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ERK Activation and Nuclear Signaling Induced by the Loss of Cell/Matrix Adhesion Stimulates Anchorage-Independent Growth of Ovarian Cancer Cells

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ABSTRACT

Ovarian cancer metastasis involves the sloughing of epithelial cells from the ovary into the peritoneal cavity, where the cells can survive and proliferate in peritoneal ascites under anchorage-independent conditions. For normal epithelial cells and fibroblasts, cell adhesion to the extracellular matrix is required to prevent apoptosis and for proper activation and nuclear signaling of the ERK MAP kinase. The mechanisms of ERK regulation by adhesion have been determined by our lab and others. In this report, we elucidate a novel means of ERK regulation by cellular adhesion in ovarian cancer cells. We demonstrate that ERK and its activator MEK are robustly stimulated after cell detachment from a substratum in several ovarian cancer cell lines, but not a benign ovarian cell line, independent of serum and FAK or PAK activity. MEK and ERK activation was sustained for 48 h after detachment, while activation by serum or growth factors in adherent cells was transient. Re-attachment of suspended ovarian cells to fibronectin restored basal levels of MEK and ERK activity. ERK activity in suspended cells was dynamically controlled through an autocrine stimulatory pathway and prevalent phosphatase activity. Suspended cells demonstrated higher levels of ERK nuclear signaling to Elk1 compared to adherent cells. Inhibition of ERK activation with the MEK inhibitor U0126 had minor effects on adherent cell growth, but greatly decreased growth in soft agar. These data demonstrate a unique regulation of ERK by cellular adhesion and suggest a mechanism by which ERK may regulate anchorage-independent growth of metastatic ovarian cancer cells. J. Cell. Biochem. 105: 875–884, 2008.

KEY WORDS: ERK; OVARIAN; SUSPENSION; DETACHMENT; SIGNALING

Extracellular regulated kinases (ERKs) are members of the MAP Kinase family of protein kinases that regulate many aspects of cell biology including, proliferation, differentiation, migration, survival, and gene expression [Pearson et al., 2001]. Inactive ERKs reside in the cytoplasm, but following activation translocate to various cellular locations including the nucleus [Chen et al., 1993; Lenormand et al., 1993] where they phosphorylate a variety of substrates to affect gene transcription. ERK activation and nuclear translocation are required for cell proliferation [Brunet et al., 1999] and cell/matrix adhesion has been reported to be a requirement for ERK nuclear translocation and activation of the Elk1 transcription factor [Aplin et al., 2002]. Cell adhesion is also required for ERK activation by mitogens, as cells in suspension fail to activate ERK in response to growth factors [Lin et al., 1997; Renshaw et al., 1997]. Overexpression of an activated mutant of focal adhesion kinase (FAK) [Renshaw et al., 1999] or p21 activated kinase (PAK) [Howe and Juliano, 2000] can stimulate anchorage independent activation of ERK, demonstrating that these proteins act as adhesion-dependent sensors for ERK activation. FAK is activated in adherent cells through autophosphorylation of Tyr397 [Lipfert et al., 1992; Calalb et al., 1996]. Activation of Raf, MEK and ERK and functional coupling between Raf and MEK and MEK and ERK upon cell attachment to fibronectin is dependent upon activation of PAK [Eblen et al., 2002; Slack-Davis et al., 2003; Edin and Juliano, 2005] through a FAK and Src dependent pathway [Slack-Davis et al., 2003]. This mechanism occurs in part through PAK phosphorylation of MEK1 on S298, which is required for MEK1 activation and stimulates MEK1/ERK complex formation in newly adherent cells [Eblen et al., 2002; Slack-Davis et al., 2003].

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Ovarian cancer is the fifth leading cause of cancer deaths in women and the deadliest form of gynecological cancer. In humans most ovarian cancer originates from the ovarian surface epithelium (OSE), which is a poorly differentiated mesothelium with a tenuous attachment to the underlying basement membrane [Dietl and Marzusch, 1993]. Dissemination of ovarian cancer cells to the peritoneal cavity involves the loss of attachment of the OSE from the underlying basement membrane or their extravasation from inclusion cysts that form within the ovary [Auersperg et al., 1998]. Ovarian cancer patients often develop peritoneal ascites fluid containing metastatic cells growing individually or as multicellular spheroids, in addition to factors that promote tumor cell growth [Mills et al., 1990; Westermann et al., 1997, 1998]. Ascitic cells survive and proliferate under anchorage-independent conditions until they attach to the mesothelium of the peritoneal cavity via fibronectin and hyaluronan produced by peritoneal mesothelial cells [Lessan et al., 1999; Casey et al., 2001; Burleson et al., 2004].

We examined the regulation of ERK activation by loss of cellular adhesion, an early step in cancer metastasis, in several ovarian cancer cell lines. We demonstrate a strong, sustained activation of MEK and ERK in response to loss of cell/matrix adhesion. Re-attachment of cells to fibronectin-coated dishes restored basal levels of active ERK. Conditioned media from suspended cells could stimulate ERK activation in serum-starved adherent cells, suggesting an autocrine mechanism for ERK activation in suspended cells. ERK signaling to the nucleus was enhanced in suspended cells compared to adherent cells. MEK inhibition had a minor effect on adherent cell growth, but had a profound effect on growth in soft agar. These studies demonstrate a novel means of ERK regulation by cellular adhesion and suggest that upregulation of ERK activation through an autocrine mechanism promotes the ability of ovarian cancer cells to grow anchorage-independent.

MATERIALS AND METHODS

CELLS AND REAGENTS

SKOV-3 cells were purchased from American Type Culture Collection (Manassas, VA) and grown in McCoy's 5A media supplemented with 10% fetal calf serum. IOSE cells were generously provided by Dr. Nellie Auersperg. CAOV3, ES2, TOV21G, and OV2008 cells were a kind gift from Dr. Runzhao Li. OVCAR3 cells were a kind gift from Dr. Kristen Atkins. ERK2 antibody was kindly provided by Dr. Michael J. Weber. Phospho-ERK antibody was purchased from Sigma (St. Louis, MO). FAK and phospho-Y397 FAK antibodies were purchased from Cell Signaling (Danvers, MA). Phospho-MEK, MEK, phospho-AKT, AKT, tubulin and MKP-1 antibodies were purchased from Invitrogen (Carlsbad, CA).

