

# Suppression of cell proliferation and collagen production in cultured human hypertrophic scar fibroblasts by Sp1 decoy oligodeoxynucleotide

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**Abstract.** Hypertrophic scars are characterized by the abnormal proliferation of fibroblasts and an overproduction of collagen. The Sp1 transcription factor is involved in the stimulation of collagen synthesis. A decoy oligonucleotide (ODN) targeting Sp1 was designed and transfected into hypertrophic scar fibroblasts (HSFs) cells using cationic liposomes. The transfection efficiency was determined by flow cytometry and was observed to be  $85\pm 7\%$  ( $n=5$ ). Specific binding of the Sp1 decoy ODN was monitored with an electrophoretic mobility shift assay (EMSA). Following transfection with the decoy ODN to Sp1, cell viability and cell proliferation, which were examined by the cell counting kit WST-8, were decreased by 80% compared with untreated cells. Transforming growth factor- $\beta$  (TGF- $\beta$ ) mRNA and collagen mRNA expression were also reduced by 48% in the transfection decoy ODN group. The cell viability of HSFs after 48 h of transfection with 25, 50, 100 and 150 nM Sp1 decoy ODN was  $0.9331\pm 0.0203$ ,  $0.7479\pm 0.0868$ ,  $0.577\pm 0.0347$  and  $0.4703\pm 0.0147$ , respectively. The 100 nM dose of the Sp1 decoy ODN inhibited the expression of types I and III collagen by 32 and 28%, respectively (both  $P<0.01$ ). TGF- $\beta$  mRNA expression was also effectively suppressed by the 100 nM Sp1 decoy ODN ( $P<0.01$ ). The Sp1 decoy ODN inhibited cell proliferation and the expression of types I and III collagen. Therefore, Sp1 decoy ODNs may be a promising tool for developing and testing novel therapeutic applications for treating hypertrophic scars.

## Introduction

Hypertrophic scars are a malformation frequently encountered by plastic surgeons. Characteristic morphological and ultrastructural changes in hypertrophic scars include the abnormal

and excessive deposition of extracellular matrix (ECM). Excessive scar fibrosis results from the increased expression of several ECM proteins, including collagen. The collagen genes pro-COL1A1 and pro-COL1A2 are responsible for the synthesis of pro1 and pro2 polypeptides of type I collagen and they are synthesized at a ratio of 2:1 to form a stable triple helical type I collagen molecule in the extracellular space (1). Fibrosis, the excessive accumulation of collagen, is induced by persistent pro-fibrotic growth factor signaling, including fibroblast growth factor (FGF), platelet-derived growth factor (PDGF) and, most predominantly, transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1). Sp1 is a transcription factor that regulates the expression of a variety of ECM genes and is involved in numerous growth factor-related signal transduction pathways, including TGF- $\beta$ 1, FGF and PDGF signaling (2).

Sp1 belongs to a family of ubiquitous transcription factors associated with GC-rich promoters that are inducible and involved in inflammation (3). Sp1 exists in a variety of isoforms which bind with varying affinities to consensus sequences that form a transcriptional network to fine-tune gene expression. Sp1-dependent transcription is altered during cell growth through increased gene expression.

Treating fibroblasts with TGF- $\beta$ 1 promotes the synthesis of type I collagen (4). Chronic exposure to TGF- $\beta$ 1 in lungs increases the deposition of type I collagen, causing excessive fibrosis, which may increase morbidity and mortality. Direct silencing of the COL1A1 gene is one method to reduce collagen type I synthesis. A novel double-stranded (ds) DNA decoy with phosphorothioate (PT) linkages containing the TGF- $\beta$  cis-element has been observed in the distal promoter region of the COL1A1 gene (5). This decoy blocks collagen type I synthesis in human hypertrophic scar fibroblasts (HSFs) stimulated by TGF- $\beta$ 1 (6). These findings support the concept that a decoy ODN may inhibit collagen type I synthesis at the transcriptional and translational levels. Therefore, we performed this *ex vivo* study to determine whether a Sp1 decoy ODN could inhibit HSF proliferation, TGF- $\beta$  signaling and collagen production.

