

# Inactivation of the Mitogen-Activated Protein Kinase Pathway as a Potential Target-Based Therapy in Ovarian Serous Tumors with KRAS or BRAF Mutations

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## Abstract

**Activation of mitogen-activated protein kinase (MAPK) occurs in response to various growth stimulating signals and as a result of activating mutations of the upstream regulators, KRAS and BRAF, which can be found in many types of human cancer. To investigate the roles of MAPK activation in tumors harboring KRAS or BRAF mutations, we inactivated MAPK in ovarian tumor cells using CI-1040, a compound that selectively inhibits MEK, an upstream regulator of MAPK and thus prevents MAPK activation. Profound growth inhibition and apoptosis were observed in CI-1040-treated tumor cells with mutations in either KRAS or BRAF in comparison with the ovarian cancer cells containing wild-type sequences. Long serial analysis of gene expression identified several differentially expressed genes in CI-1040-treated MPSC1 cells harboring an activating mutation in BRAF (V599L). The most striking changes were down-regulation of cyclin D1, COBRA1, and transglutaminase-2 and up-regulation of tumor necrosis factor-related apoptosis-induced ligand, thrombospondin-1, optineurin, and palladin. These patterns of gene expression were validated in other CI-1040-treated tumor cells based on quantitative PCR. Constitutive expression of cyclin D1 partially reversed the growth inhibitory effect of CI-1040 in MPSC1 cells. Our findings indicate that an activated MAPK pathway is critical in tumor growth and survival of ovarian tumors with KRAS or BRAF mutations and suggest that the CI-1040 induced phenotypes depend on the mutational status of KRAS and BRAF in ovarian tumors. (Cancer Res 2005; 65(5): 1994-2000)**

## Introduction

Ovarian cancer is one of the most lethal malignant diseases in women and of these, serous carcinoma is the most common type (1). Our previous studies have shown that a subset of serous tumors that include low-grade serous carcinoma and its precursor, serous borderline tumor (2–4) harbor mutations in either KRAS or BRAF (2, 3). Based on mutational analysis, we found that mutations in either BRAF or KRAS occur in 65% to 88% of these low-grade tumors. Furthermore, we found that mutations in BRAF at codon 599 and KRAS at codons 12 and 13 were mutually exclusive (2, 5), a finding consistent with the data in melanoma and colorectal

carcinoma (6, 7). This lends further support to the view that BRAF and KRAS mutations have an equivalent effect on tumorigenesis. Mutations in either KRAS or BRAF occur very early in the development of low-grade serous tumors as the same mutations of KRAS or BRAF can be detected in both serous borderline tumor and adjacent cystadenoma epithelium, a presumed precursor of serous borderline tumor (5). In contrast, mutations in KRAS and BRAF are rare in high-grade serous carcinomas, which are characterized by frequent (>50%) p53 mutations (8).

Mutations of either BRAF or KRAS lead to constitutive activation (phosphorylation) of the downstream target, mitogen-activated protein kinase (MAPK), also known as extracellular signal-regulated protein kinase (ERK; refs. 9, 10). In a previous study, we showed a correlation between mutations in BRAF or KRAS and overexpression of activated MAPK in ovarian tumor tissues further supporting the above view (11). Activation of MAPK activates downstream cellular targets (12, 13) including a variety of cellular and nuclear proteins. Although the function and downstream effectors of the RAS/RAF/MEK/MAPK (ERK) pathway have been recently studied (14), the biological role of this pathway in the development of ovarian serous tumors has not been explored.

Based on our recent studies demonstrating that mutations in KRAS or BRAF occur very early in the tumor development (5) and that these mutations are associated with activation of MAPK in ovarian serous tumors (11), we hypothesize that constitutive activation of MAPK plays a key role in the development of serous tumors containing mutant KRAS or BRAF. To test this hypothesis, we compared the phenotypes and gene expression profiles in cultured ovarian serous tumor cells after treatment with a highly potent and selective inhibitor of MEK1/2, CI-1040 (formerly known as PD184352; refs. 15–18) that prevents the activation (phosphorylation) of MAPK. One of the differentially expressed genes, *cyclin D1*, was the most dramatically and consistently down-regulated by CI-1040 and accordingly, it was selected for further study for its biological role in mediating MAPK activation and cell proliferation.

