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13. ABSTRACT (Maximum 200 Words)

We are studying the radiation response of prostate tissues in relation to the sensing and repair of DNA breaks. Specific aims relate to determining the interaction of DNA repair proteins in vitro using immunoflorescent confocal microscopy and biochemical DNA rejoining assays under both hypoxic and oxic conditions (given in vivo tumour cell populations). An in vivo program of prostate xenograft radioresponse and patient biopsy studies will determine the level of DNA repair *in situ* using immunohistochemistry and immunoflorescent markers. Our studies show that DNA repair protein expression is abnormal in malignant versus normal prostate epithelial cultures, and that particularly the Rad51 protein is defective in localizing to the nucleus following DNA damage. We have accrued 13 patients onto a pre-operative radiotherapy trial and post-irradiation immunohistochemistry supports an induction of p53-pathway signaling following 25Gy in 5 fractions. Current experiments are designed to determine whether DNA protein focal interactions using 2-photon microscopy can predict the radioresponse of prostate xenografts and human tumors, in vivo. Our studies support the use of novel molecular based therapies that target DNA repair for prostate cancer therapy.

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Introduction:

The current funded study entitled "Molecular mechanisms of radioresistance in prostate cancer", is investigating the role of DNA break repair in the radiation response of normal and malignant prostate epithelium. The overall hypothesis of this project is that the radiation response of normal and cancerous prostate tissues can be correlated to the appropriate sensing and repair of DNA breaks by repair complexes following exposure to ionizing radiation. Specific aims relate to determining the interaction of DNA repair proteins in vitro using immunoflorescent confocal microscopy and biochemical DNA rejoining assays under both hypoxic and oxic conditions (given in vivo tumour cell populations). An in vivo program of prostate xenograft radioresponse is also being initiated to determine the level of DNA repair *in situ* using immunohistochemistry and immunoflorescent markers. These initial studies will determine the heterogeneity in fractionated response in a series of prostate xenografts as relates to DNA repair capacity, which may be translated to novel markers for radiation response in patients who receive prostate radiation therapy. The relevance of this project is that this *in vitro* to *in vivo* pre-clinical approach may derive clinical biomarkers of radiation response which can predict which patients will most benefit from radiation therapy for prostate cancer. The project will also determine the molecular mechanisms behind radiation response, in general, in prostate epithelial tissues.

Body:

Task 1: Initally, the first task was to primarily complete in vitro studies on PC-3; LNCaP, DU145 and normal prostate (PRSE and PREC) cells relating to senescent populations which was quantitated and found to be dose responsive and correlated to their clonogenic survival (using a novel fluorescent flow cytometric proliferation assay). This work has been published in ***Prostate Cancer and Prostate Diseases (see Appendix)***. **THIS SUB-TASK IS NOW COMPLETE as outlined in last year's report.**

Task one projects were also completed for all 5 cell lines in determining the ability for the cells to repair double strand breaks, single strand breaks and DNA base damage (and oxidative damage) following ionizing radiation using the comet assay. We observed a series of novel observations which currently suggests that malignant prostate cancer cells have a DNA repair defect in the repair of DNA-dsbs, DNA-ssbs and DNA-base damage and oxidative damage in relation to the two normal prostate epithelial and stromal cell cultures. Primary prostatic cultures were initiated as prostatectomy specimen cultures available through Coriell Laboratories. We were successful in culturing both the prostate stromal and the prostate epithelial cell cultures in vitro to be utilized in a radiobiology and DNA repair experiments. DNA double stand break repair complexes have been determined in these cultures and are part of the data set contained in the manuscript in Appendix 1. **This has not been reported before. THIS SUB-TASK IS NOW COMPLETE and a manuscript has been conditionally accepted to Cancer Research (see Appendix I).**
We have therefore completed all endpoints for Task 1.

Task 2: Complete in vivo radiobiologic studies on human prostate xenografts (months 12-24).

We have determined that there are cell cycle phase specific changes in Rad51 or H2AX foci formation in relation to DNA-dsb rejoining, by irradiating cells under both asynchronous and G0-G1 synchronized conditions. Counterstaining with PCNA and CENP-F has determined that foci are specifically forming in a cell-cycle phase specific manner in malignant prostate cells.

Single dose (2 and 10Gy) and fractionated experiments (5 x 2 Gy) have been completed for the PC-3 and DU-145 xenografts. We have now hired a neew technician to re-develop the Rotterdam xenograft program. All current xenografts were removed and stained for immunohistochemical markers pertaining to p53, apoptosis related genes (bax, BCL2 and TUNEL assay as well as survivin) and DNA repair markers (RAD50, BRCA1, BRCA2, RAD50, $DNAPK_{cs}$, KU70, KU80, ATM, P21, RB, MYC and RPA). The xenograft histology was also stained for proliferation markers such as MIB-1, KI-67 and PCNA. Our results continue to suggest that RAD51, and H2AX, but not Ku70, increase in expression both in terms of the nuclear intensity of staining as well as a number of cells positively staining for protein following 10 Gy single dose or 5 x 2 Gy fractioned irradiation *in vivo* at 1-24 hours. The Rad51 data is consistent with cells moving into the G2 phase of the cell cycle during an irradiation-induced checkpoint. **This has not been reported in the literature before.** We have collaborated with Dr. Peter Glazer's laboratory at Yale University to show that Rad51 expression is decreased under hypoxia conditions in vitro. We have confirmed these findings in vivo as Rad51 staining in hypoxic areas (as determined with EF-5 and CA-IX) is decreased, suggesting an inverse relationship between HR-related DNA repair proteins and hypoxia. This may explain in part hypoxia-mediated genetic instability. **THIS SUB-TASK IS COMPLETE. A manuscript has been submitted to Molecular and Cell Biology regarding these findings** (Ranjit S. Bindra, Paul J. Schaffer, Alice Meng, Jennifer Woo, Kårstein Måseide, Matt E. Roth, Paul Lizardi, David W. Hedley, Robert G. Bristow, and Peter M. Glazer. ***Down-Regulation of Rad51 and Decreased Homologous Recombination in Hypoxic Cancer Cells.*** *Submitted).* We continue to attempting to utilize 2-photon microscopy for better resolution of the innate foci with indirect immunofluorescence.

We have increased the total number of patients accrued to the preoperative radiotherapy study from 6 to 13. Accrual will be complete at 15 patients and this sub-task is complete. We have sequenced all patients for their p53 gene status for all 11 exons and all patients are wild-type for p53. Immunohistochemistry for p53, p21, TUNNEL, Bax, Bcl-2, BRCA1/2/Rad51/DNA-PKcs/KU70/KU80 and PCNA is completed. We have quantitated the staining pre and post-radiotherapy using the Image Pro Plus analysis program which shows that ATM and p53 are phosphorylated following fractionated radiotherapy in patients. We are currently writing up this set of data for a manuscript. **THIS SUB-TASK IS COMPLETE and a manuscript is in preparation.**

Unfortunately, due to a contamination of our animals from our supplier with *Pseudomonas Aeruginosa,* our entire colony of mice (greater than 70 animals) bearing Rotterdam-xenografts had to be sacrificed before we could complete in vivo fractionation experiments in 2003-2003. We have hired a new technician (Helen Zhao; October 1, 2004) who will be regrowing the prostate xenograft facility to encompass fractionated and hypoxia experiments in vivo.**THIS SUB-TASK IS INCOMPLETE but is ongoing.**

Task 3: (Months 18-36)

Task 3 is to primarily complete the clinical studies undergoing radiotherapy, and to determine DNA repair foci in human clinical biopsies pre and post radiotherapy in phase 3 and Phase 1 trials. Preliminary experiments suggest heterogenous Rad51, BRCA2 and BRCA1 levels in tissue arrays containing normal, PIN and malignant prostate cancer specimens. **As stated above the Phase I trial is complete.**

Once the xenograft program is rebuilt we will continue our xenograft studies as it pertains to fractionated and single dose related growth delay experiments and DNA repair-associated markers. Some components of Task 3 will run long-term and parallel as patient biopsies are accrued for the assays.

KEY RESEACH ACCOMPLISHMENTS for YEAR 3:

1. DNA repair complexes can be visualized using confocal microscopy and the appearance and disappearance of these foci correlate to the kinetics of DNA biochemical rejoining assays (ie. Comet assay).
2. Malignant prostate epithelium may have an effect has an inherent DNA defect in terms of DNA double strand break repair, single strand break repair and base damage repair. This is correlated to increased expression of proteins involved in each of these pathways (APE/REF1. RAD51, BRCA2, PARP, etc.). In the case of Rad51, altered expression is discordant from altered function and we believe that there is an intracellular trafficking defect in Rad51 related to post-translational modification.
3. Continued accrual to the phase I pilot study of preoperative radiotherapy and Phase III neoadjuvant prostate radiotherapy trails. Immunohistochemical markers for DNA repair and apoptosis related proteins has been completed. ATM/p53 activation occurs in vivo with fractionated treatments.

Reportable Outcomes:

Manuscripts:

1. **Bristow R** on behalf of the CARO Task Force Members: "Recommendations for the future of translational radiobiology research: A Canadian perspective". Radiotherapy and Oncology 70(2); 159-164, 2004. **(Principal Author)**

2. Parker C, Milosevic M, Toi A, Sweet J, Panzarella T, **Bristow R**, Catton C, Catton P, Crook J, Gospodarowicz M, McLean M, Warde P, Hill R: Polarographic electrode study of tumor clinically localized prostate cancer. Int J. Radiation Oncology Biol Phys. 58(3) 750-757, 2004. **(Collaborator)**

3. Bayley AJ, Catton CN, Haycocks T, Kelly V, Alasti H, **Bristow R**, Catton P, Crook J, Gospodarowicz MK, McLean M, Milosevic M, Warde P: A Randomized Trial of Supine vs. Prone Positioning in Patients Undergoing Escalated Dose Conformal Radiotherapy for Prostate Cancer. Radiotherapy and Oncology 70(1) 37-44, 2004. **(Collaborator)**

4. Cuddihy A and **Bristow RG.** The p53 Protein Family and Radiosensitivity: Yes or No? Cancer Metastasis Reviews; 23(3-4) 237-257; 2004. **(Senior Responsible Author)**

5. Finlay M, Laperriere N, **Bristow R.** Radiotherapy and Marfan Syndrome: A Report of Two Cases. Clinical Oncology, *In Press,* August 2004. **(Senior Responsible Author)**

6. Catton C, Milosevic M, Warde P, Bayley A, Crook J, **Bristow R,** Gospodarowicz M: Recurrent prostate cancer following external beam radiotherapy: follow-up strategies and management. Urol Clin North Am. 2003 Nov; 30(4): 751-63. **(Collaborator)**

Book Chapters:

1. **Bristow RG** and Harrington L. Genetic Instability and DNA Repair; Chapter 5 in Basic Science of Oncology, 4th Edition (Eds Tannock IF, Hill RP, Harrington L and Bristow RG); *In Press*, 2004. **(Principal Author)**

2. **Bristow RG** and Hill RP. Molecular and Cellular Radiobiology; Chapter 14 in Basic Science of Oncology, 4th Edition (Eds Tannock IF, Hill RP, Harrington L and Bristow RG); *In Press*, 2004**(Principal Author)**

3. **Bristow RG** and Hill RP. The Scientific Basis of Clinical Radiotherapy; Chapter 15 in Basic Science of Oncology, 4th Edition (Eds Tannock IF, Hill RP, Harrington L and Bristow RG); *In Press*, 2004**(Collaborator)**

Submitted Manuscripts

1. Bindra RS, Schaffer P, Meng A, Woo J, Maseide K, Roth ME, Lizardi P, Hedley DW, **Bristow RG**, Glazer P. Down-Regulation of Rad51 and Decreased Homologous Recombination in Hypoxic Cancer Cells. Molecular and Cellular Biology, Revised Version *Submitted*, July, 2004. **(Primary Collaborator)**

