p70 S6 Kinase Promotes Epithelial to Mesenchymal Transition through Snail Induction in Ovarian Cancer Cells

Yuen L. Pon, Hong Y. Zhou, Annie N.Y. Cheung, Hextan Y.S. Ngan, and Alice S.T. Wong

Abstract

p70 S6 kinase (p70S6K) is a downstream effector of phosphatidylinositol 3-kinase and is frequently activated in human ovarian cancer. Here we show that p70S6K functions in epithelial to mesenchymal transition (EMT) responsible for the acquisition of invasiveness during tumor progression. This tumorigenic activity is associated with the ability of p70S6K to repress E-cadherin through the up-regulation of Snail. p70S6K activation induced phenotypic changes consistent with EMT in ovarian cancer cells: The cells lost epithelial cell morphology, acquired fibroblast-like properties, and showed reduced intercellular adhesion. Western blot showed that p70S6K activation led to decreased expression of the epithelial marker E-cadherin and increased expression of mesenchymal markers N-cadherin and vimentin. Inhibition of p70S6K by a specific inhibitor or small interfering RNA reversed the shift of EMT markers. Importantly, p70S6K activation also stimulated the expression of Snail, a repressor of E-cadherin and an inducer of EMT, but not other family members such as Slug. This induction of Snail was regulated at multiple levels by increasing transcription, inhibiting protein degradation, and enhancing nuclear localization of Snail. RNA interference-mediated knockdown of Snail suppressed p70S6K-induced EMT, confirming that the effect was Snail specific. Furthermore, phosho (active)-p70S6K staining correlated with higher tumor grade. We also showed a significant positive correlation between p70S6K activation and Snail expression in ovarian cancer tissues. These results indicate that p70S6K may play a critical role in tumor progression in ovarian cancer through the induction of EMT. Targeting p70S6K may thus be a useful strategy to impede cancer cell invasion and metastasis.

Introduction

Ovarian cancer is a highly metastatic cancer. Current therapies for advanced stage or metastatic ovarian cancer have little effect, with poor 5-year survival rates of only 16% to 28% (1). Understanding the molecular mechanisms underlying the progression of ovarian cancer may provide insights into new therapeutic targets.

p70 S6 kinase (p70S6K) is a downstream effector of the phosphatidylinositol 3-kinase/PI3K pathway, which is frequently activated in human ovarian cancer. p53CA, the gene that encodes the p110α catalytic subunit of phosphatidylinositol 3-kinase, is increased in copy number in 40% of ovarian cancer (2). AKT1 and AKT2 are both activated in a large number of ovarian carcinomas, with the activation being associated with high-grade tumor and aggressive clinical behavior (3–5). Constitutive activation of p70S6K is significantly more often in malignant ovarian tumors than in benign or borderline lesions (6). p70S6K is also activated by a number of growth factors including epidermal growth factor and hepatocyte growth factor (HGF), which are potent inducers of p70S6K, in ovarian cancer cells (7, 8). These observations underline the importance of understanding the role of p70S6K in ovarian cancer. In addition to its well-documented role in cell survival, our recent findings show for the first time that p70S6K may be involved in other aspects of ovarian tumor progression, including invasion and metastasis (8–10). However, the molecular mechanisms by which p70S6K may affect the motility and metastasis of ovarian cancer cells are poorly understood.

Epithelial to mesenchymal transition (EMT) is an essential morphologic conversion occurring during embryonic development. Increasing evidence also proposes that a similar process occurs during cancer progression by which tumor cells acquire the capacity to migrate, invade, and metastasize (11). Loss of cell-cell adhesion is a prerequisite of EMT and involves functional loss of E-cadherin. The zinc finger transcription factors of the Snail/Slug family have been implicated in this repression (12–14). In this study, we show for the first time a role for p70S6K in EMT of human ovarian cancer cells. We also present evidence suggesting that this tumorigenic activity is associated with the ability of p70S6K to repress E-cadherin through mechanisms that modulate the expression, localization, and function of Snail.

