
Original Articles

Downregulation of ribosomal protein S6 inhibits the growth of non-small cell lung cancer by inducing cell cycle arrest, rather than apoptosis

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ABSTRACT

Ribosomal protein S6 (rpS6), a component of the small 40S ribosomal subunit, has been found to be associated with multiple physiological and pathophysiological functions. However, its effects and mechanisms in non-small cell lung cancer (NSCLC) still remain unknown. Here, we showed that expressions of total rpS6 and phosphorylation rpS6 (p-rpS6) were both significantly overexpressed in NSCLC. Further survival analysis revealed the shortened overall survival (OS) and relapse-free survival (RFS) in p-rpS6 overexpressed patients and confirmed it as an independent adverse predictor. Stable downregulation of rpS6 in lung adenocarcinoma A549 and squamous cell carcinoma H520 cell lines was then achieved by two specific small hairpin RNA (shRNA) lentiviruses separately. Subsequent experiments showed that downregulation of rpS6 dramatically inhibited cell proliferation in vitro and tumorigenicity in vivo. Moreover, loss of rpS6 promoted cells arrested in G0-G1 phase and reduced in G2-M phase, along with the expression alterations of relative proteins. However, no notable change in apoptosis was observed. Collectively, these results suggested that rpS6 is overactivated in NSCLC and its downregulation suppresses the growth of NSCLC mainly by inducing G0-G1 cell cycle arrest rather than apoptosis.

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Introduction

Lung cancer remains one of the most lethal human cancers worldwide, with 80% being non-small cell lung cancer (NSCLC) [1]. Despite prominent recent advances in the introduction of new therapeutic agents for NSCLC, its outcome is still dismal with a 5-year survival rate of approximately 15% [1]. It is now widely accepted that NSCLC develops from bronchial or alveolar epithelial cells with multiple genetic mutations, which finally leads to dysfunctions of various proteins. Ribosome, consisting of several kinds of RNA (rRNA) and structural proteins, is one of the most important intracellular organelles, with the essential function of synthesizing proteins in accordance with mRNA instructions. Of all known ribosomal proteins, ribosomal protein S6 (rpS6), a component of the 40S ribosomal subunit, is by far the most extensively studied one, and has been functionally implicated in the stimulation of protein translation efficiency, mainly by combining with the mRNAs rich in 5′-terminal oligopyrimidine tracts (TOP mRNAs) [2,3]. Although still in controversy, the most recognized mechanism for rpS6 activation is the mTOR-dependent phosphorylation at the evolutionary conserved C-terminal serine residues of Ser235, Ser236, Ser240, Ser244 and Ser247, in response to different growth factor stimulations [2–5]. Importantly, the biological effect of rpS6 phosphorylation was found by the persistent small size of embryonic fibroblasts from the rpS6 knock-out mice, in which all rpS6 phosphorylatable serine residues were substituted by alanines [6]. Further studies provided more
evidence to stress the rpS6 phosphorylating as a critical event in the cell survival, glycometabolism and many other bioactivities [2,7,8]. In line with the physiological effects, anomalous overactivation of rpS6 is frequently observed in numerous tumors [9–15], especially phosphorylated at the site of Ser235/236 [11,13–15]. Studies even showed that rpS6 dephosphorylation suppressed the proliferation of pancreatic cancer [14] and breast cancer [16] cell lines. Moreover, the antiproliferative efficacy of mTOR inhibitors that prevent the phosphorylation of rpS6 is currently being evaluated as a new treatment for several benign and malignant tumors [17,18]. These suggest that rpS6, mainly with the phosphorylated activated form, might be a powerful biomarker in tumors. However, an obvious inverse finding that the high level of rpS6 phosphorylation indicated a low mouse embryonic cell proliferation was also observed to contradict the traditional viewpoint [2]. As for NSCLC, the expression, biological functions and basic mechanisms of rpS6 are even little known. Here, in this study we firstly detected the expressions of total rpS6 and its phosphorylation (p-rpS6, Ser235/236) in NSCLC clinical tissues via immunohistochemical (IHC) stainings and Western blot assays. Their clinical relevance and prognostic impacts on the overall survival (OS) and relapse-free survival (RFS) in NSCLC patients were evaluated as well. Secondly, two small hairpin RNA (shRNA) lentiviruses specifically silencing rpS6 were constructed to stably inhibit the expression of rpS6/p-rpS6 in adenocarcinoma A549 cell line and squamous cell carcinoma H520 cell lines. Thirdly, the effects of rpS6 on the growth and senescence of NSCLC cell lines were detected both in vitro and in vivo, followed by mechanisms explorations, including cell cycle distribution and cell apoptosis.

