

# Hypoxia sustains glioblastoma radioresistance through ERKs/DNA-PKcs/HIF-1 $\alpha$ functional interplay

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**Abstract.** The molecular mechanisms by which glioblastoma multiforme (GBM) refracts and becomes resistant to radiotherapy treatment remains largely unknown. This radioresistance is partly due to the presence of hypoxic regions, which are frequently found in GBM tumors. We investigated the radiosensitizing effects of MEK/ERK inhibition on GBM cell lines under hypoxic conditions. Four human GBM cell lines, T98G, U87MG, U138MG and U251MG were treated with the MEK/ERK inhibitor U0126, the HIF-1 $\alpha$  inhibitor FM19G11 or  $\gamma$ -irradiation either alone or in combination under hypoxic conditions. Immunoblot analysis of specific proteins was performed in order to define their anti-oncogenic or radiosensitizing roles in the different experimental conditions. MEK/ERK inhibition by U0126 reverted the transformed phenotype and significantly enhanced the radiosensitivity of T98G, U87MG, U138MG cells but not of the U251MG cell line under hypoxic conditions. U0126 and ERK silencing by siRNA reduced the levels of DNA protein kinase catalytic subunit (DNA-PKcs), Ku70 and K80 proteins and clearly reduced HIF-1 $\alpha$  activity and protein expression. Furthermore, DNA-PKcs siRNA-mediated silencing counteracted HIF-1 $\alpha$  activity and downregulated protein expression suggesting that

ERKs, DNA-PKcs and HIF-1 $\alpha$  cooperate in radioprotection of GBM cells. Of note, HIF-1 $\alpha$  inhibition under hypoxic conditions drastically radiosensitized all cell lines used. MEK/ERK signal transduction pathway, through the sustained expression of DNA-PKcs, positively regulates HIF-1 $\alpha$  protein expression and activity, preserving GBM radioresistance in hypoxic condition.

## Introduction

Glioblastoma (GBM) is the most common and aggressive type of primary brain cancer in adults (1). Despite multimodality treatment consisting of maximally safe resection, adjuvant chemoradiation with temozolomide, median survival remains dismal at 12-15 months (2), where less than 2% of patients survive 3 years post-diagnosis (3). Infiltrating cancer cells in the surrounding brain that prevent complete resection and their intrinsic resistance to chemoradiation treatment cause the poor prognosis of the GBM patient (4,5). GBM that arise *de novo* usually occurs as the result of progression from lower grade astrocytomas. Several histological changes occur during transition to GBM and these reflect a profound alteration in the tumor vascular biology. Particularly, the main change is represented by the appearance of hypoxic areas bounded by regions with a high rate of cell proliferation (6,7). In GBM hypoxia and its microenvironment are predominant features associated with the tumor growth, progression and resistance to chemo- and radiation therapies (8-10). Furthermore, it has been shown that hypoxia favors the maintenance of GBM stem cell population that is intrinsically resistant to standard therapies (11,12). Thus, targeting the molecular mechanism regulated by hypoxia could represent a valid alternative strategy in order to chemoradio-sensitize GBM cells (13). One of the main early cellular events evoked upon exposure to hypoxia is activation of HIF-1 transcription factor that, through the binding of hypoxia-responsive elements (HREs), induces the expression of several target genes involved in

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tumor angiogenesis, invasion, cell survival and glucose metabolism (14). Very little is known about the molecular mechanisms implicated in hypoxia-induced resistance to therapies, although the central role of HIF-1 $\alpha$  in promoting the cancer cells resistance to therapies has been reported. Eukaryotic cells respond to hypoxia through the modulation of several downstream effector pathways, including intracellular signal transduction cascades, which in turn interfere with gene expression regulation. In particular, members of the family of mitogen activated protein (MAP) kinases were shown to be involved in the transduction of the hypoxic signals (15-17). Even though the mitogenic Ras/Raf/MEK/ERK cascade signalling pathway, which responds to growth factors and factors inducing cellular differentiation, such as epidermal growth factor (EGF) and platelet derived growth factor (PDGF), has been intensively studied (18), little is known about its relationship to hypoxia (19). It has been shown that ERKs, activated during hypoxia, positively regulate HIF-1 $\alpha$  gene expression (20), phosphorylate HIF-1 $\alpha$  protein regulating its activity (21) and are needed for its activity as a transcription factor (22). Resistance to chemo- and radiotherapy may be caused primarily by DNA repair mechanisms (23). The main deleterious damage induced by chemo- and radiotherapy is DNA-double-strand breaks (Dsbs) and DNA repair remains one of the main responses through which cancer cells guarantee their own survival thus contributing to tumor chemo- and radioresistance. The major mechanism underlying the repair of DNA-Dsbs in mammalian cells requires the DNA-PKcs, a serine-threonine protein kinase that forms a complex of 450,000 kDa catalytic subunit (DNA-PKcs), in a heterodimeric complex composed of the proteins Ku70 (70,000 Da) and Ku80 (86,000 Da). Ku binds to both ends of a double-strand break and recruits DNA-PKcs to the DNA end (24,25). Functional interplay has been shown between HIF-1 $\alpha$  and DNA-PKcs, which could contribute to radioresistance and chemoresistance in hypoxic tumor cells (26). GBM is characterized by several aberrantly activated signalling pathways such as EGF, vascular endothelial growth factor receptor (VEGF) and PDGF pathways (27,28). All these pathways converge on the RAS-MEKs-ERKs signal transduction activation that plays a key role in the regulation of tumor progression and treatments response (29). Herein, the role of RAS-MEKs-ERKs signal transduction pathway in controlling GBM transformed phenotype and response to radiotherapy treatment was investigated under hypoxic condition. Our results showed that RAS/MAPK pathway, through the regulation of DNA-PKcs/HIF-1 $\alpha$  interplay, governs the GBM transformed phenotype and regulates the hypoxia-mediated increase of GBM radioresistance. Particularly we showed for the first time that DNA-PKcs is an upstream regulator of HIF-1 $\alpha$  protein expression, which is the determinant in sustaining GBM refractoriness to radiation therapy.

