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MEK inhibitor GSK1120212-mediated radiosensitization of pancreatic cancer cells involves inhibition of DNA double-strand break repair pathways

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Purpose: Over 90% of pancreatic adenocarcinoma PC express oncogenic mutant KRAS that constitutively activates the Raf-MEK-MAPK pathway conferring resistance to both radiation and chemotherapy. MEK inhibitors have shown promising anti-tumor responses in recent preclinical and clinical studies, and are currently being tested in combination with radiation in clinical trials. Here, we have evaluated the radiosensitizing potential of a novel MEK1/2 inhibitor GSK1120212 (GSK212, or trametinib) and evaluated whether MEK1/2 inhibition alters DNA repair mechanisms in multiple PC cell lines.

Methods: Radiosensitization and DNA double-strand break (DSB) repair were evaluated by clonogenic assays, comet assay, nuclear foci formation (γH2AX, DNA-PK, 53BP1, BRCA1, and RAD51), and by functional GFP-reporter assays for homologous recombination (HR) and non-homologous end-joining (NHEJ). Expression and activation of DNA repair proteins were measured by immunoblotting.

Results: GSK212 blocked ERK1/2 activity and radiosensitized multiple KRAS mutant PC cell lines. Prolonged pre-treatment with GSK212 for 24-48 hours was required to observe significant radiosensitization. GSK212 treatment resulted in delayed resolution of DNA damage by comet assays and persistent γH2AX nuclear foci. GSK212 treatment also resulted in altered BRCA1, RAD51, DNA-PK, and 53BP1 nuclear foci appearance and resolution after radiation. Using functional reporters, GSK212 caused repression of both HR and NHEJ repair activity. Moreover, GSK212 suppressed the expression and activation of a number of DSB repair pathway intermediates including BRCA1, DNA-PK, RAD51, RRM2, and Chk-1.

Conclusion: GSK212 confers radiosensitization to KRAS-driven PC cells by suppressing major DNA-DSB repair pathways. These data provide support for the combination of MEK1/2 inhibition and radiation in the treatment of PC.

Introduction

Pancreatic adenocarcinoma is the ninth most commonly diagnosed malignancy and the fourth leading cause of cancer-related death in the US.[1] According to the National Cancer Institute, an estimated ~46,000 new cases of pancreatic cancer were diagnosed and ~40,000 deaths occurred in the US in 2014.[2] At present, few therapeutic options are available for patients with this deadly disease, and there is an urgent need for the development of novel therapies for pancreatic carcinoma (PC). Approximately 80-85% of the patients are diagnosed with locally-advanced or metastatic disease precluding an option of surgical resection. Radiation alone or in combination with chemotherapy is an available treatment for locally advanced disease, and is sometimes used in the pre-surgical setting. It is remarkable that 90% or more of tumors harbor an oncogenic mutant KRAS, which other studies have shown confers resistance to radiation both *in vitro* and *in vivo*.[3-5] Therefore, an in-depth understanding of how deregulated KRAS contributes to the development of radioresistance in pancreatic as well as other malignancies expressing activated RAS oncogenes is critical for developing effective treatments.

Under physiologic conditions, growth factor-activated KRAS recruits and activates the RAF family of serine/threonine kinases, which in turn, activate the dual protein kinases MEK1 and MEK2 that subsequently activate MAP kinases ERK1 and ERK2. Then, activated ERK1 and ERK2 phosphorylate their target substrates promoting cell proliferation, survival, and differentiation.[6,7] Aberrant activation of mutant KRAS results in the constitutive activation of this signaling pathway, which enhances

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the growth, survival and migration of pancreatic cancer cells.[8,9] The oncogenic mutant KRAS also activates the PI3K-AKT/mTOR pathway that plays many critical roles in tumorigenesis.[10,11] Importantly, we and others have demonstrated that KRAS-induced persistent activation of both MEK-ERK and PI3K-Akt pathways confers resistance to radiation and chemotherapy in pancreatic cancer.[5,12-14] Although radiation therapy significantly improves local control in pancreatic cancer, radiation induces an activation of the MEK-ERK pathway, possibly promoting the proliferation and survival of tumor cells *in vitro*.[12,15-17] Because there are apparent difficulties in targeting deregulated KRAS activity in pancreatic cancer, we and others have recently investigated the radiosensitizing potential of MEK inhibitors both *in vitro* in tumor cell lines and *in vivo* in mouse models.[12,18-20] Although multiple MEK1/2 inhibitors have exhibited promising outcomes in preclinical studies, many early trials did not demonstrate benefit from MEK1/2 inhibition.[21-23] This may be due to a narrow therapeutic window to spare non-tumor cells that rely on ERK1/2 signaling for their survival with older generation MEK inhibitors or lack of significant efficacy in unselected populations. However, more recent trials with more modern MEK1/2 inhibitors have shown substantial efficacy or improvements in clinical outcomes in BRAF/NRAS mutated melanoma, or KRAS mutated non-small cell lung cancer.[24-27]

A clinically useful MEK inhibitor is expected to produce prolonged MEK inhibition in tumor cells with minimal systemic toxicity resulting from targeting of non-tumor cells. To this end, GSK1120212 (GSK212 or trametinib), a potent and selective allosteric inhibitor of MEK1/2, has shown very promising antitumor activity in BRAF-driven melanoma.[25-27] This ATP-non-competitive inhibitor produces sustained inhibition of ERK1/2 activity in human tumor cell lines expressing oncogenic BRAF and RAS *in vitro* and suppresses ERK1/2 activity in multiple tumor models when orally delivered in a single dose per day.[28]

While MEK1/2 inhibitors have shown activity pre-clinically as a radiosensitizer, a better characterization of the DNA repair pathways altered by MEK inhibition is needed. In the current study, GSK212 has been evaluated for its radiation sensitizing potential *in vitro* in human pancreatic cancer cells. DNA double-strand break (DSB) is recognized as the primary mechanism for radiation-induced tumor cell killing through the formation of chromosomal aberrations.[29,30] Homologous recombination (HR) and non-homologous end-joining (NHEJ) are the 2 major pathways for repairing DSBs in mammalian cells and radioresistance is associated with upregulation of these pathways.[30] Currently it is not clear whether MEK1/2 inhibitors sensitize cancer cells to radiation through alteration of DNA repair pathways. Here, we report that GSK212 significantly downregulates activity of both HR and NHEJ pathways through various DNA repair intermediates, thereby conferring radiosensitization to human pancreatic cancer cells.