## Materials and methods

**Cell culture.** Control human fibroblasts were isolated from normal human skin (the individual biopsies ranged in size

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Table II. Primer design and product lengths for RT-PCR products.

Gene	Primer	Length (bp)	Tm
COL1A1	5-AAAGACGGGAGGGCGAGTG-3 5-GCCATAGGACATCTGGGAAGCAA-3	242	62
COL1A2	5-CTCCAAGGAAATGGCAACTCA-3 5-AGGAACGGCAGGCGAGAT-3	288	58
COL3A1	5-CCCACAGCCTTCTTCTACACCT-3 5-ACCCATTCCTCCCACTCC-3	241	60
FN	5-GCCGAATGTAGATGAGGAG-3 5-GTCGAGTCGCACTGGTAGA-3	249	54
GAPDH	5-GTCGTGGAGTCTACTGGCGTCTT-3 5-CAGTCTTCTGAGTGGCAGTGATGG-3	280	58

RT-PCR, reverse transcription-polymerase chain reaction; bp, base pair; COL1A1, collagen I $\alpha$ 1; COL1A2, collagen I $\alpha$ 2; COL3A1, collagen III $\alpha$ 1; FN, fibronectin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

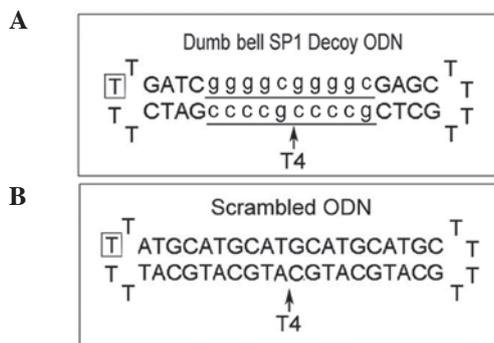


Figure 1. (A) Dumbbell Sp1 decoy oligonucleotide (ODN) and (B) scrambled ODN. The transcription factor Sp1 binding site is underlined. The arrow indicates the gap connected by T4 DNA ligase after annealing and the box indicates the site labeled by carboxyfluorescein (FAM).

transcriptase Moloney Murine Leukemia Virus (reverse transcriptase M-MLV) and 25 units ribonuclease inhibitor (Takara Bio, Inc., Shiga, Japan) and subjected to PCR amplification with the primers listed in Table II. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified as the internal control. RT products (0.5-1  $\mu$ g) were amplified with 1 unit *Taq* DNA polymerase (Takara Bio, Inc.) and 1 mM of each primer in 50  $\mu$ l of reaction mix containing 50 mM KCl, 10 mM Tris-HCl, 1.5 mM MgCl<sub>2</sub> and 0.02 mM each of dNTPs as follows: initial denaturation for 3 min at 94°C, 30 cycles of amplification, 1 min of denaturation at 94°C, different annealing temperatures for each pair of primers, 1 min of extension at 72°C and a final elongation for 5 min at 72°C. Parallel RT-PCR assays without reverse transcriptase were performed for each sample to confirm that the PCR products resulted from cDNA rather than from genomic DNA. The PCR products (10  $\mu$ l) were analyzed by 2% agarose gel electrophoresis. Relative mRNA abundance was calculated by densitometric analysis using computer software (Kodak Digital Science 1D Image Analysis software; Eastman Kodak Co., Rochester, NY, USA).

