Up-regulation of Tumor Susceptibility Gene 101 Conveys Poor Prognosis through Suppression of p21 Expression in Ovarian Cancer

Travis W. Young,1 Daniel G. Rosen,2 Fang C. Mei,1 Nan Li,1 Jinsong Liu,2 Xiao-Fan Wang,3 and Xiaodong Cheng1

Abstract

Purpose: The function of tumor susceptibility gene 101 (TSG101) in ovarian carcinogenesis is largely unexplored. The aim of this study is to investigate the role of TSG101 in human ovarian cancer development, to examine the expression levels of TSG101 in ovarian carcinomas, and to correlate the results with clinicopathologic variables and survival.

Experimental Design: Human ovarian cancer tissue arrays that contain duplicates of 422 cases of primary ovarian carcinoma were used to probe the expression levels of TSG101 and p21 in epithelial ovarian cancer. In vitro studies in ovarian cancer cells using TSG101-specific small interfering RNA (siRNA) were done to further elucidate the mechanism of TSG101-mediated p21 regulation.

Results: We show that TSG101 is increasingly overexpressed in borderline tumors and low-grade and high-grade carcinomas. Patients with low expression of TSG101 survive longer than those with high expression. Suppressing TSG101 by siRNA in ovarian cancer cells led to growth inhibition, cell cycle arrest, and apoptosis with concurrent increases in p21 mRNA and protein. Consistent with this negative association between TSG101 and p21, expression levels of these two markers are inversely correlated in ovarian cancer.

Conclusions: TSG101 negatively regulates p21 levels, and up-regulation of TSG101 is associated with poor prognosis in ovarian cancer.

The tumor susceptibility gene 101 (TSG101), originally known as CC2, was initially identified as a coiled-coil domain-containing protein that interacts with stathmin in a yeast two-hybrid screen (1). The TSG101 gene encodes a multidomain protein that contains a putative DNA-binding motif at its COOH terminus and can act as a transcriptional cofactor to repress or activate nuclear hormone receptor–mediated transactivation (2). On the other hand, the NH2-terminal region of TSG101 shares extensive sequence homology to the Ub domain of ubiquitin-conjugating enzyme but lacks a critical active-site cysteine essential for enzymatic activity, which suggests a role for TSG101 in the regulation of ubiquitin-mediated protein degradation (3–5). Several recent studies show that TSG101 is an important cellular factor that specifically recognizes mono-ubiquitylated proteins and mediates endosomal trafficking important for membrane receptor endocytosis and retroviral budding (6–11).

The implied tumor suppressor function of TSG101 was proposed based on a random functional knock-out study, in which inactivation of TSG101 in NIH3T3 mouse fibroblasts led to oncogenic transformation (12). Subsequently, several studies suggested that TSG101 was often mutated in human breast cancers, and its aberrant splice variants were frequently detected in different tumor types (13–19). However, it was correctly determined later that these so-called mutations were in fact alternative splice variants generated exclusively by exon skipping (20). Further studies showed that the deletion of TSG101 in cell cultures and TSG101 conditional knock-out in mice models did not lead to uncontrolled cell growth and neoplastic transformation. On the contrary, homozygous deletion of TSG101 in genetically engineered mice resulted in embryonic lethality, whereas excision of TSG101 in mammalian cells resulted in cell cycle arrest and cell death. These results suggest that TSG101 is required for cell survival, and loss of TSG101 is not a promoting event for tumorigenesis as would be expected for the loss of a genuine tumor suppressor.

Using functional proteomic approaches, we have recently revealed that TSG101 is overexpressed in a large number of ovarian cancer patients (21). Although TSG101 was initially...
recognized as a potential tumor suppressor (12), the precise role of TSG101 in tumor formation, development, and its relevance to ovarian carcinomas in the clinical settings are largely unknown. The purpose of the present study was to investigate the functional role of TSG101 in human ovarian cancer development, to examine the expression levels of TSG101 in ovarian carcinomas, and to correlate the results with clinicopathologic variables and survival.