Results

GSK212 produces dose- and time-dependent inhibition of ERK1/2 phosphorylation/activation and radiosensitization in multiple pancreatic cancer cell lines *in vitro*

To establish that the MEK1/2 inhibitor GSK1120212 (GSK212 or trametinib) sensitizes pancreatic cancer cells to ionizing radiation, we determined its minimum effective concentration(s) that completely inhibit(s) phosphorylation/activation of ERK1/2 by an early time point (1 hr). We found that 5–10 nM GSK212 completely inhibited ERK1/2 phosphorylation at 1hr in 2 representative KRAS mutant pancreatic cancer cell lines MIAPaCa-2 and AsPC-1 (**Fig. 1A**). Next, we measured the kinetics of ERK1/2 inhibition by 10 nM GSK212 in these 2 cell lines. We found that ERK1/2 phosphorylation was almost completely inhibited within 30 to 60 min at 10 nM and that complete inhibition was sustained for 24 hr in the continuous presence of the inhibitor (**Fig. 1B**). Similar results were obtained with additional

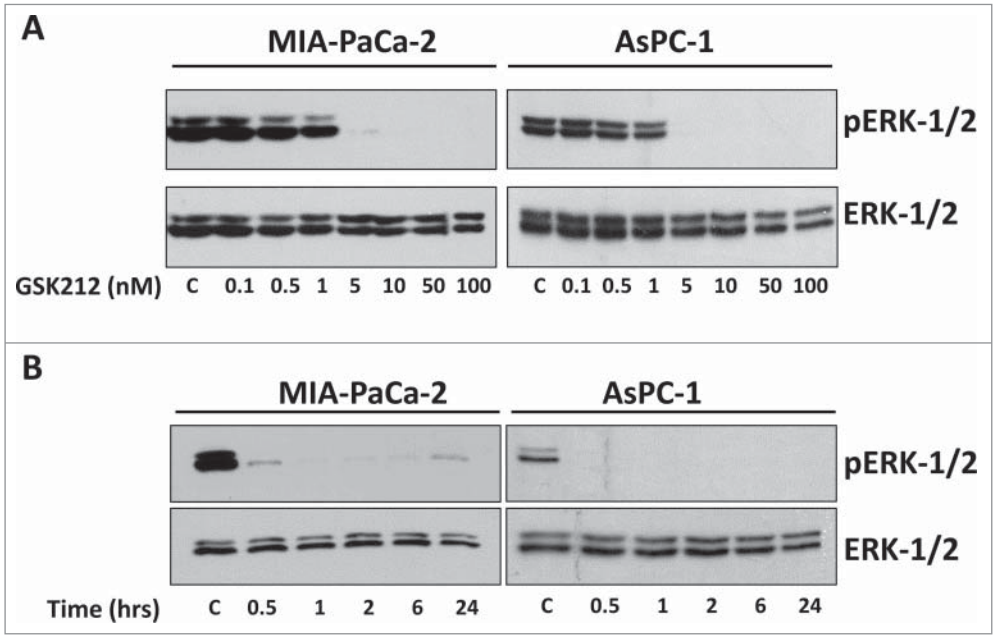

Figure 1. GSK212 is a potent MEK-1/2 inhibitor, demonstrating dose and time dependent inhibition of ERK-1/2. (**A**) Western blot images showing expression of total ERK-1/2 and phospho-ERK-1/2 in MIAPaCa-2 and AsPC1 cells treated for 1 hour with increasing doses of GSK212. ERK-1/2 activity is potently inhibited at 5–10 nM. (**B**) Western blot images showing rapid inhibition of ERK-1/2 activity within 30 min of treatment with 10 nM GSK212 that is sustained throughout 24 hours. C= control (DMSO). Multiple independent experiments were performed which showed similar results.

pancreatic cancer cell lines (AsPC1 and BxPC3). These data clearly demonstrate that GSK212 at 10 nM concentration acts as an effective inhibitor of MEK1/2 with rapid and potent suppression of ERK1/2 activity *in vitro* in the pancreatic cancer cell lines examined.

To determine the radiosensitizing potential of GSK212 in MIAPaCa-2 cells under conditions where ERK1/2 activity remained fully suppressed, radiation clonogenic survival assays were carried out. Our results found that treatment of MIAPaCa-2 cells for 1 hr with a low-dose of 10 nM GSK212 prior to delivering radiation (**Fig. 2A**) or 1 hr after irradiation (**Fig. 2B**) failed to radiosensitize cells in the continuous presence of GSK212 for 24 hr. However, when pretreated with 10 nM GSK212 for 24 hr (**Fig. 2C**) or 48 hr (**Fig. 2D**), MIAPaCa-2 cells became sensitized to radiation. These observations were confirmed in 2 other pancreatic cancer cells lines, AsPC-1 and BxPC-3 (**Fig. S1A-B**). As a control, GSK212 (10 nM) treatment for 48 hr prior to irradiation failed to radiosensitize a normal small intestinal epithelial cell line FHs74Int (**Fig. S1C**).

Taken together, these data demonstrate that GSK212 at 5–10 nM concentrations completely inhibited the constitutive activation of ERK1/2 by 1 hr, and that sustained MEK inhibition for 24–48 hr is required for induction of significant radiosensitization compared with shorter time periods at which GSK212 produced very little or undetectable radiosensitization.

Effect of GSK212-mediated MEK1/2 inhibition on global DNA damage response pathways

The goal of radiation therapy is to eradicate tumor cells by producing a variety of lesions in DNA, including single-strand breaks and DSBs. To test if GSK212 alters the global DNA repair process in irradiated pancreatic cancer cells, neutral comet assays were conducted. The comet assay, also known as single cell gel electrophoresis (SCGE) assay, is a simple and sensitive technique for detecting DNA damage at the level of individual cells.[31] We observed that pretreatment of MIAPaCa-2 cells with 10 nM GSK212 for 24 hr significantly delayed global DNA repair at 4 hr after exposure to 10 Gy radiation (**Fig. 3A**).

Efficient repair of DNA damage induces resistance to radiotherapy in cancer cells. The histone variant H2AX is a critical component of DNA repair process, which is rapidly phosphorylated by phophatidyl-inositol-3-kinase-like family Ser/Thr kinases including ATM, ATR and DNA-PK, on a unique C-terminal serine residue (Ser-139) to form γH2AX foci at nascent DSB sites.[32] This is required for the mobilization of DNA repair proteins to the DSB sites.[33] Thus, an accumulation of a large number of γH2AX molecules creates a focus at an individual DSB site, which allows the detection of DSBs with anti-γH2AX antibody.[34] As DSBs are repaired, γH2AX foci are resolved. Here, the impact of GSK212 (10 nM) pretreatment for 24 hr on γH2AX foci resoluton was determined in MIAPaCa-2 and AsPC-1 cells at 1, 6, and 24 hr after 3 or 6 Gy radiation. We found that GSK212 pretreatment for 24 hr (followed by its continuous presence) delayed the resolution of radiation-induced γH2AX foci in multiple pancreatic cancer cell lines at 6 and 24 hr, suggesting that MEK1/2 activity is required for efficient repair of radiation-induced DSBs in pancreatic cancer cells (**Fig. 3B-D**).

