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Degradation of BRCA2 in alkyltransferase-mediated DNA repair and its clinical implication

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Abstract

Germline mutations in *BRCA2* have been linked to early-onset familial breast cancer. BRCA2 is known to play a key role in repairing double-strand breaks (DSBs). Here, we describe the involvement of BRCA2 in O^6-alkylguanine DNA alkyltransferase (AGT)—mediated repair of O^6-methylguanine (O^6-mG) adducts. We demonstrate that BRCA2 physically associates and undergoes repair-mediated degradation with AGT. In contrast, BRCA2 with a 29-amino acid deletion in an evolutionarily conserved domain does not bind to alkylated AGT, the two proteins are not degraded and mouse embryonic fibroblasts (MEFs) are specifically sensitive to alkylating agents that result in O^6-mG adducts. We demonstrate that O^6-benzylguanine (O^6BG), a non-toxic inhibitor of AGT, can also induce BRCA2 degradation. BRCA2 is a viable target for cancer therapy because BRCA2-deficient cells are hypersensitive to chemotherapeutic DNA damaging agents. We show a marked effect of O^6BG pretreatment on cell sensitivity to cisplatin. We also demonstrate the efficacy of this approach on a wide range of human tumor cell lines, which suggests that chemo-sensitization of tumors by targeted degradation of BRCA2 may be an important consideration when devising cancer therapeutics.

Keywords

Mouse Models; BRCA2; O^6-Alkylguanine-DNA alkyltransferase (AGT); MGMT; O^6-benzylguanine

INTRODUCTION

Breast cancer is the most frequently diagnosed cancer in women and one of the leading causes of lethality. Among the various factors responsible for the development of this cancer, a family history of the disease seems to play a major role. Women who inherit loss-of-function mutations

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in *BRCA1* or *BRCA2* have up to 80% risk of developing breast cancer by the age of 70 (1). In addition to breast cancer, mutations in *BRCA1* and *BRCA2* also result in increased susceptibility to ovarian, pancreatic and prostate cancers (1). Mutations in *BRCA2* have also been implicated in Fanconi anemia, a rare autosomal recessive disease (2).

To gain an insight into the *in vivo* functions of BRCA2, several knockout mouse models have been generated that revealed its role in DSB repair (3,4). Due to a critical role of *Brca2* in normal embryonic development, loss-of-function mutations have resulted in lethality of mutant embryos during mid-gestation (5-7). Hypomorphic alleles of *Brca2* that result in truncated BRCA2 protein have also been generated (8-13). Mutant mice are viable, but have several developmental defects and develop thymic lymphomas a few months after birth. Cells homozygous for these hypomorphic alleles are hypersensitive to genotoxins and show chromosomal aberrations. Conditional loss of *Brca2* in mammary glands results in a higher incidence of tumors after extended latency (14).

In a complementary approach, we are generating mutant alleles of *Brca2* in mice, each bearing either a small deletion in an evolutionarily conserved region or a disease-associated missense mutation. Mutations are introduced in the *Brca2* gene cloned in a bacterial artificial chromosome (BAC). Functional analysis of the mutations is performed in transgenic mice lacking the endogenous *Brca2* gene. The *Brca2*-deficient mice were generated previously by replacing exon 10 and a portion of exon 11 by gene targeting method (5). We have rescued the mutant phenotype of the *Brca2* null mice ($Brca2^{brdm1}/Brca2^{brdm1}$, denoted hereafter as *Brca2Ko/Ko*) by introducing into their germline a BAC clone (RPCI 22-421A23 with a 220kb insert) that contains the complete mouse *Brca2* locus (data not shown).

BRCA2 contains eight BRC repeats located in the middle of the protein, each repeat consisting of 30–40 amino acids. Six of the eight repeats have been shown to interact with RAD51 (15, 16). An evolutionarily conserved 29-amino acid region (amino acids 1088–1116) is located between repeats 1 and 2 (Fig. 1A). Here, we describe the functional analysis of a mutant allele of *Brca2* with an in-frame deletion of 87 bases in exon 11, encoding these 29 amino acids. We have generated transgenic mice expressing mutant *Brca2* and found the mutant embryonic fibroblasts to be specifically sensitive to alkylating agents that result in O^6-mG adducts. BRCA2 deficient cells are known to be hypersensitive to a wide range of DNA damaging agents due to its key role in RAD51-mediated DNA repair. The specific sensitivity of the cells expressing the mutant BRCA2 with a 29 amino acid deletion led us to uncover the involvement of BRCA2 in repair of O^6-mG adducts. Our results validate the advantage of using hypomorphic alleles to uncover novel functions of BRCA2. We illustrate the utility and clinical relevance of this new finding.

MATERIALS AND METHODS

Generation of BAC transgenic mice

An 87 bp deletion in *Brca2* was generated using recombineering and transgenic mice were generated as described previously (17).

Genotyping mice by Southern analysis and genetic crosses

The *Brca2* knockout mice were generated previously by replacing exon 10 and a portion of exon 11 with human *Hprt* minigene (5). A single base-pair alteration in intron 11 of *Brca2* was generated in the BAC to disrupt an endogenous *Eco*RV site, as described previously (17). This was used to distinguish the transgene from the wild-type allele using a 1.5 kb probe specific to *Brca2* exon 11 (nucleotides 5208-6710 of NM 009765).

Genotoxin sensitivity assays

Drug sensitivity assays were performed using MEFs between passage 2 and 5 by trypan blue exclusion method. Cell proliferation assay was performed using a coulter counter to test sensitivity of MEFs to cisplatin in response to O^6BG treatment. Alamar blue and clonogenic survival assays were used to screen various tumor cell lines for increased cytotoxic response to cisplatin in presence of O^6BG. All assays were performed in triplicates and repeated at least twice. The assays are described in detail in supplementary information.

Western blots

Total cell extracts were prepared in RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP40, 1% sodium deoxycholate, 0.1% SDS, 2 mM PMSF) supplemented with protease inhibitors. Protein concentrations were determined by BCA protein assay (Pierce Biotechnology, Rockford, IL). Total cell extracts (60 μg) were resolved on 4%–12% denaturing gradient SDS polyacrylamide gels and transferred onto PVDF membranes. Blots were probed with Actin (1:5000), AGT (1:500) or BRCA2 (1:500) primary antibodies for 1 hour at 25 °C followed by peroxidase conjugated secondary antibody against mouse or rabbit IgG (1:2000, Amersham Biosciences, UK) and detected by the ECL Plus detection system (Amersham Pharmacia Biotech, UK).

In vitro AGT activity

AGT activity measurements were carried out as described previously (18). See Supplementary information for details.

Gel filtration and immunoprecipitation

Cells were extracted in RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP40, 1% sodium deoxycholate, 0.1% SDS, 2 mM PMSF) supplemented with protease inhibitors. Cell lysates (2–3mg total proteins) were treated with O^6BG in the presence of 5 mM NEM and 25 μM MG132 at 4 °C for 1 hour. Untreated or O^6BG-treated extracts were run through a Superdex 200 HR10/30 column (Pharmacia) on an AKTA FPLC system (Pharmacia) at a flow rate of 0.5 ml min-1. Fractions (1 ml) were collected and analyzed for the presence of AGT or BRCA2 by Western blot. To test the elution profile of free AGT, 1 μg of recombinant human AGT in RIPA buffer was fractionated as described above. Recombinant AGT was then mixed with cell extracts (untreated and O6-BG treated) and loaded on the column. A fraction containing both BRCA2 and AGT (#7) was used for the co-IP experiment with anti-BRCA2 antibody (Pep3), and precipitates were probed for AGT using Western blot analysis. The fraction containing AGT but lacking BRCA2 (#12) from O^6BG-treated wild type cell extracts served as the negative control.

Reciprocal co-IPs were performed in BXPC3 cells. 1 mg total proteins from untreated or O^6BG treated cells was immunoprecipitated with mouse monoclonal anti-AGT antibody (Neomarkers), followed by immunobloting with rabbit polyclonal anti-AGT (Novus Biologicals) or rabbit polyclonal anti-BRCA-2 antibody (CalBiochem). A pull-down with normal mouse IgG (Southern Biotech) served as the negative control.

^{3}H-labeled alkylated AGT binding

To test the interaction of BRCA2 with alkylated AGT, ^{3}H-labeled alkylated AGT was made by incubating purified histidine-tagged human AGT with excess [^{3}H]methylated calf thymus DNA followed by protein purification on a metal affinity resin column. ^{3}H-labeled alkylated AGT (20 ng) was mixed with *Brca2+/+* or *Brca2*$^{\Delta 87}$ cell extracts in a buffer containing 5 mM NEM and 25 μM MG132. BRCA2 was then immunoprecipitated with anti-BRCA2 antibody (Pep3), and the binding was estimated by measuring the ^{3}H counts in the pulled-down complex.

Counts in the IgG control were used as 1, and the values are depicted as a fold-increase over control. The experiment was performed in triplicate.

