

Anti-fibrotic Effects via Regulation of Transcription Factor Sp1 on Hepatic Stellate Cells

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Key Words

Transcription factor Sp1 • Decoy oligodeoxynucleotide • Hepatic stellate cells • Hepatic fibrosis • Proliferation

Abstract

Background: Hepatic stellate cells (HSCs), the central cells in hepatic fibrosis, are characterized by sustaining activation, a process that consists in increased proliferation and over-expression of fibrotic genes. Transcription factor Sp1 mediates the expression of a variety of fibrotic genes expression and thereby play an important role in fibrosis. In addition, previous reports have indicated that Sp1 binding activity is greatly increased in activated HSCs. Thus, our aim was to investigate the anti-proliferative and anti-fibrotic effects of the oligonucleotide decoy of the transcription factor Sp1, ODN, a potent inhibitor of Sp1-activated transcription. **Methods:** We optimized Lipofectamin 2000 (LF2000):ODN DNA ratio for the transfection of hepatic stellate cells HSC-T6. Then we measure the effect of transfected ODN on HSC-T6 cells' proliferation and fibrotic gene expression, and study the mechanism involved. **Results:** At a DNA concentration of 1 μ M and a ratio ODN DNA:LF2000 of 1:3, HSC-T6 cells have the maximal transfection

efficiency with the lowest toxicity. Transfected ODN effectively blocks Sp1 binding to the promoter regions of cell cycle regulatory proteins cyclin D1, p27^{KIP1} and fibrotic genes, including transforming growth factor (TGF)- β 1, Platelet-derived growth factor (PDGF)-BB, α -SMA, α 1 (I) collagen and tissue inhibitor of metalloproteinases-1 (TIMP-1). ODN inhibits HSC-T6 proliferation and fibrotic genes expression *in vitro*. **Conclusion:** Sp1 is a key transcription factor that mediates proliferation and fibrotic gene synthesis of HSC-T6, inhibition of Sp1 with decoy ODN may be an effective approach to prevent the progression of hepatic fibrosis.

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Introduction

Hepatic fibrosis, a major medical problem worldwide, is characterized by the accumulation of excessive collagen and other extracellular matrix components, resulting in the destruction of the liver parenchyma architecture and

loss of function. The hepatic stellate cell (HSC) is the key cellular element in hepatic fibrosis. Upon liver injury, HSCs become active, undergoing phenotypic change from quiescent cells to myofibroblast-like cells. Activated HSCs are characterized by enhanced cellular proliferation, overproduction of extracellular matrix components and a variety of growth factors and cytokines, appearance of the characteristic activation marker smooth muscle α -actin (α -SMA), thereby playing a central role in the development and maintenance of hepatic fibrosis [1, 2]. Because of its importance in the fibrotic process, there is considerable interest in establishing the molecular events that regulate HSCs function in fibrosis [3]. HSCs function at the level of gene transcription requires several key transcription factors include NF- κ B, AP-1, PPAR- γ and Kruppel-like factors (KLFs).

Sp1 is a family member of Kruppel-like factors which is largely associated with GC-rich promoters [4]. In addition to its participation in regulating a great variety of housekeeping genes, there is growing evidence that Sp1 plays an important regulatory role in other diverse cellular functions including cell proliferation and fibrosis [5-8]. Sp1 regulates the expression of several genes relevant to fibrosis process, including TGF- β 1, VEGF, COL1A2, and downstream targets of TGF- β 1, such as PAI-1, fibronectin and MMPs [9-13]. In addition, previous work by other groups have shown that Sp1 DNA-binding activity is increased in various form of fibrosis include activated HSCs [14, 15].

Recently, some novel anti-fibrotic approaches have been developed in an attempt to reduce the activity of specific fibrotic genes. Introduction of the oligonucleotide decoys with high affinity for their target transcription factors into specific cells leads to the selective down-regulation of the specific gene expression at DNA level [16, 17]. In these decoy ODNs, Sp1 decoy ODN is found to suppress the expression of VEGF, TGF- β 1 and fibronectin in kidney fibroblasts and *in vivo* [18]. In another report, Park et al [19] have reported that Sp1 decoy inhibits the expression of TGF- β 1 and its downstream target genes *in vivo* model of liver fibrosis, but its action in hepatic stellate cells remains to be elucidated.

In the present study, we investigated the role of Sp1 in HSC-T6 cells (an SV40-immortalized rat HSC line) using decoy ODN containing the Sp1-binding site to specifically inhibit the expression of Sp1. This investigation was performed to 1) optimize the Sp1 decoy ODN transfection conditions in HSC-T6; 2) test the hypothesis that transfection of Sp1 decoy ODN prevents HSC-T6

proliferation and fibrotic genes expression *in vitro*; 3) disclose its possible mechanism underlying proliferation of HSC-T6 cells.

Materials and Methods

Cell culture

HSC-T6, an immortalized rat hepatic stellate cell line, exhibits an activated HSC phenotype [20]. The cells, purchased from Cancer Institute and Hospital, Chinese Academy of Medical Sciences (Beijing, China), were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, NY, USA) supplemented with 100U/mL penicillin, 100 μ g/mL streptomycin, and 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Hyclone, Thermo Scientific, Waltham, Mass), in a humidified atmosphere containing 5% (v/v) CO₂ at 37°C.

