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DNA MISMATCH REPAIR AND LOW DOSE HYPER-RADIOSENSITIVITY IN PROSTATE CANCER CELLS

BY

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SUBMITTED IN FULFILMENT OF THE REQUIREMENTS

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DECLARATION

I hereby certify that this thesis submitted for the degree of Doctor of Philosophy to the University of Dublin, has not been previously submitted for a degree or diploma to this or any other University. The work presented here is entirely my own, except where stated otherwise.

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SUMMARY

Low-dose hyper-radiosensitivity (HRS) describes the excessive sensitivity of cells to radiation doses less than 0.3Gy. Evidence suggests that HRS may influence tumour response and normal tissue reactions after intensity modulated radiotherapy and low dose rate brachytherapy, and that the response may potentially be exploited to improve therapeutic efficacy. Prostate cancer (PCa) is particularly likely to be affected by HRS due to the increasing use of protocols that involve low doses of radiation or low dose rates used in the radiotherapeutic management of the disease. While accumulating evidence indicates that DNA repair and G2 checkpoint responses are involved in the manifestation of HRS, the underlying mechanism(s) remains unknown. Elucidation of the mechanism(s) of the response is fundamental to understanding the true implications of HRS for radiotherapy practice.

The overall aim of this thesis was to investigate the molecular mechanism(s) of HRS in PCa cells. To this end, a panel of prostate cell lines (DU145, PC3, 22RV1, PWR1E, RWPE1) were investigated for evidence of HRS. Clonogenic survival assays and mathematical modelling demonstrated that both PC3 and RWPE1 cell lines expressed distinct HRS responses. No correlation was found between HRS and intrinsic radioresistance, or static cell cycle distribution in these cells. Low density arrays comparing endogenous and induced (0.2Gy) DNA repair gene expression 2hr after irradiation (0.2Gy) indicated no obvious correlation between gene expression and HRS.

PC3 cells exposed to low concentrations of Temozolomide (O6MeG inducer), showed a similar survival response to HRS, suggesting a potential role for O6MeG in HRS. A similar trend was observed in gliomas cells (T98G, U373). Western blotting and qRT-PCR demonstrated that HRS correlated with weak MGMT expression, however pre-treatment with MGMT inhibitor O6-benzylguanine did not abrogate HRS. Western blotting of DNA mismatch repair (MMR) proteins (MSH2, MLH1, PMS1, PMS2, MSH6), revealed a correlation between HRS and MMR proficiency in prostate and gliomas cells.

To investigate the role of DNA mismatch repair protein MSH2 in HRS, we examined the low-dose radioresponse of endometrial carcinoma cell lines, proficient and deficient in MSH2, for evidence of a hypersensitive response using cell sorter clonogenic assays and mathematical modelling. Defects in cell cycle checkpoint activation in irradiated cells (0.2Gy) were investigated using flow cytometry (phospho-H3) and western blotting (Chk1 and Chk2). DNA damage response was investigated 2 and 24hr post 0.2Gy by high content screening for molecular markers of mitosis, DNA end resection, DNA double strand breaks, and homologous recombination, using phospho-H3, MRE11, γH2AX, and RAD51 respectively. HRS was expressed solely in MSH2+ cells and was associated with efficient activation of the early G2 checkpoint. Maintenance of the arrest was associated with phosphorylated Chk2, and persistent MRE11, γH2AX, RAD51 foci at 2hr. Persistent MRE11 and RAD51 foci were also evident 24hr after 0.2Gy. These data suggested that MSH2 status significantly affects cellular responses to low doses of IR. MSH2 may enhance cell radiosensitivity to low dose IR through inhibition of homologous recombination (via regulation of RAD51). Similarly, a role for MLH1 in the HRS survival response was demonstrated in isogenic colorectal cells proficient and deficient in the expression of MLH1.

Finally, to determine whether HRS in PC3 prostate cancer cells is derived from a similar MSH2-dependent mechanism to that delineated in endometrial carcinoma cells, we examined a number of endpoints of the proposed mechanism in PC3 cells. Flow cytometry and high content screening validated the mechanism proposed previously, demonstrating that PC3 cells arrest at the early G2 checkpoint, and display persistent H2AX and RAD51 foci 2 and 24hr after irradiation (0.2Gy). These data suggest that MSH2 protein expression may be a useful prognostic marker, and may indeed contribute to a prognostic panel for the outcome of individuals undergoing radiotherapy.

ABBREVIATIONS

17-AAG- 17-alylamine-17-demothoxy geldanamycin

3D-CRT- Three dimensional conformal radiotherapy

BASC- BRCA1 associated genome surveillance complex

BER- Base excision repair

DMSO- Dimethyl sulfoxide

DNA-PK- DNA-dependent protein kinase

DSB- Double strand break

DSBR- Double strand break repair

DTT- Dithiothreitol

EBRT- External beam radiotherapy

EDTA- Ethylenediaminetetraacetic acid

EXO1- Exonuclease 1

FA- Franconi anemia

FSC- Forward scatter

GC- Gene conversion

HDR- High dose rate

HR- Homologous recombination

HRS- Hyper radiosensitivity

IDRE- Inverse dose rate effect

IMRT- Intensity modulated radiotherapy

IR- Ionising radiation

IRR- Increased radioresistance

LDR- Low dose rate

LET- Linear energy transfer

LQ- Linear quadratic

LDFRT- Low dose fractionated radiotherapy

MESF- Molecules of Equivalent Soluble Fluorophor

MGMT- Methylguanine methyltransferase

MMR- Mismatch repair

MNNG- N-methyl-N'-Nitro-N-Nitrosoguanidine

NER- Nucleotide excision repair

NHEJ- Non homologous end-joining

O6BG- O6-Benzylguanine

O6MeG- O6-Methylguanine

PARP- Poly-ADP ribose polymerase

PCa- Prostate Cancer

PDAR- Pre-defined assay reagent

PE- Plating efficiency

PMSF- Phenyl-methyl-sulfonyl-fluoride

PSA- Prostate specific antigen

PVDF- Polyvinylidene fluoride

RFC- Replication factor C

RPA- Replication protein A

RT- Radiotherapy

SCC- Side scatter

SCCHN- Squamous cell carcinoma of the head and neck

SDSA- Synthesis dependent strand annealing

SF- Surviving fraction

TCP- Tumour control probability

TMZ- Temozolomide

UF- Ultrafractionated

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PUBLICATIONS

ORIGINAL RESEARCH PAPERS (FROM THIS THESIS)

Martin LM., Marples B., Coffey M., Lawler M., Hollywood D., Marignol L. "Recognition of O6MeG lesions by MGMT and Mismatch Repair Proficiency may be a Prerequisite for Low Dose Radiation Hypersensitivity" (*Radiation Research*, 172(4):405-413 (2009))

Martin LM., Marples B., Davies AM., Atzberger A., Edwards C., Coffey M., Lawler M., Lynch TH., Hollywood D., Marignol L. "DNA mismatch repair protein MSH2 may dictate the cellular survival in response to low dose radiation" (Manuscript submitted)

REVIEWS (FROM THIS THESIS)

Martin LM., Coffey M., Lawler M., Hollywood D., Marignol L. "DNA Mismatch Repair and the transition to Hormone Independence in Breast and Prostate Cancer" (Mini-Review, *Cancer Letters,* 291, 142-9 (2009))

Martin LM., Marples B., Coffey M., Lawler M., Hollywood D., Marignol L. "DNA mismatch repair and the DNA damage response to Ionizing Radiation: Making sense of conflicting data" (Review, *Cancer Treatment Reviews*, 36(10) 518-527 (2010))

Martin LM., Marples B., Coffey M., Lawler M., Hollywood D., Marignol L. "Low dose hyper radiosensitivity and prostate cancer radiotherapy" (Review article in preparation)

ORIGINAL RESEARCH PAPER (FROM WORK OTHER THAN IN THIS THESIS)

Forde JC., Perry AS., Brennen K., **Martin LM.**, Lawler MP., Lynch TH., Hollywood D., Marignol L. "Docetaxel maintains its cytotoxic activity under hypoxic conditions in prostate cancer cells" (*Urologic Oncology: Seminars and Original Investigations, In Press*)

Forde JC., Maginn E., McNamara G., **Martin LM.,** Campiani G, Williams C, Zisterer D., McElligott A., Lawler MP., Lynch TH., Hollywood D., Marignol L. "Microtubule-Targeting-

Compound PBOX-15 Radiosensitizes Cancer Cells In Vitro" (*Cancer Biology and Therapy, In Press)*

AWARDS

2010 Awarded a **Scholars in Training Travel Grant** to attend the Radiation Research Annual meeting in Maui, September 25th-30th 2010.

Awarded 1 of 7 **Health Research Board awards** for Irish citizens to attend the **National Cancer Institute course in Cancer Prevention 2010**, Washington DC. August 9-13th. The grant covered travel, accommodation and subsistence costs.

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Wolfsberg Travel Award to attend the 11th International Wolfsberg Meeting, Switzerland, June

Student bursary (European Association for Cancer Research) to attend the Association for Radiation Research Annual Meeting, Glasgow, June

Best Poster award (European Association for Cancer Research) at the Association for Radiation Research Annual Meeting, Glasgow, June

Travel bursary to attend Cancer 2009 Conference, Dublin, May

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Best Poster award at Association for Radiation Research, Belfast

Oncology Scholars Travel Award from the Irish Cancer Society to attend the ARR annual conference in Belfast

PRESENTATIONS

ORAL PRESENTATION

Martin LM., Marples B., Davis T., Coffey M., Lynch TH., Hollywood D., Marignol L. "Prostate cancer cells can be killed by low doses of radiation through a mechanism that involves MSH2; implications for improved multimodal treatment of prostate cancer" Radiation Research Annual Meeting, Maui September 25th – 30th 2010

ORAL POSTER

Martin LM., Marples B., Coffey M., Lawler M., Hollywood D., Marignol L. "Prostate cancer cells can be killed by low doses of radiation through a mechanism that involves MSH2; implications for improved multimodal treatment of prostate cancer" Irish Association for Cancer Research, 2010 Annual Meeting, Galway March 3-5th 2009

POSTER

Martin L., Marples B., Coffey M., Lawler M., Hollywood D., Marignol L. "Prostate cancer cells can be killed by low doses of radiation through a mechanism that involves MSH2; implications for improved multimodal treatment of prostate cancer" Institute of Molecular Medicine 12th Annual Meeting, November 13-14th 2009

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Martin L., Marples B., Coffey M., Lawler M., Hollywood D., Marignol L. "Processing of O6MeG lesions by the mismatch repair system may represent a novel mechanism for Low-Dose Radiation Hypersensitivity" 11th International Wolfsberg Meeting on Molecular Radiation Biology/Oncology, Switzerland, June 27th-29th 2009

Martin L., Marples B., Coffey M., Lawler M., Hollywood D., Marignol L. "Processing of O6MeG lesions by the mismatch repair system may represent a novel mechanism for Low-Dose Radiation Hypersensitivity"Association for Radiation Research Annual Meeting, Glasgow, June 22nd-24th 2009

Martin L., Marples B., Coffey M., Lawler M., Hollywood D., Marignol L. "DNA Mismatch Repair Proficiency may be a prerequisite for Low-Dose Radiation Hypersensitivity" Irish Association for Cancer Research Annual Meeting, Athlone, Co. Meath, 5th-6th March 2009

Martin L., Marples B., Coffey M., Lawler M., Hollywood D., Marignol L. "Role of O6MeG lesions in low-dose hyper-radiosensitivity in prostate cancer" 4th All-Ireland Cancer Conference, Dublin, November 30th to December 3rd 2008

Martin L., Marples B., Coffey M., Lawler M., Hollywood D., Marignol L. "Role of O6MeG lesions in low-dose hyper-radiosensitivity in prostate cancer" 54th Annual Radiation Research Society Meeting, Boston, MA, 21-24th September, 2008

TABLE OF CONTENTS

LIST OF FIGURES

Chapter 1: Introduction

Chapter 3: Characterisation of low dose radiation hypersensitivity in prostate cells

Chapter 4: Recognition of O6MeG lesions by MGMT and MMR proficiency may be a prerequisite for low-dose radiation hypersensitivity

Chapter 5: DNA Mismatch Repair protein MSH2 participates in low dose radiation hypersensitivity via regulation of MRE11 and the early G2 checkpoint

Chapter 6: DNA Mismatch Repair protein MSH2 sensitises cells to low doses of radiation by suppressing RAD51 recombination repair of DNA Double Strand Breaks

Chapter 7: Validation of the proposed MSH2-dependent mechanism of HRS in PC3 prostate cancer cells

Chapter 8: Discussion

LIST OF TABLES

Chapter 1: Background and Introduction

1.1 INTRODUCTION

Radiotherapy (RT) has been used in the treatment of cancer for over 100 years. RT is currently utilised in the treatment of approximately half of all oncology patients during the course of their illness. The therapeutic intent of radiation oncology is to deliver a sufficiently lethal dose to the target volume to achieve local tumour control while minimising the harmful effects to normal tissues, in order to avoid treatment-related acute side effects and late morbidity. This is typically achieved in clinical RT by fractionating the radiation dose, which exploits the radiobiological concept that the radiation repair capability of the sublethal damage is deficient in tumour cells compared with normal cells. Moreover, it exploits the difference in the alpha/beta ratios between the tumour and the surrounding normal tissue.

RT was originally delivered using live sources of radiation such as radium, which was used in various forms until the mid-1900s, when cobalt and caesium units came into use. Medical linear accelerators have been used as sources of radiation since the 1940s. The invention of computed tomography by Godfrey Hounsfield in 1971, allowed the development of three-dimensional treatment planning which is responsible for the shift from 2-D to 3-D protocols. Today, while orthovoltage machines still have specific uses, cobalt machines have largely been replaced with megavoltage linear accelerators, which are not only safer due to the absence of a physical radiation source, but also allow greater tissue penetration. The development of new imaging technologies such as magnetic resonance imaging (MRI) in the 1970s, and positron emission tomography (PET) in the 1980s, has dramatically changed the delivery of RT, and made possible the move from 3D-conformal radiotherapy (3D-CRT) to intensity modulated radiotherapy (IMRT) and image guided radiotherapy (IGRT). These techniques allow better visualisation and delineation of the treatment target, and have consequently resulted in improved treatment outcomes, and sparing of organs at risk (Bernier et al, 2004).

The radiation doses prescribed in current practice are based on the clinically determined radiation tolerance of the surrounding normal tissues, and a trade-off between normal tissue toxicity and

tumour control is often required. A number of factors can influence normal tissue tolerance including dose, fractionation, the volume irradiated as well as individual variation in radiation sensitivity (McKay and Peters 1997). RT protocols have evolved to limit the proportion of highly radiosensitive adverse reactions to about 0.5–5% of cases (Norman et al. 1988). Despite great clinical progress in the field there remains a small proportion of individuals which develops severe normal tissue reactions, the underlying molecular basis for which is currently imperfectly understood.

There is accumulating evidence that in certain tumours, RT needs to be delivered in higher than 'conventional' fractionated doses in order to achieve improved tumour control probability (TCP). To achieve this, an increasing number of RT techniques including 3D-CRT and IMRT, use multiple beams of radiation to conform the dose to the three-dimensional shape of the tumour, allowing an increased dose to be delivered to the target volume, while minimising the dose to the surrounding normal tissue. However, concern has been raised regarding the carcinogenic potential of exposing a large volume of normal tissue to such low doses of ionising radiation (IR) (Hall and Wuu 2003; Kry et al. 2005; Hall 2006; Ruben et al. 2008; Hall 2009).

1.2 PROSTATE CANCER AND RADIOTHERAPY

Prostate cancer (PCa) is the second most frequently diagnosed malignancy in men worldwide (13.8% of total) after lung cancer, currently affecting approximately 900,000 men worldwide (Ferlay et al. 2010). The death rate from PCa is significant, being the sixth leading cause of death from cancer in men (6.1% of total) with the estimated deaths from the disease in 2008, being 258,000 (Ferlay et al. 2010). The advent of prostate specific antigen (PSA) testing has transformed the presentation of PCa. The proportion of patients presenting with early stage disease has increased from 58%, in the mid-1980s (Stanford et al. 1999), to 80% (Horner et al. 2009) meaning that more and more patients are presenting at a stage when curative treatment is available.

Curative treatment options include radical prostatectomy, interstitial brachytherapy and external beam radiotherapy (EBRT). Radical prostatectomy offers complete removal of the tumour and

surgical staging; however, modern radical RT can now offer comparable disease control rates, at least in the short term, with less morbidity (D'Amico et al. 1998; Potters et al. 2004). Dose escalation to 80Gy or higher is currently indicated for local and biochemical control of localized PCa based on retrospective and randomized studies (Zelefsky et al. 1998; Vicini et al. 2001; Hanks et al. 2002; Pollack et al. 2002; Kupelian et al. 2005; Sathya et al. 2005; Zietman et al. 2005; Pinkawa et al. 2009). In practice however, doses are restricted due to the incidence of normal tissue complications such as late rectal bleeding, which has been linked to the volume of normal tissue irradiated (Lee et al. 1996; Odrazka et al. 2010).

EBRT is used in the treatment of approximately 64% of patients undergoing radiotherapeutic management for PCa (Zelefsky et al. 2004). Intensity-modulated radiation therapy (IMRT) is an EBRT technique that allows delivery of escalated doses to the target volume. Dosimetric studies have determined that IMRT is superior to 3D-CRT in terms of target coverage, conformity, and functional sparing of critical organs. In addition, IMRT is superior with respect to loco-regional control and offers survival outcomes equivalent to those with 3D-CRT (Zelefsky et al. 2001; Koontz et al. 2009; Vergeer et al. 2009; Digesu et al. 2010).

While IMRT offers superior normal tissue complication probabilities compared to 3D-CRT, it may increase the risk of a second fatal cancer by a factor of 1.2–8 (Verellen and Vanhavere 1999; Hall and Wuu 2003; Kry et al. 2005). This may occur for two reasons. Firstly, an increased volume of normal tissue will receive a lower, more carcinogenic radiation dose because of the increased number of radiation fields employed in IMRT protocols (Nutting et al. 2001). Secondly, the total body dose due to leakage radiation is likely to be increased relative to conventional 3D-CRT because an increased number of monitor units are required to keep the accelerator energized for enough time to deliver a specified dose to the isocenter from a modulated field (Hall 2006).

The use of brachytherapy as a curative treatment approach is becoming increasingly prevalent. Lee et al. reported the results of the 1999 patterns of care study, and observed a significant increase in the number of patients treated with brachytherapy, from 3% in 1994 to 36% in 1999 (Lee et al.

2003). Brachytherapy can be delivered using a low-dose-rate (LDR) (i.e. seed implantation) or high dose rate (after-loading procedure), either as a monotherapy for low-risk disease, or combined with EBRT or hormonal therapy for patients with adverse prognostic factors. The indications for these treatments differ, but their outcomes compare favourably in terms of quality of life and biochemical control (reviewed in (Koukourakis et al. 2009)). Allocation of patients according to risk categories prior to the commencement of therapy is thus particularly important for successful brachytherapy treatment.

Both low dose ionising radiation (IR) and LDR IR exposures are subject to recently discovered radiobiological phenomena that may potentially increase tumour cell kill, cancer risk or normal tissue reactions. These include; low dose radiation hypersensitivity, the adaptive response, the bystander effect and the inverse dose rate effect (IDRE) (Mullenders et al. 2009).

1.3 LOW DOSE HYPER-RADIOSENSITIVITY

Low dose hyper-radiosensitivity (HRS) is a recently described biological phenomena, that is characterized by an increased sensitivity to radiation doses less than 0.3Gy, which is followed by a more radioresistant response per unit dose between 0.3-0.6Gy termed increased radioresistance (IRR).

1.3.1 HRS AND CARCINOGENESIS

(HRS) is thought to be one of the mechanisms that may increase cell kill following irradiation and subsequently increase normal tissue reactions and protect against carcinogenesis by eliminating damaged cells following low dose IR (Marples and Collis 2008). The risk of carcinogenesis following low doses of radiation has long since been a subject of much controversy. The discovery of phenomena that may modulate the risk of carcinogenesis were thus of particular interest to the radiation community and it was for this reason that the phenomena of HRS became the focus of many scientific studies.

Clinical exposure to low doses of IR was originally considered harmless. Estimations of the stochastic effects were based on linear extrapolation from high-dose survival data as is currently recommended by standards organizations, such as the *International Commission on Radiological Protection* (Wrixon 2008). The data on which these estimates are based, are derived from the A-bomb database, a record of the health and mortality of the survivors of the Hiroshima and Nagasaki atomic bombs –which is considered the gold standard when assessing the health risks of radiation over the dose range of 0.1-2.5Gy. Data from this database suggest that the underlying dose–response relationship of IR and its stochastic effects is linear without any thresholds. However, a number of recently identified phenomena specific to the low dose region of the dose response curve have brought into question the validity of the linear no threshold model to predict cancer risk in the low dose region (Little 2010)(Fig. 1-1). Whereas the adaptive response suggests that this procedure would overestimate the risks of low dose exposures, the bystander effect, and concept of sensitive subpopulations suggest that such approximations may grossly underestimate the risk of low doses

of radiation. The nature of the dose-response relationship in this low dose region is thus a topic of lively debate.

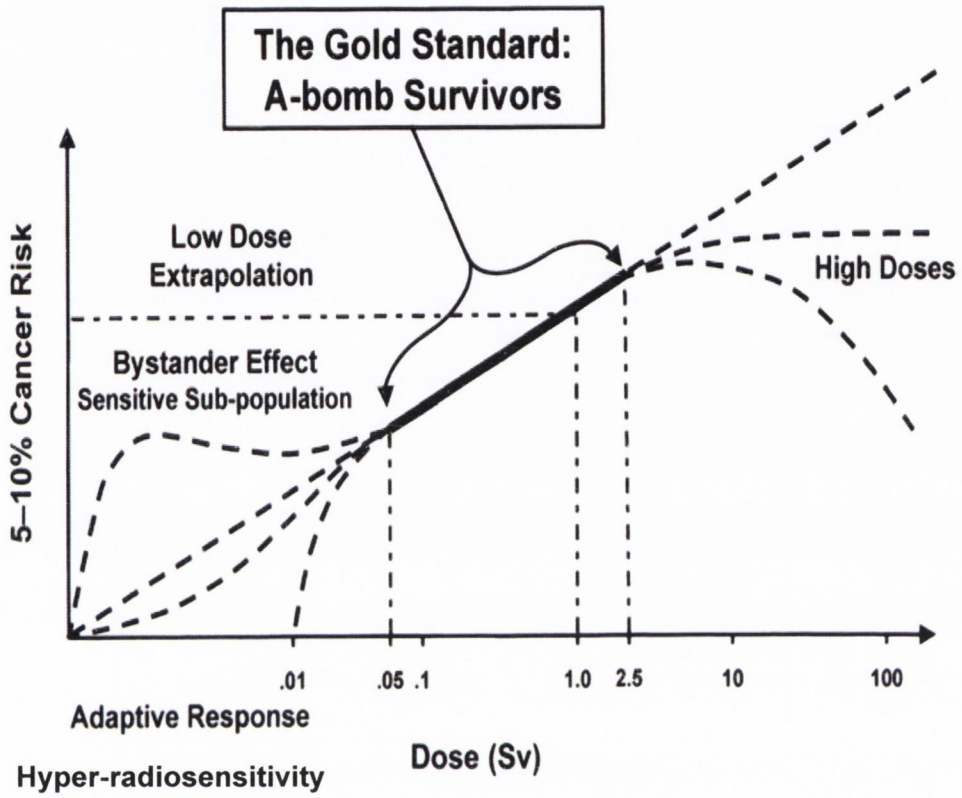

Fig. 1-1: Illustration of the dose–response relationship for radiation induced carcinogenesis in humans. The atomic-bomb data represents the "gold standard," data when quantifying the relative radiation risk over a dose range from about 0.1 to 2.5Gy. Uncertainty exists concerning the dose– response relationship at both high and low doses of radiation outside of this dose range. Image adapted from Hall et al. (Hall 2006).

1.3.2 *Evidence of HRS*

The HRS/IRR response can be defined or confirmed mathematically using the induced repair model (Fig. 1-2). Since its identification more than two decades ago (Joiner and Denekamp 1986), it has been demonstrated *in vitro* in approximately 75% of the 50 mammalian normal and malignant cell lines tested to date (Table 1-1) (Joiner et al. 2001) including a number of PCa cell lines (Fig. 1-3)(Table 1-1, and references therein). HRS has also been demonstrated *in vivo* in skin (Joiner et al. 1986), in lung and kidney tissue, metastatic tumour nodules (Harney et al. 2004), and normal human epidermis (Simonsson et al. 2008).

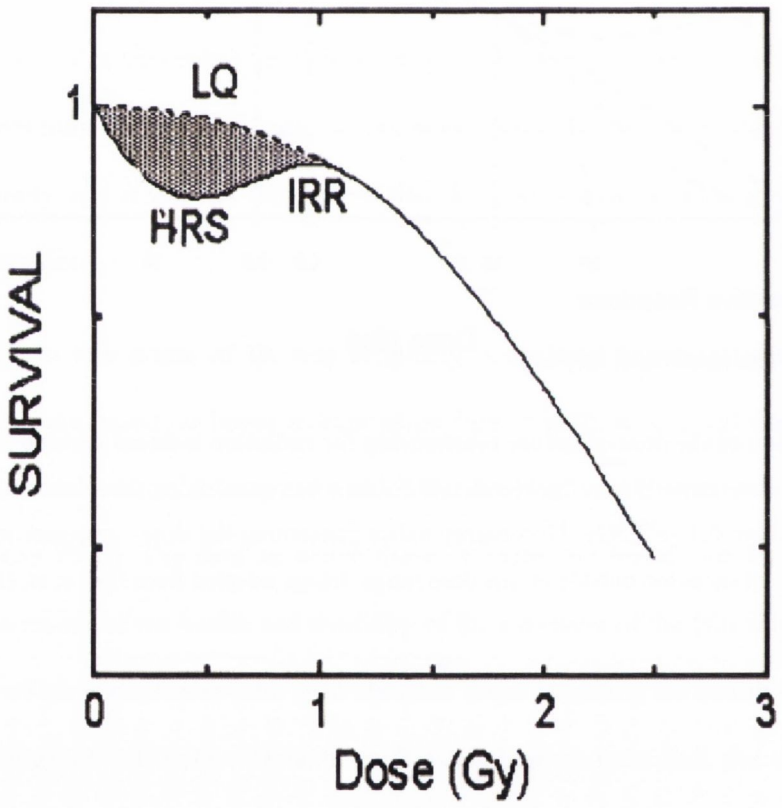

Fig. 1-2: Typical cell survival curve with evidence of hyper-radiosensitivity (HRS).
Broken line shows low-dose extrapolation from linear quadratic (LQ) model applied to high-dose survival data. Solid line shows induced repair fit. Image adapted from (Skov 1999)

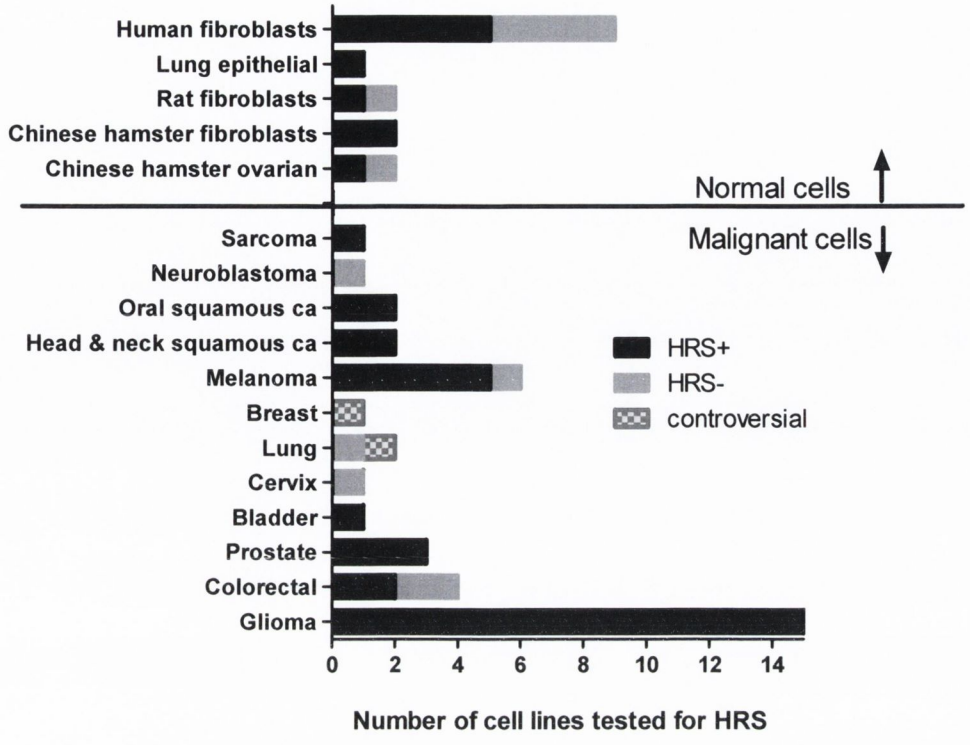

Fig. 1-3: Evidence for HRS *in vitro* by cell origin

To date, 53 cell lines from 16 different cell types (14 mammalian, 2 hamster) have been investigated for evidence of HRS. The most recent data indicate that 75% of cell lines (40/53) tested to date express the response.

Table 1-1: Prevalence of HRS by tumour origin/ cell type

	Cell Origin	HRS+ Cell lines	HRS- cell lines	Authors
Malignant cells	Glioma	T98G, CAL58, A7, HGL21, U123, BMG1, PECA-4451, U87-MG, DBTRG, MO59K, MO59J/Fus1, G5, G111, G142, G152	U373, U87-MG, MO59J, CL35 (subclone of G5)	(Short et al. 1999; Short et al. 1999; Joiner et al. 2001; Short et al. 2001; Chandna et al. 2002; Marples et al. 2002; Beauchesne et al. 2003; Wykes et al. 2006)
	Colorectal	HT29, RKO	HCT116, SW48	(Lambin et al. 1993) (Lambin et al. 1994) (Lambin et al. 1994) (Lambin et al. 1996) (Wouters et al. 1996) (Wouters and Skarsgard 1997)
	Prostate	DU145, PC3, LnCaP	DU145, PC3	(Wouters and Skarsgard 1994; Wouters et al. 1996; Garcia et al. 2006) (Hermann et al. 2008) (Mothersill et al. 2002) (Lin and Wu, 2005)
	Bladder	RT112		(Lambin et al. 1994; Lambin et al. 1996)
	Cervix		Siha	(Wouters et al. 1996)
	Lung	A549	A549, H460	(Enns et al. 2004) (Wouters and Skarsgard 1997; Beauchesne et al. 2003; Dai et al. 2009)
	Breast	MCF7	MCF7	(Beauchesne et al. 2003; Enns et al. 2004)
	Melanoma	MeWo, Be11, M4Be, A375P, SKMel2	U1	(Lambin et al. 1994) (Lambin et al. 1996) (Beauchesne et al. 2003)
	Head & neck squamous	SCC-61, SQ20B		(Dey et al. 2003)
	Oral Squamous	PECA-4451, PECA-4197		(Chandna et al. 2002)
	Neuroblastoma		HX142	(Vaganay-Juery et al. 2000)
	Human sarcoma	HS633T (soft tissue sarcoma)	ATBr1(osteosarcoma)	(Short et al. 2002) (Mothersill et al. 2002)
Normal cells	Chinese hamster ovarian	CHOAA8	CHO	(Bartkowiak et al. 2001)
	Chinese hamster fibroblasts	V79, V79379A,		(Marples and Joiner 1993) (Marples and Joiner 1995) (Lambin et al. 1994) (Marples et al. 1996; Garcia et al. 2006)
	Rat fibroblasts (oncogene-transformed)	MR4	3.7	(Wykes et al. 2006)
	Human fibroblasts	MSU-1, GS3, GM0639 cells (ATM+/+, termed GMcells), MRC5, HeLax skin human fibroblast hybrid cells (CGL1, CGL3), AT22IJE-TJ EBS7YZ5 (ATM complemented)	2800T, AT5BIVA cells (ATM-/-, termed AT cells) AT22IJE-TJ EBS7 (AT)	(Enns et al. 2004) (Ryan et al. 2009) (Joiner et al. 2001; Mothersill et al. 2002; Beauchesne et al. 2003; Redpath et al. 2003; Wykes et al. 2006; Xue et al. 2009)
	Human Keratinocyte		HaCAT, HPV-G	(Mothersill et al. 2002)
	Lung epithelial	L132		(Singh et al. 1994; Mothersill et al. 2002)

1.3.3 HRS/IRR IS ALSO RELEVANT AT HIGH DOSES AND LOW DOSE RATES

HRS appears to be a widespread phenomenon in the low dose radioresponse of mammalian cells. It has been observed in response to acute dose rate negative pi-mesons (Marples et al. 1996), high linear energy transfer (LET) radiation given at a low dose rate (Marples and Skov 1996), low dose neutrons (Dionet et al. 2000), protons (Schettino et al. 2001) and carbon ions (Xue et al. 2009). IRR however, is only evident after low and intermediate LET radiation exposures. Moreover, it has been demonstrated that the excess in cell killing observed at very low dose rates termed the "inverse dose rate effect" (IDRE), appears to be derived from the same radioprotective mechanism as HRS/IRR, and in fact, IDRE is thought to be a dose rate-dependent manifestation of HRS/IRR (Leonard 2007). In both instances, irradiated cells experience radioprotective transitions in cell killing from hypersensitive states to radioresistant states at discrete dose rate (for IDRE) and dose (for HRS/IRR) thresholds. Leonard et al. have demonstrated that IDRE only occurs in cell lines that express HRS (Leonard 2007).

Finally, it has been demonstrated that HRS may influence survival following EBRT if daily 2Gy radiation doses are delivered using multiple low dose fractions that add up to 2Gy. The authors demonstrated that delivery of partial fractions of a RT treatment such that the smaller fractions (<0.5Gy) are delivered before larger fractions (>0.5Gy), can increase cell kill *in vitro* (Lin and Wu 2005) and may therefore also have clinical relevance *in vivo*.

1.3.4 HRS MAY INFLUENCE NORMAL TISSUE REACTIONS

The potential implications of HRS for normal tissue reactions are an area of current debate. While the evidence indicates that the response has potential to be exploited in cancer cells, in normal cells it may be dose limiting. Much of what is known regarding the effects of HRS on normal tissues stems from studies utilizing human skin as a model for normal tissues. The importance of studying the effect of low radiation doses in human skin is clear; irradiation of skin is inevitable in RT treatments, it is readily observable and the propensity of skin to develop early and late reactions at radiation doses >1Gy/fraction following RT has been well documented. While skin does not always

represent the critical limiting factor for RT protocols, it serves as a valid model which will inform the radioresponse of other acute-reacting normal tissues.

Using a variety of endpoints (kidney function, clonogenic survival, basal cell density (BCD), proliferation, erythema, micronucleus assay, growth arrest, γH2AX foci), HRS has been demonstrated in lung epithelial cells and murine kidney, salivary glands (Joiner et al. 2001) as well as in various components of human skin including the epidermis, keratinocytes, and fibroblasts (see Table 1-2 and references within). This HRS response was observed after both single and repeated low dose fractions, indicating that HRS reactions are likely to occur following RT protocols incorporating low doses of radiation (e.g. IMRT). Modeling has shown that HRS in normal tissues will tend to increase the normal tissue complication probability, particularly in tissues with a pronounced volume effect and may thus affect treatment planning for IMRT (Honore and Bentzen 2006). However, the potential implications of this response in terms of normal tissue reactions are unclear. In skin, HRS appears to be relatively rare, occurring in only 6 of 40 cervical cancer patients treated with IR (Slonina et al. 2006), which did not correlate with acute or late normal tissue complications in these patients (Slonina et al. 2008). The influence of HRS as regards normal tissue complications in other models remains to be seen.

Table 1-2: Studies investigating the incidence of HRS in irradiated human normal tissues using various endpoints

Model	No. of subjects	Fractionation	Functional Endpoint	Results	Authors
Human skin	117 pts total: 65 pts 52 pts	treated palliatively with 5, 10, 12 and 20 daily treatment fractions (varying thicknesses of bolus, various body sites) undergoing prostatic irradiation for localised carcinoma of the prostate (no bolus, 30-32 fractions)	skin doses between 0.4 to 5.2Gy evaluated for erythema	LQ model significantly underpredicted peak erythema values at doses less than 1.5Gy per fraction	(Hamilton et al. 1996)
Human epidermis of prostate ca patients	47 pts	0.45Gy/fraction v 1.1Gy/fraction	Basal cell density Ki67, p53, p21	Greater loss of basal cells post 0.45Gy, growth arrest, increased p53 and p21 expression	(Turesson et al. 2001)
Human skin	24 (23 male, 1 female) 48 biopsies	3-field technique, 6-12MV photons bolus used to correct skin dose to 0.48Gy, 1.22Gy and 0.74Gy	Basal cell density	Greater reduction in BCD after 0.48Gy skin doses	(Harney et al. 2004) Study 1 of 2
Human skin	8 pts with metatstatic tumour nodules	1xdaily dose ~0.5Gy and >1Gy UF (0.5Gyx12 daily doses)	BCD & proliferation (Ki-67, Cyclin A)	Greater BCD reduction in high dose/# arm	(Harney et al. 2004) Study 2 of 2
Normal cells of cervical C.a patients	Skin fibroblasts and keratinocytes derived from 40 pts	Survival curve 0.05-4Gy	the fraction of binucleated cells with micronuclei	Low incidence of HRS in fibroblasts (2/40) and keratinocytes (4/40)	(Slonina et al. 2006) (Slonina et al. 2007)
Keratinocytes and fibroblasts of cervical Ca. patients	skin fibroblasts from 21 pts & keratinocytes derived from 23 pts	0.25Gy TDS, 4h interval, 0.75Gy x1, 0.5Gy TDS, 4h interval, 1.5Gy x1	Micronucleus assay	Inverse fractionation effect observed in pts expressing HRS after multiple low doses	(Slonina et al. 2007)
Human epidermis	64 Skin biopsies from 5 PCa pts	Samples taken 30 min after the first dose fraction at locations of the skin corresponding to approximately 0.1, 0.2, 0.45 and 1.1Gy. Additional biopsies, corresponding to 0.45 and 1.1Gy, were taken 2hr after the first fraction and after 1 wk of txt.	H2AX foci, apoptotic cells	γH2AX foci pattern in biopsies taken 30 min after a single fraction revealed HRS below 0.3Gy no decrease in foci observed at 2hr post IR	(Simonsson et al. 2008)
Keratinocytes and fibroblasts of cervical Ca. patients	40 pts, 32 of which received RT	0.25Gy TDS, 4h interval, 0.75Gy x1, 0.5Gy TDS, 4h interval, 1.5Gy x1	Micronucleus assay Maximum grade of acute and late tissue reactions	no significant relationship found between MN induction, either in fibroblasts or keratinocytes, and acute and late effects	(Slonina et al. 2008)
Keratinocytes of prostate ca patients	89 pts 25 pts	7 weeks, EBRT	1& 6.5 wks after txt Growth arrest (p21) mitosis (p-H3) cell death (γH2AX)	HRS induced: increase in p21 decrease in mitosis persistent foci	(Turesson et al. 2009)

1.4 THE MECHANISM OF HRS

The potential for an increased risk of carcinogenesis due to HRS is unclear and is very much dependent on the mechanism behind the low-dose–response. Accordingly, much work has been carried out to elucidate the mechanism of HRS. It is generally believed that defects in a number of components of the DNA damage response to IR may be responsible for the HRS response, and while the underlying mechanism remains to be elucidated, the consensus is that the relative increase in cell kill observed is derived from a protective mechanism that has evolved to prevent genomic instability by removing those cells at risk of mutation. At higher doses then, where the entire population is at risk, the biological focus is thought to switch from that concerning potentially lethal damage to a means to preserve the entire population (Marples and Collis 2008).

The studies in the field fall into one of three categories: (1) those suggesting a role for DNA repair (2) those suggesting a role for the cell cycle, and (3) those investigating the clinical implications/relevance of the effect.

1.4.1 EVIDENCE SUPPORTING THE INVOLVEMENT OF DNA REPAIR PROCESSES

Extensive evidence suggests a role for DNA repair in the HRS/IRR response (Fig. 1-4). Support for the involvement of DNA repair processes stems primarily from work demonstrating the dependence of HRS on LET (discussed in 1.3.1), a role for the non homologous end-joining (NHEJ) repair pathway in IRR, and evidence of persistent RAD51 foci, an essential recombinase in the HR double strand break (DSB) repair pathway, following exposure to low radiation doses.

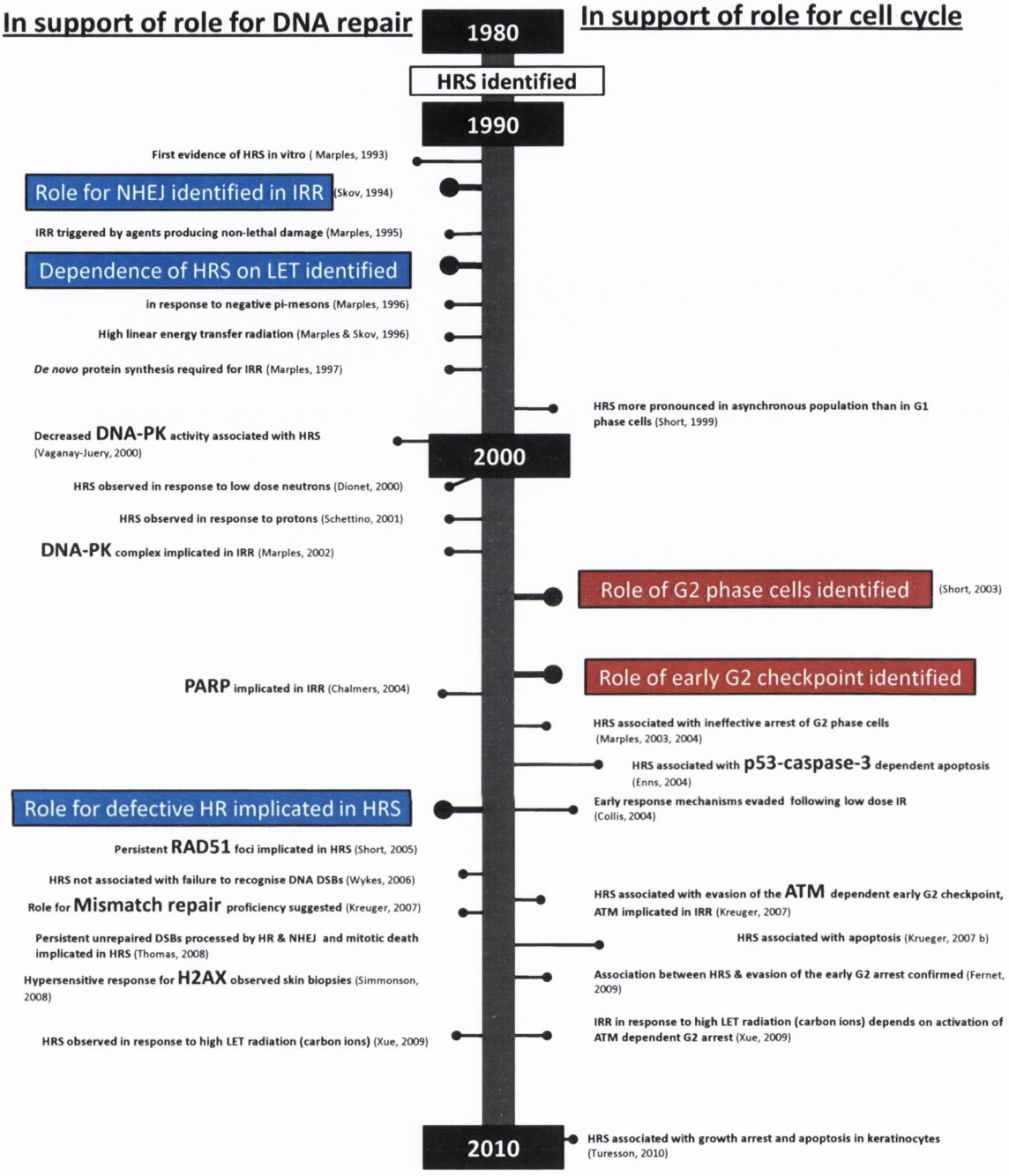

Fig. 1-1: Studies informing the current understanding of the mechanism of low dose radiation hypersensitivity

1.4.1.1 IRR is dependent on a functional NHEJ response

The dependence of IRR on a functional NHEJ response was one of the first reported attributes of the HRS/IRR phenomena (Skov et al. 1994). NHEJ is the primary DNA double strand break repair pathway in mammalian cells active during G0, G1 and early S phases of the cell cycle (reviewed in (Kass and Jasin 2010). A specific role for two components of this pathway; PARP (Poly-ADP ribose polymerase) (Chalmers et al. 2004) and DNA-PK (DNA-dependent Protein Kinase) has been demonstrated (Vaganay-Juery et al. 2000; Marples et al. 2002) in IRR, following observations that the IRR response is eliminated in PARP and DNA-PK deficient cell lines. Vaganay-Juery et al. reported decreased DNA-PK activity in association with HRS. Indeed, a number of other studies also suggest that the transition to IRR is the result of an inducible repair mechanism; a response similar to IRR is triggered by non-lethal doses of DNA-damaging agents (Marples and Joiner 1995). Moreover, the IRR response has been demonstrated to require *de novo* protein synthesis (Marples et al. 1997).

1.4.1.2 HRS is associated with persistent RAD51 foci

Evidence that persistent RAD51 foci are common at late time points after low dose IR exposure also implicates a role for DNA repair in HRS. Short et al. (Short et al. 2005) reported that RAD51 foci co-localised with BRCA2 foci, are common following low doses of radiation in glioma cells hypersensitive to low radiation doses. Co-localisation of RAD51/BRCA2 foci is thought to be indicative of HR repair. Consistent with this observation, Thomas et al. recently demonstrated a higher frequency of unrepaired DNA DSB processed by the NHEJ and by the RAD51-dependent HR pathways in hypersensitive compared with non-hypersensitive cells derived from the same tumour (Thomas et al. 2008). However, it may also represent sites of inefficient or dysregulated HR, for example sites at which there has been failure to locate a homologous partner for exchange, inappropriate RAD51 binding or self-self interaction as a result of high RAD51 protein levels (Short et al. 2005; Schild and Wiese 2009). While not the most widely demonstrated response associated with hypersensitive cells, a model in which the RAD51 recombination pathway may

have an important influence on the survival following low doses of radiation is consistent with observations that HRS is most pronounced in G2 phase cells (Marples et al. 2003; Krueger et al. 2010). Moreover, it may provide an explanation for the prevalence of HRS in radioresistant cancer cells (Joiner et al. 2001) given that RAD51 is over-expressed in many tumours (Klein 2008).

1.4.1.3 DNA double strand breaks and HRS

A particular type of DNA damage has yet to be definitively affiliated with HRS. While, it has been demonstrated that HRS is not the result of a failure to recognise DNA DSBs (Wykes et al. 2006), a number of observations implicate DNA DSB repair pathways in the HRS/IRR response. These include an inducible NHEJ response associated with IRR, and persistent RAD51 foci evident at late time points after low dose IR as mentioned previously. In addition, a hypersensitive response for H2AX has been observed in skin biopsies (Simmonson, 2008) suggesting a role for DSBs specifically in HRS in epidermal cells at least.

1.4.1.4 DNA MMR and HRS

DNA mismatch repair (MMR) was most recently implicated in HRS following observations that an absent HRS response was associated with MMR-deficiency in a panel of 3 cell lines (Krueger et al. 2007). DNA MMR is a highly conserved DNA repair pathway that recognizes and repairs base-pairing errors that arise during DNA replication or recombination (Stojic et al. 2004; Surtees et al. 2004). This pathway has been largely studied in the DNA damage response to chemotherapeutic agents, but the activation of the MMR system after IR remains controversial (Brown et al. 2003; Cejka et al. 2004). Accumulating data suggest that MMR proteins may be involved in the DNA damage response to IR, via recognition of IR induced DNA damage including oxidative damage, clustered base damage, and DSBs, either directly or indirectly as a component of the BRCA1 Associated Genome Surveillance (BASC) complex (reviewed in (Martin et al. 2010)). Recognition of these lesions may then promote differential cell cycle and apoptotic responses. A role for MMR-proficiency was recently observed in the preferential response of cancer cells to prolonged LDR-IR (Yan et al. 2009), and may therefore also have relevance to HRS.

1.4.2 EVIDENCE SUPPORTING THE INVOLVEMENT OF THE CELL CYCLE

The dependence of IRR on functional DNA repair and the importance of cell cycle phase for the efficiency and propensity for particular types of DNA repair (Branzei and Foiani 2008) prompted investigation into the differential low dose radiosensitivity of cells in various phases of the cell cycle (Fig. 1-4). Early studies demonstrated that G1 phase cells were less sensitive to low dose IR than were the entire population (Short et al. 1999), later studies investigated the HRS effect in each cell cycle phase and demonstrated a more pronounced HRS response in G2 phase enriched cells, with little (Short et al. 2003) or no evidence of HRS in other cell cycle phases (Marples et al. 2003).

Mechanistic studies have also revealed a role for cell cycle checkpoints in HRS. Cell cycle checkpoints are surveillance mechanisms that block cell cycle transitions, and function to block cell cycle progression to allow repair of DNA damage prior to cell entry and transit through mitosis (Hartwell and Weinert 1989). This arrest is only released when repair is completed. Where repair is not possible, damaged cells are removed by programmed cell death (apoptosis) (Houtgraaf et al. 2006). Two distinct G2/M phase cell cycle checkpoints are known to be activated following exposure to IR (Xu et al, 2002), namely the early and late G2/M phase checkpoints. The first and so-called early G2/M checkpoint, is the response to DNA damage in cells that are already in G2 at the time of irradiation, and reflects the failure of these cells to progress to mitosis. The second G2/M checkpoint is activated at late time points after exposure and reflects the delay of cells in other phases of the cell cycle (G1, S) at the time of irradiation.

HRS has been repeatedly correlated with evasion of the early G2 checkpoint (Marples et al. 2003; Collis et al. 2004; Marples 2004; Krueger et al. 2007; Fernet et al. 2009; Xue et al. 2009; Krueger et al. 2010), with the transition from HRS to IRR reflective of the activation of the early G2 checkpoint, and thus inducible repair mechanisms (Krueger et al. 2007; Fernet et al. 2009; Xue et al. 2009; Krueger et al. 2010). However, a maintained growth arrest has also been associated with HRS in keratinocytes (Turesson et al. 2001; Turesson et al. 2010). These data contradict the most

recent working hypothesis for the mechanism of HRS indicating that HRS is the apoptotic response of cells that evade the early G2/M checkpoint (Krueger et al. 2010), and raises the question of whether the mechanism of HRS may be cell type dependent. It will therefore be important for future studies to resolve the involvement of cell cycle responses and reconcile this with DNA repair processes.

1.5 CLINICAL RELEVANCE

1.5.1 PCA RADIOTHERAPY AND HRS

A number of important mechanistic observations suggest that HRS may have critical relevance for the treatment of PCa. Numerous studies suggest that HRS is expressed in prostate cancer cells (Skov et al. 1994; Garcia et al. 2006; Hermann et al. 2008). Although it remains for the response to be mathematically defined in prostate cancer cells, the fact that IDRE has been demonstrated to occur in metastatic PCa cells (PC3)(Mitchell et al. 2002) suggests that both HRS and IDRE will be relevant to PCa treatment. Moreover, the use of ultrafractionated (UF) RT has been demonstrated to be of benefit in the treatment of a variety of tumour types and may therefore also be of benefit in the treatment of PCa. The rationale for UF is that repeated hypersensitive reactions to low doses of IR will have a cumulative effect, allowing increased cell kill.

UF (0.5Gy 3 times per day (TDS), 4h intervals, 7 days wk/2wks) has been demonstrated to significantly extend the tumour growth delay of metastatic tumours compared to conventional treatment (CT) (1.68Gy once daily (OD), 5 days/wk x 4wks) in a study of 40 matched tumour nodules from 8 patients (Harney et al. 2004). This study demonstrated various effects of UF in its participants, however when examining only those patients with tumour nodules expected to exhibit HRS, based on *in vitro* data (sarcoma, melanoma), it was observed that UF enhanced growth delay by 24% (range, 11–45%) in sarcoma nodules and 11% (range, -28% to +45%) in melanoma nodules. Use of UF for the treatment of metastatic prostate tumours will therefore depend on whether tumour cells are more susceptible to low doses than are the normal tissues.

HRS is however, also expressed in normal tissues. Moreover, while numerous groups have attempted to exploit the G2 phase dependent nature of HRS for a therapeutic gain using either low dose fractionated radiotherapy (LDFRT), or chemotherapeutic agents to increase the proportion of G2-phase cells in the tumour, the same may not necessarily be possible for the treatment of PCa. The radiobiology of PCa is thought to be unique in that it is thought to have a low alpha/beta ratio of 1.5Gy (although this is somewhat controversial), with a potential doubling time ranging from 16 to 61 days, the largest potential doubling time measured in human tumours (Haustermans et al. 1997; Marcu 2010), meaning that it is more likely to benefit from multiple high dose fractions (hypofractionated RT treatment) rather than the conventionally used 2Gy per fraction dose schedule EBRT, protracted low dose rate treatments (LDR brachytherapy) or multiple low doses of radiation (UF). However, until a consensus regarding the true alpha/beta ratio for PCa is reached it will be difficult to ascertain the true potential that UF may provide in the treatment of this tumour subsite.

1.6 HRS/IDRE AND NORMAL TISSUE REACTIONS

With respects to brachytherapy, it is thought that 50-80% of adjoining normal tissues is a reasonable estimate for the proportion of these tissues that will experience IDRE from permanent implanted LDR seeds (Leonard and Lucas 2009). Leonard et al. (Leonard and Lucas 2009) have shown that, certain normal tissues adjacent to prostate and cervical carcinomas (i.e. bladder, rectum, urinary tract and small bowels etc) are likely to experience an increase in cell killing following decay of the sources used, if they exhibit IDRE radioprotection at the higher dose rates used during calibration of the LDR treatment. This could then induce significant post-irradiation complications in those tissues (Leonard and Lucas 2009). Comparison of the side effects reported following high dose rate (HDR) brachytherapy treatment versus LDR brachytherapy at present is difficult given that total dose, additional treatments administered, number of seeds implanted and the distribution must be taken into account. However, it has been demonstrated that HDR brachytherapy treatment compared favourably to LDR brachytherapy in a study conducted at the Beaumont Hospital, Royal Oak, Michigan (Grills et al. 2004). They report the complications of

LDR (84 patients) vs HDR (65 patients) treatments as being higher with respect to: acute Grade 1-3 dysuria (64% and 33%); urinary frequencies (46% and 8%); rectal pain (96% and 80%); long-term urinary complications (50% and 32%); and 3-year impotency (45% and 15%). Should LDR treatments prove to induce more significant side-effects it may be possible that this could in part, be explained by the IDRE effect. It may stand to reason then, in such a scenario, that HDR brachytherapy would be a viable alternative for patients with tissues that express IDRE within the treatment field.

The field of radiogenomics and the characterisation of molecular profiles that predict normal tissue damage and tumour radioresponse are gaining rapid momentum. Biomathematical modeling has demonstrated that overall gains in therapeutic ratio could be achieved theoretically if dosage prescriptions were varied according to individual or subgroup sensitivities (Mackay and Hendry 1999). Elucidation of the mechanism of HRS as it relates to hypersensitive normal tissue reactions may therefore allow stratification of patients based on their likelihood of developing adverse tissue reactions. It may then be possible to escalate the total dose in the remaining majority of patients to achieve improved tumour control. Based on mechanistic studies a second crucial question is whether HRS/IDRE is a protective response, as potential failure to express these effects may actually be associated with carcinogenesis.

There is a trend for the increased use of HDR brachytherapy, regardless of the risk category, alone or in combination with EBRT. Providing trends continue in this direction, HRS is likely to be of greater concern in EBRT treatments, however elucidation of the mechanism will be relevant to both EBRT and LDR brachytherapy for as long as LDR brachytherapy is still in use.

1.7 SUMMARY

The HRS/IRR phenomenon has been extensively demonstrated in the past decade. The balance of evidence suggests it will have far reaching and varied implications for radiotherapy practices and public health. In particular, PCa is likely to be affected by HRS given the increasing use of protocols that involve low doses of radiation or LDR radiation (brachytherapy) in the

radiotherapeutic management of this disease. Evidence suggests that HRS may influence normal tissue reactions after IMRT and following LDR brachytherapy.

While *in vivo* studies continue to provide insight into the potential clinical implications of the effect in terms of exploitation of the response for a therapeutic benefit and implications for normal tissue reactions, no changes in current practices can be made until the mechanism is fully understood. Indeed, if the HRS response is mostly related to growth arrest and cell survival as is suggested by the most recent data in epidermal cells, the risk of mutagenesis is potentially greater than expected in patients lacking HRS. This may allow time for alternative more error-prone repair mechanisms to be triggered or may trigger a senescent state. On the other hand, if cell death governs the HRS response to low doses, this risk is eliminated. Thus, the most pressing and fundamental issue to resolve is how the DNA damage response affects life or death versus genomic stability following low doses of radiation (Turesson et al. 2010). In particular we believe the potential role of DNA MMR proteins in HRS may prove to be of interest in this respect. The potential role of these caretaker proteins in the HRS mechanism is further examined in this thesis.

1.8 SPECIFIC AIMS

In light of the potential implications of the low dose response, an investigation into the underlying mechanism of low dose radiation hypersensitivity was warranted. Given the accumulating evidence suggesting a role for MMR proteins in IR-induced DNA damage recognition, signalling and repair (reviewed in (Martin et al. 2010)), the role of MMR in this mechanism was of particular interest.

The specific aims of this thesis include:

1. To characterise the HRS response in a panel of prostate cells. In addition, we aimed to gain a greater understanding of the DNA repair pathways differentially activated in HRS-positive and HRS-negative cells after exposure to 0.2Gy using Taqman® Low Density Arrays.

2. To test our primary hypothesis that O6MeG lesions and MGMT and MMR proficiency may be involved in HRS in prostate cells.

3. To test our refined hypothesis, that thDNA MMR proteins MSH2 and MLH1 may be required for expression of HRS, and regulation of early G2 cell cycle checkpoint signalling.

4. To further determine the role of MSH2 in the efficiency of DNA repair and induction of cell death after 0.2Gy.

5. And finally, to validate the refined proposed mechanism for HRS in PC3 prostate carcinoma cells.

Chapter 2: MATERIALS AND METHODS

2.1 CELL CULTURE

2.1.1 CELL LINES

Malignant DU145, PC3, LnCaP and 22RV1 PCa cell lines, and prostate epithelial PWR1E and RWPE1 cell lines were obtained from the ATCC (UK). DU145, PC3, 22RV1 and LnCaP cells were grown in RPMI 1640 media supplemented with 10% FBS, and 1% penicillin/streptomycin (Invitrogen, Dublin, Ireland). Both PWR1E and RWPE1 cells are virally transfected; PWR1E cells contain Adenovirus 12 and SV40 DNA viral sequences, and RWPE1 cells contain a single copy of the human papillomavirus 18 (HPV-18). Both prostate normal cell lines were grown in keratinocyte serum free media supplemented with bovine pituitary extract, epidermal growth factor (Gibco, Paisley UK) and 1% penicillin/streptomycin (Invitrogen).

HEC59 and HEC59+chr2 cells were kindly provided to us by Dr. Thomas A. Kunkel (NIEHS, National Institutes of Health, Research Triangle Park, NC), in which chromosome 2 (containing wild-type *MSH2*) was introduced into *MSH2*-deficient human endometrial HEC59 carcinoma cells to create HEC59+chr2 cells. HCT116 and HCT116+chr3 cells were also generously provided to us by Dr. Thomas A. Kunkel, in which a normal human chromosome 3, which contains the *MLH1* gene, was introduced by microcell fusion into an MLH1-deficient human colon cancer cell line, HCT116. Both endometrial and colorectal cancer cell lines were maintained in advanced DMEM/F-12 media containing 10% FBS and 1% penicillin/streptomycin (Invitrogen). Chromosome corrected cells were supplemented with geneticin selective antibiotic (G418) (Sigma Aldrich, Wicklow, Ireland) at a concentration of 400μg/mL.

For routine maintenance, each cell line was grown as a monolayer in a cell incubator maintained at 37°C under 5% CO_2 and subcultured once or twice weekly to maintain exponential growth.

2.1.2 SUBCULTURING OF CELL LINES

All cell culture work was carried out aseptically in a Telstar Bio-II-A laminar flow cabinet. Cells were examined daily using an inverted phase contrast Nikon Eclipse TS100 microscope (Nikon, Melville, USA). Cells were sub-cultured when culture flasks reached 70-80% confluency, so as to maintain exponential growth. Initially, the growth medium was decanted from the culture flask and cells were rinsed with 3mL of 0.01M phosphate-buffered saline (PBS, 13.8 mM NaCl, 2.7mM KCL, pH 7.4) preheated at 37°C in order to remove residual FBS. Cells were detached using 1mL ($25cm^2$ flask) or 3mL ($75cm^2$ flask) of 1 x trypsin-ethylenediamine tetra acetic acid (EDTA, 0.05% (w/v) trypsin, 0.02% (w/v) EDTA) and incubated for approximately 5-10min at 37°C until all cells had detached from the flask. Culture medium (5mL) was added to neutralise the trypsin and cells in suspension were transferred to a sterile tube and pelleted by centrifugation at 1300rpm for 3min in a Megafuge 1.0 centrifuge (Kendro Laboratory Products, Germany). Following centrifugation, the supernatant was discarded and the pellet was resuspended in 3-5mL complete media. Cells were subsequently seeded and maintained in 10mL ($25cm^2$ flask) or 20mL ($75cm^2$ flask) of the appropriate cell culture media in a cell incubator (section 2.1.1).

2.1.3 PREPARATION OF FROZEN CELL CULTURE STOCKS

Cell stocks were prepared from early passage cells growing in exponential phase. Cells were harvested with trypsin as described (section 2.1.2). Cells were then pelleted by centrifugation and resuspended in complete culture medium containing an addition 10% FBS and 5% (w/v) dimethyl sulfoxide (DMSO). Aliquots of 1mL were then transferred to cryovials and stored in a Nalgene® Mr. Frosty freezing container (Thermo Scientific Nalgene, Dublin, Ireland) prefilled with 2-isopropanol (Sigma Aldrich) at -80°C overnight. This provides the critical, repeatable, 1°C/min cooling rate required for successful cryopreservation of cells. After this time, cryovials were transferred to a liquid nitrogen tank (Forma Scientific Inc., OH, USA) for long term storage.

2.1.4 REVIVAL OF FROZEN STOCKS

Cryovials containing frozen cell culture stocks were removed from liquid nitrogen at -80°C and thawed rapidly at 37°C in a water bath with gentle agitation. Cell suspensions were transferred into sterile tubes and 10mL of complete medium was added. The cells were pelleted by centrifugation at 1300rpm for 3min to remove residual DMSO. After centrifugation, the cell pellet was resuspended in 2mL of complete medium and transferred to a $25cm^2$ flask. Cells were allowed to adhere overnight and non-viable cells were removed the following day by replacing the cell culture medium with fresh complete media pre-heated to 37°C. Flasks were maintained and subcultured as previously described (section 2.1.2).

2.1.5 CELL COUNTING

Cells were counted using a bright-line haemacytometer (Hausser Scientific, PA, USA). Cell viability was assessed using a trypan blue dye exclusion assay. 10µL of cells in suspension was added to 90µL of 0.4% (w/v) Trypan blue solution (Gibco), and allowed to stand for 1 to 2min before 10µL of the suspension was added to the counting chamber of the slide. Viable cells exclude trypan blue and are unstained, whereas dead cells stain blue due to their disrupted membranes. The number of viable cells was counted in each of the four corners of the grid. The following equation was used to calculate the total number of viable cells per mL:

(Number of cells counted x 10, 000)/4 = number of cells/mL

2.1.6 MYCOPLASMA TESTING OF THE CELL LINES

Cell lines were tested for mycoplasma every three months using MycoAlert® mycoplasma detection kit (Cambrex, UK). Briefly, working solutions were prepared by addition of Buffer to both reagent and substrate. A medium sample was collected from cells cultured for several passages in antibiotic free medium. This sample was centrifuged for 5min to remove cell debris, and then a 100µL aliquot was placed in a 96-well plate. To begin the assay, 100µL of MycoAlert® reagent was added, and the samples were incubated for 5min. A reading of the cells was taken with a luminometer (Reading A) and then 100µL of the substrate was added. After 10min incubation,

the luminescence was determined again (Reading B). A ratio was then calculated (Reading A/Reading B). A value below 1 was considered negative.

2.1.7 CHEMICALS AND CELL TREATMENTS

Temozolomide (TMZ) and O6-benzylguanine (O6BG) (Sigma, Wicklow, Ireland) were solubilised in DMSO (Sigma) as a stock solution of 100mM and 10mM respectively. Temozolomide was further diluted in cell culture media and cells were treated for 72h. O6BG (10μM) was given 1h before TMZ treatment in order to deplete MGMT, and was replaced in fresh medium once TMZ conditioned medium was removed. O6BG was also given 1h before irradiation and left on the plates in order to inactivate any newly synthesized methylguanine methyltransferase (MGMT).

Cisplatin (Sigma Aldrich) was diluted in distilled water to obtain a stock solution at 1mg/mL. Cells were treated at a final concentration of 25μM for 72hr by adding 37μL of the 1mg/mL solution to 5mL culture medium.

A commercially available Chk2 specific inhibitor was used (Sigma Aldrich). Chk2 inhibitor II is a cell-permeable and reversible benzimidazolo compound that acts as a potent and ATP-competitive inhibitor of Chk2 with an IC50 of 15nM and a Ki of 37nM. It displays ~ 1,000-fold greater selectivity over Cdk1/B and CK1 (IC50 = 12μM and 17μM, respectively) and only weakly affects the activities of a panel of 31 kinases (< 25% inhibition at 10μM). It was diluted in DMSO and distilled H_2O (dH_2O) to obtain a stock solution of 1mM. A working solution at 0.1mM was made and cells were treated at a final concentration of 15nM for 1h before irradiation and for 24hr after irradiation. At 24hr the media was replaced with fresh complete culture media preheated to 37°C.

17-allylamino-17-demethoxygeldanamycin (17-AAG) (Sigma Aldrich) was diluted in DMSO and dH_2O to make a stock solution of 1mM. A working dilution of 0.1mM was used.

Mirin (Sigma Aldrich) was diluted in PBS to obtain a stock solution at 2.27mM. Serial dilutions were performed to obtain a working stock dilution at 100nM.

Hoechst 33342 stock solution at 10mg/mL in water (Molecular Probes, H3570) was diluted to a 1:1000 solution before use. 0.1mL Triton-X 100 was diluted in 50mL PBS to obtain a 1:500 solution (0.2% solution).

All stock solutions were aliquoted and stored at -20°C for no longer than 3 months.

2.2 IRRADIATION PARAMETERS

Two different dose rates were used to deliver doses from 0-6Gy to ensure accurate dosimetry at the lowest doses. The change in dose rate was necessary to ensure accurate dosimetry at the lowest doses. Cells were irradiated as monolayers in 6-well plates or $25cm^2$ flasks at a dose rate of 0.75Gy min^{-1} (0-1Gy) or at 3.25Gy min^{-1} (2-6Gy) at room temperature using an Xstrahl RS225 molecular research system (Gulmay Medical Ltd. U.K.).

2.3 CLONOGENIC SURVIVAL ASSAY

Cell survival was evaluated using a standard colony-forming assay. A total of 500-6000 cells were plated per well in 6-well plates or $25cm^2$ flasks for low to high doses of radiation (0-6Gy). After incubation at 37°C for 7-9 days for PWR1E and RWPE1, 9-10 days for DU145, PC3, HEC59, HEC59+chr2 HCT116 and HCT116+chr3, and 10-14 days for 22RV1 cells, the resultant colonies were stained with crystal violet in 95% ethanol, and those consisting of greater than 50 cells were scored as representing surviving cells using ColCount™ (Oxford Optronix Ltd., Oxford, UK). The surviving fraction was calculated using the plating efficiency (PE) of irradiated cells/unirradiated cells.

Surviving Fraction (SF) = Plating efficiency of treated cells

Plating efficiency of untreated cells

2.3.1 DATA ANALYSIS FOR SURVIVAL ASSAYS

Surviving fractions measured at the doses tested were fitted with the Induced-Repair equation (Eqn. 1) as described previously (Marples and Joiner 1993).

$$S = \exp\left\{-\alpha_r\left(1+\left(\alpha_s/\alpha_r - 1\right)e^{-d/d_c}\right)d - \beta d^2\right\} \qquad \text{Eqn. 1}$$

Where d is dose, and α_s represents the low-dose value of α (derived from the response at very low doses), α_r is the value extrapolated from the conventional high-dose response, d_c is the 'transition' dose point at which the change from the very low-dose HRS to the IRR response occurs (i.e. when α_s to α_r is 63% complete) and β is a constant as in the LQ equation. All parameters were fitted simultaneously and estimates of uncertainty were expressed as likelihood confidence intervals. The presence of low dose hyper-radiosensitivity is deduced by values of α_s and α_r whose confidence limits do not overlap and a value of d_c (the change from low to high dose survival response) significantly greater than zero.

2.4 MOLECULAR BIOLOGY

2.4.1 *RNA ISOLATION*

Total RNA was isolated from 3-5 x 10^6 cells using the RNeasy Mini Kit (QIAGEN, Valencia, USA). After harvesting, the cells were centrifuged to remove cell debris. The supernatant was removed and the cell pellet was then vortexed and lysed by the addition of 350µL RLT buffer, a highly denaturing guanidine-thiocyanate–containing buffer. This immediately inactivates RNases to ensure purification of intact RNA. The cells were then added to a QIAshredder column and centrifuged at 13,000rpm for 2min to homogenize the sample. Ethanol (70%) was then added to provide appropriate binding conditions, and the sample was applied to an RNeasy Mini spin column. 350µL of RW1 buffer was then applied to the column and the sample was centrifuged at 10,000 rpm for 15sec. The flow through was then discarded. DNase (80µL) was then added to the RNeasy column and allowed to sit at room temperature for 15min. The RNeasy column was then transferred to a new 2mL collection tube and 500µL RPE buffer was added to the column. The sample was again centrifuged at 10,000rpm for 15sec and the supernatant was discarded. A second volume of RPE buffer (500µL) was added to the column, and the sample was centrifuged at 13,000rpm for 2min. The centrifuge step was repeated for 1min to remove any liquid in the column. High-quality RNA was then eluted in 30–100µl RNAse-free water following centrifugation. The yield of total RNA obtained was determined using the Nanodrop.

