p21-activated kinase 4 regulates ovarian cancer cell proliferation, migration, and invasion and contributes to poor prognosis in patients

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Ovarian cancer is a lethal gynecological malignancy, and to improve survival, it is important to identify novel prognostic and therapeutic targets. In this study, we present a role for p21-activated kinase 4 (Pak4) in ovarian cancer progression. We show a significant association between increased expression of Pak4 and its activated form, phosphorylated (p)-Pak4 Ser⁴⁷⁴, with metastasis of ovarian cancers, shorter overall and disease-free survival, advanced stage and high-grade cancers, serous/clear cell histological subtypes, and reduced chemosensitivity. Pak4 overexpression was also observed in ovarian cancer cell lines. Pak4 and p-Pak4 expression were detected both in the nucleus and cytoplasm of ovarian cancer cells, in vitro as well as in vivo. Stable knockdown of Pak4 in ovarian cancer cell lines led to reduced cell migration, invasion, and proliferation, along with reduced c-Src, ERK1/2, and epidermal growth factor receptor (EGFR) activation and decreased matrix metalloproteinase 2 (MMP2) expression. Conversely, Pak4 overexpression promoted ovarian cancer cell migration and invasion in a c-Src, MEK-1, MMP2, and kinase-dependent manner, and induced cell proliferation through the Pak4/c-Src/EGFR pathway that controls cyclin D1 and CDC25A expression. Stable knockdown of Pak4 also impeded tumor growth and dissemination in nude mice. This report reveals the association between Pak4 and important clinicopathologic parameters, suggesting Pak4 to be a significant prognostic marker and potential therapeutic molecular target in ovarian cancer. The implied possible cross-talk between Pak4 and EGFR suggests the potential of dual targeting of EGFR and Pak4 as a unique therapeutic approach for cancer therapy.

prognostic marker | therapeutic target

Ovarian cancer is a gynecological cancer associated with high mortality worldwide (1), and its incidence in Asian countries, including China and Japan, is on the rise (2). The poor prognosis is attributed to its commonly subtle symptoms until widespread metastases develop, and the significant failure rate of chemotherapy in patients with advanced disease (3). To improve the survival of patients with ovarian cancer, it is important to investigate genes governing metastasis and to identify novel prognostic markers and therapeutic targets.

p21-activated serine/threonine kinases (Paks) are major effectors of the small Rho GTPases Rac1 and Cdc42, which play important roles in cell morphology, cytoskeletal reorganization, apoptosis, survival, and angiogenesis—all prerequisite steps for metastasis (4). Six mammalian Paks have been identified and classified into group I (Paks1–3) and group II (Paks4–6) (4). We have recently identified Pak1 as an independent prognostic factor in ovarian cancer, affecting cell migration and invasion via the p38 pathway (5). Pak4 was first identified as an effector of Cdc42 essential for regulating cytoskeleton reorganization (6). Subsequent studies indicated that Pak4 could protect cells from apoptosis (7), inhibit cell adhesion (8), and promote cell migration (9–11) and anchorage-independent growth (8, 12). Many of these functions

are dependent on Pak4 kinase activity. Moreover, Pak4 is up-regulated in most cancer cell lines (12). In athymic mice, overexpression of Pak4 leads to tumor formation and depletion of Pak4-inhibited tumorigenesis (13). The Pak4 gene is localized to a region of chromosome 19 (12), where amplification is frequently observed in ovarian cancer (14). Indeed, amplification of Pak4 gene was found in a fraction of cancers (15). However, it is still unclear if Pak4 expression may relate to cancer progression and clinical outcome.

Herein, ovarian cancer was adopted as the model for the study of Pak4. The expression and subcellular localization of Pak4 and phosphorylated(p)-Pak4 Ser⁴⁷⁴ (the activated form) (12) in ovarian cancer cell lines and tumors was assessed and correlated with clinicopathological parameters. We also investigated the potential role and downstream pathways of Pak4 in ovarian cancer cell migration, invasion, and proliferation, and the induction effect of follicle stimulating hormone (FSH) and hepatocyte growth factor (HGF) on Pak4. Our findings suggest that Pak4 may play a role in the progression of ovarian tumors, and is a potentially useful molecular prognostic marker and therapeutic target.

