Targeting PI3K and RAD51 in Barrett's Adenocarcinoma: Impact on DNA Damage Checkpoints, Expression Profile and Tumor Growth

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Abstract. *Phosphatidylinositol 3-kinase (PI3K)/v-akt murine thymoma viral oncogene homolog 1 (AKT) signaling in cancer is implicated in various survival pathways including regulation of recombinase (RAD51). In this study, we evaluated PI3K and RAD51 as targets in Barrett's adenocarcinoma (BAC) cells both in vitro and in vivo. BAC cell lines (OE19, OE33, and FLO-1) were cultured in the presence of PI3K inhibitor (wortmannin) and the impact on growth and expression of AKT, phosphorylated-AKT (P-AKT), and RAD51 was determined. Wortmannin induced growth arrest and apoptosis in two BAC cell lines (OE33 and OE19), which had relatively higher expression of AKT. FLO-1 cells, with lower AKT expression, were less sensitive to treatment and investigated further. In FLO-1 cells, wortmannin suppressed ataxia telangiectasia and Rad3-related protein (ATR)-checkpoint kinase 1 (CHK1)-mediated checkpoint and multiple DNA repair genes, whereas RAD51 and CHK2 were not affected. Western blotting confirmed that RAD51 was suppressed by wortmannin in OE33 and OE19 cells, but not in FLO-1 cells. Suppression of RAD51 in FLO-1 cells down-regulated the expression of CHK2 and CHK1, and reduced the proliferative potential. Finally, the suppression of RAD51 in FLO-1 cells, significantly increased the anticancer activity of wortmannin in these cells, both in vitro and in vivo. We show that PI3K signaling and hsRAD51, through distinct roles in DNA damage response and repair pathways, provide survival advantage to BAC cells. In cells with inherent low expression of AKT, RAD51 is unaffected by PI3K suppression and provides an additional survival pathway. Simultaneous suppression of PI3K and RAD51, especially in cells with lower AKT expression, can significantly reduce their proliferative potential.*

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Phosphatidylinositol 3-kinase (PI3K) is a lipid kinase implicated in the generation of phosphatidylinositol 3,4,5-trisphosphate, an important second messenger which plays vital roles in several critical signaling pathways, including those associated with cell survival and proliferation (1). Deregulated or elevated PI3K activity has been observed in many types of cancer including small cell lung, ovarian, urinary tract, head and neck, and cervical cancer, and is implicated in oncogenesis (2) and resistance to radiation therapy (3). Aberrations in the PI3K pathway (4) and activation of AKT (5), a downstream effector of PI3K, have also been implicated in the etiology of esophageal cancer. Several anticancer agents have been shown to inhibit PI3K or its target (AKT) (6). Inhibitors of PI3K therefore hold promise as anticancer therapeutics and need to be evaluated for their impact on the mechanisms which enable cancer cells to acquire unique features for survival and continued proliferation *in vivo*. Wortmannin, a fungal metabolite, inhibits PI3K and related enzymes (7, 8) and is more potent than another commonly used PI3K inhibitor, LY294002.

PI3K/AKT signaling represents an important survival pathway which is implicated not only in the process of oncogenesis but also in the development of resistance to therapy. PI3K/AKT signaling is negatively regulated by the lipid phosphatase, phosphatase and tensin homolog deleted on chromosome ten (PTEN), which serves as an antioncogene and is mutated in many types of cancer. PTEN is also

implicated in the regulation of important components of genome maintenance, including p53 and homologous recombination protein RAD51, and therefore plays an important role in ensuring genomic integrity in normal cells (9). AKT, the downstream effector of PI3K, has also been implicated in the regulation of RAD51 (10). Wortmannin, can block DNA damage responses mediated via RAD51 (10) and also from DNA-dependent protein kinase (DNA-PK) (11), affecting non homologous DNA end joining (NHEJ) (12). Wortmannin has been shown to suppress the expression of DNA double-strand break repair protein DNA-PKcs (DNA-dependent protein kinase catalytic subunit) in lung cancer cells and increases their sensitivity to ionizing radiation, indicating that PI3K function is also involved in DNA recombination, repair and genome maintenance (13). PI3K-like kinases, ataxia telangiectasia and Rad3-related protein (ATR) and ataxia telangiectasia mutated (ATM), are the key components of the DNA damage response and are implicated in the maintenance of the genomic integrity of cells (14). DNA inside a cell is constantly being damaged by intrinsic and extrinsic factors. Recognition of DNA lesions by sensor proteins activates ATR or ATM which subsequently activate checkpoint effector kinases checkpoint kinase 1 (CHK1) and checkpoint kinase 2 (CHK2). CHK1 primarily mediates intra-S and G_2/M phase checkpoints, whereas CHK2 is implicated in intra-S and G_1/S checkpoints (15). These checkpoint kinases, not only allow the repair of otherwise lethal DNA damage, but also activate recombination and repair proteins to ensure genomic integrity.

Genetic recombination, through its vital roles in damaged DNA repair (16) and proper chromosomal segregation (17), significantly contributes to the maintenance of the genomic integrity of a cell. However, if the recombination pathway becomes aberrant or overactive, it can also harm genomic integrity by inducing unwanted genomic rearrangements, which may lead to amplifications (18), deletions associated with loss of heterozygosity (19) and translocation of chromosomal segments (19-21). Chemicals and other agents with carcinogenic potential have been shown to increase the rate of recombination in human cells (20, 22). We have shown that elevated homologous recombination activity significantly contributes to genomic instability in multiple myeloma cells (23). Recently, we also showed that moderate suppression of recombinase (RAD51) is associated with significantly reduced genomic instability in Barrett's adenocarcinoma (BAC) cells (24).

Genomic instability enables cancer cells to acquire new mutations which provide unique characteristics for progression of disease to advanced stages, including the emergence of metastatic phenotype. It is evident from the literature that genomic instability in BAC develops early at the stage of Barrett's esophagus (25-27) and is associated with progression to cancer (28, 29). Ongoing accrual of mutations is not only implicated in disease progression but may also provide survival advantage by allowing the development of resistance to treatment. On the other hand, an unstable genome can also make cancer cells vulnerable to treatment with inhibitors of DNA damage response, checkpoint, and repair pathways.

The two main pathways of genetic recombination utilized in repairing DNA damage are homologous (HR) and nonhomologous (NHEJ) recombination. Homologous recombination, which is based on extensive sequence homology, repairs damaged DNA either by copying the missing information from a homologous chromosome (G_1 repair) or from a sister chromatid in the G_2 phase of the cell cycle. According to one model (30), NHEJ is primarily an end-joining ligation reaction in which two DNA duplexes with their single-stranded ends are brought together by homologous pairing and are joined together. This model therefore suggests that NHEJ is mediated through short sequence homologies. The pathways of homologous and NHEJ recombination may overlap. The choice of a specific repair pathway may also depend on the phase of the cell cycle, the nature of the DNA damage, and specific proteins such as CTBP-interacting protein (31).

