SPANDIDOS PUBLICATIONS

Stat3 as a molecular target in RNA interference-based treatment of oral squamous cell carcinoma

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Abstract. Constitutive activation of signal transducer and activator of transcription 3 (Stat3) has been observed in many human malignancies. Using the sequence-specific RNA interference (RNAi) method to switch off Stat3 expression, it may be possible to arrest cancer growth. In this study, we aimed to identify the most effective sequence of a synthetic small interfering RNA (siRNA) specific for Stat3 (Stat3-siRNA) and the effect of Stat3 suppression on the growth of oral squamous cell carcinoma cells. Ten designed siRNAs with known sequences were screened for the best RNAi effect at the working concentrations of 1 and 10 nM. The range of reduction of Stat3 expression varied from 21 to 67% for 10 nM siRNAs, and from 13 to 73% for 1 nM siRNAs. Three out of the 10 screened siRNAs reduced Stat3 expression to lower levels compared with the GFP-siRNA control. The interferon response of some siRNAs was observed at a concentration of 10 nM. However, at 1 nM, the mRNA levels of interferon response genes (OAS1, OAS2, MX1 and ISFG3γ) remained unchanged. The growth of GFP-SAS, HSC-3, HSC-4 and KB cells was strongly inhibited by the use of three effective Stat3-siRNAs in comparison with other Stat3-siRNAs and GFP-siRNA. Moreover, the mRNA levels of genes for which transcription is activated by Stat3 were markedly suppressed. These results suggest that targeting Stat3 using siRNA may constitute a useful approach for the treatment of oral squamous cell carcinoma.

Introduction

Signal transducer and activator of transcription 3 (Stat3) is a key molecule for many biological processes such as embryonic development, organogenesis, innate and adaptive immune responses, regulation of cell differentiation and cell cycle progression, and apoptosis (1,2). However, a significant number of studies have suggested that phosphorylated Stat3 protein is involved in promoting tumorigenesis. Moreover, a constitutively active mutant of Stat3 has been demonstrated to transform fibroblasts and induce tumour formation in nude mice (3). Constitutive activation of Stat3 signalling has been reported in many human cancer types (1,4-9). In our previous research, 74% of oral squamous cell carcinoma (OSCC) tissues examined immunohistochemically exhibited a markedly elevated phosphorylation of Stat3 (10). Persistent signalling of Stat3 has been demonstrated to contribute to oncogenesis by stimulating cell proliferation and preventing apoptosis by the up-regulation of genes encoding cell cycle regulators, survivin and apoptosis inhibitors (11-17).

Stat3 itself serves as a molecular target in cancer therapy. The blockade of Stat protein signalling can be achieved by various means, including dominant-negative mutants, anti-sense methods (18,19), inhibition of upstream signalling (20), phosphotyrosyl peptides (21) and the double-stranded DNA (dsDNA) decoy method (22). A further strategy for abrogating protein expression in human cells is based on destroying mRNA by RNA interference (RNAi) (23), using either small hairpin RNA (shRNA) (24) or small interfering RNA (siRNA) (15).

In the current study, we examined the influence of siRNAs specific for the Stat3 sequence on the expression of Stat3 and followed its effects in OSCC cells. The aim was to identify the most effective sequence of Stat3-siRNA in order to obtain a powerful tool for targeting Stat3 expression in RNAi-based human cancer treatment.

Materials and methods

Cells and cell culture. We used human OSCC cell lines (HSC-2, HSC-3, HSC-4, KB, Ca9-22, GFP-SAS, Scc9, Scc25, Scc66 and Scc11) and a human epidermal keratinocyte cell line (HaCaT). GFP-SAS cells were generated by transfecting SAS cells with a gene encoding green fluorescent protein (GFP). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS; Biosource International, Camarillo, CA), 100 U/ml penicillin, and 100 μg/ml strepto-

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mycin (Invitrogen) (referred to as complete medium), and incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37˚C. For evaluation of the constitutive activation of Stat3 in these cells, the growth medium was replaced with a plain one.