Preparation of decoy ODN

The sequences of decoy ODN including phosphorothioated double-stranded ODN (PS-Sp1 decoy ODN) and mutated Sp1 decoy ODN (M-Sp1 decoy ODN) were as follows: PS-Sp1 decoy (consensus sequences are underlined), 5'-A_sTT ACG GGG CGG GGC GAA T_sC_s-3'; M-Sp1 decoy ODN, 5'-GTA CCG ATC TTT TGA TCG GTA CCG TAC GAG CTT TTG CTC GTA CC-3'; The ODNs were annealed for 2 h while the temperature was decreased from 80°C to 25°C.

Optimization of transfection condition

HSC-T6 cells were trypsinized and used to seed 24-well gelatin-coated plates (10⁵ cells/well), 500 μ l of growth medium without antibiotics was added so that cells will be 70%-80% confluent at the time of transfection. Lipofectamine 2000 (LF2000; Invitrogen, San Diego, CA, USA) was used for all transfections. DNA (0.4-8 μ g) was diluted in Opti-MEM (Invitrogen) to a final volume of 50 μ l. LF2000 (1.2-24 μ l) was diluted in Opti-MEM to a final volume of 50 μ l. The two solutions were incubated at room temperature for 20min and then added to each well containing cells and 500ul of serum-free medium. The final concentration of decoy ODN was from 0.1 μ M to 2 μ M. A DNA (μ g): LF2000 (μ l) ratio of 1:3 was routinely used according to the optimization result, unless otherwise specified.

Microscopy and fluorescence detection

After transfection, HSC-T6 cells were washed with PBS, observed using a Bx51 fluorescence microscope (Olympus, Japan) and photographed with Olympus DP-70 digital camera (Olympus, Japan). The FITC was excited by an argon laser (488 nm) and the fluorescence was detected through a bandpass filter (510-550 nm).

Flow cytometry

After a 6h transfection period, HSC-T6 cells were washed, trypsinized, dispersed and then transferred into 500 μ l PBS. Untransfected cells were included in every experiment and typically exhibited minimal autofluorescence. In this report, cellular uptake efficiencies refer to the percentage of cells that

captured the fluorescent ODN. The mean fluorescence intensity refers to the average of fluorescence intensity expressed by the population of cells that captured ODN.

For detection of cell cycle, HSC-T6 cells cultured 48h after transfection were washed twice with PBS and fixed in cold ethanol for 30 min, and then incubated with propidium iodide (PI) for 30 min. Thereafter, cells ($1-2 \times 10^5$) were analyzed for each sample by flow cytometry (BD, USA). Cell death was measured using the Annexin-PE Apoptosis Detection Kit I (BD Bioscience).

Assay of cell viability and lactate dehydrogenase activity

Cell viability was determined by trypan blue exclusion. HSC-T6 cells were seeded in 96-well plates at 1×10^4 cells/well. After transfection, HSC-T6 cells were washed and harvested in PBS to prepare a single-cell suspension. 50 μ l of the suspension was added to 0.95 ml 0.4% trypan blue solution. The total number of living and dead cells was counted with a haemocytometer. The activity of lactate dehydrogenase (LDH) in cultured supernatant was assayed by a LDH assay kit (Jiancheng Biology Engineering Institute, Nanjing, China) according to the manufacturer's instruction.

Electrophoretic mobility shift assay (EMSA)

EMSA was performed with the Gel Shift assay system (Promega, Madison, WI). In a typical experiment, DNA probe containing an Sp1 binding site (5'-ATT CGA TCG GGG CGG GGC GCG-3') (Promega) end-radiolabeled with [γ - 32 P]ATP (3000Ci/mmol; Perkin-Elmer Life Technology, Waltham, MA) and T4 polynucleotide kinase (Promega) were incubated with nuclear extract, 100 μ g/ml poly dI-dC, 10mM Tris/HCl (pH 7.5), 50mM NaCl, 0.5mM EDTA, 0.5mM DTT, 1mM MgCl₂, and 4% glycerol. After the incubation, samples were charged on 4% native polyacrylamide gels with a 0.5 \times TBE running buffer (45mM Tris, 45mM boric acid, and 1mM EDTA) and visualized by autoradiography. In some experiments, a 100-fold molar excess of unlabeled competitor was added to the reaction mixture before adding the nuclear extracts.

Cell proliferation

HSC-T6 cells were seeded on 96-well plates at an initial density of 1×10^4 cells/well. At 40-60% confluence, cells were rendered quiescent by incubation for 24 h in growth medium deficient in serum. To evaluate the effect of the Sp1 decoy ODN on HSC-T6 cell proliferation, PS-Sp1 decoy ODN, M-Sp1 decoy ODN and LF2000 alone were added to the wells, cells cultured with serum-free medium were used as control. HSC-T6 Cells were then incubated at 37°C for a further 6 h and replace fresh growth medium after transfection. Cell proliferation was determined with a WST cell counting kit (Wako, Osaka, Japan) and cell counting.