In this study, we evaluated the effect of wortmannin and the impact of RAD51 suppression on DNA damage response genes and proliferation of BAC cells *in vitro* and *in vivo*.

Materials and Methods

Cell lines. BAC cell line FLO-I, described previously (24, 32), was provided by Dr. David G. Beer, University of Michigan, Ann Arbor, MI, USA. BAC cell lines OE33 and OE19, described previously (24, 33), were purchased from Sigma-Aldrich (St. Louis, MO, USA). OE19 and OE33 cells were grown in RPMI-1640 medium, supplemented with 2 mM L-glutamine and 10% fetal bovine serum (FBS). For FLO-1 cells, Dulbecco's modified Eagle's medium (DMEM) (Sigma Chemical CO., St. Louis, MO, USA) supplemented with 10% FBS was used. Cells were cultured at 37˚C in humidified air with 5% CO_2 and maintained in a state of logarithmic growth. Wortmannin and lentivirus particles producing either non-targeted control (CS) shRNAs or those targeting human RAD51 (RS4) were purchased from Sigma-Aldrich.

Cell viability and apoptosis assays. Cells, plated at constant numbers, were treated with wortmannin at different concentrations (0.125-20 μM) for 1-12 days and evaluated for cell viability and apoptosis. For cell viability, the attached cells were harvested at various time points and counted, and the viable cell number was confirmed by trypan blue exclusion. Small aliquots of cells were removed for various molecular analyses and the remaining cells replated at same cell number and at the same concentration of drug. The fraction of apoptotic cells at day 3rd was detected by annexin labeling, using Fluorescein isothiocyanate-Annexin Apoptosis Detection Kit (Oncogene Research Products, Boston, MA, USA). Approximately, 200 cells, representing at least five different microscopic fields, were examined to assess the fraction of apoptotic (FITC-labeled) cells for each sample.

Lentiviruses and transductions. Lentiviruses producing non-targeted control or RAD51 specific shRNAs were purchased from Sigma-Aldrich and transduced into FLO-1 cells as described previously (24). Transduced cells were selected in puromycin (at 1 μg/ml) for seven days and suppression of RAD51 was confirmed by western blotting at different time points.

Gene expression profiling. BAC cells (FLO-1), untreated or treated with 20 μM wortmannin for 24 h were harvested and total RNA was isolated utilizing an RNeasy kit (Qiagen Inc., Valencia, CA, USA), as described by the manufacturer. Gene expression profiling was conducted using Agilent whole Human Genome arrays (4×44k format) as described previously (24). Data was extracted with Agilent's Feature Extraction software and analyzed using Rosetta Luminator Software (Rosetta Biosoftware, Seattle, WA, USA) and dChip (34, 35).

Western blot analyses. Rabbit polyclonal anti-hsRAD51 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), rabbit polyclonal anti-AKT and rabbit monoclonal anti-phosphorylated AKT (Cell Signaling Technology Inc., Danvers, MA, USA), mouse monoclonal anti-phospho histone H2A.X (Upstate Cell Signaling Solutions Inc., Temecula, CA), rabbit polyclonal anti-poly(ADP-ribose) polymerase (PARP; Cell Signaling, Technology, Inc.) and horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology, Inc.) were purchased commercially. Protein extracts were made and processed as described previously (24, 36). Specific proteins were detected using an enhanced chemiluminescence system, according to the instructions provided by the manufacturer (Amersham Life Sciences Inc., Arlington Heights, IL, USA).

Telomere length analysis. Genomic DNA from untreated and wortmannin-treated cells was isolated using DNeasy Tissue Kit (Qiagen Inc.), as described by the manufacturer. A modification of a previously described real-time PCR assay on an Applied Biosystems 7900HT Thermocycler (37) was used to determine the average relative telomere length, indicated as telomere repeat and single-copy gene copy-number (T/S) ratio, as described elsewhere (38).

Impact of wortmannin on tumor growth in vivo. Six week old male CB-17 SCID mice, purchased from the National Cancer Institute-Frederick Animal Production area (Frederick, MD), were maintained following guidelines of the Institutional Animal Care and Use Committee (IACUC) and all experimental procedures were approved by the IACUC and the Occupational Health and Safety Department of Dana Farber Cancer Institute, Boston, MA, USA. FLO-1 cells, 3.0×10^6 in 100 μl saline, were injected subcutaneously into the interscapular area of each mouse. After the appearance of palpable tumors (~7-10 days), mice were treated with normal saline or wortmannin at 0.75 mg/kg, intraperitoneal daily injections. To evaluate if efficacy of wortmannin can be enhanced by RAD51 suppression, FLO-1 cells transduced with control or RAD51-specific shRNAs were cultured for ~50 days to reduce RAD51 suppression and then injected subcutaneously into the mice as described above. Following the appearance of tumors, mice were treated with a lower dose of wortmannin (0.75 mg/kg, injecting only twice a week instead of daily injections). Tumor sizes were measured twice a week and animals were sacrifized when tumors reached 2 cm^3 in volume or when paralysis or major compromise in their quality of life occurred.

Results

Impact of wortmannin on proliferation of BAC cells. Wortmannin induced time- and dose-dependent decline in the survival of OE33 and OE19 cells. Exposure to 8.0 μM drug led to 87% and 100% cell death in OE19 cells, in a period of 3 and 6 days, respectively (Figure 1A). A marked antiproliferative activity was also observed due to wortmannin at lower concentrations of 2.0 and even 0.5 μM, which killed 100% and 74% of OE19 cells in 6 days, respectively (Figure 1A). The drug at 0.125 μM was also quite effective in OE19 cells and led to 63% cell death in 12 days. In OE33 cells, the treatment with wortmannin at 0.1 and 0.5 μM concentrations for 5 days led to 50% and 64% cell death respectively (Figure 1B). The treatment with 1.0 and 5.0 μM wortmannin was associated with 65% and 70% cell death in 3 days, respectively. The exposure of these cells to 1.0 and 5.0 μM wortmannin for 5 days was associated with 72% and 80% cell death, respectively. Death of these cells was induced by apoptosis, as indicated by annexin labeling. Treatment of OE19 cells with both 0.5 and 1.0 μM wortmannin led to annexin labeling in ≥50% of treated cells, whereas 12% of untreated cells were positively stained for apoptosis (Figure 1C). Apoptotic cell death was also seen in OE33 cells (data not shown).

FLO-1 cells were less sensitive to wortmannin. In these cells the drug reduced the proliferative potential by 31 and 48%, only when used at higher concentrations of 10 and 20 μM, respectively (Figure 1D). Because of the lack of sensitivity to wortmannin, FLO-1 cells were investigated in further detail.