Materials and Methods

Cell lines and culture conditions. The human ovarian cancer cell lines CaOV-3 and SKOV-3 were obtained from Dr. N. Auersperg (University of British Columbia, Vancouver, British Columbia, Canada). Cell lines were cultured in Media 199/MCDB105 (1:1) supplemented with 100 units/mL penicillin, 100 µg/mL streptomycin, and 5% fetal bovine serum. Cultures were maintained in a humidified incubator of 5% CO2 at 37°C. To express constitutively active p70S6K cDNA constructs (∆Np53∆Cint or Δp53E-cis) kindly provided by Dr. G. Thomas, Genome Research Institute, University of Cincinnati, Cincinnati, OH; ref. 15), cells were transiently transfected with 1.5 µg of plasmid DNA per well in six-well plates using Lipofectamine 2000 (Invitrogen). For HGF experiments, CaOV-3 and SKOV-3 cells were plated at subconfluency and incubated with 10 ng/mL of recombinant human HGF (R&D Systems) for 24 h (8). To confirm the role of p70S6K, cells were pretreated with 20 nmol/L rapamycin (Calbiochem) for 30 min before the addition of HGF.

Immunohistochemistry. Formalin-fixed, paraffin-embedded tissues that include 3 normal ovarian tissues (age, 23–47 y; mean, 31.3 y), 5 benign tumors (ages, 20–67 y; mean age, 36.8 y), and 32 ovarian carcinomas (ages, 35–69 y; mean age, 46.9 y; 12 serous, 10 endometrioid, 5 mucinous, and 5 clear cell) were obtained with Internal Review Board approval from the archives of the Department of Pathology of Queen Mary Hospital, The
Five-micrometer-thick sections were deparaffinized in xylene and hydrated in graded alcohol and water before being placed in 5% H2O2 blocking solution, which was followed by antigen unmasking (critic acid buffer, pH 6.0). Then they were incubated with primary antibodies, either phospho-p70s6k (T389; Cell Signaling; 1:100) or Snail (Santa Cruz; 1:50). The sections were stained using the ABC Elite avidin-biotin peroxidase detectionsystem (Zymed) and counterstained with hematoxylin. Omission of primary antibodies or substitution with normal

**Figure 1.** p70s6k activation induces EMT in ovarian cancer cells. A, CaOV-3 and SKOV-3 cells transfected with constitutively active p70s6k, ΔNAC, or D3E-E389 for 24 h were assessed for morphologic changes consistent with EMT. Left, the presence of spindle-shaped cells with loss of epithelial cell morphology, increased intercellular separation, and pseudopodia was noted in ΔNAC- or D3E-E389-expressing cells but not in control cells. Right, immunofluorescent microscopy for ZO-1 confirmed the loss of polarity by revealing the loss of ZO-1–associated tight junctions. B, left, scattered colonies were scored. Columns, mean of three experiments done in duplicate; bars, SD. Right, transfected cells were collected for invasion assay through Matrigel. After 24 h of incubation, cells in the upper side of the filter were removed and the invaded cells were fixed, stained, and counted. Columns, mean number of invaded cells of five fields of triplicate wells from three independent experiments; bars, SD. Inset, whole-cell lysates were analyzed for the levels of phosphorylated (p-) and total forms of p70s6k by Western blot analysis. C, expression of the epithelial cellular adhesion molecule E-cadherin and expression of the mesenchymal markers N-cadherin and vimentin were assessed by Western blotting. β-Actin was included as a loading control. Immunoblots were quantified by densitometry and relative expression to β-actin was indicated. D, immunofluorescent microscopy for E-cadherin revealed the loss of E-cadherin–associated adherens junctions (left). Whole-cell lysates were also immunoprecipitated (IP) with anti-β-catenin antibody, and Western blotted (WB) with anti-E-cadherin (right). *, P < 0.05, compared with untransfected controls.
mouse or rabbit IgG was used as a negative control. Scoring was assigned according to the intensity and percentage of positive staining and graded negative, low, or high as described (16).

**Scattering assay.** Subconfluent cultures of CaOV-3 and SKOV-3 were transfected with pcDNA3-ΔN54ΔC104 or ΔE-E398 construct by using Lipofectamine 2000 reagent (Invitrogen) and selected with G418 (400 μg/mL). Scattered colonies were judged by a typical change in morphology, characterized by cell-cell dissociation and the acquisition of a migratory, fibroblast-like phenotype. Scattering activity was measured in the total number of scattered colonies from 50 colonies under a light microscope.