Methods

Patient information and tissue specimens
A cohort of 131 surgically resected primary NSCLC patients and their paraffin-embedded archived tumor samples, together with 68 adjacent normal lung tissues, were collected in the Department of Pathology of West China Hospital of Sichuan University (WCHU), Chengdu, China from 2006 to 2007, for the immunohistochemical stainings (IHC) for total rpS6 and p-rpS6 (Ser235/236). Clinical information was obtained by chart review and the survival data were procured by telephone interviews. In agreement with the WHO classification for NSCLC and histological gradings and clinical stages were determined according to the WHO classification for NSCLC and the International Union Against Cancer’s tumor–node–metastasis system [19–21]. There were 50 squamous cell carcinomas (SCC), 62 adenocarcinomas (ADC) and 19 other types, mainly including adenosquamous carcinomas and large-cell carcinomas in the study. Another group of 36 fresh NSCLC tissues and controls from the tumor resection surgeries in the Department of Thoracic Surgery of WCHU in 2009 were also gathered up for the Western blot analysis, to confirm the results of IHC. All patients in the whole study were adjudged therapy-free before surgeries. Prior approval from the Ethics Committee of Sichuan University, China and patients’ consents were obtained as well.

Immunohistochemical (IHC) stainings

Envision immunohistochemical staining kit (DAKO) was used for the IHC detections [22]. Total rpS6 (CST, #2217, 1:100 dilution) and p-rpS6 (Ser235/236, CST, #4858, 1:200 dilution) were performed for the patients’ specimens and xenografted tumors; while Ki-67 was used in the xenografted tissues (CST, #9027, 1:400 dilution). The known colorectal cancer tissues were utilized as positive controls; whereas a negative control was obtained by replacing the primary antibody with phosphate buffered saline (PBS) (0.01 mol/l, pH 7.4). IHC images were read semiquantitatively, including both the staining intensities and positive cells’ proportions. Ten fields were randomly selected at 200× magnification. The intensities were classified into four levels from 0 to 3. That was 0 indicating invisible positive stainings, 1 for weak stainings, 2 for moderate and 3 for strong stainings. The proportion of positive cells was scored from 0 to 3 as well, which meant that 0 indicated none of the tumor cells positively stained, 1 for less than 20% positive cells, 2 for 20% to 50% positive cells and 3 for over 50% positive cells. Scores for the intensity and proportion were multiplied to get the IHC scores. In the final statistical analysis, scores of 0–2, including the completely negative ones or slightly weak expressions, were classified as negative; whereas scores of 3–9 were divided into the positive group (3–5 scores for moderate and 6–9 scores for strong expressions).

Western blot

Western blot for the total proteins from patients’ tissues, xenografted tumors and cultured cells was carried out according to the procedures described previously [23]. Controls were referred to β-actin (CST, #4970, 1:1000 dilution). The main antibodies in Western blot included total rpS6 (CST, #2217, 1:1000 dilution), p-rpS6 (Ser235/236, CST, #4858, 1:2000 dilution), p16INK4A (CST, #2407, 1:1000 dilution), p21WAF1 (CST, #2947, 1:1000 dilution), p27KIP1 (CST, #3968, 1:1000 dilution), and p53Trans (CST, #2557, 1:1000 dilution), total Rb (CST, #9013, 1:1000 dilution), p-Rb (CST#780, CST, #3590, 1:1000 dilution), cyclin D1 (CST, #2978, 1:1000 dilution), cyclin A (CST, #4656, 1:2000 dilution), cyclin E (SAB, #29030, 1:1000 dilution), Bax (CST, #2764, 1:1000 dilution), Bcl-xl (CST, #5023, 1:1000 dilution), caspase 3 (CST, #9665, 1:1000 dilution), brpase 8 (CST, #4927, 1:1000 dilution) and cyt-c (CST, #111904, 1:1000 dilution). Expressions of each protein were analyzed with the Biometrics digitized image software and recorded as integrated densities (ID). Ratios of ID(unknown)/ID β-actin were the final results for the protein expressions.

Cell lines and cell culture

NSCLC cell lines, including A549 for adenocarcinoma, H520 for squamous carcinoma and H460 for large cell carcinoma, and human bronchial epithelial cell line (HBE) purchased from the American Type Culture Collection (ATCC) were used in the study. As per the manufacturers’ instructions and previous reports [24,25], NSCLC cell lines were maintained in RPMI-1640 (HyClone, USA) supplemented with fetal bovine serum (FBS, Gibco, USA), 2 mmol/l-glutamine, 1000 U/ml penicillin and 1 mg/ml streptomycin (LifeTech, UK). HBE was cultured in keratinocyte-serum free medium with 5 mg/ml human recombinant EGF and 0.05 mg/ml bovine pituitary extract and supplemented with 0.005 mg/ml insulin, 500 ng/ml hydrocortisone, penicillin and streptomycin (LifeTech, UK). All cells were grown in a sterile humidified incubator with 5% CO2 at 37°C.

