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The Critical Role of Rab31 in Cell Proliferation and Apoptosis in Cancer Progression

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Abstract Rab31, a member of the Ras superfamily, is reported to play a role in tumor development and progression. However, the detailed role of Rab31 in proliferation and apoptosis of cancer cells is still unclear. Here, we used different cell lines, such as glioblastoma, and cervical cancer, to investigate the role of Rab31 in cancer progression. We found that Rab31 promotes U87 and SiHa cell proliferation via activation of G1/S checkpoint transitions, accompanied with an increase of cyclin D1, cyclin A, and cyclin B1. Meanwhile, Rab31 inhibits U87 and SiHa cell apoptosis, and decreased the BAX and PIG3 expression, but enhanced BCL2 expression. In addition, Rab31 induces N-cadherin, Vimentin, and Snail expression, and inhibits E-cadherin expression to regulate proliferation and migration. Besides, we observed that ERK1/2 and PI3k/AKT pathways are required for Rab31-induced cell proliferation and migration. In vivo, the knockdown of Rab31 suppresses tumor mass growth. In conclusion, our data highlight the crucial role of Rab31 in cancer progression, proliferation, and apoptosis, and indicates that Rab31 may be a useful and effective target for the clinical therapy of most cancers.

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Introduction

Rab31 acts as a member of Rab family, consisting of 194 amino acids, which is derived from human platelets [1–3]. As reported, the high expression of Rab31 in breast cancer tissues renders cell proliferation robust [4]. Additionally, Rab31 protein was also reported to be related with overall survival in patients with cervical, ovarian [5], or liver [6, 7] cancer. However, few publications reported the molecular mechanisms of Rab31 in cancer proliferation and apoptosis.

In this study, we selected the glioblastoma and other cancers like cervical cancer as the samples. As reported, glioma is the most common one in primary cerebral tumors, and its treatment mainly consists of surgical resection in combination with radio- and chemotherapy [8]. Glioblastoma is characterized as extensive brain invasion [9, 10], whose underlying molecular mechanisms were not elucidated in detail. At the same time, cervical cancer is the second most common malignancy in women worldwide, and it remains a leading cause of cancer-related death for women in developing countries [11]. About 30 % of patients with International Federation of Gynecology and Obstetrics stage IB2 to stage IV will ultimately recur [12]. Therefore, the investigation of the mechanisms of tumor invasion and metastasis will provide deep insights into the occurrence of different cancers.

Here, we investigated and analyzed the biological roles of Rab31 in cancer proliferation and apoptosis. We analyzed and summarized the expression profile and the biological functions of Rab31 with emphasis on the clinical value and advantages of developing efficient anti-Rab31 strategies.

Materials and Methods

Cell Culture

Human U87 and SiHa cervical cancer cell lines were obtained from Shanghai Institute for Biological Sciences, Chinese Academy of Sciences. Cells were cultured in DMEM supplemented with 10 % fetal bovine serum and antibiotics (Gibco BRL, Rockville, MD) in a humidified atmosphere of 5 % CO_2 at 37 °C.

Plasmids and Reagents

We inserted the amplified Rab31 fragment into a pcDNA3 vector (Invitrogen) that is linked with FLAG tag at the amino terminus to establish flag-tagged Rab31 expression vector. And then, using pCDH plasmid (System Biosciences), stable cells that overexpress Rab31 were finished by way of lentiviral transduction. Besides, stable Rab31 knockdown cell lines were also constructed by Rab31 siRNAs cloning.

Anti-Rab31, anti-BAX, anti-PIG-3, Anti-E-cadherin and anti-N-cadherin, anti-cyclin D1, anti-ERK1/2, anti-AKT, anti-phos-AKT (T308), and anti-phos-ERK1/2(T202/Y204), anti-Vimentin, anti-caspase-3, and anti-PARP were all purchased from Santa Cruz Biotechnology.

Transfection

Cells were seeded in 24-well plates containing DMEM with 10 % fetal bovine serum. The cells were transfected with the indicated plasmids according to the manufacturer's instructions (Vigorous Biotechnology).

