Tumorigenesis and Neoplastic Progression

Activation of Mitogen-Activated Protein Kinase Is Required for Migration and Invasion of Placental Site Trophoblastic Tumor

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Placental site trophoblastic tumor (PSTT) is a gestational neoplasm derived from the extravillous (intermediate) trophoblast of the implantation site. PSTT is characterized by a highly invasive phenotype, but the molecular mechanisms are poorly understood. In this report, we demonstrate that PSTTs expressed the activated (phosphorylated) form of mitogen-activated protein kinase (MAPK) in 84% of cases, whereas the normal extravillous trophoblastic cells did not. To characterize the role of MAPK activation in PSTT, we established the first PSTT cell culture, IST-2, from a surgically resected PSTT. IST-2 cells expressed HLA-G and Mel-CAM but not E-cadherin, an immunophenotype characteristic of PSTT. IST-2 cells were highly motile and invasive in culture as compared to choriocarcinoma JEG-3 cells and normal extravillous trophoblastic cells. Based on wound assay, time-lapse videomicroscopy for cell tracking, and invasion chamber assays, we found that the motility and invasion of IST-2 cells were significantly reduced ($P < 0.01$) after treatment with the MEK inhibitors CI-1040 and PD59089, which prevent activation of MAPK. In contrast, neither compound had any effect on normal extravillous trophoblastic cells or JEG-3 cells. In conclusion, our findings demonstrate a functional role of MAPK activation in the motility and invasion of PSTT. (*Am J Pathol* 2005, 167:879–885)

Gestational trophoblastic diseases represent a unique group of lesions because they derive from the conceptus and not from the patient. Gestational trophoblastic diseases encompass a diverse group of lesions with spe-

cific pathogenesis, morphological characteristics and clinical features.¹ The various forms of gestational trophoblastic disease can be defined and related to discrete pathological aberrations occurring at different stages of trophoblastic differentiation. In the modified World Health Organization classification,² gestational trophoblastic diseases can be broadly divided into molar lesions and nonmolar lesions. The molar lesions include partial and complete hydatidiform moles and invasive moles. The nonmolar lesions include choriocarcinoma and lesions derived from implantation site extravillous (intermediate) trophoblast [exaggerated placental site and placental site trophoblastic tumor (PSTT)] and those from the chorionic-type intermediate trophoblast (placental site nodule and epithelioid trophoblastic tumor). PSTT is a relatively uncommon form of gestational trophoblastic disease that is composed of neoplastic implantation site intermediate trophoblastic cells because the morphological, biological, and molecular features of the tumor are similar to those of extravillous (intermediate) trophoblastic cells in the placental site.^{2–5} In contrast to the normal extravillous (intermediate) trophoblastic cells in which invasion is highly regulated and is confined to the inner third of myometrium, the tumor cells of PSTT are highly invasive as they infiltrate deep into the myometrium and occasionally penetrate through the myometrium of the uterus.

Clinically, patients of PSTT usually are in their reproductive ages and can present with either amenorrhea or abnormal vaginal bleeding.² In contrast to choriocarcinoma, which is often associated with a complete mole, PSTT occurs most commonly after a normal pregnancy or nonmolar abortion, while there is a clinical history of complete mole in only 5 to 8% of patients.² It has been thought that PSTT is generally benign but behaves in an aggressive manner in ~15% of cases at presentation. Molecularly, PSTT contains either genetic markers from Y-chromosome and/or novel alleles not belonging to the

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patient, confirming its trophoblastic origin. Most PSTTs studied are diploid based on flow cytometric DNA analysis.²

The RAS/RAF/MEK/MAPK signaling pathway is known to play a major role in various cellular activities including proliferation, differentiation, apoptosis, angiogenesis, and migration.^{6–12} Suppression of MAPK activity by inhibitors, dominant-negative MEK1 mutants, and antisense nucleotides reduced the migratory ability of several cell types in response to extracellular growth stimulation.^{13–17} Given the important role of MAPK in cell function, we investigated whether the RAS/RAF/MEK/MAPK pathway is involved in the development of PSTT. In this study, we assessed the activation of MAPK in PSTT tissues, and examined effects of MEK inhibitors on cell proliferation, motility, and invasion.