## Materials and Methods

### Cell Culture and Cell Lines

OVCAR3, SKOV3, and CAOV3 ovarian cancer cell lines were obtained from American Tissue Culture Center (Rockville, MD). MPSC1 cell line was established from a low-grade serous carcinoma. A set of primary cultures was established from ovarian serous tumors, including OVPC-1, OVPC-2, OVPC-3, OVPC-4, OVPC-5, OVPC-6, OVPC-7, OVPC-8, and OVPC-9. In addition, primary cultures of normal ovarian surface epithelium and ovarian stroma were also obtained from ovarian tissues without neoplastic diseases. The acquisition of anonymous tissue specimens was approved by the local institutional review board. The diagnoses were confirmed by a surgical pathologist (I.S.) before harvesting tumor samples

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for experiments. Primary tumor cultures were established from freshly isolated tumor samples by immunosorting or trypsinization. For immunosorting, fresh tumor tissues were minced and incubated with collagenase A (2 mg/mL) at 37°C for 40 minutes. After filtration through sieve membranes (with 100- $\mu$ m pores), tumor cells were immunosorted using an anti-Ep-CAM antibody bound to the Dynal beads (Dynal, Oslo, Norway) following the vendor's instructions. For direct trypsinization, large fragments of fresh serous borderline tumor tissues (~1 cm) were incubated with trypsin-EDTA (Life Technologies, Grand Island, NY) at 37°C for 10 minutes with agitation. The detached epithelial cells were harvested by centrifugation. This procedure significantly minimized stromal cell contamination by detaching tumor epithelial cells directly from the tumor surface whereas keeping the underlying stroma intact. Freshly isolated tumor cells were allowed to grow in culture and were used for experiments within two passages. Culturing ovarian surface epithelium was done by gently scraping the surface of normal ovaries after incubation with trypsin-EDTA at 37°C for 15 minutes. The purity of epithelial cells was determined by the cytokeratin-8 immunoreactivity. Samples with >98% cytokeratin-8-positive cells were used. The culture of ovarian stromal cells was established by mincing a piece of normal ovary after removing the surface epithelium. All cultures were maintained in RPMI 1640 supplemented with 10% fetal bovine serum and 1% antibiotics. Selection of pCMV/cyclin D1 stable clones was done by minimal dilution in a selection medium containing 18  $\mu$ g/mL of Blasticidin (Sigma, St Louis, MO).

### Mutational Analysis of BRAF and KRAS

Genomic DNA was purified from all the cell lines and primary cultures using a Qiaquick PCR purification kit (Qiagen, Valencia, CA). PCR was then done followed by nucleotide sequencing at the Agencourt Bioscience (Beverly, MA). Exon 1 of *KRAS* and exon 15 of *BRAF* were both sequenced as each exon harbors almost all mutations of both genes (2–4, 6). The primers for PCR and sequencing were manufactured by GeneLink (Hawthorne, NY) and their sequences were described in a previous report (5). The sequences were analyzed using the Lasergene program, DNASTAR (Madison, WI).

**LongSAGE Library Construction.** Total RNA was isolated from MPSC1 cells after 9-hour incubation with 5  $\mu$ mol/L CI-1040 (a gift from M. Kolodney, Department of Medicine, UCLA School of Medicine, Los Angeles, CA; ref. 19) and DMSO control. The concentration of CI-1040 was used because this was the minimal concentration that abolished the expression of active MAPK in MPSC-1 cells based on Western blot analysis in MPSC-1 cells. LongSAGE was done with 2  $\mu$ g mRNA using the standard SAGE protocol that has been detailed at [http://www.sagenet.org/sage\\_protocol.htm](http://www.sagenet.org/sage_protocol.htm) with the modifications previously described (20). Linkers containing the *MmeI* recognition site were ligated to 3' cDNA ends after *NlaIII* digestion [linker 1A (5'-TTTGGATTGCTGGTGCAGTACAACCTAGGCTTAATATCCGACATG-3') and linker 1B (5'-TCGGATATTAAGCCTAGTTGTACTGCACCAGCAAATCCC7-amino-modified-3')] followed by annealing together and ligation to half the cDNA population, and linker 2A (5'-TTTCTGCTCGAATCAAGCTTCAACGATGTACGTCGACATG-3') and linker 2B (5'-TCGGACGTACATCGTTAGAAGCTTGAATTCGAGCAGC7-amino-modified-3') were annealed together and ligated to the remaining half of the cDNA. Linker tag molecules were released from the cDNA using the *MmeI* type IIS restriction endonuclease (21). Digestion was done at 37°C for 2.5 hours using 40 units *MmeI* in 300  $\mu$ L of 10 mmol/L HEPES (pH 8.0), 2.5 mmol/L potassium acetate, 5 mmol/L magnesium acetate, 2 mmol/L DTT, and 40  $\mu$ mol/L *S*-adenosylmethionine. The linker 1 tag and linker 2 tag molecules were not polished and were directly ligated together in a 6  $\mu$ L reaction containing 4 units T4 DNA ligase (Invitrogen, Carlsbad, CA) in the supplied buffer for 2.5 hours at 16°C. The ligation mixture was used to transform bacteria and ~3,840 clones were sequenced to obtain ~50,000 to 60,000 tags from each LongSAGE library (Agencourt, MA). The SAGE software was modified to allow extraction of 21-bp tags from sequences of concatemer clones.