SUSPENSION ASSAY

Six-well dishes coated with 1% agarose were prepared by diluting 4% agarose in PBS into three parts serum-free McCoy's 5A media. Adherent cells growing in 10% fetal calf serum were washed once with PBS and detached with trypsin-EDTA. The trypsin was inhibited with 1 mg/ml soybean trypsin inhibitor (Sigma) in PBS. The cells were collected, counted, pelleted by centrifugation, and resuspended in serum-free media at a concentration of 1×10^6 cells/ml for all experiments. Two milliliters of cells were added to each well. The cells were then incubated for the indicated time at 37°C and 5% CO_2 before harvest by gentle pipetting into 15 ml conical tubes containing 10 ml of cold PBS on ice. Centrifuged cells were snap frozen and stored at −70°C until lysis. For replating experiments, fibronectin was coated onto dishes overnight at 4°C at a concentration of 10 μg/ml. Replated cells were put onto these dishes in the same serum-free media that they were suspended in and incubated at 37°C and 5% CO_2 until harvest. Citric saline experiments were performed in a similar manner, except that adherent cells were washed, incubated with citric saline for 15 min and collected by gentle pipetting. The cells were centrifuged, washed in PBS, resuspended in serum free media, and incubated on agarose-covered dishes.

WESTERN BLOTTING

Cells were lysed in M2 lysis buffer [Eblen et al., 2001] and protein concentrations determined using the BCA kit (Pierce, Rockford, IL). Typically, 20 μg of protein was run on an SDS–PAGE gel and transferred to nitrocellulose. The membranes were blocked in 5% milk and incubated with antibodies from 1 h to overnight. Secondary antibodies conjugated to horseradish peroxidase were followed by enhanced chemiluminescence (Pierce).

MTT ASSAY

SKOV-3 or IOSE cells were plated in triplicate onto 12 well dishes at a concentration of 1×10^3 cells/well. The following day (day 0) the cells were treated in duplicate with either 10 μM UO126 or DMSO vehicle control. On each of the next 4 days, triplicate wells of cells were washed and incubated in MTT reagent for 2 h before harvest in DMSO. MTT activity was measured by reading the absorbance at 570 nm and reference wavelength of 630 nm. These experiments were performed three times. Statistical analysis was performed using the Student's *t*-test and significance determined as $P < 0.05$.

LUCIFERASE ASSAYS

SKOV-3 cells were transfected using LipofectAMINE (Invitrogen) with 1 μg of 5× Gal4 Luciferase, 50 ng of Gal4-Elk1 [Roberson et al., 1995], 100 ng of TK Renilla luciferase, and either 100 ng of empty vector or 100 ng of mutationally activated MEK1 (MEK1 S218/222D) per 6 cm dish. After a 5 h transfection in serum-free McCoy's 5A medium the cells were washed once with PBS and allowed to recover overnight in 10% serum containing media. The following day the cells were washed twice in PBS and either incubated in serum-free media or trypsinized and suspended in serum-free media as described above. After 24 h, both adherent and suspended cells were harvested, lysed and luciferase activity (Renilla and firefly) measured. The ratio of firefly/Renilla luciferase was determined for each sample. Activity in the adherent cells in the absence of activated MEK1 was set to 1 for each experiment. The results are a combination of five separate experiments all performed in triplicate. Statistical analysis was performed using the Student's *t*-test and significance determined as $P < 0.05$.

SOFT AGAR ASSAY

SKOV-3 cells were mixed at a density of 5×10^3 cells/ml in 0.4% bacto agar, 10% serum, and either 10 μM U0126 or DMSO control. One milliliter of this mixture was overlayed onto 35 mm dishes containing a 0.6% agar plug. Assays were performed in triplicate. The cells were incubated at 37°C and 5% CO_2. U0126 or DMSO was administered to the cells every 3 days by dissolving 1 μl DMSO or 1 μl of a 10 mM stock solution of U0126 into 100 μl of serum-free media and overlaying it onto the 1 ml of cells in agar, allowing for drug absorption at a final concentration of 10 μM. After 5 weeks, four random fields of cells from each plate were photographed under a 2.5× objective. Distance on the plate was determined using a known standard. The images were analyzed using Image J software (website: http://rsb.info.nih.gov/ij/) in order to quantitate colony number and individual colony size. Colonies above 400 μm^2 in four fields from each triplicate dish were counted. Results from each triplicate were averaged and the standard deviation determined. A Student's *t*-test was performed between DMSO control and U0126 treated dishes and significance determined as $P < 0.05$. The experiment was performed a total of three times with similar results. An identical experiment performed with IOSE cells did not yield any soft agar colony growth.

RESULTS

LOSS OF CELL ANCHORAGE ACTIVATES ERK IN OVARIAN CANCER CELLS

Cellular adhesion is required for efficient activation of ERK by mitogens in most epithelial cells and fibroblasts [Meredith et al., 1996; Lin et al., 1997; Renshaw et al., 1997]. To determine if serum could stimulate ERK activation in suspended ovarian cancer cells, adherent SKOV-3 cells were detached with trypsin followed by treatment with soybean trypsin inhibitor. The cells were then put in suspension culture for 3 h in fresh media with or without 10% serum. Cells under these suspended conditions remain rounded, do not attach to the agarose, and are easily collected by gentle pipetting (data not shown). Adherent SKOV-3 cells in serum had modest ERK activation as measured by ERK phosphorylation with a phospho-specific antibody that recognizes the two MEK phosphorylation sites on ERK, Thr183, and Tyr185. Cells suspended for 3 h had robust activation of both ERK and MEK (Fig. 1A). Surprisingly, activation in detached cells was serum independent. Detachment of cells with either typsin or citric saline resulted in ERK activation (Fig. 1B), demonstrating that ERK activation was not an artifact of trypsinization. A short time course of cell detachment revealed that ERK was not activated until 1 h after cell detachment using either trypsin (Fig. 1C) or citric saline (Fig. 1D).

