**Down-Regulation of Overexpressed Sp1 Protein in Human Fibrosarcoma Cell Lines Inhibits Tumor Formation**

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Abstract

Sp1 is a transcription factor for many genes, including genes involved in tumorigenesis. We found that human fibroblast cells malignantly transformed in culture by a carcinogen or by stable transfection of an oncogene express Sp1 at 8-fold to 18-fold higher levels than their parental cells. These cell lines form fibrosarcomas in athymic mice with a very short latency, and the cells from the tumors express the same high levels of Sp1. Similar high levels of Sp1 were found in the patient-derived fibrosarcoma cell lines tested, and in the tumors formed in athymic mice by these cell lines. To investigate the role of overexpression of Sp1 in malignant transformation of human fibroblasts, we transfected an Sp1 1UnsRNA/Ribozyme into two human cell lines, malignantly transformed in culture by a carcinogen or overexpression of an oncogene, and into a patient-derived fibrosarcoma cell line. The level of expression of Sp1 in these transfected cell lines was reduced to near normal. The cells regained the spindle-shaped morphology and exhibited increased apoptosis and decreased expression of several genes linked to cancer, i.e., epithelial growth factor receptor, urokinase plasminogen activator, urokinase plasminogen activator receptor, and vascular endothelial growth factor. When injected into athymic mice, these cell lines with near normal levels of Sp1 failed to form tumors or did so only at a greatly reduced frequency and with a much longer latency. These data indicate that overexpression of Sp1 plays a causal role in malignant transformation of human fibroblasts and suggest that for cancers in which it is overexpressed, Sp1 constitutes a target for therapy. (Cancer Res 2005; 65(3): 1007-17)

Introduction

It is now commonly accepted that cancer results from multiple genetic changes. Many of the genes involved have been identified, but whether all of the primary genes involved in the malignant transformation of any type of human cancer have been identified remains unclear. To address such concerns, McCormick and Maher and their colleagues have utilized human fibroblasts in culture to study the process by which these cells become malignant. By means of sequential clonal selection, they identified many genetic changes required for malignant transformation of human fibroblasts. They began these studies with a finite life span human fibroblast cell line, designated LG1, derived from the foreskin of a normal neonate. LG1 cells were transfected with a vector carrying a v-Myc oncogene and a selectable marker. Several oncogene transfectant clones, vector controls, and an LG1 clone were expanded to the end of their life span. From a population derived from a clone of cells that expressed the v-Myc oncoprotein, a telomerase positive, infinite life span, chromosomally stable, diploid cell strain arose which was designated MSU-1.0. A more rapidly growing, chromosomally stable variant of MSU-1.0 cells arose spontaneously, and this strain was designated MSU-1.1 (1). Transfection of MSU-1.1 cells with an H-Ras (2), N-Ras (3), or v-K-Ras (4) oncogene, in vectors engineered for high expression of the oncogene, caused the 1.1 cells to become malignantly transformed, i.e., able to form sarcomas when injected s.c. into athymic mice. A single exposure of MSU-1.1 cells to cobalt-60 γ-radiation (5, 6) or a chemical carcinogen (7, 8) transformed them into cells able to form distinct foci on a monolayer of cells. When the cells from the foci are expanded and injected into athymic mice, they form sarcomas after a short latency. Collectively, the cell lines/strains from LG1 cells to the tumor-derived malignant cells are referred to as the MSU1 lineage. A strong advantage of this lineage is that it allows one to identify genetic or epigenetic changes that occur during the transformation process by comparing the malignant cell lines with the normal founder cell population, LG1, and the nontumorigenic intermediate cell lines derived from it, MSU-1.0 and MSU-1.1. Using this approach, we have been able to correlate many of the steps in the transformation process with specific genetic changes. Examples include loss of wild-type p53 (6, 8) and overexpression of MET and Sp1 (9). In 1983, Sp1 was identified as a general transcription factor (10). Sp1 was the first transcription factor to be purified, cloned, and characterized in mammalian cells (11). Ubiquitously expressed, it binds the GC-box (GGCGGG) and GT-box (CACCC) via its Cys-His zinc-finger DNA binding domain (12). Sp1 belongs to the Sp Krüppel-like family, consisting of 21 members that share high homology in their DNA-binding domains (13). These proteins are present in species ranging from Caenorhabditis elegans to humans (14–17). Recently, overexpression or higher binding activity of Sp1 was found in human pancreatic cancer cell lines and cancer tissue (18), breast cancer cell lines and cancer tissue (19), gastric carcinoma (20), and thyroid carcinoma (21). Several of these studies also showed that overexpression of Sp1 protein or up-regulation of Sp1 transacti

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overexpressed, and that four of the six also overexpressed Sp1, a transcription factor for met. In addition, three of the five patient-derived fibrosarcoma cell lines examined showed a high level of Sp1 compared with normal human fibroblasts, suggesting that Sp1 plays a role in the malignant transformation of human fibroblasts, not only in culture, but also in the human body.

Here we report that we designed and constructed an Sp1 U1snRNA/Ribozyme and stably transfected it into two human fibrosarcoma cell lines found to express high levels of Sp1. These cell lines had been derived from tumors formed in athymic mice by injection of MSU-1.1 cells that we had transformed by transfection of the H-Ras oncogene (2) or by γ-irradiation (6). We also transfected the Sp1 U1snRNA/Ribozyme into a patient-derived fibrosarcoma cell line (SHAC), which expresses a high level of Sp1 protein. From all three groups, we identified transfectants in which the expression of Sp1 had been reduced to the level found in nontransformed parental MSU-1.1 cells. We tested them for their ability to produce large-sized colonies in agarose. None of them could do so. When injected into athymic mice, these cell lines with near normal levels of Sp1 failed to form tumors or did so only at a greatly reduced frequency and with a much longer latency. We also found that the inhibition of the tumorigenicity of these cell lines correlates with decreased expression of specific proteins known to play a role in the malignant transformation of such cells, i.e., EGFR, uPA, uPAR, and VEGF.

