Tumorigenesis and Neoplastic Progression

p21-Activated Kinase-1 Promotes Aggressive Phenotype, Cell Proliferation, and Invasion in Gestational Trophoblastic Disease

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Gestational trophoblastic disease (GTD) includes hydatidiform mole (HM), which can develop persistent gestational trophoblastic neoplasia requiring chemotherapy; choriocarcinoma, which is a frankly malignant tumor; placental site trophoblastic tumor; and epithelioid trophoblastic tumor. p21-Activated kinases (PAKs) promote malignant tumor progression. Therefore, this study investigated PAK1, PAK2, and p-PAK2 Ser²⁰ in the pathogenesis of GTD. By real-time PCR, PAK1 mRNA was significantly higher in HMs, particularly metastatic HMs ($P = 0.046$) and HMs that developed persistent disease ($P = 0.011$), when compared with normal placentas. By immunohistochemistry, significantly increased cytoplasmic PAK1 immunoreactivity in cytotrophoblasts was also detected in HMs ($P = 0.042$) and choriocarcinomas ($P = 0.003$). In addition, HMs that developed persistent disease displayed higher PAK1 immunoreactivity than those that regressed ($P = 0.016$), and elevated PAK1 immunoreactivity was observed in placental site trophoblastic tumors. Indeed, there was significant positive correlation between PAK1 expression and the proliferative indices Ki-67 ($P = 0.016$) and MCM7 ($P = 0.026$). Moreover, higher PAK1 mRNA and protein expression was confirmed in the choriocarcinoma cell-lines JEG-3 and JAR; however, PAK2 mRNA and p-PAK2 immunoreactivity showed a similar expression pattern in normal first trimester placentas and GTD. Knockdown of PAK1 in JEG-3 and JAR reduced cell proliferation, migration, and invasion ability, up-regulated p16, and down-regulated vascular endothelial growth factor and MT1-MMP expression. This is the first report revealing the involvement of PAK1 in the pathogenesis and clinical

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Gestational trophoblastic disease (GTD) is a disease of the placenta resulting from abnormal trophoblastic proliferation. It encompasses hydatidiform mole (HM), choriocarcinoma (CCA), placental site trophoblastic tumor (PSTT), and epithelioid trophoblastic tumor. The latter three are malignant tumors, whereas HMs are nonneoplastic lesions with an increased risk of malignant transformation. Furthermore, 8% to 30% of HMs may persist after suction evacuation and develop gestational trophoblastic neoplasia (GTN), hence requiring chemotherapy. Although the response of GTD to chemotherapy is in general very good, chemoresistant cases still exist despite advances in chemotherapy. While there is growing understanding in the molecular biology of GTD, the precise molecular pathways of its development should be further explored.^{1–3}

p21-Activated kinases (PAKs) were initially discovered as effector molecules of Rho GTPases, Rac, and CDC42,⁴ and are increasingly recognized as important mediators for a wide variety of cellular functions, including cell morphogenesis, motility, mitosis, apoptosis, and angiogenesis.^{5,6} PAKs have been divided into group I (PAKs1-3) and group II (PAKs4-6), based on structural organization and mode of regulation.⁵ In group I PAKs, the p21-binding domain in the N-terminal domain binds small GTPases Rac and CDC42 to cause autophosphorylation of certain sites in the N-terminal inhibitory domain, leading to conformational change that releases the autoinhibition and activates the kinase activity.⁵ Phosphorylation of PAK1 at Ser²¹ regulates the binding of PAK1 to Nck, resulting in PAK1 cycling between membrane and

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cytosol and finally facilitates cell migration.^{7,8} Its corresponding phosphorylation site at PAK2 is Ser²⁰.

Overexpression of PAK1 has been observed in various human cancers,⁶ such as breast, colorectal, and ovarian cancers.⁹ PAK1 was found to regulate the invasiveness of breast cancer cells and the malignant progression of colorectal carcinomas to metastasis.^{10,11} PAK2 has also been found to be involved in Rac3-mediated breast cancer cell proliferation.¹² The recent development of kinase inhibitors for the PAK family molecules^{13,14} is intriguing since they may be explored as potential therapeutic targets in chemoresistant GTD.