Immunofluorescence analysis

Cells plated on coverslips at 50%–60% confluency were treated with 2 μM MNNG for 1 hour and allowed to recover for varying times. Paraformaldehyde fixed cells were permeabilized with 0.1% Triton X-100 solution and blocked with 5% goat serum before staining with primary antibody (rabbit anti-mouse AGT, in a humidifying chamber at 4 °C overnight. After washing, cells were stained with the secondary antibody (Alexa Fluor 568 goat anti-rabbit IgG [H+L] 1:1000). After extensive washing, nuclei were counterstained with DAPI (0.5 μg/ml) and coverslips mounted onto glass slides using Vectashield mounting medium (Vector, CA). Cells were visualized by three-dimensional confocal microscopy using a Zeiss LSM 510 microscope and a 100 X oil objective.

RESULTS

Generation of mice with a 29-amino acid deletion in BRCA2

We generated an in-frame deletion of 87 bases, encoding 29 amino acids encoded by codons 1088–1116 in exon 11 of *Brca2* in BAC *RP22-421A2* as described previously (17). This modified BAC [*TgN(RP22-421A23Δ87)2sks*, denoted as $Tg^{\Delta 87}$] was used to generate transgenic mice. We used three lines that had two or three copies of the BAC and crossed them to *Brca2Ko*/+ mice (5). We then crossed the *Brca2Ko*/+; $Tg^{\Delta 87}$/+ to *Brca2Ko*/+ mice to obtain *Brca2Ko/Ko;* $Tg^{\Delta 87}$/+ (also referred to as $Brca2^{\Delta 87}$) offspring from all three lines (Fig. 1 B, C). These rescued homozygous mutant mice are viable and phenotypically indistinguishable from their littermates (*Brca2*+/+, *Brca2*+/+; $Tg^{\Delta 87}$/+, *Brca2Ko*/+ or *Brca2Ko*/+; $Tg^{\Delta 87}$/+). These mice produce healthy offspring when intercrossed, suggesting that the 29-amino acid region is not essential for normal growth and development.

$Brca2^{\Delta 87}$ mice are predisposed to tumorigenesis

To examine if these mice had any late onset phenotype, we monitored a cohort of 30 mutant and 30 control mice from one founder transgenic line for 25 months. The $Brca2^{\Delta 87}$ mutant mice showed significantly reduced survival compared to the control mice ($P \leq 0.0033$ by Wilcoxon test; Fig. 1D). Twenty-six mutant mice were found to have tumors, including 20 that had a neoplasm as the cause of death, while only 12 in the control group developed any neoplasm (Supplementary Table 1A). In addition, 13 mutant mice had multiple neoplasms, compared to only 5 in the control group. A variety of neoplasms were seen in both groups (Supplementary Table 1B). Distinct differences in incidence of specific neoplasms between mutant and control animals was observed for hematopoietic neoplasm, including B cell lymphoma and histiocytic sarcoma, (17 in mutant compared to 5 in control mice, P= 0.0067), and pituitary adenomas (9 in mutant compared to 0 in control mice, P=0.0027). These results suggested that the mutant mice were predisposed to tumorigenesis and the $Brca2^{\Delta 87}$ allele was not fully functional.

Mutant MEFs are sensitive to SN_1-alkylating agents

To understand the functions of BRCA2 that may be disrupted by the 29-amino acid deletion, we generated mouse embryonic fibroblasts (MEFs) from $Brca2^{\Delta 87}$ mutant and control (*Brca2* +/+, *Brca2*+/+; $Tg^{\Delta 87}$/+, *Brca2Ko*/+ or *Brca2Ko*/+; $Tg^{\Delta 87}$/+) embryos. We observed no significant differences when proliferation of mutant and control primary MEFs were compared (data not shown). Since the 29-amino acid region is located between the first two BRC repeats known to interact with RAD51, we first determined whether the mutation affected DSB repair by testing the sensitivity of MEFs to various doses of γ-radiation (Fig. 2A). The mutant and

control MEFs did not show any difference in their sensitivity to γirradiation, suggesting that the ability of mutant BRCA2$^{\Delta 87}$ to repair DSB was not impaired. We also found that RAD51 foci formation in response to γirradiation was not affected in the BRCA2 mutant cells (Supplementary Fig. 1), further suggesting that there was no apparent defect in the DSB repair function. We then challenged the MEFs with various other genotoxins, including methyl methanesulfonate (MMS); UV-radiation; cisplatin; N-acetyl-2-acetylaminofluorene (AAF) and methyl-N'-nitro-N-nitrosoguanidine (MNNG) to determine whether any other DNA repair processes were affected. Sensitivity of the mutant and control MEFs were comparable in all cases except when treated with MNNG (Fig. 2B & Supplementary Fig. 2A—D). MEFs derived from *Brca2*$^{\Delta 87}$ embryos were more sensitive to MNNG compared to control cells (Fig. 2B). Although the mutant MEFs were moderately sensitive to MNNG, the sensitivity was consistently observed in independently generated primary MEFs from three different transgenic founder lines. We also confirmed the sensitivity of mutant MEFs to MNNG by a clonogenic survival assay that was performed by co-culturing primary MEFs with mitotically inactivated feeder cells (data not shown).

MNNG is an S_N1-type of alkylating agent that methylates guanine nucleotides, resulting in the formation of O^6-mG adducts. Surprisingly, *Brca2*$^{\Delta 87}$ mutant MEFs were not sensitive to other alkylating agents like MMS, which produces 3-alkyladenine adduct or AAF, which modifies at the C^8 position of guanine. However, *Brca2*$^{\Delta 87}$ mutant MEFs were also sensitive to N-methyl nitrosourea (MNU), another alkylating agent known to result in O^6-mG adducts (Supplementary Fig. 2E). This finding suggests that the mutant cells are specifically sensitive to agents that alkylate at the O^6 position of guanine.

Defect in AGT-mediated repair

O^6-mG adducts are specifically repaired by a single protein, O^6-alkylguanine DNA alkyltransferase (AGT), also known as O^6-methylguanine DNA methyl transferase (MGMT) (19). Since AGT is the only protein known to function in reversal of O^6-mG adducts, we looked at the expression of AGT in wild type and mutant MEFs. AGT protein was detected in both mutant and control cells by Western blot analysis (Fig. 2C) and there was no difference in the expression levels. To determine whether the protein was functional, we measured its activity using an *in vitro* assay. The amount of ^{3}H-labeled methyl group that is transferred from calf thymus DNA to AGT present in the cell extract has been used to estimate AGT activity (18). The results of this assay indicate that the AGT protein present in mutant cell extract is fully functional and has an *in vitro* activity comparable to the control cells (Fig. 2D). AGT functions by transferring the alkyl group from the O^6 position of guanine in DNA to a cysteine residue within its active site (20). AGT irreversibly binds to the alkyl group and is functionally inactivated. Subsequently, the inactive, alkylated AGT undergoes suicidal degradation via the ubiquitin proteolytic pathway (21). We therefore looked at degradation of AGT protein by immunostaining in *Brca2*$^{\Delta 87}$ mutant and control MEFs at different time intervals following MNNG treatment as a measure of its *in vivo* activity. Control and mutant cells exhibited predominantly nuclear staining of AGT in untreated cells. In the control cells, a gradual loss of the AGT staining occurred after MNNG treatment (Fig. 3A, upper panel). Surprisingly, in the mutant cells, AGT did not appear to undergo degradation (Fig. 3A, lower panel). We examined 200 nuclei and found that, after 4 hours of MNNG treatment, all of the wild-type cells exhibited a marked reduction in AGT staining compared to the untreated cells, whereas only 20% of the mutant cells showed some reduction in AGT staining. After 8 hours, none of the wild-type cells showed any AGT staining, but about 80% of the mutant cells still displayed strong nuclear AGT staining. We confirmed these results by western blot analysis. In wild type cells, but not in the mutant, degradation of AGT is detected as early as 2 hours after MNNG treatment, reaches its peak at 8 hours, then by 16 hours, a normal level of AGT is restored through re-synthesis (Fig. 3B, top panel). Interestingly, we also detected a reduction in BRCA2

level as seen with AGT in wild type cells after MNNG treatment but no significant change was observed in mutant cells (Fig. 3B, middle panel). These results support a role for BRCA2 in the AGT mediated repair process and indicate that BRCA2 is co-degraded with AGT after alkylation.