To determine if activation of ERK in detached cells also occurred in benign ovarian epithelial cells, we compared the level of ERK activation in detached SKOV-3 cells to IOSE cells, a benign cell line derived by SV40 large T antigen immortalization of normal ovarian surface epithelium [Auersperg et al., 1994]. After 3 h in suspension in serum free media, the basal level of ERK activation in adherent IOSE cells was lost, while ERK activation was strongly induced in SKOV-3 cells (Fig. 1E). We also observed an induction of ERK1 and ERK2 activation at 1 and 3 h after detachment in three of five other ovarian cancer cell lines (Fig. 1F). ERK activation upon detachment was strongest in SKOV-3 cells, which have wild-type *ras* and *b-raf* genes [Yang et al., 2003], but was also observed in OVCAR3, TOV21G, and CAOV3 cells. Like IOSE cells, ERK activation in adherent OV2008 cells was lost upon cell detachment, as has been reported in many benign and cancerous cell lines [Lin et al., 1997; Renshaw et al., 1997; Howe and Juliano, 2000; Eblen et al., 2002; Fukazawa et al., 2002; Howe et al., 2002; Kraus et al., 2002; Slack-Davis et al., 2003; Honma et al., 2006; Zhang et al., 2006] while ERK activation in adherent ES2 cells remained unchanged after detachment. Activation of ERK in suspension was also observed in cells incubated for 3 h in the presence of 1% bovine serum albumin, 4 mM EGTA, or a combination of the two, suggesting that ERK stimulation was not due to cell/cell contact or changes in osmolarity (Fig. 1G).

PROLONGED ERK ACTIVATION IS FAK AND PAK INDEPENDENT

Growth factor stimulation of ERK in adherent cells is transient, often returning to basal levels within hours of stimulation. SKOV-3 cells are resistant to anoikis and can survive prolonged times in suspension culture [Frankel et al., 2001]. To determine the duration of MEK and ERK activation in suspended SKOV-3 cells, we performed a 48 h time course and observed activation of both proteins for the entire time course (Fig. 2A). Microscopic observation (data not shown) as well as immunoblotting for the phosphorylated, activated FAK demonstrated that the cells were not attached to the agar (Fig. 2A), as Y397 phosphorylation of FAK does not occur in detached cells [Calalb et al., 1996]. We have previously reported that MEK1 and ERK activation by cellular adhesion to the extracellular matrix requires phosphorylation of MEK1 on Ser 298 by PAK [Eblen et al., 2002; Slack-Davis et al., 2003], is negatively regulated by ERK feedback phosphorylation of MEK1 on T292 [Eblen et al., 2004] and that both are cell adhesion-dependent [Slack-Davis et al., 2003; Eblen et al., 2004]. The prolonged activation of MEK and ERK in suspended cells was independent of PAK signaling due to the absence of Ser 298 phosphorylation (Fig. 2A). Interestingly, Thr 292 phosphorylation of MEK1 had become anchorage independent due to the enhanced activation of ERK. These data demonstrate that MEK and ERK activation in suspended ovarian cells is independent of signaling from FAK and PAK and occurs even in the presence of a negative feedback loop. MEK and ERK activation were equally robust in suspended cells under both serum and serum-free conditions at 3, 24, or 48 h (Fig. 2B). Phosphorylation of AKT was not affected by cell detachment.

ACUTE MITOGEN TREATMENT OF ADHERENT CELLS STIMULATES TRANSIENT ERK ACTIVATION

Mitogen stimulation of cells typically results in robust, transient ERK activation in adherent cells. To determine if the prolonged activation of ERK in suspension also occurred in adherent cells stimulated with mitogens, we stimulated serum-starved adherent SKOV-3 cells with increasing concentrations of serum, epidermal growth factor (EGF), and hepatocyte growth factor (HGF) for 10 min (Fig. 3A). Serum was a poor stimulator of ERK activity at this time point, while EGF induced robust ERK activation at all concentrations. HGF was a moderate activator of ERK at the highest

