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Disruption of signaling through SEK1 and MKK7 yields differential responses in hypoxic colon cancer cells treated with oxaliplatin

Irina A. Vasilevskaya, Muthu Selvakumaran and Peter J. O'Dwyer

Abramson Family Cancer Center, University of Pennsylvania, Philadelphia PA 19104

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Running Title: Differential input of MKK7 and SEK1 in oxaliplatin responses

Corresponding author: Dr. Irina A. Vasilevskaya, University of Pennsylvania, 1020 BRB II/III, 421 Curie Blvd, Philadelphia PA 19104. Phone: (215) 573-7300; FAX: (215) 573-7049; e-mail: vasilevs@mail.med.upenn.edu

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The abbreviations used are: AP-1, activator-protein 1; NF-κB, nuclear factor kappa B; HIF-1, hypoxia-inducible factor-1; CREB, cAMP response element-binding protein; L-OHP, oxaliplatin; 17-AAG, 17-allylamino-17-demethoxygeldanamycin; MAPK, mitogen-activated protein kinase; JNK, Jun NH_2-terminal kinase; MEK (or MKK), MAPK kinase; MEKK, MAPK kinase kinase; SEK1 (MKK4), MAPK kinase 4; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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ABSTRACT

Transcriptional changes in response to hypoxia are regulated in part through MAP kinase signaling to AP-1, and thus contribute to resistance of cancer cells to therapy, including platinum compounds. A key role for JNK in pro-apoptotic signaling in hypoxic cells has previously been established. Here we analyze hypoxic signaling through MAPK kinases to AP-1/c-Jun in the HT29 colon adenocarcinoma cell line, and observe activation of stress-activated pathways mediated predominantly by SEK1 and MKK7. In transient transfection assays, introduction of dominant negative constructs for both MKK7 and SEK1 abolished hypoxia-induced AP-1 activation. Functional studies of the pathway using HT29-derived cell lines stably expressing mutant SEK1 or MKK7 showed impaired activation of JNK and AP-1 in response to hypoxia, more marked in MKK7-deficient than SEK1-deficient cells. Inhibition of SEK1 rendered hypoxic cells more sensitive to oxaliplatin in vitro, while the opposite effect was observed in MKK7-deficient cells. The mutant cell lines grown as mouse xenografts were treated with oxaliplatin, bevacizumab or both. The SEK1-deficient tumors exhibited greater sensitivity to all treatments, while MKK7-deficient cells were resistant in vivo, consistent with in vitro observations. These data support a positive contribution of MKK7/JNK to oxaliplatin cytotoxicity, and identify SEK1 as a potential target for reversal of hypoxic resistance to oxaliplatin.

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INTRODUCTION

Hypoxia is a characteristic of most solid tumors (Bush et al., 1978), and is recognized as a contributing factor to resistance of cancer to chemotherapy, including alkylating agents, platinum compounds, and radiation (Vaupel et al., 1989). Many of the adaptive responses set in motion by hypoxic exposure are regulated by hypoxia-inducible factor-1 (HIF-1), an oxygen-sensitive transcription factor that induces expression of genes involved in angiogenesis, proliferation and metastasis (Semenza, 2002). Other transcription factors, including activator protein -1 (AP-1), are also activated by hypoxia and contribute to the altered gene expression profile of hypoxic cells (Cummins and Taylor, 2005).

Oxaliplatin (L-OHP), a third generation platinum compound, has a major role in the treatment of colorectal cancer (Andre et al, 2004; Goldberg et al, 2006). The induction of oxaliplatin resistance by hypoxia has been described in testicular cancer cell lines (Koch et al., 2003) and in a series of hypoxia-conditioned HT29 colon cancer cell lines (Yao et al., 2005a). In previous work we have demonstrated induction of AP-1 family genes and increased AP-1 transcriptional activity in hypoxic HT29 cells (Yao et al., 1994; O'Dwyer et al., 1994). We have also demonstrated the induction of AP-1 transcriptional activity by oxaliplatin in a panel of colon cancer cell lines (Rakitina et al., 2003). Hence, these pathways may be relevant to tumor responses under hypoxic conditions.

The AP-1 transcription factor consists of homo- and heterodimers of the Jun, Fos, ATF and MAF protein families that stimulate the expression of a broad range of genes involved in proliferation, differentiation, and apoptosis (Eferl and Wagner, 2003). Activation of AP-1 by various signals, including hypoxia, occurs primarily through parallel three-tier signaling cascades terminating in a group of mitogen-activated protein kinases (MAPKs) (Derijard et al., 1995).

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MAP kinases in turn are activated by upstream regulators - MAPK kinases (MEKs or MKKs): MEK1 and MEK2 activate extracellular signal-regulated kinases (ERK1/2) mostly in response to mitogenic stimuli (Zheng and Guan, 1993); MKK3 and MKK6 are responsible for activation of p38 MAPK (Enslen et al., 1998); while MKK4 (also known as SEK1) and MKK7 both activate Jun NH_2-terminal kinase (JNK). Studies in vitro have shown that MKK4 can also phosphorylate p38 MAPK, whereas MKK7 is specific towards kinases of JNK subgroup (Davis, 2000; Tournier et al., 2001). Activation of MEKs involves multiple kinases (MEKKs or MKKKs), acting on different substrates dependent upon particular extra- or intra-cellular signals (Fanger et al., 1997). Data on oxaliplatin-induced signaling through MAP kinases and their effects on cytotoxicity of the drug are scarce. Our previous results suggest that both JNK and p38 MAPK contribute to oxaliplatin-induced cell death in colon cancer cell lines (Rakitina et al., 2003).

We previously found that short-term hypoxic exposure activates SEK1 and JNK, leading to up-regulation of c-Jun in HT29 cells (Vasilevskaya and O'Dwyer, 1999). Now we show that JNK activation results from hypoxia-induced signaling through both SEK1 and MKK7, while MEK1/2 and MKK3/6 remain constitutively active. In a functional analysis of this induced signaling we isolated HT29-derived cell lines stably expressing control vector (HTpc3) or dominant negative constructs for SEK1 or MKK7 (HTS13 and HTM9, respectively). We found that signaling through MKK7/JNK/c-Jun cascade is the major mechanism for hypoxic activation of AP-1 in HT29 cells. We also show that modulation of these pathways affects the sensitivity of hypoxic HT29 cells to oxaliplatin: the inhibition of MKK7 leads to an increase in oxaliplatin-resistance upon hypoxic exposure, whereas inhibition of SEK1 renders cells more sensitive under these circumstances. Confirmation of the potential biological relevance of this observation

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was obtained in in vivo experiments using xenografts of stably transfected cell lines. These data implicate differential effects of signaling through MKK7 and SEK1 in resistance and sensitivity to oxaliplatin in hypoxic tumor cells: that hypoxic signaling to SEK1 contributes to oxaliplatin resistance, thus identifying SEK1 as a potential target for reversal of hypoxic resistance. Conversely, absence of MKK7 has the potential to render a tumor resistant to agents that require hypoxia signaling to exert their therapeutic effects. These results may further help to address some of the conflicting data regarding the role of signaling through JNK to influence apoptosis (Vasilevskaya and O'Dwyer, 2003).

