

Effects of FOXM1 inhibition and ionizing radiation on melanoma cells

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Abstract. Metastatic melanoma can be highly refractory to conventional radiotherapy and chemotherapy but combinatorial-targeted therapeutics are showing greater promise on improving treatment efficacy. Previous studies have shown that knockdown of Forkhead box M1 (*FOXM1*) can sensitize various tumor types to radiation-induced cell death. The effect of combining radiation with a small molecule FOXM1 inhibitor, Siomycin A, on growth, death and migration of a metastatic melanoma cell line (SK-MEL-28) that overexpresses this pleiotropic cell cycle regulator was investigated. Siomycin A (SIOA) was found to be a strong inducer of apoptosis, and inhibitor of proliferation and migration in a scratch wound assay in this cell line. Induction of apoptosis occurred at concentrations $>1 \mu\text{M}$ in association with reductions in the constitutive FOXM1 and anti-apoptotic B-cell lymphoma 2 protein levels found in these cells. Single doses of ionizing radiation (0-40 Gy) delivered by linear accelerator caused inhibition of growth and migration without significant induction of cell death. Pretreatment with SIOA did not increase the sensitivity of this melanoma cell line to radiation as observed in other tumor types. These data confirm that as a single agent, SIOA is an effective inducer of cell death and inhibitor of migration in metastatic melanoma cells expressing constitutive *FOXM1*. In combination with radiation, SIOA pre-treatment, however, may not be of added benefit.

Introduction

Melanoma is one of the deadliest forms of cancer known to humans and has the highest propensity to metastasize to the brain. Current therapeutics for metastatic brain tumours include surgery and radiation therapy but despite technological advancement on improving treatment safety and efficacy with these modalities, brain metastases remain a major cause of death in patients with metastatic melanoma (1,2).

In search of more effective therapeutics, an oncogenic signalling pathway driven by the transcription factor Forkhead box M1 (*FOXM1*) has emerged as a promising anti-metastatic target in recent years. *FOXM1* protein belongs to a class of highly evolutionary conserved mammalian transcription factors which underlie the regulation of many fundamental homeostatic and developmental processes (3,4). In normal cells, *FOXM1* serves as a key regulator of cell cycle progression and cellular development, mediating G1-S and G2-M phase transition and maintaining a balance between cell proliferation and apoptosis in developing cells (4). While its expression is turned off in terminally differentiated cells, an aberrant gain of *FOXM1* function has been shown to link to tumorigenesis (3,4). Emerging evidence has demonstrated that *FOXM1* is upregulated in a multitude of solid tumours including breast, lung, basal cell, pancreatic, hepatocellular, ovarian and prostate carcinomas, rendering it one of the most overexpressed genes in human cancers (5,6). More importantly, recent studies have indicated that the oncogenic mechanisms of *FOXM1* involve not only cell cycling dysregulation as predicted from its physiological function but the control of a wide array of oncogenic pathways and events including inhibition of cell differentiation, apoptosis and DNA repair and promotion of cell invasion, migration and angiogenesis, quintessential of the expression of the metastatic phenotypes (4). Conceivably targeting *FOXM1* could result in an across-the-board inhibition of many of the pro-metastatic events and combining with existing ablative treatment with focused irradiation, highly selective and localized cancer cell death can potentially be induced with low toxicity to normal tissue.

Recently several lines of evidence have lent support to the roles of *FOXM1* expression in inducing metastatic melanoma and suppression of *FOXM1* via RNA interference, blocking

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peptides, or chemotherapeutic agents has proven successful in countering tumorigenesis (7-10). Importantly new evidence has indicated that combined treatment of *FOXMI* inhibition with a small molecule inhibitor Siomycin A (SIOA) and irradiation can achieve a higher rate of cell death in glioblastoma cell lines, thus raising the possibility of harnessing *FOXMI* inhibitors as a radiosensitizing agent (11). Currently there is a dearth of information in the role of FOXM1 inhibition in sensitizing radioresistant melanoma and we therefore sought to elucidate the cellular effects of irradiation and SIOA on metastatic melanoma cells and determine the effects of pretreatment with SIOA on cellular response to irradiation.

Materials and methods

Cell culture and irradiation. A panel of ATCC-derived (SK-MEL-28, A375) or cultured, short-term patient-derived (WMD009, WMD046, MM200, SMU027) melanoma cells were kindly provided by Professor H. Rizos (Macquarie University, Sydney, Australia) to identify a FOXM1 overexpressing strain. All cell lines were previously published except for WMD046 which is a short term patient derived sample sourced from Professor Rizos (12-16). Cells were maintained in Dulbecco's Modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal bovine serum (FBS), 20 mM HEPES and 4 mM L-glutamine and cultured at 37°C in humidified air with 5% carbon dioxide. Cells were routinely passaged at 80% confluence with 0.1% Trypsin/EDTA (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Cells were seeded in 8-well chamber slides, 96- or 6-well plates for irradiation with X-rays (0-40 Gy) generated by a 6 MV linear accelerator (LINAC; Elekta Synergy, Crawley, UK) at Macquarie University Hospital (Sydney, Australia) or for SIOA treatment. SIOA was purchased from Sigma-Aldrich; Merck KGaA and dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich; Merck KGaA). DMSO constituted a final concentration of 0.1% in all assays.

