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Rab5a overexpression promoting ovarian cancer cell proliferation may be associated with APPL1-related epidermal growth factor signaling pathway

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Rab5a is a regulatory guanosine triphosphatase that is associated with the transport and fusion of endocytic vesicles, and participates in regulation of intracellular signaling pathways embraced by cells to adapt to the specific environment. Rab5a is also correlated with lung, stomach, and hepatocellular carcinomas. Here, we detected Rab5a in paraffin-embedded samples of 20 ovarian cysts, 20 benign cystadenomas, and 39 ovarian cancers by immunohistochemistry, and observed that Rab5a expression was significantly higher in ovarian cancer (P = 0.0001). By setting up stable HO-8910 cell lines expressing Rab5a or dominant negative Rab5a (Rab5a:S34N), we found that Rab5a overexpression enhanced the cell growth by promoting G1 into S phase. In contrast, Rab5a:S34N inhibited this process. Additionally, APPL1 (adaptor protein containing PH domain, PTB domain, and Leucine zipper motif), a downstream effector of Rab5a, was also involved in promoting HO-8910 cell cycle progress. But this function was blocked by Rab5a:S34N. Laser scanning confocal microscopy represented the colocalization of APPL1 and Rab5a in the plasmolemma, which changed with the time of epidermal growth factor (EGF) stimulation. We also found APPL1 could transfer from the membranes into the nucleus where it interacted with NuRD/MeCP1 (the nucleosome remodeling and histone deacetylase multiprotein complex). NuRD is reported to be involved in the deacetylation of histone H3 and H4 to regulate nuclear transcription. So Rab5a promoted proliferation of ovarian cancer cells, which may be associated with the APPL1-related epidermal growth factor signaling pathway. (*Cancer Sci* 2010; 101: 1454–1462)

Ovarian cancer is characterized by few early symptoms, resulting in diagnosis at an advanced stage associated with poor survival.[1,2] As a result, it is the most lethal of the gynecologic malignancies. Ovarian carcinogenesis is a multistep process involving many genetic changes and epigenetic events that are not well characterized.[3] Identifying variation in oncogene and other related genes remains an important challenge in understanding the pathogenesis of this neoplasm. Emerging evidence implicates alterations in the Rab proteins, their associated regulatory proteins and effectors in many human diseases including cancer.[4] Rab proteins are small GTPases, localized to distinct cellular compartments, regulating specific steps of intracellular membrane trafficking. Some of these proteins are under the control of signal molecules, and participate in the regulation of numerous signal transduction pathways profoundly influencing cell proliferation, cell nutrition, innate immune response, fragmentation of compartments during mitosis, and apoptosis.[5] As a prototypical member of the Rab family, Rab5 is a key regulator of epidermal growth factor (EGF) receptor-mediated endocytosis.[6–8] More recently, work from many labs has suggested that the nature of the signaling response to EGF may be influenced by the quality of the internalization event controlled by Rab5.[9,10] On the other hand, with EGF engagement, the EGF receptor can control the activity of Rab5 to regulate the rate of its own endocytosis.[9,11] Once internalized, the EGF receptor is directed to the early endosome, in which Rab5 and other proteins could determine which downstream signaling pathway would be preferentially activated.[5,9,10,12] This will therefore control proliferation, differentiation, and other biological responses that cells embrace to adapt to the specific intra- and extracellular environment.[5,13–15] Recently, Miaczynska *et al.*[15] identified a pathway directly linking Rab5 to signal transduction and mitogenesis. This pathway operates via APPL1 (adaptor protein containing PH domain, PTB domain, and Leucine zipper motif), one of the Rab5 effectors, which reside on a subpopulation of endosomes. In response to EGF stimuli, APPL1 translocates from the membranes to the nucleus where it interacts with the nucleosome remodeling and histone deacetylase multiprotein complex NuRD/MeCP1, an established regulator of chromatin structure and gene expression.

Rab5 consists of three isoforms, Rab5a, Rab5b, and Rab5c. All of them are ubiquitously expressed in endosomal membranes and all enhance endocytosis when overexpressed in a variety of cells.[16] But research has shown that the three Rab5 proteins were selectively induced by serum or interferon. They underwent different post-translational modification, and were differentially recognized by different kinases. These findings suggest that the three Rab5 isoforms most likely have different functions.[17–19] Of the three isoforms, Rab5a has been widely studied in physiological conditions. But little was known about its intrinsic effect on carcinomas. Recent experiments confirmed that the overexpression of the *Rab5a* gene was correlated with the degree of malignancy and metastatic potential of lung and stomach cancer.[20,21] It was also involved in migration in hepatocellular carcinomas.[22] What is the mechanism that this protein performs in cancer cells? Is Rab5a associated with ovarian cancer?

In this study, we found that Rab5a was overexpressed in ovarian cancer compared with ovarian cystadenomas and ovarian cysts. Overexpression of Rab5a promoted tumor cell growth, but dominant negative Rab5a (Rab5a:S34N) blocked this progression. Confocol microscopy represented the colocalization between APPL1 and Rab5a in the plasmolemma and endosomes, which changed with the time of EGF stimulation. We

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also found APPL1 could transfer from the cytoplasm into the nucleus where it colocalized with NuRD complex. These data suggest that the promotion of ovarian cancer cell proliferation by Rab5a may be associated with the APPL1-related EGF signaling pathway.

Materials and Methods

Patients and tissue samples. Ovarian tissues specimens were obtained from 79 patients who underwent surgical resection at Ruijin Hospital between January 2003 and December 2006, including 39 primary ovarian cancers, 20 ovarian cystadenomas, and 20 ovarian cysts. Patients who were unable or unwilling to give informed consent or who had undergone chemotherapy or radiation therapy before surgery were excluded. All investigations described in this study were done after informed consent was obtained and in accordance with the guidelines from the Institutional Review Board and the Ethics Committee of Ruijin Hospital (Shanghai, China). The ovarian cancer patients were composed of 16 papillary, 19 endometrioid, one squamous, one mucinous, and two mixed forms, of which 41% were staged as early disease (stages I and II) according to the International Federation of Gynecology and Obstetrics (FIGO) staging system; 59% as advanced disease (stages III and IV); and 23% (9/39) patients with peritoneal metastasis. With regard to the histological grade, nine were graded as G1, 19 were G2, and 11 were G3. The ages ranged from 23 to 83 years (51 ± 14, mean ± SD) (Tables 1,2).