Construction of small hairpin RNA (shRNA) lentivirus and cell transfection

The lentiviral packaging plasmid of pGG3 with the marker of green fluorescent protein (GFP) was bought from Genechem, Shanghai, China. The two targeted rpS6 knockdown sequences and their negative control (NC) were 5′-GCCAGAATGTGCTAACATT3′ (sh1), 5′-TGAAACGCAAACTTCGCTACT-3′ (sh2) and 5′-TTCCTCCGACCTGTCAGCT-3′ respectively. Cell transfections and the stable-transfected clones’ selections were conducted as previous researches [26,27]. Briefly, cells were seeded as 150,000 cells/well on six-well culture dishes until 30% of confluence, and then transfected with lentivirus at the optimal multiplicity of infection (MOI). The GFP under fluorescence microscopy was observed to demonstrate the progression of transfections. Nearly five days after the initial transfection, cells were collected for the selection by fluorescence activated cell sorting (FACS) to obtain the stably transfected cells.

Quantitative real-time PCR (RT-PCR) analysis

Total RNA was abstracted using the RNAsimple Total RNA Kit (Tiangen, China) as described by the manufacturer. β-actin was used for controls. Genomic DNA was amplified by real-time RT-PCR using the specific rpS6 gene amplifying primers. The forward and reverse PCR primers for rpS6 were 5′-CCTATTTCCTCAAGTACCTTCA-3′ and 5′-GAATTACCTGATTCACCCG-3′ respectively; whereas for β-actin the forward primer was 5′-CCATCACTCATCCCTCGAG-3′, and reverse was 5′-ATGATGCTTCCACCATG-3′. Samples were amplified in an RT-PCR System (Applied Biosystems) as the following steps: denaturation at 94°C for 30 s, cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 30 s another 72°C for 6 min. The final relative expressions of rpS6 mRNA were compared in 2−ΔΔCt [27,28].

Xenografted tumor model

The experiments involving animals were also approved by the Ethics Committee of Sichuan University, China. Only A549 with rpS6 knockdown by the first shRNA was selected for the xenografted tumor model. Six to eight week-old BALB/c-nu/nu nude female mice 18–22 grams in weight were obtained from the Animal Centre of Sichuan University, China. Each of them was injected with 3 × 106 cells subcutaneously in the right flank and divided into the three groups: A549 with sh1-rpS6 (A549 + sh1-rpS6), A549 with sh-NC (A549 + sh-NC) and A549 without any interference (A549). There were six mice in each group. The xenografted tumors were measured by calipers for the dimensions twice a week after 7 days of injection. Growth curves of the xenografted tumors were measured by calipers for the dimensions twice a week after 7 days of injection. Volumes were calculated using the formula V = length × width2 for the tumor growth curves. At the end of the experiment (on the 28th day after the cell implantation), all mice were euthanized to measure the tumor weights and other parameters, such as the expressions of p-p rpS6/p-rpS6, Ki-67 and TUNEL in paraffin-embedded tissues [28]. The rest of xenografted tissues were stored at -80°C until use.
Cell proliferation assays

Cell proliferation in vitro was detected with the Cell Counting Kit-8 (CCK-8, Dojindo, Japan). Briefly, cells were cultured in 96-well plates repeated six times for full adherent. Then cells were treated with CCK-8 for 1–2 hours at the time points of 24 h, 48 h, 72 h, 96 h, 120 h and 144 h after the initial seeding. Cell absorbance at 450 nm was measured with a microplate reader (Thermo Scientific, USA) [30].

IHC staining of Ki-67 was performed to determine the proliferation rates of xenografted tumors in vivo, which was the same as the IHC protocol described above.

Cell cycle analysis

Collected cells were washed with cold PBS and fixed by 70% ethanol at 4 °C overnight. Then the staining buffer with 1 mg/ml PI and 10 mg/ml RNase A (Sigma, USA) was used to incubate the cells at room temperature for 30 min in the dark. Flow cytometry equipped with CXP software Modifit LT 3.1 (BD, USA) was applied to determine the percentage of cells in different phases of cell cycles [31].

Cell cycle distributions in vitro and in vivo were both confirmed by the Western blot assays for the relative proteins, including p16Ink4A, p21Cip1, p27Kip1, p57Kip2, total Rb, p-Rb (Ser780), cyclin D1, cyclin A and cyclin E.

Apoptosis analysis

Apoptosis prevalence in vitro was tested with the stains of Annexin V-APC/7AAD (Becton Dickinson) and FACS detections. All cells in complete culture medium were plated into 24-well plates overnight, and then harvested with EDTA-free trypsin for the incubation with Annexin V-APC and 7AAD. FACS and FlowJo software were applied for the Annexin V-APC detections with excitation of 633 nm and emission of 660 nm; whereas for the 7-AAD positive cells, the excitation and emission were 546 nm and 647 nm. Only the Annexin V positive but 7AAD negative cells were considered as apoptotic cells [32].

Xenografted tumors in vivo were measured by TUNEL (terminal deoxynucleotidyl transferase mediated dUTP nick end labeling) assay with the In situ Cell Death Detection kit (TMR red, Roche, Shanghai, China), which was aimed at the detections of the free 3′OH DNA ends in early apoptotic cells. Sections from paraffin-embedded xenografted tumors were reacted with TdT and TMR-dUTP after the permeabilization of 0.1% Triton X-100. TMR-dUTP would attach with the 3′OH ends in DNA catalyzed by TdT to produce red fluorescence. Moreover, cellular nuclei were stained with DAPI (4′, 6-diamidino-2-phenylindole) in blue. The final apoptotic rate was the ratio of the red cells to the total cells with DAPI staining [33].