Synthetic small interfering RNAs (siRNAs). Cells were transfected with small interfering RNAs specific for Stat3 (Stat3-siRNA: Smartpool® siRNA reagents, Cat. No. M-003544-00-05; Dharmacon, Lafayette, CO) as well as with 10 designed Stat3-siRNAs (B-Bridge International, Inc., Mountain View, CA), and green fluorescent protein (GFP-siRNA: Smartpool siRNA reagents, Cat. No. D-001300-01-20) as a control at working concentrations of 1 and 10 nM using lipofection (Lipofectamine™ 2000; Invitrogen) according to the manufacturer's instructions. After a 72 h gene knockdown, the cells were recovered by treatment with 0.05% trypsin-0.53 mM EDTA (Invitrogen) and used for RNA isolation and cell growth assays. The target sequences of 10 Stat3-siRNAs were: 5'-CUU GGA UUG AGA GUC AAG A-3' (no. 1), 5'-GAG AUU GAC CAG CAG UAU A-3' (no. 2), 5'-GUU UCU UCA GAG CAG GUA U-3' (no. 3), 5'-GUA GAG AAU CUC CAG GAU G-3' (no. 4), 5'-GGA GAA GCA UCG UGA GUG A-3' (no. 5), 5'-CCU AGA UCG GCU AGA AAA C-3' (no. 6), 5'-UCC AGU UCA CUA CUA AAG U-3' (no. 7), 5'-GCA GAA UUC AAA CAC UUG A-3' (no. 8), 5'-GGA GCA GAG AUG UGG GAA U-3' (no. 9) and 5'-CCA ACG ACC UGC AGC AAU A-3' (no. 10).

Cell growth assay. Synthetic siRNA-transfected cells were seeded in complete medium in flat-bottomed 6-well plates (Falcon; BD Biosciences, San Jose, CA) at a density of $5x10^4$ cells per well. The cells were recovered by treatment with 0.05% trypsin-0.53 mM EDTA and counted using a Z1 Coulter counter (Beckman Coulter, Fullerton, CA).

Western blot analysis. Cells grown in monolayers were harvested at subconfluency and lysed with CelLytic M cell lysis reagent (Sigma-Aldrich, St. Louis, MO) containing a protease inhibitor cocktail tablet (Roche Diagnostics, Mannheim, Germany) and phosphatase inhibitor cocktails I and II (Sigma-Aldrich). The samples were centrifuged at 12,000 x g for 10 min at 4˚C. Samples of the resulting protein supernatant were electrophoresed on sodium dodecyl sulfate (SDS)-polyacrylamide gels (Bio-Rad, Hercules, CA) and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were blocked with 5% non-fat dry milk (Wako, Osaka, Japan) in 1X T-TBS [25 mM Tris-HCl, 125 mM NaCl and 0.1% Tween-20 (Sigma)] overnight at 4˚C, probed with primary antibodies overnight at 4˚C and then with horseradish peroxidase-conjugated secondary antibodies for 1 h. The immune complexes were visualized using an enhanced chemiluminescence (ECL) Plus or ECL advance kit (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's protocol. For the internal control, the blots were stripped with 62.5 mM Tris-HCl (pH 6.8) buffer containing 0.7% 2-mercaptoethanol and 2% SDS (Sigma) at 50˚C for 30 min and reprobed with mouse anti-ß-tubulin monoclonal antibody (BD Biosciences). The primary antibodies used were polyclonal antibodies against Stat3 and phospho-Stat3 (Tyr705) (Cell Signaling Technology, Inc., Beverly, MA), and we used secondary antibodies against mouse or rabbit IgG (Amersham Biosciences).

RNA isolation and real-time quantitative RT-PCR. Total RNA was extracted using the RNA isolation solution, Isogen (Nippon Gene, Toyama, Japan), according to the manufacturer's instructions. Reverse transcriptase-polymerase chain reaction (RT-PCR) amplification was performed using a QuantiTect™ Probe RT-PCR Kit (Qiagen GmbH, Hilden, Germany). The LightCycler cycling conditions were set as: reverse transcription at 55˚C for 10 min, initial denaturation at 95˚C for 30 sec followed by 55 cycles at 95˚C for 1 sec, 60˚C for 15 sec and 72˚C for 13 sec. Specific cDNAs, along with porphobilinogen deaminase (PBGD) cDNA as an internal control, were amplified separately using an oligonucleotide probe labelled with a 5'-fluorescent reporter and a 3'-quencher dye. The 5'-fluorescent reporter dye was cleaved from the cDNA by the 5'-nuclease activity of Taq DNA polymerase and its fluorescence was detected with LightCycler (Roche Diagnostics). Automated calculations were performed by the second derivative maximum method (LC software, version 3.5). The relative mRNA levels were measured using the comparative CT method (ΔΔCT method). Taq Man gene expression assays for Stat3, PBGD, 2',5'-oligoadenylate synthetases 1 and 2 (OAS1 and 2), interferon-induced myxovirus-resistance protein 1 (MX1), interferon-stimulated gene factor 3γ (ISGF3γ), cyclin D1 (CCND1), vascular endothelial growth factor (VEGF) and matrix metalloprotease-10 (MMP-10) were all purchased from Applied Biosystems (Foster City, CA).

