Response gene to complement-32 promotes cell survival via the NF-κB pathway in non-small-cell lung cancer

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Abstract. Response gene to complement (RGC)-32 regulates the cell cycle in response to complement activation. The present study demonstrated that the expression level of RGC-32 is higher in human non-small-cell lung cancer (NSCLC) tissues compared with health controls. Overexpressing RGC-32 induced p65 nucleus translocation, significantly increased nuclear p65 levels and promoted the proliferation of A549 cells. Knockdown of RGC-32 by short hairpin RNA decreased the expression level of nuclear p65 and inhibited cell proliferation. The increase in cell proliferation induced by RGC32 could be abolished by the NF-κB inhibitor pyrrolidine dithiocarbamate. Mechanistic studies indicated that RGC32 mediated NF-κB downstream genes, including vascular cell adhesion protein 1, interleukin-6, cyclin dependent kinase inhibitor 2C, testin and vascular endothelial growth factor A. In summary, the present study demonstrated a novel role of RGC-32 in the progression of NSCLC via the NF-κB pathway and p65. Therefore, RGC-32 could be a potential therapeutic target for NSCLC.

Introduction

Cancer is one of the leading causes of mortality worldwide and lung cancer is the highest cause of mortality among all cancer types (1-5). Various strategies have been used to combat lung cancer (6-8). For example, cisplatin-based compounds have been widely used as therapeutics (9,10). However, long-term exposure to cisplatin can induce drug resistance, which compromises the therapeutic intervention. Revealing and understanding the fundamental mechanisms of lung cancer is essential for modifying current methods and improving

treatment. Therefore, there is a requirement to identify new targets to improve the therapeutic index from current therapeutics.

Multiple-factors mediate the tumor microenvironment including inflammation, immune, hemostasis/coagulation and metabolism, which serves a critical role in the generation and progression of lung cancer (11,12). The inflammatory response to various infections and injuries is a critical cause of cancer through the initiation of molecular and epigenetic regulatory mechanisms (13).

The nuclear factor-κB (NF-κB) family is a family of transcription factors that serve a key role in the regulation of inflammation (14). NF-κB regulates cell growth, differentiation, proliferation and apoptosis (15). The NF-κB complex includes p65, RelB, c-Rel, p50 and p52 (16,17). p65 is activated by kinase-mediated phosphorylation and is translocated into the nucleus to mediate gene expression levels (18,19).

Response gene to complement (RGC)-32 is a previously identified molecule that regulates the cell cycle in response to complement activation (20,21). RGC-32 may serve a critical role in the control of the cell cycle, complement-mediated inflammation, tumor metastasis, cell proliferation, migration and tubulointerstitial fibrosis (22-25). RGC-32 is widely expressed in various organs, including the liver, skeletal muscle, heart, brain, kidney, pancreas, vessels and placenta (26). RGC-32 gene methylation is reported to be associated with the development of lung cancer (27). However, the mechanism on how RGC-32 mediates non-small-cell lung cancer (NSCLC) cell proliferation remains unclear.

The present study demonstrated that RGC-32 is involved in the progression of lung cancer by regulating the NF-κB/p65-mediated inflammatory pathway. Therefore, RGC-32 is a potential target for effective lung cancer therapy.

Materials and methods

Human samples (date range: 2016.1-2018.12). A total of 17 male (age range, 48-68 years) and six female (age range, 51-69 years) patients with NSCLC (adenocarcinoma or squamous carcinoma) were recruited to the present study (Table I). The study was performed according to the International Ethical Guidelines for Biomedical Research Involving Human Subjects published by The Council for International

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Organizations of Medical Sciences and was approved by the Institutional Review Board of Shiyan Taihe Hospital, Hubei University of Medicine. Written informed consent was obtained from all participating individuals. Lung tissues specimens were collected during intraoperative and postoperative examinations with informed consent from the patients or their family members.

Immunohistochemistry. The pulmonary cancer tissue samples obtained from patients with lung cancer were sliced for the immunohistochemistry assay. The cancer tissue and normal adjacent tissue samples were fixed in 4% paraformaldehyde for 24 h at room temperature and embedded in paraffin. The tissue was then sliced to a thickness of 5-μm. Following rehydration and antigen retrieval, sections were blocked with 5% goat serum (Sigma-Aldrich; Merck KGaA) for 1 h at room temperature, permeabilized with 0.01% Triton X-100 in PBS and incubated with RGC-32 antibody (cat. no. 4445000; Novus Biologicals, LLC; 1:100) overnight at 4˚C. Following incubation with horseradish peroxidase-conjugated secondary antibody (cat. no. 111-055-003; Jackson ImmunoResearch Laboratories, Inc.; 1:200) for 30 min at room temperature, the sections were counterstained with hematoxylin for 2 sec at room temperature. Cross-sectional images were captured with a Nikon light microscope at room temperature (magnification, x200).