Western Blot

Western blot analysis was conducted according to the protocol. A 10 % SDS-PAGE was performed, and 30 mg of protein of each sample were analyzed. Proteins in the SDS gels were transferred to a polyvinylidene difluoride membrane by an electroblot apparatus. Primary antibodies were used as primary antibodies. Mouse or rabbit IgG antibodies coupled to horseradish peroxidase were used as secondary antibodies. An enhanced chemiluminescence kit (Pierce, Rockford, IL, USA) was used for detection.

Cell Growth and Colony Formation Assays

Cell growth was assessed by way of the CCK-8 Kit according to the manufacturer's protocol. As to colony formation assay, transfected cells were seeded in six-well plates at 2000 cells per well. Two weeks later, cells were fixed with 4 % paraformaldehyde and stained with crystal violet for half an hour. The number of colonies was recorded.

Quantitative Reverse-Transcription PCR (RT-qPCR)

Total RNA was extracted from cultured cells and reverse-transcribed to cDNA using the RNeasy Mini kit based on the manufacturer's protocols (Qiagen). The expression of mRNAs was determined using SYBR Premix Ex Taq Master Mix. The relative expression was defined by the comparative Ct value.

Primers Used for Real-Time RT-PCR

cyclin A:
Forward (5′–3′) CATACCTCAAGTATTTGCCATC
Reverse (5′–3′) GGTCCATGAGACAAGGCTTAAG
cyclin D1
Forward (5′–3′) CCGCCTCACACGCTTCCTCTC
Reverse (5′–3′) TCCTCCTCGGCGGCCTTGGGG
cyclin B1
Forward (5′–3′) GTCGGCCTCTACCTTTGCACTTCCTTC
Reverse (5′–3′) GAGTTGGTGTCCATTCACCATTATCCAG
E-cadherin
Forward (5′–3′) CCCATCAGCTGCCCAGAAAATGAA
Reverse (5′–3′) CTGTCACCTTCAGCCATCCTGTTT
N-cadherin
Forward (5′–3′) CGAGCCGCCTGCGCTGCCAC
Reverse (5′–3′) CGCTGCTCTCCGCTCCCCGC
Vimentin
Forward (5′–3′) GACAATGCGTCTCTGGCACGTCTT
Reverse (5′–3′) TCCTCCGCCTCCTGCAGGTTCTT
β-actin
Forward (5′–3′) ATCACCATTGGCAATGAGCG
Reverse (5′–3′) TTGAAGGTAGTTTCGTGGAT

Luciferase Reporter Assay

The cultured cells seeded into 24-well plates were co-transfected with Rab31 and luciferase reporter constructs with different promoter regions. Cells were harvested and analyzed for luciferase and b-galactosidase activities. All transfection experiments were performed in triplicates.

Cell Migration and Invasion Assays

Wound-healing assays were performed to assess cell migration. Briefly, transfected cells cultured in six-well plates as confluent monolayers were mechanically scratched using a 1-ml pipette tip to create the wound. The wound closure was recorded every day. For invasion assay, Tranwell assay was conducted according to the instructions.

In Vivo Tumor Growth

Animal studies were approved by the Institutional Animal Care Committee of Affiliated Hospital of Shandong Academy of Medical Sciences. Cells stably with si-control or si-Rab31 were injected subcutaneously in the dorsal of each animal (5-week-old male nude mice). Tumor size was measured at indicated times using calipers. Tumor volume was estimated according to the following formula: volume 5 (longest diameter × shortest diameter^2)/2. The mice were sacrificed 3 weeks after the first intratumoral injection, and tumors were excised, measured, and weighed.

Statistical Analysis

Data were expressed as mean±SEM. Significant differences between the two groups were assessed using χ^2 analysis, Student's t test, and one-way ANOVA. In our study, the $p<0.05$ was considered significant.

Results

Rab31 Promotes U87 and SiHa Cell Proliferation In Vitro

In this work, the effect of overexpression or knockdown of endogenous Rab31 protein on anchorage-dependent growth of U87 and SiHa cells was investigated. U87 transfected with flag-tagged Rab31 grew much faster than those transfected with empty vector (Fig. 1a). In contrast, cells infected with Rab31 siRNA grew more slowly than those infected with control siRNA, indicating that Rab31 knockdown in U87 and SiHa cells reduces cell proliferation (Fig. 1a). Consistent with U87, the data from SiHa identified that cells transfected with flag-tagged Rab31 grew much faster than those transfected with empty vector (Fig. 1b). In contrast, cells infected with Rab31 siRNA grew more slowly than those infected with control siRNA. These results reveal that Rab31 increases the proliferation of U87 and SiHa cells.