Senescence-associated β-galactosidase (SA-β-gal) staining assessment

Senescence-associated β-galactosidase kit (Beyotime, Shanghai, China) was used for the SA-β-gal staining according to the manufacturer’s recommendations. Cells in different groups were separately seeded in 6-well plates with $5 \times 10^4$/well. After two days of culture, the medium was discarded and cells were rinsed by PBS. One milliliter of fixative was then added to each well for fixing for 15 min. Washed cells were subsequently reacted with the working solution of β-galactosidase with X-Gal for incubating at 37 °C overnight. The senescent cells with positive staining were observed in an optical microscope and counted from ten random fields of vision to calculate the positive rate.

Frozen xenografted tumor tissues were brought out to prepare the frozen sections. Tissues were cut for 4 μm by cryotome and mounted onto positively charged glass slides on dry ice. Then sections were fixed in the fixative at room temperature for 15 min. After rinsing with PBS, sections were incubated with the fresh SA-β-gal staining solution overnight at 37 °C. A standard light microscope was used to count the SA-β-gal positive cells.

Fig. 1. Expression and clinical significance of rpS6 and p-rpS6 in NSCLC. A. IHC analysis in 131 paraffin-embedded human NSCLC tumors showed the overexpressions of total rpS6 and p-rpS6 (Ser235/236) in tumor cytoplasm, both in adenocarcinomas (ADC) and squamous cell carcinomas (SCC); while the protein expressions in normal controls were much weaker ($\times 200$). B. Western blot assays in another 36 NSCLC patients’ tissues confirmed the upregulation of rpS6 and p-rpS6 (Ser235/236) in NSCLC. C. Compared with HBE (human normal bronchial epithelia), rpS6 and p-rpS6 (Ser235/236) were also elevated in NSCLC cell lines for different histopathological types. And the expressions in A549 (lung adenocarcinoma) and H520 (lung squamous carcinoma) were much stronger than those in H460 (large cell lung carcinoma). D. Kaplan-Meier survival curves illustrated the overall survival (OS) among NSCLC patients with the expressions of rpS6 and p-rpS6. RpS6 did not influence the OS for patients (left); while p-rpS6 (Ser235/236) overexpression indicated a significantly unfavorable prognosis (right). E. Relapse-free survival (RFS) among NSCLC patients on the basis of rpS6 and p-rpS6 expressions. RFS in patients with high expression of p-rpS6 (Ser235/236), rather than rpS6, was significantly short than those with low expression.
Univariate Cox regression analysis of rpS6/p-rpS6 expressions and other clinicopathological covariates for the overall survival and relapse-free survival of NSCLC patients.

**Table 2**

<table>
<thead>
<tr>
<th>Covariates</th>
<th>Positive expressions of rpS6</th>
<th>Positive expressions of p-rpS6</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>n (%)</td>
<td>P values</td>
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<tr>
<td>Gender</td>
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<tr>
<td>Male (n = 102)</td>
<td>75 (73.5)</td>
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<tr>
<td>Female (n = 29)</td>
<td>26 (98.7)</td>
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<td>&lt;60 (n = 55)</td>
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<tr>
<td>≥60 (n = 76)</td>
<td>60 (78.9)</td>
<td>0.001</td>
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<td>Histological types</td>
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<td>ADC (n = 62)</td>
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<td>SCC (n = 50)</td>
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<tr>
<td>Other (n = 19)</td>
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<td>Histological differentiation</td>
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<td>Poor (n = 47)</td>
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<td>Tumor size/cm</td>
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<td>T1 (n = 17)</td>
<td>15 (95.3)</td>
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<tr>
<td>T2 + T3 + T4 (n = 114)</td>
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<td>0.001</td>
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<td>Lymph node invasion</td>
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<td>N1 + N2 + N3 (n = 61)</td>
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<td>Distant metastasis</td>
<td>M0 (n = 121)</td>
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<td></td>
<td>M1 (n = 10)</td>
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<td>Clinical stage</td>
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<td></td>
<td>II + III + IV (n = 84)</td>
<td>68 (81.0)</td>
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</table>

ADC, adenocarcinoma; SCC, squamous cell carcinoma. *P < 0.05.