In this study, we investigated the expression and localization of PAK1, PAK2, and p-PAK2 Ser²⁰ in normal human placenta and in GTD to determine whether PAK1 or PAK2 are involved in development and malignant progression of GTD. We also correlated their expression with clinical outcome and examined the *in vitro* effects and potential downstream targets of PAK1 on CCA cells.

Materials and Methods

Selection of Clinical Samples

Sixty-seven fresh frozen trophoblast samples were collected in Queen Mary Hospital, the University of Hong Kong including 16 first trimester placentas, 6 term placentas, 29 regressive moles (HMs that spontaneously regressed), 13 persistent moles (HMs that subsequently progressed to persistent GTN), and 3 CCAs. Among the 13 persistent moles, 6 cases developed metastasis. Forty archival paraffin blocks were also retrieved including 7 first-trimester placentas, 5 term placentas, 15 regressive moles, 8 persistent moles, and 5 CCAs. Approval was obtained from the Institutional Research Board. The gestation age of the HMs ranged from 5 weeks to 24 weeks (means 12.5 weeks). Persistent GTN was diagnosed if there was a plateau in human chorionic gonadotrophin level for 4 weeks or if there was a further increase in human chorionic gonadotrophin for three consecutive weeks when pregnancy was excluded.¹⁵ All tissue sections were reviewed using generally agreed and accepted diagnostic criteria.¹⁻³ Most HM cases have previously been assessed by proliferation indices Ki-67¹⁶ and MCM7¹⁷ and ploidy analysis by fluorescent microsatellite genotyping after microdissection and chromosome *in situ* hybridization.^{18,19}

Cell Lines

The human CCA cell lines, JEG-3 and JAR, were obtained from American Type Culture Collection (Rockville, MD). The normal trophoblast cell line, TEV1, was previously established and characterized in our center.²⁰ All cells were grown in Minimum Essential Medium Eagle (Sigma St. Louis, MO) supplemented with 10% fetal bovine serum (JRH Biosciences, Lenexa, KS) and 100 U/ml penicillin and streptomycin (Invitrogen, San Diego, CA).

Table 1. Primers Used for Real-Time PCR

Gene	Primer direction	Primer sequence
PAK1	Forward	5'-AGTTTCAGAAGATGAGGATGATGA-3'
	Reverse	5'-AATCACAGACCGTGTGTATACAG-3'
PAK2	Forward	5'-AGAAACAGCTTGCATGGATGAA-3'
	Reverse	5'-TCTCTGTGGATCACTTGATTAGCAT-3'
p16	Forward	5'-CATAGATGCCGCGGAAGGT-3'
	Reverse	5'-GGATTAGGGCTTCTCTTGGG-3'
CDC25A	Forward	5'-AGATAGCAGTGAACCAAG-3'
	Reverse	5'-TGCATCGGTTGTCAAGG-3'
p21	Forward	5'-GCAGACCAGCATGACAGATTTC-3'
	Reverse	5'-GGATTAGGGCTTCTCTTGGG-3'
CYCLIN D1	Forward	5'-GCTGCTCCTGGTGAACAAGC-3'
	Reverse	5'-TTCAATGAAATCGTGCGGG-3'
CYCLIN D2	Forward	5'-TGATGGGGAAGGAGTACAAA-3'
	Reverse	5'-TTGGAAGAAGAAGCAGGATG-3'
CYCLIN B1	Forward	5'-CTTGACAACGGTGAATGGAC-3'
	Reverse	5'-TGACAGTCATGTGCTTTGTGA-3'
PCNA	Forward	5'-AGGAAGCTGTACCATAGAGA-3'
	Reverse	5'-ACAACAAGGGGTACATCTGC-3'
c-ERB-B-2	Forward	5'-GCACCAAGGTGACACCGGC-3'
	Reverse	5'-ATTGGTGGGCGAGGTAGGTGAGTTC-3'
VEGF	Forward	5'-AACCATGAACCTTCTGCTGTCTTG-3'
	Reverse	5'-TTCACCACTTCGTGATGATTCTG-3'
MT1-MMP	Forward	5'-ACGGAGGTGATCATCATTTGAGG-3'
	Reverse	5'-AGATGGGGCTGGACAGACACA-3'
GAPDH	Forward	5'-TCCATGACAACTTTGGTATCGTG-3'
	Reverse	5'-ACAGTCTTCTGGGTGGCAGTG-3'

