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RESEARCH ARTICLE

The role of Crk/Dock180/Rac1 pathway in the malignant behavior of human ovarian cancer cell SKOV3

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Abstract Small GTPases, particularly the Rho family, are key regulators of cell motility and migration. Dock180 was well known for the main target of signal adaptor protein Crk and acted as a guanine-nucleotide exchange factor for small GTPase Rac1. In the present study, Dock180 was found to combine primarily with CrkI other than CrkII, and its association with Elmo1 was also demonstrated in ovarian cancer cell SKOV3. To evaluate the role of Dock180 in human ovarian cancer cell, we performed RNAi-mediated knockdown of Dock180 in SKOV3 cells using small interfering RNA expression vector. In Dock180 knockdown cells, we found that Elmo1 expression and Rac1 activity were decreased simultaneously. By contrast, the expressions of both another Crk-combining molecule C3G and Rap1 activity were observed to increase obviously. Accordingly, all Dock180 knockdown cells present with evident change in cell morphology, reduced cell proliferation, and attenuated cell migration. Taken together, these results suggest that signal transfer of Crk/Dock180/Rac1 is implicated in actin cytoskeleton reorganization and thus in the cell proliferation, motility, invasion, and of human ovarian cancer cell line SKOV3.

Keywords Crk · DOCK180 · Rac1 · C3G · Human ovarian cancer

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Introduction

The inherent invasive nature of ovarian cancer contributes to the prominent metastatic potential in general and primarily exhibit intraperitoneal dissemination in patients. In spite of multimodal therapies, including surgery, chemotherapy, and radiation, 90% of patients with ovarian cancer died of metastasis eventually [1]. It is established that the mechanisms under how cell motility is regulated are fundamental to the invasive phenotype of tumor cells. The maintenance of cell morphology, motility, and migration requires dynamic rearrangement of the actin cytoskeleton, which is regulated by the Rho family of small GTPases, including Rac1, Cdc42, and RhoA [2].

Signaling adaptor protein Crk, which is comprised of SH2 and SH3 domains, was originally isolated as an oncogene product of the CT10 chicken retrovirus [3]. Through alternative splicing, human Crk gene is translated into two products, CrkI and CrkII [4]. CrkII possesses one SH2 domain and two SH3 domains, while its alternative splicing product CrkI is composed of one SH2 domain and one N-terminal SH3 domain. Crk has been shown to transmit signals to small GTPases by association with its downstream effectors such as Dock180 and C3G, which are the guanine-nucleotide exchange factors (GEFs) for Rac and Rap, respectively [5–8]. It has been reported that Crk is overexpressed in human cancers including various kinds of carcinomas and sarcomas

[9]. Expression of CrkI in tumor cells induced transformation that stimulated cell migration and invasion concomitant with poor prognosis in patients [10, 11].

The role of adaptor protein Crk has been studied in two ovarian cancer cell line MCAS [12] and SKOV3 (Wang et al. submitted and being revised) and both with Crk knocked down presented with reduced Rac activity and migration. Dock180 was originally identified as a SH3 domain-binding protein of adaptor protein Crk and associated with cell morphology [13]. Studies in *Caenorhabditis elegans* and *Drosophila* reveal that Dock180 homologues modulate various functions including phagocytosis, cell migration, and actin cytoskeletal organization through the activation of Rac1 [8, 14–19]. And as a result, the Dock180 family of proteins has been suggested to function as an upstream regulator of the small GTPase Rac. The best characterized Rac activation mediated by Dock family is through Dock180–Elmo1 complex [20, 21]. The association of Dock180 with the adaptor protein Crk in activating Rac1 upon stimulation through integrin [8, 22–26] was also reported. All of the results indicated that Dock180 stimulates cell migration in normal mammalian cells. However, whether it plays a critical role in cancer cell migration and invasion has not been clearly investigated. Because of its contribution to Dock180-dependent Rac1 activation and formation of lamellipodia and filopodia, Dock180 has been suggested to be involved in the formation of focal adhesion and cell migration, tumor invasion, and metastasis [27–29], which rank first among all the reasons of tumor death. While there is little direct evidence about the role of Dock180 in tumor invasion and metastasis, its mechanism remains largely unknown.

