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TSG101, identified by screening a cancer cDNA library and soft agar assay, promotes cell proliferation in human lung cancer

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Abstract Understanding the genesis and development of tumors is an essential component in cancer research. It is of interest to discover unknown genes that are responsible for cellular transformation. A cDNA library of a highly metastatic lung adenocarcinoma cell line was constructed. This library was introduced into the NIH3T3 mouse embryonic fibroblast cell line to screen for cDNAs that increase anchorage-independent colony formation in soft agar. The expression of *TSG101* in lung cancer cell lines and specimens was confirmed using reverse transcription-polymerase chain reaction. The level of TSG101 protein in transfected A549 cells was determined by western blotting. Cell-cycle distribution was analyzed using a FACStar Plus flow cytometer. One of the candidate cDNAs that increases anchorage-independent colony formation was shown to correspond to the *TSG101* cDNA sequence. Levels of *TSG101* mRNA were higher in lung cancer cell lines and specimens compared to matched normal lung tissues. Ectopic expression of *TSG101* in the A549 lung adenocarcinoma cell line increased the numbers of cells in S phase, suggesting an increased cell proliferation rate. These results indicate that *TSG101* may induce the malignant phenotype of cells.

Keywords *TSG101* gene · Human lung cancer · Proliferation ability · Malignancy phenotype

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Introduction

Tumor susceptibility gene 101 (*TSG101*) was originally identified as a tumor suppressor gene that causes transformation of NIH3T3 cells when it was inactivated by a random antisense strategy [1]. The human homologue *TSG101* has been isolated and mapped to chromosome 11p, bands 15.1–15.2 [2], a region known to commonly exhibit loss of heterozygosity in a variety of human malignancies [3, 4]. No genomic deletion has been reported in the *TSG101* gene, however, which casts doubt on the role of *TSG101* as a classical tumor suppressor. Although *TSG101* is essential for cell proliferation, cell survival, and embryonic development under normal physiological conditions [5–8], the role of *TSG101* in tumor formation and development is complex and remains controversial. Studies have suggested that *TSG101* levels are increased in human cancers, including thyroid cancers [9], human ovarian carcinomas [10], human breast cancers [11], malignant gastrointestinal stromal tumors [12], and vincristine-resistant human gastric adenocarcinoma cells [13]. Moreover, its targeted overexpression in transgenic mice reveals weak oncogenic properties for mammary cancer initiation [11]. Reduction of *TSG101* protein has a negative impact on tumor-cell growth. Silencing of the *TSG101* leads to growth arrest and cell death in breast, prostate [14], and ovarian cancer cells [10], rather than the growth promotion that would be expected from loss of a true tumor suppressor. siRNA constructs of *TSG101* can also effectively reverse the resistant phenotype of vincristine-resistant human gastric adenocarcinoma cells [13]. *TSG101* is an important factor

for maintaining normal cellular homeostasis and elevated *TSG101* expression contributes to oncogenic transformation. Normal *TSG101* expression is stringently controlled within a narrow range [15]; deficiency or overexpression of *TSG101* can cause neoplastic formation [1]. However, the mechanism by which perturbation of *TSG101* expression leads to neoplasm is currently unclear. One report on *tsg101*-knockout mice indicates that *tsg101* deficiency results in p53 accumulation, growth arrest, and early embryonic lethality [5]. Other reports have identified TSG101 as part of the MDM2/p53 regulatory circuit [16], which is a well-recognized circuit whose deregulation results in tumorigenesis. Given these findings, the role of *TSG101* gene as a tumor-suppressor gene should be re-evaluated and its function in tumor formation and development should be further studied.

In this study, NIH3T3 cells were transfected with a cDNA library originally constructed from the highly metastatic adenocarcinoma cell line Anip973 and screened for cDNAs that could increase colony formation and generate irregular-shaped colonies in soft agar. One of the candidate cDNA sequences corresponded to *TSG101*. This result supports the idea that *TSG101* promotes proliferation and the malignant phenotype of NIH3T3 cells. Further studies have shown that *TSG101* mRNA levels are increased in lung cancer cells compared to matched normal lung tissue. Ectopic expression of *TSG101* cDNA in a lung adenocarcinoma cell line, A549, resulted in an increased cell proliferation rate as indicated by increased numbers of cells in S phase. These studies indicate that *TSG101* overexpression is associated with human tumors and that the cloning of cDNA libraries in NIH3T3 cells and subsequent screening for loss of contact inhibition in soft agar is a viable tool for identifying tumor-related genes.

Materials and methods

Cell lines and treatments

Lung cancer cell lines used in these studies were obtained from the Shanghai Institute for Biological Sciences, Chinese Academy of Sciences, except for three cell lines, AGZY83-a, Anip973, and HB-99, that came from the Harbin Medical University in China. For information on the 15 lung cancer cell lines see Table 1. NIH3T3 cells were maintained in DMEM (Life Technologies, Inc.) containing 10% fetal bovine serum and A549 cancer cells were grown in F12K medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum. Other cells, including SPC-1-A, GLC-82, LTEP-a-2, PAa, A549, L-18, 95C, 95D, PG, LH7, QG-56, NCI-H460, AGZY83-a, Anip973, and HB-99 cells, were cultured in RPMI 1640 supplemented with 10% fetal bovine serum at 37°C in a 5% CO_2 incubator.