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MATERIALS AND METHODS

Cells and reagents. The human colon adenocarcinoma cell line HT29 was from ATCC (Manassas, VA). Cells were grown in DMEM medium supplemented with 10% FBS and antibiotic-antimycotic reagent (Invitrogen, Carlsbad, CA). Cultures were maintained in a humidified incubator at 37°C in 5%CO_2-95% air. Chemical inhibitors for JNK (SP600125) and p38 (SB203580) and c-jun antisense oligonucleotides were purchased from Biomol (Plymouth Meeting, PA). The MEK1/2 inhibitor UO126 was purchased from Promega (Promega Corp., Madison, WI). 17-allylamino-17-demethoxygeldanamycin (17-AAG) and oxaliplatin were purchased from LKT Labs (St. Paul, MN).

Plasmids and isolation of stably transfected cell lines. The FLAG-tagged dominant negative mutant of MKK7 (pcDNA3-Flag-MKK7 (ala)) was kindly provided by Dr. Roger Davis (University of Massachusetts Medical Center, Worcester, MA); the HA-tagged dominant negative mutant of SEK1, (HA-SEK-AL/pcDNA3.1) was a gift from Dr. Jim Woodgett (Ontario Cancer Institute, Toronto, ON); HA-tagged dominant negative mutants of JNK1 and JNK2 (HA-JNK1-APF and HA-JNK2-APF), cloned into LNCX vector, were kindly provided by Dr. Tomas Berl (University of Colorado, Boulder). To isolate cell lines stably expressing empty vector, dnSEK1 and dnMKK7, transfectants were cultivated at low density in media containing G418 (0.75 mg/ml, Invitrogen), surviving colonies were isolated using glass cylinders, propagated and assessed for expression of tag protein.

Hypoxic treatment. Exposure of cells to acute hypoxia was achieved by incubation in anaerobic chamber (Forma Scientific, Inc., Marietta, OH) filled with gas mixture consisting of 5% CO_2, 9% H_2 and 86% N_2. Oxygen content (below 0.5%) was monitored by PROOX 110 oxygen sensor (BioSpherix, Redfield, NY). Cells were plated in 100 mm glass Petri dishes to a

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density of 2 x 10^6 cells per dish and subjected to hypoxia within 36 hours when the cells had reached 70% confluence. Inhibitors at concentrations of 10 μM were added 2 hours prior to hypoxia treatment, 0.01% DMSO was added to control samples. The cells were harvested at various time points for further isolation of protein extracts.

Protein extract preparation. Total protein extracts were prepared as follows: after hypoxia, cells were washed twice with PBS and lyzed inside the chamber in cell lysis buffer (Cell Signaling Technology, Beverly, MA), supplemented with complete protease inhibitor cocktail (Roche Applied Sciences, Indianapolis, IN) and 1mM PMSF (Sigma, St. Louis, MO). The contents of scraped dishes were transferred into microcentrifuge tubes, taken out of hypoxia chamber and placed in a shaker for 30 min at 4^{o}C. Lysates were then centrifuged for 10 min at 10,000 rpm (4^{o}C) and the protein concentration of cleared cellular extracts was measured using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA).

Western blotting. For protein electrophoresis in SDS- polyacrylamide gels, protein extracts were used in amounts of 10 μg per lane. Western blotting was carried out according to standard procedures, using horseradish peroxidase-conjugated secondary antibodies purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and the ECL+Plus detection system (Amersham, Arlington Heights, IL). The primary antibodies used were: antibodies against MKK7 and phosphorylated forms of JNK, p38, SEK1 and MEK1/2 from Cell Signaling Technology; antibody against phospho-MKK7 from Upstate (Lake Placid, NY) and c-Fos antibody from Oncogene (La Jolla, CA). The remaining antibodies were purchased from Santa Cruz Biotechnology.

Transfections and luciferase reporter assays. Control and reporter, pTA-Luc and pAP1-Luc, plasmids were from PathDetect AP-1 Cis-Reporting system (Stratagene, La Jolla,

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CA). Cells were plated in 6 well plates at the density of $1.5x10^5$ per well and after 24 hours were transfected using FuGENE 6 transfection reagent (Roche) with the mixture of dominant negative MAPKK or JNK construct (1 μg), control or reporter plasmid (1 μg) and pRL-CMV vector, expressing *Renilla* luciferase (Promega, 40 ng), unless otherwise noted. Antisense c-jun oligonucleotides, and scrambled control, were used at a concentration of 100 μM. Twenty-four hours after transfection, cells were subjected to hypoxia for 8 or 24 hours, collected and assayed for luciferase activity using the Dual-Luciferase Reporter Assay System (Promega), according to the manufacturer's protocol. Luciferase activity was measured by TD20/20 tube luminometer (Turner Design, Sunnyvale, CA). The relative light units (RLUs) were calculated as ratio of *Firefly* luciferase to *Renilla* luciferase luminescence. Non-specific luciferase activation determined in parallel experiments with control vector was subtracted. All experiments were performed at least twice in duplicate.

Colony-forming assays. For clonogenic assays, HTpc3, HTM9 and HTS13 cells were plated in 6-well plates at a density of 300 cells per well; after 24 hours oxaliplatin was added at specified concentrations immediately prior to transfer of the plates to the hypoxia chamber. After 24 hours of hypoxia, plates were returned to oxic conditions, and, following the addition of fresh media, cultivated for 10-14 days; colonies were then fixed in 75% ethanol, stained with Coomassie Blue (Sigma) and counted manually. All experiments were performed at least three times in duplicate.

Retroviral delivery of dnSEK1 construct into colon cancer cell lines. The DNA fragment coding for dnSEK1 from HA-SEK-AL/pcDNA3.1 plasmid was cloned into the LPNX plasmid (Clontech Laboratories Inc., Mountain View, CA). The resulting vector (or empty LPNX as a control) was transfected into the Phoenix-Ampho packaging cell line, kindly

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provided by Dr. Anil Rustgi from the University of Pennsylvania, using Fugene 6 reagent (Roche). Media was exchanged 24 hours later and incubation continued further. Supernatants containing control (RC) or recombinant (RS-) virus were collected at 48, 72 and 96 hours post-transfection, combined, filtered through 0.45 μm sterile filters, and stored at -80^{o}C. Colon cancer cells plated into 6 well plates were infected in the presence of polybrene (8 μg/ml, Chemicon International Inc, Temecula, CA), and 48 hours later selective media (1 μg/ml of puromycin, Sigma) was added. Surviving cells were pooled, evaluated for expression of HA-tag and used in MTT assays to assess oxaliplatin sensitivity under hypoxic conditions.

