

MEK/ERK Inhibitor U0126 Increases the Radiosensitivity of Rhabdomyosarcoma Cells *In vitro* and *In vivo* by Downregulating Growth and DNA Repair Signals

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Abstract

Multimodal treatment has improved the outcome of many solid tumors, and in some cases the use of radiosensitizers has significantly contributed to this gain. Activation of the extracellular signaling kinase pathway (MEK/ERK) generally results in stimulation of cell growth and confers a survival advantage playing the major role in human cancer. The potential involvement of this pathway in cellular radiosensitivity remains unclear. We previously reported that the disruption of c-Myc through MEK/ERK inhibition blocks the expression of the transformed phenotype; affects *in vitro* and *in vivo* growth and angiogenic signaling; and induces myogenic differentiation in the embryonal rhabdomyosarcoma (ERMS) cell lines (RD). This study was designed to examine whether the ERK pathway affects intrinsic radiosensitivity of rhabdomyosarcoma cancer cells. Exponentially growing human ERMS, RD, xenograft-derived RD-M1, and TE671 cell lines were used. The specific MEK/ERK inhibitor, U0126, reduced the clonogenic potential of the three cell lines, and was affected by radiation. U0126 inhibited phospho-active ERK1/2 and reduced DNA protein kinase catalytic subunit (DNA-PKcs) suggesting that ERKs and DNA-PKcs cooperate in radioprotection of rhabdomyosarcoma cells. The TE671 cell line xenotransplanted in mice showed a reduction in tumor mass and increase in the time of tumor progression with U0126 treatment associated with reduced DNA-PKcs, an effect enhanced by radiotherapy. Thus, our results show that MEK/ERK inhibition enhances radiosensitivity of rhabdomyosarcoma cells suggesting a rational approach in combination with radiotherapy. *Mol Cancer Ther*; 10(1); 159–68. ©2011 AACR.

Introduction

Rhabdomyosarcoma (RMS) is a rare malignancy. Nonetheless, it is a common childhood cancer, constituting more than 50% of all soft tissue sarcomas. In contrast, rhabdomyosarcoma is exceedingly infrequent in adults;

soft tissue sarcomas make up less than 1% of all adult malignancies, and RMS accounts for 3% of all soft tissue sarcomas (1). Treatment for this malignancy requires a multimodality approach combining surgery with radiotherapy (RT) and/or chemotherapy. Although overall outcomes have improved considerably, the outcome for patients with high-risk disease remains relatively poor, which points to a clear need for new therapeutic strategies. Most RMSs are not amenable to complete surgical resection, and for the majority (70%) of patients recurrence occurs within the first 2 years after treatment (1, 2). In this scenario RT is a major tool in the treatment of RMS. It can eradicate residual tumor cells, especially when the surgical eradication is not complete or limited by the anatomic position such as in the RMS of head and neck and the pelvis region (3). However, local recurrence remains a significant clinical obstacle and represents a common pattern for treatment failure for RMS. One of the major goals of local control for tumors in patients already treated with surgery and chemotherapy is to enhance the sensitivity of RMS tumor cells to the cytotoxicity of ionizing radiation. The substantial experimental body of evidence demonstrates that radiation resistance is associated with the

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abnormal expression of activated oncogenes, including Ras (4–5) and c-Myc (6). The Ras/Raf/mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) cascade regulates proliferation, differentiation, survival, motility, and tissue formation (7–16). Mutated forms of Ras are found in 30% of human cancers (9–11) including RMS (12), and mutations produce proteins that remain locked in a constitutively active state, thereby relaying uncontrolled signals (13). The Ras-MAPK pathway, when constitutively activated, mediates resistance to ionizing radiation (14) through epidermal growth factor (EGF) induction and epidermal growth factor receptor (EGFR)-mediated activation of prosurvival PI3K-AKT pathway, which in turn activates DNA protein kinase catalytic subunit (DNA-PKcs; ref. 15). The Myc/Max transcription factor family plays a role in human cancer (16). In conditional transgenic models, Myc-induced tumors can regress on reduction in Myc transgene expression (17). The Ras/Raf/MEK/ERKs pathway induces c-Myc stability and GSK-3 β reduces its stability (18–19). Accordingly, Ras/MEK/ERK activation/phosphatidylinositol 3-kinase/AKT-mediated GSK-3 β inactivation leads to c-Myc accumulation (20). Interestingly, the radiation-resistant phenotype of cells transformed by mutated Ras is enhanced by the c-Myc oncogene (6, 21). The Ras/Raf/MEK/ERKs pathway has been considered as a target for the radiosensitization of cancer cells (22) but no data have been reported in RMS as radiosensitizer agents.

DNA double-strand break is critical in DNA lesions induced by radiation. In mammalian cells the repair of these lesions occurs by nonhomologous end joining requiring Ku70/Ku86 and the recruitment of DNA-PKcs, which phosphorylates and regulate proteins involved in ligation processes. DNA-PKcs determines radioresponsiveness of human glioblastoma cell lines (23–24). DNA-PKcs also plays a role in cell cycle checkpoint control, cell death, and protein stabilization such as p53 and c-Myc (25, 26). DNA-PKcs is necessary for genomic stability whereas abnormal levels in cancer cells may contribute to cell proliferation, radioprotection, and change in c-Myc levels, eventually contributing to oncogenic phenotype. The relationship between DNA-PKcs level and chemosensitivity and radioresponse has been documented (27–29). Moreover, the human cell lines which are deficient in DNA-PKcs are radiosensitive because of the inefficient DNA DSB repair (30).

We previously showed in cultured embryonal rhabdomyosarcoma (ERMS)-derived cell lines (RD) that the transformed phenotype could be reversed by disrupting c-Myc through the specific MEK/ERK inhibitor, U0126 (31), which prevented activation of MEK1/2 (32) and ERK pathways. U0126 induces growth arrest in *in vitro* and *in vivo* ERMS models as seen in other tumor types (9).

We investigated whether MEK/ERK inhibition, by U0126, affects the radiosensitivity of ERMS tumor in an *in vitro* and *in vivo* model of xenograft. Herein, U0126 suppressed colony formation of RD xenograft-derived cell line RD-M1 and TE671 ERMS cell lines.

U0126 increased the radiosensitivity of ERMS-xenografted nude mice. Both *in vitro* and *in vivo* models of MEK/ERK inhibition downregulated c-Myc and DNA-PKcs. These results corroborate the idea that Ras/Raf/MEK/ERKs pathway may be a useful target for enhancement of RT effect. It is therefore essential to conclude that MEK inhibitors may have an important role in combination with RT for patients with ERMS.