Immunohistochemistry. All tissue sections were deparaffinized, rehydrated, and blocked as described by Tamaki *et al.*,[23] then sections were incubated with an polyclonal rabbit antihuman Rab5a antibody (1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. After washing in PBS, the sections were incubated with horseradish peroxidase-labeled goat antirabbit antibody (1:200; Santa Cruz Biotechnology) for 1 h at room temperature. After repeated washing, the antigen–antibody complex was visualized with substrate chromogen (DAB; Dako Cytomation, Carpinteria, CA, USA) and counterstained with hematoxylin. A negative control was obtained by replacing the primary antibody with non-immunized rabbit serum. Results of immunohistochemical staining were determined in terms of grades by assessing the percentage of stained cells as described.[23] Samples were considered negative if none of the cells stained for Rab5a. Weak positive was defined as positive staining in <10% of the tumor cells, moderate positive as positive staining in 10–50% of the tumor cells, and strong positive as positive staining in >50% of the tumor cells.

Construction of recombinant. To get Rab5a fusion protein, two primers were designed as follows: 5′-CGGCAAGCTTTTGGACATGGCTAGTC-3′ and 5′-GCGGGATCCGAGTTACTACAACACTGAT-3′. They contained Hind III and BamH I sites, respectively. A full-length human Rab5a cDNA was amplified by PCR, and digested and inserted into pEGFP-N1 (GFP) to construct recombinant (GFP-Rab5a:WT). For the dominant negative mutant of Rab5a (GFP-Rab5a:S34N),[10] another two mutagenic primers were synthesized including 5′-GAAGCACTAGGCTGTTTTTGCCAAC-3′ and 5′-GTTGGCAAAAACAGCCTAGTGCTTC-3′. They were responsible for the substitution mutation S34N (from serine to asparagine at residue 34). The vectors were verified by sequencing at the Invitrogen DNA sequencing facility (Shanghai, China).

Table 1. Expression of Rab5a in ovarian cancers, ovarian cystadenomas, and ovarian cysts

Histological type	Number of cases (%)	Age mean ± SD	Rab5a				
			–	+	++	+++	*P*-value
Ovarian cancer	39 (49.4)	51 ± 14	0	16	16	7	0.0001*
Cystadenoma	20 (25.3)	45 ± 17	5	11	4	0	0.163**
Ovarian cyst	20 (25.3)	37 ± 9	9	9	2	0	

*Statistical analysis of Rab5a expression between ovarian cancers and non-ovarian cancers (ovarian cystadenomas and ovarian cysts) by Kruskal–Wallis. **Statistical analysis of Rab5a expression between ovarian cystadenomas and ovarian cysts by Kruskal–Wallis.

Table 2. The relationship between Rab5a expression and clinicopathological variables in ovarian cancers

Variables	Number of cases (%)	Age mean ± SD	Rab5a				
			–	+	++	+++	*P*-value
Histological type							
Serous papillary	16 (41.0)	52 ± 11	0	10	6	0	0.005*
Endometrioid	19 (48.7)	53 ± 14	0	4	10	5	
Others	4 (10.3)	31 ± 9	0	2	0	2	
Histological grade							
I + II	28 (71.8)	52 ± 15	0	10	13	5	0.428
III	11 (28.2)	50 ± 13	0	6	3	2	
FIGO stage							
I + II	16 (41.0)	51 ± 11	0	7	6	3	0.865
III + VI	23 (59.0)	52 ± 16	0	9	10	4	
Metastasis							
Without	30 (76.9)	50 ± 13	0	10	14	6	0.113
With	9 (23.1)	55 ± 13	0	6	2	1	

*Statistical analysis of Rab5a expression between serous papillary carcinoma and endometrioid carcinoma by Kruskal–Wallis. FIGO, International Federation of Gynecology and Obstetrics.

Cell culture and transfection. The HO-8910 ovarian cancer cell line was purchased from the cell bank at the Institute of Biochemistry and Cell Biology (Shanghai Institute for Biological Sciences, Chinese Academy of Sciences, Shanghai, China). The cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere of 5% CO_2 at 37°C. GFP-Rab5a or GFP-Rab5a:S34N was transfected into HO-8910 cells using Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer's protocol. The control cells received the empty vector (GFP). G418-resistant colonies were isolated in the selection media for 1 month. The stable transfection cells were collected using a FCM flow cytometer (BD FAC-SAria Cell Sorter; BD Bioscience, San Jose, CA, USA) and further expanded.

Knockdown of APPL1 in HO-8910 cells. APPL1 siRNA was designed as described by Miaczynska *et al.*[15] APPL1 siRNA and control siRNA (unspecific siRNA) were introduced into HO-8910 cells using Lipofectamine 2000 (Invitrogen) according to the protocol. Cells were collected for experiments after 3 days of the transfection.

Immunoblot analysis. Lysates prepared from ovarian cancer tissue and cells were respectively extracted, separated, and transferred to polyvinylidene fluoride (PVDF) membranes as previously reported.[22,24] The membranes incubated with rabbit antihuman Rab5a antibody (1:200; Santa Cruz Biotechnology) or rabbit antihuman APPL1 antibody (1:200; Santa Cruz Biotechnology) for 2 h. After washing, they were incubated with HRP-conjugated goat antirabbit IgG (1:1000) for 1 h. After further washing, the immunoblots were developed using the ECL reagents (Amersham Life Science, Piscataway, NJ, USA). Each experiment was repeated three times.

Cell proliferation assay. Cell proliferation and viability were determined by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay. Cells (5×10^4cells per well) were seeded in 96-well microtiter plates and incubated for 48 h at 37°C with 5% CO_2. One hundred micrograms of MTT (Sigma,

St. Louis, MO, USA) was added to each well and the plates were incubated for an additional 4 h at 37°C, and then 100 μL DMSO was added to each well. The absorbance was read at 570 nm on an automatic microwell plate reader.

Cell cycle analysis. Cells were collected, washed with PBS, and fixed with 70% ethanol overnight. After washing and centrifugation, the pellets were suspended in 0.5 mL RNaseA (0.1 mg/mL) at 37°C for 30min. Then propidium iodide (PI) dye (0.2 mg/mL) was added and the mixture was kept in the dark for 10 min. The cells were subsequently analyzed on a FACScan flow cytometer (BD Biosciences) for DNA content. The percentage of cells in different phases of the cell cycle was sorted using the ModFit 5.2 computer program (Verity Inc., Topsham, ME, USA). Each experiment was repeated three times.