Materials and Methods

Cells and Cell Culture. The derivation of human fibroblast cell line MSU-1.1 has been described (1). PH2MT cells were derived from a tumor formed in athymic mice by injection of MSU-1.1 cells malignantly transformed by an overexpressed H-Ras oncogene (2). γ2-3A/SB1 cells were similarly derived from a tumor formed by MSU-1.1 cells malignantly transformed by γ-irradiation (6). SHAC cells are derived from a patient's fibrosarcoma. The cells were routinely cultured in Eagle's MEM, supplemented with 10% fetal calf serum (Hyclone, Logan, UT), hydrocortisone (1 μmol) (modified Eagle's medium), and 10% supplemented calf serum (Hyclone, Logan, UT), hydrocortisone (1 μmol), t-serine (0.2 mmol), and pyruvate (1 mmol) (modified Eagle's medium), and 10% supplemented cell serum (Hyclone, Logan, UT), hydrocortisone (1 μmol), t-serine (0.2 mmol), and streptomycin (100 μg/mL) (culture medium), at 37°C in a humidified incubator with 5% CO2. For selection of transfected cell strains, blasticidin (10 μg/mL) was added to this culture medium. To be sure that the cells used in each experiment maintained the drug resistance and presumably Sp1 ribozyme expression, 10 or more vials of each cell strain were frozen before experiments were carried out. When they were used, they were cultured in medium containing blasticidin (10 μg/mL) for at least 3 days before experiments were carried out. A new vial was used to provide cells for each experiment.

Preparation of Sp1 Ribozyme Antisense Construct. The Sp1 U1snRNA/Ribozyme construct was prepared following a published procedure (22). The complementary oligonucleotides that encode the antisense sequence of human Sp1 (Genbank number, AF272134), including the hammerhead ribozyme, were synthesized, and the double-stranded DNA was inserted between the EcoRI and SpeI sites of the pU1 vector containing the human U1snRNA and its endogenous promoter sequences. The U1snRNA/Sp1 antisense/hammerhead ribozyme fragment was excised with BamHI digestion and inserted into BamHI site of pCMV/Bsd vector (Invitrogen, Carlsbad, CA). The construct was sequenced using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). The structure of the chimeric RNA (U1snRNA/Sp1 antisense/hammerhead ribozyme) was analyzed using MulFold and Loop-D-Loop programs.

Transfection. Transfection was done using LipofectAMINE (Invitrogen) following the manufacturer's procedures. Transfectants were selected in medium containing 10 μg/mL blasticidin, and their Sp1 protein levels were determined by Western blot analysis.

Western Blot Analysis. Whole cell lysates were prepared using single-detergent lysis buffer as described by Liang et al. (9). Conditioned medium was prepared as described below. Protein content was quantified using the bicinchoninic acid protein assay reagent kit (Pierce, Rockford, IL), and 50 μg total protein or 20 μg conditioned medium was loaded and separated by 7.5% SDS-PAGE. Protein was transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA), and Western blot analysis was done using standard techniques. The signal was detected using SuperSignal reagent (Pierce). Antibodies against Sp1, Sp3, HGF, uPA, uPAR, and EGFR were purchased from Santa Cruz (Santa Cruz, CA); cMET, from Upstate (Waltham, MA); H-Ras (23), from Oncogene; Ku80, from Serotec (Raleigh, NC); and β-actin, from Sigma (St. Louis, MO). The latter two proteins served as loading controls. Blots were quantified by densitometry, and the signal of each band was normalized to that of its loading control. All experiments were repeated at least thrice.

Preparation of Conditioned Medium. Cells were plated in culture medium at a density of 5 × 10^4 cells per 100-mm-diameter culture dish. After 24 hours, the medium was changed to serum-free medium. After another 48 hours, the medium was collected and concentrated 15-fold to 20-fold using concentrators (Vivaspin 20ML, 5,000 MWCO, Vivascience AG, Hannover, Germany).

ELISA. The level of secreted VEGF in the medium was determined using the DuoSet ELISA Development System (R&D, Minneapolis, MN) following the manufacturer's procedures. To determine the level of VEGF in each sample, 100 μL of concentrated conditioned medium was used. To create a standard curve, a series of 2-fold serial dilutions of recombinant human VEGF (2,000-125 ng/mL) was included in each set of samples assayed. The concentration of VEGF in each sample was calculated by comparing the absorbance of each sample to that of the standard curve and then normalizing that value to the protein concentration of each sample.

Reverse Transcription-PCR Analysis of Sp1 mRNA. Total RNA was extracted from logarithmically growing cells, and 1 g of total RNA was transcribed into cDNA using oligo dT(16-18) Sp1 cDNA was amplified by PCR for 26 cycles with the following primers: 5′-TAATGTTGTTGCTGCTT-3′ and 5′-GAGATATGTCGACAGCCATT-3′, which span the proposed hammerhead cutting site. β-actin, which served as an integrity and loading control, was amplified for 21 cycles (β-actin primers, 5′-AGGCCAACCAGCGAGAAGATGACC-3′ and 5′-GAGTCCAGGCGAGATGACC-3′). The PCR products were separated by 2% agarose gel, and the gel was stained with ethidium bromide.