To examine whether BRCA2 was involved only in repair of DNA O^6mG adducts or it also had a role in subsequent degradation of alkylated AGT, we tested the effect of O^6-benzylguanine (O^6BG), a pseudo substrate of AGT (22). O^6BG alkylates AGT by transferring a benzyl group to the active site of AGT, which initiates its proteolytic degradation in a process similar to the degradation induced by the removal of an alkyl adduct from DNA (22,23). If BRCA2 is involved only in repair of damaged DNA repair by AGT, AGT should undergo degradation after O^6BG treatment in wild type as well as *Brca2*$^{\Delta 87}$ mutant MEFs as O^6BG directly inactivates AGT without involvement of any DNA damage. In contrast, if BRCA2 is also involved in degradation of the alkylated AGT, AGT should undergo degradation after O^6BG treatment in wild type but not in *Brca2*$^{\Delta 87}$ mutant MEF extracts as was observed with MNNG treatment. Therefore, we examined degradation of AGT after O^6BG treatment by Western blot analysis and found that the level of AGT decreased in extracts from control MEFs (Fig. 3C,upper panel), while remained unchanged in the mutant MEF extracts, suggesting that AGT was not degraded. As seen with MNNG, BRCA2 was co-degraded with AGT in response to O^6BG treatment in wild type cells but not in mutant cells (Fig. 3C,middle panel). These results suggest a potential role for BRCA2 in degradation of inactivated AGT, which was blocked in *Brca2*$^{\Delta 87}$ mutant cells.

Since BRCA2 degradation upon treatment with alkylating agents has not been shown previously, we tested whether this was dependant on AGT. We used AGT-deficient MEFs and two AGT deficient cell lines HelaS3 and TK6 to check BRCA2 degradation in response to O^6BG treatment. BRCA2 was degraded in wild type MEFs and Hela cells upon O^6BG treatment but not in the AGT deficient cells (Fig. 3D, middle panels), indicating that BRCA2 degradation by alkylating agents depends on the presence of AGT.

AGT is associated with a BRCA2 containing complex

Because of the involvement of BRCA2 in AGT-mediated repair process, we tested by gel filtration chromatography whether the two proteins interact physically. Total proteins from wild type and mutant MEFs were fractionated on a size exclusion column. After fractionation, each fraction was tested by Western blot analysis for the presence of BRCA2 and AGT. We found that BRCA2 eluted as a component of a very large molecular weight complex (>670 kDa). Interestingly, while the AGT levels were very low in these BRCA2-containing fractions in untreated cell extracts, the amount of AGT in these fractions increased after O^6BG treatment (Fig. 4A, right panel see fractions 7&8). In contrast, in the mutant extracts, a small amount of AGT co-eluted with BRCA2 even in the untreated extracts and there was no significant change in the amount of AGT in the BRCA2-containing fraction upon alkylation. These observations suggested that while interaction between wild type BRCA2 and AGT is increased in response to alkylation, interaction between AGT and mutant BRCA2$^{\Delta 87}$ is not affected by alkylation.

To examine the elution profile of pure AGT, we fractionated human recombinant AGT protein, which has 77% amino acids sequence similarity with mouse AGT. The pure protein (24 kDa) was not present in the high molecular weight fraction and eluted in much lower fractions with a molecular weight range of 44 – 200 kDa (Fig. 4B, lower panel). When mixed with protein cell extracts, the recombinant AGT eluted in a high-molecular-weight complex with BRCA2. In addition, the amount of AGT in BRCA2 containing fractions increased after O6BG treatment (Fig. 4B, right panels see fractions 7&8) compared to the fraction from untreated extracts (Fig. 4B, left panels) in the wild type cells. In untreated mutant cell extracts, the level of AGT in BRCA2 containing fractions appeared higher when compared with the wild type cells. The

difference became even more evident when we co-immunoprecipitated AGT with BRCA2-specific antibodies from BRCA2-containing fractions. The amount of AGT pulled down with BRCA2 antibody from wild type cells was greatly increased following O^6BG treatment, suggesting that there is an increase in interaction between BRCA2 and alkylated AGT (Fig. 4C). In contrast, AGT efficiently co-immunoprecipitated with BRCA2$^{\Delta 87}$ in mutant extracts even without O^6BG treatment.

Mutant BRCA2 does not bind to alkylated AGT

AGT undergoes alkylation in *Brca2*$^{\Delta 87}$ mutant MEFs based on its normal *in vitro* activity as well as on the observed change in the amount of AGT in the low molecular fraction after alkylation (Fig. 4A). However, it is not known whether alkylated AGT can bind to mutant BRCA2$^{\Delta 87}$. To investigate this, we performed an assay to assess the ability of wild type and mutant BRCA2 to specifically bind the alkylated form of AGT. H^3-radiolabeled recombinant alkylated AGT was added to cell extracts, and we subsequently performed coimmunoprecipitations. Based on the amount of radioactivity obtained after immunoprecipitation using anti-BRCA2 antibody, we estimated the amount of alkylated AGT bound to BRCA2. Over six-fold more (compared to an immunoglobulin (Ig) control) alkylated AGT immunoprecipitated in wild-type cell extracts, whereas in the mutant cell extracts, the amount of H^3-radiolabeled recombinant alkylated AGT was similar to the background level (Fig. 4D). These results demonstrate that while wild-type BRCA2 is able to bind to alkylated AGT, mutant BRCA2$^{\Delta 87}$ fails to bind alkylated AGT. It is possible that the deletion of 29 amino acids causes a subtle conformational change in BRCA2 that results in altered interaction with unalkylated AGT, which may interfere with the *in vivo* function of AGT causing moderate sensitivity of mutant MEFs to MNNG. The altered interaction with unalkylated AGT may disrupt the interaction between BRCA2$^{\Delta 87}$ and alkylated AGT.

BRCA2 and AGT interaction in human cells

With the aim of validating the observations made in MEFs, and also to determine whether BRCA2 plays a role in AGT-mediated repair in human cells, we examined the interaction between BRCA2 and endogenous AGT in human BxPC3 cells. BxPC3 is a pancreatic adenocarcinoma cell line that expresses functional BRCA2 (24). We performed co-immunoprecipitation using anti-AGT antibody and probed with anti-BRCA2 antibodies. We found that BRCA2 coimmunoprecipitated with AGT in BxPC-3 cells (Fig. 5A, lane1). We observed an increase in the amount of BRCA2 pull-down with AGT antibodies following O6BG treatment in BxPC3 cells (Fig. 5A, lane 2). These results demonstrated that BRCA2 interacts with endogenous alkylated as well as unalkylated AGT in human cells. We then examined the repair-mediated degradation of BRCA2 and AGT in human BxPC3 cells following MNNG treatment. Consistent with the results obtained in MEFs, BRCA2 and AGT were both degraded in BXPC3 cells following MNNG treatment (Fig. 5B, left panels).

AGT degradation in BRCA2 deficient cells

To examine if BRCA2 was essential for degradation of AGT, we treated BRCA2-deficient human cancer cell line Capan-1 with MNNG. Capan-1 cells express a truncated form of BRCA2 that is only localized in the cytoplasm (25). We found that in Capan-1 cells, AGT gradually degraded with time after MNNG treatment (Fig. 5B, right panels). These observations were further confirmed by using BRCA2-deficient mouse embryonic stem cells, which also showed AGT degradation after O^6BG treatment (Fig. 5C). These results indicated that although degradation of BRCA2 is dependent on the presence of AGT, BRCA2 is not essential for AGT degradation. It is possible that the altered interaction between AGT and BRCA2$^{\Delta 87}$ interferes with AGT degradation in BRCA2$^{\Delta 87}$ mutant MEFs.

Clinical Implication of O^6BG mediated BRCA2 degradation

Although BRCA2 may not be essential for degradation of alkylated AGT, the fact that BRCA2 can be degraded by O^6BG suggests that the interaction between AGT and BRCA2 can be of clinical relevance. The compromised DNA repair function of BRCA2-deficient cells is being explored as a potential target for treating such tumors (26-28). Recent studies have shown that the targeted inhibition of poly(ADP-ribose) polymerase can kill BRCA2 mutant cells with high specificity (29,30). Because BRCA2-deficient cells exhibit radiation-hypersensitivity, we tested the effect of O^6BG on γ-radiation sensitivity in wild-type MEFs. We found that O^6BG rendered cells more sensitive to radiation (data not shown). However, the degree of sensitivity was not very high, which we attribute to the fact that we do not completely deplete BRCA2 by O^6BG. We then tested the effect of O^6BG on cisplatin sensitivity because cells expressing hypomorphic alleles of *Brca2* in mice have been shown to be more sensitive to DNA interstrand cross-linking agents compared to -irradiation (8,10,13). Because cisplatin is a potent DNA cross-linking agent commonly used for chemotherapy, enhancing the sensitivity of tumor cells to cisplatin by O^6BG will be clinically relevant (31). To test this hypothesis we used wild type and mutant MEFs with and without O^6BG pretreatment, exposed them to different doses of cisplatin and measured the effect on cell proliferation. The wild type cells exhibited a marked increase in sensitivity to cisplatin after O^6BG pretreatment compared to cells that were not pretreated with O^6BG (Fig. 6A). As expected, *Brca2*$^{\Delta 87}$ mutant MEFs showed no significant difference in their sensitivity to cisplatin in response to O^6BG pretreatment because BRCA2$^{\Delta 87}$ does not get degraded in these cells and is able to repair the DNA damage.