Materials and Methods

Cell cultures, treatments, and radiation exposure

The human ERMS RD cell lines were obtained from American Type Culture Collection (ATCC) in 2004. The ERMS TE671 (HTL97021) cell lines were obtained from Interlab Cell Line Collection in 2006. RD cell line was tested and authenticated by ATCC for the expression of myoglobin and myosin ATPase cellular products. TE671 cell line were tested and authenticated by Interlab Cell Line Collection for the expression of nicotinic acetylcholine receptor, acetylcholine receptor, and peripheral type benzodiazepine receptor. Tumor-derived RD-M1 cell lines were obtained from RD cells xenografted on nude mice as previously described (29) and tested and authenticated by our laboratory for the expression of myoglobin and myosin ATPase cellular products. The cell lines are tested in our laboratory every year for the expression of specific markers by Western blot analysis and were last tested in 2009. The cell lines were cultured as previously described (29). Treatment with 10 μ mol/L MEK/ERK inhibitor U0126 (1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene; Promega) was done for the times shown in the figures and started before radiation lasting for 24 hours. Radiation was delivered at room temperature using an x-6 MV photon linear accelerator. The total single dose of 4 Gy was delivered with a dose rate of 2 Gy/min using a source-to-surface distance (SSD) of 100 cm. Doses of 200 kV X-rays (Yxlon Y.TU 320; Yxlon) were filtered with 0.5 mm Cu. The absorbed dose was measured using a Duplex dosimeter (PTW). The dose rate was approximately 1.3 Gy/min and applied doses ranged from 0 to 6 Gy.

Colony formation assay

For clonogenic survival assay, exponentially growing RD, RDM1, and TE671 cells in 25-cm² flasks were harvested by exposure to trypsin and counted. They were diluted serially to appropriate densities and plated in triplicate in 6 multiwell plates with 2 mL of complete medium in each well in the presence or absence of 10 μ mol/L U0126 or vehicle for 24 hours [final dimethyl sulfoxide (DMSO) concentration of 0.1%; we confirmed that this DMSO concentration did not affect the proliferation of RD and TE671 and RD-M1 cell lines]. After incubation for 24 hours, the cells were exposed at room temperature to various doses of radiation as already described. The cells were then washed with PBS, cultured in drug-free medium for 14 days, fixed with methanol/

acetic acid (10:1, v/v), and stained with crystal violet. Colonies containing more than 50 cells were counted. The plating efficiency (PE) was calculated as the number of colonies observed/the number of cell plated; the surviving fraction (SF) was calculated as follows: colonies counted/cells seeded \times (PE/100).

Immunoblot analysis

Western blotting was conducted as described previously (33). Briefly, cells from cultures were lysed in 2% SDS containing phosphatase, and protease inhibitors (Roche) sonicated for 30 seconds. Proteins of whole-cell lysates were assessed using the method described by Lowry and colleagues (34), and equal amounts were separated on SDS-PAGE. Tumors were crushed in nitrogen, and the powder was collected and resuspended in 2% SDS containing phosphatase and protease inhibitors, sonicated for 30 seconds, and clarified by centrifugation. Aliquots of tissue extracts were used for total protein evaluation. SDS-PAGE analysis was done as for cultured cells. The proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell, Bioscience) by electroblotting. The total protein level balance was confirmed by staining the membranes with Ponceau S (Sigma). Immunoblottings were done as described previously (35) with the following antibodies: anti-c-Myc (N-262), anti-phospho-ERK1/2 (E-4), anti-ERK2 (C-14), anti-phospho-GSK3, anti-Cyclin D1, and GAPDH (all from Santa Cruz Biotechnology), and DNA-PKcs (from Abcam). Peroxidase-conjugate antimouse or antirabbit IgG (Amersham GE Healthcare) were used for enhanced chemiluminescence detection.

In vivo experiments, U0126, and radiotherapy

Female CD1 athymic nude mice (Charles River) were maintained under the guidelines established by our Institution (University of L'Aquila, Medical School and Science and Technology School Board Regulations, in compliance with the Italian government regulation n.116 January 27, 1992 for the use of laboratory animals). Before any invasive manipulation, mice were anesthetized with a mixture of ketamine (25 mg/mL)/xylazine (5 mg/mL). For xenotransplants, exponentially growing TE671 cells were detached by trypsin-EDTA, washed twice in PBS, and resuspended in saline solution at cell densities of 1×10^6 /mL. Xenotransplants were done in 45-day-old female nude CD1 mice by subcutaneous injection in the leg using a 21-gauge needle on a tuberculin syringe. Treatments started when tumors reached a volume of 0.2 to 0.5 cm³.

U0126 solution was prepared in DMSO as a stock solution of 10 μ mol/L and the amount of drug to be injected into a set of mice was diluted with carrier solution (40% DMSO in physiologic solution). The U0126 dose here used has already been tested and found to be nontoxic to mice and efficient in inducing downregulation of ERK1/2 in the tumors (36). U0126 was administered 3 times per week, the day before RT. This protocol was chosen because a full inhibition of ERK activation is

guaranteed *in vivo* after 24 hours and was documented after this time (36). Mice were irradiated at room temperature using an Elekta 6-MV photon linear accelerator. Five fractions of 2 Gy were delivered over 5 consecutive days for a total dose of 10 Gy. A dose rate of 1.5 Gy/min will be used with an SSD of 100 cm. Prior to irradiation, mice were anesthetized and were protected from off-target radiation by a 3-mm lead shield. Before tumor inoculation, mice were randomly assigned to 4 experimental groups. Each group was composed of 8 mice. One control group received intraperitoneal injection of 200 μ L carrier solution; 1 group received intraperitoneal injection of 200 μ L U0126 solution at the dose of 25 μ mol/kg; 1 group received RT (6 fractions of 2 Gy delivered 3 times per week to a total dose of 12 Gy); 1 group received 200 μ L U0126 solution at the dose of 25 μ mol/kg coupled with radiotherapy (6 fractions of 2 Gy delivered 3 times per week to a total dose of 12 Gy) delivered 24 hours after the beginning of treatment with U0126 (Fig. 4). Experiments were stopped 12 days after the last RT treatment and mice were sacrificed by carbon dioxide inhalation. Tumors were directly frozen in liquid nitrogen for protein analysis and biochemical evaluation.

Evaluation of treatment response *in vivo*

The effects on tumor growth of different treatments were evaluated as follows: (a) tumor volume was assessed every 4 days and at the end of the experiment with a Vernier calliper (length \times width), expressed in mm³ according to the formula $4/3\pi r^3$; (b) tumor weight was measured at the end of experiment; and (c) for assessment of time to progression (TTP), tumor progression (TP) was defined as an increase of greater than 100% of tumor volume with respect to baseline.

