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14. ABSTRACT

Radiotherapy is a common therapeutic modality for the treatment of human prostate cancer. However, tumors often demonstrate resistance to ionizing radiation and continue to proliferate under genotoxic stress. The goal of this project is to determine whether silencing of MEK5 will sensitize prostate cancer cells to ionizing radiation. Previously, we showed that depletion of MEK5 sensitizes prostate cancer cells to γ-radiation as determined by both clonogenic survival and cell proliferation assays. Furthermore, MEK5 silencing impairs phosphorylation of DNA-PKcs in response to IR, delays resolution of IR-induced γH2AX and 53BP1 foci, and reduces DNA repair by non-homologous end-joining. In the current funding period, we have extended our *in vitro* findings by conducting *in vivo* experiments. We show that MEK5 knockdown in PC3 human prostate cancer cells combined with x-ray irradiation leads to a significant reduction in tumor growth in mice.

15. SUBJECT TERMS
MEK5, DNA-PKcs, non-homologous end-joining, ionizing radiation, prostate cancer

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INTRODUCTION

Radiation is a critical therapeutic modality for treating human prostate cancer. However, tumor recurrence and therapy resistance often ensue. Moreover, radiation is not free of other serious unwanted risks, including the promotion of secondary cancer (1), as well as increased tumor-associated angiogenesis and metastasis, especially when cells are subjected to sublethal doses of radiation. A major cause of failure in radiation treatment is intrinsic and therapy-induced radioresistant tumor cells. The major mechanisms for radiotherapeutic resistance are the DNA damage and repair pathway, as well as cell membrane-associated prosurvival pathways such as EGF receptor and phosphoinositide 3-kinase (PI3K)/AKT. Although the delivery of higher doses of ionizing radiation improves local control (2-4), there are constraints due to dose-limiting toxicities to noncancerous tissues (5-7). Thus, lowering radiation dose, while preserving therapeutic index, is a goal in both the laboratory research setting and the clinic.

Mitogen-activated protein kinase kinase 5 (MAP2K5 or MEK5), belongs to the family of MAP kinases. It is activated by the upstream kinases MEKK2 and MEKK3 at S311/T315, or in some cases directly by c-Src (8-11). MEK5, in turn, phosphorylates and activates extracellular signal-regulated kinase 5 (ERK5 or BMK1) at T218/Y220 (8). Targeted deletion of *Mek5* or *Erk5* genes in mice is embryonic lethal due to defects in blood vessel formation and cardiac development (12-14). Initial vascularization in *Erk5*$^{-/-}$ embryos occurs normally; however, subsequent remodeling and/or maintenance of vasculature (angiogenesis) is adversely affected by the absence of ERK5 (15). Most importantly, the MEK5/ERK5 signaling pathway is essential for tumor-associated angiogenesis (16). Tumor xenografts growing in *Erk5* knockout mice show a significantly reduced vascular density and tumor growth than growing in *Erk5* wild type mice (16).

The MEK5/ERK5 pathway can be activated by various stimuli such as oxidative stress, growth factors, and mitogens downstream of receptor tyrosine kinases, and G protein-coupled receptors, and culminates in the activation of a large number of transcription factors, including MEF2 (myocyte enhancer factor 2), c-JUN, NF-κB, CREB, and transcription factors that control the epithelial-mesenchymal transition (EMT) program (17-22).

We have shown that MEK5 downregulation enhances radiosensitization in human prostate cancer cells. Furthermore, we have demonstrated that MEK5 silencing modulates the activation of DNA-PKcs, major player of the DNA repair pathway, impairs non-homologous end-joining, and delays resolution of ionizing radiation induced γH2AX and 53BP1 foci. We have extended our in vitro observations to mouse xenografts and show that combination of MEK5 downregulation with ionizing radiation leads to a dramatic reduction in tumor growth *in vivo*.

KEYWORDS

MEK5, DNA-PKcs, non-homologous end-joining, ionizing radiation, prostate cancer, xenograft model

ACCOMPLISHMENTS

What were the major goals of the project?

Specific Aim 1

Task 3: MEK5 silencing combined with irradiation attenuates prostate tumor growth *in vivo*

Specific Aim 2

Task 2: Perform global gene expression analysis. Confirm by quantitative real time (qRT)-PCR.