***In vivo* experiments.** To assess the efficacy of oxaliplatin treatment with or without bevacizumab (anti-VEGF antibody) in HTpc3- , HTS13- and HTM9-derived tumors, six to eight week old female mice (C.B.17 SCID) were used. To produce tumors, control, HTS13 and HTM9 cells (2 x10^{6} cells) were injected subcutaneously into the left flank of the mice. When the tumors reached approximately 100mm^{3}, mice were divided into four groups (6 mice per group) for treatment with bevacizumab (Genentech, Inc., San Francisco, CA), oxaliplatin (Eloxatin, Sanofi Aventis, Bridgewater, NJ), the combination of bevacizumab and oxaliplatin, and a vehicle control group. Mice in the bevacizumab treatment group received 5 mg/kg of bevacizumab by intraperitoneal injection every 3 days for 30 days. The oxaliplatin treatment group was injected intraperitoneally with 10 mg/kg of oxaliplatin per week for two weeks. The combination treatment group received bevacizumab (every 3 days, 5 mg/kg for 30 days) and oxaliplatin (weekly for 2 weeks, 10 mg/kg). The control group received saline intraperitoneally on the every three days schedule. Tumor volume and body weight were measured every 3 days. Tumor volume was calculated using the formula $V=AB^2/2$, where A is the largest diameter and B is the smallest diameter. Tumor growth delay was calculated as the difference in the time for

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control and treated tumors to grow from 100 to 600 mm^3. All animal experiments were done according to an IACUC (Institutional Animal Care and Use Committee) protocol approved by the University of Pennsylvania.

Statistical analysis. Data were analyzed with unpaired Student's test: $P < 0.05$ was accepted as a statistically significant difference compared with corresponding control. In figures: *, $P < 0.05$; **, $P < 0.01$, ***, $P<0.001$.

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RESULTS

Signaling through JNK is required for hypoxic induction of AP-1 in HT29 cells. We previously showed induction of AP-1 family genes and activation of AP-1 proteins (primarily c-Jun homodimers and c-Jun/c-Fos heterodimers) by hypoxia in HT29 cells (Yao et al., 1994; Vasilevskaya and O'Dwyer, 1999). The initial approach to identify the MAP kinase signaling pathways involved using pharmacological inhibitors. Concentrations of inhibitors were selected in preliminary model experiments, using known activators of respective pathways (for example, anisomycin to activate JNK pathway, sorbitol for activation of p38MAPK pathway). Chosen concentrations of inhibitors were sufficient to cause detectable responses in signaling (based on western analysis of the downstream targets), after relatively short exposure (1-3 hrs). HT29 cells were pretreated for 2 hours with inhibitors of ERK (UO126), p38MAPK (SB203580) and JNK (SP600125), all at 10 μM, then subjected to hypoxia for up to 5 hours. Total protein extracts were used to assess the activation status of MAPK pathways and the content of AP-1 proteins. 17-AAG (Hsp90 inhibitor, 1 μM) was also used to inhibit both ERK and JNK signaling (Vasilevskaya and O'Dwyer, 1999). We found that inhibition of the MEK1/ERK cascade by either UO126 or 17-AAG did not alter c-Fos levels after 5 hours of hypoxia and, consequently, had no likely impact on its role in the c-Fos/c-Jun dimer (Figure 1A). There was no inhibition of activating transcription factor 2 (ATF2) phosphorylation in the presence of SB203580 (Figure 1B). This inhibitor does not affect phosphorylation of p38 MAPK itself by up-stream kinases (Kumar et al., 1999), but its efficacy in our system is supported by hypo-phosphorylation of CREB and of MAPKAP-2, known down-stream targets (Figure 1B). In contrast, phosphorylation of both c-Jun and ATF2 was inhibited in the presence of SP600125, confirming our previous results (Vasilevskaya and O'Dwyer, 1999) that the activation of these AP-1

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proteins in HT29 during hypoxic exposure is dependent mostly on JNK, not p38 MAPK. We also have consistently observed robust phosphorylation of JNK during hypoxia in the presence of SB203580. Activation of JNK upon inhibition of p38 MAPK was described for macrophages (Hall and Davis, 2002) and in our case could also contribute to the active status of ATF2. These data demonstrate that both induction of c-Jun expression (through activation of c-Jun/ATF2) and its activation during hypoxia in HT29 depend predominantly on functional JNK.

MKK7 is a key up-stream mediator of hypoxia signaling to AP-1 in HT29 cells. We previously found that SEK1 was activated in HT29 cells subjected to hypoxia for 3 and 6 hours, whereas MEK1/2 and MKK3/6 were constitutively active, and not further induced (Vasilevskaya and O'Dwyer, 1999). To further dissect hypoxia signaling to c-Jun/AP-1, we examined the role of another up-stream regulatory kinase - MKK7. We subjected HT29 cells to hypoxia for various time intervals (ranging from 30 min to 5 hours), collected protein extracts and assessed activation of MKK's by Western blot analysis. Hypoxia activates both MKK7 and SEK1, with a marked increase in phosphorylation by 3 hours, whereas changes in MKK3/6 and MEK1/2 were minimal (Figure 2A). We therefore focused our further study on the (SEK1/MKK7)/JNK/c-Jun cascade. To assess the effect of modulating the pathway on AP-1 transcriptional activity we carried out luciferase reporter assays: HT29 cells were co-transfected with a reporter plasmid (pAP1-Luc) and plasmid constructs expressing dominant-negative forms of SEK1, MKK7 or JNK1/2. We also used c-jun antisense oligonucleotides (along with corresponding scrambled controls) as a negative control for AP-1 activation. Results of AP-1- Luc assays performed after 8 hours of hypoxia (Figure 2B) demonstrate that although both SEK1 and MKK7 are important for activation of AP-1 by hypoxia, inhibition of MKK7 (64 % decrease) impacts activation more

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than inhibition of SEK1 (43 % decrease), while inhibition of JNK's resulted in 70 % decrease in AP-1 activation by hypoxia. These data suggest that the importance of functional MKK7 for the maintenance of basal AP-1 activity and its activation by hypoxia in HT29 cells is comparable to that of JNK. We have to note, however, that since transformation efficiency in HT29 cells is low, it would be appropriate to consider these results more qualitatively than quantitatively. Accordingly, to study the roles of SEK1 and MKK7 further we opted to isolate cell lines stably expressing the dominant negative forms of these kinases.

Differential effects of hypoxia on AP-1 activation in cell lines stably expressing dominant negative MKK's confirms major role for MKK7. Derivatives of the HT29 cell line stably expressing empty vector (HTpc3), dnSEK1 (HTS13) and dnMKK7 (HTM9) were isolated as described in Materials and Methods. The expression of dominant negative mutants was confirmed by Western blot analyses for tag-proteins: HA for dnSEK1 and FLAG for dnMKK7 (Figure 3A, left and right panels, respectively). When the derivative cell lines were subjected to hypoxia, profound inhibitory effects of dnMKK7 on both JNK and c-Jun phosphorylation were observed (Figure 3B), whereas the inhibition of SEK1 was less effective, consistent with the results above (Fig. 2B). Again, since the hypoxic activation of ATF2 in HT29 cells depends primarily on JNK, phosphorylation of ATF2 is altered in a manner similar to that of JNK. We then assessed activation of AP-1 in these cell lines using reporter gene assays during prolonged hypoxia: cells were co-transfected with 2.5 μg of control or reporter pAP1-Luc plasmid and pRL-CMV vector, and 24 hours post-transfection subjected to hypoxia for another 20 hours. The importance of SEK1 and MKK7 signaling is shown in the lower levels of AP-1 transcription even under oxic conditions. With hypoxia, however, significant hypoxic induction of AP1 was

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observed in SEK1-deficient cells, whereas in MKK7-deficient cell line it was completely abolished (Figure 3C).