Transient Silencing of PAK1 in JEG-3 and JAR

The siGENOME Smart-pool for PAK1 and siControl non-targeting short interfering (si)RNA pool (100 nmol/L each; Dharmacon, Lafayette, CO) was used for transfection using SilentFect (Bio-Rad Laboratories, Hercules, CA). Specific knockdown of PAK1 was determined by real-time (q)PCR and Western blot analysis at 24 hours and 48 hours after transfection respectively. Cells were plated for MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide), migration and invasion assays 48 hours after transfection.

qPCR

Total RNA was extracted using Trizol reagent (Invitrogen). A total of 2.5 μ g of total RNA was used to synthesize first strand cDNA using the SuperScript Reverse Transcriptase (Invitrogen). qPCR was performed on the ABI Prism 7700 sequence detection system.^{21,22} Primer sequences for each of the genes were listed in Table 1.

Western Blot Analysis

Cell lysates were prepared in lysis buffer (0.125 mol/L Tris, pH 6.8 at 22°C containing 1% NP-40 (v/v), 2 mmol/L EDTA, 2 mmol/L N-ethylmaleimide, 2 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L sodium orthovanadate, and 0.1 μ mol/L sodium okadaate). The concentration of the protein was determined by detergent compatible protein assay (Bio-Rad Laboratories, Hercules, CA). Equal amounts (35 μ g) of protein were resolved by SDS-polyacrylamide gel electrophoresis, transferred electrophoretically onto polyvinylidene difluoride membrane, and hybridized with corresponding antibodies.^{21,22} Antibodies used were listed in Table 2.⁹

Table 2. Sources and Working Dilutions of Antibodies Used in this Study

Antibodies	Catalog #	Companies	Applications*
Rabbit anti-PAK1	2602	Cell Signaling Technology, Inc. (Beverly, MA)	IHC (1:40), WB (1:1000)
Rabbit anti-PAK1/2 [†]	sc-881	Santa Cruz Biotechnology Inc. (Santa Cruz, CA)	WB (1:400)
Rabbit anti-p-PAK2 Ser ²⁰	2607	Cell Signaling Technology, Inc.	IHC (1:50), WB (1:1000)
Rabbit anti MT1-MMP	M3927	Sigma (St. Louis, MO)	WB (1:1000)
Goat anti-ACTIN	sc-1616	Santa Cruz Biotechnology Inc.	WB (1:200)

*IHC, immunohistochemistry; WB, western blot analysis. [†]This antibody against PAK1 also cross-reacted with PAK2.

Immunohistochemical Staining

Immunohistochemical staining was performed as previously described.^{21,23} Briefly, 5 μ m formalin-fixed paraffin sections were cut, deparaffinized and stained with antibodies against PAK1 and p-PAK2 Ser²⁰ (as listed in Table 2) using UltraVision LP Value Detection System (Lab Vision Corporation, Fremont, CA). These antibodies were known to cross-react with the corresponding target proteins in humans as indicated by the manufacturers and were used in our previous study.⁹ Antigen recovery was performed at 95°C for 10 minutes using 10 mmol/L sodium citrate buffer, pH 6.0. Negative control was prepared by replacing the primary antibody with Tris-buffered saline or pre-immune IgG serum.

Assessment of Immunohistochemical Staining

The immunostaining was assessed by two individual pathologists. Staining intensity was scored on an arbitrary scale: 0, no immunoreactivity; 1, weak; 2, moderate; 3, intense. The percentage of positive cells was graded as: 0, negative; 1, <33%; 2, 33 to 67%; 3, >67%. The immunoreactivity was obtained by the products of the above two parameter to give a composite "Histoscore," with a maximum score of 9.²⁴

MTT Assay

Cell proliferation/viability was assessed by MTT assay (Sigma).^{9,22} Cells (2000 cells/well) were seeded in 96-well plates. Ten μ l of MTT was added to each well at day 5. Plates were incubated at 37°C for 4 hours, followed by addition of 100 μ l dimethyl sulfoxide to each well for dye extraction. Cell viability was determined by measuring the absorbance of samples at 570 nm with 630 nm as the reference wavelength.