Trypan blue viability assay. Viable-to-dead cell ratios were determined 1-5 days after drug or radiation treatment using the trypan blue viability assay. Briefly, floating and adherent cells were collected, washed and stained with trypan blue for 10 min before automated counting of white (live) or blue (permeable, dead) cells with an automated cell counter (Countess II FL Automated Cell Counter; Thermo Fisher Scientific, Inc.).

MTT proliferation assay. Cells were seeded in 96-well plates at 5×10^3 cells per well in 5% serum-containing medium and allowed to adhere overnight before treatment with drug or radiation. Treatment proceeded for 24, 48, 72 or 120 h. At least 8 replicate wells were used for each dose and time point within each independent experiment. Five h prior to the end of each incubation period, 20 μ l of MTT (Thermo Fisher Scientific, Inc.) was added to each well (0.5 mg/ml) and the plates incubated at 37°C for a further 5 h. The medium was then discarded by inversion and the cells resuspended in 200 μ l of DMSO per well to dissolve the formazan product. The plates were mechanically shaken for 5-10 min and the

absorbance read at 560/670 nm within 1 h using a microplate reader (BMG Labtech PHERAstar FS; Thermo Fisher Scientific, Inc.).

Scratch wounding cell migration assay. A scratch wound assay was used to examine cellular migration after SIOA and radiation treatment. Cells were seeded in 8-well chamber slides (Nunc; Thermo Fisher Scientific, Inc.) or 6-well culture plates to obtain 90-100% confluence. Cells were irradiated with single doses of radiation (0-40 Gy) or SIOA (0-5 μ M) for 1 h prior to scratch wounding of the cell layer with a 1 ml pipette tip. The medium was replaced with or without the thiazole antibiotic to remove floating cells and debris. The scratch wound area was immediately imaged with an upright inverted microscope and reimaged at 24, 48 and 72 h post-wounding. When sequential combination treatments were given, a period of 24 h was given before addition of the second treatment.

Images were analyzed using Image J (Rasband, W.S., ImageJ, National Institutes of Health, Bethesda, Maryland, USA; <https://imagej.nih.gov/ij/>, 1997-2016). For every scratch wound, a series of 4 images were taken along the length of the wounded area and measured by blinded observer. For each image, a region of interest (ROI; rectangle) was drawn that aligned with the upper and lower edges of the image and the observed division between cell regrowth and denudation. The area within the ROI was recorded for each image and divided by the area measured immediately after scratch wounding on day 0 to give wound area as a percentage of the original scratch wound area. These wound area percentages for each dose and time point from 3 independent experiments were then averaged (n=3) and plotted.

Western blot analysis. Protein lysates were prepared in immunoprecipitation buffer (50 mM Tris-HCl, pH 7.5, 150 mM sodium chloride, 0.5% deoxycholate, 0.1% sodium dodecyl sulphate, 1% NP40 substitute, 5 mM EDTA) supplemented with freshly prepared protease inhibitor cocktail (GE Healthcare, Chicago, IL, USA). Protein concentration was determined using the BCA protein assay (Pierce Biotechnology Inc.; Thermo Fisher Scientific, Inc.). Whole cell protein extracts (30 μ g) were resolved by SDS-PAGE, transferred to a PVDF membrane using the iblot transfer system (Thermo Fisher Scientific, Inc.) and probed with primary antibodies, species-specific HRP-conjugated secondary antibodies and detected by enhanced chemiluminescence. The following antibodies were used: Anti-FOXM1 (H-19 sc-501 rabbit polyclonal); anti-FOXM1 (G-5 sc-376471 mouse monoclonal; both Santa Cruz Biotechnology, Inc., Dallas, TX, USA); anti-cleaved poly (ADP-ribose) polymerase 1 (PARP1; ab2321 rabbit polyclonal; Abcam, Cambridge, UK); anti-B-cell lymphoma 2 (Bcl2; sc-492 rabbit polyclonal, Santa Cruz Biotechnology, Inc.); anti-phospho-AKT (Ser473) (cs-4060S rabbit monoclonal; Cell Signalling Biotechnology, Inc., Danvers, MA, USA); anti-superoxide dismutase 2 (Sod2; ab13534 rabbit polyclonal; Abcam). All membranes were probed with antibodies targeting either β -actin (A5060.5 rabbit polyclonal; Sigma-Aldrich; Merck KGaA) or GAPDH (ab181602 rabbit monoclonal; Abcam) as protein loading controls.