Quantitative real-time PCR assay

Quantitative PCR was carried out using SYBR[®] Premix Ex Taq[™] (Takara) in the LightCycler 480 (Roche Applied Science, Indianapolis, IN). The real-time PCR primers of each gene are as follows: p27^{kip1} Sense: 5'-CGG CTG GGT TAG CGG AG-3',

antisense: 5'-GAT TCT TCT TCG CAA AAC AAA AGG-3'; Cyclin D1 sense: TGG CCT CTA AGA TGA AGG AGA, antisense: AGG AAG TGT TCG ATG AAA TCG T; TGF- β 1 sense: 5'-GGA CTA CTA CGC CAA AGA AG-3', antisense: 5'-TCA AAA GAC AGC CAC TCA GG-3'; PDGF-BB sense: 5'-GAA GCC AGT CTT CAA GAA GGC CAC-3', antisense: 5'-AAC GGT CAC CCG AGT TTG AGG TGT-3'; α -SMA sense: 5'-GAT CAC CAT CGG GAA TGA ACG, antisense: 5'-CTT AGA AGC ATT TGC GGT GGA C-3'; α 1(I)collagen sense: 5'-ACC TCA AGA TGT GCC ACT CTG AC-3', antisense: 5'-AAT CGA CTG TTG CCT TCG CC-3'; TIMP-1 sense: 5'-CTG GCA TCC TCT TGT TGC TA-3', antisense: 5'-AGG GAT CTC CAG GTG CAC AA-3'; β -actin sense: 5'-TTC AAC ACC CCA GCC ATG T-3', antisense: 5'-GCA TAC AGG GAC AAC ACA GCC-3'. The PCR cycles were 95°C for 30 seconds, followed by 40 cycles of 95°C for 10 seconds and 60°C for 30 seconds. Each reaction was performed in triplicate and analyzed individually, relative to β -actin (a normalization control), calculated using the 2^{- $\Delta\Delta$ CT} method. Thereafter, data for transcript expression levels were expressed as fold difference relative to that of negative control cells.

Western blot and antibodies

HSC-T6 cells from each group were centrifuged into pellets and extracted in ice-cold cell lysis buffer (Cell Signaling Technology, Inc., Danvers, Mass). After centrifugation for 10 min at 4°C, the supernatants were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were incubated for 1h at room temperature with blocking solution (5% nonfat milk; Sigma-Aldrich Corporation, St. Louis, Mo), followed by incubation overnight at 4°C with primary antibodies. Next, the membranes were washed with 1 \times tris-buffered saline with Tween-20 solution and incubated with a horseradish peroxidase conjugated secondary antibody. Antibody-antigen complexes on the membranes were detected using an ECL system (Amersham Life Sciences, Buckinghamshire, UK), and the relative intensities of the protein bands were quantitated with Quantity One software (Bio-Rad).

Statistical analysis

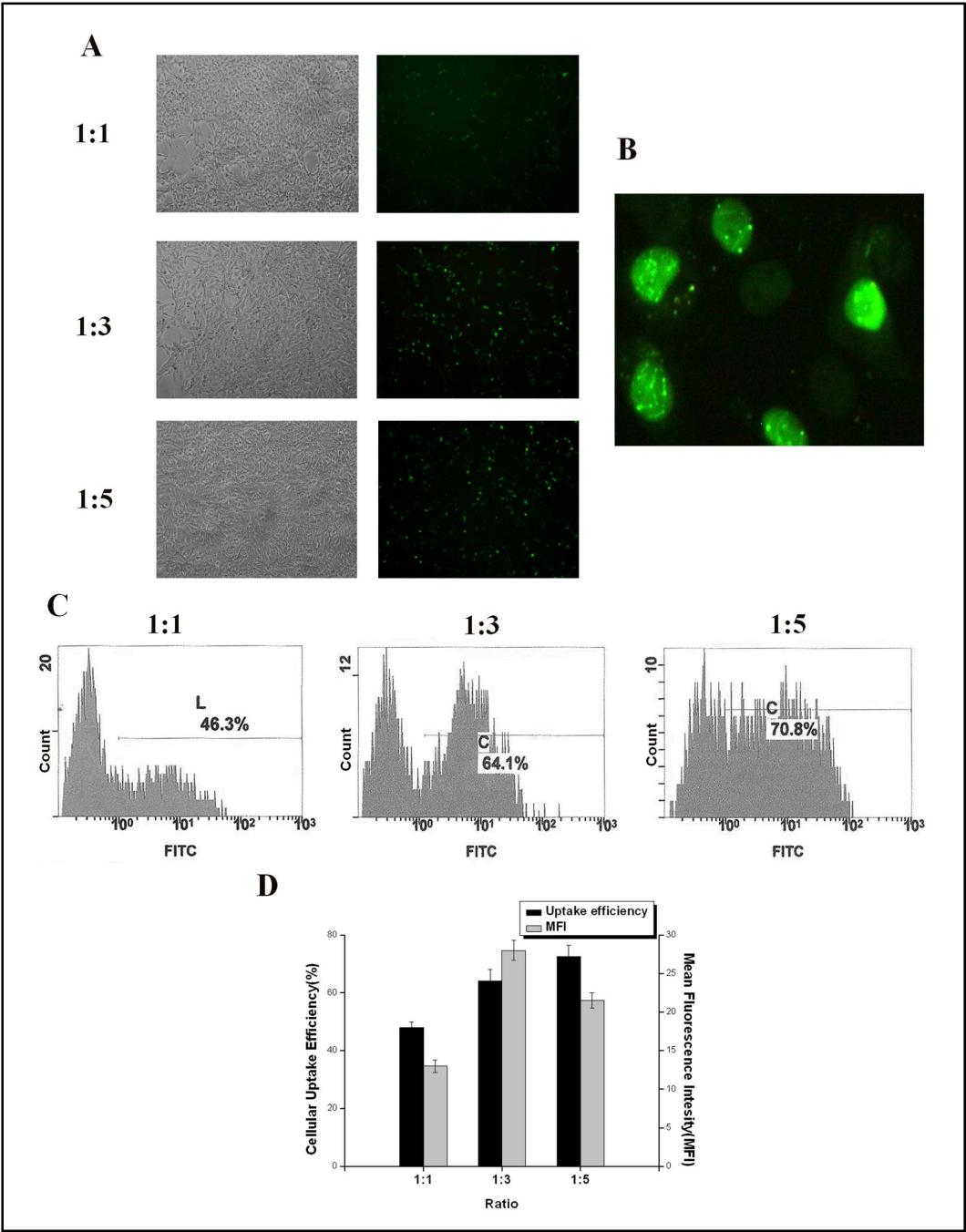
Data are presented as means \pm SD of at least three separate experiments. Difference between two groups was analyzed by two-tailed Student's t-test, and difference among three or more groups was analyzed by one-way ANOVA multiple comparisons. Statistical values of $p \leq 0.05$ were considered to be significant.