Signaling through MKK7 enhances oxaliplatin cytotoxicity in hypoxic HT29 cells, whereas SEK1 mediates protective responses. We have reported recently that hypoxia-induced transcriptional changes are a factor in the cellular responses of colon cancer cell lines to cisplatin and oxaliplatin (Yao et al., 2005a; Yao et al., 2005b). The importance of the AP-1 transcription factor in cellular resistance to platinum drugs has also been described in various cellular models (Bonovich et al., 2002; Piret et al., 2006). Therefore we went on to determine if inhibition of AP-1 signaling through down-regulation of SEK1 or MKK7 could affect cell growth and survival during hypoxia and/or treatment with oxaliplatin. We conducted MTT assays to establish the IC_{50} for oxaliplatin in hypoxic HT29 cells: oxaliplatin was added immediately before placing the cells into hypoxia chamber for 24 hours, followed by cultivation in oxic conditions for the rest of the 72 hours exposure to oxaliplatin. We found differences in oxaliplatin sensitivity of HTpc3, HTS13 and HTM9 cell lines even under oxic conditions, with IC_{50} of 0.6, 0.3 and 0.9 μM, respectively (Table 1). Hypoxia alone did not affect significantly the proliferation rate of cell lines: all three lines demonstrated MTT readings comparable to those obtained from cells cultivated in oxic conditions (data not shown). Differences in drug sensitivity between the lines were more pronounced under hypoxic conditions: while oxaliplatin resistance of HTpc3 and HTS13 cell lines was increased two-fold, inhibition of MKK7/JNK/c-Jun pathway led to much greater resistance in HTM9 cell line, with IC_{50} changed from 0.9 μM to 5 μM (Table 1). The results of MTT assays, however, are influenced by oxaliplatin effects on both survival and cell cycle regulation of HT29-derived cells. The growth inhibitory effects

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might be especially significant in HTM9 cells, since involvement of MKK7 in the coupling of stress signaling and cell cycle progression has been reported (Wada et al, 2004). Therefore, to establish the impact of SEK1 and MKK7 inhibition on cell survival (and also to separate these from the effects of growth inhibition reflected in the results of MTT assays) we then carried out colony-forming assays. HTpc3, HTS13 and HTM9 cell lines were treated with the range of oxaliplatin concentrations for 72 hours, with or without hypoxia for first 24 hours similar to previous MTT assays. The sensitivity to oxaliplatin in a panel followed the patterns of MTT assays in general: HTS13 cells exhibited the lowest IC_{50} concentration, HTM9 cells - the highest (not shown). To further illustrate the differences in oxaliplatin sensitivity of HTpc3, HTS13 and HTM9 cells lines we carried out additional colony-forming assays after exposing them to the equimolar concentration of oxaliplatin (1 μM, which falls in a medium range of clinically achievable concentrations) for 24 hours with or without simultaneous hypoxic exposure (Figure 4). Sensitivity to oxaliplatin did not vary dramatically among the cell lines when cultivated in normoxic conditions; however, when cells were subjected to both oxaliplatin treatment and hypoxia, a marked decrease in survival was observed in the HTS13 cell line, whereas HTM9 cells were most resilient. Our data suggest that MKK7-mediated activation of the JNK/c-Jun pathway is essential for cell death of HT29 cells treated with oxaliplatin under hypoxia, whereas SEK1 may be involved in the mechanisms related to oxaliplatin resistance. To determine that the effects we observed are not HT29 specific, we expanded the cellular model to incorporate multiple colon cancer cell lines with varying genetic backgrounds. We used a retrovirus-based delivery system for the introduction of dominant-negative SEK1 into these cells. The results of cytotoxicity assays in SW480 and HCT15 cell lines corroborate these in HT29, while BE cells were not sensitized (Table 2).

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Intact MKK7 is required for therapeutic effects of oxaliplatin and bevacizumab in vivo. Finally, we studied the effects of SEK1 and MKK7 inhibition in a mouse xenograft model. SCID mice were injected subcutaneously with $2x10^6$ HTpc3, HTS13 or HTM9 cells and subjected to treatments with bevacizumab and oxaliplatin alone, or in combination. The doses/schedules of the in vivo experiments were selected based on our own previous work (Selvakumaran et al., 2008) and also from other published works in mice models of colon cancer that are known to work in those doses/schedules. Both the anti-VEGF and oxaliplatin treatments demonstrated growth inhibitory effects on control HTpc3 tumors (Figure 5AB), with the combination therapy being the most effective. Figure 5A demonstrates that inhibitory effects of the therapies on the growth of HTM9-derived tumors were modest, and differed significantly from control tumors treated with bevacizumab alone ($P<0.01$), oxaliplatin alone ($P<0.001$), or with the combination ($P<0.001$). On the other hand, HTS13- derived tumors grew slower than control tumors with all treatments (Figure 5B). These differences in response to therapy were even more evident when we calculated tumor growth delay (Table 3), the time difference between untreated and treated tumors to grow from 100 to 600 mm^3. The tumor growth delay in bevacizumab-treated mice was 2.7 and 8 days for HTM9 and HTS13 tumors, respectively, versus 5.4 days for HTpc3 tumors. In the oxaliplatin-treated group, the tumor growth delay was 2 and 10 days versus 7.5 days, respectively. Finally, with the combination treatment, the growth delay for HTM9 tumors was only 1.4 days, dramatically different from 10.9 days for the control tumors, whereas HTS13 tumors demonstrated a growth delay of 13 days. These results show that MKK7-induced activation of JNK provides a direct pro-death signal in hypoxic cells exposed to oxaliplatin, while SEK1 has in addition a preponderant survival influence in this context, and may be a target for therapeutic intervention.

DISCUSSION

Hypoxia is hallmark of a majority of solid tumors and is considered a key driving force for cancer progression and development of therapeutic resistance. The HIF-1 transcription factor plays a central role in the cellular processes underlying adaptive, proliferative and anti-apoptotic responses to low oxygen pressure; as a consequence there is a focus on approaches to target it for cancer therapy (Semenza, 2003). However, the multitude of other transcription factors activated by hypoxia directly or indirectly (Cummins and Taylor, 2005), may in turn regulate a wide variety of transcriptional responses, and may be important for an understanding of the biology of tumor hypoxia in general and of hypoxic resistance to cancer therapies in particular. Activation of the AP-1 transcription factor by hypoxia was reported for multiple cancer cell lines, including human colon adenocarcinoma (Yao et al., 1994; Vasilevskaya and O'Dwyer, 1999), hepatic and ovarian carcinomas (Minet et al., 2003; Xu et al., 1999), among others. MAP kinase pathways, as major activators of AP-1-regulated transcription, might therefore be considered as potential targets to influence cellular responses to hypoxia alone, and in combination with chemotherapy. In this paper we present results detailing the input of individual MAP kinases and their upstream activators, MAPK kinases, on AP-1 induction by hypoxia in HT29 cells, as well as the effects of selective disruption of the pathways on sensitivity to hypoxia and oxaliplatin.