Table 1 The informations of 15 lung cancer cell lines

Name of cell lines	Pathology characteristics
AGZY83-a	Adenocarcinoma of lung, low metastatic ability
Anip973	Adenocarcinoma of lung, derived from AGZY83-a, high metastatic ability
SPC-1-A	Adenocarcinoma of lung, derived from pleural metastatic patient
GLC-82	Adenocarcinoma of lung, poorly differentiated
LTEP-a-2	Adenocarcinoma of lung
PAa	Adenocarcinoma of lung, low metastatic ability
A549	Adenocarcinoma of lung, have some metastatic ability
L-18	Adenocarcinoma of lung, high infiltrate ability
95C	Lung giant-cell carcinoma, low metastatic ability
95D	Lung giant-cell carcinoma, derived from 95C, high metastatic ability
PG	Lung giant-cell carcinoma
LH7	Lung giant-cell carcinoma, derived from PG, low metastatic ability
HB-99	Lung squamous carcinoma
QG-56	Lung squamous carcinoma
NCI-H460	Large cell carcinoma of lung

Lung cancer tissues and adjacent normal lung tissues were obtained from surgical biopsy or resection (from The Tumor Hospital of Harbin Medical University in China) and stored at −80°C.

Construction of cDNA library and transfection, soft agar assay, identification of DNA recombinants in cells

As previously described [17], a cDNA library was constructed from Anip973, a human lung adenocarcinoma cell line with high metastatic potential. Then transfection of the cDNA library constructs into NIH3T3 cells was performed with Fugene 6 transfection reagent (Roche). Single clones were obtained after culture in selection media containing 200 μg/ml G418 for 7–10 days. Soft agar colony assays were performed with these stable NIH3T3 transfectants. Fourteen days later certain cell colonies formed, which had special morphology. These foci were isolated and transferred into 96-well plates for expansion. Genomic DNA was isolated from these altered growth morphologies clones. Inserted sequences were obtained by PCR and identified further by sequencing.

RNA isolation and RT-PCR analysis

Total RNA extraction of 15 cell lines was performed using Trizol® reagent (Gibco-BRL) according to the

manufacturer's instructions. Briefly, cell pellets obtained from cell lines were resuspended in 1 ml of Trizol® reagent by pipetting up and down while frozen lung tissues (50–100 mg weight) were homogenized mechanically in 1 ml of Trizol® reagent. Three microgram of total RNA was used to synthesize first-strand cDNA with oligo-dT using the Reverse Transcription System (Promega) according to the manufacturer's instructions.

Multiplex-PCR was performed in a 25-μl volume containing 1 μl of tenfold diluted cDNA, 1× PCR buffer, 0.2 mM dNTP mixture, 1.5 mM $MgCl_2$, 3% DMSO, 0.4 μM each primer and 2.5 units of Taq DNA polymerase (Gibco-BRL). The expression of *TSG101* (GenBank gi:12803336) was studied by PCR amplification with oligonucleotide primers (F45: 5′-TGCGATTGTGTGGGAC GGTCTG-3′; R796:5′-CCTCGCTGATTGTGCCATCCC TAC-3′) which generated 752-bp PCR products. The product covers exon 1 to exon 8 out of 10 exons. PCR amplification consists of 25 cycles of 94°C for 30 s, 61°C for 30 s and 72°C for 50 s. The integrity of cDNA was confirmed by amplifying actin with oligonucleotide primers (F: 5′-ACT-CTTCCAGCCTTCCTTCC-3′, R: 5′-CATACTCCTGCT TGCTGATCC-3′) to generate a 308-bp PCR product.

Plasmid construction and transfection

Human *TSG101* cDNA from normal human lung tissue cDNA was obtained by PCR amplification using the oligonucleotide primers *TSG101*Ex/F 5′-GTCATGGCGGT GTCGGAGAG-3′ and *TSG101*Ex/R 5′-GTAGAGGTC ACTGAGACCGGCAG-3′. The resultant fragments were cloned into the pcDNA3.1/V5- His©TOPO®TA expression vector (Invitrogen) and confirmed by sequencing.

One day prior to the transfection A549 cells were seeded without antibiotics. This corresponded to 40–50% confluence at the time of transfection. Transfection of the pcDNA3.1/V5-*TSG101* plasmid or empty vector into A549 cells were performed with Fugene 6 transfection reagent (Roche) following the manufacturer's instructions. Twenty-four h after transfection, transfectants were selected in medium containing 500 μg/ml G418 for 7–10 days and individual clones of transfectants were picked for further analysis.