To further confirm the exact role of Dock180 and its mechanism, in this study, endogenous expression of Dock180 was silenced in ovarian cancer cell line SKOV3. The features of the Dock180 knockdown cells demonstrated that the endogenous Elmo1 expression was reduced simultaneously, and thus the reduction of Rac1-GTP level develops accordingly. As compared, the expression level of C3G, the guanine-nucleotide exchange factor for Rap-GTP, was found to increase evidently, and increase in Rap1 activity was found correspondingly. And Dock180 knockdown cells presented with attenuation in spreading, membrane ruffling formation, decrease in cell proliferation, cell migration, and invasion.

Materials and methods

Cell line and antibodies

SKOV3, a human serous cystadenocarcinoma cell line, was obtained from Department of Pathology, Chongqing Medical University (P. R. China) and maintained in Roosevelt Park Memorial Institute Medium(RPMI 1640) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and streptomycin. The mouse monoclonal antibody for Crk was purchased from BD Biosciences. Antibodies for C3G (H300), CrkL (C20), DOCK180 (H4), Elmo1 (N-20), and actin (I-19) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Plasmids and reagents

Empty vector pSUPER.retro.neo-GFP, a short hairpin RNA (shRNA) expression vector, was purchased from Oligoengine Inc. Two different Dock180 small interfering RNA (siRNA) DNA oligonucleotides were designed using Oligoengine RNAi design software. The shRNA for human Dock180 was designed to target 19 nucleotides of the human Dock180 transcript (nucleotides 2932–2950, 5′-TGAGGACTG ATGTGGTAGA-3′), the shRNA targeting another region of the human Dock180 transcript (nucleotides 1259–1277, 5′-TCCGCATTTAGTGGACAGG-3′). According to the insert sequence of MISSION Non-Target shRNA Control Vector of Sigma Corporation (5′-CAACAAGATGAAGAGCACCAA-3′), a control Non-Target shRNA was designed. These oligonucleotides were ordered from IDT (Integrated DNA Technologies, USA). By using two different Dock180 siRNA and one Non-Target shRNA segment subcloned in the pSUPER.retro.neo-GFP vector, respectively, we successfully constructed two pSUPER.retro.neo-GFP-iDock180 vectors and one new pSUPER.retro.neo-GFP-NT vector.

Immunoprecipitation and immunoblotting

Cells were lysed with lysis buffer containing150 mM NaCl, 10 mM Tris hydrocloride (pH 7.5), 1 mM sodium orthevanadate, 1 mM phenylmethysulfonyl fluoride, 0.5% Nonidet P-40, 50 mM sodium fluoride, 5 mM EDTA, and 10% glycerol. Lysates were incubated with antibodies for more than 1 h at 4°C and precipitated with protein G-sepharose beads (Sigma, USA). The immunoprecipitants were separated by SDS–polyacrylamide gel electrophoresis (PAGE) and immunoblotted to polyvinylidene fluoride membrane (Millipore Corporation, Bedford, MA, USA). The membranes were blocked, incubated with the indicated primary antibodies, and subsequently probed with peroxidase-labeled secondary antibodies. The reacted proteins were visualized by enhanced chemiluminescence reaction (Millipore Corporation).

Establishment of DOCK180 knockdown SKOV3 cells

Cells were transfected with 3 μg of pSUPER.retro.neo-GFP-iDock180 plasmids, followed by selection with G418 (700 μg/ml) for 2 weeks. Cells were observed under

fluorescence microscope. Colonies were isolated, and Dock180 expression levels of were analyzed by immunoblotting.

Fluorescence staining

Subconfluent cells grown on a glass coverslip were fixed with 3.7% formaldehyde in PBS for 10 min at room temperature and permeabilized with 0.1% Triton X-100 in PBS for 5 min and then washed three times with PBS. To visualize actin filaments (F-actin), cells were stained with Texas Red-X phalloidin (1 U/200 μl) for 20 min and observed under a confocal laser scanning microscope (LEICA TCS SP2, Germany).