Luciferase Assay. The luciferase assay was carried out using the procedure described by Liang et al. (9). Cells were transiently transfected using FuGene 6 (Roche, Indianapolis, IN), following the manufacturer's procedures. Briefly, cells were grown in triplicate to 50% to 60% confluence in six-well plates. pRL-TK vector (Promega, Madison WI), 0.5 μg was added to the cells in 1.5 μL FuGene 6 transfection reagent (1:3). In parallel wells, 0.5 μg of plasmid DNA (0.01 μg pRL-CMV vector and 0.49 μg pG2-Basic vector; Promega) were added to cells to serve as transfection efficiency controls. The cells were incubated for 48 hours, and cytosolic fractions were prepared with passive lysis buffer (Promega). The luciferase activity was analyzed using the Dual-Luciferase Reporter Assay System (Promega) and a luminometer. The luciferase activity of each sample was normalized to the protein concentration. The luciferase activity of each sample (pRL-TK) was normalized to the luciferase activity of control (pRL-CMV) to adjust for transfection efficiency.

Assay for Anchorage-Independence. Cells were assayed for ability to form colonies in 0.33% agarose essentially as described (23). Briefly, 5,000 cells were plated in 0.33% top agarose per 60-mm diameter culture dish, and that layer was covered with 2 mL of culture medium. The culture medium was replaced weekly. After 11 days, colonies were stained using a negative controls. After 3 weeks, the cells were fixed with 2.5% glutaraldehyde, and the colonies in five randomly chosen areas of each dish were photographed using NIH Image 1.62 software. The numbers and size of the colonies in each area were calculated by Quantity One software (www.biorad.com). All experiments were carried out at least thrice.
Assay for Tumorigenicity. Cells were assayed for the ability to form tumors in athymic mice as described by Liang et al. (9), except that the mice were examined weekly for tumor growth, and the tumors were removed when they reached 1 cm in diameter. If no tumor was observed in 6 months following injection, the mice were sacrificed.

Cell Morphology. Cells were plated in culture medium at a density of $2 \times 10^5$ cells per well of a chamber slide and incubated at 37°C in a humidified incubator with 5% CO$_2$. When the cells reached ~90% confluence, they were fixed with neutral-buffered formalin at 4°C for 10 minutes. The cell morphology was observed under a Nikon eclipse TE300 microscope, and the images were recorded with a digital camera.

Cell Death Assay. Cells were plated in culture medium at 5x10$^5$ cells per 100-mm-diameter culture dish (total six dishes per cell line per experiment) and incubated at 37°C as above. After 24 hours, the medium in half of the dishes was changed to serum-free medium; the cells in the other half received fresh culture medium. After 48 hours of incubation, the cells floating in the medium were collected and counted, and then the attached cells were dislodged with trypsin and counted.

Apoptosis Assay. Cells were plated at a density of $2 \times 10^5$ to $5 \times 10^5$ cells per 60-mm-diameter culture dish and incubated as described. After 24 hours at 37°C, the medium was removed in order to remove any unattached cells and fresh medium was added to the culture dishes. After 24 hours, the cells were detached with trypsin, stained with Annexin V-EGFP following the manufacturer’s procedures (Clontech, Palo Alto, CA), and assayed for evidence of apoptosis using flow cytometry. Apoptotic cells are stained by EGFP. Nonstained cells and Fas antibody–treated cells served as negative and positive controls, respectively. The cells were stained with propidium iodide to determine whether or not the cell membrane was intact. All experiments were carried out three or four times.

Results

Overexpression of Sp1 in Human Fibrosarcoma Cell Lines. To confirm the report by Liang et al. (9) that the level of Sp1 protein is higher in human fibrosarcoma cell lines PH2MT and γ2-3A/SB1 than in their parental MSU-1.1 cells, we carried out Western blot analysis using lysates from these three cell lines, as well as from LG1, the finite life span parental cell line from which the MSU-1.1 cells were derived (1). The results showed that the Sp1 level was ~2-fold higher in the MSU-1.1 cells than in the LG1 cells (Fig. 1A and B). The PH2MT cells had an Sp1 level 3-fold to 6-fold higher than the Sp1 level in the MSU-1.1 cells, and the γ2-3A/SB1 cells had an Sp1 level 7-fold to 10-fold higher than that of the MSU-1.1 cells.

Construction of the Sp1 U1snRNA/Ribozyme Vector. To determine whether the high expression of Sp1 observed was causally involved in the malignant transformation of these cells, we designed and constructed an Sp1-specific ribozyme to down-regulate Sp1 expression. The Sp1 U1snRNA/Ribozyme consists of three parts, an Sp1-specific antisense sequence with the hammerhead ribozyme in its center, and the two flanking regions of the U1snRNA (Fig. 1C). The Sp1-specific antisense is complementary to the 165 to 205 sequence of human Sp1 mRNA (Genbank accession No. AJ272134) and contains a hammerhead ribozyme sequence in its center, and the two flanking regions of the U1snRNA (Fig. 1C). The Sp1-specific antisense is complementary to the 165 to 205 sequence of human Sp1 mRNA (Genbank accession...
number AJ272134). A BLAST search showed that there is no significant similarity between this sequence and that of other genes. Use of the Mulfold and Loop-D-Loop programs (Fig. 1D; refs. 24–26) to analyze the Sp1 U1snRNA/Ribozyme structure revealed that the U1snRNA structure is well conserved. To make an expression vector, we inserted the Sp1 U1snRNA/Ribozyme with the human U1snRNA endogenous promoter into the BamHI site of the pCMV/Bsd vector containing the gene for blasticidin drug resistance.