Our analysis of hypoxia signaling indicates that in HT29 cells, hypoxic induction of AP-1 activity can be abolished by inhibition of JNK. The MEK/ERK pathway seems to play no role in this response: i) MEK1/2 and ERK1/2 are constitutively active in oxic HT29 cells, and not affected by hypoxic exposure; ii) pharmacological inhibition of this pathway by UO126 did not translate into inhibition of the expression or phosphorylation status of the major AP-1 proteins (Figure 1A, B). In contrast, dramatic changes occurred in the activity of SEK1 and MKK7

kinases. Hypoxia also strongly induced signaling to p38 and JNK, which have been both shown to be pro-apoptotic under low oxygen (Sun et al., 2006; Betigeri et al., 2006). A pro-apoptotic role for these kinases in cisplatin-treated cells has also been demonstrated in various cellular models (Roos and Kaina, 2006), including lung, ovarian and human colon cancer (Brozovic et al., 2004; Vasilevskaya et al., 2004). Much less is known about the induction of signaling pathways by oxaliplatin, and the involvement of stress-regulated MAP kinases in oxaliplatin-induced cell death. We have reported significant activation of p38 MAPK and JNK by cisplatin and oxaliplatin in a panel of colon cancer cell lines (Rakitina et al., 2003; Vasilevskaya et al., 2004; Vasilevskaya et al., 2003), and showed that inhibition of JNK and p38 MAPK by pharmacological inhibitors results in decrease of oxaliplatin cytotoxicity in colony-forming assays (Rakitina et al., 2003). Despite the pro-apoptotic properties of these stress-activated MAPK pathways induced by both hypoxia and platinum drugs, the efficacy of cytotoxic drugs is diminished in hypoxic conditions (Koch et al., 2003; Yao et al., 2005a). Therefore we have tried to further delineate the role of signaling through these pathways to better understand hypoxic resistance to oxaliplatin.

The pair of kinases activating JNK, SEK1 and MKK7, although acting synergistically (Fleming et al., 2000), display several differences (Wang et al., 2006). One key difference is the ability of SEK1 to phosphorylate p38 MAPK in addition to JNK. They also show variable substrate specificity: SEK1 preferentially phosphorylates the tyrosine residue in Thr-Pro-Tyr motif of JNK, whereas MKK7 acts on the threonine residue. This is relevant to our results in that while both kinases are necessary for full activation of JNK (Kishimoto et al., 2003), phosphorylation on the threonine residue alone (by MKK7) has been shown to be sufficient to increase JNK activity (Tournier et al., 2001). The results of MTT assays using HT29-derived

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cell lines, HTpc3, HTS13 and HTM9, point to differential functions of SEK1 and MKK7 in HT29 cells during hypoxia and oxaliplatin exposure: while the survival of none of the cell lines was affected by hypoxia alone, HTM9 cells were 3-fold more resistant to oxaliplatin in oxic conditions then HTS13 cells, confirming that JNK activation is required for oxaliplatin cytotoxicity. The dramatic difference in survival between HTM9 and HTpc3 and HTS13 cell lines after hypoxia in the presence of oxaliplatin demonstrated by both MTT (3- and 10-fold, respectively, Table 1) and colony-forming assays suggest that inhibition of SEK1 results in specific alterations of hypoxic signaling rendering cells more sensitive to oxaliplatin. In HT29 cells with impaired MKK7 function inhibition of the JNK/c-Jun/AP-1 cascade prevents cell death under hypoxia/oxaliplatin treatment, whereas the function of SEK1 seems to be more complex: although the overall activation of JNK and c-Jun in HST13 cell line is lower than in parental cells, they still demonstrate higher sensitivity to all treatments, as compared to other two lines. The observed increased mortality in the SEK1-deficient cell line, therefore, could not be solely attributed to the functional MKK7 present in these cells.

Hypoxic induction of p38 MAPK was unaltered in HTM9 and, more important, in HTS13 cell line (Figure 3B), suggesting that its activation in this situation most likely does not involve SEK1. In colony-forming assays, when these cell lines were treated with hypoxia/oxaliplatin in the presence of a pharmacological inhibitor of p38 MAPK (SB203580), resistance to oxaliplatin was uniformly increased to the same degree in all cell lines, irrespectively of SEK1 and MKK7 status (data not shown). The additional importance of signaling through MKK3/6 to p38 MAPK for oxaliplatin cytotoxicity is evident therefore, and detailed studies are currently underway. Here we focus on SEK1 and MKK7 to demonstrate that their action is not redundant, and leads to differential responses to oxaliplatin in cell lines

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deficient in either of these kinases.

Taken together, our data suggest that a survival pathway normally induced by hypoxia is not activated when SEK1 is inhibited by over-expression of a dominant-negative mutant. This was somewhat surprising, in light of the identification of SEK1 (MKK4) as a tumor suppressor (Wang et al., 2006). Our results, however, are indirectly supported by studies suggesting a pro-oncogenic role for SEK1. Wang et al used breast and pancreatic cancer cell lines with different basal levels of SEK1 to modulate kinase expression by introducing either recombinant protein or siRNA construct into cells (Wang et al., 2004). They showed that expression of SEK1 (MKK4) in MKK4-negative cell lines stimulated cell proliferation and invasion, whereas inhibition of SEK1 (MKK4) expression in MKK4-positive cells resulted in growth inhibition and higher sensitivity to serum deprivation and suppressed growth in mouse xenograft model. A recent study of MKK4-/- cell lines derived from the pancreatic cell line PL5 (Cunningham et al., 2006), demonstrated that deletion of SEK1 leads to inhibition of tumor growth in vivo, and also results in fewer lung metastases when cells were injected intravenously. Our data indicate that SEK1 is involved in the hypoxia-induced signaling pathway mediating increased oxaliplatin resistance. Signaling through this pathway (pathways) could involve not only targets of SEK1 other than JNK or p38 MAPK, and consequently the AP-1 transcriptional factor, but also up-stream mediators of signaling to MAP kinases, which specifically interact with SEK1, but not with MKK7.

The very striking effect of modulation of these signaling pathways in cell culture was demonstrated also in vivo with the SEK1 and MKK7 inhibitory constructs. Signaling through MKK7 was required to elicit the therapeutic effects of bevacizumab, of oxaliplatin and of their combination. Inhibition of SEK1 on the other hand, delayed the growth of untreated cells, and

potentiated the activity of the treatments. These data identify SEK1 as a potential target for reversal of hypoxic resistance to oxaliplatin. We have previously proposed a role for the induction of hypoxia in the action of bevacizumab (Selvakumaran et al., 2008). In HT29 colon adenocarcinoma sublines grown as xenografts (Yao et al., 2005a), hypoxia induction was observed to a comparable degree in all the cell lines, but growth inhibition was proportional to the susceptibility of the cell lines in vitro to hypoxia. The results of the in vivo experiments presented here confirm and extend these findings, and show that a determinant of the outcome of bevacizumab treatment in this model is hypoxia-induced signaling through MKK7. The activity of this pathway is similarly required for the action of oxaliplatin: the MKK7-deficient line is resistant to oxaliplatin also. We hypothesize that the integrity of this pathway may be important for the activity of both bevacizumab and oxaliplatin in human colon cancer, and clinical studies are in progress to this end. One implication of this observation is that tumors may exhibit cross-resistance to both bevacizumab and oxaliplatin under conditions of impaired MKK7 signaling. The acquisition of MKK7 mutations would be expected to be favored in resistant colon tumors, whereas mutations of SEK1 would be advantageous for efficacy of oxaliplatin.