MTT assay for cell proliferation

To measure cell growth rates, 2×10^3 cells were dispensed into each well of 96-well plates with RPMI 1640 containing 10% FBS and incubated in a humidified culture incubator at 37°C for 1, 2, 3, 4, 5, 6, and 7 days, respectively, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay was performed by adding 20 μl of MTT (5 mg/ml in PBS store at −20°C) for 4 h. When MTT incubation had been completed, supernatants were removed, and 200 μl of DSMO was added. The absorbance value (OD) of each well was measured with microplate reader set at 570 nm. All experiments were performed in triplicate.

Plate colony formation assay

Cells (1×10^2) were added to 24-well plates with RPMI 1640 containing 10% FBS, and each cell group contained three wells. After incubation at 37°C for 14 days, the cells were washed twice with PBS and stained with Wright's solution. The number of colonies containing ≥50 cells was counted under a microscope [plate clone formation efficiency= (number of colonies/number of cells inoculated)×100%].

Wound healing assay

Cell migration was evaluated by scratched wound healing assay on the plastic plate wells. In brief, a total of 5×10^5 cells were seeded on each well and incubated for 12 h. The surface of confluent cells of monolayer was wounded by a tip. The cellular migration was evaluated by measuring the width of the wound at the same point immediately and following up at the same time intervals as indicated after wounding. Each assay was carried out in triplicate.

Invasion assay

The lower surface of the filter BD Bio Coat Matrigel Invasion Chamber (BD Biosciences, Franklin Lakes, NJ, USA) was used. Control and Dock180 knockdown cells were cultured in 100-mm dishes to subconfluence. Cells (1×10^5) were seeded in each of the upper chambers (24-well chambers) in 0.4 ml of RPMI1640 medium, and the bottom chambers contained the medium with 10%FBS as a chemoattractant. The cells were then incubated for 24 h in a humidified culture incubator, at 37°C. The non-invading cells on the upper surface of the filters were removed by wiping with a cotton swab. Remaining cells at the bottom side of the membranes were fixed with ethanol and stained with HE. The number of cells invading through the Matrigel membrane was counted in microscopic fields at ×400 magnification. To minimize the bias, at least three randomly selected fields were counted. Data were averages of triplicate determinants for each condition.

Pull-down assay for Rac1 and Rap1 activation

GTP loading of Rac1 was measured using the Rac1 Activation Assay Kit (Upstate, Millipore Technology) according to the manufacturer's instructions. Cells were lysed in ice-cold magnesium lysis buffer and cleared with glutathione–agarose beads. Cell extracts were then incubated with GST-PAK-1 RBD agorose beads, pelleted, and washed. The beads were resuspended in sample buffer and separated by 10% PAGE. GTP-bound Rac1 was analyzed by immunoblotting with anti-Rac1 antibody.

GTP loading of Rap1 was measured using the Rap1 Activation Assay Kit (Upstate, Millipore Technology) according to the manufacturer' s instructions. Cells were lysed in ice-cold rap1 activation lysis buffer and cleared with glutathione–agarose beads. Cell extracts were then incubated with GST-RalGDS RBD agarose beads, pelleted, and washed. The beads were resuspended in sample buffer and separated by 10% PAGE. GTP-bound Rap1 was detected using an anti-Rap1 antibody.

Statistical analysis

All experiments were repeated at least three times. SAS 9.1 software was used for statistical analysis. All assays were tested using ANOVA. Differences were considered statistically significant when $P<0.05$.

Results

Association of Dock180 with Crk in SKOV3 cells

To evaluate protein–protein interaction of Dock180, we performed co-precipitation experiments in ovarian cancer cell SKOV3 using anti-Dock180 monoclonal antibody.

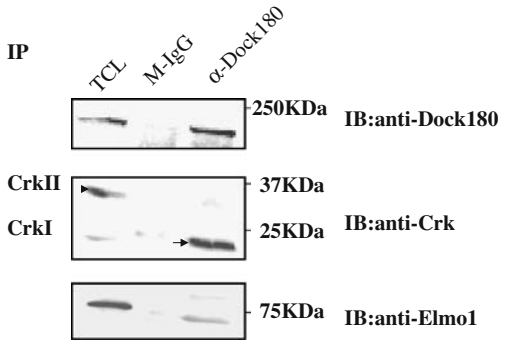

Fig. 1 Crk, Elmo1, and Dock180 were found to precipitate simultaneously

Proteins bound to DOCK180 were analyzed by Western blotting with antibodies against Crk.