Results

Optimization of transfection condition

Lipoplexes condensation is the first limiting step in lipofection and determines the proportion of DNA that becomes available for uptake. In order to obtain the best delivery efficiency, we tested several LF2000:ODN ratios. Lipoplexes were formed at 1:1, 1:3, and 1:5 DNA: LF2000 ratios, using 0.5 μ M of FITC-labeled decoy ODN. After

Fig. 1. Optimization of DNA: LF2000 ratio. HSC-T6 cells were transfected with 0.5 μ M Sp1 decoy ODN using three different DNA: LF2000 ratios (1:1, 1:3 and 1:5). (A) Representative images of different ratios by fluorescence microscope (100 \times). (B) FITC-labeled-Sp1 decoy ODN was observed in both the nuclei and the cytoplasm (400 \times). (C) The percentage of FITC/DNA-positive cells and the mean fluorescence intensity (uptake per cell) were determined by flow cytometry. Statistical column diagram from flow cytometry plots was shown in (D). Mean values and standard error were calculated from three independent transfections.



transfection of HSC-T6 cells, increasing LF2000 concentrations lead to a greater proportion of cells taking up DNA (Fig. 1A, C and D), suggesting the production of greater numbers of lipoplexes. However, at the highest LF2000 concentration, DNA uptake per cell decreases. Taken together, the delivery of Sp1 decoy ODN was optimal at a DNA: LF2000 ratio of 1:3.

DNA concentration is another key step in lipofection. Insufficient concentrations of DNA will result in poor transfection efficiencies, while an excess of lipoplexes will compromise cell viability. To determine the optimal

DNA concentration, HSC-T6 was transfected with increasing concentrations of FITC-labeled Sp1 decoy ODN using an optimal DNA: LF2000 ratio (1:3). As illustrated in Fig. 2A-C, DNA uptake per cell was proportional to lipoplex concentration at all concentrations tested. At DNA concentrations below 1 μ M, transfection efficiency increased proportionally to DNA concentration. However, exposing HSC-T6 to concentrations above 1 μ M resulted in no further increase in cellular uptake efficiency. Moreover, concentration above 1 μ M of ODN was shown toxic to HSC-T6 in culture (Fig. 2D). These

Fig. 2. Optimization of Sp1 decoy ODN concentration. HSC-T6 cells were exposed to 0.5-2.0 μ M Sp1 decoy ODN using a constant DNA: LF2000 ratio of 1:3. Representative images of different concentrations were shown in (A) by fluorescence microscope (100 \times). (B) The percentage of FITC/DNA-positive cells and the mean fluorescence intensity were determined by flow cytometry. (C) Statistical column diagram from flow cytometry plots. (D) Cell viability and LDH liberation to the media by the cells. Mean values and standard error were calculated from three independent transfections. *: P<0.05 compared with 0 μ M Sp1 decoy group. Δ : P<0.01 compared with 0 μ M Sp1 decoy group.