2.4.2 *cDNA SYNTHESIS*

Total RNA (2µg) was converted to cDNA with a first strand High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Ltd., Warrington, Cheshire, UK). Samples were processed at a final volume of 25µL following the addition of RNase-free water. The samples were then vortexed, centrifuged briefly, heated at 70°C for 10min to denature the RNA and subsequently cooled on ice for 5min. An appropriate volume of reverse-transcription reaction master mix was then prepared. For each sample the following was required: 5µL 10x RT reaction buffer, 2µL 100mM dNTPs, 5µL 10x RT random primers, 2.5µL multiscribe reverse transcriptase and 10.5µL

sterile distilled water. Master mix (25μL) was added to each sample. The sample was then briefly vortexed and centrifuged. The sample was then placed in an automated DNA thermal cycler and the following programme was run: 10min at 25 °C. 2hr at 37°C, and 85°C for 5sec. The cDNA was then stored at -20°C until required.

2.4.3 *Quantitative RT-PCR*

Quantitative RT-PCR was used to compare the expression levels of *MGMT* between cell lines. Initially all reagents and cDNA samples were thawed on ice, vortexed and centrifuged briefly before use. An appropriate volume of PCR master mix was then prepared. For each sample the following was required: 5μL Taqman® PCR master mix (2x), 0.5μL Pre-defined Taqman® assay reagent (PDAR) (MGMT or PGK1), and 2μL RNase-free water. 7.5μL of this solution was then added to the required number of wells in a 96 well plate. 100ng cDNA in 2.5μL was then added to the appropriate wells. The plate was then covered with an optical adhesive cover and centrifuged briefly to remove any air bubbles. The *MGMT* cDNA and internal control cDNA (*PGK1*) were then PCR-amplified separately using the 7900HT system (Applied Biosystems). Initial template concentration was derived from the cycle number at which the fluorescent signal crossed a threshold in the exponential phase of the PCR reaction. Relative gene expression was determined by the threshold cycles for the *MGMT* gene and the *PGK1* gene.

2.4.4 *Analysis of gene expression using Taqman Gene expression arrays*

High-throughput analysis of gene expression was performed using TaqMan® Low Density arrays (LDA) (Applied Biosystems). These are 384-well micro-fluidic cards onto which selected TaqMan® assays have been pre-formatted (a list of genes in included in Appendix I). Assays were present in quadruplicate on each card, and the assay for GAPDH gene expression was used as the endogenous control. TaqMan® Universal PCR Master Mix, No AmpErase® UNG (50μL) was added to thawed cDNA (100ng) and DNase/RNase-free H2O (to give a total volume of 100μL). Samples were mixed by pipetting, and pico-centrifuged. Samples were loaded into the ports of each LDA card, the temperature of which had been allowed to adjust to room temperature. Prior to analysis, cards were centrifuged twice (1200rpm, 1min), to ensure equal distribution of cDNA

across the TaqMan® assay reactions on each card. Loading ports were then cut away. Cards were analysed immediately on a Prism 7900HT Fast Real-Time PCR System, fitted with a TaqMan® array thermal cycling block adaptor, (Applied Biosystems).

2.4.5 CALCULATION OF RQ VALUES

Data analysis was performed using the SDS v2.1 program (Applied Biosystems). For all samples, analysis was initially carried out using default parameters (i.e. automatic baseline and threshold). If necessary, the C_T was set to manual, and the threshold line adjusted. The threshold was set above the baseline, the initial PCR cycles in which there is no significant amplification, and therefore little change in fluorescence. The threshold was also set within the exponential range of the amplification curve, in order to exclude background fluorescence, but before the plateau phase of the reaction.

Relative changes in gene expression (RQ values) were calculated as follows, using results from untreated cells as the calibrator reference sample.

$\Delta C_T = C_T$ (target gene) - C_T (endogenous reference gene)

$\Delta \Delta C_T = \Delta C_T$ (test sample) - ΔC_T (calibrator sample)

$RQ = 2^{-\Delta \Delta CT}$

2.5 WESTERN BLOTTING

2.5.1 PREPARATION OF PROTEIN EXTRACTS

Initially, cell culture medium was decanted from the flasks and cells were washed 3 times with cold 1x PBS. In experiments examining proteins implicated in apoptosis or autophagy, cell culture supernatant was collected and centrifuged at 1300rpm for 3min to recover floating dead cells. These were then combined with adherent cells in the appropriate flasks. Cell pellets were subsequently lysed in cold RIPA lysis buffer (50mM Tris-HCl, pH 7.4, 150mM NaCl, 1mM dithiothreitol (DTT), 0.25% sodium deoxycholate, 0.1% NP-40) (Santa Cruz Biotech., Santa Cruz, CA, USA.) containing 1mM phenylmethysulfonyl fluoride (PMSF), 1mM sodium orthovanadate, and protease inhibitors cocktail (Roche, Indianapolis, IN). Cell lysis was performed on ice for 15min. Clear protein extracts were obtained by centrifugation for 15min at 4°C. The supernatant was stored at -70°C.

2.5.2 BRADFORD ASSAY

Serial dilutions of BSA at 0.1 mg/μL (0 to 32mg/μL) were obtained by diluting BSA into 500μL of Bradford reagent (Sigma-Aldrich, Wicklow, Ireland) and making up the solution to 1mL with distilled water. 200μL of each of these standards was transferred in triplicate to a 96 well plate and the absorbance was read at 595nm using a plate reader. 1μL of each sample was then added to a vial containing 500μL water and 500μL Bradford reagent and replicates of each solution were added to the plate. The results were used to generate a standard curve from which the samples protein concentration could be estimated.

2.5.3 PROTEIN ELECTROPHORESIS

The percentage of the separating gel (6-15%) was chosen in accordance with the molecular weight of the protein under investigation. The separating gel and stacking gel were then prepared according to protocol. Protein (50μg) from whole cell extracts in laemmni buffer (Sigma) was heated to 90°C for 10min and then loaded onto the separating gel and transferred onto a PVDF transfer membrane for 1h at 100V in transfer buffer with cooling.

2.5.4 PROTEIN DETECTION

Membranes were blocked for 1h at room temperature in 5% (wt/vol) fat-free milk powder in PBS containing 0.1% Tween 20, incubated overnight with the primary antibody (1:100-1:1000 dilution), washed three times with 0.1% Tween in PBS, and incubated for 1h with a horseradish peroxidase-coupled secondary antibody 1:2000. The following primary antibodies were used: MLH1 (C-20), MSH2 (N-20), PMS1 (C-20), PMS2 (C-20) and goat anti-rabbit IgG-HRP (Santa Cruz Biotech.); MSH6 (Bethyl Laboratories, Montgomery, TX, USA); MGMT, and α/ß Tubulin, p-Chk1, p-Chk2, p-p53, caspase-3, Beclin-1, LC3B (Cell Signaling Technology, Wicklow, Ireland). After final washing with 0.1% Tween 20 in PBS (3×10min each) blots were developed using SuperSignal chemiluminescent substrate (Pierce, Rockford, IL, USA). Nuclear extract from CEM-CCRF cells (human leukemic lymphoblasts) and HeLa cells (human cervical carcinoma cells) were used as positive controls for MGMT protein expression and MMR protein expression respectively.

2.6 FLOW CYTOMETRY

2.6.1 CELL CYCLE ANALYSIS BY PROPIDIUM IODIDE STAINING

For cell cycle analysis with propidium iodide, cells were seeded at 0.5 x 10^6 cells /dish, grown overnight and harvested by trypsinisation. Cells were then fixed in 90% ethanol and stored at 4°C until analysis. Cells were treated with 10µg/mL RNase (Qiagen) and 5µg/mL propidium iodide (Invitrogen, Molecular Probes). DNA profiles were obtained by flow cytometry and relative numbers of cells in each phase of the cell cycle were ascertained by CellQuest software (BD Biosciences, Mountain View, CA).

2.6.2 ASSESSMENT OF ANTI-PHOSPHO-HISTONE H3 (SER28) STAINING

Asynchronously growing cells were irradiated in complete culture medium, trypsinised, fixed in 70% ethanol, and stained with a 1:200 dilution anti-phospho histone H3 primary antibody (Cell Signaling) at room temperature for 1h. Cells were fixed at 2hr after radiation exposure. After incubation, the samples were washed by centrifugation with 3mL phosphate-buffered saline (PBS). The supernatant was removed and the pellet resuspended in 200µL of PBS with 0.3% bovine serum albumin, and 0.3% Triton-X containing a 1:100 dilution of Alexa-fluor 488, conjugated secondary antibody (Invitrogen, Dublin, Ireland) and incubated for 30min at room temperature in the dark. The cells were washed again in PBS and resuspended in 200µL PBS containing 30µg propidium iodide (Molecular Probes). The samples were analysed on a CyAn™ ADP flow cytometer (Beckman Coulter) and additional analysis was carried out using Summit software v4.3 (Dako, Fort Collins, CO).

2.6.3 DETERMINATION OF MITOTIC RATIO

The mitotic ratio was determined by calculating the ratio of irradiated versus unirradiated cells that stained positive for anti-phospho-histone H3 in matched cell cultures. Flow cytometry gates procedures were used to determine the percentage of cells in each sample that were positive for phospho-H3 by comparison against similarly stained samples that were not incubated with the secondary phospho-H3 antibody.

2.7 CELL SORTING

Cells were harvested by trypsinisation as described in section 2.1.2, resuspended at a dilution of $1x10^6$ cells/ml of complete medium and filtered using a 40µM nylon mesh cell strainer (BD biosciences) to remove debris and clumped masses of cells. Propidium iodide (100µg/mL) and 20µL of this was added per 1ml cell suspension, immediately before sorting, to allow exclusion of dead cells/cell debris by dead-cell gating. Cell sorting was performed using a MoFlo XDP (Beckman Coulter) cell sorter. The cells were gated first on forward scatter-height/propidium iodide-height (FSC-H/PI-H) to gate out dead cells and debris, then on forward scatter-width/ side

scatter-height (FSC-W vs SSC-H) to gate out doublets and clumps. The laser was at 488nm. 500-6000 cells were sorted using a 100μm nozzle tip and pressure of 30psi, and captured in 25cm^2 flasks containing 10ml of complete media pre-heated to 37°C.

2.8 High Content Screening

2.8.1 Preparation of the cells

Cells were grown, treated, fixed and stained directly in 384 multiwell plates (Black/Clear Bottom, Flat Bottom, Tissue Culture Treated w/ lid, Sterile #4332 (Matrix Technologies, Thermo Fisher Scientific, Cheshire, U.K.)) Cells were initially harvested as described in 2.1.2. Cells (10,000-15,000) were plated per well in 100μL complete phenol red-free media and allowed to adhere to the plate overnight. The cells were then treated as required (irradiated with 0.2Gy with or without pre-incubation with 17-alylamine-17-demothoxy geldanamycin (17-AAG) for 24hr, or left untreated). At the appropriate time-points after treatment the cells were prepared for fixation in a fume-hood. All wells were initially rinsed with PBS 3 times, following aspiration of the PBS, the cells were covered to a depth of 2-3mm with 4% formaldehyde in PBS (Pierce Technologies, Thermo Fisher Scientific, Dublin, Ireland). The cells were allowed to fix for 15min at room temperature. The fixative was aspirated, and the cells were rinsed 3 times with PBS. After formaldehyde fixation, the cells were covered to a depth of 3-5mm with ice-cold 100% methanol and incubated for 10min in a -20°C freezer and the cells were again washed in PBS 3 times. Finally, the wells containing cells and all outside wells of the plate were filled with PBS, and the plates were wrapped in parafilm and stored at 4°C until labelling.

2.8.2 Labelling of the cells

Plates were stained in bulk after collection of cells fixed at various time-points after irradiation. All subsequent incubations were carried out at room temperature, unless otherwise noted in a humid light-tight box wrapped in tinfoil to prevent drying and fluorochrome fading. Initially, the PBS was aspirated from the wells and the cells were blocked in 5% normal serum from the same species as the secondary antibody (either goat serum or rabbit serum) in PBS/Triton for 60min. While

blocking, the primary antibodies were prepared by diluting in PBS/Triton to the required concentrations as previously determined during the optimisation process. The plates were incubated with anti-phospho-histone H3(ser28)(1:600 dilution)(Santa Cruz Biotech.), anti-MRE11(1:100)(EMD Biosciences, Nottingham, U.K.), anti-phospho histone H2AX(ser139)(1:50) (Cell Signalling), or anti-RAD51(1:50)(Abcam, Dublin, Ireland), overnight at 4°C, washed in PBS for 3 × 10min, and incubated with Alexa Fluor 488-conjugated goat anti rabbit secondary antibody (Invitrogen)(1:1000) containing Hoechst 33342 (Sigma Aldrich), for 1h at room temperature. Cells were washed in PBS, 3 × 10min. Following the incubation of cells with the appropriate secondary antibodies and Hoechst the cells were again washed with PBS 3 times, and the wells were filled with PBS prior to analysis using the IN Cell Analyzer Platform 1000.

2.8.3 *AUTOMATED IMAGE CAPTURE AND ANALYSIS*

The IN Cell Analyzer 1000 automated fluorescent imaging system (GE Healthcare, Piscataway, NJ) was used for automated image acquisition. The system is based on an inverted epifluorescence microscope (Zeiss, Thornwood, NY) equipped with a 200-W mercury-xenon lamp; the instrument automatically focuses samples and scans fields by means of a motorised stage. The images were acquired with a high-resolution thermoelectrically cooled 12-bit charge-coupled device (CCD) camera by exposing the samples for a constant time. Images were acquired with either a 10× or 20× objective, using 340/40-nm and 480/40-nm excitation filters, a Q505LP dichroic mirror and 460/40-nm and 535/50-nm HQ emission filters. Cells seeded in 384 well plates were located by means of nuclear fluorescence, which is accepted or rejected in an automated manner based on nuclear area and shape, the fluorescence intensity, and the position of the nucleus in the field. The parameters set for nuclei recognition ensured that any artefacts or cell clusters were absent in the populations analysed. The images were automatically stored and analysed by the IN Cell Analyzer 1000 software. To score targets of interest in a high-content throughput, we used the Multi-Target Analysis (MTA) algorithm (GE Healthcare, Investigator v3.5) to identify individual cells and mitotic cells (phospho-H3 positive). The Dual-target Analysis algorithm was used to identify individual cells and foci in these cells (MRE11). The nucleus was segmented via a top-hat method

($30\mu m^2$ minimum area). Inclusion thresholds were set such that only MRE11 foci of the size induced by radiation were counted (0.3-1μm) and only nuclei with >5 foci were counted as positive. The thresholds chosen were validated by use of a positive control (2Gy IR) and negative control (MRE11 inhibitor, Mirin). At least 8 fields were analysed in each well, with a 10x objective (phospho-H3) or 15 fields in each well with a 20x objective (MRE11), corresponding to at least 1600 cells counted. 3 wells were analysed per experiment and experiments were performed in triplicate, therefore each data point is the mean of a minimum of 14,400 cells counted. Experiments with secondary antibodies alone were performed to verify the specificity of the signals.

Chapter 3: CHARACTERISATION OF LOW DOSE RADIATION HYPERSENSITIVITY IN PROSTATE CELLS

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3.1 INTRODUCTION

Ionizing radiation readily destroys a cell's capacity for proliferation, more so than any other cellular function. A cell's ability to give rise to a clone of similar cells is a well-known marker of cellular viability and as such, has long since been used as a means to evaluate the viability of micro-organisms. This concept has also been adopted by radiobiologists when considering the effects of radiation on mammalian cells. Loss of cell viability, in that sense, has become synonymous with "cell death", and the "lethal" effect of radiation is that which induces loss of proliferative capacity. Conversely "survivors" are those cells which, after irradiation, retain "viability" ie. the capacity to originate a clone of similar cells.

Techniques for counting viable cells, or "survivors" after exposure to IR were described in mammalian cells about 80 years later than for prokaryotes. Relationships between radiation dose and survival, or "dose-response" relationships in bacteria and viruses were therefore found long before those in mammalian cells. The assessment of this relationship was initiated in 1956 by Puck and Marcus (Puck et al. 1956) who published a seminal paper describing a cell culture technique for the assessment of the clone- or colony forming ability of single mammalian cells plated in culture dishes. The authors carried out experiments that yielded the first radiation–dose survival curve for HeLa cells in culture irradiated with X-rays. They showed that these mammalian cells were much more radiosensitive than assumed earlier for cells in tissues, with mean lethal doses in the range of 1–2Gy. Now, best known as the clonogenic assay or colony formation assay this *in vitro* cell survival assay remains the gold standard when assessing the effects of IR on cell viability. A viable colony is defined as one consisting of at least 50 cells and the assay essentially tests every cell in the population for its ability to undergo "unlimited" division.

The biological problems associated with the measurement of survival following administration of low doses of radiation, are such that the task was initially described, as to some extent, *"like seeking the pot of gold on the far side of rainbow"* (Hall 1975). These problems include statistical uncertainty associated with plating a number of cells per dish, variations in sensitivity with cell

age, and statistical problems related to variable plating efficiencies. It is precisely because of these limitations, that in the past, cellular survival following exposure to radiation doses less than 1Gy was routinely estimated based on back-extrapolated from data obtained in response to high dose survival data, rather than directly evaluated.

Thirty-five years later, the measurement of low dose radiation survival is now commonplace. The outlined limitations have been largely overcome thanks to efforts focused on more accurately evaluating cell survival after exposure to radiation doses less than 1Gy, e.g. The statistical uncertainty associated with plating a number of cells per dish, routinely achieved with serial dilution, was initially overcome by accurately counting the number of plated cells, which at this time involved isolating cells individually with the aid of a micropipette, and counting them one at a time as they were plated (Bedford and Griggs 1975). The practicality of this experimental approach was later improved by use of flow cytometry cell sorting to define the exact number of cells plated (Durand 1986). Later still, an entirely different approach was developed using microscopy to locate and track individual cells once plated (Spadinger et al. 1989). The ensuing years have also seen the use of the gel micro-drop assay and clonogenic assay by high content analysis. The latter is an automated cell biology method drawing on optics, chemistry, biology and image analysis to permit rapid, highly parallel biological research and drug discovery. The statistical uncertainty associated with manually counting surviving colonies has been vastly reduced by use of an automated system, meaning the practicalities of the assay no longer prohibit the study of cellular survival in response low dose radiation.

The advent of such techniques has allowed the identification of dose response relationships that are specific to low doses of radiation, such as low dose radiation hypersensitivity (HRS). HRS describes the survival response of mammalian cells whereby approximately 5-10% of the population of cells undergoes cell death greater than that which is predicted by back-extrapolation of high dose survival data; meaning that low doses of radiation may actually be inducing greater cell kill than previously accounted for.

It is possible to quantify the HRS response using mathematical modeling, which is applied to survival data obtained in response to IR, to allow prediction of the dose response using non-linear regression. While initially considered a *"supreme exercise in futility"* (Hall 1975), mathematical models such as the linear-quadratic (LQ) formulation $e^{-(\alpha D+\beta D2)}$, are now routinely used to describe the response of cells to IR and to quantify the variation in the response of cells to radiation in the presence of various modifiers. is often used to model biological response to radiation. For instance, when applied to single fraction cell survival studies the surviving fraction (SF) is generally expressed as:

$$SF = e^{-(\alpha D+\beta D2)} \quad \text{Eqn. 1}$$

where D is the dose in Gy, α is the cell kill per Gy of the initial linear component (on a log-linear plot) and β the cell kill per Gy^2 of the quadratic component of the survival curve.

An adaptation of this formula, termed the Induced Repair model, has been developed to define the HRS response and provide a means to quantify it.

The Induced-Repair equation (Eqn. 1) (Marples and Joiner 1993) is as follows:

$$S = \exp\left\{-\alpha_r\left(1+\left(\alpha_s/\alpha_r - 1\right)e^{-d/d_c}\right)d - \beta d^2\right\} \quad \text{Eqn. 2}$$

Where d is dose, and α_s represents the low-dose value of α (derived from the response at very low doses), α_r is the value extrapolated from the conventional high-dose response, d_c is the 'transition' dose point at which the change from the very low-dose HRS to the IRR response occurs (i.e. when α_s to α_r is 63% complete) and β is a constant as in the LQ equation (see Fig. 3-1 for graphical representation). The presence of low dose hyper-radiosensitivity is deduced by values of α_s and α_r whose confidence limits do not overlap and a value of d_c (the change from low to high dose survival response) significantly greater than zero.

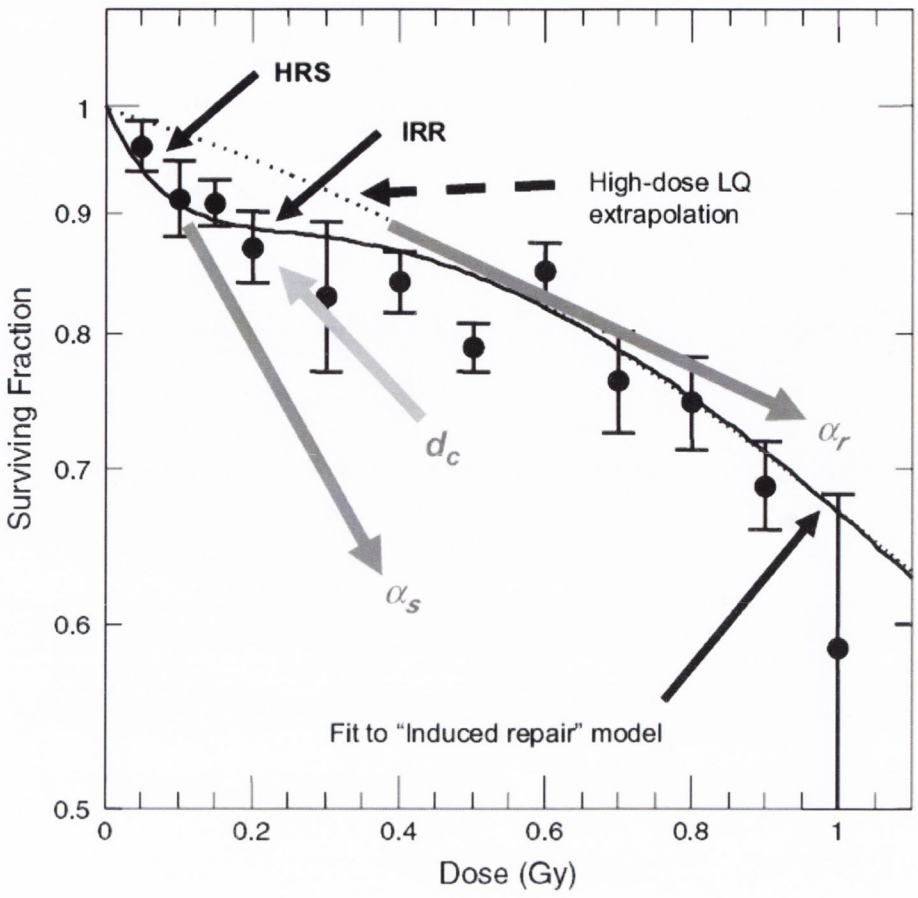

Fig. 3-1: Typical cell survival curve with evidence of hyper-radiosensitivity (HRS).

Broken line shows low-dose extrapolation from linear quadratic (LQ) model applied to high-dose survival data. Solid line shows induced repair fit. Derivation of a_s, a_r, and d_c parameters are shown in Eqn. 1. Image from (Marples and Collis 2008).

HRS has been reported in over 40 mammalian cells to date, only three of which are prostate cells. However, these studies did not define HRS using the Induced Repair model meaning the reported HRS status of these cell lines could potentially be brought into question. Evaluation of the mechanism of HRS in prostate cells will require a robust model in which to study the response. Therefore, in order to study the mechanism of HRS in prostate cells, the manifestation of HRS in a panel of prostate cells had to be evaluated and validated using the induced repair model prior to commencement of mechanistic studies. A number of factors can influence radiosensitivity including radiation dose, the intrinsic capacity to repair DNA damage, the doubling time and a number of environmental conditions. Moreover, involvement of DNA repair pathways in the HRS response appears likely (discussed in chapter 1). Characterisation of these components in HRS+ and HRS- prostate cells was undertaken in this project to provide mechanistic insight into the HRS response.

3.2 OBJECTIVES AND METHODS

The aim of this chapter is to establish a valid model in which to study the mechanism of HRS in PCa cells. To this end we set out to examine a panel of prostate cells for evidence of HRS. In addition we wanted to characterise the cell lines in terms their expression of DNA repair genes, intrinsic radiosensitivity and cell cycle distribution in order to gain a greater understanding of what might be contributing to a hypersensitive response. For this purpose, a panel of prostate cells were chosen (22RV1, DU145, PC3, PWR1E, RWPE1) which are representative of cell lines with different intrinsic DNA repair capabilities, radiosensitivities, and different origins. Specifically, 22RV1 prostate carcinoma cells are derived from a primary carcinoma and are relatively sensitive to IR, DU145 and PC3 prostate carcinoma cells are of metastatic origin and are relatively resistant to IR, and PWR1E and RWPE1 are prostate epithelial cells whose radiosensitivity is unknown. LNCap cells (androgen sensitive prostate carcinoma cells) were assessed for their suitability for this study but were excluded due to the propensity of the cells to detach from the culture plate upon movement. The specific objectives of this chapter include:

- To evaluate the survival response of a number of prostate cell lines (malignant PC3, 22RV1, DU145 and endothelial RWPE1, PWR1E) across a range of radiation doses (n=7). In the absence of access to a fluorescence-activated cell sorter, high precision clonogenic assays were used to assess sensitivity to low dose radiation. Colonies were counted using an automated colony counter.

- To identify cells that express low dose radiation hypersensitivity as can be achieved by using mathematical modeling.

- To identify DNA repair pathways differentially activated in PCa cells using gene expression profiling of a panel of DNA repair genes. The panel of genes included in the array include those involved in the following pathways: non-homologous end joining (NHEJ), homologous recombination (HR), DNA mismatch repair (MMR), base excision repair (BER), nucleotide excision repair (NER), the Fanconi Anemia (FA) pathway.

- To compare the surviving fraction at 2Gy across the panel of cell lines to evaluate the role of intrinsic radiosensitivity in the HRS response.

- To evaluate the asynchronous distribution of cells in cell cycle phases.

3.3 RESULTS

3.3.1 IDENTIFICATION OF HRS+ CELL LINES

A panel of normal (PWR1E, RWPE1) and malignant (22RV1, DU145, PC3) prostate cell lines was assessed for evidence of low-dose hyper-radiosensitivity. Presence of HRS was determined by mathematical analysis of clonogenic cell survival in response to radiation doses of up to 6Gy. The survival curves were fitted with either the induced repair model (Marples and Joiner 1993), developed to describe the response of cells to low doses of radiation, or the linear quadratic model (Fig. 3-2).

All parameters were fitted simultaneously and estimates of uncertainty were expressed as likelihood confidence intervals. The cell survival curves of DU145, 22RV1 and PWR1E were best described by the linear-quadratic model and showed no evidence of HRS. PC3 and RWPE1 cells both exhibited distinct HRS and increased radioresistant responses as defined by the IR model (HRS+) (Table 3-1). Hypersensitivity was deduced in PC3 and RWPE1 cells by values of α_s and α_r whose confidence limits did not overlap, and d_c values significantly greater than zero.

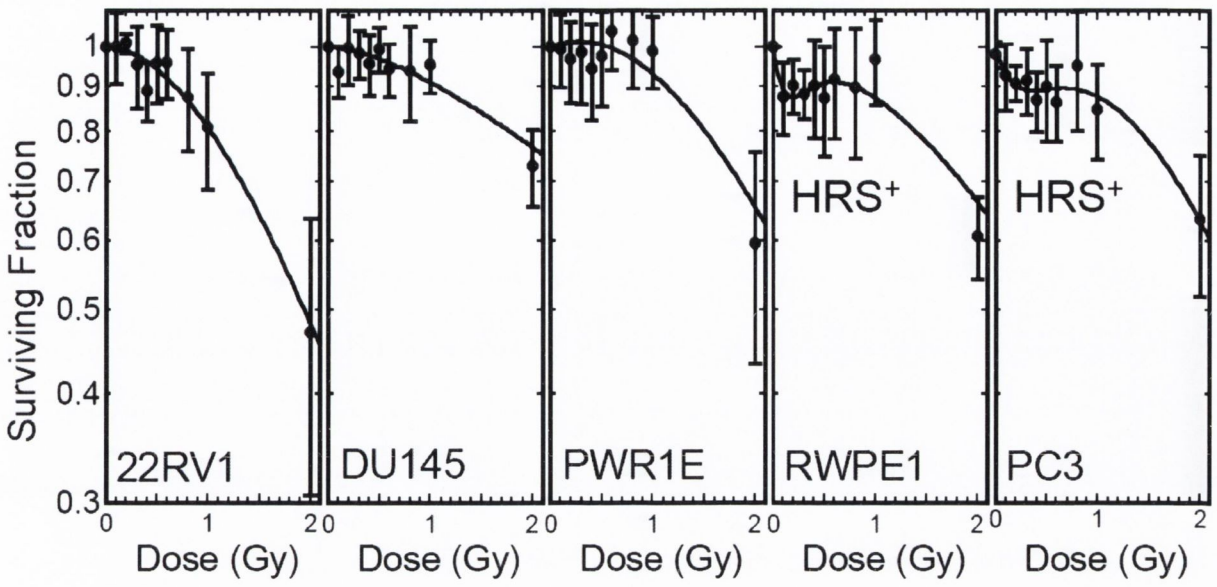

Fig. 3-2: Clonogenic survival of prostate cells and after X irradiation

Clonogenic survival of prostate cancer (DU145, PC3, and 22RV1) and prostate epithelial (RWPE1 and PWR1E) cells after X-irradiation. The data points show the mean survival from four to seven individual experiments (± SEM). The line shows the fit of the data to the induced repair model. Figure published in Martin et al, *Radiation Research*, 2009.

Table 3-1: Values of the parameters obtained from mathematical modeling of prostate cell lines using the Induced Repair model

	α_s (± SE)	α_r (± SE)	ß (± SE)	d_c (± SE)	d_c (± CL)
DU145	wnc	0.07± 0.02	0.02± 0.001	wnc	wnc
PC3	0.77±0.51	0.12± 0.05	0.03 ± 0.02	0.18 ± 0.14	0.02-0.39
22RV1	wnc	0.06±0.04	0.15±0.03	wnc	wnc
PWR1E	wnc	0.47±1.11	0.08±0.01	wnc	wnc
RWPE1	2.10±1.06	0.06±0.04	0.07±0.02	0.16±0.06	0.07-0.30

Wnc = would not converge

3.3.2 Comparison of endogenous DNA repair gene expression in HRS- prostate carcinoma cells relative to HRS+ prostate carcinoma cells

To determine if the underlying DNA repair background could play a causal role in the manifestation of HRS we next compared the endogenous gene expression of 41 distinct DNA repair genes in HRS+ PC3 cells relative to HRS- 22RV1 or DU145 cells (see Methods 2.4.4, and Table 9-6 for full list of the genes tested). These genes included those involved in the following pathways: DSB detection, homologous recombination, NHEJ, NER, BER, the Fanconi Anemia pathway. *GAPDH* was used as the endogenous control.

The results are shown in Fig. 3-3 (RQ values are shown in Tables 9-6 and 9-7 in the Appendix). In total seven genes were up-regulated >2 fold in PC3 relative to each DU145 and 22RV1. Five genes were commonly upregulated in PC3 cells relative to both DU145 and 22RV1 cells namely *XRCC5, ERCC3, ERCC2, TP53BP1*, and *RAD51C*. The pathway associations of these genes include NER, BER, and NHEJ. A total of eleven and thirteen genes were down-regulated in PC3 relative to 22RV1 and DU145 respectively (Fig. 3-3). Of these eight were commonly down-regulated in PC3 relative to the other cell lines. These genes were *RAD51, EXO1, CHEK2, H2AFX, XPA, FANCF, FANCG, FANCC*.

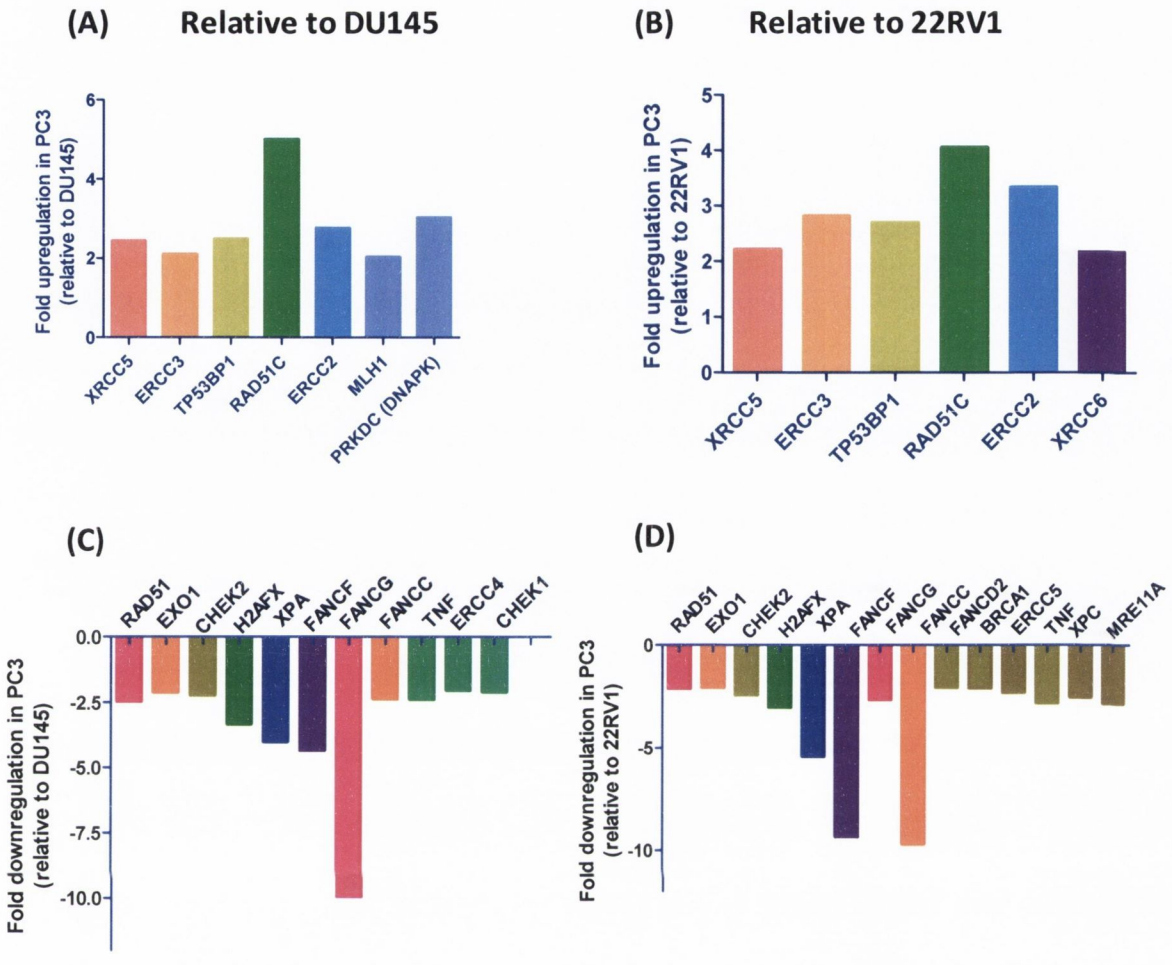

Fig. 3-3: Identification of differential endogenous expression of DNA repair genes in HRS+ PC3 cells relative to either DU145 cells (A, C) or 22RV1 cells (B, D)

Genes upregulated in PC3 relative to either DU145 (A) or 22RV1 (B) are shown in the top panels. Genes down-regulated in PC3 relative to either DU145 (C) or 22RV1 (D) are shown in the lower panels.

3.3.3 INDUCED DNA REPAIR GENE EXPRESSION OF MALIGNANT PROSTATE CELLS EXPOSED TO 0.2GY

Exposure to low dose radiation (0.2Gy) induced very few DNA repair genes overall (Table 3-2, see also Tables 9-8 to 9-10). Whereas exposure to low doses of radiation did not induce any substantial gene changes in HRS+ PC3 cells, or HRS- DU145 cells, irradiation of 22RV1 cells changed the expression (>2 fold up-regulation) of four genes (*H2AFX, XRCC6, RAD52, TP53BP1*)(Table 3-2). No genes were down-regulated greater than 2- fold in any cell line.

Table 3-2: Number of genes significantly changed by irradiation with 0.2Gy in PC3, DU145, and 22RV1 cells

>2 fold greater expression in PC3 cells	**0**
>2 fold greater expression in DU145 cells	0
>2 fold greater expression in 22RV1 cells	4 *H2AFX* (RQ: 2.25) *XRCC6* (RQ: 2.56) *RAD52* (RQ: 2.09) *TP53BP1* (RQ: 2.18)

3.3.4 HRS IS NOT CORRELATED WITH RADIORESISTANCE IN PROSTATE CELLS

HRS has previously been correlated with radioresistance (Joiner et al. 2001). To assess whether this correlation was also true in prostate cells, clonogenic survival assays were used to compare the surviving fraction at 2Gy (SF2) across the panel of cell lines. However, HRS did not correlate with overall radioresistance (i.e. high SF2) in these prostate cell lines (Fig. 3-4). While the SF2 of HRS+ RWPE1 was not significantly different when compared to other HRS- cell lines, the SF2 of HRS+ PC3 was significantly lower than that of HRS- DU145 cells (t-test, p=0.005).

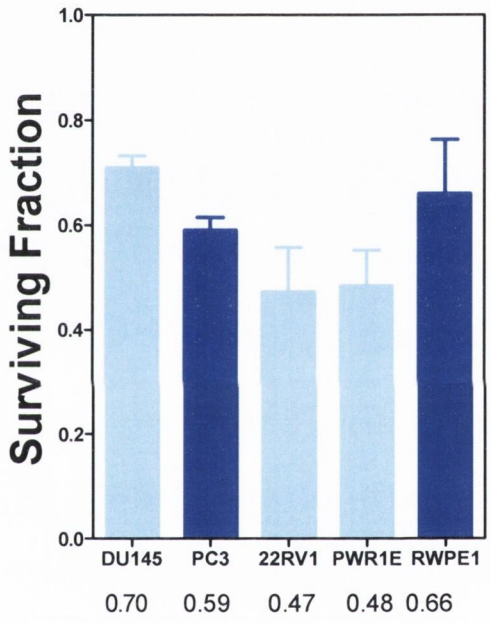

Fig. 3-4: Clonogenic response of prostate cells to 2Gy of radiation

Surviving fraction at 2Gy (SF2) of a panel of prostate cell lines. HRS+ cells are shown in dark blue. Mean ± SEM, n=3. Figure adapted from the published image in Martin et al, *Radiation Research*, 2009.

3.3.5 HRS+ CELL LINES WERE NOT ASSOCIATED WITH AN ENRICHED G2/M POPULATION

HRS has been previously related to the movement of cells through the G2 phase of the cell cycle. Consequently, in the current study, the proportion of G2/M cells in unirradiated cell populations was assessed by flow cytometry (Fig. 3-5). No relationship was seen between the static proportional of G2/M cells and HRS, although the G2/M phase cell population of HRS+ PC3 was significantly higher than that of HRS- DU145 ($p=0.005$), it was not significantly different to that of HRS- 22RV1 cells ($p=0.8$).

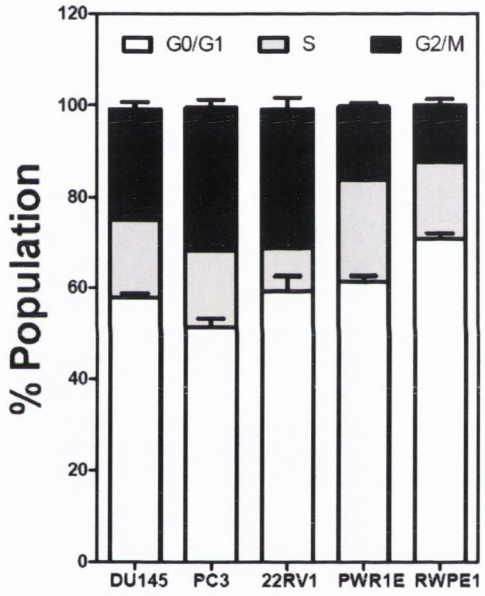

Fig. 3-5: HRS does not correlate with an enriched G2/M population

Cell cycle distributions of unirradiate malignant prostate and normal prostate cell lines. Mean ± SEM, n=3. Figure published in Martin et al, *Radiation Research*, 2009.

3.4 Discussion

Using high-precision clonogenic assays, we demonstrated HRS in PC3 PCa cells and in a normal prostate epithelial cell line (RWPE1). To our knowledge, HRS in normal prostate epithelial cell lines has not been reported previously, although HRS has been demonstrated in normal human epidermal cells (21, 22). No firm consensus exists regarding evidence of HRS in prostate tumour cell lines (23–27). The absence of HRS in DU145 cells in our study confirms the observations of Lin and Wu (25). However, the PC3 results presented in the current study contradict results published previously (26) but corroborate findings of Mothersill et al. (Mothersill et al. 2002). This difference could be reconciled by considering the criteria used to define HRS in each study. In the present work, unlike the previous report, the HRS status of PC3 cells was defined using a stringent mathematical approach. These data are also consistent with reports that HRS may be observed in metastatic cells (Thomas et al. 2007). While the radioresponse of these cells lines is poorly documented, especially in response to low doses of radiation, our results are in concordance with previously published data, where available (Bromfield et al. 2003).

Comparison of endogenous DNA repair gene expression revealed that five DNA repair genes relating to HR, NHEJ and NER were upregulated in HRS+ PC3 cells, relative to HRS- prostate cells (DU145, 22RV1). A total of eight genes were commonly down-regulated in PC3 cells relative to DU145 and 22RV1, relating to the following pathways (HR, Fanconi anemia pathway, ATM mediated signalling). The pathway to carcinogenesis is multi-mechanistic and can involve dysregulation of many cell signalling pathways involved in maintaining genomic fidelity. Dysregulated DNA repair pathways are a feature of aggressive PCa (Chan et al. 2007). Dysregulated DNA repair pathways as observed in PC3 may be the result of an accumulation of mutations. DNA repair rates may be increased to allow increased recovery rates after DNA damage as is often observed via overexpression of RAD51, an essential recombinase in the HR pathway (Mitra et al. 2009). Alternatively, genes may be down-regulated leading to decreased repair rates, an accrual of DNA damage, and subsequent further mutation and resistance. The differences in the endogenous DNA repair gene expression are greater between primary PCa cells (22RV1) and

metastatic PC3 cells, than between DU145 and PC3 (both metastatic) which is likely reflective of their differences in stage of carcinogenesis and may potentially play a causal role in the manifestation of HRS. *XRCC5* is down-regulated in HRS- cells relative to HRS+ cells. NHEJ is error prone in cells deficient in the *XRCC5* gene product Ku-80 (Feldmann et al. 2000). This may have relevance for the mechanism of HRS in future work.

In the literature, cells deficient in DNA-PK, and *ATM* (Wykes et al. 2006) were demonstrated to be HRS-. An association between MMR deficiency and HRS- was also proposed (Krueger et al. 2007). In our panel of prostate cells, no marked difference in DNA-PK *(*protein of *PRK-DC)* or *ATM* expression was observed in HRS- cells compared to HRS+ cells at a basal level. However, it was observed that HRS- DU145 cells harbour down-regulated *MLH1* (DU145), relative to HRS+ PC3 cells. These findings are consistent with reports that MMR-deficiency is evident to varying degrees in PCa (Martin et alc, 2009). Down-regulation of *MLH1* in DU145 may reflect the methylation status of *MLH1* in DU145 cells which has been reported to be partially methylated at the promoter site. While the MMR status of these cell lines will need validation by examination of MMR protein expression, it is likely that the MSH2 and MLH1 protein expression will also be down-regulated in these cell lines.

Taqman® Low Density Arrays did not reveal any obvious correlation between HRS and low dose IR induced DNA repair gene expression. Whereas HR repair genes are upregulated in HRS- cell line (22RV1) exposed to 0.2Gy (2 h), this is not evident in HRS+ PC3 or HRS- DU145 cells. The reasons for this are as yet unknown but may reflect compromised DNA damage sensing and repair pathways as these are commonly reported in metastatic PCa cells (Chan et al. 2007).

Finally, HRS status was compared to both intrinsic radiosensitivity as evaluated by comparing SF2 values, and the distribution of asynchronous cells in various phases of the cell cycle. However, no correlation between HRS and radioresistance or basal cell cycle distribution was observed. These data do not support the correlation observed between radioresistance and HRS reported previously,

but are consistent with documented reports of SF2 values and cell cycle distributions of these cell lines where available.

This detailed analysis of HRS in prostate cells has demonstrated that HRS is indeed expressed in some, but not all prostate cell lines of both malignant and epithelial origin, thus establishing a valid model in which to study the mechanism of HRS in prostate cells. In these cells, HRS is associated with absent upregulation of HR gene expression in response to low doses of radiation, but is not correlated with intrinsic radioresistance or significant variations in basal cell cycle distribution.

Chapter 4: Recognition of O6MeG Lesions by MGMT and Mismatch Repair Proficiency may be a Prerequisite for Low-Dose Radiation Hypersensitivity

Chapter 4 is adapted from a paper of the same title

Radiation Research 172(4):405-413 (2009)

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Donal Hollywood, and Laure Marignol

4.1 INTRODUCTION

The molecular signalling response to low dose radiation exposures appears to mimic the damage response to O6-Methylguanine (O6MeG) lesions. Parallels between these pathways include evasion of the early G2 arrest, the observation that down-regulation of ATM and PARP can sensitise cells to O6MeG (Debiak et al. 2004), the importance of G2 phase cells, and the observation that HRS-positive cells exhibit persistent DNA damage at late time points post IR. Cells exposed to O6MeG lesions typically bypass the early G2 checkpoint before arresting after the second round of replication, following accumulation of DSBs at late time points.

O6MeG lesions are responsible for the cytotoxicity of a number of chemotherapeutic agents. The DNA repair protein O6-methylguanine-DNA methyltransferase (MGMT) is the primary cellular defence against these lesions. It has been demonstrated that MGMT is upregulated in response to ionising radiation via a mechanism that may involve p53 (Grombacher et al. 1998). It is, however, known that MGMT is upregulated stiochiometrically in response to O6MeG lesions. In the absence of MGMT, the fate of cells exposed to O6MeG relies on the formation of O6-MeG:T and O6-MeG:C mispairs during the course of DNA duplication and the subsequent engagement of the MMR system (Papouli et al. 2004).

The DNA mismatch repair (MMR) system is a highly conserved post replicative editing process that maintains genomic fidelity through recognition and repair of incorrectly paired nucleotides (for recent reviews see (O'Brien and Brown 2006; Wang and Edelmann 2006)). In brief, this pathway involves four key processes: recognition of the erroneous bases or insertion–deletion loops, excision of these lesions, substitution of the lesion with the correct sequence, and religation of the DNA. Mismatch recognition is mediated by either of two MutS heterodimers; MutSα (comprised of MSH2 and MSH6) which binds to mismatches and small insertion-deletion loops, or MutSβ (comprised of MSH2 and MSH3) which binds to larger insertion-deletion loops (for a review see (Iyer et al. 2006; Jiricny 2006)). MutL (a heterodimer of MLH1 and either PMS2 or PMS1) is subsequently recruited by the MSH2 protein to form a ternary complex with one of the MutS complexes and promote intracellular signalling to initiate excision and repair of the mismatch.

Additional proteins involved in this process may include exonuclease 1 (EXO1), possibly helicase(s), replication protein-A (RPA), replication factor C (RFC), proliferating cell nuclear antigen (PCNA), and DNA polymerases α and β (Li 2008) (Fig. 4-1). In combination, these complexes provoke both checkpoint and apoptotic responses (Davis et al. 1998; Hirose et al. 2003; Hickman and Samson 2004; Meyers et al. 2004; Adamson et al. 2005; Li et al. 2008), although controversy remains regarding the mechanism involved.

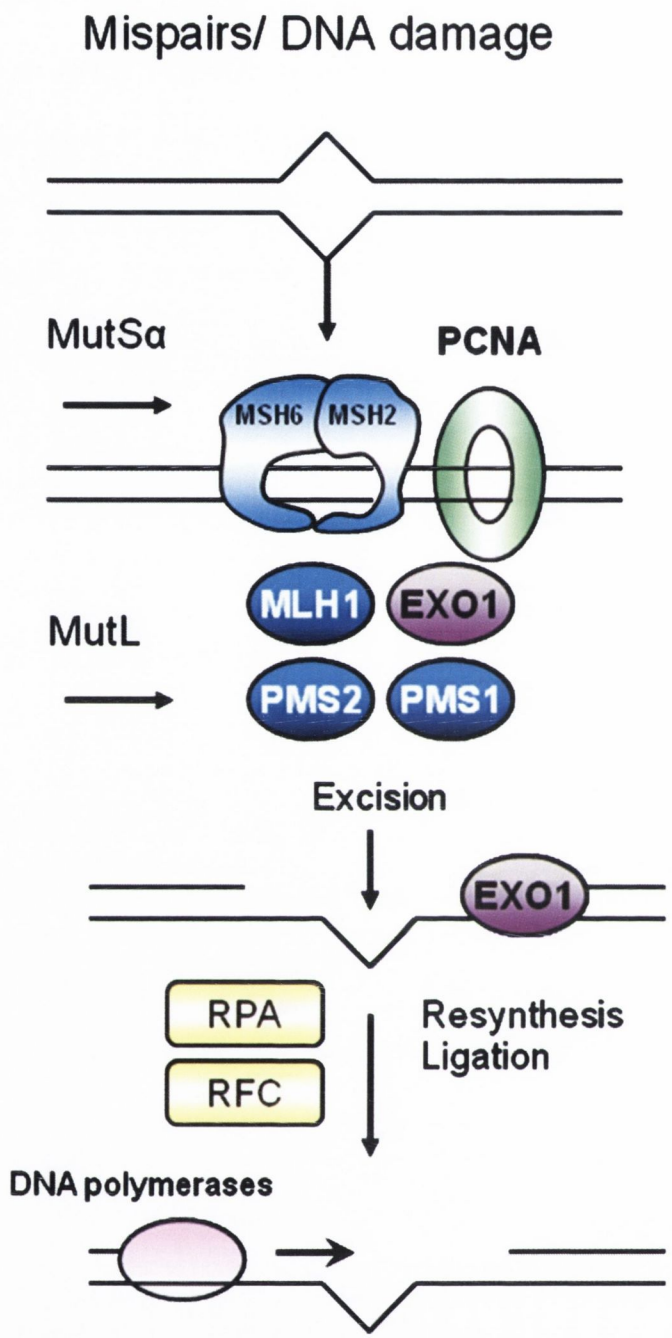

Fig. 4-1: Overview of mismatch repair mediated removal of base-base mismatches.

DNA mismatch repair is initiated when the MutSα (MSH2/MSH6) heterodimer binds to the mismatched DNA. Heterodimers of MutL homologues, such as MLH1, PMS1 and PMS2, as well as the EXO1, RPA, RFC, and DNA polymerases are then recruited to this complex to complete excision of the mismatches and resynthesis of the DNA strand. Figure published in Martin et al, *Cancer Treatment Reviews*, 2010.

MMR proteins can also recognize DNA damage distortion and bind to adducts produced by the presence of DNA damaging agents including 6-thioguanine (Hawn et al. 1995), N-Methyl-N'-Nitro-N-Nitrosoguanidine (MNNG) (Carethers et al. 1996), cisplatin (Duckett et al. 1996; Fink et al. 1996; Yoshikawa et al. 2000; Zdraveski et al. 2002), carboplatin (Fink et al. 1996), 5-fluorouracil (Tajima et al. 2004) and halogenated-thymidine-analog iododeoxyuridine (Berry et al. 2003).

Two mechanisms have been proposed for the involvement of DNA MMR in this process. The "futile repair" model (Karran and Bignami 1994) proposes that the MMR system undergoes reiterated futile attempts at repair upon recognition of the O6-MeG:T and O6-MeG:C mispairs, leading to the formation of gaps in the newly synthesized DNA strand and ultimately the creation of double strand breaks following replication. This damage then provokes a G2 cell cycle arrest after the second round of DNA synthesis (Hirose et al. 2001; Hirose et al. 2003) and ultimately cell death. Alternatively, according to the 'direct signalling' model (Fishel 1999), after the recognition of O6 MeG:T and O6-MeG:C mispairs, the MMR system transmits the damage signal directly to the checkpoint machinery, without the need for DNA processing (Fishel 1999). Therefore, we hypothesized that low-dose HRS may result from futile mismatch repair of O6MeG lesions in cells lacking sufficient MGMT activity to remove the lesions directly.

4.2 Objectives and Methods

The purpose of this chapter is to test the proposed mechanism for HRS in a panel of prostate cells in terms of the sensors, transducers and effectors of the pathway involved.

The specific objectives of this chapter were as follows:

- To evaluate the effect of varying concentrations of Temozolomide (TMZ) or 2Gy of radiation on the survival response of PCa cells using clonogenic assays.
- To determine MGMT gene expression patterns in non hypersensitive cells and compare them to those in hypersensitive cells using quantitative RT-PCR. Western blots were used to determine MGMT protein expression in all cell lines.
- To investigate the effect of MGMT inactivation on radiation survival by pre-treating hypersensitive PC3 cells with the MGMT inhibitor O6-benzylguanine before administering radiation.
- To compare mismatch repair protein expression (MSH2, MLH1, MSH6, PMS1, PMS2) across all prostate cell lines using western blotting and to validate the expression pattern using a panel of glioma cell lines (T98G, U87MG, U373).

4.3 RESULTS

4.3.1 HYPERSENSITIVITY IS EVIDENT IN LOW DOSE TEMOZOLOMIDE SURVIVAL RESPONSE IN CHEMORESISTANT PROSTATE CANCER PC3 CELLS

TMZ, an alkylating agent that induces O6MeG lesions, was used to determine whether substructure was also evident in the low dose survival response after chemotherapy similar to that observed after low dose X-irradiation. A range of concentrations of TMZ (0-1μM) were chosen such that the yield of cell kill observed was similar to that induced by low doses of radiation. This was determined using clonogenic survival assays (data not shown). Clonogenic survival measurements demonstrated that 0.3μM TMZ induced significantly more cell death than did 0.6μM TMZ (t-test, p=0.041) (Fig. 4-2A)). However, this was not observed in HRS- cell lines (DU145, 22RV1) which were significantly more sensitive to TMZ than were PC3 cells at the same concentration (one-way ANOVA, p=0.003). This trend was also evident at a higher drug concentration (30μM) (Fig. 4-2B)) but was not statistically significant (one-way ANOVA, p=0.093).

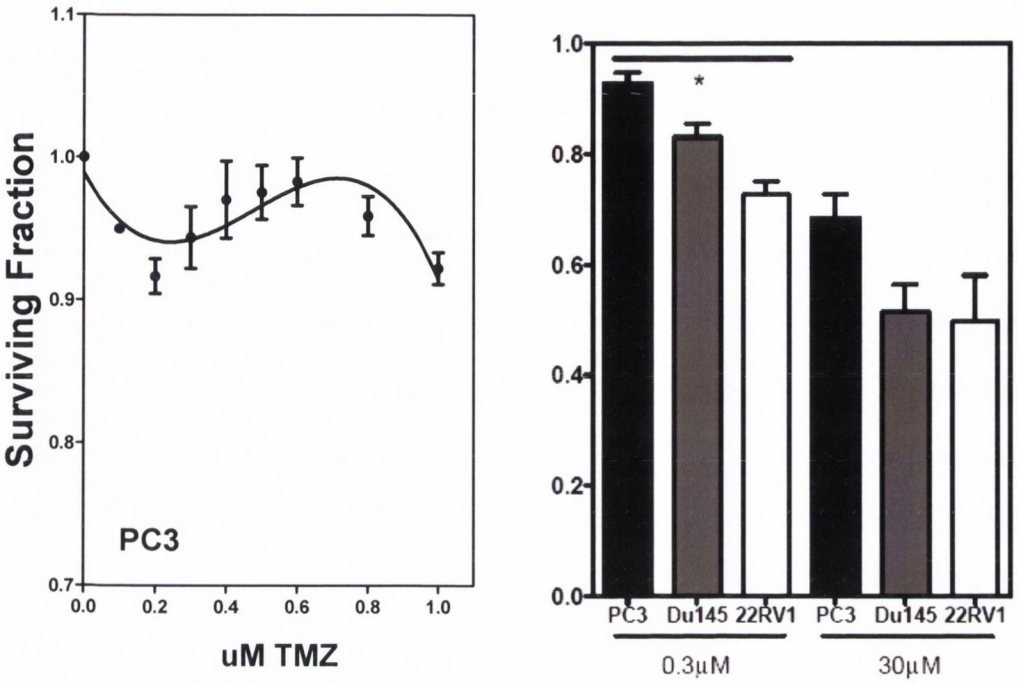

Fig. 4-2: Clonogenic survival of prostate cells in response to TMZ exposures

(A) Clonogenic survival of HRS+ PC3 cells following 3 day treatment with low concentrations of TMZ. The data points show mean survival ± SEM, n=4. (B) Clonogenic survival of prostate cells (PC3, DU145, 22RV1) following 3 day treatment with 0.3μM and 30μM TMZ. Mean ± SEM, n=3. Figure published in Martin et al, *Radiation Research*, 2009.

4.3.2 VALIDATION OF LOW DOSE HYPERSENSITIVITY SURVIVAL RESPONSE TO TEMOZOLOMIDE IN GLIOMA CELLS

Given that the HRS response is most pronounced in glioma cells, we chose to validate the survival response observed in two glioma cell lines whose HRS status has been reported previously (T98G, U373) (Short et al. 1999; Short et al. 1999; Short et al. 2001; Marples et al. 2002). Glioma cells required higher concentrations of TMZ (0-10μM) to achieve a yield of cell kill similar to that observed in response to low doses of radiation, than did prostate cells. Survival data revealed that HRS+ glioma cells (T98G) tended to be more sensitive to low concentrations of TMZ (3μM) than relatively higher concentrations (10μM) (Fig. 4-3). However the results were not significant. This hypersensitive response was not observed in HRS- U373 cells (Fig. 4-3).

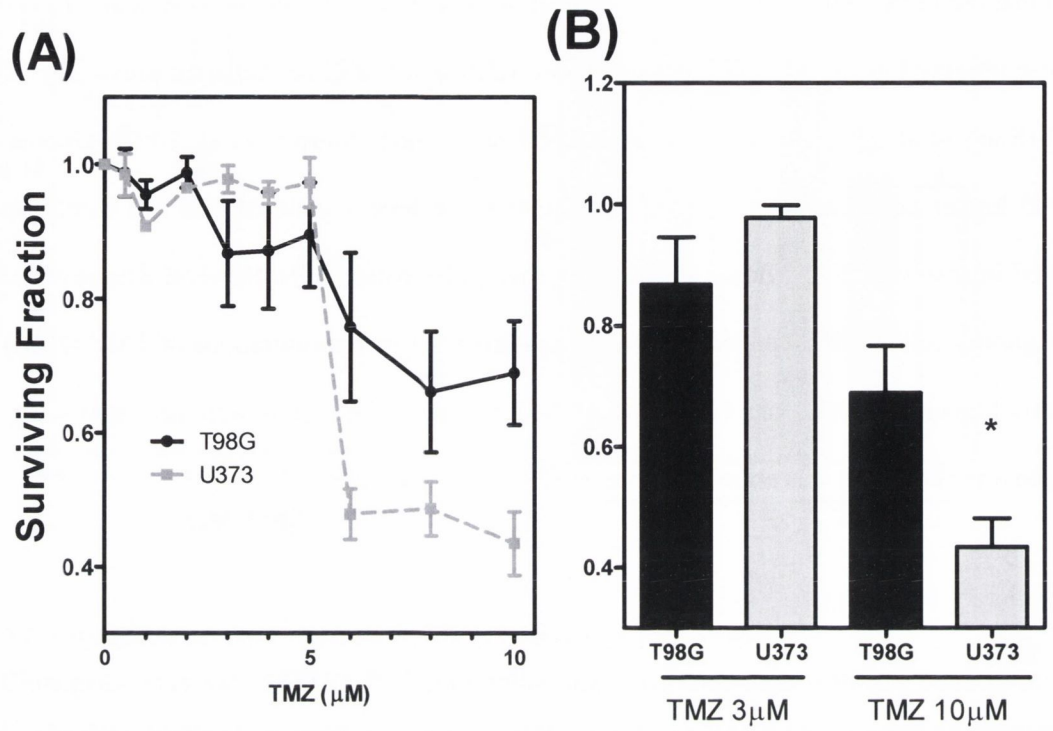

Fig. 4-3: Hypersensitivity is evident in low-dose Temozolomide survival response in chemoresistant T98G cells.

(A) Clonogenic survival of HRS+ T98G cells and HRS- U373 cells following 3 day treatment with TMZ (0-10μM). The data points show mean survival ± SEM, n=4. (B) Clonogenic survival of glioma cells (T98G, U373) following 3 day treatment with 3μM and 10μM TMZ. Mean ± SEM, n=3.

4.3.3 *MGMT* GENE AND PROTEIN EXPRESSION IS UPREGULATED IN HRS+ CELLS RELATIVE TO HRS- CELLS

HRS has previously been associated with radioresistance (Joiner et al. 2001). While HRS in these prostate cells was not associated with radioresistance (see section 3.3.4) the data above (section 4.3.1) indicated that HRS may be associated with TMZ resistance. We therefore next investigated whether HRS was related to *MGMT*, a gene that confers resistance to TMZ. *MGMT* gene expression patterns were determined in HRS- cell lines and compared to RWPE1 and PC3 HRS+ cell lines using quantitative RT-PCR (Fig. 4-4). *MGMT* gene expression was down-regulated in HRS- 22RV1 cells, compared to HRS+ RWPE1 cells (7±1.1-fold) and PC3 cells, (5±1.2). *MGMT* expression was also down-regulated in HRS- PWR1E cells relative to RWPE1 (2.1±0.5-fold) but upregulated (2±0.8-fold) when compared to PC3 cells. In contrast, *MGMT* expression was upregulated in HRS- DU145 relative to RWPE1 and PC3 (3.5 ±1.3, 7±2.4-fold, respectively). Western blots were used to determine MGMT protein expression in all cell lines (Fig. 4-4). With the exception of DU145, both HRS+ cell lines (PC3, RWPE1) weakly expressed MGMT, whereas HRS- cells (22RV1, PWR1E) did not. Nuclear extracts from human lymphocytic leukaemia cells (CEM-CCRF cells) and LnCap cells were used as positive controls.

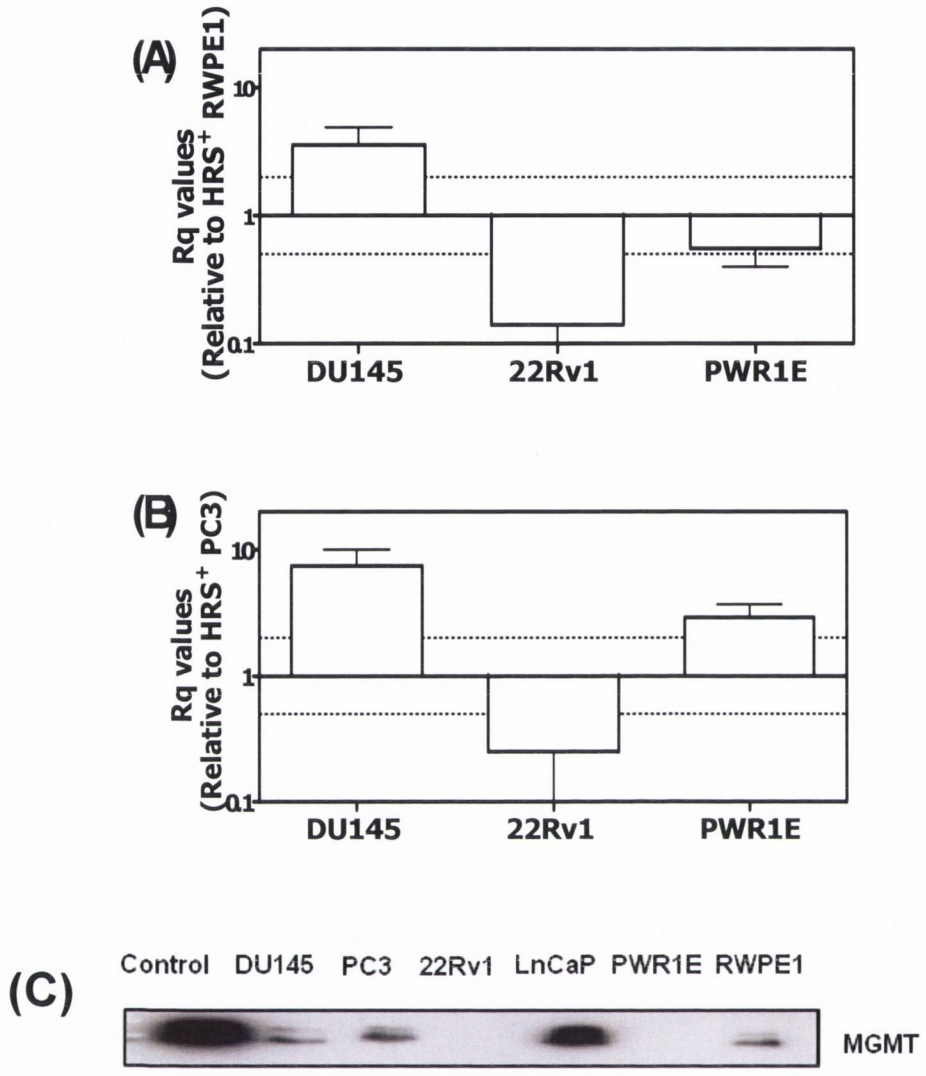

Fig. 4-4: MGMT gene and protein expression in prostate cells

(A-B) MGMT gene expression: Relative quantification (Rq) value of MGMT in HRS- cell lines (DU145, 22RV1, PWR1E), compared to the HRS+ cell lines RWPE1 (A) and PC3 (B). Mean ± SEM, n=2.

(C) MGMT protein expression in prostate cancer (DU145, PC3, 22Rv1, LnCaP) and prostate epithelial cells (PWR1E, RWPE1). CEM-CCRF nuclear extract and LnCaP were used as positive controls.

Figure adapted from the published image in Martin et al, *Radiation Research*, 2009.

4.3.4 *INHIBITION OF MGMT HAS NO SIGNIFICANT EFFECT ON SURVIVAL IN RESPONSE TO LOW DOSES OF RADIATION*

Since both HRS+ PC3 and RWPE1 cell lines express MGMT unlike the HRS- cell lines (22RV1, PWR1E), the influence of MGMT inactivation on radiation survival was investigated by treating HRS+ PC3 cells with the MGMT inhibitor O6-Benzylguanine (O6BG). Inactivation of MGMT via pre-treatment with O6BG has been shown to increase cellular sensitivity to TMZ (Hermisson et al. 2006). Here, we validated the inactivation of MGMT, by showing that pre-treatment of PC3 cells (MGMT+) with O6BG followed by 3 day treatment with either 15μM or 30μM TMZ. Pre-treatment with O6BG significantly increased cell kill by 50% (± 0.07%) (t-test, p=0.022) at a dose of 15μM, and 19.81% (± 0.08%) at the higher dose of 30μM (t-test, p=0.041) (Fig. 4.5). Inactivation of MGMT by O6BG did not appear to inhibit the induction of HRS within the low dose range (Fig. 4.5).

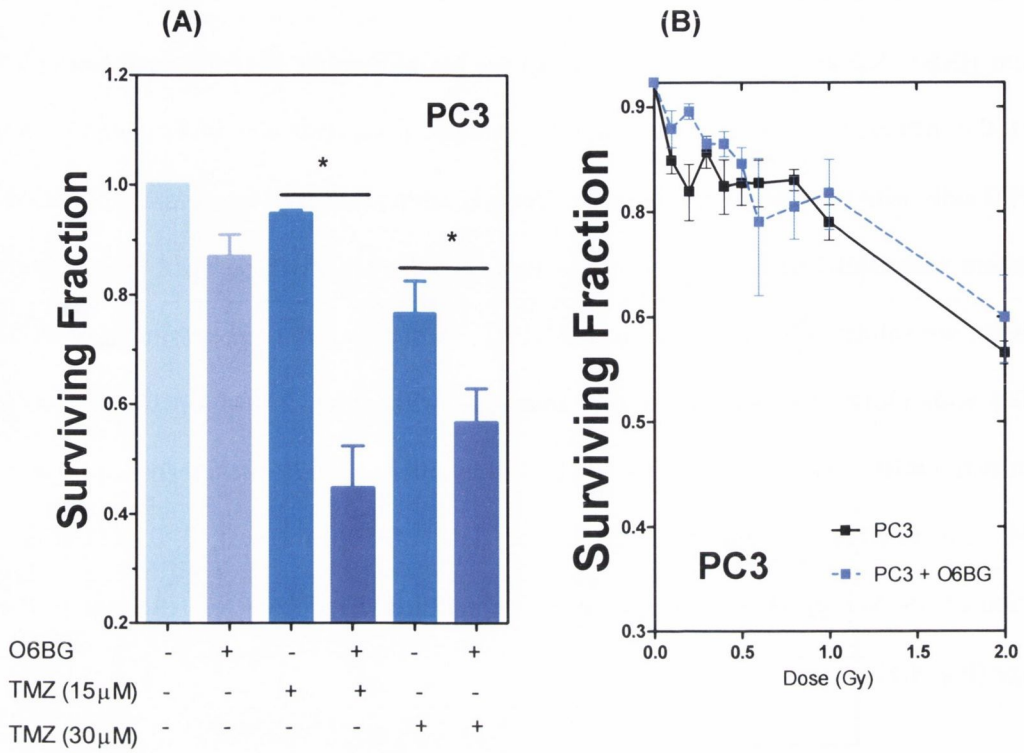

Fig. 4-5: Effect of MGMT inhibition on the survival of PC3 prostate cancer cells

A.Clonogenic survival of PC3 prostate cancer cells after 3-day treatments with TMZ (15 or 30μM) with and without pretreatment with 10 μM OBG. Means ± SEM, n=3).