As shown in Fig. 1, Crk, Elmo1 and Dock180 were found to precipitate simultaneously. As for its association with Crk, endogenous Dock180 prefers to combine with CrkI rather than with CrkII.

Establishment of Dock180 knockdown cell lines in SKOV3 cells

To silence endogenous Dock180 expression, siRNA was employed. By using two different Dock180 siRNA subcloned in the pSUPER.retro.neo-GFP vector, respectively, we successfully established two independent Dock180 knockdown cells, respectively, which were designated as Dock180i-1 and Dock180i-2. As demonstrated in Fig. 2, with the Dock180 expression knocked down, its bipartite sibling member Elmo1 was also nearly depleted. While in contrast, C3G, another downstream binding partner of adaptor protein Crk, was found to hold significantly increased expression level in all Dock180 knockdown cells, as compared with that of their mock counterparts. However, its most well-known upstream combining protein Crk remains unchanged as were those of actin. Endogenous protein levels of CrkL were unchanged as were those of actin.

To further investigate the feature of Dock180 knockdown cells, one strain of SKOV3 cell line with Dock180 knockdown, DOCKi-1, was used as representatives in the experiments. And the control cells were named as NT.cont and Empty.cont cells.

Effect of Dock180 knockdown upon cell morphology

As shown in Fig. 3, consistent with the change in Crk knockdown of SKOV3 cells as previously described, using actin staining, Dock180 knockdown of SKOV3 cells presented with decrease in formation of lamellipodia and filopodia, as compared with their control cells.

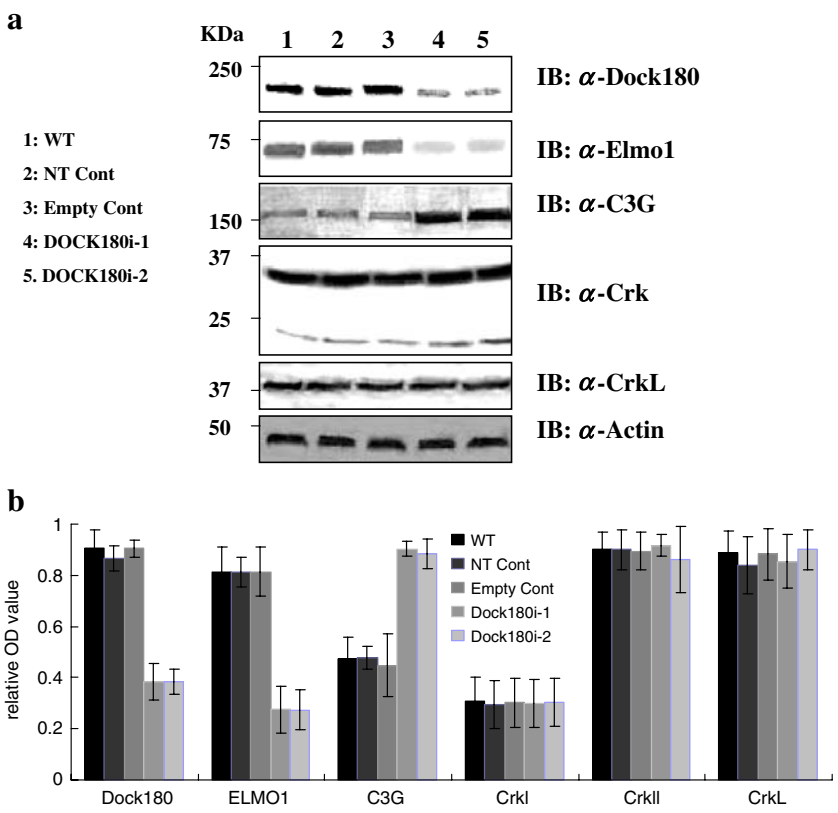

Fig. 2 **a**–**b** The Dock180 expression was knocked down, and its bipartite sibling member Elmo1 was also nearly depleted