**Results**

RpS6 and p-rpS6 are significantly overexpressed in NSCLC specimens and p-rpS6 upregulation indicates unfavorable OS and RFS

Based on the previous findings that Ser235/236 was the frequent residue for rpS6 phosphorylation in tumors [11,13,14], we assessed the expressions of total rpS6 and p-rpS6 (Ser235/236) in 131 NSCLC tissues and 68 adjacent normal lung tissues by IHC. As shown in Fig. 1A, NSCLC tissues were rich in rpS6 and p-rpS6, specifically in tumor cell cytoplasm; whereas normal tissues were much less frequently detected. The positive rate of rpS6 was significantly higher in NSCLC than that in controls (82.4% vs. 55.8%, P < 0.001). A similar result for p-rpS6 was also found, with 62.6% in NSCLC and 35.3% in controls (P < 0.001). Western blot analysis in another 36 fresh NSCLC tissues and controls confirmed the upregulation of rpS6 and p-rpS6 in NSCLC tissues (Fig. 1B, all P < 0.010). Further detections in different cell lines, including A549 (lung adenocarcinoma), H520 (lung squamous carcinoma), H460 (large cell lung carcinoma) and HBE (human normal bronchial epithelia), provided more evidence of the elevation of rpS6 and p-rpS6 in NSCLC (Fig. 1C, all P < 0.05), though certain distinctions were also observed (Fig. 1C). Details of the relationships between rpS6/p-rpS6 expressions and patients’ clinical characteristics were summarized in Table 1. Only the high level of p-rpS6 was strongly associated with poor tumor histological differentiation (P = 0.047), lymph node invasion (P < 0.001) and intermediate or advanced clinical stage (P < 0.001). The results suggested that p-rpS6 might be abnormally overactivated in NSCLC, rather than simply overexpressed.

To determine the clinical significance of rpS6/p-rpS6 in NSCLC, the OS and RFS for NSCLC patients with the expressions of rpS6 and p-rpS6 were analyzed by Kaplan–Meier method and compared by log-rank test. Subjects with a high expression of p-rpS6 had much shorter median OS and RFS time than those with a low expression (Fig. 1D and E, right; 10 months vs. 60 months; 21 months vs. 49 months, respectively; both P < 0.001), whereas cases with
positive or negative expressions of total rpS6 did not show significantly different OS or RFS (Fig. 1D and E, left; 30 months vs. 43 months; 26 months vs. 40 months, respectively; both \( P > 0.05 \)). In the following univariate Cox proportional-hazard regression analysis, the high expression of p-rpS6, as well as poor tumor histological differentiation, progressive TNM stage and free from chemotherapy or biotherapy, was shown as the risk factor for OS and RFS in NSCLC patients (Table 2, all \( P < 0.05 \)). Multivariate Cox model including the above covariates confirmed the positive expression of p-rpS6 and distant metastasis as independent risk factors for OS (Table 3; hazard ratio: 5.129 and 2.044, respectively; both \( P < 0.05 \)); while biotherapy was a protective factor (hazard ratio: 0.333, \( P < 0.001 \)). Regarding RFS, overexpression of p-rpS6 and advanced clinical stage were independent risk factors and biotherapy showed protective effects as well (Table 3; hazard ratio: 3.064, 4.693 and 0.434, respectively; all \( P < 0.010 \)).

RpS6 and p-rpS6 are significantly silenced in NSCLC cell lines by shRNA lentivirus

Based on the prominent expressions of rpS6 and p-rpS6, adenocarcinoma A549 and squamous cell carcinoma H520 cell lines were selected for the follow-up cytological and animal experiments. The two shRNA lentiviruses with rpS6 targeted inhibition (sh1-rpS6 and sh1-rpS6) and their negative control (NC) were constructed to transfect the NSCLC cell lines. Fig. 2A demonstrated the strong expressions of labeling green fluorescence protein (GFP) in stably transfected cells, which were then selected by FACS. Subsequently, the suppression efficacies were evaluated by RT-PCR and Western blot separately. The expressions of rpS6 mRNA drastically reduced in sh-rpS6 A549 and H520 cell lines (Fig. 2B, \( P < 0.001 \)) and showed a similar efficacy for the two target sequences (Fig. 2B, \( P > 0.05 \)). Correspondingly, the protein profiles of rpS6 and p-rpS6 in sh-rpS6 A549 and H520 cells were also decreased by nearly 90.0% (Fig. 2C, all \( P < 0.001 \)). But there was almost no difference in the blank cells and NC groups (Fig. 2B and C, all \( P > 0.05 \)), suggesting an ideal interference without any influence from the lentiviruses vector.

Downregulation of rpS6 significantly inhibits the growth and promotes senescence of NSCLC both in vitro and in vivo

CCK-8 assay displayed that although the proliferation rates of two rpS6 silenced A549 cells were basically the same as the controls for the first 48 hours after the initial seeding (Fig 3A, left; at 24 h and 48 h, all \( P > 0.05 \)), they were finally remarkably suppressed from the point of 72 h to the end (Fig. 3A, left; at 72 h, 96 h, 120 h and 144 h, all \( P < 0.001 \)). The proliferation rates of the A549 and NC groups were always similar to each other (Fig. 3A, left; all \( P > 0.05 \)). Similar growth suppression was also observed in the two downregulated H520 cells, even only after 48 hrs from the cell culture (Fig. 3A, right; at 48 h, 72 h, 96 h, 120 h and 144 h, all \( P < 0.001 \)).