## Materials and methods

*Cell cultures in conventional and hypoxic conditions: treatments and radiation exposure.* The human glioblastoma T98G and U138MG cell lines were obtained from American Type Culture Collection (Manassas, VA, USA). The human

glioblastoma U87MG cell lines were from Life Technologies (Frederick, MD, USA). The human glioblastoma U251MG cell lines were obtained from Sigma-Aldrich (St. Louis, MO, USA). The T98G U138MG and U251MG cell lines were cultured in Eagle's minimum essential medium containing fetal bovine serum to a final concentration of 10%. The U87MG cell lines were cultured in RPMI-1640 medium containing fetal bovine serum to a final concentration of 10%. 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 2012. For hypoxia experiments, cultures were maintained at 37°C in a humidified incubator in an atmosphere of 20% O<sub>2</sub>, 5% CO<sub>2</sub> and 75% N<sub>2</sub>. The Xvivo Closed Incubation System (Xvivo system 300 C, BioSpherix, New York, NY, USA) was used in this study in order to accurately maintain different oxygen tensions in different chambers. After 24 h of cultivation in conventional cell culture (allowing cells to attach onto the flasks), the cells were transferred into different chambers with 0.1% O<sub>2</sub>, 5% CO<sub>2</sub> and 94.9% N<sub>2</sub> for variable periods of time before being harvested for additional analysis. Treatment with 10  $\mu$ mol/l MEK/ERK inhibitor U0126 (1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene; Promega, Madison, WI, USA) or 300 mM HIF-1 $\alpha$  inhibitor FM19G11 [2-oxo-2-(p-tolyl)ethyl] 3-[(2,4-dinitrobenzoyl)amino]benzoate, 3-[(2,4-dinitrobenzoyl)amino]-benzoic acid 2-(4-methylphenyl)-2-oxoethyl ester were done for the times shown in the figures and started before radiation, lasting for 24 h. Radiation was delivered at room temperature using an x-6 MV photon linear accelerator as already described (30). The total single dose of 400 cGy 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, Copenhagen, Denmark) filtered with 0.5 mm Cu. The absorbed dose was measured using a Duplex dosimeter (PTW, Freiburg, Germany). The dose-rate was approximately 1.3 Gy/min and applied doses ranged from 0 to 600 cGy.

*Immunoblot analysis.* Immunoblot analysis was performed as described (31). Briefly, cells were lysed in 2% SDS containing phosphatase and protease inhibitors sonicated for 30 sec. Proteins of whole cell lysates were assessed using the method of Lowry *et al* (32), and equal amounts were separated on SDS-PAGE. The proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell, BioScience GmbH, Dassel, Germany) by electroblotting. Immunoblot analyses were performed with the following antibodies: anti-c-Myc (9E10), anti-N-Myc (C-19), anti-phospho ERK1/2 (E-4), anti-ERK2 (C-14 positive also for ERK1), anti-cyclin-D1 (M-20), anti-HIF-1 $\alpha$  (28b), anti-DNA-PKcs (28b),  $\alpha$ -tubulin (B-7) all from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and anti-Ku70/Ku80 (ab53126) from Abcam (Cambridge, UK). Peroxidase-conjugate anti-mouse or anti-rabbit IgG (Amersham-Pharmacia Biotech, Amersham, UK or Santa Cruz) were used for enhanced chemiluminescence (ECL) detection.

*Cell proliferation, soft agar assays and FACS analysis.* Cells from adherent and suspension culture were counted using hemocytometer, and tested for exclusion of trypan blue. Data

are expressed as mean  $\pm$  SE of experiments performed in triplicate. Suspension culture proliferation assay was performed by using polyHema assay as already described (36). Briefly, polyHEMA-coated 96-well plates were used. A total of 50  $\mu$ l of polyHEMA solution (5 mg/ml in 95% ethanol) was overlaid into wells and dried for 2 days with lids in place. Cells were inoculated in a volume of 135  $\mu$ l at a density of 5,000 cells per well. Cells were cultured for 4 days in the presence or absence of inhibitors. At the end of the treatment, 15  $\mu$ l of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide solution (5 mg/ml in PBS) was added, and the mixture was further incubated for 4 h. The resulting 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide formazan was solubilized by addition of 100  $\mu$ l of SDS solution (20% in 10 mM HCl), and the absorbance was measured after 24 h at 570 nm and a reference wavelength of 690 nm using a microplate reader (33). Colony-forming in soft agar assays were based on standard methods. Briefly,  $2 \times 10^4$  cells were resuspended in 4 ml of 0.33% special Noble agar (Difco, Detroit, MI, USA) and plated (6-cm plate) in growth medium-containing 0.5% soft agar. Colonies were photographed 14 days after plating. FACS analysis was performed as described (34). Briefly, cells were harvested by trypsin-EDTA and washed; pellets were resuspended in 0.3 ml 50% FCS in PBS, additioned with 0.9 ml 70% ethanol and left O/N in the dark at 4°C before FACS analysis (Coulter Epics XL Flow Cytometer, Beckman Coulter, Brea, CA, USA).

**Invasion and migration assays.** Transwell membrane (Corning Costar Corporation, Corning, NY, USA) was used. Cancer cells were trypsinized, washed and kept suspended in the appropriate medium without FCS. Migration-inducing medium (with 10% FCS) was added to the lower wells of the chambers, while the upper wells were filled with serum-free medium with cells (20,000 cells per well) in the absence, as controls, or in the presence of the appropriate treatments. After 8 h, filters were removed and fixed with methanol and subsequently wiped on the cells on the upper side using the Q-tip. Filters were stained with 20% Giemsa solution. Evaluation of completed transmigration was performed under a microscope, and random fields were scanned (four fields per filter) for the presence of cells at the lower membrane side only. Invasion assays were done in a similar manner as the migration assays described above, unless the inserts were pre-coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA).

**HIF-1 $\alpha$  transcription factor assay.** HIF-1 $\alpha$  transcription factor activity was evaluated by HIF-1 $\alpha$  Transcription Factor Assay (ab133104) from Abcam®. Transfection experiments were performed with siRNA for ERK1 and ERK2 or DNA-PKcs (Sancta Cruz Biotechnology) using Lipofectamine 2000 reagent (Invitrogen, San Giuliano Milanese, Milan, Italy), according to the manufacturer's instructions. Briefly, cells were plated at 40-50% confluence and transfected 24 h later with 100 nM siRNA, which we ascertained to be sufficient to detect maximum fluorescence using fluorescein-conjugated control siRNA.