Luciferase reporter assay. Luciferase reporter assay was performed according to the reports of Barbieri *et al.*[10] and Lin *et al.*[25] Cell lines (expressing GFP or GFP-Rab5a) were transfected with 1.0 μg luciferase reporter plasmid (cyclin D1) and 0.1 μg β-galactosidase plasmid as an internal control. After 12 h of transfection, the cells were serum starved overnight, and then incubated with or without EGF (100 ng/mL) for 6 h. After cells were lysed, luciferase and β-galactosidase activities were measured using a standard luciferase and β-galactosidase detection kit (Promega, Madison, WI, USA).

Confocal microscopy analysis. HO-8910 cells were grown on coverslips overnight, then transfected with pEGFP-Rab5a:WT or GFP-Rab5a:S34N. After 48 h, the cells were serum-starved overnight and incubated with 1 μg/mL EGF for 5, 10, or 30 min at 37°C as indicated in the Figures. The control cells were cultured with 10% FBS. The slips were fixed in 4% paraformaldehyde for 15 min, and then penetrated with 0.1% Triton-100 for 10 min. After blocking with 3% BSA, the slips were stained with rabbit antihuman APPL1 antibody and mouse antihuman MTA2 antibody (1:200; Santa Cruz Biotechnology) for 1 h. After washing with PBS, the slips were incubated with Cyc3-conjugated antirabbit IgG and Cyc5-conjugated antimouse IgG for 0.5 h. Then slips were washed with PBS and examined by confocal microscopy (LSM510; Carl Zeiss, Jena, Germany).

Immunoprecipitation assay. Immunoprecipitation was operated using a commercial kit (Active Motif, Carlsbad, CA, USA) First, HO-8910 cells expressing GFP or GFP-Rab5a were collected in the lysate buffer. Proteins of interest were immunoprecipitated by overnight incubation with APPL1 antibody at 4°C with constant rotation (about 200 μg protein for each reaction). Immunecomplexes were recovered by 2 h incubation with Protein Gagarose beads (Roche, Basel, Switzerland) at 4°C with rotation, followed by centrifugation, and washing in a wash buffer. Next, samples were incubated at 95°C for 5 min with Laemmli buffer and subjected to immunoblotting.

Statistical analysis. Data analysis was performed using the SAS 6.12 software package, (SAS Institute, Cary, NC, USA). Patients were divided into two groups: cancers and noncancers (cystadenomas and cysts). The ovarian cancers were divided into two groups based on clinicopathological features. Statistical analysis was done using the Kruskal–Wallis rank test. For cell proliferation assay, statistical analysis was done using the Student's *t*-test. All statistic differences were considered significant at $P < 0.05$.

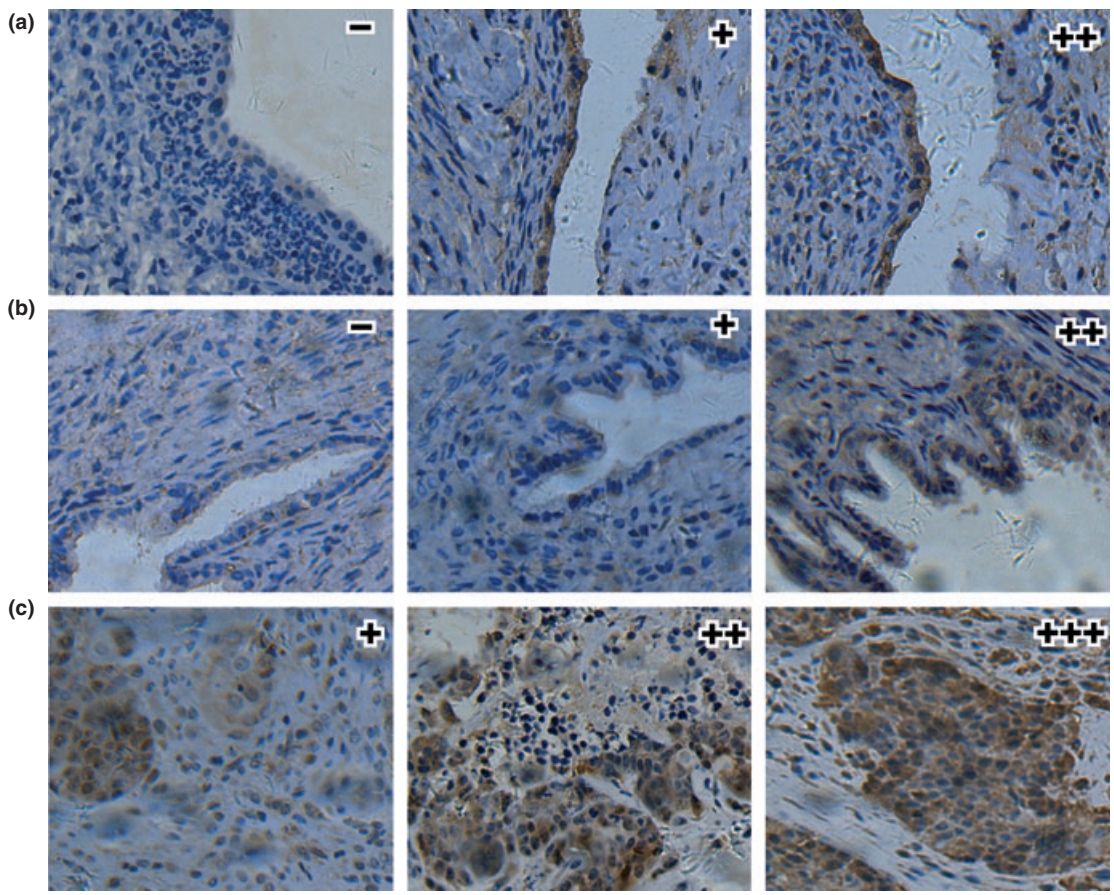

Fig. 1. Representative images of immunohistochemical staining of Rab5a in ovarian cancer and noncancer tissue sections. (a) Negative (–), weak positive (+), and moderate positive (++) cases of ovarian cyst tissue sections. (b) Negative (–), weak positive (+), and moderate positive (++) cases of ovarian cystadenoma tissue sections. (c) Weak positive (+), moderate positive (++), and strong positive (+++) cases of ovarian cancer tissue sections. Original magnification: ×200.