Statistical methods

Continuous variables were summarized as mean and SD or as median and 95% confidence interval for the median. For continuous variables, statistical comparisons between control and treated groups were established by carrying out the Kruskal-Wallis test (a parametric 1-way analysis of variance for independent groups) or the Mann-Whitney test (in the case of 2 independent groups). Dichotomous variables were summarized by absolute and/or relative frequencies. For dichotomous variables, statistical comparisons between control and treated groups were established by carrying out the exact Fisher's test. For multiple comparisons the level of significance was corrected by multiplying the *P* value by the number of comparisons performed (*n*) according to Bonferroni correction. TTP was analyzed by Kaplan-Meier curves and Gehan's generalized Wilcoxon test. When more than 2 survival curves were compared, the Log-rank test 10 for trend was used. This tests the probability that there is a trend in survival scores across the groups. All tests were 2-sided and were determined by Monte Carlo significance. *P* < 0.05 was considered statistically significant.

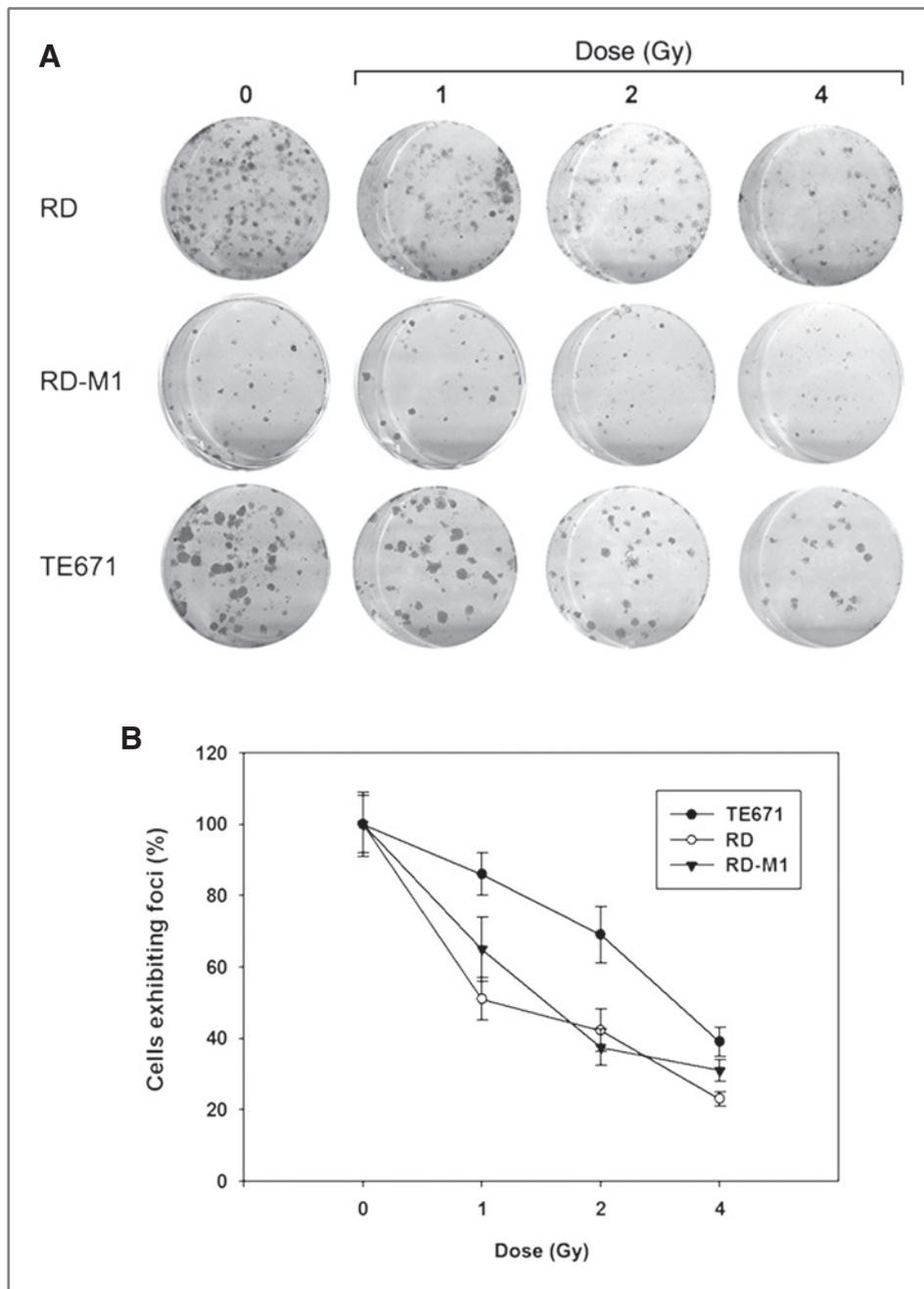


Figure 1. Clonogenic assay of embryonal RMS cell lines, RD, RD-M1, and TE671 at different Gy doses. **A**, Crystal violet-stained cultures 15 days after irradiation at indicated doses (1, 2, 4 Gy); **B**, dose-dependent colony formation, each point of the 3 curves is the average \pm SEM of 3 samples. Similar results were obtained in 3 experiments.

The effects of the treatments were examined as previously described by Prewett and colleagues (37). The effect on tumor growth was measured by taking the mean tumor volume on day 24 for the different treatment groups: controls, treatment with RT (treatment a); treatment with U0126 (treatment b); and treatment with RT + U0126 (treatment a + b). For tumor volume, fractional tumor volume (FTV) for each treatment group was calculated as the ratio between the mean tumor volumes of treated and untreated tumors. For TTP, fractional TTP (FTTP) for each treatment group was calculated as the ratio between

the median TTP of untreated and treated tumors. This was done for treatment a, treatment b, and treatment a + b. The expected FTV or FTTP for the a + b combination was defined as $FTV_{a-observed} \times FTV_{b-observed}$ or as $FTTP_{a-observed} \times FTTP_{b-observed}$. The ratio $FTV_{a+b-expected} / FTV_{a+b-observed}$ OR $FTTP_{a+b-expected} / FTTP_{a+b-observed}$ was the combination index (CI). If combination index > 1 , there are supra-additive effects and if combination index < 1 infra-additive ones. Strictly additive effects are observed if combination index = 1. All statistical analyses were performed using the SPSS statistical analysis software package, version 10.0.