**Quantitative PCR Analysis of Candidate Genes.** Single-stranded cDNA was synthesized from CI-1040 and DMSO-treated MPSC1, OVPC-1, OVPC-3, SKOV3, and OVCAR3 cells at 24 hours using the Superscript II reverse

transcriptase (Invitrogen) following the manufacturer's protocol, and mock template preparations were prepared in parallel without the addition of reverse transcriptase. After analysis of LongSAGE data, we selected the top 20 candidates of CI-1040 induced up-regulated and down-regulated genes for real-time PCR analysis. As we expected that most tags would correspond to the last exon of the candidate LongSAGE genes, primers were designed using the Primer 3 interface ([http://www-genome.wi.mit.edu/cgi-bin/primer/primer3\\_www.cgi](http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi)) to span a 100- to 200-bp region that included the tag, and were synthesized by GeneLink (Hawthorne, NY). Quantitative PCR was done using an iCycler (Bio-Rad, Hercules, CA) using Pico Green dye (Molecular Probes, Eugene, OR), and threshold numbers were collected using the iCycler software version 1.0. Averages in the threshold cycle number (Ct) of duplicate measurements were obtained. The results were expressed as the difference between the Ct of the gene of interest and the Ct of a control gene (*APP*) for which expression is relatively constant among the SAGE libraries analyzed (22).

### Western Blot Analysis

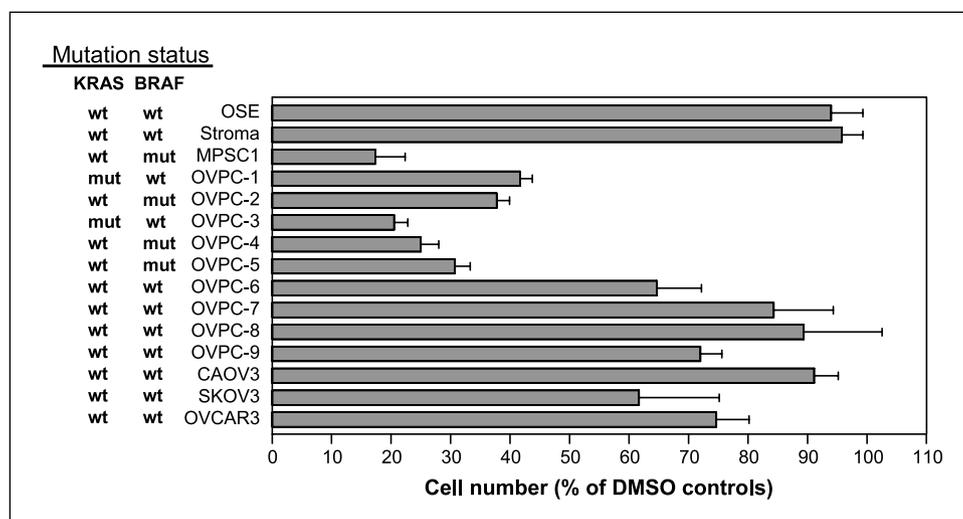
Cell lysates were prepared by dissolving cell pellets in the Laemmli sample buffer (Bio-Rad) supplemented with 5% of  $\beta$ -mercaptoethanol (Sigma). Western blot analysis was done on ovarian cancer cell lines/cultures, including MPSC1, OVCAR3, SKOV3, and OVPC-5. Similar amounts of total protein from each lysate were loaded and separated on 10% Tris-Glycine-SDS polyacrylamide gels (Novex, San Diego, CA) and electroblotted to the Millipore Immobilon-P polyvinylidene difluoride membranes. The membranes were probed with the anti-active MAPK antibody (pTEpY, 1:5,000; Promega, Madison, WI) or an anti-cyclin D1 antibody (CD1.1, 1:200; abcam, Cambridge, MA) followed by a peroxidase conjugated anti-mouse or anti-rabbit immunoglobulin (1:20,000). The same membrane was probed with an antibody that reacts with glyceraldehyde-3-phosphate dehydrogenase for loading controls. Western blots were developed by chemiluminescence (Pierce, Rockford, IL).

### Cell Growth and Cell Cycle Assays

For cell growth assay, cells were plated at the same density ( $10^5$  cells per well) in 24-well plates and cell growth assay was done by counting the number of viable cells 72 hours after treating the cells with CI-1040 at 5  $\mu$ mol/L and DMSO (control). The data was expressed as percentage of the DMSO control. The mean and SD were obtained from three experiments. To assess if cyclin D1 can revert the growth inhibitory effect of CI-1040, we constructed a mammalian expression vector, pCMV/cyclin D1 with a V5 tag at the COOH terminus. The cDNA of cyclin D1 was prepared from the MPSC1 cells, PCR and cloned to a mammalian expression vector, pcDNA6/V5-His A (Invitrogen). The clone was sequenced to ensure a wild-type coding sequence of cyclin D1. pCMV/cyclin D1 was stably transfected into MPSC1 cells using the Nucleofector II electroporator (Amaxa, Köln, Germany) under a selection marker of Blasticidin (18  $\mu$ g/mL). MPSC1 cells with both pCMV/cyclin D1- and pcDNA6/V5-His A-transfected cells were treated with either CI-1040 or DMSO (control). Cell number was counted at 0, 24, 48, and 72 hours. For cell cycle analysis, both attached and floating cells were harvested for study. Approximately  $3 \times 10^5$  cells were resuspended in 50  $\mu$ L of PBS, which was then mixed with 350  $\mu$ L of staining solution containing 0.6% NP40, 3% paraformaldehyde, and 10  $\mu$ g/mL 4',6-Diamidino-2-phenylindole. 4',6-diamidino-2-phenylindole-stained cells were subject to cell cycle analysis using a BD-LSR flow cytometer (Becton Dickinson, Mountain View, CA). The sub-G<sub>1</sub> population in the cell cycle analysis was defined as a fraction of apoptotic cells. To confirm the presence of apoptotic cells, 4',6-diamidino-2-phenylindole-stained cells were also examined under a Nikon fluorescence microscope or stained with Annexin V dye.

