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c-Met Overexpression Contributes to the Acquired Apoptotic Resistance of Nonadherent Ovarian Cancer Cells through a Cross Talk Mediated by Phosphatidylinositol 3-Kinase and Extracellular Signal–Regulated Kinase 1/2[1,2]

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Abstract

Ovarian cancer is the most lethal gynecologic cancer mainly because of widespread peritoneal dissemination and malignant ascites. Key to this is the capacity of tumor cells to escape suspension-induced apoptosis (anoikis), which also underlies their resistance to chemotherapy. Here, we used a nonadherent cell culture model to investigate the molecular mechanisms of apoptotic resistance of ovarian cancer cells that may mimic the chemoresistance found in solid tumors. We found that ovarian cancer cells acquired a remarkable resistance to anoikis and apoptosis induced by exposure to clinically relevant doses of two front-line chemotherapeutic drugs cisplatin and paclitaxel when grown in three-dimensional than monolayer cultures. Inhibition of the hepatocyte growth factor (HGF) receptor c-Met, which is frequently overexpressed in ovarian cancer, by a specific inhibitor or small interfering RNA blocked the acquired anoikis resistance and restored chemosensitivity in three-dimensional not in two-dimensional cultures. These effects were found to be dependent on both phosphatidylinositol 3-kinase (PI3K)/Akt and extracellular signal–regulated kinase (ERK) 1/2 signaling pathways. Inhibitors of PI3K/Akt abrogated ERK1/2 activation and its associated anoikis resistance in response to HGF, suggesting a signaling relay between these two pathways. Furthermore, we identified a central role of Ras as a mechanism of this cross talk. Interestingly, Ras did not lie upstream of PI3K/Akt, whereas PI3K/Akt signaling to ERK1/2 involved Ras. These findings shed new light on the apoptotic resistance mechanism of nonadherent ovarian cancer ascites cells and may have important clinical implications.

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Introduction

Ovarian cancer is the second most common type of female reproductive tract malignancy, but it has the highest mortality of all the gynecologic cancers [1]. This is largely because more than 70% of patients have their conditions diagnosed at an advanced stage, with tumor spread beyond the ovary and throughout the peritoneal cavity as ascites, which contain free floating tumor cells that exist either as single cells or as multicellular aggregates. Current therapies for advanced or metastatic ovarian cancer are not effective owing to a high degree of inherent and acquired chemoresistance [2]. Thus, unraveling the molecular mechanisms of this process is crucial for effectively targeting such cells therapeutically.

Several lines of evidence suggest that the c-Met oncogene encoded tyrosine kinase receptor and its ligand hepatocyte growth factor (HGF)

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play an important role in the aggressive behavior of ovarian cancer. First, c-Met is found to be expressed in approximately 70% of human ovarian carcinomas and overexpressed in 30% of cases and cell lines [3–5]. There was a significant inverse correlation between c-Met expression and a poor prognosis [6]. Second, high levels of HGF are found in ovarian cancer ascitic fluids [7,8]. Third, we and others have shown that HGF strongly promotes the growth and invasiveness of ovarian cancer cells [7,9]. Fourth, blocking the effects of HGF by neutralizing antibodies, the HGF antagonist, or knocking out c-Met expression by small interfering RNA (siRNA) was shown to inhibit peritoneal dissemination and ascites formation of ovarian cancer cells *in vivo* [6,10,11].

The ability to resist anoikis is a prerequisite for ovarian cancer cells to survive in the ascites. However, the mechanism for activation of anoikis resistance is not clear. Furthermore, ovarian cancer cells are more resistant to drug-induced apoptosis when grown in three-dimensional cultures than in monolayers [12,13]. Although significant progress has been made in understanding the signaling mechanisms of chemotherapeutic agents–induced apoptosis and resistance, most studies are performed on monolayer cultures, which are different from the clinical setting and presumably use different molecular mechanisms. This may also help to explain why although the *in vitro* findings are encouraging, they have not easily been translated into successful therapies [14].

In the present study, using a three-dimensional suspension culture, we report for the first time that overexpression of c-Met enhanced survival of ovarian cancer cells and increased resistance to the front-line chemotherapeutic agents, cisplatin and paclitaxel, currently used in the clinic. We also explored the molecular mechanisms by which HGF inhibited apoptosis and that was dependent on phosphatidylinositol 3-kinase (PI3K)/Akt upstream of Ras to regulate extracellular signal–regulated kinase (ERK)-1/2 signaling.

Materials and Methods

Cell Lines and Reagents

The OVCAR-3, CaOV-3, and SKOV-3 human ovarian carcinoma cell lines (generously provided by Dr. Nelly Auersperg, Department of Obstetrics and Gynecology, University of British Columbia, Vancouver, British Columbia, Canada) were grown in a 1:1 mixture of medium 199 (Invitrogen, Carlsbad, CA) and MDCB 105 (Sigma, St Louis, MO) supplemented with 5% fetal bovine serum (Invitrogen). The growth medium was supplemented with 50 μg/ml penicillin-streptomycin (Invitrogen). Cell cultures were maintained at 37°C in a humidified incubator with an atmosphere of 5% CO_2.

Recombinant human HGF was purchased from R&D Systems, Inc (Minneapolis, MN). PD98059, wortmannin, LY294002, rapamycin, and K252a were from Calbiochem (San Diego, CA). Cisplatin and paclitaxel were also obtained from Calbiochem. The pRS2-containing human c-Met complementary DNA (cDNA) was kindly provided by Dr. Vande Woude (Van Andel Research Institute, Grand Rapids, MI) [15]. siRNA oligonucleotides targeting c-Met (5′-GUCAUAGGAAGAGGGCAUU-3′) [9], Akt (5′-UAAUGUGCCCGUCCUUGUC-3′), ERK1 (5′-UAAAGGUUAACAUCCGGUC-3′), ERK2 (5′-AAUAAGUCCAGAGCUUUGG-3′), and a nonspecific duplex oligo (5′-GGCTACGTCCAGAGCGCA-3′) used as a negative control were purchased from Dharmacon (Lafayette, CO). Ras-specific siRNA duplex (5′-AAGGACUCGGAUGACGUGC-3′) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). A total of 10 nM of each siRNA were transfected into CaOV-3 and SKOV-3 cells using siLentfect (Bio-Rad, Hercules, CA) according to the manufacturer's protocols. E17K Akt was generated by polymerase chain reaction (PCR)–based site-directed mutagenesis using the forward primer 5′-**GGATCC**ATGAGCGACGTGGCTATTGTGAAGG-3′, and reverse primer 5′-GGTCTTGATGTACTTCCCTCGTTTG-3′, and the mutation incorporating oligonucleotide primers 5′-CAAACGAGGGA-AGTACATCAAGACC-3′ and 5′-**GAATTC**TCAGGCCGTGCCGC-TGGCCGAG-3″ (the mutated residue is underlined and restriction sites of *Bam*H1 and *Eco*R1 are in bold) and subcloned into pcDNA3.1. The mutation was verified by sequencing. Transiently transfected cells were grown for 24 hours and replated on agar-coated six-well plates for expression and cell death analyses.

Induction of Anoikis

To prevent cell adhesion, six-well plates were coated by applying a 10-mg/ml solution of polyHEMA (polyhydroxyethylmethacrylate; Sigma) dissolved in ethanol [16]. Three milliliters of polyHEMA was added to each well and allowed to completely evaporate at 37° C for at least 3 days. To induce anoikis, 2×10^5 cells were plated on polyHEMA-coated dishes with or without HGF (10 ng/ml). Seventy-two hours later, the cells were collected, and any cell aggregate was dispersed by trypsin before apoptosis detection assays.