Results

Constitutive activation of Stat3 in human OSCC cell lines. In order to determine whether Stat3 is constitutively activated in OSCC cell lines, we first examined its expression and the phosphorylation of tyrosine 705 by Western blot analysis. Various levels of Stat3 phosphorylation were found in the OSCC cells in the absence of FBS. However, the GFP-SAS, HSC-4, HSC-3 and KB cell lines exhibited relatively high Stat3 activation (Fig. 1).

Knockdown of Stat3 expression in GFP-SAS cells by Smartpool Stat3-siRNA. The high transfection efficiency and the RNAi effect of GFP-expressing SAS cells were previously demonstrated in our laboratory (data not shown). These cells were subjected to knockdown experiments with Smartpool Stat3-siRNA at the working concentration of 10 nM. Transfection of GFP-SAS cells with this siRNA resulted in a significant and reproducible decrease in Stat3 expression. The degree of knockdown ranged from 80 to 95% as determined by the levels of mRNA using real-time quantitative RT-PCR (Fig. 2A), and from 75 to 80% as determined by Western blot analysis (Fig. 2B). Transfection with siRNA specific for the GFP sequence used as a control did not reduce Stat3 expression. Interestingly, the diminished Stat3 expression was followed by a decrease of the number of cells in the culture maintained under growth conditions in comparison to the controls (Fig. 2C).

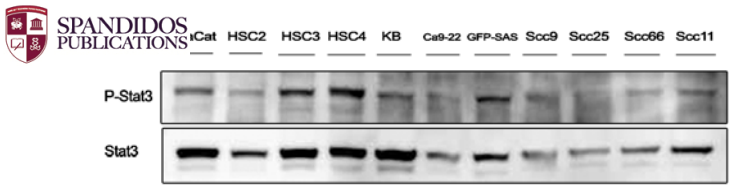

Figure 1. Phosphorylation of Stat3 in human OSCC cells. Protein samples were loaded onto a 10% SDS-polyacrylamide gel. Stat3 expression and activation were examined by Western blot analysis.

Knockdown of Stat3 expression by 10 designed synthetic Stat3-siRNAs. Ten double-stranded siRNAs specific for Stat3 were synthesized and tested for their ability to knock down Stat3 expression in human OSCC cells. The range of reduction of Stat3 expression in GFP-SAS cells varied from 21 to 67% for 10 nM siRNAs, and from 13 to 73% for 1 nM siRNAs, as assessed by the levels of mRNAs in real-time quantitative RT-PCR (Fig. 3A). A successive decrease in protein synthesis was observed by Western blot analysis 72 h after transfection (Fig. 3B). Three out of the 10 screened siRNAs reduced Stat3 expression to lower levels compared with the GFP-siRNA control.

Interferon-γ response to transfection of synthetic siRNAs. In order to examine the activation of the interferon-γ (IFN-γ) pathway in response to the 10 designed siRNAs, mRNA levels of the following four interferon response genes were subjected to analysis: OAS1, OSA2, MX1 and ISGF3γ. Increased activation of the above-mentioned genes was observed in cases of siRNA no. 9 and 10 at the working concentration of 10 nM. Transfection performed at a concentration of 1 nM did not cause a significant elevation of any of the four mRNAs (Fig. 3C).