## Results

*MEK/ERK inhibition blocks G0/G1 cell cycle phase transition and reverts transformed phenotype of glioblastoma cancer*

*cells.* Our experiments were started by verifying the role of MEKs/ERKs pathway through controlling cell cycle progression of growing GBM cancer cells in hypoxic conditions. For this purpose, T98G, U87MG, U138MG and U251MG GBM cell lines maintained under hypoxic conditions were treated with either U0126 (5, 10 or 20  $\mu$ M) or vehicle and counted 4 after days. MEKs/ERKs inhibition by U0126 arrested T98G, U87MG, U138MG in a concentration dependent manner while barely affecting the U251MG cells growth (Fig. 1A). FACS analysis showed that U0126 (10  $\mu$ M) induced accumulation of T98G, U87MG, U138MG tumor cells in the G1 phase of cell cycle with weak effects on U251MG cells (Fig. 1B). Analysis of phospho/active-ERKs status and markers of G1 arrest was performed (Fig. 2A). As shown in Fig. 2, U0126 induced a rapid (12 h), highly persistent (24 h) ERK de-phosphorylation/inactivation in T98G, U87MG, U138MG cells while a much weaker phospho/active-ERK inhibition was observed in U251MG cells. ERK inhibition was concomitant to c-Myc and cyclin D1 downregulation while N-Myc levels were not modified with the exception of U251 line which expresses N-Myc at higher levels than the other cell lines. We tested the effects of MEK/ERK inhibition assessing the ability of GBM cells to grow, migrate and/or invade in an anchorage-independent manner under hypoxic conditions. As show in Fig. 2, U0126 (10  $\mu$ M) drastically reduced the ability of T98G, U87MG and U138MG to grow in suspension (Fig. 2B) and form colonies in soft agar (Fig. 2C), while these effects were less evident in U251MG cancer cells. Finally, U0126 (10  $\mu$ M) significantly inhibited T98G, U87MG and U138MG invasion and migration at both 3 and 24 h post-treatment time points (Fig. 2D and E). Therefore, we concluded that MEK/ERK inhibition by U0126 induces growth arrest, which blocks the molecular mechanism responsible for G1 progression and reduces the tumorigenic and metastatic potential of GBM tumor cell growth under hypoxic conditions.

*The hypoxia-induced increase of radioresistance and HIF-1 $\alpha$  activity are counteracted by U0126-mediated MEK/ERK inhibition.* It has been shown that cancer cells exhibit different response to radiation therapy under hypoxia and normoxia. Since GBM usually acquires the ability to progress in hypoxic conditions, we tested whether U0126 affected radiosensitization ability under these two conditions. Tumor cells were cultured in the presence or absence of U0126 (10  $\mu$ M) in normoxic or hypoxic conditions for 12 h before the delivery of increasing doses of ionizing radiation (0-6 Gy) (Fig. 3, left panels). After radiation treatment U0126 was removed and a colony assay was then performed. All cell lines were basically radioresistant under normoxic conditions (Fig. 3, left panels, normoxia+RT), while the hypoxic conditions highly increased (Fig. 3, left panels, hypoxia+RT) the intrinsic levels of radioresistance. U0126 increased the radiosensitivity of T98G, U87MG and U138MG with effects evident both in normoxic and hypoxic conditions (Fig. 3, left panels, normoxia+U0126+RT, hypoxia+U0126+RT). No statistically significant differences were observed in the U251MG cell line (Fig. 3, left panels). We analyzed the HIF-1 $\alpha$  activity present in the GBM cells after RT treatment (Fig. 3, right panels). HIF-1 $\alpha$  basal activity increased under hypoxic condition in T98G, U87MG and U138MG while it was drasti-

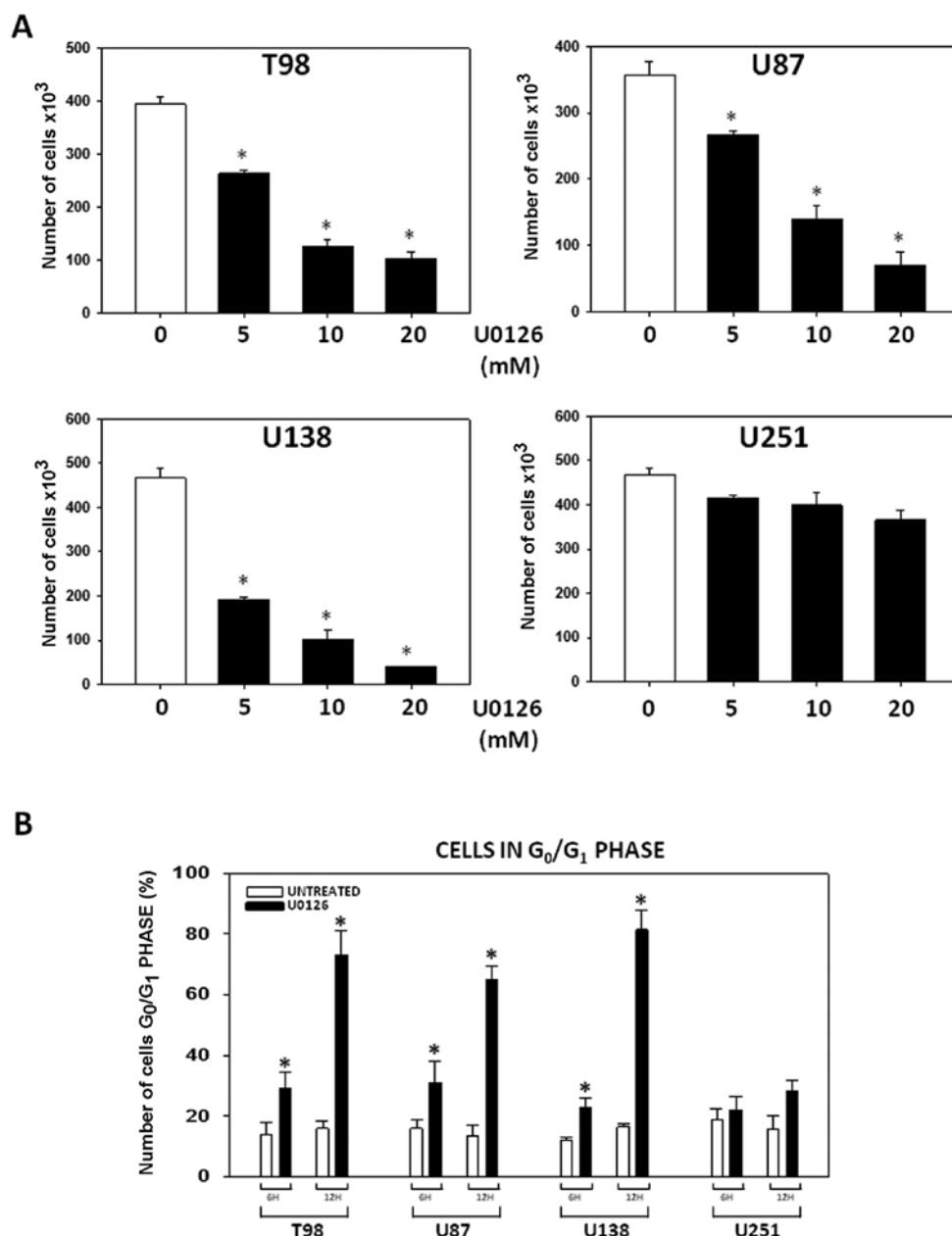


Figure 1. Effects of MEK/ERK inhibition in GBM cell lines. (A) Growth curve of cultured T98G, U87MG, U138MG and U251MG GBM cell lines under hypoxia condition, in the presence (black bar) and absence (white bar) of 5, 10 or 20  $\mu$ mol/l U0126 for 4 days. (B) FACS analysis of untreated and 6 or 12 h U0126 10  $\mu$ mol/l U0126-treated T98G, U87MG, U138MG and U251MG GBM cell lines under hypoxia condition. Mean  $\pm$  SE of triplicates of a representative experiment (\* $p$ <0.05 vs. untreated cells).

cally reduced upon MEK/ERK inhibition combined treatment (Fig. 3, right panels) which, by contrast, had no effect on HIF-1 $\alpha$  activity status of U251MG cancer cells (Fig. 3, right panels). Noteworthy, RT treatment alone increased the HIF-1 $\alpha$  basal activity both in normoxic and hypoxic conditions (Fig. 3, right panels).