What was accomplished under these goals?

(1) We have completed successfully the *in vivo* experiments with MEK5 knockdown and radiation.

(2) We have completed the DNA microarray experiments using shMEK5 alone or in combination with irradiation in PC3 and DU145 cells.

Specific Aim 1

Task 3: Evaluate the efficacy of MEK5 knockdown combined with radiation in the inhibition of prostate tumor growth *in vivo.*

To examine the impact of MEK5 knockdown on the radiosensitization of prostate cancer cells, we used PC3 stably expressing a shRNA targeting MEK5 (clone 12), a clone that shows the greatest MEK5 downregulation, or a non-targeting, control, shRNA. We confirmed MEK5 downregulation by Western blotting (**Figure 1**). We performed *in vitro* proliferation assays using PC3/shControl and PC3/shMEK5 and demonstrated silencing MEK5 in cells cultured in complete medium (10% fetal bovine serum) grew at similar rates as control PC3 cells (**Figure 2**). Next, we evaluated the efficacy of MEK5 knockdown combined with radiation in the inhibition of prostate tumor growth *in vivo*. All procedures were approved by the Columbia University Institutional Animal Care and Use Committee. Six-week old male athymic NU/J mice (Jackson Laboratory) were injected subcutaneously with $3X10^6$ PC3 cells expressing control (shControl) or MEK5 (shMEK5) shRNA. When tumors reached a volume of approximately ~200 mm^3, mice were randomized to one of the following groups: (i) shControl, unirradiated; (ii) shControl; irradiated; (iii) shMEK5; unirradiated; (iv) shMEK5; irradiated. Mice were either left untreated or irradiated with 4 Gy using the Small Animal Radiation Research Platform (SARRP; Xstrahl, Suwanee GA) irradiator as previously described (25). Briefly, mice were anesthetized with 100 mg/kg ketamide and 10 mg/kg xylazine in 0.9% saline by intraperitoneal injection and underwent cone beam computed tomography (CBCT) imaging using the onboard imager of the SARRP for image guided localization of the tumor. A single beam was designed in the sagittal arrangement to deliver 4 Gy radiation through a 10x10 mm^2 collimator prescribed to the isocenter. Radiation was delivered at a potential of 220 kVp and a filament current of 13 mA. Detailed radiation dosimetry and radiation planning information is provided **Figure 3**. Tumor growth was measured twice weekly with a caliper and the volume was estimated according to

the formula *Length x Width^2 x 0.50*, where length is the longest dimension and width the corresponding perpendicular dimension.
PC3/shControl cells were resistant to 4 Gy γ-rays and grew as fast as the control unirradiated cells (**Figure 4**). In contrast, PC3/shMEK5 cells exposed to radiation grew very slowly compared with unirradiated PC3/shMEK5 cells ($p < 4.7E-04$). Interestingly, in contrast to the in vitro proliferation data, unirradiated PC3/shMEK5 cell growth was significantly impaired when compared with unirradiated PC3/shControl cell growth ($p = 0.022$).
We conclude that MEK5 knockdown sensitizes PC3 cells to ionizing radiation not only *in vitro*, but also in xenografts.

Specific Aim 1 has been completed.

Specific Aim 2

Task 2: Perform global gene expression analysis.

PC3/shControl and PC3/shMEK5, as well as DU145/shControl and DU145/shMEK5 were exposed to 4 Gy of γ-rays and RNA was isolated 6 h and 24 h post-irradiation. RNA was purified using RNeasy mini kit and RNA yields were quantified using the NanoDrop ND1000 spectrophotometer (Thermofisher) and RNA quality was checked by the 2100 Bioanalyzer (Agilent). High quality RNA with an RNA integrity number of at least 9.0 was used for microarray hybridization. Cyanine-3 labeled cRNA was prepared using the One-Color Low Input Quick Amp Labeling kit (Agilent). Dye incorporation and cRNA yield were measured with a NanoDrop ND1000 spectrophotometer (Thermofisher). Labeled cRNA was fragmented and hybridized to Agilent Human Gene Expression 4x44K v2 Microarray Kit (G4845A). Slides were scanned with the Agilent DNA microarray scanner (G2505B) and the images were analyzed with Feature Extraction software (Agilent) using default parameters for background correction and flagging non-uniform features.