**Matrigel invasion assay.** *In vitro* invasion was assayed by determining the ability of cells to invade through Matrigel as previously reported by us (8). Results were presented as the mean cell number of five fields (±SD) of triplicate wells from three independent experiments.

**Western blot analysis and immunoprecipitation.** Twenty micrograms of total proteins were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was then incubated with antibodies specific for E-cadherin (Zymed; 1:1,000), N-cadherin (Zymed; 1:1,000), vimentin (BD Transduction Laboratories; 1:1,000), β-catenin (BD Transduction Laboratories; 1:1,000), Snail (Santa Cruz; 1:200), or anti–β-actin (Sigma; 1:1,000) overnight at 4°C. Bound antibodies were detected by anti-IgG conjugated with peroxidase and developed by enhanced chemiluminescence (Amersham). The density of the bands was quantified by densitometric analysis using the ImageTool (version 3.0) system. Immunoprecipitation was done as previously described (17). In brief, cell lysates (400 μg) were preincubated with 20 μL of 1:1 slurry of protein A/G agarose (Santa Cruz) for 2 h at 4°C, and then immunoprecipitated with anti–β-catenin or anti-Snail antibodies overnight at 4°C. The immune proteins were revealed by Western blotting with anti-E-cadherin or anti-ubiquitin antibodies (DAKO).

**Immunofluorescence microscopy.** The cellular location of proteins was determined by using indirect immunofluorescence. Briefly, cells grown on glass coverslips and transfected with constitutively active p70S6K were fixed in methanol at −20°C for 20 min. Cells were blocked with 5% normal goat serum; incubated with anti–ZO-1 (1:100), anti–E-cadherin (1:1,000), or anti-Snail (1:50); washed twice with PBS; and then incubated with the appropriate FITC-conjugated secondary antibodies. For negative controls, cells were treated with nonspecific control IgG instead of primary antibody. Coverslips were mounted on glass slides with fluorescence mounting medium (Vector Laboratories) and viewed using a Nikon Eclipse E600 fluorescence microscope.

**Secondary interfering RNA transfection.** p70S6K-specific small interfering RNA (siRNA) SMARTpool (1#, 5′-CCAGGCUAACGUGAAGCA-3′; 2#, 5′-CAUGAAGAAUUGGUAGAAA-3′; 3#, 5′-GACAAUUCUCAAAUGUA-3′; 4#, 5′-GCAGAGGUGUUUGAAGAU-3′; refs. 18, 19), Snail-specific siRNA duplex [1#, 5′-GCAGGCGUGACGACCUUA-3′ (20), or #2, 5′-AGGACUUCUACCA-GAGU-3′ (ref. 21)], and a nonspecific duplex oligo (5′-GGTACGTCCAGGAGCCA-3′) used as a negative control were purchased from Dharmacon. We also used green fluorescent protein siRNA duplex (Dharmacon; 5′-UAGGCAGAACAAACAUCAUA-3′) as a control. siRNA at 20 nmol/L was transfected into CaOV-3 or SKOV-3 cells using siLectFect following the manufacturer's instruction (Bio-Rad). At 24 h posttransfection, the cells were harvested for reverse transcription-PCR (RT-PCR), Western blotting, or immunofluorescence microscopy.

**Semiquantitative RT-PCR.** RT-PCR was done as previously described (8). The primer sequences used were E-cadherin, 5′-GATTTGACTGAAAATCAAT-CATT-3′ (sense) and 5′-GGGGGACGAGAATGACGTTT-3′ (antisense); Snail, 5′-TTGCAAGGCACAAATGGACGAG-3′ (sense) and 5′-GCGACTTCGTTCTGTTGGA-3′ (antisense); Slug, 5′-GGCCCTCAACGACGAGAG-3′ (sense) and 5′-AACATGAGCGGAGGAG-3′ (antisense); E-cadherin, 5′-GGCCCTCTGAACCTTGA-3′ (sense) and 5′-GCGAGTAACTCTCCTTGT-CATCCT-3′ (antisense). Amplification was done as follows: denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 45 s. Semiquantitative RT-PCR was conducted using the housekeeping gene β-actin as an internal standard. The number of amplification cycles, during which PCR product formation was limited by template concentration, was determined in pilot experiments.