Impact of wortmannin on expression of AKT, phosphorylated-AKT, and RAD51 proteins in BAC cells. BAC cell lines were treated with wortmannin for 72 h and protein levels of AKT, phosphorylated-AKT, and RAD51 were detected by western blotting. Of all three untreated cell lines, relative amounts of total and phosphorylated-AKT were the lowest in FLO-1 cells (Figure 2). Following wortmannin treatment, the fraction of phosphorylated-AKT was reduced by 40-60% in all three cell lines (Figure 2A and B). In contrast, the amount of RAD51, although reduced by wortmannin in OE33 and OE19 cells, remained unchanged in FLO-1 cells (Figure 2A). These data indicate that in the presence of wortmannin, RAD51 appeared to provide alternative survival mechanism for FLO-1 cells.

Impact of wortmannin on the genome-wide expression profile in FLO-1 cells. To further evaluate the impact of wortmannin in the less sensitive FLO-1 cells, we treated cells with wortmannin (20 μM) for 24 h and conducted a genome wide expression profile, using Agilent whole Human Genome arrays (4×44k format). Treatment with wortmannin led to

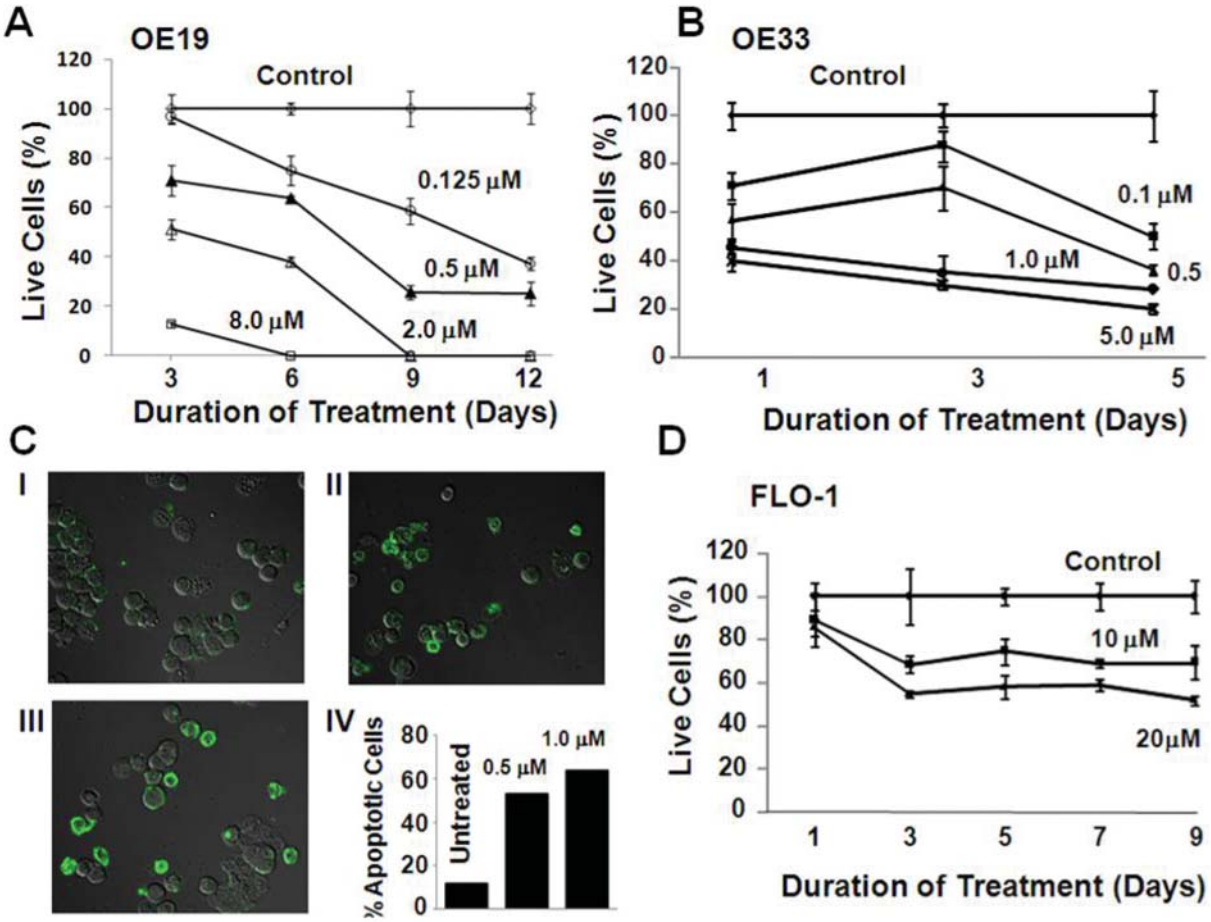

Figure 1. *Wortmannin inhibits proliferation of Barrett's adenocarcinoma (BAC) cells and induces apoptotic cell death. BAC cells were cultured in the presence or absence of wortmannin (WM) and the viable cell number was determined at different time points as indicated. A and B: The growth curves of OE19 and OE33 cells, showing the mean of three independent experiments, with S.E.M. C: OE19 cells were treated with WM for 3 days and was treated with fluorescein isothiocyanate (FITC)-labeled annexin; apoptotic cells appear bright green. Photomicrographs of: untreated OE19 cells (I); OE19 cells treated with 1 μM WM (II); OE19 cells treated with 2 μM WM (III); A total of ~200-300 cells representing different microscopic fields were evaluated to determine the percentage of apoptotic cells, shown as bar graphs (IV); D: The growth curves of FLO-1 cells, show the mean of three independent experiments, with S.E.M.*

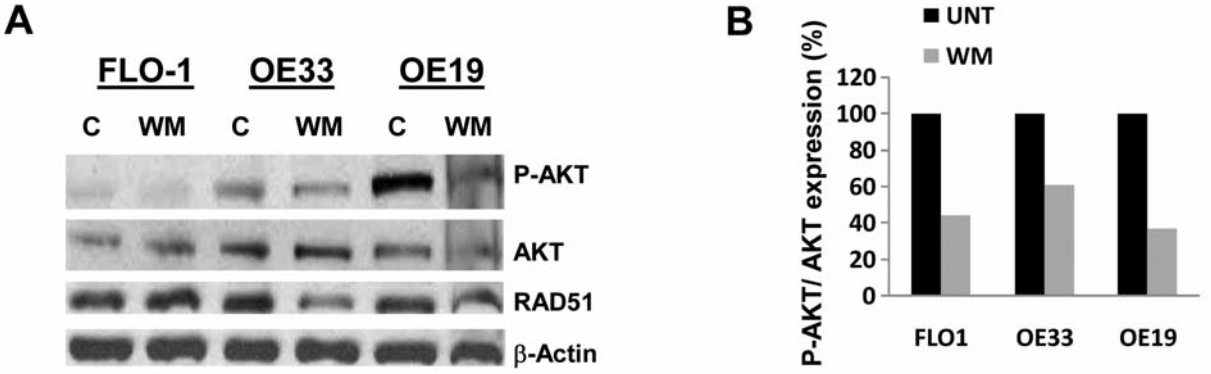

Figure 2. *Impact of wortmannin on protein levels of v-akt murine thymoma viral oncogene homolog 1 (AKT), phosphorylated-AKT, and recombinase (RAD51) in Barrett's adenocarcinoma (BAC) cells. A: BAC cell lines OE33, OE19, and FLO-1 were treated with wortmannin (WM) at 1 μM, 2 μM, or 20 μM respectively, for 72 h. The expression of proteins was analyzed by western blotting, using rabbit polyclonal antibodies against human AKT, P-AKT, RAD51, and horse radish-labeled goat anti-rabbit secondary antibodies. B: Bar graph showing phosphorylated AKT (P-AKT) relative to AKT expression in untreated (UNT) and WM-treated cells.*