Results

Overexpression of Pak4 and Activated Pak4 Correlates with Progression of Ovarian Cancer and Prognosis of Patients. By immunohistochemistry, we observed mild nuclear and strong cytoplasmic Pak4 (Fig. S1A) and strong nuclear and moderate cytoplasmic p-Pak4 (Fig. 1A) immunoreactivity in ovarian cancer tissue and ascitic fluid samples (Fig. S1B). In contrast, there was only moderate staining in borderline tumors and weak or no staining in benign cystadenomas and inclusion cysts (Fig. 1). Indeed, significantly higher nuclear and cytoplasmic Pak4 and cytoplasmic p-Pak4 immunoreactivity was detected in ovarian cancers and borderline tumors than in benign cystadenomas/inclusion cysts (all $P < 0.05$; Table S1). At mRNA level, significantly higher Pak4 was also found in ovarian cancers and borderline tumors than in benign cystadenomas as evaluated by qPCR (all $P < 0.05$; Fig. 1B). Up-regulation of Pak4 mRNA and protein was also observed in ovarian cancer cell lines compared with normal ovarian epithelium cell lines (Fig. 1C). Moreover, increased nuclear and cytoplasmic Pak4 and p-Pak4 expression were significantly associated with serous/clear cell histological subtypes and shorter overall and disease-free survival (all $P < 0.05$; Table S1, Fig. S1D). Multivariate analysis

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Fig. 1. Overexpression of Pak4 and p-Pak4 (the activated form) in ovarian cancer. (A) Immunohistochemical staining of p-Pak4 in serous (i) and mucinous (v) benign ovarian cystadenomas, serous (ii) and mucinous (vi) borderline ovarian tumors and serous (iii), mucinous (vii), clear-cell (iv), and endometrioid (viii) ovarian carcinomas. (Insets) Regions with higher magnification. (B) qPCR analysis of Pak4 mRNA in ovarian tumors. The fold change of Pak4 mRNA was calculated with respect to the lowest expression of Pak4 in cystadenomas. (C) mRNA (Left) and protein (Right) expression of Pak4 in immortalized epithelial cell lines and ovarian cancer cell lines as determined by qPCR (* $P < 0.05$ compared with HOSE 11-12) and immunoblot analysis, respectively.

revealed that cytoplasmic Pak4, nuclear and cytoplasmic p-Pak4, disease stage, and chemosensitivity remained significant predictors for overall survival (all $P < 0.05$; Table S2), whereas cytoplasmic Pak4 and p-Pak4, disease stage, and chemosensitivity continued to be significant predictors for disease-free survival (all $P < 0.05$; Table S2). Significantly higher cytoplasmic Pak4 and p-Pak4 expression was found in carcinomas of advanced stages (stages III and IV) and poor histological differentiation (grade 3) and at metastatic foci (all $P < 0.05$; Table S1). Furthermore, high nuclear and cytoplasmic Pak4 expression was significantly associated with resistance to chemotherapy (all $P < 0.05$; Table S1).

Pak4 and p-Pak4 Localization in the Nucleus and Cytoplasm of Ovarian Cancer Cells. Immunofluorescent staining displayed strong nuclear and moderate cytoplasmic Pak4 and p-Pak4 immunoreactivity in OVCAR-3. Besides, nuclear Pak4, but not p-Pak4, was colocalized with C23-nucleolin, a nucleolar protein involved in nucleolar RNA biogenesis (Fig. 2A). Four possible nuclear localization signals (NLSs), responsible for nuclear accumulation (16), and a leucine zipper pattern, responsible for dimerization and DNA binding (17), were identified when the Pak4 amino acid sequence was analyzed using the NLS-recognizing program PSORTII (16) (Fig. 2B). Subcellular expression of Pak4 and p-Pak4 in cytoplasmic and nuclear fractions of OVCAR-3 was confirmed by immunoblot analysis (Fig. 2C). Moreover, ectopic expression of Flag-tagged wt Pak4 in SKOV-3 revealed that exogenous Pak4 was localized to both the cytoplasm and the nucleus (Fig. 2D, ii and vi). In contrast, no immunofluorescence was detected in the negative control, untransfected cells immunostained with anti-Flag antibody. Furthermore, cells with ectopically expressed Flag-tagged wt Pak4 were double stained with anti-Flag and anti-Pak4/anti-p-Pak4 antibodies (Fig. 2D). The resulting immunofluorescent signals were colocalized in both the cytoplasm and the nucleus, further verifying the specificity of the anti-Pak4 and anti-p-Pak4 antibodies.