**Reporter gene assays.** Subconfluent human ovarian cancer cells cultured in six-well plates were transiently transfected with 1.5 μg of the Snail reporter construct (a kind gift of Dr. A. Garcia de Herreros, Universitat Pompeu Fabra, Barcelona, Spain; ref. 22) and cotransfected in the presence or absence of constitutively active p70S6K (ΔN54ΔC104 or ΔE-E398) together with 15 ng of β-galactosidase expression plasmid (pSVβ-gal; Promega), using the Lipofectamine 2000 reagent (Invitrogen). At 24 h after transfection, luciferase activities were assayed according to the manufacturer’s protocol using the Luciferase Reporter Assay kit (Promega). β-Galactosidase activity was used to normalize the transfection. The promoterless luciferase vector (pGL3-Basic) was an internal control. Luciferase units were calculated as luciferase activity/β-galactosidase activity and are presented as mean ± SD of three individual experiments with triplication. The fold change was calculated by comparison with the activity of promoter alone.

**Statistical analysis.** All experiments were done in duplicate or triplicate and repeated at least thrice, with each experiment yielding essentially identical results. Data were expressed as mean ± SD. The statistical significance of differences and associations was analyzed by one-way ANOVA followed by Tukey’s least significant difference test for post hoc analysis or Fisher’s exact test (GraphPad Software). P < 0.05 was considered significant.

**Results**

**p70S6K** activation induces morphologic changes consistent with EMT. The cell morphology and behavior after p70S6K activation were followed by optical microscopy in the absence or presence of constitutively active p70S6K. Figure 1A shows that CaOV-3 and SKOV-3 cells transfected with constitutively active p70S6K (ΔN54ΔC104 or ΔE-E398 refs. 8, 15) lost their epithelial cell morphology; they were dispersed and assumed a fibroblast-like appearance. These changes are typical of cells with a mesenchymal phenotype (Fig. 1A, left). We confirmed the loss of polarity by immunofluorescent staining for ZO-1 (Fig. 1A, right). They also suggest enhanced cell scattering (a response that represents increased cell migration; Fig. 1B, left) and invasive capacity (Fig. 1B, right) compared with untransfected controls. The expression of constitutively active p70S6K was confirmed by Western blot (Fig. 1B, inset).

**p70S6K** activation induces changes in the localization of cellular EMT markers. To determine whether the molecular alternations of an EMT occurred in these cells, we examined the expression of epithelial and mesenchymal markers by immunoblotting. As shown in Fig. 1C, active p70S6K-expressing CaOV-3 cells induced a loss of expression of E-cadherin, indicating a reduction in epithelial marker expression. In contrast, the expression of mesenchymal markers, including N-cadherin and vimentin, was strongly induced. Furthermore, in correlation with our findings from Western blotting, changes consistent with breakdown of E-cadherin–mediated cell adhesion complex were observed by immunofluorescence microscopy (Fig. 1D, left). Despite E-cadherin down-regulation, the levels of total β-catenin were not significantly altered (Fig. 1D, left). The amount of E-cadherin coimmunoprecipitated with β-catenin, however, was reduced by exogenous p70S6K expression (Fig. 1D, right). Similar experiments with SKOV-3 revealed changes in expression indistinguishable from those in CaOV-3 (data not shown).

HGF activates and integrates multiple intracellular signaling pathways involved in cellular migration and invasion, including p70S6K (8–10). The HGF receptor Met is frequently overexpressed or constitutively activated in human ovarian tumors and correlated with increased metastatic potential (8, 23). To further confirm the role of p70S6K on EMT, we inhibited p70S6K using a specific

inhibitor rapamycin or siRNA. The effectiveness of this siRNA to deplete p70S6K expression was confirmed by Western blot (Fig. 2A)