We next examined if O^6BG can be used to render human tumor cells sensitive to cisplatin. Since cisplatin is commonly used to treat wide range of tumors, we tested the effect of O^6BG on cisplatin sensitivity on various tumor cell lines including BxPC3 and others derived from lung (NCI-H460, A549), melanoma (MDA-MB-435, SK-Mel-28, A375, UACC-62, MALME-3E), ovary (SK-OV-3), and prostate (DU145) tumors. MDA-MB-435 was previously considered to be a breast adenocarcinoma cell line, but recently it has been reclassified as a melanoma cell line (32)**.** Out of the ten cell lines we tested, eight exhibited a significant reduction in cell proliferation due to O^6BG pretreatment (Supplementary Fig. 4). As shown in Figure 6B, there was a marked reduction in IC_{50} of these cells due to pretreatment of cells by O^6BG compared to cells that were not pretreated. One melanoma and a prostate tumor cell line did not show any reduction in IC_{50} in response to O^6BG treatment. O^6BG alone did not have any toxic effect on these cells. We further confirmed these results by clonogenic survival assay and found that O^6BG pretreatment significantly increased the sensitivity of all cell lines (except for SK-Mel-28) to cisplatin (Supplementary Figure 5, Fig. 6C). Taken together, these results support our hypothesis that O6BG can be used to increase the sensitivity of tumor cells to chemotherapeutic agents like cisplatin.

DISCUSSION

We have generated a small deletion in the mouse *Brca2* gene and found MEFs derived from mutant embryos to be specifically sensitive to SN_1-type alkylating agents. Since the identification of AGT more than two decades ago, it has been proposed to function alone in repairing the O^6mG adducts caused by these agents (33-35). We show that AGT is part of an *in vivo* complex containing BRCA2 and the two proteins physically interact We also show that like AGT, BRCA2 is also degraded in the process. Our observation that AGT is degraded in BRCA2-deficient cells suggests that BRCA2 is not essential for degradation of alkylated AGT. However, BRCA2 is known to be part of a multiprotein complex including BRCA1 and BARD1 that have ubiquitin E3 ligase activity (36). Therefore, it is possible that BRCA2 may have a regulatory or non-essential function in signaling ubiquitination and degradation of alkylated AGT.

The functional significance of the interaction between BRCA2 and AGT remains to be elucidated. We speculate that the involvement of BRCA2 in AGT-mediated repair process may provide specificity and increase the fidelity of the process. Degradation of BRCA2 may be due to the fact that it is bound to alkylated-AGT and the two proteins may be targeted together as a complex for degradation. Alternatively, AGT bound to BRCA2 may not be accessible for degradation. Therefore, removal of BRCA2 may be necessary to gain access to alkylated-AGT. However, in BRCA2-deficient cells, AGT is accessible and is able to undergo degradation. In contrast, we postulate that the 29- amino acid deletion results in a conformational change in BRCA2, which alters its interaction with unalkylated AGT and interfere with its binding to alkylated AGT and degradation. Although AGT present in *Brca2*$^{\Delta 87}$ mutant cells has normal activity based on the i*n vitro* activity assay, we cannot rule out the possibility that BRCA2 may play a role in the *in vivo* function of AGT. It may be involved in the localization of AGT to the site of DNA damage similar to its role in localization of RAD51 to the site of a double strand break (8).

We show that alkylated AGT fails to undergo degradation in *Brca2*$^{\Delta 87}$ mutant cells. How a defect in this process results in increased tumor predisposition of mutant mice is unclear. It is possible that alkylated AGT remains bound to DNA and interferes with DNA replication, transcription or DNA repair activities. It has also been suggested that degradation of the inactivated AGT proteins provides a signal for synthesis of additional protein (37,38). Therefore, though the *Brca2*$^{\Delta 87}$ mutant cells have functional AGT, the accumulation of alkylated form could be causing the mild sensitivity and tumor predisposition by interfering with DNA repair and synthesis of new AGT. AGT-deficient mice do not develop tumors spontaneously. However, when exposed to a very low dose of MNU, these mice develop thymic lymphomas as well as lung adenomas (39-41). We cannot rule out the possibility that the tumor predisposition of *Brca2*$^{\Delta 87}$ mice is due to some other function of BRCA2 that is currently unknown, which is disrupted by the 29-amino acid deletion.

DNA alkylating agents are widely used chemotherapy drugs, however tumor cells frequently acquire resistance to these drugs by over expressing AGT. Depletion of AGT by its inhibitors prior to treatment with O^6-alkyalting agents has been suggested to increase the efficacy of such therapy (42,43) . In a parallel approach, depletion of BRCA2 by O^6BG can be used to increase the sensitivity of tumor cells to DNA crosslinking agents like cisplatin. Some previous studies have shown that O^6BG has the ability to sensitize cells to cisplatin, but the exact mechanism for this sensitivity was not clear (44-46). One possibility was that O6BG and other guanine derivatives could be used to inhibit CDK2 and enhance sensitivity of head and neck tumor cells lines to cisplatin (45,46). In another study, the enhancement was shown to be independent of glutathione or glutathione-S-transferase activity and also nucleotide excision repair proteins (44). We have provided a new mechanism for the effect of O^6BG on cisplatin sensitivity and also shown its efficacy on a wide range of tumor cell lines.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

1. Rahman N, Stratton MR. The genetics of breast cancer susceptibility. Annu Rev Genet 1998;32:95–121. [PubMed: 9928476]
2. Howlett NG, Taniguchi T, Olson S, et al. Biallelic inactivation of BRCA2 in Fanconi anemia. Science 2002;297:606–9. [PubMed: 12065746]
3. Venkitaraman AR. Cancer susceptibility and the functions of BRCA1 and BRCA2. Cell 2002;108:171–182. [PubMed: 11832208]
4. Evers B, Jonkers J. Mouse models of BRCA1 and BRCA2 deficiency: past lessons, current understanding and future prospects. Oncogene 2006;25:5885–97. [PubMed: 16998503]
5. Sharan SK, Morimatsu M, Albrecht U, et al. Embryonic lethality and radiation hypersensitivity mediated by Rad51 in mice lacking Brca2. Nature 1997;386:804–10. [PubMed: 9126738]
6. Suzuki A, de la Pompa JL, Hakem R, et al. Brca2 is required for embryonic cellular proliferation in the mouse. Genes Dev 1997;11:1242–52. [PubMed: 9171369]
7. Ludwig T, Chapman DL, Papaioannou VE, Efstratiadis A. Targeted mutations of breast cancer susceptibility gene homologs in mice: lethal phenotypes of Brca1, Brca2, Brca1/Brca2, Brca1/p53, and Brca2/p53 nullizygous embryos. Genes Dev 1997;11:1226–41. [PubMed: 9171368]
8. Patel KJ, Yu VP, Lee H, et al. Involvement of Brca2 in DNA repair. Mol Cell 1998;1:347–57. [PubMed: 9660919]
9. McAllister KA, Bennett LM, Houle CD, et al. Cancer susceptibility of mice with a homozygous deletion in the COOH-terminal domain of the Brca2 gene. Cancer Res 2002;62:990–4. [PubMed: 11861370]
10. Donoho G, Brenneman MA, Cui TX, et al. Deletion of Brca2 exon 27 causes hypersensitivity to DNA crosslinks, chromosomal instability, and reduced life span in mice. Genes Chromosomes Cancer 2003;36:317–31. [PubMed: 12619154]
11. Connor F, Bertwistle D, Mee PJ, et al. Tumorigenesis and a DNA repair defect in mice with a truncating Brca2 mutation. Nat Genet 1997;17:423–30. [PubMed: 9398843]
12. Friedman LS, Thistlethwaite FC, Patel KJ, et al. Thymic lymphomas in mice with a truncating mutation in Brca2. Cancer Res 1998;58:1338–43. [PubMed: 9537225]
13. Atanassov BS, Barrett JC, Davis BJ. Homozygous germ line mutation in exon 27 of murine Brca2 disrupts the Fancd2-Brca2 pathway in the homologous recombination-mediated DNA interstrand cross-links' repair but does not affect meiosis. Genes Chromosomes Cancer 2005;44:429–37. [PubMed: 16127665]
14. Ludwig T, Fisher P, Murty V, Efstratiadis A. Development of mammary adenocarcinomas by tissue-specific knockout of Brca2 in mice. Oncogene 2001;20:3937–48. [PubMed: 11494122]
15. Wong AK, Pero R, Ormonde PA, Tavtigian SV, Bartel PL. RAD51 interacts with the evolutionarily conserved BRC motifs in the human breast cancer susceptibility gene brca2. J Biol Chem 1997;272:31941–4. [PubMed: 9405383]
16. Chen PL, Chen CF, Chen Y, Xiao J, Sharp ZD, Lee WH. The BRC repeats in BRCA2 are critical for RAD51 binding and resistance to methyl methanesulfonate treatment. Proc Natl Acad Sci USA 1998;95:5287–92. [PubMed: 9560268]
17. Swaminathan S, Ellis HM, Waters LS, et al. Rapid engineering of bacterial artificial chromosomes using oligonucleotides. Genesis 2001;29:14–21. [PubMed: 11135458]
18. Kanugula S, Pegg AE. Novel DNA repair alkyltransferase from Caenorhabditis elegans. Environ Mol Mutagen 2001;38:235–243. [PubMed: 11746760]
19. Demple B, Jacobsson A, Olsson M, Robins P, Lindahl T. Repair of alkylated DNA in Escherichia coli. Physical properties of O6-methylguanine-DNA methyltransferase. J Biol Chem 1982;257:13776–80. [PubMed: 6754717]
20. Pegg AE. Repair of O(6)-alkylguanine by alkyltransferases. Mutat Res 2000;462:83–100. [PubMed: 10767620]