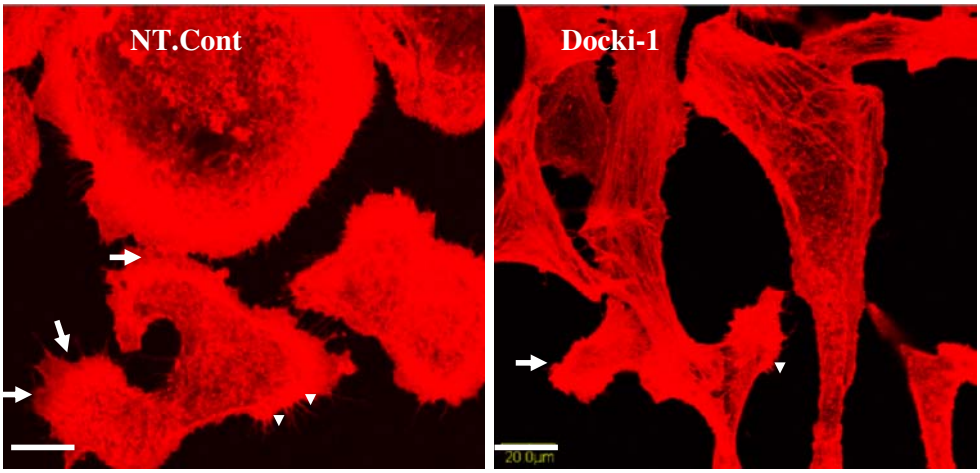

Fig. 3 Dock180 knockdown of SKOV3 cells presented with decrease in formation of lamellipodia and filopodia

Effect of Dock180 knockdown on cell proliferation

To clarify if the change of cell migration mediated by Dock180/Elmo1 complex has any interference with cell proliferation, usually the first prominent characteristic of malignancies, the effect of Dock180 protein reduction on the proliferation of ovarian cancer cells was determined by MTT assay and plate clone formation assay. As shown in Fig. 4a, NT control and empty vector control cells showed in vitro growth ability approximately equal to that of wild-type (WT) cells, and Docki-1and Docki-2cells have reduced growth ability as compared with NT control cells, empty vector control cells, and WT control cells. The results suggested that in vitro cell growth ability correlates with Dock180 expression. Figure 4b shows the plate colony formation of NT control cells and Docki-1 cells. Figure 4c shows that Docki-1 and Docki-2 cells, compared with WT, NT, and empty vector control cells, had a significant reduction in their ability to form colonies.

Analysis of migration and invasion of Dock180 knockdown cells

Cell migration and invasion are basic characteristics of tumor metastasis. Dock180 has been reported to act an essential role in promoting cell migration and invasion