The nonspecific siRNA had no effect. Here we showed that inhibition of p70S6K activity by the inhibitor rapamycin or by transfecting the cells with p70S6K siRNA blocked the migratory activity of HGF (Fig. 2B). Under these conditions, HGF was also unable to repress E-cadherin and gain expression of N-cadherin and vimentin (Fig. 2C). To exclude any possible off-target effects of the p70S6K siRNA, we repeated the experiments with four individual p70S6K siRNA duplexes, and consistent with the observations above, there was a shift in expression of mesenchymal markers to epithelial markers and a decrease in cell scattering and migration (Supplementary Fig. S1). We also included a siRNA that is active against an irrelevant target, such as green fluorescent protein, as a negative control and showed no effect on p70S6K-mediated activities (Supplementary Fig. S1). Together, these data show a critical role of p70S6K activation in the induction of EMT and metastatic phenotypes.

p70S6K activation induces transcriptional up-regulation of Snail. Snail is a zinc finger–containing transcription factor that induces EMT and represses E-cadherin gene transcription (12–14). Because p70S6K inhibited E-cadherin expression, we investigated whether p70S6K up-regulates expression of Snail. As shown in Fig. 3A, analysis by RT-PCR revealed that p70S6K induced an increase in Snail mRNA, which was noted at 4 hours, reaching a peak at 8 hours. The increase in Snail expression correlated with the down-regulation of its target gene, E-cadherin, with a first decline at 8 hours and further reduction gradually afterwards. We also examined expression of Slug, a zinc finger protein that is closely related to Snail and also represses E-cadherin gene transcription. However, our results revealed that p70S6K activation did not affect Slug expression (Fig. 3A), suggesting that Snail was the most prominent E-cadherin transcriptional repressor activated by p70S6K in cells. To further elucidate the involvement of p70S6K in Snail up-regulation, p70S6K was repressed by the use of specific inhibitors. Rapamycin completely blocked HGF-induced Snail expression in cells. Similar to the effect of rapamycin, p70S6K-specific siRNA markedly reduced Snail mRNA. No inhibition was observed for nonspecific siRNA (Fig. 3B).

To test whether the effect of p70S6K on Snail mRNA expression was the result of increased mRNA stability, we performed actinomycin D chase experiments to determine the half-life and
stability of Snail mRNA. Cells were transfected with constitutively active p70S6K for 24 hours. Figure 3C shows that p70S6K did not affect the decay rate of the Snail mRNA (which would be reflected in a decreased slope), showing that increased Snail mRNA level in response to p70S6K is not due to an increase in Snail mRNA stability.

To determine whether the Snail gene is a downstream target of p70S6K, CaOV-3 cells were transiently cotransfected with

![Graph showing the effect of p70S6K on Snail mRNA stability and synthesis.](image)

Figure 3. p70S6K increases Snail mRNA synthesis through modulation of transcription but not mRNA stability. A, CaOV-3 cells were transfected with plasmids encoding ΔNΔC or ΔNΔC for the indicated time periods. Total RNA was extracted and semiquantitative PCR was done using sequence-specific primers to Snail, E-cadherin, and Slug. β-Actin was included as an internal control. The signal intensities were determined by densitometry, and the amount was normalized for the amount of β-actin.

B, CaOV-3 cells were untreated or treated with 10 ng/mL HGF in the presence or absence of 20 nmol/L rapamycin or expression of 20 nmol/L nonspecific siRNA or p70S6K siRNA. Cells were then harvested for semiquantitative PCR with specific primers to Snail. β-Actin served as an internal control.

C, cells untransfected or transfected with plasmids encoding ΔNΔC or D3E-E389 were incubated with actinomycin D (ActD; 4 μg/mL) over a time course of 0, 2, 4, and 6 h. Total RNA was then extracted and reverse transcribed followed by PCR with Snail and β-actin sequence-specific primers. The signal intensity of Snail mRNA was quantified by densitometry, and the amount was normalized for the amount of β-actin present. The Snail mRNA levels before addition of actinomycin D were set to be 100%.

D, cells were transfected with 1.5-kb Snail promoter construct and 15-ng β-galactosidase plasmid. Twenty-four hours after transfection, cells were treated with 10 ng/mL HGF, 20 nmol/L rapamycin, or 20 nmol/L p70S6K siRNA, or transfected with vectors encoding ΔNΔC or ΔNΔC, when indicated, for a further 24 h and harvested. Luciferase and β-galactosidase activities were assayed, and the luciferase activity of each sample was normalized by β-galactosidase activity. The relative luciferase activities were calculated relative to promoter alone, which was arbitrarily assigned a value of 1. Experiments were repeated thrice. Columns, mean; bars, SD.

