A PROMISING SET OF CANDIDATE BIOMARKERS OF RADIORESISTANT PROSTATE CANCER

BY

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DECLARATION

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ABSTRACT

Radiotherapy (RT) has a prominent role when used as a single modality or in combination with androgen deprivation therapy in the management of prostate cancer (CaP) patients. However, even with currently available high-dose RT protocols, many patients develop recurrence or “biochemical failure” associated with a rise in prostate specific antigen (PSA), and ultimately reduced overall survival. As key regulators of cancer cell behavior, dysregulation of miRNAs expression is associated with both prostate cancer pathogenesis and treatment resistance. The identification of miRNA biomarkers have shown great promise in a number of cancers and in CaP show potential as both novel markers of radiation therapy failure and as targets for the re-sensitisation of radioresistant tumours.

The overall aim of this thesis was the identification of novel biomarkers which may distinguish between a radio sensitive and resistant phenotype as a prerequisite for the future development of a novel pre-treatment assay for the identification of radiotherapy prostate cancer patients at risk of biochemical failure. This was achieved through the generation of an isogenic model of resistance and subsequent miRNA microarray profiling of a panel of cell line models of radiation resistance.

The development of *in vitro* isogenic models of radioresistance through exposure to fractionated radiation is an increasingly used approach to investigate the mechanisms of radioresistance in cancer cells and a valuable tool for biomarker discovery programs. An isogenic model of radioresistance of the 22Rv1 non-metastatic CaP cell line was generated using 2Gy fractionated ionising radiation to a cumulative dose of 60Gy. This process selected for 22Rv1 cells with increased proliferative potential following radiation exposure (1.3 fold increase in clonogenic survival after 2Gy and 2.2 fold increase after 10Gy) when compared to wild type and aged matched control 22Rv1 cells. This radiation resistant 22Rv1 model was enriched in S-phase cells, less

ABSTRACT

susceptible to radiation-induced apoptosis and DNA damage coupled with increased repair capacity. Exposure to fractionated radiation progressively selected for 22Rv1 cells with enhanced oncogenic properties protective against radiation injury and supportive of tumour invasion. While this data must be considered within the context of one cell line, the phenotypic modifications observed support the clinical relevance of this model to enable further study of the mechanisms of radioresistance in prostate cancer cells.

Microarray analysis of this model combined with hypoxic and cancer stem like cell models resulted in the selection and validation of a panel of miRNAs and the selection of hsa-miR-4284 as a potential novel marker of radioresistance in CaP. While re-expression of miR-4284 did not result in the re-sensitisation of radiation resistant cells to treatment, investigation of predicted downstream targets resulted in the identification of two targets: *RLIM* and *RASGEFA1* as well as a potential key regulatory protein (YB-1).

This work identifies a set of candidate biomarkers of radioresistant CaP with potential for the development of companion prognostic assays for radiotherapy CaP patients.

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ABBREVIATIONS

ACA	Alkaline Comet Assay
adj.p.value	Adjusted P Value
AMC	Age Matched Control
AUC	Area Under The Curve
CaP	Prostate Cancer
cDNA	Complementary DNA
CSC	Cancer Stem Cells
ddH_2O	Double Distilled H_2O
DDR	DNA Damage Response
DNA	Deoxyribonucleic Acid
DSB	Double Strand Break
EC	Endogenous Control
EMT	Epithelial To Mesenchymal Transition
FC	Fold Change
FDR	False Discovery Rate
FFPE	Formalin Fixed Paraffin-Embedded
G1 phase	Gap 1 Phase
G2 Phase	Gap 2 Phase
G2/M Phase	Gap2/Mitosis
GEF	Guanine Nucleotide Exchange Factors
Gy	Gray
h	Hour
HIF1α	Hypoxia-Inducible Factor-1-α
HNSCC	Head And Neck Squamous Cell Carcinoma
HPV	Human Papillomavirus