Cellular senescence, which is characterized by an increased activity of SA-β-gal, is generally associated with the cells' proliferative capacity. In this study, as shown in Fig. 3B, the positive staining for SA-β-gal in the two rpS6-silenced A549 cells was approximately 50% (51.0% ± 7.3%; 45.6% ± 6.8%), much more than that in the two controls (8.9% ± 2.9%; 7.4% ± 3.2%; all \( P < 0.05 \)). Additionally, the senescent cells were enlarged and flattened in morphology. Likewise, the number of SA-β-gal positive cells also increased in the knockdown H520 cells (all \( P < 0.05 \)).

Because of the similar results for the two specific silence sequences in A549 and H520 cell lines in vitro, only the first transfected A549 cells (A549 + sh1-rpS6) were used to develop a xenograft mouse model to detect the effects of rpS6 downregulation in tumorigenic abilities in vivo. Curves for tumor growth in volumes revealed the distinctly slower tumor formations in the rpS6 knockdown group (Fig. 3C, right; all \( P < 0.001 \)). Importantly, one of the sh-rpS6 A549 mice came up with a palpable tumor nodule even after 21 d with inoculation. Extracted tumors in the rpS6 silenced group also weighed much less than the two controls (Fig. 3C, left and middle; both \( P < 0.001 \)). The follow-up H&E detections with rpS6 and p-rpS6 for the xenografted tissues not only confirmed the effective rpS6/p-rpS6 downregulation in cell proliferation in vivo (Fig. 3D, left; all \( P < 0.001 \)), but also exhibited the malignant histological features of the xenografted nodules, that was with clear hyperchromatic nuclei, high nucleocytoplasmic ratio and salient mitosis. Further Western blot assays for the xenografted tissues revealed the stable loss of rpS6/p-rpS6 in the knockdown group as well (Fig. 3C, right; all \( P < 0.001 \)). In addition, Ki-67 staining was performed to evaluate the cell proliferation in vivo. As shown in Fig. 3E, cells with positive nucleus expression of Ki-67 were dramatically reduced in the sh-rpS6 group, compared with those in the A549 and NC groups (20.5% ± 3.9% vs. 79.2% ± 5.6% \( P < 0.001 \); 20.5% ± 3.9% vs. 76.2% ± 7.6% \( P < 0.001 \); respectively).

SA-β-gal staining was also employed to detect the xenografted tissue senescence. The number of SA-β-gal-positive cells was nearly threefold higher in the rpS6 downregulated group than that in the two controls (36.6% ± 4.9%, 9.8% ± 1.0%, 8.4% ± 0.7%, respectively; both \( P < 0.010 \)).

The above concordant results in vitro and in vivo confirmed the important role of rpS6 in NSCLC growth. However, its underlying mechanism was still unknown, which inspired us to conduct the follow-up explorations.

The cell growth inhibition in rpS6 downregulated cells is greatly associated with cell cycle arrest, but not apoptosis inducing

Cell cycle redistribution and apoptotic regulation are generally considered as the primary mechanisms in cell survival, growth and proliferation. From the flow cytometry analysis for cell cycle distribution (Fig. 4A), we found that rpS6 downregulation dramatically increased the number of A549 cells in the G0-G1 phase (73.6% ± 3.6% vs. 52.7% ± 4.2% for the comparisons between A549 + sh1-rpS6 and A549, \( P < 0.001 \); 71.6% ± 3.0% vs. 52.7% ± 4.2% for A549 + sh2-rpS6 and A549, \( P < 0.001 \)) and decreased cells in the G2-M phase (13.6% ± 2.1% vs. 22.4% ± 2.6% for A549 + sh1-rpS6 and A549, \( P = 0.003 \); 13.7% ± 2.4% vs. 22.4% ± 2.6% for A549 + sh2-rpS6 and A549, \( P = 0.003 \)), along with the reduction in S phase (12.8% ± 1.6% and 14.6% ± 1.8% for the two knockdown groups; 25.0% ± 6.8% for A549, \( P = 0.026 \) and 0.048 respectively). Similar alterations were also found in H250 cells for G0-G1 and G2-M phases (all \( P < 0.05 \)), although the change in the S phase did not reach statistical significance (all \( P > 0.05 \)). Western blot for the cell cycle relative proteins was conducted subsequently. As expected, levels of phosphorylation Rb (p-Rb) and cyclin D1 were remarkably reduced in sh-rpS6 cells, accompanied by
significant increase of p16Ink4A, p21Cip1, p27Kip1 and p57Kip2 (all \( P < 0.05 \)). However, no alteration of cyclin A, cyclin E and total Rb was observed (Fig. 4B, left; all \( P > 0.05 \)). Consistently, similar results appeared again in the xenografted tissues (Fig. 4B, right).

Moreover, flow cytometry analysis for apoptotic cells in vitro showed that though the apoptosis rate in the two rpS6 silenced A549 cells (3.6% ± 0.7%; 3.1% ± 0.7%) seemed to increase from the controls (3.0% ± 1.0%; 2.7% ± 0.8%), no statistical significance was proved (Fig. 5A, all \( P > 0.05 \)). For the xenografted tissues, TUNEL assay revealed the slight increase of apoptotic cells (red staining) in sh-rpS6 tissues without any significance neither (Fig. 5B, 11.3% ± 2.4% vs. 8.5% ± 2.0% for A549, \( P = 0.611 \); 11.3% ± 2.4% vs. 9.3% ± 2.7% for NC, \( P = 0.610 \); respectively). Relative protein expressions, such as Bcl-xl, Bax, caspase 3, caspase 8 and cyto-c, did not change either. Collectively, these results suggested that downregulation of rpS6 predominantly induced the cell cycle arrest in G0-G1, but did not effectively promote cell apoptosis.