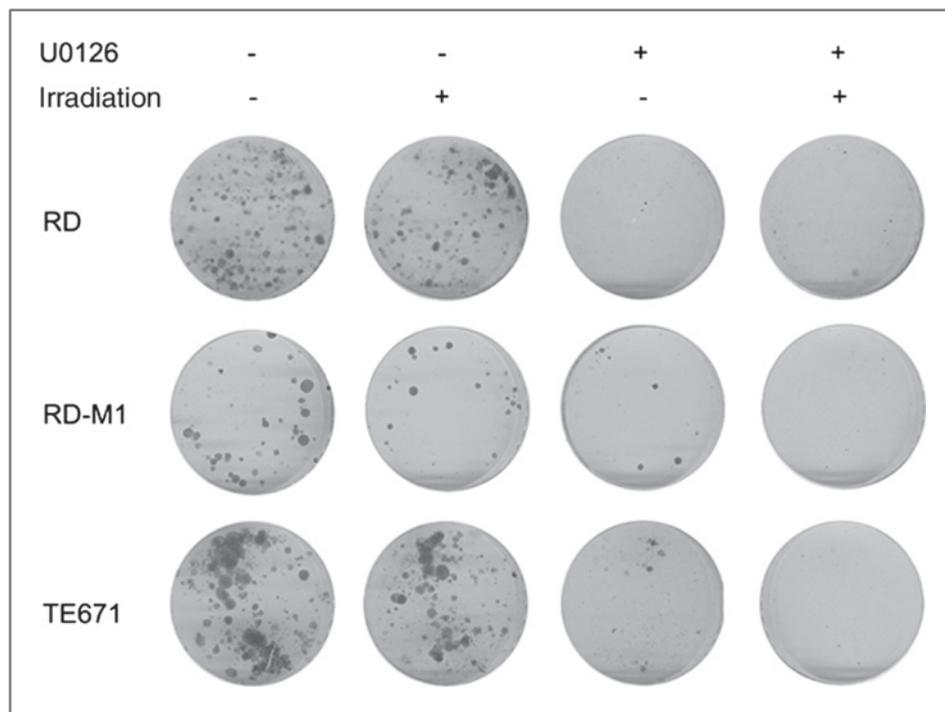


Figure 2. Clonogenic assay of the 3 embryonal cell lines with (+) or without (-) U0126 and 4-Gy irradiation treatments. Crystal violet stained cultures 15 days after U0126, RT, or combined treatment.

Results

Radiation decreases clonogenic survival of embryonal RMS cell lines

To explore the differential effect of radiation on RD, RD-M1, and TE671 cell lines, clonogenic survival was determined on treatment with increasing radiation doses (0–6 Gy; Fig. 1). Increasing doses of radiation significantly ($P < 0.05$) decreased the number of colony formations compared with control (Fig. 1A). This was associated with a reduction of clonogenicity in all tested cell lines (Fig. 1B). RD and RD-M1 cell lines showed the greatest sensitivity to RT (Fig. 1 A and B).

MEK/ERK inhibition synergistically increases the radiosensitivity of RMS cell lines

As previously reported (31, 36) MEK/ERK inhibition reverses the transformed phenotype of ERMS cells. Clonogenic survival was determined in tumor cell cultures

on treatment with a potent MEK/ERK inhibitor U0126 1 hour before radiation of RD, RDM1, and TE671 (Fig. 2; Table 1). The rationale of treating the cells before radiation was to assess whether the inactivation of MEK/ERK pathway positively enhanced radiosensitization of the tumor cell lines (Fig. 2).

As shown in Table 1, the more radioresistant tumor cell line was TE671 with SF of 0.83. Although the antitumor effect of U0126 was evident in all cell lines, the U0126 synergistically increased the radiosensitivity in all 3 cell lines (Table 2).

MEK/ERK inhibition inhibits growth and radioresistant signals in RD and TE671 cells

DNA-PKcs is upregulated in many tumors and is important in the radiation response. DNA-PKcs abundance was assessed by Western blot in response to U0126 (Fig. 3A and B). The U0126 treatment alone and in combination with radiation reduced (Fig. 3A and B; see also supplementary data) DNA-PKcs in parallel with c-Myc inhibition. The combined treatment of U0126 with radiation inhibited cyclin D1 expression more than either treatment alone. U0126 alone inhibited phospho-active ERKs but not GSK3- β .

U0126 with radiation results in synergistic antitumor effects *in vivo*

For *in vivo* experiments, the TE671 cell line was chosen due to its intrinsic radioresistance in comparisons with the other tumor cells (Fig. 1). When tumor volume reached 0.5 to 1.0 cm³ (T_0), U0126 was intraperitoneally administered and followed by radiation 1 day later (Fig. 4). Tumor volumes were measured every 4 days for a period of

Table 1. Surviving fraction after treatments

Cell line	Plating efficiency	RT surviving fraction	U0126 surviving fraction	U0126 + RT surviving fraction
RD	0.8	0.67	0.17	0.08
RD-M1	0.67	0.42	0.3	0.14
TE671	0.84	0.83	0.24	0.11

Abbreviation: RT, radiotherapy.

Table 2. Synergism of additivity of combined treatment

Cell line	RT observed effect	U0126 observed effect	U0126 + RT observed effect	U0126 + RT expected effect	Combination index
RD	0.84	0.22	0.10	0.18	Synergism (1.8)
RD-M1	0.63	0.45	0.21	0.28	Synergism (1.3)
TE671	0.76	0.29	0.13	0.22	Synergism (1.7)

24 days in untreated (control), U0126-treated (U0126), irradiated (RT), and U0126/Irradiated (RT + U0126) tumors (Fig. 4A). Tumor masses from irradiated mice grew rapidly from day 12 of treatments but at lesser extent than control mice (Fig. 4A). U0126-treated mice grew at significantly less extent (45% of inhibition at end point; ref. 36). The treatment with U0126 before RT decreases growth further with an 80% inhibition. Computed CI value, in terms of tumor volume as outcome measure, was determined on day 24 after cell implantation and indicated synergistic effect for the combination of RT with U0126 (CI = 1.92). Tumor weights in mice treated with U0126 and RT decreased significantly ranging from 60% to 80% with respect to controls (Fig. 4B). Treatments with U0126 or RT alone were not as effective as in combination (Fig. 4B).

U0126 synergizes with radiation in delaying time to progression

The number of mice with tumor progression significantly differed across the groups and this was confirmed by the Kaplan–Meier curves (Fig. 5A) and the median values of TTP (Fig. 5B). In the control group, tumor

progression occurred within 14 days after the beginning of treatment (Fig. 5A) with a median TTP of 13 days (95% confidence interval, 11.3–13.7). On RT treatment, a negligible improvement in the TTP was documented compared with controls ($P = 0.10$). In the RT group, the tumor progression occurred within 15 days after RT with a median TTP of 13.5 days (95% confidence interval, 12.3–14.7). The treatment with U0126 significantly improved the TTP compared with controls ($P = 0.0002$) or RT ($P = 0.0002$). In the group treated by U0126, tumor progression occurred from day 17 after the beginning of treatment and completed before day 20. The median TTP after this treatment was 18 days (95% confidence interval, 17.0–19.7). The most evident improvement was documented when U0126 was coupled with RT. This combined treatment significantly improved the TTP compared with RT ($P < 0.0001$) or U0126 ($P < 0.0001$) with a clear synergistic effect (CI = 1.27). For this group, the median TTP was 24 days (95% confidence interval, 22.0–24.0) and the tumor progression was documented only from day 22 after the beginning of treatments.