B. Clonogenic survival following X-irradiation of PC3 cells pre-treated with O6-Benzylguanine for 1 hour (dashed line), relative to survival following X irradiation alone (solid line). The data points show mean survival from 3 to 5 individual experiments (± SEM). n=3. Figure adapted from the published image in Martin et al, *Radiation Research*, 2009.

4.3.5 *MMR PROFICIENCY MAY BE A PREREQUISITE FOR HRS*

In the absence of MGMT or sufficient MGMT to remove O6MeG lesions, MMR proficiency is required for removal of the lesions by apoptosis (Stojic et al. 2004), and has also been implicated in the processing of IR induced DNA damage (reviewed in Martin et al. 2010). Moreover, MMR has been recently implicated in HRS (Krueger et al. 2007). To evaluate a potential role for MMR in HRS, we examined protein expression patterns in HRS+ (PC3, RWPE1) and HRS- (DU145, 22Rv1, PWR1E) cell lines using western blots (Fig. 4-6). As shown in Fig. 4-6, HRS+ cell lines (PC3, RWPE1) expressed MGMT and all five MMR proteins, whereas all HRS- cell lines (DU145, 22Rv1, PWR1E) lacked at least one protein. PMS1 was expressed in all cell lines tested. In HRS- MGMT+ DU145 cells, loss of PMS2 and MLH1 also appeared to prevent induction of HRS.

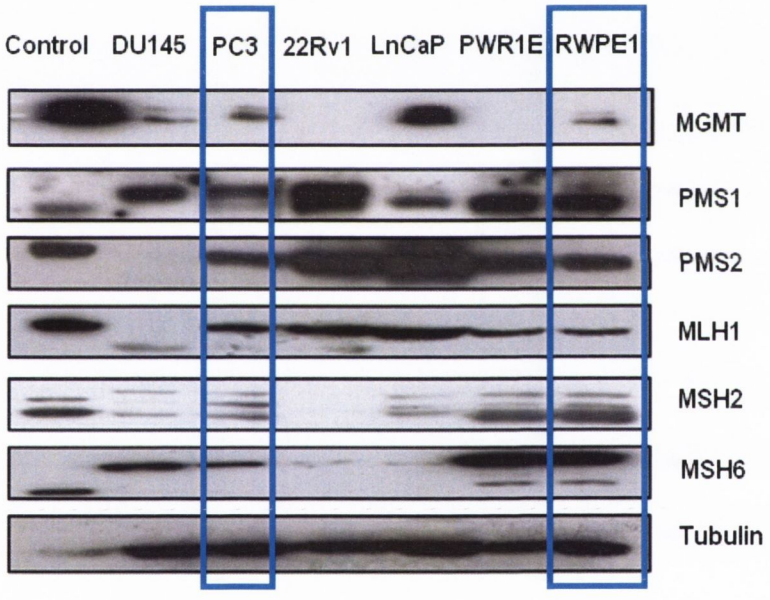

Fig. 4-6: HRS+ is associated with MMR proficiency and MGMT in prostate cells

(A) Representative western blots for MGMT and the mismatch repair proteins PMS1, PMS2, MLH1, MSH2 and MSH6 in a panel of prostate cell lines. HRS+ cells are represented by a blue box. CEM-CCRF and Hela nuclear extracts were used as positive controls for MGMT and MMR proteins respectively. Detection of Tubulin was used as a loading control. LnCap was used as a negative control for MSH2. Figure published in Martin et al, *Radiation Research*, 2009.

4.3.6 *VALIDATION OF MMR PROTEIN EXPRESSION PANEL IN GLIOMA CELLS*

Given that the majority of work on HRS has been conducted in glioma cells which have been demonstrated to show the most extreme HRS responses to date, we chose to validate this protein expression pattern in a panel of glioma cell lines (T98G, U87-MG and U373) whose HRS expression status has been previously reported. Western blots investigating the MMR and MGMT status of glioma cells (Fig. 4-7) corroborate the protein expression pattern observed in prostate cells; HRS+ T98G cells expressed all 5 MMR proteins and MGMT, whereas non-hypersensitive U87-MG and U373 lacked expression of PMS1, MGMT or both.

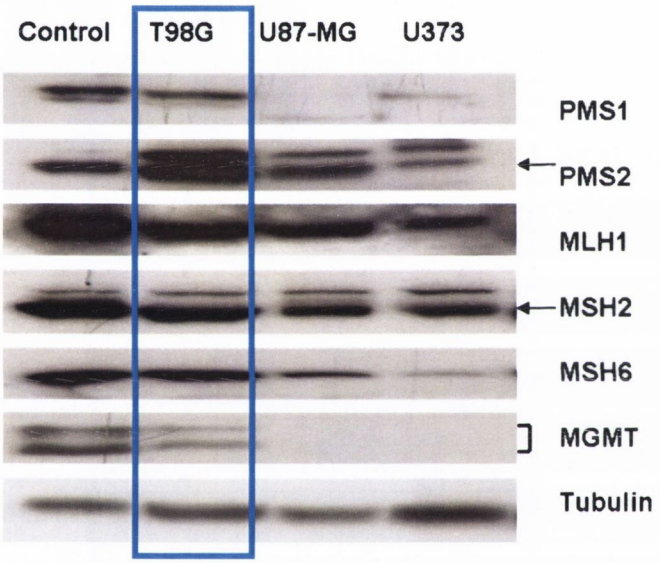

Fig. 4-7: HRS+ is associated with MMR proficiency and MGMT in glioma cells

(A) Representative western blots for MGMT and the mismatch repair proteins PMS1, PMS2, MLH1, MSH2 and MSH6 in a panel of glioma cell lines. HRS+ cells are represented by a blue box. CEM-CCRF and Hela nuclear extracts were used as positive controls for MGMT and MMR proteins respectively. Detection of Tubulin was used as a loading control.

4.4 Discussion

The molecular signalling response of cells exposed to low doses of ionizing radiation, appears to mirror the signalling response of cells to O6-Methylguanine damage. Thus, we hypothesized that MGMT and MMR proteins may play a role in promoting low-dose hyper-radiosensitivity or overcoming HRS. In this model, we propose, that in the absence of MGMT, recognition and repair of the lesions by the mismatch repair system leads to the induction of DNA double strand breaks, the subsequent induction of apoptosis, leading to the low-dose HRS survival response (Fig. 4-8).

Given that the response of cells to low dose radiation appeared to mimic the cellular response to O6MeG lesions we investigated whether low dose hypersensitivity was evident in response to TMZ, an alkylating agent that creates O6MeG lesions. Treatment with low concentrations of TMZ resulted in a survival response not dissimilar to HRS in HRS+ PC3 cells (Fig. 4-2). Moreover, this sensitivity was not observed in HRS- cell lines. Given that TMZ resistance may be associated with HRS we next investigated whether HRS could be correlated with MGMT, a gene that confers resistance to TMZ. *MGMT* gene expression and protein expression was upregulated in HRS+ cells relative to HRS- cells (Fig. 4-4).

While HRS was not correlated with absent MGMT gene expression as might be predicted if O6MeG lesions are involved, MGMT and all MMR proteins (MSH2, MSH6, PMS1, PMS2, MLH1) were expressed only by the HRS+ cell lines (PC3, RWPE1, T98G). Loss of at least one of the MMR proteins was co-incident with a HRS- phenotype, with the exception of PWR1E (Fig. 4-6, Fig. 4-7). In MMR-proficient cells, loss of MGMT but not its inactivation appears sufficient to inhibit the induction of HRS. Moreover, since treatment with the MGMT inhibitor O6-Benzylguanine did not prevent induction of HRS, it may be postulated that the number of O6MeG lesions produced by low doses of radiation are below the activation threshold of the enzyme. It must however be noted that levels of MGMT in these HRS+ cell lines were low and it is possible that these levels are stochiometrically insufficient to remove the lesions (Fig. 4-4) (Pegg et al. 1985). It has, in addition, been suggested that MGMT may protect against background DNA damage (e.g. endogenous single strand breaks (SSBs)) following demonstration of a significant

correlation between background SSBs and the MGMT polymorphism 84Phe in lymphocytes *in vitro* (Rzeszowska-Wolny et al. 2005). Thus, it may alternatively be postulated that the cumulative DNA damage following low dose radiation in MGMT+ cells is less than that in MGMT- cells, keeping overall damage sufficiently low for HRS to occur.

The MMR protein expression pattern of 22RV1, PWR1E and RWPE1 prostate cells has, to our knowledge, not previously been reported. Our data suggest the involvement of MMR proteins in the hypersensitivity response (Fig. 4-6). This hypothesis is supported by the documented expression of HRS in five MMR-proficient cell lines (T98G, SNB19 (glioblastoma), A549 (lung), HT29 (colorectal), MeWo (melanoma) and the absence of HRS in three MMR-deficient cell lines (SW48, HT116, RKO (colorectal))(Joiner et al. 2001).

In our working model, we propose that enhanced cell kill following low doses of radiation may result from low levels of O6MeG lesions, leading to formation of mispairs and induction of apoptosis following processing by the MMR system, in cells with low levels of background DNA damage as afforded by low levels of MGMT. Since inhibition of MGMT did not prevent induction of increased radioresistance (IRR), it may be postulated that IRR results not only from increasing levels of DNA damage but rather a shift in the type of critical DNA damage above doses of 0.5Gy. The increased activation of ATM (ser1981) above doses of 0.3Gy as demonstrated by Marples and colleagues (Krueger et al. 2007) could therefore reflect a shift in the hierarchy of critical DNA damage and the subsequent signalling pathways (MGMT, MMR) from the processing of O6MeG lesions at low doses, to DNA double strand breaks at higher doses, rather than an accumulation of the same type of damage. Moreover, reduced or absent ATM expression has been shown to sensitise cells against O6MeG (Debiak et al. 2004), so decreased ATM activity at low doses may even be an active protective response to facilitate removal of these critical toxic lesions. The transition from HRS to IRR may therefore require both inactivation of signalling pathways (as previously proposed by Marples and Joiner (Marples et al. 1997)) in response to O6MeG as well as activation of DNA repair pathways. The increase in mitotic index reported in HRS+ cells relative to HRS- cells (Marples et al. 2004; Krueger et al. 2007) could be explained by the fact that

mismatch repair dependent processing of O6MeG lesions and the associated G2 arrest occurs after the second S phase after damage (Bean et al. 1994; Galloway et al. 1995). Interestingly, among the cell lines most sensitive to low radiation doses are a large number of glioma cell lines (Short et al. 1999; Short et al. 2001; Chandna et al. 2002) which express low levels of MGMT (Lorente et al. 2008), are MMR+ and are consequently highly sensitive to O6MeG lesions. In this regard, defects in DNA-dependent protein kinase (DNA-PK) also sensitise cells to O6MeG (Roos et al. 2007). Down-regulation of DNA-PK in response to low radiation doses may therefore also reflect an active protective response to maintain cell repair fidelity.

In the present chapter, we investigated the role of O6MeG lesions in HRS by measuring the survival of prostate cell lines in response to low dose Temozolomide ,an agent that induces O6MeG lesions. Our cell lines showed differing MGMT and MMR expression patterns which correlated with the response of these prostate cells to low dose irradiation. Experiments involving the inactivation of MGMT were conducted to examine the role of MGMT in HRS. Our results support the involvement of MMR-dependent processing of damage induced by low-dose radiation in PCa cells (Fig. 4-8).

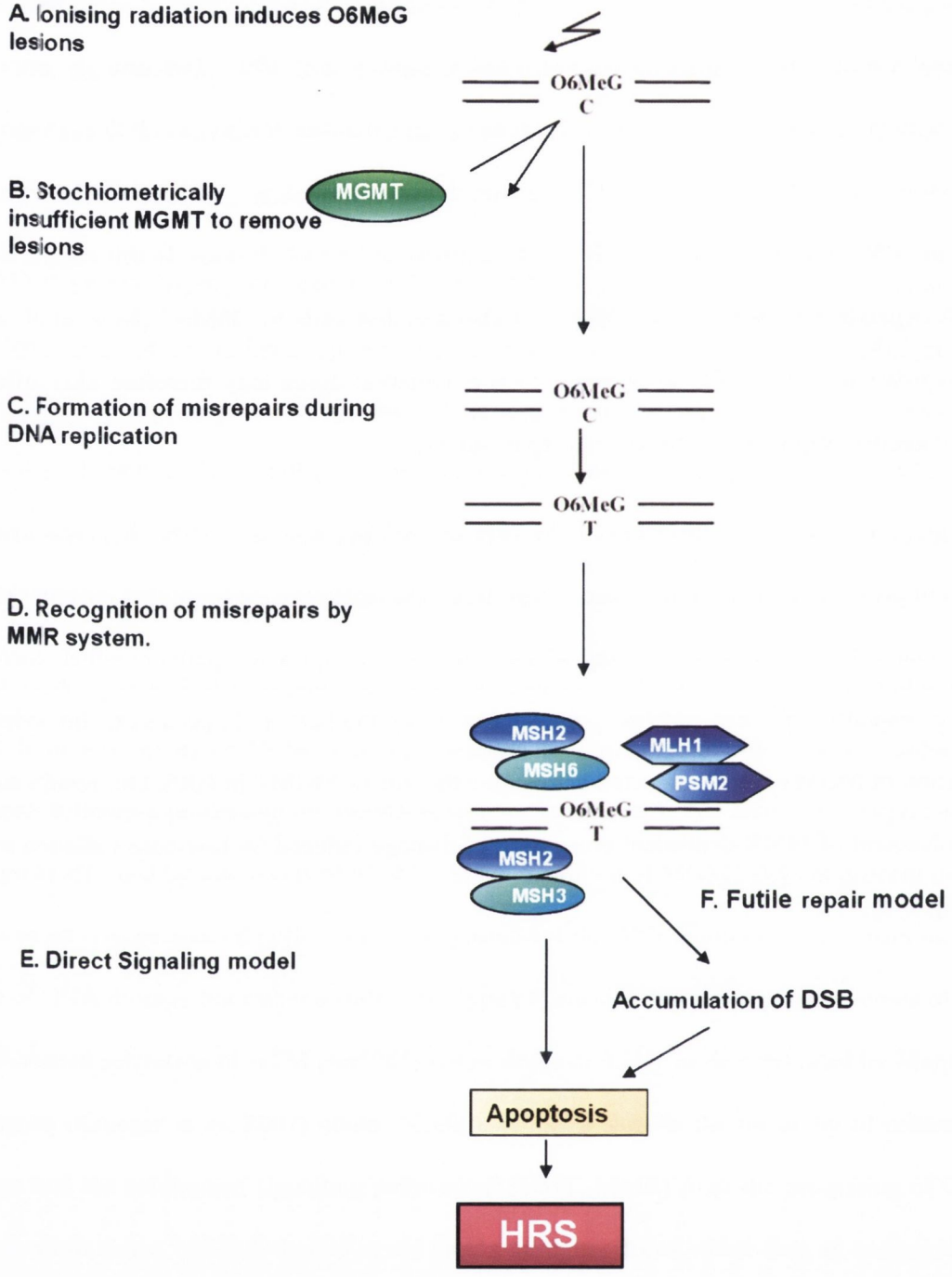

Fig. 4-8: Model of O6MeG triggered low-dose hyper-radiosensitivity.

Current working hypothesis A. Ionising radiation induces O6MeG lesions. B. There is stochiometrically insufficient MGMT to remove these lesions. C. Formation of mispairs by the MMR system. D. Recognition of mispairs by the MMR system E. Direct signalling model: MMR proteins may signal apoptosis directly. F. Futile repair model: Reiterated futile attempts at repair upon recognition of the O6-MeG:T and O6-MeG:C mispairs, ultimately leads to the creation of double strand breaks following replication and cell death by apoptosis. Figure adapted from the published image in Martin et al. *Radiation Research 2009.*

Chapter 5: DNA Mismatch Repair Protein MSH2 Participates in Low Dose Radiation Hypersensitivity via Regulation of MRE11 and the Early G2 Checkpoint

Chapter 5 is adapted from the

submitted manuscript:

"DNA MMR protein MSH2 may dictate the cellular survival to low dose radiation"

Lynn M. Martin, Brian Marples, Anthony Davies, Ann Atzberger, Connla Edwards, Mary Coffey, Thomas H. Lynch, Donal Hollywood, and Laure Marignol

5.1 INTRODUCTION

Two distinct G2/M phase cell cycle checkpoints are known to be activated following exposure to IR (Xu et al, 2002) namely the early and late G2/M phase checkpoints. The first and so-called early G2/M checkpoint is the response to DNA damage in cells that are already in G2 at the time of irradiation, and reflects the failure of these cells to progress to mitosis. This checkpoint is typically activated within two hours of irradiation, is transient, independent of the dose of IR used in the range 1-10Gy (Xu et al, 2002), and has a distinct activation threshold. The second G2/M checkpoint is activated at late time points after exposure and reflects the delay of cells in other phases of the cell cycle (G1, S) at the time of irradiation.

HRS has been repeatedly correlated with evasion of the early G2/M checkpoint (Marples et al. 2003; Collis et al. 2004; Marples 2004; Krueger et al. 2007; Fernet et al. 2009; Xue et al. 2009; Krueger et al. 2010). The transition from HRS to IRR appears to be reflective of the activation of the early G2 checkpoint, and thus inducible repair mechanisms (Krueger et al. 2007; Fernet et al. 2009; Xue et al. 2009; Krueger et al. 2010). However, HRS in keratinocytes is associated with a maintained growth arrest (Turesson et al. 2001; Turesson et al. 2010).

The early G2/M checkpoint can be initiated by the MRN complex (Fig. 5-1), consisting of the highly conserved proteins MRE11, RAD50, and NBS1, which are essential for activation of the ATM kinase (Carson et al. 2003; Bi et al. 2005); the latter activates the checkpoint effector kinases Chk1 (via ATR) and Chk2 (directly) (Chaturvedi et al. 1999), which ultimately control entry into mitosis. The delay afforded by the arrest in proliferation is thought to allow vital time for the orderly and timely repair of mutagenic lesions created by DNA damaging agents, prior to cell entry and transit through mitosis (Hartwell and Weinert 1989). The arrest is only released when repair is completed. Where repair is not possible, damaged cells are removed by programmed cell death (apoptosis)(Houtgraaf et al. 2006).

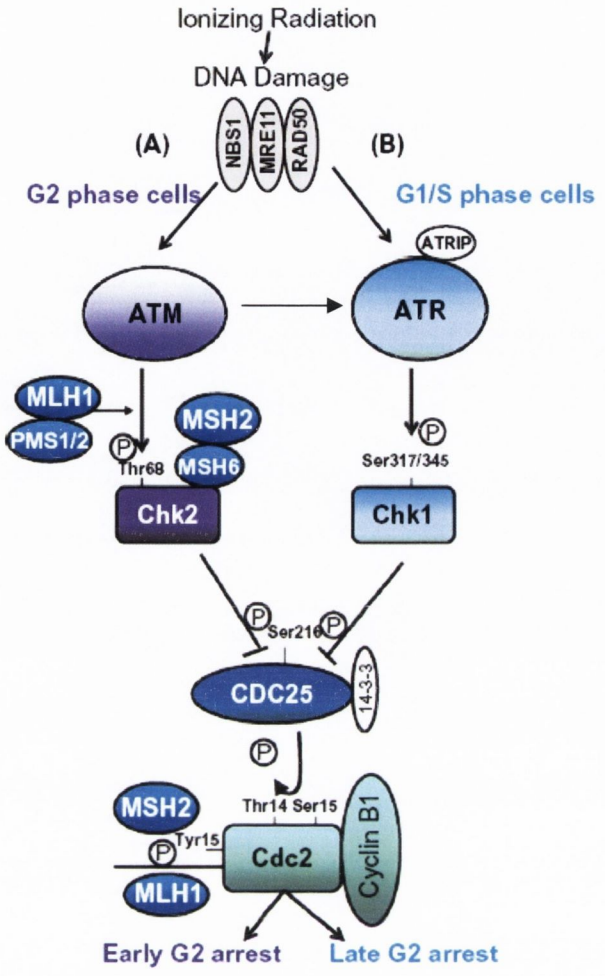

Fig. 5-1: Ionizing radiation induced activation of the G2/M checkpoint

(A) Cells in G2 phase at the time of radiation undergo a rapid transient G2 arrest (early G2 arrest). Ataxia telangiectasia mutated (ATM) is recruited to sites of DNA damage following ionizing radiation. ATM responds to double strand breaks and an activating role for the MRE11/RAD50/NBS1 (MRN) complex has been suggested. This arrest is mediated by ATM dependent phosphorylation of checkpoint kinase Chk2 and CDC25 phosphatase. This prevents dephosphorylation of Cdc2–CyclinB, which is required for progression into mitosis. Evidence suggests that MLH1 (which forms a heterodimer with PMS1/PMS2) interacts with ATM and MSH2 (which forms a heterodimer with MSH6) interacts with Chk2 indicating a possible role for these proteins in the early G2 arrest. (B) Cells in G1/S phase at the time of irradiation are thought to undergo a late G2 arrest. This is ATM independent and likely to be primarily activated by ataxia telangiectasia and RAD3 related (ATR) kinase. ATR mediates this arrest by phosphorylation of Chk1 and Cdc25 which prevents dephosphorylation of Cdc2–CyclinB and progression into mitosis. A role for MLH1 and MLH2 has been suggested in the regulation of Cdc2 signalling pathway and these proteins may therefore have a possible role in the activation of the late G2/M arrest. Figure published in Martin et al, *Cancer Treatment Reviews* 2010

As previously mentioned, DNA mismatch repair (MMR) is a post-replicative editing process, primarily involved in repairing errors that arise during replication. Inherited mutations in MMR genes give rise to a cancer predisposition syndrome called hereditary non-polyposis colorectal cancer (HNPCC) which is associated with DNA damage tolerance and resistance to a wide variety of chemotherapeutic agents (Stojic et al. 2004; Kinsella 2009). The role that MSH2 and MLH1 proteins play in the DNA damage response to IR remains controversial. Conflicting studies report that MMR-proficiency either confers radiosensitivity, radioresistance, or has no effect on cellular radiosensitivity (Martin et al. 2010)). For example, MSH2-proficiency has been associated with radiosensitivity in mouse embryo fibroblast (MEF) cell lines (Fritzell et al. 1997) (Xu et al. 2001) and mouse embryonic stem cells (DeWeese et al. 1998). Similarly, PMS2-proficiency was associated with radiosensitivity in MEFs in response to IR (Zeng et al. 2000). However, MSH2-proficiency has also been associated with radioresistance (Franchitto et al. 2003; Bucci et al. 2005; Barwell et al. 2007).

MMR is essential for the activation of the early G2-M cell cycle arrest following treatment with a variety of anti-cancer agents (Stojic et al. 2004; Li 2008). Accumulating evidence indicates that efficient G2-M checkpoint activation following exposure to IR also requires MMR. Immunohistochemistry studies have revealed that following exposure to IR, components of the MMR system interact with ATM both directly (Brown et al. 2003) and possibly indirectly, via localization of MRE11 (Franchitto et al. 2003), thus facilitating the phosphorylation of Chk2 (Brown et al. 2003). Defects in the IR triggered activation of the G2-M checkpoint have thus been attributed to dysregulated MSH2-dependent localization of MRE11, as well as incomplete activation of checkpoint kinases Chk1 and Chk2 (Franchitto et al. 2003).

In Chapter 4, we implicated MMR-dependent processes in the HRS response, demonstrating that HRS is associated with MMR-proficiency in a panel of prostate (Martin et al. 2009) and glioma cell lines (unpublished results), and is associated with the response of cells to O6MeG lesions. Given that inhibition of MGMT had no significant effect on survival in the low dose range, it is reasonable to infer that HRS could not rely solely on the processing of these lesions, and a direct

role for MMR is certainly possible, given the accumulating evidence suggesting that MMR is involved in recognition of IR induced damage, and checkpoint responses. Our previous studies have suggested that direct MMR-dependent processes might be required for the expression of HRS. The enhanced low-dose cell killing was likely attributable to an inactivation of the early G2 arrest. Here, we test our hypothesis that MMR proteins may be involved in HRS by examining the low-dose radiation response of isogenic cell lines proficient and deficient in the expression of MSH2.

5.2 Objectives and Methods

Pre-established human isogenic disease models are valid research tools to evaluate the influence of a particular gene in response to a cytotoxic agent. These isogenic models are a family of cells that are selected or engineered to accurately model the genetics of a specific patient population, *in vitro*. They are coupled with genetically matched 'normal cells' to provide an isogenic system to research disease biology and novel therapeutic agents.

In this instance, use of pre-established isogenic models was considered the most suitable approach to evaluate the role of MMR genes in HRS. The alternative approach would be to conduct knock-down studies using the MMR-proficient PC3 cell line used in the previous chapters. We came to this conclusion based on several observations: first, any one of the MMR genes could potentially be involved in the HRS response and it would be very limiting to the scope of the study to invest the considerable time it would take to validate and test each individual gene for its involvement in the HRS response. Second, it was unclear at which time-point such an involvement would take place. The HRS cell survival response observed in PC3 PCa cells was observed following a 14-day incubation post IR. In theory, the MMR genes could be involved in either the early response to DNA damage, or in the late stages in terms of induction of cell death, or both, and so a stable knock-down of the MMR gene of interest would be preferable to either siRNA, or lentiviral knock-down methods, as these would not achieve gene knock-down for this time period. Moreover, the level of expression of MMR genes is known to alter their function in terms of cell cycle checkpoint signalling ((Stojic et al. 2004) and refs. within) and so knock-down studies may not answer the research question posed being whether these genes are involved in the HRS response if the gene is not fully knocked down. Finally, the MMR-proficient gene signature was associated with HRS in prostate cells and glioma cells, and appeared valid in other models based on previous studies. Therefore, we believed that the use of pre-established isogenic cell lines would not detract from the study of the HRS mechanism in prostate cells, as the mechanism appeared likely to be similar, and the "proof of principle" experiments to be tested, could be validated later in prostate cells (Chapter 7)

Thus, to investigate the role for mammalian MMR genes MSH2 and MLH1 in HRS, and in the regulation of the early G2 checkpoint following exposure to low dose IR, we studied whether absence of the MMR protein could result in an altered survival and cell cycle response to DNA damage. To this end, we used endometrial carcinoma cells proficient or deficient in MSH2, and colorectal carcinoma cells proficient and deficient in MLH1.

Specifically, we set out:

- To confirm the differential MMR protein expression of isogenic cells lines differing in the expression of MSH2 (endometrial cancer; HEC59 and HEC59+chr2) or MLH1 (colorectal cancer; HCT116 and HCT116+chr3) using western blotting.

- To evaluate the sensitivity of these cell lines to IR using high precision clonogenic assays, incorporating use of the cell sorter to accurately plate the cells.

- To determine the kinetics of the temporal activation of the early cell cycle response of MSH2 isogenic cells using flow cytometry and to further validate these results using high content screening.

- To examine cell cycle distributions of HEC59 (MSH2-, HRS-) and HEC59+chr2 (MSH2+, HRS+) cells at various time-points after exposure to 0.2Gy radiation for accumulation of cells in G2 phase.

- To evaluate the role of MRE11 in the differential activation of the early G2 arrest using western blotting and high content analysis of MRE11 foci induced by 0.2Gy.

- And finally to determine the effect of MSH2 status on the activation of checkpoint protein kinases Chk1 and Chk2 in response to 0.2Gy radiation using western blotting.

5.3 RESULTS

5.3.1 *MSH2* AND *MLH1* PROFICIENT CELLS DISPLAY HYPERSENSITIVITY TO LOW DOSE RADIATION

To evaluate the specific role of two components of the DNA MMR pathway (MSH2 and MLH1) in the HRS response, we exposed matched MMR-proficient (HEC59+chr2, HCT116+chr3) and MMR-deficient (HEC59, HCT116) cells to low doses of X-rays and then assessed the cytotoxic response using clonogenic assays.

Western blotting confirmed the differential MMR MSH2 and MLH1 status of the two isogenic cell lines (Fig. 5-2A, Fig. 5-3A). Addition of chromosome 2 to HEC59 cells restored MSH2 expression and addition of chromosome 3 to HCT116 and HCT116+chr3 cells restored MLH1 expression. High resolution clonogenic survival experiments demonstrated that MSH2-proficient HEC59+Chr2 (MSH2+) cells exhibited a distinct HRS response that was absent in the native HEC59 cells (MSH2-) (Fig. 5.2B). Similar results were found in MLH1-proficient HCT116 (MLH1+) and native HCT116 (MLH1-) cells (Fig. 5.3B).

Surviving fractions measured at the doses tested were fitted with the induced repair equation (Marples and Joiner 1993), as described previously (Chapter 3). All parameters were fitted simultaneously and estimates of uncertainty were expressed as likelihood confidence intervals. Mathematical modeling confirmed that the cell survival response of MSH2+ and MLH1+ cells were best described by the induced repair model, whereas those of MSH2- and MLH1- cells were best described by the linear quadratic model (Table 5-1). These data indicate that in mammalian cells, MMR proficiency confers hypersensitivity to low doses of radiation (<0.2Gy).

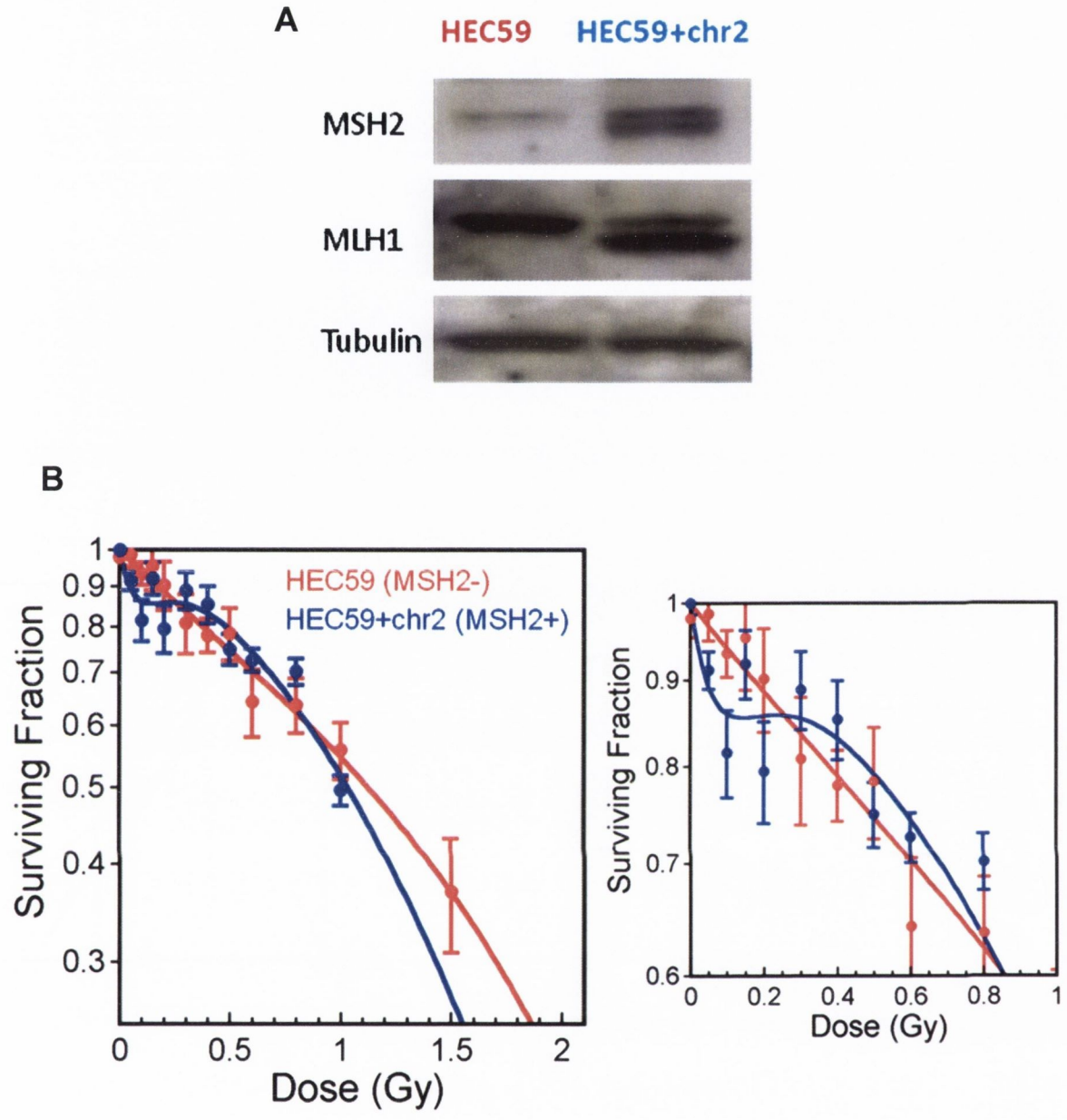

Fig. 5-2: MSH2-proficient cells display HRS

A. Comparison of mismatch repair protein expression in cells proficient and deficient in the expression of MSH2 (HEC59+chr2, HEC59) B. Clonogenic survival of HEC59 and HEC59+chr2 cells in response to low doses of radiation. An expanded image of the low dose region (0-1Gy) is shown on the right. Data points represent the mean ± SEM of at least 6 independent experiments.

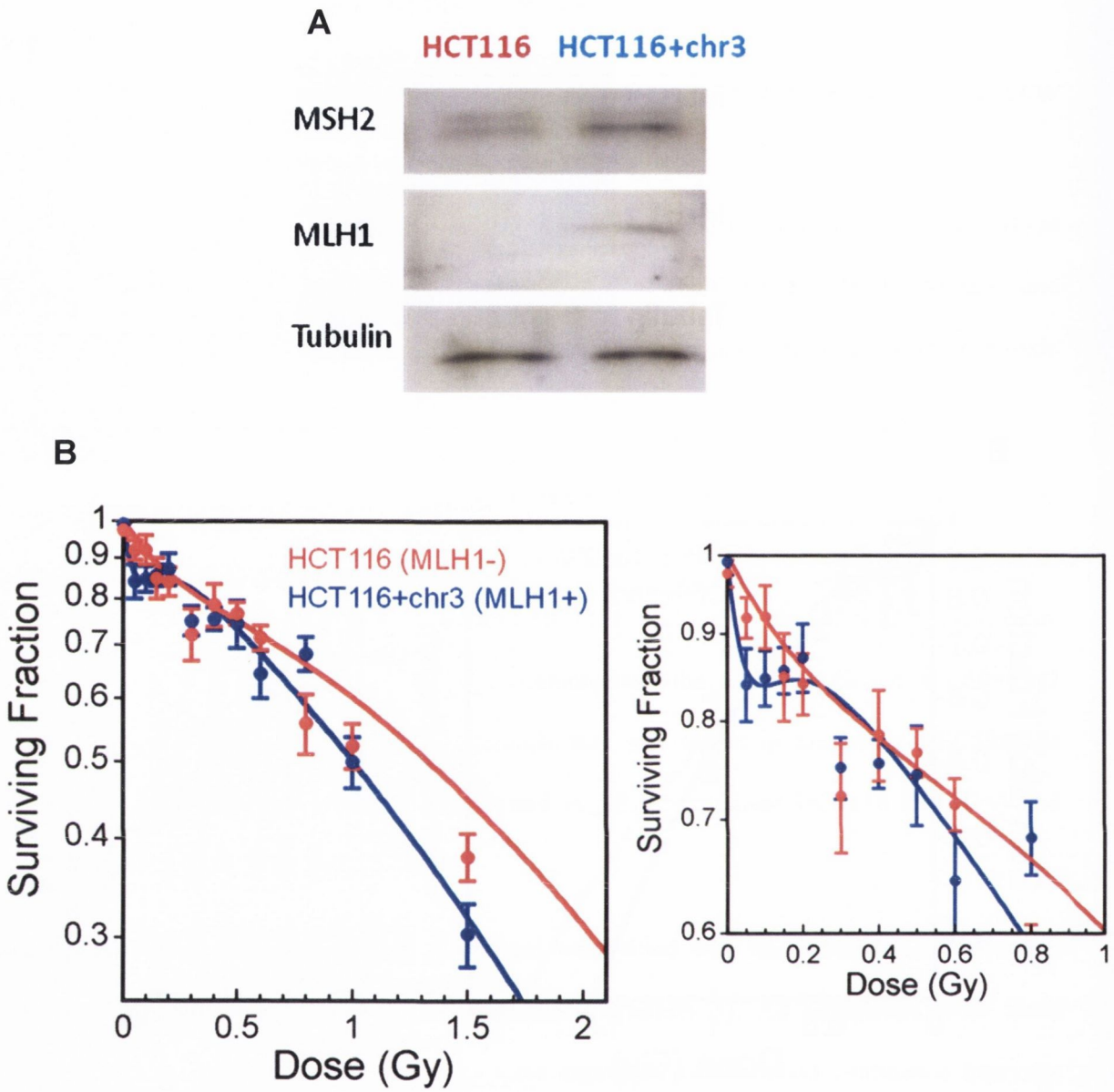

Fig. 5-3: MMR-proficient cells display HRS

A. Comparison of mismatch repair protein expression (MSH2, MLH1) in cells proficient and deficient in the expression of MLH1 (HCT116+chr3, HCT116) B. Clonogenic survival of HCT116, HCT116+chr3 cells in response to low doses of radiation. An expanded image of the low dose region (0-1Gy) is shown on the right. Data points represent the mean ± SEM of at least 6 independent experiments.

Table 5-1: Values of the parameters obtained from mathematical modeling of matched MMR-proficient and MMR-deficient cell lines using the Induced Repair model

Only MMR-proficient cells fulfilled the criteria for distinct hypersensitive responses (i.e. values of α_s and α_r whose confidence limits do not overlap and a value of d_c significantly greater than zero.)

	α_s (± SE)	α_r (± SE)	ß (± SE)	d_c (± SE)	d_c (± CL)
HEC59 (MSH2-)	wnc	0.53±0.09	-0.10±0.08	wnc	wnc
HEC59+chr2 (MSH2+)	3.75±1.51	0.23±0.09	-0.43±0.09	0.11±0.03	0.05±0.2
HCT116 (MLH1-)	wnc	0.58±0.07	0.07 ±0.06	wnc	wnc
HCT116+chr3 (MLH1+)	12.29±8.06	0.57 ±0.06	0.12 ±0.06	0.04±0.013	0.02±0.032

Wnc = would not converge

5.3.2 *MSH2* FUNCTION IS REQUIRED FOR AN EFFICIENT EARLY-ACTING G2/M CHECKPOINT RESPONSE TO LOW DOSES OF RADIATION

5.3.2.1 Phospho-histone H3 Analysis using flow cytometry

The mitotic ratio of a population of cells can be determined by distinguishing the number of cells that stain positive for phospho-H3 within the G2/M cell population determined by PI staining. Phosphorylation of histone H3 (ser28) is tightly correlated with chromosome condensation in mitosis (Hendzel et al, 1997), and has been successfully used for assessment of the early G2 checkpoint (Krueger et al. 2007). Compared to MSH2- cells, untreated MSH2+ cells showed a significantly lower basal mitotic population (p=0.0036)(Fig. 5-4, Fig. 5-5A). The mitotic ratio of irradiated MSH2- and MSH2+ cells increased 15min after exposure to 0.2Gy (Fig. 5-5B). Thereafter, the ratio decreased in both cell lines over the next 120min. However, at 2hr only MSH2+ cells showed a significantly decreased in comparison to sham irradiated control cells (p=0.0328) (Fig. 5-5C).

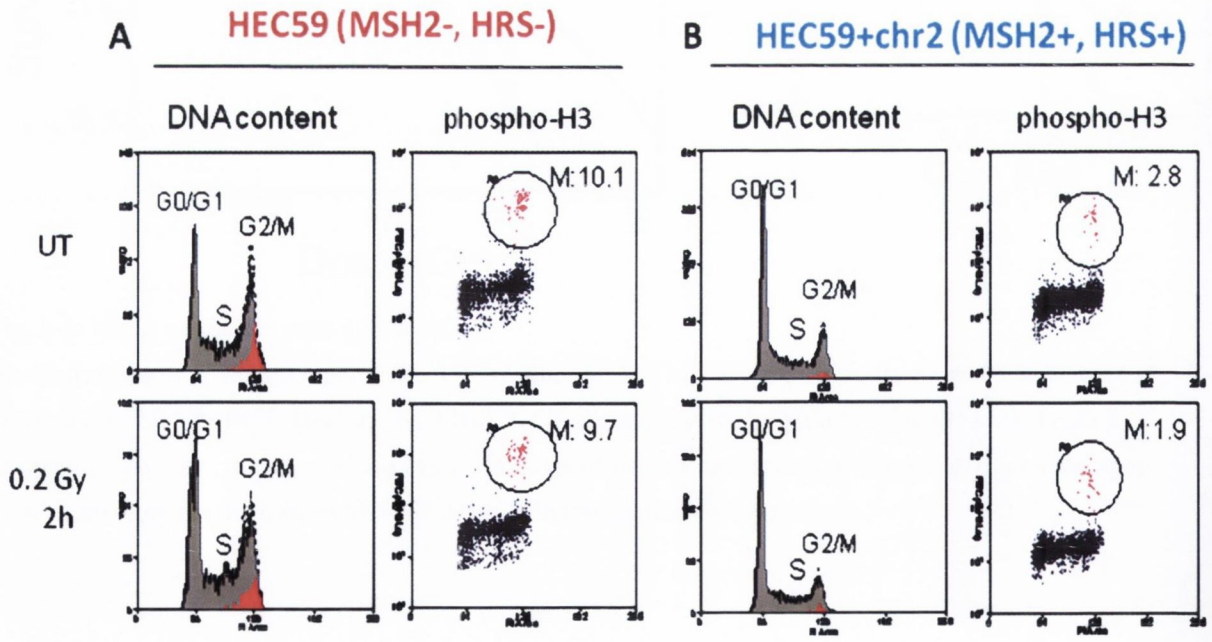

Fig. 5-4: Detection of mitotic cells based on staining with the mitotic marker phospho-histone H3 before and after exposure to ionizing radiation (0.2Gy) using flow cytometry

Representative images for HEC59 (A) and HEC59+chr2 cells (B) either untreated (UT) or 2hr after exposure to radiation (0.2Gy) stained with propidium iodide and phospho-histone H3 (red).

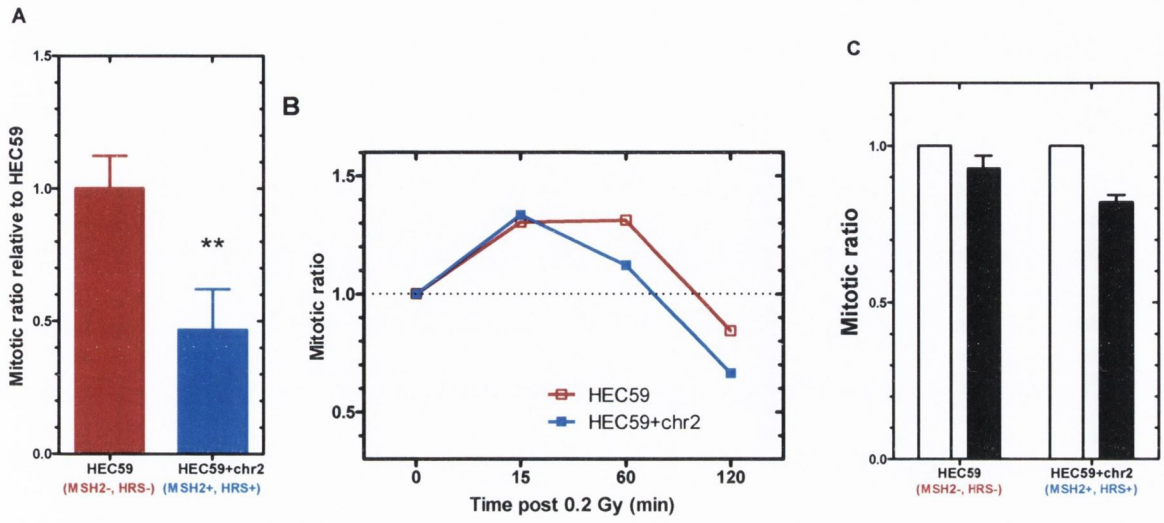

Fig. 5-5: Analysis of the mitotic index of HEC59 and HEC59+chr2 cells following irradiation.

A. Comparison of the basal mitotic ratio of HEC59 cells shown in blue (MSH2-, HRS-) to the mitotic index of HEC59+chr2 cells shown in red (MSH2+, HRS+), following quantification of the data obtained in Fig. 5-3 (mean ± SEM of at least 3 independent experiments are shown). Image analysis was performed with Summit Software v4.3 (Dako).

B. Comparison of the mitotic index of HEC59 and HEC59+chr2 cells up to 2hr after irradiation with 0.2Gy. Standard errors are not shown on B. but were always < 10% of the respective mean value.

C. Comparison of the mitotic index of HEC59 and HEC59+chr2 cells 2hr after irradiation with 0.2Gy. Shown are untreated cells (white bars) compared to cells irradiated with 0.2Gy (black bars).

5.3.2.2 Phospho-histone H3 Analysis using high content screening

We chose to validate these results using high content screening. Hoechst was used to stain nuclei of cells, and anti-phospho histone H3 (phospho-H3) was used to identify mitotic cells. To score mitotic cells in a high-content throughput, we used the Multi-Target Analysis (MTA) algorithm (GE Healthcare, Investigator v3.5) to identify individual cells and mitotic cells (phospho-histone H3 positive). A filter was incorporated into the algorithm to calculate the percentage of cells staining positive for phospho-histone H3 (i.e. mitotic cells). This was facilitated by the inclusion of a nuclear intensity threshold for Alexa-fluor 488 such that cells with a nuclear intensity greater than 200 MESFs (Molecules of Equivalent Soluble Fluorophor) were identified as phospho-histone H3 positive. This value was chosen based on evaluation of positive and negative controls. The mitotic spindle inhibitor Hsp90 inhibitor 17-AAG was used as a positive control to ensure that this algorithm could identify an accumulation of mitotic cells. This segmentation algorithm analyzes grey scale images of the data, and outlines and counts phospho-H3 positive cells as green, and phospho-H3 negative cells (those with a nuclear intensity <200 MESFs) as red, as shown in Fig. 5-6.

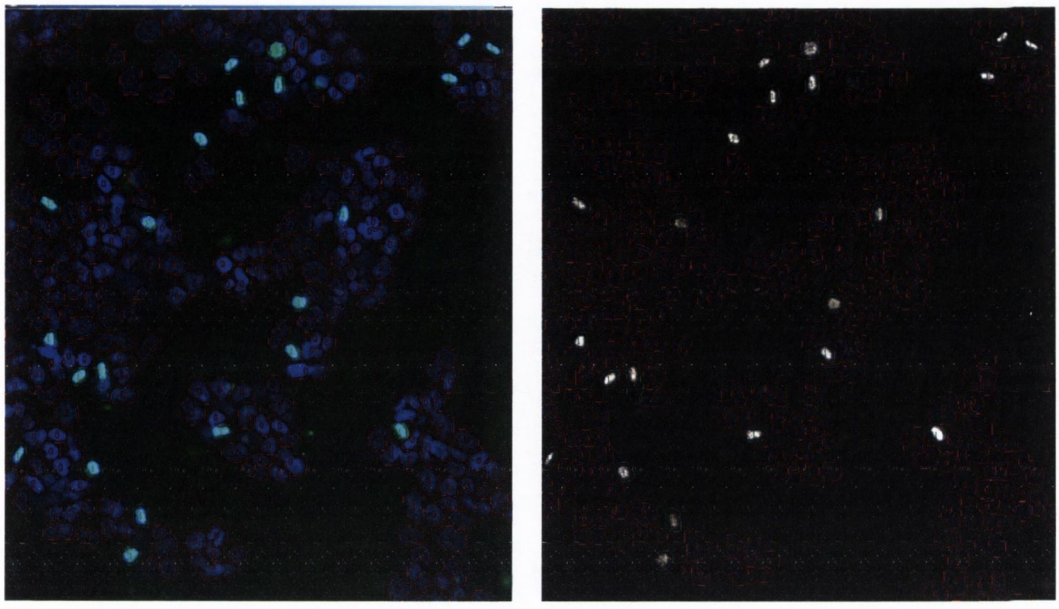

Fig. 5-6: Representative images of phospho-histone H3 staining using high-content analysis
(Left panel) Positive control (HEC59 cells treated with a mitotic spindle inhibitor) B. In cell analyzer software was used to count mitotic cells (green) and non-mitotic cells (red).

Analysis of phospho-H3 by high content analysis, corroborated those obtained previously by flow cytometry, revealing a significantly decreased mitotic ratio 2hr post IR ($p=0.0115$), that was not evident in native HEC59 cells ($p=0.1439$) (Fig. 5-7-Fig.5-9).

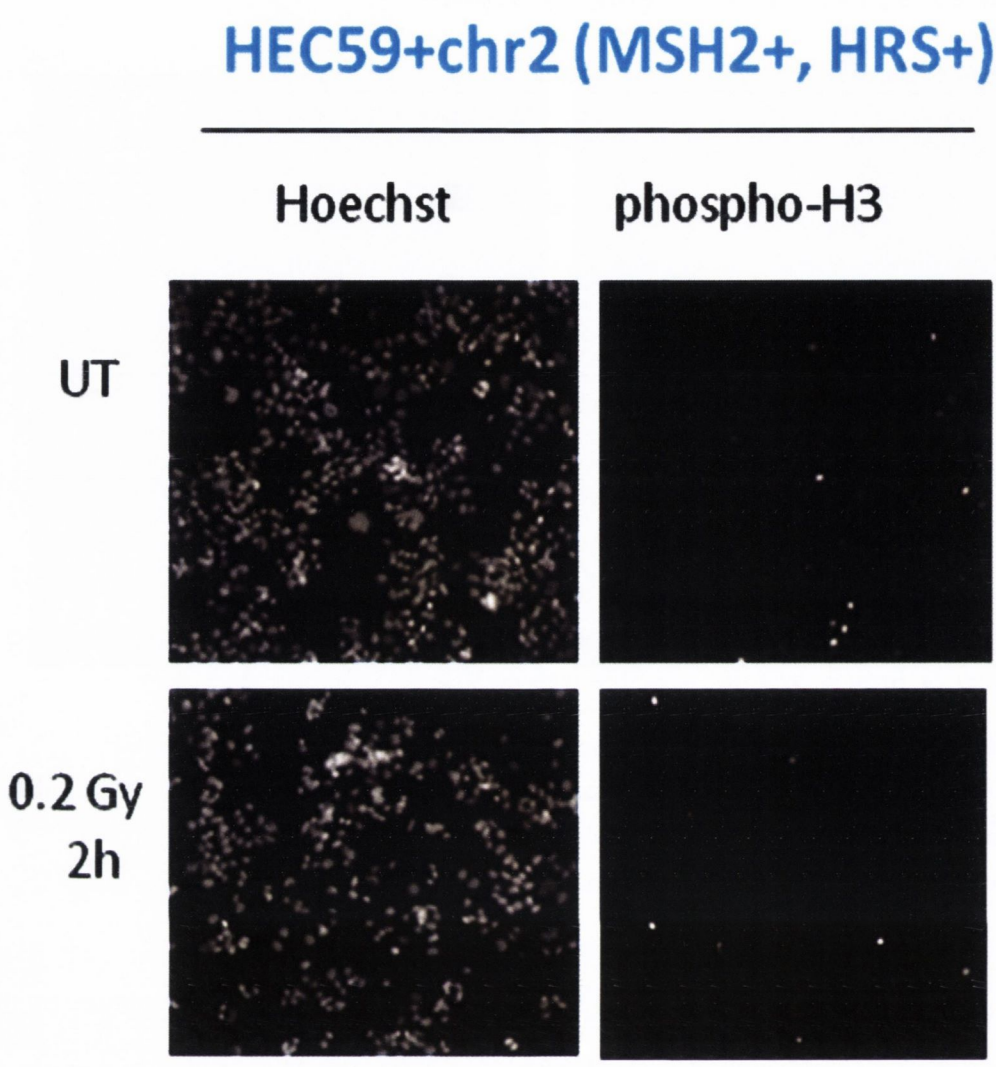

Fig. 5-7: Detection of mitotic cells in HEC59+chr2 cells, based on staining with the mitotic marker phospho-histone H3 before and after exposure to ionizing radiation (0.2Gy) using high content analysis.

Representative fields of HEC59+chr2 cells either untreated (UT) (upper panels) or 2hr after exposure to radiation (0.2Gy) (lower panels) stained with Hoechst (left panels) and anti-phospho histone H3 antibody (right panels). Images were acquired with IN Cell Analyzer 1000 automated fluorescent imaging system (GE Healthcare, Piscataway, NJ) by exposing fields for fixed times using a using a 340/40-nm and 480/40-nm excitation filters, a Q505LP dichroic mirror and 460/40-nm and 535/50-nm HQ emission filters (objective×10).

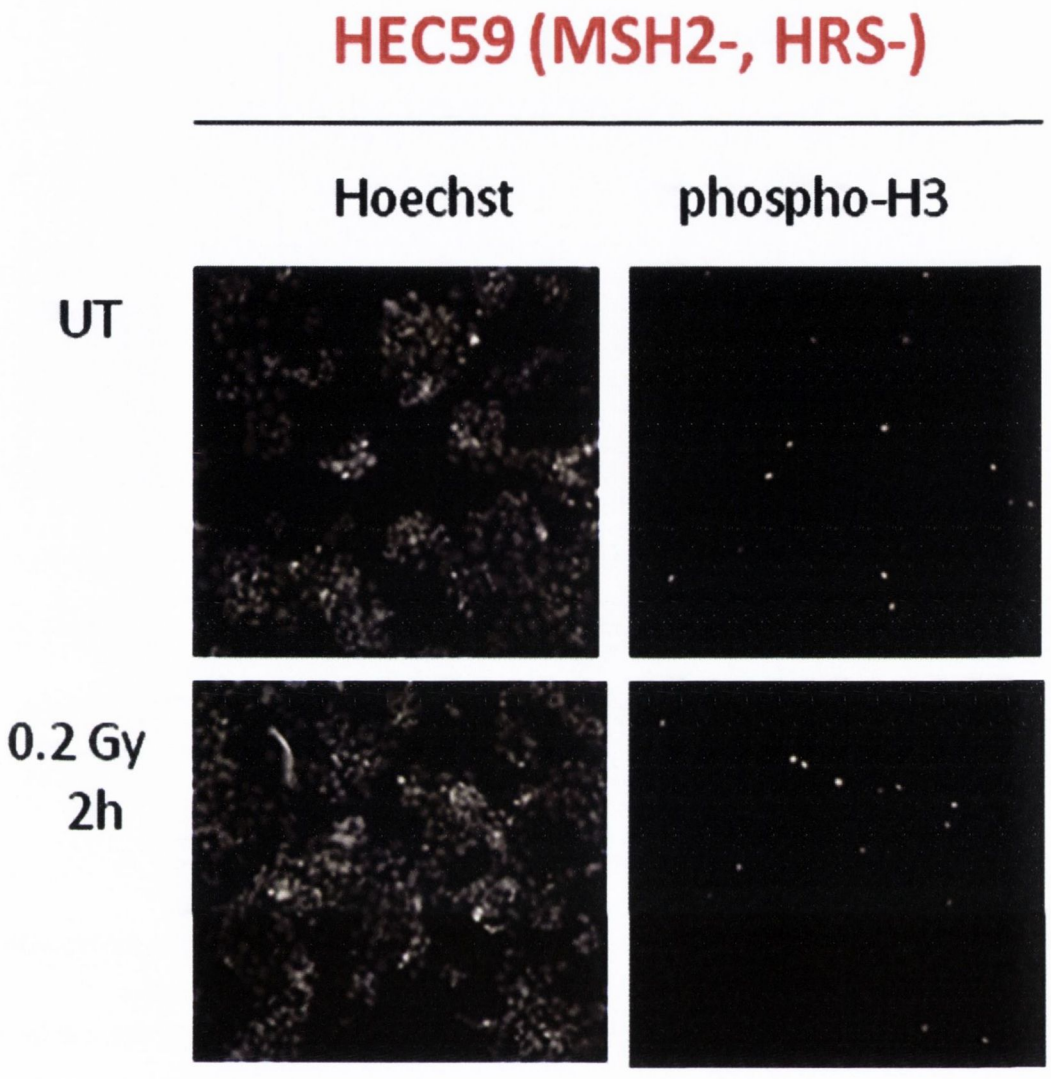

Fig. 5-8: Detection of mitotic cells in HEC59 cells, based on staining with the mitotic marker phospho-histone H3 before and after exposure to ionizing radiation (0.2Gy) using high content analysis.
Representative fields of HEC59 cells either untreated (UT) (upper panels) or 2hr after exposure to radiation (0.2Gy) (lower panels) stained with Hoechst (left panels) and anti-phospho histone H3 antibody (right panel). Images were obtained as described previously (Fig. 5-7).

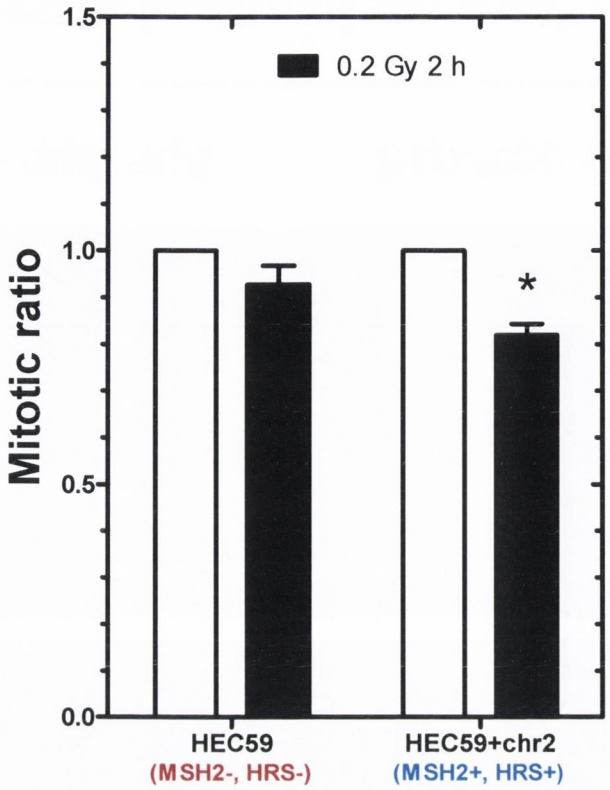

Fig. 5-9: MSH2+ endometrial cells have a reduced percentage of mitotic cells relative to MSH2- cells and arrest preferentially at the early G2 checkpoint following exposure to 0.2Gy radiation

Analysis of the mitotic index following quantification of the data obtained in Fig.5-7 and Fig. 5-8 (mean± standard error of the mean of at least 3 independent experiments are shown). Image analysis was performed with IN Cell Analyzer 1000 using a Multi-target analysis algorithm.

5.3.3 HRS IS NOT ASSOCIATED WITH G2 ACCUMULATION AT LATE TIME POINTS AFTER 0.2GY

Time points up to 72hr after IR were also evaluated using PI staining, however, no significant differences were observed in G2 phase cells or any other cell cycle phase at the time points investigated (8, 24, 48 or 72h) (Fig. 5-10).

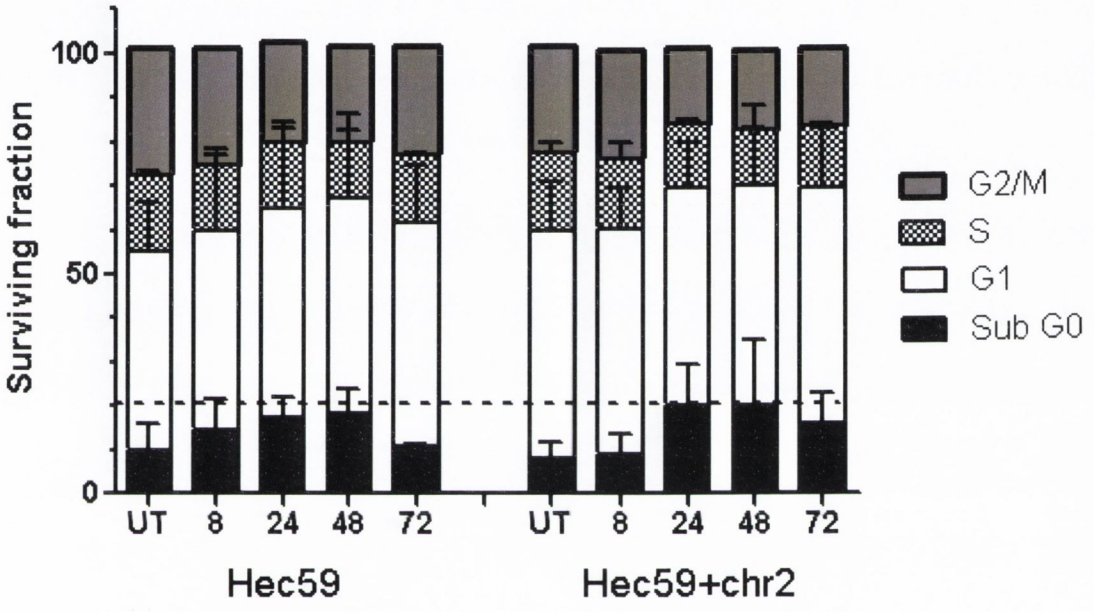

Fig. 5-10: Cell cycle distributions of HEC59 (MSH2-, HRS-) and HEC59+chr2 (MSH2+, HRS+) cells at various time-points after exposure to 0.2Gy radiation

Cells were fixed in 70% ethanol at indicated time points after exposure to 0.2Gy and subsequently stained with propidium iodide for analysis of DNA content by flow cytometry. Images are representative of 3 independent experiments. Data analysis was performed using CyAn ADP flow cytometer (Beckman Coulter) and additional analysis was performed using Summit software (Dako).

5.3.4 THE MMR SYSTEM IS REQUIRED FOR LOW DOSE RADIATION INDUCED MRE11 FOCI INDUCTION

MSH2 has been reported to be required for efficient cell cycle arrest and MRE11 localisation following irradiation with high doses of radiation (4-8Gy)(Franchitto et al. 2003). To investigate the role of MRE11 in MMR after low dose exposures to IR the number of MRE11 foci was measured in the two cell lines at 2 and 24hr post irradiation using high content screening. MRE11 foci were counted with an algorithm developed to count foci induced by radiation (Fig. 5-11). The algorithm used was validated by demonstrating that use of a specific inhibitor of MRE11 (Mirin), significantly reduced the number of MRE11 foci counted post IR.

MSH2+ cells displayed a more substantial increase in the number of cells containing MRE11 foci, relative to control sham irradiated cells (mean difference= 90.4±31.48, p=0.0454) (Fig. 5-11). The number of cells containing persistent MRE11 foci 24hr after IR was also significantly greater in MSH2+ cells relative to MSH2- cells (p=0.0368) (Fig. 5-11).

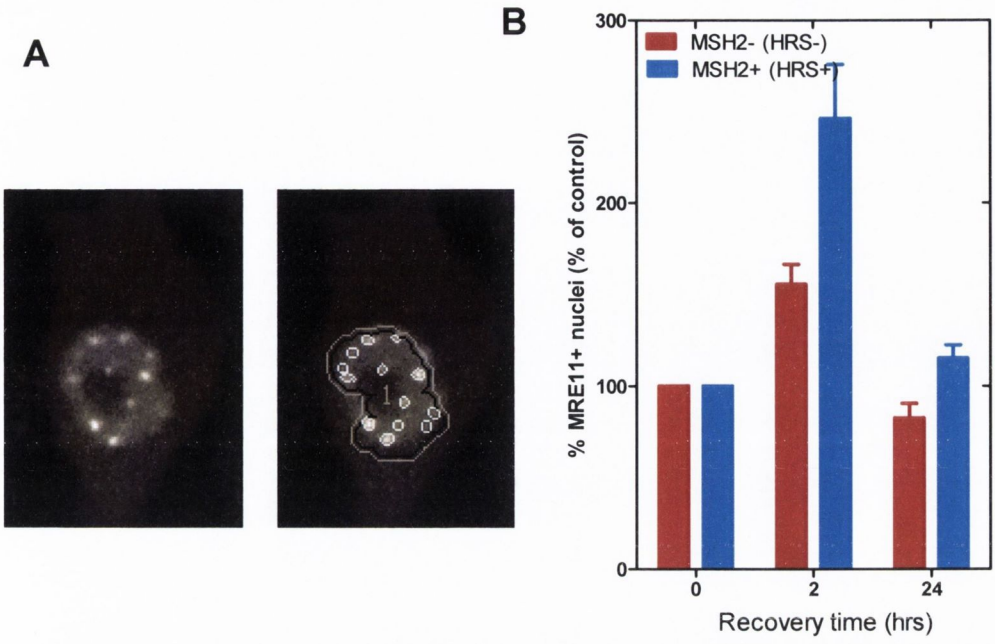

Fig. 5-11: Low dose radiation activates MRE11 in an MSH2-dependent manner

(A) Representative image of MRE11 foci scoring using the IN Cell Analyzer 1000 software The Dual-target Analysis algorithm was used to identify individual cells and foci in these cells (MRE11). The nucleus was segmented via a top-hat method (30μm^2 minimum area). MRE11 foci in the nucleus were segmented using a multiscale top hat method measuring granules of 0.3 to 1 μm in size. At least 15 fields were analysed in each well with a 20x objective, corresponding to at least 1600 cells counted. (B) Comparison of the percentage of cells with greater than 5 MRE11 foci after 0.2Gy of radiation in MSH2- HRS- (HEC59) cells shown in red, versus MSH2+ HRS+ (HEC59+chr2) cells shown in blue.

5.3.5 Effect of MRE11 inhibition on the mitotic index of HEC59+chr2 cells

To further evaluate the role of MRE11 foci formation in the induction of the early G2 arrest in MSH2+ cells we examined the mitotic ratio of MSH2+ cells pre-treated with the MRE11 inhibitor Mirin, following exposure to 0.2Gy. The concentration of Mirin used was validated by demonstrating its ability to significantly inhibit MRE11 focus formation. Pre-treatment with Mirin (10nM, 30min) significantly decreased the mitotic ratio relative to control irradiated cells ($p=0.0441$)(Fig. 5-12).

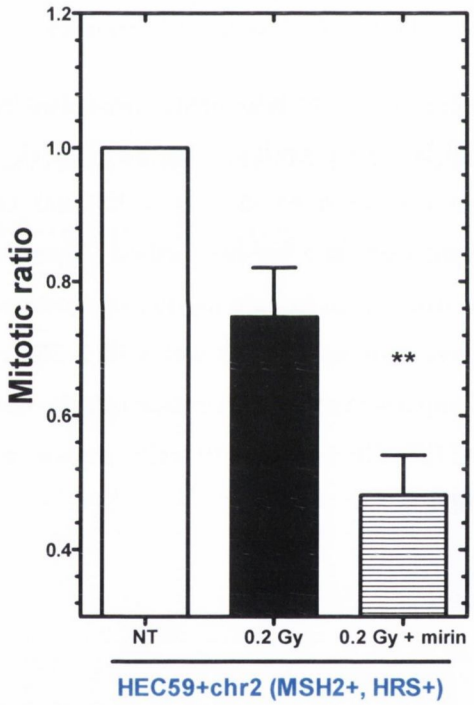

Fig. 5-12: Analysis of the influence of MRE11 inhibition on the mitotic index of HEC59 and HEC59+chr2 cells following exposure to ionizing radiation (0.2Gy).

Cells were treated with 10nM Mirin for 30min before irradiation. (Mean± standard error of at least 3 independent experiments are shown).

5.3.6 THE MMR SYSTEM IS REQUIRED FOR LOW DOSE RADIATION INDUCED EFFICIENT CHECKPOINT SIGNALLING

We next examined MSH2+ and MSH2- cells for activation of Chk1 and Chk2 by immunoblotting with phospho-specific Chk1(ser296), and Chk2(Thr68) antibodies. Whereas MSH2-positive cells (HEC59+chr2) clearly showed detectable levels of phosphorylated Chk1 and Chk2, matched MSH2-negative (HEC59) cells showed only detectable levels of phosphorylated Chk1 (Fig. 5-13).

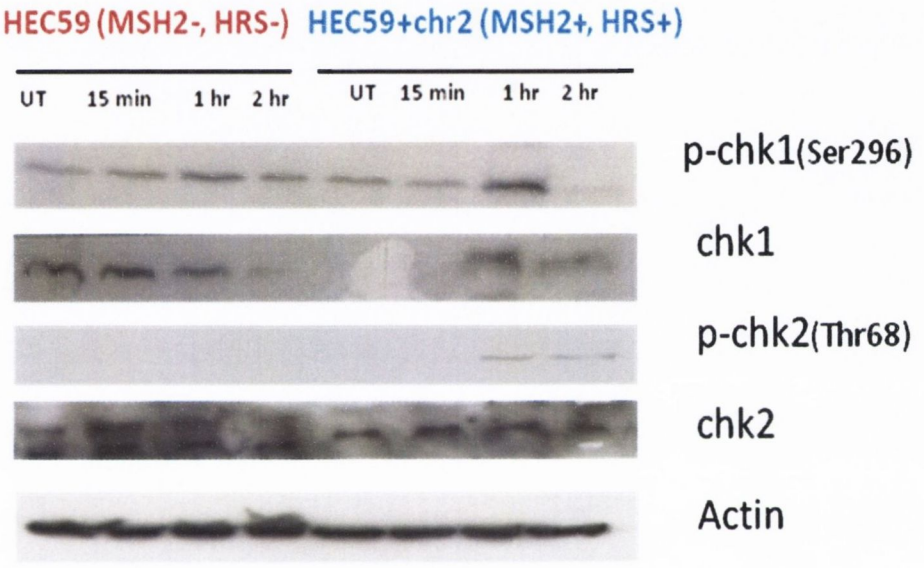

Fig. 5-13: Low dose radiation activates MRE11 and Chk2 kinase in an MSH2-dependent manner
(B) Western blots of phosphorylated Chk1(Ser296), total Chk1, phosphorylated Chk2 (Thr68), total Chk2 and actin. Cells were harvested at various time points after 0.2Gy and assessed by western blotting for phosphorylated Chk1(Ser296), total Chk1, phosphorylated Chk2 (Thr68), total Chk2. Actin is a loading control. Data are representative of experiments performed at least twice.