**Discussion**

RpS6 is key to protein synthesis and has been reported to be involved in cell size and survival [2]. In this study, we demonstrated that the total rpS6 and its phosphorylation form p-rpS6 were both significantly upregulated in NSCLC tissues from patients. However, further analysis revealed a probably more important effect of p-rpS6 than rpS6, because only the high level of p-rpS6 was primarily related to the poor differentiation, lymph node metastasis and intermediate or advanced clinical stage. In particular, we also found that the hyperphosphorylation of rpS6 predicted shortened OS and RFS in NSCLC patients, and it was a powerful independent prognostic factor. Such results indicated that rpS6 might be mainly overactivated, rather than merely overexpressed in NSCLC. It would have certain instructional implications for future work in rpS6 targeted interventions. Consistent with our results, studies in oral cavity carcinoma [9], esophageal squamous cell carcinoma [10], chronic
myelogenous leukemia [34] and hepatocellular carcinoma [35] also revealed the vital roles of rpS6 phosphorylation.

How does rpS6/p-rpS6 work in NSCLC? Due to the lack of commoditized specific inhibitor for rpS6 phosphorylation so far, two specific shRNA lentiviruses were used to knock down the expression of total rpS6 in adenocarcinoma A549 and squamous cell carcinoma H520 cell lines respectively, which led to the corresponding decrease of p-rpS6 inevitably. As shown by the CCK-8 assay and xenografted experiments in immunocompetent mice, rpS6 silence led to a slow cell growth, as well as a weak tumorigenicity, suggesting a possible crucial role of rpS6 in promoting NSCLC proliferation and growth. This is supported by the study that loss of rpS6 phosphorylation with p70S6K suppression caused a growth inhibition in breast cancer cells [16]. Recent research in acute myeloid leukemia and prostate cancer also showed the p-rpS6 dependent cell survival and growth [36,37]. Data from another NSCLC study revealed the elevated expressions of AKT substrates, including rpS6, in cetuximab-resistant NSCLC clones. Further inhibition of AKT signaling by siAKT1/2 or the allosteric AKT inhibitor MK-2206 put a brake on the cell proliferation in all cetuximab-resistant clones, together with the reduced expressions of rpS6 and p-rpS6 [38]. These results provided strong evidence that rpS6 plays a crucial role in cell growth. However, mice embryonic cells surprisingly showed a slower proliferation with an elevated level of p-rpS6 [2]. This is so far the only opposite report. Such inconsistency might be roughly attributed to the cell species disparities and seems to hardly challenge the current accepted roles of rpS6 in most cases.

During the process of cell culture, we observed that cells with rpS6 knockdown displayed enlarged and flattened morphological characteristics, which were represented features of the senescence-associated differentiation. So SA-β-gal activity, an important marker of senescence, was examined to verify the predominant senescence existence for rpS6 silence. The distinct influence of rpS6 phosphorylation on cellular aging/senescence was also found in previous studies [39,40] to support the active role of rpS6 in senescence regulation.

In the follow-up experiments, specific ways by which rpS6 exerts biological functions in NSCLC were explored. Eukaryotic cell growth and proliferation are largely through cell mitosis, with controls from various regulatory proteins. Flow cytometry analysis for cell cycle distributions in vitro demonstrated the notable accumulation of G0/G1 cells and synchronous reduction of G2-M cells in the sh-rpS6 groups. Interestingly, remarkable reduction in S phase was only found in the A549 rpS6 knockdown cells, without any significant alteration in H520 cells. We speculate that such discordant results might partly be due to the essential biological characteristics of the two NSCLC cell lines. A549 is for lung adenocarcinoma and H520 is derived from a patient with lung squamous cell carcinoma. It is probable that proteins involved in the S phase in the two types of NSCLC are not fully identical and ones related to A549 cells cell cycle distribution are much more likely to be dependent on the regulation of rpS6. Additionally, the elevation of G0-G1 phase cells in A549 interfered cells was much greater than that in H520 cells, which possibly contributes to the notable decrease of the A549 cells in the S phase as well. In accordance with our present findings, breast cancer cells were also found to be arrested in G1 phase to some extent by the rpS6 dephosphorylation [16]. It is possible that cell cycle redistribution is the primary way in which rpS6 affects the cell growth. Further Western blot assays for cell cycle relative proteins were performed to prove the inference. Rb is a well-known tumor suppressor gene encoding the Rb protein as a transcription factor. It is a truth generally acknowledged that phosphorylation or dephosphorylation is the dominant approach for Rb to regulate cell growth and differentiation. To be specific, Rb phosphorylation greatly facilitated G2-M phase progression, whereas its dephosphorylation induced G0-G1 arrest, with the regulation of the p16ink4a/cyclin D1-Rb signaling pathway [41,42]. There are several other potent cyclin-dependent kinase inhibitors (CKI), such as p21waf1, p27kip1 and p57kip2, and cyclins involved in the cell cycle regulations. As expected, in our study the Western blot assays for the cells in vitro and xenografted tissues showed the distinct downregulation of p-Rb and cyclin D1, together with elevated p16ink4a, p21waf1, p27kip1 and p57kip2 with rpS6/p-rpS6 inhibition. The slight changes in cyclin A, cyclin E and total Rb in H520 cells might be presumably explained by the inconspicuous alterations of S phase, because they were all physiologically associated with the S phase DNA synthesis. Indeed, the close relationship between rpS6/p-rpS6 was also proved in chondrosarcomas by the genome-wide array-comparative genomic hybridization (CGH) [43] and in synchronized mammalian cells by RT-PCR [44]. Another study even demonstrated that the phosphorylation of rapamycin-resistant rpS6 at ser240/244 was induced via serum factors in a cell cycle-dependent manner [45]. Taken together, our findings confirmed that the mechanism of rpS6-mediated cell growth was greatly associated with the cell cycle redistributions.