*MEK/ERK pathway is an upstream regulator of HIF-1 $\alpha$  protein expression in GBM cancer cells.* Since radiation increased HIF-1 $\alpha$  activity in hypoxic conditions and MEK/ERK inhibition combined with radiation affected HIF-1 $\alpha$  activity, we investigated whether MEK/ERK pathways regulate HIF-1 $\alpha$  protein expression in hypoxic conditions. GBM cell lines were

cultured with or without U0126 (10  $\mu$ M) in hypoxic conditions for 12, 24 or 36 h; analysis of HIF-1 $\alpha$  protein expression levels was performed (Fig. 4, right panels). MEK/ERK inhibition by U0126 dramatically and persistently (12 to 36 h) reduced HIF-1 $\alpha$  protein expression levels. In T98G as well as in U87MG and in U138MG cell lines the protein expression was completely abrogated (Fig. 4, left panels). U0126 did not affect HIF-1 $\alpha$  protein expression levels in U251MG GBM cancer cells. MEK/ERK pathway functioning upstream of HIF-1 $\alpha$  in GBM, as suggested by U0126 experiments, was further demonstrated by RNA interference experiment with ERK1/ERK2- and scramble-siRNA in transient transfection. Three days after ERK1/ERK2 siRNA transfection, we

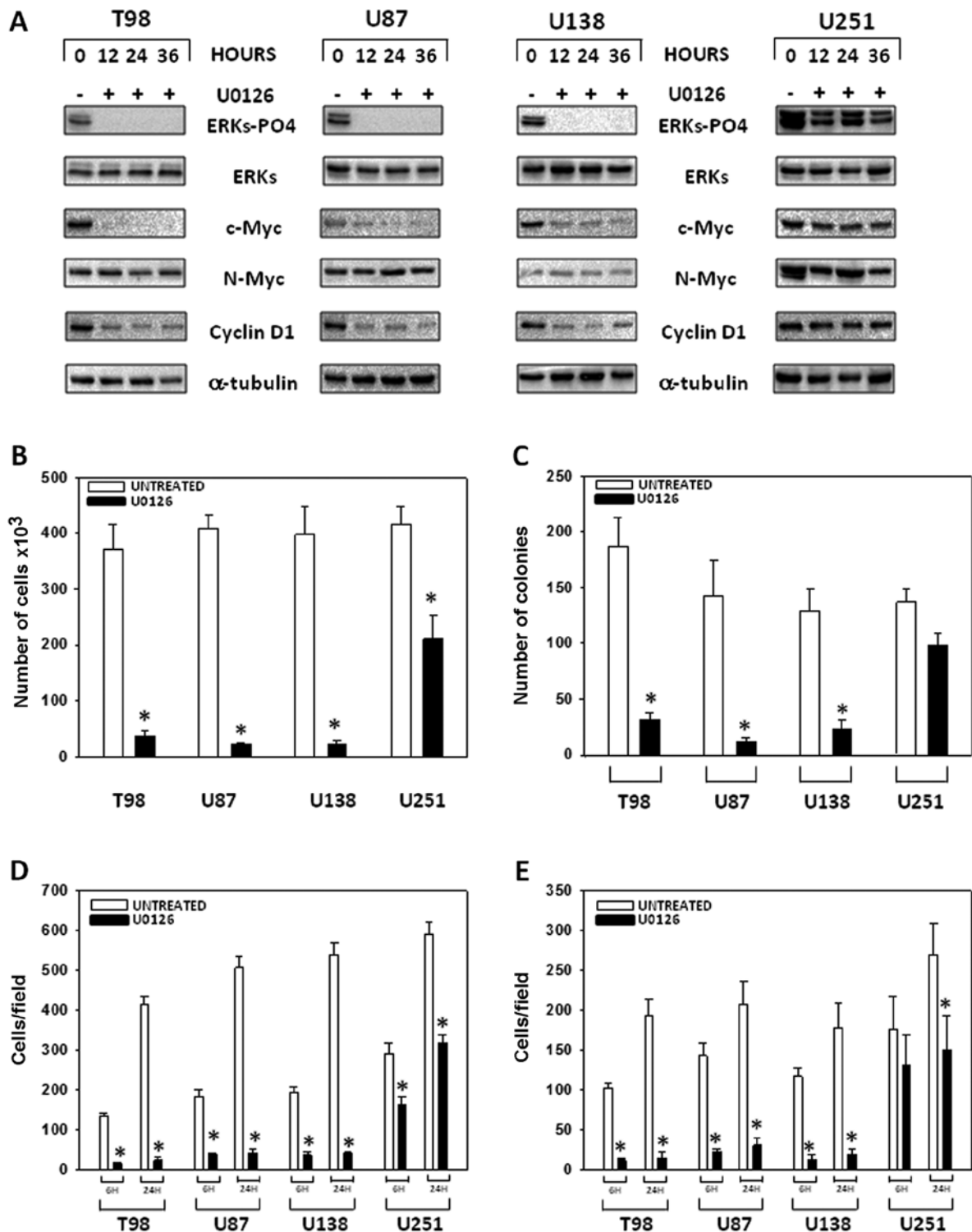


Figure 2. MEK/ERK inhibition by U0126 affects G1 cell cycle phase marker proteins and oncogenic potential of GBM cell lines. (A) Cell lysates from T98G, U87MG, U138MG and U251MG GBM cell lines untreated (-) or treated (+) with 10  $\mu$ mol/l U0126 for indicated times were analysed by immunoblot analysis with specific antibodies for indicated proteins.  $\alpha$ -tubulin expression shows the loading of samples. (B) Growth curve in Poly-HeMa coated plates of T98G, U87MG, U138MG and U251MG GBM cell lines untreated (white bar) or treated (black bar) with 10  $\mu$ mol/l U0126. Cells were counted after 6 days. (C) T98G, U87MG, U138MG and U251MG GBM cell lines untreated (white bar) or treated (black bar) with 10  $\mu$ mol/l U0126 were tested for growth in soft agar. Colonies were photographed after 14 days. (D) Transwell migration or (E) invasion assays of T98G, U87MG, U138MG and U251MG GBM cell lines untreated (white bar) or treated (black bar) with 10  $\mu$ mol/l U0126 for 6 or 24 h. Cells number/field were counted. Similar results were obtained in three experiments. The data shown are the mean  $\pm$  SEM of triplicates of a representative experiment (\* $p$ <0.05 vs. untreated cells).

observed a downregulation of total ERKs and a drastic reduction of HIF-1 $\alpha$  in T98G, U87MG, U138MG and U251MG transfected cells (Fig. 4, right panels).