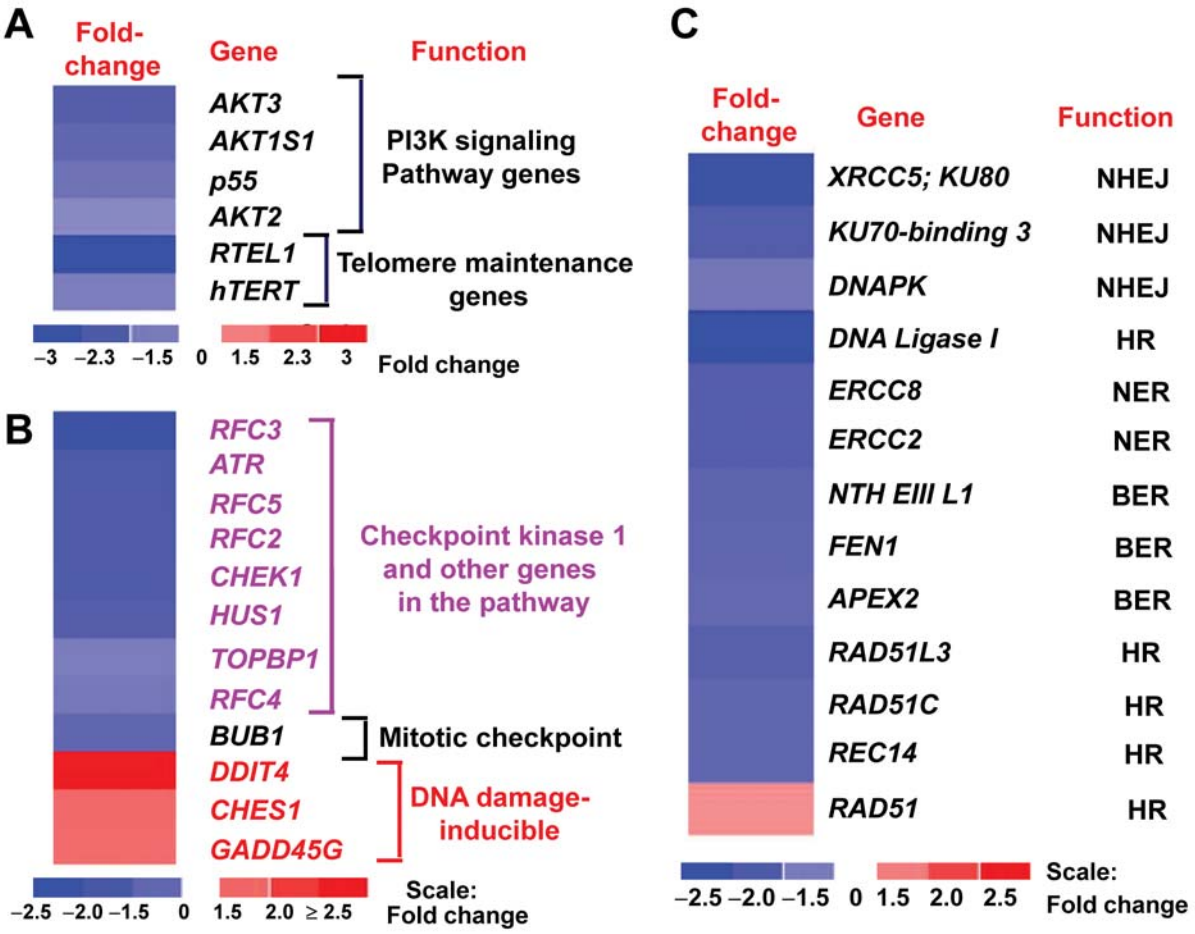

Figure 3. *Impact of wortmannin on genome-wide expression profile of FLO-1 cells. FLO-1 cells were treated with wortmannin at 20 μM for 24 h and evaluated for gene expression, using Agilent whole Human Genome arrays (4×44k format). The color scale at the bottom of the figure represents the fold change in expression of genes in treated, relative to control cells. A: Phosphatidylinositol 3-kinase (PI3K)/v-akt murine thymoma viral oncogene homolog 1 (AKT) pathway genes; B. ataxia telangiectasia and Rad3 related (ATR)–Checkpoint kinase 1 (CHEK1) pathway and related genes; C. DNA repair-related genes. NHEJ, Non homologous end joining; BER, base excision repair, NER, nucleotide excision repair, HR, homologous recombination.*

reduced expression of several key genes implicated in the PI3K/AKT pathway; these included PI3K regulatory subunit (p55), *AKT3, AKT1S1*, and *AKT2* (Figure 3A). A prominent (3-fold) reduction in telomere maintenance gene regulator of telomere elongation helicase 1 (*RTEL1*) and a slight decrease in the expression of telomerase (*hTERT*) was also observed (Figure 3A). Reduced hTERT expression was consistent with similar reduction in telomerase activity (data not shown). Treatment of FLO-1 cells with wortmannin was also associated with reduced expression of at least eight members of the ATR-checkpoint kinase 1 (*CHEK1*) pathway [replication factor C subunit 3 (RFC3), *ATR, RFC5, RFC2, CHEK1,* HUS1 checkpoint homolog (*HUS1*), DNA topoisomerase 2-binding protein 1 (*TOPBP1*), and RFC4; Figure 3B) and a mitotic checkpoint gene budding uninhibited by benzimidazoles 1 homolog (*BUB1*). The expression of DNA damage-inducible genes *DDIT4*, checkpoint suppressor