Western-blot analysis

A549 cell pellets were collected after transfection with pcDNA3.1/V5-*TSG101* or an empty vector. Cell lysates were prepared by suspending cell pellets in ice-cold 1× phosphate-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 0.4 units/ml aprotinin, 1 mM NaF, and 0.1 mM sodium orthovanadate. Protein concentrations were measured using the Bradford assay (Pierce) according to the manufacturer's protocols. An equal amount of protein (100 μg) was loaded onto an SDS–polyacrylamide gel for electrophoresis and then transferred onto a polyvinylidene difluoride membrane (Invitrogen). The membranes were blocked in 1× Tris-buffered saline containing 0.1% Tween 20 and 5% non-fat dry milk for 1 h. Membranes were then incubated with antibody against Tsg101 (C-2; Santa Cruz) in blocking buffer at 4°C overnight with gentle agitation. After washing with buffer (1× Tris-buffered saline, 0.1% Tween 20) three times for 15 min each, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies in blocking buffer for 1 h at room temperature. Membranes were then washed in buffer for 15 min three times and once for 15 min in 1× Tris-buffered saline without Tween 20. Signals were visualized using ECL kit for Western-blot analysis (Amersham Biosciences).

Cell-cycle analysis

A549 cells transfected with pcDNA3.1/V5-*TSG101* or pcDNA3.1/V5-*LacZ* were starved in medium containing 0.2% FBS for 48 h and then cultured in medium containing 10% FBS for 24 h. Cells were collected by trypsinization at different times and fixed in ice-cold 70% ethanol. After washing in PBS, cells were incubated with 10 μg/ml RNase and 50 μg/ml propidium iodide for 30 min. Cell-cycle distribution was analysed using a FACStar Plus flow cytometer (Becton–Dickinson).

Statistical analysis

Significant differences in *TSG101* mRNA levels between cancer and lung tissues were identified using a one-way paired *t*-test. Data were presented as the mean ± SD, and P values <0.05 were considered to be statistically significant. Statistical analyses were performed using the Statistical Package for Social Science software program (version 11.0; SPSS Inc., IL). Data shown are representative of four or more independent experiments.

Results

Anip973 cDNA expression library

Total RNA was extracted from 5×10^7 cultured cells using Trizol reagent. mRNA was further isolated for cDNA library construction. The quality of the cDNA was checked by 1.1% agarose gel electrophoresis after it was synthesized, amplified, and purified. A typical gel profile of the double-stranded cDNA showed a moderately strong smear from 0.5 to 6 kb (Fig. 1a), which indicated that the quality

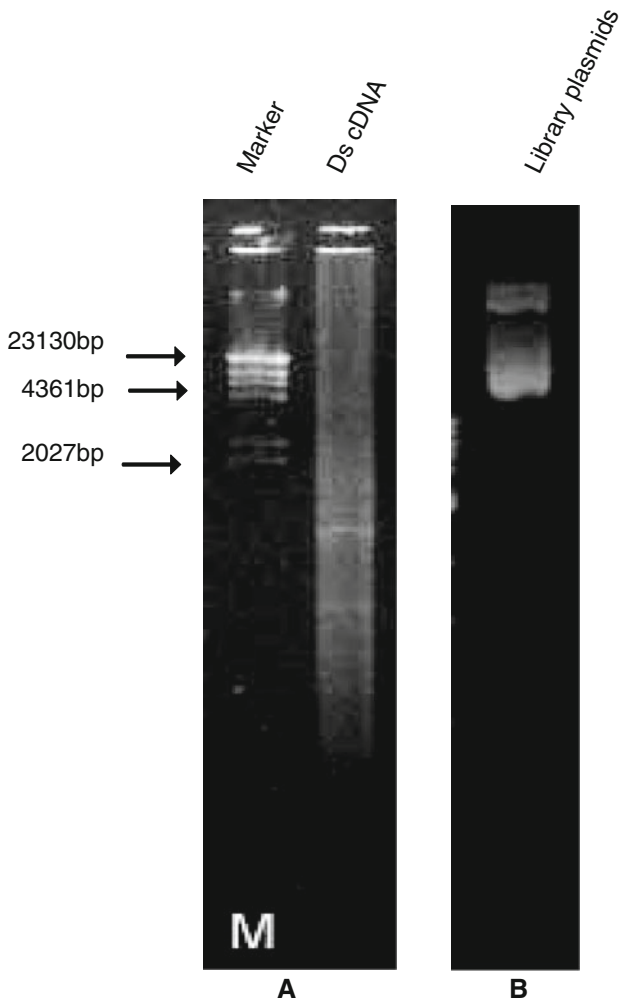


Fig. 1 Characterization of the cDNA library. mRNA was isolated from total RNA of Anip 973 cells. 0.5 μg of mRNA was used as the starting material for first-strand cDNA synthesis. 2 μl of single-strand cDNA was used as the template for second-strand synthesis by long-distance PCR. The PCR products were purified and cloned into the pcDNA3.1/V5- His©TOPO®TA expression vector. The plasmids were obtained after they were transformed into Top 10 *Escherichia coli* cells and cultured overnight. **a** 1% agarose gel electrophoresis of double-strand cDNA after PCR and purification. *M HindIII*-digested λDNA; **b**1% agarose gel electrophoresis of library plasmids

of cDNA library was good. Library plasmids also display a smear rather than a single strand because they consist of multiple recombinants (Fig. 1b).