2. Fan R, Kumaravel TS, Jalali F, Bayani J, Squire J and **Bristow RG.** Defective DNA Strand Break Repair Following DNA Damage in Prostate Cancer Cells: Implications for Genetic Instability and Prostate Carcinogenesis. Cancer Research, Revised Version Conditionally Accepted, June 2004 **(Senior Responsible Author)**

3. Nichol A, Warde P, and Bristow RG. Intermediate Risk Prostate Cancer and Radical Radiotherapy: clinical and translational Issues. Submitted to CANCER, August 2004. **(Senior Responsible Author)**

4. Wiltshire K, **Bristow RG**, Warde P, Gospodarowicz M. Letter to Editor, Radiotherapy and Oncology; Re. Vikram, Radiotherapy and Oncology, *Submitted* August 2004 **(Senior Responsible Author)**

Manuscripts in preparation

1. Coleman A, Smith K, Trachtenberg J, Ozelich, Narod S and **Bristow RG.** BRCA2 mutations, DNA Repair and Prostate Cancer: Implications for Local and Systemic Management (Review). **(Senior Responsible Author)**

Abstracts and Presentations:

Meng A, Cole H, Syed A, Billings S, Chung P, Sweet J, Milosevic M, Hedley D, Woo J, Maseide K, Hill RP, **Bristow RG**. Hypoxia-Induces Gene Expression within Prostate Cancer: In Vitro and In Vivo Studies. CARO, Annual Scientific Meeting 2004. **(Supervisor; Senior Responsible Author)**

Tabassum A, Venkateshwaran V, Klotz L, Fleshner NE, **Bristow RG**. Selenium: A Beneficial or Harmful Antioxidant in Adjunct to Prostate Radiotherapy? CARO, 2004. **(Supervisor; Senior Responsible Author)**

Coleman A, Jonkman J, **Bristow R**. DNA-dsb Repair *In Situ* In Normal and Malignant Prostate Cells. CARO Annual Scientific Meeting, 2004. **(Supervisor; Senior Responsible Author)**

Faulhaber O, Blin N, Bristow R. HDAC Inhibitors and Prostate Cancer Radiosensitivity. CARO Annual Scientific Meeting, 2004. **(Supervisor; Senior Responsible Author)**

Nichol A, Jaffray D, Catton C, Haycocks T, Lockwood G, Milosevic M, Bayley A, McLean M, **Bristow R**, Crook J, Gospodarowicz M, Warde P. "A Cohort Study using Cinematic Magnetic Resonance Imaging of a Bowel Regimen to Reduce Intra-fraction Prostate Motion". ASTRO Annual Scientific Meeting, 2004. **(Collaborator)**

Bristow R, Al-Rashid S. "Radiation-Induced Phosphoforms of p53 as Biomarkers of DNA-dsbs In Situ". ASTRO Annual Scientific Meeting, 2004. **(Principal Author)**

Other reportable outcomes:

There is continued development of the tissue bank relating to irradiated prostate specimens as it relates to the phase I and phase III clinical trials; the latter has accumulated > 200 biopsies of patients having undergone radical radiotherapy at PMH-UHN. Our REBs has been re-approved for all human studies. Funding CONTINUES FROM the Ontario Cancer Research Network (OCRN) to test anti-sense to Rad51 as an experimental prostate therapy, based on our Rad51-overexpression data from this US Army grant. We were successful and received a grant totaling more than $ 420,000 CAN for pre-clinical studies based on the data derived from our US Army grant.

The prostate related studies also led TWO NEW GRANTS:

(1) Canadian Cancer Society Research Scientist Award to the PI (Bristow) worth more than $ 500,000 over 6 years is salary support and
(2) a new NCIC Operating grant on hypoxia and DNA repair worth > $ 500,000 over 5 years to PI(Bristow).

Conclusions:

We have gained excellent momentum regarding the importance of DNA-dsb repair as an important endpoint in the radiation response of prostate cancer and potentially, prostate carcinogenesis. A number of manuscripts have been accepted, submitted or are in press. We have also leveraged the original US Army grant to more than 1.5 million Canadian dollars in external funding.

Out studies suggest that there are defects in DNA repair relating to intracellular trafficking or chaperoning of DNA repair factors to the nucleus. This is a novel concept and could give rise to new

treatments targeting nuclear import and export of proteins in prostate cancer. Other DNA repair pathways amenable to study and targeting are the DNA-ddb and base excision repair (BER) pathways which also are abnormal in prostate cancer cells. Our data with human biopsies pre and post-clinical radiotherapy also supports the quantification of DNA damage signaling pathways and repair factors as potential determinants of radioresponse.

Defective DNA Strand Break Repair Following DNA Damage in Prostate Cancer Cells: Implications for Genetic Instability and Prostate Carcinogenesis[1]

Rong Fan[2], Tirukalikundram S Kumaravel[2,3], Farid Jalali, Paula Marrano, Jeremy A. Squire and Robert G Bristow[4 1]

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(University Health Network; *R.F., T.S.K., F.J., P.M., J.A.S., R.G.B.*) and
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Running Title: *DNA Repair and Prostate Cancer*

Key Words: homologous recombination, chromosomal aberrations, comet assay

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[1]Supported by grants from the US Army DOD Prostate Program (DAMD17-01-1-0110 (A-10308)), Canadian Foundation for Innovation and the G.H. Woods and PMH Foundations

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[5]Abbreviations: DNA-dsb, DNA double-strand break, DNA-ssb, DNA single strand break; DNA-PK, DNA protein kinase; ATM, ataxia telangiectasia mutated; HR, homologous recombination; NHEJ, non-homologous recombination/end-joining; BER, base excision repair; MRN, Mre11/Rad50/Nbs1 complex; IR; ionizing radiation; MMC, mitomycin C.

ABSTRACT

Together with cell cycle checkpoint control, DNA repair plays a pivotal role in protecting the genome from endogenous and exogenous DNA damage. Although increased genetic instability has been associated with malignant prostate cancer progression, the relative role of DNA-dsb repair during prostate carcinogenesis is not known. In this study, we determined the RNA and protein expression of a series of DNA-dsb genes in both normal (PREC-epithelial and PRSC-stromal) and malignant (LNCAP, DU-145 and PC-3) prostate cultures. Expression of genes downstream to ATM following IR-induced DNA damage reflected the p53 status of the cell lines. In the three malignant prostate cell lines, the mRNA and protein levels of the HR-related Rad51, XRCC3, Rad52, Rad54 genes were elevated ~2-5 fold in comparison to normal PrEC cells. In contrast, there were no consistent differences in gene expression relating to NHEJ repair. In addition, the XRCC1, DNA polymerase–beta and -delta proteins were also elevated. Despite increased expression of DNA repair genes, malignant prostate cancer cells had defective repair of DNA-dsbs, DNA-ssbs, alkali-labile sites and excision of oxidative base damage based on the comet assay. Furthermore, following IR- and MMC-induced DNA damage, chromosomal aberration assays confirmed that malignant prostate cells had defective DNA-dsb repair. This discordance between DNA repair gene expression and function in malignant prostate cancer cells supports the hypothesis that the process of prostate carcinogenesis may reflect aberrant DNA repair. Novel chemoprevention strategies designed to re-instate normal DNA repair may therefore be useful in high-risk cohorts.

INTRODUCTION

The genetic determinants of prostate carcinogenesis are still poorly understood (1). However, numerous models of prostate carcinogenesis suggest that increasing chromosomal instability and the acquisition of mutations and chromosomal aberrations drives progression from pre-neoplasia to neoplasia (2). In support of these models, increased levels of chromosomal aberrations can be associated with decreased telomere length in high-grade prostatic intraepithelial neoplasia (PIN); a precursor to prostate cancer (3). Human cells have therefore evolved complex signaling responses to both endogenous and exogenous DNA damage in order to preserve genomic integrity. Tumor progression in a number of epithelial malignancies has been associated with the sequential loss of function of genes involved in cell cycle checkpoint control, DNA repair and cell death; all processes which protect against DNA damage. For example, in response to DNA-double strand breaks (DNA-dsbs), the ATM (ataxia telangiectasia mutated) protein is activated and stabilizes the p53 tumor suppressor gene. This leads to the upregulation of p53-dependent genes (e.g. p21WAF, Bax, Gadd45) and post-translational modifications of CHK2, BRCA1 and NBS1 all acting together to induce G1, S and G2 cell cycle arrests, DNA repair or activation of cell death pathways (apoptosis, mitotic catastrophe or terminal growth arrest), depending on the cellular context (4-6).

Defective DNA repair as a risk factor for prostate cancer has not been extensively studied. Several groups have shown mismatch repair defects in prostate cancer cell lines (7, 8) and recent data also suggests that polymorphisms in selected DNA repair enzymes (i.e. XRCC1, OGG1, DNA polymerase-beta) which are involved in base excision repair (BER) or DNA-ssb repair may be a risk factor for prostate cancer (7, 9, 10). However, functional data is lacking concerning the homologous recombination (HR) and non-homologous recombination (ie. end-joining/NHEJ) pathways, which

involved in the repair of DNA-dsbs. NHEJ repair requires little or no homology on the ends of the strands being joined and involves two main discrete repair protein complexes: (1) the DNA-PK/XRCC4/LigIV complex and (2), the MRE11/RAD50 complex (11). In homologous recombination, extensive homology is required between the region of the DNA-dsb, and the sister chromatid or homologous chromosome from which repair is directed. HR involves the BRCA2, Rad51, Rad52, Rad54, Rad55-57 and RPA proteins and the Rad51 paralogs: XRCC2/3 and Rad51B/C/D; a separate HR pathway involves single-strand annealing(SSA). The HR pathway predominates in the late S/G2 phases of the cell cycle and provides relatively error-free repair. In contrast, the NHEJ pathway predominates in the G1 phase of the cell cycle (12). However, recent data suggests an interplay between the two pathways which may be dependent on cell type and early versus late times following induction of DNA-dsbs (11). Cells defective for either HR or NHEJ proteins show increased rates of mutagenesis and chromosomal instability, which could relate to the propensity for acquired genetic instability during prostate carcinogenesis. Furthermore, there is evidence that BRCA1 and BRCA2 mutations may increase the risk of prostate carcinogenesis (2, 12-14).

Our laboratory has previously shown that normal and malignant prostate cells preferentially respond to DNA damage by undergoing growth arrest rather than apoptosis (15). This may allow for attempted DNA repair by normal cells during the cell cycle arrests following DNA damage. However, in malignant cells with aberrant cell cycle checkpoint control, defective DNA-dsb repair could increase DNA mutation and lead to genetic instability. We therefore hypothesized that one of the critical steps in prostate carcinogenesis may be the loss of the normal response to DNA damage and that specific defects in DNA-dsb repair could further drive tumor progression. Herein, we show that malignant prostate cancer cells have increased expression of HR-related and BER-related genes independent of p53 status, G1 cell cycle checkpoint control and relative cell proliferation. However, despite expressing high levels of DNA repair proteins, malignant cells have a decreased capacity for DNA-dsb, DNA-ssb

and base excision repair and acquire chromosomal aberrations which reflect a DNA-dsb repair defect following exposure to DNA damaging agents. Our findings support inappropriate DNA repair as a potential determinant of prostate carcinogenesis.

METHODS AND MATERIALS

Prostate Cell Cultures and DNA-damaging Treatments. All cell cultures were incubated in vented tissue culture flasks under 5% CO_2 and 37°C culture conditions as previously described (15). LNCaP cells were maintained in T-media (Gibco-BRL) and supplemented with 10% FCS. PC-3 and DU-145 cells were purchased from ATCC and maintained as suggested in Ham's F12K, and alpha-Modified Eagles Medium respectively, supplemented with 10% FCS. PrEC (normal prostate epithelial cells) and PrSC (normal prostate stromal cells) were purchased from Clonetics and maintained using suggested PrEGM and SCGMmedia, respectively (Clonetics Inc, San Diego, CA). The latter cell cultures have limited lifespan and proliferative potential in culture and decrease in proliferation following passage 5-8 from frozen stock. Approximate doubling times for cell cultures under these conditions were as follows: PrEC: 48-72 hours, PrSC: 18 hours, LNCaP: 36 hours, PC-3: 24 hours and DU-145: 18 hours.