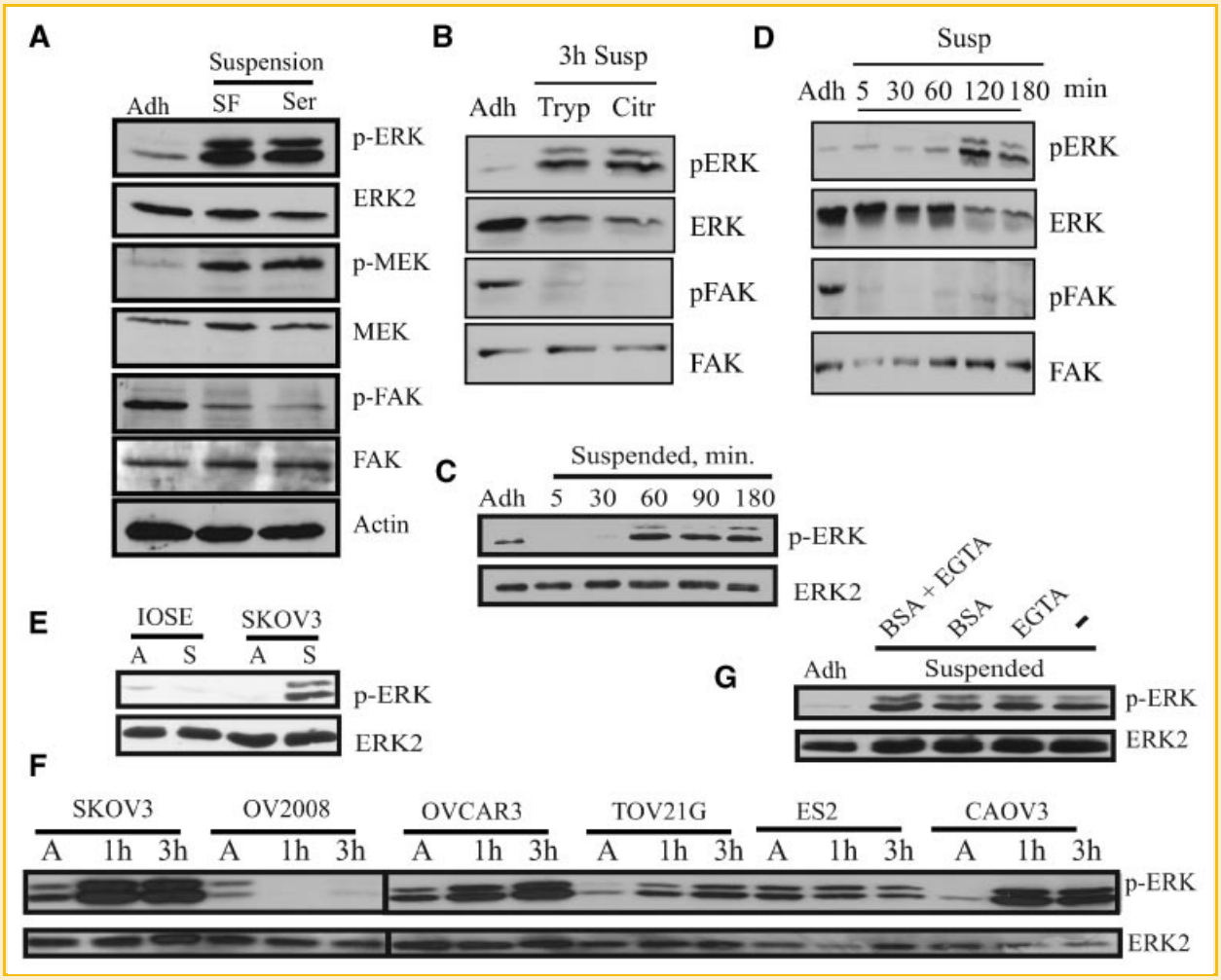

Fig. 1. Activation of ERK in suspended ovarian carcinoma cells. A: Adherent (Adh) SKOV-3 cells were detached with trypsin and treated with soybean trypsin inhibitor. The cells were centrifuged, resuspended in either serum free medium (SF) or media containing 10% FBS, and put in suspension culture for 3 h before harvest. Cell lysates were immunoblotted for ERK2, MEK, FAK, and the activated forms of these proteins. B: Adherent SKOV-3 cells were detached with either trypsin or citric saline and put in suspension culture for 3 h in serum-free media. C,D: Adherent SKOV-3 cells were detached with trypsin (C) or citric saline (D) and put in suspension culture in serum free media for the indicated time. E: Adherent (A) IOSE and SKOV-3 cells were detached with trypsin and put in suspension (S) for 3 h in serum free media. Lysates were immunoblotted for ERK2 and active ERK. F: Six adherent (A) ovarian cancer cell lines were detached with trypsin and placed in suspension in serum-free media for 1 or 3 h. G: Adherent (Adh) SKOV-3 cells were detached with trypsin and put in suspension for 3 h in either serum-free media (-), or serum-free media containing 0.5% bovine serum albumin (BSA), 4 mM EGTA, or BSA and EGTA.

concentration tested. To determine the duration of ERK activation by each mitogen, a time course was performed after stimulation with 1 ng/ml EGF, 10 ng/ml HGF (Fig. 3B), or 10% serum (Fig. 3C). ERK activation with each mitogen was transient, returning to basal levels within several hours. These data demonstrate a fundamental difference in the duration of ERK activation stimulated by growth factor addition in adherent cells versus activation due to loss of cell attachment.

CELL/MATRIX RE-ATTACHMENT RESTORES BASAL MEK AND ERK ACTIVITY

To directly test the effects of extracellular matrix attachment on ERK activation, SKOV-3 cells were detached and put into suspension for 3 h and were then either left in suspension for additional time or allowed to re-attach to fibronectin-coated dishes (Fig. 4A). Fibronectin is one of the main extracellular matrix proteins that is produced by mesothelial cells of the peritoneal cavity and is important for attachment and migration of ovarian cancer cells to the mesothelium [Lessan et al., 1999; Casey et al., 2001]. Replating suspended SKOV-3 cells onto fibronectin resulted in a restoration of basal MEK and ERK activity within 2 h, while both proteins remained active in parallel cultures of cells kept in suspension. Phosphorylation of MEK1 on Ser298 and FAK on Tyr397 were both restored upon cell adhesion. A similar experiment was performed to directly compare IOSE to SKOV-3 cells (Fig. 4B). Unlike in SKOV-3 cells, ERK and MEK activation in adherent IOSE cells was lost in detached cells, but restored upon adhesion of the cells to fibronectin.

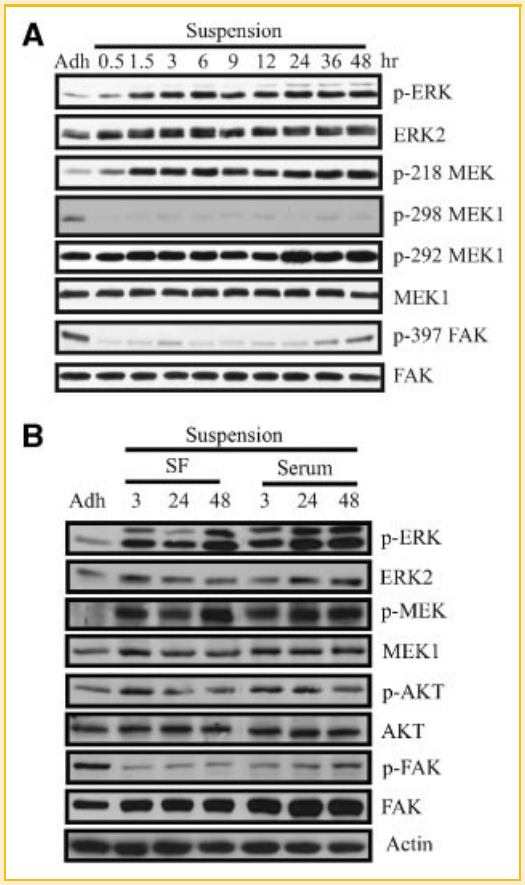

Fig. 2. Sustained activation of MEK and ERK in suspended cells. A: Adherent (Adh) SKOV-3 cells were trypsinized and suspended in serum-free media for the indicated time. Cell lysates were immunoblotted as indicated. The p-218 MEK antibody recognizes the active form of the kinase, while the p-298 and p-292 antibodies recognize the PAK and ERK phosphorylation sites on MEK1, respectively. B: Adherent (Adh) SKOV-3 cells were put in suspension for 3, 24, or 48 h in 10% serum or serum-free media. Lysates were immunoblotted as indicated.