Effect of Stat3 knockdown on the growth of human OSCC cells. The effect of Stat3 reduction on the proliferation of GFP-SAS, HSC-4, HSC-3 and KB cells was examined. For this experiment, the most effective Stat3-siRNAs (no. 2, 5 and 10) were applied at a working concentration of 1 nM. For the negative control GFP- and Stat3-siRNAs no. 1 and 7 were used. The total cell number in the culture was markedly decreased 72 h after transfection and varied depending on the siRNA sequence and the cell line. The reduction of the cell number range was 59-89% for no. 2, 61-91% for no. 5, 74-96% for no. 10, 0-4% for no. 1 and 1-37% for no. 7 (Fig. 4A). Consistent with these observations, fewer cells were observed under the microscope (Fig. 4B).

Effect of Stat3 knockdown on the expression of downstream genes. In order to investigate the functional influence of Stat3-siRNAs, the expression levels of the two genes regulated by Stat3 were examined. The reduction in Stat3 expression was followed by a decrease in the levels of CCND1 and VEGF. The reduction varied depending on the siRNA sequence and the cell line. For CCND1, the reductions observed were 41-62% for no. 2, 41-67% for no. 5, 52-72% for no. 10, 0-8% for no. 1 and 4-25% for no. 7, while for VEGF, the reductions were 13-68% for no. 2, 23-59% for no. 5, 35-64% for no. 10, 0-28% for no. 1 and 0-47% for no. 7 (Fig. 5). In contrast, the expression of MMP-10 was not significantly affected by any of the examined siRNAs.

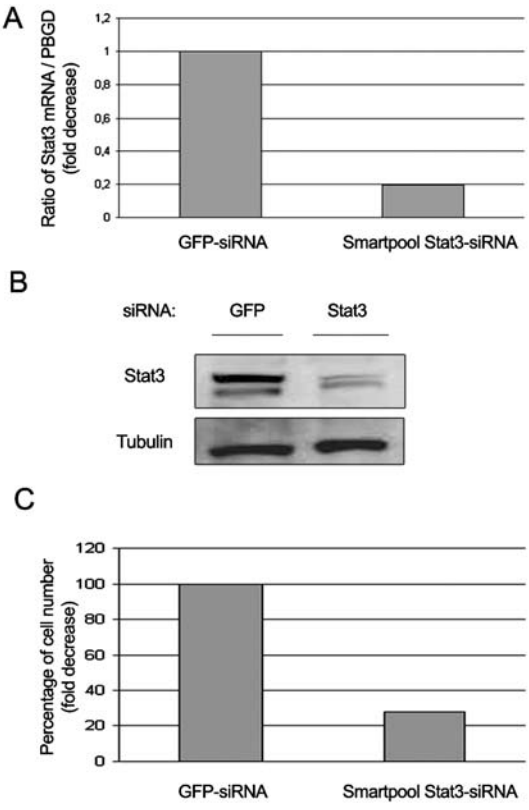

Figure 2. Effects of Smartpool Stat3-siRNA on GFP-SAS cells. (A) Decrease in Stat3 mRNA levels mediated by 10 nM Stat3-siRNA treatment 72 h after the onset of the experiment, detected by real-time quantitative RT-PCR. Stat3 mRNA levels were normalized by PBGD expression. (B) Decrease in Stat3 expression observed by Western blot analysis. (C) Decrease in cell numbers in cultures. The values were normalized to 100%.

Discussion

Stat3, a signal transducer and activator of transcription, functions as a downstream effector of cytokine and growth factor receptors (1,18). It has been demonstrated that the TGFα/EGFR-mediated growth of transformed epithelial cells is dependent on the activation of Stat3, and that interrupting Stat3 signalling abrogates the TGFα-induced growth of these cells (18). Targeting constitutively active Stat3 has repeatedly been demonstrated to inhibit tumour cell growth *in vitro* and *in vivo* (4,11,12,14-16,18,25,26). Moreover, although mice lacking Stat3 die before birth (27), studies on astrocytes have revealed that the inhibition of Stat3 signalling does not kill normal cells (15). Therefore, targeting Stat3 provides a powerful method for therapeutic intervention in human cancer.