**Down-Regulation of Sp1 Level and Transactivating Activity.** To test the ability of the Sp1 U1snRNA/Ribozyme to down-regulate Sp1 expression, we stably transfected PH2MT cells, γ2-3A/SB1 cells, and SHAC cells with the Sp1 U1snRNA/Ribozyme expression vector, or with the pCMV/Bsd empty vector as a control, and selected for drug resistance. Twelve independent empty vector-transfected clonal populations of the PH2MT cell line were isolated. Eleven (92%) exhibited high levels of Sp1 protein equal to that of the parental cell line; one had a medium level. Fifty-five independent Sp1 ribozyme-transfected clonal populations of the PH2MT cell line were isolated. Twenty-five (45%) expressed low levels of Sp1 protein, 8 (15%) expressed medium levels, and 22 (40%) expressed the high level found in the parental cells. When the same type of experiment was then carried out with the MW7.3A2/SB1 cell line, the number of independent clones assayed was reduced in view of the very high percentage of empty vector-transfectants exhibiting the expected high level of Sp1. Six of six (100%) of the vector control clonal populations expressed Sp1 at the same level as the parental cell line. Twelve of 26 (46%), of the Sp1 ribozyme-transfected cells expressed low

**Figure 2.** Evidence that stable transfection of malignant cell lines with the Sp1 U1snRNA/Ribozyme reduces expression of Sp1 protein and its transactivating activity, and expression Sp3 protein, but does not affect that of a H-ras oncogene. A, Western blot analysis of Sp1 and Sp3 protein expression in whole cell lysates (50 μg/lane) of tumor-derived cell line PH2MT, two derivative cell strains transfected with an empty vector as controls (V1 and V2), and two Sp1 ribozyme-transfected clonal derivative strains (SpR1 and SpR2). β-Actin was used as a loading control. B, same type of analysis as in A, except tumor-derived cell line γ2-3A/SB1 was used as the parental cell line and was compared with two of its derivative strains transfected with an empty vector as controls, and three Sp1 U1snRNA/Ribozyme-transfected clonal derivatives. Ku80 was used as a loading control. C, Sp1/Sp3 transactivational activity found in the series of cell lines in A. The cells were grown to 50% confluence and transiently transfected with an HSV-TK promoter luciferase construct and a control vector. After 48 hours, whole cell lysates were prepared, and the luciferase activity was analyzed as described in Luciferase Activity. D, same type of analysis as in C, but using the series of cell lines in B. E, level of Sp1 mRNA in the cell lines in A. Total RNA was extracted, and the relative level of Sp1 mRNA was assayed by reverse transcription-PCR. β-Actin served as the control for quantitation and determination of the integrity of the RNA. Only cell strain SpR1 showed a decreased level of Sp1 mRNA. F, same type of analysis as in E, except that cell line γ2-3A/SB1 and its derivatives in B were used. Only SpR1 showed a decreased level of Sp1 mRNA. G, Western blot analysis of the level of expression of H-ras oncoprotein in MSU-1.1 cells and in the five cell strains in A (30 μg protein/lane). Ku80 was used as the loading control.
levels of Sp1 protein, 6 of 26 (23%) expressed medium levels, 8 of 26 (31%) expressed levels like the parental cells. The fact that, taken together, >95% of the empty vector transfectants in these three experiments did not exhibit a decrease in the level of expression of Sp1 indicates that the reduction in the level of expression of Sp1 protein observed in the Sp1 U1snRNA/Ribozyme-transfected clonal populations is not the result of random variation between clonal populations, but rather results from the expression of the Sp1 U1snRNA/Ribozyme.

Figure 2A shows the results of a Western blot for Sp1 and Sp3 expression for the PH2MT cell line, two empty vector-transfected cell strains, and two ribozyme-transfected cell strains. Figure 2B shows the results of a similar Western blot for the γ2-3A/SB1 cell line, two empty vector-transfected cell strains, and three ribozyme-transfected cell strains. These five clonal populations of Sp1 ribozyme-transfected cell strains, as well as the two parental cell lines, and two empty vector clonal populations from each parental strain, were used for further study.

To determine whether the Sp1 transactivating activity is reduced by down-regulation of Sp1 protein levels, we transiently transfected the same cell strains as shown in Fig. 2A and B with a luciferase reporter construct in which the Renilla luciferase gene is driven by an HSV-1 TK promoter, which responds to the level of Sp1 protein. As shown in Fig. 2C and D, Sp1 transactivating activity was reduced 70% to 90% in these Sp1 ribozyme-transfected cell strains, compared with their respective parental cell line. The vector-transfected control cell strains, designated V1 from each cell line, exhibited Sp1 activity levels equal to that of their respective parental cell strain; the strains designated V2 exhibited an intermediate level of Sp1 activity. These results show that the Sp1 U1snRNA/Ribozyme down-regulates the level of Sp1 protein and that this reduced level correlates with reduced transactivating activity.

### Down-Regulation of Sp1 Expression Reduces Expression of Sp3

Sp3 is a ubiquitously expressed transcription factor that binds to the same DNA responsive element as Sp1 and with the same affinity (27). Unlike Sp1, which always acts as a transcription activator, Sp3 can function as an activator or a repressor of transcription, depending on modifications to the Sp3 protein (28-30). Because both Sp1 and Sp3 are ordinarily expressed in mammalian cells, the expression of genes that have the Sp1/Sp3 response elements is modulated by the combined action of Sp1 and Sp3. To determine the relative expression levels of Sp1 and Sp3, we probed the Sp1 blots shown in Fig. 2A and B with an antibody specific for the human Sp3. As shown in Fig. 2A and B, the level of Sp3 expressed correlated with the level of Sp1 expressed, and the cell strains exhibiting down-regulation of Sp1 exhibited a parallel down-regulation of Sp3. We carefully examined the antisense sequence of the Sp1 ribozyme to determine whether there was a homologous sequence in the Sp3 gene. None was found.