Materials and Methods

Establishment of IST-2 Cell Culture and Extravillous Trophoblastic (EVT) Cells from a Normal Placenta

A fresh PSTT tissue was collected from an anonymous patient undergoing hysterectomy who was previously diagnosed to have uterine PSTT by endometrial curettage. The tumor specimen was washed in cold phosphate-buffered saline (PBS) and a representative frozen section of the microdissected PSTT was stained with hematoxylin and eosin (H&E) to ensure that the selected material contained the tumor. The tumor was transferred to a 10-cm Petri dish and was minced into ~1-mm³ tissue fragments that were then incubated with collagenase A (2 mg/ml) at 37°C for 30 minutes. After filtration through sieve membranes (with 100- μ m pores), cells were plated on culture flasks in RPMI 1640 medium supplemented with 10% fetal bovine serum (Life Technologies, Grand Island, NY) and penicillin/streptomycin (50 U/ml). The purity of tumor cells was determined by the cytokeratin-8 immunoreactivity.

To obtain normal EVT culture, we collected fresh human placental tissue from a patient undergoing elective abortion at 7 weeks of gestation using a method we previously described.¹⁸ Briefly, the specimen was carefully microdissected to remove fetal parts and endometrium. The chorionic villi were minced into ~1-mm³ villous explants and were plated on dishes in RPMI 1640 medium supplemented with 10% fetal bovine serum. After 5 days of incubation at 37°C the EVT cells grew out from the chorionic villous explants. They were then harvested for assays.

Western Blot Analysis

A panel of antibodies was used to characterize IST-2 cells including MN-4 that reacts with Mel-CAM (CD146);¹⁹ 4H84 that reacts with HLA-G;²⁰ and antibodies that react to E-cadherin (clone HECD-1; Zymed, San Francisco, CA) and GAPDH (clone 6C5; Research Diag-

nostics, Flanders, NJ). The primary IST-2 cells were scraped from culture flasks, washed three times with PBS, and then lysed by vortexing vigorously in RIPA lysis buffer containing 20 mmol/L Tris-HCl (pH 8.3), 150 mmol/L NaCl, 2 μ mol/L phenylmethyl sulfonyl fluoride, 5 μ mol/L ethylenediamine tetraacetic acid, 1% deoxycholic acid, and 1% Triton X-100 for 10 minutes. After centrifugation, the supernatants were subject to Western blot analysis on an 8% sodium dodecyl sulfate-polyacrylamide gel and the membrane was probed with the antibodies. Immunoreactive bands were detected by chemiluminescence (Amersham, Arlington Heights, IL).

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue including 15 normal human placentas with implantation sites ranging from 7 to 12 weeks of gestation and 25 PSTTs were evaluated for the expression of activated (phosphorylated) MAPK and total MAPK using immunohistochemistry. The antibody used was a rabbit polyclonal antibody, pTEpY that specifically reacted with the active (phosphorylated) but not the unphosphorylated MAPK (Promega, Madison, WI),²¹ and an anti-ERK1/2 polyclonal antibody that reacted with total MAPK (Promega). The specificity of pTEpY in immunohistochemistry was previously reported.^{21–23} Immunohistochemistry was performed on tissue sections at a dilution of 1:500 followed by the EnVision+System using the peroxidase method (DAKO, Carpinteria, CA). The percentage of positive cells was estimated by randomly counting ~500 tumor cells from three different high-power fields ($\times 40$) within one specimen. A positive reaction was defined as discrete localization of the brown chromogen in the nucleus or cytoplasm. Cases in which more than 5% of the tumor cells showed detectable immunoreactivity were scored as positive. The intensity of immunoreactivity was scored from 0 (negative) to 3+.