Apoptosis Assays

Apoptosis assays were carried out essentially as described [17]. Briefly, cells were washed once with PBS, and fixed with Carnoy's fixative (methanol/acetic acid, 3:1) for 10 minutes at room temperature. Ten microliters of this suspension was placed on a glass slide, and Hoechst 33258 (50 ng/ml; Molecular Probes, Eugene, OR) was added. The slides were subjected to fluorescence microscopic examination at an excitation wavelength of 350 nm. Apoptotic nuclei were identified by the condensed chromatin or a total fragmented nuclear bodies. At minimum, 200 cells were counted in five different fields, and the percentage of apoptotic nuclei was determined.

Further characterization of apoptosis was performed using a commercially available *In Situ* Cell Death Detection kit (Roche, Indianapolis, IN) to find DNA breaks according to the manufacturer's instructions. Briefly, fixed cells were incubated with the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) reaction mix containing 10 U of terminal deoxyribosyltransferase, 10 mM dUTP-biotin, and 2.5 mM cobalt chloride in 1× terminal transferase reaction buffer (Boehringer Mannheim, Mannheim, Germany) for 1 hour at 37°C. The reaction was stopped by rinsing slides with PBS. Omission of the terminal deoxyribosyltransferase enzyme was used as the negative control. The number of TUNEL-positive cells was counted in five different fields, and representative fields were photographed. The experiment was performed in duplicate and repeated three times.

Western Blot Analysis

Equal amounts (20 μg) of protein extracts from nonadherent cells separated by 7.5% SDS-PAGE were transblotted to nitrocellulose, blocked with 3% nonfat dry milk for 30 minutes at room temperature, and then incubated overnight with antibodies to phospho-Akt Ser^{473}, total Akt, phospho-$p70^{S6K}$ Thr^{389}, total $p70^{S6K}$, phospho-ERK1/2 Thr^{202}/Tyr^{204}, total ERK1/2, phospho-c-Met $Tyr^{1230/1234/1235}$ (Invitrogen), c-Met (Cell Signaling Technologies, Austin, TX), Ras (C-20; Santa Cruz Biotechnology), and β-actin (1:1000 dilution; Sigma) at 4°C.

After washing in phosphate-buffered saline–Tween 20, the membrane was incubated with appropriate horseradish peroxidase–conjugated secondary antibodies (1:5000 dilution; Bio-Rad) for 1 hour. Protein bands were detected by enhanced chemiluminescence (Amersham, Arlington Heights, IL). The density of the bands was quantified by densitometric analysis using an Image Tool System (Version 3.0; San Antonio, TX).

Reverse Transcription–PCR

Total RNA was extracted by the TRIzol reagent, and reverse transcription was performed using the SuperScript II kit (Invitrogen) following the manufacturer's instruction. Quantitative analysis of cDNA amplification was assessed by incorporation of SYBR Green (Bio-Rad) into double-strand DNA. PCR primers for c-Met are as follows: forward primer 5′-GGTGAAGTGTTAAAAGTTGGA-3′ and reverse primer 5′-ATGAGGAGTGTGTACTCTTG-3′. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression (forward 5′-ATGTTCGTCATGGGTGTGAACCA-3′ and reverse 5′-TGGCAGGTTTTTCTAGACGGCAG-3′) was analyzed in the same run as internal controls. The authenticity of the PCR products was verified by melting curve analysis and gel electrophoresis. Relative messenger RNA expression was determined by dividing the threshold of each sample by the threshold of the internal control GAPDH. These experiments were carried out in duplicate and repeated three times.

Statistical Analysis

Data were expressed as mean ± SD of three independent experiments. Statistical analysis was done using one-way analysis of variance followed by Tukey least significant difference *t* test for *post hoc* analysis. All tests of significance were set at $P < .05$.

Results

c-Met Overexpression Promotes Anoikis Resistance in Ovarian Cancer Cells

PolyHEMA, due to its nonionic character, prevents cell attachment to the substratum and matrix deposition, and thus keeps the cells in suspension similar to single cells or multicellular aggregates isolated from patients' ascitic fluid [16]. Considering the status of c-Met overexpression in malignant ovarian tumors and association with metastasis, we determined whether HGF/c-Met signaling might provide protection against apoptosis induced by a loss of matrix attachment. To test it, we compared the sensitivity of three well-characterized human ovarian carcinoma cell lines CaOV-3, SKOV-3, and OVCAR-3 as representative of the most common serous histologic subtypes of this tumor to suspension-induced apoptosis: CaOV-3 and SKOV-3, which express high levels of c-Met, and OVCAR-3, in which c-Met is almost undetectable [9]. As shown in Figure 1, a loss of cell adhesion strongly induced apoptosis in ovarian cancer cells as determined by Hoechst and TUNEL staining. In contrast, significantly less apoptotic cells were detected when CaOV-3 and SKOV-3 were treated with HGF, whereas OVCAR-3 had virtually no effect (Figure 1). More than 21% of CaOV-3 and 11.5% SKOV-3 cells were dead 72 hours after a loss of matrix contact, but only 10.5% of CaOV-3 and 4.5% SKOV-3 cells treated with HGF were dead (Figure 1; $P < .05$). Consistent with previous observations [18], HGF induced either little or no apoptotic changes on monolayers (Figure 1).

Next, we sought to determine whether suppression of c-Met expression would abrogate the induction of anoikis. CaOV-3 and SKOV-3 were transfected with the c-Met-specific siRNA. Suppression of c-Met expression (up to 90%) was confirmed by Western blot analysis, which detected active c-Met at 140 kDa, and real-time PCR (Figure 2*A*). c-Met siRNA, but not nonspecific siRNA, significantly inhibited HGF-mediated resistance to apoptosis of both cell lines in nonadherent but not adherent cultures (Figure 2*B*). Similar results were obtained with the specific c-Met inhibitor K252a (Figure 2*B*), which inhibited tyrosine phosphorylation and thus the kinase activity of c-Met (Figure 2*C*) [19]. No inhibition was observed for siRNA or K252a alone (Figure 2*B*). To investigate a possible direct cause effect of increasing c-Met expression on cellular apoptotic response, we overexpressed c-Met in OVCAR-3 and tested whether this was sufficient to induce resistance to anoikis. Overexpression of c-Met was confirmed by Western blot analysis (Figure 2*D*, *inset*). Consistent with our biochemical data, c-Met–transfected OVCAR-3 cells showed an increased resistance to apoptosis in response to HGF stimulation. There was no effect in empty vector–transfected control cells (Figure 2*D*). These results suggest a key role for c-Met to this resistance.

c-Met–Overexpressing Cells Show Increased Resistance to Drug Treatment

One of the major modes of action of chemotherapeutic drugs is through the induction of apoptosis. We therefore tested whether resistance to anoikis may confer chemoresistance in ovarian cancer cells. The role of c-Met expression in mediating chemoresistance in ovarian cancer cells is of particular interest because current chemotherapeutic agents have limited efficacy in patients with metastatic ovarian cancer [2]. For these analyses, we focused on the effects of cisplatin and paclitaxel because these two agents are the first-line chemotherapeutic drugs used in the treatment of ovarian cancer. Compared with monolayers, nonadherent cultures of both CaOV-3 and SKOV-3 cells were consistently more resistant to cisplatin and paclitaxel at the clinically relevant dose of 50 μM and 100 nM, respectively (Figure 3*A*) [20]. Moreover, nonadherent CaOV-3 and SKOV-3 showed increased resistance to these drugs when cotreated in the presence of HGF (Figure 3*A*). Decreasing c-Met expression using c-Met siRNA in CaOV-3 and SKOV-3 cells enhanced their susceptibility to chemotherapy (Figure 3*B*). No inhibition was observed for siRNA alone (data not shown). These results indicate that c-Met overexpression promotes ovarian cancer cell chemoresistance.