Aim 2 is still in progress.

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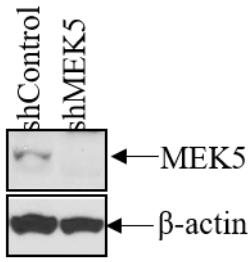


Figure 1: PC3 clone 12 stably expressing shMEK5. PC3 cells were infected with lentiviral particles containing pLKO.1-shRNA against MEK5 or a control shRNA. shMEK5 clones were isolated after puromycin (1 µg/ml) selection. Cell lysates from control or clone 12 were immunoblotted with anti-MEK5 and anti-β-actin (loading control) antibodies.

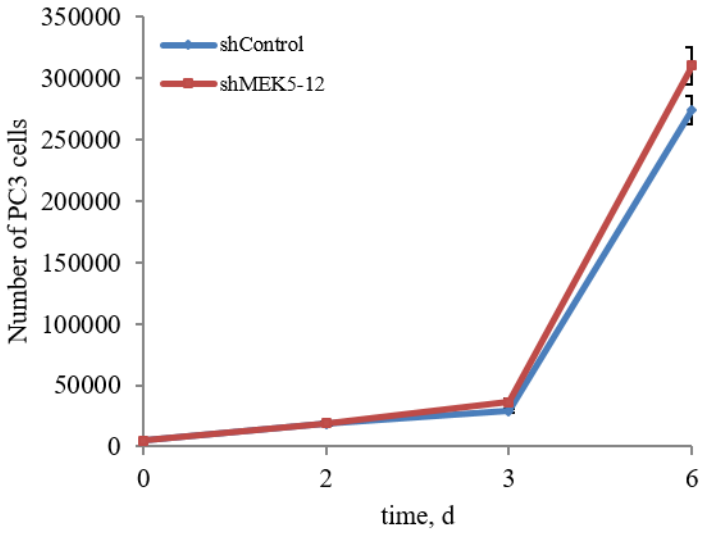
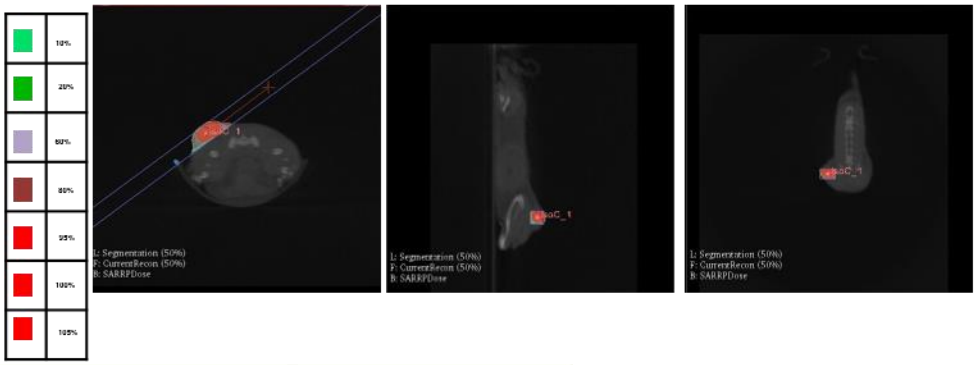


Figure 2: Cell proliferation. PC3 (shControl, shMEK5 clone 12) were seeded in 12-well plate (5,000 cells/well). Cells were trypsinized and counted with a hemocytometer at days 0, 2, 3, and 6.

A



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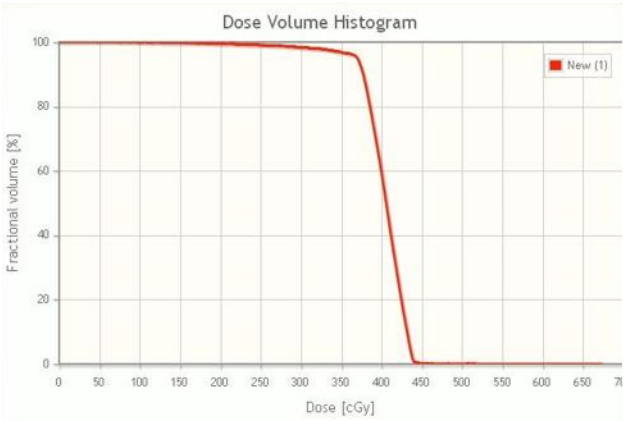