Screening of clones with special morphology in soft agar

NIH3T3 cells were transfected with the cDNA library construct and selected by growth in the presence of 200 μg/ml G418 for 3 weeks, resulting in a set of stably transfected NIH3T3 cell populations. Stable NIH3T3 transfectants were seeded into soft agar and incubated for 2 weeks

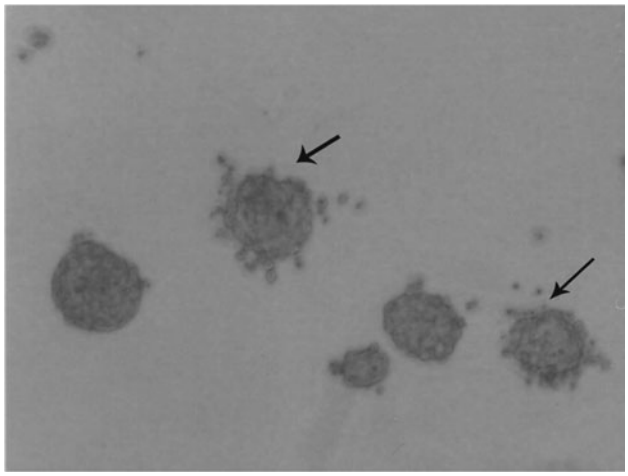


Fig. 2 Colonies with altered morphology in soft agar. Transfection of the cDNA library constructs into NIH3T3 cells was performed. After 7–10 days of culture in selection media containing 200 μg/ml G418, cells were re-suspended and a soft agar colony assay was performed. Cells transfected with library plasmids were observed using an inverted microscope (×400) in soft agar for 14 days. Some cell colonies were large (diameter >1 mm) and had unusual growth patterns (*arrow*)

before a significant number of foci were observed. The size of these foci ranged from a few clumps of cells to up to 1 mm in diameter. They were evenly distributed throughout the agar, suggesting that the individual cells from which these foci originated overcame their inherent characteristics. "Special morphology" was defined as a large (diameter >1 mm), spread out colony with some individual cells visible around the maternal colony. These putative colonies (Fig. 2, arrow) were expanded for further analysis.

Identification of recombinants

Inserts from the recombinants were recovered using PCR. More than one product was often obtained in a single PCR reaction, which indicated that plasmids containing different cDNAs were sometimes transfected into a single cell. As the goal was to link 3T3 transformation to a single, specific gene, all PCR fragments were isolated and sequenced individually. Sequence analysis identified a known gene, *TSG101*, It is by far the best represented, accounting for 10% of all recombinants (Table 2; Online Resource 1). In this paper the first 6 sequences (% representation of the recombinants was from 10 to 4%) were displayed and the others are listing in Online Resource 1.

Five recombinants which contain *TSG101* plasmid were selected randomly and real-time PCR was done to validate the mRNA level of *TSG101* It can be seen that the expression of *TSG101* in recombinants are higher than control cells (Fig. 3; Online Resource 2).

Table 2 Identification of part insertion sequences in recombinants

	Name of insertion sequences	% representation of the recombinants
1	Homo sapiens TSG101 (tumor susceptibility gene101)	10
2	Homo sapiens hypothetical protein FLJ22104	6
3	Homo sapiens ribosomal protein L23	6
4	Homo sapiens serine protease inhibitor, kazal type6	4
5	COX2(mt)	4
6	Homo sapiens heparan sulfate 6-O-sulfotransferase 2	4

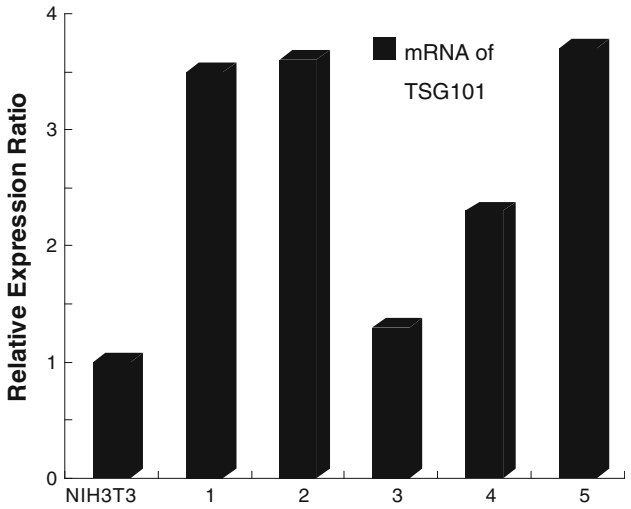


Fig. 3 Expression level of *TSG101* in five recombinants. Five recombinants which contain *TSG101* plasmid were selected randomly and real-time PCR was done to validate the mRNA level of *TSG101*. It can be seen that the expression of *TSG101* in recombinants are higher than control cells

Increased expression of TSG101 mRNA in lung cancer cells

cDNA from normal lung specimens exhibited only a slight PCR product, whereas variable expression of *TSG101* is visible in 15 lung cancer cell lines. Quantification of the signals was performed using a densitometric scanner (Scion image software, Table 3, Table 4; Online Resource 3). The value obtained by densitometry scanning of the *TSG101* signal was normalized to the value obtained from the *β-actin* internal control signal in the corresponding sample. *TSG101* expression levels are higher in the 15 lung cancer lines than in normal lung tissue, although there was some variation (Fig. 4a, b).