In Vitro Migration and Invasion Assays

Migration and invasion assays were performed as previously described.^{9,22} Briefly, 1.25×10^5 cells were plated on the upper compartment of a Transwell chamber and allowed to migrate through an 8- μ m pore size membrane or invade through a Matrigel-coated membrane. After 24 hours (migration assays) or 48 hours (invasion assays), cells on the upper side of the membrane were removed and the migrated or invaded cells were fixed, stained, and counted.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 3.0 for Windows (GraphPad Software, San Diego, CA). Mann-Whitney test (non-parametric unpaired *t*-test) was used for continuous data. Correlation between Histoscore and proliferation indices Ki-67 and MCM7 was performed using Pearson test. *P* value of less than 0.05 was considered as statistically significant.

Results

PAK1 Was Overexpressed in HMs, CCAs, and PSTT, and Contributed to Development of GTN Among HMs

By real-time PCR, PAK1 mRNA was significantly evaluated in HMs when compared with normal first-trimester (*P* = 0.038) and term placentas (*P* = 0.025) (Figure 1A). The mRNA expression of PAK1 in patients who developed persistent GTN requiring chemotherapy was significantly higher than those that had spontaneous regression of molar pregnancy (*P* = 0.011) (Figure 1A). We also observed significantly higher PAK1 mRNA in the HMs that developed metastasis than those without metastasis (*P* = 0.046) (Figure 1B). Moreover, PAK1 mRNA in CCAs was significantly higher when compared with normal first trimester (*P* = 0.007), term placentas (*P* = 0.02) and HMs (*P* = 0.005) (Figure 1A). Increase of PAK1 mRNA and protein expression was also detected in CCA cell lines, JEG-3 and JAR, as compared with the normal trophoblast cell line, TEV-1 (Figure 1, C and D).

By immunohistochemistry, PAK1 expression was predominantly confined to the cytoplasm with occasional nuclear staining detected. The immunoreactivity was localized predominantly in cytotrophoblasts and villous intermediate trophoblasts in both normal trophoblastic and GTD tissues (Figure 2). Syncytiotrophoblasts showed negative staining and PAK1 was nearly nondetectable in term placentas (Figure 2B). In cytotrophoblasts, significantly higher PAK1 expression was revealed in HMs (*P* = 0.042) and CCAs (*P* = 0.003) when compared with normal placentas (Figure 2, A, C–E, and H). Consistent with the mRNA finding, we also detected significantly higher PAK1 expression in the HMs that subsequently developed persistent GTN than the HMs that spontaneously regressed (*P* = 0.016) (Figure 2, C, D, and H). Moreover, PAK1 expression was found to correlate with proliferation

Figure 1. mRNA and protein expression of PAK1 in GTD clinical samples and cell lines. **A:** PAK1 mRNA profile in GTD clinical samples by qPCR. PAK1 expression was normalized with that of TBP, a housekeeping gene, and analyzed by Mann-Whitney test. **B:** A comparison of PAK1 mRNA expression in HMs with or without metastasis. **C and D:** mRNA and protein expression of PAK1 and PAK2 in a normal extravillous trophoblast cell line (TEV-1) and two CCA cell lines, JEG-3 and JAR, as determined by qPCR (Bars = means $\pm$ SD of three experiments. * $P < 0.05$ compared with TEV-1, Mann-Whitney test) and Western blot analysis respectively.

index Ki-67 (correlation coefficient = 0.453, $P = 0.009$, Pearson test) and MCM7 (correlation coefficient = 0.473, $P = 0.026$, Pearson test) in the group of HMs. Besides CCAs, PAK1 expression was also higher in PSTT than its normal trophoblast counterpart, extravillous implantation site intermediate trophoblast (Figure 2, F and G).