**Statistical analysis.** Statistical Package for Social Sciences (SPSS, Inc., Chicago, IL, USA), version 13.0, was used for

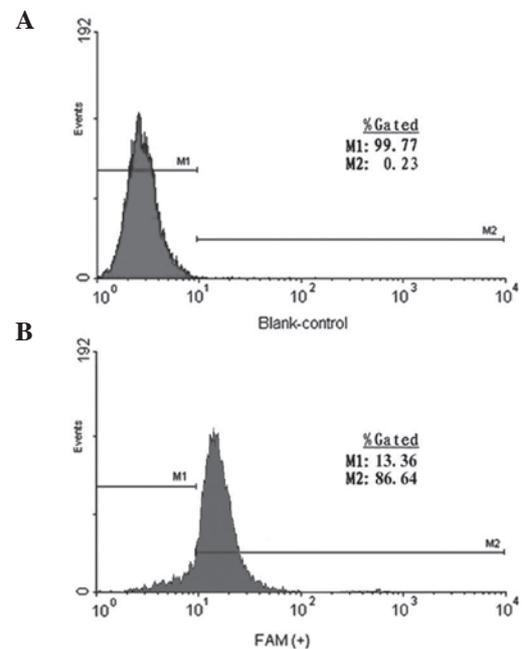


Figure 2. Transfection efficiency of the cells transfected with 100 nM carboxyfluorescein (FAM)-labeled oligonucleotides (ODNs). (A) Blank control. (B) FAM-labeled ODN in HSFs. The transfection efficiency was >99% at 4 h after ODN transfection. HSFs, hypertrophic scar fibroblasts.

statistical analyses and the Wilcoxon signed rank test was used to determine if there were differences between the groups.  $P < 0.05$  was considered to indicate a statistically significant result.

## Results

**Design and identification of Sp1 decoy ODNs.** Dumbbell Sp1 decoy ODNs and scrambled Sp1 ODNs were designed and the structure and sequence of the ODNs are shown in Fig. 1. Cells transfected with 100 nM FAM-labeled ODNs showed positive results by flow cytometry, with the positive efficiency being >99%. No fluorescence was detected in cells transfected with the blank control (Fig. 2).

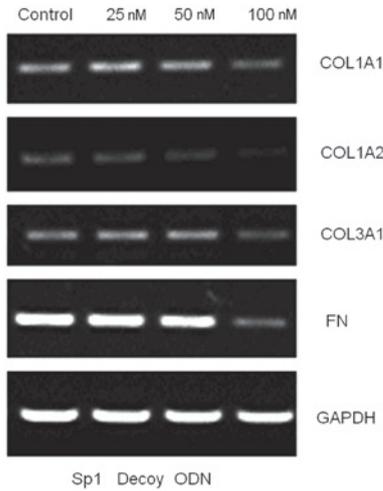


Figure 3. Extracellular matrix mRNA expression was inhibited by the Sp1 decoy oligonucleotide (ODN). COL1A1, collagen I $\alpha$ 1; COL1A2, collagen I $\alpha$ 2; COL3A1, collagen III  $\alpha$ 1; FN, fibronectin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

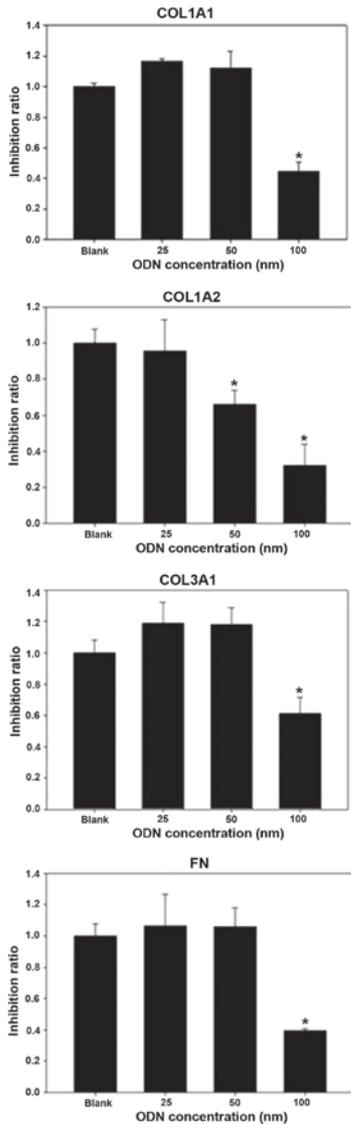


Figure 4. Effects of 25, 50 and 100 nM Sp1 decoy oligonucleotides (ODNs) on COL1A1, COL1A2, COL3A1 and fibronectin (FN) expression. The sample size was n=3 per group for each concentration; \*P<0.05 vs. the blank control.