Five lung cancer and corresponding control normal lung tissue cDNA samples were amplified. To compare the amounts of the amplified *TSG101* products in lung cancer tissues and normal lung tissue, we performed quantification of the signals (Table 5; Online Resource 3) using a densitometric scanner (Scion image software). The value obtained by densitometry scanning of the *TSG101* signal was normalized to the value obtained from the *β-actin* internal control signal in the corresponding sample. As indicated in Fig. 4c, the relative amount of *TSG101* expression in the tumor sample was greater than that found in normal sample (SPSS software). The mean ± SD of normal tissues was 0.7720 ± 0.15156, whereas in cancer tissues it was 1.0220 ± 0.19728, $t = -6.118$, $P = 0.004$.

These results show that *TSG101* expression levels are higher in lung cancer cell lines (15/15) and in all cancer samples (5/5) compared to normal lung tissue.

Table 3 The value of RT-PCR in eight lung cancer cells and tissue obtained by densitometry scanning

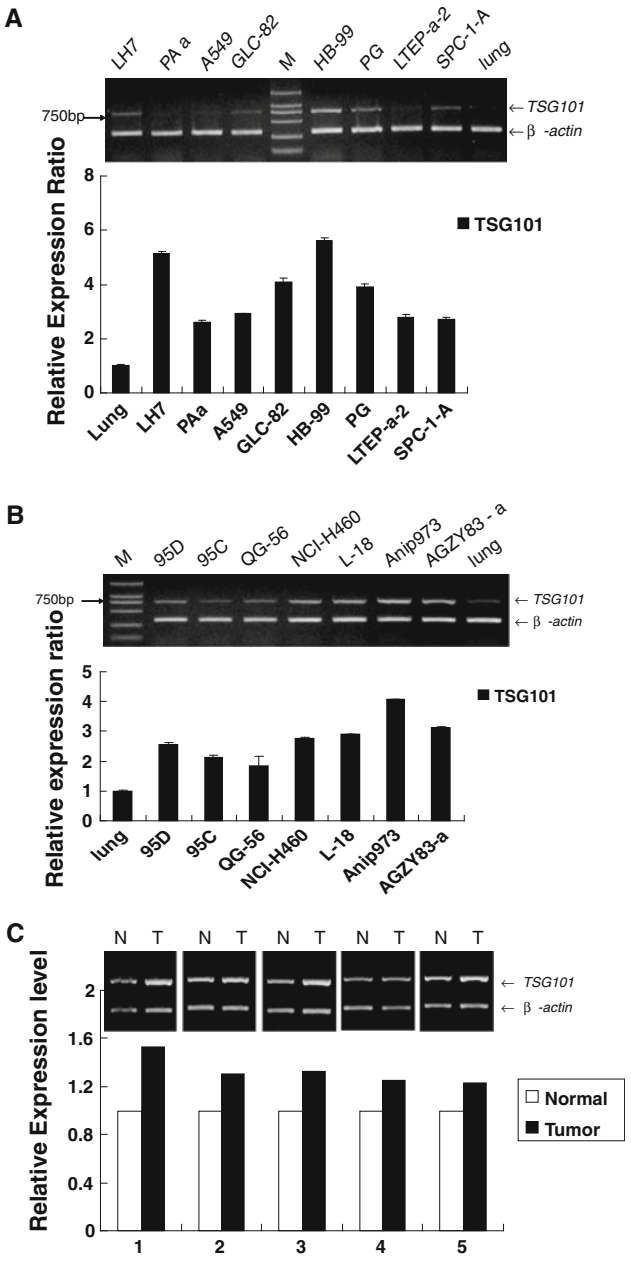
	Ratio (cancer cells/lung tissue)
LH7	5.11
PAa	2.58
A549	1.99
GLC-82	4.06
HB-99	4.78
PG	3.90
LTEP-a-2	2.56
SPC-1-A	2.66
Lung	1

Table 4 The value of RT-PCR in seven lung cancer cells and tissue obtained by densitometry scanning

	Ratio (cancer cells/lung tissue)
95D	2.53
95C	2.12
QG-56	2.45
NCI-H460	2.75
L-18	2.88
Anip973	4.06
AGZY83-a	3.13
Lung	1