### The Sp1 U1snRNA/Ribozyme Acts as a Ribozyme and as Antisense

To analyze the mechanisms involved in the inhibition of Sp1 expression by the Sp1 U1snRNA/Ribozyme, we determined the Sp1 mRNA levels by semiquantitative reverse transcription-PCR. Total RNA extracted from the cell strains with reduced Sp1 levels and from their parental and vector control cell strains were used for reverse transcription and PCR. After DNA quantitation, six to eight 5′-primers and one 3′-primer were used to amplify Sp1 mRNA from the RNA samples using Taq DNA polymerase. The primers used were the same as those used to prepare the antisense construct. Total RNA was extracted from the cell strains with reduced Sp1 levels and from their parental and vector control cell strains by using the TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. The Sp1 mRNA levels were calculated and plotted as a percentage of the total number. Bars, SE.

### Table 1. Inhibition of tumor formation by down-regulation of Sp1 expression in malignant cell lines

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Relative Sp1 level</th>
<th>Frequency (tumors/sites)</th>
<th>Latency (wk)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PH2MT</td>
<td>1.0</td>
<td>6/6</td>
<td>4</td>
</tr>
<tr>
<td>V1</td>
<td>0.9</td>
<td>4/4</td>
<td>4-6</td>
</tr>
<tr>
<td>V2</td>
<td>1.2</td>
<td>6/6</td>
<td>4-6</td>
</tr>
<tr>
<td>SpR4</td>
<td>0.7</td>
<td>4/6</td>
<td>5-8</td>
</tr>
<tr>
<td>SpR3</td>
<td>0.6</td>
<td>4/4</td>
<td>8-10</td>
</tr>
<tr>
<td>SpR5</td>
<td>0.4</td>
<td>6/16</td>
<td>12-31</td>
</tr>
<tr>
<td>SpR 1</td>
<td>0.2</td>
<td>0/6</td>
<td>NA</td>
</tr>
<tr>
<td>SpR 2</td>
<td>0.2</td>
<td>0/6</td>
<td>NA</td>
</tr>
<tr>
<td>γ2-3A/SB1</td>
<td>1.0</td>
<td>6/6</td>
<td>5-6</td>
</tr>
<tr>
<td>V1</td>
<td>1.0</td>
<td>5/6</td>
<td>4-5</td>
</tr>
<tr>
<td>V2</td>
<td>1.0</td>
<td>6/6</td>
<td>4-5</td>
</tr>
<tr>
<td>SpR1</td>
<td>0.1</td>
<td>0/6</td>
<td>NA</td>
</tr>
<tr>
<td>SpR2</td>
<td>0.1</td>
<td>0/6</td>
<td>NA</td>
</tr>
<tr>
<td>SpR3</td>
<td>0.1</td>
<td>0/6</td>
<td>NA</td>
</tr>
</tbody>
</table>

NOTE: Cells (10^6) were injected s.c. into the right and left rear flank regions of athymic mice (two sites per mouse). Mice were examined weekly for tumors. When tumors reached ~1 cm in diameter, they were removed. V, vector control; SpR, transfectants expressing Sp1 U1snRNA/Ribozyme.

*Relative Sp1 level determined by Western blotting.

†Time required for tumors to reach ~1 cm in diameter.

‡No tumors formed within the 26 to 30-week observation period.

§Not applicable.

Figure 3. Evidence that down-regulation of overexpressed Sp1 protein inhibits anchorage-independent growth. A, the cell lines/strains in Fig. 2A were assayed for the ability to form colonies in agarose, as described in Materials and Methods. After 21 days, photographs were taken of the colonies in five randomly chosen areas of each of the five dishes used per cell strain, and the average number of colonies with the diameters of the designated sizes were calculated and plotted as a percentage of the total number. B, same type of analysis as in A, but using the cell lines/strains shown in Fig. 2B. Bars, SE.
H-Ras expression vector contains SV40 enhancers that are Sp1 level in PH2MT cells decreases H-Ras V12 expression, we expected, the MSU-1.1 cells didn’t express H-Ras V12. The H-Ras, as carried out Western blot analysis. As shown in Fig. 2, RasV12 levels in PH2MT cells and the transfectants showed no change in whole cell lysates (50 μg/lane) from the cells shown in A-E, with Ku80 as the loading control; L, same as in K except using the cells shown in F-J.

subjected to reverse transcription, and the levels of Sp1 mRNA were determined using a pair of Sp1-specific primers which amplify the DNA fragment spanning the proposed ribozyme cutting site (5’GUC3’). β-Actin served as the loading control.

Two of the five cell strains with reduced Sp1 expression, PH2MT, SpR1 and γ2-3A/SB1, SpR1 showed reduced Sp1 mRNA levels compared with the parental and vector control cell strains. However, the other three cell strains showed no change. These data suggest that in the former cell strains, the Sp1 U1snRNA/Ribozyme cut the Sp1 mRNA, resulting in degradation of the mRNA, whereas in the latter three cell strains, the antisense sequence inhibited translation of the Sp1 mRNA. The latter mechanism has been reported for other ribozymes (31).

H-Ras Expression in PH2MT Cell Line and Its Derivatives. The PH2MT cells overexpress the oncogene H-RasV12 (2). The H-Ras expression vector contains SV40 enhancers that are regulated by Sp1 (32, 33). To determine if down-regulation of Sp1 level in PH2MT cells decreases H-RasV12 expression, we carried out Western blot analysis. As shown in Fig 2G, as expected, the MSU-1.1 cells didn’t express H-RasV12. The H-RasV12 levels in PH2MT cells and the transfectants showed no change [the relative H-Ras levels range from 0.9 to 1.0 in the transfectants, compared with that in PH2MT cells (1.0)]. This result indicates that the down-regulation of Sp1 protein level doesn’t cause loss of transformed characteristics by reducing H-RasV12 expression.