Cell Proliferation Assay Using SYBR Green Dye

To measure the effect of MEK inhibitors on cell proliferation, we plated 4000 IST-2 cells per well in 96-well plates. The medium contained 5 μ mol/L CI-1040 (Pfizer, Providence, RI), 5 μ mol/L PD98059 (Promega) or dimethyl sulfoxide (DMSO) (control). At different time points, we replaced the culture medium with 50 μ l of 2% sodium dodecyl sulfate and the cells were incubated for 2 hours at 37°C. SYBR Green I nucleic acid gel stain (150 μ l) was added to each well at a dilution of 1:1000 (Molecular Probes, Eugene, OR). The fluorescence intensity from each well in the 96-well plate was then analyzed using a microplate reader (BMG Durham, NC). The data were expressed as the mean $\pm$ 1 SE from five replicates.

Wound Assay to Assess Cell Motility

IST-2, EVT, and JEG-3 cells were allowed to grow confluent in 24-well plate in the presence of 5 μ mol/L CI-

Table 1. Immunoreactivity of MAPK in Trophoblast

Immunointensity	Activated MAPK				Total MAPK			
	0	1+	2+	3+	0	1+	2+	3+
Normal placenta (<i>n</i> = 10)								
Cytotrophoblast	6	4	0	0	2	8	0	0
Syncytiotrophoblast	10	0	0	0	10	0	0	0
Extravillous trophoblast	5	5	0	0	1	9	0	0
PSTT (<i>n</i> = 25)	0	2	5	18	0	25	0	0

1040, 5 μ mol/L PD98059, or DMSO control. A linear wound was created by scraping the wells with an ART-1000E pipette tip (Molecular BioProduct, San Diego, CA). The floating cells were removed by gentle washes in culture medium. The wound was observed 24 hours later and the number of individual cells in the wound was quantified as an average from multiple fields (at least five) at $\times 200$ magnification for each experiment.

Time-Lapse Videomicroscopy for Cell Tracking

The experiment was performed as previously described.²⁴ Cells were incorporated within three-dimensional collagen lattices containing 1.5 mg/ml purified native type I dermal bovine collagen (Vitrogen; Cohesion Technologies, Palo Alto, CA) in minimal essential medium adjusted to pH 7.4. This suspension was allowed to polymerize (37°C for 30 minutes, 5% CO₂) and the collagen lattices were monitored by bright-field time-lapse digital microscopy ($\times 100$) throughout a time period of 48 hours. For cell tracking, the paths of individual cells were followed using the Multitrack 4.2 software (Mediquant, Halle, Germany). The time interval from step to step was 10 minutes.

BD BioCoat Matrigel Invasion Assay

The invasion chamber assay was performed according to the manufacturer's instructions. Briefly, Matrigel-precoated transwell chambers with PET membranes containing 8- μ m pores (BD Bioscience, Bedford, MA) were soaked in Dulbecco's modified Eagle's medium and incubated for 60 minutes at 37°C. IST-2 and EVT cells were pretreated with 5 μ mol/L CI-1040, 5 μ mol/L PD98059, or DMSO (as vehicle control) for 30 minutes. For each well, 5×10^4 cells in 0.5 ml of culture medium were added to the upper compartment of the transwell chambers. As a control, an equal number of uncoated BD Falcon TC companion plates were seeded with cells in parallel. After 24 hours of incubation in the medium containing CI-1040, PD98059, or DMSO, non-invaded cells of the upper compartment were removed using a cotton-tipped swap. Viable cells were stained with H&E and photographs ($\times 200$ magnification) were taken. Cells were counted in several fields of triplicate membranes. Data were expressed as the percentage of cells that invaded through the Matrigel matrix-coated membrane relative to the cells that migrated through the control membrane.

Results

Expression of Active MAPK in Normal Trophoblast and PSTTs

The expression of active MAPK and total MAPK in human trophoblasts was evaluated by immunohistochemistry using antibodies that specifically reacted with the active (phosphorylated) form of MAPK and the total MAPK.²¹ We demonstrated that 92% of PSTTs expressed high levels of activated MAPK (2+ and 3+) (Table 1). The immunoreactivity was diffuse in PSTT and it was predominantly located in the cytoplasm. In contrast, cytotrophoblastic cells and extravillous (intermediate) trophoblastic cells in normal placentas expressed low levels (1+) of active MAPK in 40% and 53% of specimens, respectively. Syncytiotrophoblastic cells did not express detectable immunoreactivity of active MAPK in any of the specimens examined. The immunoreactivity of total MAPK was weak (1+) in all PSTTs and was not detectable in syncytiotrophoblastic cells from all normal placentas and cytotrophoblastic cells from two placentas. The placentas with negative total MAPK immunoreactivity were also negative for active MAPK staining. Representative photomicrographs of active MAPK immunostaining are shown in Figure 1.