5.3.7 *INHIBITION OF CHK2 ENHANCES G2-M ARREST AND DECREASES SENSITIVITY TO LOW DOSE RADIATION*

To determine whether the MSH2-dependent phosphorylation of Chk2 mediates G2-M arrest and increases sensitivity to low doses of IR, we inhibited Chk2 activity with the drug Chk2 inhibitor II (Sigma) that specifically inhibits this kinase. Pre-treatment of MSH2+ (HEC59+chr2) cells with Chk2 inhibitor II prior to exposure to 0.2Gy induced a robust decrease in mitotic ratio 2hr post irradiation (p=0.0200)(Fig. 5-14, Fig. 5-15). To further assess the role of Chk2 in HRS we exposed MSH2+ cells pre-treated with the Chk2 inhibitor to low doses of radiation. Chk2 inhibition significantly increased survival of irradiated cells (p=0.0130) (Fig. 5-14).

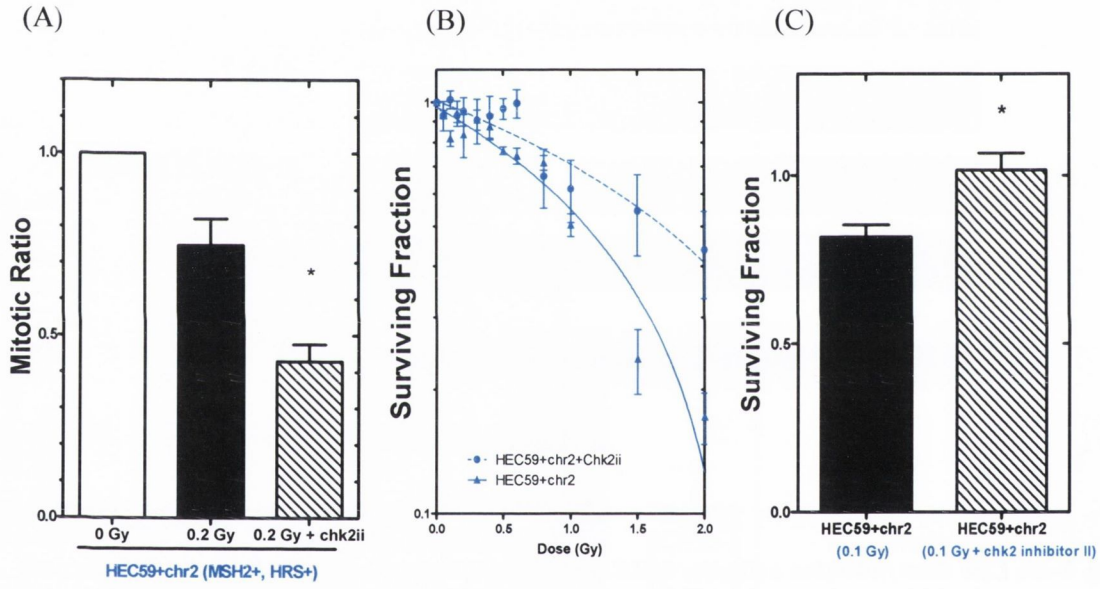

Fig. 5-14: Inhibition of Chk2 decreases the mitotic ratio and induces radioresistance in MSH2+ cells exposed to 0.2Gy

A. Mitotic index of HEC59+chr2 cells treated with 0.2Gy alone or in combination with chk2 inhibitor II (mean± standard deviation of at least 3 independent experiments are shown). Image analysis was performed with IN Cell Analyzer 1000 using a Multi-target analysis algorithm. C. Clonogenic survival of HEC59+chr2 cells in response to increasing doses of ionizing radiation alone (solid line) or with pre-treatment with Chk2 Inhibitor II (Sigma) 15nM for 30min prior to irradiation. Shown are the mean±SEM of 4 independent experiments.

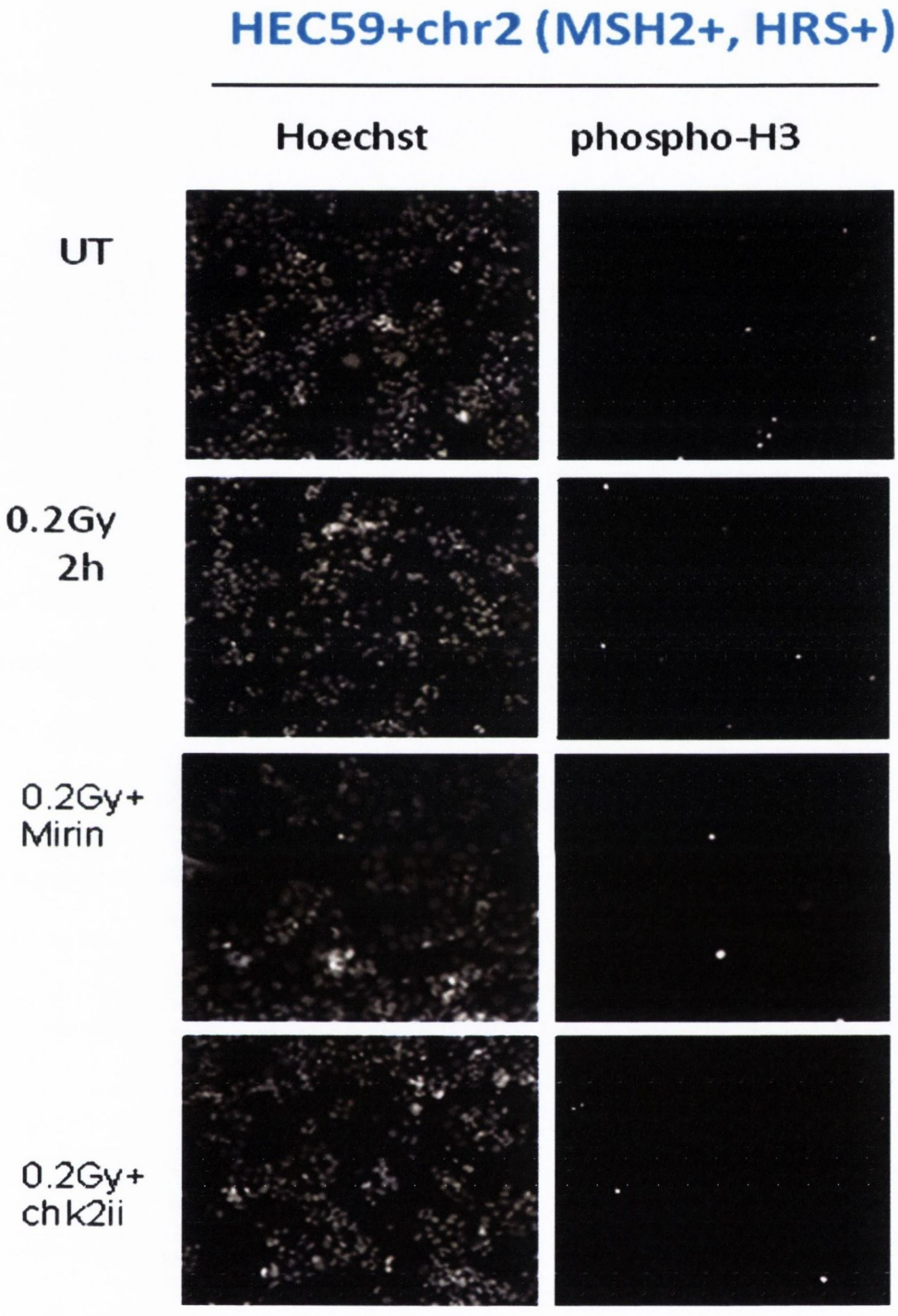

Fig. 5-15: Influence of MRE11 and Chk2 inhibition on the mitotic index of irradiated HEC59+chr2 cells

Representative fields of HEC59+chr2 cells stained with Hoechst and anti-phospho-H3 antibody either untreated (UT), 2hr after exposure to radiation alone (0.2Gy) or in combination with the MRE11 inhibitor Mirin or Chk2ii inhibitor II (chk2ii).

5.4 Discussion

In this chapter, we report that MSH2+ and MLH1+ cells are preferentially sensitive to low doses of radiation. Sensitivity in MSH2+ cells is associated with efficient activation of the early G2 checkpoint.

Using matched MMR-proficient and MMR-deficient cells, we demonstrate that MMR-proficient carcinoma cells are in fact more radiation sensitive to low doses of radiation than their MMR-deficient counterparts. These data corroborate previous findings that indicate that MLH1-deficient HCT116 colorectal cells do not display HRS, and are supported by our previous observations in prostate and glioma cells, showing that HRS is associated with MMR-proficiency (Chapter 4). This argument is substantiated by the documented expression of HRS in five MMR-proficient cell lines in the literature (T98G, SNB19 (glioblastoma), A549 (lung), HT29 (colorectal), MeWo (melanoma) and the absence of HRS in 3 MMR-deficient cell lines (SW48, HT116, RKO (colorectal))(Joiner et al. 2001).

HRS in glioma and fibroblast cells has been associated with evasion of the ATM-dependent early G2 checkpoint (Krueger et al. 2007; Fernet et al. 2009; Xue et al. 2009). In contrast, we observed that HRS was, in fact, associated with induction of the early G2 checkpoint 2hr post irradiation. While it is surprising that HRS correlates with induction of the arrest in this model, these data are consistent with an early role for MSH2 in the cell-cycle arrest process in response to a number of DNA damaging agents, including IR, as has been suggested (Franchitto et al. 2003; Marquez et al. 2003). These data also corroborate recent findings in skin biopsies correlating HRS with a growth arrest (Turesson et al. 2010).

According to the model proposed by Franchitto and colleagues, the MSH2-associated early-acting delay may engage MRE11-dependent pathways (Franchitto et al. 2003). Our results are in keeping with findings that irradiation results in a reduced and shorter G2 arrest in MSH2-deficient cells, compared with MSH2-proficient cells, and that this is functionally realised through defective MRE11 signalling (Franchitto et al. 2003). This defective MRE11 signalling may be the result of a

mutation in the MRE11 gene, as mutations in MRE11 are frequently observed in MMR-deficient cancers (Giannini et al. 2002; Giannini et al. 2004). Franchitto and colleagues demonstrate that this defective relocalisation is observed in the G2 phase but not in S phase of the cell cycle. Such an analysis was not possible in this study. The relative yield of toxicity associated with Hoeschst staining prior to cell sorting, as well as that induced by chemical synchronisation with Nocodazole, was too high relative to the yield of damage under investigation, and consequently prevented segmentation of our results by cell cycle phase (data not shown). Therefore while not investigated in this study, these processes may be specific to G2 phase cells. Counterstaining cells with a cell cycle marker such as cyclin B1 by immunofluorescence should resolve this in future studies. Of note, a G2 specific response would be in keeping with the observed behaviour of HRS+ cells (Short et al. 2003).

The MRE11 complex may carry out the checkpoint response via regulation of ATM and the checkpoint effector kinases Chk1 and Chk2. The MRE11 complex is required for both ATM activation and the ATM-dependent early G2/M checkpoint in response to DSBs (Carson et al. 2003). In support of a role for MRE11-ATM dependent process in the IR induced MSH2-dependent G2 arrest, we observed that HRS+ MSH2+ cells induced phosphorylation of both Chk1 and Chk2 (signalling events downstream of ATM) at time points co-incident with induction of the G2 arrest. The checkpoint kinase Chk2 was not properly activated in MSH2- cells, consistent with premature release from the early G2/M checkpoint. Both Chk1 and Chk2 kinases are required to activate the G2 checkpoint after IR (Matsuoka et al. 1998) Sanchez et al, 1997). Chk1 appears to be essential in triggering the checkpoint, whereas Chk2 is needed to maintain the arrest (Hirao et al. 2000). Our observations corroborate those reported by others (Franchitto et al. 2003), suggesting that MSH2 is not necessary to impose this arrested state, but is required to maintain the arrested state, possibly acting somewhat indirectly in later steps of the G2 checkpoint activation.

An MRE11 inhibitor (Mirin) and Chk2 inhibitor (Chk2 inhibitor II) were used to validate the involvement of MRE11 and Chk2 in the activation of the arrest. We observed an enhanced decrease in mitotic ratio following treatment with either inhibitor rather than abrogation of the

arrest as expected. This anti-proliferative effect observed in response to Chk2 inhibition has been previously documented in response to Chk2 inhibitor PV1019 (Jobson et al. 2009). Jobson et al report that PV1019 and Chk2 small interfering RNAs can exert anti-proliferative activity themselves in cancer cells with high Chk2 expression in the NCI-60 screen (Jobson et al. 2009). The cells used in this study expressed high levels of the Chk2 protein. This may therefore provide a reasonable explanation for the anti-proliferative effect observed. Given that MRE11 inhibition should also inhibit activation of Chk2, the decrease in mitotic index observed following treatment with Mirin, may be a result of Chk2 inhibition. This anti-proliferative effect is thought to occur if cells have become somewhat dependent on Chk2 expression to drive proliferation. It is therefore difficult to ascertain whether these proteins (Chk2, MRE11) are definitively involved in the activation of this arrest.

To further evaluate the role of Chk2 activation in the HRS response we exposed MSH2+ cells to low doses of IR following pre-treatment with the Chk2 inhibitor and evaluated the cytotoxic response. Consistent with a role for Chk2 in HRS, Chk2 inhibition significantly increased the survival of cells exposed to low doses of radiation.

These data provide insight into the cellular function of MSH2, indicating that MSH2 is involved in the early G2 checkpoint in response to low levels of DNA damage in endometrial cells, through what appears to be an MSH2-MRE11-Chk2 dependent pathway that likely includes the ATM kinase.

In summary, we have identified a role for MSH2 in the HRS response, indicating that MMR+ cells are genetically susceptible to low dose radiation exposures. We demonstrate that cells proficient in MSH2 function exhibit increased sensitivity to radiation doses less than 0.2Gy. Elucidation of the mechanism behind the enhanced sensitivity revealed that the activation of the DNA damage sensor MRE11, Chk2 kinase, and the induction of the G2-M arrest triggered by low dose radiation exposure, required MSH2 function. Together these findings indicate that the MMR system sensitizes cells to low dose IR, in part, by activating an efficient G2-M arrest. In clear contrast,

MSH2- cells fail to activate this response, transit into mitosis with a damaged genome and subsequently fail to initiate the HRS response.

Chapter 6: DNA MISMATCH REPAIR PROTEIN MSH2 SENSITISES CELLS TO LOW DOSES OF RADIATION BY SUPPRESSING RAD51 RECOMBINATION REPAIR OF DNA DOUBLE STRAND BREAKS

CHAPTER 6 IS ADAPTED FROM THE

SUBMITTED MANUSCRIPT:

"DNA MMR protein MSH2 may dictate the cellular survival to low dose radiation"

LYNN M. MARTIN, BRIAN MARPLES, ANTHONY DAVIES, ANN ATZBERGER, CONNLA EDWARDS, MARY COFFEY, THOMAS H. LYNCH, DONAL HOLLYWOOD, AND LAURE MARIGNOL

6.1 INTRODUCTION

DNA double strand breaks (DSBs) are considered the most deleterious of IR induced lesions, with the potential to lead to cell death or genomic instability. In mammalian cells, DSB repair is widely executed by either of two mechanisms; the non-homologous end joining (NHEJ) pathway or homologous recombination (HR) pathway. HR is a high fidelity repair mechanism that is predominant in S and G2 phases of the cell cycle (Branzei and Foiani 2008). The initial step in HR involves processing the broken chromosome ends to give 3' single-stranded DNA (ssDNA) tails, which then invade sister chromatids or a homologous chromosome to copy genetic information into the donor chromosome (Fig. 6-1) (San Filippo et al. 2008). The MRN complex (composed of MRE11, RAD50 and NBS1) appears to be the major regulator of DSB-end resection (Mimitou and Symington 2009), whereas RAD51 and RAD54 proteins act during the pairing and strand invasion steps by forming a nucleoprotein filament along the 3' single-stranded tails. Recombination intermediates are processed further in reactions that involve DNA synthesis and nick ligation. Resolution of the exchanged DNA strands can result in either of two outcomes; synthesis dependent strand annealing (SDSA) or double strand break repair (DSBR) by crossover or gene conversion (GC). GC involves formation of a tract around the DSB, followed by unidirectional transfer of sequence information from the unbroken donor DNA molecule to the broken DNA molecule. Inappropriate template usage during the HR process can cause deleterious genomic rearrangements, such as deletions, translocations, duplications and loss of heterozygosity (Surtees et al. 2004).

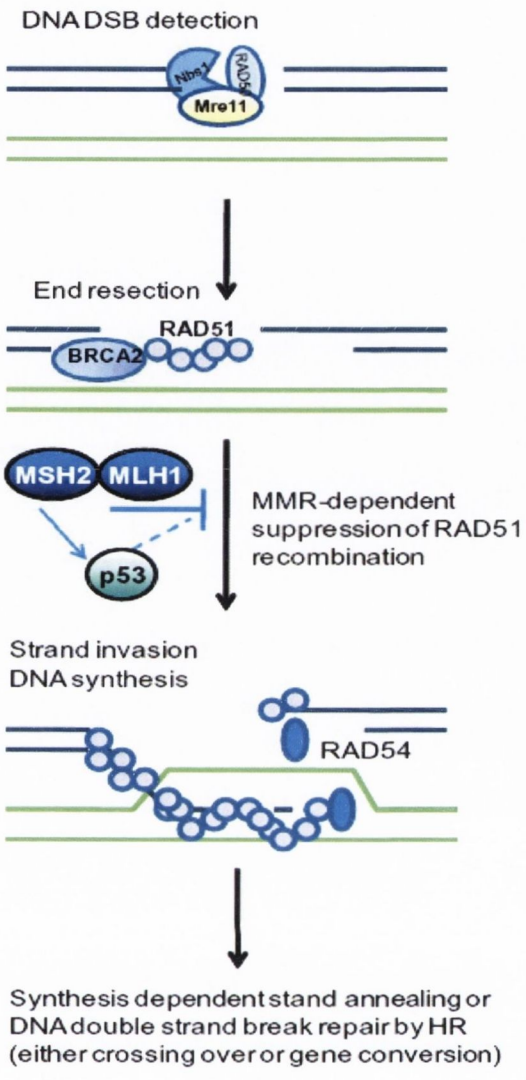

Fig. 6-1: An overview of the interplay between the homologous recombination repair pathway and mismatch repair proteins

Following the induction of DNA double strand breaks (DSBs) in S/G2 phase cells, the MRE11/RAD50/NBS1 complex is recruited to initiate DSB end resection. RAD51 is then recruited and loaded onto single stranded DNA with the assistance of BRCA2. In the absence of functional MMR, the RAD51 and RAD54 proteins act during the pairing and the strand exchange steps by forming a nucleoprotein filament along the 3' single-stranded tails. Resolution of the exchanged DNA strands can result in synthesis dependent strand annealing or DNA double strand break repair by either crossing over or gene conversion. In the presence of functional mismatch repair proteins, MSH2 and MLH1 co-ordinate to suppress the RAD51 recombination pathway at sites of divergent DNA sequences, possibly through p53 dependent pathways. Figure published in Martin et al, *Cancer Treatment Reviews* 2010.

While primarily involved in the repair of errors that arise during DNA replication, DNA MMR components are also involved in the repair of errors that arise post recombination. For example, while HR by GC is generally considered to be error free, HR at divergent sequences can produce insertions and deletions at sites adjacent to the damage site. MMR prevents this occurrence by aborting strand exchange between divergent sequences (de Wind et al. 1995; Abuin et al. 2000; Elliott and Jasin 2001; Zhang et al. 2009). A role for MSH2 in particular has been indicated in HR (Elliott and Jasin 2001; Villemure et al. 2003; Surtees and Alani 2006) along with MSH3 (Surtees et al. 2004), via suppression of RAD51. Recent results suggest that MSH2 may also co-ordinate with p53 to monitor the fidelity of HR during S phase (Zink et al. 2002).

MMR-dependent suppression of RAD51 sensitises cells to low dose rate IR (Yan et al. 2009). We have previously demonstrated that MSH2 and MLH1-proficiency confer hyper-radiosensitivity to low doses of IR (Chapter 5). MMR-dependent sensitivity to IR may occur via recognition of otherwise mutagenic lesions (8-oxoguanine, DSBs, OCDL and possibly O6MeG) alone or in combination with the BRCA1 associated genome surveillance (BASC) complex, which may then promote a G2 cell cycle arrest, aberrant HR and induction of apoptosis and/or autophagy (see Martin et al, 2010 for review, and references within). Our previous studies (Chapter 5) demonstrated that MSH2-dependent HRS was associated with efficient activation of the early G2 arrest, as well as nuclear retention of the MRE11 complex 2 and 24hr post IR (0.2Gy), that was not observed in MSH2- cells, thus implying deficient DSB repair.

Deficient repair of DSBs has recently been implicated in HRS; Thomas et al. report persistent unrepaired DSBs processed by HR & NHEJ pathways at late time points after IR (Thomas, 2008), and a hypersensitive γH2AX response has been documented in skin biopsies, which was correlated with infrequent apoptosis (Simonsson et al. 2008). Additional evidence implicating deficient DSB repair in HRS comes from a study by Yan et al (Yan et al. 2009) investigating the inverse dose rate effect, which has been established to be a dose-rate dependent manifestation of HRS/IRR (Leonard 2007). In this study, sensitivity to low dose rate IR was correlated with increased MLH1 and decreased RAD51 protein expression at late time points after IR, which correlated with increased

expression of p53 and the autophagy marker LC3B-II. This sensitivity was attributed to MLH1-dependent suppression of RAD51 recombination and activation of p53-dependent autophagic processes (Yan et al. 2009). To date, HRS has been associated with a modest induction of apoptosis (Enns et al. 2004), that in some but not all cases appears to be p53-dependent (Enns et al. 2004). The involvement of autophagy in HRS remains unknown.

Thus, while a role for MMR in the regulation of RAD51 following low dose IR/LDR-IR appears likely, the functional relationship between MMR and HRR in relation to HRS remains to be seen. Hence, from the outlined observations we hypothesized (i) that retention of MRE11 foci at 2 and 24hr post IR may be indicative of persistent DNA damage, (ii) MSH2-dependent persistent DNA damage may occur as a result of suppression of RAD51 HRR, and (iii) unrepaired DSBs by the HR pathway may initiate cell death in an MSH2-dependent manner.

6.2 OBJECTIVES AND METHODS

To investigate the role for mammalian MSH2 in DSB repair following low dose IR, we studied whether absence of the MSH2 protein resulted in an altered DNA damage response. To this end, we used the same endometrial carcinoma cells proficient or deficient in MSH2 used in the previous chapter (HEC59, HEC59+chr2). Use of this endometrial carcinoma isogenic pair allowed "proof of principle" investigations into the role of MSH2 in DSB repair. The results of these experiments will be validated in prostate cancer cells (Chapter 7).

In this chapter we set out:

- To determine the effect of MSH2 status on the induction and resolution of γH2AX foci 2hr and 24hr after exposure to 0.2Gy using high content analysis of cells stained with anti-phospho-histone H2AX (ser139).
- To compare RAD51 protein expression as well as MMR protein expression in HEC59 isogenic cell lines before and after exposure to 0.2Gy 2hr after irradiation using western blotting.
- To determine the effect of MSH2 status on the induction and resolution of RAD51 foci 2 and 24hr after exposure to 0.2Gy using high content analysis of cells stained with anti-RAD51.
- To examine HEC59 (MSH2-, HRS-) and HEC59+chr2 (MSH2+, HRS+) cells at various time-points after exposure to 0.2Gy radiation for changes in the expression of MSH2, phospho-p53, and LC3B-II.

6.3 RESULTS

6.3.1 MSH2-DEPENDENT HYPERSENSITIVITY CORRELATES WITH AN INCREASED NUMBER OF γH2AX + CELLS AT BOTH 2 AND 24HR FOLLOWING EXPOSURE TO 0.2GY RADIATION

In light of observations that the MRN complex associates with IR-induced DSBs, we inferred that the observed retention of MRE11 under these conditions was associated with persistent DNA DSBs. IR-induced DSBs rapidly phosphorylate histone H2AX (γH2AX) (Rogakou et al. 1998) which is considered to be a sensitive and selective signal for the existence of a DNA double-strand break (Modesti and Kanaar 2001).

γH2AX foci were counted using high content screening. A representative image of the algorithm used to count γH2AX foci is shown in Fig. 6-2.

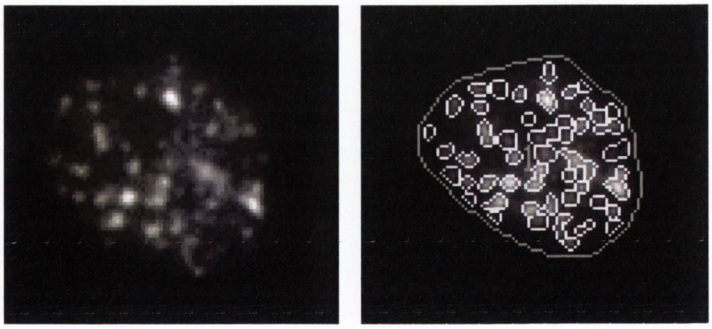

Fig. 6-2: Representative image of the algorithm used to count γH2AX foci

Image analysis was performed using the In Cell Analyzer 1000 software. The Dual-target Analysis algorithm was used to identify individual cells and foci in these cells (γH2AX). The nucleus was segmented via a top-hat method (30μm^2 minimum area). γH2AX foci in the nucleus were segmented using a multiscale top hat method measuring granules of 0.3 to 1μm in size. At least 15 fields were analysed in each well with a 20x objective, corresponding to at least 1600 cells counted.

We observed a greater number of cells containing >10 γH2AX foci in MSH2+ cells 2hr after exposure to 0.2Gy, than were evident in matched MSH2- cells (p=0.0327) (Fig. 6-3B). In addition, modestly higher levels of γH2AX were observed 24hr post IR in MSH2+ cells, relative to untreated controls, than were evident in MSH2- cells however, this was not significant (p=0.425) .

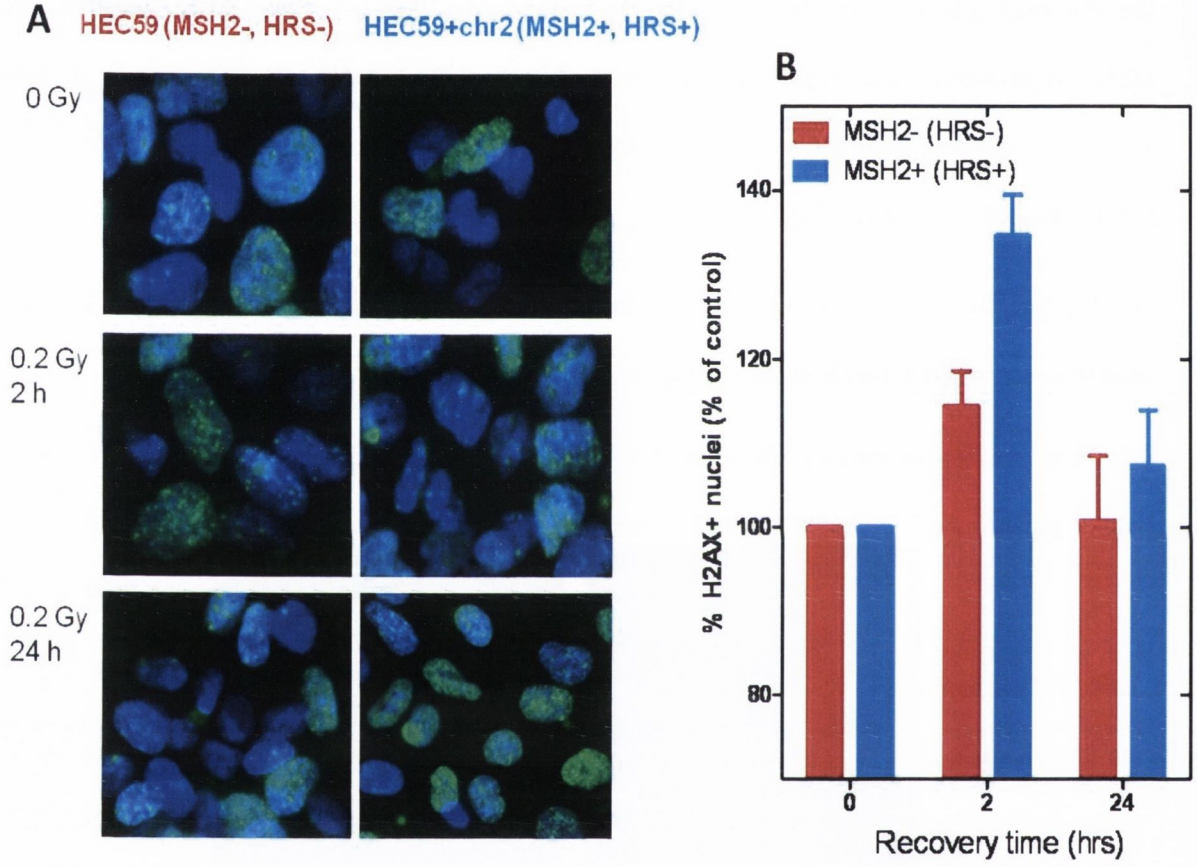

Fig. 6-3: MSH2-dependent hypersensitivity correlates with a higher number of γH2AX 2hr and persistent γH2AX foci following exposure to 0.2Gy

(A) Detection of γH2AX+ cells based on staining with the DSB marker phosphorylated-H2AX (ser139) (green) and nuclear staining with Hoechst (blue) before and after exposure to 0.2Gy using high content analysis. A: Representative fields of HEC59 and HEC59+chr2 cells either untreated (UT) 2 or 24hr after exposure to 0.2Gy (B) Analysis of the percentage of cells expressing greater than 10 γH2AX foci following quantification of the data obtained in A-B. (mean± SEM of at least 3 independent experiments are shown). Image analysis was performed with IN Cell Analyzer 1000 using a Multi-target analysis algorithm.

6.3.2 *MSH2-DEPENDENT HYPERSENSITIVITY CORRELATES WITH A INCREASED NUMBER OF RAD51 FOCI AT BOTH 2 AND 24HR FOLLOWING EXPOSURE TO 0.2GY RADIATION*

To investigate whether the observed enhanced MRE11 activity and γH2AX foci could be related to DSB repair by HR, retention of RAD51 recombinase was analysed by high content screening, 2hr after 0.2Gy. Only nuclei with greater than five foci were scored as RAD51+. Fig. 6-4 shows a representative image of the algorithm used to count foci. The algorithm used was validated by demonstrating that irradiation increased the number of RAD51 foci, and these foci were inhibited by a compound that is known to inhibit RAD51 (Hsp-90 inhibitor 17-AAG).

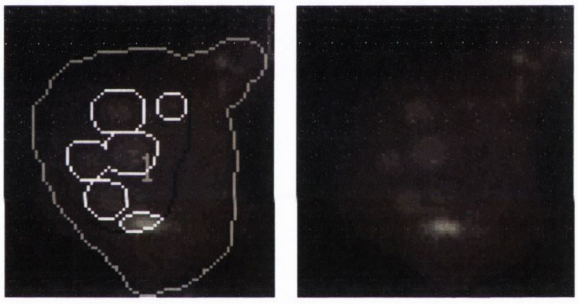

Fig. 6-4: Representative image of RAD51 foci scoring using the IN Cell Analyzer 1000 software
The Dual-target Analysis algorithm was used to identify individual cells and foci in these cells (RAD51). The nucleus was segmented via a top-hat method (30μm^2 minimum area). RAD51 foci in the nucleus were segmented using a multiscale top hat method measuring granules of 2 to 3 μm in size.

After exposure to 0.2Gy (2h), the percentage increase in RAD51+ nuclei was higher in MSH2+ cells (29.92±1.647) than in MSH2- cells (22.65±1.816) (Fig. 6-5, A-B). This was supported by RAD51 protein expression as determined by western blotting (Fig. 6-5C). In addition, elevated RAD51 foci levels were evident 24hr after IR in MSH2+ cells relative to control cells (15.97±5.3, p=0.0196). The same was not observed in MSH2- cells (p=0.3621).

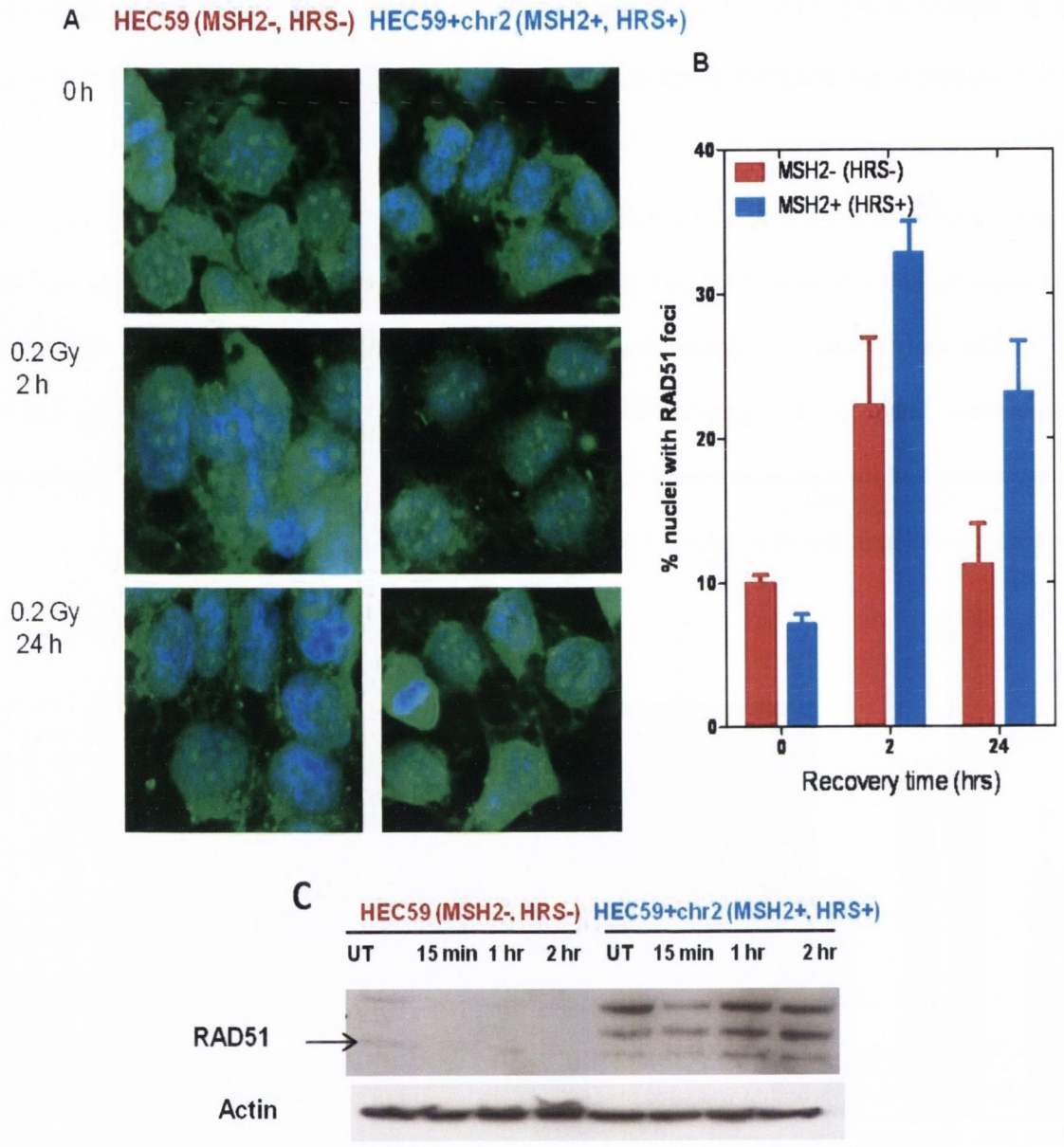

Fig. 6-5: MSH2-dependent early G2 arrest correlates with a higher number of RAD51 foci 2hr following exposure to 0.2 Gy radiation

Detection of RAD51+ cells based on staining with RAD51 before and after exposure to ionizing radiation (0.2Gy) using high content screening (A): Representative fields of HEC59 (red) and HEC59+chr2 cells (blue) either untreated (0hr), 2hr or 24hr after exposure to radiation (0.2Gy) (B): Analysis of the percentage of cells expressing greater than 5 foci following quantification of the data obtained in B. (mean± standard deviation of at least 3 independent experiments are shown). Image analysis was performed with IN Cell Analyzer 1000 using a Multi-target analysis algorithm. (C): Western blots of RAD51 and actin. Cells were harvested at various time points after 0.2Gy and assessed by western blotting for RAD51. Actin is a loading control. Data are representative of experiments performed at least twice.

6.3.3 Low dose radiation (0.2 Gy) increased MSH2, p53, and LC3-II levels to greater extents in MSH2+ HRS+ (HEC59+chr2) cells compared with MSH2- HRS- (HEC59) cells.

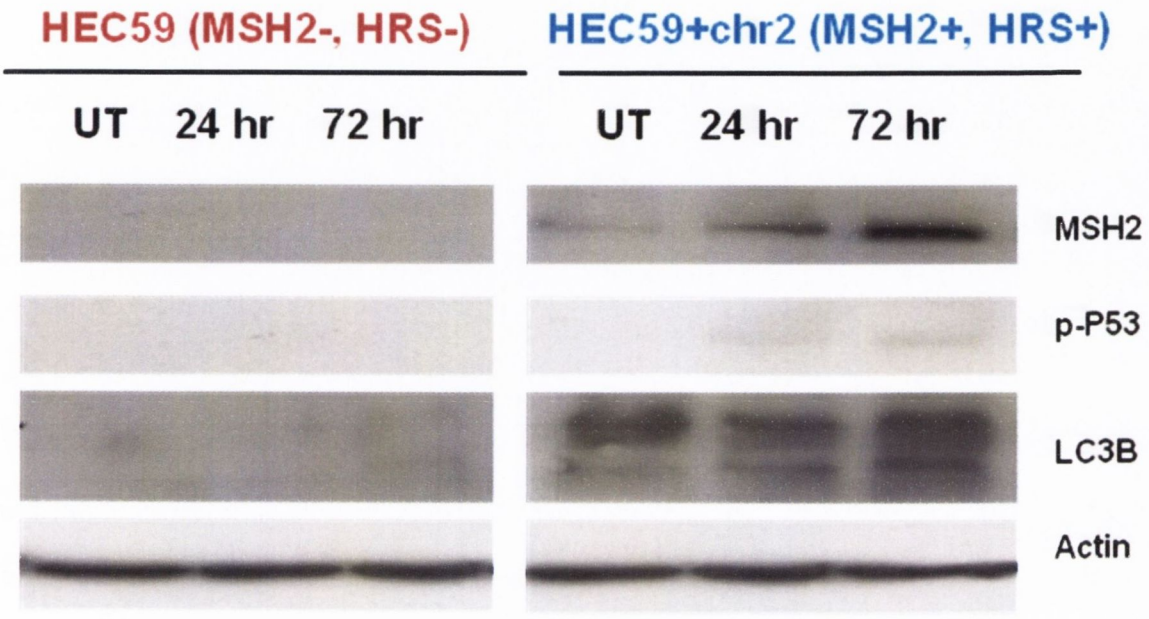

Fig. 6-6: Differential autophagic response observed in HRS+ and HRS- endometrial cells after 0.2Gy
Western blots of MSH2, phospho-p53, and LC3B and Actin. Cells were harvested at various time points after 0.2Gy and assessed by western blotting for MMR proteins. Actin is a loading control. Data are representative of experiments performed at least twice.

Radiation is typically associated with apoptosis while fewer studies report activation of cell death by autophagy. No significant difference in apoptosis levels was observed up to 24hr after irradiation with 0.2Gy in this study (data not shown) and so autophagic processes were investigated. The conversion of LC3B to the lower migrating form LC3B-II is indicative of autophagy (Kabeya et al. 2004). We examined the protein expression of LC3B-II (i.e. the lower migrating form of LC3B thought to be indicative of autophagy) as well as phospho-p53 at time-points up to 72hr after 0.2Gy. A modest increase in the expression LC3B-II protein levels (lower band) was evident solely in MSH2+ cells after IR (Fig. 6-6). No significant changes in the levels of p-p53 were observed. This correlated with a relative increase in MSH2 protein expression.

6.4 Discussion

We report that MSH2-dependent HRS is associated with persistent γH2AX and RAD51 foci, and activation of autophagy following exposure to low dose IR.

Using matched MSH2+ and MSH2- endometrial carcinoma cells, previously demonstrated to differentially express HRS (Chapter 5), we demonstrate that MSH2-dependent HRS is associated with a greater proportion of cells with >10 DSBs 2hr post IR (0.2Gy), than was evident in native MSH2- cells. Recently, it was demonstrated that a threshold of ~10-20 DSBs exists for checkpoint activation and maintenance (Deckbar et al. 2007). Consistently, our previous studies (Chapter 5) demonstrated that these cells arrest at the early G2 checkpoint at this time point. These data also implicated inefficient DSB repair in MSH2+ cells. In keeping with these findings, in this study, γH2AX foci persisted in MSH2+ cells 24hr post exposure to 0.2Gy, the observation of which is thought to be reflective of the fraction of cells that fail to divide and form colonies (Banath et al. 2004), (Klokov et al. 2006). These data corroborate the findings of others in matched progressive (i.e. showing tumourigenicity and metastatic potential) and regressive cells (i.e. showing neither tumourigenicity nor metastatic potential) isolated from a rat colon tumour (Thomas et al. 2008), and skin biopsies (Simonsson et al. 2008), suggesting that HRS is associated with persistent γH2AX foci at late time points after IR.

Persistent γH2AX foci and enhanced MRE11 activity (as observed in MSH2- cells in Chapter 5) could theoretically indicate upregulated NHEJ or HR. However, a role for MSH2 in DSB repair has been envisaged only in HR. Moreover, defective HR has been implicated in the HRS response (Thomas et al. 2008) (Simonsson et al. 2008). Consistent with a role for MSH2 in HR, we observed a higher endogenous formation of spontaneous RAD51 foci, as well as increased retention of RAD51 following IR in MSH2+ cells, at both 2 and 24hr after irradiation, indicating the presence of unrepaired DSBs by the HR pathway. These results are consistent with reports that RAD51 foci persist at late timepoints after low doses of IR (Short et al. 2005; Thomas et al. 2008). In addition, our observations are in keeping with findings that MSH2 can suppress HR via regulation of RAD51. These data are, however, in contrast to previous reports that indicate MSH2 is required for

efficient HR repair after exposure to IR (Franchitto et al. 2003), but this may possibly be explained by the difference in dose used. In the present study we used a radiation dose 10 times smaller than that used in the study by Franchitto et al. While not the most widely demonstrated response associated with hypersensitive cells, a model in which the RAD51 recombination pathway may have an important influence on the survival following low doses of radiation is consistent with HRS being most marked in G2 phase cells. Moreover, it may provide an explanation for the prevalence of HRS in radioresistant cancer cells (Joiner et al. 2001) given that RAD51 is over-expressed in many tumours (Klein 2008).

The DNA MMR system initiates autophagy in response to a variety of anti-cancer agents (Zeng et al. 2007). Consistently, we observed differential activation of the autophagic marker LC3B-II that correlated with differential MSH2 expression. A significant role for p53 in this process appears unlikely. In contrast to Yan et al. (Yan et al. 2009) who reported that sensitivity in response to low dose rate IR was dependent on MLH1 dependent suppression of RAD51 and activation of LC3B-II, our data indicate a role for MSH2 in this process. While not investigated in this study, a role for MLH1 in this process cannot be ruled out. In the recognition of mismatches, MSH2 functions to recruit MLH1. It may therefore be possible that MLH1 also acts downstream of MSH2 in the signalling cascade to activate autophagy.

In summary, in this chapter we have further characterised the MSH2-dependent HRS response in endometrial carcinoma cells. We have demonstrated that persistent γH2AX and RAD51 foci are associated with the maintained early G2 arrest evident 2hr post IR (0.2Gy) in these cells, and that these foci persist at late time points, and correlate with induction of autophagy. From these data, we conclude that that MSH2 suppresses RAD51-recombination of DSBs after exposure to low dose IR, which may consequently induce autophagy.

Chapter 7: VALIDATION OF THE PROPOSED MSH2-DEPENDENT MECHANISM OF HRS IN PC3 PROSTATE CANCER CELLS

7.1 INTRODUCTION

The HRS/IRR phenomenon has been extensively demonstrated in the past decade (Table 1-1). The balance of evidence suggests it will have far reaching and varied implications for radiotherapy practices and public health. As previously mentioned, PCa is likely to be affected by HRS given the increasing use of brachytherapy in the radiotherapeutic management of the disease (Lee et al. 2003). It has therefore become increasingly important to understand the mechanism of HRS in PCa cells, so as to enable effective exploitation of the response for a therapeutic gain, as well as allow adequate precautions to be made should the risk of genomic instability be greater following the administration of low radiation doses. Obtaining a greater understanding of the mechanism of HRS in prostate cells was thus the major goal of this thesis.

In Chapter 1, we outlined current progress in the field and established that observations in glioma and fibroblast cells form the basis of our understanding of the mechanism of the response. In these models, cells exposed to low doses of radiation are observed to evade early cellular response mechanisms. Cell death is thought to occur as a result of the mitotic entry of G2 phase cells with damage (see Chapter 1 and refs. within).

In chapters 4-6 we demonstrated what appears to be an independent mechanism for HRS in cells up to three times more radiosensitive than glioma cells. In Chapter 4, we investigated the role of O6MeG lesions in HRS in prostate cells and determined that a similar survival response is observed in response to an agent that induces O6MeG (TMZ), indicating that these lesions may potentially be responsible for the HRS response observed in PCa cells. Proteins known to sense these lesions, the MMR proteins, were demonstrated to be required for the expression of HRS using isogenic endometrial carcinoma cells proficient and deficient in the expression of MSH2 (Chapter 5). Our results suggest an alternative working model for the mechanism of HRS, in which HRS appears to be dictated by the resolution of the early G2 arrest rather than evasion of early cellular response mechanisms e.g. an absent mitotic delay. Following irradiation with 0.2Gy, these cells undergo cell death as a consequence of MSH2-dependent maintenance of the early G2 arrest

post IR, which occurs via increased localisation of MRE11 and Chk2 phosphorylation. The early G2 arrest is coupled with the abortion of promiscuous homologous recombination via suppression of RAD51 recombination and subsequent cell death.

In order to determine if the manifestation of HRS in PCa cells is consistent with the MSH2-dependent mechanism outlined above, we chose to evaluate a number of endpoints predicted by the proposed MSH2-dependent model in PC3 PCa cells. These cells were demonstrated to exhibit a distinct HRS response as defined by the induced repair model (Chapter 3), but are somewhat more resistant to radiation than are the endometrial cells used to explore the MSH2-dependent mechanism. Given the correlation between MMR-proficiency and HRS observed in the panel of prostate cells tested, we reasoned that HRS in PCa cells may be generated from the same radioprotective mechanism observed in endometrial cells, despite their difference in intrinsic radiosensitivities. We thus hypothesised that HRS in PC3 PCa cells would correlate with (i) maintenance of the early G2 checkpoint during the repair phase after IR (2h), (ii) persistent MRE11 foci, (iii) persistent γH2AX foci, (iv) and finally persistent RAD51 foci at both 2 and 24hr post irradiation with 0.2Gy.

7.2 AIMS AND METHODS

The purpose of this chapter was to test the hypotheses outlined above. To this end, we employed high content screening:

- To determine whether the early G2 checkpoint is active 2hr after exposure to 0.2Gy.
- To determine whether HRS correlates with persistent MRE11, γH2AX, and RAD51 foci 2 and 24hr post 0.2Gy.
- To compare the results obtained for PC3 cells to those obtained previously in endometrial carcinoma cells in order to determine if the mechanism of HRS in prostate cells is likely to be the MSH2-driven mechanism delineated previously.

7.3 RESULTS

7.3.1 HRS+ PC3 CELLS ARREST AT THE EARLY G2 ARREST 2HR POST 0.2GY

HRS in endometrial cells correlates with activation of the early G2 checkpoint. To investigate whether the early G2 checkpoint is active 2hr after 0.2Gy, we determined the mitotic ratio of irradiated PC3 cells and compared results to those obtained in endometrial cells. Results revealed that PC3 cells have a mitotic fraction similar to MSH2- HEC59 cells (p=0.93) (Fig. 7-1A), but significantly different to that of HEC59+chr2 cells (p=0.0492). In addition, the mitotic ratio of HRS+ PC3 cells was significantly reduced 2hr after 0.2Gy, indicating an activated early G2 checkpoint (p=0.002) (Fig. 7-1B).

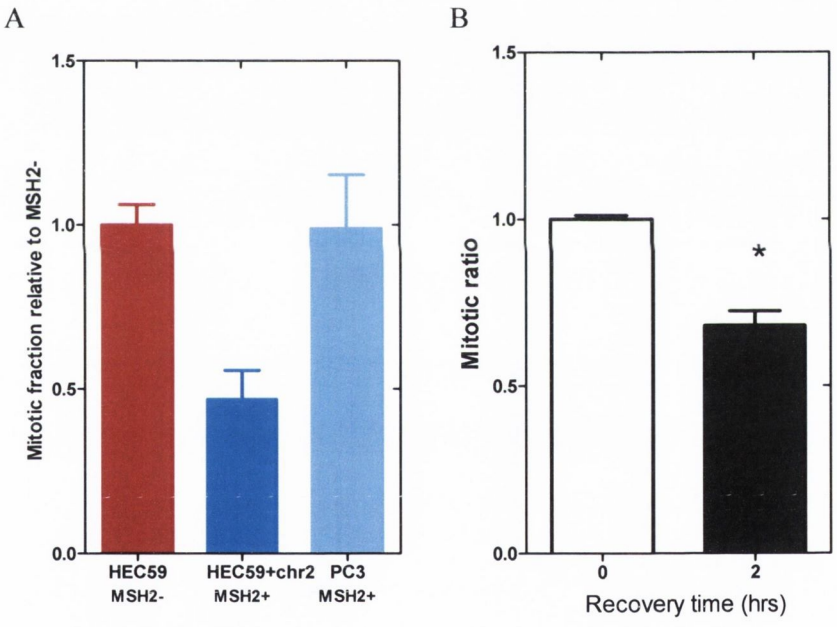

Fig. 7-1: MSH2+ PC3 cells arrest at the early G2 checkpoint after 0.2Gy

(A) Mitotic fraction of untreated PC3 prostate carcinoma cells compared to that of MSH2-(HEC59) and MSH2+ cells (HEC59+chr2). (B) Mitotic ratio of PC3 cells both before (0hr) and 2hr after exposure to 0.2Gy. (mean± SEM of at least 3 independent experiments are shown)

7.3.2 PERSISTENT MRE11 FOCI ARE EVIDENT IN HRS+ PC3 CELLS

In HRS+ MSH2+ endometrial cells we observed elevated levels of persistent MRE11 foci 2hr and 24hr post IR. To investigate the role of MRE11 in the observed HRS response in PC3 cells, we evaluated MRE11 foci in PC3 cells post IR (Fig. 7.2A) and compared results to those obtained in endometrial cells (Fig. 7-2B). Results revealed a significant elevated level of MRE11 foci in PC3 cells, at both 2hr ($p=0.0008$) and 24hr post IR ($p=0.0168$) relative to sham irradiated control PC3 cells.

The extent of MRE11 elevation observed in PC3 cells was similar that observed in HRS+ endometrial cells (Fig. 7-2B), and consistent with the trend of persistent MRE11 foci observed in HEC59+chr2 (HRS+ MSH2+) endometrial cells.

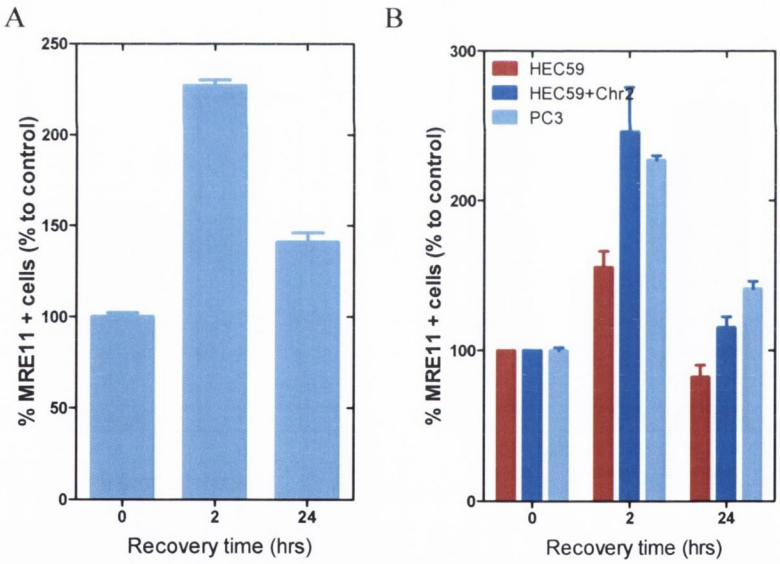

Fig. 7-2: HRS in PC3 cells correlates with persistent MRE11 foci, as had been observed in endometrial carcinoma cells.

Comparison of the percentage of cells with greater than 5 MRE11 foci after 0.2Gy of radiation in (A) PC3 cells and (B) PC3 cells relative to HEC59 cells (HRS-, MSH2-), and HEC59+chr2 cells (HRS+ MSH2+).

7.3.3 PERSISTENT γH2AX AND RAD51 FOCI ARE EVIDENT IN HRS+ PC3 CELLS

HRS in endometrial cells correlated with a trend for elevated γH2AX levels, and persistent RAD51 foci at 24hr post IR. To determine whether the same trend was evident in HRS+ PC3 cells, we investigated the resolution of γH2AX and RAD51 foci at both 2 and 24hr post IR with 0.2Gy (Fig. 7-3). We observed a significant increase in the percentage of γH2AX+ cells 2hr post IR ($p=0.0461$), as well as a modest population of cells retaining γH2AX foci were evident 24hr after 0.2Gy, however this was not significant. Consistent with results in Chapter 6, maintenance of the early G2 arrest 2hr post IR correlated with a greater percentage of cells containing greater than 10 γH2AX foci in PC3 cells relative to (MSH2-) HEC59 cells ($p=0.036$) (Fig. 7-4A).

In addition, we observed that a significant increase in the percentage of RAD51+ cells 2h post IR ($p=<0.0001$), as well as a significant population of cells retaining RAD51 foci evident 24hr after 0.2Gy ($p=0.0077$) (Fig. 7-4B).

These observations were consistent with those reported in endometrial cells, with a trend towards persistent γH2AX and RAD51 foci evident in HRS+ MSH2+ cells (HEC59+chr2, PC3) 2hr and 24hr post IR (Fig. 7-5).

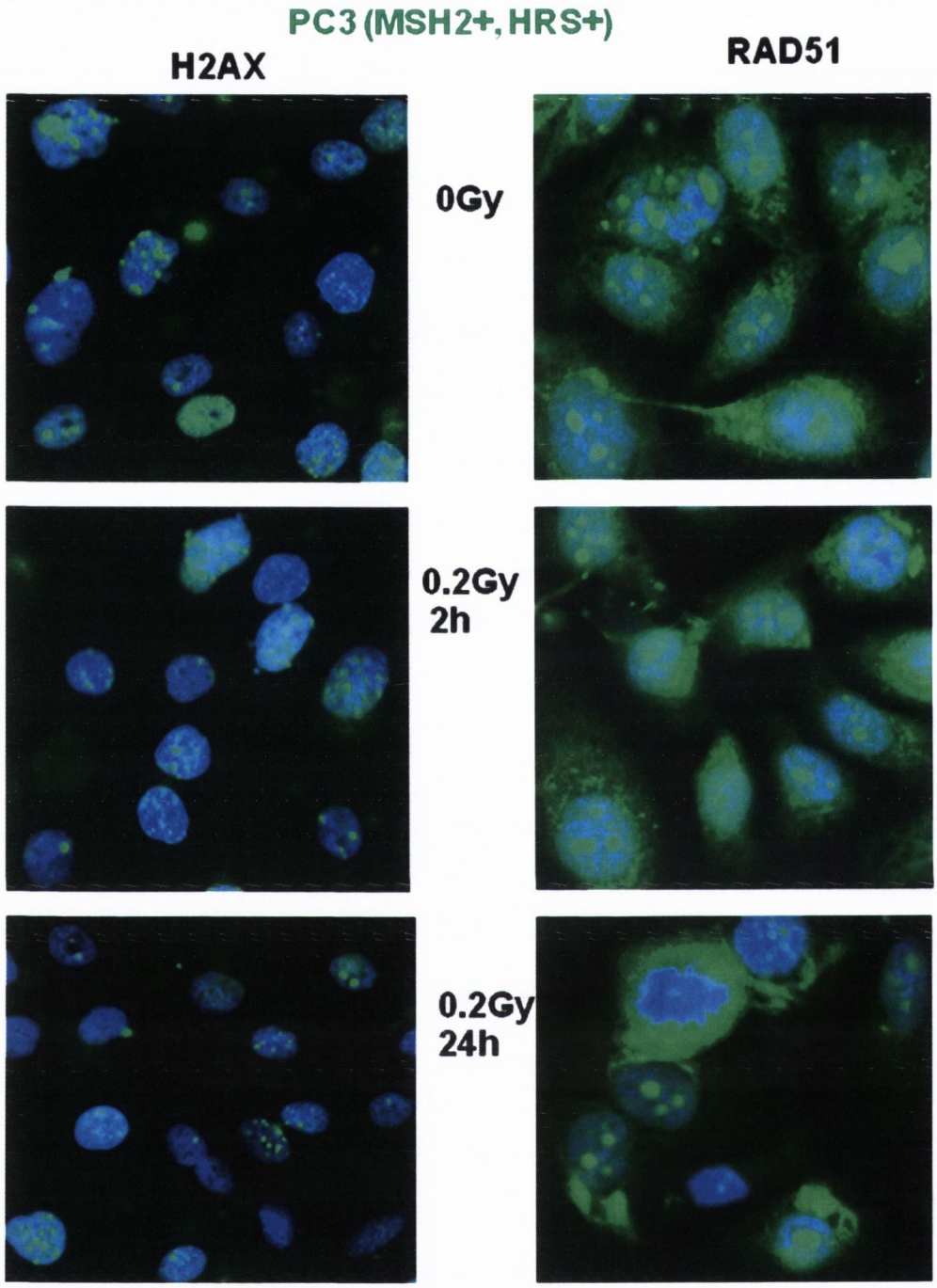

Fig. 7-3: Detection of γH2AX foci and RAD51 foci in PC3 prostate cancer cells before and after irradiation with 0.2Gy

Representative images showing the detection of γH2AX+ cells (Left panel) or RAD51+ cells (right panel) based on staining with the DSB marker phosphorylated-H2AX (ser139) or the anti-RAD51 antibody (green) and nuclear staining with Hoechst (blue) before and after exposure to 0.2Gy using high content analysis.

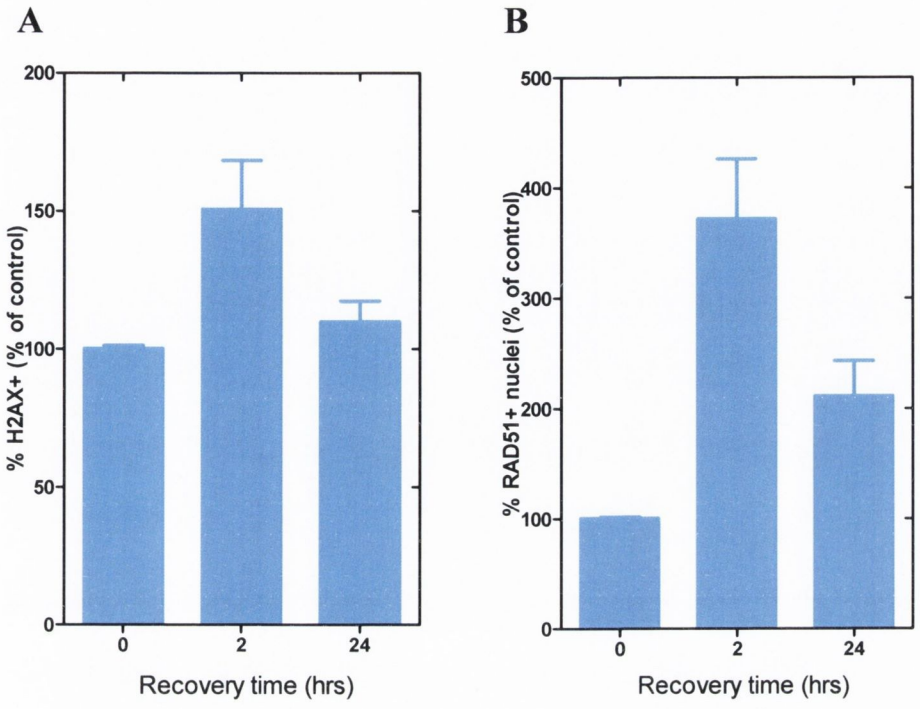

Fig. 7-4: HRS in PC3 prostate carcinoma cells correlates with persistent γH2AX and RAD51 foci.
(A): Quantification of the percentage of cells containing greater than 10 γH2AX foci in PC3 cells; untreated (0h), 2 or 24hr after exposure to 0.2Gy. (B): Quantification of the percentage of cells containing greater than five RAD51 foci in PC3 cells; untreated (0h), 2 or 24hr after exposure to 0.2Gy (mean ± standard deviation of at least 3 independent experiments are shown).

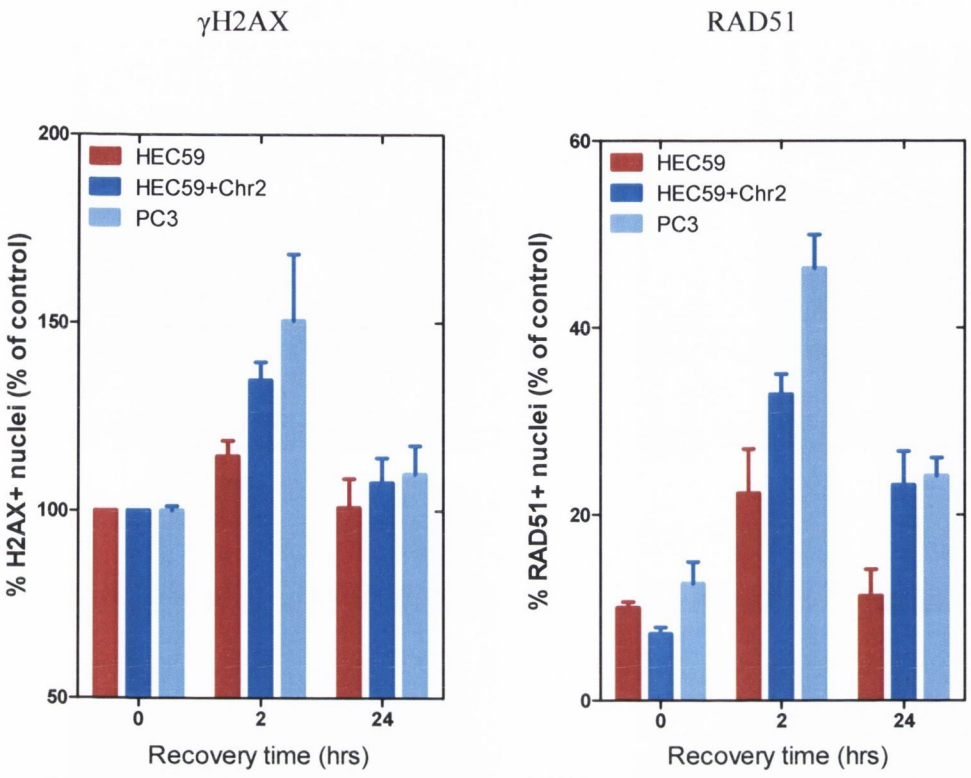

Fig. 7-5: Capacity of PC3 cells in comparison to that of HEC59 and HEC59+chr2 cells to resolve γH2AX and RAD51 foci at 2 and 24hr post irradiation with 0.2Gy.

7.4 Discussion

In this chapter, we demonstrate that the mechanism of HRS in PC3 PCa cells is consistent with the MSH2-dependent mechanism delineated in endometrial cells (Chapters 5 & 6).

In keeping with our observations in endometrial carcinoma cells, we observed that HRS+ PC3 cells arrested at the early G2 checkpoint 2hr after exposure to 0.2Gy. Maintenance of this arrest correlated with an increased number of cells containing persistent MRE11, γH2AX and RAD51 foci, as had been demonstrated for endometrial cells. These data indicate that HRS in PC3 cells correlates with activation and maintenance of the early G2 checkpoint during the repair phase (up to 2hr) post IR, and that this is likely the result of persistent unrepaired DSBs. In addition, in keeping with a role for defective HR in HRS, we observed persistent γH2AX and RAD51 foci 24hr after IR.

While we did not evaluate all of the same end-points investigated in endometrial carcinoma cells, the points of the pathway investigated for validation in PC3 cells indicate that the mechanism of HRS is consistent with the MSH2-dependent mechanism previously identified in endometrial cells.

In future studies, it will be important to confirm whether the mechanism of HRS in PC3 cells is in fact MSH2-dependent. We have determined that the signalling pathway activated by low doses of radiation appears to mimic the MSH2-dependent mechanism delineated in endometrial cells. However, we have yet to determine whether MSH2 is responsible for the early G2 arrest, and cell death observed in prostate cells following exposure to low doses. It will therefore be important in future studies to address this question, which could be done by creating a stable knockdown of MSH2 in PC3 cells, and observing whether the defect in resulting clones negates the early G2 arrest, and deficient DSB repair associated with the HRS effect.

Chapter 8: Discussion

8.1 INTRODUCTION

The advent of prostate-specific antigen (PSA) testing has transformed the field of PCa detection and management. Screening with both digital rectal examination and measurement of serum PSA, now means that PCa can be diagnosed at an earlier stage than was previously possible, meaning that more and more patients are presenting at a stage when curative treatment is available (Horner et al. 2009). In the past, a number of factors limited the success of radical RT, including poor localization and staging and the fact that it was only possible to deliver treatment with square or rectangular fields that irradiated large volumes of normal tissue. This in turn limited the dose prescription due to the risk of incurring serious adverse normal tissue reactions.

The last 20 years have witnessed remarkable advances in imaging, radiotherapy delivery systems and methods of outcome analysis. These have transformed the prospects for the increasing numbers of men now being diagnosed with localised potentially curable prostate disease. The increased use of multiple beams of RT, with lower incident doses of IR in conformal radiotherapy techniques has enabled dose escalation to the target volume while minimising the dose to the surrounding normal tissue. The benefit of dose escalation has recently been demonstrated both in terms of local tumour control and biochemical recurrence (Zelefsky et al. 1998; Vicini et al. 2001; Hanks et al. 2002; Pollack et al. 2002; Kupelian et al. 2005; Sathya et al. 2005; Zietman et al. 2005; Pinkawa et al. 2009). Despite the obvious success of dose escalation in the treatment of PCa, late rectal toxicity and proctitis remain serious complications for a great number of patients (Storey et al. 2000; Odrazka et al. 2010). In practice, dose prescriptions are limited by the likelihood of developing normal tissue reactions, and have evolved to limit severe adverse reactions to 0.5-5% of patients (Norman et al. 1988). It therefore stands to reason that if we could determine the underlying reason for such adverse reactions we could stratify patients so that those likely to develop such reactions could be identified before treatment, and treated appropriately. This would allow dose escalation in the majority of the patients, which would be likely to increase local tumour control and survival.

Following these advanced RT protocols, normal tissues receive a lower dose than would have been previously delivered using conventional techniques. Normal tissues, may therefore be subject to the influence of biological phenomena that are specific to low dose radiation, which may contribute to or may be responsible for these adverse reactions. These phenomena include low dose radiation hypersensitivity (HRS), the adaptive response, the bystander effect and the inverse dose rate effect (IDRE). HRS in particular is a widespread phenomenon in the low dose radioresponse of mammalian cells, occurring in approximately 75% of cell lines tested to date (Table 1-1 and refs. therein), and in response to acute low dose radiation, protracted low dose rate IR (LDR brachytherapy), as well as in response to high dose rate IR delivered using a low dose rate (reviewed in section 1.3.1). Results to date suggest the underlying mechanism is independent of the cells origin. Mechanistic work has determined that HRS, the adaptive response (Joiner et al. 1999) and IDRE (Leonard 2007) appear to be derived from the same underlying protective response, however, the underlying mechanism for the response remains to be elucidated. In addition, the true potential for HRS to increase normal tissue reactions, reduce carcinogenesis or improve cancer therapy is unclear.

It is apparent that the biological outcome of HRS both in terms of normal tissue toxicity and carcinogenesis, can only be determined following elucidation of the mechanism(s) underlying the response. Thus far, evasion of early cellular responses such as the ATM-dependent early G2 cell cycle arrest (Krueger et al. 2007) and impaired DNA DSB repair (Simonsson et al. 2008; Thomas et al. 2008), appear to have causal roles in the manifestation of HRS. Thus previous work has set the precedent to reconcile the early G2 checkpoint response with DNA repair in mechanistic studies.

8.2 IDENTIFICATION OF HRS

In Chapter 3 of this thesis, I set out to identify HRS in a panel of prostate cell lines using mathematical modeling. While the mechanism of HRS may be relevant to all tumour types, we believed elucidation of this mechanism in prostate cells would be of particular interest given that this disease subsite is particularly suitable for treatment with conformal therapy, and modern techniques are often piloted in PCa before other tumour types. In addition, the relevance of the mechanism of HRS to that of IDRE (Leonard 2007), meant that elucidation of the mechanism will also be relevant to those undergoing LDR-brachytherapy. As previously mentioned, the presence of HRS is defined by the induced repair model (Marples and Joiner 1993). To date, HRS has been documented but not defined in a number of prostate cell lines (Wouters and Skarsgard 1994; Wouters et al. 1996; Garcia et al. 2006; Hermann et al. 2008). Study of the mechanism of HRS in these cells thus required a more robust classification of PCa cells by HRS status than was previously carried out. We determined the HRS status of three prostate carcinoma cell lines and two prostate epithelial cell lines using high resolution clonogenic assays and mathematical modeling (Chapter 3). Whereas HRS responses were evident in prostate epithelial (RWPE1) and prostate carcinoma (PC3) cell lines as defined by the IR model, HRS was not evident in DU145, 22RV1 or PWR1E cells. In these prostate cells, HRS was associated with absent upregulation of HR repair pathways and did not correlate with either intrinsic radioresistance, or asynchronous cell cycle distribution.

8.3 PROCESSING OF DNA DAMAGE

In Chapter 4, I investigated the role of O6MeG lesions and MGMT in HRS, using TMZ as sensitivity to TMZ as a surrogate endpoint for sensitivity to O6MeG. The survival response to low concentrations of TMZ mimicked that in response to low doses of radiation, indicating a possible role of O6MeG lesions in the response. HRS was also observed to correlate with weak MGMT expression, which is the primary defence against these lesions. If O6MeG lesions were involved in the response inhibition of MGMT would likely sensitise cells to low dose IR, however following

inhibition of MGMT, no such sensitisation was observed. HRS did however, correlate with the expression profile of proteins known to sense these lesions, the DNA MMR proteins, in the panel of prostate cell lines tested.