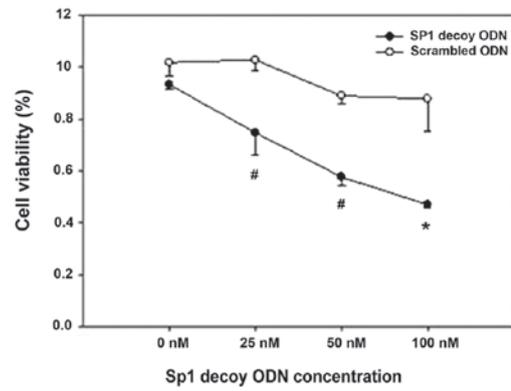


Figure 5. Cell viability was reduced in hypertrophic scar fibroblasts (HSFs) exposed to increasing concentrations of the Sp1 decoy oligonucleotide (ODN). There were no significant differences with the scrambled ODN control. The sample size was n=5 per group for each concentration. \*P<0.05 vs. 0 nM and #P<0.05 vs. the respective scrambled ODN.

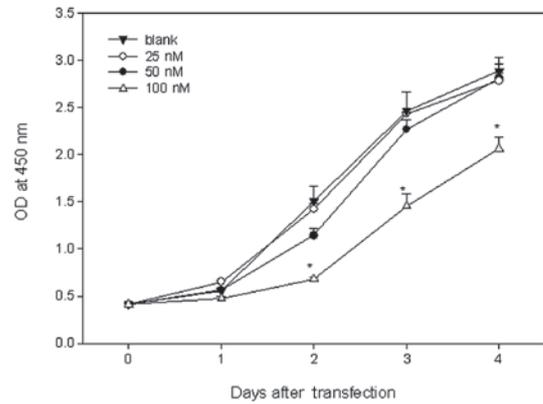


Figure 6. Cell proliferation was reduced in hypertrophic scar fibroblasts (HSFs) transfected with 100 nM of the Sp1 decoy oligonucleotide (ODN). The sample size was n=8 per group for each concentration. \*P<0.05 compared with the blank control.

*Inhibition of collagen type I and III expression by Sp1 decoy ODNs.* At 48 h after transfection with Sp1 decoy ODNs, COL1A1 and COL3A1 mRNA levels were significantly reduced (both P<0.01; Fig. 3). However, the control ODNs had no inhibitory effect on the expression of collagen type I or III (Fig. 4; P>0.05).

*Suppression of cell proliferation by Sp1 decoy ODNs.* Cultured HSFs were transfected with FAM-labeled ODNs targeting Sp1 transcriptional factor, with the negative control containing a random sequence. Transfection with 100 nM Sp1 decoy ODN resulted in minimal cell viability (Fig. 5). There were significant differences between cells transfected with the Sp1 decoy ODN and scrambled ODN at the 25, 50 and 100 nM doses. No significant difference was observed between cells transfected with the Sp1 decoy ODN and scrambled Sp1 ODN effect at 0 nM (P=0.21). HSFs treated with 100 nM Sp1 ODN had minimal growth potential. There were significant differences between cells treated with 100 nM Sp1 ODN and the other concentrations at 2 days after transfection. There were no significant differences between the cells treated with the 25 and 50 nM dose (P=0.23; Fig. 6).