Pak4 Regulation of Gene Transcription. Having found the nuclear localization of Pak4, we attempted to determine the effect of Pak4 on gene transcription. A GAL4-Luc assay revealed that a construct expressing wt Pak4 as a fusion protein with a GAL4-DNA binding domain significantly increased the GAL4-Luc activity by 1.5- to 2-fold in SKOV-3. The expression of the fusion protein (~93 kDa) was detected by immunoblot analyses (Fig. S2A). Moreover, a dose-dependent activation was observed when the concentration of construct used for transfection was increased, suggesting that Pak4 may influence gene transcription (Fig. 2E). To further investigate the effect of the NLSs for Pak4 nuclear accumulation, four Pak4 NLS mutants were generated. The GAL4-Luc activities were significantly reduced in Pak4 NLS1 and NLS3 mutants when compared with wt Pak4 (Fig. 2F). Mutation of NLS1 and NLS3 also inhibited exogenous Pak4 nuclear accumulation as detected by immunoblotting using nuclear fractions, suggesting that NLS1 and NLS3 may play roles for Pak4 nuclear accumulation (Fig. 2G).

Pak4 Regulation of Cell Migration and Invasion Is Dependent on PAK4 Kinase Activity, c-Src, MEK-1/ERK1/2, and MMP2. The specific transient (siPak4; Fig. S3A) and stable (shPak4; Fig. 3A) knockdown of Pak4 in OVCAR-3 and OVCA420, ovarian cancer cell lines with relatively high Pak4 expression, was detected. In a wound-healing assay, a slower migration rate was found in siPak4 OVCA420 as compared with control cells (Fig. 3B). In Transwell migration and invasion assays, significantly reduced migration and invasion were observed in OVCAR-3 and OVCA420 upon siPak4 (Fig. S3B and C) or shPak4 (Fig. 3C and D) knockdown. We then aimed to unravel Pak4 downstream signaling pathways. Focal adhesion kinase (FAK), c-Src, and extracellular signal-regulated kinase (ERK1/2) together with other signaling and adaptor proteins form large cell-matrix adhesion complexes that constitute a key machinery in cancer cell migration, invasion, and metastasis, in part through the regulation of matrix metalloproteinase (MMP) expression (18, 19). Interestingly, knockdown of Pak4 in OVCA420 reduced c-Src and ERK1/2 activity, but not FAK or p38 activity (Fig. 3E). The MMP2

Fig. 2. Localization of Pak4 and p-Pak4 in the nucleus and cytoplasm of ovarian cancer cells and activation of gene transcription by Pak4. (A) Subcellular localization of Pak4 and p-Pak4 in OVCAR-3 determined by confocal microscope. Cells were stained with rabbit anti-Pak4 antibody (i) or rabbit anti-p-Pak4 antibody (v) along with mouse anti C23-nucleolin antibody (ii and vi). Merge images displays Pak4 colocalization with C23-nucleolin (iii). p-Pak4 was not colocalized with C23-nucleolin in merge image (vii). White arrowheads indicated the cytoplasmic localization of Pak4 and p-Pak4. (iv and viii) DAPI staining of nuclei. (B) Schematic drawing illustrating four possible nuclear localization signals (NLSs 1–4) and a leucine zipper pattern and their positions on Pak4 protein. (C) Pak4 and p-Pak4 in subcellular protein fractions (T, total cell lysate; C, cytoplasmic fraction; N, nuclear fraction) of OVCAR-3. (D) Immunofluorescent staining of Flag-tagged wt Pak4 overexpressing SKOV-3 cells with rabbit anti-Pak4 antibody (i) or rabbit anti-p-Pak4 antibody (v) along with mouse anti-Flag tag antibody (ii and vi). Exogenous wt Pak4 was detected in the nucleus and cytoplasm of SKOV-3 cells and colocalized with fluorescent staining as detected by anti-Pak4 antibody (iii) or rabbit anti-p-Pak4 antibody (vii). (iv and viii) DAPI staining of nuclei. (E Upper) Schematic illustration of GAL4-Luc reporter plasmid, GAL4-BD vector (control), and GAL4-BD-wt Pak4 construct. (Lower) The GAL4-Luc activity of the reporter gene as fold of control; $n = 3$; $*P < 0.05$. (F) The GAL4-Luc activity in SKOV-3 cells expressing wt Pak4 or four NLS Pak4 mutants as fold of control; $n = 3$. (G) Immunoblot analyses of exogenous Pak4 in nuclear fractions extracted from SKOV-3 cells expressing wt Pak4 or Pak4 NLS mutants using anti-GAL4 DNA-BD antibody.