While a role for MMR proficiency was suggested here, further work was required to determine its specific involvement. As in the processing of alkylation damage, a role for MMR was possible either indirectly via futile processing of damage induced by low doses of radiation ("futile" repair model), or directly via signalling cascades initiated by the MMR system (direct signalling model). A number of current observations support the "futile repair" model. As HRS+ cells do not undergo an early G2 arrest (<3h) following low doses of radiation in the same way as HRS- cells (Marples et al. 2004; Krueger et al. 2007), this could reflect the progression of cells through the cell cycle that will undergo a G2 arrest following the second S phase after irradiation. Apoptosis has been shown to occur in HRS+ cells following irradiation, which is a known mode of cellular death following MMR processing. Direct signalling may however also be involved. The MMR protein MSH2 has recently been shown to be required for correct MRE11 and RAD51 relocalisation and for efficient cell cycle arrest induced by ionizing radiation in G2 phase and in particular is required to maintain this G2 arrest when induced (Franchitto et al. 2003). Moreover, MSH2 proficiency may be required for efficient repair of clustered DNA damage induced by radiation (Holt et al. 2009).

8.4 DNA MMR PROTEIN MSH2 MAY DICTATE THE CELLULAR SURVIVAL IN RESPONSE TO LOW DOSE IR

This apparent correlation between MMR proficiency and HRS observed in Chapter 4, prompted investigation into the direct role of the MMR proteins (MSH2 and MLH1) in the hypersensitive response. Using isogenic endometrial carcinoma cells proficient and deficient in the expression of MSH2 I demonstrated that MSH2 is required for the expression of HRS survival response (Chapter 5), and that HRS in these cells was associated with activation of the early G2 arrest, and was maintained by phosphorylation of Chk2, in the presence of persistent MRE11 and γH2AX, and RAD51 foci. Persistent foci (MRE11, RAD51, γH2AX) were also evident 24hr post IR, as was

activated LC3B-II protein which is indicative of autophagic processes. These results are in contrast to previous reports suggesting that evasion of the early G2 checkpoint underpins HRS (Krueger et al. 2007), but corroborate findings that persistent RAD51 foci are evident at late time-points after IR (Short et al. 2005; Thomas et al. 2008). Activation of HRS in our MSH2-proficient model was associated with induction of the arrest. Resolution of the arrest thus appears to be an important and perhaps defining factor of the HRS response, rather than simply induction or evasion of the arrest. In this instance, activation of the early G2 arrest appears to be related to cell death, and indeed persistent MRE11 foci were observed in HRS+ cells only. The MRN complex has recently been demonstrated to dictate DSB repair independently of γH2AX (Yuan and Chen 2009). It therefore does not seem unreasonable to imply that inefficient repair of DNA damage must be taking place.

It may be possible, that MSH2 also suppresses HR at the dose the transition between HRS and IRR occurs. The transition point then could reflect the dose at which the cells are forced to use NHEJ to repair DSBs. Consistently it has been reported that NHEJ could serve as a backup system for DSB repair when HR is impaired (Fukushima et al, 2001) and indeed a role for NHEJ, and in particular PARP (Chalmers et al. 2004) and DNA-PK (Vaganay-Juery et al. 2000; Marples et al. 2002) have been reported in IRR.

Importantly, these results challenge the current theory that HRS is a measure of the clonogenic death of G2 phase cells that enter mitosis with unrepaired DNA DSBs. Certainly it appears that in endometrial carcinoma cells, HRS is the manifestation of a protective mechanism that results from MSH2-dependent activation of the early G2 checkpoint, and abortion of promiscuous homologous recombination. The more data that becomes available, the more evident it becomes that the protective mechanism induced in response to low doses radiation may manifest in different ways in different biosystems.

Intriguingly, we have demonstrated that inclusion of MLH1 in HCT116 colorectal cancer cells also allows HRS to manifest in response to low dose IR, a response that is not observed in native HCT116 cells.

8.5 VALIDATION IN PROSTATE CANCER CELLS

During the course of our studies we have demonstrated what appears to be an independent mechanism for HRS (Chapters 4-6) to that previously documented in glioma cells (reviewed in (Marples and Collis 2008)). In Chapter 7, we provide data to support the involvement of the MSH2-dependent mechanism demonstrated previously in Chapters 5-6, in the manifestation of HRS in PC3 PCa cells. We demonstrate that HRS+ PC3 cells arrest at the early G2 checkpoint 2hr after 0.2Gy, and that this arrest is correlated with persistent MRE11, γH2AX, and RAD51 foci. Persistent RAD51 and γH2AX foci were also evident in HRS+ PC3 cells 24hr after 0.2Gy. From all of these data, we conclude that the DNA damage signalling response to low doses of IR in PC3 cells mimics the MSH2-dependent mechanism delineated in Chapters 5 and 6, thus indicating that it is likely that the means by which HRS manifest in both models is derived from the same mechanism which is MSH2-dependent.

Based on these data, we propose the model shown in Fig. 8-1 to represent how MSH2 mediates cellular responses to low dose radiation: Briefly, low dose IR-induced lesions may trigger recruitment of MRE11 to resect DNA ends. MSH2 may then promote retention of MRE11, and ATM-dependent phosphorylation of Chk2, while also suppressing RAD51 recombination (possibly at divergent sequences), preventing completion of DSB repair. Ultimately, this action would then promote removal of the damaged cell by autophagy, mediated by MSH2, p-53 and LC3B-II, thus inducing cell death, which may manifest as HRS. In this way, MSH2 can maintain genomic fidelity by preventing incorporation of mismatched bases into DNA.

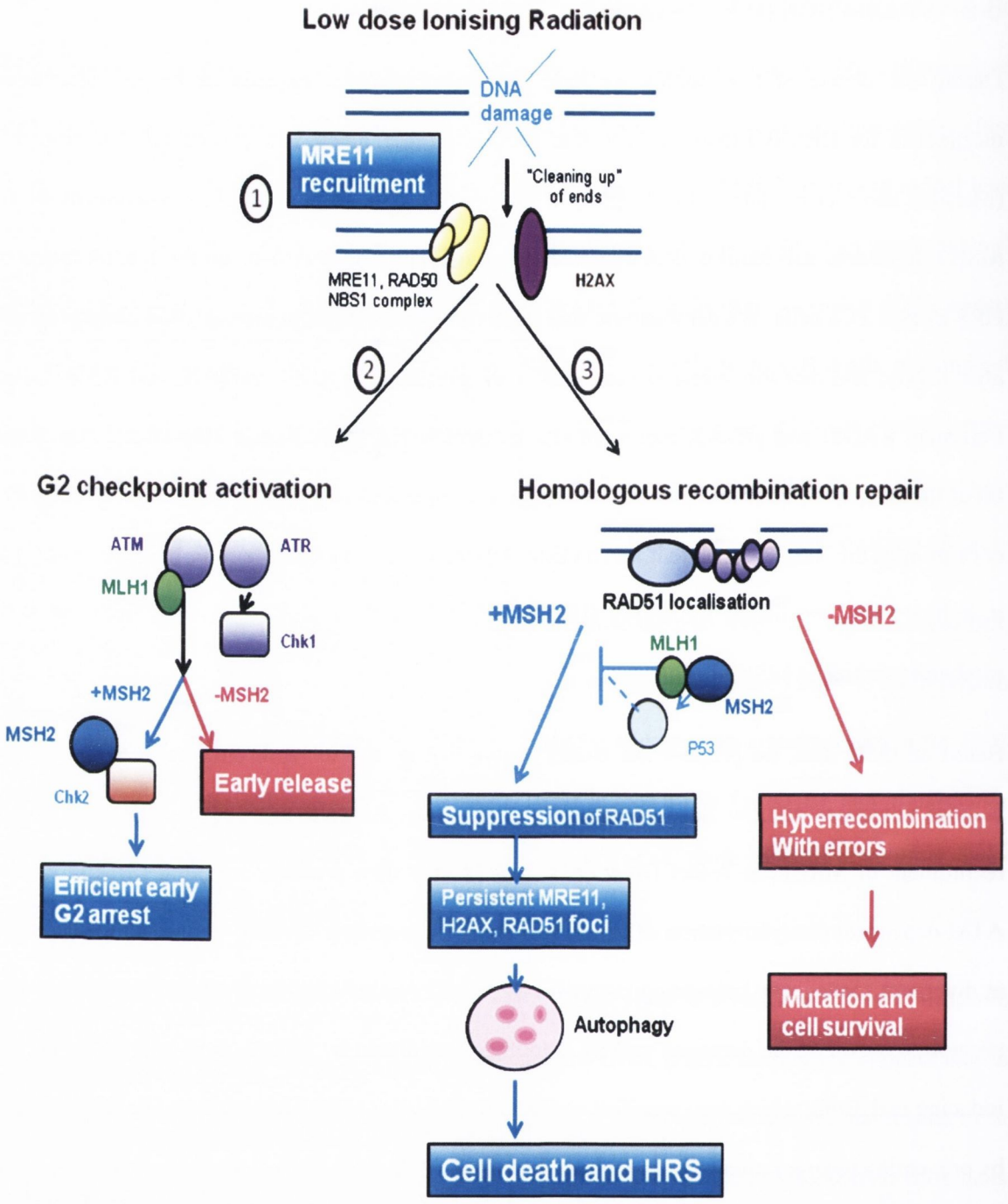

Fig. 8-1: Proposed model for the mechanism of low dose radiation hypersensitivity (HRS) in prostate cancer cells

(1) MRE11 is recruited to DNA ends to initiate repair following exposure to low dose radiation. (2) ATM, γH2AX, and the ensuing signalling cascade are activated promoting phosphorylation of checkpoint effector kinases Chk1 and Chk2. MSH2-dependent phosphorylation of Chk2 promotes maintenance of the early G2 arrest. (3) RAD51 is localised to damage sites. MSH2 suppresses RAD51, preventing completion of DSB repair. This action promotes removal of the damaged cell by autophagy, mediated by MSH2, p-53 and LC3B-II, thus inducing cell death, which manifests as HRS. Shown in blue are the MSH2-dependent events signalling events, red are events likely to occur in MSH2- cells.

8.6 Future Mechanistic Perspectives

The data provided in this thesis provide further characterization of the mechanism of HRS, in particular the cell cycle checkpoint, DNA repair and survival responses activated in response to low doses of IR. Our findings suggest that DNA MMR proteins play a critical role in the co-ordination of DNA damage signalling in response to low dose IR with regards to induction of the early G2 checkpoint, DNA repair and autophagic responses. These studies increased our understanding of the mechanism of low dose radiation hypersensitivity both in terms of cell cycle G2 arrest and how this relates to DNA repair processes and cell death. However, the results from this thesis generated an even larger number of more interesting questions regarding the outcome of the MSH2-dependent mechanism:

In future work, one important issue to address is how cells resolve the early G2 checkpoint? Micronuclei have been successfully employed as a biological endpoint that is indicative of a hypersensitive response in skin (Slonina et al. 2007), which would imply progression through or evasion of the early G2 arrest. Yet, a growth arrest has been demonstrated in skin. It will be interesting to determine whether HRS- cells display increased mutation rates or MSI after low dose treatment.

This research has also raised important questions regarding the role of other members of the MMR family in HRS including: whether MSH2 is the key player in HRS and whether lack of MSH6, PMS1 and PMS2 could also negate the HRS effect?

In addition, it will be important to determine if the G2 arrest observed in MSH2+ cells is ATM-dependent. While a role for ATM is implicated here, because MRE11 is linked to phosphorylated Chk2 via ATM, we have yet to confirm a role for this protein in this study. This could be achieved using western blotting for total and phosphorylated ATM to assess changes in protein expression, high content screening for ATM foci, alone or co-localised with phosphorylated Chk2 foci or MSH2. The direct role of ATM in the early G2 arrest and radiation survival could be assessed

using an ATM inhibitor and observing whether inhibition of ATM negates the early G2 arrest and HRS response.

It will also be important to determine the influence of hypoxia on HRS in this model. Hypoxia is known to inhibit MMR (Rodriguez-Jimenez et al. 2008), yet HRS in glioma cells has been demonstrated to occur in hypoxic irradiated cells.

8.7 CLINICAL RELEVANCE

During our studies we investigated the mechanism of low dose radiation mediated hypersensitivity in prostate and endometrial carcinoma cells. In the process we elucidated the signalling pathway of the low dose IR induced early G2 cell cycle checkpoint and evaluated the efficiency of low dose IR induced DNA DSB repair by the HR repair pathway and the activation of autophagic responses. These data have important clinical-translational relevance for the radiotherapeutic management of PCa.

8.7.1 TRANSLATIONAL RELEVANCE AND FUTURE PERSPECTIVES OF AN MSH2-DEPENDENT MECHANISM FOR HRS

As previously mentioned, the field of radiogenomics and the characterisation of molecular profiles that predict normal tissue damage and tumour radioresponse are gaining rapid momentum (Rutman and Kuo 2009; West et al. 2010). Biomathematical modeling has demonstrated that overall gains in therapeutic ratio could be achieved theoretically if dosage prescriptions were varied according to individual or subgroup sensitivities (Mackay and Hendry 1999). Elucidation of the mechanism of HRS as it relates to hypersensitive normal tissue reactions may therefore allow stratification of patients based on their likelihood of developing adverse tissue reactions. It may then be possible to escalate the total dose in the remaining majority of patients to achieve improved tumour control.

In practice, it will be important to identify a patient's likelihood to develop HRS before treatment, so that therapy may be adjusted to account for the HRS effect. Detection of HRS in patients is only possible thanks to knowledge of the mechanism pertaining to the effect. To date, HRS has been

identified *in vivo* using a variety of endpoints including kidney function, basal cell density (BCD), proliferation, erythema, the micronucleus assay, growth arrest, and γH2AX foci. HRS was observed after both single and repeated low dose fractions, indicating that HRS reactions are likely to occur following RT protocols incorporating low doses of radiation (e.g. IMRT). Such cell-based assays have the advantage of measuring a parameter thought to be more directly relevant for therapy than, for example, gene expression. However, these measurements are subject to *in vitro* artefacts, are labour intensive, and are time consuming. Moreover, assessing the response of internal organs such as the rectum or bladder would not be feasible. Thus, although cell based assays are still used in some studies, the consensus is that they will not have a role in routine testing, but may prove useful in the validation and interpretation of new technologies. Molecular markers of HRS may allow identification of the response by imaging techniques in the future but, for the present, assessment of predictive markers in patient biopsies or blood prior to treatment will prove most useful.

We have determined that MSH2 proficiency in tumour cells may increase the efficacy of cancer treatment regimens that use low doses of IR, such as IMRT, or LDR brachytherapy. However, increasing evidence suggests that MMR deficiency may be a feature of PCa. Microsatellite instability (MSI) is a hallmark of MMR deficiency in HNPCC, and results from mutations in the MMR genes MLH1 or MSH2 or from gene inactivation associated with DNA promoter hypermethylation. Microsatellites are short nucleotide sequences (1–5 base pairs, repeated 15–30 times) which are normally relatively stable. MSI (or replication error positive, RER+) is defined as loss or gain of microsatellite repeats at two or more loci (Wheeler et al. 2000). In HNPCC, a single mutation in one allele of a MMR gene is inherited in the germline; however, MSI only follows inactivation of the other allele. MSI occurs in 8% (4/50 cases) (Azzouzi et al. 2007) to 35% (14/40 cases) (Dahiya et al. 1997; Perinchery et al. 2000) of prostate tumours, with more aggressive cancers showing more frequent MSI (4/47 MSI+ and poorly differentiated) (Watanabe et al. 1996). Yet, other studies suggest that MSI may be an early event in prostate carcinogenesis, but not a

marker for progression or prognosis (Dahiya et al. 1997; Perinchery et al. 2000; Ahman et al. 2001).

The majority of immunohistochemistry studies reported suggest a causal link between MSH2 down-regulation and the pathogenesis of PCa (Table 8-1 and refs. therein).

Table 8-1: Reduced expression of MMR genes in cases of Prostate Cancer (*In vivo*). Table adapted from (Martin et al. 2009).

Investigators	MMR Gene studied	Proportion (%) of tumours showing decreased MMR expression
(Hirata et al. 2008)	MSH3	---
(Chuang et al. 2008)	MLH1	**5.6% (4/71)**
(Burger et al. 2006)	MLH1	**22% (9/41)**
	MSH2	**39.6% (23/58)**
(Prtilo et al. 2005)	MSH2	**39% (88/243)**
(Velasco et al. 2002)	MSH2	**29% (21/73)**
(Strom et al. 2001)	MLH1	**53% (37/70)**
	MSH2	**53% (37/70)**
(Chen et al. 2003)	**PMS1**	**86% (11/13)**

In vivo, Prtilo et al. found MSH2 expression to be reduced in 39% (88/243) tumours using a tissue microarray (Prtilo et al. 2005). Similarly, Burger et al found MSH2 to be down-regulated in 39.6% PCa cases (23/58) (Burger et al. 2006). In another study, absent to low staining for the MSH2 protein was documented in 30% of well to moderately differentiated prostate carcinoma (Gleason score 5–6) and 29% of poorly differentiated PCa (Gleason score 7–10) specimens (Velasco et al. 2002).

A minor role for MLH1 in prostate carcinogenesis has also been suggested. *In vitro*, MLH1 protein expression is retained in androgen-sensitive PCa cell lines (22RV1, LnCaP) and expression is lost in androgen-independent cells (DU145, DUPro) (Chen et al. 2001; Yeh et al. 2001). Similar results have been documented *in vivo*. Burger et al. reported decreased MLH1 protein expression in 9/41 cases (22%) using a tissue microarray (Burger et al. 2006). In another study of 70 cases and 97 controls Strom et al. reported a significantly lower expression of *MLH1* in PCa cases (37/70, 53%) than in controls (47.8%) (P=0.003) using multiplex RT-PCR. This was determined to be a statistically significant risk factor for PCa (OR=4.31, P=0.004) (Strom et al. 2001). However, the findings of a recent study contradict those found earlier, showing a significant increase (P<0.0001) in MLH1 immunoreactivity in prostatic adenocarcinoma (benign, 5.6% (4/71); high grade prostatic intraepithelial neoplasia, 46.2% (12/26); grade 3, 75.0% (27/36); grade 4/5, 74.2% (23/31)) (Chuang et al. 2008).

The fact that the majority of studies indicate that approximately one third of PCa patients present with MMR-deficient tumours, suggests that immunophenotyping of tumours before RT treatments may be required to identify patients that may benefit from low-dose protocols. MMR status is currently routinely tested in the treatment of colorectal tumours using immunostaining, therefore this would certainly be a cost-effective and feasible means to stratify patients. Because the germline MMR status will also be relevant as regards normal tissue toxicity it will be important to determine the MMR status of both the tumour and the surrounding normal tissues. This could potentially be done by assessing MMR gene expression in DNA obtained from lymphocytes and circulating cancer cells.

Testing of a panel of MMR proteins (MSH2, MLH1 and their heterodimer partners MSH6 and PMS2) is considered superior to testing of any one protein alone for the purpose of diagnosing germline MMR deficiencies. e.g,, HNPCC. The same is therefore likely to be true for MMR testing for radiotherapeutic purposes. The addition of MSH6 and PMS2 to the IHC panel increases the sensitivity of IHC to that of MSI testing (Shia 2008). MSI is also routinely used to identify MMR defects. High levels of MSI are suggestive of an MMR defect but the exact gene involved may only be defined by IHC. IHC alone can determine retention or loss of MLH1, MSH2, MSH6, and PMS2 protein expression. The likelihood of MMR gene involvement in the tumour is very low if all four proteins are present. However, approximately 5% of tumours will display MSI but have normal protein expression for these four genes and so MMR gene involvement cannot be excluded (Baudhuin et al. 2005; Shia et al. 2005; Lindor et al. 2006). The use of IHC with all four recommended markers MSH2/MSH6, MLH1/PMS2 (Shia 2008; Zhang 2008) should therefore be considered alone or in combination with MSI analysis in future studies. Where high throughput analysis is required, tissue microarrays may be useful in testing expression of MMR protein expression, as they allow staining of consecutive slides with different antibodies, allowing screening of protein expression of multiple genes (approximately 20) on many tumours (approximately 150) (West et al. 2005).

Use of a single prognostic marker is rarely sufficient for clinical use. Combining MSH2 expression with additional markers such as its functional targets Chk2 and Chk1 may therefore improve the power of such a panel to predict for HRS.

Prediction of normal tissues complications is more complex than prediction of tumour response. It is not always possible to obtain biopsies or to determine which tissue/cell type is most responsible for the manifested response. Many studies have therefore used peripheral blood lymphocytes as an easy-to-obtain surrogate tissue, with the rationale that genetic factors affecting radiosensitivity in a particular organ will also be reflected globally in all cell types. Study of genes in unirradiated cells for predictive markers, as well as changes in gene expression after irradiation have and will continue to shed light on the role of HRS in normal tissue reactions (Turesson et al. 2010). To date,

studies investigating the effect of HRS on normal tissues have analysed skin biopsies from PCa or cervical cancer patients undergoing RT. An alternative would be to culture cells taken from the patient *in vitro* and irradiate them. Such an approach would not be practical in the clinic due to the time-consuming nature of the assay, however, such studies may prove useful in determining the prevalence of HRS *in vivo*.

Clinical radiosensitivity of normal tissues is hypothesised to be a so-called complex trait dependent on the cumulative effect of many minor genetic determinants (Andreassen et al. 2002). Now that a large body of evidence is available for HRS, analysis of gene expression using microarrays should allow selection of candidate genes. Future studies could use a candidate gene approach with gene selection based on *in vitro* screening data from human cell lines and animal models. Genes could be selected using three criteria: genes with expression profiles showing statistically significant associations with cellular radiosensitivity, genes induced or suppressed after irradiation, and known radiosensitivity-related genes. SNP genotyping of these genes in individuals exhibiting early adverse skin reactions in response to low dose IR may allow correlation of HRS-dependent normal tissue reaction to SNP markers.

8.7.2 *Potential contribution of this MSH2-depenendent hypersensitive response for a therapeutic gain*

Numerous groups have attempted to exploit the G2 phase-dependent nature of HRS for a therapeutic gain using either low dose fractionated radiotherapy (LDFRT), or chemotherapeutic agents to increase the proportion of G2-phase cells in the tumour, thereby enhancing the HRS response of the tumour. The benefits of LDFRT treatment as regards cell killing have been demonstrated *in vitro*, alone or as a means to potentiate paclitaxel or taxotere treatment in squamous cell carcinoma of the head and neck (SCCHN) (Beauchesne et al. 2003; Dey et al. 2003; Spring et al. 2004). The success of this treatment approach prompted a clinical trial of biweekly combined gemcitabine and paclitaxel with 50 to 80cGy twice daily (ClinicalTrials.gov NCT 00176241), the results of which are ongoing. Modelling studies also suggest benefits of LDFRT in the treatment of gliomas *in vivo* in terms of tumour control probability (TCP) using ten 0.2Gy dose fractions delivered 3 minutes apart (Tome and Howard 2007).

While the alpha beta ratio of PCa remains controversial, it is difficult to ascertain whether PCa will benefit from a low dose fractionated approach. However, given that we have demonstrated that HRS can manifest in metastatic PCa tumours, it may be possible that a LDFRT approach could potentiate docetaxel treatment of PCa metastases.

8.8 Significance and final concluding remarks

This thesis contributes to the greater understanding of the mechanism of HRS. The significance and specific contributions to this research field are the following: First, it was shown that prostate cells of both epithelial and malignant origin display hypersensitivity to low radiation doses (HRS). Second, it was demonstrated that HRS is associated with the response to O6MeG lesions, as well as MGMT and MMR proficiency, suggesting a role for these lesions and MMR proteins in the response. Third, it was demonstrated for the first time that MMR components (specifically MSH2, MLH1) are required for the expression of HRS in endometrial carcinoma cells, and efficient induction of the early G2 arrest after low doses of radiation. Fourth, MSH2-dependent sensitivity

was linked to low dose IR to suppression of homologous recombination via inhibition of RAD51 and induction of an autophagic response. Overall, these data indicate that MSH2 status significantly affects cellular responses to low doses of IR, and that MSH2 may enhance cellular radiosensitivity to low dose IR through inhibition of homologous recombination (through inhibition of RAD51 recombination).

The important clinical-translational relevance of this data are 2-fold: first, MSH2 proficiency in tumour cells may increase the efficacy of the cancer treatment regimens that use low doses of IR, such as IMRT, or LDR brachytherapy. Second, MSH2 proficiency may reduce cancer susceptibility by preventing potentially mutagenic lesions from being passed on to progeny after low dose IR exposure via elimination of damaged cells (increased cell kill). Furthermore, the data presented in this thesis suggest that use of LDFRT may be contra-indicated when treating the tumours of patients with germline deficiencies in MSH2 (e.g. hereditary non-polyposis colorectal cancer). In these circumstances, increased HR rates may result in increased resistance to radiotherapy due to damage tolerance in tumours that lack MMR activity. Furthermore, MMR-deficient cells could, in fact, be selected for by such low dose IR treatments. In addition, such resistant tumours may have greatly increased rates of genetic instability due to IR induced DNA lesions, which are MMR resistant. This condition could increase genetic instability, resulting in increased tumour heterogeneity and selection for more malignant and invasive tumour cells.

8.9 OVERALL CONCLUSION

The research aims of this thesis were achieved in the sense that a mechanism for low dose radiation hypersensitivity in PCa cells was hypothesised, tested, refined and validated. We initially hypothesised that O6MeG lesions may be involved in HRS, and envisaged a role for DNA MMR proteins in the futile repair of low dose IR induced damage. After testing this hypothesis, our data suggested a more direct role for DNA MMR proteins in the processing of IR induced damage. We consequently refined our working hypothesis and re-tested it. We have demonstrated that the low dose radioresponse of endometrial cells is governed by the DNA mismatch repair gene MSH2. We have elucidated a pathway for this response that involves activation of the early G2 checkpoint, and suppression of homologous recombination repair (via suppression of RAD51). We have since validated this working model for HRS by demonstrating that the low dose radioresponse of PCa cells is mechanistically similar to this MSH2-dependent mechanism. The balance of evidence therefore suggests that MSH2 protein expression may be a useful prognostic marker, and may indeed contribute to a prognostic panel for the outcome of individuals undergoing low dose radiotherapy treatment.

Chapter 9: APPENDICES

9.1 DOSIMETRY FOR LOW DOSE RADIATION EXPOSURES

Medical dosimetry was performed annually by Peter McLoone, a medical physicist in St. Luke's hospital, to verify the output of the X-ray machine. A low dose rate was required to accurately deliver radiation doses in the range of 0.05Gy – 0.8Gy. This was achieved by adjusting the dose rate to 0.75Gy/min which required the amperage to be changed from 15 mA to 3 mA while maintaining the voltage at 200 kV. The dose rate was validated by exposing thermodosimeter badges to various radiation doses, which were then compared to standards irradiated in St. Luke's hospital.

9.2 MYCOPLASMA TESTING OF CELL LINES

All prostate cells were tested for Mycoplasma following 14 days in culture in antiobiotic-free media using the Micoalert® kit, as described in section 2. 1.6.

Table 9-1: Mycoplasma test results

Cell line	Ratio	Result
22RV1	0.86	Negative
DU145	0.79	Negative
PC3	0.80	Negative
PWR1E	0.49	Negative
RWPE1	0.71	Negative
LnCaP	0.68	Negative
T98G	0.48	Negative
U373	0.40	Negative
U87-MG	0.45	Negative
HEC59	0.51	Negative
HEC59+chr2	0.45	Negative
HCT116	0.61	Negative
HCT116+chr3	0.53	Negative

9.3 OPTIMISATION OF CLONOGENIC SURVIVAL ASSAYS

Clonogenic assays are based on the premise that a cell that can produce progeny to produce a colony size of a minimum of 50 cells is viable. A minimum of 100 colonies are required for accurate determination of the percentage of viable cells post irradiation. Various cell numbers were tested to determine both the plating efficiency, and number of cells to be plated to allow for a minimum of 100 colonies to be counted post IR.

For each cell type and container (6 well plates or T25 flask) the following cell concentrations were tested to determine the number of cells required for each dose point, such that cells were not too clustered (which would complicate colony counting due to overlap) or too far apart (which would inhibit optimal cell growth).

Table 9-2: Cell density optimisation for clonogenic assays

Radiation dose (Gy)	Cell numbers
0	250, 500, 1000
1Gy	500, 1000, 2000, 4000
6Gy	4000, 6000, 10000

The cell numbers chosen for plating differed according to cell type, due to different doubling times and intrinsic radiosensitivities.

Colonies were counted using the Oxford Optronix automated colony counter. The preset colony size parameters were modified to adjust for the differing size of the colonies produced (tiny, discrete, disperse) so as to ensure accurate and reproducible counting of the colonies.

Table 9-3: Antibodies conditions used for western blotting

Protein	Size	AB dilution	Dilution buffer	Secondary AB	Product No.
MSH2	100	1:500	Milk	Mouse	Ab52266 Sc-494
MSH6	160	1:200		Rabbit	A300-023A Bethyl-laboratories
MLH1 (C-20)	75	1:100			Sc-582
PMS1 (C-20)	115	1:200			Sc-615
PMS2 (C-20)	110	1:200			Sc-618
MGMT	21kDA	1:1000	BSA		#2739
Phospho-Chk1 (Ser296)	56kDa	1:1000	BSA	rabbit	#2349
Total Chk1		1:1000			#2345
Phospho-Chk2 (Thr68)	62kDa	1:1000	Milk		#2661
Total Chk2		1:1000	Milk		#2662
RAD51 (Rabbit Abcam)				Rabbit	AB63801
MRE11 (Rabbit EMD)				Rabbit	PC388
Phospho-p53(Ser15)	53kDa	1:1000	Milk	Mouse	#9286
LC3B	14, 16kDa	1:1000	BSA	Rabbit	#3868
α/β Tubulin	55kDa	1:1000	BSA	Rabbit	#2148
Actin					
Goat anti-rabbit IgG-HRP		1:2000	Milk		#7074
Goat anti-mouse IgG-HRP		1:2000	Milk		#7076

Table 9-4: Antibody conditions used for high content screening

Protein	Primary dilution	Secondary	Secondary dilution	Product no.
Phospho-histone H3	1:600	Goat-anti-rabbit Alexa fluor 488	1:1000	#9713
Phospho histone H2AX (Ser139) 488 conjugate	1:50	Alexa fluor 488 conjugate	1:1000	#9719
RAD51	1:100	Goat-anti-rabbit Alexa fluor 647	1:1000	Ab63801
MRE11	1ug/ml	Goat-anti-rabbit Alexa fluor 488	1:1000	PC388 EMD biosciences
Goat-Anti-rabbit IgG (H+L) F(ab')2 Fragment Alexa Fluor 647 conjugate	1:100			#4414
Goat-Anti-rabbit IgG (H+L) F(ab')2 Fragment Alexa Fluor 488 conjugate	1:1000			A-11070 Invitrogen

Table 9-5: Studies investigating the benefits of ultrafractionation schedules (UF) over conventional treatment (CT)

Model	No. of subjects	Fractionation	Functional Endpoint	Results	Authors
A7 gliomas in vivo	7–14-weekold female and male NMRI (nu/nu) mice 23 (UF arm) 32 (CT arm) 126 tumours total	UF (0.4Gy x3, 6h min interval, per day x 21days) CT (30 fractions in 6 weeks, 1.68Gy per fraction)	tumour growth delay & local tumour control (top-up TCD50) 180 days after the end of treatment	UF significantly less efficient than CT, benefits *in vivo* cannot be extrapolated from *in vitro* data	(Krause et al. 2003)
SCCHN cell lines	N/A, Cell lines	LDFRT (0.5Gy x4, 8h interval)	Clonogenic survival	LDFRT potentiated paclitaxel treatment ER=4.3 & ER=3.43 *	(Dey et al. 2003)
Glioma cells, G152 cells	21 (female nude mice, Swiss nu/nu) 7 in each tx arm	UF (0.8Gy x3,4h intervals per day, 4days/wk x2 wks) CT (2Gy OD, or 2.4Gy OD) 4days/wk x2 wks	Tumour growth delay, measured once weekly up to 12 wks post txt	UF treatment significantly increased tumour growth delay compared to CT	(Beauchesne et al. 2003)
SCCHN tumours	78 nude mice, 6-8 wks old,	LDFRT (0.5Gy x4, 8h interval, 1#/wk x6 wks) LDFRT+ paclitaxel, Paclitaxel only, 2Gy once weekly, 2Gy +paclitaxel	Tumour growth delay 8 weeks after txt apoptosis (tunel assay, bcl-2 & bax expression, cytochrome c release)	LDFRT potentiated taxotere treatment of SCCHN cells correlated with: Increased apoptosis, upregulated bax, enhanced cytochrome c release	(Spring et al. 2004)
Metastatic tumour nodules to skin	40 matched tumour nodules 36 evaluable from 8 pts: with metastatic malignant melanoma (3), metastatic leio-myosarcoma (2), metastatic breast cancer (1), and advanced non-Hodgkin lymphoma (NHL) (2)	UF (0.5Gy TDS: 4-h gap) x12 days CT (1.5Gy/day) x12 days	Tumour growth delay measured on days 0, 5, 8, 12, and 26 and monthly until regrowth occurred	UF significantly increased tumour growth delay	(Harney et al. 2004)
Glioma cells, T98G, HGL21	168 animals, 7-14 wk old female and male NMRI nu/nu mice 89 (T98G) 108 (HGL21)	UF (0.4Gy x3, 4h intervals, per day x 21 days) w/o top-up IR CT (1.68Gy x1, 5 days/wk x 4wks)	Tumour growth delay Tumour control probability 50%	UF is less efficient than conventional RT in glioma cells	(Krause et al. 2005)a
Murine DDL1 lymphoma	219 tumours 129 used for comparison	UF (0.4Gy x3, per day,7 days/wk, 6 wks) CT (1.68Gy x1, 5 days/wk x 4wks)	Tumour growth delay, time that tumors needed to reach fivefold the starting volume (GDV5).	UF does not improve the results of RT	(Krause et al. 2005)b
Modeling of response of glioma cell *in vivo* based on *in vitro* data	N/A gliomas	LDFRT (0.2Gy x10, 3 min intervals)	Tumour control probability	LDFRT could yield increased cell kill in	(Tome and Howard 2007)

Table 9-6: DNA repair gene expression of PC3 cells relative to DU145 prostate cancer cells

Gene	RQ value (DU145 NT)	RQ value (PC3 NT)	>2 Fold downregulation (1/RQ)	Gene	RQ value (DU145 NT)	RQ value (PC3 NT)	>2 Fold downregulation (1/RQ)
XRCC2	1	0.9174		IL6	1	und	
ERCC2	1	**2.7517**		FANCF	1	0.4255	**2.3501**
H2AFX	1	0.2997	**3.3367**	ERCC5	1	1.0475	
XRCC3	1	0.6226		XPA	1	0.2477	**4.0371**
RAD51C	1	**4.8781**		RAD51C	1	1.0873	
AICDA	1	und		PMS2	1	0.6010	
PRKDC	1	**2.9815**		ERCC1	1	1.1064	
MLH1	1	**2.0228**		FANCC	1	0.1008	**9.9206**
PMS2	1	0.6444		ATM	1	0.9946	
ERCC4	1	0.4910	**2.0366**	FANCG	1	0.2304	**4.3403**
XPC	1	0.7251		RAD52	1	1.1932	
TNF	1	0.4199	**2.3815**	TP53BP1	1	0.8073	
MRE11A	1	1.4564		XRCC6	1	0.5274	
BRCA2	1	1.0169		CHEK2a	1	0.4488	**2.2281**
TP53BP1	1	**2.5187**		CHEK1	1	0.4771	**2.0960**
UNG	1	1.0071		CHEK2b	1	0.8762	
AICDA	1	und		EXO1	1	0.4077	**2.4527**
BM2	1	1.8615		BRCA1	1	0.6301	
MSH2	1	1.0836		ERCC3	1	**2.0839**	
RAD51	1	0.4749	**2.1057**	FANCD2	1	0.5356	
XRCC6	1	0.6930		XRCC5	1	**2.4401**	
MSH6	1	0.8607		NBN	1	1.3328	
BRCA1	1	0.6180					
FANCE	1	0.5686					
FANCA	1	0.8206					

und=undetermined

Table 9-7: DNA repair gene expression in PC3 cells relative to 22RV1 prostate cancer cells

Gene	RQ value (PC3 NT)	RQ value (22RV1 NT)	>2 Fold downregulation (1/RQ)	Gene	RQ value (PC3 NT)	RQ value (22RV1 NT)	>2 Fold downregulation (1/RQ)
XRCC2	1	0.6584		IL6	1	und	-
ERCC2	1	**3.3795**		FANCF	1	0.1071	**9.3305**
H2AFX	1	0.3297	**3.0322**	ERCC5	1	0.4344	**2.3021**
XRCC3	1	1.4934		XPA	1	0.1845	**5.4204**
RAD51C	1	**4.0460**		RAD51C	1	0.8016	
AICDA	1			PMS2	1	0.5565	
PRKDC	1	1.8434		ERCC1	1	1.7129	
MLH1	1	0.5146		FANCC	1	0.1031	**9.6920**
PMS2	1	0.6552		ATM	1	1.7536	
ERCC4	1	0.5161		FANCG	1	0.3792	**2.6376**
XPC	1	0.3932	**2.5434**	RAD52	1	1.0305	
TNF	1	0.3537	**2.8270**	TP53BP1	1	0.3993	**2.5047**
MRE11A	1	0.3537	**2.8266**	XRCC6	1	0.8377	
BRCA2	1	0.9081		CHEK2a	1	0.4137	**2.4172**
TP53BP1	1	**2.6686**		CHEK1	1	0.5492	
UNG	1	0.7274		CHEK2b	1	1.2205	
AICDA	1	und		EXO1	1	0.4739	**2.1099**
BM2	1	**3.4118**		BRCA1	1	0.5429	
MSH2	1	1.6587		ERCC3	1	**2.8176**	
RAD51	1	0.4841	**2.0657**	FANCD2	1	0.4842	**2.065**
XRCC6	1	**2.1786**		XRCC5	1	**2.2061**	
MSH6	1	1.6584		NBN	1	1.3696	
BRCA1	1	0.4786	**2.0894**				
FANCE	1	1.1470					
FANCA	1	1.1906					

und=undetermined

Table 9-8: DNA repair gene expression before and after irradiation with 0.2Gy in PC3 prostate cancer cells

Gene	RQ value (PC3 NT)	RQ value (0.2Gy 2hr)	Gene	RQ value (PC3 NT)	RQ value (0.2Gy 2hr)
XRCC2	1	0.9931	IL6	1	und
ERCC2	1	0.9062	FANCF	1	0.7895
H2AFX	1	0.7617	ERCC5	1	0.9517
XRCC3	1	0.9983	XPA	1	0.9849
RAD51C	1	0.8602	RAD51C	1	0.8478
AICDA	1	und	PMS2	1	1.0266
PRKDC	1	0.9037	ERCC1	1	0.9553
MLH1	1	0.9485	FANCC	1	0.8470
PMS2	1	0.9624	ATM	1	0.9767
ERCC4	1	0.9840	FANCG	1	0.9765
XPC	1	und	RAD52	1	1.0668
TNF	1	1.0150	TP53BP1	1	0.9920
MRE11A	1	0.8826	XRCC6	1	1.0290
BRCA2	1	1.2234	CHEK2a	1	0.9780
TP53BP1	1	0.9616	CHEK1	1	0.9286
UNG	1	1.0758	CHEK2b	1	0.8337
AICDA	1	und	EXO1	1	0.8468
BM2	1	und	BRCA1	1	0.8849
MSH2	1	0.8849	ERCC3	1	0.9305
RAD51	1	0.9145	FANCD2	1	0.8159
XRCC6	1	0.8217	XRCC5	1	0.9310
MSH6	1	0.8412	NBN	1	0.9284
BRCA1	1	0.8813			
FANCE	1	0.9591			
FANCA	1	0.9708			

und=undetermined

Table 9-9: DNA repair gene expression before and after irradiation with 0.2Gy in DU145 prostate cancer cells

Gene	RQ value (DU145 NT)	RQ value (0.2Gy 2hr)	Fold downregulation (1/RQ)	Gene	RQ value (DU145 NT)	RQ value (0.2Gy 2hr)	Fold downregulation (1/RQ)
XRCC2	1	0.9647		IL6	1	1.6798	
ERCC2	1	0.8698		FANCF	1	0.8806	
H2AFX	1	0.7707		ERCC5	1	1.0577	
XRCC3	1	0.9954		XPA	1	0.8477	
RAD51C	1	1.2044		RAD51C	1	1.0811	
AICDA	1	und		PMS2	1	0.8655	
PRKDC	1	0.9726		ERCC1	1	0.7802	
MLH1	1	1.0587		FANCC	1	0.9273	
PMS2	1	0.8183		ATM	1	1.1337	
ERCC4	1	0.9593		FANCG	1	0.8864	
XPC	1	1.0614		RAD52	1	1.0001	
TNF	1	0.6714		TP53BP1	1	1.5100	
MRE11A	1	1.0286		XRCC6	1	0.7587	
BRCA2	1	0.9615		CHEK2a	1	1.1863	
TP53BP1	1	1.0359		CHEK1	1	0.7958	
UNG	1	0.8984		CHEK2b	1	0.9114	
AICDA	1	und		EXO1	1	0.7617	
BM2	1	0.9806		BRCA1	1	0.9245	
MSH2	1	0.9411		ERCC3	1	0.9249	
RAD51	1	0.8899		FANCD2	1	0.9855	
XRCC6	1	0.8820		XRCC5	1	0.8083	
MSH6	1	1.01		NBN	1	0.78	
BRCA1	1	1.0149					
FANCE	1	0.81					
FANCA	1	0.80					

und=undetermined

Table 9-10: DNA repair gene expression before and after irradiation with 0.2Gy in 22RV1 prostate cancer cells

Gene	RQ value (22RV1 NT)	RQ value (0.2Gy 2hr)	Gene	RQ value (22RV1 NT)	RQ value (0.2Gy 2hr)
XRCC2	1	1.2899	IL6	1	und
ERCC2	1	1.1599	FANCF	1	1.0944
H2AFX	1	**2.2484**	ERCC5	1	1.1268
XRCC3	1	1.6937	XPA	1	1.3528
RAD51C	1	1.1525	RAD51C	1	0.9469
AICDA	1	und	PMS2	1	1.2247
PRKDC	1	1.1850	ERCC1	1	1.6950
MLH1	1	1.1255	FANCC	1	1.0687
PMS2	1	1.0879	ATM	1	1.5836
ERCC4	1	0.9920	FANCG	1	1.1188
XPC	1	1.1533	RAD52	1	**2.0910**
TNF	1	0.8374	TP53BP1	1	**2.1801**
MRE11A	1	1.6975	XRCC6	1	1.5996
BRCA2	1	1.2944	CHEK2a	1	1.0278
TP53BP1	1	0.9674	CHEK1	1	1.0421
UNG	1	0.9587	CHEK2b	1	1.5286
AICDA	1	und	EXO1	1	0.9502
BM2	1	1.2614	BRCA1	1	1.4732
MSH2	1	1.1759	ERCC3	1	1.1932
RAD51	1	1.2625	FANCD2	1	1.8284
XRCC6	1	**2.5476**	XRCC5	1	0.9249
MSH6	1	1.7434	NBN	1	0.9094
BRCA1	1	1.1594			
FANCE	1	0.9805			
FANCA	1	1.1788			

und=undetermined

Chapter 10: REFERENCES

Adamson, A. W., D. I. Beardsley, W. J. Kim, Y. Gao, R. Baskaran and K. D. Brown, Methylator-induced, mismatch repair-dependent G2 arrest is activated through Chk1 and Chk2. *Molecular Biology of the Cell* **16**, 1513-26 (2005).

Ahman, A. K., B. A. Jonsson, J. E. Damber, A. Bergh and H. Gronberg, Low frequency of microsatellite instability in hereditary prostate cancer. *British Journal of Urology International* **87**, 334-8 (2001).

Andreassen, C. N., J. Alsner and J. Overgaard, Does variability in normal tissue reactions after radiotherapy have a genetic basis--where and how to look for it? *Radiotherapy and Oncology* **64**, 131-40 (2002).

Azzouzi, A. R., J. W. Catto, I. Rehman, S. Larre, M. Roupret, K. M. Feeley, O. Cussenot, M. Meuth and F. C. Hamdy, Clinically localised prostate cancer is microsatellite stable. *British Journal of Urology International* **99**, 1031-5 (2007).

Banath, J. P., S. H. Macphail and P. L. Olive, Radiation sensitivity, H2AX phosphorylation, and kinetics of repair of DNA strand breaks in irradiated cervical cancer cell lines. *Cancer Research* **64**, 7144-9 (2004).

Bartkowiak, D., S. Hogner, W. Nothdurft and E. M. Rottinger, Cell cycle and growth response of CHO cells to X-irradiation: threshold-free repair at low doses. *International Journal of Radiation Oncology Biology Physics* **50**, 221-7 (2001).

Barwell, J., L. Pangon, S. Hodgson, A. Georgiou, I. Kesterton, T. Slade, M. Taylor, S. J. Payne, H. Brinkman, J. Smythe, N. J. Sebire, E. Solomon, Z. Docherty, R. Camplejohn, T. Homfray and J. R. Morris, Biallelic mutation of MSH2 in primary human cells is associated with sensitivity to irradiation and altered RAD51 foci kinetics. *Journal of Medical Genetics* **44**, 516-20 (2007).

Baudhuin, L. M., L. J. Burgart, O. Leontovich and S. N. Thibodeau, Use of microsatellite instability and immunohistochemistry testing for the identification of individuals at risk for Lynch syndrome. *Familial Cancer* **4**, 255-65 (2005).

Bean, C. L., C. I. Bradt, R. Hill, T. E. Johnson, M. Stallworth and S. M. Galloway, Chromosome aberrations: persistence of alkylation damage and modulation by O6-alkylguanine-DNA alkyltransferase. *Mutation Research* **307**, 67-81 (1994).

Beauchesne, P. D., S. Bertrand, R. Branche, S. P. Linke, R. Revel, J. F. Dore and R. M. Pedeux, Human malignant glioma cell lines are sensitive to low radiation doses. *International Journal of Cancer* **105**, 33-40 (2003).

Bedford, J. S. and H. G. Griggs (1975). The estimation of survival at low doses and the limits of resolution of the single-cell-plating technique. *Cell survival after low doses of radiation: Theoretical and clinical implications*. A. T. London, John Wiley & Sons: p34-39.

Bernier, J., E. J. Hall and A. Giaccia, Radiation oncology: a century of achievements. *Nature Reviews Cancer* **4**, 737-47 (2004).

Berry, S. E., T. Loh, T. Yan and T. J. Kinsella, Role of MutSalpha in the recognition of iododeoxyuridine in DNA. *Cancer Research* **63**, 5490-5 (2003).

Bi, X., M. Gong, D. Srikanta and Y. S. Rong, Drosophila ATM and Mre11 are essential for the G2/M checkpoint induced by low-dose irradiation. *Genetics* **171**, 845-7 (2005).

Branzei, D. and M. Foiani, Regulation of DNA repair throughout the cell cycle. *Nature reviews Molecular Cell Biology* **9**, 297-308 (2008).

Bromfield, G. P., A. Meng, P. Warde and R. G. Bristow, Cell death in irradiated prostate epithelial cells: role of apoptotic and clonogenic cell kill. *Prostate Cancer and Prostatic Diseases* **6**, 73-85 (2003).

Brown, K. D., A. Rathi, R. Kamath, D. I. Beardsley, Q. Zhan, J. L. Mannino and R. Baskaran, The mismatch repair system is required for S-phase checkpoint activation. *Nature Genetics* **33**, 80-4 (2003).

Bucci, B., I. D'Agnano, D. Amendola, A. Citti, G. H. Raza, R. Miceli, U. De Paula, R. Marchese, S. Albini, A. Felsani, E. Brunetti and A. Vecchione, Myc down-regulation sensitizes melanoma cells to radiotherapy by inhibiting MLH1 and MSH2 mismatch repair proteins. *Clinical Cancer Research* **11**, 2756-67 (2005).

Burger, M., S. Denzinger, C. G. Hammerschmied, A. Tannapfel, E. C. Obermann, W. F. Wieland, A. Hartmann and R. Stoehr, Elevated microsatellite alterations at selected tetranucleotides (EMAST) and mismatch repair gene expression in prostate cancer. *Journal of Molecular Medicine* **84**, 833-41 (2006).

Carethers, J. M., M. T. Hawn, D. P. Chauhan, M. C. Luce, G. Marra, M. Koi and C. R. Boland, Competency in mismatch repair prohibits clonal expansion of cancer cells treated with N-methyl-N'-nitro-N-nitrosoguanidine. *Journal of Clinical Investigations* **98**, 199-206 (1996).

Carson, C. T., R. A. Schwartz, T. H. Stracker, C. E. Lilley, D. V. Lee and M. D. Weitzman, The Mre11 complex is required for ATM activation and the G2/M checkpoint. *The EMBO Journal* **22**, 6610-20 (2003).

Cejka, P., L. Stojic, G. Marra and J. Jiricny, Is mismatch repair really required for ionizing radiation-induced DNA damage signaling? *Nature Genetics* **36**, 432-3; author reply 434 (2004).

Chalmers, A., P. Johnston, M. Woodcock, M. Joiner and B. Marples, PARP-1, PARP-2, and the cellular response to low doses of ionizing radiation. *International Journal of Radiation Oncology Biology and Physics* **58**, 410-9 (2004).

Chan, N., M. Milosevic and R. G. Bristow, Tumor hypoxia, DNA repair and prostate cancer progression: new targets and new therapies. *Future Oncology* **3**, 329-41 (2007).

Chandna, S., B. S. Dwarakanath, D. Khaitan, T. L. Mathew and V. Jain, Low-dose radiation hypersensitivity in human tumor cell lines: effects of cell-cell contact and nutritional deprivation. *Radiation Research* **157**, 516-25 (2002).

Chaturvedi, P., W. K. Eng, Y. Zhu, M. R. Mattern, R. Mishra, M. R. Hurle, X. Zhang, R. S. Annan, Q. Lu, L. F. Faucette, G. F. Scott, X. Li, S. A. Carr, R. K. Johnson, J. D. Winkler and B. B. Zhou, Mammalian Chk2 is a downstream effector of the ATM-dependent DNA damage checkpoint pathway. *Oncogene* **18**, 4047-54 (1999).

Chen, Y., J. Wang, M. M. Fraig, K. Henderson, N. K. Bissada, D. K. Watson and C. W. Schweinfest, Alterations in PMS2, MSH2 and MLH1 expression in human prostate cancer. *International Journal of Oncology* **22**, 1033-43 (2003).

Chen, Y., J. Wang, M. M. Fraig, J. Metcalf, W. R. Turner, N. K. Bissada, D. K. Watson and C. W. Schweinfest, Defects of DNA mismatch repair in human prostate cancer. *Cancer Research* **61**, 4112-21 (2001).

Chuang, S. T., B. Adley, M. Han, F. Lin, X. J. Yang and W. J. Catalona, Mutant L Homologue 1 (MLH1): a possible new immunohistochemical marker for prostatic cancer. *Histopathology* **52**, 247-50 (2008).

Collis, S. J., J. M. Schwaninger, A. J. Ntambi, T. W. Keller, W. G. Nelson, L. E. Dillehay and T. L. Deweese, Evasion of early cellular response mechanisms following low level radiation-induced DNA damage. *The Journal of Biological Chemistry* **279**, 49624-32 (2004).

D'Amico, A. V., R. Whittington, S. B. Malkowicz, D. Schultz, K. Blank, G. A. Broderick, J. E. Tomaszewski, A. A. Renshaw, I. Kaplan, C. J. Beard and A. Wein, Biochemical outcome after radical prostatectomy, external beam radiation therapy, or interstitial radiation therapy for clinically localized prostate cancer. *JAMA: the Journal of the American Medical Association* **280**, 969-74 (1998).

Dahiya, R., C. Lee, J. McCarville, W. Hu, G. Kaur and G. Deng, High frequency of genetic instability of microsatellites in human prostatic adenocarcinoma. *International Journal of Cancer* **72**, 762-7 (1997).

Dai, X., D. Tao, H. Wu and J. Cheng, Low dose hyper-radiosensitivity in human lung cancer cell line A549 and its possible mechanisms. *Journal of Huazhong University of Science and Technology* **29**, 101-6 (2009).

Davis, T. W., C. Wilson-Van Patten, M. Meyers, K. A. Kunugi, S. Cuthill, C. Reznikoff, C. Garces, C. R. Boland, T. J. Kinsella, R. Fishel and D. A. Boothman, Defective expression of the DNA mismatch repair protein, MLH1, alters G2-M cell cycle checkpoint arrest following ionizing radiation. *Cancer Research* **58**, 767-78 (1998).

Debiak, M., T. Nikolova and B. Kaina, Loss of ATM sensitizes against O6-methylguanine triggered apoptosis, SCEs and chromosomal aberrations. *DNA Repair (Amst)* **3**, 359-68 (2004).

Deckbar, D., J. Birraux, A. Krempler, L. Tchouandong, A. Beucher, S. Walker, T. Stiff, P. Jeggo and M. Lobrich, Chromosome breakage after G2 checkpoint release. *Journal of Cell Biology* **176**, 749-55 (2007).

DeWeese, T. L., J. M. Shipman, N. A. Larrier, N. M. Buckley, L. R. Kidd, J. D. Groopman, R. G. Cutler, H. te Riele and W. G. Nelson, Mouse embryonic stem cells carrying one or two defective Msh2 alleles respond abnormally to oxidative stress inflicted by low-level radiation. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 11915-20 (1998).

Dey, S., P. M. Spring, S. Arnold, J. Valentino, D. Chendil, W. F. Regine, M. Mohiuddin and M. M. Ahmed, Low-dose fractionated radiation potentiates the effects of Paclitaxel in wild-type and mutant p53 head and neck tumor cell lines. *Clinical Cancer Research* **9**, 1557-65 (2003).

Digesu, C., S. Cilla, A. De Gaetano, M. Massaccesi, G. Macchia, E. Ippolito, F. Deodato, S. Panunzi, C. Iapalucci, G. C. Mattiucci, E. D'Angelo, G. D. Padula, V. Valentini, N. Cellini, A. Piermattei and A. G. Morganti, Postoperative intensity modulated radiation therapy in high risk prostate cancer: A dosimetric comparison. *Medical Dosimetry* (2010).

Dionet, C., A. Tchirkov, J. P. Alard, J. Arnold, J. Dhermain, M. Rapp, V. Bodez, J. C. Tamain, I. Monbel, P. Malet, F. Kwiatkowski, D. Donnarieix, A. Veyre and P. Verrelle, Effects of low-dose neutrons applied at reduced dose rate on human melanoma cells. *Radiation Research* **154**, 406-11 (2000).

Duckett, D. R., J. T. Drummond, A. I. Murchie, J. T. Reardon, A. Sancar, D. M. Lilley and P. Modrich, Human MutSalpha recognizes damaged DNA base pairs containing O6-methylguanine, O4-methylthymine, or the cisplatin-d(GpG) adduct. *Proceedings of the National Academy of Sciences USA* **93**, 6443-7 (1996).

Durand, R. E., Use of a cell sorter for assays of cell clonogenicity. *Cancer Research* **46**, 2775-8 (1986).

Elliott, B. and M. Jasin, Repair of double-strand breaks by homologous recombination in mismatch repair-defective mammalian cells. *Mol Cell Biol* **21**, 2671-82 (2001).

Enns, L., K. T. Bogen, J. Wizniak, A. D. Murtha and M. Weinfeld, Low-dose radiation hypersensitivity is associated with p53-dependent apoptosis. *Molecular Cancer Research* **2**, 557-66 (2004).

Feldmann, E., V. Schmiemann, W. Goedecke, S. Reichenberger and P. Pfeiffer, DNA double-strand break repair in cell-free extracts from Ku80-deficient cells: implications for Ku serving as an alignment factor in non-homologous DNA end joining. *Nucleic Acids Research* **28**, 2585-96 (2000).

Ferlay, J., H. R. Shin, F. Bray, D. Forman, C. Mathers and D. M. Parkin, Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *International Journal of Cancer* (2010).

Fernet, M., F. Megnin-Chanet, J. Hall and V. Favaudon, Control of the G2/M checkpoints after exposure to low doses of ionising radiation: implications for hyper-radiosensitivity. *DNA Repair (Amst)* **9**, 48-57 (2009).

Fink, D., S. Nebel, S. Aebi, H. Zheng, B. Cenni, A. Nehme, R. D. Christen and S. B. Howell, The role of DNA mismatch repair in platinum drug resistance. *Cancer Research* **56**, 4881-6 (1996).

Fishel, R., Signaling mismatch repair in cancer. *Nature Medicine* **5**, 1239-41 (1999).

Franchitto, A., P. Pichierri, R. Piergentili, M. Crescenzi, M. Bignami and F. Palitti, The mammalian mismatch repair protein MSH2 is required for correct MRE11 and RAD51 relocalization and for efficient cell cycle arrest induced by ionizing radiation in G2 phase. *Oncogene* **22**, 2110-20 (2003).

Fritzell, J. A., L. Narayanan, S. M. Baker, C. E. Bronner, S. E. Andrew, T. A. Prolla, A. Bradley, F. R. Jirik, R. M. Liskay and P. M. Glazer, Role of DNA mismatch repair in the cytotoxicity of ionizing radiation. *Cancer Research* **57**, 5143-7 (1997).

Galloway, S. M., S. K. Greenwood, R. B. Hill, C. I. Bradt and C. L. Bean, A role for mismatch repair in production of chromosome aberrations by methylating agents in human cells. *Mutation Research* **346**, 231-45 (1995).

Garcia, L. M., J. Leblanc, D. Wilkins and G. P. Raaphorst, Fitting the linear-quadratic model to detailed data sets for different dose ranges. *Physics in Medicine and Biology* **51**, 2813-23 (2006).

Giannini, G., C. Rinaldi, E. Ristori, M. I. Ambrosini, F. Cerignoli, A. Viel, E. Bidoli, S. Berni, G. D'Amati, G. Scambia, L. Frati, I. Screpanti and A. Gulino, Mutations of an intronic repeat induce impaired MRE11 expression in primary human cancer with microsatellite instability. *Oncogene* **23**, 2640-7 (2004).

Giannini, G., E. Ristori, F. Cerignoli, C. Rinaldi, M. Zani, A. Viel, L. Ottini, M. Crescenzi, S. Martinotti, M. Bignami, L. Frati, I. Screpanti and A. Gulino, Human MRE11 is inactivated in mismatch repair-deficient cancers. *EMBO Reports* **3**, 248-54 (2002).

Grills, I. S., A. A. Martinez, M. Hollander, R. Huang, K. Goldman, P. Y. Chen and G. S. Gustafson, High dose rate brachytherapy as prostate cancer monotherapy reduces toxicity compared to low dose rate palladium seeds. *The Journal of Urology* **171**, 1098-104 (2004).

Grombacher, T., U. Eichhorn and B. Kaina, p53 is involved in regulation of the DNA repair'gene O6-methylguanine-DNA methyltransferase (MGMT) by DNA damaging agents. *Oncogene* 17, 845-51 (1998).

Hall, E. J. (1975). Biological problems in the measurement of survival at low doses. *Cell survival after low doses of radiation: Theoretical and clinical implications*. A. T. London, John Wiley & Sons: p13-24.

Hall, E. J., The inaugural Frank Ellis Lecture--latrogenic cancer: the impact of intensity-modulated radiotherapy. *Clinical oncology (Royal College of Radiologists (Great Britain))* **18**, 277-82 (2006).

Hall, E. J., Intensity-modulated radiation therapy, protons, and the risk of second cancers. *International Journal of Radiation Oncology Biology and Physics* **65**, 1-7 (2006).

Hall, E. J., Is there a place for quantitative risk assessment? *Journal of Radiological Protection* **29**, A171-84 (2009).

Hall, E. J. and C. S. Wuu, Radiation-induced second cancers: the impact of 3D-CRT and IMRT. *International Journal of Radiation Oncology Biology and Physics* **56**, 83-8 (2003).

Hamilton, C. S., J. W. Denham, M. O'Brien, P. Ostwald, T. Kron, S. Wright and W. Drr, Underprediction of human skin erythema at low doses per fraction by the linear quadratic model. *Radiotherapy and Oncology* **40**, 23-30 (1996).

Hanks, G. E., A. L. Hanlon, B. Epstein and E. M. Horwitz, Dose response in prostate cancer with 8-12 years' follow-up. *International Journal of Radiation Oncology Biology and Physics* **54**, 427-35 (2002).

Harney, J., N. Shah, S. Short, F. Daley, N. Groom, G. D. Wilson, M. C. Joiner and M. I. Saunders, The evaluation of low dose hyper-radiosensitivity in normal human skin. *Radiotherapy and Oncology* **70**, 319-29 (2004).

Harney, J., S. C. Short, N. Shah, M. Joiner and M. I. Saunders, Low dose hyper-radiosensitivity in metastatic tumors. *International Journal of Radiation Oncology Biology and Physics* **59**, 1190-5 (2004).

Hartwell, L. H. and T. A. Weinert, Checkpoints: controls that ensure the order of cell cycle events. *Science* **246**, 629-34 (1989).

Haustermans, K. M., I. Hofland, H. Van Poppel, R. Oyen, W. Van de Voorde, A. C. Begg and J. F. Fowler, Cell kinetic measurements in prostate cancer. *International Journal of Radiation Oncology Biology and Physics* **37**, 1067-70 (1997).

Hawn, M. T., A. Umar, J. M. Carethers, G. Marra, T. A. Kunkel, C. R. Boland and M. Koi, Evidence for a connection between the mismatch repair system and the G2 cell cycle checkpoint. *Cancer Research* **55**, 3721-5 (1995).

Hendzel, M. J., Y. Wei, M. A. Mancini, A. Van Hooser, T. Ranalli, B. R. Brinkley, D. P. Bazett-Jones and C. D. Allis, Mitosis-specific phosphorylation of histone H3 initiates primarily within pericentromeric heterochromatin during G2 and spreads in an ordered fashion coincident with mitotic chromosome condensation. *Chromosoma* **106**, 348-60 (1997).

Hermann, R. M., H. A. Wolff, H. Jarry, P. Thelen, C. Gruendker, M. Rave-Fraenk, H. Schmidberger and H. Christiansen, In vitro studies on the modification of low-dose hyper-radiosensitivity in prostate cancer cells by incubation with genistein and estradiol. *Radiation Oncology* **3**, 19 (2008).

Hermisson, M., A. Klumpp, W. Wick, J. Wischhusen, G. Nagel, W. Roos, B. Kaina and M. Weller, O6-methylguanine DNA methyltransferase and p53 status predict temozolomide sensitivity in human malignant glioma cells. *Journal of Neurochemistry* **96**, 766-76 (2006).

Hickman, M. J. and L. D. Samson, Apoptotic signaling in response to a single type of DNA lesion, O(6)-methylguanine. *Molecular Cell* **14**, 105-16 (2004).

Hirao, A., Y. Y. Kong, S. Matsuoka, A. Wakeham, J. Ruland, H. Yoshida, D. Liu, S. J. Elledge and T. W. Mak, DNA damage-induced activation of p53 by the checkpoint kinase Chk2. *Science* **287**, 1824-7 (2000).

Hirata, H., Y. Hinoda, K. Kawamoto, N. Kikuno, Y. Suehiro, N. Okayama, Y. Tanaka and R. Dahiya, Mismatch repair gene MSH3 polymorphism is associated with the risk of sporadic prostate cancer. *Journal of Urology* **179**, 2020-4 (2008).

Hirose, Y., M. S. Berger and R. O. Pieper, Abrogation of the Chk1-mediated G(2) checkpoint pathway potentiates temozolomide-induced toxicity in a p53-independent manner in human glioblastoma cells. *Cancer Research* **61**, 5843-9 (2001).

Hirose, Y., M. Katayama, D. Stokoe, D. A. Haas-Kogan, M. S. Berger and R. O. Pieper, The p38 mitogen-activated protein kinase pathway links the DNA mismatch repair system to the G2 checkpoint and to resistance to chemotherapeutic DNA-methylating agents. *Molecular Cell Biology* **23**, 8306-15 (2003).

Holt, S. M., J. L. Scemama, M. I. Panayiotidis and A. G. Georgakilas, Compromised repair of clustered DNA damage in the human acute lymphoblastic leukemia MSH2-deficient NALM-6 cells. *Mutation Research* **674**, 123-30 (2009).

Honore, H. B. and S. M. Bentzen, A modelling study of the potential influence of low dose hypersensitivity on radiation treatment planning. *Radiotherapy and Oncology* **79**, 115-21 (2006).

Horner, M., L. Ries, M. Krapcho, N. Neyman, R. Aminou and N. Howlader, SEER Cancer Statistics Review, 1975-2006. *Bethesda, MD: National Cancer Institute* (2009).

Houtgraaf, J. H., J. Versmissen and W. J. van der Giessen, A concise review of DNA damage checkpoints and repair in mammalian cells. *Cardiovascular Revascularization Medicine : including molecular interventions* **7**, 165-72 (2006).

Howard, A., Wallner K, Han B, Dominitz J, Schneider B and S. S, Clinical course and dosimetry of rectal fistulas after prostate brachytherapy. *Journal of Brachytherapy International* **17**, 37–42 (2001).

Iyer, R. R., A. Pluciennik, V. Burdett and P. L. Modrich, DNA mismatch repair: functions and mechanisms. *Chemical Reviews* **106**, 302-23 (2006).

Jiricny, J., The multifaceted mismatch-repair system. *Nature reviews Molecular Cell Biology* **7**, 335-46 (2006).

Jobson, A. G., G. T. Lountos, P. L. Lorenzi, J. Llamas, J. Connelly, D. Cerna, J. E. Tropea, A. Onda, G. Zoppoli, S. Kondapaka, G. Zhang, N. J. Caplen, J. H. Cardellina, 2nd, S. S. Yoo, A. Monks, C. Self, D. S. Waugh, R. H. Shoemaker and Y. Pommier, Cellular inhibition of checkpoint kinase 2 (Chk2) and potentiation of camptothecins and radiation by the novel Chk2 inhibitor PV1019 [7-nitro-1H-indole-2-carboxylic acid {4-[1-(guanidinohydrazone)-ethyl]-phenyl}-amide]. *The Journal of Pharmacology and Experimental Therapeutics* **331**, 816-26 (2009).

Joiner, M. C. and J. Denekamp, The effect of small radiation doses on mouse skin. *The British Journal of Cancer. Supplement* **7**, 63-6 (1986).

Joiner, M. C., J. Denekamp and R. L. Maughan, The use of 'top-up' experiments to investigate the effect of very small doses per fraction in mouse skin. *International Journal of Radiation Biology and Related Studies in Physics, Chemistry, and Medicine* **49**, 565-80 (1986).

Joiner, M. C. and H. Johns, Renal damage in the mouse: the response to very small doses per fraction. *Radiation Research* **114**, 385-98 (1988).

Joiner, M. C., P. Lambin and B. Marples, Adaptive response and induced resistance. *Comptes rendus de l'Academie des sciences. Serie III, Sciences de la vie* **322**, 167-75 (1999).

Joiner, M. C., B. Marples, P. Lambin, S. C. Short and I. Turesson, Low-dose hypersensitivity: current status and possible mechanisms. *International Journal of Radiation Oncology Biology and Physics* **49**, 379-89 (2001).

Kabeya, Y., N. Mizushima, A. Yamamoto, S. Oshitani-Okamoto, Y. Ohsumi and T. Yoshimori, LC3, GABARAP and GATE16 localize to autophagosomal membrane depending on form-II formation. *Journal of Cell Science* **117**, 2805-12 (2004).

Karran, P. and M. Bignami, DNA damage tolerance, mismatch repair and genome instability. *Bioessays* **16**, 833-9 (1994).

Kass, E. M. and M. Jasin, Collaboration and competition between DNA double-strand break repair pathways. *FEBS Letters* **584**, 3703-8 (2010).

Kinsella, T. J., Coordination of DNA mismatch repair and base excision repair processing of chemotherapy and radiation damage for targeting resistant cancers. *Clinical Cancer Research* **15**, 1853-9 (2009).

Klein, H. L., The consequences of Rad51 overexpression for normal and tumor cells. *DNA Repair (Amst)* **7**, 686-93 (2008).

Klokov, D., S. M. MacPhail, J. P. Banath, J. P. Byrne and P. L. Olive, Phosphorylated histone H2AX in relation to cell survival in tumor cells and xenografts exposed to single and fractionated doses of X-rays. *Radiotherapy and Oncology* **80**, 223-9 (2006).

Koontz, B. F., S. Das, K. Temple, S. Bynum, S. Catalano, J. I. Koontz, G. S. Montana and J. R. Oleson, Dosimetric and radiobiologic comparison of 3D conformal versus intensity modulated planning techniques for prostate bed radiotherapy. *Medical Dosimetry* **34**, 256-60 (2009).

Koukourakis, G., N. Kelekis, V. Armonis and V. Kouloulias, Brachytherapy for prostate cancer: a systematic review. *Advances in Urology*, 327945 (2009).

Krause, M., F. Hessel, J. Wohlfarth, D. Zips, C. Hoinkis, H. Foest, C. Petersen, S. C. Short, M. C. Joiner and M. Baumann, Ultrafractionation in A7 human malignant glioma in nude mice. *International Journal of Radiation Biology* **79**, 377-83 (2003).

Krause, M., J. Prager, J. Wohlfarth, F. Hessel, D. Dorner, M. Haase, M. C. Joiner and M. Baumann, Ultrafractionation does not improve the results of radiotherapy in radioresistant murine DDL1 lymphoma. *Strahlentherapie und Onkologie* **181**, 540-4 (2005).

Krause, M., J. Wohlfarth, B. Georgi, N. Pimentel, D. Dorner, D. Zips, W. Eicheler, F. Hessel, S. C. Short, M. C. Joiner and M. Baumann, Low-dose hyperradiosensitivity of human glioblastoma cell lines in vitro does not translate into improved outcome of ultrafractionated radiotherapy in vivo. *International Journal of Radiation Biology* **81**, 751-8 (2005).

Krueger, S. A., S. J. Collis, M. C. Joiner, G. D. Wilson and B. Marples, Transition in survival from low-dose hyper-radiosensitivity to increased radioresistance is independent of activation of ATM Ser1981 activity. *International Journal of Radiation Oncology Biology and Physics* **69**, 1262-71 (2007).