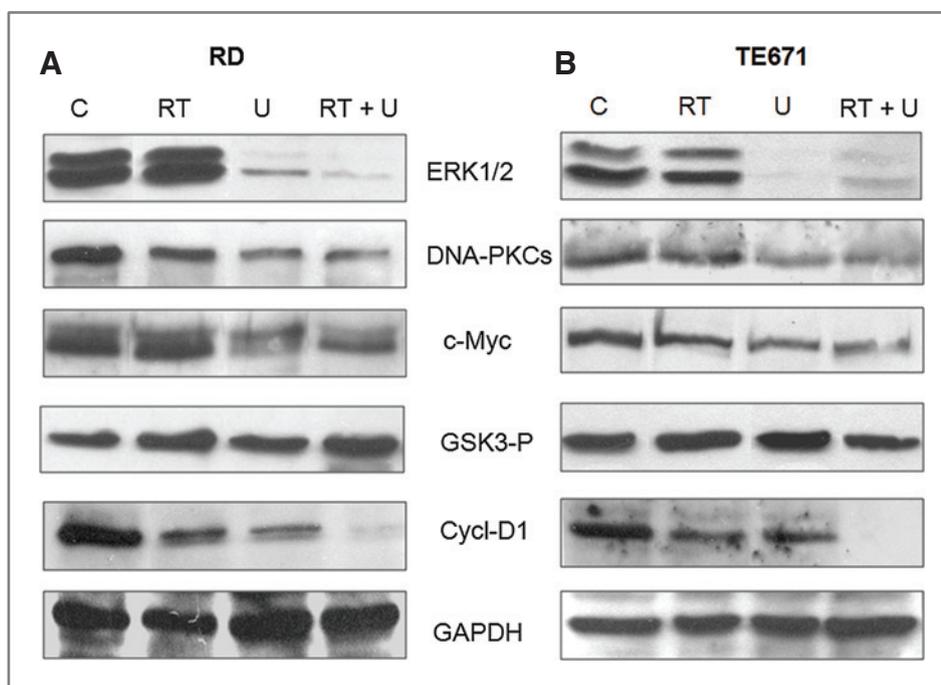
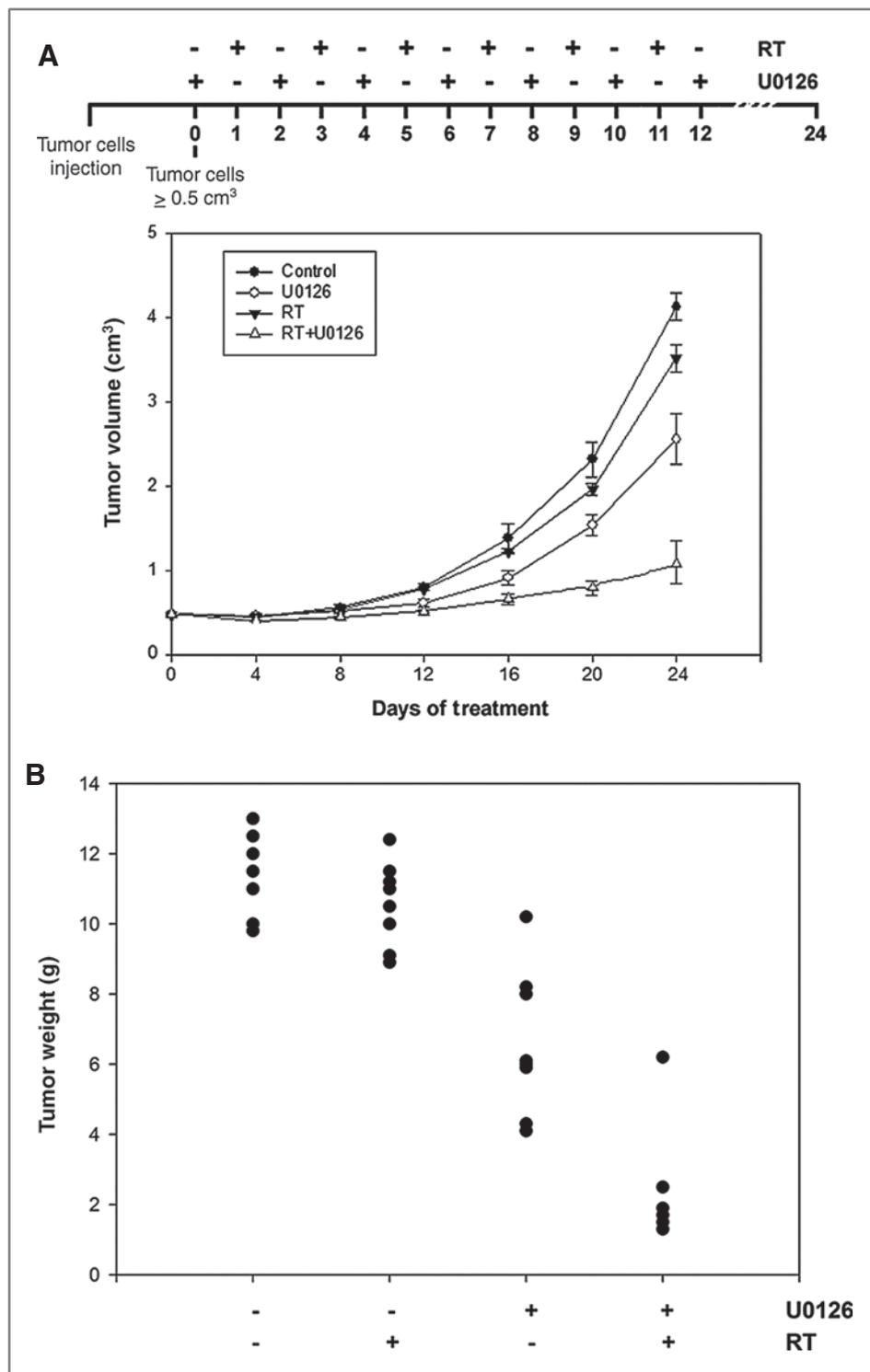


Figure 3. Expression of protein kinases (ERK1/2-P04, DNA-PKcs, GSK3-beta-P04), c-Myc, and cyclin D1 in untreated (C), irradiated (RT), U0126-treated (U), and U0126-pretreated and irradiated (RT + U) RD cells (A) and TE671 (B). GAPDH was blotted as loading control.