* P < 0.05, compared with untreated or untransfected controls.
constitutively active p70\textsuperscript{S6K} and a reporter construct in which the human Snail promoter sequence was cloned upstream of the firefly luciferase gene. p70\textsuperscript{S6K} activation increased Snail promoter activity \textasciitilde 3.5-fold (Fig. 3D). Inhibition of p70\textsuperscript{S6K} activity by either rapamycin-treated or p70\textsuperscript{S6K} siRNA–transfected cells significantly decreased Snail promoter gene expression (Fig. 3D), indicating that p70\textsuperscript{S6K} signaling induces transcription of the Snail gene.

**p70\textsuperscript{S6K} induces Snail stabilization and accumulation in the nucleus.** Snail is a highly unstable protein, with a half-life of \textasciitilde 25 minutes (16). Similarly, we found that Snail protein was barely detectable in CaOV-3 cells (data not shown). In contrast, Snail protein was stabilized by expression of p70\textsuperscript{S6K} and the half-life was estimated to be \textasciitilde 2 hours, as deduced from kinetics of Snail degradation after treatment of cells with the protein synthesis inhibitor cycloheximide (Fig. 4A). These results indicate that p70\textsuperscript{S6K} decreases the turnover of Snail. Because Snail can be degraded by the ubiquitin-proteasome pathway, we therefore wished to determine whether p70\textsuperscript{S6K} regulates ubiquitination of Snail. Treatment with the proteasome inhibitor MG132 alone increased the amount of the ubiquinated form of Snail, suggesting that Snail levels are regulated by the ubiquitin-proteasome–dependent proteolysis. Expression of constitutively active p70\textsuperscript{S6K} led to a significant increase of Snail protein and there was less Snail-ubiquitinated species compared with control (Fig. 4B). Furthermore, p70\textsuperscript{S6K} activation decreased accumulation of ubiquitinated forms of Snail stimulated by MG132. These results suggest that p70\textsuperscript{S6K} interferes with the ubiquitin-proteasome degradation pathway to stabilize Snail protein.

Snail function could be affected by its subcellular localization because the cytosolic distribution of Snail inhibits its activity as a transcription repressor (24). We therefore set out to gain additional insights into the regulation of Snail’s subcellular localization by p70\textsuperscript{S6K}. Here immunofluorescent staining showed prominent nuclear localization of Snail when active p70\textsuperscript{S6K} was expressed, whereas inhibition of p70\textsuperscript{S6K} activity with either rapamycin (Fig. 4C) or p70\textsuperscript{S6K} siRNA (data not shown) significantly inhibited HGF-induced increase of Snail in the nucleus, suggesting a role for p70\textsuperscript{S6K} in regulating the location and function of Snail.

**Snail is required for p70\textsuperscript{S6K}-induced EMT.** To assess whether Snail up-regulation was required for p70\textsuperscript{S6K}-induced EMT, we used siRNA to ablate Snail expression. Transfection with Snail siRNA (#1; ref. 20), but not with nonspecific siRNA, revealed a significant decrease in Snail level (Fig. 5A). Importantly, Snail siRNA–transfected cells exhibited a discernible reduction in p70\textsuperscript{S6K}-induced mesenchymal phenotype, indicating that Snail function is required for this process (Fig. 5B). The relocalization of E-cadherin at the plasma membrane confirmed this observation (Fig. 5B). Similar results were obtained with a different siRNA duplex (#2) shown previously to deplete Snail (21), confirming that the effect was Snail specific (Supplementary Fig. S2).