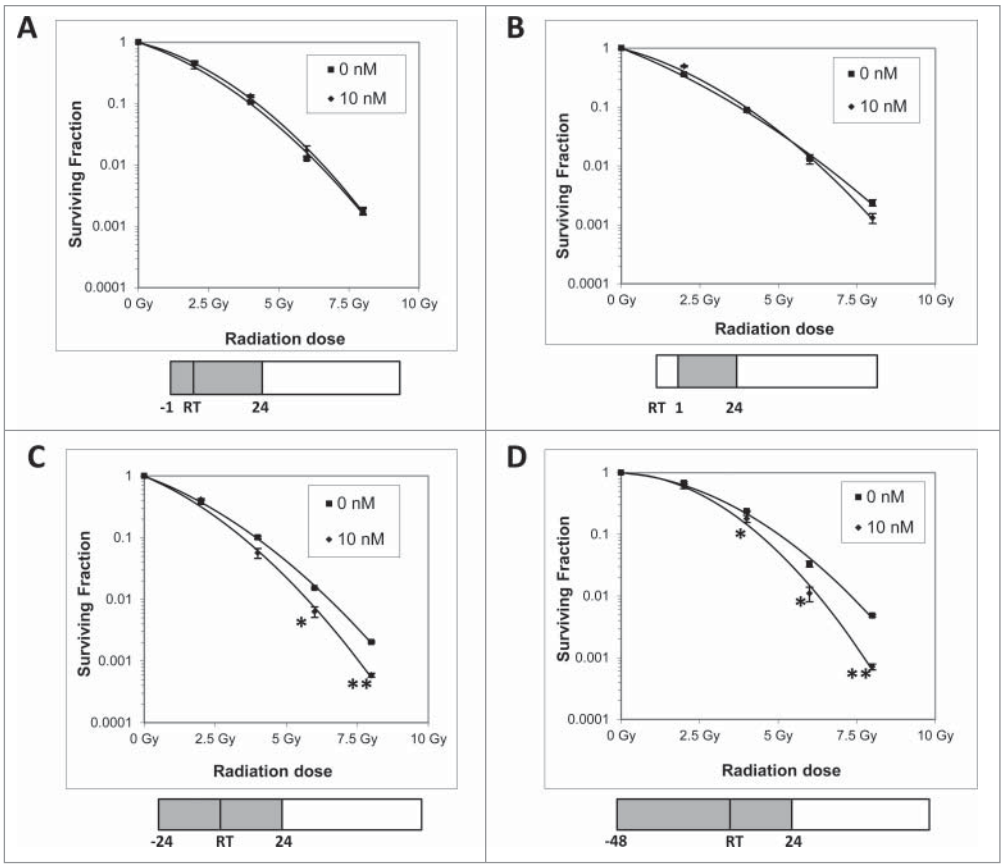

Figure 2. Prolonged MEK1/2 inhibition is required for effective radiosensitization. MIAPaCa-2 cells treated with 10 nM GSK212 or DMSO ("0 nM") for 1 hour before radiation (**A**) or 1 hour after radiation (**B**) does not result in radiosensitization in clonogenic survival assays. However, pretreating cells with GSK212 for 24 (**C**) or 48 (**D**) hours prior to radiation results in significant radiosensitization. In all cases, GSK212 was left on for an additional 24 hours after radiation before removal from the medium. (*p <0.01; **p<0.001) For all assays, multiple independent experiments were performed which showed similar results.

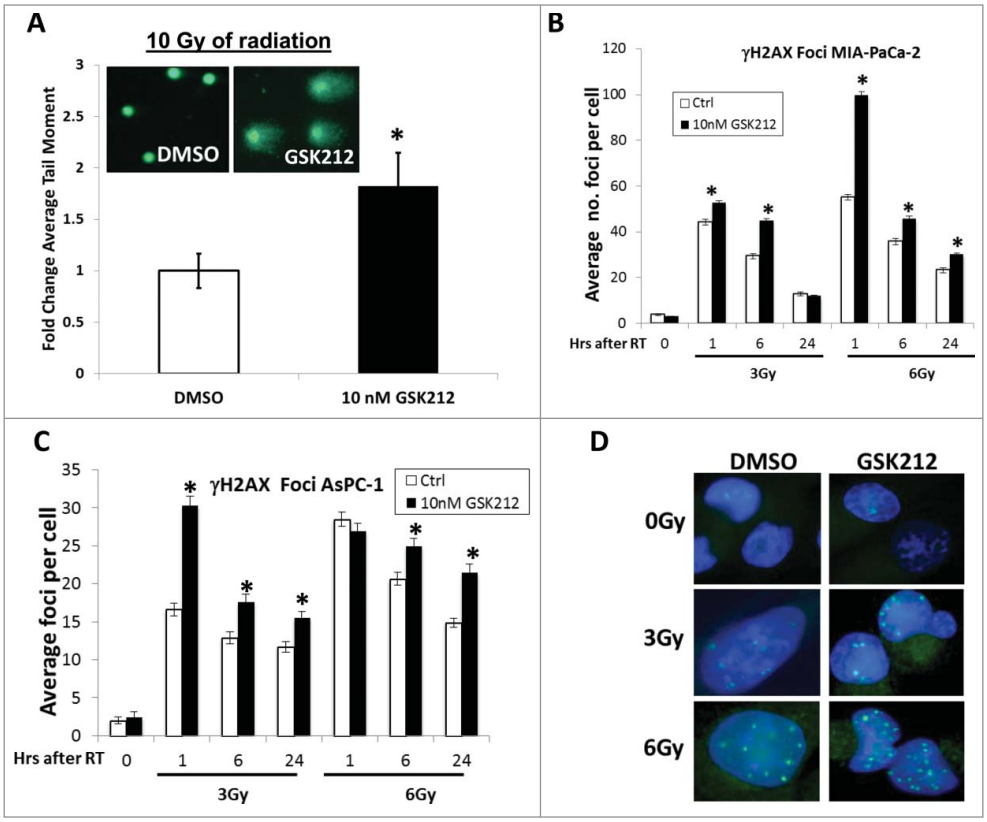

Figure 3. GSK212 treatment attenuates cellular DNA repair mechanisms. (**A**) Pretreatment of MIAPaCa-2 cells with 10 nM GSK212 for 24 hours prior to 10 Gy results in delayed repair compared to DMSO-treated cells, as measured 4 hours after radiation with a neutral comet assay (*p <0.05). Inset shows representative photos of comet tails taken with fluorescence microscopy at 4 hr after 10 Gy. (**B-C**) Pre-treatment of MIAPaCa-2 (B) or AsPC-1 (C) cells with 10 nM GSK212 for 24 hr followed by 3 or 6 Gy results in delayed resolution of nuclear γH2AX foci at 1, 6, and 24 hr after radiation compared to DMSO-treated ("Ctrl") cells. (*p<0.001) (**D**) Representative photos of γH2AX foci at 24 hr after radiation for DMSO or 10 nM GSK212-treated MIAPaCa-2 cells. For all assays, multiple independent experiments were performed which showed similar results.

Effect of GSK212-mediated MEK1/2 inhibition on DSB repair by HR

Since the development of radioresistance in human tumor cells can be caused by an up-regulation of DSB repair pathways, we examined the impact of GSK212 on both HR and NHEJ repair pathways in irradiated MIAPaCa-2 and AsPC-1 cells. BRCA1, encoded by the breast cancer associated gene 1 (*BRCA1*), plays an essential role in the HR repair pathway, and has controversial roles in the NHEJ and nucleotide excision repair pathways.[30,35,36] *In vitro* studies reveal that BRCA1 deficiency is associated with increased radiosensitization resulting from defects in the HR repair pathway.[37] Moreover, embryonic fibroblasts derived from *BRCA1*$^{-/-}$ mice are susceptible to radiation, implicating a central role of BRCA1 in DNA repair.[30] These findings prompted us to examine the effects of GSK212 on radiation-induced BRCA1 foci formation in the nuclei of MIAPaCa-2 and AsPC-1 cells. Our data reveal that MEK1/2 inhibition in pancreatic cancer cells caused a substantial reduction in nuclear BRCA1 foci after irradiation (**Figs. 4A-B**). These data demonstrate that BRCA1 foci formation, which is required for facilitating HR repair in response to radiation-induced DSBs,[38] was impaired by GSK212-mediated inhibition of MEK1/2.