ABBREVIATIONS

HR	Homologous Recombination
hsa-miR-1	miR-1
hsa-miR-185-5p	miR-185
hsa-miR-210	miR-210
hsa-miR-423-5p	miR-423
hsa-miR-4284	miR-4284
hsa-mR-200a-3p	miR-200a
kDA	Kilodalton
LogFC	Log Fold Change-
LOWESS	LOcally WEighted Scatterplot Smoothing
M phase	Mitotic Phase
min	Minutes
miR	miRNA
miRNA	microRNA
MRI	Magnetic Resonance Imaging
NHEJ	Non-Homologous End-Joining
NSCLC	Non Small Cell Lung Cancer
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PI	Propidium Iodine
pO2	Oxygen Partial Pressure
PSA	Prostate Specific Antigen
q-PCR	Real Time Polymerase Chain Reaction
q-RTPCR	Real-Time Reverse Transcription Polymerase Chain Reaction

ABBREVIATIONS

RNA	Ribionucleic Acid
ROS	Reactive Oxygen Species
RQ	Relative Quantification
RR	Radiation Resistant
rRNA	Ribosomal RNA
RT	Radiation Therapy/Radiotherapy
RTPCR	Reverse Transcription Polymerase Chain Reaction
S	Seconds
SA-β-Gal	Senescence-Associated B-Galactosidase
SD	Standard Deviation
SEM	Standard Error Mean
snRNA	Small Nuclear RNA
S-phase	Synthetic Phase
SSB	Single Strand Break
Sub-G1 phase	Sub-Gap 1 Phase
TGF-β	Transforming Growth Factor Beta 1
VEG-F	Vascular Endothelial Growth Factor
WT	Wild Type
β-Gal	Beta-Galactosidase

RESEARCH OUTPUTS

PUBLICATIONS

McDermott N, Meunier A, Mooney B, Nortey G., Hernandez C, Hurley S, Lynam-Lennon N, Barsoom SH, Bowman KJ, Marples B, Jones GDD, Marignol L. Fractionated radiation exposure amplifies the radioresistant nature of prostate cancer cells. Scientific Reports. October 2016; 6, 34796

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RESEARCH OUTPUTS

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McDermott N, Meunier A, Christopher Hernandez, Bowman KJ , Jones GD, Lynch TH, Marignol L. **“**Identification of a miRNAs signature of radioresistance in a prostate cancer model” American Association Of Cancer Research Annual Meeting. Philadelphia, USA, April 2015

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McDermott N, Meunier A, Christopher Hernandez, Bowman KJ , Jones GD, Lynch TH, Marignol L. "Generation and Characterisation of an Isogenic Cell Line Model for Radiation Resistant Prostate Cancer" The 9th International TCD Cancer Conference. Dublin Ireland, September 2014

McDermott N, Meunier A, Christopher Hernandez, Bowman KJ , Jones GD, Lynch TH, Marignol L. "Generation and Characterisation of an Isogenic Cell Line Model for Radiation Resistant Prostate Cancer" The Irish Association of Cancer Research Annual Meeting. Galway, Ireland, February 2014

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McDermott N, Meunier A, Lynch TH, Marignol L. **"**Generation and Characterisation of an Isogenic Cell Line Model for Radiation Resistant Prostate Cancer" 6th Annual Trinity College School of Medicine Postgraduate Research Day. Dublin Ireland, November 2013

RESEARCH OUTPUTS

Awards

Trinity College Dublin Travel Grant Award Scheme, 2015

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CHAPTER 1: BACKGROUND AND INTRODUCTION

1.1 RADIOTHERAPY FOR PROSTATE CANCER

Prostate cancer (CaP) is the fourth most common malignancy diagnosed worldwide and second only to lung cancer in men, with diagnosis rates highest in the western world (Bray et al., 2013). It is the most common non-cutaneous male cancer in Ireland accounting for ~30% of newly diagnosed cancers each year, with a risk of 1:7 men being diagnosed with the disease in their lifetime and an anticipated incidence increase of up to 288% by 2040 (National Cancer Registry Ireland). The number of patients receiving radiotherapy (RT) for CaP has increased significantly over the years, with a 115% increase in patients undergoing this treatment modality, while those receiving surgery decreased by 35% in the same 5 year time period (Comber and Walsh, 2008).