Asynchronous cultures were irradiated, or treated with MMC, at 16 to 20 hours post-plating to reduce the immediate effects of trypsinization. Cells were either mock irradiated or irradiated with 0 to 10Gy under aerobic conditions using a ^{137}Cs irradiator at ~ 1Gy/min at room temperature. MMC was prepared as a stock solution of 0.5 mg/ml in distilled water prior to each use.

Quantification of Gene Expression by Ribonuclease Protection Assays (RPA). Asynchronously growing cells were harvested at 70-80% growth confluence from either non-irradiated cultures or 0-24

hours following irradiation. RPAs were carried out as per manufacturer's instructions (Pharmingen Inc.). Total RNA was extracted using Trizol reagent (Sigma Inc.). Antisense riboprobes (RAD50, MRE11, RAD52, RAD54, RAD51, XRCC2, XRCC3, RAD51B, RAD51C, RAD51D, L32 and GAPDH from DBSR1 set; ATM, NBS1, XRCC2, XRCC3, XRCC9, Ligase IV, XRCC4, Ku70, DNA-PK, Ku86, L32, GAPDH from DBSR2 set; Bcl-x, p53, Gadd45, c-fos, p21, Bax, Bcl-2, Mcl-1, L32, GAPDH from HSTRESS set; all sets from PharMingen Inc.) were synthesized with the RoboQuant multi-probe template sets and purified by MAXIscript (Ambion Inc.). 5ug of sample RNA was hybridized with 2000cpm of the synthesized multi-riboprobe in a single tube with 10 ml hybridization buffer, digested with RNase and the resulting RNA pellet centrifuged before re-suspension in 4ul loading buffer prior to acrylamide electrophoresis (1200-1500V for 1-2hr). The sample gel was mounted on a 3MM paper and dried at 80C° for 1hr. The cpm value of the individual band on the gel was counted by Phosphor Imager (Storm 840, Molecular Dynamic Co.). The statement level of the GAPDH gene was used as an internal control for relative statement levels of other individual genes calculated as a ratio (i.e. the observed total volume for the target gene divided by the volume of GAPDH). Three different total RNA extracts were analyzed in triplicate for each cell line. The results of the three separate experiments are expressed as the mean value +/- 1 standard error of the mean (S.E.).

Western blot Analyses for Relative Expression of HR, NHEJ and BER Proteins. Logarithmically-growing cells were either mock-irradiated or irradiated and then lysed on ice for 20 minutes with E7 lysis buffer (PBS, 0.1%SDS, containing protease inhibitors; Roche Molecular Biochemicals. Indianapolis, IN) to prepare lysates for western blotting as previously described (15). SDS-PAGE was performed using 7-12% bis-acrylamide (29:1) gels with a 4% stacking gel run in a Novex X-cell semi-dry Mini Cell western blotting apparatus at room temperature. Each well was loaded with 20*u*g of total protein plus loading buffer (final concentration 1x-6% glycerol, 0.83% b-mercaptoethanol, 1.71% Tris-

HCl pH 6.8, 0.002% Bromophenol Blue) after boiling for 3 minutes. Samples were resolved by electrophoresis at 80-110 Volts for 1.5-2.5 hrs were transferred onto nitrocellulose (Schleicher&Schuell, Inc.) and pre-hybridization staining with Ponceau S confirmed equal loading and transfer between running lanes. Post-transfer, membranes were rinsed with TBST and incubated in appropriate secondary antibodies and immersed in a chemi-luminescence solution before exposure to film (Amersham Pharmacia). Relative expression was determined by densitometry (Molecular Dynamics Computing Densitometer, ImageQuant Mac v.1.2) and equal lane loading was confirmed based on relative α–tubulin levels. Primary antibodies were used at dilutions ranging from 1:200 to 1:1000 as suggested by the supplier and included: Rad51, RPA, α–tubulin (Oncogene Research Products, Inc.), Rad50, Nbs1(p95), XRCC3, Rad51C, Rad51D (Novus Biologicals, Inc), Rad52, Rad54, Ku86, DNA polymerase-beta, APE/REF1 (Santa Cruz Biotechnology Inc.), p21 (1:500; OP64; Ab-1; Oncogene Research Products, Inc.), Ku70 (1:500; SC-1486; Santa Cruz), XRCC1(Abcam), PARP (Biomol), DNApolymerase-delta (BD Biosciences), Mre11 (Genetex) and phospho-H3 (Upstate Technologies). Relative protein expression amongst PrSc, LNCaP, DU-145 and PC-3 cells was normalized to PrEC expression (e.g. arbitrary relative value of one) and western blots shown are representative of 2 independent experiments.

Single-Cell Gel Electrophoresis (Comet Assay) to Detect DNA Strand Breaks and Base Damage. Repair of DNA-dsbs, DNA-ssbs, DNA base damage and repair of FPG-sensitive sites was quantitated using the Comet assay following irradiation as previously described (16, 17) Kumaravel: www.cometassay.com). For the neutral comet assay, 10 and 25 Gy doses were used; for the alkaline comet and FPG-comet assays, a dose of 6 Gy was used. For the determination of DNA-dsbs by the neutral comet assay, approximately 10^5 cells were mixed with 100ul of 0.7 % LMA at 45°C and spread

on regular glass microscope slides precoated with 1% normal melting agarose. After gelling, the slides were treated with lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Trizma base, 10% DMSO and 1% Triton X-100, pH 9) at pH 9.0 overnight. The slides were placed in 1x TBE and electrophoresed for 15 minutes at 32V (current ~25mA). After electrophoresis the slides were dried and stored until scoring. The slides were stained with ethidium bromide (2 ng/ml) and comets scored under a Zeiss fluorescence microscope (with TRITC filters) coupled to KOMET 5.0 software (Kinetic Imaging Ltd). The alkaline comet assay detects alkali-labile sites in the DNA which is a global assessment of DNA-ssbs, DNA-dsbs and DNA base damage. In brief, cells were suspended in 0.5% low-melting point agarose (LMA; Sigma) and spread on glass microscope slides precoated with 1% normal agarose. After immersion in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Trizma base, 10% DMSO and 1% Triton X-100) at 4°C for a minimum period of 1 h to remove cellular proteins, the slides were immersed in electrophoresis buffer [300 mM NaOH, 1 mM EDTA (pH > 13)] for unwinding DNA, and subjected to electrophoresis (25 V; 300 mA) for 20 min. Neutralized, dehydrated slides were stained with ethidium bromide (2 ng/ml) before scoring.

Finally, repair of sites of DNA base modification was scored by treating the DNA with a lesion-specific glycosylase (FPG; Formamidopyrimidine-DNA Glycosylase). This enzyme recognizes oxidative damage manifest as DNA base modifications including 8 oxo-dG (8-oxo-7,8-dihydro-2'-deoxyguanosine), 7-methyl-guanine, 5-OH-cytosine and 5-OH-uracil Cells were suspended in 0.5% low-melting point agarose (LMA; Sigma) and spread on glass microscope slides precoated with 1% normal agarose. After immersion in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Trizma base, 10% DMSO and 1% Triton X-100) at 4°C for a minimum period of 1 h, to remove cellular proteins. After lysis, the slides were equilibrated with enzyme reaction buffer (HEPES 9.5g; KCl 7.5 g; EDTA 0.5mM; in 1 lit; and BSA fraction V added 0.2 per ml, pH 8.0) for 10 minutes 2 times on ice. The slides were treated with FPG enzymes (obtained from Dr. Karel Angelis, Institute of Experimental

Botany, Praha, Czech Republic; 1:100 dilution in enzyme reaction buffer) and placed in 37°C for 30 minutes in a moist box. The enzyme treatment was terminated by dipping the slides in denaturing solution (0.3 M NaOH, 5mM EDTA, pH 12.1) for 20 minutes, followed by neutralization in 1 X TBE for 5 minutes and finally electrophoresis. The ratio of DNA migration between the enzyme treated and buffer control slides gives an estimate of the FPG sensitive sites in the sample

For all comet assays, the comet parameter, "Olive Tail Moment" or "OTM" (i.e. % DNA x distance of center of gravity of DNA) was used as the indicator of DNA damage (17). One hundred consecutive cells were scored at random from the middle of each slide for 2-3 independent experiments and the final result expressed as the (mean of the median OTM values) ± SE of the medians). For graphical purposes in the FPG assay, the difference between the OTM for FPG–treated slides versus the OTM of same population treated with buffer alone were plotted as the final endpoint of FPG-sensitive sites. Statistical differences in OTM or gene expression profiles between cell lines was determined using the non-parametric Mann-Whitney analysis.

Chromosomal Aberration Assays. Frequencies of spontaneous, IR-induced (4Gy) or MMC-induced (40 ng/ml for 24 hours) chromosomal aberrations were determined in exponentially growing cell cultures of PRSC, LNCAP, DU145 and PC3. The cells were harvested by trypsinization 24hrs after treatment and incubation with 1 μg of Colcemid/ml for 2 hours to collect metaphase spreads for analysis. The cells were fixed, after treatment with hypotonic solution (0.6% sodium citrate), in ethanol-glacial acetic acid (3:1). Air-dried preparations were made and slides were stained DAPI/antifade mixture (Vectashield Burlingame, Inc.). For chromosomal aberrations, 25 mitotic cells were analyzed for each treatment per cell line.

Immunohistochemistry (IHC) of Prostate Xenografts. For xenograft studies, 1 x 10^6 PC3 or DU-145 cells were injected i.m. into the gastronemius muscle of Balbc/nu mice. Tumors were mock-irradiated or irradiated at a weight of 0.5 gms with 20 Gy using a 250KvP X-ray unit without anaesthesia. For irradiation, the mouse was lightly restrained in a lucite holder box with lead shielding, such that only the tumor-bearing hind leg was within the irradiation volume. Tumors were excised from mock-irradiated and irradiated and placed immediately in formalin for subsequent fixation and immunostaining using primary Rad51 (Ab-1, Oncogene Research Products) and secondary horseradish peroxidase (HRP) antibodies for immunodetection. All studies were in ethical compliance with the PMH-UHN Animal Care Committee.

RESULTS

Gene Expression Relating to ATM-p53 DNA Damage Pathways In Prostate Cell Cultures. To initially test the utility of ribonuclease protection assays (RPA) in quantifying gene expression in our panel of 2 normal (PrEC-epithelial and PrSC-stromal) and 3 malignant (LNCAP, PC3 and DU-145; all epithelial) prostate cell lines, we first quantitated ionizing radiation (IR)-induction of genes associated with the ATM-p53 DNA damage signaling cascade. We have previously reported (15) the apoptotic and p21WAF responses in these cell cultures. All 5 cell cultures undergo minimal and non-differential apoptosis (<5%) after doses of up to 20 Gy (measured 12-96 hours post-IR); yet the three malignant cell lines have the highest clonogenic survival (i.e. mean SF2Gy values ranging from 0.4-0.55 relative to the values for PrSC and PrEC, at 0.25 and 0.1, respectively). PrSC, PrEC and LNCaP cells express two wild type p53 alleles and have an intact G1 cell cycle arrest checkpoint with increased $p21^{WAF}$ protein expression following irradiation. PC-3 cells were devoid of p53 protein expression due to chromosome 17p hemizygosity and a mutation in the remaining allele at codon 138 that results in a

premature stop codon at position 169. DU-145 cells express high levels of trans-dominant, mutant (MT) p53 proteins due to missense mutations at codons 223 and 274. Both these latter cell lines lack a G1 checkpoint and do not upregulate p21WAF following DNA damage(15).