In our study, the targeted disruption of Stat3 was achieved by the RNAi method using synthetic siRNA in OSCC cells, which are characterized by a high constitutive phosphorylation of Stat3. Knockdown of Stat3 expression was obtained with the Smartpool Stat3-siRNA. However, since this product is a mixture of four siRNAs, the most effective sequence

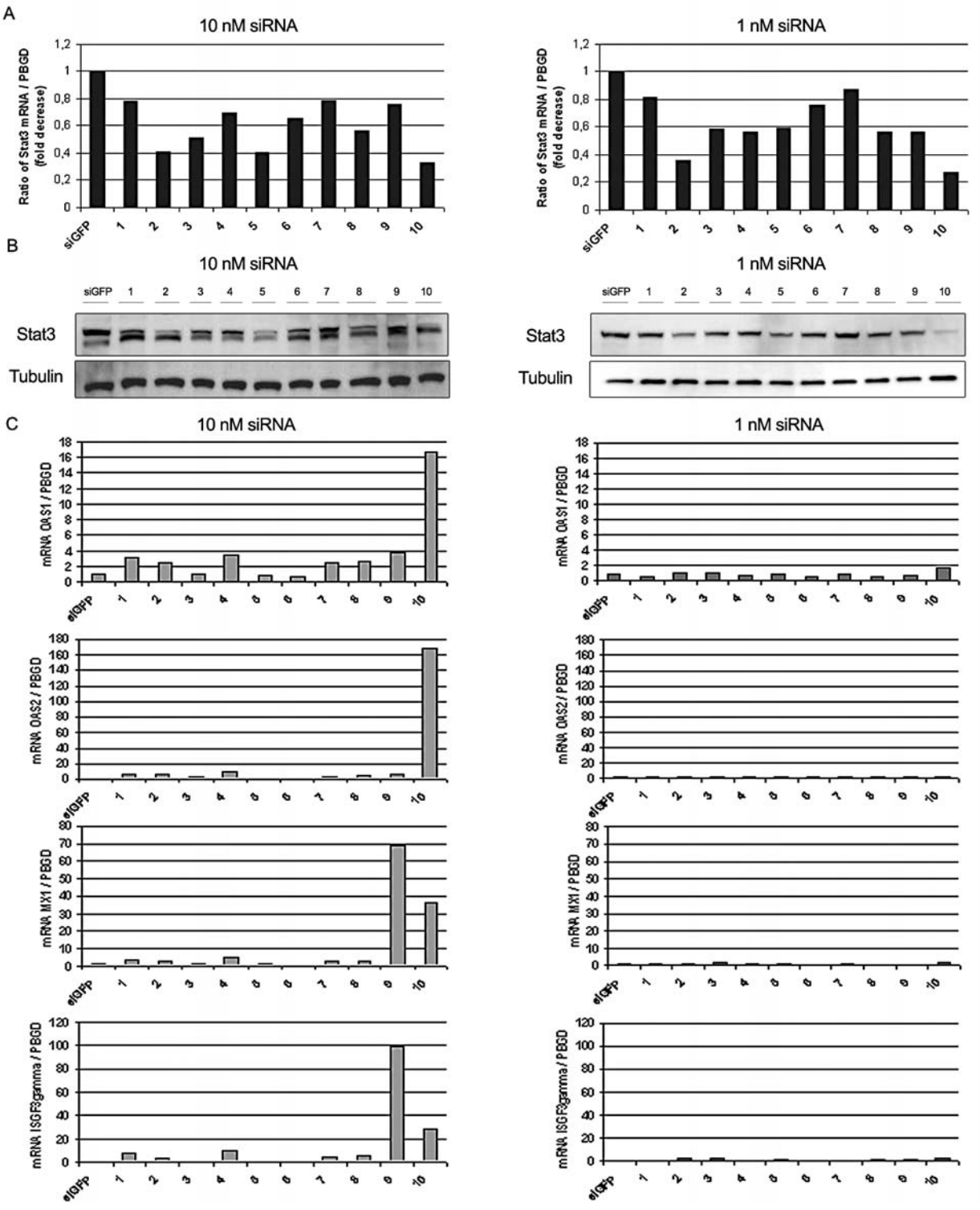

Figure 3. Knockdown of Stat3 expression by designed Stat3-siRNAs. (A) Stat3 mRNA detection after treatment of GFP-SAS cells with 1 and 10 nM of siRNAs. (B) Western blot detection of Stat3 expression. (C) IFN response to treatment with 10 designed siRNAs. Relative mRNA levels were normalized by PBGD expression and the values were adjusted to 100%.