Materials and Methods

Patients and specimens. Formalin-fixed, paraffin-embedded primary ovarian cancer specimens were obtained from patients who underwent surgery for primary epithelial ovarian cancer (EOC) at the M.D. Anderson Cancer Center (MDACC). All tissue specimens were collected under Institutional Review Board–approved Health Insurance Portability and Accountability Act–compliant protocols at MDACC. Histopathologic diagnoses were based on WHO criteria (22), and tumor grading was based on Gynecologic Oncology Group criteria (23). In this system, the grading method employed depends on the histologic type of the tumor. Endometrioid carcinomas are graded similar to the International Federation of Gynecology and Obstetrics (FIGO) system used for endometrioid endometrial carcinomas. Based on the amount of solid tumor component, endometrioid carcinomas of the ovary are graded as grade 1 when it has <5% solid areas, grade 2 with 5% to 50% solid areas, and grade 3 with more than 50% of solid areas. Transitional cell carcinomas of the ovary are graded in a manner similar to that for transitional cell carcinomas of the urinary bladder as low and high grade tumors. Grading of serous carcinomas was done by using a two-tier system according to the new criteria proposed by Malpica et al. (24). Disease staging was assigned according to the FIGO system (25).

Construction of the tissue microarrays. Tissue blocks were constructed as previously described (26). The final tissue microarray consisted of two blocks. All samples were spaced 0.5 mm apart. Sections of 5 μm were obtained from the microarray and stained with H&E to confirm the presence of tumor and to assess the tumor histology. Tumor samples were randomly arranged on the blocks. The first tissue microarray block was constructed to analyze under the same conditions 5 normal ovaries, 7 serous tumors of low malignant potential, 7 mucinous tumors of low malignant potential, 10 low-grade serous carcinomas, 19 high-grade serous carcinomas, 5 low-grade and 7 high-grade endometrioid carcinomas, and 6 clear cell carcinomas. The second tissue microarray contained duplicates of 422 cases of primary ovarian carcinoma. The array was read according to the given tissue microarray map; each core was scored individually, and the results were presented as the mean of the two replicate core samples. Cases in which no tumor was found or no cores were available were excluded from the final data analysis.

Immunohistochemical analysis. The tissue microarray slides were subjected to immunohistochemical staining using antibodies against TSG101 (1:100, Clone 4A10, Novus Biologicals) and p21 (1:50, Neomarkers). TSG101 staining and analysis were carried out as described previously (21). For p21, a positive nuclear area above 2% was considered as overexpression, and an area below 2% was considered as low expression.

Statistical analysis. Descriptive statistics were calculated; Spearman rank correlation methods were used to estimate the pairwise associations of the markers; χ² tests or, if there were five or fewer observations in a cell, Fisher’s exact tests were done to assess the association between two categorical variables; Wilcoxon rank sum nonparametric methods were used to assess the association between TSG101 and clinical variables and between p21 and clinical variables. All P values presented were two sided. Kaplan-Meier method was for survival analysis. All statistical analyses were carried out in SAS 8.0 or Splus 6.1 or StatXact 4 as appropriate. Results were considered statistically significant at the P < 0.05 level.

Flow cytometry analysis. SKOV-3 cells transfected with either TSG101 or control small interfering RNA (siRNA) were harvested 4 and 6 days after transfection. About 1 x 10⁶ cells were trypsinized from 10-cm plates and transferred to fluorescence-activated cell sorting (FACS) tubes, washed twice with PBS, and then fixed with 70% ethanol for 2 h on ice. Ethanol was removed by centrifugation, and cells were rehydrated in PBS at room temperature for 5 min. Cells were stained with 1 mL of propidium iodide staining solution (0.1% Triton X-100, 0.2 mg/mL RNAase A, 0.05 mg/mL propidium iodide, prepared fresh) for 1 h at room temperature. Cellular DNA content was determined on a Becton Dickinson FACSscan apparatus.

Nuclear DNA content staining. About 2 to 3 x 10⁵ SKOV-3 cells were plated in 3-cm tissue culture plates containing polylysine-coated coverslips and transfected with either control or TSG101 siRNA. Cells on coverslips were stained 5 days post-transfection with Hoechst 22658 for 30 min, rinsed with PBS, and sealed onto glass slides. Nuclear DNA condensation was examined using a fluorescence microscope (Olympus BX51) equipped with a Hamamatsu digital camera (C4742-95).