Using the RPA analyses at 4 hours following 10Gy, we observed increased expression of p21WAF, Gadd45, Bax and Bcl-2 in the WTp53-expressing cells (ie. PrSC, PrEC and LNCaP), whereas similar increases in gene expression were not observed for the null-p53 and MTp53-expressing, PC3 and DU-145 cells (see Figure 1A/1B). The RPA assay also confirmed a previous observation that the relative upregulation of p21WAF in PrEC epithelial cells was attenuated in comparison to PrSC stromal cells (15). Neither ATM, nor p53, RNA was increased following irradiation consistent with post-translational modification as the basis for activation of these proteins(5). Furthermore, the level of PC3 (null-p53) p53 RNA was at background. These results confirmed previous p21WAF protein expression data from our laboratory for the same cell lines (15). We also confirmed a dose- and time-dependent (i.e. maximal induction at 4-6 hours post-IR) induction of p21WAF in the LNCaP cell line using RPA assays (Figure 1B insert and data not shown). We conclude that the RPA assay is a sensitive indicator of gene expression under the conditions of DNA damage and repair within the panel of normal and malignant prostate cell cultures.

Expression and Functional Assessment of DNA-dsb and DNA-ssb Repair Proteins in Prostate Cultures. The initial data relating to ATM/p53 stress response provided confidence in the use of RPA assays for quantification of gene expression relating to the non-homologous and homologous recombination pathways of DNA-dsb repair. We observed a significant and differential increase (~2-3 fold) in HR-complex associated genes (ie. Rad51, Rad54, XRCC3 and Rad52) in malignant prostate cell cultures at the RNA and protein levels, when compared to PrEC cells (see Figure 2; western bot shown supported in Suppl. Figure 1B). Gene expression relating to the MRN complex

(Mre11/Rad50/Nbs1-p95) was not differentially expressed. In contrast, we found little evidence for increased basal mRNA or protein expression of the NHEJ-related Ku70, Ku80, DNA-PKcs, XRCC4 or Ligase IV genes (see Figures 3A and Suppl. Figure 1A and 1C). We also observed elevated levels of DNA-ssb repair protein XRCC1 (but not PARP) in the malignant cultures (Figure 3A). In contrast to a previous report (18), we observed that HR and NHEJ expression in malignant prostate cell lines was invariant at both the RNA and protein levels following irradiation (Figure 2A; also supported by ratio of IR-induced : basal levels of RNA in Suppl. Figure 1A-lower panel and data not shown). We conclude that the endogenous expression of the HR-related Rad51, Rad52, Rad54, XRCC3 and XRCC1 genes are increased at RNA and/or protein levels in malignant prostate cell lines.

We next compared the panel of cell cultures for their relative ability to repair DNA-dsbs, DNA-ssbs and alkali labile sites using the comet assay under neutral and alkaline lysis conditions (Figure 3B and 3C). Despite similar amounts of initial DNA damage, under both neutral and alkaline lysis conditions, the malignant cultures had significantly decreased capacity in repairing IR-induced DNA damage. These data suggest that despite high levels of HR-related and XRCC1 proteins; malignant prostate cells are defective in the rejoining of DNA-dsbs and alkali-labile sites including DNA-ssbs.

Base Excision Repair in Malignant Prostate Cultures. As the alkaline comet assay also scores abasic sites, we used the FPG (formamidopyrimidine-DNA glycosylase) comet assay to directly determine whether the malignant cultures had relatively increased spontaneous and IR-induced levels of oxidative damage. Treating DNA with FPG unmasks non-repaired oxidative damage as 8-oxo-dG (8-oxo-7,8-dihydro-2'-deoxyguanosine), 7-methyl-guanine, 5-OH-cytosine and 5-OH-uracil DNA lesions (17). The results shown in Figure 4A are consistent with a decreased capacity for base excision repair of these lesions in the malignant cultures. This defect was not related to decreased levels of the BER-related p53, APE/REF1 or OGG1 proteins; in fact, the malignant cells lines had increased levels

of DNA-polymerase–beta and delta; two key enzymes involved in short-patch and long-patch BER ((19); Figure 4B/4C). We also assessed whether increased expression of HR-related or BER-related proteins could be secondary to cell cycle bias given these proteins are optimally expressed in S and G2 phases of the cell cycle (12). In comparing the cell culture doubling times *in vitro* with mitotic and S-phase biomarkers (phosphorylated-histone 3 and RPA proteins, respectively); we did not observe any correlation between increased DNA repair protein expression and the varied cell proliferation indices amongst the five cultures (Figure 4B).

Chromosomal Repair and Heterogeneity of Rad51 Protein Expression *In Vivo*. HR and NHEJ events can be scored using integrated genetic substrates or characterization of distinct types of chromosomal aberrations following cellular exposure to MMC or IR (to induce DNA cross-links or DNA strand breaks, respectively). We used the latter assay as it can compare directly the capacity for HR and NHEJ in both malignant and normal cells following DNA damage as normal PrSc and PrEC cells would be difficult to transfect and select for integrated chromosomal reporter repair substrates (20). The metaphase spreads in Figure 5A show representative structural cytogenetic aberrations which are quantified in Table 1 for four of the five cultures; the protracted doubling time of PrEC precluded their use in this assay. All three malignant cell lines show an increased incidence of a variety of aberrations associated with aberrant HR during damage occurring in the S and G2 cell cycle phases including: chromatid breaks, double minutes, tri-, quad- and complex-radial chromosomes, abnormal telomeric associations and centromere fissions. Furthermore, NHEJ-associated defects occurring within the G1 cell cycle phase are also appreciable as increased chromosomal breaks and di- or tricentric chromosomes in the malignant cultures. These data further support our hypothesis that defects in DNA-dsb repair can be observed in malignant cultures and augment our findings with the comet assay.

Our results are consistent with elevated HR-related protein expression in vitro; we therefore wished to compare the expression of similar proteins in vivo within xenografts derived from the same malignat cells lines (e.g. PC-3 and DU-145). We therefore immunostained for Rad51 protein in sections derived from xenografts prior to and following 20Gy irradiation *in vivo* (see Figure 5B). Similar to a previous report in pancreatic cancer (21), we observed intratumoral heterogeneity with respect to endogenous Rad51 expression. Both cytoplasmic and nuclear Rad51 expression was observed and a similar observation was made in DU-145 xenografts (not shown). However, following irradiation, nuclear Rad51 expression increased. Taken together, our data suggests that Rad51 expression in vivo can be further modified by intratumoral biology and physiology.

DISCUSSION

This is the first study to report altered DNA-dsb, DNA-ssb and base damage repair defects in malignant prostate cancer cells with an associated increase in the DNA damaged-induced chromosomal aberrations and genetic instability. Our data is consistent with HR-related and BER-related protein dysfunction in malignant cells which fails to provide protection from genomic instability following cytotoxic insult. These findings are supported by previous data which suggested that the LNCaP cell line had a decreased ability to repair restriction-enzyme mediated DNA-dsbs using a fluorescence – based plasmid reconstitution assay (22). Additionally, during the writing of this manuscript, Trzeciak and colleagues (23) reported that PC-3 and DU-145 cells have defective incision of oxidative lesions with altered levels of superoxide dismutase and glutathione peroxidase, which supports our observation of decreased BER, based on our alkaline and FPG-comet assay data. This group also did not observe altered OGG1 or APE/Ref1 protein levels within the two cell lines. Other reports in the literature with in panels of cell lines with varying histopathologic type have reported heterogeneity in DNA polymerase-beta activity and elevated PARP expression (24-26).

Altered stoichiometry of repair proteins relative to functional protein-protein complexes or a disconnect between cell cycle checkpoint control and DNA repair may be the basis for the discordance between repair protein expression and function (12, 27). Altered HR-related protein expression might indirectly lead to altered NHEJ activity given the interplay between the two pathways during DNA-dsb repair (28, 29). There have been previous reports of elevated levels of Rad51 mRNA and protein expression occurring de novo within human tumour cell lines (30, 31). However, to our knowledge this is the first direct comparison of DNA-dsb gene expression within normal and malignant cells from the same histopathologic type linked to functional assessments of DNA-dsb repair. Maintenance of survival in malignant cells despite high levels of DNA-dsbs and chromosomal aberrations following DNA damage is probably secondary to loss of potentially deleterious accentric fragments or other chromosomal abnormalities within micronuclei at 48-72 hours post-irradiation.

In isogenic systems, the relationship between Rad51 expression, homologous recombination and induction of chromosomal aberrations remains controversial (30, 32-34) This may relate to variability in genetic background in isogenic systems or plasmid HR reporter substrates used for study (20). Transfection studies with forced overexpression of Rad51 has led to observations of both increased and decreased frequencies of homologous recombination (33, 35) arguing for Rad51 acting as either a promoter or repressor of genetic instability and tumor progression. Other transfection studies suggested that Rad51, XRCC3 and XRCC1 over-expression leads to increased $p21^{WAF}$ expression and decreased apoptotic response with resulting radioresistance (21, 36, 37). However, our data does not support such direct correlations given our HR-overexpressing malignant cultures have varying G1 checkpoint control, p21WAF expression and all 5 cultures are resistant to apoptosis. Indeed, the increased endogenous DNA repair protein expression observed in all three malignant cell lines was invariant following irradiation and independent of relative: p53 status, G1-checkpoint control, androgen-responsiveness, clonogenic radiation cell survival, cell proliferation and susceptibility for radiation-

induced apoptosis (15, 18, 38).

It had also been hypothesized that Rad51 overexpression might abrogate Rad51-p53 interactions and over-ride the G1 checkpoint leading to aneuploidy and that a loss of p53 leads to high levels of homologous recombination and elevated Rad51 levels (33, 39). Although this study was not designed to characterize an isogenic cell panel, the decreased levels of MMS- and IR-induced HR-related chromosomal aberrations in the WTp53-expressing and G1 checkpoint-proficient LNCaP cultures, relative to the G1 checkpoint-deficient DU-145 and PC-3 cultures, would support this hypothesis. Additionally, our observed over-expression of all of Rad51, XRCC3, Rad52 and Rad54 genes at the RNA and protein level suggests that loss of control of HR-expression in malignant cells is operational at the transcriptional level and may be secondary to altered activity of transcription factors. RAD51 overexpression can be mediated by BCR/ABL and STAT5-dependent transcription in addition to inhibition of caspase-3-dependent Rad51 cleavage (40, 41); similar mechanisms may exist for other HR-related genes. Indeed, preliminary data in our laboratory suggests that endogenous BRCA1 and BRCA2 expression is also elevated in the three malignant cell lines.[2]

Our results implicate DNA repair, and particularly DNA-dsb repair and homologous recombination, as a potential factor in prostate carcinogenesis. Importantly, our data may have implications for both prostate cancer diagnosis and therapy as we would hypothesize that prostate intraepithelial neoplasia (PIN) may have altered frequencies of homologous recombination and defective DNA-dsb repair when compared to nonmalignant epithelium. Clustered DNA repair protein expression profiles may be useful as a biomarker of genetic instability and increased risk for malignant progression in high-risk clinical cohorts (42, 43). Prostate tissue arrays will be useful to confirm that our *in vitro* findings are

[2] Fan, R; Jalali, F and Bristow R.G; unpublished observations

operational is human prostate tissues *in vivo* and whether loss of checkpoint control and DNA repair activity is correlated to tumor progression such as that reported for breast cancer (44). However, the data presented in Figure 5B suggest that interpretation of tissue arrays may be difficult without added information pertaining to cell cycle phase, oxygenation, nutritional status or clonal variation which may increase the intratumoural heterogeneity of protein expression. For example, the relative increase in expression following irradiation *in vivo* may be secondary to altered cell cycle phase as increased fraction of cells might undergo a G2 phase arrest or due to microenviromental factors. Indeed, in a separate study, we have observed that Rad51 expression can be altered under conditions of intratumoral hypoxia[3]. Further experiments with defined doses and timepoints following irradiation in LNCaP, PC3 and DU-145 xenografts may clarify these issues and glean more information regarding Rad51 expression within solid tumours.