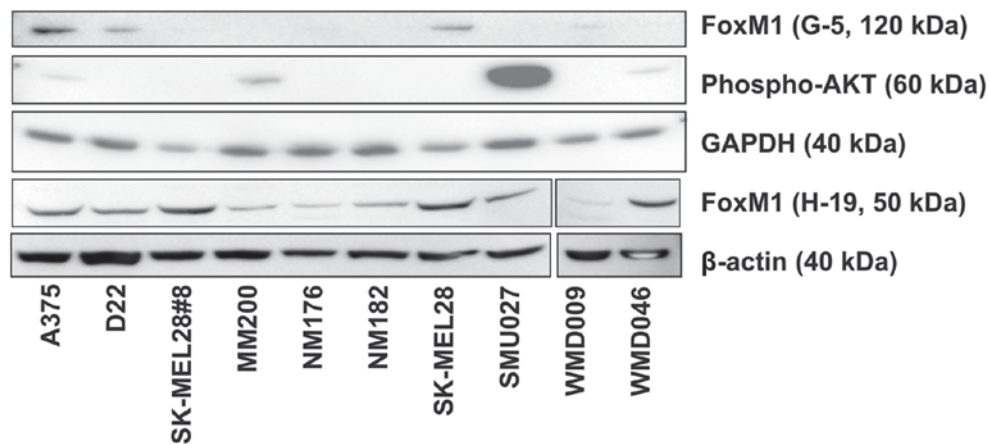


Figure 1. FOXM1 expression is variable in melanoma cell lines. Whole cell protein lysates were prepared from cultured melanoma cells as listed. Proteins (30 μ g) were separated by SDS-PAGE prior to western transfer to PVDF membrane. Membranes were probed with antibodies targeting FOXM1 and phospho-AKT (Ser473). HRP-tagged secondary antibodies were detected with enhanced chemiluminescence. Antibodies targeting GAPDH or β -actin were used to determine equal loading. MELRMU8 (SK-MEL-28#8) is a subclone of original ATCC strain. FOXM1, Forkhead box M1.

Statistical analysis. Data are presented as mean \pm standard error of the mean (SEM). Effects of single treatments over time were analyzed by two-way ANOVA with Dunnett's post-hoc analysis for multiple group comparisons using Prism 6.04 software (Graphpad Software, Inc., La Jolla, CA, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Constitutive FOXM1 expression in melanoma cell lines. FOXM1 expression has been noted to be constitutively activated in many cancers including melanoma and to contribute to tumorigenesis and chemo- and radio-resistance (5,7-9). A series of 10 melanoma cell lines were tested for FOXM1 protein expression by Western blotting with two different FOXM1 antibodies. A mouse monoclonal anti-FOXM1 antibody (G-5) recognized a single band of approximately 120 kDa, consistent with the full length FOXM1 protein, in strains A375, D22, SK-MEL-28 and faintly in WMD009 (Fig. 1). In contrast, a polyclonal rabbit anti-FoxM1 antibody (H-19) identified a primary band on western blots at approximately 50 kDa with a faint band at 80 kDa (Fig. 1). The pattern of expression of the 80 kDa band in strains A375, D22 and SK-MEL-28 was consistent with that of the 120 kDa band (G-5). The 50 kDa band was expressed in all cell lines but was considered non-specific as it did not respond to SIOA treatment. In view of these, the H-19 mouse monoclonal antibody was selected for further studies.

As constitutive activation of the AKT pathway can influence FOXM1 expression (8), we also examined the relative levels of expression of phosphorylated AKT in the 10 melanoma cell lines. Expression of phosphorylated AKT (Ser473) was observed only in A375, MM200, SMU027 and WMD046 (Fig. 1). There appeared to be no correlation between the relative expression levels of FOXM1 and phospho-AKT. Collectively the SK-MEL-28 cell line was chosen for further studies given its high expression levels of FOXM1.

SIOA inhibits SK-MEL-28 proliferation and induces cellular apoptosis. SK-MEL-28 cells were treated with SIOA at concentrations of 0-10 μ M for up to 5 days. Cell proliferation

and death were measured using trypan blue viability assays (Fig. 2A and B) and MTT proliferation assays (Fig. 2C). Vehicle controls (0.1% DMSO) continued to grow over the 72 h period. At a dose of 0.6 μ M SIOA, cell growth was inhibited 1.5-fold by 72 h ($P < 0.0001$) while doses $> 1.2 \mu$ M completely inhibited growth and significantly increased the proportion of dead cells in the first 48 h period. The IC_{50} was determined as 0.67 μ M (Fig. 2D).

SIOA inhibits SK-MEL-28 cell migration. Cellular migration was examined in a scratch wound assay. At concentrations $> 1.2 \mu$ M, SIOA primarily induced cell death across the entire plate of cells (see phase-contrast images, Fig. 2E). At sub-lethal concentrations ($\leq 0.6 \mu$ M), cellular migration was inhibited without significant cell death after 3 days (Fig. 2E and F).

SIOA decreases FOXM1 and BCL2 protein expression in SK-MEL-28 melanoma cells with a concomitant increase in PARP-1 cleavage. Protein expression of FOXM1 and two FOXM1-regulated genes, the anti-oxidant enzyme, SOD2, and the anti-apoptotic protein, BCL2, were examined in response to SIOA (Fig. 3). FOXM1 (full length protein, 120 kDa) was abruptly reduced at concentrations between 1.2 and 2.5 μ M SIOA. Levels of SOD2 increased dose-dependently, however BCL2 decreased between 1.2 and 2.5 μ M SIOA, in line with the decreases in FOXM1 expression observed. Expression of the p65 subunit of the NF κ B transcription factor decreased in a linear fashion with SIOA dose. The late apoptotic marker, cleaved PARP1, was evident at concentrations above 1.2 μ M SIOA up to 48 h, consistent with the level of cell death observed.