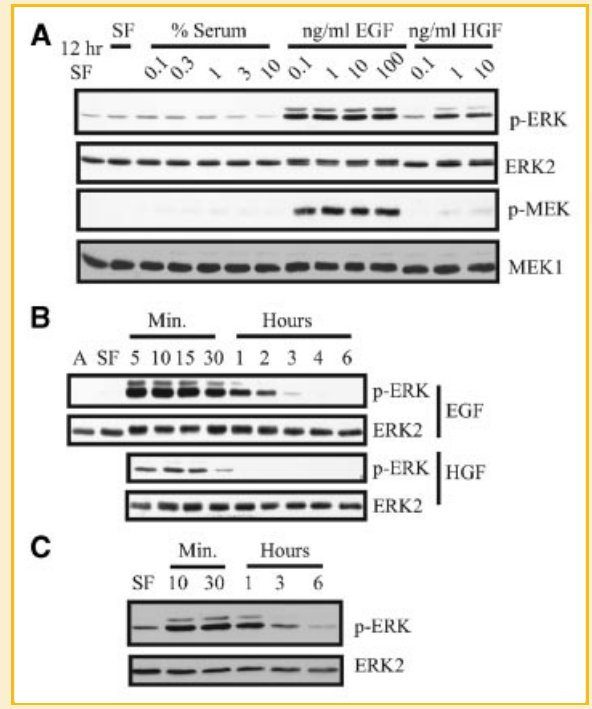

Fig. 3. A: Adherent SKOV-3 cells were serum-starved for 12 h and then treated with either fresh serum free media (SF) or increasing concentrations of serum, EGF, or HGF for 10 min. Cell lysates were immunoblotted for total and active ERK and MEK. B: Adherent (A) SKOV-3 cells were washed twice and incubated in serum-free (SF) media overnight. The cells were then stimulated for the indicated time with either 1 ng/ml EGF (top) or 5 ng/ml HGF (bottom). Cell lysates were immunoblotted for phosphorylated ERK and ERK2. C: Adherent cells were serum-starved overnight (SF) and then stimulated with 10% serum for the indicated times. Cell lysates were immunoblotted for phosphorylated ERK and ERK2.

Protein levels of MKP-1, a nuclear MAP Kinase phosphatase that is down-regulated in ovarian cancer [Denkert et al., 2002], were much lower in SKOV-3 cells compared to IOSE cells, but did not significantly vary with the attachment state of the cells. Collectively, these data directly demonstrate that cellular adhesion to extracellular matrix and/or cell spreading acts to down-regulate ERK in malignant, but not benign, ovarian cells.

DYNAMIC REGULATION OF ERK ACTIVATION BY AN AUTOCRINE MECHANISM

If ERK activation in suspended ovarian cancer cells is due to low expression or activity of ERK-specific phosphatases, such as MKP-1, suspended cells should have low MEK activity and there should be a slow turnover of ERK phosphorylation. To address this, SKOV-3 cells were put in suspension for 3 h and treated with the MEK inhibitor U0126 for the last 2 min, 5 min, 10 min, 15 min, 30 min or the entire 3 h before harvest to prevent upstream signaling to ERK (Fig. 5A). Interestingly, inhibition of MEK activity for as little as 2 min before harvest caused the complete dephosphorylation of active ERK. Similar results were seen with 2 min of U0126 addition over a 48 h time course in suspension (Fig. 5B), demonstrating that ERK activation in suspended cells is a dynamic process involving activation by MEK and inactivation by phosphatases.

Dynamic ERK activation suggests a possible autocrine stimulatory pathway; therefore we performed a conditioned media experiment. SKOV-3 cells were put in suspension for 3 or 24 h in serum-free media. Activation of ERK was evident at both time points, with increased activation at 24 h (Fig. 6A). Fresh serum-free media or the conditioned media from the suspended cells was then added to adherent, serum-starved SKOV-3 cells. Addition of conditioned media from suspended cells, but not fresh serum-free media, resulted in a rapid activation of MEK and ERK in the adherent cells, equal to the level observed in suspended cells. Activation was reduced at 15 and 60 min after addition, but was much higher than basal levels. Performing a longer time course of ERK activation using conditioned media from 24 h suspended cells demonstrated that basal levels of ERK activation were restored between 2 and 6 h

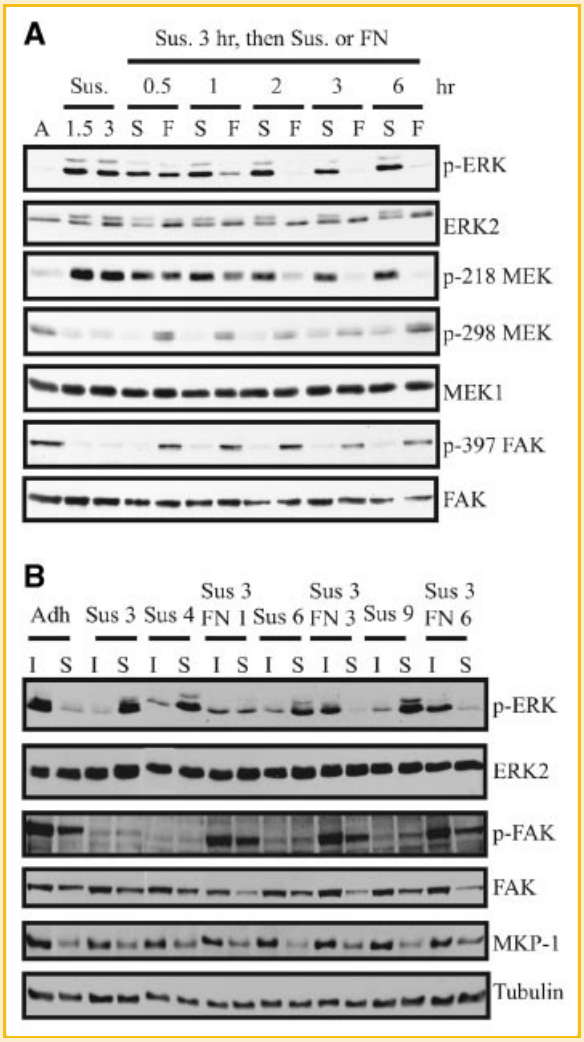

Fig. 4. Re-attachment of SKOV-3 cells to an extracellular matrix restores down-regulation of ERK activity. A: Adherent (A) SKOV3 cells were detached with trypsin and suspended in serum-free media for 90 min or 3 h. After 3 h the cells were either left in suspension for the additional time indicated or replated onto fibronectin-coated dishes in parallel. Cell lysates were prepared and immunoblotted for total and the activated forms of ERK, MEK, and FAK. B: Adherent (A) IOSE and SKOV-3 cells were detached with trypsin and put in suspension for 3 h. The cells were either kept in suspension for the number of hours indicated or replated onto fibronectin-coated dishes for the number of hours indicated. Cell lysates were immunoblotted for total and the activated forms of ERK, MEK, and FAK as well as MKP-1 and tubulin.