21. Srivenugopal KS, Yuan XH, Friedman HS, Ali-Osman F. Ubiquitination-dependent proteolysis of O6-methylguanine-DNA methyltransferase in human and murine tumor cells following inactivation with O6-benzylguanine or 1,3-bis(2-chloroethyl)-1-nitrosourea. Biochemistry 1996;35:1328–34. [PubMed: 8573590]
22. Moschel RC, McDougall MG, Dolan ME, Stine L, Pegg AE. Structural features of substituted purine derivatives compatible with depletion of human O6-alkylguanine-DNA alkyltransferase. J Med Chem 1992;35:4486–91. [PubMed: 1447749]
23. Dolan ME, Moschel RC, Pegg AE. Depletion of mammalian O6-alkylguanine-DNA alkyltransferase activity by O6-benzylguanine provides a means to evaluate the role of this protein in protection against carcinogenic and therapeutic alkylating agents. Proc Natl Acad Sci USA 1990;87:5368–72. [PubMed: 2164681]
24. Abbott DW, Freeman ML, Holt JT. Double-strand break repair deficiency and radiation sensitivity in BRCA2 mutant cancer cells. J Natl Cancer Inst 1998;90:978–85. [PubMed: 9665145]
25. Spain BH, Larson CJ, Shihabuddin LS, Gage FH, Verma IM. Truncated BRCA2 is cytoplasmic: implications for cancer-linked mutations. Proc Natl Acad Sci USA 1999;96:13920–5. [PubMed: 10570174]
26. Donawho CK, Luo Y, Penning TD, Bauch JL, et al. ABT-888, an orally active poly(ADP-ribose) polymerase inhibitor that potentiates DNA-damaging agents in preclinical tumor models. Clin Cancer Res 2007;13:2728–37. [PubMed: 17473206]
27. Hay T, Jenkins H, Sansom OJ, Martin NM, Smith GC, Clarke AR. Efficient deletion of normal Brca2-deficient intestinal epithelium by poly(ADP-ribose) polymerase inhibition models potential prophylactic therapy. Cancer Res 2005;65:10145–8. [PubMed: 16287996]
28. De Soto JA, Wang X, Tominaga Y, et al. The Inhibition and Treatment of Breast Cancer with Poly (ADP-ribose) Polymerase (PARP-1) Inhibitors. Int J Biol Sci 2006;2:179–85. [PubMed: 16810332]
29. Bryant HE, Schultz N, Thomas HD, et al. Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. Nature 2005;434:913–7. [PubMed: 15829966]
30. Farmer H, McCabe N, Lord CJ, et al. Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. Nature 2005;434:917–21. [PubMed: 15829967]
31. Rabik CA, Dolan ME. Molecular mechanisms of resistance and toxicity associated with platinating agents. Cancer Treat Rev 2007;33:9–23. [PubMed: 17084534]
32. Rae JM, Ramus SJ, Waltham M, et al. Common origins of MDA-MB-435 cells from various sources with those shown to have melanoma properties. Clin Exp Metastasis 2004;21:543–52. [PubMed: 15679052]
33. Margison GP, Santibanez-Koref MF. O6-alkylguanine-DNA alkyltransferase: role in carcinogenesis and chemotherapy. Bioessays 2002;24:255–66. [PubMed: 11891762]
34. Gerson SL. MGMT: its role in cancer aetiology and cancer therapeutics. Nat Rev Cancer 2004;4:296–307. [PubMed: 15057289]
35. Sabharwal A, Middleton MR. Exploiting the role of O6-methylguanine-DNA-methyltransferase (MGMT) in cancer therapy. Curr Opin Pharmacol 2006;6:355–363. [PubMed: 16777483]
36. Dong J, Chang-Claude J, Wu Y, Schumacher V, Debatin I, Tonin P, Royer-Pokora B. A high proportion of mutations in the BRCA1 gene in German breast/ovarian cancer families with clustering of mutations in the 3′ third of the gene. Hum Genet 1998;103:154–61. [PubMed: 9760198]
37. Pegg AE, Dolan ME, Moschel RC. Structure, function, and inhibition of O6-alkylguanine-DNA alkyltransferase. Prog Nucleic Acid Res Mol Biol 1995;51:167–223. [PubMed: 7659775]
38. Pegg AE, Wiest L, Mummert C, Stine L, Moschel RC, Dolan ME. Use of antibodies to human O6-alkylguanine-DNA alkyltransferase to study the content of this protein in cells treated with O6-benzylguanine or N-methyl-N'-nitro-N-nitrosoguanidine. Carcinogenesis 1991;12:1679–83. [PubMed: 1893528]
39. Glassner BJ, Weeda G, Allan JM, et al. DNA repair methyltransferase (Mgmt) knockout mice are sensitive to the lethal effects of chemotherapeutic alkylating agents. Mutagenesis 1999;14:339–47. [PubMed: 10375003]
40. Tsuzuki T, Sakumi K, Shiraishi A, et al. Targeted disruption of the DNA repair methyltransferase gene renders mice hypersensitive to alkylating agent. Carcinogenesis 1996;17:1215–20. [PubMed: 8681434]

41. Sakumi K, Shiraishi A, Shimizu S, Tsuzuki T, Ishikawa T, Sekiguchi M. Methylnitrosourea-induced tumorigenesis in MGMT gene knockout mice. Cancer Res 1997;57:2415–8. [PubMed: 9192819]

42. Gerson SL. Clinical relevance of MGMT in the treatment of cancer. J Clin Oncol 2002;20:2388–99. [PubMed: 11981013]

43. Rabik CA, Njoku MC, Dolan ME. Inactivation of O6-alkylguanine DNA alkyltransferase as a means to enhance chemotherapy. Cancer Treat Rev 2006;32:261–76. [PubMed: 16698182]

44. Fishel ML, Gamcsik MP, Delaney SM, et al. Role of glutathione and nucleotide excision repair in modulation of cisplatin activity with O6-benzylguanine. Cancer Chemother Pharmacol 2005;55:333–42. [PubMed: 15723259]

45. Fishel ML, Newell DR, Griffin RJ, et al. Effect of cell cycle inhibition on Cisplatin-induced cytotoxicity. J Pharmacol Exp Ther 2005;312:206–13. [PubMed: 15304523]

46. Fishel ML, Delaney SM, Friesen LD, et al. Enhancement of platinum-induced cytotoxicity by O6-benzylguanine. Mol Cancer Ther 2003;2:633–40. [PubMed: 12883036]