Krueger, S. A., M. C. Joiner, M. Weinfeld, E. Piasentin and B. Marples, Role of apoptosis in low-dose hyper-radiosensitivity. *Radiat Research* **167**, 260-7 (2007).

Krueger, S. A., G. D. Wilson, E. Piasentin, M. C. Joiner and B. Marples, The effects of G2-phase enrichment and checkpoint abrogation on low-dose hyper-radiosensitivity. *International Journal of Radiation Oncology Biology and Physics* **77**, 1509-17 (2010).

Kry, S. F., M. Salehpour, D. S. Followill, M. Stovall, D. A. Kuban, R. A. White and Rosen, II, The calculated risk of fatal secondary malignancies from intensity-modulated radiation therapy. *International Journal of Radiation Oncology Biology and Physics* **62**, 1195-203 (2005).

Kupelian, P., D. Kuban, H. Thames, L. Levy, E. Horwitz, A. Martinez, J. Michalski, T. Pisansky, H. Sandler, W. Shipley, M. Zelefsky and A. Zietman, Improved biochemical relapse-free survival with increased external radiation doses in patients with localized prostate cancer: the combined experience of nine institutions in patients treated in 1994 and 1995. *International Journal of Radiation Oncology Biology and Physics* **61**, 415-9 (2005).

Lambin, P., J. Coco-Martin, J. D. Legal, A. C. Begg, C. Parmentier, M. C. Joiner and E. P. Malaise, Intrinsic radiosensitivity and chromosome aberration analysis using fluorescence in situ

hybridization in cells of two human tumor cell lines. *Radiation Research* **138**, S40-3 (1994).

Lambin, P., B. Fertil, E. P. Malaise and M. C. Joiner, Multiphasic survival curves for cells of human tumor cell lines: induced repair or hypersensitive subpopulation? *Radiation Research* **138**, S32-6 (1994).

Lambin, P., E. P. Malaise and M. C. Joiner, The effect of very low radiation doses on the human bladder carcinoma cell line RT112. *Radiotherapy and Oncology* **32**, 63-72 (1994).

Lambin, P., E. P. Malaise and M. C. Joiner, Might intrinsic radioresistance of human tumour cells be induced by radiation? *International Journal of Radiation Biology* **69**, 279-90 (1996).

Lambin, P., B. Marples, B. Fertil, E. P. Malaise and M. C. Joiner, Hypersensitivity of a human tumour cell line to very low radiation doses. *International Journal of Radiation Biology* **63**, 639-50 (1993).

Lee, W. R., J. Moughan, J. B. Owen and M. J. Zelefsky, The 1999 patterns of care study of radiotherapy in localized prostate carcinoma: a comprehensive survey of prostate brachytherapy in the United States. *Cancer* **98**, 1987-94 (2003).

Leonard, B. E., Thresholds and transitions for activation of cellular radioprotective mechanisms - correlations between HRS/IRR and the 'inverse' dose-rate effect. *International Journal of Radiation Biology* **83**, 479-89 (2007).

Leonard, B. E. and A. C. Lucas, LDR brachytherapy: can low dose rate hypersensitivity from the "inverse" dose rate effect cause excessive cell killing to peripherial connective tissues and organs? *The British Journal of Radiology* **82**, 131-9 (2009).

Li, G. M., Mechanisms and functions of DNA mismatch repair. *Cell Research* **18**, 85-98 (2008).

Li, L. S., J. C. Morales, A. Hwang, M. W. Wagner and D. A. Boothman, DNA mismatch repair-dependent activation of c-Abl/p73alpha/GADD45alpha-mediated apoptosis. *The Journal of Biological Chemistry* **283**, 21394-403 (2008).

Lin, P. S. and A. Wu, Not all 2 Gray radiation prescriptions are equivalent: Cytotoxic effect depends on delivery sequences of partial fractionated doses. *International Journal of Radiation Oncology Biology and Physics* **63**, 536-44 (2005).

Lindor, N. M., G. M. Petersen, D. W. Hadley, A. Y. Kinney, S. Miesfeldt, K. H. Lu, P. Lynch, W. Burke and N. Press, Recommendations for the care of individuals with an inherited predisposition to Lynch syndrome: a systematic review. *JAMA: The Journal of the American Medical Association* **296**, 1507-17 (2006).

Little, M. P., Do non-targeted effects increase or decrease low dose risk in relation to the linear-non-threshold (LNT) model? *Mutation Research* **687**, 17-27 (2010).

Lorente, A., W. Mueller, E. Urdangarin, P. Lazcoz, U. Lass, A. von Deimling and J. S. Castresana, RASSF1A, BLU, NORE1A, PTEN and MGMT Expression and Promoter Methylation in Gliomas and Glioma Cell Lines and Evidence of Deregulated Expression of de novo DNMTs. *Brain Pathology* (2008).

Mackay, R. I. and J. H. Hendry, The modelled benefits of individualizing radiotherapy patients' dose using cellular radiosensitivity assays with inherent variability. *Radiotherapy and Oncology* **50**, 67-75 (1999).

Marcu, L. G., Altered fractionation in radiotherapy: From radiobiological rationale to therapeutic gain. *Cancer Treatment Reviews* (2010).

Marples, B., Is low-dose hyper-radiosensitivity a measure of G2-phase cell radiosensitivity? *Cancer Metastasis Reviews* **23**, 197-207 (2004).

Marples, B., H. Adomat, C. J. Koch and K. A. Skov, Response of V79 cells to low doses of X-rays and negative pi-mesons: clonogenic survival and DNA strand breaks. *International Journal of Radiation Biology* **70**, 429-36 (1996).

Marples, B., N. E. Cann, C. R. Mitchell, P. J. Johnston and M. C. Joiner, Evidence for the involvement of DNA-dependent protein kinase in the phenomena of low dose hyper-radiosensitivity and increased radioresistance. *International Journal of Radiation Biology* **78**, 1139-47 (2002).

Marples, B. and S. J. Collis, Low-dose hyper-radiosensitivity: past, present, and future. *International Journal of Radiation Oncology Biology Physics* **70**, 1310-8 (2008).

Marples, B. and M. C. Joiner, The response of Chinese hamster V79 cells to low radiation dose: evidence of enhanced sensitivity of the whole cell population. *Radiation Research* **133**, 41-51 (1993).

Marples, B. and M. C. Joiner, The elimination of low-dose hypersensitivity in Chinese hamster V79-379A cells by pretreatment with X rays or hydrogen peroxide. *Radiation Research* **141**, 160-9 (1995).

Marples, B., P. Lambin, K. A. Skov and M. C. Joiner, Low dose hyper-radiosensitivity and increased radioresistance in mammalian cells. *International Journal of Radiation Biology* **71**, 721-35 (1997).

Marples, B. and K. A. Skov, Small doses of high-linear energy transfer radiation increase the radioresistance of Chinese hamster V79 cells to subsequent X irradiation. *Radiation Research* **146**, 382-7 (1996).

Marples, B., B. G. Wouters, S. J. Collis, A. J. Chalmers and M. C. Joiner, Low-dose hyper-radiosensitivity: a consequence of ineffective cell cycle arrest of radiation-damaged G2-phase cells. *Radiation Research* **161**, 247-55 (2004).

Marples, B., B. G. Wouters and M. C. Joiner, An association between the radiation-induced arrest of G2-phase cells and low-dose hyper-radiosensitivity: a plausible underlying mechanism? *Radiation Research* **160**, 38-45 (2003).

Marquez, N., S. C. Chappell, O. J. Sansom, A. R. Clarke, J. Court, R. J. Errington and P. J. Smith, Single cell tracking reveals that Msh2 is a key component of an early-acting DNA damage-activated G2 checkpoint. *Oncogene* **22**, 7642-8 (2003).

Martin, L., M. Coffey, M. Lawler, D. Hollywood and L. Marignol, DNA mismatch repair and the transition to hormone independence in breast and prostate cancer. *Cancer Letters* **291**, 142-9 (2009).

Martin, L., B. Marples, M. Coffey, M. Lawler, D. Hollywood and L. Marignol, Recognition of O6MeG Lesions by MGMT and Mismatch Repair Proficiency may be a Prerequisite for Low-Dose Radiation Hypersensitivity. *Radiation Research* **172**, 405-13 (2009).

Martin, L. M., B. Marples, M. Coffey, M. Lawler, T. H. Lynch, D. Hollywood and L. Marignol, DNA mismatch repair and the DNA damage response to ionizing radiation: Making sense of apparently conflicting data. *Cancer Treatment Reviews* **36**, 518-527 (2010)) (2010).

Matsuoka, S., M. Huang and S. J. Elledge, Linkage of ATM to cell cycle regulation by the Chk2 protein kinase. *Science* **282**, 1893-7 (1998).

McKay, M. and L. Peters, Genetic determinants of radiation response. Report on a symposium held at Peter MacCallum Cancer Institute, Melbourne, Australia, 8 August 1996. *International Journal of Radiation Biology* **71**, 225-9 (1997).

Meyers, M., A. Hwang, M. W. Wagner and D. A. Boothman, Role of DNA mismatch repair in apoptotic responses to therapeutic agents. *Environmental Molecular Mutagen* **44**, 249-64 (2004).

Mimitou, E. P. and L. S. Symington, DNA end resection: many nucleases make light work. *DNA Repair (Amst)* **8**, 983-95 (2009).

Mitchell, C. R., M. Folkard and M. C. Joiner, Effects of exposure to low-dose-rate (60)co gamma rays on human tumor cells in vitro. *Radiation Research* **158**, 311-8 (2002).

Mitra, A., C. Jameson, Y. Barbachano, L. Sanchez, Z. Kote-Jarai, S. Peock, N. Sodha, E. Bancroft, A. Fletcher, C. Cooper, D. Easton, R. Eeles and C. S. Foster, Overexpression of RAD51 occurs in aggressive prostatic cancer. *Histopathology* **55**, 696-704 (2009).

Modesti, M. and R. Kanaar, DNA repair: spot(light)s on chromatin. *Curr Biol* **11**, R229-32 (2001).

Mothersill, C., C. B. Seymour and M. C. Joiner, Relationship between radiation-induced low-dose hypersensitivity and the bystander effect. *Radiation Research* **157**, 526-32 (2002).

Mullenders, L., M. Atkinson, H. Paretzke, L. Sabatier and S. Bouffler, Assessing cancer risks of low-dose radiation. *Nature Reviews Cancer* **9**, 596-604 (2009).

Norman, A., A. R. Kagan and S. L. Chan, The importance of genetics for the optimization of radiation therapy. A hypothesis. *American Journal of Clinical Oncology* **11**, 84-8 (1988).

Nutting, C. M., D. J. Convery, V. P. Cosgrove, C. Rowbottom, L. Vini, C. Harmer, D. P. Dearnaley and S. Webb, Improvements in target coverage and reduced spinal cord irradiation using

intensity-modulated radiotherapy (IMRT) in patients with carcinoma of the thyroid gland. *Radiotherapy and Oncology* **60**, 173-80 (2001).

O'Brien, V. and R. Brown, Signalling cell cycle arrest and cell death through the MMR System. *Carcinogenesis* **27**, 682-92 (2006).

Odrazka, K., M. Dolezel, J. Vanasek, M. Vaculikova, M. Zouhar, J. Sefrova, P. Paluska, M. Vosmik, T. Kohlova, I. Kolarova, Z. Macingova, P. Navratil, M. Brodak and P. Prosvic, Time course of late rectal toxicity after radiation therapy for prostate cancer. *Prostate Cancer and Prostatic Diseases* **13**, 138-43 (2010).

Papouli, E., P. Cejka and J. Jiricny, Dependence of the cytotoxicity of DNA-damaging agents on the mismatch repair status of human cells. *Cancer Research* **64**, 3391-4 (2004).

Pegg, A. E., M. E. Dolan, D. Scicchitano and K. Morimoto, Studies of the repair of O6-alkylguanine and O4-alkylthymine in DNA by alkyltransferases from mammalian cells and bacteria. *Environmental Health Perspect*ives **62**, 109-14 (1985).

Perinchery, G., D. Nojima, R. Goharderakhshan, Y. Tanaka, J. Alonzo and R. Dahiya, Microsatellite instability of dinucleotide tandem repeat sequences is higher than trinucleotide, tetranucleotide and pentanucleotide repeat sequences in prostate cancer. *International Journal of Oncology* **16**, 1203-9 (2000).

Pinkawa, M., C. Attieh, M. D. Piroth, R. Holy, S. Nussen, J. Klotz, R. Hawickhorst, W. Schafer and M. J. Eble, Dose-escalation using intensity-modulated radiotherapy for prostate cancer--evaluation of the dose distribution with and without 18F-choline PET-CT detected simultaneous integrated boost. *Radiotherapy and Oncology* **93**, 213-9 (2009).

Pollack, A., G. K. Zagars, G. Starkschall, J. A. Antolak, J. J. Lee, E. Huang, A. C. von Eschenbach, D. A. Kuban and I. Rosen, Prostate cancer radiation dose response: results of the M. D. Anderson phase III randomized trial. *International Journal of Radiation Oncology Biology Physics* **53**, 1097-105 (2002).

Potters, L., E. A. Klein, M. W. Kattan, C. A. Reddy, J. P. Ciezki, A. M. Reuther and P. A. Kupelian, Monotherapy for stage T1-T2 prostate cancer: radical prostatectomy, external

beam radiotherapy, or permanent seed implantation. *Radiotherapy and Oncology* **71**, 29-33 (2004).

Prtilo, A., F. S. Leach, R. Markwalder, A. Kappeler, F. C. Burkhard, M. G. Cecchini, U. E. Studer and G. N. Thalmann, Tissue microarray analysis of hMSH2 expression predicts outcome in men with prostate cancer. *Journal of Urology* **174**, 1814-8; discussion 1818 (2005).

Puck, T. T., P. I. Marcus and S. J. Cieciura, Clonal growth of mammalian cells in vitro; growth characteristics of colonies from single HeLa cells with and without a feeder layer. *Journal of Experimental Medicine* **103**, 273-83 (1956).

Redpath, J. L., S. C. Short, M. Woodcock and P. J. Johnston, Low-dose reduction in transformation frequency compared to unirradiated controls: the role of hyper-radiosensitivity to cell death. *Radiation Research* **159**, 433-6 (2003).

Rodriguez-Jimenez, F. J., V. Moreno-Manzano, R. Lucas-Dominguez and J. M. Sanchez-Puelles, Hypoxia causes downregulation of mismatch repair system and genomic instability in stem cells. *Stem Cells* **26**, 2052-62 (2008).

Rogakou, E. P., D. R. Pilch, A. H. Orr, V. S. Ivanova and W. M. Bonner, DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. *Journal of Biological Chemistry* **273**, 5858-68 (1998).

Roos, W. P., L. F. Batista, S. C. Naumann, W. Wick, M. Weller, C. F. Menck and B. Kaina, Apoptosis in malignant glioma cells triggered by the temozolomide-induced DNA lesion O6-methylguanine. *Oncogene* **26**, 186-97 (2007).

Ruben, J. D., S. Davis, C. Evans, P. Jones, F. Gagliardi, M. Haynes and A. Hunter, The effect of intensity-modulated radiotherapy on radiation-induced second malignancies. *International Journal of Radiation Oncology Biology Physics* **70**, 1530-6 (2008).

Rutman, A. M. and M. D. Kuo, Radiogenomics: creating a link between molecular diagnostics and diagnostic imaging. *European Journal of Radiology* **70**, 232-41 (2009).

Ryan, L. A., C. B. Seymour, M. C. Joiner and C. E. Mothersill, Radiation-induced adaptive response is not seen in cell lines showing a bystander effect but is seen in lines showing HRS/IRR response. *International Journal of Radiation Biology* **85**, 87-95 (2009).

Rzeszowska-Wolny, J., J. Polanska, M. Pietrowska, O. Palyvoda, J. Jaworska, D. Butkiewicz and R. Hancock, Influence of polymorphisms in DNA repair genes XPD, XRCC1 and MGMT on DNA damage induced by gamma radiation and its repair in lymphocytes in vitro. *Radiation Research* **164**, 132-40 (2005).

San Filippo, J., P. Sung and H. Klein, Mechanism of eukaryotic homologous recombination. *Annual Reviews in Biochemistry* **77**, 229-57 (2008).

Sathya, J. R., I. R. Davis, J. A. Julian, Q. Guo, D. Daya, I. S. Dayes, H. R. Lukka and M. Levine, Randomized trial comparing iridium implant plus external-beam radiation therapy with external-beam radiation therapy alone in node-negative locally advanced cancer of the prostate. *Journal of Clinical Oncology* **23**, 1192-9 (2005).

Schettino, G., M. Folkard, K. M. Prise, B. Vojnovic, A. G. Bowey and B. D. Michael, Low-dose hypersensitivity in Chinese hamster V79 cells targeted with counted protons using a charged-particle microbeam. *Radiation Research* **156**, 526-34 (2001).

Schild, D. and C. Wiese, Overexpression of RAD51 suppresses recombination defects: a possible mechanism to reverse genomic instability. *Nucleic Acids Research* (2009).

Shia, J., Immunohistochemistry versus microsatellite instability testing for screening colorectal cancer patients at risk for hereditary nonpolyposis colorectal cancer syndrome. Part I. The utility of immunohistochemistry. *Journal of Molecular Diagnostics* **10**, 293-300 (2008).

Shia, J., D. S. Klimstra, K. Nafa, K. Offit, J. G. Guillem, A. J. Markowitz, W. L. Gerald and N. A. Ellis, Value of immunohistochemical detection of DNA mismatch repair proteins in predicting germline mutation in hereditary colorectal neoplasms. *American Journal of Surgical Pathology* **29**, 96-104 (2005).

Short, S., C. Mayes, M. Woodcock, H. Johns and M. C. Joiner, Low dose hypersensitivity in the T98G human glioblastoma cell line. *International Journal of Radiation Biology* **75**, 847-55 (1999).

Short, S. C., S. Bourne, C. Martindale, M. Woodcock and S. P. Jackson, DNA damage responses at low radiation doses. *Radiation Research* **164**, 292-302 (2005).

Short, S. C., J. Kelly, C. R. Mayes, M. Woodcock and M. C. Joiner, Low-dose hypersensitivity after fractionated low-dose irradiation in vitro. *International Journal of Radiation Biology* **77**, 655-64 (2001).

Short, S. C., J. Kelly, C. R. Mayes, M. Woodcock and M. C. Joiner, Low-dose hypersensitivity after fractionated low-dose irradiation in vitro. *International Journal of Radiation Biology* **77**, 655-64 (2001).

Short, S. C., S. A. Mitchell, P. Boulton, M. Woodcock and M. C. Joiner, The response of human glioma cell lines to low-dose radiation exposure. *International Journal of Radiation Biology* **75**, 1341-8 (1999).

Short, S. C., M. Woodcock, B. Marples and M. C. Joiner, Effects of cell cycle phase on low-dose hyper-radiosensitivity. *International Journal of Radiation Biology* **79**, 99-105 (2003).

Simonsson, M., F. Qvarnstrom, J. Nyman, K. A. Johansson, H. Garmo and I. Turesson, Low-dose hypersensitive gammaH2AX response and infrequent apoptosis in epidermis from radiotherapy patients. *Radiotherapy and Oncology* **88**, 388-97 (2008).

Singh, B., J. E. Arrand and M. C. Joiner, Hypersensitive response of normal human lung epithelial cells at low radiation doses. *International Journal of Radiation Biology* **65**, 457-64 (1994).

Skov, K., B. Marples, J. B. Matthews, M. C. Joiner and H. Zhou, A preliminary investigation into the extent of increased radioresistance or hyper-radiosensitivity in cells of hamster cell lines known to be deficient in DNA repair. *Radiation Research* **138**, S126-9 (1994).

Skov, K. A., Radioresponsiveness at low doses: hyper-radiosensitivity and increased radioresistance in mammalian cells. *Mutation Research* **430**, 241-53 (1999).

Slonina, D., B. Biesaga, K. Urbanski and Z. Kojs, Low-dose radiation response of primary keratinocytes and fibroblasts from patients with cervix cancer. *Radiation Research* **167**, 251-9 (2007).

Slonina, D., B. Biesaga, K. Urbanski and Z. Kojs, The response of primary keratinocytes and fibroblasts from cancer patients to multiple low-dose irradiations. *Radiation Research* **168**, 631-6 (2007).

Slonina, D., B. Biesaga, K. Urbanski and Z. Kojs, Comparison of chromosomal radiosensitivity of normal cells with and without HRS-like response and normal tissue reactions in patients with cervix cancer. *International Journal of Radiation Biology* **84**, 421-8 (2008).

Slonina, D., B. Biesaga, K. Urbanski, Z. Kojs and M. Waligorski, Evidence of low-dose hyper-radiosensitivity in normal cells of cervix cancer patients? *Radiation Protection Dosimetry* **122**, 282-4 (2006).

Spadinger, I., S. S. Poon and B. Palcic, Automated detection and recognition of live cells in tissue culture using image cytometry. *Cytometry* **10**, 375-81 (1989).

Spring, P. M., S. M. Arnold, S. Shajahan, B. Brown, S. Dey, S. M. Lele, J. Valentino, R. Jones, M. Mohiuddin and M. M. Ahmed, Low dose fractionated radiation potentiates the effects of taxotere in nude mice xenografts of squamous cell carcinoma of head and neck. *Cell Cycle* **3**, 479-85 (2004).

Stanford, J., R. Stephenson, L. Coyle, J. Cerhan, R. Correa, J. Eley, F. Gilliland, B. Hankey, L. Kolonel, C. Kosary, R. Ross, R. Severson and D. West (1999). Prostate Cancer Trends 1973-1995, SEER Program, National Cancer Institute. Bethesda, MD **NIH Pub. No. 99-4543**.

Stojic, L., R. Brun and J. Jiricny, Mismatch repair and DNA damage signalling. *DNA Repair (Amst)* **3**, 1091-101 (2004).

Storey, M. R., A. Pollack, G. Zagars, L. Smith, J. Antolak and I. Rosen, Complications from radiotherapy dose escalation in prostate cancer: preliminary results of a randomized trial. *International Journal of Radiation Oncology Biology Physics* **48**, 635-42 (2000).

Strom, S. S., M. R. Spitz, Y. Yamamura, R. J. Babaian, P. T. Scardino and Q. Wei, Reduced expression of hMSH2 and hMLH1 and risk of prostate cancer: a case-control study. *The Prostate* **47**, 269-75 (2001).

Surtees, J. A., J. L. Argueso and E. Alani, Mismatch repair proteins: key regulators of genetic recombination. *Cytogenetics Genome Research* **107**, 146-59 (2004).

Tajima, A., M. T. Hess, B. L. Cabrera, R. D. Kolodner and J. M. Carethers, The mismatch repair complex hMutS alpha recognizes 5-fluorouracil-modified DNA: implications for chemosensitivity and resistance. *Gastroenterology* **127**, 1678-84 (2004).

Thomas, C., J. Charrier, C. Massart, M. Cherel, B. Fertil, J. Barbet and N. Foray, Low-dose hyper-radiosensitivity of progressive and regressive cells isolated from a rat colon tumour: impact of DNA repair. *International Journal of Radiation Biology* **84**, 533-48 (2008).

Thomas, C., B. Fertil and N. Foray, Very low-dose hyper-radiosensitivity: impact for radiotherapy of micrometastases. *Cancer Radiotherapy* **11**, 260-5 (2007).

Tome, W. A. and S. P. Howard, On the possible increase in local tumour control probability for gliomas exhibiting low dose hyper-radiosensitivity using a pulsed schedule. *British Journal of Radiology* **80**, 32-7 (2007).

Turesson, I., R. Bernefors, M. Book, M. Flogegard, I. Hermansson, K. A. Johansson, A. Lindh, S. Sigurdardottir, U. Thunberg and J. Nyman, Normal tissue response to low doses of radiotherapy assessed by molecular markers--a study of skin in patients treated for prostate cancer. *Acta Oncologica* **40**, 941-51 (2001).

Turesson, I., J. Nyman, F. Qvarnstrom, M. Simonsson, M. Book, I. Hermansson, S. Sigurdardottir and K. A. Johansson, A low-dose hypersensitive keratinocyte loss in response to fractionated radiotherapy is associated with growth arrest and apoptosis. *Radiotherapy and Oncology* **94**, 90-101 (2010).

Vaganay-Juery, S., C. Muller, E. Marangoni, B. Abdulkarim, E. Deutsch, P. Lambin, P. Calsou, F. Eschwege, B. Salles, M. Joiner and J. Bourhis, Decreased DNA-PK activity in human cancer cells exhibiting hypersensitivity to low-dose irradiation. *Brritish Journal of Cancer* **83**, 514-8 (2000).

Velasco, A., P. S. Albert, H. Rosenberg, C. Martinez and F. S. Leach, Clinicopathologic implications of hMSH2 gene expression and microsatellite instability in prostate cancer. *Cancer Biology and Therapy* **1**, 362-7 (2002).

Velasco, A., S. M. Hewitt, P. S. Albert, M. Hossein, H. Rosenberg, C. Martinez, A. I. Sagalowsky, J. D. McConnell, W. Marston and F. S. Leach, Differential expression of the mismatch

repair gene hMSH2 in malignant prostate tissue is associated with cancer recurrence. *Cancer* **94**, 690-9 (2002).

Verellen, D. and F. Vanhavere, Risk assessment of radiation-induced malignancies based on whole-body equivalent dose estimates for IMRT treatment in the head and neck region. *Radiotherapy and Oncology* **53**, 199-203 (1999).

Vergeer, M. R., P. A. Doornaert, D. H. Rietveld, C. R. Leemans, B. J. Slotman and J. A. Langendijk, Intensity-modulated radiotherapy reduces radiation-induced morbidity and improves health-related quality of life: results of a nonrandomized prospective study using a standardized follow-up program. *International Journal of Radiation Oncology Biology Physics* **74**, 1-8 (2009).

Vicini, F. A., A. Abner, K. L. Baglan, L. L. Kestin and A. A. Martinez, Defining a dose-response relationship with radiotherapy for prostate cancer: is more really better? *International Journal of Radiation Oncology Biology Physics* **51**, 1200-8 (2001).

Wang, J. Y. and W. Edelmann, Mismatch repair proteins as sensors of alkylation DNA damage. *Cancer Cell* **9**, 417-8 (2006).

Watanabe, M., H. Imai, H. Kato, T. Shiraishi, T. Ushijima, M. Nagao and R. Yatani, Microsatellite instability in latent prostate cancers. *International Journal of Cancer* **69**, 394-7 (1996).

West, C., B. S. Rosenstein, J. Alsner, D. Azria, G. Barnett, A. Begg, S. Bentzen, N. Burnet, J. Chang-Claude, E. Chuang, C. Coles, K. De Ruyck, D. De Ruysscher, A. Dunning, R. Elliott, L. Fachal, J. Hall, K. Haustermans, C. Herskind, T. Hoelscher, T. Imai, M. Iwakawa, D. Jones, C. Kulich, J. H. Langendijk, P. O'Neils, M. Ozsahin, M. Parliament, A. Polanski, B. Rosenstein, D. Seminara, P. Symonds, C. Talbot, H. Thierens, A. Vega and J. Yarnold, Establishment of a Radiogenomics Consortium. *International Journal of Radiation Oncology Biology and Physics* **76**, 1295-6 (2010).

West, C. M., M. J. McKay, T. Holscher, M. Baumann, I. J. Stratford, R. G. Bristow, M. Iwakawa, T. Imai, S. M. Zingde, M. S. Anscher, J. Bourhis, A. C. Begg, K. Haustermans, S. M. Bentzen and J. H. Hendry, Molecular markers predicting radiotherapy response: report and

recommendations from an International Atomic Energy Agency technical meeting. *International Journal of Radiation Oncology Biology and Physics* **62**, 1264-73 (2005).

Wheeler, J. M., W. F. Bodmer and N. J. Mortensen, DNA mismatch repair genes and colorectal cancer. *Gut* **47**, 148-53 (2000).

Wouters, B. G. and L. D. Skarsgard, The response of a human tumor cell line to low radiation doses: evidence of enhanced sensitivity. *Radiation Research* **138**, S76-80 (1994).

Wouters, B. G. and L. D. Skarsgard, Low-dose radiation sensitivity and induced radioresistance to cell killing in HT-29 cells is distinct from the "adaptive response" and cannot be explained by a subpopulation of sensitive cells. *Radiation Research* **148**, 435-42 (1997).

Wouters, B. G., A. M. Sy and L. D. Skarsgard, Hypoxic cell sensitization: low-dose intrinsic radiosensitivity is predictive for etanidazole efficacy in a panel of human tumour cell lines. *International Journal of Radiation Biology* **70**, 719-33 (1996).

Wouters, B. G., A. M. Sy and L. D. Skarsgard, Low-dose hypersensitivity and increased radioresistance in a panel of human tumor cell lines with different radiosensitivity. *Radiation Research* **146**, 399-413 (1996).

Wrixon, A. D., New ICRP recommendations. *Journal of Radiological Protection* **28**, 161-8 (2008).

Wykes, S. M., E. Piasentin, M. C. Joiner, G. D. Wilson and B. Marples, Low-dose hyper-radiosensitivity is not caused by a failure to recognize DNA double-strand breaks. *Radiation Research* **165**, 516-24 (2006).

Xu, X. S., L. Narayanan, B. Dunklee, R. M. Liskay and P. M. Glazer, Hypermutability to ionizing radiation in mismatch repair-deficient, Pms2 knockout mice. *Cancer Research* **61**, 3775-80 (2001).

Xu, B., S. T. Kim, D. S. Lim and M. B. Kastan, Two molecularly distinct G(2)/M checkpoints are induced by ionizing irradiation. *Mol Cell Biol* **22**, 1049-59 (2002).

Xue, L., D. Yu, Y. Furusawa, J. Cao, R. Okayasu and S. Fan, ATM-dependent hyper-radiosensitivity in mammalian cells irradiated by heavy ions. *International Journal of Radiation Oncology Biology and Physics* **75**, 235-43 (2009).

Yan, T., Y. Seo and T. J. Kinsella, Differential Cellular Responses to Prolonged LDR-IR in MLH1-Proficient and MLH1-Deficient Colorectal Cancer HCT116 Cells. *Clinical Cancer Research* (2009).

Yeh, C. C., C. Lee and R. Dahiya, DNA mismatch repair enzyme activity and gene expression in prostate cancer. *Biochemical and Biophysical Research Communications* **285**, 409-13 (2001).

Yoshikawa, K., T. Ogawa, R. Baer, H. Hemmi, K. Honda, A. Yamauchi, T. Inamoto, K. Ko, S. Yazumi, H. Motoda, H. Kodama, S. Noguchi, A. F. Gazdar, Y. Yamaoka and R. Takahashi, Abnormal expression of BRCA1 and BRCA1-interactive DNA-repair proteins in breast carcinomas. *International Journal of Cancer* **88**, 28-36 (2000).

Yuan, J. and J. Chen, MRE11-RAD50-NBS1 complex dictates DNA repair independent of H2AX. *Journal of Biological Chemistry* **285**, 1097-104 (2009).

Zdraveski, Z. Z., J. A. Mello, C. K. Farinelli, J. M. Essigmann and M. G. Marinus, MutS preferentially recognizes cisplatin- over oxaliplatin-modified DNA. *Journal of Biological Chemistry* **277**, 1255-60 (2002).

Zelefsky, M. J., Z. Fuks, M. Hunt, H. J. Lee, D. Lombardi, C. C. Ling, V. E. Reuter, E. S. Venkatraman and S. A. Leibel, High dose radiation delivered by intensity modulated conformal radiotherapy improves the outcome of localized prostate cancer. *Journal of Urology***166**, 876-81 (2001).

Zelefsky, M. J., S. A. Leibel, P. B. Gaudin, G. J. Kutcher, N. E. Fleshner, E. S. Venkatramen, V. E. Reuter, W. R. Fair, C. C. Ling and Z. Fuks, Dose escalation with three-dimensional conformal radiation therapy affects the outcome in prostate cancer. *International Journal of Radiation Oncology Biology and Physics* **41**, 491-500 (1998).

Zelefsky, M. J., J. Moughan, J. Owen, A. L. Zietman, M. Roach, 3rd and G. E. Hanks, Changing trends in national practice for external beam radiotherapy for clinically localized prostate cancer: 1999 Patterns of Care survey for prostate cancer. *International Journal of Radiation Oncology Biology and Physics* **59**, 1053-61 (2004).

Zeng, M., L. Narayanan, X. S. Xu, T. A. Prolla, R. M. Liskay and P. M. Glazer, Ionizing radiation-induced apoptosis via separate Pms2- and p53-dependent pathways. *Cancer Research* **60**, 4889-93 (2000).

Zeng, X., T. Yan, J. E. Schupp, Y. Seo and T. J. Kinsella, DNA mismatch repair initiates 6-thioguanine--induced autophagy through p53 activation in human tumor cells. *Clinical Cancer Research* **13**, 1315-21 (2007).

Zhang, L., Immunohistochemistry versus microsatellite instability testing for screening colorectal cancer patients at risk for hereditary nonpolyposis colorectal cancer syndrome. Part II. The utility of microsatellite instability testing. *Journal of Molecular Diagnostics* **10**, 301-7 (2008).

Zietman, A. L., M. L. DeSilvio, J. D. Slater, C. J. Rossi, Jr., D. W. Miller, J. A. Adams and W. U. Shipley, Comparison of conventional-dose vs high-dose conformal radiation therapy in clinically localized adenocarcinoma of the prostate: a randomized controlled trial. *JAMA: The Journal of the American Medical Association* **294**, 1233-9 (2005).

Zink, D., C. Mayr, C. Janz and L. Wiesmuller, Association of p53 and MSH2 with recombinative repair complexes during S phase. *Oncogene* **21**, 4788-800 (2002).

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Recognition of O6MeG Lesions by MGMT and Mismatch Repair Proficiency may be a Prerequisite for Low-Dose Radiation Hypersensitivity

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Martin, L., Marples, B., Coffey, M., Lawler, M., Hollywood, D. and Marignol, L. Recognition of O6MeG Lesions by MGMT and Mismatch Repair Proficiency may be a Prerequisite for Low-Dose Radiation Hypersensitivity. *Radiat. Res.* 172, 405–413 (2009).

Low-dose hyper-radiosensitivity (HRS) is the phenomenon whereby cells exposed to radiation doses of less than ~0.5 Gy exhibit increased cell killing relative to that predicted from back-extrapolating high-dose survival data using a linear-quadratic model. While the exact mechanism remains to be elucidated, the involvement of several molecular repair pathways has been documented. These processes in turn are also associated with the response of cells to O6-methylguanine (O6MeG) lesions. We propose a model in which the level of low-dose cell killing is determined by the efficiency of both pre-replicative repair by the DNA repair enzyme O6-methylguanine methyltransferase (MGMT) and post-replicative repair by the DNA mismatch repair (MMR) system. We therefore hypothesized that the response of cells to low doses of radiation is dependent on the expression status of MGMT and MMR proteins. MMR (MSH2, MSH6, MLH1, PMS1, PMS2) and MGMT protein expression signatures were determined in a panel of normal (PWR1E, RWPE1) and malignant (22RV1, DU145, PC3) prostate cell lines and correlated with clonogenic survival and cell cycle analysis. PC3 and RWPE1 cells (HRS positive) were associated with MGMT and MMR proficiency, whereas HRS negative cell lines lacked expression of at least one (MGMT or MMR) protein. MGMT inactivation had no significant effect on cell survival. These results indicate a possible role for MMR-dependent processing of damage produced by low doses of radiation. © 2009 by Radiation Research Society

INTRODUCTION

The cytotoxicity of a number of chemotherapeutic agents is associated with damage at the O6 position of guanine, creating O6-methylguanine (O6MeG) lesions. The DNA repair protein O6-methylguanine-DNA methyltransferase (MGMT) is the primary cellular defense against these lesions. In the absence of MGMT, the fate of cells exposed to O6MeG relies on the formation of O6-MeG:T and O6-MeG:C mispairs during the course of DNA duplication and the subsequent engagement of the MMR system (*1*).

The DNA mismatch repair (MMR) system is a highly conserved post-replicative editing process that maintains genomic fidelity through recognition and repair of incorrectly paired nucleotides [for recent reviews see refs. (*2, 3*)]. Mismatch recognition is facilitated by hMutSalpha (heterodimer of hMSH2 and hMSH6) or hMutSbeta (heterodimer of hMSH2 and hMSH3) and MutL, a heterodimer of hMLH1 and hPMS2 In combination, these complexes provoke both checkpoint and apoptotic responses (*4–9*), although controversy remains regarding the mechanism involved.

The "futile repair" model (*10*) proposes that the MMR system undergoes reiterated futile attempts at repair upon recognition of the O6-MeG:T and O6-MeG:C mispairs, leading to the formation of gaps in the newly synthesized DNA strand and ultimately the creation of double-strand breaks after replication. This damage then provokes a G_2 cell cycle arrest after the second round of DNA synthesis (*5, 11*) and ultimately cell death. Alternatively, according to the "direct signaling" model (*12*), after the recognition of O6 MeG:T and O6-MeG:C mispairs, the MMR system transmits the damage signal directly to the checkpoint machinery, without the need for DNA processing (*12*).

Low-dose hyper-radiosensitivity (HRS) describes the response of cells to low doses of radiation whereby radiation doses less than ~0.5 Gy produce cell killing greater than that predicted by the linear-quadratic model. This is followed by a period of increased radioresistance (IRR) from ~0.5–1 Gy (*13, 14*). Several DNA repair mechanisms have been implicated in the transition from HRS to IRR (*15, 16*) with activation of the ataxia telangiectasia mutated (ATM)-dependent early G_2-phase cell cycle checkpoint (*15, 16*) shown to be a key requirement. Mismatch repair was recently implicated in

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the low-dose response after demonstration of the absence of HRS in three MMR-deficient cell lines (*16, 17*).

Our work has demonstrated a number of parallels between the molecular signaling response to low-dose radiation exposures and damage response to O6MeG lesions, for example the identified roles for ATM and PARP and importance of G_2-phase cells. Therefore, we hypothesized that low-dose HRS may result from futile mismatch repair of O6MeG lesions in cells lacking sufficient MGMT activity. To test this, HRS was defined in a panel of normal and malignant prostate cell lines and compared with the expression of MGMT and MMR proteins. Cell survival was also determined after inhibition of MGMT activity by O6-benzylguanine and subsequent X irradiation. We report that the presence of a full complement of MMR proteins correlated with the expression of HRS and was associated with an $MGMT^+$ phenotype. Inactivation of MGMT with O6-benzylguanine, however, did not significantly alter survival after irradiation. We suggest that recognition of O6MeG lesions by MGMT and the subsequent processing by a functional MMR system may represent a novel mechanism for the induction of enhanced cytotoxicity after low doses of radiation.

MATERIALS AND METHODS

Cell Lines

Cells of the malignant DU145, PC3, LnCaP and 22RV1 prostate cancer cell lines and normal PWR1E and RWPE1 prostate cell lines were obtained from the ATCC. DU145, PC3, 22RV1 and LnCaP cells were grown in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin (Invitrogen, Dublin, Ireland). Both PWR1E and RWPE1 cells are virally transfected. PWR1E cells contain adenovirus 12 and SV40 DNA viral sequences, and RWPE1 cells contain a single copy of the human papillomavirus 18 (HPV-18). Both prostate normal cell lines were grown in keratinocyte serum free medium supplemented with bovine pituitary extract, epidermal growth factor (Gibco, Paisley, UK) and 1% penicillin/streptomycin. For routine maintenance, each cell line was grown as a monolayer at 37°C in 95% air/5% CO_2 and subcultured once or twice weekly to maintain exponential growth. All cells were confirmed to be mycoplasma free before use and at bimonthly intervals thereafter.

Clonogenic Survival Assay

Cell survival was evaluated using a standard colony-forming assay. A total of 500–6000 cells were plated per well in six-well plates for exposure to low to high doses of radiation (0–6 Gy). After incubation at 37°C for 7–9 days for PWR1E and RWPE1 cells, 9–10 days for DU145 and PC3 cells, and 10–14 days for 22RV1 cells, the resultant colonies were stained with crystal violet in 95% ethanol, and those consisting of greater than 50 cells were scored as representing surviving cells using ColCount™ (Oxford Optronix Ltd., Oxford, UK). The surviving fraction was calculated using the plating efficiency (PE) of irradiated cells/unirradiated cells. The average surviving fractions are shown, and the error bars are the standard errors of the mean.

Irradiation Parameters

Two different dose rates were used to deliver doses from 0–6 Gy to ensure accurate dosimetry at the lowest doses. For the cell survival assays, cells were irradiated as monolayers in six-well plates at a dose rate of 0.75 Gy min^{-1} (0–1 Gy) or at 3.25 Gy min^{-1} (2–6 Gy) using an Xstrahl RS225 molecular research system (Gulmay Medical Ltd., UK).

Data Analysis for Survival Assays

Surviving fractions measured at the doses tested were fitted with the Induced-Repair equation (Eq. 1) as described previously (*18*).

$$S = \exp\left\{-\alpha_r\left(1 + \left({}^{\alpha_s}/_{\alpha_r} - 1\right)e^{-{}^{d}/_{d_c}}\right)d - \beta d^2\right\}, \qquad (1)$$

where d is the dose, α_s represents the low-dose value of α (derived from the response at very low doses), α_r is the value extrapolated from the conventional high-dose response, d_c is the "transition" dose at which the change from the very low-dose HRS to the IRR response occurs (i.e., when α_s to α_r is 63% complete), and β is a constant as in the LQ equation. All parameters were fitted simultaneously and estimates of uncertainty were expressed as likelihood confidence intervals. The presence of low-dose hyper-radiosensitivity is deduced by values of α_s and α_r whose confidence limits do not overlap and a value of d_c (the change from low- to high-dose survival response) significantly greater than zero.

Cell Cycle Analysis

Cells were seeded at 5×10^5 cells/dish, grown overnight and harvested by trypsinization, fixation in 90% ethanol, and treatment with 10 μg/ml RNase (Qiagen) and 5 μg/ml propidium iodide (Invitrogen, Molecular Probes). DNA profiles were obtained by flow cytometry, and the relative numbers of cells in each phase of the cell cycle were determined using CellQuest software (BD Biosciences, Mountain View, CA).

Drugs and Drug Treatment

Both temozolomide (TMZ) and O6-benzylguanine (O6BG) (Sigma, Wicklow, Ireland) were solubilized in DMSO (Sigma) as stock solution of 100 m*M* and 10 m*M*, respectively, divided into aliquots, and stored at −20°C until use. Temozolomide was further diluted in cell culture medium, and cells were treated for 72 h. O6BG (10 μ*M*) was given 1 h before TMZ treatment to deplete MGMT, and the medium was replaced with fresh medium once the TMZ-conditioned medium was removed. O6BG was also given 1 h before irradiation and was not removed to inactivate any newly synthesized MGMT.

RNA Isolation and Quantitative RT-PCR Analysis

Quantification of MGMT mRNA levels was performed by a real-time fluorescence detection method as described previously (*31, 32*). In brief, after RNA isolation with an RNeasy Mini Kit (Qiagen, Valencia, CA), 2 μg of total RNA was converted to cDNA with a first-strand High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Ltd., Warrington, Cheshire, UK). The *MGMT* cDNA and internal control cDNA (*PGK1*) were amplified separately by PCR (Applied Biosystems). Initial template concentration was derived from the cycle number at which the fluorescent signal crossed a threshold in the exponential phase of the PCR reaction. Relative gene expression was determined by the threshold cycles for the *MGMT* gene and the *PGK1* gene.

Preparation of Protein Extracts

Cell pellets of treated and untreated samples were washed with cold PBS and subsequently lysed in cold RIPA lysis buffer [50 m*M* Tris-HCl, pH 7.4, 150 m*M* NaCl, 1 m*M* dithiothreitol (DTT), 0.25% sodium deoxycholate, 0.1% NP-40] (Santa Cruz Biotechnology, Santa Cruz, CA) containing 1 m*M* phenylmethysulfonyl fluoride (PMSF), 1 m*M*

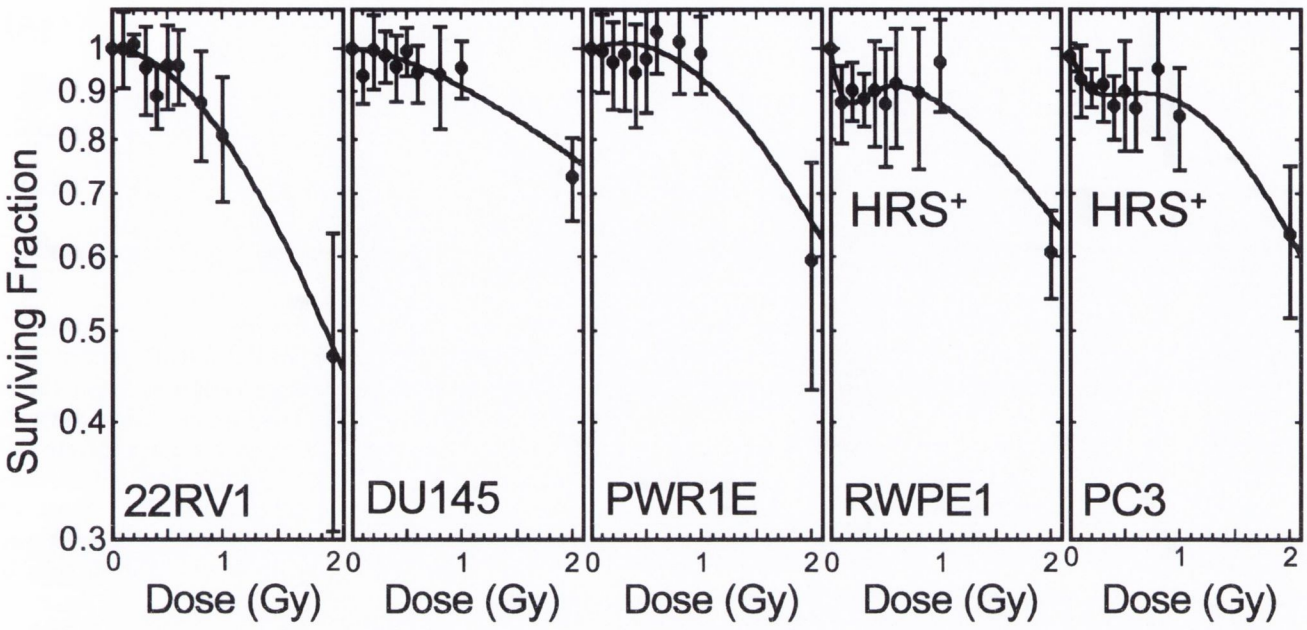

FIG. 1. Clonogenic survival of prostate cancer (DU145, PC3 and 22RV1) and prostate epithelial (PWR1E and RWPE1) cells after X irradiation. The data points show mean survival from four to seven individual experiments (±SEM). The line shows the fit of the data to the Induced Repair model.

sodium orthovanadate, and a protease inhibitor cocktail (Roche, Indianapolis, IN). Cell lysis was performed on ice for 30 min. Clear protein extracts were obtained by centrifugation for 30 min at 4°C.

Western Blot Analysis

The method used was described previously by Renart *et al.* (*19*). Protein (50 μg) from cell extracts was separated in 6%/15% SDS polyacrylamide gels and blotted onto a PVDF transfer membrane for 1 h. Membranes were blocked for 1 h at room temperature in 5% (wt/vol) fat-free milk powder in PBS containing 0.1% Tween 20, incubated overnight with the primary antibody (1:100–1:1000 dilution), washed three times with 0.1% Tween in PBS, and incubated for 1 h with a horseradish peroxidase-coupled secondary antibody 1:2000. The following primary antibodies were used: MLH1 (C-20), MSH2 (N-20), PMS1 (C-20), PMS2 (C-20) and goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology); MSH6 (Bethyl Laboratories, Montgomery, TX); ATM (D2E2), MGMT, and α/β tubulin (Cell Signaling Technology, Wicklow, Ireland). After final washing with 0.1% Tween 20 in PBS (three times for 10 min each), blots were developed using SuperSignal chemiluminescent substrate (Pierce, Rockford, IL). CEM-CCRF cell nuclear extract and HeLa cell nuclear extract were used as positive controls for MGMT protein expression and MMR protein expression, respectively.

Statistics

All experiments were performed in triplicate unless otherwise stated. Unpaired *t* tests were used to compare means. A *P* value of <0.05 was considered statistically significant.

RESULTS

Identification of HRS$^+$ Cell Lines

A panel of normal (PWR1E, RWPE1) and malignant (22RV1, DU145, PC3) prostate cell lines was assessed for evidence of low-dose hyper-radiosensitivity. The presence of HRS was determined by mathematical analysis of clonogenic cell survival in response to radiation doses of up to 6 Gy. The survival curves were fitted with either the Induced Repair (IR) model (*18*), which was developed to describe the response of cells to low doses of radiation, or the linear-quadratic model (Fig. 1). The cell survival curves of DU145, 22RV1 and PWR1E cells were best described by the linear-quadratic model and showed no evidence of HRS. PC3 and RWPE1 cells both exhibited distinct HRS and increased radioresistant responses as defined by the IR model (HRS$^+$) (Table 1).

HRS$^+$ Cell Lines were not Associated with an Enriched G_2/M Population

HRS has previously been related to the movement of cells through the G_2 phase of the cell cycle. Consequently, in the current study, the proportion of G_2/M cells in unirradiated cell populations was assessed by flow cytometry (Fig. 2). No relationship was seen been the static proportion of G_2/M cells and HRS. Although the G_2/M-phase cell population of HRS$^+$ PC3 cells was significantly higher than that of HRS$^-$ DU145 cells ($P = 0.005$), it was not significantly different from that of HRS$^-$ 22RV1 cells ($P = 0.8$).

Hypersensitivity is Evident in Low-Dose Temozolomide Survival Response in Chemoresistant PC3 Cells

Temozolomide, an alklyating agent that induces O6MeG lesions, was used to determine whether

TABLE 1
Values of the Parameters Obtained from Mathematical Modeling of Prostate Cell Lines using the Induced Repair Model

Cell line	α_s (± SE)	α_r (± SE)	β (± SE)	d_c (± SE)	d_c (CL)
DU145	wnc[a]	0.07 ± 0.02	0.02 ± 0.001	wnc	wnc
PC3	0.77 ± 0.51	0.12 ± 0.05	0.03 ± 0.02	0.18 ± 0.14	0.02–0.39
22RV1	wnc	0.06 ± 0.04	0.15 ± 0.03	wnc	wnc
PWR1E	wnc	0.47 ± 1.11	0.08 ± 0.01	wnc	wnc
RWPE1	2.10 ± 1.06	0.06 ± 0.04	0.07 ± 0.02	0.16 ± 0.06	0.07–0.30

[a] Would not converge.

substructure was also evident in the low-dose survival response after chemotherapy similar to that observed after low-dose X irradiation. Clonogenic survival measurements demonstrated that 0.3 μM TMZ induced significantly more cell death than 0.6 μM TMZ (t test, $P = 0.041$) (Fig. 3A). However, this was not observed in HRS$^-$ cell lines (DU145, 22RV1), which were significantly more sensitive to TMZ than were PC3 cells at the same concentration (one-way ANOVA, $P = 0.003$). This trend was also evident at a higher drug concentration (30 μM) (Fig. 3B) but was not statistically significant (one-way ANOVA, $P = 0.093$). Since HRS$^+$ cells were more resistant to TMZ than HRS$^-$ cells, we next explored whether hypersensitivity may be a default mechanism to cytotoxic insult in radioresistant and chemoresistant cells. Clonogenic survival assays were used to compare the surviving fraction at 2 Gy (SF2) across the panel of cell lines. HRS did not correlate with overall radioresistance (high SF2) in these prostate cell lines (Fig. 3C). While the SF2 of HRS$^+$ RWPE1 cells was not significantly different compared to those of other HRS$^-$ cell lines, the SF2 of HRS$^+$ PC3 cells was significantly lower than that of HRS$^-$ DU145 cells (t test, $P = 0.005$).

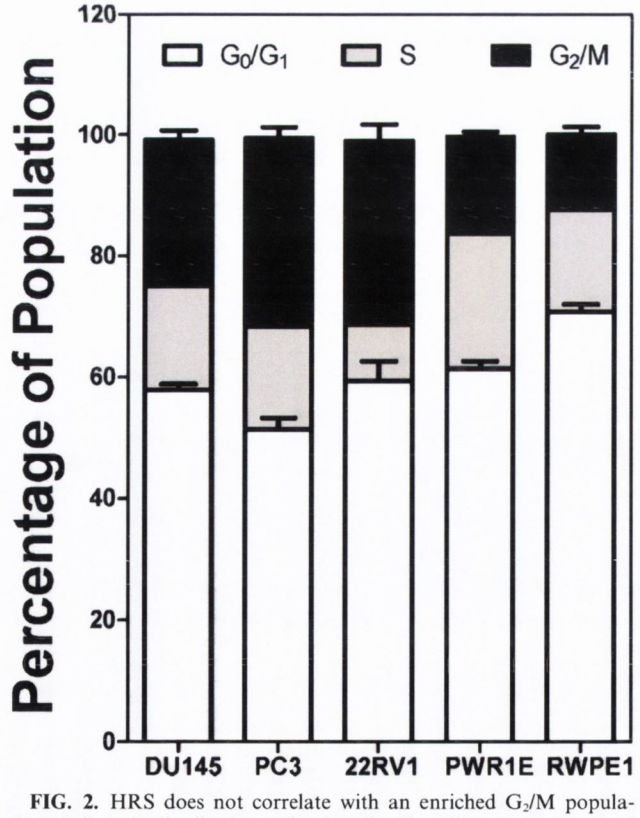

FIG. 2. HRS does not correlate with an enriched G_2/M population. Cell cycle distributions of unirradiated malignant prostate and normal prostate cell lines. Means ± SEM, $n = 3$.

A Potential Role for MGMT

Since these data indicated that TMZ resistance may be associated with HRS, we next investigated whether HRS was related to *MGMT*, a gene that confers resistance to TMZ. *MGMT* gene expression patterns were determined in HRS$^-$ cell lines and compared to those in RWPE1 and PC3 HRS$^+$ cell lines using quantitative RT-PCR (Fig. 4A, B). *MGMT* gene expression was down-regulated in HRS$^-$ 22RV1 cells compared to HRS$^+$ RWPE1 cells (7 ± 1-fold) and PC3 cells (5 ± 1-fold). *MGMT* expression was also down-regulated in HRS$^-$ PWR1E cells relative to RWPE1 cells (2.1 ± 0.5-fold) but was up-regulated (2 ± 1-fold) compared to PC3 cells. In contrast, MGMT expression was up-regulated in HRS$^-$ DU145 cells relative to RWPE1 (3.5 ± 1.3-fold) and PC3 cells (7 ± 2.4-fold). Western blots were used to determine MGMT protein expression in all cell lines (Fig. 4B). Since both HRS$^+$ PC3 and RWPE1 cell lines express MGMT, unlike the HRS$^-$ cell lines (DU145, 22RV1, PWR1E), the influence of MGMT inactivation on radiation survival was investigated by treating HRS$^+$ PC3 cells with the MGMT inhibitor O6-benzylguanine. Inactivation of MGMT by pretreatment with O6BG has been shown to increase cell sensitivity to TMZ (*20*). Here we validated the inactivation of MGMT by showing that pretreatment of PC3 cells (MGMT$^+$) with O6BG sensitized cells to further treatment with either 15 μM or 30 μM TMZ. Pretreatment with O6BG significantly increased cell killing by 50% (±7%) (t test, $P = 0.022$) at a dose of 15 μM and 20% (±8%) at the higher dose of 30 μM (t test, $P = 0.041$) (Fig. 4C). Inactivation of MGMT by O6BG did not appear to inhibit the induction of HRS within the low-dose range (Fig. 4D).

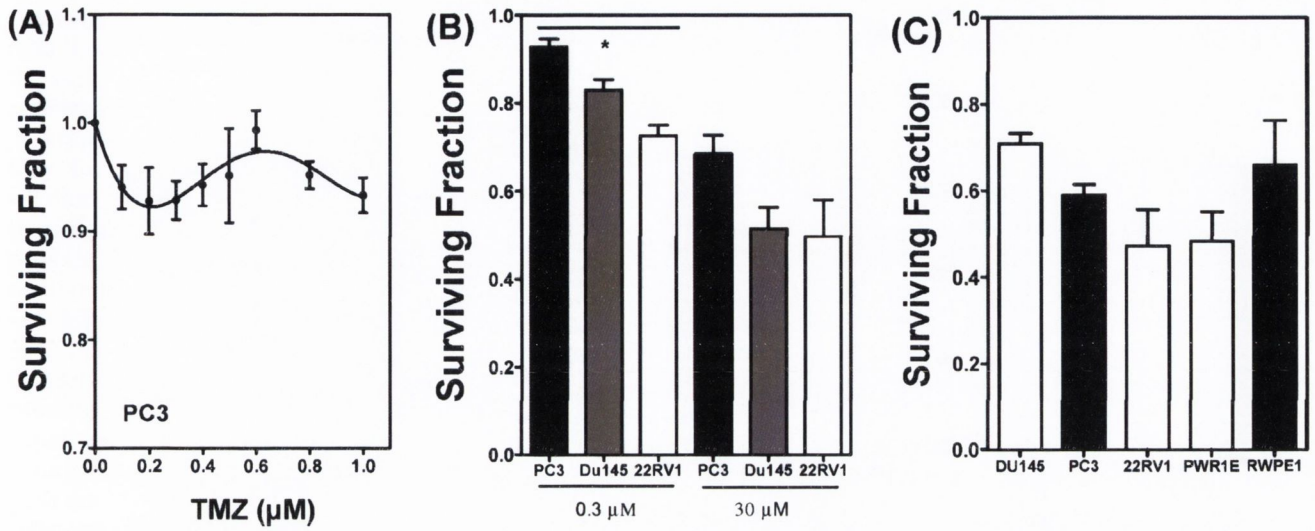

FIG. 3. Clonogenic survival of prostate cells in response to TMZ or radiation. Panel A: Clonogenic survival of HRS$^+$ PC3 cells after 3-day treatment with low concentrations of TMZ. The data points show mean survival ± SEM, $n = 4$. Panel B: Clonogenic survival of prostate cells (PC3, DU145, 22RV1) after 3-day treatments with 0.3 μ*M* and 30 μ*M* TMZ. Means ± SEM, $n = 3$. Panel C: Surviving fraction at 2 Gy (SF2) of a panel of prostate cell lines. HRS$^+$ cell lines are shown in black.

MMR Proficiency may be a Prerequisite for HRS

In the absence of MGMT or sufficient MGMT to remove O6MeG lesions, MMR proficiency is required for removal of the lesions by apoptosis. Moreover, MMR has recently been implicated in HRS (*16*). We therefore examined MMR protein expression patterns in HRS$^+$ (PC3, RWPE1) and HRS$^-$ (DU145, 22Rv1, PWR1E) cell lines using Western blotting (Fig. 5) to determine the association between HRS and both MGMT and MMR proficiency. As shown in Fig. 5, HRS$^+$ cells (PC3, RWPE1) expressed MGMT and all five MMR proteins, whereas all HRS$^-$ cells (DU145, 22Rv1, PWR1E) lacked at least one protein. PMS1 was expressed in all cell lines tested. In HRS$^-$ MGMT$^+$ DU145 cells, loss of PMS2 and MLH1 also appeared to prevent induction of HRS.

DISCUSSION

The molecular signaling response of cells exposed to low doses of ionizing radiation mirrors the signaling

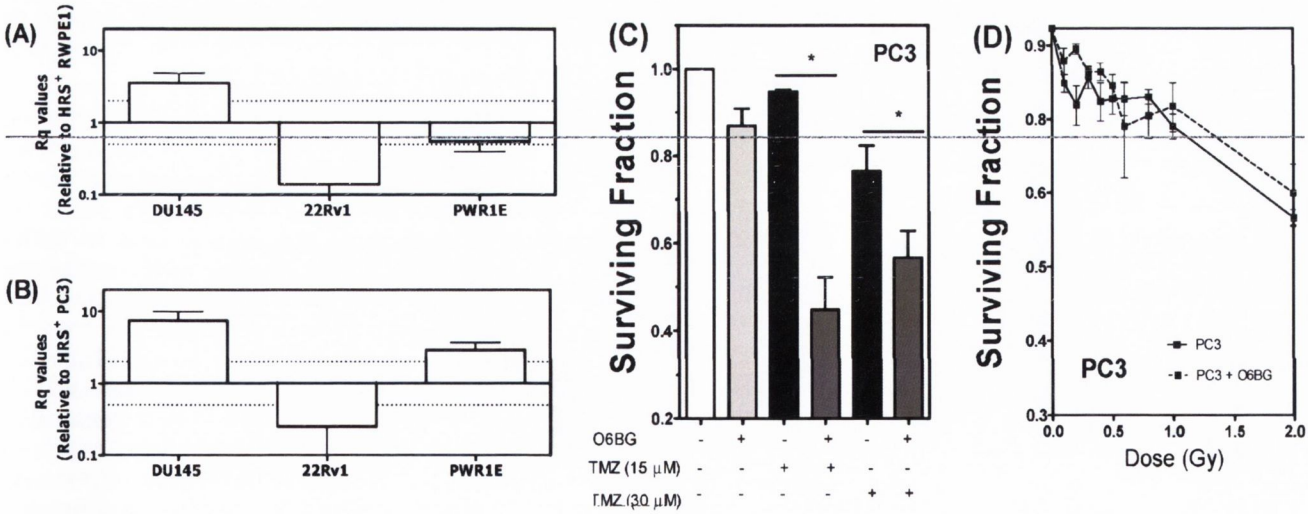

FIG. 4. MGMT gene expression and effect of O6BG on cell survival in response to TMZ or radiation exposures. Panels A and B: Relative quantification (Rq) value of MGMT in HRS$^-$ cell lines (DU145, 22RV1, PWR1E), compared to the HRS$^+$ cell lines RWPE1 (panel A) and PC3 (panel B). Means ± SEM, $n = 2$. Panel C: Clonogenic survival of PC3 prostate cells after 3-day treatments with TMZ (15 or 30 μ*M*) with and without pretreatment with 10 μ*M* O6BG. Means ± SEM, $n = 3$. Panel D: Clonogenic survival after X irradiation of PC3 cells pretreated with O6 benzylguanine for 1 h (dashed line) relative to survival after X irradiation alone (solid line). The data points show mean survival from three to five individual experiments (±SEM).

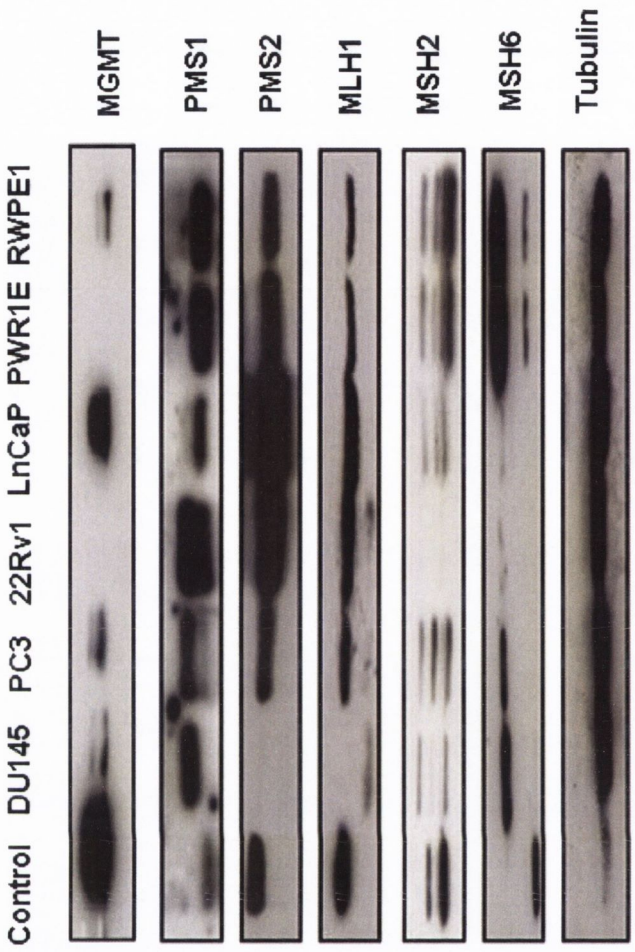

FIG. 5. HRS$^+$ is associated with MGMT and MMR proficiency. Panel A: Representative Western blots for MGMT and the mismatch repair proteins PMS1, PMS2, MLH1, MSH2 and MSH6 in a panel of prostate cell lines. CEM-CCRF and HeLa cell nuclear extracts were used as positive controls for MGMT and MMR proteins, respectively. Tubulin was used as a loading control. LnCap was used as a negative control for MSH2.

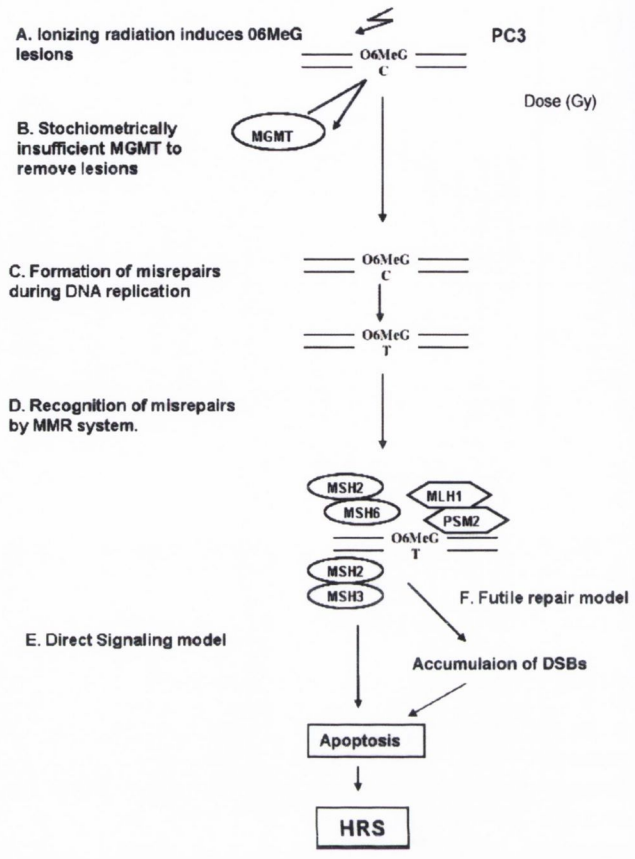

FIG. 6. Model of O6MeG triggered low-dose hyper-radiosensitivity. Current working hypothesis A: Ionizing radiation induces O6MeG lesions. B: There is stochiometrically insufficient MGMT to remove these lesions. C: Formation of mispairs by the MMR system. D: MMR proteins may signal apoptosis directly. E: Futile repair model: Reiterated futile attempts at repair upon recognition of the O6-MeG:T and O6-MeG:C mispairs ultimately leads to the creation of double-strand breaks after replication and cell death by apoptosis.

response of cells to O6-methylguanine damage. Thus we hypothesized that MGMT and mismatch repair proteins may play a role in promoting low-dose hyper-radiosensitivity or in overcoming HRS. In this model, we propose that, in the absence of MGMT, recognition and repair of the lesions by the mismatch repair system leads to the induction of DNA double-strand breaks and the subsequent induction of apoptosis, leading to the low-dose HRS survival response (Fig. 6).

Using high-precision clonogenic assays, we demonstrated HRS in PC3 prostate cancer cells and in a normal prostate epithelial cell line (RWPE1) (Fig. 1). To our knowledge, HRS in normal prostate epithelial cell lines has not been reported previously, although HRS has been demonstrated in normal human epidermis cells (*21, 22*). No firm consensus exists regarding evidence of HRS in prostate tumor cell lines (*23–27*). The absence of HRS in DU145 cells in our study confirms the observations of Lin and Wu (*25*). However, the PC3 results presented in the current study contradict results published previously (*26*). This difference could be reconciled by considering the criteria used to define HRS in each study. In the present work, unlike the previous report, the HRS status of PC3 cells was defined using a stringent mathematical approach.

Given that the response of cells to low-dose radiation appeared to mimic the cellular response to O6MeG lesions, we investigated whether low-dose hypersensitivity was evident in response to temozolomide (TMZ), an alkylating agent that creates O6MeG lesions. Treatment with low concentrations of TMZ resulted in a survival response not dissimilar to HRS in HRS$^+$ PC3 cells (Fig. 3A). Moreover, this sensitivity was not observed in HRS$^-$ cell lines. Because HRS$^+$ cells were therefore more resistant to TMZ than HRS$^-$ cells, we investigated whether hypersensitivity may be a default mechanism to

cytotoxic insult in radioresistant and chemoresistant cells. Clonogenic survival assays were used to compare the surviving fraction at 2 Gy (SF2) across the panel of cell lines. HRS did not correlate with a high SF2 in these prostate cell lines (Fig. 3C). Given that TMZ resistance may be associated with HRS, we next investigated whether HRS could be correlated with *MGMT*, a gene that confers resistance to TMZ. *MGMT* gene and protein expression was up-regulated in HRS^+ cells relative to HRS^- cells (Fig. 4A, B, Fig. 5).

While HRS was not correlated with the lack of *MGMT* gene expression as might be predicted if O6MeG lesions are involved, MGMT and all MMR proteins (MSH2, MSH6, PMS1, PMS2, MLH1) were expressed only by the HRS^+ cell lines (PC3, RWPE1). Loss of least one of the MMR proteins was coincident with an HRS^- phenotype (Fig. 5). In MMR-proficient cells, loss of MGMT but not its inactivation appears sufficient to inhibit the induction of HRS. Moreover, since treatment with the MGMT inhibitor O6-benzylguanine did not prevent induction of HRS, it may be postulated that the amount of O6MeG lesions produced by low doses of radiation are below the activation threshold of the enzyme. It must be noted, however, that levels of MGMT in these HRS^+ cell lines were low, and it is possible that these levels are stochiometrically insufficient to remove the lesions (Fig. 4A, B) (*28*). It has also been suggested that MGMT may protect against background DNA damage [e.g. endogenous single-strand breaks (SSBs)] after demonstration of a significant correlation between background SSBs and the MGMT polymorphism 84Phe in lymphocytes *in vitro* (*29*). Thus it may alternatively be postulated that the cumulative DNA damage after low-dose irradiation in $MGMT^+$ cells is less than that in $MGMT^-$ cells, keeping overall damage sufficiently low for HRS to occur.