To provide further evidence of dysregulation of the HR repair pathway, we studied the formation of RAD51 foci. RAD51 molecules accumulate at DSB sites and form nucleoprotein filaments which can be visualized by immunofluorescence microscopy as RAD51 foci.[39] Because overexpression of RAD51 upregulates the HR repair process and increases radioresistance in eukaryotic cells,[40] we examined if GSK212 compromises RAD51 foci formation in irradiated pancreatic cancer cells. We found that GSK212 also significantly decreased the number of RAD51 foci after irradiation of MIAPaCa-2 cells (**Fig. S2**).

To further demonstrate that GSK212-mediated MEK1/2 inhibition suppresses HR repair in irradiated pancreatic cancer cells, we used a previously validated MIAPaCa-2 stable cell line bearing a green fluorescent protein (GFP) reporter construct DR-GFP.[41] This DR-GFP construct measures homology-directed repair of I-SceI endonuclease-induced DSBs within the genome-integrated DR-GFP reporter construct.[42] MIAPaCa-2-DR-GFP cells were infected with adenovirus expressing I-SceI, to measure HR repair activity in the presence and absence of GSK212, by determining the percentage of GFP-positive cells 42 hr post-infection by flow cytometry. Our data confirm that GSK212 markedly decreased functional HR repair in cells when MEK1/2 inhibition occurred at the time of DNA damage induced by I-SceI, compared with MEK1/2 inhibition applied after induction of DNA damage (**Fig. 4C, D**).

Taken together, our data demonstrate that GSK212-mediated radiosensitization of pancreatic cancer cells is associated with the downregulation of DSB repair activity through the HR repair pathway.

Effect of GSK212-mediated MEK1/2 inhibition on DSB repair by NHEJ

NHEJ is another dominant repair pathway for radiation-induced DSBs in mammalian cells in which Ku70/Ku80 heterodimer recruits the catalytic subunit of DNA-dependent protein

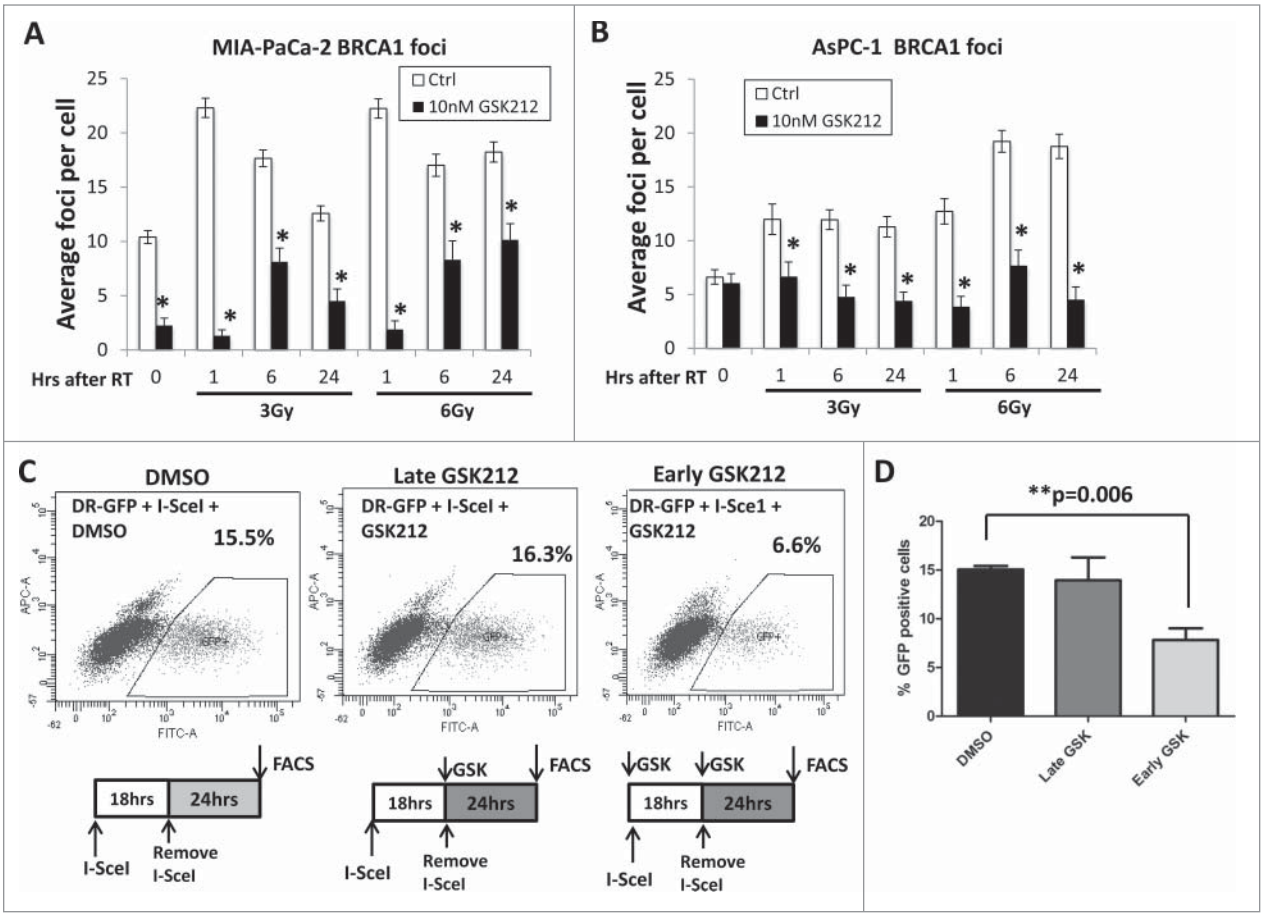

Figure 4. MEK1/2 inhibition results in attenuation of homologous recombination DNA double strand break repair. (**A-B**) Pre-treatment of MIAPaCa-2 (**A**) or AsPC-1 (**B**) cells with 10 nM GSK212 for 24 hr followed by 3 or 6 Gy results in defects in the formation of nuclear BRCA1 foci at 1, 6, and 24 hr after radiation compared to DMSO-treated ("Ctrl") cells. (*p <0.001) (**C**) MIAPaCa-2-DR-GFP cells were treated with 5 μL of I-SceI adenovirus to induce DNA double-strand breaks in the target DNA recognition sequence, and subjected to flow cytometry either in the presence of DMSO (vehicle), or 10 nM GSK212 for either 24 hr ("late GSK212") or 42 hr ("early GSK212"). Red indicates the presence of GSK212 in the medium of cells in the schematics accompanying each panel. Percent GFP-positive cells were quantified by flow cytometry. Note that early treatment of GSK212, but not late treatment, results in defects in functional HR repair as measured by a decrease in the %GFP positive cells compared to DMSO-treated cells (15.5% DMSO, 16.3% late GSK212, 6.6% early GSK212). (**D**) Mean of 3 independent experiments shown, which independently showed similar results.

kinase catalytic subunit (DNA-PKcs) to DSB sites. Subsequent dimerization of DNA-PKcs upregulates its catalytic activity through autophosphorylation and phosphorylation of other proteins involved in the NHEJ repair process.[30,43,44] Since upregulation of DNA-PK enhances the DSB repair process rendering tumors radioresistant in preclinical [45] and clinical studies,[46-48] we determined the effects of GSK212 on radiation-induced DNA-PK foci formation in MIAPaCa-2 and AsPC-1 cells. We noted that GSK212 significantly increased the presence of DNA-PK foci at various time points after radiation in both cell lines, including persistence of nuclear foci at 6 and 24 hr after radiation (**Figs. 5A-B**).