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FOOTNOTES

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Requests for reprints should be addressed to Dr. Irina A. Vasilevskaya at the University of Pennsylvania, 1020 BRB II/III, 421 Curie Blvd, Philadelphia PA 19104. Phone: (215) 573-7300; FAX: (215) 573-7049; e-mail: vasilevs@mail.med.upenn.edu

FIGURE LEGENDS

Figure 1. Induction of AP-1 by hypoxia depends mainly on JNK activity. Activation of AP-1 family members in hypoxic HT29 cells is decreased most markedly by the inhibition of JNK, whereas inhibition of ERK has no effect.

HT29 cells were treated with the inhibitors of ERK (UO126), p38 (SB803580) and JNK (SP600125) at concentrations of 10 μM for 2 hours prior to hypoxia treatment (0.01% DMSO was added to control samples). 17-AAG was used at the concentration of 1 μM (established in previous experiments) to inhibit both JNK and ERK activities. **A** and **B**, HT29 cells were subjected to hypoxia with (1.5, 3 and 5 hours) or without MAPK inhibitors (5 hours), total protein extracts were then isolated at the indicated time points and subjected to Western blot analysis to assess the activation status of ERK pathway (A), or that of stress-activated MAPK pathways (B). The picture shown is representative of three independent experiments.

Figure 2. Hypoxia differentially activates MAPK kinases in HT29 cells, and MKK7 has a key role in signaling to AP-1. While hypoxia induces both SEK1 and MKK7 (exerting lesser effects on activities of MKK3/6 and MEK1/2), inhibition of MKK7 is more effective in down-regulation of hypoxic AP-1 activation than that of SEK1.

A, HT29 cells were subjected to hypoxia for indicated time intervals, followed by total protein extract isolation and Western blot analysis to assess the activation status of MAPK kinases. The picture shown is representative of two independent experiments. **B**, HT29 cells were transfected with AP1-Luc-reporter and plasmids encoding dominant-negative constructs for MKK7, SEK1 or JNK1/2 (or empty vector) and 24 hours post-transfection subjected to hypoxia for 8 hours, followed by Luc-reporter gene assays as described in Materials and Methods.

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Antisense oligonucleotides to c-jun were used as a negative control of AP-1 activation. Changes in AP-1 activity are presented as -fold of control (luciferase activity in oxygenated, pAP1-Luc-transfected cells was given the value of 1), with the values from non-specific luciferase activation subtracted; graphs represent the average of values derived from two independent experiments in triplicate; bars represent standard deviation.

Figure 3. Effects of hypoxia on AP-1 activation in cell lines stably expressing dominant negative MKK's confirms major role for MKK7. HT29-derived cell lines stably expressing dominant negative constructs for SEK1 or MKK7 differ in the activation of JNK/c-Jun/AP-1 pathway by hypoxia.

A, Expression of dominant negative HA-SEK1 or FLAG-MKK7 was confirmed by western blot analysis of tag proteins in HT29-derived cell lines expressing empty vector (HTpc3) and mutant kinases (HTS13 and HTM9, respectively). **B**, HTpc3, HTS13 and HTM9 cells were subjected to hypoxia for indicated time intervals, followed by protein extracts preparation and western blot analysis to assess function of stress-activated MAP kinases. The picture shown is representative of four independent experiments. **C**, HTpc3, HTS13 and HTM9 cell lines were transfected with AP1-Luc (or control) vector and 24 hours later subjected to hypoxia for 20 hours. Cellular extracts were collected and Luciferase assays were carried out as described in Materials and Methods. Graphs represent the average of values for relative light units (RLUs) - readings of *Firefly* luciferase luminescence normalized to *Renilla* luciferase luminescence. Non-specific luciferase activation determined in parallel experiments with control vector was subtracted. Open and hatched columns represent oxic and hypoxic conditions, respectively. All experiments were performed three times in duplicate; *, P values (oxic versus hypoxic).

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Figure 4. Inhibition of MKK7 confers resistance to oxaliplatin treatment under condition of hypoxia/reoxygenation. HTS13 cells display high sensitivity, whereas HTM9 cells demonstrate enhanced survival in colony-forming assays, when treated with oxaliplatin under hypoxia.

HTpc3, HTS13 and HTM9 cells were plated in 6-well plates at a density of 300 cells per well and treated with oxaliplatin for 24 hours under hypoxia as described in Material and methods. Cells were then cultivated under normal conditions, fixed and stained. Graphs present the averages of values from three independent experiments in duplicate, columns reflect the surviving colonies' counts, presented as % of control (no oxaliplatin treatment), with both oxic and hypoxic controls given the value of 100%; bars represent standard deviation; *, P values (L-OHP treated, oxic versus hypoxic conditions). The ratios of survival for hypoxic/oxic controls were 1.25 for HTpc3 and HTS13 cells, and 1.18 for HTM9 cells.

Figure 5. Efficacy of oxaliplatin alone or in combination with bevacizumab is diminished in HTM9-derived mouse xenografts.

A and **B**, Tumor growth curves for HTpc3- , HTM9- and HTS13-derived tumors. Mice were injected subcutaneously with HTpc3 (A and B), HTM9 (A) or HTS13 (B) cells as described in Materials and methods. After formation of tumors animals were treated with bevacizumab (Bev, 5mg/kg every 3 days for 30 days), oxaliplatin (L-OHP, 10 mg/kg weekly once for 2 weeks), or the combination of bevacizumab and oxaliplatin. Tumor size was measured every 3 days, and presented as average volume; bars represent standard deviation (n=6); *, $P < 0.05$; **, $P < 0.01$, ***, $P<0.001$ (HTM9 or HTS13 tumors versus control).

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Table 1. Sensitivity to oxaliplatin in dnSEK1- and dnMKK7-expressing cell lines (HTS13 and HTM9, respectively) increases differentially during hypoxia

	Oxic	Hypoxic
HTpc3	0.62 ± 0.08	1.67 ± 0.3
HTS13	0.33 ± 0.06	0.47 ± 0.07
HTM9	0.9 ± 0.1	5.1 ± 0.4

Shown are the IC_{50} concentrations (μM) derived from MTT assays; data are presented as the averages of values +/- SD (n=7)

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Table 2. Sensitivity to oxaliplatin in colon cancer cell lines infected with control (RC) or dnSEK1-expressing (RS-) retrovirus under normal and hypoxic conditions[a]

	Oxic	Hypoxic
SW480.RC[b]	0.68±0.02	1.2±0.1
SW480.RS-	0.5±0.02	0.39±0.01
HCT15.RC	2.4±0.1	7.5±0.2
HCT15.RS-	1.7±0.06	4.7±0.1
HT29.RC	0.76±0.04	1.45±0.06
HT29.RS-	0.62±0.01	0.95±0.04
BE.RC	1.3±0.1	2.3±0.1
BE.RS-	1.1±0.04	2.7±0.08

[a] Shown are the IC_{50} concentrations (μM) derived from MTT assays; data are presented as the averages of values +/- SD (n=9)

[b] Puromycin-resistant pools of SW480, HCT15, HT29 and BE cells collected after infection with control or recombinant virus were used in MTT assays

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Table 3. Effect of bevacizumab, oxaliplatin, and the combination therapy of bevacizumab and oxaliplatin on the growth delay of HTpc3-, HTM9- and HTS13-derived tumors

Treatment	Tumor Growth Delay[a]				
	HTpc3	HTM9	P value*	HTS13	P value*
Bevacizumab	5.4[b]	2.7[c]	< 0.01	8[c]	< 0.01
Oxaliplatin	7.5	2	<0.001	10	<0.01
Bevacizumab + Oxaliplatin	10.9	1.4	< 0.001	13	<0.05

[a] Tumor growth delay was calculated as time (days) needed for treated tumor to grow from 100 to 600 mm^3 minus the time needed for control tumor to grow to the same size;

[b] Mean values from the twelve tumors;

[c] Mean values from the six tumors;

* P values (treated versus untreated tumors).