Our study supports the use of agents that either augment DNA repair in normal and pre-malignant prostate epithelium or inhibit the hyper-recombinogenic phenotype of malignant cells. For example, the anti-oxidant selenium has been implicated as a chemoprotective agent for high-risk prostate cancer and has recently been shown to increase DNA repair and chemoprotect normal cells in response to DNA damaging agents (reviewed by Klein(45)). Additionally, molecular-targeted agents which decrease aberrant Rad51 or other DNA repair expression, such as STI571-Gleevec (41, 46) or siRNA approaches (47), may be a potentially novel approach to prostate cancer chemoprevention and also allow for sensitization of malignant prostate cancer cells to radiotherapy and chemotherapy (48, 49).

[3] Ranjit S. Bindra, Paul J. Schaffer, Alice Meng, Jennifer Woo, Kårstein Måseide, Matt E. Roth, Paul Lizardi, David W. Hedley, Robert G. Bristow, and Peter M. Glazer. Down-Regulation of Rad51 and Decreased Homologous Recombination in Hypoxic Cancer Cells. *Submitted.*

REFERENCES

1. Karayi MK and Markham AF. Molecular biology of prostate cancer. *Prostate Cancer Prostatic Dis*, 7: 6-20, 2004.
2. Elliott B and Jasin M. Double-strand breaks and translocations in cancer. *Cell Mol Life Sci*, 59: 373-385, 2002.
3. Vukovic B, Park PC, Al-Maghrabi J, Beheshti B, Sweet J, Evans A, Trachtenberg J, and Squire J. Evidence of multifocality of telomere erosion in high-grade prostatic intraepithelial neoplasia (HPIN) and concurrent carcinoma. *Oncogene*, 22: 1978-1987, 2003.
4. Gangopadhyay S, Jalali F, Reda D, Peacock J, Bristow RG, and Benchimol S. Expression of different mutant p53 transgenes in neuroblastoma cells leads to different cellular responses to genotoxic agents. *Exp Cell Res*, 275: 122-131, 2002.
5. Kastan MB, Lim DS, Kim ST, and Yang D. ATM--a key determinant of multiple cellular responses to irradiation. *Acta Oncol*, 40: 686-688, 2001.
6. Roninson IB, Broude EV, and Chang BD. If not apoptosis, then what? Treatment-induced senescence and mitotic catastrophe in tumor cells. *Drug Resist Updat*, 4: 303-313, 2001.
7. Chen L, Elahi A, Pow-Sang J, Lazarus P, and Park J. Association between polymorphism of human oxoguanine glycosylase 1 and risk of prostate cancer. *J Urol*, 170: 2471-2474, 2003.
8. Yeh CC, Lee C, and Dahiya R. DNA mismatch repair enzyme activity and gene expression in prostate cancer. *Biochem Biophys Res Commun*, 285: 409-413, 2001.
9. Rybicki BA, Conti DV, Moreira A, Cicek M, Casey G, and Witte JS. DNA repair gene XRCC1 and XPD polymorphisms and risk of prostate cancer. *Cancer Epidemiol Biomarkers Prev*, 13: 23-29, 2004.
10. Xu J, Zheng SL, Turner A, Isaacs SD, Wiley KE, Hawkins GA, Chang BL, Bleecker ER, Walsh PC, Meyers DA, and Isaacs WB. Associations between hOGG1 sequence variants and prostate cancer susceptibility. *Cancer Res*, 62: 2253-2257, 2002.
11. Valerie K and Povirk LF. Regulation and mechanisms of mammalian double-strand break repair. *Oncogene*, 22: 5792-5812, 2003.
12. West SC. Molecular views of recombination proteins and their control. *Nat Rev Mol Cell Biol*, 4: 435-445, 2003.
13. Takata M, Sasaki MS, Tachiiri S, Fukushima T, Sonoda E, Schild D, Thompson LH, and Takeda S. Chromosome instability and defective recombinational repair in knockout mutants of the five Rad51 paralogs. *Mol Cell Biol*, 21: 2858-2866, 2001.
14. Liede A, Karlan BY, and Narod SA. Cancer risks for male carriers of germline mutations in BRCA1 or BRCA2: a review of the literature. *J Clin Oncol*, 22: 735-742, 2004.
15. Bromfield GP, Meng A, Warde P, and Bristow RG. Cell death in irradiated prostate epithelial cells: role of apoptotic and clonogenic cell kill. *Prostate Cancer Prostatic Dis*, 6: 73-85, 2003.
16. Bristow RG, Hu Q, Jang A, Chung S, Peacock J, Benchimol S, and Hill R. Radioresistant MTp53-expressing rat embryo cell transformants exhibit increased DNA-dsb rejoining during exposure to ionizing radiation. *Oncogene*, 16: 1789-1802, 1998.
17. Olive PL. The comet assay. An overview of techniques. *Methods Mol Biol*, 203: 179-194, 2002.
18. Yuan R, Fan S, Wang JA, Meng Q, Ma Y, Schreiber D, Goldberg ID, and Rosen EM. Coordinate alterations in the expression of BRCA1, BRCA2, p300, and Rad51 in response to genotoxic and other stresses in human prostate cancer cells. *Prostate*, 40: 37-49, 1999.
19. Dianov GL, Sleeth KM, Dianova, II, and Allinson SL. Repair of abasic sites in DNA. *Mutat Res*, 531: 157-163, 2003.
20. Willers H, Xia F, and Powell SN. Recombinational DNA Repair in Cancer and Normal Cells: The Challenge of Functional Analysis. *J Biomed Biotechnol*, 2: 86-93, 2002.

21. Henning W and Sturzbecher HW. Homologous recombination and cell cycle checkpoints: Rad51 in tumour progression and therapy resistance. *Toxicology*, 193: 91-109, 2003.
22. Collis SJ, Sangar VK, Tighe A, Roberts SA, Clarke NW, Hendry JH, and Margison GP. Development of a novel rapid assay to assess the fidelity of DNA double-strand-break repair in human tumour cells. *Nucleic Acids Res*, 30: E1, 2002.
23. Trzeciak AR, Nyaga SG, Jaruga P, Lohani A, Dizdaroglu M, and Evans MK. Cellular repair of oxidatively induced DNA base lesions is defective in prostate cancer cell lines, PC-3 and DU-145. *Carcinogenesis*, 2004.
24. Bhattacharyya N, Chen HC, Wang L, and Banerjee S. Heterogeneity in expression of DNA polymerase beta and DNA repair activity in human tumor cell lines. *Gene Expr*, 10: 115-123, 2002.
25. Dobashi Y, Shuin T, Tsuruga H, Uemura H, Torigoe S, and Kubota Y. DNA polymerase beta gene mutation in human prostate cancer. *Cancer Res*, 54: 2827-2829, 1994.
26. McNealy T, Frey M, Trojon L, Knoll T, Alken P, and Michel MS. Intrinsic presence of poly (ADP-ribose) is significantly increased in malignant prostate compared to benign prostate cell lines. *Anticancer Res*, 23: 1473-1478, 2003.
27. Venkitaraman AR. Cancer susceptibility and the functions of BRCA1 and BRCA2. *Cell*, 108: 171-182, 2002.
28. Allen C, Halbrook J, and Nickoloff JA. Interactive competition between homologous recombination and non-homologous end joining. *Mol Cancer Res*, 1: 913-920, 2003.
29. Richardson C and Jasin M. Coupled homologous and nonhomologous repair of a double-strand break preserves genomic integrity in mammalian cells. *Mol Cell Biol*, 20: 9068-9075, 2000.
30. Lambert S and Lopez BS. Inactivation of the RAD51 recombination pathway stimulates UV-induced mutagenesis in mammalian cells. *Oncogene*, 21: 4065-4069, 2002.
31. Raderschall E, Stout K, Freier S, Suckow V, Schweiger S, and Haaf T. Elevated levels of Rad51 recombination protein in tumor cells. *Cancer Res*, 62: 219-225, 2002.
32. Kim PM, Allen C, Wagener BM, Shen Z, and Nickoloff JA. Overexpression of human RAD51 and RAD52 reduces double-strand break-induced homologous recombination in mammalian cells. *Nucleic Acids Res*, 29: 4352-4360, 2001.
33. Richardson C, Stark JM, Ommundsen M, and Jasin M. Rad51 overexpression promotes alternative double-strand break repair pathways and genome instability. *Oncogene*, 23: 546-553, 2004.
34. Vispe S, Cazaux C, Lesca C, and Defais M. Overexpression of Rad51 protein stimulates homologous recombination and increases resistance of mammalian cells to ionizing radiation. *Nucleic Acids Res*, 26: 2859-2864, 1998.
35. Bertrand P, Lambert S, Joubert C, and Lopez BS. Overexpression of mammalian Rad51 does not stimulate tumorigenesis while a dominant-negative Rad51 affects centrosome fragmentation, ploidy and stimulates tumorigenesis, in p53-defective CHO cells. *Oncogene*, 22: 7587-7592, 2003.
36. Raderschall E, Bazarov A, Cao J, Lurz R, Smith A, Mann W, Ropers HH, Sedivy JM, Golub EI, Fritz E, and Haaf T. Formation of higher-order nuclear Rad51 structures is functionally linked to p21 expression and protection from DNA damage-induced apoptosis. *J Cell Sci*, 115: 153-164, 2002.
37. Yanagisawa T, Urade M, Yamamoto Y, and Furuyama J. Increased expression of human DNA repair genes, XRCC1, XRCC3 and RAD51, in radioresistant human KB carcinoma cell line N10. *Oral Oncol*, 34: 524-528, 1998.
38. Carlomagno F, Burnet NG, Turesson I, Nyman J, Peacock JH, Dunning AM, Ponder BA, and Jackson SP. Comparison of DNA repair protein expression and activities between human fibroblast cell lines with different radiosensitivities. *Int J Cancer*, 85: 845-849, 2000.

39. Kumari A, Schultz N, and Helleday T. p53 protects from replication-associated DNA double-strand breaks in mammalian cells. *Oncogene*, 23: 2324-2329, 2004.
40. Slupianek A, Schmutte C, Tombline G, Nieborowska-Skorska M, Hoser G, Nowicki MO, Pierce AJ, Fishel R, and Skorski T. BCR/ABL regulates mammalian RecA homologs, resulting in drug resistance. *Mol Cell*, 8: 795-806, 2001.
41. Skorski T. BCR/ABL regulates response to DNA damage: the role in resistance to genotoxic treatment and in genomic instability. *Oncogene*, 21: 8591-8604, 2002.
42. Bishay K, Ory K, Olivier MF, Lebeau J, Levalois C, and Chevillard S. DNA damage-related RNA expression to assess individual sensitivity to ionizing radiation. *Carcinogenesis*, 22: 1179-1183, 2001.
43. Goode EL, Ulrich CM, and Potter JD. Polymorphisms in DNA repair genes and associations with cancer risk. *Cancer Epidemiol Biomarkers Prev*, 11: 1513-1530, 2002.
44. Gonzalez R, Silva JM, Dominguez G, Garcia JM, Martinez G, Vargas J, Provencio M, Espana P, and Bonilla F. Detection of loss of heterozygosity at RAD51, RAD52, RAD54 and BRCA1 and BRCA2 loci in breast cancer: pathological correlations. *Br J Cancer*, 81: 503-509, 1999.
45. Klein EA. Selenium: epidemiology and basic science. *J Urol*, 171: S50-53; discussion S53, 2004.
46. Russell JS, Brady K, Burgan WE, Cerra MA, Oswald KA, Camphausen K, and Tofilon PJ. Gleevec-mediated inhibition of Rad51 expression and enhancement of tumor cell radiosensitivity. *Cancer Res*, 63: 7377-7383, 2003.
47. Collis SJ, Tighe A, Scott SD, Roberts SA, Hendry JH, and Margison GP. Ribozyme minigene-mediated RAD51 down-regulation increases radiosensitivity of human prostate cancer cells. *Nucleic Acids Res*, 29: 1534-1538, 2001.
48. Collis S, Swartz M, and DeWeese T. siRNA-silencing of DNA repair factors results in enhanced radiation and chemotherapy-mediated killing of human cancer cells. *Int J Radiat Oncol Biol Phys*, 57: S144, 2003.
49. Taki T, Ohnishi T, Yamamoto A, Hiraga S, Arita N, Izumoto S, Hayakawa T, and Morita T. Antisense inhibition of the RAD51 enhances radiosensitivity. *Biochem Biophys Res Commun*, 223: 434-438, 1996.