Construction of adenovirus (Ad) expression vectors for RCG-32 and short hairpin (sh)RCG-32. Construction of RGC32 overexpression or short hairpin RNA (shRNA) adenoviral vector were prepared as previously described (22). shRGC-32 was designed by OriGene Technologies, Inc. The shRNA sequences were as follows: 5'-CTGAATTCTCCAACAGACT-3'. A vector expressing RGC-32 was constructed as described previously (22) and cells were transfected with Ad-RGC32 or Ad-shRGC32 for 48 h [10^{-10} PFU/ml; 100 multiplicity of infection (MOI)], according to the manufacturer's protocol. Under a fluorescent microscope, it was determined that >95% of the cells were transfected with different MOIs (Ad-RGC32 or Ad-shRGC32) to determine the optimal MOI. Ad-RGC32 was purified by gradient density ultracentrifugation with cesium chloride.

Cells proliferation assay. A549 cells (cat. no. CCL-185; American Type Culture Collection) and H460 cells (kindly provided by Stem Cell Bank, Chinese Academy of Sciences) were cultured in DMEM (Gibco; Invitrogen; Thermo Fisher Scientific, Inc.) containing 10% FBS (Thermo Fisher Scientific, Inc.). Cells were transfected with either Ad-RGC32 or Ad-shRGC32 for overexpression or knockdown of RGC32. NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC) was used to evaluate the effect of RGC32 overexpression. The transfection of adenoviral vector (Null) was used as a control. Briefly, the cells were seeded into a 96-well plate at a density of $2x10^3$ cells/well and incubated for 24 h at 37˚C in 5% CO$_2$ incubator. After transfection, cells were cultured for 24, 48 or 72 h for a time-dependent study. Subsequently, the culture medium was discarded and the wells were washed twice with PBS, followed by the addition of 10 μl Cell Counting Kit-8 reagent (cat. no. CK04; Dojindo Molecular Technologies,

Inc.) or 10 μl MTT dye (0.5 mg/ml; Beijing Solarbio Science & Technology, Co., Ltd.) to each well, respectively. The cells were incubated for a further 2 h at 37˚C. The samples were then detected at 450 nm using a Microplate reader (Bio-Rad Laboratories, Inc.). Each treatment was replicated and each experiment was repeated 3-5 times.

Immunofluorescence assay. To observe NF-κB p65 nuclear translocation, A549 cells transfected with Ad-Null, Ad-RGC32 or Ad-shRGC32 were rinsed with PBS and fixed with 4% paraformaldehyde for 20 min at room temperature, followed by blocking with 5% goat serum in 0.01% Triton X-100 in PBS for 1 h at room temperature The cells were then incubated with anti-NF-κB p65 antibody (cat. no. sc-109; Santa Cruz Biotechnology, Inc.; 1:100) overnight at 4˚C, followed by tetramethylrhodamine-conjugated secondary antibody (cat. no. 711-585-152; Jackson ImmunoResearch Laboratories, Inc.; 1:200) for 1 h at room temperature. The cellular nucleus was then counterstained with DAPI (Sigma-Aldrich; Merck KGaA) for 10 min at room temperature. Cells were subsequently imaged using fluorescence microscopy (magnification, x400).

Nuclear protein extraction. CHEMICON® Nuclear Extraction kit (EMD Millipore) was used. Briefly, A549 cells ($4x10^4$ cells/well) were washed with cold PBS buffer, followed by incubation with cytoplasmic lysis buffer. The whole lysis solution was centrifuged at 4˚C at a speed of 1,800 x g for 5 min and the cellular pellet was further incubated with nuclear extraction buffer. The supernatant was then used for western blot analysis.