expression was also reduced in shPak4 OVCA420 both in protein lysates (Fig. 3E) and in conditioned media (Fig. 3F).

To further investigate the effect and signaling of Pak4 in ovarian cancer cell migration and invasion, SKOV-3 cells, an ovarian cancer cell line with relatively low Pak4 expression, were stably transfected with Flag-tagged wt Pak4, constitutively active (ca) Pak4, or kinase-dead Pak4. Wt Pak4 and ca Pak4 significantly increased cell mi-

gration and invasion when compared with the FLAG control with greater effect found of ca Pak4 (Fig. 4A), whereas kinase-dead Pak4 inhibited cell migration and invasion (Fig. S4C), indicating that Pak4 kinase activity promotes cell migration and invasion. In addition, c-Src and ERK1/2 activity were induced together with MMP2 expression, and this induction was further enhanced by ca Pak4 kinase activity (Fig. 4B). To test the effect of Pak4-induced c-Src and ERK1/2 activities on cell migration and invasion, and to elucidate possible interrelations between c-Src, ERK1/2, and MMP-2, wt or ca Pak4 overexpressing SKOV-3 cells were treated with a c-Src inhibitor (PP1), two MEK-1 inhibitors (U0126 and PD98059), or a MMP2 inhibitor (OA-Hy). PP1 inhibited both basal and Pak4-mediated cell migration and invasion (Fig. 4A). By immunoblot analyses, PP1 abolished c-Src activation, but did not alter ERK1/2 activation or MMP2 expression in SKOV-3 cells with or without Pak4 overexpression (Fig. 4B), suggesting that Pak4-induced ERK1/2 activation and MMP2 expression is independent of c-Src. However, U0126 attenuated Pak4-induced migration and invasion (Fig. 4A) and also abrogated Pak4-induced MMP2 expression but did not change c-Src activation, suggesting that MEK-1/ERK1/2 mediates Pak4-induced MMP2 expression (Fig. 4B). PD98059 (Fig. 4C) and OA-Hy (Fig. 4D) also showed inhibition on Pak4-mediated migration and invasion. In addition, specific siRNAs of c-Src, MEK-1, or MMP2 were used to treat wt, ca, or kinase-dead Pak4 overexpressing cells, and all of these siRNAs inhibited Pak4-mediated migration and invasion (Fig. S4A–C). These findings show that Pak4 acts through at least two downstream pathways to promote ovarian cancer cell migration and invasion—one including c-Src and the other including MEK-1/ERK1/2 signaling to MMP2. We also tested the effect of NLS mutants on cell migration and invasion, but no significant difference was found between cells transiently transfected with NLSs mutants and with wt Pak4 (Fig. S2B).