Gene expression analysis. Total RNA was isolated from cells using the TRizol reagent (Invitrogen). Semi quantitative reverse transcription-PCR (RT-PCR) was carried out using 1 μg of isolated total RNA. Primers used for RT-PCR were TSG101 forward 5’-TGGATGTCCGGATCCTGC- TATTCT-3’, reverse 5’-TGTCCTCCTCTACTCCGATCT-3’, p21 forward 5’-GCAGTGTGATGCGGATATG-3’, reverse 5’-CGTTCCTGACCCTGAGA-3’.

Immunoblotting analysis. Cells transfected with either control or TSG101 siRNA were harvested on the appropriate days by trypsinizing cells, washing with PBS, and then lysis in SDS lysis buffer. Protein concentration was determined using Bio-Rad protein assay reagent. Equal amounts of protein (10-20 μg) were loaded onto 12% SDS polyacrylamide gels and transferred to polyvinylidene difluoride membranes. Polyvinylidene difluoride blots and the remaining polyacrylamide gels were stained with Ponceau S and Coomassie Blue, respectively, to ensure equal loading and even transfer of the samples. After being blocked overnight in 5% milk in TBS-Tween, blots were incubated with p21 antibody (1:2,000; Cell Signaling) or TSG101 (1:2,000) for 1.5 h at room temperature, followed by incubation in antimouse immunoglobulin G secondary antibody (1:2,000; Bio-Rad). Immunoblots were developed by enhanced chemiluminescence (Pierce).

Chromatin immunoprecipitation assays. We used a chromatin immunoprecipitation (ChIP) kit from Upstate Cell Signaling. Experiments were done with SKOV-3 cells transfected with control or TSG101 siRNA according to the manufacturer’s instruction with minor modifications. PCR was done using the following primers for the p21 promoter, forward 5’-CTGCGTGGTGGTGGTGAGCTAGA-3’, reverse 5’-CTGTTGGTGTCCTGCGCTT-3’.

Results

Up-regulation of TSG101 in human ovarian cancer samples. We previously identified TSG101 as a downstream target of the RAS oncogene in a genetically defined human ovarian cancer model (27). To investigate the clinical implication of RAS-mediated TSG101 up-regulation in human ovarian surface epithelial cells, we probed the expression levels of TSG101 in EOC using human ovarian cancer tissue arrays. We used an affinity-purified mouse anti-human TSG101 monoclonal antibody (clone 4A10) that has been successfully used for immunohistochemical staining (28, 29). Overall, localization of the TSG 101 was mostly found in the cytoplasmic compartment of epithelial cells. Although normal human
ovarian surface epithelium (HOSE) did not show significant expression for TSG101 (Fig. 1A1), the expression of TSG101 was increasingly positive in borderline tumors and low-grade and high-grade carcinomas compared with normal HOSE (Fig. 1). In a transition zone between normal HOSE and a high-grade serous carcinoma, we found that normal HOSE was negative, whereas the tumor showed strong cytoplasmic staining for TSG101 (Fig. 1A2). Similar findings were observed in a mucinous low malignant potential (LMP) tumor where an area of mucinous cystadenoma weakly expressed the marker compared with the adjacent LMP lesion (Fig. 1A3). The levels of expression for TSG101 varied among ovarian carcinomas showing a wide range of variability from totally negative (26.3%) to strongly positive (23%) with more than 70% samples showing certain degrees of TSG101 up-regulation (Fig. 1B).

**Prognostic value of TSG101 expression levels in EOC.** As shown in Table 1, TSG101 was most frequently up-regulated in serous carcinoma, undifferentiated carcinomas, and MMMT as opposed to the other histotypes ($P = 0.0083$). Patients with grade 3 ovarian cancer had a significantly higher percentage of high TSG101 as compared with patients with grade 1 or grade 2 ($P = 0.044$), and patients with stage 3 or stage 4 had a significantly higher percentage of high TSG101 as compared with patients with stage 1 or stage 2 ($P = 0.027$). Furthermore, suboptimal cytoreduction was significantly correlated with high TSG101 ($P < 0.014$), whereas no significant difference was observed in patient’s age at the time of diagnosis between high and low TSG101 levels.

The impact of high TSG101 concentration on ovarian cancer survival was investigated. Kaplan-Meier survival curves for ovarian cancer patients with either low or high TSG101 level revealed that patients with low expression of TSG101 survive longer than those with high expression of TSG101 (Fig. 2).