## Results

**Effects of MAPK Inactivation on Ovarian Serous Tumors.** A panel of ovarian serous tumor cell lines and primary cultures were first analyzed for their mutation status in the *KRAS* and *BRAF* genes. Their mutational status was correlated with growth

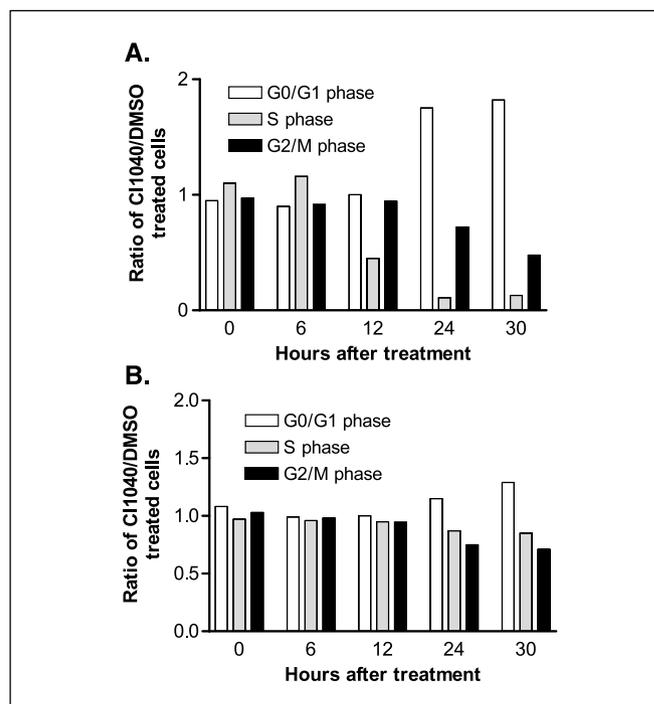


**Figure 1.** Effects of CI-1040 on cell proliferation. Cell number was counted after 72 hours of CI-1040 or DMSO (control) treatment. The mutational status of KRAS and BRAF for each sample is shown next to the tumor cell cultures and cell lines. Ovarian tumors with mutations in either KRAS or BRAF are more sensitive to growth inhibition by CI-1040 than those with wild-type sequences.

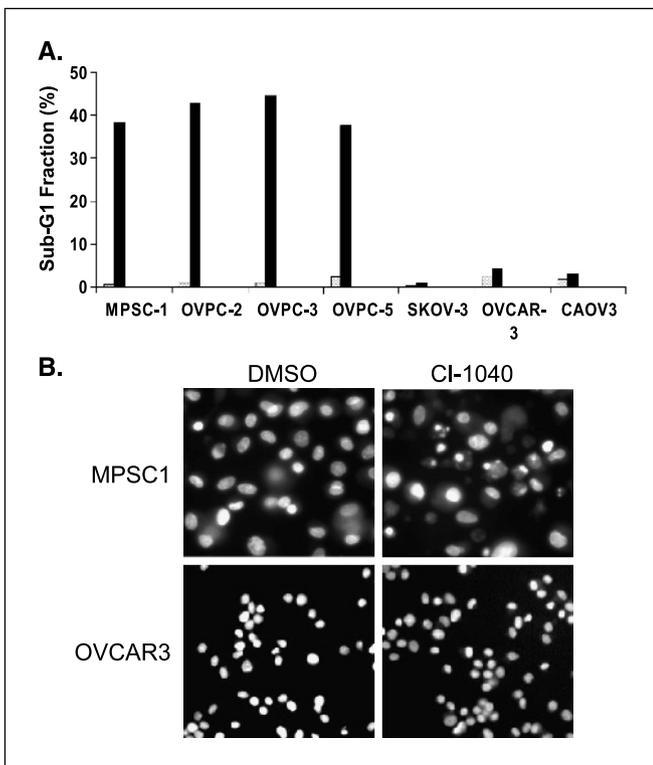
inhibition and apoptosis induction by CI-1040 which inhibits MEK and thus prevents activation of its downstream target, MAPK (ERK). Western blot analysis showed a dose-dependent effect on the expression of active MAPK in MPSC1 cells and active MAPK was not detectable 6 hours after treating the cells with CI-1040 at 5  $\mu\text{mol/L}$ , which was the concentration used in this study. As shown in Fig. 1, six of the tumors harboring either KRAS or BRAF mutations showed a marked reduction (<50% of DMSO control) in the cell number in CI-1040-treated group as compared with the other seven tumors containing wild-type KRAS and BRAF ( $P < 0.001$ ). Normal cells including ovarian surface epithelial cultures and ovarian stromal cells did not show a significant effect on growth inhibition by CI-1040. Cell cycle analysis showed a time-dependent cell cycle arrest at  $G_1$  phase in MPSC1 cells that contained an activating mutation in BRAF (V599L), but such arrest was not apparent in SKOV3 and OVCAR3 cells which did not have mutations in KRAS or BRAF (Fig. 2). Besides, CI-1040 induced apoptosis was more remarkable in the cell cultures and cell lines with either KRAS or BRAF mutations than those with wild-type sequences (Fig. 3).