HGF Suppresses Anoikis through the PI3K/Akt and ERK1/2 Pathways

We next wished to determine the molecular mechanisms by which HGF/c-Met modulates the acquired survival. On binding of c-Met, HGF activates several key signaling pathways including the PI3K/Akt pathway. PI3K/Akt has been described as a central mediator of antiapoptotic signals [21]. For these studies, we used the specific PI3K inhibitors. Treatment with 200 nM wortmannin (or another PI3K inhibitor, LY294002; data not shown) caused a significant increase in apoptosis as measured by Hoechst or TUNEL staining (Figure 4*A*). These data suggest a role of the PI3K signaling pathway in this process.

The ERK1/2 mitogen-activated protein kinase (MAPK) has also been associated with increased survival of cells and resistance to anoikis in some systems [22,23]. To determine whether ERK1/2 activation played a role in HGF-mediated inhibition of anoikis, we used a specific MEK1/2 inhibitor, PD98059, to block the activation of ERK1/2. As shown in Figure 4*A*, pretreatment with 50 μM PD98059 also significantly rendered CaOV-3 and SKOV-3 cells sensitive to anoikis despite HGF stimulation ($P < .05$). Moreover, treatment with either inhibitor

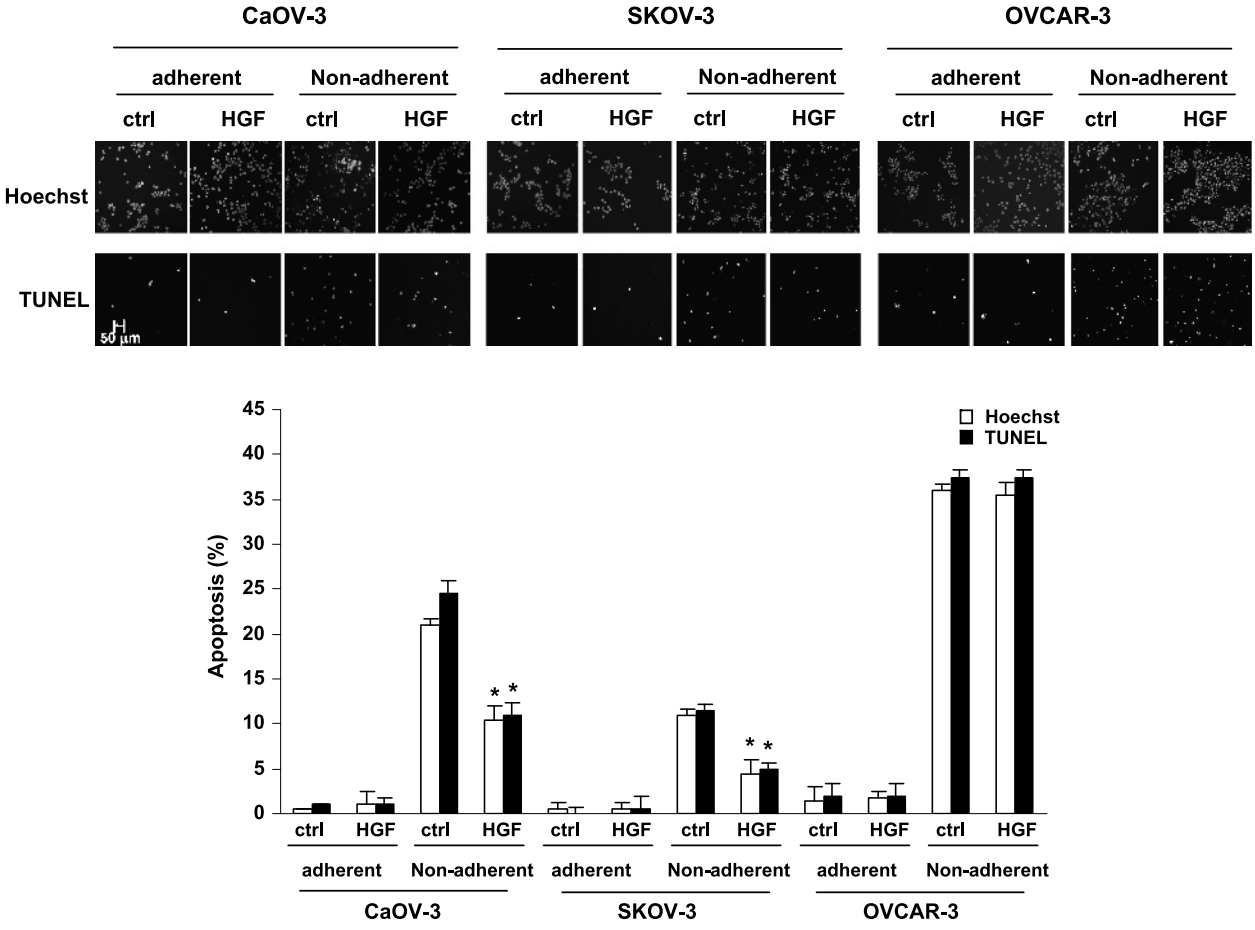

Figure 1. HGF protected ovarian cancer cell from apoptosis induced by loss of cell adhesion. CaOV-3, SKOV-3, and OVCAR-3 cells were plated on plastic Petri dishes (adherent) or polyHEMA-coated dishes (nonadherent) in the presence or absence of 10 ng/ml HGF for 72 hours. Representative results from Hoechst staining (nuclear morphology) and TUNEL labeling were shown. Bar, 50 μm. The number of apoptotic cells were counted, and the bar diagram summarized the percentage of apoptotic cells from triplicate determination. Error bars indicate the SD of the mean. *$P < .05$ *versus* untreated controls.

alone did not affect cellular apoptosis (data not shown). These results suggest that HGF activates both PI3K and MAPK signaling pathways to provide protection against anoikis. Similarly, CaOV-3 and SKOV-3 cells preincubated with wortmannin and PD98059 had little effect on the apoptotic response of monolayers but consistently reduced the resistance of nonadherent cells to cisplatin and paclitaxel than that of the control cells (Figure 4*B*), demonstrating the essential role of these signaling pathways also in chemoresistance.

HGF Stimulates Cross Talk Between the PI3K and MAPK Signaling Pathways

To investigate any role of PI3K in regulating ERK1/2 activity, we examined the effect of PI3K inhibitor on ERK1/2 phosphorylation using phosphospecific antibodies in nonadherent CaOV-3 cells. As shown in Figure 5*A*, strong phosphorylation of ERK1/2 was observed after HGF stimulation. Preincubation of the cells with PD98059 suppressed HGF-induced ERK1/2 phosphorylation (Figure 5*A*). Interestingly, pretreatment with wortmannin or LY294002 (data not shown) partially but significantly inhibited the HGF-stimulated activating phosphorylation of ERK1/2 (Figure 5*A*). These data suggest that PI3K, in addition to other factor(s), contributes to MAPK activity in response to HGF.