Tests for the prevalence of apoptotic cells were conducted as well in our study. Detections both in vitro by Annexin V-APC/7AAD staining and in vivo with TUNEL assay revealed a little increase of apoptosis with rpS6 knockdown, but neither of them reached a statistical significance. However, controversial results have been observed. The inhibition of rpS6 induced apoptosis in K-ras mutation mouse [46]. To more complicate matters, Jeon et al. performed a functional genetic study in several tumor cells to elucidate the relationship between the expression/phosphorylation of rpS6 and the apoptosis evoked by TRAIL (tumor necrosis factor-related apoptosis-inducing ligand, as a potent inducer of apoptosis in tumor cells). Reduction of rpS6 expression in Jurkat (acute T cell leukemia cell line), HeLa (cervical cancer) and SKHeP-1 (hepatocarcinoma) cells attenuated apoptosis induced by TRAIL. Consistently, the apoptosis sensitivity to TRAIL was increased by the ectopic expression of wild-type rpS6. However, regarding the phosphorylation status, low level or deficiency in rpS6 phosphorylation increased the TRAIL-induced apoptosis, except for the phospho-mimic rpS6 mutant. They concluded that rpS6, especially in its unphosphorylated form, was a selective mediator of TRAIL-induced apoptosis [47]. Considering the inconsistent results above, including our present study, the exact role of rpS6 in apoptosis seems to be bafflingly complicated for further in-depth study and we roughly believe the essential characteristic disparities of different tumors might be another reason for the discrepant results.

In summary, our study suggests that rpS6 is excessively activated as p-rpS6 in NSCLC. Downregulation of rpS6 greatly inhibits...
Fig. 4. Downregulation of rpS6 greatly promotes the G0-G1 cell cycle arrest. A. The PI staining and FACS detection in vitro showed a significant increase of G0-G1 cells in the rpS6 downregulated A549 and H520 groups, correspondingly decreased in G2-M cells. B. Western blot assays for the cell lysates in vitro revealed the expected overexpressions of p16Ink4A, p21Cip1, p27Kip1, p57Kip2, downregulation of p-Rb and cyclin D1 in the rpS6 silenced groups, though no remarkable alteration was found in cyclin A, cyclin E and total Rb (left). The same alternations were found from the xenografted tissues in vivo as well (right). Data were expressed as the mean ± SD for the six repetitions. *: vs. A549, P < 0.05; #: vs. A549 + NC, P < 0.05.
Fig. 5. Downregulation of rpS6 scarcely induces cells apoptosis. A. Annexin V-APC/7AAD with FACS showed no obvious change in apoptosis prevalence in vitro with the inference of rpS6, neither in A549 nor H520 cells. Q4 (positive in Annexin V and negative in 7AAD) indicated apoptotic cells. B. TUNEL assay for the xenografted tissues revealed no alteration of apoptosis in vivo either (×400, arrows). All nuclei were stained in blue with DAPI, and the apoptotic nuclei are showed in red with TUNEL detections. The merged images demonstrated the apoptotic cells. C. Western blot detected no remarkable change in relative proteins, including Bcl-xl, Bax, caspase 3, caspase 8 and cyto-c neither in vitro (left) nor in vivo (right). Data were expressed as the mean ± SD for the six repetitions. *: vs. A549, P < 0.05; #: vs. A549 + NC, P < 0.05. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
the cell growth, tumorigenicity and senescence mainly by inducing G0-G1 cell cycle arrest, rather than apoptosis. Full understanding of the precise effects of rpS6 in NSCLC may provide the opportunity to develop a novel therapeutic strategy by suppressing the expression and phosphorylation of rpS6 in NSCLC.

Conflicts of interest

No potential conflicts of interest were disclosed.

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