Cell Strains with Reduced Sp1 Levels No Longer Form Large Colonies in Agarose. Cell lines PH2MT and γ2-3A/SB1 are highly tumorigenic and form large-sized colonies in agarose. In contrast, their nontumorigenic parental cell strain, MSU-1.1, forms only very small colonies (2, 6). Figure 3A and B show that the two ribozyme transfectants of PH2MT cells and the three from 2-3A/SB1 cells formed very small colonies in agarose, identical to those formed by MSU-1.1 cells, whereas the vector control cell strains formed the large-sized colonies, similar to those of their parental malignant cell lines, PH2MT and γ2-3A/SB1.

Down-Regulation of Sp1 Inhibits the Tumorigenicity of Cell Lines PH2MT and γ2-3A/SB1. To determine whether high expression level of Sp1 plays a role in tumor formation, we injected athymic mice with the five Sp1 U1snRNA/Ribozyme transfectants showing the largest reduction in Sp1 levels (Fig. 2A and B) as well as three ribozyme-transfected derivatives of PH2MT cells that exhibited intermediate levels of Sp1 (Table 1, Western blot data not shown), the two parental cell strains (Fig. 2A and B) and four vector control cell lines (Fig. 2A and B). Six months after injection, these five cell strains with markedly reduced Sp1 expressions had not produced any tumors (Table 1). In contrast, the parental and vector control cell strains formed large-sized tumors within 4 to 6 weeks and three ribozyme-transfected PH2MT strains with intermediate levels of Sp1, i.e., SpR3, R4, and R5, produced tumors in approximately half of the sites after a longer latency.

Cell Morphology Changes following the Down-Regulation of Sp1 Level. As shown in Fig. 4A-J, four cell strains with reduced Sp1 levels (D, E, I, and J; cf. Western blotting, Fig. 2A and B) showed dramatic changes in morphology compared with their malignant parental strains (A, F, and C) and the vector transfectants (B and C; G and H). The cell lines shown in D, E, and I had acquired a spindle-shaped morphology, similar to that of the
MSU-1.1 cells from which their respective parental cell strains had been derived. The cells shown in J were flatter than those shown in D, E, and I, and had a round shape, intermediate between normal fibroblasts and fibrosarcoma cells. All four transfectants with reduced Sp1 level were larger than their parental cell lines and the vector control cell lines (Fig. 4A-C and F-H). Figure 4K and L show that these changes in cell morphology in the four cell strains with reduced levels of Sp1 were not the result of changes in their levels of \( \beta \)-actin. Such levels were constant among the parental, vector control cell strains, and ribozyme transfectants with reduced Sp1 levels.

**Down-Regulation of Sp1 Expression Induces Apoptosis.** As shown in Fig. 5A and B, with or without serum in the growth medium, 8% to 22% of the Sp1 U1snRNA/Ribozyme transfectants of PH2MT and 2-3A/SB1 detached from the dish. In the parental and vector control cell strains <8% of the cells detached from the dish. To determine whether the floating cells were dead, we collected the floating cells by centrifugation and plated them in new dishes. None of the cells attached (data not shown). To determine if cell death was caused by apoptosis, the cells were grown in medium with 10% supplemented calf serum for 48 hours, collected, and labeled with Anexin V-EGFP and analyzed by flow cytometry. The ribozyme transfectants of PH2MT and 2-3A/SB1 cells displayed a 20% to 40% increase in EGFP-positive cells, indicating they died by apoptosis (Fig. 5C and D). The parental and vector control cells had <5% EGFP-positive cells (Fig. 5C and D).

These results show that down-regulation of Sp1 levels correlates with increased apoptosis.

**The Expression of HGF/MET, uPA/uPAR, EGFR and VEGF in the Cell Strains with Reduced Sp1 Levels.** Sp1 is a transcription factor and regulates more than a thousand genes, some of which play an important role in tumorigenesis (34). To determine whether the expression level of proteins coded by Sp1-regulated genes which are thought to play a role in cancer formation is reduced in Sp1 U1snRNA/Ribozyme transfectants that exhibited down-regulated Sp1 levels, whole cell lysates were prepared and analyzed by Western blotting. Conditioned medium from these cell strains were also prepared, concentrated, and analyzed for secreted proteins by Western blotting or ELISA. The level of the cMET protein, which was shown to be higher in human fibrosarcoma cell lines (9), did not change. The level of HGF protein, the ligand for cMET, also showed no change (Fig. 6A-D). However, the level of uPA, which was found to be high in 11 out of 11 human fibrosarcoma cell lines (35), was reduced in the Sp1 U1snRNA/Ribozyme transfectants (Fig. 6C and D). The level of EGFR, VEGF, and uPAR was strikingly decreased in the transfectants of y2-3A/SB1 cell line but not in those of the PH2MT cell line (Fig. 6A,B and E,F).

**Down-Regulation of Sp1 Expression in a Patient-Derived Fibrosarcoma Cell Line Inhibits Its Tumorigenicity.** Because the Sp1 ribozyme successfully blocked tumor formation by human fibrosarcoma cells malignantly transformed in culture,
we transfected the Sp1 U1snRNA/Ribozyme construct into a patient-derived fibrosarcoma cell line (SHAC), which expresses a high level of Sp1 protein, compared with normal human fibroblast cell line, LG1, and tested them for ability to form large colonies in agarose and for tumorigenicity. As shown in Fig. 7A, the SHAC cells and two vector controls expressed high levels of Sp1 protein, and formed large-sized colonies (>50 μm in diameter, at a frequency of 30-44%; Fig. 7B). The two clonal populations with the largest reduction in Sp1 protein levels (SpR1 and SpR2; Fig. 7A) formed large colonies (>50 μm in diameter, at a frequency of <10%; Fig. 7B), as did MSU-1.1 cells (>50 μm in diameter, 7%; Fig. 7B). The transfectant with an intermediate level of Sp1 (SpR3; Fig. 7A) also formed large colonies (>50 μm in diameter, at a frequency of 35%; Fig. 7B). These results show that down-regulation of Sp1 in a patient-derived fibrosarcoma cell line inhibited its ability to form large-sized colonies in agarose.