**Activation of p70\textsuperscript{S6K} correlates with up-regulation of Snail in primary cancer tissues.** To investigate whether this regulation also existed in tumor tissues, we compared the levels of phosphorylated (activated) p70\textsuperscript{S6K} and the expression of Snail in tissue samples of human ovarian cancer by immunohistochemistry. Nonspecific IgGs were used as a control to show specificity (Fig. 5C). In accordance with previous reports on p70\textsuperscript{S6K} staining (6), we also found that activated p70\textsuperscript{S6K} was highly expressed in ovarian carcinomas (including 7 of 12 serous, 6 of 10 endometrioid, 2 of 5 mucinous, and 4 of 5 clear cell cancers), with cytoplasmic and nuclear localization (Fig. 5C; Supplementary Table S1). No significant p70\textsuperscript{S6K} activation was observed in normal or benign tissues (Fig. 5C; Supplementary Table S1). Although there was no correlation with the surgical stage of the tumor, high-grade tumors showed strong immunostaining of p70\textsuperscript{S6K} (P = 0.042; Supplementary Table S1). More importantly, we observed that the same ovarian tumor region showing strong expression of activated p70\textsuperscript{S6K} was also expressing Snail (representative results are shown in Fig. 5C; P = 0.008; Table 1), suggesting that the in vitro regulation of Snail expression by p70\textsuperscript{S6K} that we showed in cell cultures might be found in situ in ovarian carcinomas.

**Discussion**

EMT is a critical cellular mechanism during tumor progression and metastasis development and results in enhanced cell motility and invasion. However, few studies have examined the possible role of EMT in ovarian cancer (25, 26). Here we show that p70\textsuperscript{S6K}, which is frequently activated in ovarian carcinoma cells, is a critical mediator of EMT. Furthermore, we have unraveled the mechanism by which p70\textsuperscript{S6K} activates Snail. The combined abilities of p70\textsuperscript{S6K} to increase Snail transcription, prevent Snail degradation, and promote Snail nuclear localization indicate that this kinase plays a central role in regulating Snail expression and thus EMT.

E-cadherin is a cell-cell adhesion molecule and the loss of its expression is a hallmark of EMT (11). Unlike most epithelial cancers, although E-cadherin is almost always present in primary ovarian tumors, it is often scanty or absent in ovarian carcinoma metastases (27). In particular, being the most common serous subtype that exhibits papillary growth, these tumor cells tend to detach from each other and disseminate within the peritoneal cavity (28). Moreover, higher levels of soluble E-cadherin have been reported in ascites and cyst fluids of malignant ovarian tumors than in benign cysts (27). This confirms that the prerequisite of EMT that is closely associated with the loss of E-cadherin expression and the acquisition of invasive phenotype exists in ovarian cancer (29). E-cadherin is known to be a major regulator of β-catenin. We showed that the levels of E-cadherin associated with β-catenin were significantly reduced by p70\textsuperscript{S6K}. Down-regulation of E-cadherin is a possible cause of the reduced levels seen associated with β-catenin. Interestingly, despite the decreased expression of E-cadherin, the levels of total β-catenin were not altered. An increased expression of additional cadherins, such as N-cadherin, may explain this observation. This increase in N-cadherin, which has been observed in some cases of ovarian cancer (29), may also facilitate the actual in vivo metastatic spread by promoting mesenchymal signaling through its interaction with stromal cells.

We report here that p70\textsuperscript{S6K}, a protein kinase linked to protein synthesis, is also involved in transcriptional regulation of the Snail gene. Currently, very little is known about the mechanism by which p70\textsuperscript{S6K} regulates gene transcription, but it has been shown that p70\textsuperscript{S6K} can enter the nucleus (30). Moreover, it can bind to and phosphorylate several transcription factors such as CREM (31) and TRAF-4 (32). Whether these transcription factors participate in the p70\textsuperscript{S6K}-dependent activation of the Snail gene remains to be determined. Interestingly, whereas activation of p70\textsuperscript{S6K} significantly increased the expression of Snail, it had no effect on Slug. This is supported by studies that Slug seems to be regulated through the mitogen-activated protein kinase kinase/extracellular...
signal-regulated kinase pathway (33). Several other EMT inducers, such as Zeb1, SIP1, E12/E47, and TWIST, have also been shown to induce EMT and metastasis through the repression of E-cadherin (34). Whereas Snail is required during the initial invasion stage, Zeb1 and other described repressors are involved in the maintenance of the dedifferentiated and motile phenotype (34). Our data showing that p70S6K induces expression of Snail suggest that p70S6K may regulate early events in ovarian carcinoma dissemination. Whether p70S6K also activates other critical EMT regulators, which have distinct, nonredundant roles from Snail, remains to be explored.