To our knowledge, the MMR protein expression patterns of 22RV1, PWR1E and RWPE1 prostate cells have not been reported previously. Our data suggest the involvement of mismatch repair proteins in the hypersensitivity response (Fig. 5). This hypothesis is supported by the documented expression of HRS in five MMR-proficient cell lines [T98G, SNB19 (glioblastoma), A549 (lung), HT29 (colorectal), MeWo (melanoma)] and the absence of HRS in three MMR-deficient cell lines [SW48, HT116, RKO (colorectal)] (*14*).

This study provides evidence for the first time that suggests a role for O6MeG lesions in HRS. In our model, enhanced cell killing after low doses of radiation may result from low levels of O6MeG lesions, leading to formation of mispairs and induction of apoptosis after processing by the MMR system in cells with low levels of background DNA damage as afforded by low levels of MGMT. Since inhibition of MGMT did not prevent induction of increased radioresistance (IRR), it can be postulated that IRR results not from increasing levels of DNA damage but rather from a shift in the type of critical DNA damage above doses of 0.5 Gy. The increased activation of ATM (ser1981) above doses of 0.3 Gy as demonstrated by Marples and colleagues (*16*) could therefore reflect a shift in the hierarchy of critical DNA damage and the subsequent signaling pathways (MGMT, MMR) from the processing of O6MeG lesions at low doses to DNA double-strand breaks at higher doses rather from than an accumulation of the same type of damage. Moreover, reduced or absent ATM expression has been shown to sensitize cells against O6MeG (*30*), so decreased ATM activity at low doses may even be an active protective response to facilitate removal of these critical toxic lesions. The transition from HRS to IRR may therefore require both inactivation of signaling pathways [as proposed previously by Marples and Joiner (*31*)] in response to O6MeG as well as activation of DNA repair pathways. The increase in the mitotic index reported in HRS^+ cells relative to HRS^- cells (*32, 33*) could be explained by the fact that mismatch repair-dependent processing of O6MeG lesions and the associated G_2 arrest occur after the second S phase after damage (*34, 35*). Interestingly, among the cell lines most sensitive to low radiation doses are a large number of glioma cell lines (*36–38*) that express low levels of MGMT (*39*), are MMR^+ and are consequently highly sensitive to O6MeG lesions. In this regard, defects in DNA-dependent protein kinase (DNA-PK) also sensitize cells to O6MeG (*40*). Down-regulation of DNA-PK in response to low radiation doses may therefore also be part of an active protective response to maintain cell repair fidelity.

While a role for MMR proficiency is suggested here, further work is required to determine its specific involvement. As in the processing of alkylation damage, MMR may be involved either indirectly through futile processing of damage induced by low doses of radiation ("futile" repair model) or directly through signaling cascades initiated by the MMR system (direct signaling model). A number of current observations support the "futile repair" model. Because HRS^+ cells do not undergo an early G_2 arrest (<3 h) after low doses of radiation in the same way as HRS^- cells (*32, 33*), this could reflect the progression through the cell cycle of cells that will undergo a G_2 arrest after the second S phase after irradiation. Apoptosis has been shown to occur in HRS^+ cells after irradiation, which is a known mode of cell death after MMR processing. However, direct signaling may also be involved. The mismatch repair protein hMSH2 has recently been shown to be required for correct MRE11 and RAD51 relocalization and for efficient cell cycle arrest induced by ionizing radiation in G_2 phase and in particular is required to maintain this G_2 arrest when induced (*41*). Moreover, MSH2 proficiency may be required for efficient repair of clustered DNA damage induced by radiation (*42*).

MLH1 has also been implicated in maintaining a prolonged G_2 arrest, although its exact involvement is poorly understood (*6*). This G_2 arrest may occur at later times than have currently been investigated.

Conclusion

In the present study, we investigated the role of O6MeG lesions in HRS by measuring the survival of cells of five prostate cell lines (of both malignant and normal origin) in response to low-dose ionizing radiation and low-dose temozolomide, an agent that induces O6MeG lesions. Our cell lines showed differing MGMT and MMR expression patterns that correlated with the response of these prostate cells to low-dose radiation. Experiments involving the inactivation of MGMT were conducted to examine the role of MGMT in HRS. Our results support the involvement of MMR-dependent processing of damage induced by low-dose radiation in prostate cancer cells and may allow us to identify those tumors that may benefit from a low-dose fractionated radiotherapy regimen in the clinic. Because loss of one or more MMR proteins is characteristic of a wide range of tumors (lung, bladder, esophagus, endometrial, head and neck, gastric) (*43, 44*), a repeated low-dose fractionated regimen would be predicted to be of limited benefit in these cases and in tumors prone to loss of MMR such as hereditary nonpolyposis colon cancer. If this hypothesis is true, then identifying those with a full MMR complement by routine immunohistochemical analysis might represent a useful prognostic biomarker for low-dose fractionated radiation response.

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REFERENCES

1. E. Papouli, P. Cejka and J. Jiricny, Dependence of the cytotoxicity of DNA-damaging agents on the mismatch repair status of human cells. *Cancer Res.* **64**, 3391–3394 (2004).
2. V. O'Brien and R. Brown, Signaling cell cycle arrest and cell death through the MMR system. *Carcinogenesis* **27**, 682–692 (2006).
3. J. Y. Wang and W. Edelmann, Mismatch repair proteins as sensors of alkylation DNA damage. *Cancer Cell* **9**, 417–418 (2006).
4. A. W. Adamson, D. I. Beardsley, W. J. Kim, Y. Gao, R. Baskaran and K. D. Brown, Methylator-induced, mismatch repair-dependent G_2 arrest is activated through Chk1 and Chk2. *Mol. Biol. Cell* **16**, 1513–1526 (2005).
5. Y. Hirose, M. Katayama, D. Stokoe, D. A. Haas-Kogan, M. S. Berger and R. O. Pieper, The p38 mitogen-activated protein kinase pathway links the DNA mismatch repair system to the G_2 checkpoint and to resistance to chemotherapeutic DNA-methylating agents. *Mol. Cell. Biol.* **23**, 8306–8315 (2003).
6. T. W. Davis, C. Wilson-Van Patten, M. Meyers, K. A. Kunugi, S. Cuthill, C. Reznikoff, C. Garces, C. R. Boland, T. J. Kinsella and D. A. Boothman, Defective expression of the DNA mismatch repair protein, MLH1, alters G_2-M cell cycle checkpoint arrest following ionizing radiation. *Cancer Res.* **58**, 767–778 (1998).
7. M. J. Hickman and L. D. Samson, Apoptotic signaling in response to a single type of DNA lesion, O(6)-methylguanine. *Mol. Cell* **14**, 105–116 (2004).
8. L. S. Li, J. C. Morales, A. Hwang, M. W. Wagner and D. A. Boothman, DNA mismatch repair-dependent activation of c-Abl/p73alpha/GADD45alpha-mediated apoptosis. *J. Biol. Chem.* **283**, 21394–21403 (2008).
9. M. Meyers, A. Hwang, M. W. Wagner and D. A. Boothman, Role of DNA mismatch repair in apoptotic responses to therapeutic agents. *Environ. Mol. Mutagen.* **44**, 249–264 (2004).
10. P. Karran and M. Bignami, DNA damage tolerance, mismatch repair and genome instability. *Bioessays* **16**, 833–839 (1994).
11. Y. Hirose, M. S. Berger and R. O. Pieper, Abrogation of the Chk1-mediated G(2) checkpoint pathway potentiates temozolomide-induced toxicity in a p53-independent manner in human glioblastoma cells. *Cancer Res.* **61**, 5843–5849 (2001).
12. R. Fishel, Signaling mismatch repair in cancer. *Nat. Med.* **5**, 1239–1241 (1999).
13. B. Marples and S. J. Collis, Low-dose hyper-radiosensitivity: past, present, and future. *Int. J. Radiat. Oncol. Biol. Phys.* **70**, 1310–1318 (2008).
14. M. C. Joiner, B. Marples, P. Lambin, S. C. Short and I. Turesson, Low-dose hypersensitivity: current status and possible mechanisms. *Int. J. Radiat. Oncol. Biol. Phys.* **49**, 379–389 (2001).
15. B. Xu, S. T. Kim, D. S. Lim and M. B. Kastan, Two molecularly distinct G(2)/M checkpoints are induced by ionizing irradiation. *Mol. Cell Biol.* **22**, 1049–1059 (2002).
16. S. A. Krueger, S. J. Collis, M. C. Joiner, G. D. Wilson and B. Marples, Transition in survival from low-dose hyper-radiosensitivity to increased radioresistance is independent of activation of ATM Ser1981 activity. *Int. J. Radiat. Oncol. Biol. Phys.* **69**, 1262–1271 (2007).
17. P. Lambin, E. P. Malaise and M. C. Joiner, Might intrinsic radioresistance of human tumour cells be induced by radiation? *Int. J. Radiat. Biol.* **69**, 279–290 (1996).
18. B. Marples and M. C. Joiner, The response of Chinese hamster V79 cells to low radiation doses: evidence of enhanced sensitivity of the whole cell population. *Radiat. Res.* **133**, 41–51 (1993).
19. J. Renart, J. Reiser and G. R. Stark, Transfer of proteins from gels to diazobenzyloxymethyl-paper and detection with antisera: a method for studying antibody specificity and antigen structure. *Proc. Natl. Acad. Sci. USA* **76**, 3116–3120 (1979).
20. M. Hermisson, A. Klumpp, W. Wick, J. Wischhusen, G. Nagel, W. Roos, B. Kaina and M. Weller, O6-methylguanine DNA methyltransferase and p53 status predict temozolomide sensitivity in human malignant glioma cells. *J. Neurochem.* **96**, 766–776 (2006).
21. I. Turesson, R. Bernefors, M. Book, M. Flogegård, I. Hermansson, K. A. Johansson, A. Lindh, S. Sigurdardottir, U. Thunberg and J. Nyman, Normal tissue response to low doses of radiotherapy assessed by molecular markers—a study of skin in patients treated for prostate cancer. *Acta Oncol.* **40**, 941–951 (2001).
22. J. Harney, N. Shah, S. Short, F. Daley, N. Groom, G. D. Wilson, M. C. Joiner and M. I. Saunders, The evaluation of low dose hyper-radiosensitivity in normal human skin. *Radiother. Oncol.* **70**, 319–329 (2004).
23. B. G. Wouters and L. D. Skarsgard, The response of a human tumor cell line to low radiation doses: evidence of enhanced sensitivity. *Radiat. Res.* **138** (Suppl.), S76–S80 (1994).
24. B. G. Wouters, A. M. Sy and L. D. Skarsgard, Low-dose hypersensitivity and increased radioresistance in a panel of human tumor cell lines with different radiosensitivity. *Radiat. Res.* **146**, 399–413 (1996).

25. P. S. Lin and A. Wu, Not all 2 Gray radiation prescriptions are equivalent: Cytotoxic effect depends on delivery sequences of partial fractionated doses. *Int. J. Radiat. Oncol. Biol. Phys.* **63**, 536–544 (2005).

26. L. M. Garcia, J. Leblanc, D. Wilkins and G. P. Raaphorst, Fitting the linear-quadratic model to detailed data sets for different dose ranges. *Phys. Med. Biol.* **51**, 2813–2823 (2006).

27. R. M. Hermann, H. A. Wolff, H. Jarry, P. Thelen, C. Gruendker, M. Rave-Fraenk, H. Schmidberger and H. Christiansen, *In vitro* studies on the modification of low-dose hyper-radiosensitivity in prostate cancer cells by incubation with genistein and estradiol. *Radiat. Oncol.* **3**, 19 (2008).

28. A. E. Pegg, M. E. Dolan, D. Scicchitano and K. Morimoto, Studies of the repair of O6-alkylguanine and O4-alkylthymine in DNA by alkyltransferases from mammalian cells and bacteria. *Environ. Health Perspect.* **62**, 109–114 (1985).

29. J. Rzeszowska-Wolny, J. Polanska, M. Pietrowska, O. Palyvoda, J. Jaworska, D. Butkiewicz and R. Hancock, Influence of polymorphisms in DNA repair genes XPD, XRCC1 and MGMT on DNA damage induced by gamma radiation and its repair in lymphocytes *in vitro*. *Radiat. Res.* **164**, 132–140 (2005).

30. M. Debiak, T. Nikolova and B. Kaina, Loss of ATM sensitizes against O6-methylguanine triggered apoptosis, SCEs and chromosomal aberrations. *DNA Repair (Amst.)* **3**, 359–368 (2004).

31. B. Marples, P. Lambin, K. A. Skov and M. C. Joiner, Low dose hyper-radiosensitivity and increased radioresistance in mammalian cells. *Int. J. Radiat. Biol.* **71**, 721–735 (1997).

32. S. A. Krueger, M. C. Joiner, M. Weinfeld, E. Piasentin and B. Marples, Role of apoptosis in low-dose hyper-radiosensitivity. *Radiat. Res.* **167**, 260–267 (2007).

33. B. Marples, B. G. Wouters, S. J. Collis, A. J. Chalmers and M. C. Joiner, Low-dose hyper-radiosensitivity: a consequence of ineffective cell cycle arrest of radiation-damaged G_2-phase cells. *Radiat. Res.* **161**, 247–255 (2004).

34. C. L. Bean, C. I. Bradt, R. Hill, T. E. Johnson, M. Stallworth and S. M. Galloway, Chromosome aberrations: persistence of alkylation damage and modulation by O6-alkylguanine-DNA alkyltransferase. *Mutat. Res.* **307**, 67–81 (1994).

35. S. M. Galloway, S. K. Greenwood, R. B. Hill, C. I. Bradt and C. L. Bean, A role for mismatch repair in production of chromosome aberrations by methylating agents in human cells. *Mutat. Res.* **346**, 231–245 (1995).

36. S. C. Short, S. A. Mitchell, P. Boulton, M. Woodcock and M. C. Joiner, The response of human glioma cell lines to low-dose radiation exposure. *Int. J. Radiat. Biol.* **75**, 1341–1348 (1999).

37. S. C. Short, J. Kelly, C. R. Mayes, M. Woodcock and M. C. Joiner, Low-dose hypersensitivity after fractionated low-dose irradiation *in vitro*. *Int. J. Radiat. Biol.* **77**, 655–664 (2001).

38. S. Chandna, B. S. Dwarakanath, D. Khaitan, T. L. Mathew and V. Jain, Low-dose radiation hypersensitivity in human tumor cell lines: effects of cell-cell contact and nutritional deprivation. *Radiat. Res.* **157**, 516–525 (2002).

39. A. Lorente, W. Mueller, E. Urdangarin, P. Lazcoz, U. Lass, A. von Deimling and J. S. Castresana, RASSF1A, BLU, NORE1A, PTEN and MGMT expression and promoter methylation in gliomas and glioma cell lines and evidence of deregulated expression of de novo DNMTs. *Brain Pathol.* **19**, 279–292 (2009).

40. W. P. Roos, L. F. Batista, S. C. Naumann, W. Wick, M. Weller, C. F. Menck and B. Kaina, Apoptosis in malignant glioma cells triggered by the temozolomide-induced DNA lesion O6-methylguanine. *Oncogene* **26**, 186–197 (2007).

41. A. Franchitto, P. Pichierri, R. Piergentili, M. Crescenzi, M. Bignami and F. Palitti, The mammalian mismatch repair protein MSH2 is required for correct MRE11 and RAD51 relocalization and for efficient cell cycle arrest induced by ionizing radiation in G_2 phase. *Oncogene* **22**, 2110–2120 (2003).

42. S. M. Holt, J. L. Scemama, M. I. Panayiotidis and A. G. Georgakilas, Compromised repair of clustered DNA damage in the human acute lymphoblastic leukemia MSH2-deficient NALM-6 cells. *Mutat. Res.* **674**, 123–130 (2009).

43. G. M. Li, Mechanisms and functions of DNA mismatch repair. *Cell Res.* **18**, 85–98 (2008).

44. J. C. Boyer, A. Umar, J. I. Risinger, J. R. Lipford, M. Kane, S. Yin, J. C. Barrett, R. D. Kolodner and T. A. Kunkel, Microsatellite instability, mismatch repair deficiency, and genetic defects in human cancer cell lines. *Cancer Res.* **55**, 6063–6070 (1995).

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Tumour Review

DNA mismatch repair and the DNA damage response to ionizing radiation: Making sense of apparently conflicting data

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SUMMARY

The DNA mismatch repair (MMR) pathway detects and repairs DNA replication errors. While DNA MMR-proficiency is known to play a key role in the sensitivity to a number of DNA damaging agents, its role in the cytotoxicity of ionizing radiation (IR) is less well characterized. Available literature to date is conflicting regarding the influence of MMR status on radiosensitivity, and this has arisen as a subject of controversy in the field. The aim of this paper is to provide the first comprehensive overview of the experimental data linking MMR proteins and the DNA damage response to IR. A PubMed search was conducted using the key words "DNA mismatch repair" and "ionizing radiation". Relevant articles and their references were reviewed for their association between DNA MMR and IR. Recent data suggest that radiation dose and the type of DNA damage induced may dictate the involvement of the MMR system in the cellular response to IR. In particular, the literature supports a role for the MMR system in DNA damage recognition, cell cycle arrest, DNA repair and apoptosis. In this review we discuss our current understanding of the impact of MMR status on the cellular response to radiation in mammalian cells gained from past and present studies and attempt to provide an explanation for how MMR may determine the response to radiation.

Introduction

The principal target of Ionizing radiation (IR) has long since been indicated to be deoxyribonucleic acid (DNA), a large molecule with a well known double helix structure, consisting of two strands held together by hydrogen bonds between the bases. IR induces a spectrum of lesions that can cause structural damage to the DNA molecule and can alter or eliminate the cell's ability to transcribe the gene that the affected DNA encodes. Single strand breaks (SSBs) are of little biological significance as regards cell killing, as they are readily repaired using the opposite stand as a template. However, if the repair is incorrect (mispair) it may result in mutation. DSBs are thought to be the most deleterious of IR induced lesions, and occur when breaks in the two strands are opposite one another, or separated by only a few base pairs. IR also induces other forms of DNA damage including cross-links, oxidative base modification[1] and clustered base damage. The numbers of DNA lesions per cell that are detected immediately after a radiation dose of 1 Gy have been estimated to be approximately greater than 1000 base damage, 1000 SSBs, 40 DSBs, 20 DNA–DNA cross-links, 150 DNA–protein cross-links and 160–320 non-DSB clustered DNA damage.[2,3]

Failure to repair DNA damage can have deleterious outcomes such as mutagenesis or cell death. Given the importance of transmitting an intact genome to daughter progeny, cells have evolved a range of mechanisms to respond to DNA damage, that link cell cycle arrest which prevents the proliferation of damaged cells with repair processes that rejoin broken DNA. Unrepaired DSBs are thought to be the main event associated with cell killing following exposure to IR. DSB are repaired by either of two mechanisms; the non-homologous end joining (NHEJ) pathway or homologous recombination (HR) pathway. Defects in DNA repair pathways are associated with altered survival responses to IR and can cause either resistance or sensitivity to IR depending on the particular gene affected.[4,5]

While the role of DSB repair pathways in the survival response to IR has been extensively studied, DNA mismatch repair (MMR) has received less attention. DNA MMR is a highly conserved DNA repair pathway that recognizes and repairs base-pairing errors that arise during DNA replication or recombination. The role of this pathway has been largely studied in the DNA damage response to chemotherapeutic agents, but the activation of the MMR system

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after IR remains controversial.[6,7] Accumulating data suggest that MMR proteins may be involved in the DNA damage response to IR. We and others have recently implicated a role for MMR-proficiency in the hypersensitive response observed in cancer cells to low radiation doses[8] and the preferential response of cancer cells to prolonged low dose rate (LDR) IR.[9] Moreover, a role for these proteins has been implicated in the processing of clustered base damage.[10] Increasing evidence suggests that this type of damage has biological significance following IR exposure,[11] thus underscoring the need to determine the biological significance of loss of these proteins on the survival response to IR.

Conflicting data in the literature indicates that MMR-proficiency confers sensitivity, resistance, or has no effect on cell killing in response to IR. Consequently, it is tempting to conclude that no association exists between MMR and IR. However, when the literature reports are considered with respect to the ability of MMR proteins to recognize and bind to IR-induced DNA damage, or produce G2 phase cell cycle arrest, apoptosis and cytotoxicity a clearer picture begins to emerge.

In this review we focus on data available regarding the role of MMR proteins in the recognition of IR-induced DNA damage, G2/M phase cell cycle arrest, apoptosis and cytotoxicity induced following exposure to IR, and draw conclusions based on the association of these events with the IR dose-rates used. In particular we are interested in how MMR proteins may participate in low dose radiation hypersensitivity (HRS). We have chosen to provide a lengthier discussion regarding the participation of MMR proteins in the activation and maintenance of the G2/M cell cycle arrest, because of the important role that abrogation of this checkpoint plays in the induction of HRS. In addition, given that HRS is associated with aberrant HR,[12,13] understanding the interplay between MMR and HR is essential to better understand how MMR proteins may participate in the DNA damage response to IR and in particular HRS, and sensitivity to LDR-IR. Finally we conclude with suggested future directions for research in this area. Due to the lack of evidence detailing the apoptotic pathways or other modes of cell death regulated by MMR proteins in response to IR, these will not be a feature of this review. For more detailed review articles regarding MMR proteins and how they signal cell cycle arrest and cell death in response to chemotherapeutic agents we recommend the following reviews.[14,15]

Repair of DNA damage by DNA mismatch repair

MMR was originally described in bacteria.[16] The most described function of MMR is in repair of replication errors that escape DNA polymerase proof-reading. If left unrepaired, these mis-paired bases can lead to mutations and microsatellite instability, a hallmark of MMR defects.[17,18] Inherited mutations in MMR genes are known to cause a cancer predisposition syndrome called hereditary non-polyposis colorectal cancer (HNPCC) and mutations in MMR genes have also been reported to increase the risk and progression of a wide-variety of sporadic cancers.[19,20] MMR proteins also participate in HR[21–23] (discussed later in Sections "Recognition of ionizing radiation induced DNA damage by DNA mismatch repair proteins" and "Sensitivity to IR and G2 arrest: a role for MMR-dependent suppression of homologous recombination?"), and play a key role in cell killing in response to a variety of DNA damaging agents.[5,15] A minimal role for MMR proteins has been suggested in the NHEJ repair pathway which repairs DSBs in G1 phase cells.[24,25] Much of what is known today regarding the role of MMR in response to DNA damage comes from early work studying repair of DNA damage after exposure to methylating agents.

Mismatch recognition is mediated by either of two MutS heterodimers; MutSα (comprised of MSH2 and MSH6) which binds to mismatches and small insertion-deletion loops, or MutSβ (comprised of MSH2 and MSH3) which binds to larger insertion-deletion loops (for a review see Refs. [17,18]). MutL (a heterodimer of MLH1 and either PMS2 or PMS1) is subsequently recruited by the MSH2 protein to form a ternary complex with one of the MutS complexes and promote intracellular signaling to initiate excision and repair of the mismatch. Additional proteins involved in this process may include exonuclease 1 (EXO1), possibly helicase(s), replication protein-A (RPA), replication factor c (RFC), proliferating cell nuclear antigen (PCNA), and DNA polymerases α and β[26] (Fig. 1). MMR proteins can also recognize DNA damage distortion and bind to adducts produced by the presence of DNA damaging agents including 6-thioguanine,[27] N-methyl-N′-nitro-N-nitrosoguanidine (MNNG),[28] cisplatin,[29–32] carboplatinum,[30] 5-fluorouracil[33] and halogenated-thymidine-analog iododeoxyuridine.[34]

Two opposing models describe how MMR proteins may be involved in cell killing. In one model, following recognition of a mismatch or drug-induced adduct, the MMR system undergoes reiterated futile attempts at repair, leading to the formation of gaps in the newly synthesized DNA stand and ultimately the accumulation of DSBs. This damage then provokes a G2/M phase cell cycle arrest and subsequent cell death by apoptosis (reviewed in[35]).

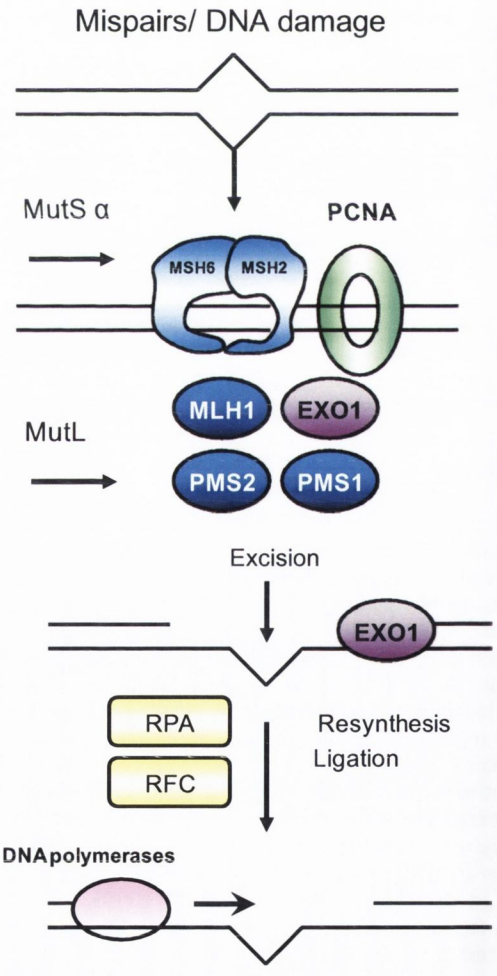

Fig. 1. Overview of mismatch repair mediated removal of base–base mismatches. DNA mismatch repair is initiated when the MutSα (MSH2/MSH6) heterodimer binds to the mismatched DNA. Heterodimers of MutL homologues, such as MLH1, PMS1 and PMS2, as well as the EXO1, RPA, RFC, and DNA polymerases are then recruited to this complex to complete excision of the mismatches and resynthesis of the DNA strand.

The alternative model proposes a direct signaling role for MMR proteins in which DNA damage is recognized by MMR proteins which in turn transmit the damage signal directly to the checkpoint and apoptotic machinery.[15]

Cells deficient in MMR protein expression display DNA damage tolerance and resistance to cell killing in response to a variety of anti-cancer agents including temozolomide (TMZ),[5,36,37] 6-thioguanine,[38] cisplatin,[39–41] etoposide,[42] 5-fluorouracil[43] and bleomycin.[44] Resistance to alkylating agent induced cell death in particular is increased approximately 100-fold in MMR-deficient cells compared to MMR-proficient counterparts. MMR-mediated resistance to these agents has been attributed to factors including the inability of the cell to recognize drug induced intra-strand adducts, suppress recombination (HR), and induce efficient G2/M cell cycle checkpoint activation and apoptosis (reviewed in[5,36]).

Recognition of ionizing radiation induced DNA damage by DNA mismatch repair proteins

The role that MMR proteins may play in the DNA damage response (DDR) to IR is unclear. However, since MMR proteins are involved in the processing of drug induced genetic damage, it is likely that MMR proteins are involved in the DDR to IR since similar DNA lesions are produced by both processes. The mechanism by which they are involved may be either via direct signaling or futile repair of IR-induced DNA damage. The first step in either pathway involves MMR proteins recognizing and binding to the mismatch or genetic damage induced following exposure to IR.

Recognition and repair of oxidative-modified bases such as 8-oxoguanine lesions (8-oxoG) by the MMR system was the first mechanism to be associated with MMR-mediated sensitivity to IR.[45] Increasing evidence suggests that these lesions can mispair with adenine during DNA replication, and that MMR recognizes and removes these mismatches.[46–51] Indeed in the absence of MMR, a buildup of 8-oxoG is observed *in vitro*[47,52–54] and *in vivo*,[55] suggesting that after exposure to IR, 8-oxoG lesions may accumulate in MMR-defective cells due to their impaired ability to remove these lesions. 8-oxoG may then be incorporated into the DNA causing mutation and disruption to the normal DDR, and subsequent radioresistance.

Support for a role for MMR proteins in the DNA damage response to IR stems from evidence that loss of MMR can lead to radioresistance and increased mutation rates (**Table 1**). These early studies also demonstrate that loss of MMR is associated with an accumulation of 8-oxoG lesions. Fritzell et al. were among the first to indicate a role for DNA MMR in the cytotoxicity of IR: loss of PMS2, MLH1 or MSH2 in mouse embryo fibroblast (MEF) cell lines was associated with a modest increase in clonogenic survival following IR (0–12 Gy, 2.25 Gy/min),[45] a higher level of single base-pair deletions and insertions in mononucleotide repeat sequences, and a slight increase in transversions than was evident in cell lines from wild-type littermates.[56] Similarly, loss of PMS2 was associated with increased clonogenic survival in MEFs in response to IR (0–8 Gy, 2.25 Gy/min).[57] Interestingly, this was also shown to be exaggerated when delivered using a LDR (0.16–0.27 Gy/hr) and the relative resistance observed was also attributed to inefficient apoptotic signaling. Loss of MSH2 has also been associated with radioresistance. DeWeese et al. report that loss of MSH2 in mouse embryonic stem cells was associated with a modest increase in survival to IR (0–10 Gy, 1 Gy/min). This resistance was enhanced (~20%) when radiation was delivered using a LDR (0–75 h, 0.004 Gy/min) and was associated with an increased accumulation of 8-oxoG.[47] The resistance observed in these MSH2-deficient cancer cells was attributed to inefficient apoptotic signaling.[47] More recently, Yan et al. reported that loss of MLH1 in colorectal cells was associated with resistance to prolonged LDR-IR. The authors attributed the radiosensitivity observed to inhibition of RAD51, an essential recombinase in homologous recombination (HR).[9]

HR is one of two DSB repair pathways important in rejoining radiation-induced strand breaks, the other being the error-prone NHEJ repair pathway. MMR has been shown to inhibit HR of DSBs by aborting strand exchange between divergent sequences,[58–60] reviewed recently in.[23] HR is a high fidelity repair mechanism that functions in S and G_2 phases and requires the assembly of a multi-protein complex.[61] RAD51 co-ordinates with a number of other proteins to elicit the HR repair process. These include; DNA damage sensor proteins MRE11, Rad50, NBS1 (MRN complex) and DNA repair proteins BRCA1, BRCA2, RAD51, and RAD52, a number of which are also known to regulate cell cycle checkpoint control. The initial step in HR involves processing the broken chromosome ends to give single-stranded DNA (ssDNA) tails, which invade a sister chromatid or homologous chromosome to copy genetic information into the donor chromosome (Fig. 2).[61] The MRN complex appears to be the major regulator of DSB-end resection[62] and has recently been reported to dictate DSB repair independent of H2AX.[63] The RAD51 and RAD54 proteins act during the pairing and the strand exchange steps by forming a nucleoprotein filament along the 3′ single-stranded tails. BRCA2 assists in the recruitment and loading of RAD51 onto ssDNA.[61] Recombination intermediates produced in this way are then processed further in reactions that involve the resolution of Holliday junctions, DNA synthesis and nick ligation. Resolution of the exchanged DNA strands can result in either of two outcomes synthesis dependent stand annealing (SDSA) or double strand break repair (DSBR) by crossover or gene conversion. SDSA occurs as a result of displacement of the invading strand after repair synthesis and the subsequent annealing of the single stranded tail on the other DSB end. DSBR is facilitated by crossover between chromatids, whereby segments of the interacting chromosomes are exchanged.[64] Inappropriate template usage during the HR process can cause deleterious genomic rearrangements, such as deletions, translocations, duplications and loss of heterozygosity.[22] A role for MSH2 in particular has been indicated in this process[21,59,65] along with MSH3,[65] and results suggest that MSH2 may co-ordinate with p53 to monitor the fidelity of HR during S phase.[66] Recent data provided by Siehler et al. demonstrate that MLH1 and its complex partners PMS1 and PMS2 downregulate conservative HR independently of DNA MMR.[67] MMR proteins are also involved in the initiation of HR via regulation of the early G2 checkpoint (discussed later in Section "Sensitivity to IR and G2 arrest: a role for MMR-dependent suppression of homologous recombination?"). Of note, a role for aberrant HR in the differential sensitivity of MMR-proficient and deficient cells to IR is consistent with commentary provided by Cejka et al., who noted that MMR proteins are more likely to be involved in the damage response to IR via repair of DSBs rather than repair of 8-oxoG, given that these are the most deleterious lesions induced by IR and can be repaired by HR, a DNA repair pathway in which MMR is involved.[7,22]

Recognition and repair of non-DSB bi-stranded oxidative clustered DNA lesions (OCDLs) by the MMR system is another possible mechanism through which MMR may be implicated in the DDR to IR. OCDLs are defined as two or more DNA lesions (including abasic sites, oxidised purines, oxidised pyrimidines or SSBs) within a short DNA fragment of 1–10 base pairs.[68] Clustered base damage occurs when two or more lesions occur within one or two helical turns of the DNA by passage of a single radiation track.[68] Non-DSB clustered DNA damage is 4–8 times more prevalent than DSBs in mammalian DNA following IR exposure. Both the frequency and complexity of DNA damage are thought to increase with increasing linear energy transfer (LET) of the radiation.[3,69,70] Radiation-induced clustered damage sites are considered less repairable than endogenously formed isolated base lesions, and are particularly harmful to cells.[3,71]

Table 1
Outcome of studies evaluating the role of DNA mismatch repair in the cytotoxicity of ionizing radiation. (See below mentioned **references** for further information.)

	Reference	MMR gene	Radiation dose	Effect on survival	Effect on cell cycle	Model
MMR-proficiency confers radiosensitivity	Fritzell et al.[45]	PMS2 MLH1 MSH2	0–12 Gy 2.25 Gy/min	PMS2-, MLH1- and MSH2-confer resistance	–	Mice fibroblast cell lines 29pms2, C18pms2, MC2Mlh1, MS57msh2, 40wt, BC1wt
	DeWeese et al.[47]	MSH2	0.004 Gy/min 1 Gy/min	MSH2-confers resistance (reduced apoptosis 24 h)	–	Mouse embryonic stem cells (MSH2+ and −)
	Zhang et al.[116]	MSH2		MSH2-confers resistance (reduced apoptosis 24 h)	–	MEFs derived from MSH2+/+ and MSH2−/− mice
	Zeng et al.[57]	PMS2	20 Gy	PMS2-confers resistance (reduced apoptosis 18, 40 h) Independent of p53 status		MEFs derived from PMS2 and p53-knockout mice CL7 (P53+/+, PMS2+/+), CL11(P53+/+, PMS2), TF6 (P53−/−, PMS2+/+), TF11 (P53−/−, PMS2−/−)
	Holt et al.[10]	MSH2	5 Gy 0.57 Gy/min	MSH2−/− confers resistance (reduced apoptosis 6–24 h)	–	Human ALL MSH2- NALM-6 cells and MSH2 + 697 cells
	Yan et al.[9]	MLH1	LDR-IR 17–1.3 cGy/h	MLH1-confers resistance	Greater late S-phase population and greater G2/M population after 72 h LDR-IR	Human colorectal cancer cells (HCT116/HCT116 + chr3)
	Xu et al.[56]	PMS2	6 Gy 2.25 Gy/min	Hypermutability	–	Mice
No difference	Aquilina et al.[84]	PMS2 MutSβ (Raji F10) MutSα (Raji F12)	2,4,6 Gy 6 Gy/min	None	Slightly enhanced G2/M arrest in parent cells Prolonged G2/M arrest in F10 and F12 clones vs parent	Human cervical cancer cells (HeLa) and MMR- clones Lymphoblastoid cells derived from Burkitts Lymphoma (Raji)
	Yan et al.[48]	MLH1 MSH2	0–6 Gy 4.1 Gy/min	None None	MMR+ cells show enhanced G2/M arrest after 6 Gy	Human ovarian cancer cells (A2780/CP70) Human colorectal cancer cells (RKO ± azacytidine treatment to reexpress hMLH1 Human endometrial cancer cells (HEC59/HEC59 + chr2)
	Papouli et al.[117]	MLH1	0.5–6 Gy survival curve	None	–	Human embryonic kidney cells (293T Lα)
	Wang et al.[118]	MLH1	137 cs radiation 0–8 Gy	None Increased mitotic recombination	–	K634 MLH1 null kidney cell line, DNA repair + K06 kidney cell line
Loss of MMR confers radiosensitivity	Davis et al.[83]	MLH1	0–6 Gy 6.1 Gy/min	MLH1-confers radiosensitivity	MMR+ cells show enhanced G2/M arrest 12–24hr post 5 Gy	Human colorectal cancer cells (HCT116/HCT116 + chr3) MEFs derived from MLH1-knockout mice and wt embryonic fibroblasts
	Franchitto and co-workers[93]	MSH2	0–8 Gy 2 Gy/min	MSH2-confers radiosensitivity	MSH2−/− cells showed inefficient G2/M checkpoint	MEFs derived from MSH2+/+ and MSH2−/− mice Mouse colorectal carcinoma cells (Colo5/Colo26)
	Bucci et al.[119]	MSH2 MLH1		Myc downregulation of MLH1 & MSH2 increases sensitivity to radiation	–	Mutant melanoma cells
	Barwell et al.[98]	MSH2	10 Gy 3.18 Gy/min	MSH2-confers radiosensitivity	–	Primary human MSH2-deficient neonatal cells, bearing a biallelic truncating mutation in MSH2

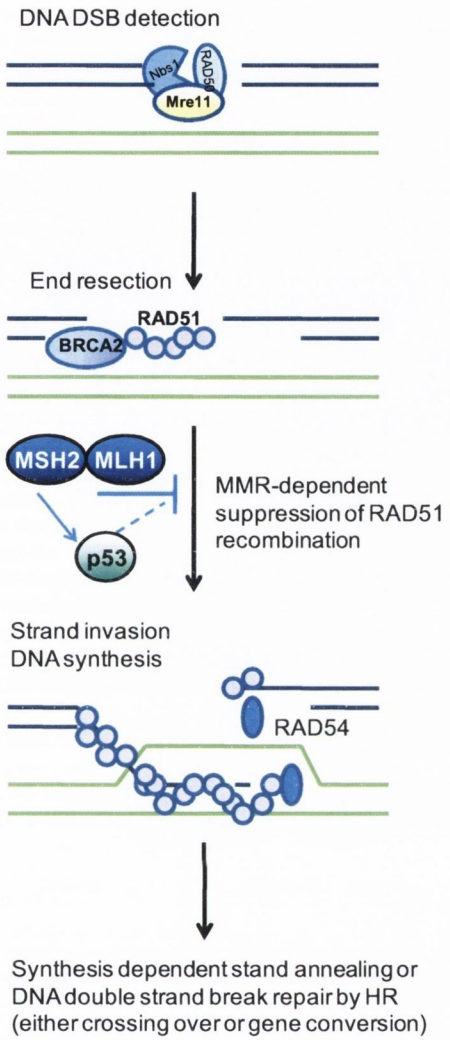

Fig. 2. An overview of the interplay between the homologous recombination repair pathway and mismatch repair proteins. Following the induction of DNA double strand breaks (DSBs) in S/G2 phase cells, the MRE11/RAD50/NBS1 complex is recruited to initiate DSB-end resection. RAD51 is then recruited and loaded onto single-stranded DNA with the assistance of BRCA2. In the absence of functional MMR, the RAD51 and RAD54 proteins act during the pairing and the strand exchange steps by forming a nucleoprotein filament along the 3′ single-stranded tails. Resolution of the exchanged DNA strands can result in synthesis dependent strand annealing or DNA double strand break repair by either crossing over or gene conversion. In the presence of functional mismatch repair proteins, MSH2 and MLH1 co-ordinate to suppress the RAD51 recombination pathway at sites of divergent DNA sequences, possibly through p53 dependent pathways.

The mechanisms of repair of clustered DNA damage remains unclear. Holt et al. recently demonstrated that loss of MSH2 is associated with resistance to IR (0.57 Gy/min) in acute lymphoblastic leukemia (ALL) cells, and a lower population of apoptotic cells. This response was attributed to a role for MSH2 in the processing of OCDLs.[10] Given that these lesions are readily induced by low doses of IR (0–1 Gy),[72,73] repair of OCDL at low doses may contribute to the enhanced cell kill observed following LDR-IR in the aforementioned studies. In addition it is possible that these data may be reconciled with early studies implicating a role for MMR in the processing of 8-oxoG lesions given that that these lesions are major contributors to clustered base damage.[74,75]

Finally, recognition of another type of genetic damage such as O6-Methylgaunine (O6MeG) or O6MeG-like lesions by MMR proteins at low doses of IR may also be possible. These lesions are typically induced by methylating agents but similarities have been observed between the DDR to O6MeG lesions and the DDR to low doses of IR in prostate cells suggesting a possible role for these lesions in the sensitivity observed in response to low doses of radiation.[8]

MMR proteins therefore appear to recognize a wide range of genetic damage including that induced by chemotherapeutic agents and IR and have consequently been proposed to serve as general sensors of DNA damage,[76] however this hypothesis is not universally supported.[77] The involvement of the MMR system in such generic damage signaling could be reconciled by considering its participation in the BRCA1-associated genome surveillance complex (BASC).[78] MMR proteins are thought to act in concert with a number of other proteins in this complex which are involved in DNA repair including BRCA1, ATM and the MRN complex protein complex.[78] Each of these proteins possesses the ability to bind abnormal DNA structures or damaged DNA, such as DSBs, base-pair mismatches, Holliday junctions, cruciform DNA, template–primer junctions, and telomere repeat sequences[79,80] suggesting that BASC may serve as sensors for these structures. These proteins have also been implicated in cell cycle checkpoint activation and DNA repair.[63,81,82]

In combination, these data support a role for MMR-proficiency in IR-induced DNA damage signaling and cell death (Fig. 3), possibly via the ability of MMR-deficient cells to recognize and repair DNA damage induced by oxidative stress; 8-oxoG or OCDLs, DSBs, or O6MeG lesions and suppress HR.

Radiation induced G2/M cell cycle checkpoint activation and DNA Mismatch repair

The preferential sensitivity of MMR-proficient cells to DNA damaging agents is typically preceded by an increased and prolonged late accumulation of cells in the G2 phase of the cell cycle that is not observed in MMR-deficient cells.[15] Fewer studies have examined the influence that differential MMR expression may have on cell cycle modulation following IR. MMR-proficiency has been shown to be required for S-phase checkpoint activation in response to IR.[6] In particular, an enhanced accumulation of cells in G2 phase in MMR-proficient cells relative to MMR-deficient counterparts has been reported, which was associated with either an increase in cell killing,[9] resistance to cell killing[83] or no effect on survival.[48,84] Thus, a role for MMR proteins in the accumulation of cells in G2 is implied, but it remains unclear how this may relate to cell killing after IR. The resolution of cell division after cell cycle arrest is likely to be important in terms of cell killing.[15]

This accumulation of MMR-proficient cells in G2 phase following IR is attributed to activation of the G2/M cell cycle checkpoint. Cell cycle checkpoints are surveillance mechanisms that block cell cycle transitions. For each phase of the cell cycle (G1, S, G2) one or more checkpoints have been identified and individual proteins may have distinct or overlapping functions in the different checkpoints.[85,86] The delay afforded by the block/arrest is thought to allow vital time for the orderly and timely repair of mutagenic lesions created by DNA damaging agents prior to cell entry and transit through mitosis.[87] This arrest is only released when repair is completed. Where repair is not possible, damaged cells are removed by programmed cell death (apoptosis).[86]

DNA mismatch repair and Ionizing radiation induced G2/M arrest

Two distinct G2/M phase cell cycle checkpoints are known to be activated following exposure to IR[88] namely the early and late G2/M phase checkpoints. The first and so-called early G2/M checkpoint is the response to DNA damage in cells that are already in G2 at the time of irradiation, and reflects the failure of these cells to progress to mitosis. This checkpoint is typically activated within

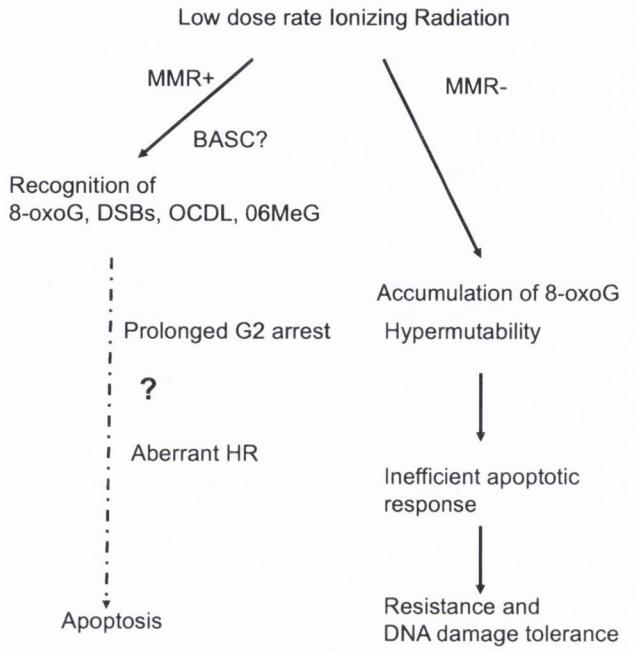

Fig. 3. Influence of MMR status on the cellular response to low dose rate ionizing radiation. Mismatch repair proteins may co-ordinate with the BRCA1-associated genome surveillance complex to recognize radiation induced DNA damage such as (8-oxoG, DSBs, OCDL, O6MeG). This may then provoke a prolonged G2 arrest, aberrant homologous recombination and ultimately signal apoptotic pathways. Alternatively, on a MMR deficient background DNA lesions such as 8-oxoG may accumulate in cells following ionizing radiation and cause hypermutability leading to an inefficient apoptotic response. Ultimately this may cause resistance to radiation and DNA damage tolerance.

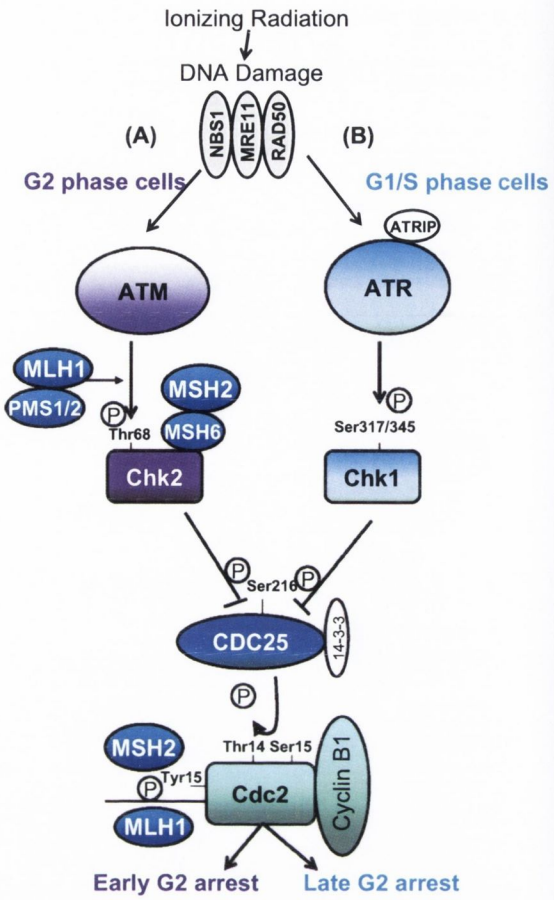

Fig. 4. Ionizing radiation induced activation of the G2/M checkpoint. (A) Cells in G2 phase at the time of radiation undergo a rapid transient G2 arrest (early G2 arrest). Ataxia telangiectasia mutated (ATM) is recruited to sites of DNA damage following ionizing radiation. ATM responds to double strand breaks and an activating role for the MRE11/RAD50/NBS1 (MRN) complex has been suggested. This arrest is mediated by ATM dependent phosphorylation of checkpoint kinase Chk2 and CDC25 phosphatase. This prevents dephosphorylation of Cdc2–CyclinB, which is required for progression into mitosis. Evidence suggests that MLH1 (which forms a heterodimer with PMS1/PMS2) interacts with ATM and MSH2 (which forms a heterodimer with MSH6) interacts with Chk2 indicating a possible role for these proteins in the early G2 arrest. (B) Cells in G1/S phase at the time of irradiation are thought to undergo a late G2 arrest. This is ATM independent and likely to be primarily activated by ataxia telangiectasia and RAD3 related (ATR) kinase. ATR mediates this arrest by phosphorylation of Chk1 and Cdc25 which prevents dephosphorylation of Cdc2–CyclinB and progression into mitosis. A role for MLH1 and MLH2 has been suggested in the regulation of Cdc2 signaling pathway and these proteins may therefore have a possible role in the activation of the late G2/M arrest.

two hours after irradiation, is transient and independent of the dose of IR used in the range 1–10 Gy.[88] It is bypassed in glioma cells hypersensitive to low doses of IR (<0.5 Gy).[89] The early G2/M checkpoint is also ATM (*Ataxia telangiectasia mutated*) dependent. ATM is one of two key phosphatidylinositol 3-like protein kinases that control induction of the DNA damage G2 cell cycle arrest, the other being ATR (*Ataxia telangiectasia-Rad3-related kinase*).[86] ATM and ATR are global regulators of the DNA damage response, signaling cell cycle checkpoint activation, DNA repair, and apoptosis. Their roles in these events partially overlap and are cooperative. ATM is considered primarily responsible for signaling IR induced DSBs whereas ATR responds to UV damage or stalled replication forks. The checkpoint function of ATM and ATR is mediated in part by a pair of checkpoint effector kinases termed Chk1 and Chk2. Both pathways converge on Cdc25, a positive regulator of cell cycle progression, which is inhibited by Chk1-mediated or Chk2-mediated phosphorylation. Activation of Cdc25 promotes its binding to 14–3-3 proteins, preventing it from dephosphorylating and activating the Cdc2–Cyclin B1 complex. This complex directly controls the transition of cells through the G2 phase of the cell cycle into mitosis. The maintenance of inhibitory phosphorylations (tyr15, thr14 or ser15-cdc2) on the cdc2–cyclinB1 complex contributes to the G2 arrest (**Fig. 4**; for review see[86]). The second, later-acting G2 checkpoint induced by IR is dose dependent, ATM-independent and represents the accumulation of cells in G2/M that had been in G1/S at the time of DNA damage.[88]

MMR proteins have been demonstrated to interact with a number of the checkpoint kinases. Support for a role for MMR proteins in the early G2/M arrest comes from evidence that MMR proteins interact with ATM and that loss of MMR results in an inefficient early G2/M checkpoint (discussed in Section entitled "Sensitivity to IR and G2 arrest: a role for MMR-dependent suppression of homologous recombination?"). Using co-immunoprecipitation methods in isogenic cell systems, Brown et al. demonstrated an interaction between MLH1 and ATM, and between MSH2 and Chk2, and that both these interactions are enhanced in response to DNA damage (**Fig. 4**;[6]). Assembly of the MMR complex at DNA damage sites has been suggested to provide a molecular scaffold that allows ATM to phosphorylate and consequently activate Chk2.[6] While Chk1 activation appears to be required for activation of the G2 arrest it has been suggested that Chk2 activation is required to sustain the arrest.[90] These results were contended by Cejka et al. who examined the response to IR of the strictly isogenic 293T Lα+ and 293T Lα- cell pair. These MMR-proficient 293T Lα+ cells differ from the MMR-deficient 293T Lα- cells solely by expression of the MMR protein MLH1. This system is considered a technical improvement over isogenic pairs because switching the MMR

status does not involve clonal selection. Cejka et al. report no differences in survival or MMR-dependent differences in phosphorylation of the checkpoint kinases Chk1, Chk2, NBS1 or BRCA1.[7] Possible explanations for these conflicting results include secondary mutations due to a mutator phenotype or differences in cell culture stains and treatment conditions but further work is required to reconcile these reports.

The role of MMR proteins in the late G2/M arrest is uncertain. MLH1 in particular has been implicated in the regulation of the G2 cell cycle phase checkpoint following IR and methylating agents.[27,83,91,92] Yan et al. demonstrated that MMR status can influence Cdc2 signaling and G2 arrest responses after 6 Gy without altering survival.[48] In this study, loss of MLH1 in colorectal cells and MEFs was associated with reduced and shorter G2 arrests after IR. Loss of MSH2 in endometrial cells was also associated with a reduced and shorter G2 arrest after IR (6 Gy) but no significant difference in cytotoxicity was observed. Immunoblotting indicated that phosphorylation of the protein Tyr15 on Cdc2 (p-Tyr15-Cdc2) increased transiently in keeping with the transient G2 arrest in both MMR-proficient and deficient cell lines, and that levels of p-Tyr15-Cdc2 remained high in MMR-proficient cells, corresponding to the slow release of IR-damaged cells from late G2 arrest.[48] Aquilina et al. also report a prolonged G2 arrest in MMR-proficient cells compared to MMR-deficient cells without an effect on survival.[84] In another study, an MLH1-dependent G2/M accumulation of human colorectal cells in G2/M was observed after IR, but in this case a deficiency in this G2/M cell cycle arrest at 12–24 h after 5 Gy was associated with sensitivity to IR. Similar responses were also noted in murine MLH1-knockout mice compared to wild-type embryonic fibroblasts.[83]

These data support a model in which MLH1 and ATM interact to initiate the early ATM dependent G2 arrest in response to IR. MSH2-dependent Chk2 activation is then required to maintain this arrest by inhibiting the activation of Cdc25 and sustaining the inhibitory phosphorylation of Tyr15 on Cdc2. This prevents the activation of the Cdc2–cyclinB1 complex, and the subsequent progression of cells into mitosis (Fig. 4). Given that the late G2/M arrest is ATM-independent, MMR may be minimally involved by maintaining the phosphorylation Tyr15 on Cdc2 but this may depend on MSH2 protein expression levels. This may partly explain the disparity between the association of a prolonged G2 arrest and survival outcome.

Sensitivity to IR and G2 arrest: a role for MMR-dependent suppression of homologous recombination?

Only one of the six studies that support a role for MMR-proficiency in the sensitivity of cells to IR also examined cell cycle distribution following IR (Table 1). This study reports that MMR-proficiency is associated with increased G2/M and S-phase populations after irradiation with LDR-IR. This was related to MLH1-dependent suppression of RAD51 proteins levels.[9] How MMR-dependent suppression of the RAD51 recombination pathway relates to sensitivity to DNA damaging agents remains unclear but it is likely due to maintenance of the G2 arrest and decreased DSB repair. Loss of MMR and its associated suppression of the RAD51 recombination pathway has been reported to increase the sensitivity of cells to a number of DNA damaging agents including IR,[93] the DNA cross-linking agent 1-(2-chloroethyl)-3-cyclohexyl-nitrosourea (CCNU),[94] mitomycin C,[95] camptothecin,[96] and DNA polymerase inhibitors,[97] temozolomide,[81] bleomycin, and acute high dose-rate IR.[93] Sensitivity to these agents is associated with loss of the early G2/M checkpoint.[81,93]

In total four studies report that loss of MMR confers sensitivity to IR (Table 1). Only two of these studies examined cell cycle modulation following IR, and in both cases sensitivity to IR was associated with an inefficient MMR-dependent G2/M arrest. Two of the four studies also report that sensitivity is associated with aberrant RAD51 foci, however both parameters were examined together in only one study. Davis et al. report that loss of MLH1 is associated with an inefficient G2/M arrest in human HCT116 colorectal cancer cells but did not examine RAD51 foci. Franchitto et al. demonstrated that loss of MSH2 function in murine cells was associated with sensitivity to IR (2 Gy/min), aberrant RAD51 and MRE11 focus formation and a higher level of chromosomal damage specifically in G2 phase cells. Moreover, these MSH2-deficient cells showed an inefficient early G2/M checkpoint and incomplete activation of checkpoint kinases Chk1 and 2.[93] Consistent with these data, Barwell et al. demonstrated loss of MSH2 compromised localisation of RAD51 but not BRCA1 to damage sites after exposure to IR (10 Gy), this was associated with an increase in chromosomal damage and subsequent cytotoxicity after IR. Interestingly, the emergence of RAD51 foci was influenced by MSH2. MSH2-proficient cells treated with control siRNA resolved RAD51 foci within 2 h, whereas primary fibroblasts treated with *MSH2* siRNA knock-down resolved foci within a shorter time of 1 h, indicating that the kinetics of RAD51 foci formation after irradiation are regulated at least in part by MSH2.[98] The observation that MSH2-dependent processes are associated with activation of these kinases is consistent with the model of MMR-dependent checkpoint activation outlined in 3.1 and Fig. 4. These data are also consistent with the observation that checkpoint function is lost in MSH2-deficient MEFs when treated with cisplatin,[99] and that the absence of MSH2 can result in both spontaneous DNA damage and uncontrolled recombination events leading to increased chromosomal damage and the higher induction of RAD51 foci following Camptothecin treatment.[96] In combination, these results suggest an involvement of MSH2 in the activation of the early G2 checkpoint as well as the early events leading to correct RAD51 relocalization after the formation of DSBs. Indeed, abrogation of the G2/M checkpoint arrest is commonly associated with sensitivity to DNA damaging agents, including low doses of radiation.[89] However, these data are in direct conflict with data suggesting that MMR-dependent G2 arrest is related to cytotoxicity outlined in Section "Repair of DNA damage by DNA mismatch repair" (Fig. 2).

The data are consistent with the concept that MMR is required for the efficient activation of the early G2 checkpoint in response to IR and may also be involved in the late G2 checkpoint. However, the role of MMR repair in the cytotoxic response to IR appears to be dependent on the radiation dose-rate. MMR-proficiency confers modest sensitivity to cell killing after high dose-rate IR, with enhanced sensitivity following LDR-IR. This low dose rate sensitivity was attributed to persistent accumulation of cells in radiation sensitive G2 phase of the cell cycle.[100] Should MMR proteins be required to activate and maintain this G2 arrest it may provide an explanation for the observed MMR-dependent sensitivity to LDR-IR. The additional role of MMR proteins in the suppression of RAD51 may then render the cell incapable of repairing DSBs via HR. Given that HR is primary mechanism of DSB repair in G2 phase, the cells would subsequently be committed to die. Following acute high dose-rate IR, cytotoxicity is associated with loss of MMR and the subsequent inefficient activation and maintenance of either the early or late G2 arrest. The absence of MMR in this instance also allows for excessive and aberrant HR leading to an increase in chromosomal damage after IR and subsequent cell death. It is possible then that cells displaying differential checkpoint activation without subsequent changes in survival may be defective in a component of the pathway required to elicit cell death (apoptosis).

Low dose radiation hypersensitivity and DNA mismatch repair

Hypersensitivity to low doses of radiation (HRS) has been observed in mammalian cells in response to radiation doses less than

0.5 Gy in a wide-variety of cell types and is considered to be the default response of mammalian cells to low dose radiation. HRS has been demonstrated in both *in vitro* and *in vivo* models and is followed by a period of increased radioresistance (IRR) as the dose increases to 1 Gy.[101] To date the molecular mechanisms of HRS/IRR have not been fully elucidated but HRS has been associated with a number of events such as a deficient early G2/M checkpoint, enhanced sensitivity in G2 phase cells,[89,102] persistent RAD51 foci, and p53-caspase 3 dependent apoptosis.[103–105] We have recently implicated a role for MMR-proficiency in the HRS response.[8] It remains a possibility that the relative contribution of different DNA repair pathways differs depending on the extent of radiation-induced damage. For example, defects in repair pathways associated with clustered DNA damage may be more relevant to cell killing than DSBs repair after low dose exposures.[60–62]

Interestingly hypersensitive cells appear to display hallmarks of MMR-deficient cells. These include a deficient early G2/M checkpoint, enhanced sensitivity in G2 phase cells[89,102] and p53-caspase-3 dependent apoptosis[103–105] as well as persistent RAD51 foci following irradiation. Short et al. report that RAD51 foci colocalised with BRCA2 foci are common following low doses of radiation in glioma cells hypersensitive to low radiation doses. Colocalisation of RAD51/BRCA2 foci is thought to be indicative of HR repair. Consistent with this observation, Thomas et al. recently demonstrated a higher frequency of unrepaired DNA DSB processed by the NHEJ and by the RAD51-dependent recombination pathways in hypersensitive compared with non-hypersensitive cells derived from the same tumour. However, it may also represent sites of inefficient or dysregulated HR, for example sites at which there has been failure to locate a homologous partner for exchange, inappropriate RAD51 binding or self-self interaction as a result of high RAD51 protein levels.[106,107] While not the most widely demonstrated response associated with hypersensitive cells, a model in which the RAD51 recombination pathway may have an important influence on the survival following low doses of radiation is consistent with HRS being most marked in G2 phase cells. Moreover, it may provide an explanation for the prevalence of HRS in radioresistant cancer cells[108] given that RAD51 is overexpressed in many tumours.[109]

Low dose radiation hypersensitivity thus appears to be an atypical response of cells to IR, displaying a DDR that is characteristic of MMR-deficient cells, yet appears to be associated with MMR-proficient phenotype.[8] The biological causes of this response are likely multifactorial, although, abrogation of the early G2/M checkpoint appears to be the most promising explanation.[89,103,110] It is unknown why cells bypass this early G2/M checkpoint. One possibility is that MMR-dependent futile repair of IR-induced damage (e.g. O6MeG lesions) because this type of repair typically causes an accumulation of cells in G2 phase after the second replication after DNA damage, and hypersensitivity has been observed following low concentrations of TMZ, an agent that induces these lesions.[8] Alternatively, hypersensitive cells may express insufficient MMR protein levels for an effective DNA damage response because a threshold of expression of MSH2 or MLH1 proteins is required for proper checkpoint and cell-death signaling, even though sub-threshold levels appear sufficient for fully functional MMR repair activity.[15] These low levels of MMR protein expression may however be sufficient to protect against the accumulation of oxidative damage such as 8-oxoG lesions thus keeping the cumulative damage below the activation threshold of the early G2/M checkpoint.

Another alternate explanation is that other essential early G2/M checkpoint proteins such as MRE11 may also be defective. This protein is required for the MMR-dependent G2 arrest of cells treated with TMZ. In this study, Mirzoeva et al. showed that TMZ exposure triggered MMR-dependent MRN foci formation and co-immunoprecipitation of MMR protein MLH1 with the MRN complex component Nbs1 specifically in response to TMZ treatment. Of particular interest, was the observation that small inhibitory siRNA-mediated silencing of *MRE11* phenocopied that of defective MMR, with silencing of either *MRE11* or *MLH1* leading to a comparable reduction of TMZ-induced G2 arrest and an increase in cellular tolerance to the drug[81] suggesting that the MMR-induced G2 arrest in response to TMZ, may also be MRE11 dependent. Indeed, increasing evidence suggests that MMR proteins and MRE11 are in fact more intimately connected than simple signaling partners. MRE11-deficiency has been repeatedly correlated with microsatellite instability and defective MMR[111–114] *in vitro* and *in vivo* suggesting that MRE11 may be co-expressed with MMR and possibly even co-regulated by MMR. Moreover it has been demonstrated that siRNA-mediated silencing of *MRE11* in cervical cancer HeLa cells results in microsatellite instability and defective 3′–5′ MMR repair,[111] Zhao et al. suggest that the integrity of hMLH1–hMRE11 complex is essential for 3′ MMR reaction and DNA damage triggered cellular response, which may possibly include the MMR-mediated G2 arrest in response to IR. Given the interplay between MLH1 and MRE11 is it conceivable that in the absence of functional MRE11 the MMR-dependent early G2 checkpoint may be compromised in response to IR. It will therefore be interesting for future studies to determine if HRS+ cell lines are deficient in MRE11, or express mutated MRE11.

Another possible explanation is that these cells do not adequately detect DSBs. It has previously been reported that failure to recognize DNA DSBs is unlikely to be the underlying reason that cells bypass the early G2 phase checkpoint and display HRS. Using the λ-H2AX assay Wykes et al. found no relationship between the initial or residual levels of DSBs and the prevalence of HRS.[115] However, a recent study by Yuan et al.[63] showing that the MRN complex may recognize DSBs independent of γ-H2AX may reopen this discussion.

Conclusion

An accumulating body of evidence supports a role for DNA MMR proteins in DNA damage signaling following IR. Specifically it is becoming increasingly clear that DNA MMR proteins may recognize and bind to IR-induced DNA damage and promote a G2/M cell cycle arrest and ultimately cell death by apoptosis. Emerging data suggest that sensitivity to IR may indeed be associated with MMR status but that the mechanism of MMR related sensitivity may depend on the dose rate of the radiation used. MMR-proficiency appears to be associated with radiosensitivity following low dose-rate IR whereas loss of MMR appears to be associated with radiosensitivity following acute high dose-rate IR. Regulation of the G2/M checkpoint and homologous recombination by the MMR system appear fundamental to these responses. Characterization of the interplay between MMR proteins and the G2/M cell cycle checkpoint and RAD51 recombination pathway following IR has and will continue to provide valuable insight into the molecular functions essential for the manifestation of radiosensitivity. Understanding when and how MMR proteins may dictate the cellular response to IR will be crucial for identifying the cause of hypersensitivity to low doses of radiation, not only so as to enable exploitation of this response to improve the efficacy of radiotherapy, but also for understanding the implications of MMR status for cancer predisposition following modern radiotherapy treatment.

References

1. Hutchinson F. Chemical changes induced in DNA by ionizing radiation. *Prog Nucleic Acid Res Mol Biol* 1985;**32**:115–54.