Identification of transfection effect of pcDNA-TSG101 in A549 cells

Normal human lung tissue was amplified using the *TSG101*Ex/Fand *TSG101*Ex/R primers, which resulted in a PCR product of 1,173 bp that included the whole open reading frame (ORF) of *TSG101* (124–1,296 bp). The fragment was inserted into the pcDNA3.1/V5- His©TOPO®TA



◀ **Fig. 4** Expression level of *TSG101* in lung cancer cell lines and tissues. **a**, **b** Expression level of TSG101 in lung cancer cell lines Representative expression profile of TSG101 mRNA in lung cancer cells compared to normal lung tissue. We performed quantification of the signals using a densitometric scanner (Scion image software, Tables 3, 4).The value obtained by densitometry scanning of the TSG101 signal was normalized to the value obtained from the β-actin internal control signal in the corresponding sample. cDNA from normal lung specimens exhibited only a slight PCR product; the expression level of TSG101 was greater in the 15 cancer cell lines than in normal lung tissue. **c** Expression level of TSG101 in lung cancer tissues. Expression profile of TSG101 mRNA in five pairs of lung cancer and corresponding control normal lung tissues. We performed quantification of the signals using a densitometric scanner (Scion image software, Table 5).The value obtained by densitometry scanning of the TSG101 signal was normalized to the value obtained from the β-actin internal control signal in the corresponding sample. A significant difference between cancer and normal lung tissue was identified using the one-way paired *t*-test (version 11.0, SPSS software). Data are presented as mean ± SD, normal tissue was 0.7720 ± 0.15156, cancer tissue was 1.0220 ± 0.19728, t = −6.118, $P = 0.004$. (*N* normal tissue, *T* cancer tissue)

expression vector and the resulting plasmid was transfected into A549 cells; control plasmid (pcDNA-*LacZ*) was transfected at the same time. Cells were screened using G418. Western-blot analyses indicated that TSG101 is stably expressed in A549 cells transfected with pcDNA-*TSG101* (see Fig. 5).

Ectopic expression of TSG101 increased cell proliferation rate

It was shown that cell-cycle synchronization was achieved by serum starvation. A total of 87.57% of A549 cells transfected with pcDNA-*TSG101* were in G_0–G_1 phase compared to 83.40% of control cells. Cells recovered by addition of 10% FBS, and after 24 h the cell-cycle distribution changed: 68.95% of A549 cells were in G_0–G_1 phase and 25.27% were in S phase, which had increased from 9.52%. A total of 63.34% of control cells were in G_0–G_1 phase and 24.72% were in S phase, which had increased from 15.5%. In conclusion, the percentage of S phase cells grew more rapidly in A549 cells transfected with pcDNA-*TSG101* (from 9.52 to 25.27%) than in control cells (from 15.5 to 24.72%). Ectopic expression of *TSG101* in A549 cells increased the number of cells in S phase, which indicates that cells transfected with pcDNA-*TSG101* have powerful proliferation and growth ability (See Fig. 6; Table 6).

Discussion

It is important to establish a cell-phenotyping system that can detect a direct correlation with the function of a gene. In this study, cDNA library screening in combination with soft agar assay was used to study the molecular mechanism of lung cancer progression. Screening of cDNA libraries can be a useful tool for the discovery of unknown genes responsible for cellular transformation. In this study, a cDNA expression library from the Anip973 cell line was transfected into NIH3T3 cells and used to detect genes that, when overexpressed, altered cell morphology in soft agar assays. This is feasible because transformed cells lose contact inhibition and change morphology in soft agar. Oncogenes such as *Ras* and sphingosine kinase [18, 19] have been detected in this manner. In the present study, we retrieved full-length, wild-type versions of the known genes ribosomal protein *L23*, *TSG101*, and *Homo sapiens*

Table 5 The value of RT-PCR in five pairs lung cancers obtained by densitometry scanning

Serial number	Pathology characteristics	Tissues	Ratio (TSG101/β-actin)
1	Adenocarcinoma of lung, no metastasis	Normal (N)	0.77
		Tumor (T)	1.17
2	Adenocarcinoma of lung, well-differentiated	Normal (N)	0.79
		Tumor (T)	1.03
3	Adenocarcinoma of lung, moderately differentiated	Normal (N)	0.72
		Tumor (T)	0.95
4	Adenocarcinoma of lung, no metastasis	Normal (N)	0.58
		Tumor (T)	0.73
5	Adenocarcinoma of lung, have metastasis	Normal (N)	1.0
		Tumor (T)	1.23

	Normal (N)	Tumor (T)
	0.77	1.17
	0.79	1.03
	0.72	0.95
	0.58	0.73
	1.0	1.23
Mean	0.7720	1.0220
SD	0.15156	0.19728