Another common marker of NHEJ repair is the 53BP1 protein. DSBs induce a rapid co-localization of the p53-binding protein 53BP1 with γH2AX and other DNA repairing proteins including BRCA1 to form discrete nuclear foci.[49,50] Because mice lacking 53BP1 are highly susceptible to radiation,[51,52] we sought to determine the effects of GSK212 on radiation-induced 53BP1 foci formation in MIAPaCa-2 and AsPC-1 cells. Here, we found that the kinetics of radiation-induced 53BP1 foci resolution were also altered by GSK212 treatment in both cell lines compared to DMSO-treated cells (**Fig. S2**). These results paralleled those observed with DNA-PK, with more 53BP1 foci observed after radiation. Taken together, these data initially suggested to us that activation of NHEJ was compensating for loss of HR repair activity, or that kinetics of DNA repair through NHEJ were altered/delayed.

To more directly determine whether GSK212 induces radiosensitivity by altering NHEJ repair, a fluorescent reporter construct that allows sensitive and quantitative measurement of NHEJ repair was employed. Similar to the DR-GFP construct for HR activity, this construct (Pem-1-NHEJ) contains an engineered GFP gene containing recognition sites for I-SceI endonuclease for induction of DSBs, which does not express GFP in the

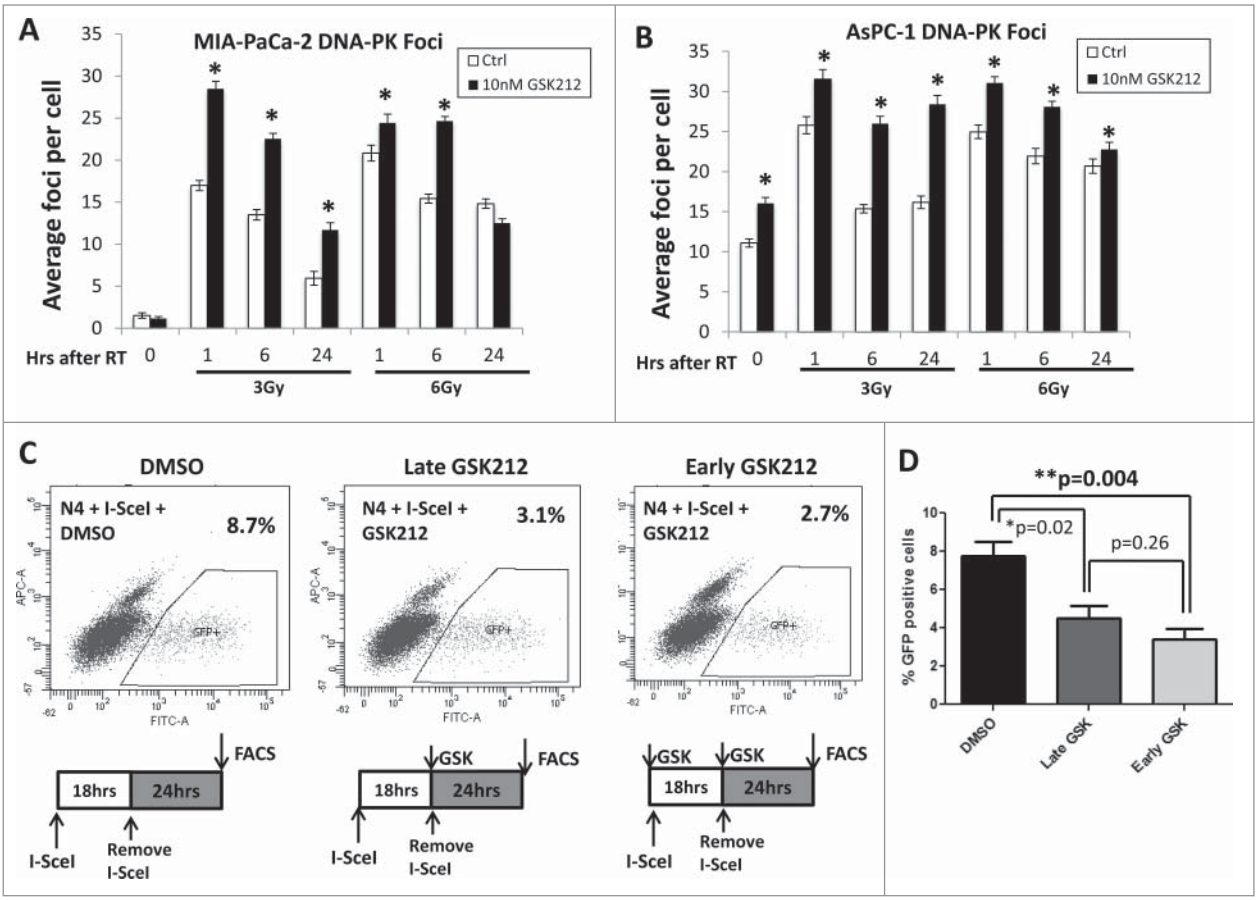

Figure 5. MEK1/2 inhibition attenuates non-homologous end joining repair DNA double strand break repair. (**A-B**) Pre-treatment of MIAPaCa-2 (**A**) or AsPC-1 (**B**) cells with 10 nM GSK212 for 24 hr followed by 3 or 6 Gy results in an increase in nuclear DNA-PK foci at 1, 6, and 24 hr after radiation in DMSO-treated ("Ctrl") cells. (*p <0.001) (**C**). MIAPaCa-2-Pem1-GFP cells were treated with 5 μL of I-SceI adenovirus to induce DNA double-strand breaks in the NHEJ reporter construct, and subjected to flow cytometry either in the presence of DMSO (control), or 10 nM GSK212 for either 24 hr ("late GSK212") or 40 hr ("early GSK212"). Percent GFP-positive cells were quantified by flow cytometry. Note that early treatment of GSK212 (but not late treatment) results in defects in functional NHEJ repair as measured by a decrease in the %GFP positive cells compared to DMSO-treated cells (8.7% DMSO, 3.1% late GSK212, 2.7% early GSK212). (**D**) Mean of 4 independent experiments shown, which independently showed similar results.

absence of NHEJ repair of I-SceI-induced DSBs. Successful repair of DSBs by NHEJ repair results in GFP expression, and the number of GFP positive cells counted by flow cytometry provides a quantitative measure of NHEJ repair activity.[53] We created stable MIAPaCa-2-Pem1-GFP cells (clone N4) and validated reporter activity with a DNA-PK inhibitor, NU-7441 (**Fig. S4**). Next, we tested MIAPaCa-2-Pem1-GFP cells with GSK212 to determine the effects of MEK1/2 inhibition on NHEJ repair. We found that GSK212 also significantly attenuated NHEJ repair both when MEK was inhibited at the time of DNA damage or after, suggesting that GSK212 also acts as an effective inhibitor of NHEJ repair in pancreatic cancer cells (**Figs. 5C,D**).