Current prostate cancer therapies are based on four elements: the risk of prostate-cancer specific mortality, tumour clinical features, the biological key to prostate tumorigenesis and metabolomics programming. Following a prostate cancer diagnosis, approximately 50% of men will receive radiation therapy. Patients with PSA >20 ng/ml or biopsy Gleason score 8–10 or T2-3N0M0 localised prostate carcinoma are recognised as high risk (Heidenreich et al., 2014) and the optimal management of these patients remains unclear. Randomized control trials recommend the combination of external beam radiotherapy with androgen deprivation therapy to improve overall survival (Cooperberg et al., 2010). While initially most patients are responsive to radiation therapy (RT) and treatment is generally effective a large subset of those treated will present with recurrence. Thirty to sixty percent of this high risk group show signs of biochemical recurrence within five years of treatment cessation (Shipley et al., 1999) that is associated with a limited chance of cure. This subset of patients destined to fail RT will present as histologically identical to those with a more successful clinical course making it difficult to anticipate relapse

post treatment. The burden of RT failure results not just in poor treatment outcome for patients but also in a significant strain on the healthcare system with the increased cost of treatment. This high risk of RT failure in CaP emphasizes the need for prognostic biomarkers that will allow for the identification and stratification of patients in order to offer the most effective treatment option.

1.2 BEYOND THE 5R'S OF RADIOBIOLOGY

While the five established radiobiological concepts (the '5Rs' - Radiosensitivity, Reassortment, Reoxygenation, Repair and Repopulation) underpin current radiotherapy delivery protocols, radiobiology needs to be expanded beyond the traditional confines of the 5Rs to continue to guide improvements in radiotherapy standards (Good and Harrington, 2013). For instance, through the discovery of new biomarkers of treatment response and the identification of novel targets for the sensitisation of tumour cells to radiation.

The radioresistant phenotype is likely the result of the tumour clinical features, intrinsic differences in cellular radiosensitivity relating to constitutive and/or radiation-induced activation of protective intracellular signalling pathways (such as altered deoxyribonucleic acid (DNA) repair, altered cell cycle check point operation, altered growth, altered reactive oxygen species (ROS) biology and altered induction of apoptosis) (Bristow et al., 2007, Skvortsova et al., 2008, Diehn et al., 2009), the tumour microenvironment (inflammation, hypoxia) (Bristow and Hill, 2008) and the cancer stem cell (CSC) population (Baumann M et al., 2009). Formation of DNA damage is the key event for cell killing by ionizing radiation (Dugle et al., 1976) and a cell's ability to repair such damage is crucial to determining fate following treatment (Bristow et al., 2007). Understanding how the DNA damage response has changed in radioresistant cancer cells after radiation exposure and the role these mechanisms play in the switch from a sensitive to resistant

cell type is essential if we are to ever truly decipher the cellular processes which lead to this phenotype.