No Significant Changes of PAK2 mRNA and p-PAK2 Ser²⁰ Protein Expression Were Found in Normal First Trimester Placentas and GTD

By real-time PCR, no significant difference in PAK2 mRNA was found between normal first trimester placentas and HMs (data not shown). PAK2 mRNA and protein expression level was also found to be similar in the normal trophoblast and CCA cell lines as detected by real-time PCR and Western blot analysis (Figure 1, C and D). By immunohistochemistry, p-PAK2 was expressed predominantly in the cytoplasm with occasional nuclear accentuation found (Figure 3). Similar level of p-PAK2 expression was detected in normal first trimester placentas, HMs and CCAs (Figure 3, A, C–E, and H). p-PAK2 was barely detectable in term placentas (Figure 3B). No significant difference in p-PAK2 expression was found between HMs that regressed and the developed GTN (Figure 3, C, D, and H). Level of p-PAK2 expression was found to be similar in PSTT (4.50 ± 2.45) and its normal trophoblast counterpart, extravillous implantation site intermediate trophoblast (4.79 ± 2.39) (Figure 3, F and G).

Knockdown of PAK1 in CCA Cells Reduced Cell Proliferation and Up-Regulated p16

To examine the functional roles of PAK1 in CCAs cells, transient knockdown of PAK1 was performed in JEG-3 and JAR by siRNA approach. After confirming the specific knockdown of PAK1 mRNA and protein (Figure 4A), the effect on cell proliferation was first determined by MTT proliferation assay. PAK1 depleted JEG-3 and JAR cells showed significantly reduced proliferation rate when

compared with control cells that transfected with control siRNA (Figure 4B). Moreover, knockdown of PAK1 in JEG-3 and JAR significantly up-regulated *cell cycle inhibitor p16* mRNA expression (Figure 4D), but have no effect on other cell cycle-related genes, including *CDC25A*, *p21*, *CYCLIN D1*, *CYCLIN D2*, *CYCLIN B1*, *proliferation cell nuclear antigen*, and *c-ERB-B-2* as evaluated by qPCR (data not shown).

Transient Knockdown of PAK1 in CCA Cells Reduced Cell Migration and Invasion Ability and Down-Regulated Vascular Endothelial Growth Factor and MT1-MMP

We next tested the effect of PAK1 depletion on cell migration and invasion by Transwell migration and invasion assays, respectively. PAK1 depleted JEG-3 and JAR cells displayed significantly reduced cell migration and invasion when compared with control cells (Figure 4C). qPCR analysis showed that reduced PAK1 expression also down-regulated mRNA expression of vascular endothelial growth factor in JEG-3 and JAR and membrane type 1-matrix metalloproteinase (MT1-MMP) in siPAK1 JEG-3 cells (Figure 4D). MT1-MMP mRNA expression was nondetectable in JAR by qPCR analysis. Western blot analysis revealed down-regulation of MT1-MMP protein expression in siPAK1 JEG-3 cells (Figure 4D).

Discussion

In this study, significantly higher PAK1 mRNA and protein expression in HMs and CCAs was detected, when compared with normal placentas. Importantly, higher PAK1 expression was associated with aggressive behavior in HMs, as manifested by its higher expression in HMs that progressed to gestational trophoblastic neoplasia, particularly those with metastasis. Increased expression of PAK1 was also found in CCA cell lines. PSTT, the gestational trophoblastic tumor derived from extravillous im-

Figure 2. PAK1 immunoreactivity in first trimester placenta (A), term placenta (B), HM that regressed (C), HM that subsequently developed persistent trophoblastic neoplasia (D), CCA (E), extravillous implantation site intermediate trophoblast (F) (arrows), and PSTT (G). PAK1 immunoreactivity was mainly found in the cytoplasm of cytotrophoblast (CT) and was absent in syncytiotrophoblast (ST) (arrows). **H:** Bar chart showing the immunoreactivity of PAK1 in first trimester placentas (First), term placentas (Term), HM that regressed (HM-R), HM that subsequently developed persistent trophoblastic neoplasia (HM-GTN), and CCAs. The immunoreactivity of PAK1 was analyzed by Mann-Whitney test.

plantation site intermediate trophoblasts, also showed higher PAK1 expression than its normal counterpart. Our findings suggested that PAK1 may play important roles in the pathogenesis of GTD including progress of HMs into GTN with even development of metastasis as well as tumorigenesis of CCA. Indeed, overexpression and oncogenic effect of PAK1 has been earlier documented in various human malignancies, including glioblastoma,²⁵ breast,²⁶ colorectal,¹⁰ ovarian,^{9,27} and hepatocellular cancers.²⁸