Radiation inhibits SK-MEL-28 growth with minimal effects on cell death. Total cell counts were determined over a period of 5 days for SK-MEL-28 melanoma cells exposed to radiation doses up to 40 Gy. Cells continued to grow in the absence of irradiation up to day 5 until growth reached 100% confluence, while radiation inhibited cell growth at all doses (Fig. 4A and B). In the irradiated cells, cell numbers increased between day 1 and day 3 at all doses, but declined moderately between days 3

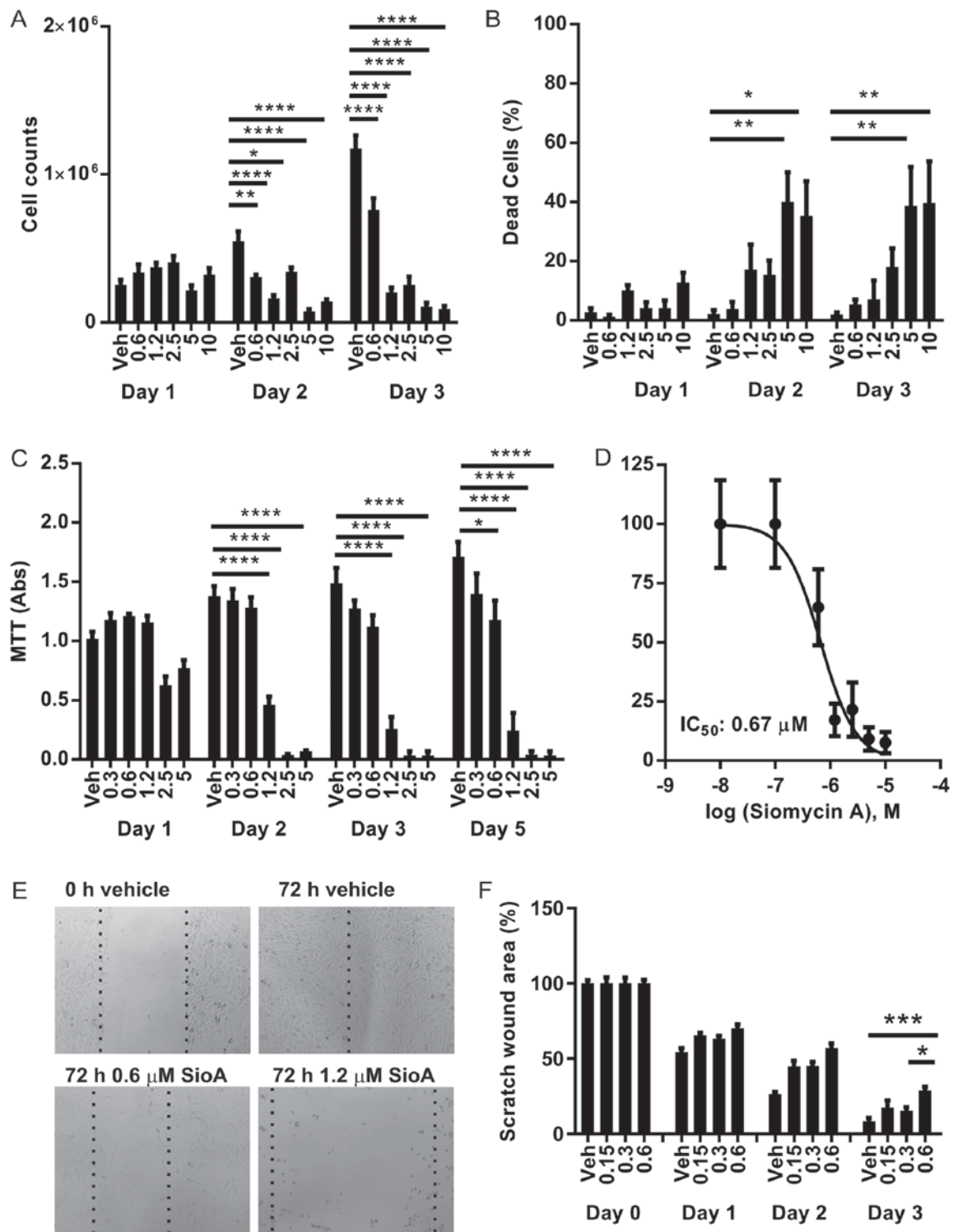


Figure 2. SIOA induces growth inhibition and cell death in SK-MEL-28 melanoma cells. SK-MEL-28 cells were treated with SIOA at doses of 0-10 μ M and monitored for viability and migration for up to 5 days. (A) Cell counts (trypan blue-negative cells/ml) by trypan blue assay and automated cell counting. Data shown as mean \pm SEM and representative of 2 independent experiments (n=2); (B) Proportion of dead cells (% of total) measured by trypan blue viability assay and automated cell counter. Data shown as mean \pm SEM and representative of 2 independent experiments (n=2); (C) Cell proliferation was examined by MTT assay. Mean \pm SEM of 3 independent assays (n=3); (D) Dose-response curve for SIOA in SK-MEL-28; (E) Representative images of wound area after treatment at day 0 and day 3. Dotted lines approximate the borders between cellular and denuded areas. Original magnification, x50. (F) Wound area (% of vehicle control at time zero) after scratch injury in response to SIOA treatment. Data represent mean \pm SEM of 3 independent experiments (n=3). Two-way ANOVA with Dunnett's post-hoc test: *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. SEM, standard error of the mean; SIOA, Siomycin A.

and 5 at the higher doses (10 and 20 Gy). The percentage of dead, trypan blue-positive cells did not change significantly over the time period in response to radiation at all doses and did not reach >8% at any time (Fig. 4C), consistent with the

inability to detect the apoptotic marker, cleaved PARP-1, on western blots (not shown). An MTT proliferation assay confirmed the predominance of growth inhibition rather than cell death at radiation doses of 10-40 Gy (Fig. 4D).