To elucidate the signaling pathway downstream of PI3K, we tested the involvement of Akt using siRNA under nonadherent conditions. The efficacy of silencing Akt was determined by immunoblot analysis (Figure 5*B*). More importantly, reduced Akt expression with siRNA significantly inhibited HGF-induced ERK1/2 activity compared with cells transfected with nonspecific siRNA (Figure 5*B*), suggesting that ERK1/2 activation is dependent on Akt. In addition, the simultaneous expression of Akt siRNA, but not nonspecific siRNA, suppressed HGF-mediated resistance to apoptosis in nonadherent but not adherent cells, confirming that the effect was Akt-specific (Figure 5*C*). The mTOR/$p70^{S6K}$ signaling pathway is another PI3K effector, which is also involved in cell proliferation and growth of several cell types. Although HGF stimulation induced $p70^{S6K}$ phosphorylation in a PI3K-dependent manner, pretreatment of the cells with rapamycin did not inhibit ERK1/2 activity (Figure 5*A*). Treatment of cells with PD98059 or ERK1/2 siRNA showed no effect on Akt kinase activity (Figure 5, *A* and *B*). Similar results were also obtained with a different MEK1 inhibitor U0126 and with a dominant-negative MEK1 (data not shown). Similar experiments with SKOV-3 revealed changes in kinase activities indistinguishable from those in CaOV-3 (Figure W1). Together, these results suggest that PI3K/Akt influences the MAPK pathway but not *vice versa*.

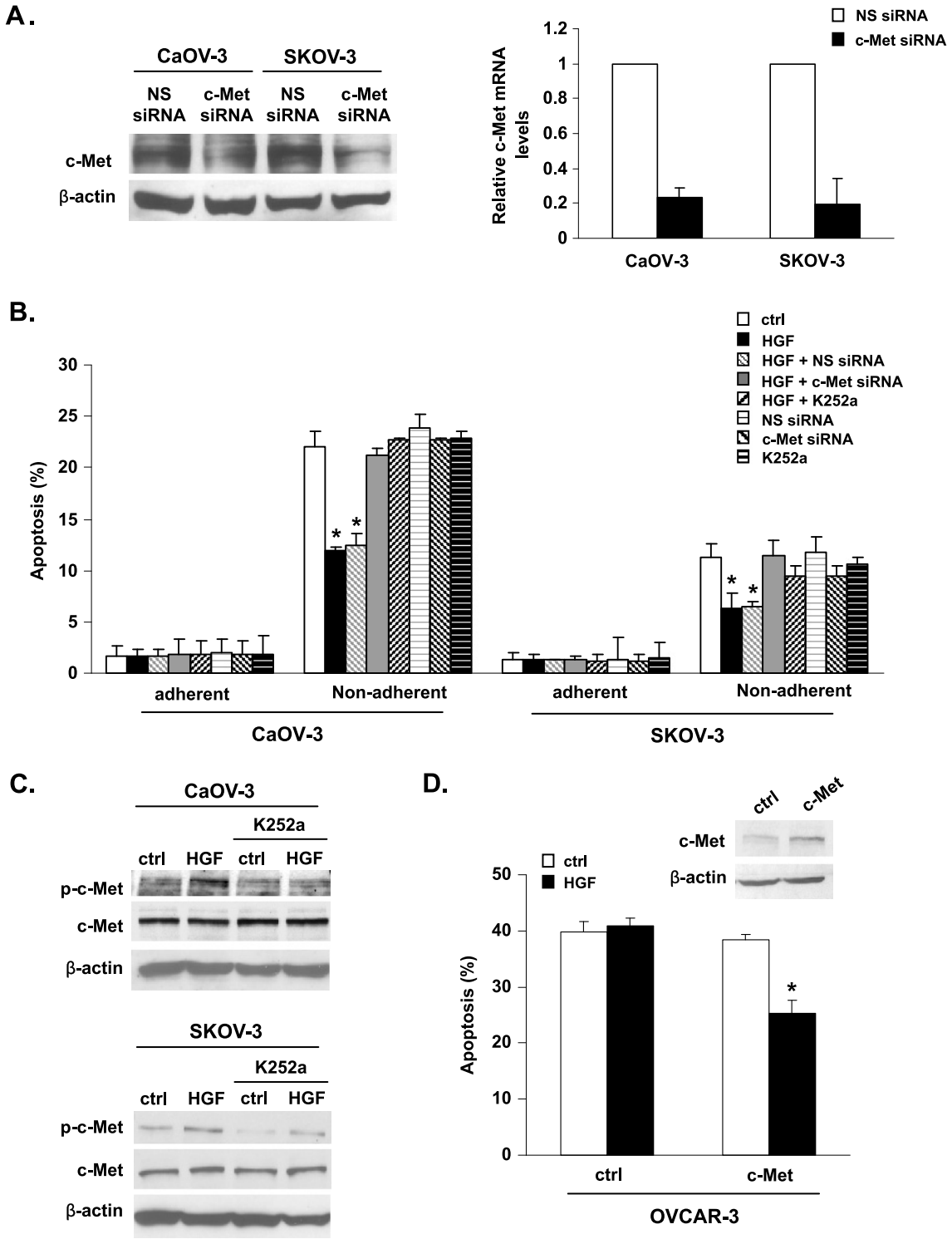

Figure 2. Effects of c-Met silencing on anchorage-independent growth and anoikis resistance. CaOV-3 and SKOV-3 cells plated on culture dishes (adherent) or polyHEMA-coated dishes (nonadherent) were transfected with 10 nM nonspecific (NS) siRNA or c-Met siRNA for 24 hours. (A) Equal amounts of protein (20 μg) were analyzed by Western blot using c-Met–specific antibodies. β-Actin was included as a loading control. Right panel: Total RNA was isolated, and real-time PCR was performed with c-Met and GAPDH sequence-specific primers. The amount of c-Met messenger RNA was normalized for the GAPDH present. (B) Cells were transfected with NS siRNA or c-Met siRNA for 24 hours or pretreated with K252a inhibitor for 30 minutes and then treated with HGF (10 ng/ml) for 72 hours. Cell apoptosis was determined with the TUNEL assay. (C) Twenty micrograms of protein was analyzed by Western blot using phospho (p)-c-Met–specific antibodies. The same membranes were stripped and reprobed with antibodies to c-Met. β-Actin was included as a loading control. (D) OVCAR-3 cells were transfected with empty vector (control) or human c-Met cDNA for 72 hours. Cells were analyzed for c-Met expression by Western blot analysis (inset) or were subjected to TUNEL assay. The number of apoptotic cells was counted, and the bar diagram summarized the percentage of apoptotic cells from triplicate determination. Error bars indicate the SD of the mean. *$P < .05$ *versus* untreated controls.