**Suppression of TSG101 in ovarian cancer epithelial cells results in growth inhibition, cell cycle arrest, and apoptosis.** We evaluated the potential mechanisms by which increased levels of TSG101 contribute to the development and progression of EOC by disrupting the expression of TSG101 in SKOV-3 using a TSG101-specific siRNA that suppresses endogenous TSG101 (as shown in Fig. 3A) led to a dramatic growth inhibition of SKOV3 cells when compared with cells transfected with a scrambled control siRNA with the same GC content as the TSG101 siRNA (Fig. 3B). To further examine the potential mechanism of TSG101 suppression-mediated cell growth inhibition, flow-cytometric cell cycle analyses of SKOV-3 cells transfected either with TSG101 or control siRNA were done. TSG101 knockdown cells exhibited a significant increase of

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**Table 1.** $\chi^2$ test or Fisher’s exact test to assess the association between TSG101 (score) and clinical variables

<table>
<thead>
<tr>
<th>Clinical variables</th>
<th>TSG101 (score), n (%)</th>
<th>$P$ value</th>
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<tbody>
<tr>
<td>Age (y)</td>
<td></td>
<td>0.81</td>
</tr>
<tr>
<td>≤55</td>
<td>39 (25.32)</td>
<td></td>
</tr>
<tr>
<td>&gt;55</td>
<td>65 (24.25)</td>
<td></td>
</tr>
<tr>
<td>Histotype</td>
<td></td>
<td>0.0083</td>
</tr>
<tr>
<td>Clear cell carcinoma</td>
<td>6 (40.00)</td>
<td></td>
</tr>
<tr>
<td>Endometrioid adenocarcinoma</td>
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<td></td>
</tr>
<tr>
<td>MMT</td>
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<td></td>
</tr>
<tr>
<td>Mucinous adenocarcinoma</td>
<td>3 (50.00)</td>
<td></td>
</tr>
<tr>
<td>Undifferentiated carcinoma</td>
<td>5 (26.32)</td>
<td></td>
</tr>
<tr>
<td>Serous carcinoma</td>
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<td></td>
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<tr>
<td>Transitional cell carcinoma</td>
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<tr>
<td>Grade</td>
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</tr>
<tr>
<td>1</td>
<td>8 (34.78)</td>
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<tr>
<td>2</td>
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<td>3</td>
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<td>Stage</td>
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<td>III</td>
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<tr>
<td>IV</td>
<td>19 (22.89)</td>
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<tr>
<td>Type of cytoreduction</td>
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<td>Optimal</td>
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<tr>
<td>Suboptimal</td>
<td>32 (21.05)</td>
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**Fig. 1.** Expression levels of TSG101 in human ovarian carcinomas. A, TSG101 immunostaining in normal and ovarian cancer samples. A1, negative expression in normal HOSE (40%). A2, negative and positive expressions of TSG101 in normal HOSE (arrowhead) and in adjacent serous carcinoma (arrow, 16%). A3, weak to negative expression in a mucinous cystadenoma (arrowhead) with strong positive expression in adjacent mucinous LMP (15%). A4, positive expression in a high-grade endometrioid carcinoma (40%). A5, strong positive expression in a high-grade serous carcinoma (40%). A6, example of a positive nuclear staining against p21 in a serous carcinoma. B, TSG101 box plot by groups. The relative absorbance of the expression of the marker was measured and analyzed using a nonparametric analysis in normal HOSE and ovarian tumors. As shown, ovarian tumors have increased expression of TSG101 compared with normal HOSE. Low-grade lesions, both endometrioid and serous, have lower average levels of expression compared with high-grade lesions. LG, low grade; HG, high grade; CCC, clear cell carcinoma.
cells in the G2-M phase and a decrease in G1-G0 phase cells when compared with control siRNA-transfected cells (Fig. 3C), indicating a block in the cell cycle at the G2-M phase. Moreover, a significant population of sub-G1 apoptotic cell debris was observed. These observations were corroborated by increases in nuclear DNA condensation/fragmentation (Fig. 3D) in TSG101 knockdown cells. Taken together, these results suggest that silencing of TSG101 results in G2-M arrest and, subsequently, cell death in SKOV-3 cells.