Figure 4. A, growth curve of tumors volumes from xenografted TE671 cell lines, untreated (●-●), U0126-treated (○-○), irradiated (RT ▼-▼), U0126-pretreated, and irradiated (RT+U ▲-▲). Tumor volumes were evaluated as described in Materials and Methods and represent the mean \pm SEM of 8 mice. The upper panel shows the sequential treatments of xenografted mice started when tumor reached a volume of approximate 0.5 cm³. U0126 was administered 1 day before each irradiation. B, tumor weights in mice untreated or treated with U0126, RT, or combined treatment.



DNA-PKcs and c-Myc tumor expression is reduced by MEK/ERK inhibition and radiation *in vivo*

Tumors from different settings at end point were analyzed for the expression levels of phospho-ERKs, cMyc, DNA-PKcs, and cyclin D1. c-Myc, DNA-PKcs, and cyclin D1 expression levels were decreased on

U0126 treatment either alone or in combination with RT (Fig. 6). ERK inhibition was evaluated in tumors that were taken 16 hours after the last U0126 administration. These results support the hypothesis that *in vivo* radiosensitivity can be recovered following growth and DNA-repair signal inhibition.

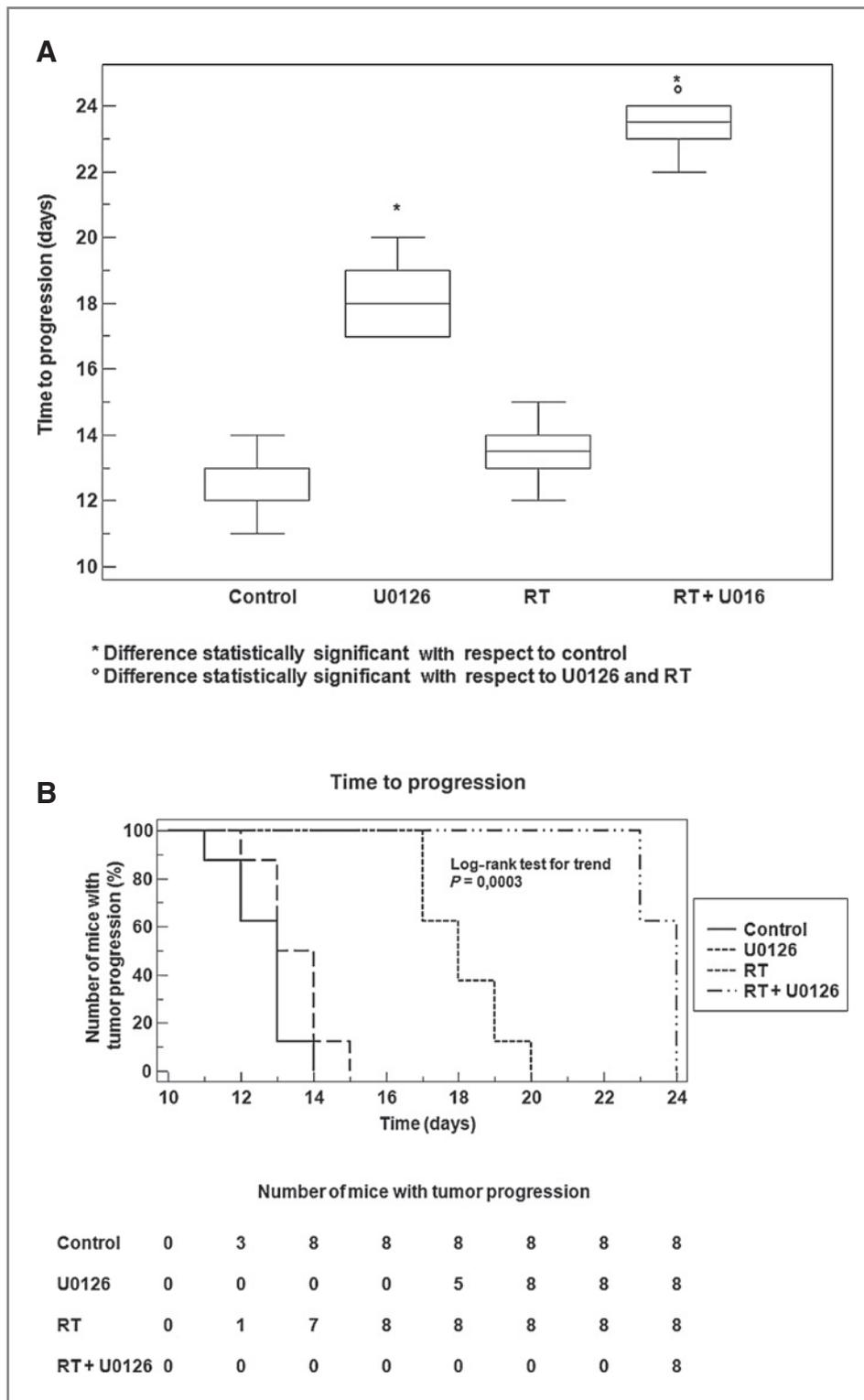


Figure 5. A, U0126 with RT combined treatment affects time to progression *in vivo* xenografted tumors. B, box plot diagram comparing median time (days) of tumor progression among treatment groups. Box represents interquartile range (IQR), bar median value, and whiskers data points within IQR.

Discussion

The Ras-MAPK pathway is important in radioresistance and activated oncogenic Ras mediates resistance to ionizing radiation (14). Active Ras-protein induces

MAPK- and PI3K/AKT-pathway via autocrine mechanisms such as the production of EGFR ligands (4, 5, 38). It has been recently reported that radiation-mediated EGFR activation induces cell survival also supported by DNADSB repair through direct interaction with DNA-

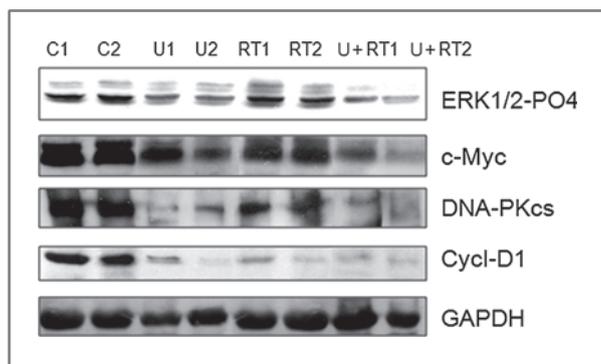


Figure 6. Expression of protein kinases (ERK1/2 and DNA-PKcs), c-Myc, and cyclin D1 in tumors untreated (C1, C2), U0126-treated (U1, U2), irradiated (RT1, RT2), and U0126-pretreated and irradiated (U + RT1, U + RT2). At the end point mice were sacrificed, and the tumor was excised and extracted as described in Materials and Methods. GAPDH was blotted as loading control.

PKcs. In this regard, the effect of Ras/MEKs/ERKs inhibition and the downstream target pathways in radiation response has not yet been studied in RMS tumors.