Rab31 Activates the G1/S Transitions in U87 and SiHa Cells

To figure out the potential reasons why Rab31 promotes U87 and SiHa cell growth, we conducted cell cycle analysis to explore the effect of Rab31 on cell cycle. Compared with the control, the overexpression of Rab31 in U87 and SiHa cells resulted in the decreased proportion in G0/G1 phase (from 65 to 33 %), but the increased proportion in G2/M phase (from 7 to 32 %) and S phase (from 28 to 35 %) (Fig. 2a). However, the knockdown of Rab31 in U87 and SiHa cell lines obviously increased the cell proportion in G0/G1 phase (70 to 89 %), but decreased the cell proportion in G2/M (7 to 5 %) phase and S phase (23 to 6 %) (Fig. 2b). These results indicated Rab31 regulated the G1/S phase transitions in U87 and SiHa cells.

Rab31 Regulates the Expression of Cell Cycle-Related Proteins in U87 Cells

Based on the above results, we further detected the expression profile of cell cycle-related proteins, including cyclin D1, cyclin A, and cyclin B1 in SiHa cells. Western blot analysis showed that the overexpression of Rab31 in U87 cells

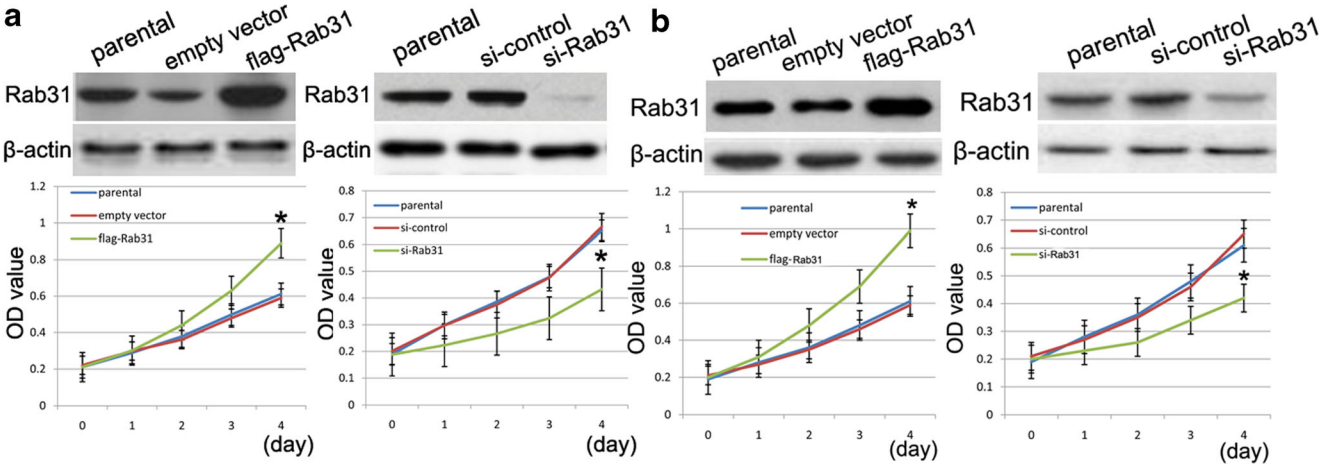

Fig. 1 Expression and significance of Rab31 in U87 and SiHa cells. **a**. U87 cells transfected with flag-tagged Rab31 or empty vector or parental U87 cells were grown in regular medium and harvested at the indicated times. Cell number was determined by CCK-8 assay. At the same time, cells infected with Rab31 siRNA were cultured and analyzed. All values shown are mean±SEM of triplicate measurements (*$p<0.05$ versus empty vector or control siRNA; one-way ANOVA or Student-Newman-Keuls.). **b**. SiHa cells transfected with flag-tagged Rab31 or empty vector or parental SiHa cells were grown in regular medium and harvested at the indicated times. Cell number was determined by CCK-8 assay. At the same time, cells infected with Rab31 siRNA were cultured and analyzed. All values shown are mean±SEM of triplicate measurements and have been repeated for three times (*$p<0.05$ versus empty vector or control siRNA; one-way ANOVA or Student-Newman-Keuls.)