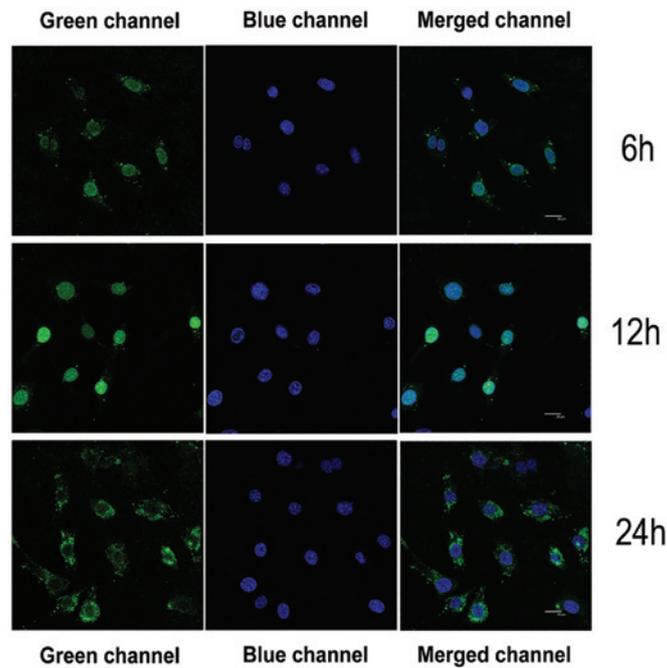


Figure 7. Hypertrophic scar fibroblasts (HSFs) were transfected with the 100 nM Sp1 decoy oligonucleotide (ODN) and imaged at 6, 12 and 24 h after transfection. At 12 h post-transfection, the yellow green fluorescence appeared in the cell nuclei of merged channel (yellow green fluorescence is a result of green and blue screen overlay), while at 24 h post-transfection the green fluorescence was observed in the cytoplasm of the infected cells.

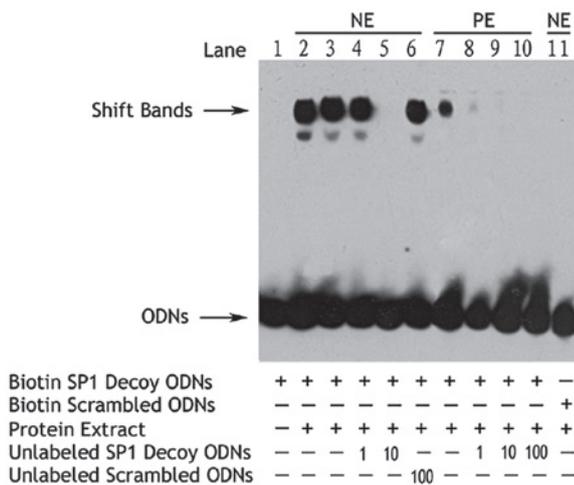


Figure 8. *In vitro*, nuclear extracts (NE; lanes 2-6 and 11) and plasma extracts (PE; lanes 7-10) were added to the biotin-labeled Sp1 decoy oligonucleotide (ODN) probe and co-cultured at room temperature before electrophoresis. Bands that were shifted are shown in lanes 2 and 3. A 10-fold increase in unlabeled Sp1 decoy ODN completely inhibited the binding of nuclear proteins and biotin-labeled Sp1 decoy ODN (lane 5). A 100-fold increase in unlabeled scrambled ODN showed no inhibition effect (lane 6). Biotin-labeled scrambled ODN could not bind with the nuclear protein extracts (lane 11).

**Location of Sp1 decoy ODNs in cells after transfection.** Yellow-green fluorescence appeared in the cell nucleus after HSFs were transfected with the 100 nM Sp1 decoy ODN for 6-12 h. By 24 h post-transfection, the yellow-green fluorescence was observed in the cytoplasm of the infected cells (Fig. 7).

The EMSA assay revealed that a 10-fold increase in the unlabeled Sp1 decoy ODN inhibited binding of the nuclear

protein and biotin-labeled Sp1 decoy ODN (Fig. 8). A 100-fold increase in the unlabeled scrambled Sp1 ODN showed no inhibitory effect, while the biotin-labeled scrambled Sp1 ODN was unable to bind nuclear protein.