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Results

Overexpression of Rab5a in ovarian cancer. The representative immunostainings for Rab5a are illustrated in Figure 1. Rab5a showed the typical cytoplasmic distribution in positive cells. The positive cells were limited to epithelial lamina in cysts and cystadenomas, but in carcinomas, positive cells were scattered and invaded into ovarian stroma. All ovarian cancer specimens were Rab5a positive (41% weak, 41% moderate, and 18% strong), but only 75% (15/20) of cystadenomas were Rab5a positive (55% weak, and 20% moderate), and 55% (11/20) of cysts were Rab5a positive (45% weak, 10% moderate). Expression of Rab5a was significantly increased in ovarian cancer compared with ovarian cystadenomas and ovarian cysts ($P = 0.0001$) (Table 1), and ovarian endometrioid carcinoma had higher Rab5a expression than ovarian serous adenocarcinoma ($P = 0.005$). However, the high expression of Rab5a did not correlate with histological grade, FIGO stage, or neoplastic metastasis in ovarian carcinoma ($P > 0.05$) (Table 2). Additionally, five paired cases of tumor and adjacent nontumor tissues from 39 ovarian cancer patients were subjected to immunoblot analysis using the anti-Rab5a polyclonal antibody. A specific band (24 kDa) was detectable from both tumor and nontumor tissues in all five samples (Fig. 2). However, Rab5a protein expression was higher in tumors than nontumors; the mean T/N (tumor/nontumor) ratio of Rab5a protein was 2.52 ± 1.33.

Rab5a may promote the growth of HO-8910 cells by activating cyclin D1 transcription. Stable HO-8910 cell lines expressing GFP, GFP-Rab5:WT, and GFP-Rab5:S34N were established as described in the Materials and Methods. Immunoblot analysis showed expression of the constructs in each of the cell lines. GFP-Rab5a:S34N protein was about 1.1 times that of endogenous Rab5a. GFP-Rab5a:WT protein was about 2.3 times that of endogenous Rab5a (Fig. 3). Cells expressing these three constructs were examined for proliferative activity by MTT assay. The expression of GFP-Rab5:WT was found to promote cell proliferation compared with cells expressing GFP ($P < 0.01$). In contrast, cells expressing GFP-Rab5:S34N showed a significant decrease of proliferation ($P < 0.01$) (Fig. 4). Cell cycle assay was performed by PI staining. As seen in Figure 5, the S-phase fraction increased from 27.26% in control GFP-expressing cells to 33.45% in GFP-Rab5a:WT-expressing cells. But in comparison to control cells, Rab5a:S34N induced an accumulation of cells in the G0-G1-phase fraction, with a decrease of the S-phase fraction from 27.26% to 18.39%. It was reported that Rab5a could activate gene transcription of cyclin D1 in mouse NR6 cells in presence of EGF.[10] To test whether the expression and activity of Rab5 affected cyclin D1 transcription in ovarian cancer cells, HO-8910 cells expressing GFP, Rab5a:WT, and Rab5a:S34N were transfected with a luciferase reporter construct driven by the proximal region of the cyclin D1 promoter. As expected, Rab5:S34N blocked the EGF-induced luciferase activity. But Rab5a:WT overexpression promoted the activation of cyclin D1 with or without EGF incubation (Fig. 6). This indicated that Rab5a may influence ovarian cancer growth, in part by regulating transcription of the cyclin D1 in EGF signaling.

Rab5a may exert feedback regulation on APPL1 gene expression. APPL1 protein in stable HO-8910 cell lines expressing GFP, GFP-Rab5a:S34N, and GFP-Rab5a:WT was measured by immunoblotting. Figure 7 shows that APPL1 protein increased about 40% in the cell line expressing Rab5a:S34N and decreased about 50% in the cell line expressing Rab5a:WT compared with the control cells. This is consistent with previous experimental results. HO-8910 cells were transfected with Rabex5 siRNA or control siRNA. After 3 days, the cells were collected for gene microarray analysis. With Rabex5 deletion, Rab5a-GTP decrease, we found that APPL1 mRNA increased 1.2-fold in Rabex5 siRNA-treated cells compared with control

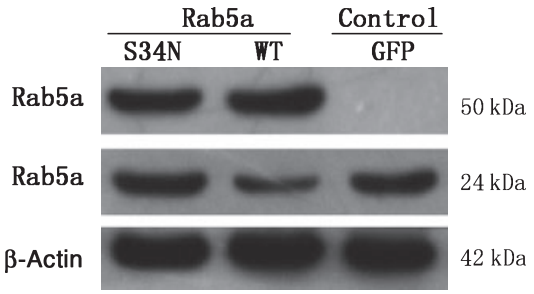

Fig. 3. Expression of Rab5a in stable HO-8910 cell lines expressing recombinants. HO-8910 cells were transfected with GFP-Rab5a:S34N, GFP-Rab5a:WT, and GFP control. Stable cell lines expressing corresponding recombinant were lysed and subjected to western blot analysis using Rab5a antibody as described in the Materials and Methods. β-Actin was detected by an anti-β-actin antibody as an internal loading control.

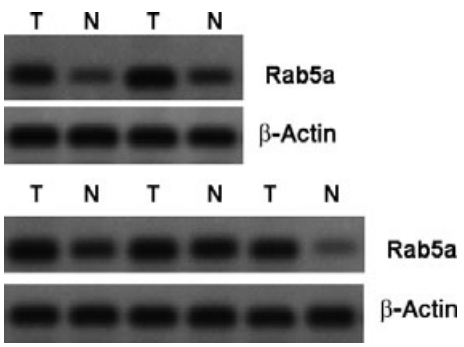

Fig. 2. Western blot analysis of Rab5a protein in tumor and adjacent nontumor. Five paired tumor and adjacent nontumor tissues in 39 ovarian cancer patients were detected by western blot analysis as described in the Materials and Methods. Lane T indicates tumor tissues, and lane N (right) indicates their adjacent nontumor tissues. β-Actin was detected as an internal loading control.

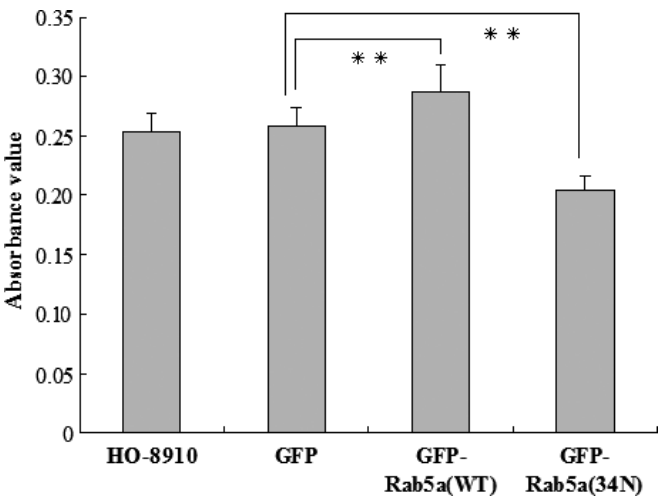

Fig. 4. Effect of Rab5a on the proliferation of HO-8910 cells by MTT assay. HO-8910 cells and stable HO-8910 cell lines expressing GFP, GFP-Rab5a:WT, or GFP-Rab5a:S34N were plated in 96-well dishes as described in the Materials and Methods. After 48 h, the cells were detected by MTT assay. Each group was incubated in 12 wells and the experiment was repeated three times. Values are expressed as mean ± SD. $^{**}P < 0.01$.