Western blotting analysis. The lung tissues were ground in liquid nitrogen and homogenized on ice with RIPA buffer containing protease inhibitors (Beyotime Institute of Biotechnology, China). Protein concentration was measured using a bicinchoninic acid protein assay kit. Subsequently, 20 μg protein from tissues and whole cell lysates was subjected to 12% SDS-PAGE and transferred to PVDF membranes (Bio-Rad Laboratories, Inc.). The blots were blocked with 5% fat-free milk at room temperature for 1 h by shaking and then incubated with rabbit anti-RGC32 (cat. no. 4445000; Novus Biologicals, LLC; 1:500) and rabbit p65 (sc-109; Santa Cruz Biotechnology, Inc.; 1:1,000) at 4˚C overnight, followed by incubation with horseradish peroxidase-conjugated secondary antibodies (cat. no. 111-035-003; Jackson ImmunoResearch Laboratories, Inc.; 1:200) at room temperature for 1 h by shaking. The immunized blots were then visualized and analyzed with chemiluminescence reagent (Novex™ ECL Chemiluminescent Substrate Reagent kit; cat. no. WP20005) and Image J 1.51 software (National Institutes of Health).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from cells using TRIzol reagent (Roche Diagnostics GmbH). RNA (1 μg) was reverse transcribed with a RevertAid RT Reverse Transcription kit (cat. no. K1691; Thermo Fisher Scientific, Inc.) to amplify the genes using the following temperature protocol: 25˚C for 5 min, 42˚C for 60 min and 70˚C for 5 min. The primers used for qPCR were as follows: Vascular cell adhesion protein 1 (VCAM1) forward, 5'-CCTGAGCCCTGTGAGTTTTG-3' and reverse,

Table I. Information of patients included in the present study.

Patients, n	Sex	Age range, years	Pathology	Stage
17	Male	48-68	Squamous carcinoma Adenocarcinoma	IIB-IIIA
6	Female	49-69	Squamous carcinoma Adenocarcinoma	IB-IIIA

A total of 17 male and 6 female patients with non-small-cell lung cancer were recruited to this study. The patients were selected based on their age, pathological analysis and tumor stage.
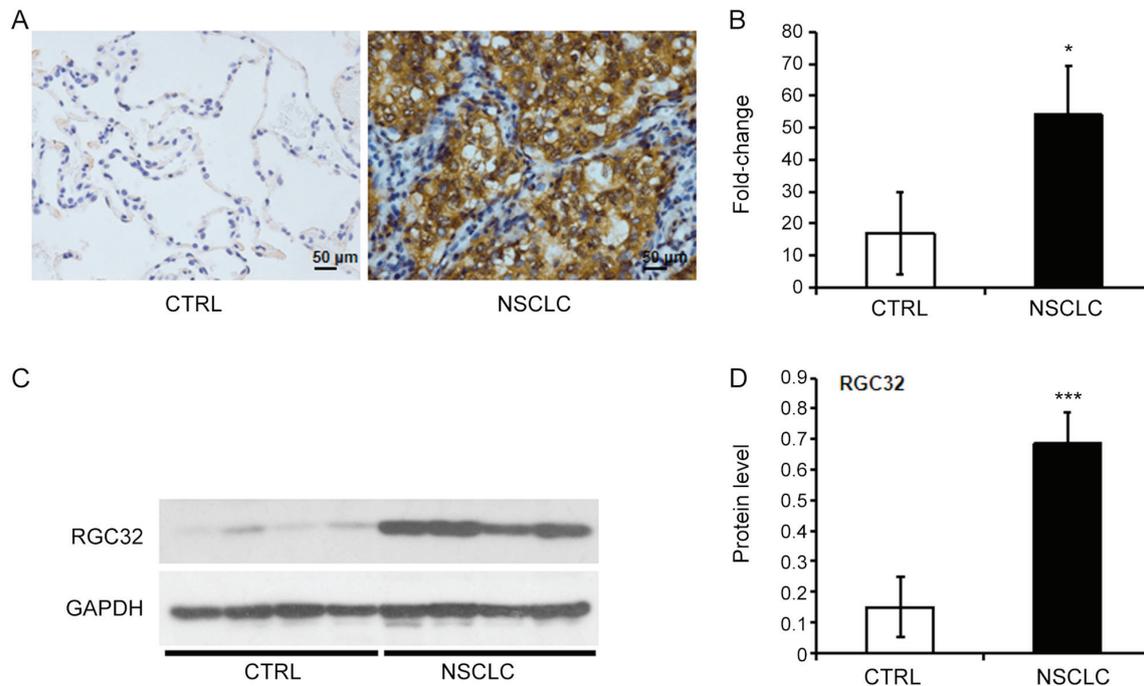
Figure 1. NSCLC tissues exhibit a higher RGC32 expression level. (A) Representative image of immunohistochemistry. (B) Semi-quantification of the immunohistochemistry assay revealed a higher expression level of RGC32 in NSCLC tissues compared with the normal lung tissues. n=23. (C) Representative immunoblot image of RGC32 expression in the normal control and NSCLC lung tissues. (D) Quantification of the western blot analysis of RGC32 expression level. n=3; *P<0.05 and ***P<0.001 vs. CTRL. NSCLC, non-small-cell lung cancer; RGC32, response gene to complement-32; CTRL, control.