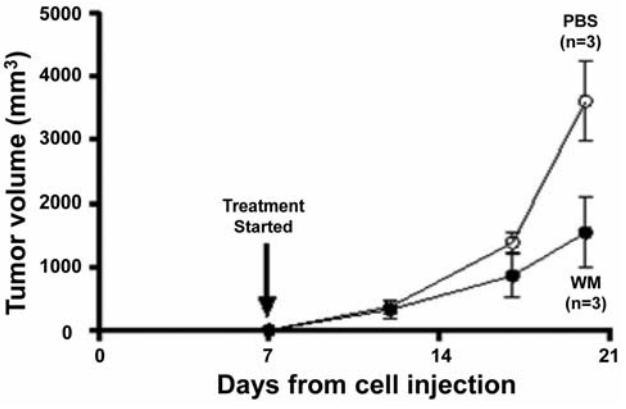

Figure 4. *Impact of wortmannin on the growth of FLO-1 cells as tumors in mice. FLO-1 cells were injected subcutaneously into the interscapular area of SCID mice and after the appearance of tumors, mice were treated with normal saline or wortmannin at 0.75 mg/kg, injecting daily, intraperitoneally.*

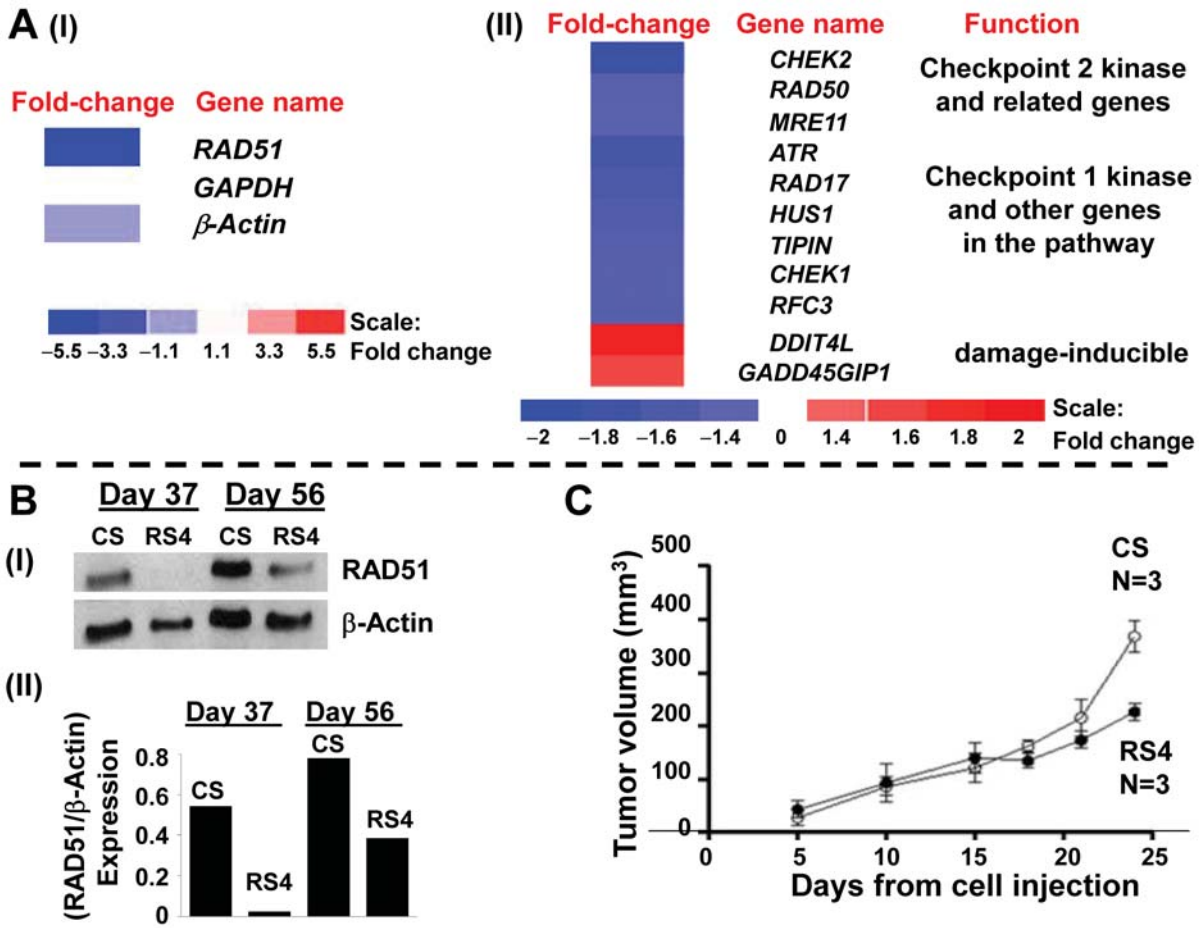

Figure 5. *Impact of recombinase (RAD51) suppression in FLO-1 cells. FLO-1 cells were transduced with control (CS) or RAD51-specific (RS4) shRNAs and selected in puromycin. A: (I) RS4 lentiviruses mediate a strong suppression of RAD51. The expression profile of RAD51-suppressed FLO-1 cells was evaluated at day 6 after transduction, using Human Gene 1.0 ST Arrays (Affymetrix) and analysis software dChip (34, 35). Panel A shows the expression of RAD51 and two house keeping genes (GAPDH and β-actin). The color scale, indicating fold-change in gene expression in RS4 relative to CS cells, is shown at the bottom of the figure. (II) Impact of RAD51 suppression on DNA damage checkpoint genes. The expression profile of RAD51-suppressed FLO-1 cells was evaluated at day 6 after transduction as described in Materials and Methods. The figure shows DNA damage checkpoint genes with altered expression. The color scale at the bottom of the figure represents the fold-change in gene expression in RS4 relative to CS cells. B: Western blot showing RAD51 suppression over a period of 56 days. Panel I is a western blot showing nearly complete suppression of RAD51 by lentivirus RS4 when evaluated at day 37 and a relatively weaker suppression at day 56. Panel II is a bar graph showing relative expression of RAD51 in CS and RS4 cells, following normalization to β-actin expression. C. Impact of RAD51 suppression on growth of FLO-1 cells as tumors in mice. The cells transduced with CS or RS4 (cultured for ~37 days) were injected subcutaneously into the mice as described in Materials and Methods and tumor sizes measured at the indicated time points.*

1 (*CHES1*), and growth arrest and DNA-damage-inducible, gamma (*GADD45G*) was elevated (Figure 3B) whereas *CHEK2* was not affected by wortmannin (data not shown). These data indicate that wortmannin down-regulates the ATR–CHEK1 pathway without affecting the key genes implicated in the CHEK2 pathway. Although several DNA damage-inducible genes were up-regulated (Figure 3B), many key DNA repair genes including DNA ligase I, *REC14*, those associated with NHEJ [(*KU80*, *KU70-binding 3, DNA-PK*), nucleotide excision repair (*ERCC8, ERCC2*), base excision repair (*NTHE3L1, FEN1, APEX2*), and two paralogs of RAD51 (*RAD51*-like protein 3, RAD51C) were down-regulated (Figure 3C). However, consistent with western blot data (Figure 2), the expression of recombinase RAD51 itself was not reduced. DNA repair genes analyzed were representative of most, if not all, of the DNA repair genes present in the microarray. Taken together, the gene expression data indicate that wortmannin targets the ATR-CHEK1 pathway and is associated with reduced expression of DNA repair genes, except RAD51.