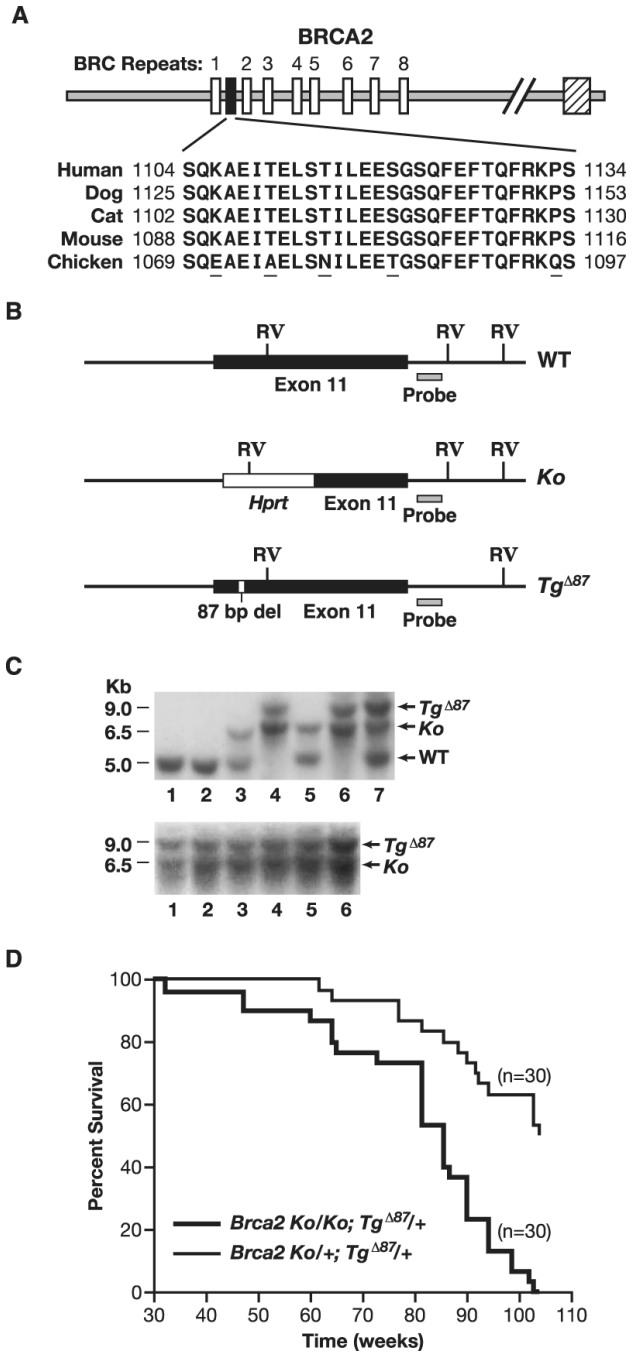

Figure 1. A small in-frame deletion in BRCA2 results in sensitivity to MNNG

A) An evolutionarily conserved 29-amino acid region (solid box) is present between the first two BRC repeats (open boxes). Non-conserved amino acids are underlined. Several of the BRC repeats interact with RAD51. An additional RAD51-binding domain is near the C-terminus of BRCA2 (hatched box). An 87-bp deletion was generated in a BAC containing a full-length BRCA2 gene that precisely deletes this 29-amino acid region. **B)** Scheme for genotyping mice by Southern analysis. The probe detects a 5.0 kb wild type (WT), 6.5 kb knockout (Ko) and 9.0 kb transgenic (Tg) fragment on Southern blot containing *Eco*RV (RV)-digested genomic DNA. **C)** Southern analysis of *Eco*RV digested genomic DNA isolated from littermates obtained from a mating between *Brca2Ko*/+; $Tg^{\Delta 87}$/+ and *Brca2Ko*/+ mice (Upper panel).

Lanes 4 and 6 show homozygous mutants as marked by the absence of the *WT* band. Lower panel shows the Southern analysis of *Eco*RV digested DNA isolated from littermates obtained from an intercross of *Brca2Ko/Ko*; $Tg^{\Delta 87}$/+ mice. All the offspring are homozygous mutant and have the transgene. **D)** Spontaneous tumor incidence in $Brca2^{\Delta 87}$ mutant mice. *Brca2Ko/Ko*; $Tg^{\Delta 87}$/+ mice show significantly reduced survival when compared to *Brca2Ko*/+; $Tg^{\Delta 87}$/+ littermates. 30 mutant and 30 control mice were monitored for neoplastic development for 25 months.

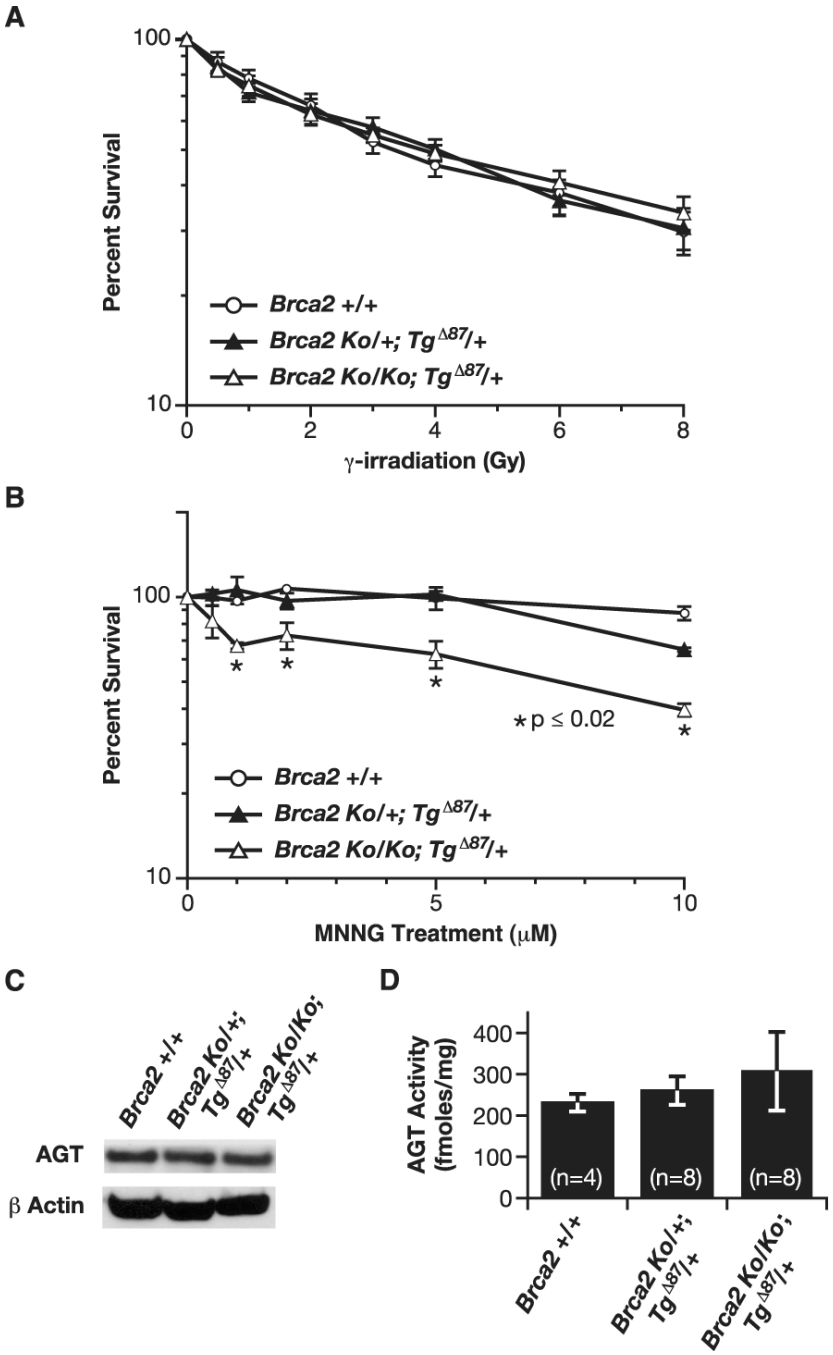

Figure 2. *Brca2*$^{\Delta 87}$ mutant cells exhibit sensitivity to MNNG and express normal levels of functional AGT

A & B) Survival of *Brca2Ko*/*Ko*; *Tg*$^{\Delta 87}$/+ compared to *Brca2*+/+ and *Brca2Ko*/+;*Tg*$^{\Delta 87}$/+ MEFs, after exposure to γirradiation **(E)** and MNNG **(F)** was measured by trypan blue exclusion method and represented as percentage over untreated cultures. Error bars represent standard deviation. **C)** AGT expression in cell extracts isolated from *Brca*2+/+, *Brca*2*Ko*/+; *Tg*$^{\Delta 87}$/+ and *Brca2Ko/Ko*; *Tg*$^{\Delta 87}$/+ MEFs (top); βactin (bottom) serves as the loading control. **D)** Measurement of AGT activity in total cell lysates obtained from *Brca*2+/+, *Brca*2*Ko*/+; *Tg*$^{\Delta 87}$/+ and *Brca*2*Ko*/+; *Tg*$^{\Delta 87}$/+ MEFs using an *in vitro* assay described in the "Methods" section.