Characterization of a PSTT Primary Culture

A PSTT primary culture, IST-2, was successfully established from one of the three PSTT specimens collected in the last 8 years. The PSTT cells in the primary culture expressed cytokeratin, confirming the epithelial nature of trophoblastic cells. IST-2 cells were cultured for 10 passages to expand the cell number for study. Under phase-contrast microscopy, IST-2 cells exhibited a pleomorphic morphology varying from triangular to fusiform in shape (Figure 2A). This morphological feature was similar to those observed in the extravillous (intermediate) trophoblastic cells grown in culture.¹⁸ IST-2 cells formed a monolayer and the growth of IST-2 cells was serum-dependent with a doubling time of ~ 2 days in RPMI 1640 medium containing 10% fetal bovine serum. A karyotype analysis revealed 38-45, X, -X, -1, -2, -3, -6, -8, -22[cp11]/46, XX. Western blot analysis demonstrated that IST-2 cells expressed HLA-G and Mel-CAM but not E-cadherin, an immunohistochemical phenotype consistent with EVT cells in a normal placental site and in PSTT tissues (Figure 2B).²⁵⁻²⁸

Figure 1. Expression of active MAPK in normal and neoplastic trophoblastic cells. Immunohistochemistry was performed using an antibody against the active (phosphorylated) form of MAPK. In chorionic villi of early gestation, cytotrophoblastic cells on the villous surface demonstrated weak immunoreactivity and the syncytiotrophoblastic cells were negative for staining. In the normal implantation site (NIS), extravillous (intermediate) trophoblastic cells (arrows) display weak immunoreactivity. In contrast, PSTT shows an intense and diffuse staining for active MAPK.

Effects of MEK Inhibitors on IST-2 Cells

To investigate the roles of MAPK activation in PSTT, we inactivated MAPK in IST-2 cells using CI-1040 and PD98059, compounds that selectively inhibit MEK and prevent activation of MAPK,^{29,30} and analyzed their effects on cell proliferation, cell motility, and invasion *in vitro*. As controls, normal EVT cells and JEG-C choriocarcinoma cells were analyzed in parallel for cell motility and invasion assays. Western blot analysis demonstrated that the active MAPK protein was not detectable after 5 μ mol/L CI-1040 treatment (Figure 3A) or PD98059 treatment. The concentrations of the inhibitors used in this

Figure 2. Characterization of IST-2 cells. **A:** Under a phase contrast microscope, the cells exhibit a pleomorphic morphology varying from triangular to fusiform in shape. **B:** Western blot analysis demonstrates that IST-2 cells express HLA-G and Mel-CAM but not E-cadherin, an immunohistochemical phenotype consistent with the extravillous trophoblast in a normal placental site and a PSTT.

Figure 3. The effects of MEK inhibitors on cell proliferation and migration. **A:** Western blot analysis shows that the active MAPK protein is not detectable after CI-1040 treatment of IST-2 cells. **B:** Inactivation of MAPK does not affect the cell growth in IST-2 cells because the growth curves are similar between CI-1040-, PD98059-, and control (DMSO)-treated IST-2 cells. The error bars represent 1 SD and may be too small to be visible. **C** and **D:** An artificial wound was created after scraping cells from a confluent monolayer of IST-2 cells. As compared with the IST-2 cells treated with DMSO, there is a significant reduction in the number of CI-1040-treated IST-2 cells that migrated into the wound.

study were based on a pilot study showing the concentrations were the lowest concentrations to completely inhibit MAPK activation. Therefore this concentration was used in the following studies. Growth curves of CI-1040-, PD98059-, and DMSO (control)-treated IST-2 cells did not show significant differences, indicating that inactivation of MAPK does not affect the cell growth of IST-2 cells (Figure 3B).