5'-GGGTACACGCTAGGAACCTT-3'; interleukin-6 (IL-6) forward, 5'-AGTCCTGATCCAGTTCCTGC-3' and reverse, 5'-CTACATTTGCCGAAGAGCCC-3'; vascular endothelial growth factor A (VEGFA) forward, 5'-GAACTTTCTGCTGTC TTGGG-3' and reverse, 5'-CTTCGTGATGATTCTGCCCT-3'; cyclin dependent kinase inhibitor 2C (CDKN2C) forward, 5'-CCCGATTTGAAAGACCGAAC-3' and reverse, 5'-TCA CCAGGAACTCCACCACC-3'; and TES forward, 5'-AAG AAGATGGGCTTAGGTC-3' and reverse, 5'-TTTTGCAAT CAGAGTGGTA-3'. All qPCR reactions (cat. no. 04913850001; Roche Diagnostics GmbH) were performed in triplicate with an ABI-7000 PCR detection machine (28). Denaturation was performed at 95°C for 10 min, followed by 40 cycles at 95°C for 30 sec and 30 sec annealing at 60°C.

Statistical analysis. The results are expressed as the mean ± standard error of the mean. A Student's t-test was used for comparisons between two groups and one-way analysis of variance was used for comparisons of more than two groups. Significance was confirmed by post-hoc analysis using Fisher's

least significant difference test. P<0.05 was considered to indicate a statistically significant difference. Each experiment was repeated three times.

Results

NSCLC tissue exhibits a high expression level of RGC-32. Immunohistochemistry staining of tissues derived from patients and healthy controls demonstrated that RGC-32 was specifically expressed in the NSCLC tissue (Fig. 1A). Semi-quantification of the image revealed that the expression of RGC-32 was 5.2-fold higher in NSCLC tissue compared with normal tissue (Fig. 1B). Western blot analysis demonstrated that the protein expression level of RGC-32 was significantly increased in NSCLC tissues compared with normal tissues (P<0.001; Fig. 1C and D). These data suggest that RGC-32 is highly expressed in NSCLC.

RGC-32 promotes lung cancer cell proliferation. To determine the role of RGC-32 in human NSCLC cells, RGC-32 was either overexpressed or knocked down in A549 cells and
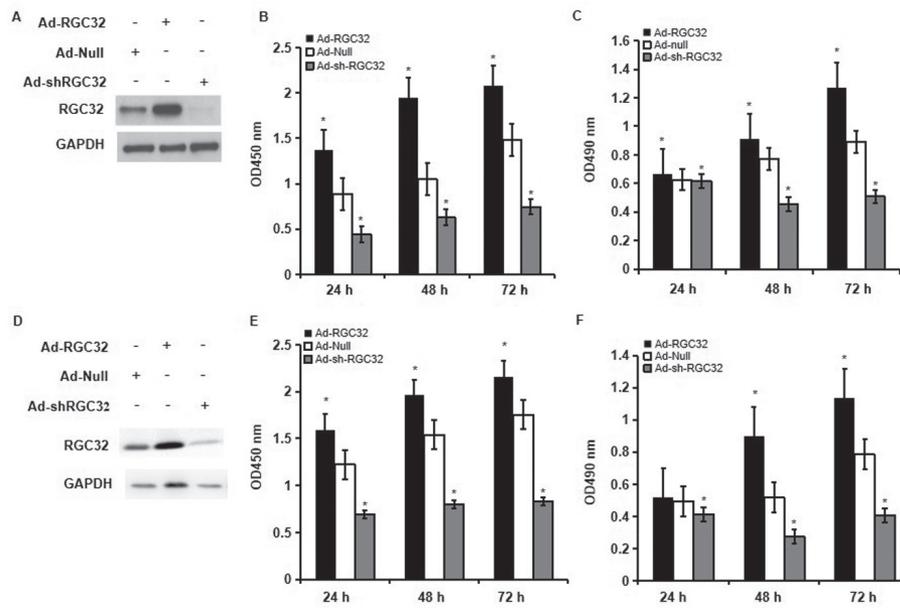
Figure 2. RGC32 promotes the proliferation of human non-small-cell lung cancer cells. Western blot analysis of the RGC-32 protein expression level in (A) A549 and (D) H460 cells following transfection with either Ad-RGC32 or Ad-shRGC32 vectors for 48 h. Overexpression of RGC32 promoted (B) A549 and (E) H460 cell proliferation, as determined by Cell Counting Kit-8 assay. *P<0.05 vs. Ad-Null. Overexpression of RGC32 promoted (C) A549 and (F) H460 cell proliferation as determined by MTT assay. *P<0.05 vs. Ad-Null. RGC32, response gene to complement-32; Ad, adenovirus; sh, short hairpin; OD, optical density.