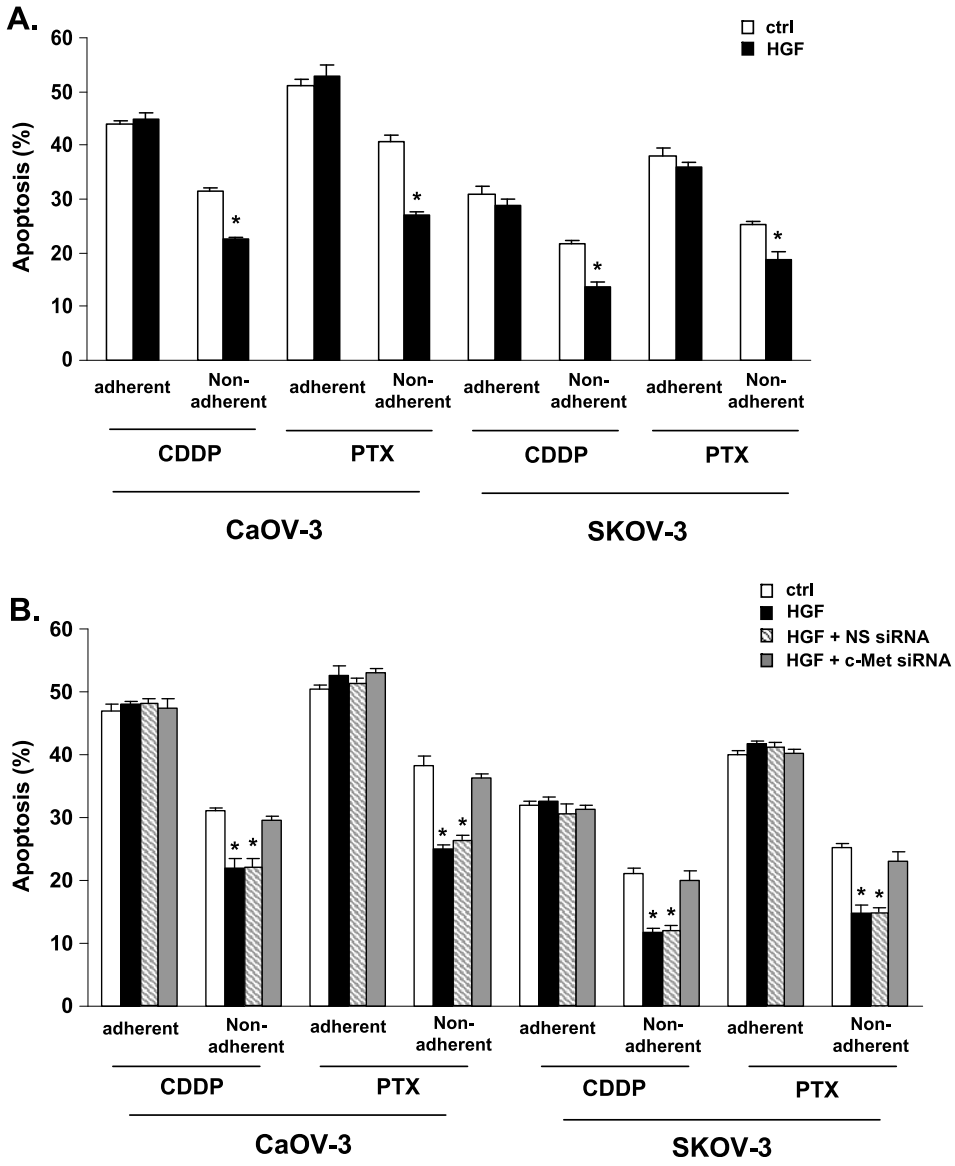

Figure 3. HGF increased resistance of ovarian cancer cells to chemotherapeutic agents in nonadherent cultures. (A) CaOV-3 and SKOV-3 were plated on culture dishes (adherent) or polyHEMA-coated dishes (nonadherent) and subjected to 50 μM cisplatin (CDDP) or 100 nM paclitaxel (PTX) treatment in the presence or absence of 10 ng/ml HGF for 72 hours. (B) To assess the specificity of c-Met, cells were transfected with NS or c-Met siRNA before cisplatin, paclitaxel, or HGF treatment. Cell apoptosis was determined with the TUNEL assay. The number of apoptotic cells was counted, and the bar diagram summarized the percentage of apoptotic cells from triplicate determination. Error bars indicate the SD of the mean. *$P < .05$ *versus* untreated controls.

PI3K/Akt–Dependent ERK1/2 Activation Is Mediated by Ras

We next investigated the possible cross talk mechanism between the PI3K/Akt and MAPK pathways. Although it is well known that Ras plays a key role in growth factor–induced activation of MAPK, it is also possible that ERK1/2 could be activated through Ras-independent pathways [24]. To explore the role of Ras in the HGF-induced activation of ERK1/2, we used Ras siRNA. Knocking down Ras by siRNA in these nonadherent cells significantly inhibited HGF-induced activating phosphorylation of ERK1/2, whereas nonspecific siRNA had no effect (Figure 6*A*). Consistently, transfection with Ras siRNA abolished HGF-mediated anoikis resistance and chemoresistance in both cell lines (Figure 6*B*). The siRNA alone had no effect (data not shown). In contrast, cells in monolayer cultures were sensitive to cisplatin- and paclitaxel-induced apoptosis after transfection with Ras siRNA (Figure 6*B*). Similar results were also obtained with a farnesyltransferase inhibitor FTI-277 used to inhibit Ras activity, confirming that the effect was Ras-specific (data not shown).

Because MAPK activation requires PI3K as demonstrated in our studies (Figure 5), we determined whether PI3K was involved in the activation of MAPK through Ras. Because multiple studies implicate a role for Ras in PI3K/Akt activation [25–27], we therefore examined whether Ras was involved in Akt activation in response to HGF. As shown in Figure 6*A*, treatment of CaOV-3 and SKOV-3 cells with Ras siRNA was unable to block HGF-stimulated Akt activity, indicating that Ras does not regulate PI3K/Akt activation by HGF. Interestingly, in contrast, the expression of a catalytically active mutant of Akt (E17K) increased the activation of ERK1/2, which could be abolished in the presence of the Ras siRNA (Figure 6*C*). The nonspecific

siRNA had no effect. Together, these results suggest that PI3K/Akt may function upstream of Ras in HGF signaling leading to MAPK activation.

Discussion

A promising strategy to kill tumor cells is the induction of apoptosis either alone or in synergy with chemotherapeutic drugs. Thus, understanding the molecular mechanisms of apoptotic resistance is of paramount importance. In recent years, it is becoming clear that the standard monolayer cultures do not fully reflect the pathological properties seen in human tumors. This may be particularly relevant to ovarian carcinomas, which exist as anchorage-independent single cells or multicellular aggregates in the ascitic fluid of the peritoneal cavity. In this study, by using three-dimensional suspension cultures, we showed for the first time that c-Met overexpression endowed ovarian carcinoma cells with an increased survival capacity that was not apparent in the same cells grown as monolayers. Also, these cells were more resistant to chemotherapy-induced apoptosis. Correspondingly, down-regulating c-Met levels by siRNA led to increased susceptibility to the chemotherapeutic agents. These examinations would more closely approximate *in vivo* conditions. Thus, these findings have important implications in killing ascitic ovarian carcinoma cells during chemotherapy.