TABLE 1 Chromosomal Aberrations in Prostate Cell Cultures In Response to DNA Damage[4]

Aberration	PRSC			LNCAP			DU145			PC3		
	Control	MMC	IR	Control	MMC	IR	Control	MMC	IR	Control	MMC	IR
Chromosome break	0	3	0	0	0	1	0	0	2	1	3	1
Chromatid break	0	0	0	1	0	1	0	4	0	0	6	1
Tri-, Quad- or Complex radial	0	1	0	3	2	8	0	2	1	0	8	9
Di- or Tri-centric	0	0	1	0	1	6	0	7	14	2	20	22
Ring chromosomes	0	0	0	0	0	0	0	0	0	0	1	3
Double minutes	0	0	0	0	0	3	0	2	14	2	4	6
Accentric fragments	0	0	1	1	0	18	3	4	15	12	10	15
Telomeric Association of 2 Acrocentric chromosomes	0	0	1	0	3	4	3	4	0	2	5	1
Centromere fission	0	1	0	0	2	0	4	0	0	0	0	1
TOTAL	**1**	**5**	**3**	**5**	**8**	**41**	**10**	**23**	**46**	**19**	**57**	**58**

[4] For each cell culture, the frequency of spontaneous (Control), IR-induced (4Gy) or MMC-induced (40 ng/ml for 24 hours) chromosomal aberrations were determined in exponentially growing cell cultures of PRSC, LNCAP, DU145 and PC3. The extremely slow doubling time of PrEC cells (> 48 hrs) precluded a similar analysis in this cell type. The cells were harvested by trypsinization 24h after treatment and after incubation with 1 μg of Colcemid/ml for 2 hours to collect metaphase spreads for analysis. 25 metaphases were scored for each culture. Both increased chromosome and chromatid types of aberrations are observed in the malignant cells relative to PrSC cells following IR or MMC treatment, consistent with defects in HR and NHEJ repair.[4]

FIGURE LEGENDS:

Fig. 1 Gene expression relating to ATM-p53 signaling in malignant and normal prostate cultures using RPA analyses. (A) Representative example of RPA blot whereby ^{32}P labeled multi-riboprobes were hybridized to the total mRNA derived from mock-irradiated (NIR) and irradiated (IR: 10Gy-4hrs) asynchronously growing prostate cultures. Specific multiprobes used in this analysis are indicated in the far left margin of the blot aside the corresponding gene band of interest. Also shown are lanes containing probed sequences within HeLa cells (positive control), Yeast(Yst; negative control) and the RNA probes themselves at far right. GAPDH was a housekeeping gene that served as internal control for densitometric quantitation of results. Note IR-induced $p21^{WAF}$ signal in WT-p53 expressing PrSC, PrEC and LNcaP cell lines consistent with wild type p53 status and intact IR-induced G1 checkpoint in these cells.

(B) Quantitation of ATM/p53-dependent stress response based on RPA analyses of mRNA in normal and malignant prostate cells. Upper figure shows basal (ie non-irradiated) levels of mRNA expression. Shown are mean gene expression values +/- 1 S.E. based on at least 3 independent experiments. Lower panel shows mean values of expression of same genes relative to basal levels at 4 hours following 10 Gy. Significantly increased expression was observed for the p21WAF gene in WTp53-expressing cells (PrSC, PrEC, LNCaP) which both time-dependent (see insert for p21WAF expression over 6 hours post-10Gy in LNCaP cells) and dose dependent from 2 to 10Gy (data not shown; Mann-Whitney test; $p<0.05$)). Bax, Bcl-2 and Gadd45 mRNA in these cell lines was also significantly increased following irradiation (Mann-Whitney test; $p<0.05$).

Fig. 2 Increased expression of homologous recombination (HR) genes, Rad51, XRCC3, Rad52 and Rad54, in malignant prostate cancer cell lines. (A) Upper panel shows relative basal HR gene expression of Rad52 epistaxis group of proteins based on RPA analyses in normal and malignant

prostate cells. Significant increased mRNA expression of Rad51, Rad54, Rad52 and XRCC3 is noted in the malignant prostate cultures in comparison to normal cell cultures (Mann-Whitney test, $p<0.05$). IR-induction of Rad51, Rad54, Rad52 and XRCC3 gene was observed solely in the PrSC stromal cell line (Mann-Whitney test; $p<0.05$), but not the other epithelial cell lines (data not shown; Supp Fig. 1A). Lower panel shows western blots of selected HR proteins in which protein expression is invariant before (NIR) and at 4 hours following (IR) a dose of 10Gy. α–tubulin is shown as the loading control and p21WAF as an irradiation control. (i.e. p21WAF protein is elevated in cell lines with wild type p53 status).

(b) Confirmation of increased basal Rad51, Rad52, Rad54 and XRCC3 protein expression in malignant prostate cells relative to PrEC cells using quantitative densitometry of western blot analyses (see example blot in Supp. Fig 1B). Similar results were obtained amongst 2 independent experiments. The dotted line represents the relative expression within the PrEC cell line. HR-related protein expression was not further induced at 4 hours following a dose 10Gy (data not shown).

Fig. 3 Expression of non-homologous end-joining NHEJ proteins and defects in DNA-dsb and global repair in malignant prostate cell cultures. (A) Quantitation of NHEJ and DNA-ssb repair pathway-related protein expression in malignant prostate cells relative to PrEC cells using quantitative densitometry of western blot analyses (see example blot in Supp. Fig 1C). Similar results were obtained amongst 2 independent experiments. The dotted line represents the relative expression within the PrEC cell line. The level of XRCC1 expression was consistently elevated in all 3 malignant cell lines in independent experiments. Neither NHEJ- nor DNA-ssb-related protein expression was further induced at 4 hours following a dose 10Gy (data not shown).

(B) Neutral comet assay of malignant and normal prostate cells before and following 10 or 25 Gy of ionizing radiation. Plotted is the "Olive Tail Moment" or "OTM" (i.e % DNA x distance of center of

gravity of DNA) on the y-axis as the indicator of the presence of DNA-dsbs for a given time and radiation dose. No significant differences exist between the 5 cells lines for OTM values at baseline or immediately following irradiation (time = 0). However, the residual number of DNA-dsbs at 24 hours following 25 Gy is greater in the three malignant cultures in comparison to the normal cultures (Mann-Whitney test; $p<0.05$).

(C) Alkaline (pH >13.0) comet assay of malignant and normal prostate cells before and following 6Gy of ionizing radiation. Similar to (B) above, the "OTM" on the y-axis as the indicator of the presence of alkali-labile sites, DNA-dsbs, DNA-ssbs and DNA base damage following irradiation. The residual damage at 24 hours following irradiation in the three malignant cultures is greater than that of the control PrEC cultures (Mann-Whitney test; $p<0.05$). For both (B) and (C), one hundred consecutive cells were scored at random from the middle of each slide for 2-3 independent experiments and the final result expressed as the (mean of the OTM median values) ± SE of the medians).

Fig. 4 Defective excision of oxidative damage in malignant prostate cancer cultures. (A) Comet assay of FPG-sensitive sites representing extent of oxidative base damage before and following 6 Gy irradiation in malignant prostate cell cultures relative to normal control (PrEC) culture. The final result expressed as the (mean of the OTM median values) ± SE of the medians). A trend towards increased endogenous oxidative lesions is observed at time 0 of FPG-treatment in the malignant cells, although only the baseline FPG-site levels of DU-145 are statistically increased when compared to levels within PrEC cultures (Mann-Whitney test; $p<0.05$). However, at 24 hours following 6 Gy, the number of remaining oxidative lesions is statistically higher in all three malignant cultures (Mann-Whitney; $p<0.05$)

(B) Western blot analyses and (C) relative densitometry of basal levels of BER- and cell cycle-associated proteins in normal and malignant cell cultures. Elevated levels of DNA polymerase-beta and –delta were consistently observed in a p53-independent manner in malignant cell cultures. Similar

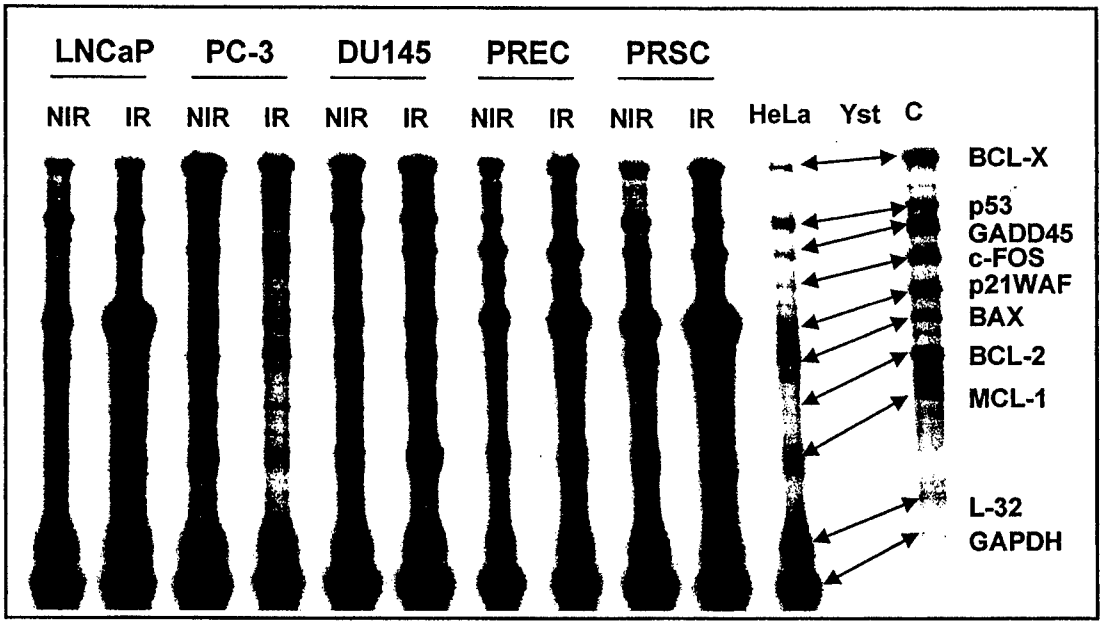
observations were made between 2 independent experiments. There is no correlation between phosphorylated-H3 (H3-P; a marker of mitosis), RPA (marker of S-phase) or cell doubling time *in vitro* (Td; hours) and levels of DNA polymerase (or HR-associated protein expression in Fig. 2), confirming that elevated levels of DNA repair proteins is not solely due to increased growth fraction of cells within S and G2 phases of cell cycle.

Fig. 5 Chromosomal damage *in vitro* and patterns of Rad51 expression *in vivo* in PC-3 following DNA damage. (A) Representative images of photomicrographs of metaphase spreads stained with DAPI from (i) untreated, and (ii), MMC-treated (30 ng/ml for 1 hour) PC-3 cells, at 24 hours following treatment. The two representative spreads in (ii) also show magnified images of quadradial, complex-radial and dicentric chromosomes (see white arrows and magnified views) consistent with defects in DNA-dsb repair. The full spectrum of chromosomal aberrations within PC-3, DU-145, LNCaP and PrSC cells following DNA damage is shown in Table 1.

(B) Immunohistochemistry for Rad51 protein within human PC3 cells grown as a solid tumor xenograft in the hind leg of a Balb/c nude mouse. Note heterogeneity of cytoplasmic and nuclear staining within tumor prior to irradiation (NIR; left panel). Following X-irradiation with a single dose of 20Gy *in vivo*, intratumoural heterogeneity of Rad51 staining is maintained, yet the number and intensity of nuclear staining increases at 24 hours post-irradiation (20Gy, right panel). Similar observations were made in DU-145 xxenografts.

Figure 1

A.


LNCaP
PC-3
DU145
PREC
PRSC
NIR
IR
HeLa
Yst
C
BCL-X
p53
GADD45
c-FOS
p21WAF
BAX
BCL-2
MCL-1
L-32
GAPDH

B.