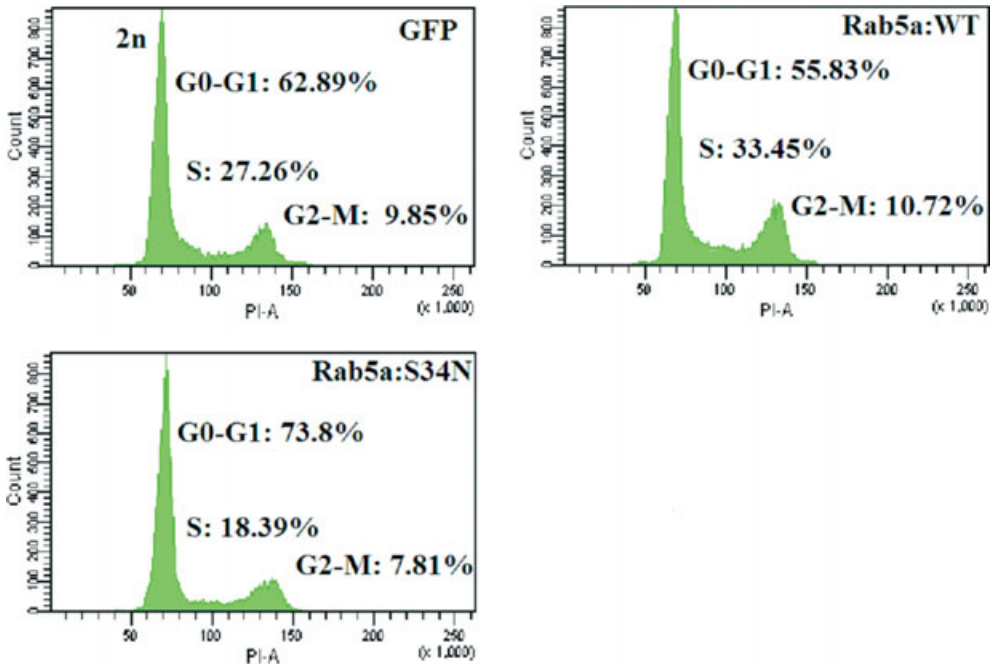

Fig. 5. Effect of Rab5a on cell cycle by flow cytometry. The stable HO-8910 cell lines expressing GFP, GFP-Rab5a:WT, or GFP-Rab5a:S34N were plated in six-well dishes. After 24 h, the cell cycle was detected by flow cytometry as described in the Materials and Methods.

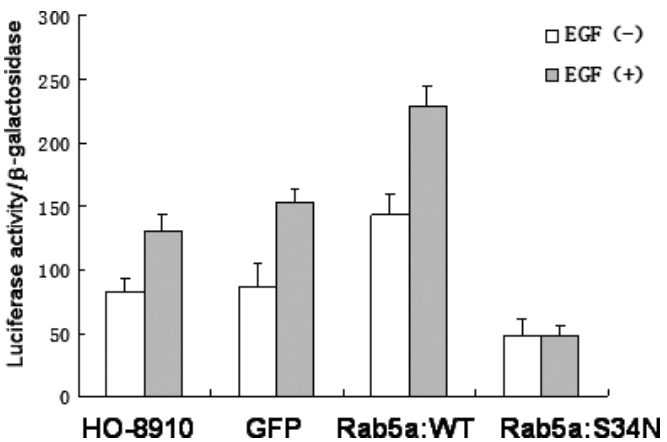

Fig. 6. Effect of Rab5a on the transcription of cyclin D1. Cells expressing GFP, Rab5:WT, and Rab5a:S34N were transfected with a luciferase construct driven by the proximal region of the cyclin D1 promoter and a β-galactosidase construct, then luciferase analysis was carried out as described in the Materials and Methods. Luciferase activity was measured and normalized by β-galactosidase activity. Results represent the mean ± SD from two independent experiments performed in triplicate.

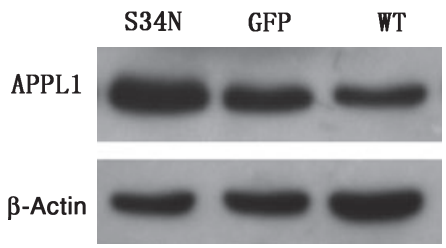

Fig. 7. Expression of APPL1 (adaptor protein containing PH domain, PTB domain, and Leucine zipper motif) in stable HO-8910 cell lines expressing recombinants. Stable HO-8910 cell lines expressing GFP, GFP-Rab5a:WT, and GFP-Rab5a:S34N were lysed and subjected to immunoblot analysis using APPL1 antibody as described in the Materials and Methods. β-Actin was detected as an internal loading control.

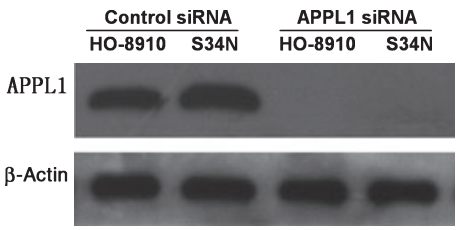

Fig. 8. Expression of APPL1 (adaptor protein containing PH domain, PTB domain, and Leucine zipper motif) in cells transfected with siRNA. The HO-8910 cell line and stable HO-8910 cell line expressing GFP-Rab5a:S34N were transfected with control siRNA or APPL1 siRNA, respectively. After 3 days the cells were lysed and subjected to western blot analysis using APPL1 antibody. β-Actin was detected as an internal loading control.

siRNA-treated cells (data not shown). Rabex-5 is a nucleotide exchange factor of Rab5. It displays GDP/GTP exchange activity on Rab5 upon delivery of the GTPase to the membrane[26].

Rab5a is required for promotion of HO-8910 cell cycle progress by APPL1. APPL1 siRNA and control siRNA were transfected into HO-8910 cells and a stable HO-8910 cell line expressing Rab5a:S34N. The deletion of APPL1 was confirmed by immunoblotting. As shown in Figure 8, APPL1 protein decreased up to 90% in both cell lines treated with APPL1 siRNA in comparison with control cells, and cell cycle assay showed that the deletion of APPL1 induced a reduction of S-phase fraction from 29.2% to 20.15% in HO-8910 cells. But APPL1 deletion did not have a significant impact on S-phase fraction in a stable HO-8910 cell line expressing GFP-Rab5:S34N (Fig. 9). These results indicate that APPL1 can promote cell cycle progression.