Pak4 Regulation of Cell Proliferation Involves c-Src and EGFR. We found that both wt and ca Pak4 significantly induced SKOV-3 cell proliferation after 12 d. More obvious effect was observed in ca Pak4 transfected cells (Fig. 5A). Conversely, significantly reduced proliferation was observed in OVCA420 12 d after shPak4 knockdown (Fig. 5E). c-Src has been reported to mediate phosphorylation of epidermal growth factor receptor (EGFR) on Tyr845 leading to modulation of receptor function, such as increase in DNA content (20). We sought to investigate if Pak4-mediated c-Src activation may lead to phosphorylation of EGFR on Tyr845 and affect ovarian cancer cell proliferation. We observed that wt Pak4, and to a higher degree ca Pak4, induced p-EGFR Tyr⁸⁴⁵ in SKOV-3 cells (Fig. 5B), whereas knockdown of Pak4 in OVCA420 reduced p-EGFR Tyr⁸⁴⁵ (Fig. 5E). The Pak4-induced EGFR phosphorylation was blocked by PP1, suggesting that c-Src signaling mediates Pak4-induced EGFR phosphorylation. Intriguingly, PP1 (Fig. 5A) and two distinct EGFR inhibitors (CL387, 785 and PD153035; Fig. 5C) all blocked the Pak4-mediated increase in SKOV-3 cell proliferation. PP1 and CL also inhibited basal cell proliferation. To further elucidate downstream targets of Pak4/c-Src/EGFR-regulated SKOV-3 cell proliferation, the mRNA expression of G1-phase-associated cyclin D1, cyclin D3, and CDC25A, and G2/M-associated cyclin B1 was determined in ca Pak4 overexpressing cells with or without PP1 or CL. We found that ca Pak4 induced cyclin D1 and CDC25A mRNA expression (Fig. 5F) but had no effect on cyclin D3 and cyclin B1 expression (Fig. S4D). Further, CL blocked ca Pak4-induced cyclin D1 and CDC25A mRNA expression (Fig. 5F). Moreover, both PP1 and CL abolished ca Pak4 induced cyclin D1 protein expression (Fig. 5G). These findings suggest that Pak4 regulation of cell proliferation involves the c-Src and EGFR pathway leading to the regulation of cyclin D1 and CDC25A expression. Next, we examined the effect of Pak4 on cell survival. TUNEL assay showed lack of significant difference in the proportion of apoptotic cells in OVCAR-3 and OVCA420 after siPak4 under regular culture conditions (Fig. S3D), implying that Pak4 may not be critical for ovarian cancer cell survival, at least in the absence of apoptotic stimulus.

Fig. 3. Pak4 abrogation inhibited ovarian cancer cell migration and invasion and reduced c-Src and ERK1/2 activation as well as MMP2 expression. (A) Stable knockdown of Pak4 mRNA and protein in OVCA420 cells as detected by qPCR (Upper) and immunoblot analysis (Lower), respectively. (B) Wound-healing assay in control and transient knockdown of Pak4 in OVCA420. In vitro migration (C) and invasion assays (D) using Transwell membrane without or with Matrigel coating, respectively. (Upper) Representative images of migrating or invading OVCA420 cells. (Lower) Cell migration or invasion from OVCA420 presented as percentage of control; $n = 3$; $**P < 0.005$. (E) Immunoblot analysis on FAK, c-Src, ERK1/2, p38, MMP2, and EGFR expression and/or activation in control and shPak4 OVCA420. (F) Immunoblot analysis of MMP2 expression using conditioned media prepared from control and shPak4 OVCA420.

Pak4 Regulation of Tumor Growth and Dissemination in Nude Mice.

To investigate the *in vivo* effects of Pak4, shPak4 ES-2, and control cells were prepared (Fig. S3E) and inoculated s.c. or i.p. to nude mice. The growth rate of s.c. tumors formed in mice inoculated with shPak4 ES-2 cells was significantly lower than in tumors formed by control cells (Fig. 6A). Examination of the abdominal cavity of mice 17 d after i.p. inoculation with control cells showed extensive dissemination, particularly throughout the mesentery with ascites formation in three of the seven mice (Fig. 6B). In contrast, mice injected with shPak4 ES-2 cells showed much less dissemination, with only focal deposits at the mesentery, and no ascites formation was observed (Fig. 6B). The total i.p. tumor weight was significantly lower in the shPak4 ES-2 cell-injected mice (0.027 ± 0.030 g) than in the control mice (0.184 ± 0.079 g; $P < 0.05$).

HGF and FSH Regulate Pak4 in Ovarian Cancer Cells. Given that most of Pak4-mediated functions are kinase dependent, and activated Pak4 was found in ovarian cancer cells, we investigated HGF as a potential activator of Pak4. HGF has been reported to exert potent effects on ovarian cancer cell migration and invasion through the activation of ERK1/2 (21), similar to what we found for Pak4 as described above. We found that HGF induced OVCA420 cell migration and invasion along with Pak4, c-Src, and ERK1/2 phosphorylation. However, the HGF-induced migration, invasion, and phosphorylations were all reversed by shPAK4 (Fig. S5). We also investigated the effect of FSH on Pak4 expression in ovarian

cancer cells because FSH and its receptor (FSHR) have been found to play important roles in ovarian cancer development (22, 23). In fact, FSH treatment caused an increased Pak4 expression in a dose-dependent manner in OVCA420 cells (Fig. S5).