Although it is generally thought that tumor or oncogene-transformed cells are anchorage-independent and therefore can proliferate even under loss of matrix adhesion [28], many human ovarian cancer cell lines underwent apoptosis when deprived of matrix attachment in cell culture. These results suggest that paracrine/autocrine growth factors produced by stromal and/or tumor cells in the tumor microenvironment may be important for cancer cell survival and progression. Our results suggest that HGF may be such a factor for ovarian cancer cells. *In vivo*, HGF is found at high levels in malignant ovarian cystic (4.1-16.0 ng/ml) and ascitic fluid (2.3-11.2 ng/ml) from women with ovarian carcinomas [10]. This is consistent with the concentration of HGF used in our experiments (10 ng/ml). The expression of HGF is primarily produced by immune cells and stromal fibroblasts. We have shown that ovarian cancer cells may also synthesize small amounts of HGF [29]. The fact that most (70%) ovarian

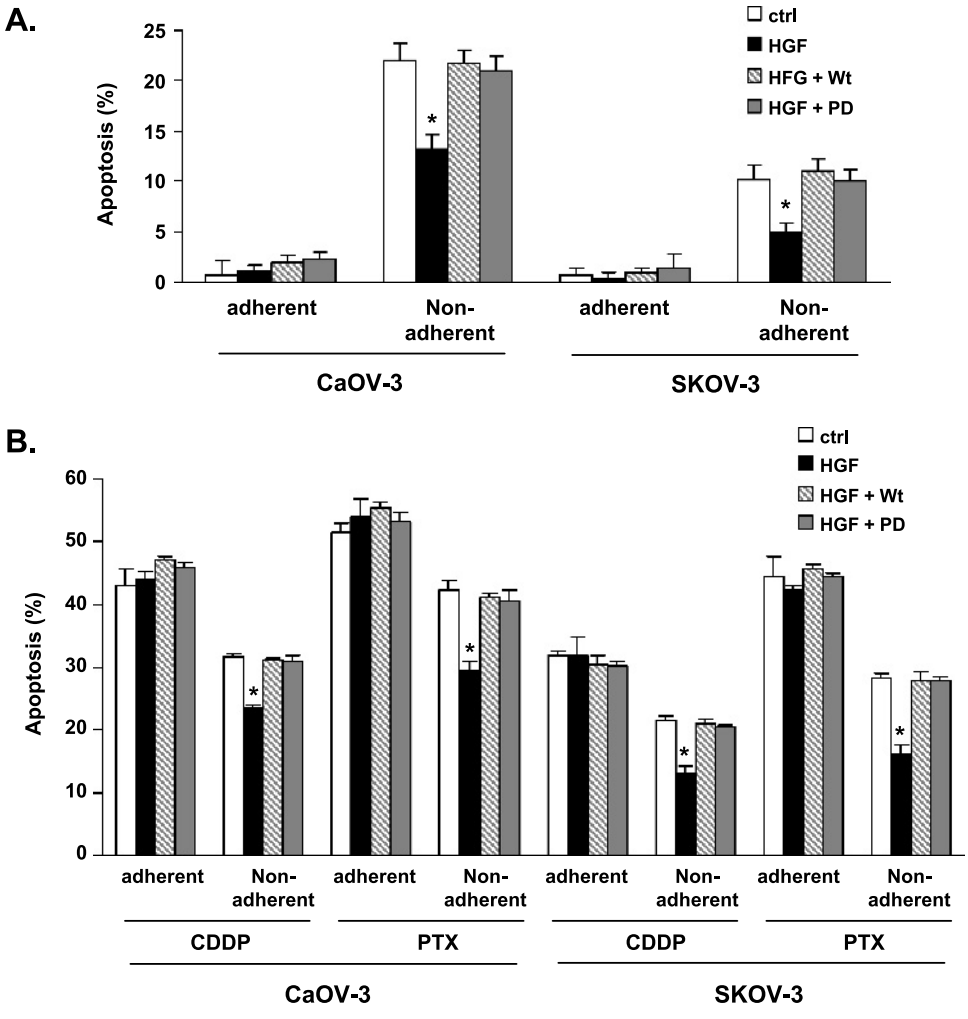

Figure 4. Signaling pathways for HGF-induced anoikis resistance and chemoresistance. (A) CaOV-3 and SKOV-3 cells plated on culture dishes (adherent) or polyHEMA-coated dishes (nonadherent) were pretreated with 200 nM wortmannin (Wt) or 50 μM PD98059 (PD) for 30 minutes and (B) subjected to 50 μM cisplatin (CDDP) or 100 nM paclitaxel (PTX) treatment in the presence or absence of 10 ng/ml HGF for 72 hours. Cell apoptosis was determined with the TUNEL assay. The number of apoptotic cells was counted, and the bar diagram summarized the percentage of apoptotic cells from triplicate determination. Error bars indicate the SD of the mean. *$P < .05$ *versus* untreated controls.