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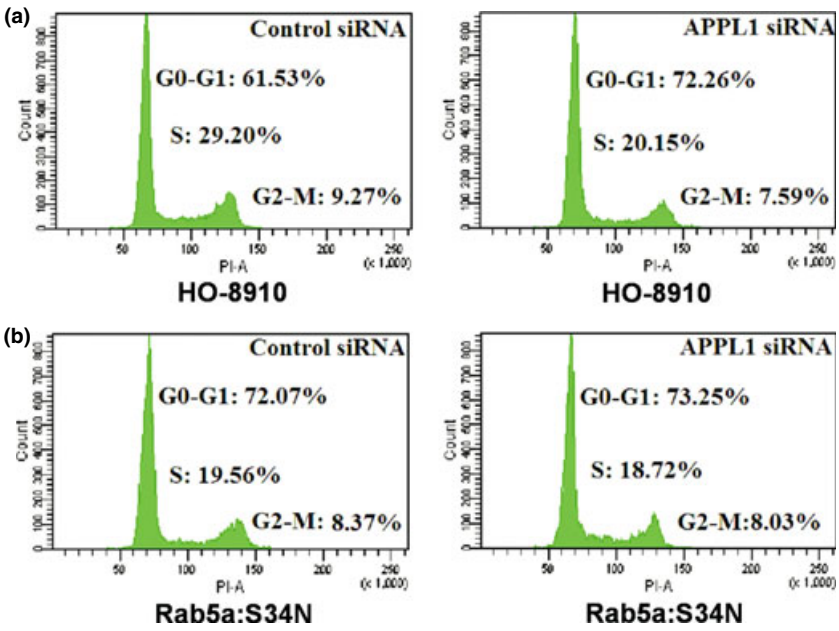

Fig. 9. Effect of APPL1 (adaptor protein containing PH domain, PTB domain, and Leucine zipper motif) on the cell cycle by flow cytometry. (a) HO-8910 cells were transfected with control siRNA or APPL1 siRNA. (b) The stable HO-8910 cell lines expressing GFP-Rab5a:S34N were transfected with control siRNA or APPL1 siRNA. After 3 days the cell cycle was detected by flow cytometry.

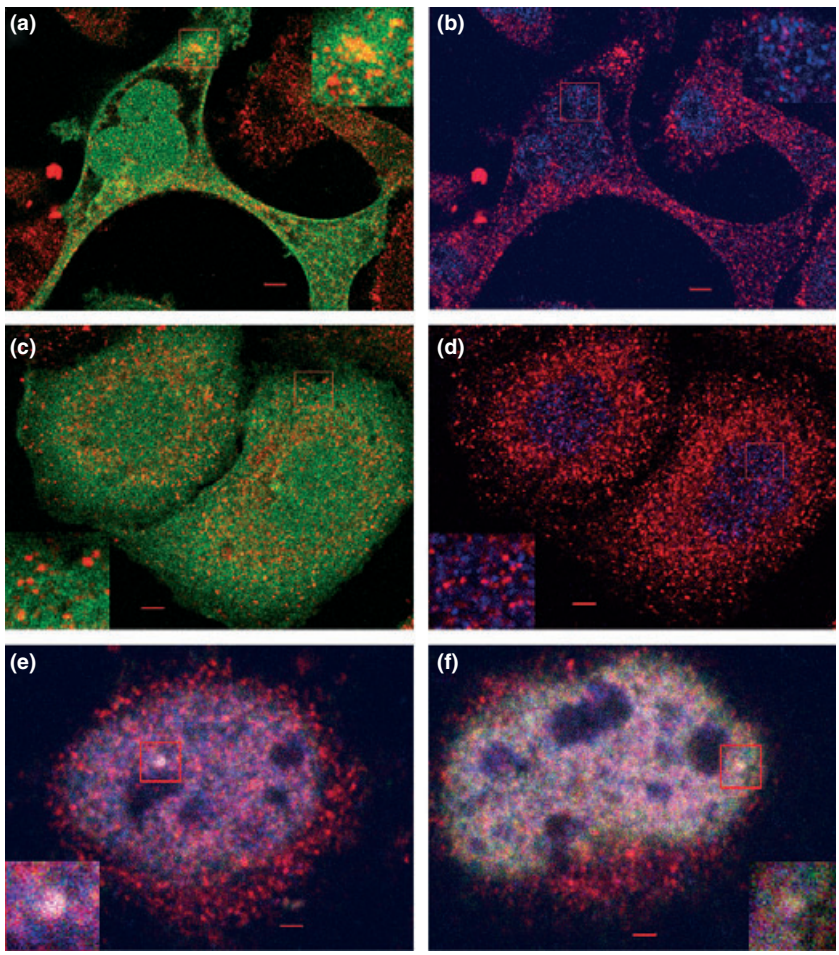

Fig. 10. Confocal microscope analysis of Rab5a, APPL1 (adaptor protein containing PH domain, PTB domain, and Leucine zipper motif), and NuRD subunits. (a,b) HO-8910 cells expressing Rab5a:WT were fixed and stained with rabbit antihuman APPL1 antibody/Cyc3-conjugated antirabbit IgG and mouse antihuman MTA2 antibody/Cyc5-conjugated antimouse IgG. (c,d) HO-8910 cells expressing Rab5a:S34N were fixed and stained with rabbit antihuman APPL1 antibody/Cyc3-conjugated antirabbit IgG and mouse antihuman MTA2 antibody/Cyc5-conjugated antimouse IgG. (e) HO-8910 cells were fixed and stained with goat antihuman HDAC1 antibody/FITC-conjugated donkey antigoat IgG. After washing with PBS, the slides were stained with rabbit antihuman APPL1 antibody/Cyc3-conjugated goat antirabbit IgG and mouse anti-human MTA2 antibody/Cyc5-conjugated goat antimouse IgG, (f) HO-8910 cells were fixed and stained with goat antihuman HDAC2 antibody/FITC-conjugated donkey antigoat IgG. After washing with PBS, the slides were stained with rabbit antihuman APPL1 antibody/Cyc3-conjugated goat antirabbit IgG and mouse antihuman MTA2 antibody/Cyc5-conjugated goat antimouse IgG. Scale bar is equal to 10 μm.

However, this function was closed when the effect of Rab5a was inhibited.