*MEK/ERK inhibition by U0126 radiosensitizes GBM cancer cell lines by affecting the DNA repair molecular mechanism.* We next investigated the molecular mechanism responsible

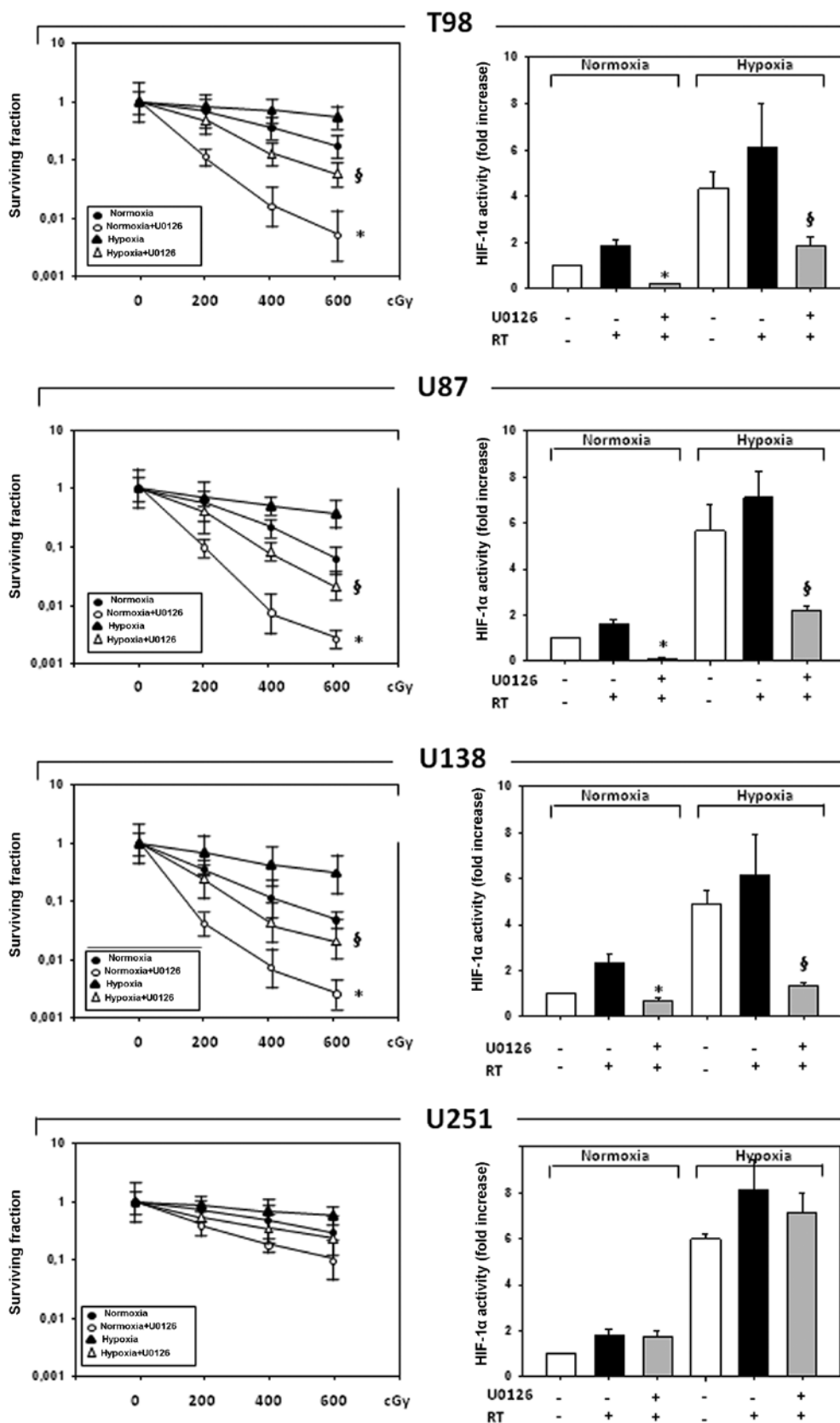


Figure 3. Effects of U0126 on GBM cancer cell line survival and on HIF-1 $\alpha$  activation status after irradiation. (Left panels) T98G, U87MG, U138MG and U251MG GBM cell lines in the exponential phase of growth under hypoxic or normoxic conditions, were exposed to the indicated doses of  $\gamma$ -radiation. Clonogenic survival was determined by counting the number of colonies containing >50 cells after 2 weeks of growth. The surviving fraction is shown in a semi-logarithmic plot against radiation dose. Each point is the mean of triplicate flasks from two to three independent experiments; bars, SE. (Right panels) T98G, U87MG, U138MG and U251MG GBM cell lines in the exponential phase of growth under hypoxic conditions, were exposed to 400 cGy of  $\gamma$ -radiation in the presence or the absence of U0126. Analysis on HIF-1 $\alpha$  activation status were performed after 1 h from  $\gamma$ -radiation. Similar results were obtained in three experiments. The data shown are the mean  $\pm$  SEM of triplicates of a representative experiment (\* $p$ <0.05 vs. untreated cells under normoxia condition, § $p$ <0.05 vs. untreated cells under hypoxia condition).