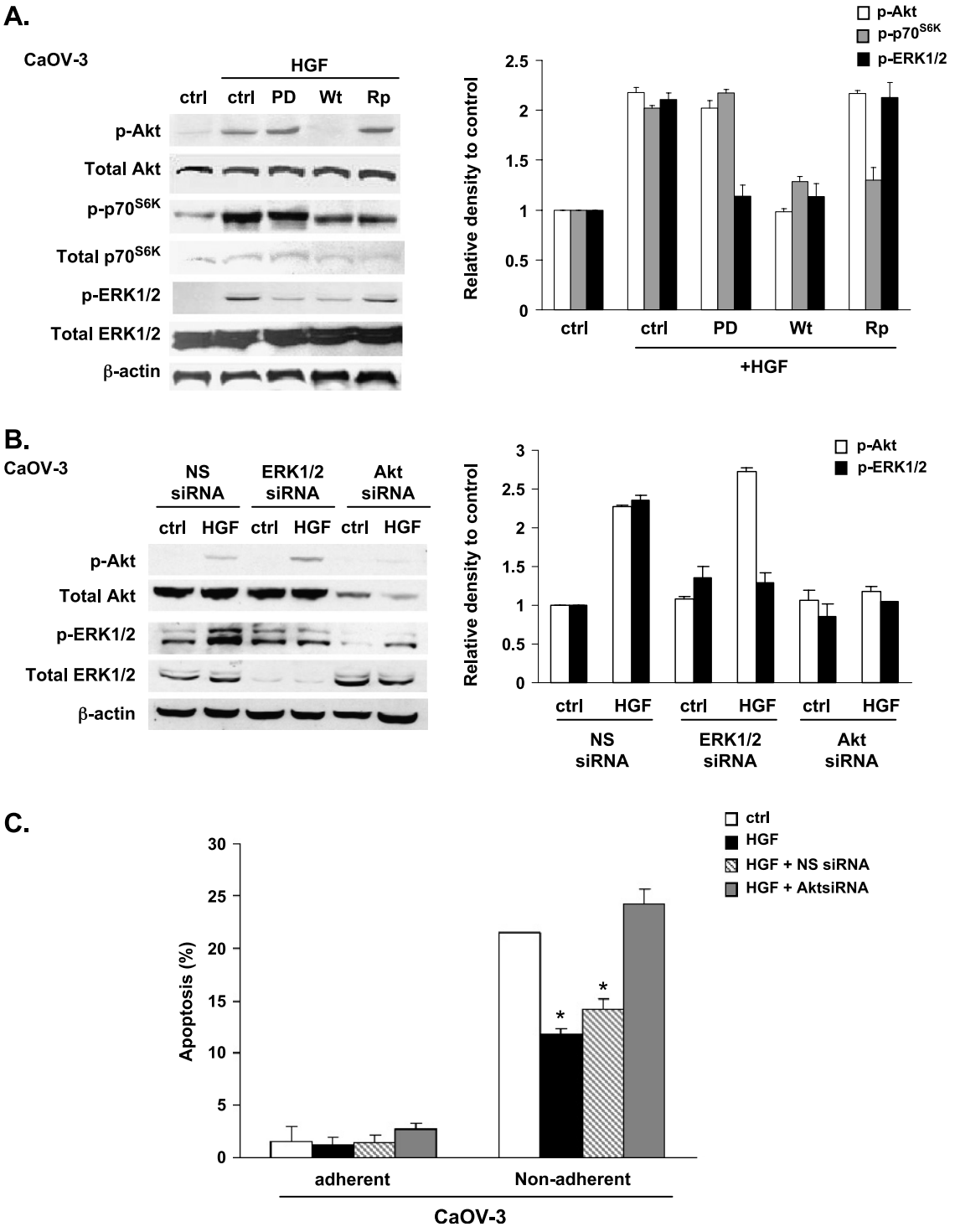

Figure 5. Cross talk between PI3K and ERK1/2 in response to HGF. (A) CaOV-3 cells plated on polyHEMA-coated dishes were pretreated with 50 μM PD98059 (PD), 200 nM wortmannin (Wt), or 20 nM rapamycin (Rp) for 30 minutes or (B) transfected with nonspecific (NS), ERK1/2 or Akt siRNA for 24 hours and then treated with HGF (10 ng/ml) for 15 minutes. Equal amounts of protein (20 μg) were analyzed by Western blot using specific antibodies recognizing phospho (p)-Akt, p-p70^{S6K}, or p-ERK1/2. The same membranes were stripped and reprobed with antibodies to total Akt, p70^{S6K}, and ERK1/2. β-Actin was also included as a loading control. The signal intensity was determined by densitometry and expressed as p-Akt, p-p70^{S6K}, and p-ERK1/2 relative to total Akt, p70^{S6K}, ERK1/2, and β-actin for each sample. (C) Cell apoptosis was determined with the TUNEL assay. The number of apoptotic cells was counted, and the bar diagram summarized the percentage of apoptotic cells from triplicate determination. Error bars indicate the SD of the mean. *$P < .05$ *versus* untreated controls.

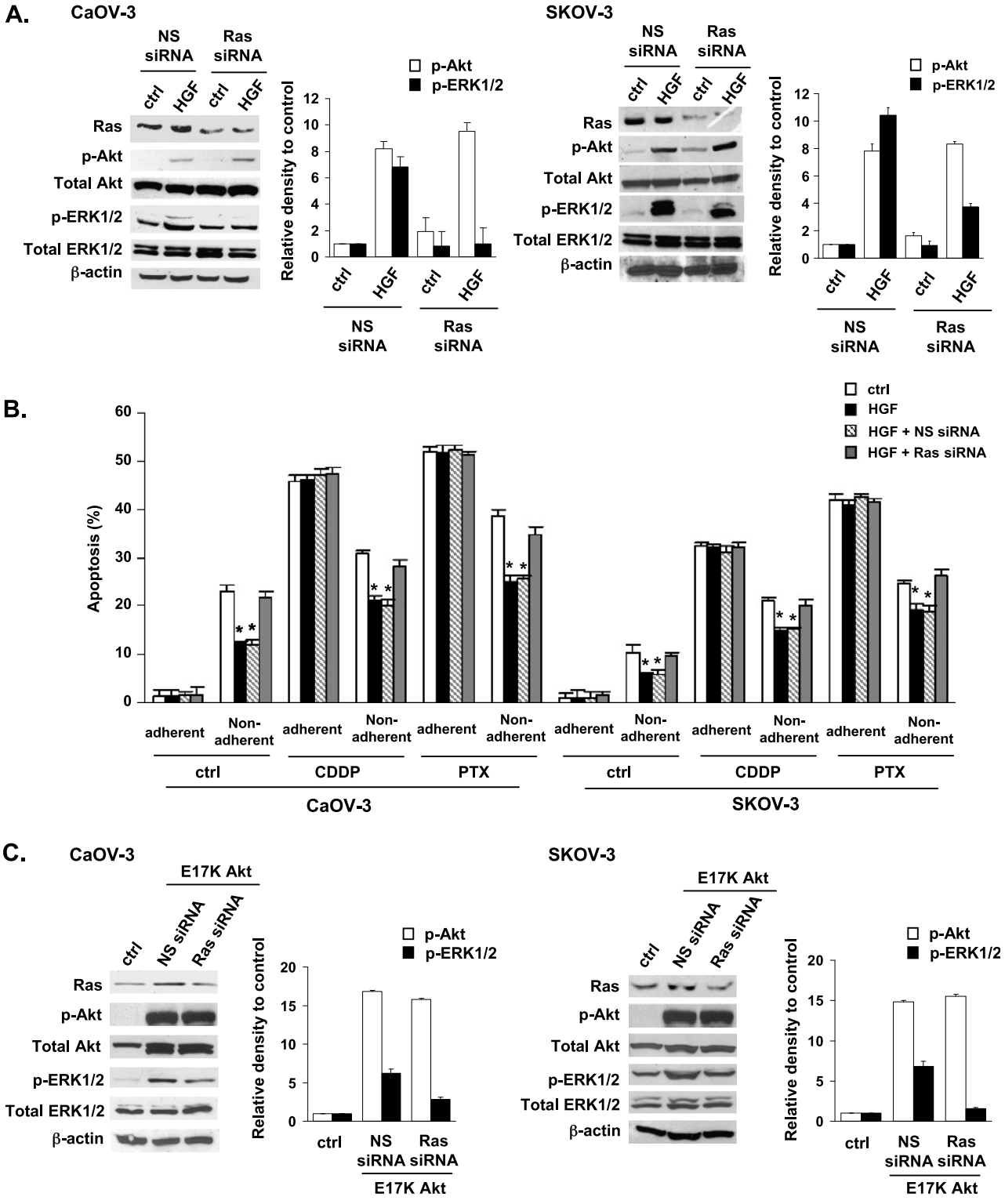

Figure 6. PI3K functions upstream of Ras in response to HGF. CaOV-3 and SKOV-3 cells were transfected with non-specific (NS) siRNA or Ras siRNA for 24 hours and then treated with HGF (10 ng/ml) for 15 minutes. (A) Ras expression was determined by Western blot. Akt and ERK1/2 activities were analyzed using anti–phospho-specific (p-) Akt and p-ERK1/2 antibodies. (B) CaOV-3 and SKOV-3 cells plated on culture dishes (adherent) or polyHEMA-coated dishes (nonadherent) were transfected with NS siRNA or Ras siRNA for 24 hours and then subjected to 50 μM cisplatin (CDDP) or 100 nM paclitaxel (PTX) for 72 hours in the presence or absence of 10 ng/ml HGF. Cell apoptosis was determined with the TUNEL assay. The number of apoptotic cells was counted, and the bar diagram summarized the percentage of apoptotic cells from triplicate determination. (C) CaOV-3 and SKOV-3 cells were cotransfected with a constitutively active Akt (E17K) and NS siRNA or Ras siRNA for 24 hours. Ras expression was determined by Western blot. Akt and ERK1/2 activities were analyzed using anti–p-Akt and p-ERK1/2 antibodies. β-Actin was also included as a loading control. The signal intensity was determined by densitometry and expressed as p-Akt and p-ERK1/2 relative to total Akt and ERK1/2 for each sample. Error bar indicates the SD of the mean. *$P < .05$ *versus* untreated controls.

carcinomas express c-Met and these are overexpressed in ~30% cases suggests that our findings are relevant to a large number of ovarian carcinomas [3,4,6]. Given its apparent role in oncogenic signals, targeted therapies that inhibit the function of HGF or c-Met alone or in combination with other receptor tyrosine kinases may lead to significant tumor inhibition [30]. In comparison to monolayer cultures, the effect of HGF/c-Met on survival became evident only in the three-dimensional setting. This may represent a redirection of signals by distinct signaling pathways or coupled to other pathways on a transition from two-dimensional to three-dimensional cultures [31–33].