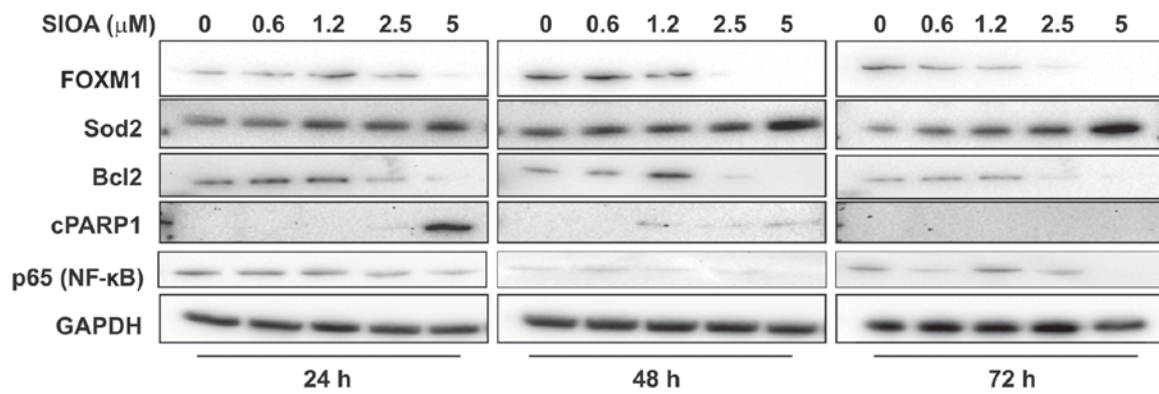


Figure 3. SIOA inhibits FoxM1 expression in SK-MEL-28 melanoma cells. SIOA (0.6-5 μ M) or vehicle (0.1% DMSO) was added to SK-MEL-28 cells and total protein harvested after 24, 48 and 72 h. Western analysis was performed with antibodies targeting FoxM1 (120 kDa), Sod2 (25 kDa), Bcl2 (26 kDa), cleaved PARP1 (85 kDa), p65 (NF κ B) (65 kDa), and GAPDH (40 kDa). Blots are representative of 3 independent experiments. SIOA, Siomycin A; DMSO, dimethyl sulfoxide; FOXM1, Forkhead box M1; Sod2, superoxide dismutase 2; Bcl2, B-cell lymphoma 2; PARP-1, polymerase 1.

Radiation inhibits SK-MEL-28 migration in a scratch wound assay. Wound area after scratch injury was measured consecutively in live cells over 3 days in response to radiation. Wound area (as a percentage of denuded area measured immediately after scratch injury) continued to decline at all doses over the time period as cells repopulated the denuded area (Fig. 4E and F). Inhibition of regrowth was seen at all doses however this reached significance at doses of 20 Gy ($P < 0.05$) and 40 Gy ($P < 0.01$).

Radiation does not alter FOXM1 expression in SK-MEL-28 melanoma cells. No significant change in FOXM1 protein expression was observed in SK-MEL-28 cells in response to radiation (Fig. 5). SOD2 and BCL2 protein expression increased transiently with radiation dose at the 24 h time point relative to the non-irradiated control. BCL2 expression was moderately reduced at the higher doses (20 and 40 Gy) at later time points, however SOD2 expression was not different to controls.

Pretreatment with SIOA at sub-lethal doses has no radio-sensitizing effect on SK-MEL-28 melanoma cells. To examine whether radiation and SIOA could act synergistically on inhibition of SK-MEL-28 cell growth and migration, cells were treated with a mid-range dose of radiation (10 Gy) combined with SIOA. Cells were treated with Siomycin A 24 h prior to radiation treatment to allow FoxM1 knockdown prior to irradiation. As earlier experiments showed complete cell loss after 2 days with concentrations of SIOA $> 1.2 \mu$ M, cells were examined at sub-lethal concentrations of 0.3 and 0.6 μ M SIOA with 10-40 Gy doses of radiation.

MTT proliferation assays were performed to establish any synergistic effects of SIOA and radiation on cell proliferation. At a dose of 0.3 μ M (Fig. 6A), Siomycin A had minimal effect on proliferation in these assays, while at 0.6 μ M, SIOA significantly reduced proliferation from day 3 onwards (Fig. 6B), consistent with earlier results (Fig. 2). In combination with radiation, no further reduction in proliferation was observed over the time period at either dose (Fig. 6A and B). Similarly, no synergistic effects of SIOA and radiation were observed on migration (Fig. 6C and D). At high doses ($> 1 \mu$ M), apoptosis was absolute within 2 days in the 8-well chamber

slides and no further contribution from radiation could be observed. At sub-lethal concentrations (0.3-0.6 μ M), there were no synergistic effects on migration at radiation doses of 10 and 20 Gy measured at 72 h after radiation (Fig. 6C and D).