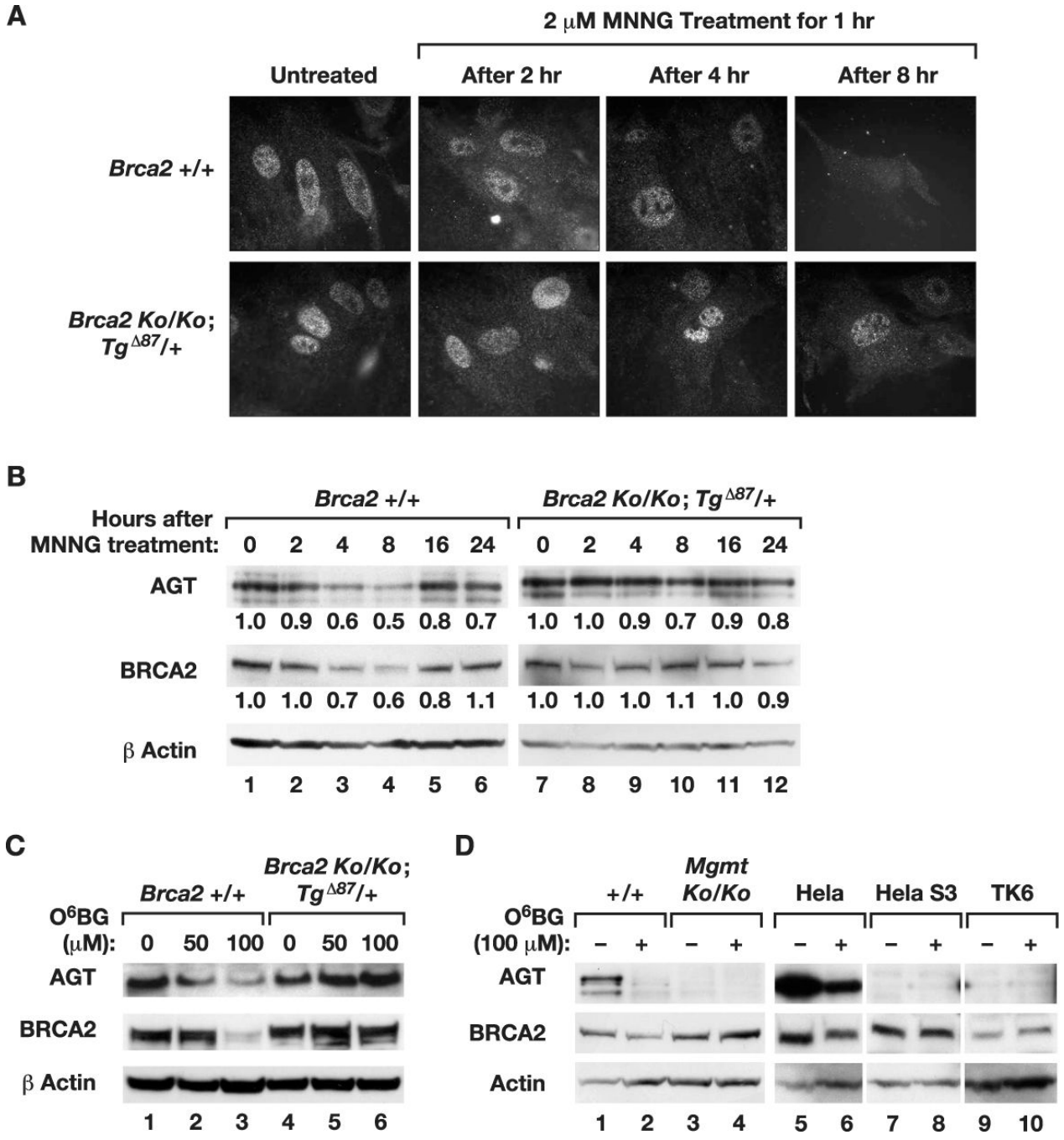

Figure 3. Degradation of AGT and BRCA2 in response to MNNG and O^{6}BG treatment in MEFs and human tumor cell lines

A) Detection of *in vivo* AGT activity at different time intervals by visualizing degradation of AGT using anti-AGT antibodies and a goat anti-rabbit IgG (H+L) Alexa 488 secondary antibody in control (upper panel) and *Brca2*$^{\Delta 87}$ mutant cells (lower panel). MEFs were treated with 2 μM MNNG for 1 hour and then immunostained after 2, 4 and 8 hours. **B)** Effect of MNNG treatment on degradation of AGT (upper panel) and BRCA2 (middle panel) in *Brca2* +/+ MEFs and *Brca2Ko/Ko*;Tg$^{\Delta 87}$/+ MEFs. βactin (lower panel) serves as the loading control. Values (in arbitrary units) represent quantitation of bands obtained by Western blot analysis using βactin for normalization. **C)** O^{6}BG treatment induces degradation of AGT (upper panel)

and BRCA2 (middle panel) in *Brca*2+/+ MEFs and not in *Brca*2*Ko*/*Ko*;$Tg^{\Delta 87}$/+ MEFs. βactin (lower panel) serves as the loading control. **D)** O^6BG treatment induces degradation of BRCA2 (middle panel) in *Mgmt*+/+ MEFs and Hela cells but not in *MgmtKo*/*Ko* MEFs and tumor cell lines Hela S3 and TK6 that lack AGT.

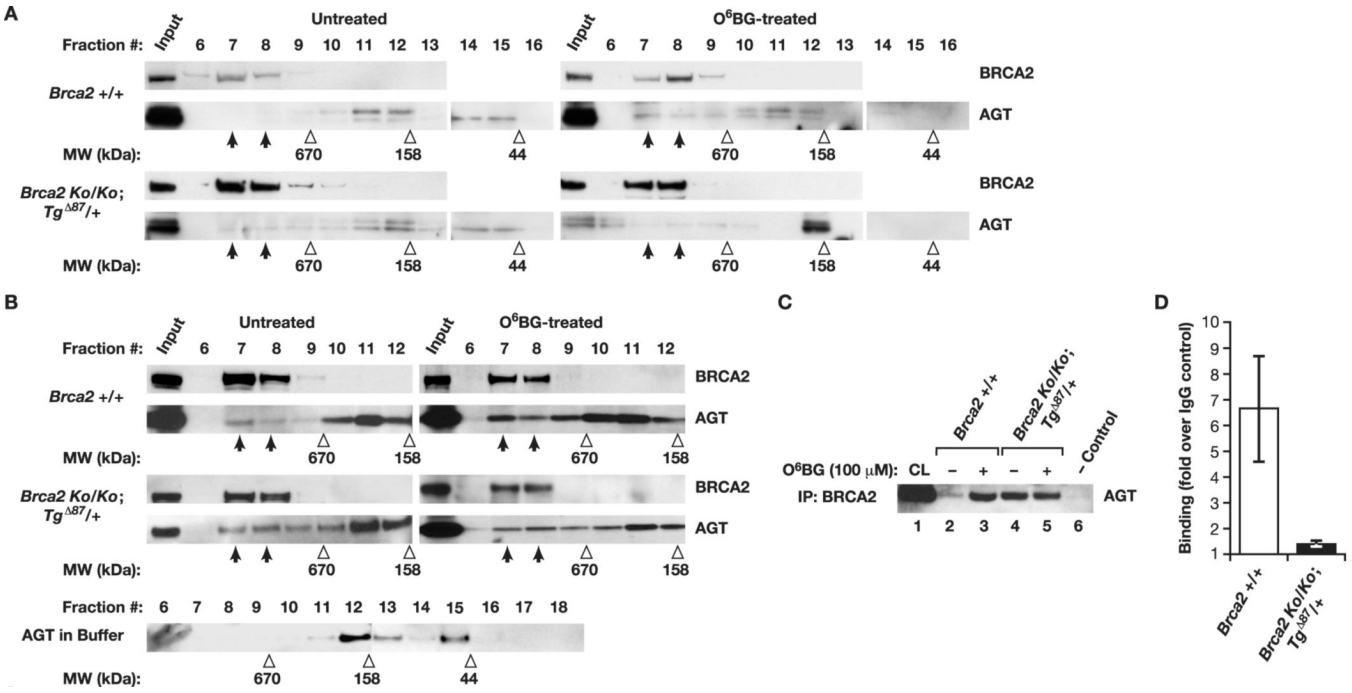

Figure 4. BRCA2 and AGT are present in a high molecular weight complex

A) BRCA2 and AGT elute in different fractions from a size exclusion column. BRCA2 is a part of a very high molecular-weight complex. In *Brca2*+/+ extract there is an increase in the amount of AGT after O^6BG treatment but in *Brca2*$^{\Delta 87}$ more AGT elutes in the higher fractions and there is no increase in AGT in these fractions after alkylation. Input represents endogenous BRCA2 and AGT in the cell extracts. Closed arrows mark the fractions in which BRCA2 and AGT coelute. **B)** When recombinant human AGT in buffer alone was loaded on the column, it was not present in the high-molecular-weight fraction (bottom panel). It co-elutes with BRCA2 in a high-molecular-weight complex before and after O6BG treatment in *Brca2*+/+ extract, there is an increase in the amount of AGT after O^6BG treatment. In *Brca2*$^{\Delta 87}$ mutant untreated extract more AGT elutes in the higher fractions compared to *Brca2*+/+ untreated extract the there is no change in AGT levels in these fractions after alkylation. Input represents endogenous BRCA2 and recombinant human AGT in cell extracts. Arrows mark the fractions in which BRCA2 and AGT co-elute. **C)** A fraction containing BRCA2 and AGT (#7 from 3e) from untreated (lanes 2, 4) and O^6BG treated (lanes 3, 5) was immunoprecipitated with anti-BRCA2 antibodies. AGT was detected by Western blot of the immunoprecipitate. A fraction that lacked BRCA2 but had AGT (#12 from 3b) served as the negative control (lane 6). Lane 1 (CL) represents cell lysate. **D)** Histogram showing interaction of ^{3}H-labeled recombinant alkylated AGT with wild type (open bars) and mutant BRCA2$^{\Delta 87}$ (closed bars). Binding was estimated by measuring the ^{3}H counts after immunoprecipitation. Counts in the IgG control were taken as 1 and the values obtained are depicted as a fold-increase over control.