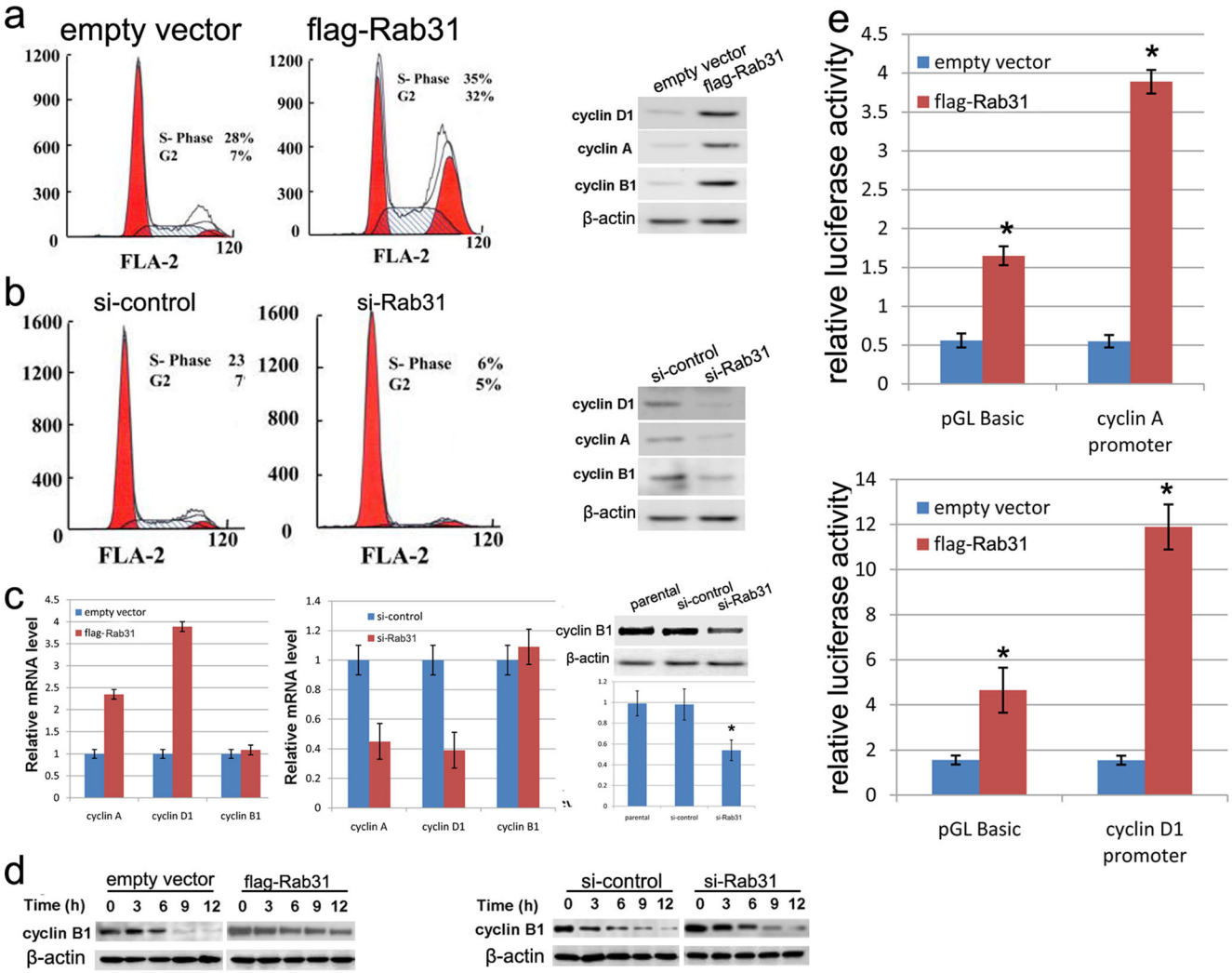

Fig. 2 Rab31 regulates the expression of cyclins and the EMT markers. **a** Flow cytometry analysis of cell cycle in U87 cells transfected with empty vector or Flag-Rab31. **b** Flow cytometry analysis of cell cycle in U87 cells infected with control siRNA or Rab31 siRNA. The experiments have been repeated three times. **c** RT-qPCR analyses for cyclin D1, cyclin A and cyclin B1 mRNA expression in Rab31-overexpressing U87 cells and Rab31 knockdown U87 cells. Data shown are mean± SEM of three independent experiments. *$p<0.05$ versus empty vector or control siRNA; *$p<0.001$, compared with control, one-way ANOVA or Student-Newman-Keuls. **d** Immunoblot analysis of cyclin B1 expression in Rab31-overexpressing U87 cells and Rab31 knockdown U87 cells at the indicated times after exposure to the protein synthesis inhibitor cycloheximide (20 mg/ml). **e** Luciferase reporter assays in U87 cells transfected with flag-tagged Rab31 and the cyclin A-Luc or cyclin D1-Luc reporter, and E-cadherin-Luc, N-cadherin-Luc or Vimentin-Luc reporter. Data shown are mean±SEM of triplicate measurements that have been repeated for three times (*$p<0.05$ vs control)

upregulated the expression of cyclin D1, cyclin A, and cyclin B1 (Fig. 2a). On the other hand, the knockdown of intracellular Rab31 protein in U87 cells decreased the expression of cyclin D1, cyclin A, and cyclin B1 (Fig. 2b). Consistent with U87, the knockdown of intracellular Rab31 protein in SiHa cells also inhibited the expression level of cyclin D1, cyclin A, and cyclin B1. Subsequently, we used qRT-PCR to investigate the effects of Rab31 on the mRNA levels of cyclin D1, cyclin A, and cyclin B1. We found that Rab31 overexpression increased the mRNA levels of cyclin A and cyclin D1 in U87 cells, while knockdown of Rab31 decreased the mRNA levels of cyclin A and cyclin D1 in U87 cells (all $p<0.01$; Fig. 2c). Unfortunately, Rab31 cannot change the mRNA level of cyclin B1 in U87 cells ($p=0.561$; Fig. 2c). Thus, we continued to investigate the impact of Rab31 on the protein levels of cyclin B1, and observed Rab31 altered the protein expression of cyclin B1 in U87 cells ($p<0.01$; Fig. 2d). The same results were also obtained from SiHa cells (data not shown). These findings suggested that Rab31 induces the expression of cyclin A and cyclin D1 at the transcriptional level, while it induces the expression of cyclin B1 at the protein level.

Next, the promoter luciferase reporter assay was used to analyze the role of Rab31 in the transcription of cyclin A and cyclin D1. We observed that the overexpression of Rab31 obviously increased the activity of cyclin A and cyclin D1 promoter (Fig. 2e). Because Rab31 regulates cyclin B1

protein expression, we explored the effect of Rab31 on the half-life of cyclin B1 protein using cycloheximide chase assay. The data verified the overexpression of Rab31 prolong the protein half-life of cyclin B1 (Fig. 2d), while the knockdown of Rab31 decreased the protein half-life of cyclin B1 (Fig. 2d), indicating the ubiquitin-proteasome pathway may be implicated in the Rab31 regulation of cyclin B1 protein stability.

Rab31 Inhibits U87 and SiHa Cell Apoptosis

Next, we investigated whether Rab31 regulated apoptosis of U87 and SiHa cells. The percentage of apoptotic cells was lower in U87 cells with Rab31-overexpressing than that in cells with the empty vector (from 7.43 to 4.23 %; $p<0.01$) (Fig. 3a). By contrast, Rab31 knockdown resulted in higher apoptosis percentage (from 7.43 to 40.86 %; $p<0.01$) (Fig. 3a). It should be noted that Rab31 overexpression inhibited the expression of BAX and PIG3, and increased the BCL2 expression (Fig. 3b). Meanwhile, Rab31 overexpression decreased the expression of cleaved caspase-3 and PARP (Fig. 3b), while Rab31 knockdown increased the expression of cleaved caspase-3 and PARP, indicating BCL2 is critical for Rab31 modulation of apoptosis. The same results were also obtained from SiHa cells.