Discussion

Overexpression of the pleiotropic transcription factor FOXM1 has been observed in many human malignancies and regulates multiple pathways important for tumorigenesis (5,6). Recent studies demonstrate FOXM1 overexpression in malignant melanoma and an association with cancer stage and prognosis suggesting FOXM1 knockdown may be a novel therapeutic target (7-9). Consistent with these studies, we observed reduction in FOXM1 at micromolar Siomycin A doses in the metastatic melanoma cell line, SK-MEL-28, with a rather rapid drop-off in FOXM1 expression at concentrations above 1 μ M. BCL2 was similarly inhibited by SIOA in the same dose range and this is of particular importance as BCL2 is a well-known anti-apoptotic factor and a downstream target of FOXM1 (17). BCL2 and FOXM1 inhibition occurred simultaneously with an increase in expression of the apoptotic marker, cleaved PARP-1. BCL2 is also a target of the NF κ B transcription factor (18), and downregulation may have been associated with the simultaneous decrease observed in constitutive NF κ B (RELA/P65) expression. In other studies, proteasome inhibitor-induced apoptosis is at least partly mediated via suppression of the NF κ B signalling pathway (19). Our results illustrated a rapid induction of apoptosis at doses between 1-2 μ M Siomycin A and are in agreement with the regulatory role of FOXM1 and BCL2.

SOD2 has been demonstrated to be both a FOXM1 (20,21) and NF κ B (22) transcriptional target but our data suggested an uncoupling of this relationship in response to Siomycin A—we found that SOD2 expression was unchanged despite a dramatic loss of FOXM1 and NF κ B expression. In addition we observed a transient induction of SOD2 in response to radiation but this did not correlate with FOXM1 expression, which remained unchanged. SOD2 is an important scavenger enzyme controlling intracellular reactive oxygen species (ROS), and overexpression is a known contributor to both chemo- and radioresistance in various tumor types (23). Silencing FOXM1 was shown to