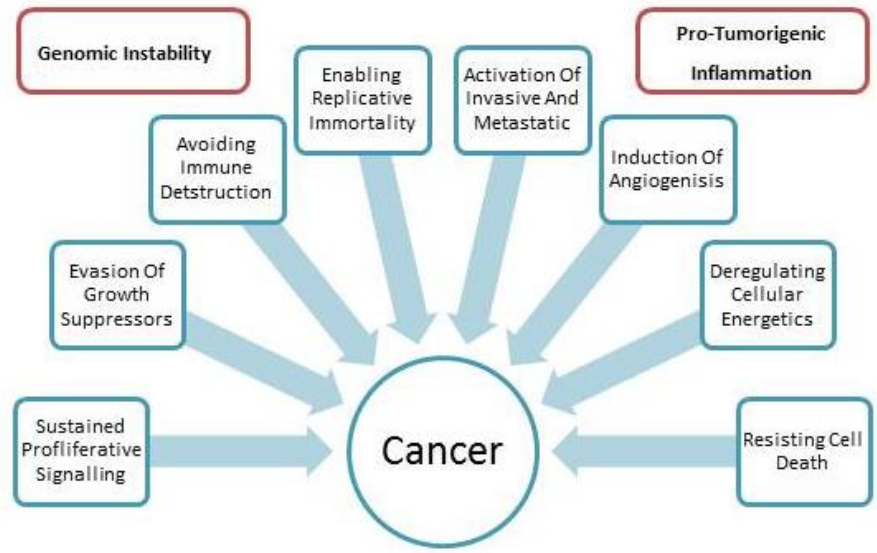

Figure 1-1: The hallmarks of Cancer

The eight hallmarks and two enabling characteristic of cancer adapted from Hanahan and Winberg 2011 (Hanahan and Weinberg, 2011)

All eight identified hallmarks of cancer (Hanahan and Weinberg, 2011) (Figure 1-1) help explain the radiobiological response of tumours (Good and Harrington, 2013). Investigation of these hallmarks following radiation exposure has identified new features and markers of the radiobiology of prostate cancer cells and patient tumours. This includes several markers of sustained proliferative signalling (Chen and Nirodi, 2007); deregulated p53 and transforming growth factor beta 1 (TGF-β) signalling (Makinde et al., 2013), increased activation of phosphatidylinositol-3-kinase/Akt/mammalian target of rapamycin (PI3K/Akt/mTOR) pathway (Chang et al., 2015), over expression of the anti-apoptotic marker B-cell lymphoma 2 (Bcl-2) (Rosser et al., 2003), loss of the tumour suppressor DOC-2/DAB2 Interacting proteins (DAB2IP) (Kong et al., 2010, Jacobs et al., 2014) and candidate tumour

suppressor caveolin-1 (CAV-1)(Klein et al., 2015) , elevated expression of the metastatic markers membrane type-matrix metalloproteinase (MMPs) (Cao et al., 2005), unlimited telomerase activity (Sawant et al., 1999), induction of autophagy (Yu et al., 2012, Koukourakis et al., 2015) and altered metabolic activity (Bol et al., 2014, Matschke et al., 2016). The development of the enabling characteristics (Hanahan and Weinberg, 2011): genomic instability (Little et al., 1997, Goldberg, 2003) and tumour promoting inflammation (Multhoff and Radons, 2012), described by Hanahan and Weinberg as necessary for the acquisition of the hallmarks of cancer and oncogenic transformation, are also frequently reported following exposure of cells to ionising radiation and implicated in the radioresistant phenotype (Little et al., 1997, Multhoff and Radons, 2012, Goldberg, 2003) further validating the importance of using these cancer defining characteristics for studying radiobiology in cancer.

1.3 ISOGENIC CELL LINE MODELS OF RADIATION RESISTANCE

The search for biomarkers of radioresistance requires models. While the comparison of cell lines of different tumour origin with differing radiosensitivities and their molecular response to radiation exposure have been used in the past to investigate the underlying mechanisms of radiation resistance in prostate cancer (Josson et al., 2008) these studies may not give an accurate picture of the molecular changes that cells undergo in order to become radioresistant due to differences in cell type. Isogenic cell lines represent attractive models for the determination of the molecular radiation response *in vitro*. They give the opportunity to study the changes that occur in cells over the course of treatment without confounding factors as they have the same origin and are genetically identical except for one characteristic, in this case resistance to *fractionated* radiation. By comparing the isogenic line to

the parent cell line it may be possible to elucidate the underlying mechanisms of the radioresistant phenotype.