**LongSAGE.** To determine the molecules that are potentially regulated by MAPK activation in ovarian serous tumors, we compared the gene expression profiles between the MPSC1 cells (with mutant BRAF) treated with CI-1040 and DMSO control using LongSAGE which is a technique modified from conventional SAGE. LongSAGE uses a different mapping enzyme to analyze longer tags that allows for direct and comprehensive comparison in gene expression profiles (20, 23). To identify the early alteration in gene expression, we did LongSAGE in MPSC1 cells 9 hours after CI-1040 treatment when the cell cycle arrest began to become evident and the active MAPK was undetectable. A total of 55,546 and 50,716 tags (transcripts) were obtained from CI-1040 and DMSO LongSAGE libraries, respectively. Tags ( $n = 168$ ) were selected based on the CHIDIST value of  $\geq 0.8896$  and among them, 93 tags were further chosen based on the ratio of tag number of each library (i.e., DMSO/ CI-1040 or CI-1040/DMSO)  $> 3$ . Thus, almost all the genes showed a similar tag count (<3-fold) in both libraries except those 93 genes that represented 0.46% of ~20,250 unique tags. We selected the top 20 genes for validation based on the largest fold difference in tag number between CI-1040 and DMSO

LongSAGE libraries. The validation was done in two steps using an independent assay, the quantitative real-time PCR. First, 14 candidate genes were selected as the pattern of gene expression was identical between LongSAGE and real-time PCR in MPSC1 cells. The gene names, unigene number, possible functions, and expression levels of LongSAGE and real-time PCR are listed in Table 1. In the second step, the genes identified in step 1 were further tested in CI-1040- and DMSO-treated OVPC-1, OVPC-3, and OVPC-5 primary cultures in which sufficient RNA was available for multiple real-time PCR analyses. Of the 14 genes, we found that 10 genes showed consistent alteration in gene expression in at least



**Figure 2.** Cell cycle analysis of MPSC1 cells (A) and OVCAR3 cells (B). CI-1040-treated MPSC1 cells harboring a BRAF mutation show a gradual loss of S and  $G_2$ -M peaks and an accumulation of cells in the  $G_1$  phase. In contrast, OVCAR3 cells with wild-type KRAS and BRAF show only a mild  $G_1$  arrest.



**Figure 3.** Detection of apoptotic cells. *A*, apoptotic cells are quantified by the sub-G<sub>1</sub> fraction in four cell cultures with mutations in KRAS or BRAF (MPSC1, OVPC-2, OVPC-3, and OVPC-5) and in three cell lines with wild-type sequence (SKOV3, OVCAR3, and CAOVS3). The experiment was done 72 hours after CI-1040 or DMSO treatment. *B*, many of the CI-1040-treated cells but not OVCAR3 cells show morphologic features typical of apoptosis.

three of four low-grade tumors including MPSC1, OVPC-1, OVPC-3, and OVPC-5 (Table 2). The up-regulated genes included tumor necrosis factor-related apoptosis-induced ligand (*TRAIL*), *thrombospondin-1*, *optineurin*, *palladin*, and *pannexin* and the down-regulated genes contained *cyclin D1*, *COBRA1* (cofactor of BRCA1), *transglutaminase-2*, *HSPC152* (not yet annotated) and protein phosphatase (*PP1R14B*). In fact, down-regulation of cyclin D1 and COBRA1 and up-regulation of pannexin were observed in all four cultures.