H460 cells. The results revealed that RGC-32-knockdown in both cell lines significantly inhibited proliferation compared with the control cells (P<0.05; Fig. 2). In addition, the effect of RGC-32 overexpression on the proliferation of A549 and H460 cells was examined by CCK-8 assay, which revealed that overexpression of RGC-32 significantly increased A549 and H460 cell proliferation (P<0.05; Fig. 2B and E). An MTT assay was used to evaluate the effect of RGC-32 on the proliferation of NSCLC cells and similar results were obtained (Fig. 2C and F). These data indicate that RGC-32 is a novel inducer of NSCLC cell proliferation.

RGC-32 promotes the proliferation of lung cancer cells via NF-κB. NF-κB promotes tumor growth and activation of NF-κB is often identified in cancer, including human hepatocellular carcinoma (29). To determine whether the NF-κB signaling pathway mediates RGC-32 function in A549 cell prolifera-tion, the NF-κB inhibitor PDTC was used to treat A549 cells expressing RGC-32. As presented in Fig. 3, overexpression of RGC-32 increased A549 cell proliferation in a time-dependent manner. A significant increase in cell proliferation was observed after incubation for 48 h with Ad-RGC32 (P<0.05). However, the NF-κB inhibitor PDTC markedly inhibited the effect of Ad-RGC32 on A549 cell proliferation and significant differ-ences were identified at the 48-h time point compared with the untreated RGC32-overexpressing control cells (P<0.05). These results suggest that NF-κB is critical in regulating RGC-32-associated A549 cell proliferation.

RGC-32 activates NF-κB by promoting the nuclear translocation of p65. Due to the involvement of NF-κB in RGC-32-induced cell proliferation, the present study further investigated the role of RGC-32 in NF-κB expression. Translocation of NF-κB from the cytoplasm to the nucleus is critical for the activation of downstream
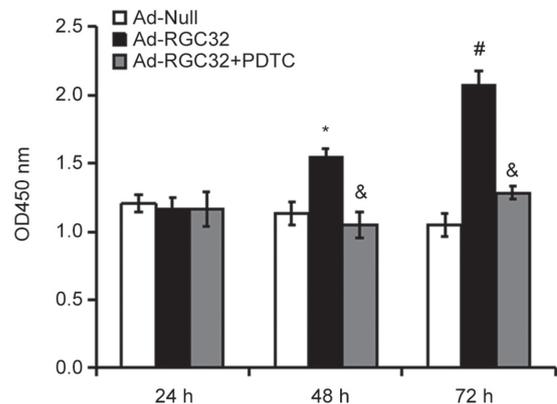
Figure 3. RGC32-induced A549 cell proliferation involves NF-κB. RGC32 enhanced A549 cell proliferation at the indicated times-point, which could be reversed by the NF-κB inhibitor (100 μM PDTC). n=6; *P<0.05 vs. 24 h control group; #P<0.01 vs. 48 h control group; &P<0.01 vs. Ad-RGC32 group. RGC32, response gene to complement-32; Ad, adenovirus; PDTC, pyrrolidine dithiocarbamate; NF, nuclear factor; OD, optical density; PDTC, pyrrolidine dithiocarbamate.