In human placentas, cytotrophoblasts can be considered as trophoblastic stem cells responsible for proliferation, which eventually differentiate into either syncytiotrophoblasts or villous intermediate trophoblasts.³

Villous intermediate trophoblasts in turn proliferate and differentiate to implantation site and chorionic type intermediate trophoblasts. Implantation site intermediate trophoblasts infiltrate the deciduas, myometrium, and spiral arteries of the implantation site to form the maternal fetal circulation. Owing to the proliferative property of cytotrophoblast and villous intermediate trophoblast, the presence of PAK1 immunoreactivity in cytotrophoblast and villous intermediate trophoblast as demonstrated in the present study concurs with the reported proliferation enhancing function of PAK1. For instance, it was documented that constitutively active PAK1 can promote anchorage-independent growth of breast cancer cells through the mitogen-activated protein kinase pathway.¹¹

Figure 3. p-PAK2 immunoreactivity in first trimester placenta (A), term placenta (B), HM that regressed (C), HM that subsequently developed persistent trophoblastic neoplasia (D), CCA (E), extravillous implantation site intermediate trophoblast (F) (arrow), and PSTT (G). p-PAK2 immunoreactivity was mainly found in the cytoplasm of cytotrophoblast (CT) and was absent in syncytiotrophoblast (ST) (arrows). **H:** Bar chart showing the immunoreactivity of p-PAK2 in first trimester placentas (First), term placentas (Term), HM that regressed (HM-R), HM that subsequently developed persistent trophoblastic neoplasia (HM-GTN), and choriocarcinomas (CCAs). The immunoreactivity of p-PAK2 was analyzed by Mann-Whitney test.

Figure 4. Transient knockdown of PAK1 impeded JEG-3 and JAR cell proliferation, migration, and invasion, up-regulated p16 and down-regulated VEGF and MT1-MMP. **A:** Specific knockdown of PAK1 (siPAK1) mRNA and protein expression in JEG-3 and JAR cells by qPCR (upper panel) and Western blot analysis (lower panel) respectively. PAK1 mRNA expression in control was arbitrarily set as 1. Bars = means $\pm$ SD of three experiments. **B:** Cell proliferation rate in control and siPAK1 JEG-3 and JAR as determined by MTT assay. The absorbance of control was arbitrarily set as 1. Bars = means $\pm$ SD of three experiments. **C:** *In vitro* migration (left panel) and invasion assays (right panel) in control and siPAK1 JEG-3 and JAR. Upper panels: representative images showing control and siPAK1 JEG-3 cells migrated or invaded to the lower chamber through the transwell membrane without or with Matrigel coating after 24 and 48 hours respectively. Lower panels: Bars = mean $\pm$ SD of cell migration or invasion as percentage of control in five fields of triplicate wells from three experiments. **D:** qPCR analysis (left panel) on the mRNA levels of p16, VEGF, and MT1-MMP in control and siPAK1 JEG-3 and JAR. Bars = means $\pm$ SD of three experiments. **E:** Western blot analysis (right panel) on MT1-MMP protein expression in control and siPAK1 JEG-3.

Another study also reported that ETK tyrosine kinase can activate PAK1 and increase proliferation, anchorage-independent growth, and tumorigenicity of breast cancer cells.²⁶ In fibroblasts, CDC2A-mediated PAK1 phosphorylation on Thr212 has been found to regulate postmitotic spreading, which is an essential step for cell cycle progression and proliferation.²⁹ In a transgenic mouse model, overexpression of constitutively activate PAK1 can stimulate downstream proliferative signaling effectors MEK1/2 and p38- mitogen-activated protein kinase and was sufficient for mammary gland tumorigenesis.³⁰ The present findings regarding the correlation of PAK1 expression with the proliferative indices Ki-67 and MCM7 in HMs and the proliferation promoting effect of PAK1 on CCA cells as revealed using siRNA approach further support the involvement of PAK1 in trophoblasts proliferation.