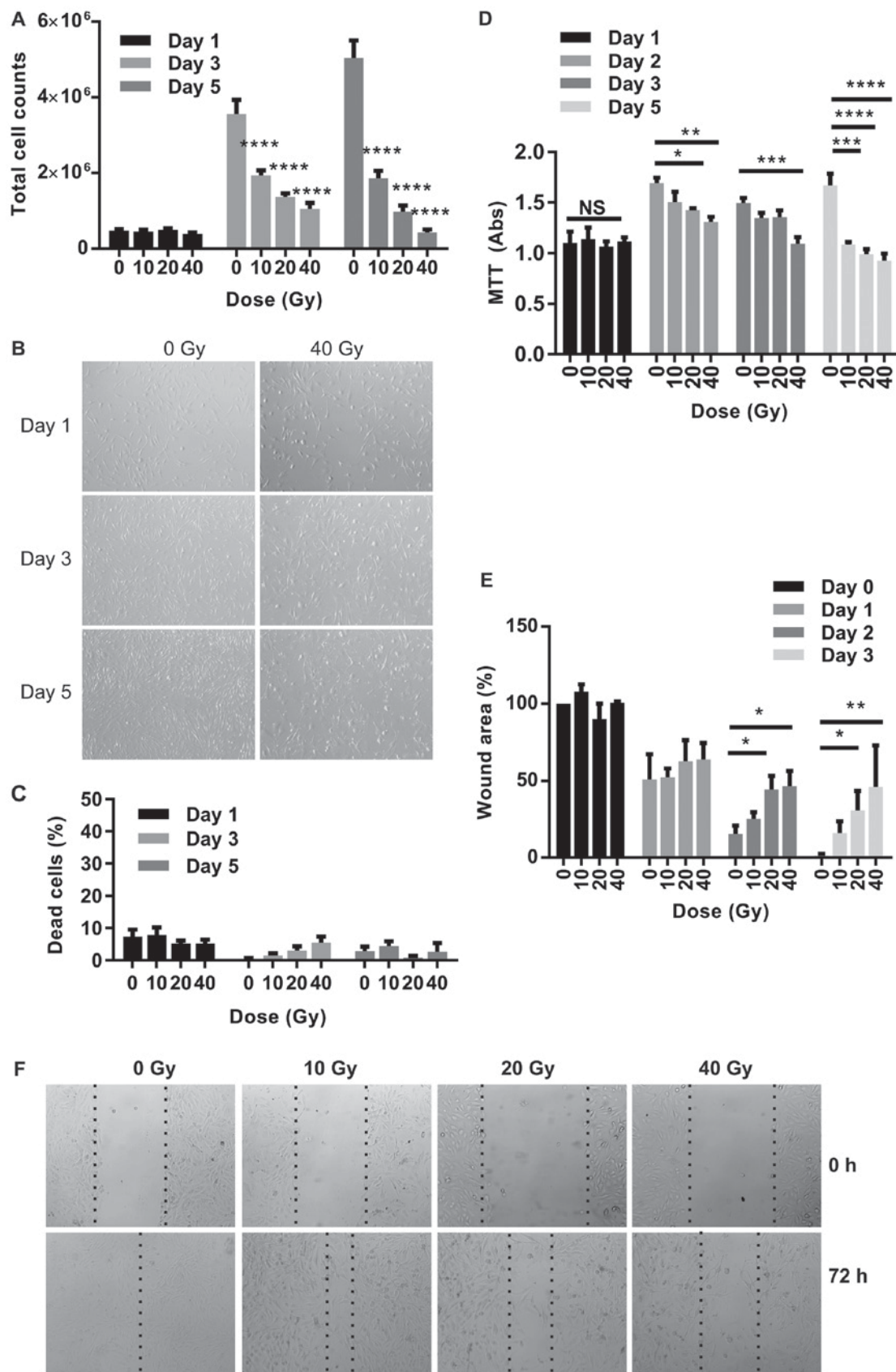


Figure 4. Radiation inhibits growth and migration of SK-MEL-28 melanoma cells without significant cell death. SK-MEL-28 cells were irradiated at doses of 0-40 Gy by linear accelerator and monitored for viability and migration for up to 5 days. (A) Cell counts (trypan blue-negative cells/ml) by trypan blue assay and automated cell counting. Data shown as mean \pm SEM and representative of 2 independent experiments; (B) Representative images of SK-MEL-28 cell morphology after irradiation. Original magnification, 25x; (C) Proportion of dead cells (% of total) measured by trypan blue viability assay and automated cell counter. Data shown as mean \pm SEM and is representative of 2 independent experiments. (D) Cell proliferation measured by MTT assay, mean \pm SEM of 3 independent experiments; (E) Wound area after scratch injury in response to radiation at 0-40 Gy dose. Data represent mean \pm SEM of 3 independent experiments; (F) Representative images of wound area after irradiation at day 0 and day 3. Dotted lines approximate the borders between cellular and denuded areas. Original magnification, x50. Two-way ANOVA with Dunnett's post-hoc test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. SEM, standard error of the mean.

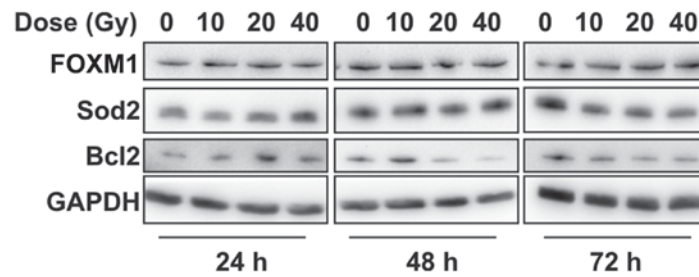


Figure 5. Radiation has no inhibitory effect on FoxM1 in SK-MEL-28 melanoma cells. Doses of 0-40 Gy X-ray radiation were delivered to SK-MEL-28 cells in 6-well plates and total protein harvested after 24, 48 and 72 h. Western analysis was performed with antibodies targeting FoxM1 (120 kDa), Sod2 (25 kDa), Bcl2 (26 kDa), and GAPDH (44 kDa). Cleaved PARP-1 was not detected at any time point (not shown). Blots are representative of 2 independent experiments. FOXM1, Forkhead box M1; Sod2, superoxide dismutase 2; Bcl2, B-cell lymphoma 2.