Discussion

In this study, we demonstrated significant up-regulation of total and phosphorylated Pak4 in ovarian cancer tissue and ascitic fluid samples and cell lines when compared with benign ovarian epithelium. More importantly, we found a significant correlation between high Pak4 expression with extent of ovarian cancers, shorter overall and disease-free survival. These findings suggest Pak4 to be a significant prognostic marker in ovarian cancer. Pak4 immunocytochemistry may also be explored to enhance the sensitivity of detecting metastatic cancer cells in ascitic fluid.

The above Pak4 expression profiling and our *in vitro* and *in vivo* studies on cell migration and invasion highlight the contribution of Pak4 to ovarian cancer progression and metastasis, concurring with the role of Pak4 in cytoskeleton reorganization and cell migration at least in part executed in the cytoplasm (6) via phosphorylation of integrin $\alpha 5$ (10) and LIMK1 (24). In this study, we were able to show a link between Pak4 and c-Src, MEK-1/ERK1/2, and MMP2 in the regulation of cell migration and invasion. In fact, our experiments on the mechanisms regarding Pak4 regulation of cell migration and invasion indicate the involvement of at least two downstream pathways, including Pak4 to c-Src and Pak4 to MEK-

Fig. 4. Pak4-mediated cell migration and invasion in ovarian cancer cells involved c-Src and MEK-1/ERK1/2/MMP2 pathways. (A, C, and D) In vitro migration (Left) and invasion (Right) assays in SKOV-3 cells stably transfected with Flag-tagged wt Pak4, ca Pak4, or control vector in the presence or absence of PP1 (c-Src inhibitor), U0126 (MEK-1 inhibitor), PD98059 (MEK-1 inhibitor), OA-Hy (MMP2 inhibitor), or DMSO (vehicle). Cell migration or invasion presented as percentage of control; $n = 3$; $*P < 0.05$, $**P < 0.005$. (B) Immunoblot analyses of exogenous Flag-tagged Pak4 and MMP2 levels; and c-Src and ERK1/2 activities in SKOV-3 cells expressing wt or ca Pak4 in the presence or absence of PP1 or U0126. (C, Lower) Immunoblot analysis of ERK1/2 activation in wt or ca Pak4 over-expressing SKOV-3 cells in the presence or absence of PD98059.

Fig. 5. Pak4-induced proliferation involved the c-Src/EGFR pathway that controls cyclin D1 and CDC25A expression. (A and C) Cell proliferation rate of wt or ca Pak4 overexpressing SKOV-3 cells in the presence or absence of PP1, CL387,785 or PD153035. (E) Cell proliferation rate of OVCA420 in control and shPak4 after 12 d displayed as fold change compared with control; $n = 3$; $**P < 0.005$. (B and D) Immunoblot analysis of p-EGFR Tyr⁸⁴⁵ and EGFR expression in SKOV-3 cells overexpressing wt or ca Pak4 in the presence or absence of PP1, CL or PD153035. (F) mRNA expression of cyclin D1 and CDC25A in ca Pak4 overexpressing cells displayed as percentage of control (Flag vector-transfected SKOV-3 cells with DMSO) in the presence or absence of CL by qPCR; $n = 3$; $*P < 0.05$. (G) Immunoblot analysis of cyclin D1 expression in ca Pak4 overexpressing cells in the presence or absence of PP1 or CL.

1/ERK1/2 to MMP2. These pathways are intimately linked with cell matrix adhesion complexes found to be essential for cancer cell metastasis (18, 19). We also showed that the Pak4 regulation of cell migration and invasion is kinase dependent.