We further elucidate p16 as one of the downstream targets of PAK1 in CCA cell lines, as manifested by its up-regulation in PAK1 depleted CCA cells. p16 is an inhibitor of the cyclin D-dependent kinases, CDK4 and

CDK6, which blocks S-phase entry through the disruption and inhibition of the complexes containing D-type cyclins and either CDK4 or CDK6.³¹ Previous studies from our group and others have documented significantly reduced expression and hypermethylation of p16 in HMs and CCAs, particularly in patients who developed persistent GTN than those with spontaneous regression.^{32,33} Aberrant methylation of p16 associated with its loss of expression in tumor cells have also been found in other tumors, including esophageal, colon, prostate, and pancreatic carcinomas, supporting the prominent tumor suppression role of p16.^{31,34,35} The reciprocal expression pattern of PAK1 and p16 in GTD suggests that PAK1 is a putative upstream negative regulator of p16.

Besides proliferation, PAK1 can also regulates cellular polarity and motility through cytoskeletal reorganization, and hence affects cell invasion.^{5,6} Overexpression of PAK1 has been correlated with human breast cancer invasiveness and tumor grade.¹¹ The present study also revealed the participation of PAK1 in the aggressive behavior of trophoblasts in HMs, CCAs, and PSTT, based

on its overexpression and the reduced CCA cell migration and invasion after knockdown of PAK1. We also found that PAK1 may control CCA cell invasion through its regulation on the expression of VEGF and MT1-MMP. VEGF is a potent pro-angiogenic growth factor and has also been found to regulate ovarian cancer cell migration and invasion, thus essential for tumor growth and metastasis.³⁶ We have indeed recently reported a similar inhibiting effect on VEGF expression after knockdown of PAK1 in ovarian cancers.⁹ The effect of PAK1 on VEGF and thus on angiogenesis is likely to be a persistent phenomenon. A recent study also documented higher VEGF expression in CCAs than in HMs.³⁷ MT1-MMP is a pericellular collagenase and also an activator of pro-MMP2 through the formation of the MT1-MMP/proMMP-2/tissue inhibitor of metalloproteinase-2 complex, thereby controlling tumor cell invasive ability via traverse the extracellular matrix.³⁸ This is the first report showing the expression of MT1-MMP in CCA cells. Stronger MMP-2 expression has already been reported in CCAs than in placentas and HMs.³⁹ Our group has also documented down-regulation and promoter methylation of tissue inhibitor of metalloproteinase-3 in CCAs when compared with placentas.⁴⁰ The inductive effect of PAK1 on VEGF and MT1-MMP further explains the mechanisms governing cell invasion enhancing effect of PAK1.

Unlike PAK1, PAK2 mRNA and p-PAK2 immunoreactivity showed similar expression pattern in normal first trimester placentas and GTD. It was documented that transfection of constitutively active Rac3 mutant into normal mammary epithelial cell lines significantly increases cell proliferation through PAK2.¹² PAK2 also displayed both pro-apoptotic or anti-apoptotic functions. The C-terminal PAK2 fragment cleaved by caspases during apoptosis activates the JNK pathway and contributes to the morphological changes in apoptosis.⁴¹ On the other hand, full-length PAK2 has been shown to promote cell survival and suppresses cell death induced by tumor necrosis factor- α , growth factor withdrawal, and UV light.⁴² Thus, PAK2 may regulate trophoblasts proliferation and apoptosis in both normal and abnormal trophoblastic tissues.

In conclusion, we have presented the first study on the expression profiles of PAK1, PAK2, and p-PAK2 in GTD and their prognostic value in predicting clinical outcome. We demonstrated that there is up-regulation of PAK1, but not PAK2 or p-PAK2 in HMs and CCAs. In particular, PAK1 expression was shown to associate with the development of aggressive GTN and metastasis of HMs. Furthermore, the involvement of PAK1 in the pathogenesis and clinical progress of GTD is likely to act through the regulation of cell proliferation, migration, and invasion. Although most GTDs are highly responsive to chemotherapy, chemoresistant cases especially in high-risk metastatic diseases do exist, causing problems in management despite combined chemotherapy, surgery, and radiation.^{43,44} Therefore, identifying novel therapeutic targets for GTDs especially those with metastatic disease is important. A recent study has identified fatty acid synthase as a potential therapeutic target for GTD via its inhibitor C93.⁴⁵ In view of the fact that kinase inhibitors for

the PAK family are being developed,^{13,14} our present findings may provide new insight into PAK1 as a potential therapeutic target in GTD.

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