Rab31 Induces Migration and EMT of U87 and SiHa Cells

In order to investigate the impact of Rab31 on U87 and SiHa cell migration, we used wound-healing assay to found the overexpression of Rab31 in U87 cells improved the ability of migration (Fig. 3c), while Rab31 knockdown by siRab31 inhibited U87 cell motility (Fig. 3c). Next, we explored the involvement of Rab31 in inducing EMT biomarkers, and observed that Rab31 overexpression enforced the mRNA levels of N-cadherin and Vimentin, but decreased the mRNA levels of E-cadherin (Fig. 3d–e). Furthermore, we applied promoter luciferase reporter methods to identify Rab31 promotes the bio-activities of N-cadherin and Vimentin promoter, but affected the bio-activities of E-cadherin promoter (Fig. 3f). Subsequently, we found Rab31 overexpression in U87 cells promoted cell proliferation and migration (Fig. 4a), and stimulated the phosphorylation of AKT and ERK1/2 as well as the expression of cyclin D1, cyclin A, and cyclin B1 (Fig. 4b).

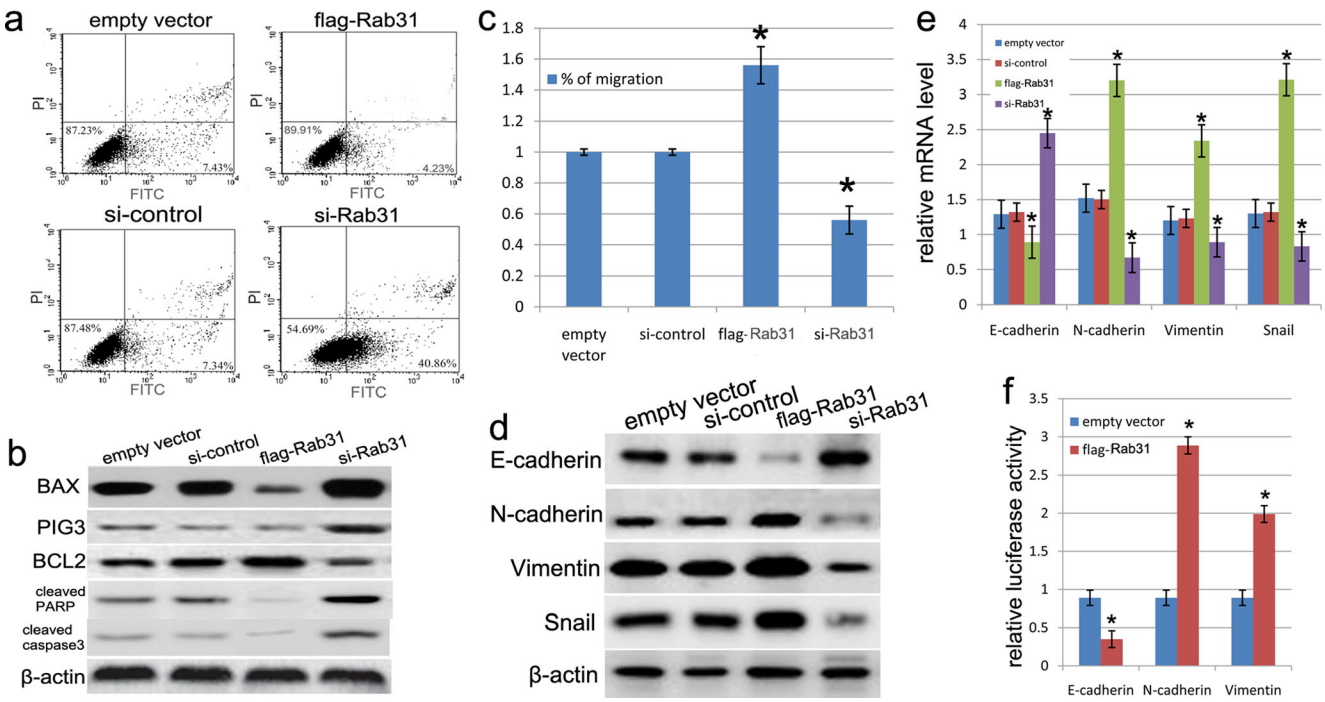

Fig. 3 Rab31 inhibits U87 cell apoptosis. **a** Representative flow cytometry analysis of apoptosis stained with Annexin V and PI in U87 cells transfected with Flag-Rab31 or U87 cells infected with Rab31 siRNA. Data shown are mean±SEM of three independent experiments. *$p<0.05$ versus empty vector or control siRNA. **b** Representative Western blots with the indicated antibodies in U87 cells transfected/infected and treated above. β-actin was used as a normalization control. **c** Migration of Rab31-overexpressing U87 cells and Rab31 knockdown U87 cells was determined by wound-healing assay. The experiments have been repeated three times. **d–e** Cell extracts were used for representative Western blot analysis with the indicated antibodies. RT-qPCR analyses for N-cadherin, E-cadherin, and Vimentin mRNA expression in Rab31-overexpressing U87 cells and Rab31 knockdown U87 cells. Data shown are mean±SEM of three independent experiments. *$p<0.05$, versus empty vector or control siRNA. **f** Luciferase reporter assays in U87 cells transfected with flag-tagged Rab31 and the E-cadherin-Luc, N-cadherin-Luc, or Vimentin-Luc reporter. Data shown are mean±SEM of triplicate measurements that have been repeated three times (*$p<0.05$ vs control)