Exposure of cancer cell models to a variety of fractionated radiation schedules has resulted in the selection of a cancer subpopulation with modified cell fate in response to subsequent radiation exposure and these isogenic models of radioresistance are increasingly used to investigate the molecular response of cells to radiation (McDermott et al., 2014).

Radioresistant models have been derived from a variety of cancer cell lines including the prostate. Isogenic models of LNCaP, PC-3 and DU145 cells were generated through 2 Gray (Gy) daily exposure over five consecutive days and associated with a dose modifying factor of 1.6, 1.5 and 1.5 respectively at 0.1% survival. (Skvortsova et al., 2008) The radiation-surviving cell population of DU145, PC-3, LnCaP and 22Rv1 cells following exposure to 35 doses of 2Gy were also isolated to examine effects on plasticity (Kyjacova et al., 2015) and neuroendocrine differentiation (Deng et al., 2008). Of the four commonly used prostate cancer cell lines, only one (22Rv1) may be representative of primary disease (Sramkoski et al., 1999) and thus might be the most suitable cell line to model the switch from a sensitive to resistant form of prostate cancer.

The need for the discovery of novel prognostic and predictive biomarkers of the radioresponse is widely recognized. A number of possible candidates for markers have been investigated using isogenic cell line models including changes in DNA damage response and cell cycle checkpoints, the development of micronuclei, apoptotic events and clonal cell survival. The potential of these models may however be underutilized and data to date indicates the selection of a stem-cell like cell population, suggesting isogenic radioresistant cell lines may present a new model for exploration of stemness properties in

radiobiology. Studies using these models show potential for the discovery of novel biomarkers of treatment response, the identification of new drug targets and the development of therapeutic agents that will increase tumour cell sensitivity to radiation therapy.

But care may need to be taken when evaluating these results. The current laboratory procedures for analysing these biological events may not be readily transferable to a clinical setting as a number of limitations prevent the reproduction of clinical radiotherapy delivery under experimental conditions, such as cell ageing and the necessity for recovery periods (McDermott et al., 2014). No standardised protocol for the generation or phenotypic validation of these cell lines currently exists resulting in different definitions of radiation resistance which may not be applicable in a clinical context. The characterisation of these cell lines in terms of the underlying mechanisms of oncogenesis and the radioresponse is therefore necessary to evaluate their utility and clinical relevance.

1.4 BIOMARKERS OF RADIATION RESISTANCE

Biomarkers are biological molecules present in tissues or body fluids which signify normal or abnormal process occurring in the body as well as pharmacological response to therapeutics and can be used accurately and reproducibly in the assessment of a disease (Strimbu and Tavel, 2010). The search for biomarkers of radiation sensitivity is difficult due to the complex nature of resistance and the lack of knowledge surrounding the exact molecular characteristics of those cells capable of initiating tumour regrowth following treatment with RT. When investigating markers of radioresistance one must take into account both the acquired and intrinsic radiosensitivity of cells. While some cancers such as renal cell cancer and melanoma are intrinsically resistant to radiation others like prostate cancer are initially controlled by treatment but eventually some patients fail and tumours recur. The

discovery of biomarkers for tumours destined to acquire resistance are necessary for the adequate control of prostate tumours and to stratify patients based on treatment outcome so those destined to fail RT are treated appropriately and do not endure unnecessary therapy and the side effects associated with it. Identification of biomarkers of radiation resistance has been conducted using a number of different approaches (Figure 1-2).