Cell motility was assessed using two independent assays. The wound migration assay measured the ability of IST-2 cells to dissociate and migrate away from the confluent monolayer and the time-lapse videomicroscopy and cell tracking, on the other hand, measured the motility rates of individual cells. For the wound assay, an artificial wound was created by scraping cells from a confluent monolayer of IST-2, EVT, and JEG-3 cells. As compared to the DMSO-treated IST-2 cells, there was a significant reduction in the number of CI-1040- or PD98059-treated IST-2 cells that migrated into the wound ($P < 0.001$, one-tailed Student's *t*-test) (Figure 3, C and D). In contrast, there was no statistical significance in DMSO-, CI-1040-, and PD98059-treated EVT and JEG-3 cells (Figure 3D). Similarly, the migration velocity of IST-2 cells was significantly reduced after CI-1040 or PD98059 treatment as compared to the control-treated cells ($P < 0.05$, one-tailed Student's *t*-test) (Figure 4). The migration velocity of control IST-2 cells varied significantly but was much higher than that in EVT or JEG-3 choriocarcinoma cells ($P < 0.05$). The invasion of IST-2 cells was evaluated by their ability to invade and penetrate through the Matrigel-coated pores on membrane inserts of transwell. As shown in Figure 5, the CI-1040- and PD98059-treated IST-2 cells demonstrated a decrease in their invasion ability as compared to DMSO-treated IST-2 cells. In contrast, there was no statistical significance in DMSO-, CI-

Figure 4. Time-lapse videomicroscopy and cell tracking. The **top** panel of the photomicrographs demonstrates the tracks of individual cell migration (**lines**) after time-lapse videomicroscopy. The migration velocity in the control IST-2 cells varied and was significantly higher than that in CI-1040- and PD98059-treated IST-2 cells. EVT and JEG-3 cells do not appear to be affected by either of the compounds.

1040-, and PD98059-treated EVT and JEG-3 cells. Furthermore, IST-2 cells showed a higher invasion capacity than the EVT and JEG-3 cells ($P < 0.001$).

Discussion

PSTT is a unique trophoblastic neoplasm that is characterized by highly invasive behavior as the tumor usually invades deep into the myometrium and uterine serosa. In this study, we report that PSTTs overexpress activated MAPK as compared to their normal counterparts. Inactivation of the MAPK signaling pathway significantly reduces motility and invasion of PSTT cells in a cell culture model. These findings suggest a critical role of the MAPK signaling pathway in controlling cell migration and invasion in PSTT.

Activation of the MAPK pathway plays a key role in several neoplasms including melanoma,^{31–33} papillary thyroid carcinoma,³⁴ colorectal carcinoma,³⁵ and low-grade ovarian serous carcinoma.^{36,37} In this study, we demonstrated that the active (phosphorylated) form of MAPK was overexpressed in the majority of PSTTs but not in their normal counterparts, suggesting that activation of MAPK pathway may be involved in the development of PSTT. We further showed that the expression of active MAPK was not likely a consequence of MAPK overexpression but rather the result of hyperactivation of MAPK in PSTTs. This observation is similar to a previous report showing that hyperactivation but not overexpression of MAPK was associated with non-small-cell lung carcinoma.³⁸ The cause of MAPK hyperactivation in PSTTs is not clear. Activation of MAPK can be a result of activating mutations in the members belonging to the RAS/MAPK pathway or it could be a result of epigenetic

Figure 5. Invasion chamber assay. The invasion of IST-2 cells was evaluated by their ability to invade and penetrate through the Matrigel-coated pores on membrane inserts of the Transwells. DMSO-treated IST-2 cells showed a significantly higher invasion capacity than the CI-1040- and PD98059-treated IST-2 cells. EVT and JEG-3 cells do not appear to be affected by both compounds.

events such as stimulation by a variety of growth factors and cytokines. For example, it has been shown in melanoma that growth factors including fibroblast growth factor and hepatocyte growth factor can mediate MAPK activation through autocrine mechanism in addition to mutations in BRAF gene.³²

To elucidate the functional roles of MAPK pathway in cell motility and invasion in PSTT, we have established a PSTT culture, IST-2. The origin of IST-2 cells was determined by evaluating expression of trophoblast-related markers including HLA-G and Mel-CAM (CD146). Furthermore, these cells did not express detectable E-cadherin. This phenotype is consistent with PSTT and normal extravillous (intermediate) trophoblastic cells from which PSTT is derived. In addition, IST-2 cells are highly motile in culture, a phenotype similar to extravillous (intermediate) trophoblastic cells in PSTT.