**Constitutive Expression of Cyclin D1.** Among the differentially expressed genes, *cyclin D1* showed the most dramatic changes in the expression level (23-fold less by LongSAGE and 11-fold less by real-time PCR) in MPSC1 cells and in all other three tumors by real-time PCR after CI-1040 treatment. It was therefore selected for further study to assess its biological role in MAPK-mediated effects. Western blot analysis showed that cyclin D1 protein was undetectable in MPSC1, OVCAR3, SKOV3, and OVPC-2 cells 24 hours after treating the cells with CI-1040 (Fig. 4). Because it has been reported that cyclin D1 promoter is related to the activation of MAPK (24, 25), we established an expression vector (pCMV/cyclin D1) that constitutively expressed cyclin D1. MPSC1 cells were transfected with pCMV/cyclin D1 or the empty vector alone (as the control) and stable clones were selected for cell growth assay. Expression of engineered cyclin D1 was detected by Western blot analysis using an anti-V5 antibody which recognized the V5-cyclin D1 fusion protein (data not shown). As shown in Fig. 5, Western blot analysis showed that the endogenous cyclin D1 was reduced to an

undetectable level after CI-1040 treatment in both pCMV/cyclin D1-transfected and control MPSC1 cells. In contrast, the engineered cyclin D1 expression was not affected by CI-1040. Cell proliferation was determined by counting the cell number at various time points. Constitutive expression of cyclin D1 did not show significant changes in cell number as compared with the controls (Fig. 5). Interestingly, expression of cyclin D1 driven by a cytomegalovirus promoter partially reversed the growth inhibitory effect of CI-1040 as the cell counts significantly increased as compared with the CI-1040-treated cells transfected with the control vector although it did not achieve to a similar level of cells treated with DMSO.

## Discussion

Although the biological roles of the RAS/RAF/MEK/MAPK (ERK) pathway in human cancer have been studied for several years, it is not known whether its role in tumor progression differs in tumors with and without activating KRAS or BRAF mutations. In this study, we did a genotype-phenotype correlation of ovarian tumor cells using a MEK inhibitor, CI-1040, and provided evidence that the biological effects of the MAPK signaling pathway depend on the mutation status of its upstream regulator (i.e., *KRAS* and *BRAF* genes). Ovarian tumors with mutations in either KRAS or BRAF were more sensitive to growth inhibition and apoptosis induction by the MAPK inhibitor, CI-1040. This observation suggests that ovarian tumors with mutations in either KRAS or BRAF depend much more on the activation of the RAS/RAF/MEK/MAPK pathway for cell proliferation and survival than those without such mutations. Thus, inactivation of MAPK results in a marked growth inhibition in ovarian tumors with mutations in KRAS or BRAF and only a modest effect in tumors with wild-type KRAS and BRAF. Our result is consistent with a recent report showing that CI-1040 significantly inhibited proliferation in melanoma cell lines harboring mutant BRAF, but not in the cell line with wild-type *KRAS* and *BRAF* genes (19). The above observations lend strong support to the view of "kinase addiction" by which the activating mutations in the kinase pathway confer susceptibility of the tumors to the inhibitor (16, 26). As ovarian serous tumors with KRAS or BRAF mutations are almost exclusively low grade (2, 3), which are generally not sensitive to conventional chemotherapeutic agents (27, 28), our findings may have practical implication. Thus, CI-1040 can become a potential drug for the treatment of ovarian tumors with KRAS or BRAF mutations. This is further supported by the effectiveness of CI-1040 in treating experimental tumors in mice with colon carcinomas of either mouse or human origin (16). In that study, efficacy was achieved with a wide range of doses and was correlated with a reduction in the levels of activated, phosphorylated MAPK in tumors excised from treated animals without overt toxicity. In this study, we focused on CI-1040 because it inhibited the common downstream target in the RAS signaling pathway and the compound may provide a convenient therapeutic strategy for patients with either KRAS or BRAF mutations. Besides, CI-1040 has a relatively high selectivity in inhibiting MEK; therefore, it provides a reliable tool to study the biological effects of KRAS/BRAF/MEK/MAPK pathway in low-grade serous tumors. Future studies will be done to address whether the inhibitors of KRAS and BRAF have similar effects on low-grade ovarian serous tumors.

**Table 1.** Differentially expressed genes after inactivation of MAPK in MPSC1 cells

Gene name	UniGene number	Possible functions	Tag count CI-1040-treated cells	Tag count DMSO-treated cells	Fold of difference by real-time PCR
<i>Cyclin D1</i>	371468	Cell cycle progression of G <sub>1</sub> phase; proto-oncogene.	1	23	10.9-fold down
<i>COBRA1</i>	410095	Binds to chromatin-unfolding domains of BRCA1 and induces chromatin decondensation	0	6	3.7-fold down
<i>HSPC152</i>	333579	Unknown function, hypothetical protein	2	12	3.1-fold down
<i>NFκB</i>	458276	Transcription factor, involved in the inflammatory and immune response	0	6	3.6-fold down
<i>PPP1R14B</i>	120197	PPP; Ser/Thr phosphatases implicated in a multitude of cellular functions	16	37	2.9-fold down
<i>PPP2R5D</i>	118244		0	7	3.5-fold down
<i>Transglutaminase-2</i>	512708	Catalyze cross-linking of proteins. Maybe involved in apoptosis.	3	27	4.4-fold down
<i>TRAIL</i>	387871	Mediator of apoptosis, immune regulation and inflammation.	17	3	2.5-fold up
<i>Thrombospondin-1</i>	164226	Inhibits angiogenesis and tumorigenesis, extracellular matrix protein	11	0	2-fold up
<i>Calnexin</i>	155560	Type-I integral membrane protein (lectin) that serves as molecular chaperone for glycoproteins in the endoplasmic.	6	0	2.3-fold up
<i>Nucleobindin</i>	172609	Might play a role in G protein- and Ca <sup>2+</sup> -regulated signal transduction	6	0	3-fold up
<i>Optineurin</i>	390162	Cellular target for adenovirus E3. Affecting cell death through the tumor necrosis factor receptor.	8	0	2.5-fold up
<i>Palladin</i>	194431	Actin-associated protein that control cell shape, adhesion, and contraction.	23	8	3.4-fold up
<i>Pannexin</i>	32163	Induced formation of intercellular channels.	7	0	2.7-fold up