remains unknown, and off-target effects were not considered. Therefore, we designed 10 different siRNAs corresponding to the Stat3 gene. The sequences were selected on the basis of studies of RNAi in mammalian cells (23). Interestingly, not all designed siRNAs efficiently decreased Stat3 mRNA levels. Our screening identified only three candidates as efficient Stat3-siRNAs. It should be noted that the disruption of Stat3 signalling was obtained at a markedly low working concentration of these siRNAs (1 nM) and, consistent with other studies, was found to be time-dependent with the maximum effect being achieved at 72 h of siRNA treatment (15). The depletion of endogenous Stat3 by RNAi influenced cancer cell growth due to the lower cell numbers in the cultures. We failed to observe any significant increase in apoptosis under Stat3-siRNA treatment (data not shown). Stat3 knockdown decreased the levels of the mRNAs of genes

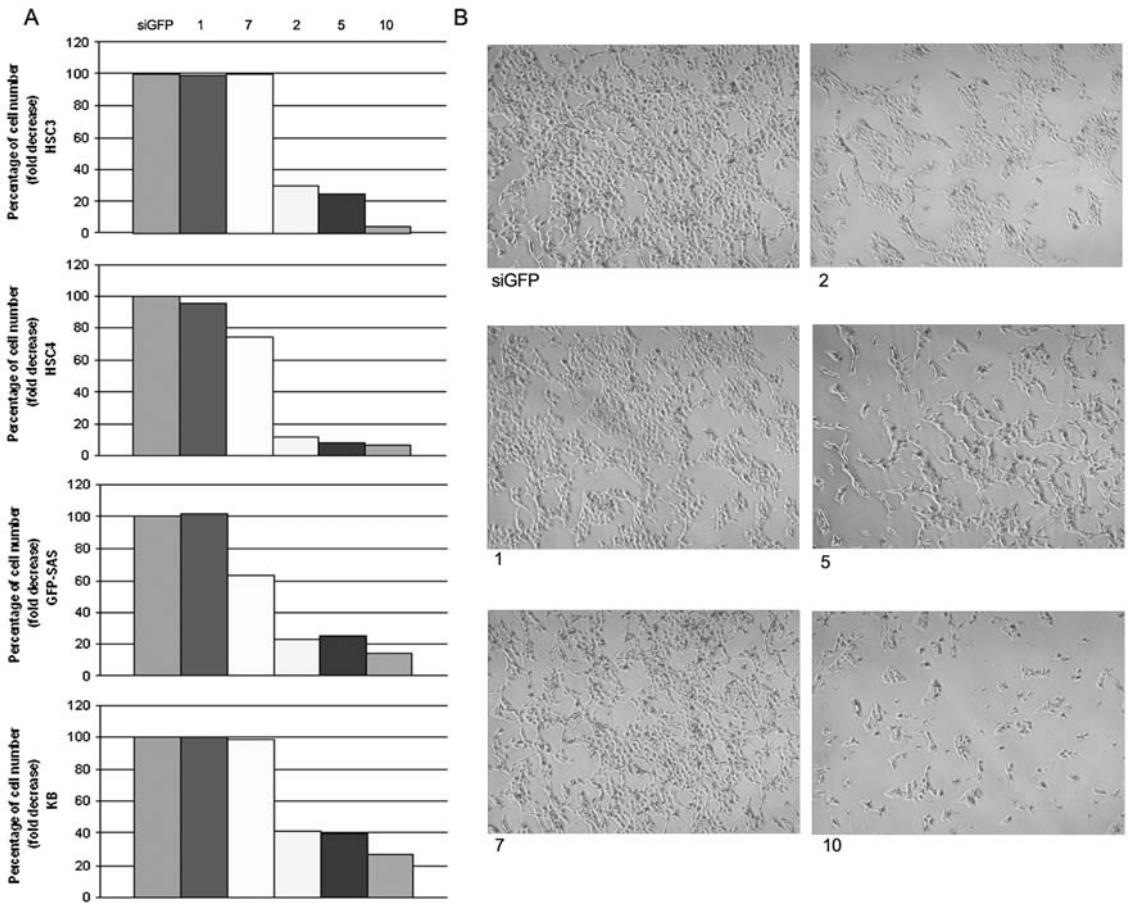

Figure 4. Growth inhibition of human OSCC cells mediated by effective Stat3-siRNAs. (A) A lower number of cells was detected in the cultures of HSC-3, HSC-4, GFP-SAS and KB cells when treated with Stat3-siRNAs no. 2, 5 or 10. GFP- and Stat3-siRNAs no. 1 and 7 were used as controls. The values were adjusted to 100%. (B) Microscopic view of HSC-3 cell cultures under the relevant Stat3-siRNA treatment. Magnification, x40.