In the present study, we successfully down-regulated Sp1 levels by >80% without significantly affecting the doubling time of the cells in culture. We found that the cell strains in which the level of Sp1 was near normal, no longer formed tumors in athymic mice and lost the ability to form colonies in agarose. In transient assays, Ishibashi et al. (36) found that an Sp1 decoy suppressed the invasive activity of human lung adenocarcinoma cell line A549 and human glioblastoma cell line U251. However, the Sp1 decoy oligonucleotides would be expected to inhibit all the members of the Sp Krüppel-like factor family that are able to bind the "Sp1 site" (GC-box or GT-box). Therefore, this study argues for a role for Sp1 site-dependent transcription rather than directly implicating the Sp1 itself. A study by Abdelrahim et al. (37) showed that transient transfection of small interfering RNA duplexes for Sp1 mRNA decreased Sp1 protein in nuclear extracts of MCF-7 cells to 30% to 50% of that of the parental cells. This was accompanied by a decrease in the percentage of cells in the S phase and an increase in the percentage in cells in G0/G1 (37). In our studies using the Sp1 down-regulation constructs, the controls. These results show that decreased expression of Sp1, caused by the Sp1 U1snRNA/Ribozyme, is effective in blocking tumor formation by a patient-derived fibrosarcoma cell line that overexpresses Sp1.

**Discussion**

In the present study, we successfully down-regulated Sp1 levels by >80% without significantly affecting the doubling time of the cells in culture. We found that the cell strains in which the level of Sp1 was near normal, no longer formed tumors in athymic mice and lost the ability to form colonies in agarose. In transient assays, Ishibashi et al. (36) found that an Sp1 decoy suppressed the invasive activity of human lung adenocarcinoma cell line A549 and human glioblastoma cell line U251. However, the Sp1 decoy oligonucleotides would be expected to inhibit all the members of the Sp Krüppel-like factor family that are able to bind the "Sp1 site" (GC-box or GT-box). Therefore, this study argues for a role for Sp1 site-dependent transcription rather than directly implicating the Sp1 itself. A study by Abdelrahim et al. (37) showed that transient transfection of small interfering RNA duplexes for Sp1 mRNA decreased Sp1 protein in nuclear extracts of MCF-7 cells to 30% to 50% of that of the parental cells. This was accompanied by a decrease in the percentage of cells in the S phase and an increase in the percentage in cells in G0/G1 (37). In our studies using the Sp1
Reducing Overexpressed Sp1 Inhibits Tumorigenicity

U1snRNA/Ribozyme which is specific for the Sp1 gene, we provide direct evidence that up-regulation of Sp1 expression is involved in the malignant transformation of the fibroblast cell lines we examined. These data are consistent with the fact that when the non-tumor-forming MSU-1.1 cells are malignantly transformed, Sp1 expression is at least increased in 65% of the cases. It is likely that the malignantly transformed cells that did not show a higher level of Sp1 became malignant by an alternative route. Further evidence of the importance of overexpression of Sp1 in malignant transformation comes our finding the Sp1 U1snRNA/Ribozyme transfectants of SHAC cells with reduced Sp1 levels formed tumors in athymic mice with a greatly increased latency and decreased frequency, indicating up-regulation of Sp1 also plays a causal role in the formation of fibrosarcoma in vivo.

We found that when we reduced the level of Sp1 by >80%, the human fibrosarcoma cells reverted to a spindle cell morphology characteristic of the nontumorigenic MSU-1.1 cells from which they were derived (2, 6). In one case, the Sp1-U1snRNA/Ribozyme-expressing cell strains exhibited a morphology intermediate between normal fibroblasts and fibrosarcoma. Malignant transformation of cells commonly results in a morphologic change (38). The data in Fig. 4 clearly indicate that the change in cell morphology in the cells with reduced Sp1 protein level is not the result of alterations of β-actin expression. It would be interesting to know if the down-regulation of Sp1 level affects the expression of other genes that are related to the regulation of cell morphology and if the down-regulation of Sp1 level causes rearrangement of cell skeleton (38).

The promoters of many proapoptotic and antiapoptotic genes contain Sp1 sites (39–47), which suggests that Sp1 and other members of Sp Krippel-like family are involved in the regulation of apoptosis. Here we found that down-regulation of Sp1 expression resulted in 20% to 40% of the cells undergoing apoptosis. However, in another type of mesenchymal cell (vascular smooth muscle), up-regulation of Sp1 activity is linked to Fas-mediated apoptosis (48). Whether the difference reflects a difference in cell type, or in the nature of the malignant change is not clear.

The HGF/SF receptor, cMET, has been shown to be overexpressed in malignant human musculoskeletal tumors as well as several other types of soft tissue sarcomas (49). Studies carried out in our laboratory (9) as well as other laboratories (50, 51) showed that the expression of MET and HGF is regulated by Sp1. Surprisingly, the expression of both the HGF/SF and MET showed no change in the transfectants with reduced Sp1 protein levels. These results suggest that other transcription factors are mainly responsible for the hgf/met promoter activity, or that a minimal level of Sp1 protein is sufficient for transcription of both genes.