**Suppressing TSG101 promotes p21 expression and activation.** To determine the molecular mechanism of TSG101 knockdown-mediated cell cycle arrest and inhibition of cell viability, we checked the expression of a panel of genes involved in controlling cell cycle progression and cell survival. The expression of one important tumor suppressor, the cyclin-dependent kinase inhibitor p21, was found significantly up-regulated both at mRNA and protein levels in SKOV-3 cells with reduced TSG101 (Fig. 4A and B).

To investigate the mechanism by which TSG101 silencing induces the accumulation of p21 mRNA, a plasmid construct, p21P, with a luciferase reporter gene under the transcriptional control of the p21 promoter, was cotransfected into the SKOV-3 cells either with TSG101-specific or control siRNA. Luciferase activity was significantly increased in TSG101 knockdown cells compared with controls (Fig. 4C), suggesting that TSG101 silencing-induced p21 mRNA accumulation is partly due to a transcriptional activation of the p21 gene. Because SKOV-3 cells are p53-null, this apparent TSG101 silencing-mediated transcriptional activation of p21 is most likely p53 independent. To further confirm this observation, we next monitored the luciferase reporter activity using a deletion p21 reporter construct, p21PΔ1.1, which contains a 1.1-kb deletion that removes the consensus p53-responsive element from the 5’ region of the 2.4-kb p21 promoter (32). As shown in Fig. 4C, the full-length p21 promoter construct and p53-deletion construct responded to TSG101 silencing to similar extents, both leading to a 6-fold induction of p21. Taken together, these data suggest that suppression of TSG101 in ovarian cancer cells leads to a p53-independent transcriptional activation of p21.

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**Fig. 2.** Prognostic significance of TSG101 expression levels in ovarian carcinomas. Kaplan-Meier survival curves for ovarian cancer patients with either high or low TSG101 expression.

**Fig. 3.** Association of suppression of TSG101 expression with cell cycle arrest and growth inhibition. A, SKOV-3 cells were transfected with TSG101 and control siRNAs as described in Materials and Methods. RNA and protein were isolated 48 h post-transfection as described. mRNA levels were determined by semiquantitative RT-PCR and protein levels by immunoblotting. Similar results were obtained from more than four independent experiments. B, SKOV-3 cells were plated at 2.5 × 10⁴ cells per well in six-well plates, and cell growth was determined by counting over successive days. Each data point represents the average of three wells. C, control and TSG101 knockdown SKOV-3 cells were stained with propidium iodide and analyzed by FACS on day 8 following siRNA transfection. Sub-G1 cells are calculated as a supplemental population. D, control and TSG101 knockdown SKOV-3 cells on day 5 following siRNA transfection were stained with Hoechst 22658, and nuclear condensation was observed on a fluorescent microscope. Cells with condensed/fragmented DNA were quantified from at least eight images and represented as a percentage of the total number of cells.
Because TSG101 contains a putative DNA-binding domain and can act as a general transcriptional suppressor (2), we hypothesized that TSG101, acting as a transcriptional corepressor, suppresses the transcriptional activation of p21 by binding to the p21 promoter. Consequently, suppression of TSG101 by siRNA leads to the transcriptional activation of p21. To test this hypothesis, we measured the amount of p21 promoter DNA immunoprecipitated using a ChIP assay with a TSG101-specific antibody and primers that amplify the p21 promoter. Association of TSG101 with p21 promoter was apparent in SKOV-3 cells treated with control siRNA, and the recruitment of TSG101 to this promoter was dramatically reduced in TSG101 knockdown SKOV-3 cells (Fig. 4D). These results not only provide the first biochemical evidence of TSG101 recruitment to the p21 promoter, but also show that reduced TSG101 binding to the p21 promoter in TSG101 knockdown SKOV-3 cells is responsible for the induction of p21 expression.

**Association between TSG101 and p21 in ovarian carcinomas.**

Given the causal relationship between TSG101 and p21 observed in ovarian cancer cells, we decided to determine whether a pairwise association between TSG101 and p21 exists in ovarian carcinomas. Spearman's rank correlation analysis revealed that markers TSG101 and p21 were significantly correlated with Spearman's rank correlation -0.11 and P value 0.04. In addition, a χ² test was done to assess the association between TSG101 and p21. As shown in Table 2, TSG101 again is significantly associated with p21. Patients with lower TSG101 score had a significantly higher rate of high p21 score. Taken together, these results confirm our prediction based on the in vitro mechanistic study: there is a significant negative correlation between the markers TSG101 and p21 in human ovarian carcinomas.