We previously reported that MEK/ERK pathway inhibition reverses the transformed phenotype of RMS in *in vivo* and *in vitro* systems (31, 36). Herein, MEK/ERK inhibition enhances radiosensitivity of ERMS cell lines. RT diminished the colony formation of 3 ERMS cell lines and pretreatment with the MEK/ERK inhibitor, U0126, synergistically enhances the RT response also in TE671 line which was more radioresistant.

U0126 treatment downregulated phospho-active ERKs cyclin D1, and c-Myc. RT alone sensibly reduced DNA-PKcs and cyclin D1 expression levels, the latter being even more affected by U0126 and strongly downregulated in combined U0126 and RT. The role of cyclin D1 in cell survival has been documented. Cyclin D1^{-/-} mouse embryonic fibroblast have enhanced response invoked by γ -irradiation (39), consistent with earlier experiments showing that cyclin D1 expression inhibited UV-induced apoptosis in human trophoblastic cells (40). A positive correlation between cyclin D1 expression and tumor radioresistance has been proposed earlier (41). Cyclin D1 has been implicated in radioresistance that can be inhibited by short interfering RNA (42). In line with these findings, U0126 pretreatment reduced cyclin D1, enhancing decrease in clonogenic survival. U0126-mediated decrease of c-Myc is consistent with the role of ERKs and DNA-PKcs in mediating c-Myc stabilization and expression (26, 43). Inhibition of DNA-PKcs suppressed tumor cell growth and enhanced cervical cancer cell sensitivity to chemotherapeutic agents (44). Consistently, in RMS the synergistic

effect of MEK/ERK inhibitor on irradiation results from the targeting of DNA-PKcs and cyclin D1 compromising DNA-repair and growth mechanisms, respectively, thus preventing oncogenic phenotype expression.

Growth of tumors from TE671 which, in the *in vitro* system, are more radioresistant than other ERMS cell lines were inhibited by U0126 by 47% after U0126 alone whereas the radiation itself was inhibited by 13% at the end points. Combined RT and MEK inhibition reduced tumor mass of 75%. Consistently, data analysis on tumor time progression provided the evidence of a synergistic effect of the MEK inhibitor when combined with RT. Immunobiochemical evaluation of tumors demonstrated concordant findings to the *in vitro* analysis. Phospho-ERK was inhibited by U0126 either alone or in combination with RT. The DNA-PKcs and c-Myc levels were decreased after U0126 but not by RT. Cyclin D1 was reduced by U0126 or RT and further inhibited by combined therapy. The role of cyclin D1 in radioprotection or radiosensitivity is still an open question although it has been reported that the sensitivity of the cyclin D1-overexpressing MCF7 human breast cancer cell line to ionizing radiation was higher than that of cells that did not overexpress cyclin D1 (45). In our system, cyclin D1 decrement by both U0126 and radiation *in vitro* and *in vivo* system might be indicative of the success of combined therapy in RMS.

This article shows for the first time that greater success in irradiation of ERMS tumors can be achieved when combined with signal transduction-based chemotherapy is applied.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

- Ferrari A, Dileo P, Casanova M, Bertulli R, Meazza C, Gandola L, et al. Rhabdomyosarcoma in adults. A retrospective analysis of 171 patients treated at a single institution. *Cancer* 2003; 98:571–80.
- Frustaci S, Gherlinzoni F, De Paoli A, Bonetti M, Azzarelli A, Comandone A, et al. Adjuvant chemotherapy for adult soft tissue sarcomas of the extremities and girdles: results of the Italian randomized cooperative trial. *J Clin Oncol* 2001; 19:1238–47.