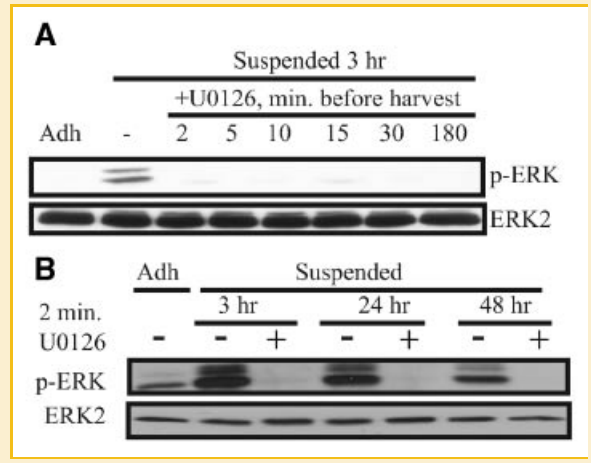

Fig. 5. Sustained activation of ERK in suspended cells is not due to loss of phosphatase activity. A: Adherent (Adh) SKOV3 cells were trypsinized and put in suspension in serum-free media for 3 h. The MEK inhibitor U0126 was added to the media at a final concentration of 10 μM for the indicated number of minutes prior to harvest of the suspended cells. Cell lysates were immunoblotted for phosphorylated ERK and ERK2. B: Adherent (Adh) SKOV-3 cells were put in suspension culture for 3, 24, or 48 h. Two minutes before harvest the cells were treated with either DMSO or 10 μM U0126. Cell lysates were immunoblotted for phosphorylated ERK and ERK2.

after addition (Fig. 6B). These data demonstrate that suspended cells produce a soluble factor that acts in an autocrine fashion to stimulate intracellular signaling to MEK and ERK.

ENHANCED ERK NUCLEAR SIGNALING IN SUSPENDED CELLS

ERK translocates to the nucleus in response to mitogens [Chen et al., 1993; Lenormand et al., 1993] and this translocation is required for cellular proliferation [Brunet et al., 1999]. It has been reported that ERK cannot signal to its nuclear substrate Elk1 in the absence of cellular adhesion [Aplin et al., 2001]. We used an Elk1-responsive luciferase reporter system [Roberson et al., 1995] and a constitutive TK Renilla luciferase control to test for the ability of ERKs to signal to the nucleus in suspended SKOV-3 cells. The following day the cells were either serum starved or put in suspension in serum free media for 24 h. The TK Renilla reporter demonstrated an overall reduction in transcription in suspended cells (data not shown). Normalizing to Renilla luciferase, ERK-responsive firefly luciferase activity was 2.5-fold higher in suspended SKOV-3 cells compared to adherent cells (Fig. 7). Co-transfection of a mutationally activated MEK1 enhanced ERK nuclear signaling in both adherent and suspended cells, demonstrating that enhanced ERK activation in adherent cell lines could stimulate luciferase production and that the difference in endogenous signaling was due to the enhanced ERK activation in suspended cells. These results demonstrate that cellular detachment in SKOV-3 cells enhances MAP Kinase signaling to the nucleus and regulation of gene transcription.

ENHANCED ROLE OF ERK IN SUSPENDED CELLS

To determine the effect of ERK activation on anchorage-dependent versus anchorage-independent cell growth, we first performed cell proliferation assays on adherent SKOV-3 and IOSE cells. Cells were plated and treated the following day with either 10 μM U0126 or DMSO control. Cells were treated with MTT reagent 2 h before harvest over the next 4 days (Fig. 8A). After 4 days of treatment, cell proliferation in U0126 treated cells was decreased 22% in IOSE cells and only 12% in SKOV-3 cells, both of which, while small differences, were statistically significant. These results suggest that SKOV-3 cells have a reduced requirement for ERK activity in regards

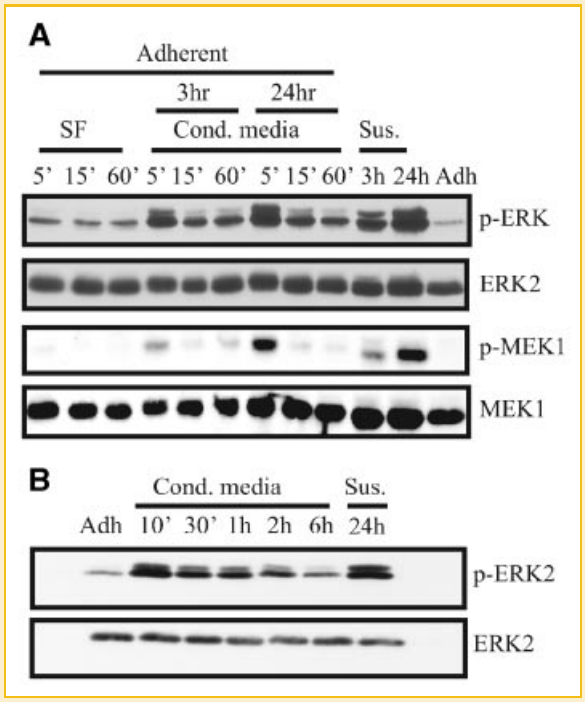

Fig. 6. Activation of ERK in suspended ovarian cells occurs through an autocrine mechanism. A: Adherent SKOV-3 cells (Adh) were put in suspension (Sus.) for 3 or 24 h. The cells were harvested and conditioned media from these cells was collected. Either fresh serum-free media (SF) or conditioned media from the suspended cells was then added to serum-starved adherent SKOV-3 cells for 5, 15, or 60 min. Cells lysates for the suspended and adherent cells were prepared and immunoblotted for ERK, MEK, and the activated forms of these proteins. B: Adherent (Adh) cells were put in suspension for 24 h (Sus.). Conditioned media from the suspended cells was collected and added to serum-starved adherent SKOV-3 cells from 10 min to 6 h. Cell lysates were immunoblotted with antibodies to ERK2 and phospho-ERK.