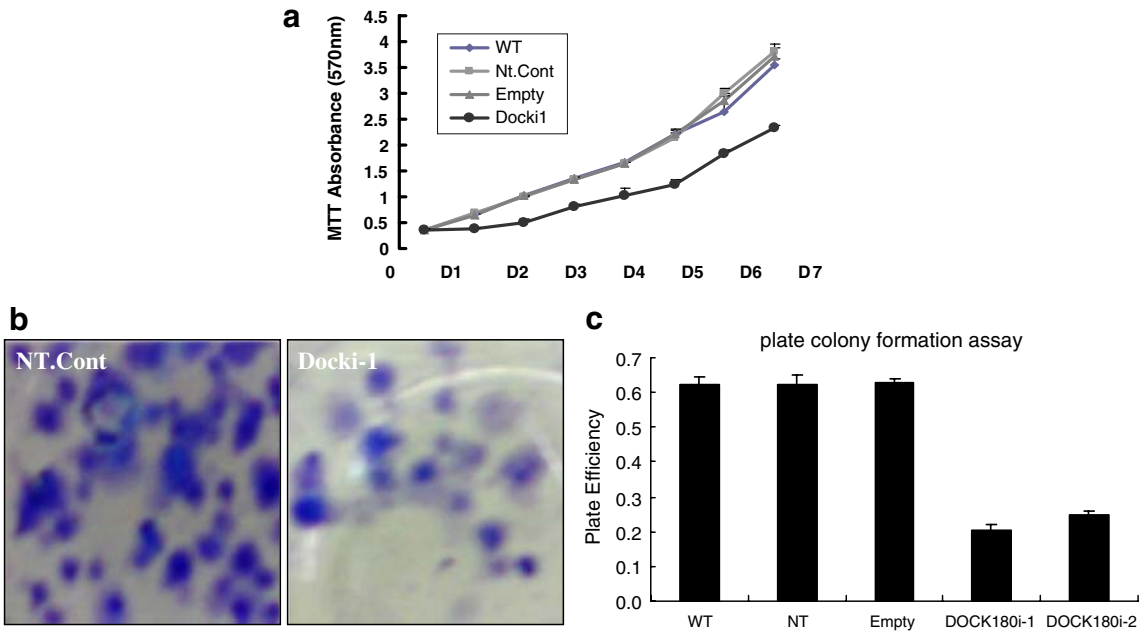

Fig. 4 **a**–**c** NT control and empty vector control cells showed in vitro growth ability approximately equal to that of WT cells

through Rac1 activation [2, 26, 30]. Thus we assessed cell migration by wound healing assay. As shown in Fig. 5b, Docki-1 cells demonstrated about 38.7% decrease in healing rate compared with their controls. Similarly, cell invasiveness was inhibited in the Dock180 knockdown cells versus the control cells by Matrigel transwell assay (Fig. 5c).

As shown in Fig. 5d, Dock180 knockdown cells, compared with WT, NT, and empty vector control cells, displayed a remarkable decrease in invasiveness. These results demonstrate that the invasive ability of ovarian cancer cells correlated with Dock180 expression, and Dock180 silencing is sufficient to attenuate invasion in ovarian cancer cells.

Analysis of Rac1 and Rap1 activity by pull-down assay

As Dock180 acts as the downstream of Crk and functions as an activator for Rac-GTP (which is the activated form of Rac), Rac activity was measured by pull-down assay. As indicated in Fig. 6a, b, Dock180 knockdown SKOV3 cells presented with significantly decreased Rac1-GTP levels despite the unchanged total Rac1 protein levels. These data suggested that Dock180 regulates the activity of Rac1-GTP. In contrast, as indicated in Fig. 6c, d, we found that the Rap1 activity was increased in Dock180 knockdown cells, in line with the increase in C3G expression.

Discussion

The poor prognosis of ovarian cancer patients is mainly due to dissemination caused by the aggressive migration activity of cancer cells, which results in invasion and metastasis. Based on our prior findings that Crk knockdown ovarian cancer cells (including SKOV3 cells) displayed reduction in Rac1 activity and limited tumorigenetic ability ([12] and another manuscript submitted elsewhere and being revised), Dock180, as the major downstream combining molecule of Crk and up-regulator of Rac1, was further investigated for its role in SKOV3 cell motility and tumorigenesis. In the present study, association between Dock180 and Elmo1 was confirmed by immunoprecipitation experiments, and we showed that Dock180 interacted predominantly with CrkI and found that inhibition of endogenous Dock180 expression attenuated the invasive behavior of SKOV3 cell lines in concomitant with a reduction in activated Rac1, which is a key regulator of actin cytoskeletal dynamics modulating cell migration and invasion. Given the previous findings that CrkI held much stronger transfor-