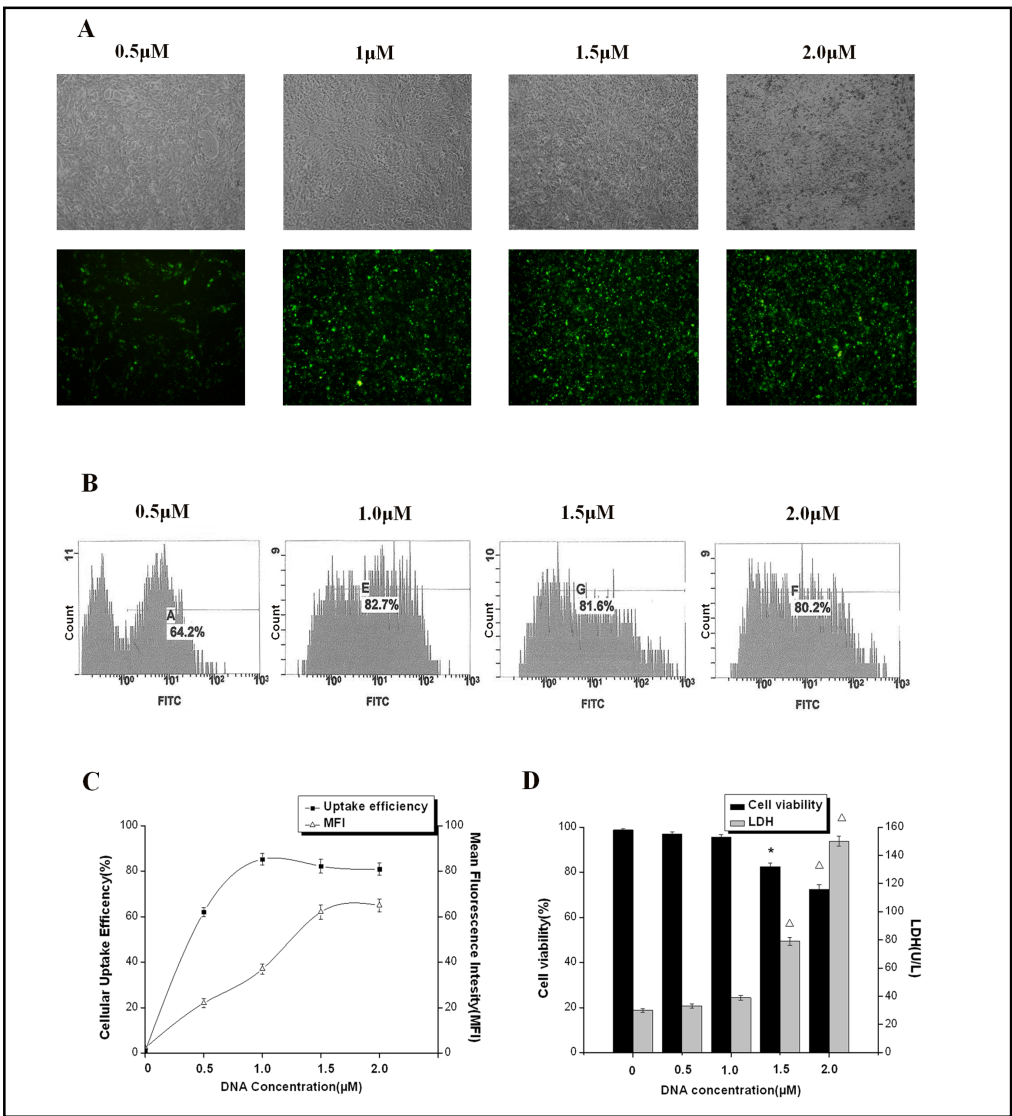
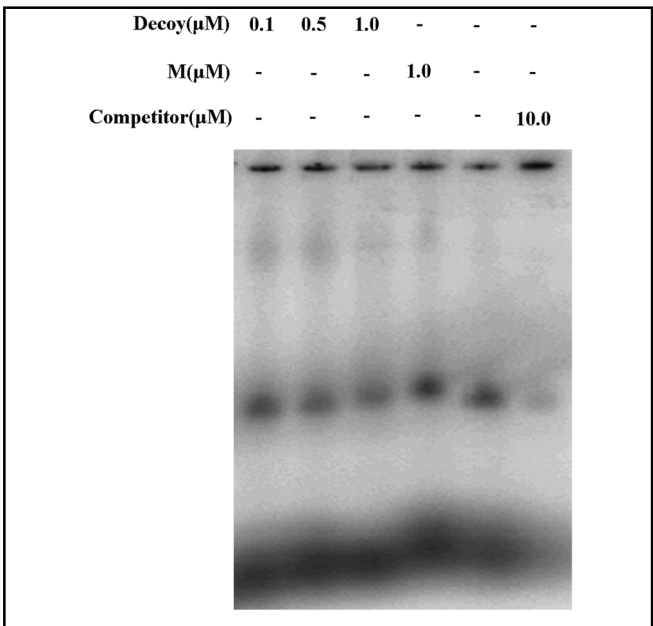


Fig. 3. Effects of Sp1 decoy ODN on the DNA binding activity of Sp1 in HSC-T6. A typical EMSA result is shown. HSC-T6 cells were treated with (in μ M) 0.1, 0.5 and 1.0 of Sp1 decoy ODN, 1.0 of mutant decoy ODN or 10.0 of unlabeled Sp1 probe. The DNA-binding activities of Sp1 were then estimated by EMSA. This experiment was repeated 3 times.

results suggest that a DNA concentration of 1 μ M will be optimal for transfection of HSC-T6. As the transfection condition (1 μ M of DNA at a DNA: LF2000 ratio of 1:3) provided maximal DNA delivery efficiency and minus toxicity for HSC-T6, this transfection condition was used in further experiments.