In addition to effects on cell migration and invasion, we also demonstrated the cell proliferation enhancement effect of Pak4 in ovarian cancer cells, in line with the tumor-promoting effect of ca Pak4 overexpression in NIH 3T3 cells in athymic mice (13). We are also excited by the identification of a downstream pathway of Pak4, the Pak4 to c-Src to EGFR pathway that controls cyclin D expression and ovarian cancer cell proliferation. EGFR is known to be important in the etiology of several common human cancers, including that of the lung, colon, stomach, head, and neck. EGFR-targeting monoclonal antibodies and small molecule tyrosine kinase inhibitors are currently in clinical use or trial for cancer therapy. However, the frequent occurrence of primary and acquired resistance to these agents limits the clinical efficacy of monospecific EGFR-targeted therapy (25, 26). For instance, targeting EGFR alone does not appear optimistic in ovarian cancer patients (27). Even in cancers with a good response, such as lung cancers, resistance often develops and subsequent salvage treatment is difficult. In this study, a cross-talk between Pak4, c-Src, and EGFR in ovarian cancer is contemplated. Exploration for dual targeting of EGFR and Pak4 may be examined in future studies on ovarian or other cancers as possible promising novel therapeutic approaches. Cyclin D1, a D-type cyclin regulating G1-phase cell-cycle progression, has been identified as a critical downstream effector of mutant EGFR signaling in non-small cell lung cancer and as an alternative target of therapy (28). In ovarian cancer, cyclin D1 and CDC25A expression were associated with patient survival (29, 30). The link we identified between Pak4, c-Src, EGFR, cyclin D1, and CDC25A not only suggests that Pak4 may regulate cell proliferation through the alteration of G1-phase cell cycle progression, but also provides insights into the use of alternative multitargeted therapy.

Anticancer therapies often induce apoptosis. Although we observed that Pak4 had no significant effect on apoptosis under regular culture conditions, Pak4 has been found to protect cells from apoptosis induced by various apoptotic stimuli, including UV irradiation, serum deprivation, and TNF α (4, 7). We also revealed a significant association between high Pak4 expression and chemoresistance, which is an important contributing factor to the high mortality in ovarian cancer patients. As such, molecular manipulation of Pak4 to enhance chemosensitivity of ovarian cancer may provide a useful direction to putative targeted therapy. However, the mechanisms by which Pak4 protects cancer cells from apoptosis induced by chemotherapy needs to be elucidated in more detail.

We have also demonstrated, at subcellular level, Pak4 and p-Pak4 immunoreactivity in both the nucleus and the cytoplasm of ovarian cancer cells. A dose-dependent increase of GAL4-Luc activity was also observed when the concentration of Pak4 construct was increased, suggesting that Pak4 may influence gene transcription. We further found that two PAK4 NLS motifs, NLS1 and NLS3, may be responsible for Pak4 nuclear accumulation. The Pak4 nuclear localization and its influence on gene transcription in ovarian cancer cells implicate a function for Pak4 in the nucleus. Pak1, another Pak isoform, was also shown to be associated with nuclear chromatin and modulate gene transcription (31). The same study also revealed nuclear localization of Pak1 upon stimulation by EGF. Moreover, nucleocytoplasmic shuttling of Pak5 has also been found to be vital for cell survival under stress (32). Studies on Pak4 nucleocytoplasmic shuttling will be worthwhile to further elucidate the nuclear functions of Pak4, as well as whether Pak4 kinase activity may affect gene transcription. We further observed nuclear Pak4 immunoreactivity in the nucleolus, a sub-nuclear organelle that forms the ribosomal DNA repeats and is responsible for the synthesis, process, and assembly of ribosomal subunits (33). Apart from cell growth and cell proliferation, the nucleolus may also play a role in aging, cell cycle control, and sensing cellular stress (33). The localization of Pak4 in the nucleolus suggests that Pak4 could potentially exert effects on such functions, including cell proliferation and cell cycle control, as shown in the present study.

We also identified HGF as a potential activator of Pak4 in ovarian cancer cells, consistent with the effect of HGF observed in other epithelial cells (34). The blockage of induced cell migration, invasion, and activation of Pak4, c-Src, and ERK1/2 in HGF-treated ovarian cancer cells after stable knockdown of Pak4 further suggests that Pak4, c-Src, and ERK1/2 are putative downstream effectors of HGF to mediate cell migration and invasion functions in ovarian cancer cells. This also concurs with

Fig. 6. Pak4 depletion impeded tumor growth and dissemination in nude mice. (A) Growth rates of s.c. tumors formed in mice inoculated with shPak4 ES-2 cells or control cells (2×10^6). (B) Representative views of the abdominal cavity of mice inoculated i.p. with shPak4 ES-2 cells or control cells. Arrows, tumors.

the effects of HGF on ovarian cancer cell migration and invasion through the activation of ERK1/2, as found in previous studies (21). Besides HGF, the up-regulation of Pak4 by FSH in ovarian cancer cells suggests that FSH is a putative upstream mediator accounting for Pak4 overexpression in ovarian cancer cells.