target genes (30). Thus, the NF-κB translocation behavior was examined. Immunofluorescence staining demonstrated that RGC-32 overexpression increased p65 nucleus translocation compared with the control group and RGC32 knockdown reduced p65 nuclear translocation (Fig. 4). Furthermore, western blot analysis revealed that the nuclear expression level of p65 protein in RGC-32-overexpressing cells was significantly increased compared with the control cells (P<0.05) and the increased level of RGC32 in the Ad-RGC32 group was also confirmed (Fig. 5). Knockdown of RGC32 by shRNA significantly decreased the nuclear p65 level (P<0.05; Fig. 5). These results indicate that RGC-32 could regulate A549 cell proliferation by activating p65.
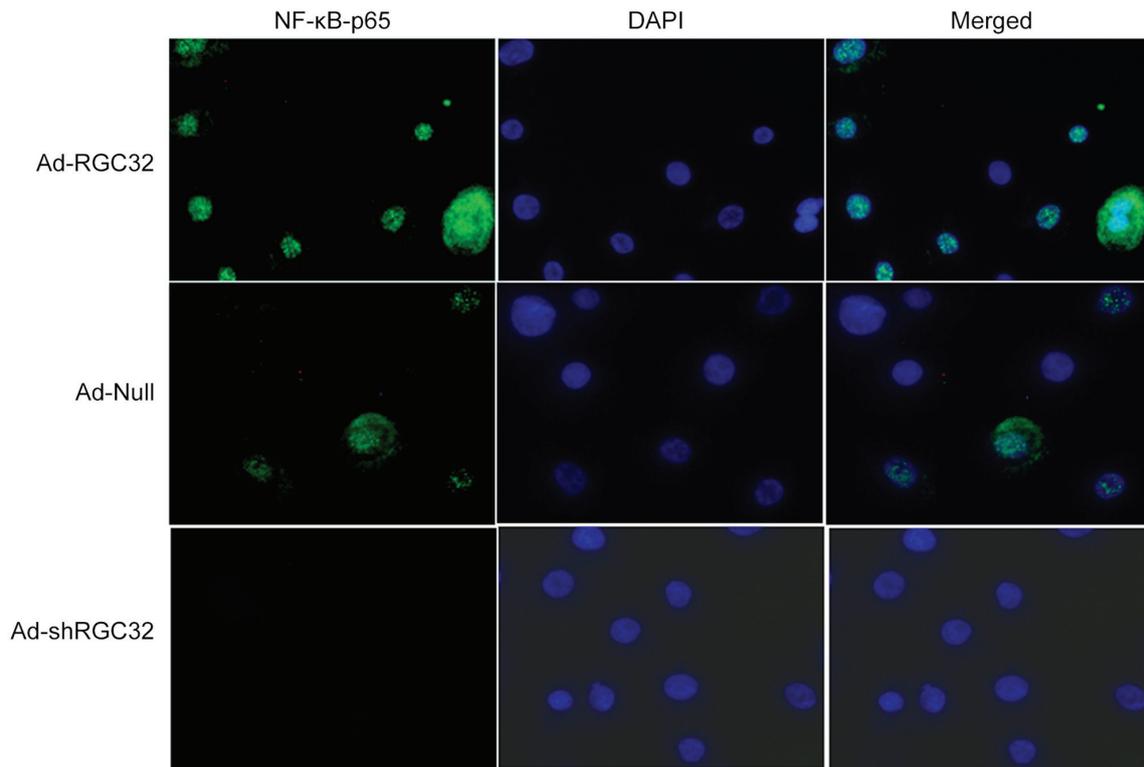
Figure 4. Representative images of p65 nuclear translocation in Ad-RGC32 and Ad-shRGC32-treated A549 cells. Immunofluorescence staining was performed to evaluate the p65 expression level. DAPI was used to stain nuclei. RGC32, response gene to complement-32; Ad, adenovirus; sh, short hairpin; NF, nuclear factor; DAPI, 4,6-diamidino-2-phenylindole.
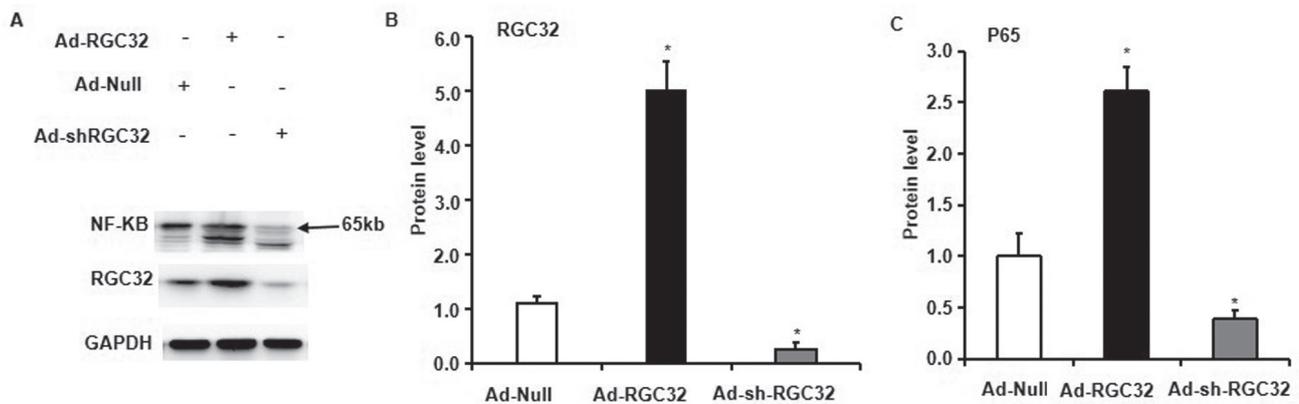
Figure 5. Overexpression of RGC32 increases the nuclear NF-κB levels in A549 cells and knockdown of RGC32 decreases the NF-κB levels. (A) Western blot analysis was used to determine the expression levels of RGC32, NF-kB p65 and GAPDH in A549 cells expressing RGC32, A549 cells with RGC32-knockdown and non-treated control cells. Quantification of (B) RGC32 and (C) NF-kB p65 expression levels from the western blot results. n=3; *P<0.01 vs. Ad-Null group. RGC32, response gene to complement-32; Ad, adenovirus; sh, short hairpin; NF, nuclear factor.