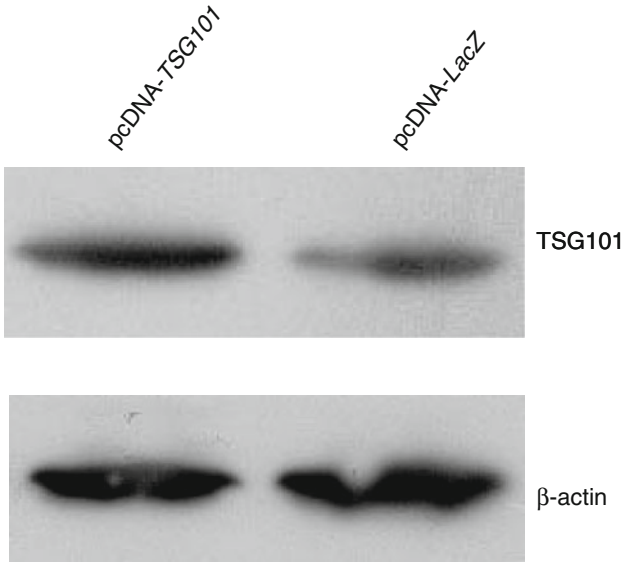


Fig. 5 Expression of TSG101 in TSG101 transfectants and control cells. Western-blot analysis showed that TSG101 is stably expressed in A549 cells transfected with pcDNA-*TSG101* plasmid compared to controls. β-actin was used as a loading control

hypothetical protein *FLJ22104*. A number of N-terminally truncated clones were also retrieved. *TSG101* was selected for further study owing to the uncertainty regarding tumor formation and development mechanisms.

TSG101 was originally discovered in a screen for potential tumor suppressors using insertional mutagenesis in immortalized fibroblasts and it was considered a candidate tumor suppressor gene [1]. Some researchers have investigated large series of breast cancers, and have shown that intragenic deletion of *TSG101* is rare [20–22]. In this report, we have shown that *TSG101* induced malignant characteristics in NIH3T3 cells in soft agar. The mechanism by which *TSG101* affects proliferation of cells was unclear. It has been reported that *TSG101* is mainly localized to the cytoplasm but upon cell-cycle progression it can be found in the nucleus and the mitotic spindle. TSG101 has several conserved protein domains that have cell-cycle regulatory functions. The coiled-coil domain at the carboxyl terminus of the TSG101 protein has been reported to interact with the cytoplasmic phosphoprotein stathmin and oncoprotein 18 [23, 24], the latter may have a role in microtubule dynamics as well as cell growth and differentiation. This region of TSG101 also has potential co-repressor activity [25, 26]. Furthermore, a proline-rich domain in TSG101 was found to act as an activation domain in transcriptional regulation. Based on its important role in cell proliferation and cell survival, overexpression of *TSG101* induces changes in the characteristics of NIH3T3 cells, as shown by the large and irregular foci in soft agar.

Recent studies have suggested that *TSG101* levels are increased in many human cancers, but in human lung cancers the expression and function of *TSG101* has not been determined. To determine the significance of *TSG101* in tumor formation and development in human lung cancers, semi-quantitative RT-PCR analysis in normal lung tissue and lung cancers was performed. The results of our