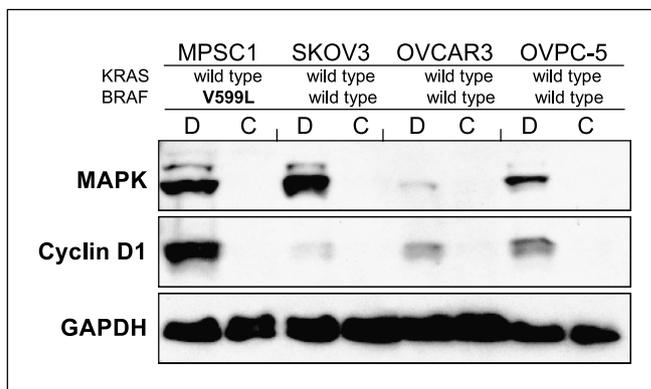
NOTE: Expression level in CI-1040- versus DMSO-treated MPSC1 cells. Abbreviation: PPP, protein phosphatase.

In this study, we applied LongSAGE as a discovery tool to identify genes in which their expression was altered shortly (9 hours) after CI-1040 treatment. Among all the differentially expressed genes, *cyclin D1* showed the greatest fold in the

alteration of gene expression. Cyclin D1 plays an important role in the cell cycle transition from G<sub>1</sub>-to-S phase by association with cyclin-dependent kinases (cdk) 4 and 6 which phosphorylate the retinoblastoma protein, blocking its growth inhibitory activity and

**Table 2.** Validation of differentially expressed genes by quantitative PCR

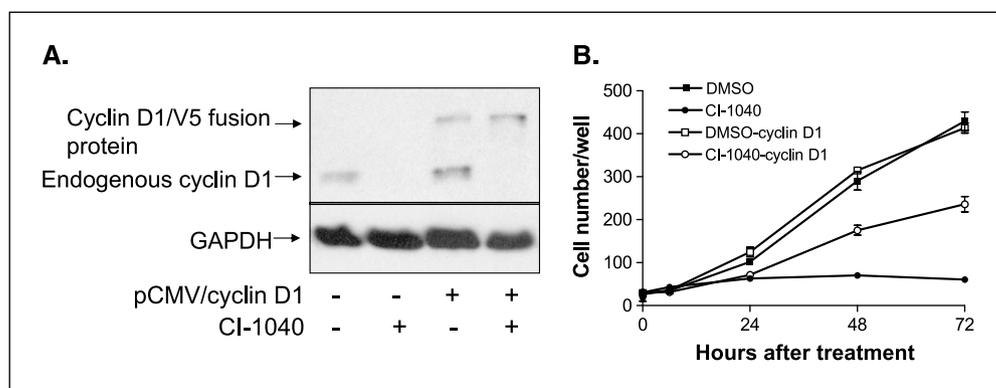
Gene name	MPSC1	OVPC-1	OVPC-3	OVPC-5
Cyclin D1	20	15	10	5
COBRA1	10	5	0	0
HSPC152	5	0	0	0
NF-KB	4	0	0	0
PPP1R14B	3	0	0	0
PPP2R5D	2	0	0	0
Transglutaminase-2	0	0	0	0
TRAIL	2	0	0	0
Thrombospondin-1	3	0	0	0
Calnexin	4	0	0	0
Nucleobindin	5	0	0	0
Optineurin	10	0	0	0
Palladin	15	0	0	0
Pannexin	20	0	0	0



**Figure 4.** Western blot analysis. Expression of active MAPK and cyclin D1 is undetectable in all CI-1040-treated samples. A similar amount of protein was loaded in CI-1040- and DMSO-treated samples as evidenced by a similar intensity of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). D, DMSO treatment; C, CI-1040 treatment.