2. Ward JF. DNA damage produced by ionizing radiation in mammalian cells: identities, mechanisms of formation, and reparability. *Prog Nucleic Acid Res Mol Biol* 1988;**35**:95–125.
3. Goodhead DT. Initial events in the cellular effects of ionizing radiations: clustered damage in DNA. *Int J Radiat Biol* 1994;**65**:7–17.
4. Jeggo P, Lavin MF. Cellular radiosensitivity: how much better do we understand it? *Int J Radiat Biol* 2009;**85**:1061–81.
5. Stojic L, Brun R, Jiricny J. Mismatch repair and DNA damage signalling. *DNA Repair (Amst)* 2004;**3**:1091–101.
6. Brown KD, Rathi A, Kamath R, et al. The mismatch repair system is required for S-phase checkpoint activation. *Nat Genet* 2003;**33**:80–4.
7. Cejka P, Stojic L, Marra G, Jiricny J. Is mismatch repair really required for ionizing radiation-induced DNA damage signaling? *Nat Genet* 2004;**36**:432–3. [author reply 434].
8. Martin L, Marples B, Coffey M, et al. Recognition of O6MeG lesions by MGMT and mismatch repair proficiency may be a prerequisite for low-dose radiation hypersensitivity. *Radiat Res* 2009;**172**:405–13.
9. Yan T, Seo Y, Kinsella TJ. Differential cellular responses to prolonged LDR-IR in MLH1-proficient and MLH1-deficient colorectal cancer HCT116 cells. *Clin Cancer Res* 2009;**15**:6912–20.
10. Holt SM, Scemama JL, Panayiotidis MI, Georgakilas AG. Compromised repair of clustered DNA damage in the human acute lymphoblastic leukemia MSH2-deficient NALM-6 cells. *Mutat Res* 2009;**674**:123–30.
11. Hada M, Georgakilas AG. Formation of clustered DNA damage after high-LET irradiation: a review. *J Radiat Res (Tokyo)* 2008;**49**:203–10.
12. Short SC, Buffa FM, Bourne S, et al. Dose- and time-dependent changes in gene expression in human glioma cells after low radiation doses. *Radiat Res* 2007;**168**:199–208.
13. Thomas C, Charrier J, Massart C, et al. Low-dose hyper-radiosensitivity of progressive and regressive cells isolated from a rat colon tumour: impact of DNA repair. *Int J Radiat Biol* 2008;**84**:533–48.
14. Kunkel TA, Erie DA. DNA mismatch repair. *Ann Rev Biochem* 2005;**74**:681–710.
15. O'Brien V, Brown R. Signalling cell cycle arrest and cell death through the MMR System. *Carcinogenesis* 2006;**27**:682–92.
16. Wildenberg J, Meselson M. Mismatch repair in heteroduplex DNA. *Proc Natl Acad Sci USA* 1975;**72**:2202–6.
17. Iyer RR, Pluciennik A, Burdett V, Modrich PL. DNA mismatch repair: functions and mechanisms. *Chem Rev* 2006;**106**:302–23.
18. Jiricny J. The multifaceted mismatch-repair system. *Nat Rev Mol Cell Biol* 2006;**7**:335–46.
19. Peltomaki P. Role of DNA mismatch repair defects in the pathogenesis of human cancer. *J Clin Oncol* 2003;**21**:1174–9.
20. Martin L, Coffey M, Lawler M, Hollywood D, Marignol L. DNA mismatch repair and the transition to hormone independence in breast and prostate cancer. *Cancer Lett* 2009;**291**:142–9.
21. Villemure JF, Abaji C, Cousineau I, Belmaaza A. MSH2-deficient human cells exhibit a defect in the accurate termination of homology-directed repair of DNA double-strand breaks. *Cancer Res* 2003;**63**:3334–9.
22. Surtees JA, Argueso JL, Alani E. Mismatch repair proteins: key regulators of genetic recombination. *Cytogenet Genome Res* 2004;**107**:146–59.
23. Zhang Y, Rohde LH, Wu H. Involvement of nucleotide excision and mismatch repair mechanisms in double strand break repair. *Curr Genom* 2009;**10**:250–8.
24. Bannister LA, Waldman BC, Waldman AS. Modulation of error-prone double-strand break repair in mammalian chromosomes by DNA mismatch repair protein Mlh1. *DNA Repair (Amst)* 2004;**3**:465–74.
25. Larijani M, Zaheen A, Frieder D, et al. Lack of MSH2 involvement differentiates V(D)J recombination from other non-homologous end joining events. *Nucleic Acids Res* 2005;**33**:6733–42.
26. Li GM. Mechanisms and functions of DNA mismatch repair. *Cell Res* 2008;**18**:85–98.
27. Hawn MT, Umar A, Carethers JM, et al. Evidence for a connection between the mismatch repair system and the G2 cell cycle checkpoint. *Cancer Res* 1995;**55**:3721–5.
28. Carethers JM, Hawn MT, Chauhan DP, et al. Competency in mismatch repair prohibits clonal expansion of cancer cells treated with N-methyl-N′-nitro-N-nitrosoguanidine. *J Clin Invest* 1996;**98**:199–206.
29. Duckett DR, Drummond JT, Murchie AI, et al. Human MutSalpha recognizes damaged DNA base pairs containing O6-methylguanine, O4-methylthymine, or the cisplatin-d(GpG) adduct. *Proc Natl Acad Sci USA* 1996;**93**:6443–7.
30. Fink D, Nebel S, Aebi S, et al. The role of DNA mismatch repair in platinum drug resistance. *Cancer Res* 1996;**56**:4881–6.
31. Yoshikawa K, Ogawa T, Baer R, et al. Abnormal expression of BRCA1 and BRCA1-interactive DNA-repair proteins in breast carcinomas. *Int J Cancer* 2000;**88**:28–36.
32. Zdraveski ZZ, Mello JA, Farinelli CK, Essigmann JM, Marinus MG. MutS preferentially recognizes cisplatin- over oxaliplatin-modified DNA. *J Biol Chem* 2002;**277**:1255–60.
33. Tajima A, Hess MT, Cabrera BL, Kolodner RD, Carethers JM. The mismatch repair complex hMutS alpha recognizes 5-fluorouracil-modified DNA: implications for chemosensitivity and resistance. *Gastroenterology* 2004;**127**:1678–84.
34. Berry SE, Loh T, Yan T, Kinsella TJ. Role of MutSalpha in the recognition of iododeoxyuridine in DNA. *Cancer Res* 2003;**63**:5490–5.
35. Meyers M, Hwang A, Wagner MW, et al. A role for DNA mismatch repair in sensing and responding to fluoropyrimidine damage. *Oncogene* 2003;**22**:7376–88.
36. Karran P. Mechanisms of tolerance to DNA damaging therapeutic drugs. *Carcinogenesis* 2001;**22**:1931–7.
37. Yip S, Miao J, Cahill DP, et al. MSH6 mutations arise in glioblastomas during temozolomide therapy and mediate temozolomide resistance. *Clin Cancer Res* 2009;**15**:4622–9.
38. Glaab WE, Risinger JI, Umar A, et al. Resistance to 6-thioguanine in mismatch repair-deficient human cancer cell lines correlates with an increase in induced mutations at the HPRT locus. *Carcinogenesis* 1998;**19**:1931–7.
39. Lin X, Kim HK, Howell SB. The role of DNA mismatch repair in cisplatin mutagenicity. *J Inorg Biochem* 1999;**77**:89–93.
40. Pani E, Stojic L, El-Shemerly M, Jiricny J, Ferrari S. Mismatch repair status and the response of human cells to cisplatin. *Cell Cycle* 2007;**6**:1796–802.
41. Topping RP, Wilkinson JC, Scarpinato KD. Mismatch repair protein deficiency compromises cisplatin-induced apoptotic signaling. *J Biol Chem* 2009;**284**:14029–39.
42. Lage H, Dietel M. Involvement of the DNA mismatch repair system in antineoplastic drug resistance. *J Cancer Res Clin Oncol* 1999;**125**:156–65.
43. Fujita H, Kato J, Horii J, et al. Decreased expression of hMLH1 correlates with reduced 5-fluorouracil-mediated apoptosis in colon cancer cells. *Oncol Rep* 2007;**18**:1129–37.
44. Vernole P, Tedeschi B, Tentori L, et al. Role of the mismatch repair system and p53 in the clastogenicity and cytotoxicity induced by bleomycin. *Mutat Res* 2006;**594**:63–77.
45. Fritzell JA, Narayanan L, Baker SM, et al. Role of DNA mismatch repair in the cytotoxicity of ionizing radiation. *Cancer Res* 1997;**57**:5143–7.
46. Zhang QM, Ishikawa N, Nakahara T, Yonei S. Escherichia coli MutY protein has a guanine-DNA glycosylase that acts on 7,8-dihydro-8-oxoguanine:guanine mispair to prevent spontaneous G:C → C:G transversions. *Nucleic Acids Res* 1998;**26**:4669–75.
47. DeWeese TL, Shipman JM, Larrier NA, et al. Mouse embryonic stem cells carrying one or two defective Msh2 alleles respond abnormally to oxidative stress inflicted by low-level radiation. *Proc Natl Acad Sci USA* 1998;**95**:11915–20.
48. Yan T, Schupp JE, Hwang HS, et al. Loss of DNA mismatch repair imparts defective cdc2 signaling and G(2) arrest responses without altering survival after ionizing radiation. *Cancer Res* 2001;**61**:8290–7.
49. Ni TT, Marsischky GT, Kolodner RD. MSH2 and MSH6 are required for removal of adenine misincorporated opposite 8-oxo-guanine in *S. cerevisiae*. *Mol Cell* 1999;**4**:439–44.
50. Mazurek A, Berardini M, Fishel R. Activation of human MutS homologs by 8-oxo-guanine DNA damage. *J Biol Chem* 2002;**277**:8260–6.
51. Macpherson P, Barone F, Maga G, et al. 8-Oxoguanine incorporation into DNA repeats in vitro and mismatch recognition by MutSalpha. *Nucleic Acids Res* 2005;**33**:5094–105.
52. Colussi C, Parlanti E, Degan P, et al. The mammalian mismatch repair pathway removes DNA 8-oxodGMP incorporated from the oxidized dNTP pool. *Curr Biol* 2002;**12**:912–8.
53. Blasi MF, Ventura I, Aquilina G, et al. A human cell-based assay to evaluate the effects of alterations in the MLH1 mismatch repair gene. *Cancer Res* 2006;**66**:9036–44.
54. Wang G, Alamuri P, Humayun MZ, Taylor DE, Maier RJ. The Helicobacter pylori MutS protein confers protection from oxidative DNA damage. *Mol Microbiol* 2005;**58**:166–76.
55. Russo MT, De Luca G, Degan P, Bignami M, Different DNA. Repair strategies to combat the threat from 8-oxoguanine. *Mutat Res* 2007;**614**:69–76.
56. Xu XS, Narayanan L, Dunklee B, Liskay RM, Glazer PM. Hypermutability to ionizing radiation in mismatch repair-deficient, Pms2 knockout mice. *Cancer Res* 2001;**61**:3775–80.
57. Zeng M, Narayanan L, Xu XS, et al. Ionizing radiation-induced apoptosis via separate Pms2- and p53-dependent pathways. *Cancer Res* 2000;**60**:4889–93.
58. de Wind N, Dekker M, Berns A, Radman M, te Riele H. Inactivation of the mouse Msh2 gene results in mismatch repair deficiency, methylation tolerance, hyperrecombination, and predisposition to cancer. *Cell* 1995;**82**:321–30.
59. Elliott B, Jasin M. Repair of double-strand breaks by homologous recombination in mismatch repair-defective mammalian cells. *Mol Cell Biol* 2001;**21**:2671–82.
60. Abuin A, Zhang H, Bradley A. Genetic analysis of mouse embryonic stem cells bearing Msh3 and Msh2 single and compound mutations. *Mol Cell Biol* 2000;**20**:149–57.
61. San Filippo J, Sung P, Klein H. Mechanism of eukaryotic homologous recombination. *Ann Rev Biochem* 2008;**77**:229–57.
62. Mimitou EP, Symington LS. DNA end resection: many nucleases make light work. *DNA Repair (Amst)* 2009;**8**:983–95.
63. Yuan J, Chen J. MRE11-RAD50-NBS1 complex dictates DNA repair independent of H2AX. *J Biol Chem* 2009;**285**:1097–104.
64. Sung P, Klein H. Mechanism of homologous recombination: mediators and helicases take on regulatory functions. *Nat Rev Mol Cell Biol* 2006;**7**:739–50.
65. Surtees JA, Alani E. Mismatch repair factor MSH2-MSH3 binds and alters the conformation of branched DNA structures predicted to form during genetic recombination. *J Mol Biol* 2006;**360**:523–36.
66. Zink D, Mayr C, Janz C, Wiesmuller L. Association of p53 and MSH2 with recombinative repair complexes during S phase. *Oncogene* 2002;**21**:4788–800.
67. Siehler SY, Schrauder M, Gerischer U, et al. Human MutL-complexes monitor homologous recombination independently of mismatch repair. *DNA Repair (Amst)* 2009;**8**:242–52.

68. Georgakilas AG. Processing of DNA damage clusters in human cells: current status of knowledge. *Mol Biosyst* 2008;**4**:30–5.
69. Nikjoo H, O'Neill P, Goodhead DT, Terrissol M. Computational modelling of low-energy electron-induced DNA damage by early physical and chemical events. *Int J Radiat Biol* 1997;**71**:467–83.
70. Nikjoo H, O'Neill P, Wilson WE, Goodhead DT. Computational approach for determining the spectrum of DNA damage induced by ionizing radiation. *Radiat Res* 2001;**156**:577–83.
71. Ward JF. The complexity of DNA damage: relevance to biological consequences. *Int J Radiat Biol* 1994;**66**:427–32.
72. Sutherland BM, Bennett PV, Sidorkina O, Laval J, Clustered DNA. Damages induced in isolated DNA and in human cells by low doses of ionizing radiation. *Proc Natl Acad Sci USA* 2000;**97**:103–8.
73. Georgakilas AG, Bennett PV, Wilson 3rd DM, Sutherland BM. Processing of bistranded abasic DNA clusters in gamma-irradiated human hematopoietic cells. *Nucleic Acids Res* 2004;**32**:5609–20.
74. Frelon S, Douki T, Cadet J. Radical oxidation of the adenine moiety of nucleoside and DNA: 2-hydroxy-2′-deoxyadenosine is a minor decomposition product. *Free Rad Res* 2002;**36**:499–508.
75. Pouget JP, Frelon S, Ravanat JL, et al. Formation of modified DNA bases in cells exposed either to gamma radiation or to high-LET particles. *Radiat Res* 2002;**157**:589–95.
76. Wu J, Zhu BB, Yu J, et al. In vitro and in vivo modulations of benzo[*c*]phenanthrene-DNA adducts by DNA mismatch repair system. *Nucleic Acids Res* 2003;**31**:6428–34.
77. O'Driscoll M, Martinelli S, Ciotta C, Karran P. Combined mismatch and nucleotide excision repair defects in a human cell line: mismatch repair processes methylation but not UV- or ionizing radiation-induced DNA damage. *Carcinogenesis* 1999;**20**:799–804.
78. Wang Y, Cortez D, Yazdi P, et al. BASC, a super complex of BRCA1-associated proteins involved in the recognition and repair of aberrant DNA structures. *Genes Dev* 2000;**14**:927–39.
79. Clark AB, Cook ME, Tran HT, et al. Functional analysis of human MutSalpha and MutSbeta complexes in yeast. *Nucleic Acids Res* 1999;**27**:736–42.
80. Alani E, Lee S, Kane MF, Griffith J, Kolodner RD. *Saccharomyces cerevisiae* MSH2, a mispaired base recognition protein, also recognizes Holliday junctions in DNA. *J Mol Biol* 1997;**265**:289–301.
81. Mirzoeva OK, Kawaguchi T, Pieper RO. The Mre11/Rad50/Nbs1 complex interacts with the mismatch repair system and contributes to temozolomide-induced G2 arrest and cytotoxicity. *Mol Cancer Ther* 2006;**5**:2757–66.
82. Yamane K, Schupp JE, Kinsella TJ. BRCA1 activates a G2-M cell cycle checkpoint following 6-thioguanine-induced DNA mismatch damage. *Cancer Res* 2007;**67**:6286–92.
83. Davis TW, Wilson-Van Patten C, Meyers M, et al. Defective expression of the DNA mismatch repair protein, MLH1, alters G2-M cell cycle checkpoint arrest following ionizing radiation. *Cancer Res* 1998;**58**:767–78.
84. Aquilina G, Crescenzi M, Bignami M. Mismatch repair, G(2)/M cell cycle arrest and lethality after DNA damage. *Carcinogenesis* 1999;**20**:2317–26.
85. Reinhardt HC, Yaffe MB. Kinases that control the cell cycle in response to DNA damage: Chk1, Chk2, and MK2. *Curr Opin Cell Biol* 2009;**21**:245–55.
86. Houtgraaf JH, Versmissen J, van der Giessen WJ. A concise review of DNA damage checkpoints and repair in mammalian cells. *Cardiovasc Revasc Med* 2006;**7**:165–72.
87. Hartwell LH, Weinert TA. Checkpoints: controls that ensure the order of cell cycle events. *Science* 1989;**246**:629–34.
88. Xu B, Kim ST, Lim DS, Kastan MB. Two molecularly distinct G(2)/M checkpoints are induced by ionizing irradiation. *Mol Cell Biol* 2002;**22**:1049–59.
89. Marples B, Wouters BG, Collis SJ, Chalmers AJ, Joiner MC. Low-dose hyper-radiosensitivity: a consequence of ineffective cell cycle arrest of radiation-damaged G2-phase cells. *Radiat Res* 2004;**161**:247–55.
90. Bartek J, Falck J, Lukas J. CHK2 kinase – a busy messenger. *Nat Rev Mol Cell Biol* 2001;**2**:877–86.
91. Cejka P, Stojic L, Mojas N, et al. Methylation-induced G(2)/M arrest requires a full complement of the mismatch repair protein hMLH1. *Embo J* 2003;**22**:2245–54.
92. Hirose Y, Katayama M, Stokoe D, et al. The p38 mitogen-activated protein kinase pathway links the DNA mismatch repair system to the G2 checkpoint and to resistance to chemotherapeutic DNA-methylating agents. *Mol Cell Biol* 2003;**23**:8306–15.
93. Franchitto A, Pichierri P, Piergentili R, et al. The mammalian mismatch repair protein MSH2 is required for correct MRE11 and RAD51 relocalization and for efficient cell cycle arrest induced by ionizing radiation in G2 phase. *Oncogene* 2003;**22**:2110–20.
94. Aquilina G, Ceccotti S, Martinelli S, Hampson R, Bignami M. N-(2-Chloroethyl)-N′-cyclohexyl-N-nitrosourea sensitivity in mismatch repair-defective human cells. *Cancer Res* 1998;**58**:135–41.
95. Fiumicino S, Martinelli S, Colussi C, et al. Sensitivity to DNA cross-linking chemotherapeutic agents in mismatch repair-defective cells in vitro and in xenografts. *Int J Cancer* 2000;**85**:590–6.
96. Pichierri P, Franchitto A, Piergentili R, Colussi C, Palitti F. Hypersensitivity to camptothecin in MSH2 deficient cells is correlated with a role for MSH2 protein in recombinational repair. *Carcinogenesis* 2001;**22**:1781–7.
97. Takahashi T, Min Z, Uchida I, et al. Hypersensitivity in DNA mismatch repair-deficient colon carcinoma cells to DNA polymerase reaction inhibitors. *Cancer Lett* 2005;**220**:85–93.
98. Barwell J, Pangon L, Hodgson S, et al. Biallelic mutation of MSH2 in primary human cells is associated with sensitivity to irradiation and altered RAD51 foci kinetics. *J Med Genet* 2007;**44**:516–20.
99. Marquez N, Chappell SC, Sansom OJ, et al. Single cell tracking reveals that Msh2 is a key component of an early-acting DNA damage-activated G2 checkpoint. *Oncogene* 2003;**22**:7642–8.
100. Muller VO, Klein M, Schultze B. X-ray induced mitotic delay and death of cells in different phases of the cell cycle. An autoradiographic study on jejunal crypt cells of the mouse using double labelling with 3H- and 14C-thymidine. *Strahlentherapie* 1983;**159**:34–40.
101. Marples B, Collis SJ. Low-dose hyper-radiosensitivity: past, present, and future. *Int J Radiat Oncol Biol Phys* 2008;**70**:1310–8.
102. Marples B, Wouters BG, Joiner MC. An association between the radiation-induced arrest of G2-phase cells and low-dose hyper-radiosensitivity: a plausible underlying mechanism? *Radiat Res* 2003;**160**:38–45.
103. Krueger SA, Collis SJ, Joiner MC, Wilson GD, Marples B. Transition in survival from low-dose hyper-radiosensitivity to increased radioresistance is independent of activation of ATM Ser1981 activity. *Int J Radiat Oncol Biol Phys* 2007;**69**:1262–71.
104. Krueger SA, Joiner MC, Weinfeld M, Piasentin E, Marples B. Role of apoptosis in low-dose hyper-radiosensitivity. *Radiat Res* 2007;**167**:260–7.
105. Enns L, Bogen KT, Wizniak J, Murtha AD, Weinfeld M. Low-dose radiation hypersensitivity is associated with p53-dependent apoptosis. *Mol Cancer Res* 2004;**2**:557–66.
106. Schild D, Wiese C. Overexpression of RAD51 suppresses recombination defects: a possible mechanism to reverse genomic instability. *Nucleic Acids Res* 2009;**38**:1061–70.
107. Short SC, Bourne S, Martindale C, Woodcock M, Jackson SP. DNA damage responses at low radiation doses. *Radiat Res* 2005;**164**:292–302.
108. Joiner MC, Marples B, Lambin P, Short SC, Turesson I. Low-dose hypersensitivity: current status and possible mechanisms. *Int J Radiat Oncol Biol Phys* 2001;**49**:379–89.
109. Klein HL. The consequences of Rad51 overexpression for normal and tumor cells. *DNA Repair (Amst)* 2008;**7**:686–93.
110. Fernet M, Megnin-Chanet F, Hall J, Favaudon V. Control of the G2/M checkpoints after exposure to low doses of ionising radiation: implications for hyper-radiosensitivity. *DNA Repair (Amst)* 2009;**9**:48–57.
111. Vo AT, Zhu F, Wu X, et al. hMRE11 deficiency leads to microsatellite instability and defective DNA mismatch repair. *EMBO Rep* 2005;**6**:438–44.
112. Giannini G, Rinaldi C, Ristori E, et al. Mutations of an intronic repeat induce impaired MRE11 expression in primary human cancer with microsatellite instability. *Oncogene* 2004;**23**:2640–7.
113. Giannini G, Ristori E, Cerignoli F, et al. Human MRE11 is inactivated in mismatch repair-deficient cancers. *EMBO Rep* 2002;**3**:248–54.
114. Wen Q, Scorah J, Phear G, et al. A mutant allele of MRE11 found in mismatch repair-deficient tumor cells suppresses the cellular response to DNA replication fork stress in a dominant negative manner. *Mol Biol Cell* 2008;**19**:1693–705.
115. Wykes SM, Piasentin E, Joiner MC, Wilson GD, Marples B. Low-dose hyper-radiosensitivity is not caused by a failure to recognize DNA double-strand breaks. *Radiat Res* 2006;**165**:516–24.
116. Zhang H, Richards B, Wilson T, et al. Apoptosis induced by overexpression of hMSH2 or hMLH1. *Cancer Res* 1999;**59**:3021–7.
117. Papouli E, Cejka P, Jiricny J. Dependence of the cytotoxicity of DNA-damaging agents on the mismatch repair status of human cells. *Cancer Res* 2004;**64**:3391–4.
118. Wang Q, Ponomareva ON, Lasarev M, Turker MS. High frequency induction of mitotic recombination by ionizing radiation in Mlh1 null mouse cells. *Mutat Res* 2006;**594**:189–98.
119. Bucci B, D'Agnano I, Amendola D, et al. Myc down-regulation sensitizes melanoma cells to radiotherapy by inhibiting MLH1 and MSH2 mismatch repair proteins. *Clin Cancer Res* 2005;**11**:2756–67.

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Mini-review

DNA mismatch repair and the transition to hormone independence in breast and prostate cancer

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ABSTRACT

The molecular basis for the progression of breast and prostate cancer from hormone dependent to hormone independent disease remains a critical issue in the management of these two cancers. The DNA mismatch repair system is integral to the maintenance of genomic stability and suppression of tumorigenesis. No firm consensus exists regarding the implications of mismatch repair (MMR) deficiencies in the development of breast or prostate cancer. However, recent studies have reported an association between mismatch repair deficiency and loss of specific hormone receptors, inferring a potential role for mismatch repair deficiency in this transition. An updated review of the experimental data supporting or contradicting the involvement of MMR defects in the development and progression of breast and prostate cancer will be provided with particular emphasis on their implications in the transition to hormone independence.

1. Introduction

Breast cancer is both the most prevalent cancer and the leading cause of cancer-related mortality in women worldwide [1]. Prostate cancer is the most commonly diagnosed malignancy in men in the United States, second only to lung cancer in cancer-related deaths [2]. These cancers are not only similar in their epidemiological patterns, but also possess similar molecular mechanisms of pathogenesis and disease progression. Both breast and prostate cancer are hormone-related diseases. Steroid hormones, such as oestrogen, progesterone, and androgen as well as exogenous hormones contribute to the initiation and promotion of multistage carcinogenesis via specific steroid hormone receptors [3]. Hormone deprivation therapies inhibit cell growth and have provided significant improvements in survival in both diseases. Currently, anti-oestrogens are the most effective treatment option for women with oestrogen receptor (ER) positive breast cancer, while androgen deprivation therapy is the prime therapeutic approach for men with advanced prostate cancer. However, hormone resistance remains a significant clinical problem and limits the benefits of these therapies in a considerable proportion of initially drug-responsive patients [4–6]. To date, curative treatments for advanced stages of both cancers are lacking. Indeed, an understanding of the underlying molecular mechanisms involved in the transition to hormone refractory disease is vital for the development of effective therapeutic and preventive strategies to combat these malignancies.

A large and compelling body of epidemiological and experimental data implicates oestrogen in the pathogenesis of breast and endometrial cancer (for review see [3,7]). Similarly, androgens have been recognised to play an important role in controlling the growth of the normal prostate gland, and in promoting benign prostate

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hyperplasia and prostatic carcinoma [8–10] however unlike breast cancer, serum sex hormone levels appear unrelated to prostate cancer risk [11]. The most commonly accepted risk factors for breast cancer include early menarche, late menopause, alcohol consumption, post-menopausal obesity and hormone replacement therapy [3]. Each of these risk factors increases one's exposure to hormones. Hormones stimulate cell proliferation and thus increased exposure to these hormones promotes the opportunity to develop and accumulate random genetic errors. While a number of select candidate genes have been identified as biomarkers for breast and prostate cancer (such as those involved in hormone biosynthesis, activation, inactivation and transport) [12,13], the molecular mechanisms involved in the progression to hormone independent disease are less well-understood.

Random genetic errors due to this increased proliferation occur simultaneously in genes not related to hormone manipulation and can drastically reduce a cell's capacity for self-protection against random excitotoxic, metabolic and oxidative insults. Mutations in DNA repair genes have been associated with a mutator phenotype and confer resistance to cancer therapies [14–16]. In particular, the role of defects in DNA mismatch repair (MMR) genes in the pathogenesis of breast and prostate cancer has been investigated in the last decade. In this review, we will describe the experimental data supporting or contradicting the involvement of MMR defects in the development of breast and prostate cancer. In addition, we will explore the possible role of MMR deficiency in the transition to hormone independence. A number of recent observations imply a direct role for oestrogen in the regulation of MMR activity, but how this may relate to disease progression and what this may imply in terms of the mechanisms of androgen independence are unclear.

2. The DNA mismatch repair system

The mismatch repair (MMR) system is made up of a number of key components. Three heterodimers are required for efficient repair. Two MutS complexes, MutSα (MSH2/MSH6) and MutSβ (MSH2/MSH3), recognise base–base mismatches (MutS) and insertion-deletion loops (MutS). The heterodimer MutLα (MLH1/PMS2) is subsequently recruited by the MSH2 protein and forms a ternary complex with one of the MutS complexes. It then promotes the repair process via its endonucleolytic activity, coordinating the interplay between the mismatch recognition complex and other proteins necessary for MMR. These additional proteins may include exonuclease 1 (EXO1), possibly helicase(s), replication protein-A (RPA), replication factor c (RFC), proliferating cell nuclear antigen (PCNA), and DNA polymerases α and β [16,17] (Fig. 1). MMR s an integral repair mechanism. Its best understood function is the repair of mismatched bases and insertion-deletion loops in DNA that may arise during replication [18,19]. In doing so, it maintains genomic fidelity by preventing mutations that may give rise to cancer. MMR proteins have also been implicated in double-strand break repair and recombination [16,20] and the transcription-coupled repair pathway [21]. MLH1 in particular has been implicated in the regulation of the G2 cell cycle phase checkpoint [14,22–24].

Lack of any one MMR protein, known as mismatch repair deficiency, reduces repair capacity [25,26]. MMR deficiency increases the risk of developing cancer, due to an elevated rate of spontaneous mutation, and has also been implicated in the differentiation, growth and invasion of cancer [27,28]. In particular, heterozygous germline defects in the DNA mismatch repair genes *MSH2* or *MLH1* cause hereditary non-polyposis colon cancer (HNPCC) or Lynch syndrome, a dominantly inherited cancer susceptibility syndrome (for review see [17]). Individuals diagnosed with HNPCC have an elevated cumulative risk of developing any HNPCC-related cancer (95% confidence interval) of 67% (47–84%) for men and 72% (48–85%) for women [29] typically colorectal, endometrium, small bowel, ureter, and renal pelvis malignancy [15]. A number of international criteria for the diagnosis of HNPCC have been established. These are known as "Amsterdam criteria I" (based on colorectal cancer) and "Amsterdam criteria II" (based on cancers of the colon and rectum, endometrium, small bowel, ureter, and renal pelvis) and rely on a number of clinical hallmarks. These include the observation that at least three relatives have a HNPCC-associated tumour, that the patient is diagnosed at an early age and that the syndrome was transmitted as an autosomal dominant trait [30]. MMR defects are also implicated in the pathogenesis of a number of sporadic tumours that do not fit the Amsterdam criteria for HNPCC. In particular, while inactivating mutations of MSH2 are less common in sporadic tumours than in HNPCC, these mutations have been demonstrated both at the genetic and immunohistochemical level in sporadic tumours of the colon [31], endometrium [32,33], stomach [34,35], head and neck [36], cervix [37], prostate [38] and breast [39].

3. MMR deficiency in the pathogenesis of breast and prostate cancer

3.1. Microsatellite instability as a marker of MMR deficiency

Microsatellite instability (MSI) is a hallmark of MMR deficiency in HNPCC and results from mutations in the mismatch repair genes *MLH1* or *MSH2* or from gene inactivation associated with DNA promoter hypermethylation. Microsatellites are short nucleotide sequences (1–5 base pairs, repeated 15–30 times) which are normally relatively stable. Microsatellite instability (or replication error positive, RER+) is defined as loss or gain of microsatellite repeats at two or more loci [40]. In HNPCC, a single mutation in one allele of a mismatch repair gene is inherited in the germline; however, microsatellite instability only follows inactivation of the other allele. MSI in sporadic breast cancer is generally accepted to be a relatively rare event. Siah et al. failed to find evidence of MSI in any of the 66 breast tumour samples studied [41]. Caldes et al. reported MSI of mono- and di-nucleotide repeats in only 6 out of 88 cases (7%) all belonging to stage II or III disease [42]. Similarly, Anbazhagen et al. failed to find evidence

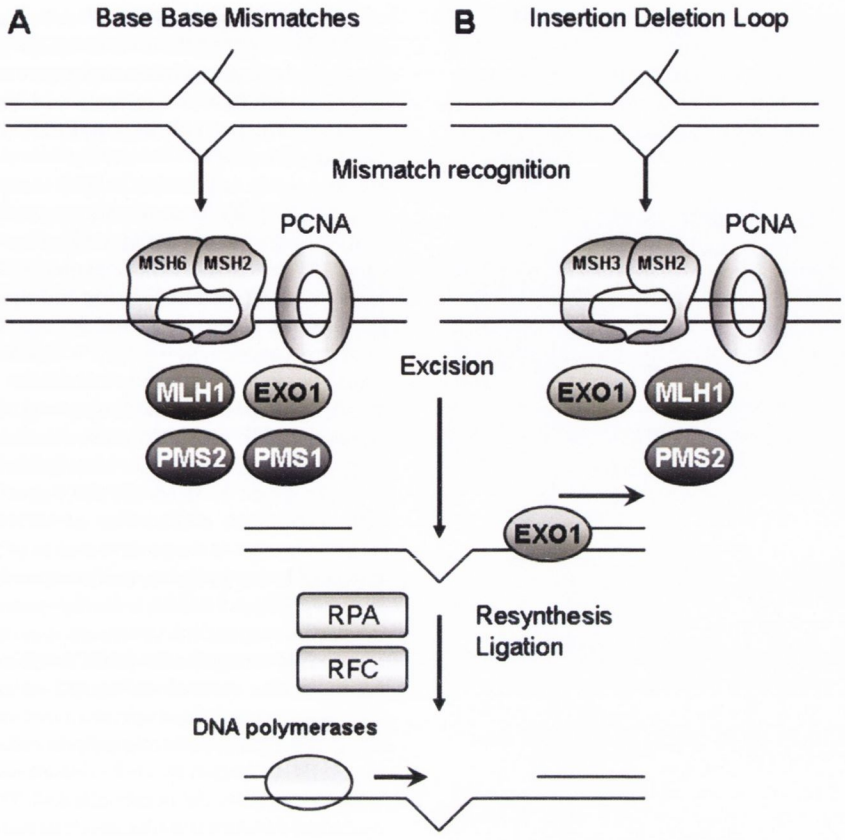

Fig. 1. Overview of the mismatch repair system. DNA mismatch repair is initiated when either a MutSα (MSH2/MSH6) or MutSβ (MSH2/MSH3) dimer binds to mismatched DNA. (A) MutSα: MSH2–MSH6 recognises and binds to base–base mismatches. (B) MutSβ: MSH3–MSH6 recognises and binds to insertion-deletion loops. Heterodimers of MutL homologues, such as MLH1–hMLH3, PMS1 and PMS2, as well as the EXO1, RPA, RFC, and DNA polymerases are then recruited to this complex to complete excision of the mismatches and resynthesis of the DNA strand.

of MSI of 104 mono-, di- or tri-nucleotide repeats in 267 presumably sporadic breast cancers [43]. Absent MSI has also been documented in medullary breast carcinomas in eight cases using eight markers [44]. Bilateral breast carcinoma appears to be the exception demonstrating MSI of di-nucleotide repeats in 15% of tumours (7/46 tumours, 23 cases) [45]. Numerous studies have suggested a role for MSI in the early stage of breast carcinogenesis because MSI was detected in in situ carcinoma (8%) [46], atypical epithelial hyperplasia [47] and immortalized and transformed human epithelial breast epithelial cell lines [48]. Other studies have suggested that MSI was involved in the later stage of breast carcinogenesis because MSI was much more frequently detected in invasive carcinoma (9/19 cases, 47.4%) compared with in situ carcinoma (2/10 cases, 20%) [49]. Recently the relative frequency of allelic imbalance in 100 breast cancer patients was found to increase significantly ($P < 0.001$) with increasing grade (well differentiated, 12%; moderately differentiated, 17%; poorly differentiated, 26%) [50].

MSI as detected by use of di-nucleotide tandem repeat sequence microsatellite markers, has been suggested to occur in 8% (4/50 cases) [51] to 35% (14/40 cases) [52,53] in prostate cancer, with more aggressive cancers showing more frequent MSI (4/47 MSI+ and poorly differentiated) [54]. Yet, other studies suggest that MSI may be an early event in prostate carcinogenesis, but not a marker for progression or prognosis [52,53,55].

Thus, no firm consensus exists regarding the implication of MMR deficiency in the pathogenesis of malignant breast or prostate cancer [41,52,55]. A common problem in determining the etiology of MMR defects in both cancers is the inconsistency in techniques used in studies to date. Earlier studies tended to examine the presence or absence of MSI rather than the underlying cause of genomic instability. The degree of MSI then varied depending on the number of microsatellites examined and the percentage of microsatellites demonstrating instability [56]. BAT25 and BAT26 are commonly used mono-nucleotide repeat microsatellites markers which have been demonstrated to accurately identify tumours with a defective MMR system [57]. If these markers are not included in the analysis, it is difficult to determine the involvement of MMR defects in carcinogenesis. Recent studies which have focused on the presence or absence of MMR by immunohistological staining, did not necessarily examine MSI in the same cohort. In addition, it is only in recent work that the value of including PMS2 and MSH6 to the traditionally used immunohistochemistry (IHC) panel of MLH1 and MSH2 has been realized. The addition of these two antibodies

to the IHC panel increases the sensitivity of IHC to that of MSI testing [58]. Ideally MSI analysis should be performed and evaluated together with MMR immunohistochemistry to gain a complete picture of the MMR deficiency status of a tumour. High levels of MSI are suggestive of an MMR defect but the exact gene involved may only be defined by IHC. IHC alone can determine retention or loss of MLH1, MSH2, MSH6, and PMS2 protein expression. The likelihood of MMR gene involvement in the tumour is very low if all four proteins are present. However, approximately 5% of tumours will display MSI but have normal protein expression for these four genes and so MMR gene involvement cannot be excluded [59–61]. Failing the use of both techniques, researchers should ensure the use of MSI markers which include BAT25 and BAT26 where determination of MMR involvement is required, or consider the use of IHC with all four recommended markers MSH2/MSH6, MLH1/PMS2 [58,62].

3.2. MMR protein analysis and MMR deficiency

MLH1 and MSH2 are the most commonly studied MMR proteins in breast and prostate cancer (Table 1). MLH1 downregulation in particular has been associated with breast cancer occurence. Murata et al. report reduced expression of MLH1 in 26/83 of cases (31.1%) with hypermethylation of the hMLH1 promoter accounting for reduced expression of MLH1 in the majority of cases [39]. In another study of 232 Indian patients with primary breast cancer, hypermethylation of the *MLH1* gene was observed in 43.5% of patients with primary breast cancer, of whom 66.9% had locally advanced breast cancer (stage IIIA, IIIB, and IIIC) ($P < 0.0001$) [63]. Moreover, the MLH1 gene variant 219II/IV has been significantly associated with breast cancer risk in caucasians ($n = 752$) (odds ratio (OR) = 1.87; 95% CI = 1.11, 3.16) [64] implying that a defect in the MMR pathway may indeed contribute to breast cancer risk. However, larger studies are warranted to draw any definitive conclusions.

A minor role for MLH1 in prostate carcinogenesis has also been suggested. *In vitro*, MLH1 protein expression is retained in androgen-sensitive prostate cancer cell lines (22RV1, LnCaP) and expression is lost in androgen-independent cells (DU145, DUPro) [25,65]. Similar results have been documented *in vivo*. Burger et al. reported decreased MLH1 protein expression in 9/41 cases (22%) using a tissue microarray [66]. In another study of 70 cases and 97 controls Strom et al. reported a significantly lower expression of *MLH1* in prostate cancer cases (37/70, 53%) than in controls (47.8%) ($P = 0.003$) using multiplex RT-PCR. This was determined to be a statistically significant risk factor for prostate cancer (OR = 4.31, $P = 0.004$) [67]. However, the findings of a recent study contradict those found earlier, showing a significant increase ($P < 0.0001$) in MLH1 immunoreactivity in prostatic adenocarcinoma (benign, 5.6% (4/71); high grade prostatic intraepithelial neoplasia, 46.2% (12/26); grade 3, 75.0% (27/36); grade 4/5, 74.2% (23/31)) [68]. While the mechanism of MLH1 downregulation in breast tumours is frequently due to promoter hypermethylation of *MLH1* [69], the mechanism of *MLH1* downregulation in prostate cancers is rarely reported. However, it may be due to somatic mutations or loss of heterozygosity as is commonly the case in HNPCC tumours [70]. A role for *MLH1* in the control of the G2-M cell-cycle checkpoint has been suggested, such that decreased levels of *MLH1* expression may lead to impaired cell cycle control, allowing cells to proceed with cell division before accurate DNA repair can be accomplished. This impaired control could overwhelm the mismatch repair mechanism, leading to the accumulation of mutations [71].

The majority of studies reported suggest a causal link between MSH2 downregulation in prostate cancer (Table 1). *In vitro*, absent MSH2 protein expression has been documented in hormone-sensitive prostate cancer cells (LnCaP) [72] but expression is upregulated in a number of hormone independent prostate cancer cell lines (DU145, PC3) [73]. *In vivo*, Prtilo et al. found MSH2 expression to be reduced in 39% (88/243) tumours using a tissue

Table 1
Reduced expression of MMR genes and risk of prostate/breast cancer (*in vivo*).

Investigators	Origin	MMR gene studied	Proportion (%) of tumours showing decreased MMR expression
Hirata et al. [79]	Prostate	MSH3	–
Chuang et al. [68]	Benign prostatic tissue	MLH1	5.6% (4/71)
Burger et al. [66]	Prostate Ca	MLH1	22% (9/41)
		MSH2	39.6% (23/58)
Prtilo et al. [74]	Prostate Ca	MSH2	39% (88/243)
Velasco Albert et al. [91]	Prostate Ca	MSH2	29% (21/73)
Velasco Hewitt et al. [56]	Prostate Ca	MSH2	29%
Strom et al. [67]	Prostate Ca	MLH1	53% (37/70)
		MSH2	53% (37/70)
Chen et al. [38]	Prostate Ca	PMS1	86% (11/13)
Balogh et al. [92]	Breast Ca	PMS2	Non-sense mutation in nucleotide 1862 in 9/20
Murata [39]	Breast Ca	MLH1	31.1% (26/83)
		MSH2	27.7% (23/83)
Naqvi et al. [63]	Breast Ca (Indian women)	MLH1	Hypermethylation 43.5%
		MSH2	Hypermethylation 16%
Smith et al. [64]	Breast Ca (caucasians)	MLH1	Polymorphism
Khilko et al. [93]	Breast Ca (DCIS IDC)	MSH2	0/211
		MLH1	0/211
Wong et al. [94]	Breast Ca	MSH2	59 Families no association

microarray [74]. Similarly, Burger et al. found MSH2 to be downregulated in 39.6% prostate cancer cases (23/58) [66]. In another study, absent to low staining for the MSH2 protein was documented in 30% of well to moderately differentiated prostate carcinoma (Gleason scores 5–6) and 29% of poorly differentiated prostate carcinoma (Gleason scores 7–10) specimens [56]. Overall, the evidence suggests that MMR defects are likely involved in breast and prostate carcinogenesis. Given that these repair defects are known to confer resistance to certain chemotherapeutic agents [75–77], it seems possible that MMR defects may be involved in the acquired hormone resistance in breast and prostate cancers.

Relatively fewer studies have examined the expression of mismatch repair proteins MSH3, MSH6, PMS2, and PMS1 in breast and prostate cancer. Allelic losses of *MSH3* have been reported in breast cancer patient samples (5/22, 23%), suggesting a role for *MSH3* in tumorigenesis through cellular functions other than replication error [78]. Moreover, *MSH3* polymorphism was recently identified as a risk factor for sporadic prostate cancer [79]. However, this study reports a finding of OR 2.1 (95% CI 1.05–4.34) based on only 110 cases and 110 controls and perhaps should be considered a hypothesis generating report rather than a definitive study. Researchers have also suggested a role for PMS2 in tumorigenesis following demonstration of truncation of the protein during neoplastic transformation of human breast epithelial cells *in vitro* [80]. Conversely, significantly elevated levels ($P < 0.0001$) of PMS2 were documented in 17 of 33 (52%) individual prostate cancer tumours from 19 patients compared to normal and benign prostate tissues [81].

4. MSH2 upregulation may be a marker of disease progression in hormone dependent cancers

While reduced MSH2 expression has been observed during development from in situ to invasive breast cancer [49], thereafter increased MSH2 expression corresponds to an unfavorable prognosis and disease progression. Koster et al. reported that the expression of MSH2 correlated significantly with the expression of p53, with the appearance of distant metastases, low differentiation and the appearance of hemangiosis carcinomatosa and lymphangiosis carcinomatosa, while it negatively correlated with the expression of the oestrogen receptor [28]. Similar results have been found in prostate cancer. Clinically, reduced or absent MSH2 immunohistochemical staining in prostate cancer specimens has been correlated with an extended overall, disease free and biochemical recurrence free interval, independent of pathologic stage or Gleason pattern [56,74]. In addition, a number of studies have reported a significant correlation between immunohistochemical staining intensity (moderate/strong staining) and reduced overall and disease free survival [66,74], increased malignancy of the tumour (Gleason score >7) [66] and detectable serum PSA after prostatectomy [56]. While reduced or absent MSH2 protein expression may be associated with an increased risk of prostate cancer, it also appears to correspond to a hormone-sensitive phenotype accounting for the comparatively favorable prognosis documented in patients with reduced or absent MSH2 expression, relative to those with moderate-strong MSH2 expression.

The increased MSH2 expression observed in aggressive phenotypes may be explained by a study from Marra et al., which reported an increased expression of MSH2 during cell proliferation [82]. Therefore tumours with high levels of MSH2 expression might display a higher proliferation rate, resulting in a more aggressive phenotype (e.g. early recurrence) [66] or alternatively those tumours with a higher proliferation rate may express higher levels of MSH2 expression as a result. Miyamoto and colleagues showed that oestrogen upregulates MMR activity in normal and malignant endometrial glandular cells [83]. Moreover, MSH2 has been shown to be a potent co-activator of oestrogen receptor alpha [84]. Therefore if MSH2 is upregulated due to increased proliferation, this may then further increase circulating oestrogen levels which in turn further increase proliferation.

Interestingly, elevated PMS2 expression also appears to be negatively correlated with prognosis in prostate cancer patients. Norris et al. recently reported elevated PMS2 expression to be an independent predictor of time to recurrence after surgery [85]. Indeed, overexpression of PMS2 is known to confer hypermutability and DNA damage tolerance [15] and so a role for PMS2 in the transition to hormone independence could be implied, but sufficient evidence is currently lacking to confirm this hypothesis.

5. Downregulation of MSH2 is associated with hormone independence

Downregulation of the *MSH2* gene has been reported during progression of in situ lesions to invasive breast cancer [86,87] and has also been associated with hormone-refractory prostate cancer [88] and so further work is required to reconcile these differences. In breast cancer, Koster et al. reported a weak negative correlation between MSH2-immuno-reactivity score (IRS) and the IRS of the oestrogen receptor (ER) [28]. Some suggested MSI was associated with negative expression of ER and PR after analysis of 10 markers in 88 patients [89] and others suggested that this is not the case [49,90]. Indeed, if *MSH2* can increase oestrogen receptor alpha, then it is likely that a decrease in MSH2 expression could in part be responsible for loss of oestrogen receptors and subsequent tumour resistance to hormonal therapy. Moreover, given that breast and prostate cancer have similar pathological characteristics, it is possible that loss of MSH2 expression also has the same effect on androgen receptor expression, but to our knowledge this to date has not been investigated (**Fig. 2**A).

MSH2 immunohistochemical staining has been observed to increase in prostate tumours between Gleason scores of 5 and 7 and decrease between Gleason scores of 7 and 10 [56] (**Fig. 2**B). While increased proliferation might account for the increase in MSH2 expression in tumours up to Gleason grade 7, the relative decrease in expression with increasing Gleason scores from 7 to 10 is relatively less well-understood. Here we speculate that MSH2 expression is somewhat cyclical; reduced in early stages of disease, in-

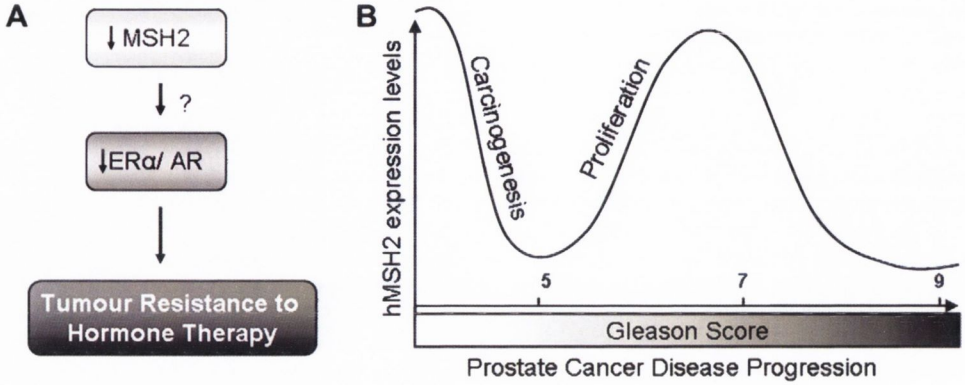

Fig. 2. (A) DNA mismatch repair protein MSH2 may participate in the transition to hormone independence. Downregulation of MSH2 protein expression may downregulate oestrogen receptor alpha (ERα) and the androgen receptor (AR) leading to tumour resistance to hormonal therapies in both breast and prostate cancer. (B) Cyclical expression of MSH2 in prostate tumours. MSH2 immunohistochemical staining is reduced during early stages of carcinogenesis, increases between Gleason scores of 5 and 7 and decreases between Gleason scores of 7 and 10.

creased as the tumour becomes more aggressive and then decreased as the tumour advances to metastatic hormone independent disease.

6. Conclusions

The mismatch repair system is a highly conserved post-replicative editing process that maintains genomic fidelity through the recognition and repair of incorrectly replicated nucleotides. A deficiency in any one of the genes involved reduces repair capacity. The involvement of MMR defects in the development of breast and prostate cancer remains unclear based on MSI analysis. However, a role for these defects in the development of a hormone independent phenotype is inferred by the apparent cyclical changes in MSH2 protein expression during the course of disease progression. The balance of evidence suggests that MSH2 and perhaps PMS2 protein expression may indeed be useful prognostic markers for the outcome of individuals with hormone dependent disease. Increased knowledge of the attributes of the MMR system and the interplay between MSH2, PMS2 and other molecular pathways is essential to better understand the fundamental mechanisms of hormone independence and to identify targets for effective preventive and therapeutic interventions.

Conflicts of interest

None declared.

References

[1] G.N. Hortobagyi, J. de la Garza Salazar, K. Pritchard, D. Amadori, R. Haidinger, C.A. Hudis, H. Khaled, M.C. Liu, M. Martin, M. Namer, J.A. O'Shaughnessy, Z.Z. Shen, K.S. Albain, The global breast cancer burden: variations in epidemiology and survival, Clin. Breast Cancer 6 (2005) 391–401.
[2] A. Jemal, R. Siegel, E. Ward, Y. Hao, J. Xu, M.J. Thun, Cancer statistics 2009, CA Cancer J. Clin. 59 (2009) 225–249.
[3] B.E. Henderson, H.S. Feigelson, Hormonal carcinogenesis, Carcinogenesis 21 (2000) 427–433.
[4] C. Huggins, C.V. Hodges, Studies on prostatic cancer: I. The effect of castration, of estrogen and of androgen injection on serum phosphatases in metastatic carcinoma of the prostate 1941, J. Urol. 168 (2002) 9–12.
[5] M. Laufer, S.R. Denmeade, V.J. Sinibaldi, M.A. Carducci, M.A. Eisenberger, Complete androgen blockade for prostate cancer: what went wrong?, J Urol. 164 (2000) 3–9.
[6] S.R. Johnston, Acquired tamoxifen resistance in human breast cancer–potential mechanisms and clinical implications, Anticancer Drug 8 (1997) 911–930.
[7] A.M. Ferreira, H. Westers, A. Albergaria, R. Seruca, R.M. Hofstra, Estrogens, MSI and Lynch syndrome-associated tumors, Biochim. Biophys. Acta (2009).
[8] W.A. Ricke, K. Ishii, E.A. Ricke, J. Simko, Y. Wang, S.W. Hayward, G.R. Cunha, Steroid hormones stimulate human prostate cancer progression and metastasis, Int. J. Cancer 118 (2006) 2123–2131.
[9] B. Chatterjee, The role of the androgen receptor in the development of prostatic hyperplasia and prostate cancer, Mol. Cell Biochem. 253 (2003) 89–101.
[10] D. Karan, B.M. Schmied, B.J. Dave, U.A. Wittel, M.F. Lin, S.K. Batra, Decreased androgen-responsive growth of human prostate cancer is associated with increased genetic alterations, Clin. Cancer Res. 7 (2001) 3472–8340.
[11] A.W. Roddam, N.E. Allen, P. Appleby, T.J. Key, Endogenous sex hormones and prostate cancer: a collaborative analysis of 18 prospective studies, J. Natl. Cancer Inst. 100 (2008) 170–183.
[12] S.K. Chatterjee, B.R. Zetter, Cancer biomarkers: knowing the present and predicting the future, Future Oncol. 1 (2005) 37–50.
[13] J.A. Ludwig, J.N. Weinstein, Biomarkers in cancer staging, prognosis and treatment selection, Nat. Rev. Cancer 5 (2005) 845–856.
[14] M.T. Hawn, A. Umar, J.M. Carethers, G. Marra, T.A. Kunkel, C.R. Boland, M. Koi, Evidence for a connection between the mismatch repair system and the G2 cell cycle checkpoint, Cancer Res. 55 (1995) 3721–3725.
[15] S.L. Gibson, L. Narayanan, D.C. Hegan, A.B. Buermeyer, R.M. Liskay, P.M. Glazer, Overexpression of the DNA mismatch repair factor, PMS2, confers hypermutability and DNA damage tolerance, Cancer Lett. 244 (2006) 195–202.
[16] G.M. Li, Mechanisms and functions of DNA mismatch repair, Cell Res. 18 (2008) 85–98.
[17] P. Peltomaki, Role of DNA mismatch repair defects in the pathogenesis of human cancer, J. Clin. Oncol. 21 (2003) 1174–1179.
[18] R.R. Iyer, A. Pluciennik, V. Burdett, P.L. Modrich, DNA mismatch repair: functions and mechanisms, Chem. Rev. 106 (2006) 302–323.
[19] J. Jiricny, The multifaceted mismatch-repair system, Nat. Rev. Mol. Cell Biol. 7 (2006) 335–346.
[20] A. Franchitto, P. Pichierri, R. Piergentili, M. Crescenzi, M. Bignami, F. Palitti, The mammalian mismatch repair protein MSH2 is required for correct MRE11 and RAD51 relocalization and for efficient cell cycle arrest induced by ionizing radiation in G2 phase, Oncogene 22 (2003) 2110–2120.

[21] K. Kobayashi, P. Karran, S. Oda, K. Yanaga, Involvement of mismatch repair in transcription-coupled nucleotide excision repair, Hum. Cell 18 (2005) 103–115.
[22] T.W. Davis, C. Wilson-Van Patten, M. Meyers, K.A. Kunugi, S. Cuthill, C. Reznikoff, C. Garces, C.R. Boland, T.J. Kinsella, R. Fishel, D.A. Boothman, Defective expression of the DNA mismatch repair protein, MLH1, alters G2-M cell cycle checkpoint arrest following ionizing radiation, Cancer Res. 58 (1998) 767–778.
[23] P. Cejka, L. Stojic, N. Mojas, A.M. Russell, K. Heinimann, E. Cannavo, M. di Pietro, G. Marra, J. Jiricny, Methylation-induced G(2)/M arrest requires a full complement of the mismatch repair protein hMLH1, Embo. J. 22 (2003) 2245–2254.
[24] Y. Hirose, M. Katayama, D. Stokoe, D.A. Haas-Kogan, M.S. Berger, R.O. Pieper, The p38 mitogen-activated protein kinase pathway links the DNA mismatch repair system to the G2 checkpoint and to resistance to chemotherapeutic DNA-methylating agents, Mol. Cell Biol. 23 (2003) 8306–8315.
[25] C.C. Yeh, C. Lee, R. Dahiya, DNA mismatch repair enzyme activity and gene expression in prostate cancer, Biochem. Biophys. Res. Commun. 285 (2001) 409–413.
[26] A.B. Mohd, B. Palama, S.E. Nelson, G. Tomer, M. Nguyen, X. Huo, A.B. Buermeyer, Truncation of the C-terminus of human MLH1 blocks intracellular stabilization of PMS2 and disrupts DNA mismatch repair, DNA Repair (Amst) 5 (2006) 347–361.
[27] Chintamani, B.P. Jha, V. Bhandari, A. Bansal, S. Saxena, D. Bhatnagar, The expression of mismatched repair genes and their correlation with clinicopathological parameters and response to neo-adjuvant chemotherapy in breast cancer, Int. Semin. Surg. Oncol. 4 (2007) 5.
[28] F. Koster, A. Schroer, D. Fischer, A.K. Horn, K. Diedrich, M. Friedrich, Immunohistochemistry of proteins for DNA mismatch repair in correlation to prognostic factors of mammarian cancer, Oncol. Rep. 17 (2007) 1223–1227.
[29] M.A. Jenkins, L. Baglietto, J.G. Dowty, C.M. Van Vliet, L. Smith, L.J. Mead, F.A. Macrae, D.J. St John, J.R. Jass, G.G. Giles, J.L. Hopper, M.C. Southey, Cancer risks for mismatch repair gene mutation carriers: a population-based early onset case-family study, Clin. Gastroenterol. Hepatol. 4 (2006) 489–498.
[30] H.F. Vasen, P. Watson, J.P. Mecklin, H.T. Lynch, New clinical criteria for hereditary nonpolyposis colorectal cancer (HNPCC, Lynch syndrome) proposed by the international collaborative group on HNPCC, Gastroenterology 116 (1999) 1453–1456.
[31] C. Schafmayer, S. Buch, J.H. Egberts, A. Franke, M. Brosch, A. El Sharawy, M. Conring, M. Koschnick, S. Schwiedernoch, A. Katalinic, B. Kremer, U.R. Folsch, M. Krawczak, F. Fandrich, S. Schreiber, J. Tepel, J. Hampe, Genetic investigation of DNA-repair pathway genes PMS2, MLH1, MSH2, MSH6, MUTYH, OGG1 and MTH1 in sporadic colon cancer, Int. J. Cancer 121 (2007) 555–558.
[32] K. Takano, Y. Ichikawa, E. Ueno, M. Ohwada, M. Suzuki, H. Tsunoda, M. Miwa, K. Uchida, H. Yoshikawa, Microsatellite instability and expression of mismatch repair genes in sporadic endometrial cancer coexisting with colorectal or breast cancer, Oncol. Rep. 13 (2005) 11–16.
[33] F. Bianchi, S. Rosati, L. Belvederesi, C. Loretelli, R. Catalani, A. Mandolesi, R. Bracci, I. Bearzi, E. Porfiri, R. Cellerino, MSH2 splice site mutation and endometrial cancer, Int. J. Gynecol. Cancer 16 (2006) 1419–1423.
[34] K. Kanemitsu, K. Kawasaki, M. Nakamura, D. Li, T. Yasuda, D. Kuroda, H. Yokozaki, T. Kamigaki, Y. Kuroda, MSI is frequently recognized among gastric cancer patients with a family history of cancer, Hepatogastroenterology 54 (2007) 2410–2414.
[35] M. Yamamoto, K. Taguchi, H. Baba, K. Endo, S. Kohnoe, T. Okamura, Y. Maehara, Loss of protein expression of hMLH1 and hMSH2 with double primary carcinomas of the stomach and colorectum, Oncol. Rep. 16 (2006) 41–47.
[36] J. Nunn, S. Nagini, J.M. Risk, W. Prime, P. Maloney, T. Liloglou, A.S. Jones, S.R. Rogers, J.R. Gosney, J. Woolgar, J.K. Field, Allelic imbalance at the DNA mismatch repair loci, hMSH2, hMLH1, hPMS1, hPMS2 and hMSH3 in squamous cell carcinoma of the head and neck, Oral Oncol. 39 (2003) 115–129.
[37] E.R. Nijhuis, H.W. Nijman, K.A. Oien, A. Bell, K.A. Ten Hoor, N. Reesink-Peters, H.M. Boezen, H. Hollema, A.G. van der Zee, Loss of MSH2 protein expression is a risk factor in early stage cervical cancer, J. Clin. Pathol. 60 (2007) 824–830.
[38] Y. Chen, J. Wang, M.M. Fraig, K. Henderson, N.K. Bissada, D.K. Watson, C.W. Schweinfest, Alterations in PMS2, MSH2 and MLH1 expression in human prostate cancer, Int. J. Oncol. 22 (2003) 1033–1043.
[39] H. Murata, N.H. Khattar, L. Gu, G.M. Li, Roles of mismatch repair proteins hMSH2 and hMLH1 in the development of sporadic breast cancer, Cancer Lett. 223 (2005) 143–150.
[40] J.M. Wheeler, W.F. Bodmer, N.J. Mortensen, DNA mismatch repair genes and colorectal cancer, Gut 47 (2000) 148–153.
[41] S.P. Siah, D.M. Quinn, G.D. Bennett, G. Casey, R.L. Flower, G. Suthers, Z. Rudzki, Microsatellite instability markers in breast cancer: a review and study showing MSI was not detected at 'BAT 25' and 'BAT 26' microsatellite markers in early-onset breast cancer, Breast Cancer Res. Treat 60 (2000) 135–142.
[42] T. Caldes, P. Perez-Segura, A. Tosar, M. De La Hoya, E. Diaz-Rubio, Low frequency of microsatellite instability in sporadic breast cancer, Int. J. Oncol. 16 (2000) 1235–1242.
[43] R. Anbazhagan, H. Fujii, E. Gabrielson, Microsatellite instability is uncommon in breast cancer, Clin. Cancer Res. 5 (1999) 839–844.
[44] S.C. Lee, K.D. Berg, M.E. Sherman, C.A. Griffin, J.R. Eshleman, Microsatellite instability is infrequent in medullary breast cancer, Am. J. Clin. Pathol. 115 (2001) 823–827.
[45] E.N. Imyanitov, A.V. Togo, E.N. Suspitsin, M.Y. Grigoriev, K.M. Pozharisski, E.A. Turkevich, K.P. Hanson, N.K. Hayward, G. Chenevix-Trench, C. Theillet, M.F. Lavin, Evidence for microsatellite instability in bilateral breast carcinomas, Cancer Lett. 154 (2000) 9–17.
[46] T. Toyama, H. Iwase, H. Iwata, Y. Hara, Y. Omoto, M. Suchi, T. Kato, T. Nakamura, S. Kobayashi, Microsatellite instability in in situ and invasive sporadic breast cancers of Japanese women, Cancer Lett. 108 (1996) 205–209.
[47] C.L. Rosenberg, P.S. Larson, J.D. Romo, A. De Las Morenas, D.V. Faller, Microsatellite alterations indicating monoclonality in atypical hyperplasias associated with breast cancer, Hum. Pathol. 28 (1997) 214–219.
[48] Y. Huang, B. Bove, Y. Wu, I.H. Russo, X. Yang, A. Zekri, J. Russo, Microsatellite instability during the immortalization and transformation of human breast epithelial cells in vitro, Mol. Carcinogen. 24 (1999) 118–127.
[49] K.L. Shen, L.S. Yang, H.F. Hsieh, C.J. Chen, J.C. Yu, N.M. Tsai, H.J. Harn, Microsatellite alterations on human chromosome 11 in in situ and invasive breast cancer: a microdissection microsatellite analysis and correlation with p53, ER (estrogen receptor) and PR (progesterone receptor) protein immunoreactivity, J. Surg. Oncol. 74 (2000) 100–107.
[50] R.E. Ellsworth, D.L. Ellsworth, B. Love, H.L. Patney, L.R. Hoffman, J. Kane, J.A. Hooke, C.D. Shriver, Correlation of levels and patterns of genomic instability with histological grading of DCIS, Ann. Surg. Oncol. 14 (2007) 3070–3077.
[51] A.R. Azzouzi, J.W. Catto, I. Rehman, S. Larre, M. Roupret, K.M. Feeley, O. Cussenot, M. Meuth, F.C. Hamdy, Clinically localised prostate cancer is microsatellite stable, BJU Int. 99 (2007) 1031–1035.
[52] R. Dahiya, C. Lee, J. McCarville, W. Hu, G. Kaur, G. Deng, High frequency of genetic instability of microsatellites in human prostatic adenocarcinoma, Int. J. Cancer 72 (1997) 762–767.
[53] G. Perinchery, D. Nojima, R. Goharderakhshan, Y. Tanaka, J. Alonzo, R. Dahiya, Microsatellite instability of dinucleotide tandem repeat sequences is higher than trinucleotide, tetranucleotide and pentanucleotide repeat sequences in prostate cancer, Int. J. Oncol. 16 (2000) 1203–1209.
[54] M. Watanabe, H. Imai, H. Kato, T. Shiraishi, T. Ushijima, M. Nagao, R. Yatani, Microsatellite instability in latent prostate cancers, Int. J. Cancer 69 (1996) 394–397.
[55] A.K. Ahman, B.A. Jonsson, J.E. Damber, A. Bergh, H. Gronberg, Low frequency of microsatellite instability in hereditary prostate cancer, BJU Int. 87 (2001) 334–338.
[56] A. Velasco, S.M. Hewitt, P.S. Albert, M. Hossein, H. Rosenberg, C. Martinez, A.I. Sagalowsky, J.D. McConnell, W. Marston, F.S. Leach, Differential expression of the mismatch repair gene hMSH2 in malignant prostate tissue is associated with cancer recurrence, Cancer 94 (2002) 690–699.
[57] C. Brennetot, O. Buhard, F. Jourdan, J.F. Flejou, A. Duval, R. Hamelin, Mononucleotide repeats BAT-26 and BAT-25 accurately detect MSI-H tumors and predict tumor content: implications for population screening, Int. J. Cancer 113 (2005) 446–450.
[58] J. Shia, Immunohistochemistry versus microsatellite instability testing for screening colorectal cancer patients at risk for hereditary nonpolyposis colorectal cancer syndrome: part I. The utility of immunohistochemistry, J. Mol. Diagn. 10 (2008) 293–300.
[59] L.M. Baudhuin, L.J. Burgart, O. Leontovich, S.N. Thibodeau, Use of microsatellite instability and immunohistochemistry testing for the identification of individuals at risk for Lynch syndrome, Fam. Cancer 4 (2005) 255–265.
[60] J. Shia, D.S. Klimstra, K. Nafa, K. Offit, J.G. Guillem, A.J. Markowitz, W.L. Gerald, N.A. Ellis, Value of immunohistochemical detection of DNA mismatch repair proteins in predicting germline mutation in

hereditary colorectal neoplasms, Am. J. Surg. Pathol. 29 (2005) 96–104.
[61] N.M. Lindor, G.M. Petersen, D.W. Hadley, A.Y. Kinney, S. Miesfeldt, K.H. Lu, P. Lynch, W. Burke, N. Press, Recommendations for the care of individuals with an inherited predisposition to Lynch syndrome: a systematic review, JAMA 296 (2006) 1507–1517.
[62] L. Zhang, Immunohistochemistry versus microsatellite instability testing for screening colorectal cancer patients at risk for hereditary nonpolyposis colorectal cancer syndrome: part II. The utility of microsatellite instability testing, J. Mol. Diagn. 10 (2008) 301–307.
[63] R.A. Naqvi, A. Hussain, S.S. Deo, H. Kukreti, M. Chauhan, R. Sarin, A. Saxena, M. Asim, N.K. Shukla, S.A. Husain, S.T. Pasha, S.F. Basir, Hypermethylation analysis of mismatch repair genes (hmlh1 and hmsh2) in locally advanced breast cancers in Indian women, Hum. Pathol. 39 (2008) 672–680.
[64] T.R. Smith, E.A. Levine, R.I. Freimanis, S.A. Akman, G.O. Allen, K.N. Hoang, W. Liu-Mares, J.J. Hu, Polygenic model of DNA repair genetic polymorphisms in human breast cancer risk, Carcinogenesis 29 (2008) 2132–2138.
[65] Y. Chen, J. Wang, M.M. Fraig, J. Metcalf, W.R. Turner, N.K. Bissada, D.K. Watson, C.W. Schweinfest, Defects of DNA mismatch repair in human prostate cancer, Cancer Res. 61 (2001) 4112–4121.
[66] M. Burger, S. Denzinger, C.G. Hammerschmied, A. Tannapfel, E.C. Obermann, W.F. Wieland, A. Hartmann, R. Stoehr, Elevated microsatellite alterations at selected tetranucleotides (EMAST) and mismatch repair gene expression in prostate cancer, J. Mol. Med. 84 (2006) 833–841.
[67] S.S. Strom, M.R. Spitz, Y. Yamamura, R.J. Babaian, P.T. Scardino, Q. Wei, Reduced expression of hMSH2 and hMLH1 and risk of prostate cancer: a case-control study, Prostate 47 (2001) 269–275.
[68] S.T. Chuang, B. Adley, M. Han, F. Lin, X.J. Yang, W.J. Catalona, Mutant L Homologue 1 (MLH1): a possible new immunohistochemical marker for prostatic cancer, Histopathology 52 (2008) 247–2450.
[69] H. Murata, N.H. Khattar, Y. Kang, L. Gu, G.M. Li, Genetic and epigenetic modification of mismatch repair genes hMSH2 and hMLH1 in sporadic breast cancer with microsatellite instability, Oncogene 21 (2002) 5696–5703.
[70] M. Ollikainen, U. Hannelius, C.M. Lindgren, W.M. Abdel-Rahman, J. Kere, P. Peltomaki, Mechanisms of inactivation of MLH1 in hereditary nonpolyposis colorectal carcinoma: a novel approach, Oncogene 26 (2007) 4541–4549.
[71] S. Raptis, M. Mrkonjic, R.C. Green, V.V. Pethe, N. Monga, Y.M. Chan, D. Daftary, E. Dicks, B.H. Younghusband, P.S. Parfrey, S.S. Gallinger, J.R. McLaughlin, J.A. Knight, B. Bapat, MLH1-93G>A promoter polymorphism and the risk of microsatellite-unstable colorectal cancer, J. Natl. Cancer Inst. 99 (2007) 463–474.
[72] F.S. Leach, A. Velasco, J.T. Hsieh, A.I. Sagalowsky, J.D. McConnell, The mismatch repair gene hMSH2 is mutated in the prostate cancer cell line LNCaP, J. Urol. 164 (2000) 1830–1833.
[73] L. Martin, B. Marples, M. Coffey, M. Lawler, D. Hollywood, L. Marignol, Recognition of O6MeG lesions by MGMT and mismatch repair proficiency may be a prerequisite for low-dose radiation hypersensitivity, Radiat. Res. 172 (2009) 405–413.
[74] A. Prtilo, F.S. Leach, R. Markwalder, A. Kappeler, F.C. Burkhard, M.G. Cecchini, U.E. Studer, G.N. Thalmann, Tissue microarray analysis of hMSH2 expression predicts outcome in men with prostate cancer, J. Urol. 174 (2005) 1814–1818 (discussion 1818).
[75] B.H. Son, S.H. Ahn, C.D. Ko, I.W. Ka, G.Y. Gong, J.C. Kim, Significance of mismatch repair protein expression in the chemotherapeutic response of sporadic invasive ductal carcinoma of the breast, Breast J. 10 (2004) 20–26.
[76] I. Casorelli, M.T. Russo, M. Bignami, Role of mismatch repair and MGMT in response to anticancer therapies, Anticancer Agent Med. Chem. 8 (2008) 368–380.
[77] A. Bellacosa, Functional interactions and signaling properties of mammalian DNA mismatch repair proteins, Cell Death Differ. 8 (2001) 1076–1092.
[78] N. Benachenhou, S. Guiral, I. Gorska-Flipot, D. Labuda, D. Sinnett, Frequent loss of heterozygosity at the DNA mismatch-repair loci hMLH1 and hMSH3 in sporadic breast cancer, Br. J. Cancer 79 (1999) 1012–1017.
[79] H. Hirata, Y. Hinoda, K. Kawamoto, N. Kikuno, Y. Suehiro, N. Okayama, Y. Tanaka, R. Dahiya, Mismatch repair gene MSH3 polymorphism is associated with the risk of sporadic prostate cancer, J. Urol. 179 (2008) 2020–2024.
[80] G.A. Balogh, I.H. Russo, J. Russo, Truncation of the mismatch repair protein PMS2 during the neoplastic transformation of human breast epithelial cells in vitro, Int. J. Oncol. 25 (2004) 381–387.
[81] A.M. Norris, R.D. Woodruff, R.B. D'Agostino Jr., J.E. Clodfelter, K.D. Scarpinato, Elevated levels of the mismatch repair protein PMS2 are associated with prostate cancer, Prostate 67 (2007) 214–225.
[82] G. Marra, C.L. Chang, L.A. Laghi, D.P. Chauhan, D. Young, C.R. Boland, Expression of human MutS homolog 2 (hMSH2) protein in resting and proliferating cells, Oncogene 13 (1996) 2189–2196.
[83] T. Miyamoto, T. Shiozawa, H. Kashima, Y.Z. Feng, A. Suzuki, M. Kurai, T. Nikaido, I. Konishi, Estrogen up-regulates mismatch repair activity in normal and malignant endometrial glandular cells, Endocrinology 147 (2006) 4863–4870.
[84] O. Wada-Hiraike, T. Yano, T. Nei, Y. Matsumoto, K. Nagasaka, S. Takizawa, H. Oishi, T. Arimoto, S. Nakagawa, T. Yasugi, S. Kato, Y. Taketani, The DNA mismatch repair gene hMSH2 is a potent coactivator of oestrogen receptor alpha, Br. J. Cancer 92 (2005) 2286–2291.
[85] A.M. Norris, M. Gentry, D.M. Peehl, R. D'Agostino Jr., K.D. Scarpinato, The elevated expression of a mismatch repair protein is a predictor for biochemical recurrence after radical prostatectomy, Cancer Epidemiol. Biomarkers. Prev. 18 (2009) 57–64.
[86] P. Karihtala, R. Winqvist, J.E. Syvaoja, V.L. Kinnula, Y. Soini, Increasing oxidative damage and loss of mismatch repair enzymes during breast carcinogenesis, Eur. J. Cancer 42 (2006) 2653–2659.
[87] N. Bock, H. Meden, M. Regenbrecht, B. Junemann, J. Wangerin, D. Marx, Expression of the mismatch repair protein hMSH2 in carcinoma in situ and invasive cancer of the breast, Anticancer Res. 20 (2000) 119–124.
[88] G. Fromont, L. Chene, M. Vidaud, G. Vallancien, P. Mangin, G. Fournier, P. Validire, A. Latil, O. Cussenot, Differential expression of 37 selected genes in hormone-refractory prostate cancer using quantitative taqman real-time RT-PCR, Int. J. Cancer 114 (2005) 174–181.
[89] T. Caldes, P. Perez-Segura, A. Tosar, M. de La Hoya, E. Diaz-Rubio, Microsatellite instability correlates with negative expression of estrogen and progesterone receptors in sporadic breast cancer, Teratogen. Carcinogen. Mutagen. 20 (2000) 283–291.
[90] J.A. Shaw, T. Walsh, S.A. Chappell, N. Carey, K. Johnson, R.A. Walker, Microsatellite instability in early sporadic breast cancer, Br. J. Cancer 73 (1996) 1393–1397.
[91] A. Velasco, P.S. Albert, H. Rosenberg, C. Martinez, F.S. Leach, Clinicopathologic implications of hMSH2 gene expression and microsatellite instability in prostate cancer, Cancer Biol. Ther. 1 (2002) 362–367.
[92] G.A. Balogh, R.C. Heulings, J. Russo, The mismatch repair gene hPMS2 is mutated in primary breast cancer, Int. J. Mol. Med. 18 (2006) 853–857.
[93] N. Khilko, P. Bourne, Y. Qi, T. Ping, Mismatch repair genes hMLH1 and hMSH2 may not play an essential role in breast carcinogenesis, Int. J. Surg. Pathol. 15 (2007) 233–241.
[94] E.M. Wong, A.A. Tesoriero, G.M. Pupo, M.R. McCredie, G.G. Giles, J.L. Hopper, G.J. Mann, D.E. Goldgar, M.C. Southey, Is MSH2 a breast cancer susceptibility gene?, Fam Cancer 7 (2008) 151–155.