a guide to its use in first-line treatment of non-small-cell lung cancer with epidermal growth factor-activating mutations. *Mol Diagn Ther* 2013; **17**: 57–62.
- 21 Favier J, Lapointe S, Maliba R, Sirois MG *et al.* HIF2 alpha reduces growth rate but promotes angiogenesis in a mouse model of neuroblastoma. *BMC Cancer* 2007; **7**: 139.
- 22 Hanna SC, Krishnan B, Bailey ST *et al.* HIF1alpha and HIF2alpha independently activate SRC to promote melanoma metastases. *J Clin Invest* 2013; **123**: 2078–93.
- 23 Yadav A, Kumar B, Datta J, Teknos TN, Kumar P *et al.* IL-6 promotes head and neck tumor metastasis by inducing epithelial-mesenchymal transition via the JAK-STAT3-SNAIL signaling pathway. *Mol Cancer Res* 2011; **9**: 1658–67.
- 24 Alvarez JV, Greulich H, Sellers WR, Meyerson M, Frank DA. Signal transducer and activator of transcription 3 is required for the oncogenic effects of non-small-cell lung cancer-associated mutations of the epidermal growth factor receptor. *Cancer Res* 2006; **66**: 3162–8.
- 25 Klampfer L. Signal transducers and activators of transcription (STATs): novel targets of chemopreventive and chemotherapeutic drugs. *Curr Cancer Drug Targets* 2006; **6**: 107–21.
- 26 Kusaba T, Nakayama T, Yamazumi K *et al.* Activation of STAT3 is a marker of poor prognosis in human colorectal cancer. *Oncol Rep* 2006; **15**: 1445–51.
- 27 Yin W, Cheepala S, Roberts JN, Syson-Chan K, DiGiovanni J, Clifford JL. Active Stat3 is required for survival of human squamous cell carcinoma cells in serum-free conditions. *Mol Cancer* 2006; **5**: 15.
- 28 Nolte A, Aufderklamm S, Scheu K *et al.* Small interfering RNA transfection against serum response factor mediates growth inhibition of benign prostatic hyperplasia fibroblasts. *Nucleic Acid Ther* 2013; **23**: 62–70.
- 29 Nolte A, Walker T, Schneider M *et al.* Small-interfering RNA-eluting surfaces as a novel concept for intravascular local gene silencing. *Mol Med* 2011; **17**: 1213–22.
- 30 Nolte A, Raabe C, Walker T, Simon P, Ziemer G, Wendel HP. Optimized basic conditions are essential for successful siRNA transfection into primary endothelial cells. *Oligonucleotides* 2009; **19**: 141–50.