**Figure 5.** Effect of engineered cyclin D1 expression in MPSC1 cells.

**A.** Western blot analysis shows expression of cyclin D1/V5 fusion protein that is independent of CI-1040 as the gene expression is driven by pCMV/cyclin D1. In contrast, the endogenous cyclin D1 is undetectable after CI-1040 treatment. **B.** pCMV/cyclin D1-transfected MPSC1 cells are less sensitive to CI-1040 treatment as shown by the growth curve.



promoting the release of bound E2F transcription factor. These events facilitate the activation of cyclin E-cdk 2 and cyclin A-cdk 2, molecules required for the entry into and completion of S phase (29, 30). Thus, the G<sub>1</sub> cell cycle arrest as observed in CI-1040-treated MPSC1 cells is consistent with the function of cyclin D1. Several studies have reported that overexpression of cyclin D1 occurs in several types of human cancer including ovarian serous tumors based on immunohistochemistry (31). In ovarian tumors, overexpression of cyclin D1 is associated with low-grade tumors (31), a finding consistent with our view that cyclin D1 is a downstream target of active MAPK which is constitutively expressed in most low-grade ovarian tumors because of their frequent activating mutations in KRAS and BRAF. In the present study, Western blot analysis showed a significant decrease of cyclin D1 in all the ovarian tumor cell lines and primary cultures examined independent of their mutational status in KRAS and BRAF. This observation is in accordance with a previous report showing that inhibition of the ERK1 and ERK2 MAPK signaling by expression of dominant-negative forms of MAPK or by MAP kinase phosphatase strongly inhibited cyclin D1 promoter (24). In addition, expression of cyclin D1 and overexpression of constitutively active MKK1 mutant dramatically increased cyclin D1 promoter activity and cyclin D1 protein expression (24). To address whether cyclin D1 is required for active MAPK-mediated cell proliferation, we established a stable MPSC1 line that constitutively expresses cyclin D1 and showed that the transfected cells were less sensitive to the growth inhibitory effect of CI-1040, providing cogent evidence to support the role of cyclin D1 as a downstream target in the MAPK pathway. Although cyclin D1 expression rescues growth suppression by CI-1040, it may not recapitulate all the functions in cell proliferation of active MAPK. This is because alterations in other genes may occur later and are therefore not detected by LongSAGE before it was done. It is interesting to note that the ovarian cancer cells with wild-type KRAS and BRAF were less dependent on cyclin D1 for cell proliferation because the expression level of cyclin D1 was down-regulated to an undetectable level by CI-1040, whereas the growth in those tumor cells was only mildly inhibited.

*TRAIL* represents a well-known gene that is up-regulated after CI-1040 treatment. *TRAIL* is a member of the death ligand family and consists of an extracellular *TRAIL* binding domain and a cytoplasmic "death domain." Binding of *TRAIL* to its receptor facilitates the induction of apoptosis (32–34). Our finding of up-regulation of *TRAIL* after MAPK inhibition in ovarian tumor cells with mutations in KRAS/BRAF but not in those with wild-type

sequences suggests that CI-1040 induced apoptosis is related to the expression of *TRAIL*. This finding complements a recent report showing that activation of MAPK suppressed the expression of *TRAIL* in nontransformed mammalian epithelial cells based on gene expression profiling using oligonucleotide expression arrays (14).

The functional roles of other CI-1040-responsive genes are less clear in the development of ovarian cancer. COBRA1 is a novel cofactor of the BRCA1 protein and has been cloned from a human ovarian cDNA library (35). It binds to the chromosome site by the first BRCT repeat of BRCA1 protein and is itself sufficient to induce chromatin unfolding. Thrombospondin is an extracellular matrix protein that plays a role in tumor progression by modifying tumor microenvironment. It has been shown that thrombospondin expression is repressed by RAS and MYC (36). Thrombospondin also inhibits tumorigenesis by suppressing the activity of matrix metalloproteinase-9 (37) and inhibits angiogenesis by binding to the CD36 receptor protein, which is present on endothelial cell surfaces (38). Thrombospondin is able to inhibit cancer cell growth and prevents metastasis in several tumor models, including breast, skin, and lung carcinomas and melanoma and malignant gliomas; its repression also promotes tumor growth (39–41). Transglutaminase-2 is an enzyme that catalyzes the post-translational modification of proteins by the formation of cross-links (42). Future studies will focus on demonstrating the functions of these genes in response to activation of the RAS/RAF/MEK/MAPK pathway in ovarian tumors.

In summary, we have shown a genotype (mutation status of KRAS/BRAF)-dependent phenotypic change (i.e., cell proliferation and apoptosis), in ovarian serous tumors in response to MAPK inactivation. The findings in this study provide new insight into the biological roles of the RAS/RAF/MEK/MAPK signaling pathway in ovarian serous tumors and have important therapeutic implication in ovarian cancer patients with KRAS or BRAF mutations. Ovarian tumors with KRAS or BRAF mutations are clinically low-grade and they are refractory to conventional cytotoxic chemotherapy (27, 28). Detection of KRAS and BRAF mutations in ovarian cancers may identify patients who will benefit from CI-1040 treatment.

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## Inactivation of the Mitogen-Activated Protein Kinase Pathway as a Potential Target-Based Therapy in Ovarian Serous Tumors with KRAS or BRAF Mutations

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