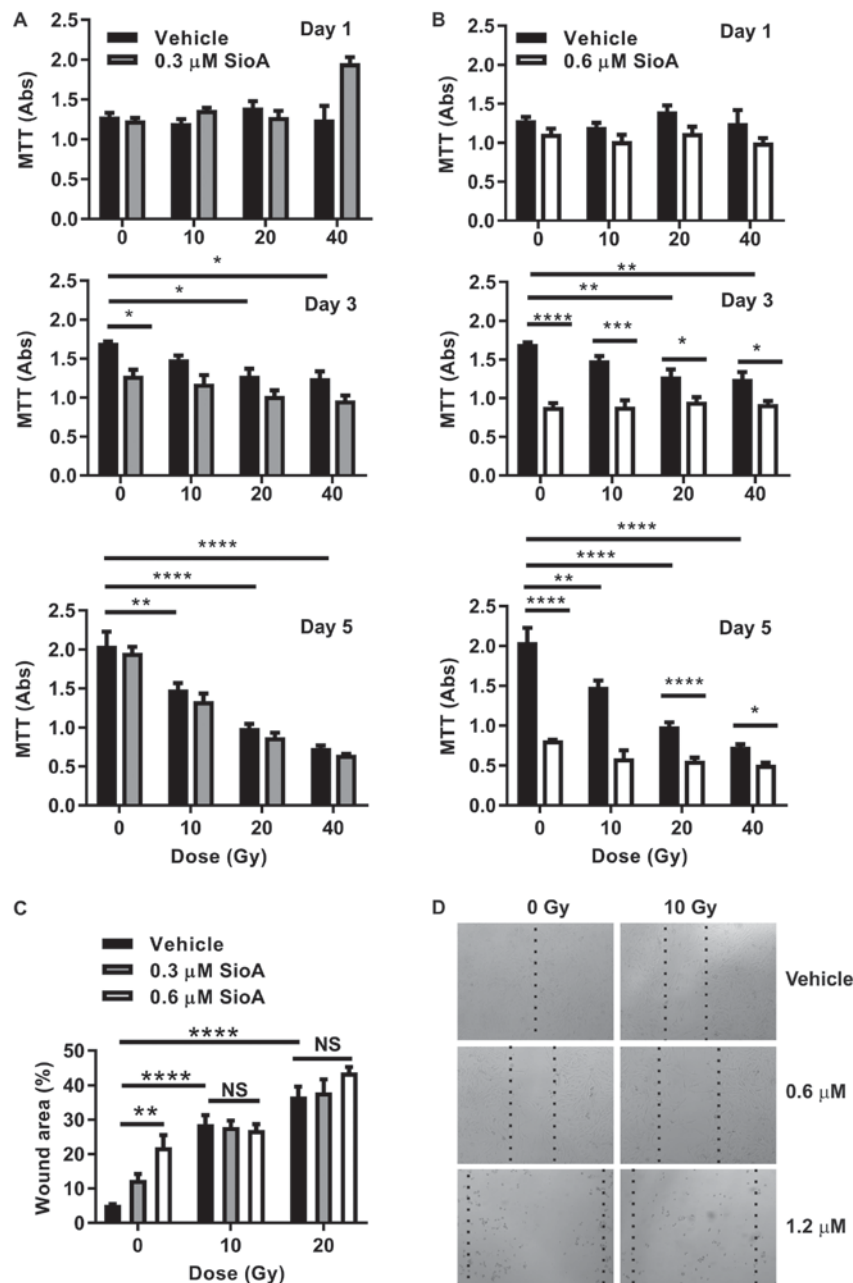


Figure 6. Combinatorial effects of SIOA and radiation on cell proliferation and migration. Cells were treated with SIOA 24 h prior to irradiation by linear accelerator. Cell proliferation was monitored at day 1, 3 and 5 post-irradiation by MTT assay with 0.3 μ M (A) or 0.6 μ M (B) SIOA. Data represent mean \pm SEM of 3 independent experiments. (C) Cell migration was monitored at day 3 post-irradiation with 0, 10 and 20 Gy doses. Mean \pm SEM of 3 independent experiments. (D) Representative images of scratch wound areas 72 h after irradiation at 0-10 Gy after pretreatment with 0.6 or 1.2 μ M SIOA. Dotted lines approximate the borders between cellular and denuded areas. Original magnification, x50. Two-way ANOVA with Dunnett's post-hoc test: * P <0.05, ** P <0.01, *** P <0.001, **** P <0.0001. SIOA, Siomycin A; SEM, standard error of the mean.

decrease *SOD2* expression, increase intracellular ROS levels and in turn the sensitivity to ROS inducers (not radiation), enhancing apoptosis *in vitro* and *in vivo* (21). We think that the observed *FOXMI-SOD2* uncoupling may have contributed to the inability of SIOA to sensitize melanoma cells to radiation. In line with this observation, others have shown that radiosensitivity can be enhanced by downregulation of *NFκB* and *SOD2* (24) and increased chemosensitivity can be induced by simultaneous *BCL2* and *SOD2* knockdown in resistant melanoma (25). Collectively further studies on *FOXMI-SOD2* interactions in tumor cells with different radiosensitivity would shed light on the underpinnings of radioresistance.

To our knowledge, this is the first study to examine the effects of combining SIOA as a *FOXMI* inhibitor with radiation in melanoma cells. Combination of *FOXMI* suppression and radiation was previously investigated in various tumor cell lines, which overall suggested a potential synergistic treatment effect (11,17,26). Our results contrast with these findings, however, in that while the basal expression of *FOXMI* was significantly induced by radiation in various non-melanoma cancer cells types, radiation appeared to have no effect on *FOXMI* expression. Similarly, robust apoptosis was induced by radiation in various non-melanoma cells but only cell stasis was observed here, even up to doses of 40 Gy. This dose range is well above that used in other combination studies and valid in the clinical setting, hence the radiation dose range does not appear a limiting factor in this study (11,17,26). Rather, it appears that the basal level of sensitivity of individual cell types to radiation and the ability to further induce *FOXMI* may predict the efficacy of combination treatment. Such notion of cell specificity is echoed by a lymphoma study in which synergistic induction of apoptosis in several cell lines treated with combined proteasome inhibitor and radiation was noted to be dependent on the p53 status with mutant or null p53 cells exhibiting a lack of sensitisation and vice versa (27). On this note, the observed resistance to radiosensitization of SK-MEL-28, being p53 mutant (28), corroborates with these findings.

It should be noted that in this study we used the MTT assay as a surrogate for the use of clonogenic survival assays. Good correlation has been demonstrated between clonogenic survival and MTT assays, especially when multiple and extended time points are examined to account for the acute and post-acute phases of the radiation response (29). However it should be made clear that our findings of lack of synergism refer to the properties of proliferation and migration and relationships to clonal survival and invasion are inferred from the important role they play in these phenotypes.

We demonstrated that *FOXMI*-overexpressing melanoma cells, SK-MEL-28, were highly susceptible to SIOA-induced apoptosis which in turn was associated with *FOXMI*, *BCL2* and *NFκB* inhibition. SIOA treatment, however, did not further sensitize these radioresistant cells to combined radiation treatment. The combination of *FOXMI* inhibition and radiation therapy may be ineffective for radioresistant melanoma.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

VSL was involved in data acquisition (all techniques), analysis and interpretation of data, manuscript drafting and revision, and the final approval of the manuscript. LSM was involved in the conception and design of the study (methodology), data acquisition (migration, western blotting), analysis and interpretation of data, manuscript drafting and revision, and the final approval of the manuscript. VM and EDS were involved in the conception and design of the study (radiation), manuscript revision, and the final approval of the manuscript. TLS was involved in the conception and design of the whole study, analysis and interpretation of data, manuscript drafting and revision, the final approval of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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