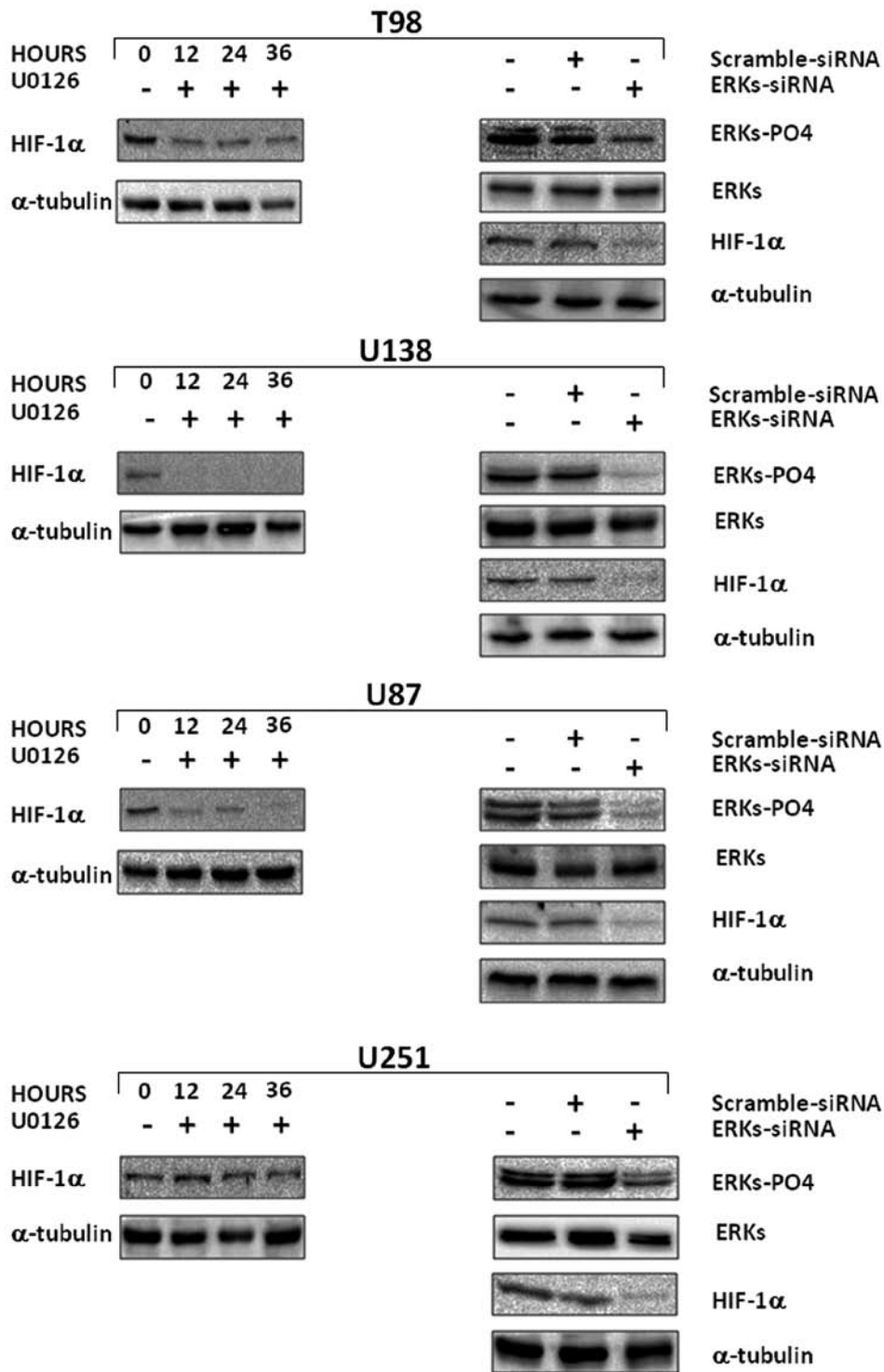


Figure 4. MEK/ERK inhibition counteracts the HIF-1 $\alpha$  protein expression in GBM cancer cell lines. (Left panels) Cell lysates from T98G, U87MG, U138MG and U251MG GBM cell lines untreated (-) or treated (+) with 10  $\mu$ mol/l U0126 for indicated times were analysed by immunoblotting with specific antibodies for indicated proteins.  $\alpha$ -tubulin expression shows the loading of samples. Similar results were obtained in three different experiments. (Right panels) Cells were transfected with control (scramble) or ERK1/2 siRNAs and cultured for 3 days under hypoxia conditions. Immunoblot analyses of total lysates were performed using specific antibodies recognizing the indicated proteins. Similar results were obtained in two experiments.

of radiosensitization induced by MEK/ERK inhibition. DNA-PKcs and Ku proteins are upregulated in various tumors and are implicated in the radiation response. DNA-PKcs, Ku-80 and Ku-70 expression levels in response to U0126 were assessed by western blot analysis (Fig. 5A). The 24 h treat-

ment with U0126 alone, and in combination with radiation reduced DNA-PKcs, Ku-80 and Ku-70 protein expression levels concomitantly with HIF-1 $\alpha$  downregulation in the T98G, U87MG and U138MG is shown in Fig. 5A. No change was observed in U0126-treated U251MG cells. RT treatment