RGC-32 regulates gene expression levels downstream of NF-κB p65. Active NF-κB is able to control the transcription of various target genes, which mediates the expression of inflammatory cytokines, chemokines and cell adhesion molecules (31). The present study examined target genes of NF-κB, including IL-6, VCAM1, VEGFA, CDKN2C and TES. As presented in Fig. 6, VCAM1, VEGFA and IL-6 mRNA levels were significantly increased in the Ad-RGC32 group (P<0.05). By contrast, knockdown of RGC32 by shRNA decreased the expression levels. Meanwhile, the expression levels of CDKN2C and TES, a cell cycle inhibitor (32-34), were

decreased in Ad-RGC32 cells expressing RGC32 compared with the untreated control cells. Knockdown of RGC32 by shRNA increased CDKN2C and TES mRNA levels. Thus, the findings suggest that RGC-32-driven tumor growth is associated with genes downstream of NF-κB p65.

Discussion

RGC-32 is a previously identified molecule that acts in response to complement activation (20). RGC-32 is a cell-cycle modulator and RGC-32 overexpression has been reported in
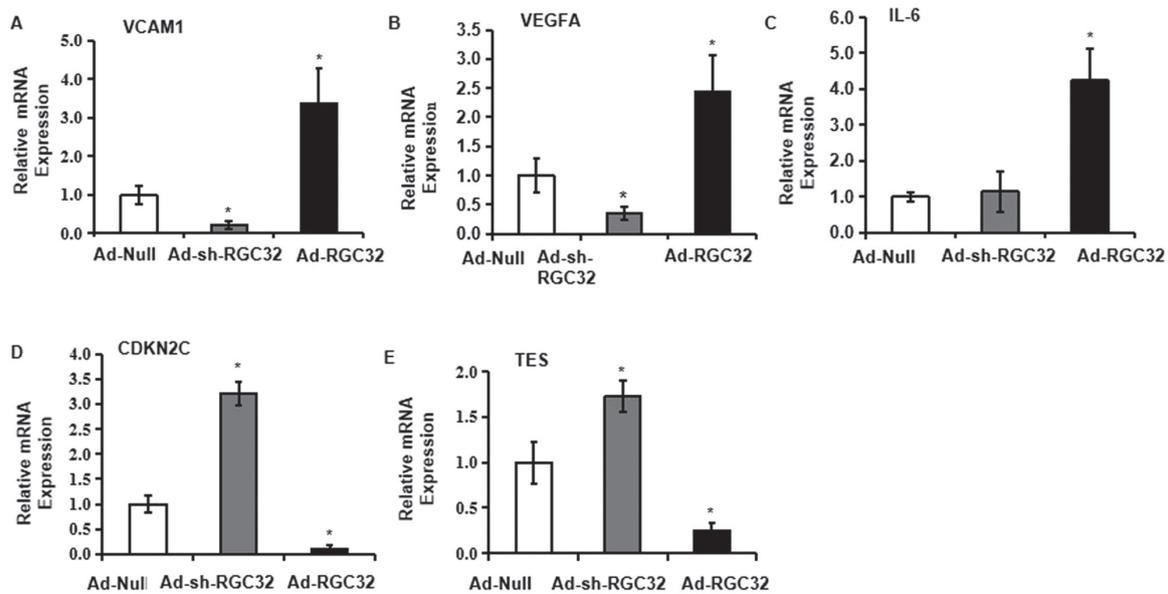
Figure 6. RGC-32 regulates the downstream genes of NF-κB p65. The expression levels of NF-κB downstream genes (A) VCAM1, (B) VEGFA, (C) IL-6, (D) CDKN2C and (E) TES in A549 cells following treatment with Ad-RGC32 or Ad-shRGC32, as determined by reverse transcription-quantitative polymerase chain reaction. n=3; *P<0.01 vs. Ad-Null group. RGC32, response gene to complement-32; VCAM1, vascular cell adhesion protein 1; VEGFA, vascular endothelial growth factor A; CDKN2C, cyclin dependent kinase inhibitor 2C; IL-6, interleukin-6; sh, short hairpin; NF, nuclear factor; Ad, adenovirus; sh, short hairpin.