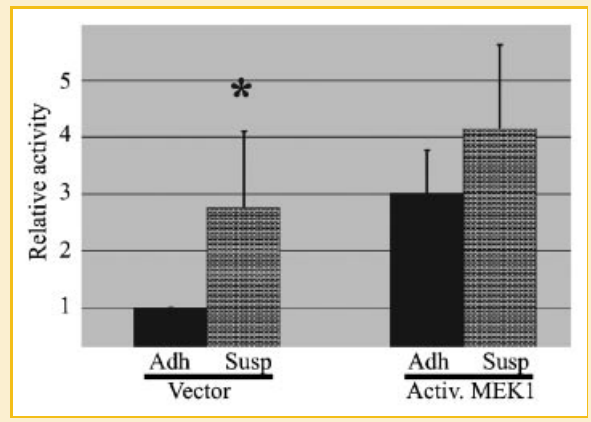

Fig. 7. ERK nuclear signaling is enhanced in suspended ovarian cancer cells. SKOV-3 cells were co-transfected with GAL4-Elk1, 5X GAL4 luciferase, TK Renilla luciferase, and either empty vector or mutationally activated MEK1. After a 5 h transfection in serum-free media, the cells were incubated in 10% serum overnight. The following day, the cells were washed twice with PBS and either incubated in serum free media or trypsinized and incubated in serum-free media in suspension. After 24 h all cells were harvested and both Renilla and firefly luciferase determined using a dual luciferase assay kit (Promega). Firefly luciferase activity was normalized to Renilla. Solid bars represent adherent cells and checked bars represent suspended cells. The results from the adherent cells transfected with the reporter construct, Gal4-Elk1, TK Renilla and empty vector (first column) was set at one. The results are an average of five experiments performed in duplicate. There was a significant difference ($^*P < 0.05$) between normalized firefly luciferase activity between adherent and suspended cells (left columns). There was not a significant difference in normalized firefly luciferase activity between the adherent and suspended cells that were transfected with mutationally activated MEK1.

to adherent proliferation compared to IOSE cells. Since we have observed an enhanced activation of ERK in detached SKOV-3 cells, we hypothesized that ERK inhibition may have a greater effect on anchorage-independent growth. Therefore, we determined the ability of IOSE and SKOV-3 cells to grow in soft agar in the presence of DMSO or U0126. Cells were treated with DMSO or U0126 at the time of plating and every 3 days afterwards. The benign IOSE cells demonstrated no growth in soft agar, as expected (data not shown). Conversely, SKOV-3 cells produced colonies in soft agar, as reported [Pegues et al., 1999; Popadiuk et al., 2006]. U0126 treatment decreased colony growth by 69% compared to DMSO treated cells (Fig. 8B,C), demonstrating that inhibition of ERK activation has profound effects on anchorage-independent growth of ovarian cancer cells.

DISCUSSION

We present the novel finding of strong and sustained activation of MEK and ERK in ovarian cancer cell lines in response to loss of cell adhesion. To our knowledge, this is the first report of loss of cell/matrix adhesion stimulating a sustained induction of ERK activity. ERK activity in suspension was dynamically regulated by autocrine activation and robust phosphatase activity. Re-attachment of suspended cells restored basal ERK activity. ERK signaling to the Elk1 transcription factor was enhanced in suspended cells and MEK activity was required for soft agar growth. These results demonstrate a unique mode of ERK regulation by cell attachment that has implications for anchorage independent growth of ovarian cancer cells.

ERK inactivation in suspended cells has been noted in several cancer cell lines, including breast cancer [Fukazawa et al., 2002], small cell lung cancer [Kraus et al., 2002], and in highly metastatic melanoma [Zhang et al., 2006] and hepatocellular carcinoma [Honma et al., 2006] cell lines. This suggests that loss of ERK activation in suspension does not just occur in benign cells, but also in many cancer cell lines as well. We observed a strong and sustained activation of MEK and ERK in four of six ovarian cancer cell lines in response to loss of matrix adhesion, but not in the benign ovarian IOSE cell line. Inactivation of ERK in suspended cells has been demonstrated to occur through a mechanism involving phosphatase activity and detachment-induced PKA activation, leading to inhibition of PAK activity [Howe and Juliano, 2000], thereby inhibiting Raf and MEK activation. This mechanism is intact in most cells and most likely accounts for the inactivation of ERK in suspended IOSE cells.