GSK212 downregulates the phosphorylation and expression of multiple DSB repairing proteins

To further elucidate the biochemical mechanisms underlying GSK212-mediated radiosensitization of pancreatic cells, we evaluated the alterations in expression/activation of a number of key proteins that are involved in the global DNA damage response and repair pathways. For example, BRCA1 is phosphorylated on specific serine residues in response to radiation, and is differentially regulated in S- and G2/M phases of the cell cycle. Ionizing radiation induces phosphorylation of Ser-988/Ser-1524 on BRCA1 during the S phase and of Ser-988/Ser-1423 during the G2/M phase, which is catalyzed by a number of Ser/Thr kinases that include ATM, ATR, cdk2, and Chk2.[54] We found that GSK212 pretreatment for 24 h markedly decreased total BRCA1 expression (and therefore the presence of radiation-induced phosphorylation of BRCA1 on Ser-1524) in MIAPaCa-2 cells (**Fig. 6A, B**). Interestingly, RAD51 expression was also markedly decreased in the presence of GSK212 (**Fig. 6A**). With regard to NHEJ repair, ionizing radiation induces rapid phosphorylation of DNA-PKcs on Thr-2609 cluster and on Ser-2056, which is critical for the repair of DSBs.[55] Our data also show that GSK212 pre-treatment decreased total DNA-PK expression (and therefore the presence of radiation-induced phosphorylation of DNA-PK on

Ser-2056) in MIAPaCa-2 cells under the same conditions (**Fig. 6A**). Conversely, 53BP1 expression was upregulated in cells treated with GSK212.

Ribonucleotide reductase (RR) catalyzes the synthesis of deoxyribonucleoside triphosphates necessary for both DNA synthesis and DNA repair, and is a heterodimer consisting of 2 subunits, M1 and M2.[56] RRM2 expression increases with the progression of cell cycle while the RRM1 expression remains at a steady-state level throughout the cell cycle.[57] We found that RRM2 baseline and radiation-induced expression were markedly decreased by GSK212 pretreatment of MIAPaCa-2 cells when compared with RRM1 expression (**Fig. 6A**). We have also examined the effects of GSK212 pretreatment on total Chk1, as well as the early phosphorylation status of Chk1 (Ser-345), Chk2 (Thr-68), p53 (Ser-15), ATR (Ser-428), and ATM (Ser-1981), which are involved in the initial regulation of DNA damage response.[30] Our data show that under the present experimental conditions, only total and phospho-Chk1 were significantly attenuated by GSK212 treatment of MIAPaCa-2 cells, with the Chk2 and p53 pathways largely unaffected (**Fig. 6A, B**). This reduction in Chk1 phosphorylation persisted throughout 24 hr (**Fig. 6A**). Earlier studies have shown that phosphorylation of Chk1 on Ser-317 and Ser-345 catalyzed by the upstream kinase ATR is critical for checkpoint activation in the S-phase and DNA damage response in the G2-phase.[58,59] We therefore evaluated this kinase as well at the ATM kinase which activates Chk2 after radiation, but detected no differences in the activation state of either kinase (**Fig. 6B**).

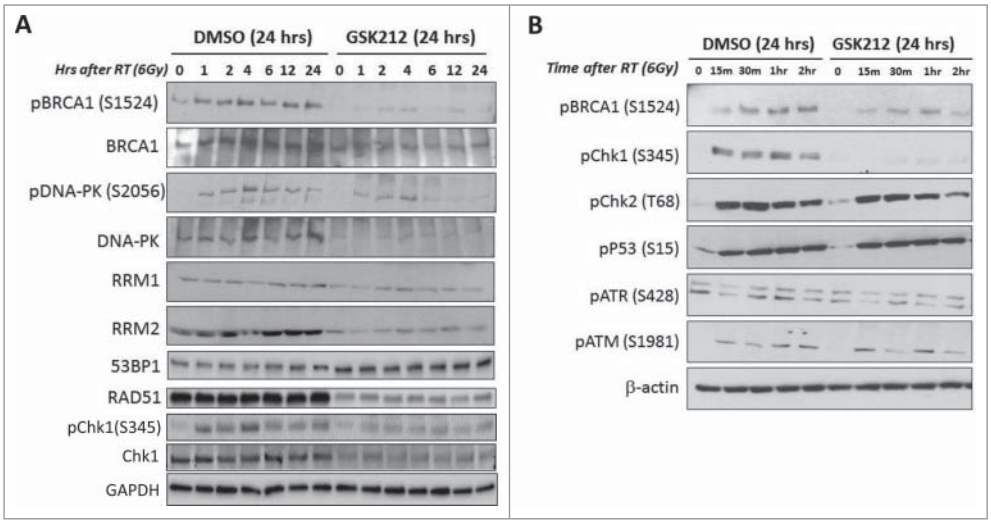

Figure 6. MEK1/2 inhibition results in inactivation or loss of multiple DNA repair pathway intermediates including BRCA1, DNA-PK, RRM2, RAD51, and Chk1. MIAPaCa-2 cells were pre-treated with DMSO (control) or GSK212 for 24 hr then radiated with 6 Gy. (**A**) At 0, 1, 2, 4, 6, 12, and 24 hours after 6 Gy, lysates were made and subjected to immunoblotting. Note the loss of radiation-induced activation of BRCA1 and DNA-PK, as well as loss of RRM2. (**B**) At 0.25, 0.5, 1, and 2 hr after 6 Gy, lysates were made and subjected to immunoblotting. Note the attenuation or loss of radiation-induced activation of BRCA1 and Chk1, that appears independent of ATM, ATR, p53, or Chk2.

Together, our data reveal that MEK1/2 inhibition led to the downregulation of multiple intermediates of the DSB repair pathways, including BRCA1, RAD51, DNA-PK, RRM2, and Chk-1 in irradiated cells, providing potential mechanisms for GSK212-mediated radiosensitization.

Discussion

The use of chemotherapy and radiotherapy remains a standard practice for the treatment of unresectable pancreatic cancer. In addition, radiation is often utilized in the post-operative management of resectable pancreatic cancer. Despite this, patient outcomes for pancreatic cancer remain dismal. Since KRAS acquires an activating oncogenic mutation in almost all pancreatic cancer, which activates downstream signaling pathways (RAF-MEK-MAPK and PI3K-Akt), conferring resistance to radiotherapy as well as chemotherapy, there is an urgent need for optimizing the current therapeutic regimens by incorporating novel agents that sensitize malignant cells to radiation and/or chemotherapy. To this end, a number of MEK inhibitors have shown promising outcome in preclinical studies and clinical trials in different disease sites.[18,24,26,28] In pancreatic cancer preclinical studies, the use of MEK inhibitors seems to show some promise either alone or in combination with other agents, such as STAT3 inhibitors or CDK4/6 inhibitors.[60-62] These combinations have been shown to be highly effective and synergistic in certain models, resulting in potent inhibition of tumor cell growth. A number of preclinical studies have demonstrated the benefit of MEK1/2 inhibition in combination with radiation with various types of MEK-1/2 inhibitors.[12,18,19,63] This finding has led to the development of a number of phase I clinical trials in the United States testing the addition of MEK1/2 inhibition in combination with radiation for rectal, lung, and brain cancer (NCT01912625; NCT02015117; NCT01740648).