**Discussion**

TSG101 is an essential protein involved in numerous cellular processes, including transcriptional regulation, protein degradation/ubiquitination, cell cycle control, vesicular sorting/transport, and viral budding. Levels of TSG101 are tightly controlled under physiologic conditions, and complete knockout of TSG101 leads to embryonic lethality in mice and cell cycle arrest and death in cultured cell lines (33–35). Although originally identified as a potential tumor suppressor gene in NIH3T3 cells (12), the precise roles of TSG101 in tumorigenesis remain undefined. Previously, we identified TSG101 as a downstream target of oncogenic RAS up-regulated during in vitro transformation of human ovarian epithelial cells (27, 36). This increased expression of TSG101 is also observed in ovarian cancer patients because more than 70% of ovarian carcinomas express elevated levels of TSG101. These observations agree with several recent studies showing that TSG101 levels are elevated in certain human cancers, including thyroid (37) and gastrointestinal tumors (38), and gene silencing of TSG101 leads to growth arrest and cell death in breast and prostate cancer cells (39). Additionally, our present study reveals that increased TSG101 concentration is associated with poor prognostic outcomes.

The tumor-promoting effects of TSG101 in EOC is mediated at least partly through the suppression of cyclin-dependent kinase inhibitor p21, as our data show recruitment of TSG101 to the p21 promoter where TSG101 acts as a corepressor of p21 transcription in ovarian cancer cells. Furthermore, the levels of TSG101 and p21 are inversely correlated in human ovarian tumor samples. Our observations that patients with lower TSG101 score have significantly higher rates of high p21 score to the p21 promoter, but also show that reduced TSG101 binding to the p21 promoter in TSG101 knockdown SKOV-3 cells is responsible for the induction of p21 expression.
and high TSG101 levels are associated with poor prognosis coincidences with earlier studies reporting that high p21 expression is a favorable disease outcome in EOC (40). p21, a mediator of p53 tumor suppression, inhibits cyclin-dependent kinases and leads to G1-S cell cycle arrest (41, 42). Although in wild-type p53-containing tumor cells, p21 is induced in p53-mediated G1 arrest and apoptosis (43), p53-independent induction of p21 has been shown in various ovarian and other cancer cells (44–46) and in p53-knock-out mice (47). TSG101 and p21 have been previously linked as a homozygous deletion of TSG101 in mice that led to a dramatic increase of p53 with concomitant accumulation of p21 and embryonic lethality (33, 34). Increased p21 expression by silencing TSG101 was believed to be mediated by p53 through an autoregulatory feedback loop between TSG101 and MDM2/p53, in which TSG101 suppresses MDM2 ubiquitination and results in consequent down-regulation of p53 protein (48). However, a more recent study suggests that whereas p21 is a mediator of the cell arrest in TSG101 knockout cells, deletion of TSG101 has no effect on MDM2 steady-state levels, and null mutation of TSG101 does not rescue the tsg101 deficiency phenotype (49), suggesting that the proposed feedback loop between TSG101 and MDM2/p53 is not likely the mechanism for TSG101-mediated p21 regulation. Alternatively, TSG101 has been implicated to negatively regulate cell proliferation through direct association and stabilization of p21 in differentiating primary keratinocytes (50). Although the p21 stabilization effect of TSG101 may be specific to differentiating primary keratinocytes, this notion is not consistent with the majority of studies, including the aforementioned gene knock-out studies that show that TSG101 is essential for cell proliferation (33–35, 49). In this regard, our results show TSG101 acting as a transcriptional suppressor of p21 and provide an important link for understanding the role of TSG101 in cell growth and proliferation.

In summary, our study reveals that TSG101 negatively regulates the tumor suppressor protein p21 and plays an important role in ovarian carcinomas because increased TSG101 protein levels are observed to associate with late-stage and high-grade EOC. In addition, TSG101 seems to contribute to tumor aggressiveness because high TSG101 protein levels are related to reduced patient survival. Taken together, our results suggest that elevated TSG101 is associated with poor prognosis and a potential therapeutic target for EOC.

References
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