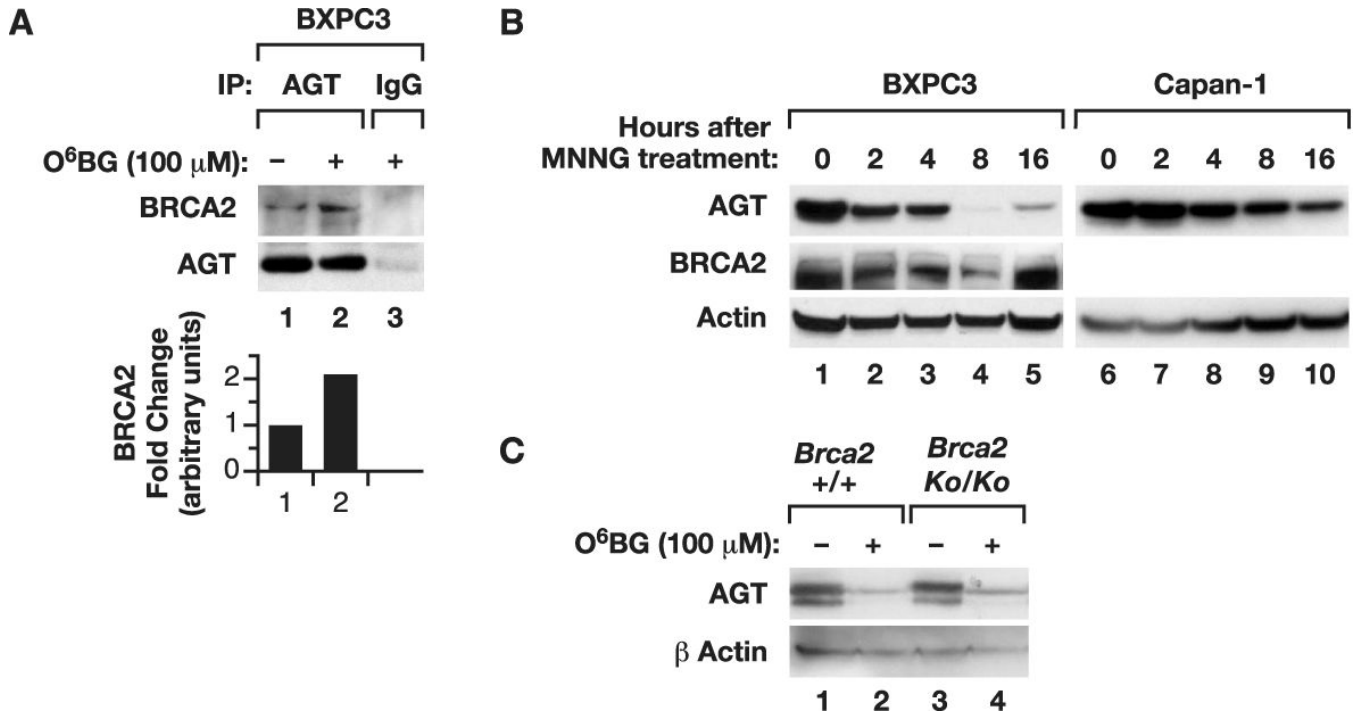

Figure 5. Interaction between BRCA2 and AGT and their degradation in human cell lines and mouse embryonic stem cells

A) BRCA2 and AGT co-immunoprecipitate in the BxPC3 cells before (lane 1) and after (lane 2) O^6BG treatment. Immunoprecipitation was performed with mouse anti-AGT antibody and then immunoblotted with rabbit anti-BRCA2 (top) and anti-AGT (bottom) antibodies. Lane 3 represents immunoprecipitation using mouse IgG, which serves as the negative control. Graph shows quantification of BRCA2 using AGT for normalization (lower panel). **B)** O^6BG-mediated degradation of AGT (top panels) and BRCA2 (middle panels) was examined by Western blot analysis in BXPC3 (left panel) and Capan-1 cells (right panel). βactin (bottom) served as loading control. **C)** O^6BG-mediated degradation of AGT (top panels) in wild-type (*Brca2*+/+) and BRCA2-deficient (*Brca2Ko*/*Ko*) ES cells was examined by Western blot, βactin (bottom) served as loading control.

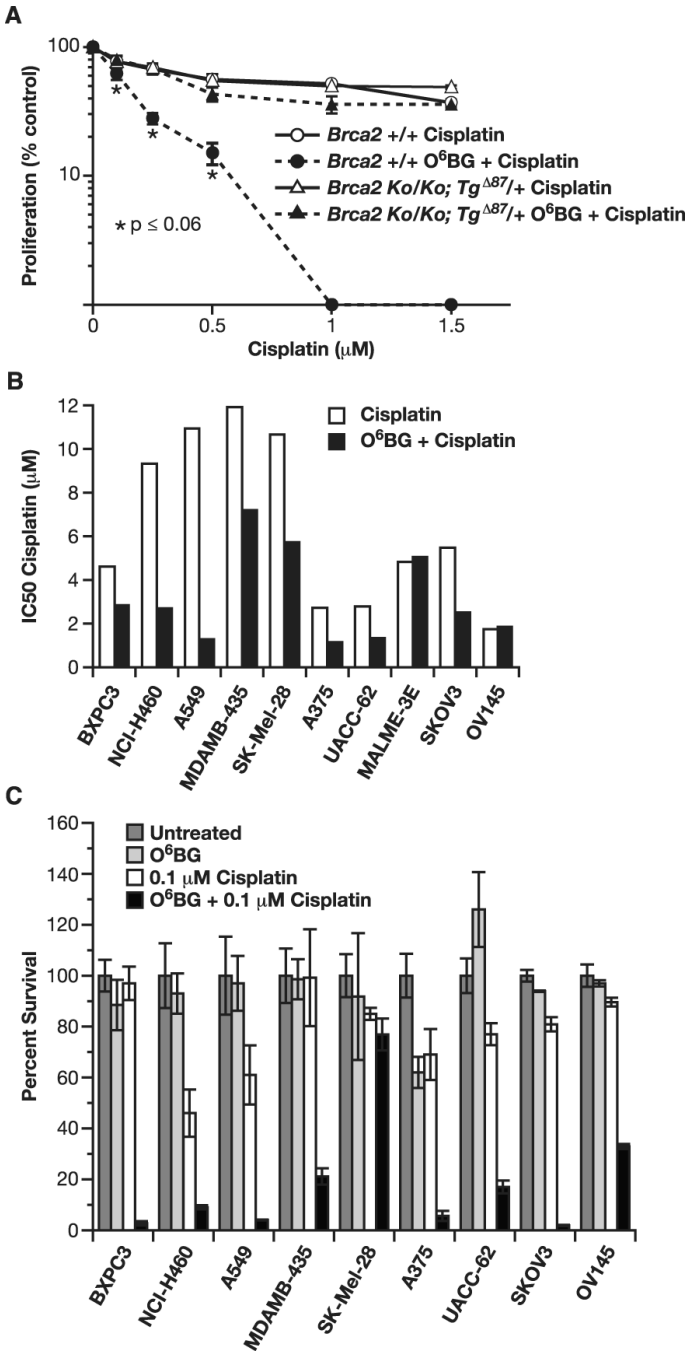

Figure 6. Degradation of BRCA2 by O^{6}BG can be used to sensitize human tumor cell lines to cisplatin

A) O^{6}BG pretreatment sensitizes *Brca2*+/+ (circle) but not *Brca2Ko*/*Ko*; *Tg*$^{\Delta 87}$/+ (triangle) MEFs to cytotoxicity of Cisplatin. Untreated (open circle, open triangles) or cells pretreated with O^{6}BG (100 M) (closed circles, closed triangles) for 16 hours were exposed to varying concentrations of cisplatin (0-5 μM) in presence or absence of 50 μM O^{6}BG. After 48 hours cells were counted. Proliferation in untreated cultures for each group was taken as 100% and data is represented as percentage of untreated controls. Error bars represent standard deviation. **B)** O^{6}BG pretreatment enhances cisplatin cytotoxicity in established tumor cell lines, semi-confluent cultures of various tumor cell lines in 96 well plates were either pretreated with

O^6BG (100 μM) for 16 hours and then treated with varying concentration of cisplatin (0-10 μM) in presence of 50 μM O^6BG (closed bars) or treated with cisplatin (0-10 μM) alone (open bars). After 48 hours cell proliferation was measured using alamar blue. Cisplatin IC_{50} values for each cell line in presence and absence of O^6BG was calculated and represented as a bar graph. O^6BG enhanced sensitivity to cisplatin in 8 out of the 10 cell lines tested. **C)** Bar graph showing survival of multiple human tumor cell lines by clonogenic assay in response to 0.1μM cisplatin with and without 100μM O^6BG pretreatment. MALME-3E cell line could not be tested because it failed to form colonies under conditions used in this assay.