3. Paulino AC. Role of radiation therapy in parameningeal rhabdomyosarcoma. *Cancer Invest* 1999;17:223–30.
4. McKenna WG, Weiss MC, Bakanauskas VJ, Sandler H, Kelsten ML, Biaglow J, et al. The role of the H-ras oncogene in radiation resistance and metastasis. *Int J Radiat Oncol Biol Phys* 1990;18:849–59.
5. Jones HA, Hahn SM, Bernhard E, McKenna WG. Ras inhibitors and radiation therapy. *Semin Radiat Oncol* 2001;11:328–37.
6. Chiang CS, Sawyers CL, McBride WH. Oncogene expression and cellular radiation resistance: a modulatory role for c-myc. *Mol Diagn* 1998;3:21–7.
7. O'Neill E, Kolch W. Conferring specificity on the ubiquitous Raf/MEK signaling pathway. *Br J Cancer* 2004;90:283–8.
8. Murphy LO, Blenis J. MAPK signal specificity: the right place at the right time. *Trends Biochem Sci* 2006;31:268–75.
9. Kohno M, Pouyssegur J. Pharmacological inhibitors of the ERK signaling pathway: application as anticancer drugs. *Prog Cell Cycle Res* 2003;5:219–24.
10. Faivre S, Djelloul S, Raymond E. New paradigms in anticancer therapy: targeting multiple signaling pathways with kinase inhibitors. *Semin Oncol* 2006;33:407–20.
11. Liu D, Liu Z, Condouris S, Xing M. BRAF V600E maintains proliferation, transformation, and tumorigenicity of BRAF-mutant papillary thyroid cancer cells. *J Clin Endocrinol Metab* 2007;92:2264–71.
12. Martinelli S, McDowell HP, Vigne SD, Kokai G, Uccini S, Tartaglia M. RAS signaling dysregulation in human embryonal Rhabdomyosarcoma. *Genes Chromosomes. Cancer* 2009;48:975–82.
13. Marshall CJ. The ras oncogenes. *J Cell Sci* 1998;Suppl 10:157–69.
14. Gupta AK, Bakanauskas VJ, Cerniglia GJ, Cheng Y, Bernhard EJ, Muschel RJ, et al. The Ras radiation resistance pathway. *Cancer Res* 2001;61:4278–82.
15. Toulany M, Baumann M, Rodemann HP. Stimulated PI3K-AKT signaling mediated through ligand or radiation-induced EGFR depends indirectly, but not directly, on constitutive K-Ras activity. *Mol Cancer Res* 2007;5:863–72.
16. Adhikary S, Eilers M. Transcriptional regulation and transformation by Myc proteins. *Nat Rev Mol Cell Biol* 2005;6:635–45.
17. Jain M, Arvanitis C, Chu K, Dewey W, Leonhardt E, Trinh M, et al. Sustained loss of a neoplastic phenotype by brief inactivation of MYC. *Science* 2002;297:102–4.
18. Sears R, Nuckolls F, Haura E, Taya Y, Tamai K, Nevins JR. Multiple Ras-dependent phosphorylation pathways regulate Myc protein stability. *Genes Dev* 2000;14:2501–14.
19. Yeh E, Cunningham M, Arnold H, Chasse D, Monteith T, Ivaldi G. A signalling pathway controlling c-Myc degradation that impacts oncogenic transformation of human cells. *Nat Cell Biol* 2004;6:308–18.
20. Bachireddy P, Bendapudi PK, Felsher DW. Getting at MYC through RAS. *Clin Cancer Res* 2005;11:4278–81.
21. McKenna WG, Weiss MC, Endlich B, Ling CC, Bakanauskas VJ, Kelsten ML, et al. Synergistic effect of the v-myc oncogene with H-ras on radioresistance. *Cancer Res* 1990;50: 97–102.
22. Shannon AM, Telfer BA, Smith PD, Babur M, Logie A, Wilkinson RW. The mitogen-activated protein/extracellular signal-regulated kinase kinase 1/2 inhibitor AZD6244 (ARRY-142886) enhances the radiation responsiveness of lung and colorectal tumor xenografts. *Clin Cancer Res* 2009;15:6619–29.
23. Allalunis-Turner MJ, Zia PK, Barron GM, Mirzayans R, Day RS 3rd. Radiation-induced DNA damage and repair in cells of a radiosensitive human malignant glioma cell line. *Radiat Res* 1995;144:288–93.
24. Lees-Miller SP, Godbout R, Chan DW, Weinfeld M, Day RS 3rd, Barron GM, et al. Absence of p350 subunit of DNA-activated protein kinase from a radiosensitive human cell line. *Science* 1995;267: 1183–5.
25. Lees-Miller SP, Sakaguchi K, Ullrich SJ, Appella E, Anderson CW, et al. Human DNA-activated protein kinase phosphorylates serines 15 and 37 in the amino-terminal transactivation domain of human p53. *Mol Cell Biol* 1992;12:5041–9.
26. An J, Yang DY, Xu QZ, Zhang SM, Huo YY, Shang ZF, et al. DNA-dependent protein kinase catalytic subunit modulates the stability of c-Myc oncoprotein. *Mol Cancer* 2008;7:32.
27. Tian X, Chen G, Xing H, Weng D, Guo Y, Ma D. The relationship between the down-regulation of DNA-PKcs or Ku70 and the chemosensitization in human cervical carcinoma cell line HeLa. *Oncol Rep* 2007;18:927–32.
28. Gerweck LE, Vijayappa S, Kurimasa A, Ogawa K, Chen DJ, et al. Tumor cell radiosensitivity is a major determinant of tumor response to radiation. *Cancer Res* 2006;66:8352–5.
29. Yan D, Ng WL, Zhang X, Wang P, Zhang Z, Mo YY, et al. Targeting DNA-PKcs and ATM with miR-101 sensitizes tumors to radiation. *PLoS One* 2010;5:e11397.
30. van Gent DC, Hoeijmakers JH, Kanaar R. Chromosomal stability and the DNA double-stranded break connection. *Nat Rev Genet* 2001;2 (3):196–206.
31. Marampon F, Ciccirelli C, Zani BM. Down-regulation of c-Myc following MEK/ERK inhibition halts the expression of malignant phenotype in rhabdomyosarcoma and in non muscle-derived human tumors. *Mol Cancer* 2006;5:31.
32. Favata MF, Horiuchi KY, Manos EJ, Daulerio AJ, Stradley DA, Feeser WS, et al. Identification of a novel inhibitor of mitogen-activated protein kinase kinase. *J Biol Chem* 1998;273:18623–32.
33. Marampon F, Casimiro MC, Fu M, Powell MJ, Popov VM, Lindsay J, et al. Nerve Growth factor regulation of Cyclin D1 in PC12 cells through a p21RAS extracellular signal-regulated kinase pathway requires cooperative interactions between Sp1 and nuclear factor-kappaB. *Mol Biol Cell* 2008;19:2566–78.
34. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951;193: 265–75.
35. Pelosi M, Marampon F, Zani BM, Prudente S, Perlas E, Caputo V, et al. ROCK2 and its alternatively spliced isoform ROCK2m positively control the maturation of the myogenic program. *Mol Cell Biol* 2007;27:6163–76.
36. Bossi G, Marampon F, Maor-Aloni R, Zani B, Rotter V, Oren M, et al. MEK/ERK inhibitor U0126 affects in vitro and in vivo growth of embryonal rhabdomyosarcoma. *Mol Cancer Ther* 2009;8:543–51.
37. Prewett MC, Hooper AT, Bassi R, Ellis LM, Waksal HW, Hicklin DJ. Enhanced antitumor activity of anti-epidermal growth factor receptor monoclonal antibody IMC-C225 in combination with irinotecan (CPT-11) against human colorectal tumor xenografts. *Clin Cancer Res* 2002;8:994–1003.
38. Toulany M, Kasten-Pisula U, Brammer I, Wang S, Chen J, Dittmann K, et al. Blockage of epidermal growth factor receptor-phosphatidylinositol 3-kinase-AKT signaling increases radiosensitivity of K-RAS mutated human tumor cells in vitro by affecting DNA repair. *Clin Cancer Res* 2006;12:4119–26.
39. Agami R, Bernards R. Distinct initiation and maintenance mechanisms cooperate to induce G1 cell cycle arrest in response to DNA damage. *Cell* 2000;102:55–66.
40. Albanese C, D'Amico M, Reutens AT, Fu M, Watanabe G, Lee RJ, et al. Activation of the Cyclin D1 gene by the E1A-associated protein p300 through AP-1 inhibits cellular apoptosis. *J Biol Chem* 1999;274:186–95.
41. Milas L, Akimoto T, Hunter NR, Mason KA, Buchmiller L, Yamakawa M, et al. Relationship between Cyclin D1 expression and poor radioresponse of murine carcinomas. *Int J Radiat Oncol Biol Phys* 2002;52:514–21.
42. Ahmed KM, Fan M, Nantajit D, Cao N, Li JJ. Cyclin D1 in low-dose radiation-induced adaptive resistance. *Oncogene* 2008;27: 6738–48.
43. An J, Xu QZ, Sui JL, Bai B, Zhou PK. Downregulation of c-myc protein by siRNA-mediated silencing of DNA-PKcs in HeLa cells. *Int J Cancer* 2005;117:531–7.
44. Tian X, Chen G, Xing H, Weng D, Guo Y, Ma D. The relationship between the down-regulation of DNA-PKcs or Ku70 and the chemosensitization in human cervical carcinoma cell line HeLa. *Oncol Rep* 2007;18:927–32.
45. Coco Martin JM, Balkenende A, Verschoor T, Lallemand F, Michalides R. Cyclin D1 overexpression enhances radiation-induced apoptosis and radiosensitivity in a breast tumor cell line. *Cancer Res* 1999; 59:1134–40.

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