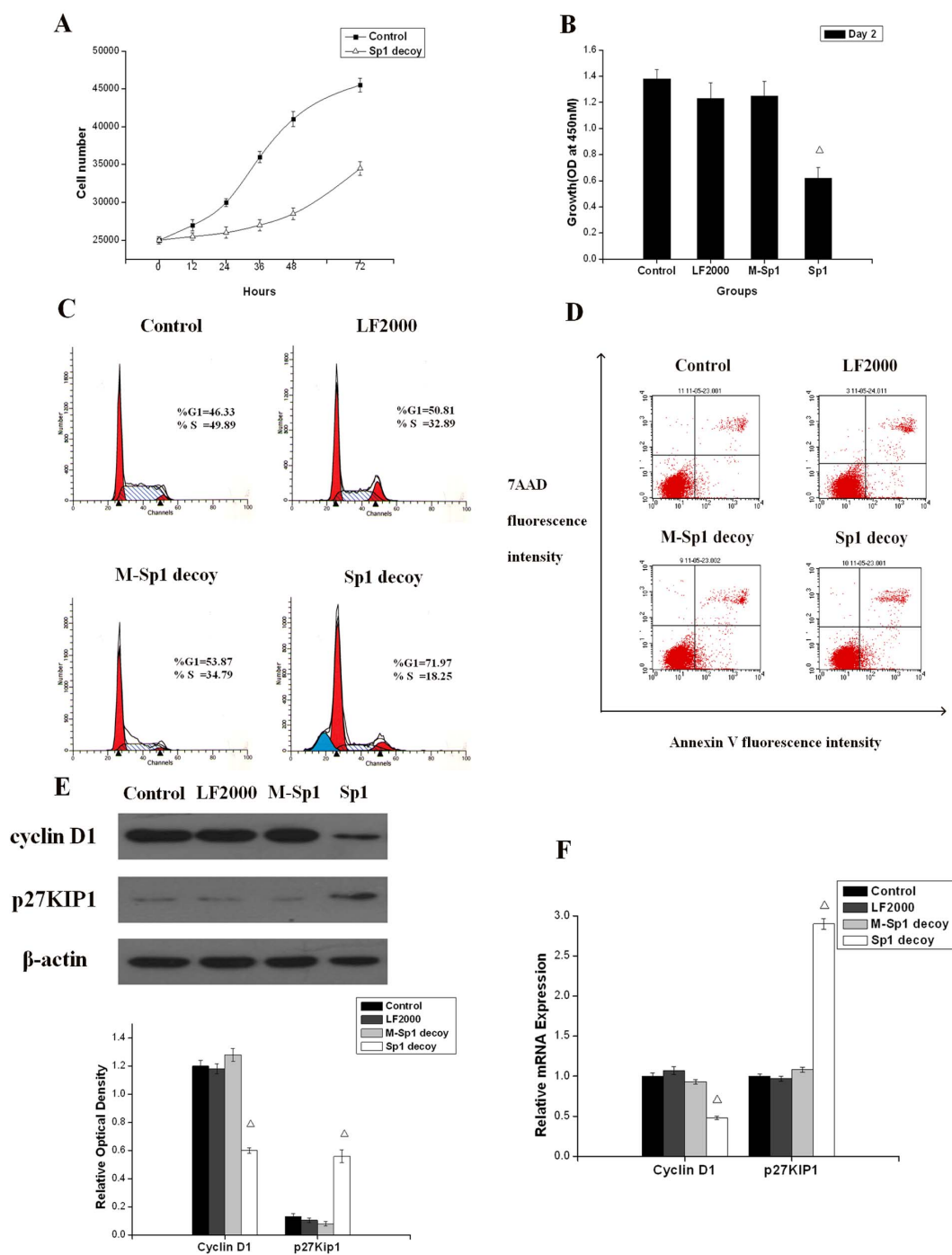
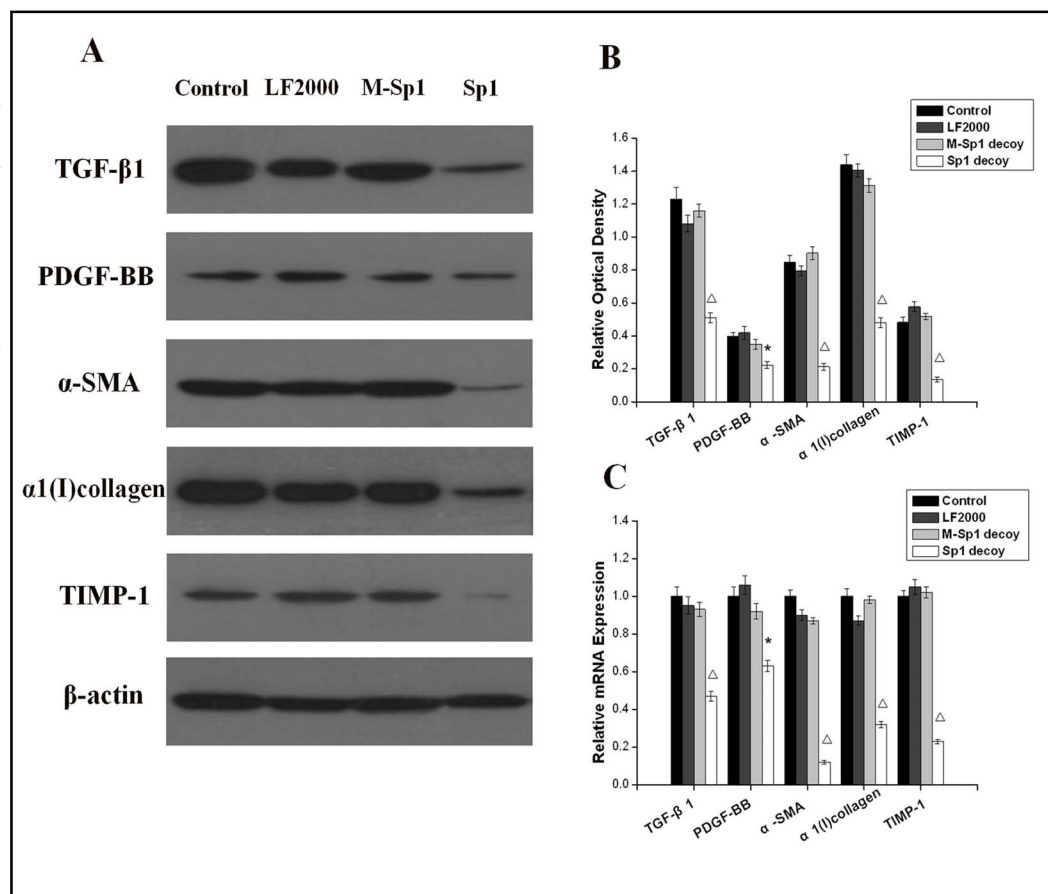