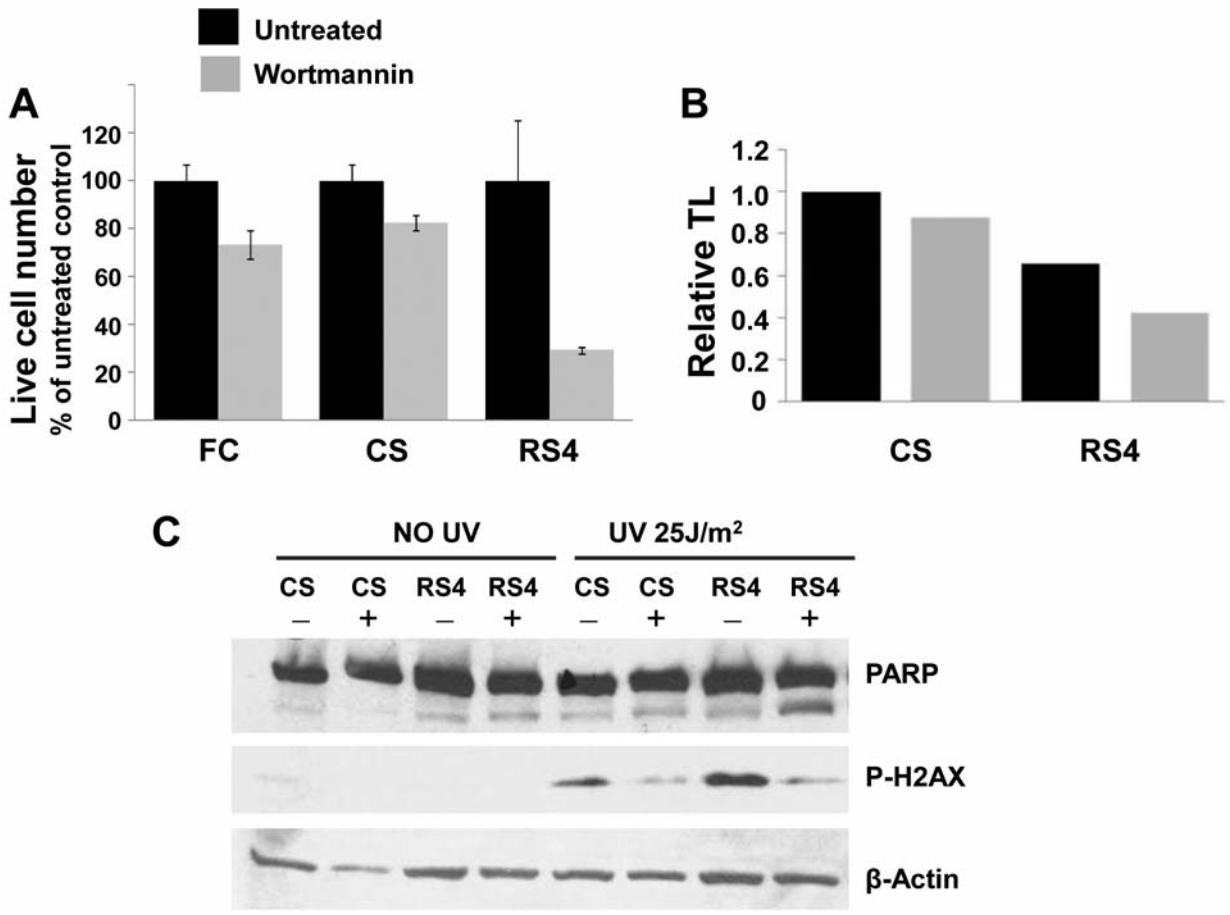

Figure 6. *Impact of RAD51 suppression on the efficacy of wortmannin (WM) in FLO-1 cells. FLO-1 cells, untransduced (FC) or transduced with control (CS) or RAD51-specific (RS4) shRNAs were treated with WM and evaluated for its impact on growth, telomere maintenance, levels of phosphorylated histone H2AX, and poly(ADP-ribose) polymerase (PARP) cleavage. A: Impact on growth. The FC, CS and RS4 cells were cultured in the presence or absence of WM for 5 days and cell viability was determined by counting the substrate attached cell number, confirmed by trypan blue exclusion. B: Impact on telomere length (TL). The CS and RS4 cells were cultured in the presence or absence of WM for 10 days and TL determined by real-time PCR as described in the Materials and Methods. C. Western blot showing phosphorylated-H2AX and PARP bands. The CS and RS4 cells were cultured in the presence (+) or absence (–) of WM for 72 h and were analyzed by western blotting for phosphorylated H2AX and PARP cleavage. H2AX and PARP were also investigated following exposure of these cells to ultraviolet (UV) light (25J/m^2). UV-treated cells were incubated for 4 h and then harvested and analyzed by western blotting.*

Impact of wortmannin on ability of FLO-1 cells to grow as tumors in SCID mice. FLO-1 cells were injected subcutaneously into the interscapular area of SCID mice and after the appearance of tumors, mice were treated daily with normal saline or wortmannin at 0.75 mg/kg. As shown in Figure 4, a significant reduction in tumor size was observed in treated mice, indicating *in vivo* antitumor activity, inspite of a relatively weak antiproliferative activity *in vitro*.

Impact of RAD51 suppression on gene expression in FLO-1 cells. We transduced FLO-1 cells with control or RAD51-specific (RS4) shRNAs and selected cells in puromycin. Lentivirus RS4 causes strong (80-100%) suppression of RAD51 in different transductions; the suppression is quite effective for the first 30-40 days and then starts declining between days 40-60 and beyond. As shown in Figure 5B, following transduction with RS4, the expression of RAD51 in FLO-1 cells was nearly completely suppressed for at least the first 37 days; however, when evaluated at day 56, the suppression was reduced to 50% that of control cells. Figure 5A shows the expression profile of RAD51-suppressed FLO-1 cells, evaluated at day 6 after transduction. Even at a transcript level evaluated at day six after transduction, the RS4 lentivirus was associated with 82% (5.3-fold) suppression of RAD51 (Figure 5A, panel I). Suppression of RAD51 was associated with a prominent (~50%) reduction

in the expression of CHK2 (Figure 5A, panel II). The expression of several genes from both the CHK1 and CHK2 pathways was also reduced in RAD51-suppressed cells; these included *RAD50, MRE11, ATR, RAD17, HUS1, TIPIN*, and *RFC3* (Figure 5A, panel II). RAD51 suppression was also associated with elevated expression of DNA damage-inducible genes *DDIT4L, GADD45GIP1* (Figure 5A, panel II). These data indicate that RAD51 suppression is associated with suppression of *CHK2* and other genes from both checkpoint pathways.