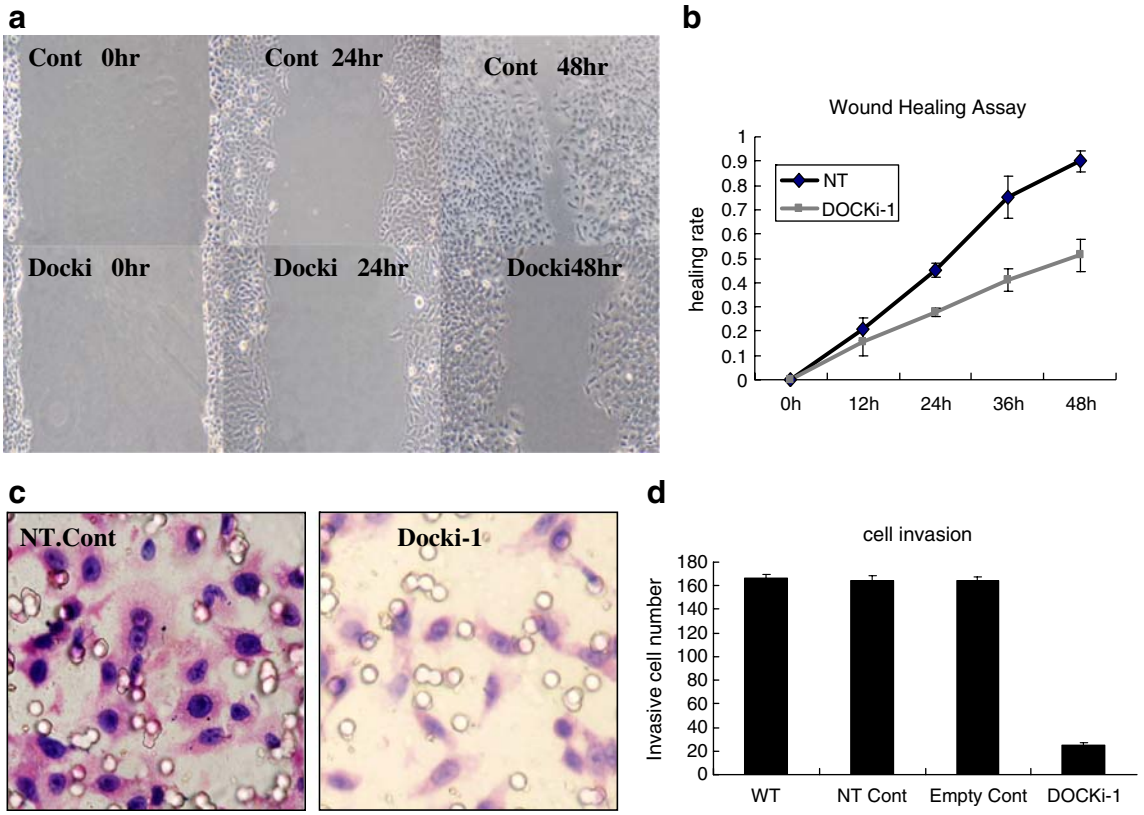

Fig. 5 **a**–**d** Docki-1 cells demonstrated about 38.7% decrease in healing rate compared with their controls