Rab5a and APPL1 work together to achieve EGF signal transduction. To analyze the effect of Rab5 on APPL1 delivery, HO-8910 cells were transfected with GFP-Rab5a:WT and GFP-Rab5a:S34N. After 48 h, the cells were stained and studied by confocal microscopy, which showed that Rab5a:WT was mainly expressed in the cytoplasm and the membrane, and interacted with APPL1 in the plasmolemma. APPL1 also could enter the nucleus in which it colocalized with metastasis-associated

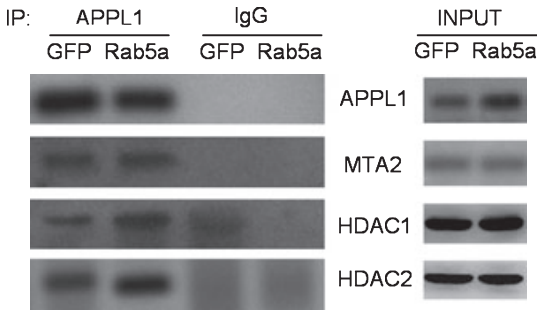

Fig. 11. Immunoprecipitation assay using APPL1 (adaptor protein containing PH domain, PTB domain, and Leucine zipper motif) antibody. Extracts from HO-8910 cells transfected with GFP or GFP-Rab5a were subjected to immunoprecipitation (IP) using anti-APPL1 antibody as described in the Materials and Methods. Non-specific antibodies (IgG) were used as negative controls. Input indicates 10% of total cell extracts used for positive control.

protein 2 (MTA2) (Fig. 10). But the colalization between Rab5a:S34N and APPL1 was not found, and the colalization of APPL1 and MTA2 was reduced significantly in the Rab5a:S34N-expressing cells compared with Rab5a:WT-expressing cells (Fig. 10). It was reported that APPL1 could not directly bind to MTA2 in the nucleus, but bound to NuRD/-MeCP1 complex (MTA2, p66, histone deacetylase 1 [HDAC1], HDAC2, RbAp46, RbAp48, and methyl-CpG binding domain protein 3 [MBD3]) depending on the HDAC2 subunit.[15,27] To test whether APPL1 was bound to NuRD complex rather than MTA2 in HO-8910 cells, immunoprecipitation assay was performed using APPL1 antibody, and then immunoblot was performed using antibodies to several components in the NuRD complex, including MTA2, HDAC1, and HDAC2. Figure 11 shows that the three subunits were all in the precipitation. Using confocal microscopy, we also observed that APPL1, MTA2, and HDAC1 (HDAC2) could colocalize in the nucleus (Fig. 10), showing that APPL1 interacted with NuRD complex instead of an individual component. To find the relationship between Rab5a and APPL1 in EGF signaling, the cells expressing Rab5a:WT were serum-starved overnight, which indicated that both colocalization of Rab5a/APPL1 and APPL1/MTA2 reduced dramatically. However, upon treatment of EGF for 5 min, the colocalization of Rab5a and APPL1 began to increase in the plasmalemma, then APPL1 transferred to the nucleolemma at 15 min, and then a corresponding colocalization increase of APPL1 and MTA2 was observed in the nucleus. But the nucleocytoplasmic shuttling of APPL1 was not observed in cells expressing Rab5a:S34N (Fig. 12). This suggested that APPL1, with the help of Rab5a, could shuttle from the plasmalemma into the nucleus and interact with NuRD complex to play a role in EGF signal transduction.

Discussion

The EGFR family members (receptor protein tyrosine kinases) have been shown to play a key role in normal ovarian follicle development, and cell growth regulation of the ovarian surface epithelium. Disregulation of these normal growth regulatory

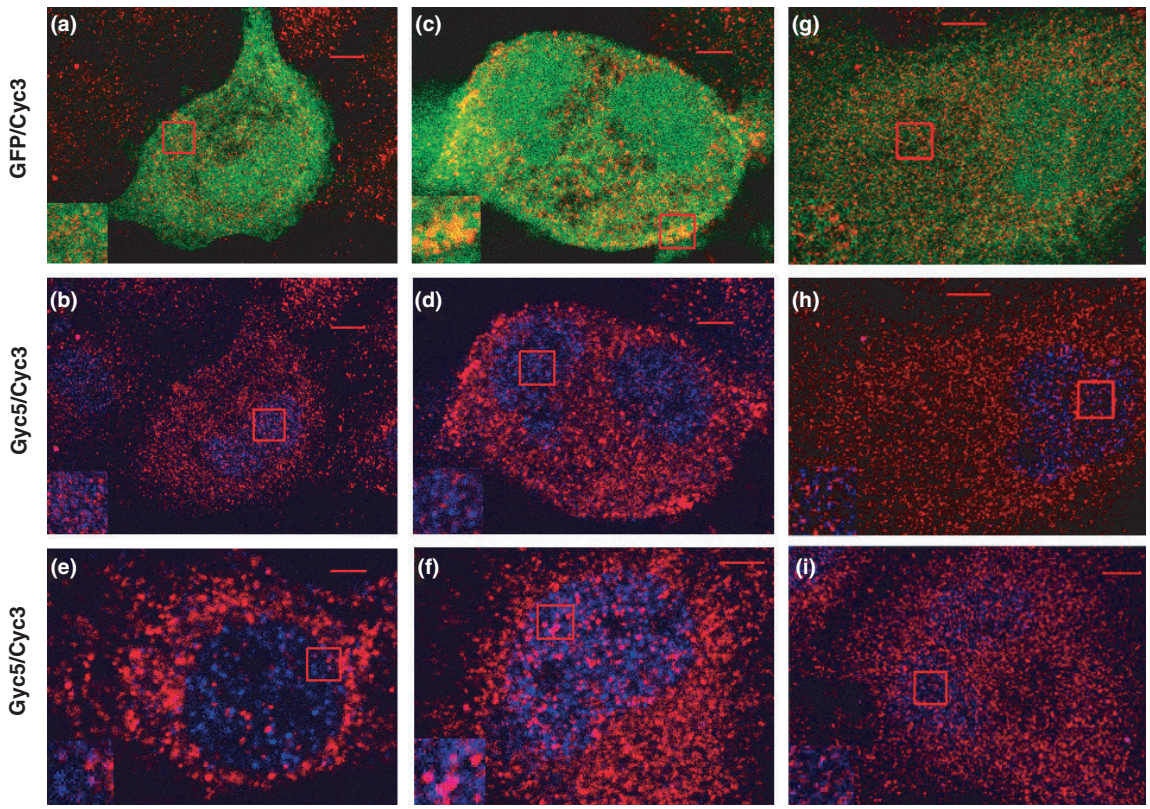

Fig. 12. Effect of APPL1 (adaptor protein containing PH domain, PTB domain, and Leucine zipper motif) and Rab5a in epidermal growth factor (EGF) signal transduction. HO-8910 cells were grown on coverslips overnight, and transfected with GFP-Rab5a:WT. After 48 h, these cells were serum-starved overnight (a,b without EGF), and incubated with 1 μg/mL EGF for 5 min (c,d), 15 min (e), and 30 min (f) at 37°C. Then cells were fixed and stained with rabbit antihuman APPL1 antibody/Cyc3-conjugated antirabbit IgG and mouse antihuman MTA2 antibody/Cyc5-conjugated antimouse IgG for confocal microscope analysis. Cells transfected with GFP-Rab5a:S34N were used as controls. (g,h) Incubated with EGF for 5 min; (i) incubated with EGF for 15 min. Scale bar is equal to 10 μm.