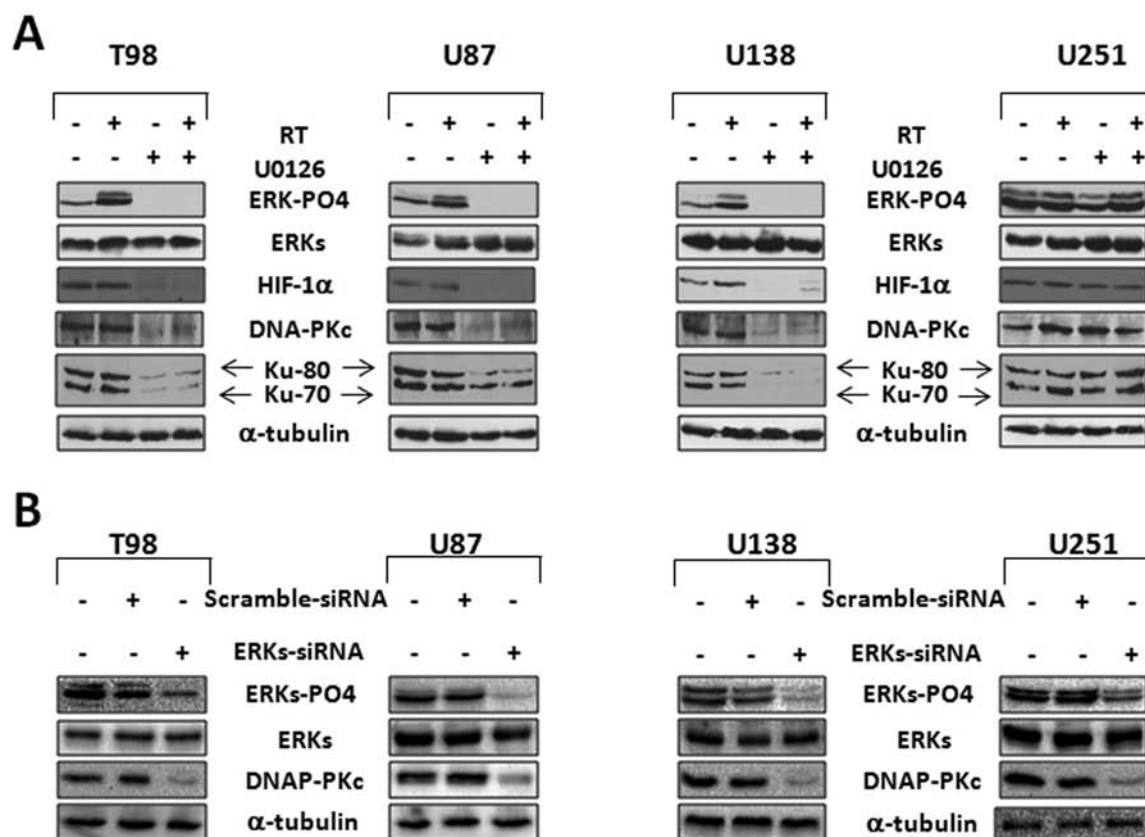


Figure 5. MEK/ERK inhibition abrogates the molecular mechanism responsible of DNA double strand break repair. (A) Cell lysates from T98G, U87MG, U138MG and U251MG GBM cell lines pre-treated (+) or not (-) with 10  $\mu$ mol/l U0126 for 24 h under hypoxic conditions and then exposed to 400 cGy of  $\gamma$ -radiation were analysed by immunoblot analysis with specific antibodies for indicated proteins.  $\alpha$ -tubulin expression shows the loading of samples. Similar results were obtained in three different experiments. (B) T98G, U87MG, U138MG and U251MG cells were transfected with control (scramble) or ERK1/2 siRNAs and cultured for 3 days under hypoxia conditions. Immunoblot analyses of total lysates were performed using specific antibodies recognizing the indicated proteins. Similar results were obtained in two experiments.

alone increased the level of phospho/active ERKs. RNA interference experiment with ERK1/ERK2- or scramble-siRNA in transient transfection confirmed that DNA-PKcs expression is under the control of ERKs (Fig. 5B). Three days after the transfection we observed a downregulation of total ERKs and a drastic reduction of DNA-PKcs in ERK1/ERK2 siRNA T98G, U87MG, U138MG and U251MG transfected cells (Fig. 5B).

*MEK/ERK pathway regulates DNA-PKcs protein expression levels and controls HIF-1 $\alpha$  protein accumulation and radioresistance of GBM cancer cells.* Since MEK/ERK inhibition by U0126 or siRNA-mediated silencing induced HIF-1 $\alpha$  and DNA-PKcs downregulation, we investigated if there was a functional correlation between DNA-PKcs and HIF-1 $\alpha$ . T98G, U87MG, U138MG and U251MG were transiently transfected with either scramble control or DNA-PKcs-siRNA. Sixty hours after transfection the cells were exposed to hypoxic condition for 12 h. Finally, total lysates were processed for western blot analysis (Fig. 6A) and HIF-1 $\alpha$  activity evaluation (Fig. 6B). DNA-PKcs silencing by siRNA reduced the HIF-1 $\alpha$  protein expression levels (Fig. 6A) and activity (Fig. 6B) in T98G, U87MG, U138MG as well as in U251MG cell line. Since HIF-1 $\alpha$  seemed to be the final regulator of the molecular mechanism governed by

MEK/ERK pathway and responsible of GBM radioresistance, we tested whether the HIF-1 $\alpha$  inhibition could radiosensitize GBM cells. For this experiment T98G, U87MG, U138MG and U251MG cancer cell lines were cultured in the presence or absence of FM19G11 (300 nM), a specific HIF-1 $\alpha$  inhibitor, in hypoxic conditions for 12 h before the delivery of increasing doses of ionizing radiation (0-6 Gy). After radiation treatment, FM19G11 was removed and colony assay was performed (Fig. 6C). To test the inhibitory efficiency of FM19G11, HIF-1 $\alpha$  activity was measured before the radiation delivery (Fig. 6D). HIF-1 $\alpha$  inhibition by FM19G11 (Fig. 6D) reduced the radioresistance of T98G, U87MG, U138MG and U251MG cancer cells (Fig. 6C).

## Discussion

Hypoxia is a negative prognostic and predictive factor, known to contribute to chemoresistance, radioresistance, angiogenesis, vasculogenesis, invasiveness, metastasis, resistance to cell death, altered metabolism and genomic instability. Given its central role in tumor progression and resistance to therapy, tumor hypoxia might well be considered the best validated target that has yet to be explored in oncology (35). Hypoxic stress has been linked to several phenotypic changes that are fundamental to GBM progres-