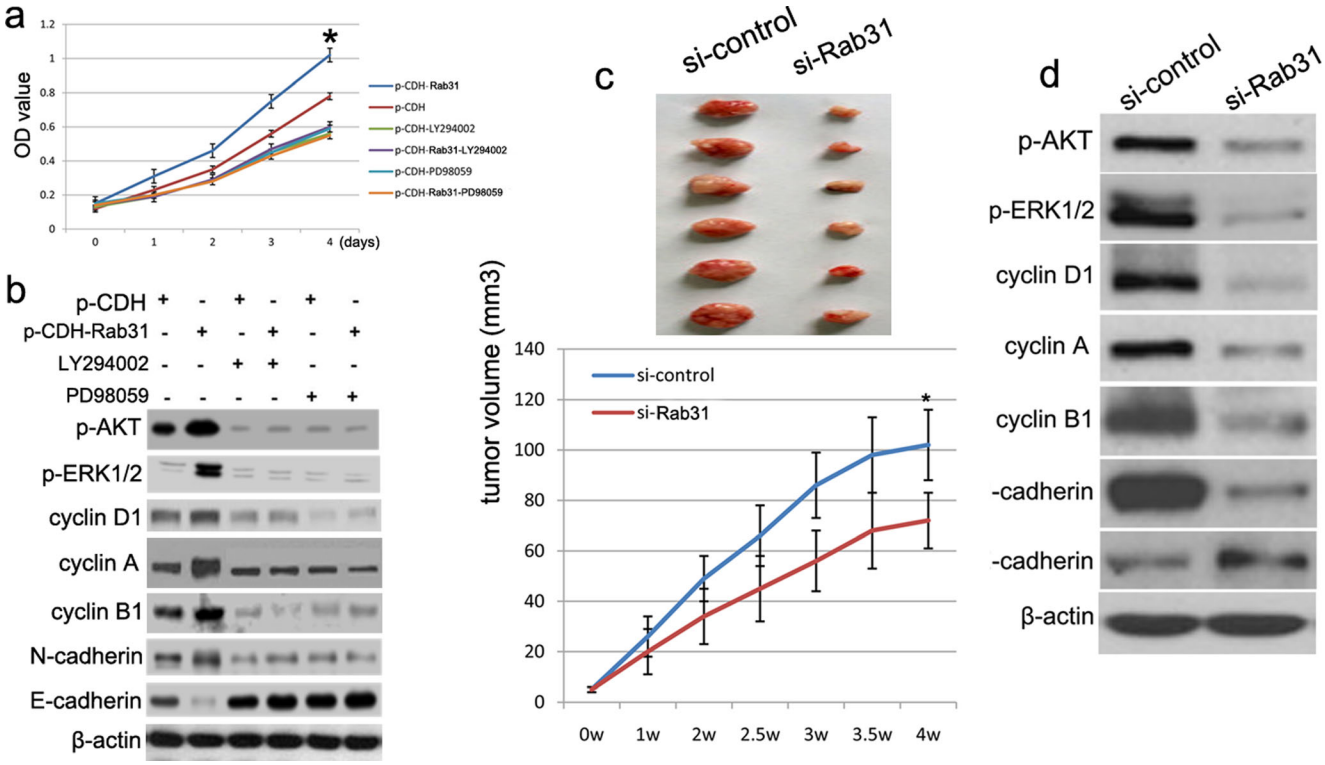

Fig. 4 Rab31 increases U87 cell proliferation, and migration through activation of MAPK and AKT. **a** U87 cells were infected with lentivirus expressing Rab31 (pCDH-Rab31) or empty vector (pCDH), and were treated for 24 h with 10 mM LY294002 or 10 mM PD98059. After 24 h, the culture medium was changed with fresh drug-free medium, and the cells were grown for the indicated times. Cell number was determined by CCK-8 assay. **b** Representative Western blot analysis of U87 cells infected and treated above. **c**. Rab31 cells stably infected with Rab31 siRNA or control siRNA cells were injected into nude mice. At the indicated times, tumors were measured (mean±SEM; $n=3$). *$p<0.01$ versus control siRNA. **d** Immunoblot analysis. *$p<0.001$, compared with control, one-way ANOVA or Student-Newman-Keuls

However, either LY294002 or PD98059 inhibited the phosphorylation of AKT and ERK1/2 as well as the expression of cyclin D1, cyclin A, and cyclin B1 and the EMT biomarkers (Fig. 4b). The same data were also obtained from SiHa cells (data not shown).

Rab31 Knockdown Represses the Growth of U87 Cell in Vivo

In this work, we further used the nude mice to investigate the impact of Rab31 knockdown on U87 cell growth. Our finding showed that in comparison with the control, Rab31 knockdown obviously affected the growth of U87 cells in nude mouse (Fig. 4c). In addition, it should be noted that Rab31 siRNA inhibited the elevated expression level of Rab31, cyclin D1, cyclin A, cyclin B1, N-cadherin, p-AKT, and p-ERK1/2, but induced the expression of E-cadherin in tumor tissues of nude mice (Fig. 4d).

Discussions

It is reported that Rab31 can promote proliferation and migration/invasion in some cancers [13–15]. However, the detailed mechanisms of Rab31 in cervical cancer proliferation and apoptosis are still unknown. In the present work, our results identified the critical role of Rab31 in cancer progression. Firstly, Rab31 expression or overexpression promotes cell growth, and inhibited U87 and SiHa cell apoptosis, and then activated cell