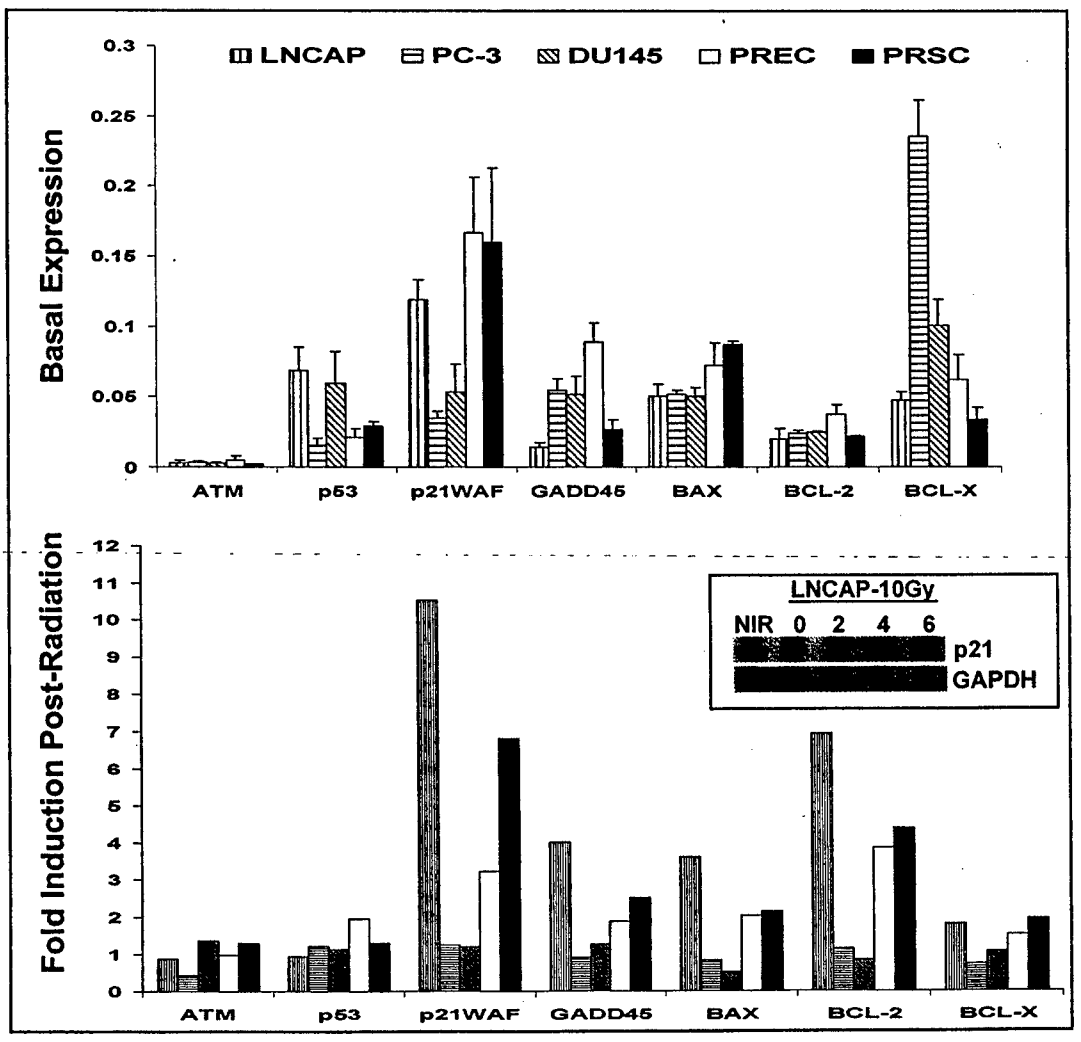
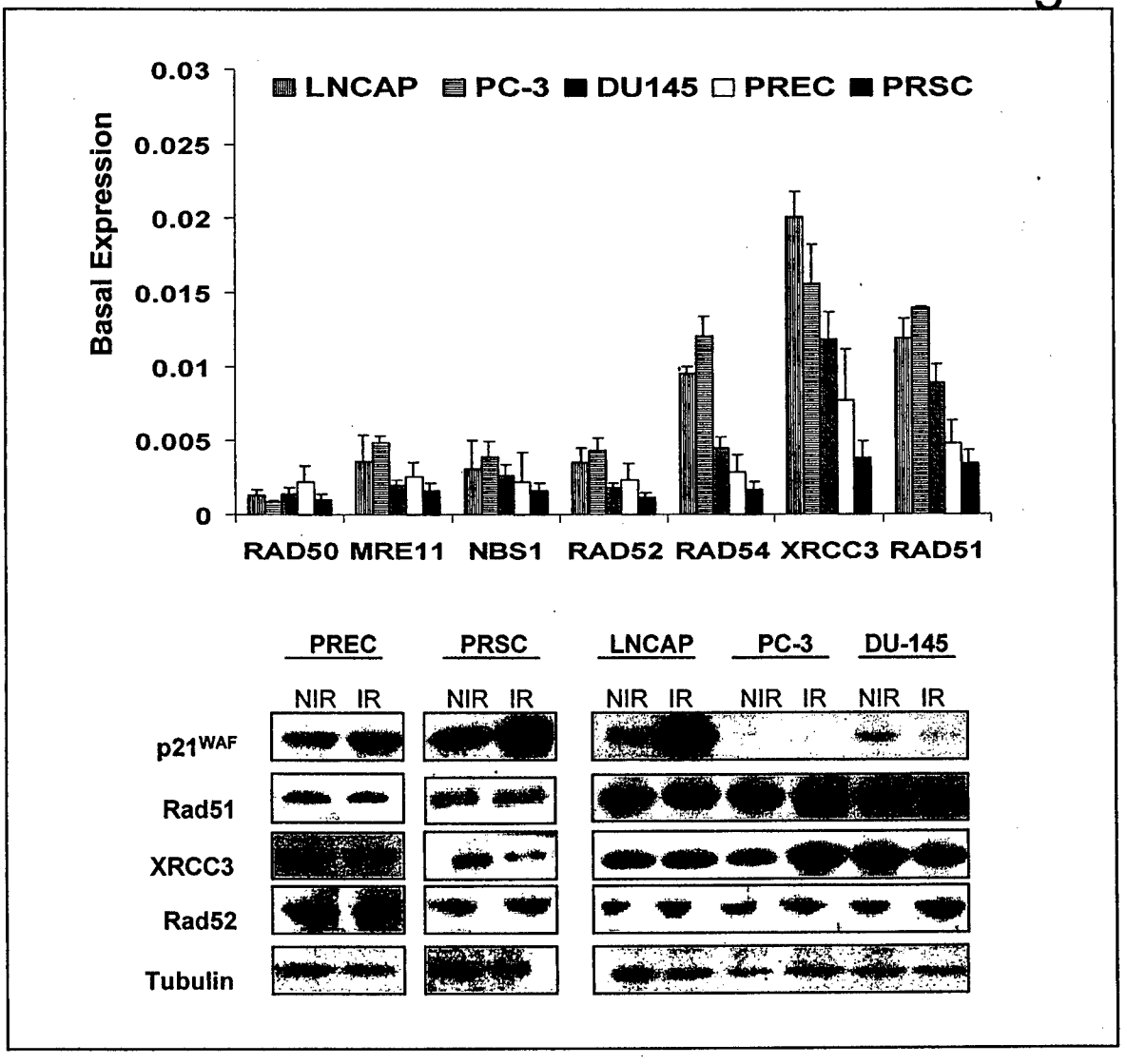

LNCAP
PC-3
DU145
PREC
PRSC
Basal Expression
0.3
0.25
0.2
0.15
0.1
0.05
0
ATM
p53
p21WAF
GADD45
BAX
BCL-2
BCL-X
Fold Induction Post-Radiation
12
11
10
9
8
7
6
5
4
3
2
1
0
LNCAP-10Gy
NIR 0 2 4 6
p21
GAPDH

Figure 2

A.


LNCAP
PC-3
DU145
PREC
PRSC
Basal Expression
0.03
0.025
0.02
0.015
0.01
0.005
0
RAD50
MRE11
NBS1
RAD52
RAD54
XRCC3
RAD51
PREC
PRSC
LNCAP
PC-3
DU-145
NIR IR
NIR IR
NIR IR
NIR IR
NIR IR
p21WAF
Rad51
XRCC3
Rad52
Tubulin

B.

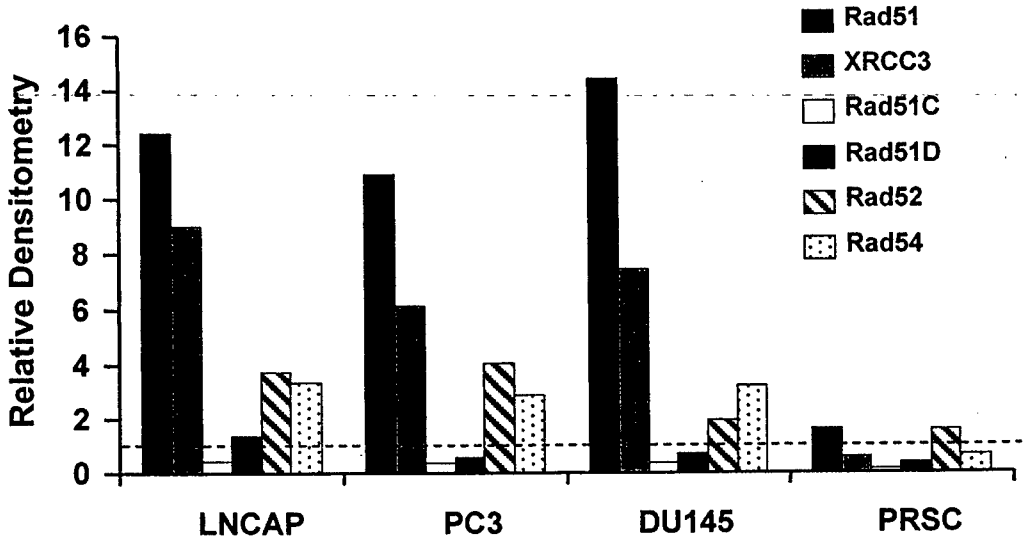
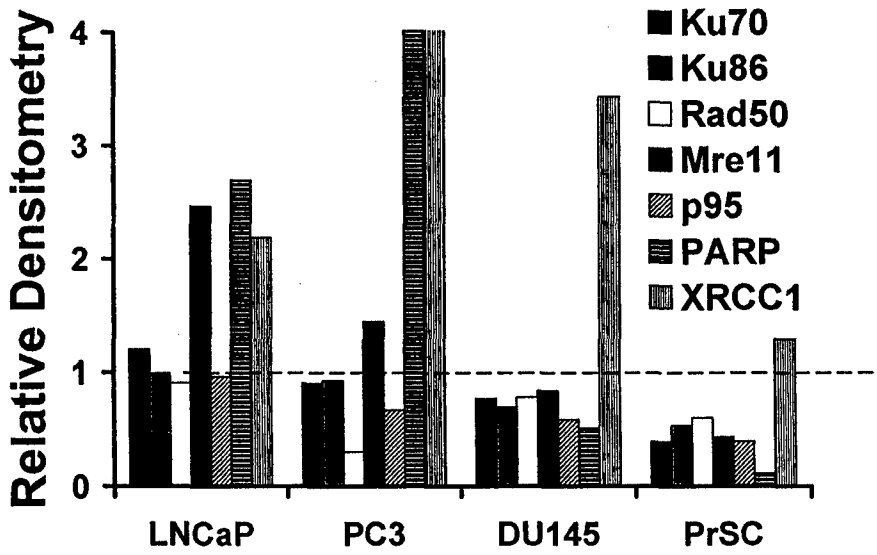
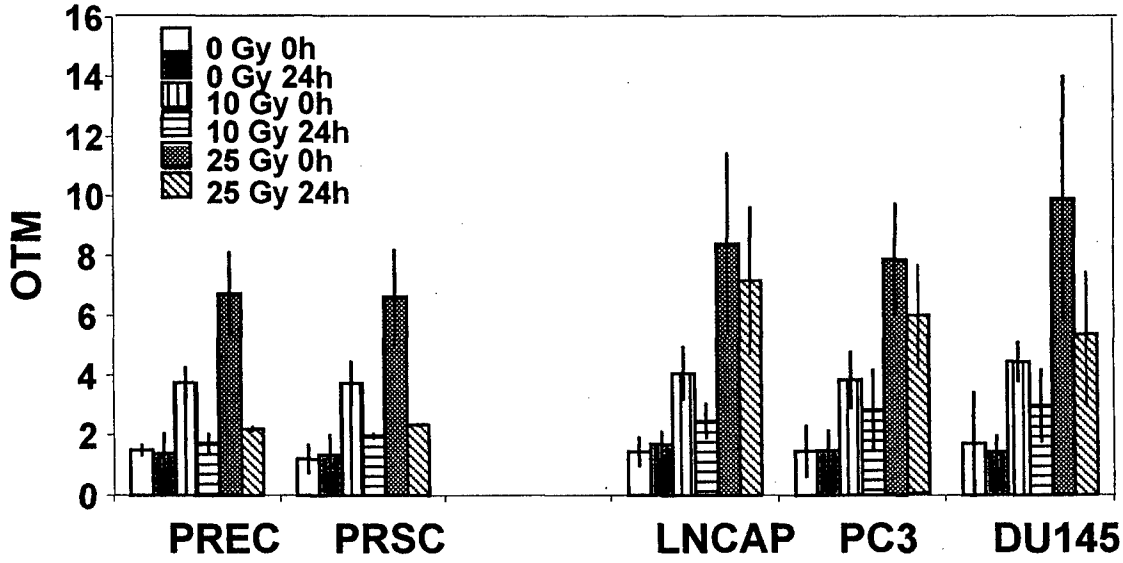