Impact of RAD51 suppression on growth of FLO-1 cells as tumors in mice. To evaluate the impact of RAD51 suppression on the growth of FLO-1 cells as tumors, cells transduced with control or RAD51-specific shRNAs (cultured for ~37 days) were injected subcutaneously into the mice as described above and tumor sizes were measured at different time points. The tumors in mice injected with RAD51-suppressed cells were significantly smaller than those injected with control cells (Figure 5C), indicating an *in vivo* antitumor effect. The growth rate of RAD51-suppressed cells *in vitro* was reduced by 20–30% (data not shown).

Impact of RAD51 suppression on efficacy of wortmannin in FLO-1 cells. Since wortmannin suppressed the expression of several important recombination/repair genes in FLO-1 cells, except RAD51, we evaluated the impact of RAD51 suppression on the proliferative potential of these cells. FLO-1 cells, transduced with control or RAD51-specific shRNAs, were cultured in the presence or absence of wortmannin and were evaluated for cell viability. Following treatment, the viability of cells transduced with control and RAD51-specific shRNAs was reduced by 18% and 71%, respectively (Figure 6A). Consistent with previous observations, the treatment of untransduced FLO-1 cells with wortmannin reduced cell viability by only 27% (Figure 6 A). These data indicate that suppression of RAD51 significantly enhances the antiproliferative activity of wortmannin in BAC cells. Telomere length in wortmannin-treated cells, RAD51-suppressed cells, and wortmannin-treated RAD51-suppressed cells was reduced by 12%, 34%, and 58%, respectively (Figure 6B). This indicates that simultaneous suppression of PI3K and RAD51 has a great impact on telomere maintenance. Transduced cells were cultured in the presence or absence of wortmannin for 72 h and analyzed by western blotting for levels of phosphorylated H2AX, a marker for DNA damage, and poly(ADP-ribose) polymerase (PARP), a protein which is involved in DNA damage response and undergoes caspase-3-mediated cleavage during apoptosis. H2AX and PARP were also investigated following exposure of these cells to UV light which induces DNA damage. Following exposure of cells to UV, a maximum amount of phosphorylated H2AX was detected in the RAD51-suppressed cells, indicating that loss of RAD51 was associated with less repair and hence more DNA damage. Within 72 h of treatment of RAD51-suppressed cells with wortmannin, 10% of PARP had already been cleaved, compared to 5% in control cells (Figure 6C). Following exposure of cells to UV, the cleavage of PARP increased to 10%, 9%, and 25% in cells subjected to wortmannin, RAD51 suppression, and their combination, respectively, within 4 h of incubation. This early cleavage of PARP seems to be direct effect of UV-induced severe DNA damage. These data indicate that simultaneous suppression of PI3K and RAD51 makes cells more vulnerable to unrepaired DNA damage, growth arrest and apoptosis.

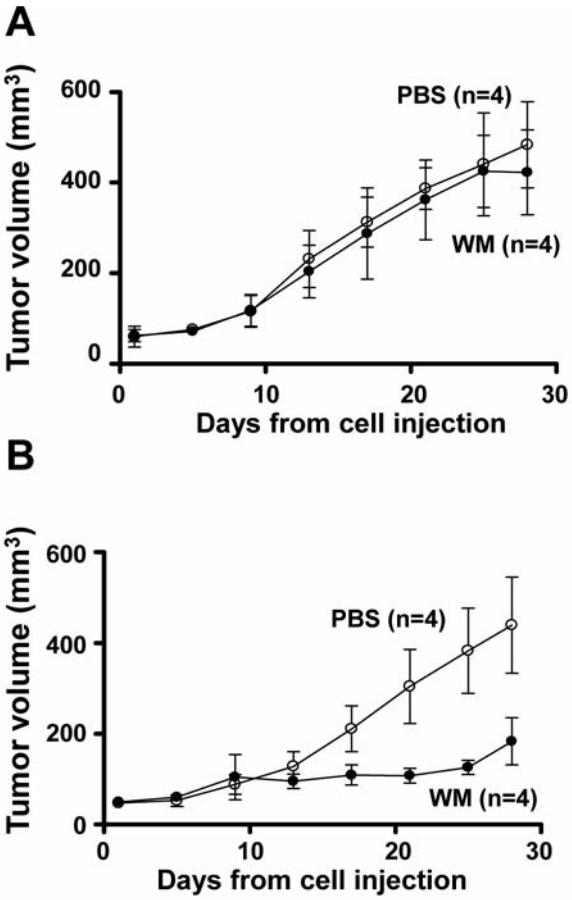

Figure 7. *Impact of RAD51 suppression on antitumor activity of wortmannin (WM): To investigate the combined effect of RAD51-suppression and wortmannin, both the wortmannin dose and RAD51 suppression were reduced so that separately these treatments did not have any effect on tumor growth. FLO-1 cells transduced with control (A) or RAD51-shRNAs (B) were cultured for ~56 days and injected subcutaneously into the interscapular area of SCID mice and after the appearance of tumors, mice were treated with normal saline or WM at a low dose (0.75 mg/kg, injecting only twice a week), intraperitoneally.*

Impact of RAD51 suppression on antitumor activity of wortmannin. To investigate the combined effect of RAD51 suppression and wortmannin, both the wortmannin dose and the RAD51 suppression were reduced so that separately, these treatments did not have any effect on tumor growth. RAD51 suppression was reduced to around 50% by culturing cells for around 50 days. FLO-1 cells, transduced with control or RAD51-shRNAs were injected subcutaneously in the interscapular area of SCID mice and after the appearance of tumors, mice were treated with normal saline or wortmannin at low dose (0.75 mg/kg, injecting only twice a week), intraperitoneally. Although separately low dose wortmannin or reduced RAD51 suppression, did not have any effect on tumor growth (Figure 7A), a significant reduction in tumor size was observed in mice injected with RAD51 suppressed cells and treated with wortmannin (Figure 7B). These data show that RAD51 suppression significantly enhances the *in vivo* antitumor activity of wortmannin against BAC cells.

Discussion

We have previously shown that elevated expression of hsRAD51 has a critical role in ongoing genomic evolution in BAC cells (24). This study reports that RAD51 is also implicated in the survival of BAC cells at multiple levels, and in combination with PI3K inhibitor, can effectively prevent their proliferation, both *in vitro* and *in vivo*.