GSK212 (trametinib or Mekinist^{TM}) is a novel and potent MEK-1/2 inhibitor, and is FDA-approved for the treatment of BRAF V600E or V600K mutated melanoma.[25,26] One of the advantages of using newer generation MEK1/2 inhibitors such as GKS212, is that they are highly selective for their targets by virtue of binding to a unique pocket that is adjacent to, but distinct from, the ATP-binding site. This offers the potential for less mutation rates compared to other small molecule inhibitors which bind to the ATP-binding site. Furthermore MAP kinases ERK1 and ERK2 are the only known

substrates for the MEK isoforms highlighting their tight specificity.[64,65] Thus, blocking MEK1/2 will likely result in fewer off-target un-intended effects.

The goal of the current study was to evaluate the radiosensitizing potential of a GSK212 in pancreatic cancer cells, which has shown promising efficacy in BRAF-mutated melanoma.[25,26] Our key findings reported in this article are the following ones: (1) MEK-1/2 inhibition by GSK212 radiosensitizes multiple pancreatic cancer cell lines including MIAPaCa-2 and AsPC-1, which both have KRAS oncogenic mutations (G12C and G12D, respectively) [66,67] (2) Longer treatment time with GSK212 is required for effective radiosensitization (which has implications for clinical implementation); (3) GSK212-mediated radiosensitization is caused by functional inhibition of both HR and NHEJ repair pathways resulting in delayed DNA repair as evidenced by comet assay and persistent γH2AX expression; and (4) GSK212-mediated MEK1/2 inhibition decreases the expression of established proteins directly or indirectly involved in DSB repair or DNA damage response signaling, which include BRCA1, RAD51, DNA-PK, RRM2, and Chk-1. These data support the clinical investigation of GSK212 in combination with radiation for pancreatic cancer, which harbors high rates of KRAS activating mutations which promote radioresistance. Furthermore, our data suggest that p-BRCA1 (S1524), BRCA1, RAD51, p-DNA-PK (S2056), DNA-PK, RRM2, Chk1, and pChk1 (S345) might serve as useful biomarkers to predict the clinical efficacy of GSK212 in clinical trials.

Ionizing radiation kills tumor cells largely by inducing DSBs in DNA leading to genomic instability, apoptosis, or post-mitotic death. These DSBs are primarily repaired by HR and NHEJ.[30] However, we and others have observed that radiation also induces MEK-ERK and PI3K-Akt pathway activation, which provides both proliferation and survival signals in pancreatic cancer cells rendering them radioresistant, as blocking either or both of these pathways induces radiosensitization.[12] Up-regulation of DNA repair pathways is recognized as a primary acquired mechanism by which malignant cells become radioresistant. In accord with this notion, we found that a potent and specific MEK1/2 inhibitor GSK212 radiosensitizes pancreatic cancer cells by inhibiting the repair of radiation-induced DSBs, by functionally decreasing efficacy of both HR and NHEJ pathways, which is a novel finding.

Our data reveal that GSK212 significantly reduces the number of nuclear foci formed by both BRCA1 and RAD51, demonstrating that the HR repair pathway is impaired by MEK1/2 inhibition. These observations were confirmed by using HR-specific functional reporter constructs in pancreatic cancer cells. Our initial data on DNA-PK and 53BP1 foci suggested to us that the cancer cells up-regulated NHEJ activity in order to compensate for reductions in HR activity with GSK212 treatment. However, on further evaluation, we found that the NHEJ repair pathway is also inhibited by GSK212 as assessed by a specific and well-characterized functional NHEJ reporter.[53] Further confirmation of the NHEJ functional reporter data was provided by immunoblotting, which showed that GSK212 treatment resulted in significant reductions in DNA-PK and pDNA-PK expression, a critical NHEJ repair protein.

With regard to homologous recombination, RAD51 is an important component of HR-mediated DNA repair, as RAD51 over-expression results in increased homologous recombination repair and resistance to radiation.[40] RAD51 has been shown to be overexpressed in the majority of pancreatic cancer, providing further rationale for the approach of combining GSK212 with radiation.[64] Our data also show that BRCA1-foci formation was significantly impaired when GSK212-treated pancreatic cancer cells are exposed to radiation. G2/M cell cycle arrest is one of the multiple functions BRCA1 performs in the cellular response to DNA damage through its interactions with different protein partners.[68] It has been previously shown that suppression of ERK1/2 activity using MEK1/2-specific inhibitor U0126 induces inhibition of BRCA1-mediated G2/M cell cycle arrest in breast cancer cells, indicating that ERK1/2 activity is required for G2/M arrest.[69] This study has further established that ERK1/2 inhibition through MEK1/2 inhibition abrogated the effects of BRCA1 on components of the G2/M checkpoint, including activation of Chk1 kinase. Our data reveal that GSK212-mediated ERK1/2 inhibition impairs the phosphorylation/activation of BRCA1 and Chk1 in pancreatic cancer cells, suggesting that GSK212-mediated ERK1/2 inhibition prevents proper radiation-induced G2/M checkpoint arrest to allow for proper DNA repair. Presumably, this could lead to lethal genomic events, as unrepaired DNA damage results in post-mitotic cell death. This notion is supported by an earlier study demonstrating that MEK2 activation is essential for progression through the G2/M checkpoint arrest in cells that are exposed to ionizing radiation.[70]

Likewise, both RRM2 and Chk1 have roles in DNA repair. RRM2 is upregulated after DNA damage, and has been shown to be important for mediating repair of a broad array of DNA damage induced by ultraviolet radiation, ionizing radiation, genotoxic stress, or genotoxic compounds such as camptothecin or doxorubicin.[71,72] Interestingly, Chk1 activation appears important for DNA damage induced upregulation of RRM2.[71] Furthermore, RRM2 expression is up-regulated by oncogenic KRAS and controls tumor cell proliferation, while downregulation of RRM2 can sensitize cells to genotoxic compounds like gemcitabine, an inhibitor of RRM1.[73,74] Chk1 has also been shown to be important in DNA repair, as Chk1 inhibition results in pancreatic cancer cell radiosensitization, through loss of the G2 cell cycle checkpoint and inhibition of HR repair, as mentioned above.[41] Together, these proteins have defined roles in DNA damage response signaling to promote DNA repair, and whose function is down-regulated by MEK-1/2 inhibitors.

In summary, our data demonstrate that sustained suppression of constitutive (resulting from activating KRAS mutation) and radiation-induced activation of ERK1/2 by the MEK1/2-specific inhibitor GSK212 sensitizes pancreatic cancer cells (but not normal small intestinal epithelial cells) to radiation by impairing proper DSB repair through altering the functionality of both HR and NHEJ pathways. Together with the *in vivo* data previously

published for MEK inhibition in combination with radiation for pancreatic cancer,[12] these data support the development of a clinical trial combining MEK-1/2 inhibition and radiation for the treatment of pancreatic cancer.