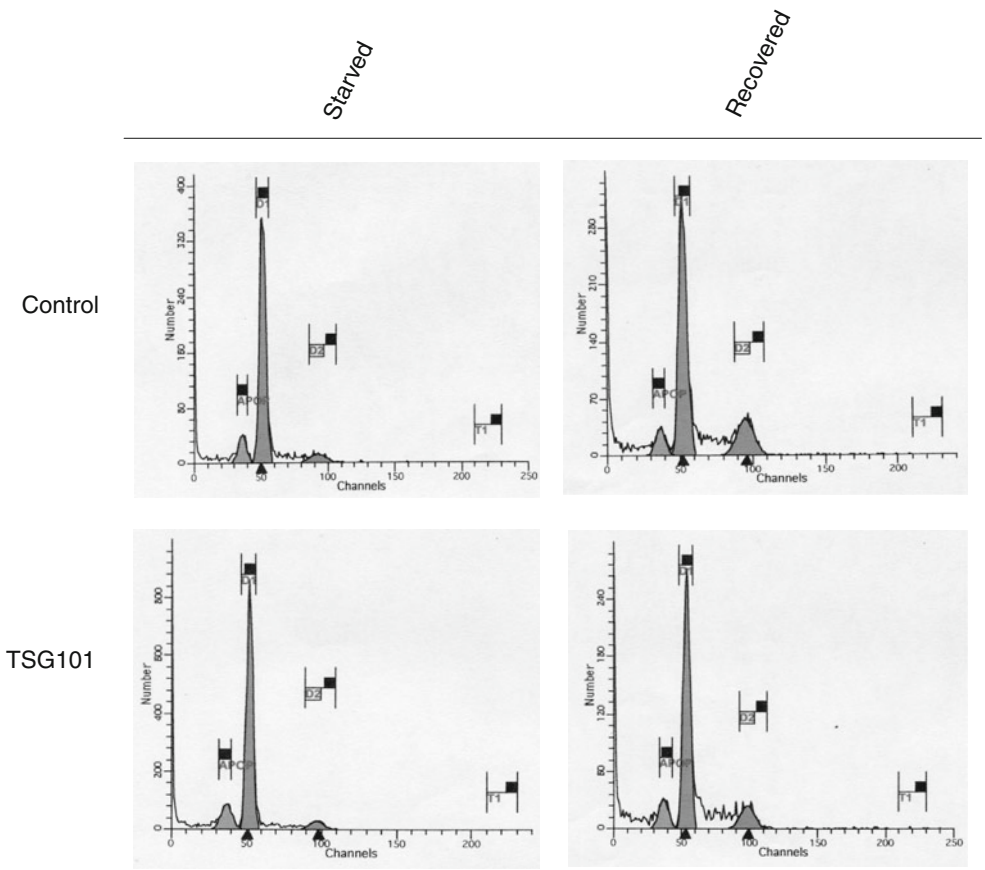


Fig. 6 Flow-cytometry analysis result. A549 cells transfected with pcDNA3.1/V5-*TSG101* or pcDNA3.1/V5-*LacZ* were starved in medium containing 0.2% FBS for 48 h and then cultured in medium containing 10% FBS for 24 h. Cells were collected and fixed, then cell cycle distribution was analysed using a FACStar Plus flow cytometer. S phase cells grew more rapidly in transfected pcDNA-*TSG101* cells (from 9.52 to 25.27%) than in control cells (from 15.5 to 24.72%)

Table 6 Cell-cycle analysis after cell synchronization

	pcDNA-*LacZ*	pcDNA-*TSG101*
Starvation for 2d		
G_0–G_1 (%)	83.40	87.57
S (%)	15.50	9.52
G2-M (%)	1.10	2.91
After starvation 2d, adding high serum for 24 h		
G_0–G_1 (%)	63.34	68.95
S (%)	24.72	25.27
G2-M (%)	11.94	5.78

Cell-cycle synchronization was achieved by serum starvation. A total of 87.57% of cells transfected with pcDNA-*TSG101* were in G_0–G_1 phase, compared with 83.40% of control cells. Cells were recovered by the addition of 10% FBS after 24 h. The number of transfected cells in G_0–G_1 phase was reduced to 68.95%; by contrast, 25.27% were in S phase, which had increased from 9.52%. Of control cells, 63.34% were in G_0–G_1 phase and 24.72% were in S phase, which had increased from 15.5%

RT-PCR analysis confirm that *TSG101* was up-regulated in lung cancer cell lines (15/15) and in lung cancer tissues (5/5). Our findings provide the first evidence for the association of *TSG101* overexpression in human lung cancers. As other reports have shown that the MDM2 protein is upregulated in a high proportion of lung cancer specimens [27, 28], the same mechanism might be responsible for mediating the effects of *TSG101* overexpression in human lung cancer cells. Through its ubiquitin-conjugating E2 variant domain [29, 30], TSG101 interacts with MDM2, inhibits MDM2 ubiquitination, and prolongs the half-life of MDM2 protein; conversely, increased levels of MDM2 promote proteolysis of TSG101 [31]. It was found that upregulation of MDM2 in cells that overexpress TSG101 decreases the amount of p53 [16] and a recent study has indicated that only p90MDM2 controls the protein levels of p53, TSG101, and MDM2 itself [32]. Another report has indicated that TSG101 negatively regulates p21 levels [10]. Together, these results might explain the mechanism by which *TSG101* affects cell-cycle control. As the MDM2/TSG101 regulatory loop modulates the cellular levels of both proteins and consequently affects MDM2 control of p53, TSG101 is both a regulator and a target of p53/MDM2 circuitry. Hence, overexpression of *TSG101* might have an oncogenic role by inactivating p53 through

MDM2 upregulation. On the other hand, proteomics analysis has identified *TSG101* as a downstream target of the *ras* oncogene [33]; this may be another pathway by which *TSG101* is overexpressed in lung cancer cells.

Deletion of *Tsg101* caused growth arrest and cell death [6], and cell-cycle arrest in *TSG101*-deficient cells is p53-dependent [8]. In our study, ectopic overexpression of *TSG101* in A549 cells (a lung adenocarcinoma cell line with low *TSG101* gene expression levels) resulted in an increased proliferation rate as indicated by increased cell-cycle distribution in S phase. Reports have indicated that TSG101 is essential for normal membrane trafficking and regulation of receptor recycling [34–37]. Forced expression of *TSG101* could suppress receptor function indirectly by interfering with the regulation of membrane trafficking and receptor recycling. Therefore, it is tempting to speculate whether aberrant receptor recycling control due to overexpression of *TSG101* might result in abnormal cell growth. It also has been shown that TSG101 can suppress transcription by direct association with DMAP1- and DNMT1-containing transcription repression complexes [38]. It is possible that the altered TSG101 protein level interferes with the mechanism by which cell growth is controlled through nuclear receptors.

Taken together, we have demonstrated that *TSG101* is overexpressed in lung cancer cells and tissues and that it is an important factor in the control of cell-cycle regulation. *TSG101* is not a primary tumor suppressor gene, but might act as a cell survival factor and promote the malignant phenotype in NIH3T3 and lung cancer cells.

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