C

Dose (cGy)	Tumor
Mean	382.2
Min	19.4
Max	392.6

Figure 3: Radiation plan dosimetry and dose-volume histograms. (A) Representative images in coronal, axial and sagittal orientation of tumor-bearing mouse with radiation target volumes (tumor, red; tumor isocenter, cyan) contoured on cone-beam computed tomography images imported into MuriPlan software (Xstrahl, Suwanee, GA). **(B, C)** Representative dose-volume histogram (DVH) and corresponding dosimetry to tumor (mean, minimum and maximum radiation dose (cGy)) for Tumor RT treatment plans.

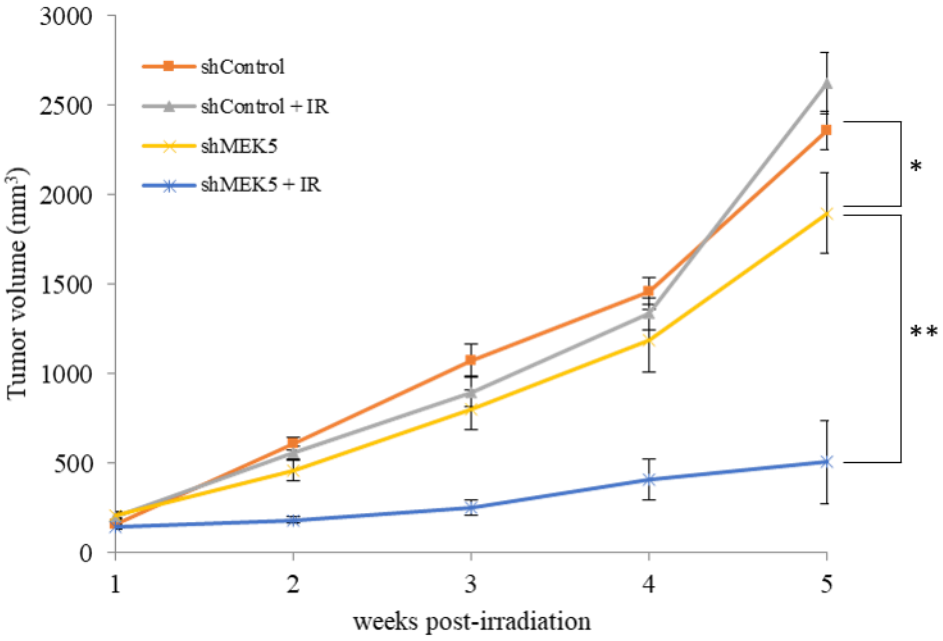

Figure 4: Tumor growth. PC3 cells stably expressing control Scrambled or MEK5 shRNA were injected subcutaneously into athymic male NU/J mice. When tumors reached ~200 mm^3, mice were irradiated with 4 Gy x-rays (IR) or they were sham irradiated. Tumor growth was measured using a caliper. Shown mean volume ± S.E.M. (n = 8 mice /treatment). * p = 0.022 ** p < 4.7E-04.

What opportunities for training and professional development has the project provided?

Nothing to Report

How were the results disseminated to communities of interest?

Results were presented in the AACR- Prostate Cancer: Advances in Basic, Translational, and Clinical Research meeting (https://www.aacr.org/Meetings/Pages/MeetingDetail.aspx?EventItemID=114).

What do you plan to do during the next reporting period to accomplish the goals?

(1) Perform immunohistochemical analysis of PC3 tumor samples from the *in vivo* experiments. Analyze proliferation and apoptosis by Ki67 and TUNEL staining.

(2) Examine the effect of MEK5 on irradiation-induced angiogenesis as outlined in the proposal. IL-1β will be tested for its ability to affect angiogenesis.

(3) Analyze DNA microarray data. Confirm expression of select differentially expressed genes by qRT-PCR.

IMPACT

What was the impact on the development of the principal discipline(s) of the project?