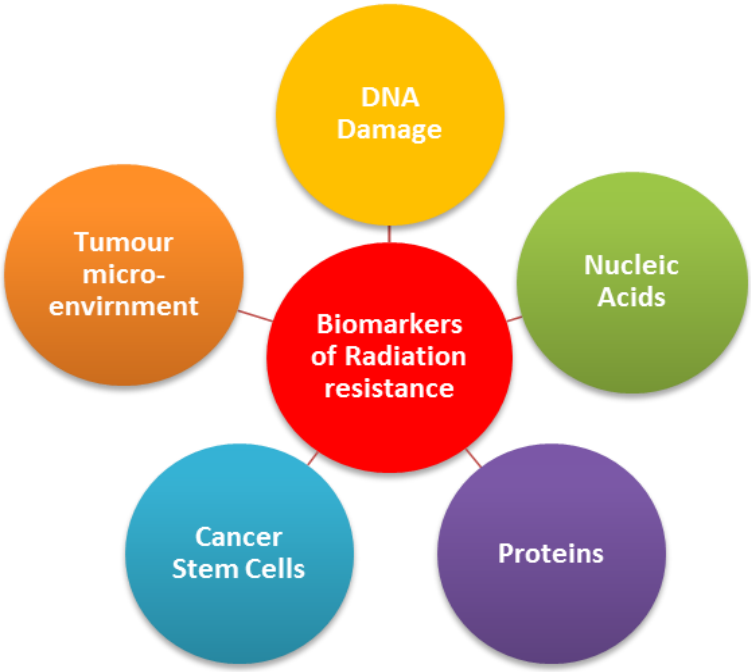

Figure 1-2: Biomarker Discovery

Sources and strategies for biomarker discovery

1.4.1 DNA DAMAGE

The study of changes commonly observed during the DNA damage response has resulted in the discovery of a number of potential biomarkers of the radioresistant phenotype. For example, use of the Comet Assay a technique which measures the formation of single (SSBs) or double strand breaks (DSBs) following exposure to radiation or genotoxic drugs, is a sensitive method which allows for the

assessment of DNA damage formation and repair capacity on a single cell level (Ostling and Johanson, 1984) both of which are commonly altered in cells with radioresistant phenotypes. This assay has been used to investigate genotoxic effects on cells (Singh et al., 1988, Olive, 1989, Olive et al., 1990, Singh et al., 1994) and as a biomarker of exposure to DNA damaging agents in bio-monitoring studies (Møller et al., 2000, Collins et al., 1997, Kopjar and Garaj-Vrhovac, 2001, Betti et al., 1994). It has been proposed as a biomarker of exposure and as a potential addition to standard biodosimetry methods for clinical workers occupationally exposed to radiation (Maluf et al., 2001, Garaj-Vrhovac and Kopjar, 2003) as well as in cases of incidental exposure (Garaj-Vrhovac et al., 2002).

The alkaline version of this assay has also been suggested as a predictive biomarker of radiation sensitivity as it is capable of detecting DNA damage caused by clinically relevant does of ionising radiation in cell lines and patient biopsy samples (McKenna et al., 2008) and has shown inverse correlation between cell line radiosensitivity and DNA damage and repair in bladder (Moneef et al., 2003, McKeown et al., 2003, McKelvey-Martin et al., 1998, McKenna et al., 2003), CaP (Dunne et al., 2003, Cardile et al., 2005), colorectal (Dunne et al., 2003), cervical (Banáth et al., 2004) (Gurska et al., 2007), lung (Bergqvist et al., 1998), ovarian (Bacova et al., 1999), renal (Smyth et al., 2007) and breast (Djuzenova et al., 2006) cancer cells as well as in normal fibroblasts which were assayed using the neutral version of the technique (Eastham et al., 1999). This assay is a quick, simple and relatively inexpensive biomarker of DNA damage and shows the potential for significant impact in the clinic.

Gamma-H2A histone family, member X (γ-H2AX) has also shown promise as a surrogate marker of radiosensitivity in cells. One of the earliest steps in the DNA damage response process is the formation of γ-H2AX, following the generation of DSBs, which results from the