Fig. 4. The functional roles of Sp1 decoy ODN on HSC-T6 cell growth, cell cycle progression and apoptosis. HSC-T6 cells were transfected with Sp1-decoy ODN (1.0 μ M), mutated decoy ODN (1.0 μ M) or LF2000 alone, cells cultured with serum-free medium were used as control. (A) Effects of Sp1 decoy ODN on cell number at different time points. (B) Sp1 decoy ODN suppressed HSC-T6 proliferation. Proliferation was determined by a WST-1 test in day 2. (C) Sp1 decoy ODN blocked the cell cycle in G1 phase at 48h after transfection. (D) Apoptosis was measured by annexin V-PE labeling; the nucleic acid dye 7-amino-actinomycin D (7-AAD) was used to detect non-viable cells. The right lower quadrant (RLQ) contains early apoptotic cells. Upper right quadrant (RUQ) contains late apoptotic and necrotic cells. The proportions of cells in RLQ+RUQ were as follows: control: 4.21%; LF2000: 6.33%; M-Sp1: 9.47%; Sp1: 9.03%. (E) Effects of Sp1 decoy ODN on the protein expression of cyclin D1 and p27^{KIP1} in HSC-T6. (F) Effects of Sp1 decoy ODN on the mRNA expression of cyclin D1 and p27^{KIP1} in HSC-T6. Beta-actin was used for the loading control. Values represent means \pm SD of three independent experiments. *: $P < 0.05$ compared with control group. Δ : $P < 0.01$ compared with control group.

Fig. 5. Effects of Sp1 decoy ODN on the expression of fibrotic genes in HSC-T6. (A) Effects of Sp1 decoy ODN on the protein expression of fibrotic genes. Quantification of the target protein bands relative to β -actin is shown in (B). Effects of Sp1 decoy ODN on the mRNA expression of fibrotic genes were shown in (C). Beta-actin was used for the loading control. Values represent means \pm SD of three independent experiments. *: $P < 0.05$ compared with control group. Δ : $P < 0.01$ compared with control group.



Sp1 decoy ODN prevents Sp1 binding activity

To investigate the effect of Sp1 decoy ODN on Sp1 DNA binding activity, EMSA was performed with nuclear extracts of HSC-T6 that had been transfected with decoy ODN. As shown in Fig. 3, compared with control HSC-T6, transfection with Sp1 decoy ODN, but not with the M-Sp1 decoy ODN, significantly attenuated the DNA binding activity. The binding was specific, as evidenced by the complete suppression of the formation of the DNA-protein complexes in the presence of an excess of unlabeled competitor ODN.

Sp1 decoy ODN inhibits HSC-T6 proliferation and induces G1 cell cycle arrest

As shown in Fig. 4A, Sp1 decoy ODN inhibited HSC-T6 cell growth and this suppression lasted for 48h. The water-soluble tetrazolium salt (WST-1) assay showed that Sp1 decoy ODN, but not Sp1 M-ODN, inhibited HSC-T6 growth 48h after transfection (Fig. 4B).

To further explore the reason for the growth inhibition, we examined the effects of Sp1 decoy ODN on cell cycle progression. As illustrated in Fig. 4C,

pretreatment of HSC-T6 with Sp1 decoy ODN blocked the cell cycle in G1 phase. The G0/G1-phase fraction increased from 46.33% (untreated) to 71.97% at 48h post-transfection with 1 μ M of Sp1 decoy ODN. However, no significant change in cell cycle arrest was seen in both cells treated with Sp1 M-ODN and LF2000 alone. Meanwhile, to evaluate whether the inhibition of cell growth might also have occurred due to apoptotic cell death, we also performed apoptosis assay using flow cytometry. However, there was no significant increased apoptosis in transfected cells, when compared with the control cells (Fig. 4D). Therefore, our results indicate that Sp1 decoy ODN inhibits the proliferation of HSC-T6 provoking the arrest of cells at the G1 phase of the cell cycle.

In addition, the molecular mechanism for cell cycle arrest in HSC-T6 was also investigated. At 48 h post-transfection, Sp1 decoy ODN but not Sp1 M-ODN, induced down-regulation of cyclin D1 simultaneously with up-regulation of p27^{kip1} in HSC-T6 (Fig. 4E). Furthermore, we also performed quantitative real-time PCR assay to measure mRNA, and the results correlated with those of Western blot (Fig. 4F). Together,

these results indicate that Sp1 decoy ODN inhibits cellular proliferation which may be associated with transcription modification of cyclin D1 and p27^{Kip1} in HSC-T6.