In summary, Pak4 is overexpressed and activated in ovarian cancers, suggesting that Pak4 expression and activation play important roles in cancer progression. The nuclear expression of Pak4 and p-Pak4 in ovarian cancer cells may imply unique nuclear Pak4 function(s), such as modulation of gene transcription. Importantly, increased Pak4 and/or activated Pak4 expression were associated with cancer metastasis, reduced patient survival, late stages, and increased resistance to chemotherapy. The mechanisms by which Pak4 affects ovarian cancer cell progression include the control of cell migration, invasion, and proliferation through the regulation of c-Src, MEK-1/ERK1/2, MMP2, and c-Src/EGFR. Given that kinase inhibitors for the Pak family are being developed as therapeutics for cancer therapy (35–37), it is of great interest to explore Pak4 as a therapeutic molecular target either alone or in combination with conventional chemotherapy or other molecular targets, such as EGFR. The latter combined approach may be particularly promising for salvage of patients with various human cancers manifesting primary or secondary resistance to EGFR antagonist, a targeted therapy of increasing use in oncology patients.

Materials and Methods

Clinical Samples, Cell Culture, in Vivo Studies, Treatments, and General Methods. Clinical samples, cell culture, subcellular proteins extraction, in vivo studies, HGF, and FSH treatments and other pertinent methods, including various functional assays, are described in *SI Materials and Methods* (5, 38).

Plasmid, Transfection, Treatments with Inhibitors or siRNAs, and Luciferase Assay. To stably express Pak4 in SKOV-3, cells were transfected with Flag-tagged wt Pak4, ca Pak4 (445N/474E), kinase-dead Pak4 (M350), or the control vector

p3XFLAG-CMV-10 (11) using Lipofectamine 2000 (Invitrogen) and then selected with G418 (800 μ g/mL) (5). For drug or siRNAs treatment, Pak4 overexpressing cells were plated 6 or 24 h before treating with the c-Src inhibitor PP1 (20 μ M), the two MEK-1 inhibitors U0126 (20 μ M) and PD 98059 (50 μ M), the two EGFR inhibitors CL387, 785 (1 μ M) and PD153035 (2 μ M), vehicle (DMSO), or siRNAs (100 nM; Ambion) of c-Src, MEK-1, MMP2, or control. All inhibitors were purchased from Calbiochem except PP1 (Biomol). After 48 h (for PP1, U0126, PD98059, and siRNAs) or 12 d (for CL and PD153035 with change of medium and drugs in every 3 d), cells were harvested for real-time PCR and/or immunoblot analyses. To generate the Pak4 fusion protein with GAL4-DNA binding domain construct, wt Pak4 was amplified and subcloned in-frame into the vector pCMV-BD (Stratagene). To generate Pak4 NLS mutants, a QuikChange Kit (Stratagene) was used, and the lysine residues in the four NLSs were mutated to alanines using pCMV-BD wt Pak4 as template. Primers used are described in *SI Materials and Methods*. Nuclear localization of wt and NLS mutants of Pak4 were determined by immunoblotting. The constructs or the control vector were transiently transfected into SKOV-3 cells along with pFR-Luc reporter plasmid (Stratagene). After 24 h, luciferase assay was performed using Promega luciferase assay system. The transcription activation domain of NF- κ B fused with GAL4 was used as positive control. To transiently silence Pak4 in OVCAR-3 and OVCA420, 100 nM each of siGENOME Smart-pool for Pak4 and siControl nontargeting siRNA pool (Dharmacon) was used. Cells were plated for migration and invasion assays 48 h after transfection. To stably silence Pak4, cells were transfected with a set of shRNA constructs against human Pak4, pRS-shPak4 (Origene), and then selected with puromycin (1.5 μ g/mL) (5, 38). The pRS vector was used as controls.

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