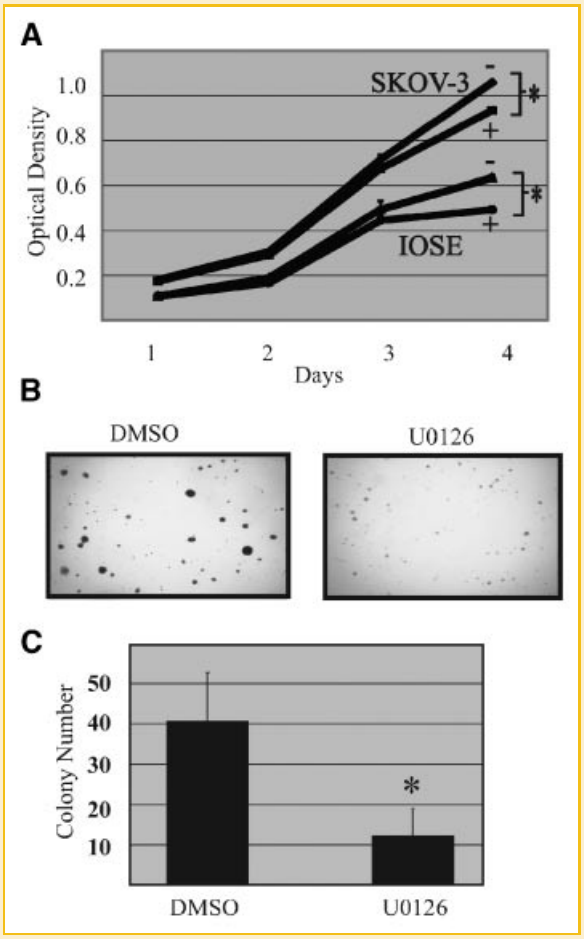

Fig. 8. Inhibition of ERK activation impairs anchorage independent growth. A: SKOV-3 and IOSE cells were plated in triplicate at a density of 1×10^4 cells per well in 12-well dishes in 10% serum-containing media. The following day the cells were treated with either 10 μM U0126 or DMSO control. MTT reagent was added for 2 h before cell harvest for the next 4 days. MTT activity was measured after harvest. The data shown is a representative experiment that was performed three times ($^*P < 0.05$). B: Adherent SKOV-3 cells were trypsinized and placed in 1ml of soft agar containing 10% serum at a density of 5,000 cells per ml. The assay was performed three times in triplicate. Cells were treated every 3 days with either 10 μM U0126 or DMSO control. Four random fields were photographed using a 2.5 objective. A representative field from each treatment is shown. C: Colony count (>400 μm^2) and size was determined using Image J software. Quantitation of the total colony count from four photographed fields per dish were averaged and then compared within each group. There was a statistical significant difference ($^*P < 0.05$) between U0126 and the DMSO control.

Expression of mutationally activated FAK [Renshaw et al., 1999] or PAK [Howe and Juliano, 2000] in suspended cells has been shown to result in ERK activation. However, FAK activation in SKOV-3 cells was inversely correlated with ERK activation, demonstrating that suspension-induced activation of ERK was FAK-independent. In addition, phosphorylation of MEK1 on Ser 298, a PAK phosphorylation site required for MEK1 activation in newly adhering cells [Slack-Davis et al., 2003], did not occur. These results suggest that signaling to MEK and ERK in suspended SKOV-3 cells is through a mechanism that does not require pathways that crosstalk for ERK activation in adherent cells. Interestingly, we observe that feedback phosphorylation of MEK1 on Ser292 [Mansour et al., 1994], which is normally adhesion dependent and acts as a negative regulatory mechanism for MEK1 activation during cell adhesion [Eblen et al., 2004], is not diminished in suspended SKOV-3 cells due to sustained activation of ERK. We currently show that it does not play an inhibitory feedback role in the context of loss of matrix adhesion.

Reduced expression of MKP-1 in ovarian tumor specimens [Denkert et al., 2002] and in malignant granulosa cells [Steinmetz et al., 2004] compared to their normal controls may enhance ERK activation in these cells and tissues. However, enhanced ERK activation in detached cells was not due to lack of phosphatase activity, even though MKP-1 levels were reduced in SKOV-3 cells compared to IOSE cells. Stimulation of ERK in adherent SKOV-3 cells by EGF, HGF, and serum demonstrated normal kinetics and there was no difference in MKP-1 protein levels in suspended versus adherent SKOV-3 cells. Moreover, we demonstrate a robust and sustained activation of MEK in suspended SKOV-3 cells and demonstrate that blocking upstream signaling from MEK to ERK by the addition of U0126 for as little as 2 min resulted in a complete dephosphorylation of ERK. Thus, there is sufficient phosphatase activity present from either MKP-1 or other ERK phosphatases [Camps et al., 2000] to stimulate rapid turnover of ERK phosphorylation. Our results demonstrate that ERK activation in detached SKOV-3 cells is dynamically regulated by a skewed balance between robust upstream activation by MEK, which is favored over and counteracted by prevalent phosphatase activity. This balance is shifted towards MEK and ERK inactivation in adherent cells to allow for inactivation of MEK and ERK after cell stimulation. The signaling pathways that restore this balance upon cell adhesion will be an interesting area to investigate, as they either regulate production of the autocrine factor or the way that the cell responds to the factor.

Aplin et al. [2001] demonstrated a requirement for cellular adhesion in order for active ERK to signal to the nucleus in NIH3T3 cells. We demonstrate that the activation of MEK and ERK in suspended SKOV-3 cells allows for 2.5-fold enhancement of ERK signaling to Elk1 compared to adherent cells. While this enhancement is not proportional to the increase in ERK activation, it shows that ovarian cancer cells have developed mechanisms to support nuclear signaling of activated ERK under conditions of anchorage independence. ERK nuclear signaling is required for cell proliferation [Brunet et al., 1999]. Our studies demonstrate that SKOV-3 cells are much more dependent on ERK activity for growth in soft agar compared to adherent growth. The effects of ERK signaling in suspended cells may not only be transcriptional, but non-genomic as well. In MCF7 cells, arrest of cells at G1/S results in transient activation of ERK in suspended cells and phosphorylation of pro-apoptotic Bim-EL [Collins et al., 2005], stimulating its degradation [Luciano et al., 2003; Ley et al., 2004; Marani et al., 2004]. However, the mechanism for anchorage-independent activation of ERK in ovarian cancer cells is apparently not due to G1 growth arrest, as MEK was inactive in the suspended MCF7 cells and activation of ERK was thought to occur through loss of phosphatase activity towards ERK [Collins et al., 2005].

We have shown that conditioned media from suspended cells was able to stimulate ERK activation in adherent cells, demonstrating the presence of an autocrine pathway activated in detached cells. This autocrine pathway most likely supports the robust activation of MEK that we observe in suspended cells. At present, the identity of the autocrine signal is unknown. Several mitogens, such as lysophosphatidic acid (LPA), platelet derived growth factor (PDGF) [Matei et al., 2006] and others have been identified in ovarian ascites fluid. LPA was shown to be secreted by peritoneal mesothelial cells, stimulating the migration of ovarian cancer cells [Ren et al., 2006]. It is doubtful that LPA is responsible for the autocrine signal, as LPA is the main mitogen in serum and serum was a weak stimulator of ERK activation in adherent SKOV-3 cells; however, we have not ruled out this possibility, as high levels of LPA may be produced from detached cells. Signaling through the ErbB family by either direct or indirect means could also be a possibility, as SKOV-3 cells overexpress ErbB family members and EGF stimulates robust ERK activation in these cells. EGFR and ErbB-2 are both overexpressed in a significant percentage of ovarian tumors and correlate with poor prognosis [Berchuck et al., 1990]. The identification of the autocrine signal is currently under investigation in our laboratory, as it could serve as a marker for metastatic ovarian cancer.

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