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pathways, including overexpression and/or mutation of EGFR family members, as well as elements of their downstream signaling pathways, have been shown to contribute to the etiology and progression of epithelial ovarian cancer.[28] EGFR overexpression is seen in 17–30% of patients who suffer from ovarian cancer, and is associated with a more invasive phenotype and a poor prognosis.[29,30] Additionally, increased EGFR activity promotes cell cycle progression from the G1 to S phase, causing disproportionate cell proliferation.[31,32] Many studies have discovered that EGFR internalization and intracellular itinerary is Rab5a dependent and is coupled to Rab5a activation.[9,10,33] Recently, a set of observations found that Rab5a was overexpressed in lung, stomach and hepatocellular carcinomas.[20–22] But its effect on the tumor cells is unclear.

In this study, we confirmed Rab5a was overexpressed in ovarian cancer compared with non-ovarian cancer, and it had different expression in ovarian endometrioid carcinoma and ovarian serous adenocarcinoma. Given that Rab5a was closely related to EGF signaling under physiological conditions, we set up a stable HO-8910 cell line expressing Rab5a and Rab5a:S34N, then analyzed cell cycle and proliferation. This illustrated that Rab5a could promote growth of tumor cells by enhancing the G1 to S phase. But Rab5a:S34N, exhibiting a defective GTP-binding activity and acting as a dominant negative mutant, caused a G1 accumulation and inhibited cell proliferation. It was reported that activated EGFR translocates to the nucleus and binds to specific AT-rich DNA sequences to activate cyclin D1 gene transcription.[25] Barbieri *et al.*[10] uncovered that Rab5:S34N blocked EGF induction of cyclin D1 transcription in mouse NR6 cell line. In contrast, overexpression of Rab5 increased cyclin D1 transcription in response to EGF. Consistent with these results, we also found that Rab5a could promote the activation of cyclin D1 with EGF incubation in HO-8910 cell lines. This indicates that Rab5 may play a universal role in EGF-induced cell proliferation.

As a small GTP enzyme, Rab5a regulates the membrane recruitment and activity of a wide range of effectors, such as Rabaptin-5/Rabex-5, early endosome antigen 1 (EEA1), phosphatidylinositol-3 kinases (PI3Ks), and APPL1,[15,26,33] of which APPL1 is closely related to EGF intercellular signal transduction.[15] When APPL1 was deleted by specific siRNA, we found that S-phase fraction reduced significantly in HO-8910 cells. But this effect of APPL1 siRNA was closed when Rab5a GTPase activity was inhibited by Rab5a:S34N. This indicates that for APPL1 to promote cell cycle progression, activation of Rab5a is required. Additionally, immunoblot and gene microarray analysis showed that inhibition of Rab5a GTPase activity induced an increase in APPL1 protein and mRNA. But overexpression of Rab5a caused a reduction of APPL1 protein. So we speculate that, as an upstream regulator, Rab5a can interact with APPL1 and may exact feedback regulation on APPL1 gene expression.

Laser scanning confocal microscopy showed the colocalization of APPL1 and Rab5a in the plasmolemma in normal culture conditions; and Rab5a:S34N mutant lost APPL1-binding capacity. So APPL1 binding to Rab5a was GTP dependent. By serum-starved and EGF incubation, we found that both Rab5a and APPL1 participated in EGF signaling, as the colocalization between Rab5a and APPL1 reduced markedly when the cells were serum-starved overnight. With stimulus of EGF, APPL1 and Rab5a combined with each other again under the membrane. Subsequently, APPL1 left Rab5a, entered the nucleus, and interacted with the NuRD complex. It was reported that about 90% of the Rab5a was in the GDP-bound form in serum-starved cells. But the GTP-bound form of Rab5a rapidly accumulated after exposure of the cells to EGF.[9] This led to an increase of the APPL1/Rab5a-GTP complex. With EGF signal delivery, Rab5a-GTP hydrolysis, APPL1 could potentially be disengaged from Rab5a. This shows that the interaction with Rab5 appeared to be part of a control mechanism to couple the transport of APPL1 to EGF signaling and trafficking.

It was previously documented that APPL1 interacts with the NuRD complex depending on the HDAC2 subunit.[15,27] In the present study, we observed APPL1 could colocalize with MTA2, HDAC1, and HDAC2 by confocal microscopy, and we also found the that three subunits could be pulled down by APPL1 with immunoprecipitation analysis. These results further confirmed Miaczynska's theory that APPL1 interacted with the NuRD complex rather than a single component in the nucleus.[27] NuRD is a multiprotein co-repressor complex with the main function of transcriptional repression. It is mediated by HDAC1 and HDAC2, and is involved in deacetylation of histone H3 and H4 tails. Through this mechanism, NuRD regulates fundamental cellular processes such as proliferation and differentiation, and thus plays important roles in development or carcinogenesis.[27,34–37] Together with our findings, this suggests that Rab5a and APPL1 may work together to link EGF extracellular stimulation to nuclear transcription controlled by the NuRD complex, so as to influence the proliferative activity of ovarian cancer cells. However, the specific mechanism remains to be further studied. Additionally, besides APPL1, APPL2 (another member of the APPL family) can interact with Rab5a and influence HeLa cell proliferation.[15] Future research should focus on whether APPL2 also participates in regulation of ovarian cancer cell growth.

In summary, Rab5a was significantly overexpressed in ovarian cancer tissues in comparison with ovarian cysts and ovarian cystadenomas. Though it was correlated with neither FIGO stage nor neoplastic metastasis, Rab5a may promote the tumor cell growth, in part by interaction with APPL1 which links EGF stimulus to nuclear transcription.

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Disclosure Statement

The authors have no conflict of interest.

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