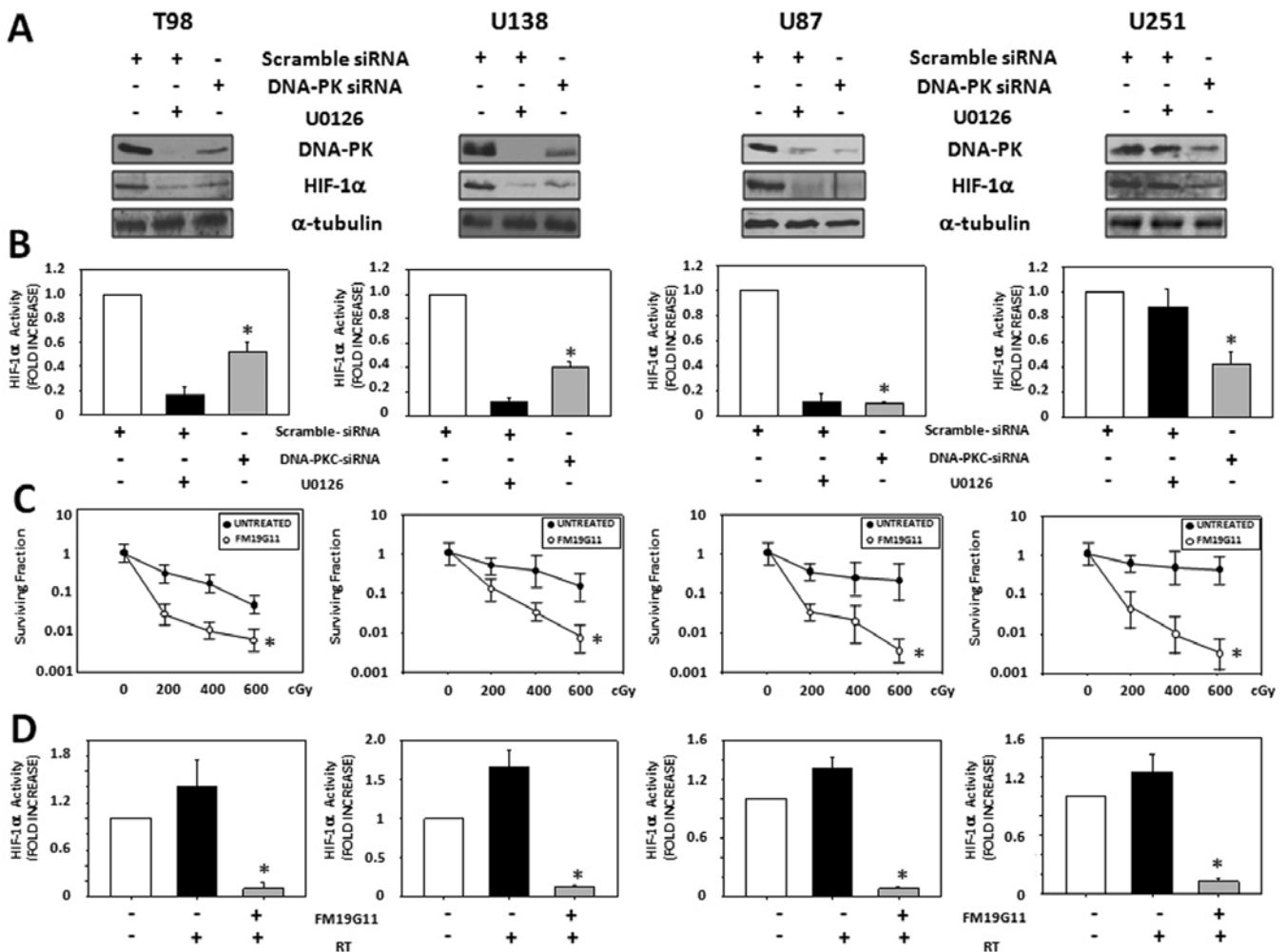


Figure 6. DNA-PKcs regulates HIF-1 $\alpha$  protein accumulation and activity which inhibition affects GBM cancer cell lines survival. (A) T98G, U87MG, U138MG and U251MG cells were transfected with control (scramble) or DNA-PKcs siRNAs and cultured for 3 days under hypoxia conditions. Immunoblot analyses of total lysates were performed using specific antibodies recognizing the indicated proteins. Similar results were obtained in three experiments. (Right panels) (B) Analysis on HIF-1 $\alpha$  activation status were performed. The data shown are the mean  $\pm$  SEM of triplicates of a representative experiment. (C) T98G, U87MG, U138MG and U251MG GBM cell lines in the exponential phase of growth under hypoxic condition, were pre-treated for 24 h with FM19G11 (300 nM) and then exposed to the indicated doses of  $\gamma$ -radiation. Clonogenic survival was determined by counting the number of colonies containing >50 cells after 2 weeks of growth. The surviving fraction is shown in a semilogarithmic plot against radiation dose. Points, means from triplicate flasks from two to three independent experiments; bars, SE. (Right panels) T98G, U87MG, U138MG and U251MG GBM cell lines in the exponential phase of growth under hypoxic condition, were exposed to 400 cGy of  $\gamma$ -radiation. (D) Analysis on HIF-1 $\alpha$  activation status were performed after 1 h from  $\gamma$ -radiation. Similar results were obtained in three experiments. The data shown are the mean  $\pm$  SEM of triplicates of a representative experiment.

sion and unresponsiveness of treatments (36). Even though Ras-MAPK pathway is important in radioresistance, where activated oncogenic Ras mediates resistance to ionizing radiation (37), the role of its inhibition under hypoxic conditions and the downstream target in radiation response has not yet been investigated in GBM. In the present study, we addressed the issue on whether MEK/ERK inhibition, through targeting HIF-1 $\alpha$ , prevents the transformed phenotype expression and radiation therapy resistance in several GBM cell lines grown under hypoxic conditions. Experimental conditions chosen, were based on data already present in the literature for which a near-maximal HIF-1 $\alpha$  expression occurs at 12 h of hypoxia at an oxygen concentration of 0.1% O<sub>2</sub>, representing a level of hypoxia that is frequently observed in solid tumors and is radiobiologically relevant (38). Our data show that MEK/ERK inhibition by U0126 reverts the transformed phenotype of T98G, U87MG and U138MG GBM cell lines by blocking

the proliferation, migration and invasion under hypoxic conditions. U251MG GBM cell line in which U0126 did not induce a persistent MEK/ERK inhibition, was unresponsive to the treatment. According to the literature (8-10), in the clonogenic assay, hypoxia increased the intrinsic levels of radioresistance in GBM cell lines that was counteracted by U0126 in T98G, U87MG and U138MG but not in U251MG cells. It has been shown that tumors deficient in the function or expression of HIF-1 $\alpha$  present an enhanced response to radiotherapy *in vivo* and *in vitro* models (39-42). In this study we show that MEK/ERK inhibition counteracted hypoxia-induced increment of HIF-1 $\alpha$  basal activity and reduced HIF-1 $\alpha$  protein expression levels in T98G, U87MG and U138MG GBM cell lines. Furthermore, we note that RT treatment, independently of oxygen concentration, increased MEK/ERK pathway activation and HIF-1 $\alpha$  activity. We speculate that GBM cancer cells respond to radiation treatment by activating a pro-survival

molecular mechanism characterized by the MEK/ERK-HIF-1 $\alpha$  interplay, which is interrupted by U0126 treatment. Thus, the molecular mechanism responsible of U0126 mediated radiosensitization was further dissected. DNA-PKcs activity is known to be essential for the repair of DSBs in the cell, and substantial scientific evidence has shown that DNA-PKcs and Ku proteins strongly correlate with radiosensitivity/resistance in several types of cancer (43,44). Consistently, in T98G, U87MG and U138MG, but not in U251MG cell line, the synergistic effect of MEK/ERK inhibitor on irradiation results from the targeting of DNA-PKcs and Ku protein expression levels, most likely compromising DNA-repair mechanism. Even though functional interplay between HIF-1 $\alpha$  and DNA-PK has been shown (26), there is no evidence on the molecular mechanism responsible for this relationship. Herein, for the first time we show that DNA-PKcs positively regulate HIF-1 $\alpha$  protein expression levels and support its accumulation. Interestingly, the selective inhibition of DNA-PKcs by siRNA downregulates HIF-1 $\alpha$  protein expression levels in all the GBM cell line tested including the U251MG cell line, which are unresponsive to U0126 treatment. Thus, HIF-1 $\alpha$  appears to be a downstream target of a complex molecular signal transduction pathway responsible of GBM radioresistance and its inhibition by FM19G11, HIF-1 $\alpha$  inhibitor, drastically radiosensitizes GBM cells. Our data strongly suggest that HIF-1 $\alpha$  is an attractive target for overcoming hypoxia-induced radioresistance. The selection of clinically promising HIF-1 $\alpha$  targeting agents represents an essential step towards identifying new radiosensitive agents. In conclusion, we demonstrated that through signal transduction-based chemotherapy it is possible to radiosensitize GBM cancer cells by interrupting the interplay between MEK/ERK and DNA-PKcs pathways and preventing HIF-1 $\alpha$ -mediated hypoxic cell survival and DNA-PKcs-mediated cancer cells radiation escape. MEK/ERK or HIF-1 $\alpha$  inhibitors combined with radiation are an efficient antitumor treatment for GBM cell lines suggesting a successful therapy to be tested *in vivo* and translated to clinic.

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