Relative Densitometry
16
14
12
10
8
6
4
2
0
Rad51
XRCC3
Rad51C
Rad51D
Rad52
Rad54
LNCAP
PC3
DU145
PRSC

Figure 3

A.


Relative Densitometry
0
1
2
3
4
Ku70
Ku86
Rad50
Mre11
p95
PARP
XRCC1
LNCaP
PC3
DU145
PrSC

B.


OTM
0
2
4
6
8
10
12
14
16
0 Gy 0h
0 Gy 24h
10 Gy 0h
10 Gy 24h
25 Gy 0h
25 Gy 24h
PREC
PRSC
LNCAP
PC3
DU145

C.

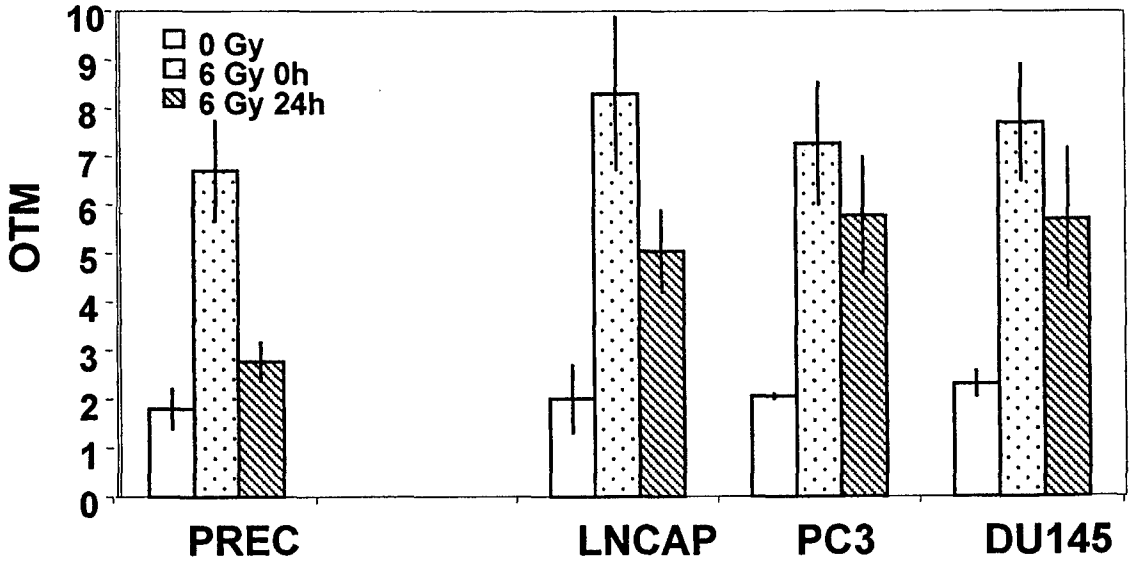
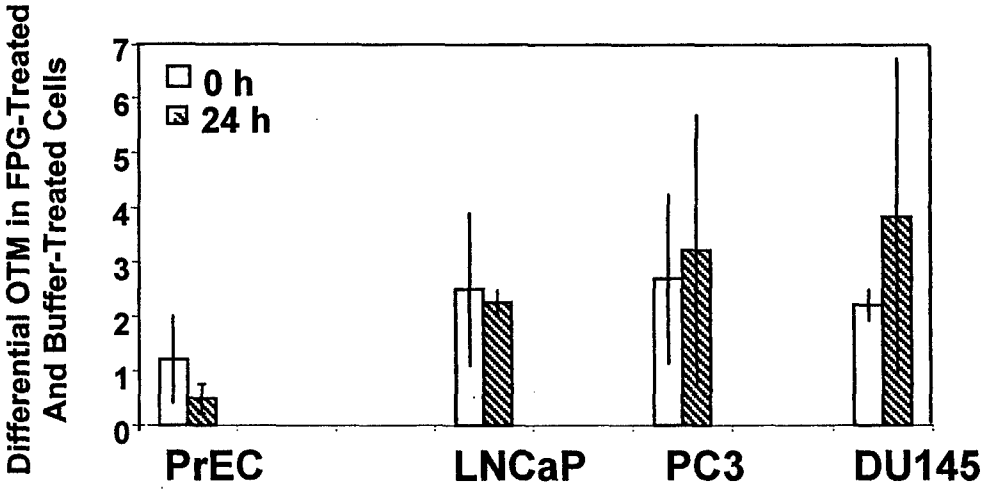
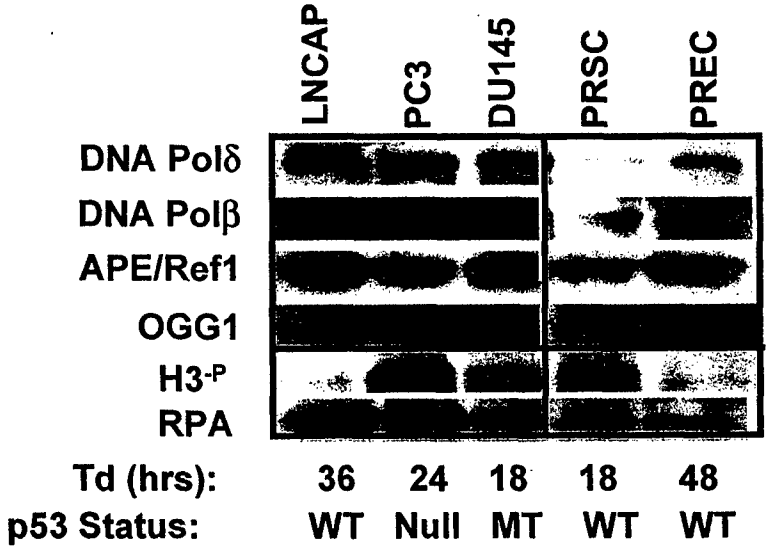

OTM
0
1
2
3
4
5
6
7
8
9
10
0 Gy
6 Gy 0h
6 Gy 24h
PREC
LNCAP
PC3
DU145

Figure 4

A.


Differential OTM in FPG-Treated And Buffer-Treated Cells
0 h
24 h
0
1
2
3
4
5
6
7
PrEC
LNCaP
PC3
DU145

B.


LNCAP
PC3
DU145
PRSC
PREC
DNA Polδ
DNA Polβ
APE/Ref1
OGG1
H3-P
RPA
Td (hrs): 36 24 18 18 48
p53 Status: WT Null MT WT WT

C.

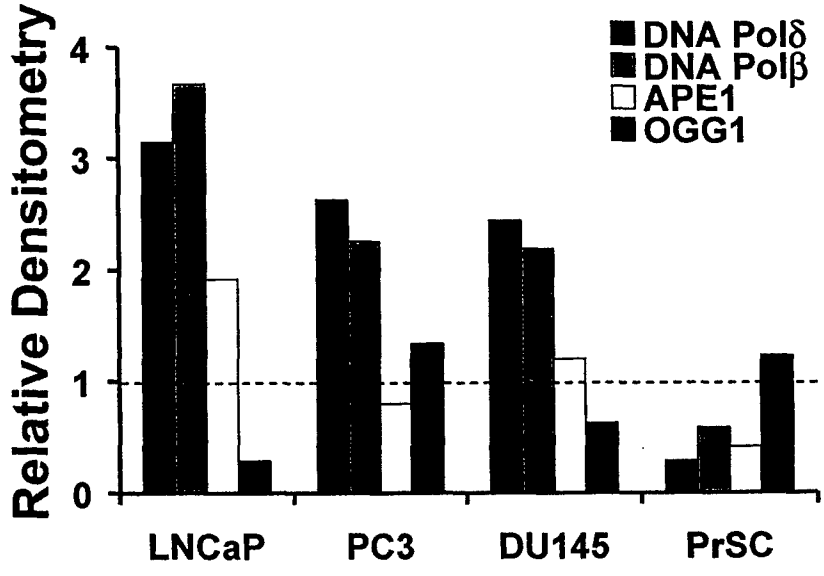
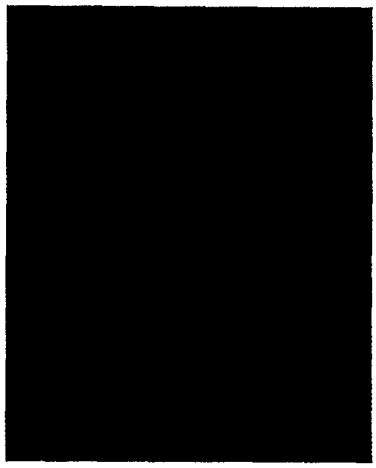

Relative Densitometry
DNA Polδ
DNA Polβ
APE1
OGG1
0
1
2
3
4
LNCaP
PC3
DU145
PrSC

Figure 5

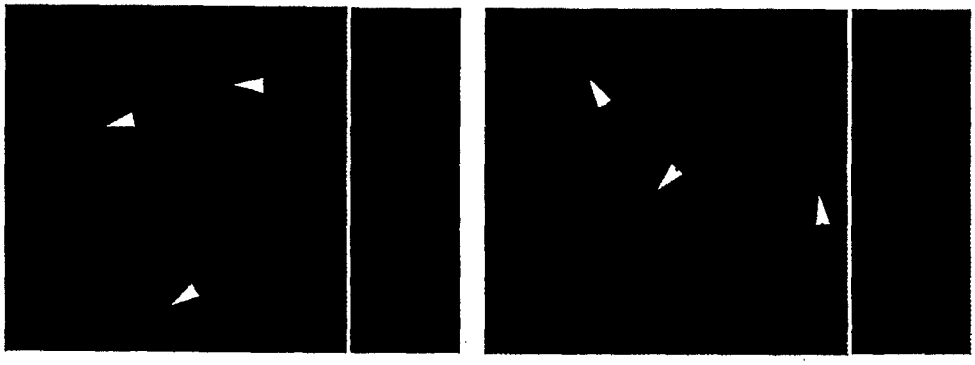
A.

(i)



PC-3 Untreated
Partial Metaphase

(ii) PC-3 MMC-treated



B.