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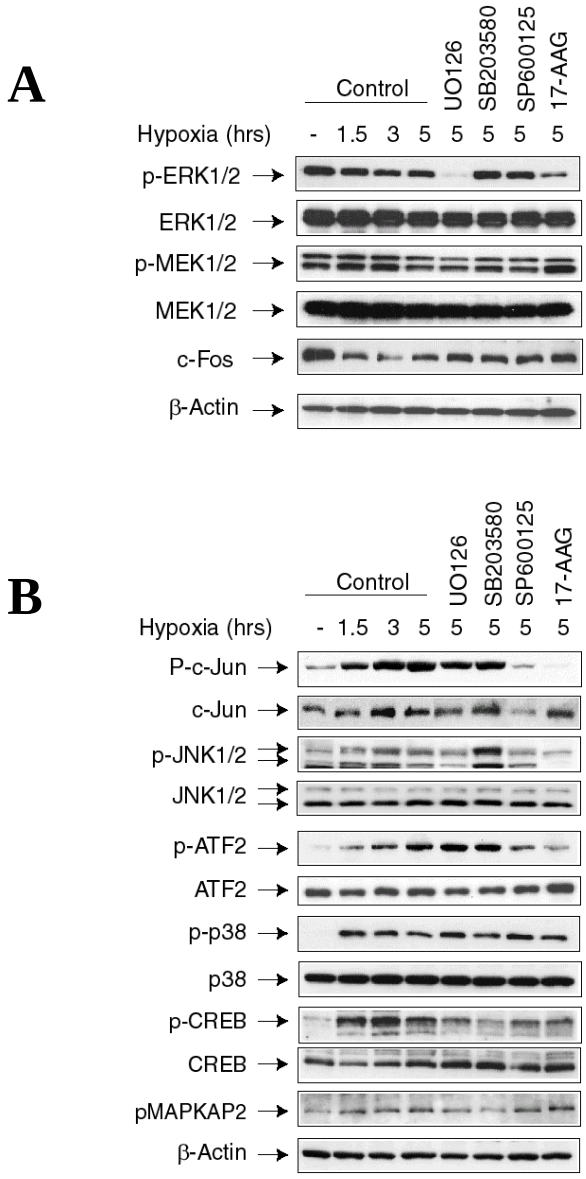

Figure 1

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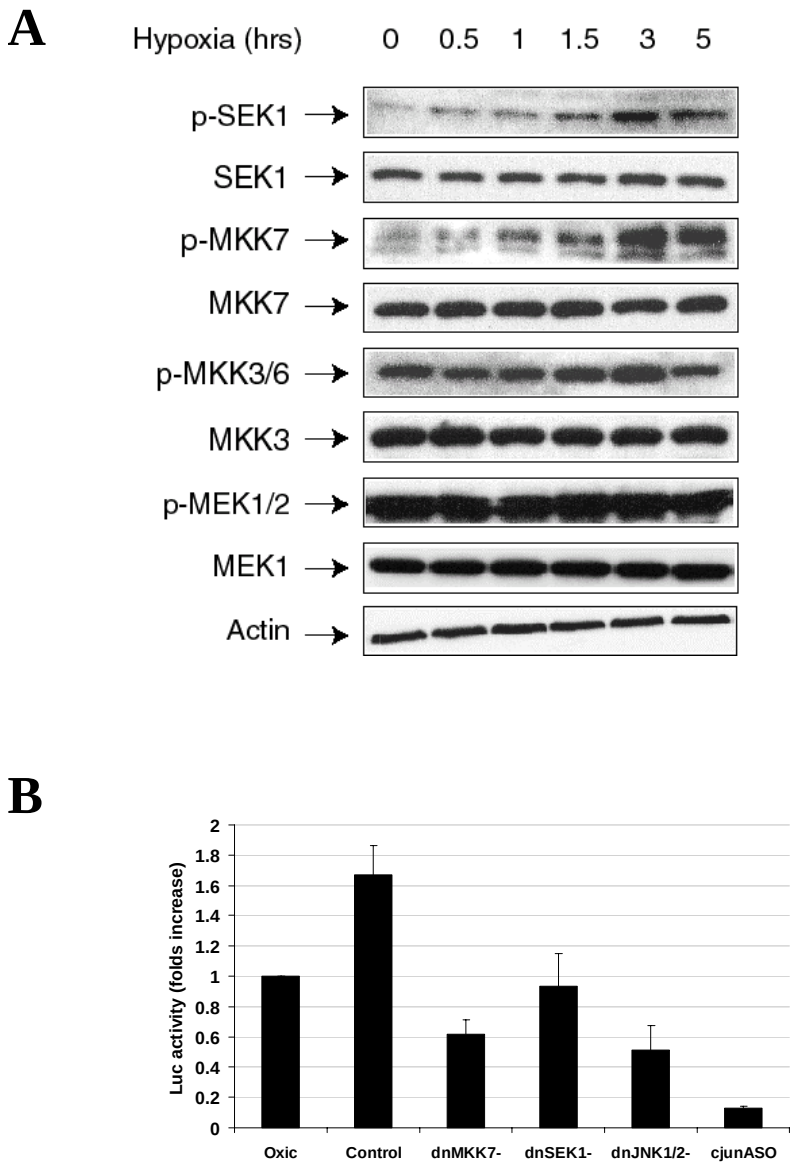

Figure 2

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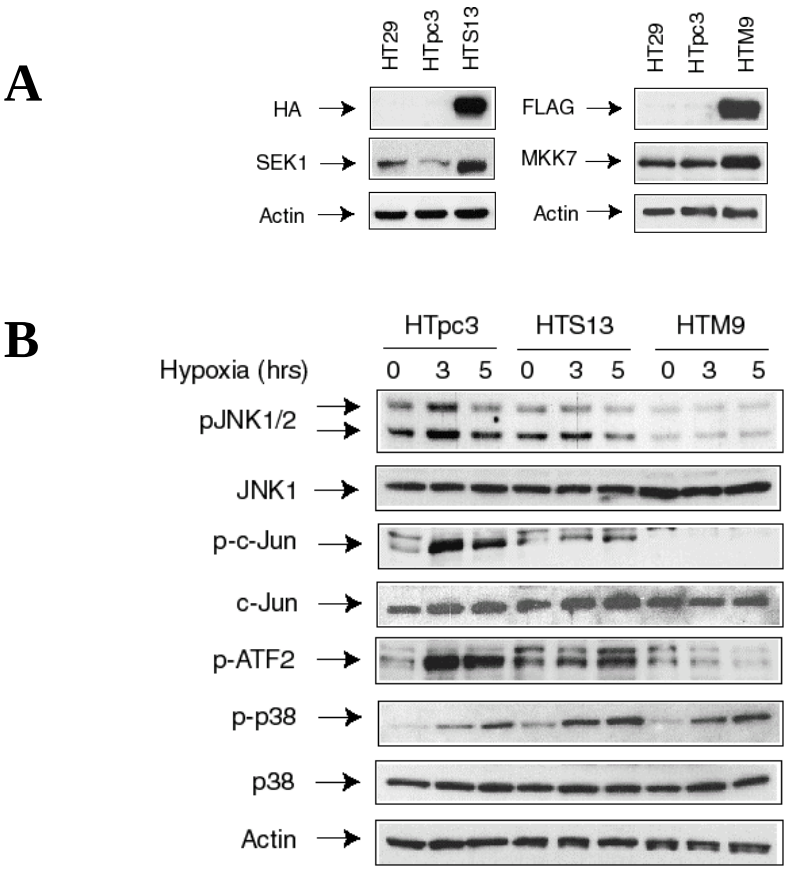