Wortmannin is known to inhibit PI3K family and related enzymes including DNA-PK, a key protein implicated in DNA repair through NHEJ and telomere maintenance (7, 8). PI3K activity, elevated or deregulated in many types of cancer (2), is implicated in signaling pathways associated with cell survival and proliferation (1), elevated telomerase activity (39), and development of drug resistance (40). Aberrations in the PI3K pathway (4) and activation of its downstream effector AKT, have also been implicated in the etiology of esophageal cancer (5, 41). Inhibitors of PI3K therefore hold promise as effective cancer therapeutics. Consistently, we observed that the treatment of BAC cells (OE33 and OE19) with wortmannin (at 1-2 μM concentration) is associated with a dose- and time-dependent decline in cell viability and induction of apoptotic cell death. The third BAC cell line (FLO-1) exhibited less sensitivity to wortmannin *in vitro*, although when injected subcutaneously in SCID mice and mice treated with wortmannin, the tumor size in treated in mice was significantly reduced.

A significant impact of wortmannin on tumor growth *in vivo* may reflect differences in environmental factors or differences in its uptake *in vitro* and *in vivo*. However, the lack of sensitivity of FLO-1 cells to wortmannin *in vitro* was interesting and therefore was further investigated. The genome-wide expression profile indicated that wortmannin was associated with reduced expression of multiple genes implicated in the PI3K/AKT pathway, as expected. Wortmannin treatment was also associated with reduced expression of telomere maintenance genes, *hTERT* (catalytic subunit of telomerase reverse transcriptase) and *RTEL1*. Although *hTERT* expression was only slightly reduced, this was consistent with a similar reduction in telomerase activity (not shown). Wortmannin also reduced the expression of several DNA repair genes including KU80, which is the DNA-binding subunit of *DNA-PK*, a key gene implicated in repair of double-stranded chromosome breaks through NHEJ. This is consistent with previous reports indicating that wortmannin can block the DNA damage signaling cascade mediated through DNA-PK (11), thus impairing the NHEJ repair pathway (42). Wortmannin has also been shown to suppress the expression of DNA-PKcs in lung cancer cells and increases their sensitivity to ionizing radiation (13). Clearly, the gene expression data is consistent with reduced repair of chromosomal breaks including double-strand break repair through NHEJ, in treated cells. Most importantly the wortmannin treatment was associated with reduced expression of at least eight members of the ATR-CHK1 checkpoint pathway. Checkpoint kinases CHK1 and CHK2 are key components of the DNA damage response which mediate cell cycle arrest following DNA damage and safeguard genomic integrity (14). ATR-CHK1 is the primary replication checkpoint and *CHK1* is an essential gene for survival; if the expression of this gene reaches a critical limit, the cells may enter mitosis with substantial DNA damage, leading to apoptotic cell death (14). It has been reported that even a 50% decrease in the amount of CHK1 could lead to cell death (43). However, it is surprising that inspite of reduced expression of eight members of the ATR-CHK1 pathway and several genes from telomere maintenance and DNA repair pathways, FLO-1 cells continued to proliferate *in vitro*.

The expression data also indicated that the level of recombinase (hsRAD51), a key DNA repair gene, was not suppressed by wortmannin in FLO-1 cells. Western blot analysis also showed that following wortmannin treatment, hsRAD51 expression did not decrease in FLO-1 cells, whereas it was suppressed in wortmannin sensitive OE33 and OE19 cells. Since AKT, a downstream effector of PI3K, is implicated in the regulation of RAD51 and wortmannin has been shown to suppress RAD51 in cancer cells (10), its suppression of RAD51 in OE33 and OE19 is expected. In FLO-1 cells, with substantially lower AKT expression (Figure 2), wortmannin was not able to affect RAD51 through AKT signaling. Therefore, the loss of RAD51 suppression by wortmannin in FLO-1 cells could be attributed to a lack or loss of its regulation by AKT. Since RAD51 is implicated in the repair of double-strand breaks (44), ongoing genomic evolution (23, 24) and telomere

maintenance (45), it could help wortmannin-treated FLO-1 cells survive under conditions of reduced NHEJ and reduced telomerase activity. We therefore suppressed RAD51 in FLO-1 cells using lentivirus-based shRNAs which mediate a strong suppression of RAD51 in these cells. The suppression of RAD51 was associated with a significant increase in the antiproliferative activity of wortmannin. Gene expression data showed that whereas wortmannin down-regulated ATR-CHK1 genes without affecting CHK2, suppression of RAD51 affected both the CHK1 and CHK2, with relatively stronger suppression of CHK2. Moreover, following exposure of transduced cells to UV, a substantial induction of phosphorylated H2AX was detected in RAD51-suppressed relative to control cells. Phosphorylation of histone H2AX is used as a marker for DNA damage (46, 47), therefore induction of phosphorylated H2AX indicates that loss of RAD51 was associated with a reduced ability of RAD51-suppressed cells to repair the damage caused by UV. Although the combination of RAD51 and PI3K suppression is expected to result in accumulation of more DNA damage than RAD51 suppression alone, the amount of phosphorylated H2AX detected was low because wortmannin inhibits H2AX phosphorylation (48, 49). Furthermore, within a short period, 10% of PARP had already been cleaved in RAD51-suppressed cells treated with wortmannin, compared to 5% cleavage in control cells. Following exposure of cells to DNA-damaging UV, the cleavage of PARP increased to ~10% in individual treatments, whereas it reached 25% in RAD51-suppressed cells treated with wortmannin, indicating increased DNA damage response. Both wortmannin treatment and RAD51 suppression were also associated with elevated expression of DNA damage-inducible genes (such as *DDIT4, GADD45G* or their isoforms *etc*.), implicated in growth arrest and/or apoptosis (50). Taken together, these data show that RAD51 suppression negatively affects important DNA damage checkpoints (mediated by CHK2 and CHK1) and reduces the ability of cells to repair double-strand breaks. Wortmannin has a specific affect on checkpoint mediated by the ATR-CHK1 pathway but also down-regulates a number of other repair proteins in FLO-1 cells, except RAD51. In cancer cells, especially BAC cells, with elevated recombination activity, the DNA breaks are introduced and then recombined at a much higher rate than in normal cells. Therefore, simultaneous suppression of PI3K function and RAD51, by targeting checkpoints and ongoing repair, may cause substantial DNA damage to go through mitosis, leading to growth arrest and ultimately apoptotic cell death.

Suppression of RAD51 also significantly increased the antitumor activity of wortmannin in FLO-1 cells in a subcutaneous tumor model. The ATR-CHK1-mediated checkpoint, down-regulated by wortmannin, is a key DNA damage checkpoint and an attractive target for cancer drug design (51). Our data show that for cells with a low AKT expression (such as FLO-1), targeting CHK1 alone may not be very effective because RAD51 seems to play a critical role, not only in genomic evolution, but also in DNA damage checkpoints and survival of these and probably other, cancer cells. Simultaneous suppression of PI3K and RAD51, especially in cells with low AKT expression, can significantly enhance growth inhibition, both *in vitro* and *in vivo*.

Conflict of Interest

Authors have no conflict of interest.

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