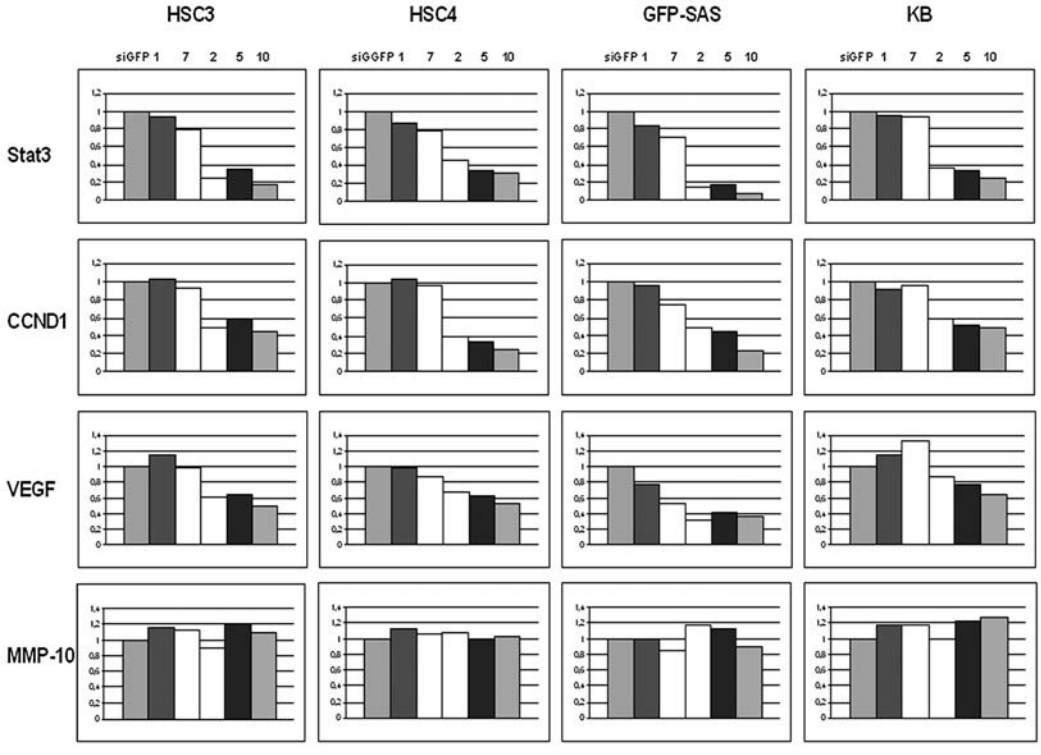

Figure 5. Suppression of CCND1 and VEGF expression by effective Stat3-siRNAs. CCND1 and VEGF mRNA levels were evaluated by real-time quantitative RT-PCR analysis. Relative mRNA levels were normalized by PBGD expression. Stat3-siRNAs no. 1 and 7 were used as controls and the values were adjusted to 1.0.

regulated by the binding of Stat3 dimers to the relevant DNA-response elements.

In addition to the gene-specific silencing, siRNA can induce non-specific effects, many of which are based on the stress reaction pathways mediating cellular antiviral responses (28-30), the levels of which depend on the concentration of siRNA and the cell line (31). Stat3 itself is connected to INF-γ signalling. It has been shown that Stat3 activation can inhibit the expression of cytokines and chemokines induced by INF-γ (32). Treatment of prostate cancer cells with INF-γ has been demonstrated to be associated with the persistent dephosphorylation of constitutively tyrosine-phosphorylated Stat3 (33). Our data clearly demonstrated that low concentrations of Stat3-siRNA did not cause the interferon response. However, at higher concentrations, sequence-specific reactions may occur in a sequence-specific manner.

In this study, we demonstrated the importance of Stat3 signalling inhibition in abrogating human OSCC growth. We also identified three Stat3-siRNA sequences and propose these to be effective tools, which may be applied separately or in combination at markedly low working concentrations, to increase the number of approaches to cancer therapy.

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