Materials and Methods

Antibodies, chemicals, and cell culture

The human pancreatic adenocarcinoma cell lines MIAPaCa-2, AsPC-1, BxPC3, and FHs74Int were obtained from American Type Culture Collection (Manassas, VA) and were maintained at 37°C in 5% CO2 in DMEM media (MIAPaCa-2, FHs74Int) or RPMI 1640 (AsPC-1 and BxPC3), and supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) and penicillin/streptomycin (Life Technologies, Grand Island, NY). GSK1120212 (GSK212) (ChemieTek, Indianapolis, IN) was dissolved in DMSO and added to the cells for 48 hours unless stated differently. Total ERK-1/2, phospho-ERK-1/2 (Thr202/Tyr204), phospho-BRCA1(Ser1524), phospho-DNAPK(Ser2056), total BRCA1, total DNAPK, phospho-H2AX, RAD51, 53BP1, RRM1,RRM2, phospho-Chk1(Ser345), phospho-Chk2(T68), phospho-p53(Ser15), phospho-ATR(Ser428), phospho-ATM (Ser1981) and β actin antibodies were purchased from Cell Signaling Technology (Danvers, MA).

Immunoblotting

Immunoblotting was performed as described previously.[75] Briefly, cell lysates were prepared using RIPA buffer (ThermoFisher, Waltham MA) supplemented with 1x protease (Complete, Roche, Indianapolis, IN) and phosphatase inhibitors (PhosSTOP, Roche, Indianapolis, IN, Roche) followed by protein quantification by the Dc protein assay kit (Bio-Rad, Hercules, CA). Equal amounts of protein were loaded and resolved by SDS/PAGE and transferred to nitrocellulose membranes. Primary antibodies were allowed to bind overnight at 4°C, and used at a dilution of 1:500-1,000. After washing in TBS-Tween, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies diluted 1:2,500-1:5,000 for 1 hour. Membranes were washed with TBS-Tween and incubated for 1 minute with enhanced chemiluminescence reagent (Immobilon, Millipore, Billerica, MA) prior to film exposure.

Radiation Clonogenic Assays

Cells were trypsinized to generate single cell suspensions and seeded onto 60 mm tissue culture plates in triplicate. Cells were incubated with DMSO or GSK212 at the indicated times and then irradiated with various doses (0–8 Gy). Twenty-four hours after radiation, medium was changed to remove DMSO or GSK212. Ten to 14 days after seeding, colonies were fixed with Methanol/Acetic Acid, stained with 0.5% crystal violet and the numbers of colonies or colony forming units (CFU) containing at least 50 cells were counted using a dissecting microscope (Leica Microsystems, Inc.. Buffalo Grove, IL) and surviving fractions calculated. Experiments were repeated multiple, independent times.

Experimental Radiation

Irradiation was performed essentially as described previously with 160kV, 25mA at a dose rate of approximately 113cGy/min using a RS-2000 biological irradiator (RadSource,GA).[75]

Neutral Comet Assay

Cells were treated with DMSO or GSK212 for different time points as stated. Neutral comet assay was performed according to the manufacturer's protocol (Trevigen, USA). Briefly, cells were suspended and mixed with low melting-point agarose at 37°C. The mixture was pipetted onto slides and placed in the dark at 4°C for 10 minutes. Slides were immersed in cold lysis solution overnight at 4°C. On the next day, slides were immersed in 50 ml of cold Neutral Electrophoresis Buffer for 30 minutes. Neutral electrophoresis was performed at 110 milliAmps, 21 Volts for 45 minutes. Slides were then placed in DNA Precipitation Solution for 30 minutes at room temperature followed by 70% ethanol for 30 minutes. Samples were dried at 37°C and then diluted SYBR Gold was added to them. Slides were then briefly rinsed and dried completely. Slides were viewed by epifluorescence microscopy. At least 50 cells were counted and imaged. CometScore software (TriTek Corp, Sumerduck, VA) was used to quantify the average tail moment.

Immunofluorescence for Nuclear Foci

For phospho-H2AX and α tubulin: Cells plated on coverslips were fixed with 2% paraformaldehyde, permeabilized with 1% Triton X-100 and blocked with 3% bovine serum albumin (BSA) in PBS. Cells were stained with anti-phospho-H2AX (Cell Signaling, Danvers, MA) or α-tubulin (Sigma, St. Louis, MO), washed and incubated with a fluorophore-conjugated secondary antibody (Biotium, Hayward, CA).

For 53BP1, BRCA1, and DNA-PK: Cells were fixed with 3%, 4%, and 2% paraformaldehyde, respectively, then permeabilized with 0.2–1% Triton X-100 and blocked with 3% BSA in PBS. 10% BSA was used for BRCA1. Cells were stained with anti-53BP1 (Cell Signaling, Danvers, MA), anti-BRCA1 (Santa Cruz Biotechnologies, Santa Cruz, CA), or anti-DNA-PK (AbCam, Cambridge, MA) followed by fluorophore-conjugated secondary antibody.

For RAD51: Cells were fixed with 4% paraformaldehyde, permeabilized with 0.25% Triton X-100 and blocked in 1% goat serum in PBS. Anti-RAD51 (Santa Cruz) was used followed by a fluorophore-conjugated secondary antibody.

Slides were examined on a Zeiss fluorescence microscope. For each experiment the total number of foci per cell was determined in at least 100 cells.

Flow cytometry

A stable clone of MIAPaCa-2 cells transfected with the pDR-GFP plasmid has been previously characterized,[41] and was used to measure HR activity. In order to measure NHEJ activity, stable clones of MIAPaCa-2 cells transfected with NHEJ-GFP-PEM1 plasmid (generously provided by V. Gorbunova), were created by transient transfection using Lipofectamine 2000 and selection with 1 mg/mL G418 according to a previously published protocol.[53] NHEJ-GFP-PEM1 clones were selected based on identifying clones with the highest percentage of green fluorescent protein (GFP) positivity 24 hr after infection with the adenovirus AdNGUS24i using flow cytometry analysis. In addition, the NHEJ-GFP-PEM1 construct was validated in MIAPaCa2 cells by treatment with an inhibitor of NHEJ, NU-7441 (Selleckchem, Houston,TX) as described. For the experiments, MIAPaCa2-DR-GFP or –NHEJ-GFP-PEM1 cells were treated with adenovirus expressing the I-SceI restriction enzyme that induces double-strand DNA breaks in the recognition sequence within the reporter construct. Cells were also pretreated with DMSO or GSK212 using different variations in scheduling as described. Cells were then washed with ice-cold PBS and fixed in cold 1% formaldehyde solution for 30 minutes. Cells were then washed and I-SceI–induced homologous recombination (HR) and non-homologous end-joining (NHEJ) were measured by flow cytometry as the percentage of GFP positive cells. Cells were sorted through BD LSR II flow cytometer.

Data Analysis

Data are presented as the mean ± standard error of the mean (s.e.m.) for clonogenic survival and foci experiments. Statistical comparisons were made between the control and experimental conditions using the unpaired 2-tailed Student's *t*-test with significance assessed at p-values <0.05.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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