The uPA protein is a key player in the regulation of cancer cell invasion and metastasis (52). Elevated levels of uPA protein and/or mRNA have been reported in colorectal cancer (53), gastric cancer (54), breast cancer (55–57), prostate cancer (58), head and neck adenoid cystic carcinoma (59), and non-small-cell lung carcinoma (60). Inhibition of uPA activity by uPA inhibitors or down-regulation of uPA expression has been shown to suppress tumor growth in vivo and cell invasiveness in vitro (61–63). An earlier study in our laboratory (35) showed that 11 out of 11 fibrosarcoma cell lines derived from the MSU1 lineage, as well as cell lines from patients’ tumors, exhibited significantly higher levels of active (receptor bound) uPA than the cell strain from which they were derived or the other nonmalignant cell strains. In the present studies, we found that uPA expression was significantly decreased with the down-regulation of Sp1 protein level in both cell lines. Taken together, these data suggest that higher uPA expression is very important for the malignant transformation of human fibroblasts.

We also found that uPAR, EGFR, and VEGF, which contribute to tumor growth and angiogenesis (64), display dramatic decreases in the transfectants of γ2-3A/SB1 cell line with reduced Sp1, but show

### Table 2. Inhibition of tumor formation by down-regulation of Sp1 expression in patient-derived fibrosarcoma cell line SHAC

<table>
<thead>
<tr>
<th>SHAC</th>
<th>Relative Sp1 level*</th>
<th>Frequency (tumors/sites)</th>
<th>Latency (wk)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parental</td>
<td>1.0</td>
<td>6/6</td>
<td>9</td>
</tr>
<tr>
<td>V1</td>
<td>0.8</td>
<td>3/4</td>
<td>6-8</td>
</tr>
<tr>
<td>V2</td>
<td>1.0</td>
<td>3/4</td>
<td>9-11</td>
</tr>
<tr>
<td>SpR3</td>
<td>0.6</td>
<td>5/12</td>
<td>11-17</td>
</tr>
<tr>
<td>SpR1</td>
<td>0.4</td>
<td>3/10</td>
<td>18-30</td>
</tr>
<tr>
<td>SpR2</td>
<td>0.4</td>
<td>1/12</td>
<td>17</td>
</tr>
</tbody>
</table>

**NOTE:** Cells (10⁶) were injected s.c. into the right and left rear flank regions of athymic mice (two sites per mouse). Mice were examined for tumors weekly. When tumors reached ~1 cm in diameter, they were removed. The data in parentheses are from a second experiment with the same cell lines. V, vector control; SpR, transfectants expressing Sp1 U1snRNA/Ribozyme.

*Relative Sp1 level determined by Western blotting.

†Time required for tumors to reach ~1 cm.
no change or only a slight decrease in the transfectants of the PH2MT cells with reduced levels of Sp1. These results suggest that uPAR, EGFR, and VEGF play different roles in the inhibition of the tumorigenicity of human fibrosarcoma cell lines caused by down-regulation of Sp1 expression. The y2-3A/SB1 cells express wild-type H-Ras and the PH2MT cells express wild-type p53. Because both types of transformed cell lines are derived from MSU-1.1 cells, they both express the v-Myc oncogene, telomerase, and perhaps other as yet unidentified genetic changes.

Sp1 belongs to human Sp Krippel-like family consisting at least 21 members (13). Among these members, Sp3 shares the same expression patterns and the same binding affinity to the same DNA responsive elements as Sp1, but has different transcriptional activity (27). We observed that Sp3 protein levels were high in the human fibrosarcoma cell lines with elevated levels of Sp1 protein, and low in the cells with low levels of Sp1 protein (data not shown). The Sp3 protein levels decreased when the expression level of Sp1 was down-regulated by the Sp1 ribozyme antisense. The inhibition of Sp3 expression cannot be caused directly by the Sp1 U1snRNA/Ribozyme because there is no similarity between the sequences of the Sp1 U1snRNA/Ribozyme sequence and the Sp3 cDNA. We hypothesize that the Sp1 acting as a transcription factor regulates the transcription of the Sp3 gene, and that the down-regulation of the Sp1 protein level reduces the level of Sp3 gene transcription. Additional studies on this problem are under way.

The finding that the transcription factor Sp1 acts as an oncoprotein when it is overexpressed is not surprising. The c-Myc oncogene encodes a transcription factor, which activates a diverse group of genes involved in the regulation of cell proliferation, differentiation and apoptosis, and acts as an oncoprotein when up-regulated (65). Other transcription factors known to act as oncogenes when up-regulated include c-JUN, and STATs (66). What is surprising is that the Sp1 protein, when functioning as an oncoprotein, can exhibit specificity in up-regulation of other genes (e.g., oncogenes) although it controls more than a thousand genes (27). Sp1 is ubiquitously expressed and regulates the expression of genes that have a single Sp1 site in their promoters as well as genes that have multiple Sp1 sites in their promoters. We propose that Sp1 functions more like a “switch” to turn on and off the transcription of genes with a single Sp1 site in their promoter, whereas in genes with multiple Sp1 sites in their promoters, function in a synergistic manner to regulate expression. This would explain why the known oncogenes (e.g., VEGF, EGFR, uPAR, and uPA) which we found to be modulated by Sp1 U1snRNA/Ribozyme expression all have multiple Sp1 sites in their promoters.

Overexpression of Sp1 or up-regulation of Sp1 binding activity has been reported in multiple cancer types or cancer cell lines, including human pancreatic cancer cell lines and pancreatic cancer tissue specimens (18), breast cancer cell lines and breast cancer tissue specimens (19), gastric cancer (20), and thyroid cancer (21). The studies presented here provide direct evidence that up-regulation of Sp1 expression plays a causal role in the malignant transformation of human fibroblasts. Given the important role of Sp1 in the regulation of cell growth, invasiveness/metastasis, angiogenesis, and cell apoptosis, these data suggest that down-regulation of Sp1 protein levels or inhibition of its transactivating activity in cancer cells in which Sp1 is overexpressed might be a useful therapeutic strategy.

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References


Down-Regulation of Overexpressed Sp1 Protein in Human Fibrosarcoma Cell Lines Inhibits Tumor Formation

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