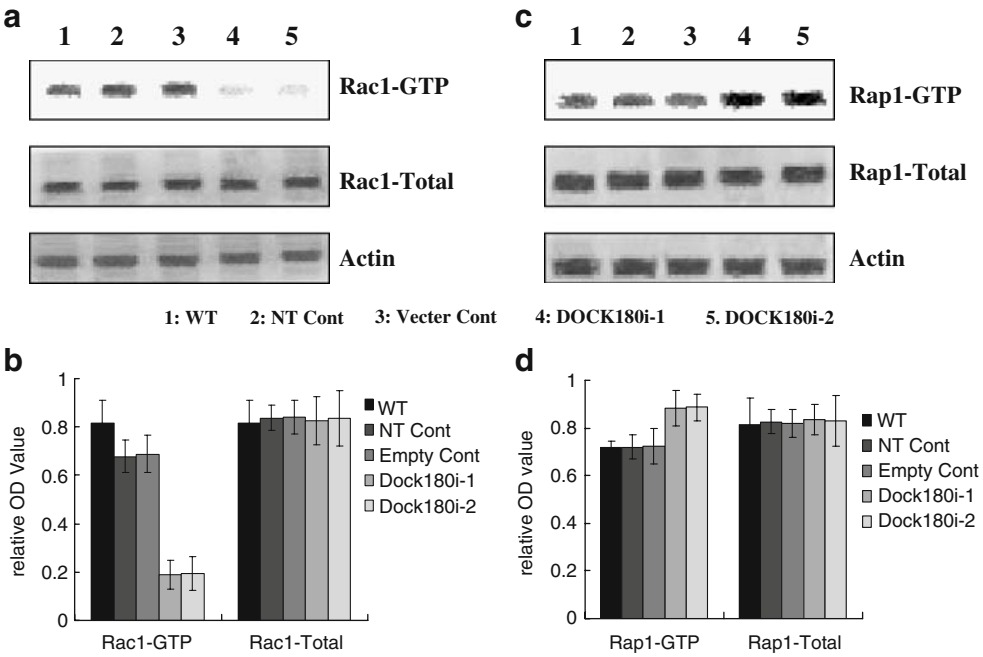

Fig. 6 **a–d** Dock180 knockdown SKOV3 cells presented with significantly decreased Rac1-GTP levels despite the unchanged total Rac1 protein levels

mative activity as compared with CrkII [10, 11], it is reasonable to speculate that it is CrkI/Dock180/Rac1 pathway other than CrkII/Dock180/Rac1 pathway that plays a key role in the carcinogenesis of SKOV3 cells. The accompanying reduced Elmo1 expression (Fig. 2 and Electronic supplementary materials figure) together with the knockdown of Dock180 expression supports that these two molecules act as a bipartite GEF for Rac1 activity, and their function would be dependent upon each other, even though the exact mechanisms by which they cooperate in vivo need further identifying.

The change in cell morphology and thus the compromised cell migration and invasion in Dock180 knockdown cells denote the important role of Dock180/Elmo1 complex in forming and maintaining membrane ruffling dynamically, which matters vastly in controlling cell motility, the prerequisite for tumor invasion and spreading. The results imply the involvement of Dock180 in the malignancy of ovarian cancer.

In contrast, the decrease in Dock180 expression and Rac1 activity were complemented with increase in C3G expression and Rap1 activity as a result. Both Dock180 and C3G are well-known Crk-SH3 (N)-domain-combining partners [6–8, 31, 32]. C3G is a guanine-nucleotide exchange factor for Rap-1, which was originally reported to be a suppressor of Ras [33] and activation of C3G reverses the Ras-induced transformation of cells, as does Rap1 [6].

Provided that the interference of overexpressed active Rap1 with Rac1 activity mediated through paxillin-Crk-Dock180 pathway [28], such reciprocal action of one another indicates the probably competitive role between Dock180/Rac1 and C3G/Rap1, even though the exact mechanism needs further identification.

Rap1 proteins have been confirmed to participate in the formation and maintenance of adherens junctions in epithelia, just as the Rho GTPases [34]. Combined with the recent findings that Rap-GTP was localized both unto the plasma membrane and in intracellular space [35], C3G-mediated Rap1 activity was involved in epithelialization [28], in contrast with the course of malignant transformation, which is epithelial–mesenchymal transformation [36]. From this point, endeavors to decrease Rac1 activity mediated by Crk/Dock180/Elmo1 complex and resultant increase in Rap1 activity through Crk/C3G pathway could favor reversing malignant transformation, and this may help to control metastatic loci and prevent recurrence efficiently.

We found that knockdown of Dock180 expression strongly inhibited in vitro cell growth and colony formation efficiency. All data indicated that Dock180 may be a positive regulator of tumor growth in ovarian cancer. Moreover, in agreement with our previous study about Crk, our experiments demonstrated that knockdown of Dock180 expression in ovarian cancer SKOV3 cells reduced their ability to invade Matrigel-coated membranes in an invasion chamber assay. These results support the involvement of Dock180 with the malignant behavior of human ovarian cancer SKOV3 cells and underline the relevance of a stable Dock180 knockdown mediated by RNAi in the impairment of the invasive ability of ovarian cancer.

Altogether, the results of this study denote that Dock180 acts as a major downstream molecule of adaptor protein

Crk to activate their effecter Rac1 in ovarian cancer SKOV3 cells. Rac1 activity mediated by Dock180 plays an important role in promoting cell migration and consequently cell invasion and dissemination. Dock180 knockdown reverses the malignant behavior of SKOV3 cell mediated through both increase in Rap1 activity and decrease in Rac1 activity.

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