Figure 3

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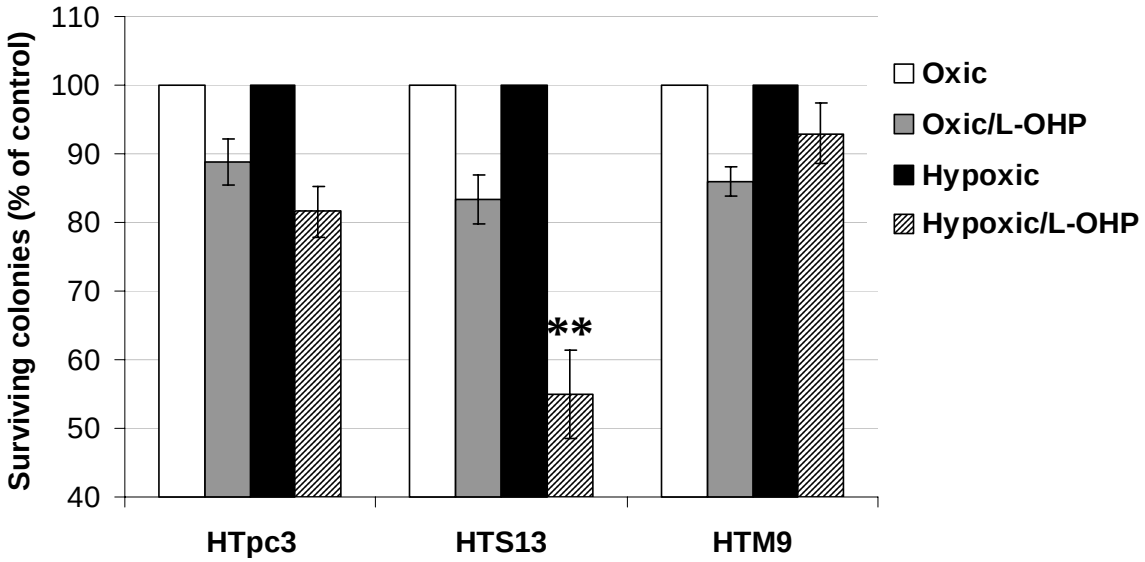

Figure 4

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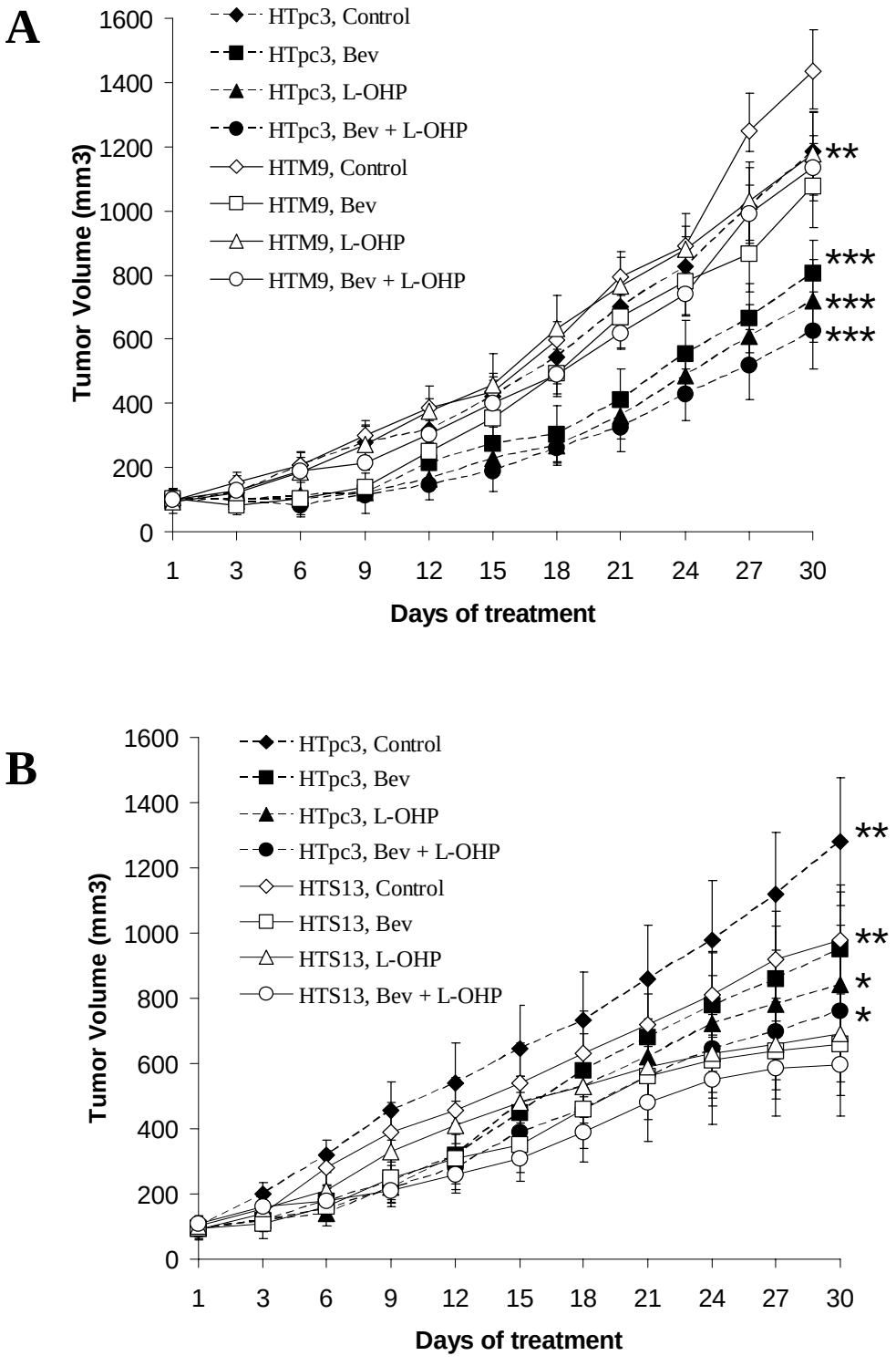

Figure 5