We have discovered that MEK5 downregulation sensitizes prostate cancer cells to radiotherapy. Mechanistically, MEK5 knockdown leads to reduced phosphorylation/activation of DNA-PKcs, delays repair of DNA double strand breaks, as judged by the persistence of γH2AX and 53BP1 IR-induced foci and impairs non-homologous-end joining. Finally, our *in vivo* experiments confirm the importance of MEK5 silencing as a radiosensitizing modality.

What was the impact on other disciplines?

Nothing to Report

What was the impact on technology transfer?

Nothing to Report

What was the impact on society beyond science and technology?

Nothing to Report

CHANGES/PROBLEMS

Changes in approach and reasons for change
Angiogenesis experiments using Human Prostate Microvascular Endothelial Cells from a commercial source were not feasible, because cells could not be propagated in culture. As an alternative, we will perform angiogenesis experiments using HUVEC cells that we are able to grow them for several passages in culture.

Actual or anticipated problems or delays and actions or plans to resolve them
None anticipated

Changes that had a significant impact on expenditures
Nothing to Report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
No change

PRODUCTS

Publications, conference papers, and presentations

Constantinos G. Broustas. MEK5 downregulation enhances radiosensitization of human prostate cancer cells by inhibiting DNA repair [abstract]. In: Proceedings of the AACR Special Conference: Prostate Cancer: Advances in Basic, Translational, and Clinical Research; 2017 Dec 2-5; Orlando, Florida. Philadelphia (PA): AACR; Cancer Res 2018;78(16 Suppl):Abstract nr B030.
http://cancerres.aacrjournals.org/content/78/16_Supplement/B030

Radiotherapy is commonly used to treat a variety of solid human tumors, including localized prostate cancer. However, treatment failure almost always ensues due to tumor intrinsic or acquired radioresistance.

Mitogen-activated protein kinase kinase 5 (MAP2K5 or MEK5), belongs to the family of MAP kinases. It is activated by the upstream kinases MEKK2 and MEKK3 at Ser311/Thr315. MEK5, in turn, phosphorylates and activates extracellular signal-regulated kinase 5 (ERK5 or BMK1) at Thr218/Tyr220. MEK5/ERK5 pathway plays a pivotal role in tumor initiation and progression. MEK5 protein is overexpressed in prostate cancer cells compared with normal prostate epithelial cells, and MEK5 levels are correlated with prostate cancer metastasis. High expression of ERK5 in prostate cancer is also found to correlate with poor disease-specific survival and can serve as an independent prognostic factor.

To determine whether the MEK5/ERK5 pathway is activated in response to ionizing radiation (IR), RNA interference was used to deplete MEK5 from PC3 and DU145 cells. Western blot analysis demonstrated that control cells with normal levels of MEK5 exposed to 3-Gy γ-rays had an increase in phospho-ERK5 levels at 5 and 15 min post-IR, diminishing at later time points. No activated ERK5 was detected in MEK5-depleted cells. Downregulation of MEK5 did not impact on cell cycle checkpoint activation in irradiated cells. In contrast, depletion of MEK5 markedly impaired phosphorylation of DNA-PKcs at Ser2056 in response to IR treatment. Furthermore, MEK5 knockdown did not influence the initial appearance of γH2AX or 53BP1 foci after irradiation, but significantly delayed the resolution of radiation-induced γH2AX and 53BP1 foci, detectable even 48 h post-irradiation, indicating a DNA repair defect. Cell based assay showed that nonhomologous end-joining is compromised in PC3 cells with ablated MEK5 protein expression. Finally, long-term clonogenic survival analyses and short-term cell growth assays indicated that MEK5 knockdown sensitized PC3 and DU145 prostate cancer cell lines to IR. Likewise, the topoisomerase II inhibitor etoposide that causes double-strand breaks also sensitized MEK5-depleted cells.

These data indicate that MEK5 influences the response of prostate cancer cells to radiation and MEK5 downregulation is associated with delayed double-strand break repair kinetics. Inhibition of MEK5 in combination with radiation may provide a strategy to improve survival of prostate cancer patients.

PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: Constantinos G. Broustas
Project Role: PI
Nearest Person month worked: 5
Contribution to Project: Designed, performed, interpreted experiments

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report

What other organizations were involved as partners?

Nothing to Report

SPECIAL REPORTING REQUIREMENTS

Nothing to Report

APPENDICES

None