We have also shown a role of proteasome-mediated turnover of Snail by p70S6K as an additional layer of regulation in Snail protein function. Although the signal for the p70S6K-mediated regulation of Snail via the ubiquitin-proteasome pathway is not yet known, phosphorylation at specific amino acid residues could lead to Snail degradation by the 26S proteasome (16). In its capacity as a transcription factor, Snail activity is known to be influenced by its subcellular localization (24). Snail can cycle between the nucleus and the cytosol by virtue of phosphorylation of a serine-rich nuclear export sequence (16, 35). We show that p70S6K can induce the accumulation of Snail in the nucleus. Thus, multiple signaling pathways that control the Snail activity are likely to be regulated by p70S6K. These data substantiate our hypothesis that p70S6K is a critical regulator of EMT and are consistent with the observation that activation of mammalian target of rapamycin in epithelial cells leads to EMT and invasion (36).

The Snail siRNA knockdown experiments presented here clearly indicate that Snail is required for p70S6K-mediated morphologic changes in EMT. It is likely that Snail might also regulate other genes required for the EMT process, in conjunction with E-cadherin down-regulation. In fact, Snail-mediated repression of adherens, tight, and desmosomal junction components has recently been reported (37). Snail can also induce the expression of the mesenchymal genes such as fibronectin and lymphoid enhancer factor 1 (13). Thus, the identification of Snail as a novel target of p70S6K activity that is relevant to the EMT is of considerable interest. It is also relevant to the clinical situation that Snail expression in ovarian carcinomas is associated with a worse prognosis and shows more aggressive biological behavior (28, 38, 39).
and progression. The observation that p70S6K was highly expressed and activated in high-grade, poorly differentiated ovarian adenocarcinomas further suggests that p70S6K activation may be associated with tumor aggressiveness (Supplementary Table S1). Our data are in agreement with the published results (6). These data are also consistent with previous reports that p70S6K inhibition in response to rapamycin treatment markedly reduced ascites formation, the most common pattern of spread of clinical ovarian epithelial cancers (40). More importantly, in line with our observations in cell cultures, p70S6K expression was found to correlate closely with that of Snail in primary ovarian tumor tissues, suggesting that p70S6K may be able to contribute to tumorigenicity through up-regulation of Snail. p70S6K can be translocated to the nucleus on serum or growth factor stimulation (30). It is likely that the accumulation of p70S6K in the nuclei of tumor tissues may reflect constitutive mitogenic activation of this kinase in tumor cells. Moreover, upstream oncogenic alterations, such as PIK3CA (2), may also contribute to increased activation of p70S6K in ovarian cancer. Regardless of the mechanism, the present results strongly suggest a role for p70S6K in ovarian tumor progression, and further studies may shed light on whether p70S6K is a true prognostic factor. It is worth noting that p70S6K activation has also been shown to correlate with malignant phenotypes in patients with advanced breast, colon, and liver cancer (41–43), suggesting that the role of p70S6K in EMT that we propose in ovarian cancer may have broader implications for other tumor cell types.

In summary, the results of this study reveal a new mechanism by which p70S6K activation might promote the aggressive behavior of ovarian carcinoma cells. This mechanism involves p70S6K-mediated up-regulation of Snail. Based on results from our previous (8–10) and present studies, it seems that p70S6K has developed different mechanisms for the invasive growth of ovarian tumor cells. These data highlight the importance of p70S6K as a potential therapeutic target. Inhibition of p70S6K may thus be a useful strategy to impede ovarian cancer cell invasion and metastasis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Table 1. Relationship of coexpression of phospho-p70S6K and Snail in surgical specimens of ovarian cancer

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<td>Negative/low High</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Snail, n (%)</td>
<td>Negative/low</td>
<td>High</td>
</tr>
<tr>
<td>Negative/low</td>
<td>8 (25.0)</td>
<td>6 (18.8)</td>
</tr>
<tr>
<td>High</td>
<td>2 (6.3)</td>
<td>16 (49.9)</td>
</tr>
</tbody>
</table>

Abbreviation: p-p70S6K, phospho-p70S6K.

*The correlation between phospho-p70S6K and Snail was analyzed using Fisher’s exact test.
Acknowledgments

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References


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