breast and colon cancers (35,36). Highly expressed RGC-32 activates the cell cycle by regulating CDC2 (35). The epigenetic modulation of RGC-32 (promoter methylation) is reported to be significantly associated with the prognosis of patients with lung cancer (27). The present study identified that RGC-32 was overexpressed in human lung cancer tissue compared with normal controls and knockdown of RGC-32 inhibited the activity of lung cancer cells, which is consistent with the previous study by Xu *et al* (37). Thus, RGC-32 could be used as an important marker for lung cancer.

Inflammation targeting endothelial cells in the pulmonary tract could act as the most common cause of various lung diseases, including lung cancer (13). NF-κB activation following an inflammatory response contributes to abnormalities of the pulmonary tract (38). NF-κB is a major factor of inflammation and serves a critical role in the progression of lung cancer (39). Therefore, it is important to identify the upstream factors associated with the NF-κB pathway that regulate lung cancer development. RGC-32 has been reported to stimulate epithelial-mesenchymal transition in lung cancer cells via the NF-κB signaling pathway (39). Similarly, the present study demonstrated that RGC-32 accelerated the translocation of p65 into the nucleus. Hence, activation of NF-κB/p65 by RGC-32 could play an important role in lung cancer development.

In addition, NF-κB/p65 can regulate downstream genes associated with cancer development, including VCAM1, IL-6, CDKN2C, TES and VEGFA (40-42). In the present study, VCAM1, VEGFA and IL-6 mRNA levels were increased in RGC32-overexpressed A549 cells. By contrast, knockdown of RGC32 by shRNA reduced the expression levels. CDKN2C and TES, cell cycle inhibitors (32-34), exhibited decreased mRNA levels in Ad-RGC32-treated A549 cells, and knockdown of RGC32 by shRNA increased CDKN2C and TES

mRNA levels. Therefore, RGC-32 could regulate lung cancer growth by regulating the expression of genes downstream of NF-κB p65. The physical interaction of RGC32 with NF-κB has been confirmed by a co-immunoprecipitation assay (unpublished data). These results suggest that RGC32 may be associated with NF-κB both functionally and physically.

In conclusion, RGC-32 may be a novel and specific marker of NSCLC. RGC-32-mediated NSCLC development has been demonstrated to involve p65 activation. Therefore, RGC-32 may be a new target for preventative and immuno-pharmacological treatments of NSCLC.

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

JZ carried out main human sample collection and detection, cell experiments and drafted the manuscript. JL carried out qPCR, cell proliferation assays and data evaluation. LY participated in the immunostaining and protein assays. RW

participated in the design of the study and manuscript writing. JY conceived of the study, participated in the experimental design and helped to draft the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The current study was approved by the Institutional Review Board of Shiyan Taihe Hospital, Hubei University of Medicine. Written informed consent was obtained from all participating individuals.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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