## Discussion

Enhanced HSF proliferation and increased collagen production are principal contributors to hypertrophic scar formation (7). This process is governed by signal transduction cascades that are finely regulated by growth factors. The transcription factor Sp1 is ubiquitously expressed and plays a significant role in signal transduction (8). The most significant findings of the current study were that the Sp1 decoy ODN inhibited cell proliferation and collagen expression. These results demonstrate that Sp1 decoy ODNs may provide a promising therapeutic option for the treatment of hypertrophic scars.

Complementary approaches have also demonstrated that Sp1 targeting may be a potentially powerful therapeutic approach for reducing ECM accumulation in hypertrophic scars (9). Recently, a number of new technologies have been developed to inhibit target gene expression in a sequence-specific manner and these technologies have been investigated as treatment modalities for a variety of diseases (10,11). While there are numerous studies on strategies targeting mRNA via antisense ODNs, ribozymes and RNA interference, targeting proteins that regulate expression of a specific gene is a relatively novel approach (12). Decoy technology has recently been developed in an attempt to reduce the activity of a specific transcription factor through the use of cis-element ds ODNs containing the consensus binding sequence of the transcription factor (13).

The greatest restriction to using ODNs is that they are easily degraded by nucleases or by readily nonspecific reac-

tions with the control strand. To circumvent these problems, various modified DNA analogs, particularly ODNs with PT-modified ODNs, are often employed. These modified DNA analogs replace the oxygen atoms in the phosphodiester bond of the terminal nucleotides with sulfur atoms. This structural modification increases resistance to nuclease attack (14). In this study, we used dumbbell Sp1 decoy ODNs to enhance the stability of the decoy. Using this method, Sp1 regulated the expression of collagen. Similar approaches have been used to deliver gene-regulating molecules, such as synthetic ds ODNs that mimic cis-acting promoter elements (15,16).

In the present study, Sp1 decoy ODNs inhibited HSF proliferation and downregulated collagen synthesis. Sp1 decoy ODNs at concentrations above 10 nM significantly inhibited HSF proliferation. In addition, the Sp1 decoy ODN suppressed TGF- $\beta$ 1 and fibronectin mRNA levels, as well as cell proliferation induced by PDGF (12). EMSA evaluation showed that the DNA-protein complex induced by PDGF induction was reduced significantly in HSFs transfected with Sp1 decoy ODNs, whereas the scramble decoy ODNs had no effect on DNA binding activity. Using reporter plasmids containing the Sp1 binding motif in the TGF- $\beta$ 1 and fibronectin promoters, the enhancement in Sp1-dependent transcription activity in response to serum stimulation was reduced significantly by the Sp1 decoy ODN (17).

We also demonstrated that the *in vitro* addition of the Sp1 decoy ODN effectively attenuated ECM mRNA induction and the resulting ECM deposition (18). Therefore, these results suggest that the Sp1 decoy ODN represents a potentially effective gene therapy strategy to ameliorate dermal ECM accumulation during hypertrophic scar formation (19). In addition, Sp1 decoy ODNs markedly suppressed collagen IV mRNA levels and fibronectin mRNA and protein in the hypertrophic scar (20).

One limitation of this study is that Sp1 regulates numerous genes, many of which are not involved in the etiology of hypertrophic scars. The present study did not analyze any of these genes. While this limitation precludes the decoy ODN approach from being useful as a systemic therapeutic strategy, this approach could be viable as a topical ointment applied directly to the skin. Whether decoy ODNs would be useful for other diseases that involve Sp1 upregulation remains to be investigated.

In conclusion, our data demonstrate that Sp1 is a key transcription factor mediating HSF proliferation and ECM gene expression that may prevent the pathogenesis of the hypertrophic scar. Although additional *in vitro* and *in vivo* studies need to be conducted to fully understand the potential of decoy ODNs in preventing excessive collagen contraction in patients with hypertrophic scars, this study provides a platform for these experiments.

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