cycle. Secondly, Rab31 overexpression prompts U87 and SiHa cancer cell migration, and induces the expression of EMT biomarkers depending on the ERK1/2 and AKT pathway. Eventually, Rab31 knockdown can inhibit the growth of U87 and SiHa cell in nude mice. These findings indicate that Rab31 might play a crucial role in the development and progression of cancers.

Till now, it is still unclear whether Rab31 has a regulatory role in balancing proliferation and apoptosis [16–18], In the present work, our data revealed that the high expression of Rab31 affected cell apoptosis, and elevated the BCL2/BAX ratio, but inhibited the PIG3 expression and the cleavage of PARP, indicating the critical role of Rab31 in balancing apoptosis and proliferation. We also explored the regulatory mechanisms of Rab31 in the cell cycle by detecting the cell cycle proteins, and showed Rab31 overexpression enforced the expression of cyclin D1, cyclin A, and cyclin B, indicating that Rab31 plays an crucial role in the regulation of cell cycle.

Besides, in the present study, the PD98059 and LY294002 treatment hampers the proliferation and migration capacity of

U87 and SiHa cells with Rab31 overexpression, and the expression of cyclin D1, cyclin A, cyclin B1, and EMT biomarkers, indicating the requirement of ERK1/2 and AKT for Rab31-induced U87 and SiHa cell proliferation, migration, and EMT. Most importantly, Rab31 knockdown significantly affected the capacity of U87 cell growth in nude mice. At the same time, the tumor tissues in nude mice treated with Rab31 siRNA decreased the expression of cyclin D1, cyclin A, cyclin B1, N-cadherin, and the phosphorylation of AKT and ERK1/2, but enforced the expression of E-cadherin.

In conclusion, Rab31 promotes cervical cancer growth and migration in vivo, inhibition of ERK1/2 and AKT or inhibition of Rab31 may be useful strategies for the treatment of Rab31-overexpressing cancers.

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Conflicts of Interest The authors indicated no potential conflicts of interest.

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