CI-1040 is a compound that has been shown to selectively inhibit MEK,³⁷ the immediate upstream regulator of MAPK, and it has been used as a reagent to explore the functions of MAPK activation in several cell systems including low-grade ovarian tumors.³⁹ Likewise, PD98059 is another MEK inhibitor and has been used in many reports to study the MAPK activation. Like PSTTs, IST-2 cells in culture expressed activated MAPK. CI-1040 and PD98059 completely abolished the immunoreactivity of

active MAPK in IST-2 cells, suggesting that IST-2 cell culture was an appropriate model to study the MAPK pathway in PSTT. We found that CI-1040 and PD98059 had no apparent effect on cell growth in IST-2 cells containing wild-type KRAS and BRAF, indicating that activation of MAPK pathway is not related to cell proliferation in IST-2 cells. This finding is consistent with our recent study showing that activated MAPK pathway is critical for tumor growth only in tumors harboring KRAS or BRAF mutations but not in tumors with wild-type KRAS and BRAF genes.³⁹ More interestingly, we observed a profound reduction of cell motility in CI-1040- and PD98059-treated IST-2 cells as evidenced by both wound assay and time-lapse videomicroscopy analysis. The lack of inhibitory effects of MEK inhibitors on motility and invasion in normal EVT cells is consistent with the fact that normal EVT cells do not express activated MAPK. Identification of the active MAPK-regulated molecules in PSTT is important to gain further insight into the role of the MAPK signaling pathway in the development of PSTT.

How could activated MAPK contribute to cell motility and invasion in PSTT? Several studies have shown that MAPK activation leads to extracellular matrix-dependent cell spreading and migration.^{9,14,16} These mechanisms for this could involve integrin activation,⁴⁰ integrin-dependent adhesion, or cytoskeletal organization and phosphorylation of myosin light chain kinase, calpain, or focal adhesion kinase (FAK).^{14–16} These activities directly or indirectly enhance cell motility. In addition to promote cell motility and migration, constitutive activation of MAPK is demonstrated to enhance matrix metalloproteinase activation, a key event during cellular invasion.⁴¹ The current study is consistent with previous reports showing that MAPK pathway inhibitors are potent in suppressing cell migration in tumor cells.^{14,42,43} Blockade of the MAPK pathway by treatment with MEK inhibitors correlated well with the inhibition of invasion by the tumor cells derived from rhabdomyosarcoma, fibrosarcoma, bladder carcinoma, colon carcinoma, prostate carcinoma, and breast carcinoma cell lines.⁴⁴ These results suggest that inactivation of the MAPK pathway can have useful anti-metastatic effects on tumor cells.

In this study, we have compared the motility and invasiveness of the IST-2 cells with JEG3, a well-known choriocarcinoma cell line frequently used for migration and invasion studies.^{45–47} Based on the results shown in this report, we found that IST-2 cells may be more suitable than JEG-3 for studying trophoblastic cell migration and invasion based on the evidence that IST-2 cells are highly motile and invasive in comparison to JEG-3 cells. This is because IST-2 was derived from PSTT, a tumor developed from extravillous (intermediate) trophoblast whereas JEG-3 cells are composed mainly of cytotrophoblast that does not show the ability to infiltrate the surrounding normal tissue *in vivo*.

In conclusion, we established the IST-2 cell culture and used it as an *in vitro* model to study the biological mechanisms underlying the motility and invasion of PSTT. Our study suggests that the regulation of migration/invasion in PSTT cells is conferred, at least in part, by the activation

of MAPK. Unlike other tumors with KRAS or BRAF mutations, the CI-1040- and PD98059-induced phenotype in PSTT is uncoupled with growth inhibition. Our results provide a new model to study the biology of PSTT and suggest a potential to apply MEK inhibitors as a new target-based therapy for PSTT.

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