Sp1 decoy ODN inhibits the expression of fibrosis genes in HSC-T6

The effects of Sp1 decoy ODN on the expression of fibrosis genes in HSC-T6 were investigated. Introduction of the Sp1 decoy ODN into HSC-T6 significantly inhibited both mRNA and protein expression of TGF- β 1, PDGF-BB, α -SMA, α 1 (I) collagen and TIMP-1 compared with the control group. However, such a significant suppressive effect was not observed in HSC-T6 treated both M-Sp1 decoy ODN and LF2000 alone. These results clearly show that R-Sp1 decoy effectively inhibits fibrotic changes in HSC-T6, indicating that the transcription of these genes are at least partly regulated by Sp1 binding site in their promoter.

Discussion

Activation of Sp1 is an important event during fibrosis where several reports have shown it regulates a great number of fibrotic gene expression in various form of fibrosis [9-13, 21]. Furthermore, the anti-fibrotic effects of Sp1 decoy ODN were observed *in vitro* and *in vivo* models of fibrosis, including renal [18], lung [22] and liver [19]. Nevertheless, no reports on the role of Sp1 decoy ODN in HSCs have been investigated. The major new finding of the present study is that an optimal transfection of Sp1 decoy ODN effectively inhibits the expression of the fibrotic genes and the proliferation of HSC-T6 and may play a role in the sustained activation of HSC-T6.

The use of decoy ODN for reducing the transactivity of transcription factor is an innovative and attractive strategy for gene therapy. However, the use of decoy ODN is limited by their generally low efficiency of transfection [23]. To circumvent this problem, we are therefore investigating the factors that limit the transfection of HSC-T6 with Sp1 decoy ODN, with a view to developing an efficient delivery method. During the condensation process, lower DNA: LF2000 ratios may increase the rate of decoy ODN encapsulation, generating greater numbers of lipoplexes with fewer ODNs being recruited to each lipoplex. Meanwhile, higher DNA: LF2000 ratios are likely to cause aggregation,

leading to the sequestering of DNA molecules in large, untransfectable particles. In our study, an optimal DNA: LF2000 ratio of 1:3 in HSC-T6 supports this interpretation. Concentration of ODN is another important factor influencing the transfection. We observed that increasing DNA concentration above 1 μ M resulted in no further increase in the proportion of cells taking up ODN, which may reflect a saturation of HSC-T6 at such concentrations. In support of this interpretation, concentration above 1 μ M of ODN was shown toxic to HSC-T6 in culture. Taken together, we find that optimal transfection condition of HSC-T6 is achieved using 1 μ M of DNA at a DNA: LF2000 ratio of 1:3.

Previous reports suggest a role for Sp1 in the cell cycle. It has been shown that Sp1 is increasingly phosphorylated in G1 phase of the cell cycle [7] and interacts with certain cell cycle regulatory proteins such as cyclin D1, EGFR, p27^{Kip1} and p21^{WAF1/cip1} [24-26]. Our results indicated that down-regulation of Sp1 was associated with a gradual decrease in proliferation of HSC-T6. Moreover, we did not detect significant apoptotic cell death after inhibition of Sp1 in HSC-T6, and the inhibition of cell growth can be partly attributed to the occurrence of G1 cell cycle arrest. These observations are consistent with up-regulation of p27^{Kip1} expression and down-regulation of cyclin D1, both of which are required for cell cycle progression in G1, suggesting that the Sp1 is involved in controlling the cell cycle in G1 phase, which is consistent with the research in epithelial cells by Grinstein et al. [27].

Among the transcription factors, Sp1 regulates the expression of a vast variety of genes, including TGF- β 1, fibrotic cytokine, and a wide variety of matrix gene [12-16, 23]. In the present study, our data showed that transfection with Sp1 decoy ODN can effectively attenuate the expression of TGF- β 1, PDGF-BB and TIMP-1 in HSC-T6. It has been reported that repeated injection of R-Sp1 decoy ODN though the mouse tail vein can effectively decrease the liver fibrosis induced by CCl4 *in vivo* [21]. Together with the present results, it may suggest that this strategy specifically suppresses Sp1-mediated transcription of fibrotic genes in liver, and thus can be used as an effective therapy for hepatic fibrosis. In addition to these results, α -SMA, the characteristic activation marker of HSCs, was decreased after the transfection of Sp1 decoy ODN. Moreover, the expression of α 1 (I) collagen, another classical markers for activated HSCs, was also down-regulated. This finding

is supposed to be an evidence of Sp1 may partly play a role in the sustained activation of HSC-T6.

In conclusion, our data clearly demonstrate that Sp1 is a key transcription factor mediated proliferation, fibrotic genes synthesis of HSC-T6. Transfection with Sp1 decoy ODN seems to be an effective strategy to limit the progression of fibrosis [28]. However, the

gene manipulation of Sp1 need to claim attention since there might be serious adverse effects relating to development, differentiation, and metabolism, especially in case targeting is insufficient [29-31]. To establish the Sp1-directed gene therapy for hepatic fibrosis, development of cell-specific gene modulation for HSCs will be essential.

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