Our results showing inhibition of MAPK phosphorylation by wortmannin and LY294002 demonstrate that HGF-stimulated MAPK is PI3K-dependent. This positive regulatory effect of PI3K on MAPK activity resulted in anoikis resistance. Conversely, inhibition of MEK1 did not affect Akt phosphorylation or activity, suggesting that PI3K influences the MAPK pathway but not *vice versa*. These data demonstrate for the first time the presence of a direct relation between PI3K and MAPK in HGF-mediated signal transduction.

One PI3K effector is Akt [21]. In contrast to the observation that growth factors, such as epidermal growth factor and insulin-like growth factor-1, do not stimulate PI3K-mediated ERK1/2 activity in a Akt-dependent manner [34,35], we found that Akt was essential for HGF-induced stimulation of ERK1/2 and this regulation is important for the suppression of anoikis in ovarian cancer cells. Unlike Akt, involvement of ERK1/2 in the HGF-mediated antiapoptotic function is variable. For example, it has been previously reported that HGF-induced ERK1/2 activation inhibited the apoptosis induced by serum starvation and UV irradiation [36]. In contrast, the presence of ERK1/2–independent antiapoptotic effects of HGF has also been demonstrated [37].

Whereas PI3K/Akt and MAPK are two parallel pathways in some cell types, we found that they were related pathways. In addressing the cross talk mechanism between these two pathways, we examined the role of Ras. Interestingly, despite striking evidence that Ras binds to and activates PI3K [25–27], we found that blocking Ras had no effect on HGF-stimulated PI3K activation. In contrast, our results suggest a role for PI3K/Akt upstream of Ras. The ectopic expression of a constitutively active form of Akt stimulated ERK1/2 activities. This activation was completely blocked by the down-regulation of Ras with siRNA. Unlike other growth factor receptors, such as epidermal growth factor receptor, there is evidence that c-Met can directly associate with and activate PI3K; perhaps it may explain why PI3K is not subjected to regulation by Ras in our studies [38]. Consistent with our results, it has been reported that PI3K is able to stimulate Ras at least in certain systems, suggesting the importance of cell context and stimuli [39–41]. Aberrant Ras activation and signaling are commonly seen in ovarian cancer. The expression of oncogenic RasV12 has been shown to abrogate anoikis *in vitro* and is tumorigenic in nude mice [42,43].

In summary, using a three-dimensional suspension culture condition, we have discovered a role for c-Met in survival that could provide a therapeutic rationale for the use of c-Met inhibitors to prevent ascitic dissemination and to resensitize ovarian tumor cells to standard chemotherapy.

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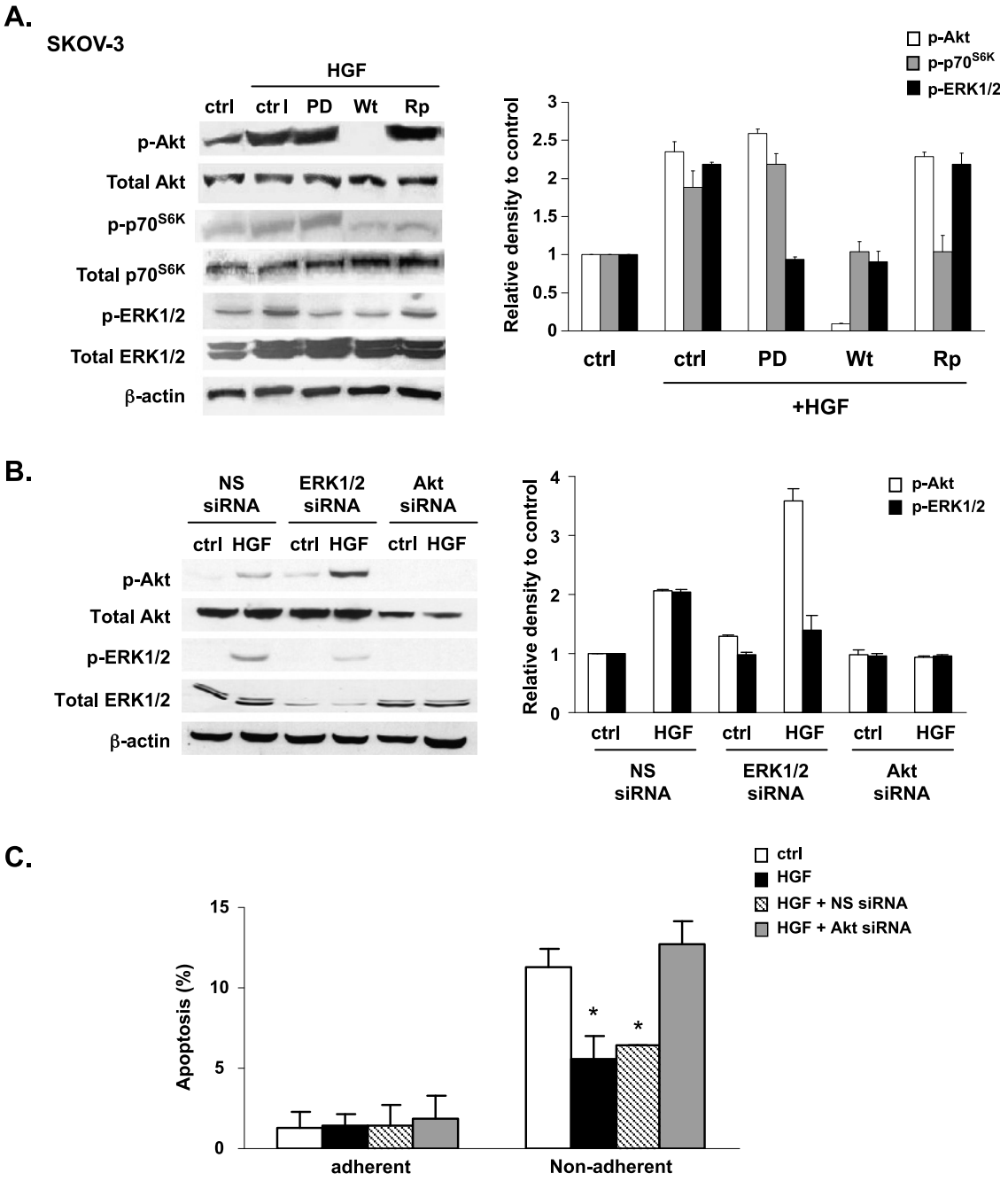

Figure W1. Cross talk between PI3K and ERK1/2 in response to HGF in SKOV-3 cells. (A) Cells plated on polyHEMA-coated dishes were pretreated with 50 μM PD98059 (PD), 200 nM wortmannin (Wt), or 20 nM rapamycin (Rp) for 30 minutes or (B) transfected with non-specific (NS), ERK1/2, or Akt siRNA for 24 hours and then treated with HGF (10 ng/ml) for 15 minutes. Equal amounts of protein (20 μg) were analyzed by Western blot using specific antibodies recognizing phospho (p)-Akt, p-p70^{S6K}, or p-ERK1/2. The same membranes were stripped and reprobed with antibodies to total Akt, p70^{S6K}, and ERK1/2. β-Actin was also included as a loading control. The signal intensity was determined by densitometry and expressed as p-Akt, p-p70^{S6K}, and p-ERK1/2 relative to total Akt, p70^{S6K}, ERK1/2, and β-actin for each sample. (C) Cell apoptosis was determined with the TUNEL assay. The number of apoptotic cells was counted, and the bar diagram summarized the percentage of apoptotic cells from triplicate determination. Error bars indicate the SD of the mean. *$P < .05$ *versus* untreated controls.