phosphorylation of serine 139 on the H2AX protein (Rogakou et al., 1998). This quantitative biological response (Rogakou et al., 1999, Sedelnikova et al., 2002, Rothkamm and Löbrich, 2003) has shown great utility as a biomarker of DNA damage and repair (Martin and Bonner, 2006, Kuo and Yang, 2008). It can be used as a biodosimeter in the assessment of the biological response to both therapeutic (Sak et al., 2007) and diagnostic (Löbrich et al., 2005, Kuefner et al., 2010) radiation and DNA damaging chemotherapeutics (Clingen et al., 2008, Kinders et al., 2010). This DNA damage marker has great potential for the personalisation of disease management as a clinical indicator of cell radiosensitivity (Olive and Banáth, 2004, MacPhail et al., 2003, Banáth et al., 2004, Taneja et al., 2004), in the selection of the most appropriate treatment for individual patients and has been proposed as a target to modulate cell radiosensitivity for the improved treatment of resistant tumours (Taneja et al., 2004). It has also been adapted for high throughput screening (RABiT (rapid Automated BIodosoiometry Tool)) capable of assessing H2AX phosphorylation from a single drop of blood for the rapid triage of individuals exposed to radiation following large scale radiological events (Garty et al., 2010) and for future clinical and academic study of DNA repair kinetics (Turner et al., 2014).

1.4.2 GENE EXPRESSION

A number of gene mutations in pathways involved in cell survival have been implicated in the radiosensitivity of cancer cells including *P53* (Lee and Bernstein, 1993, Gudkov and Komarova, 2003), *RAS* (Sklar, 1988, Bernhard et al., 2000), *Raf-1* (Kasid et al., 1989, Warenius et al., 1994) and the *BCL-2* family (Condon et al., 2002, Rosser et al., 2003) genes but no one marker has been identified which can truly discriminate sensitive from resistant tumours. The use of gene arrays to identify expression profiles specific to the radioresistant phenotype (Kim et al., 2012) has resulted in the correlation of a number gene panels which are

dysregulated in resistant relative to sensitive cells in a number of cancers such as CaP (Hümmerich et al., 2006), oesophageal (Fukuda et al., 2004, Maher et al., 2008), pancreatic (Ogawa et al., 2006), head and neck (Ishikawa et al., 2006, Akervall et al., 2014, Dumur et al., 2009, Ishigami et al., 2007), melanoma (Ishikawa et al., 2006), lung (Lee et al., 2010, Guo et al., 2005) and cervical (Tewari et al., 2005, Kitahara et al., 2002). For example, in a study of 406 CaP patients receiving radiotherapy Hümmerich et al identified a panel of nineteen differentially expressed DNA repair related genes for which high expression levels associated with reduced clinical radiosensitivity (Hümmerich et al., 2006).

Another example is mutations in the Ataxia telangiectasia gene. Ataxia telangiectasia is a neurodegenerative syndrome that effects a number of different systems and shows increased sensitivity to ionising radiation (Paterson et al., 1979, West et al., 1995) and predisposition to cancer (Swift et al., 1991). Heterozygosity for the ataxia telangiectasia gene can result in increased risk of sensitivity to RT (Varghese et al., 1999) and increased susceptibility to breast cancer (Swift et al., 1987, Easton, 1994, Iannuzzi et al., 2002, Renwick et al., 2006, Broeks et al., 2000) without other phenotypic characteristics of the disease. Ataxia telangiectasia mutant (ATM) protein levels have been suggested as a possible biomarker of increased radiosensitivity and predictive marker of severe RT-induced side effects in breast cancer (Iannuzzi et al., 2002) (Fang et al., 2010). Although the identification of prognostic profiles for RT show potential as predictive and prognostic markers of the radioresistant phenotype in the clinic, the success of gene signatures as biomarkers of RT will rely on large scale clinical validation in order to generate truly predictive signatures of treatment outcome with reproducible results (Ein-Dor et al., 2006).

1.4.3 PROTEIN MARKERS

Proteomic analysis has also shown success in identifying deregulated proteins and pathways which have potential as biomarkers of prognosis and treatment outcome for radioresistant tumours in a multitude of cancers, including CaP (Skvortsova et al., 2008, Zhang et al., 2010a), lung (Yun et al., 2016, Brognard et al., 2001), breast (Wang et al., 2005, Chevalier et al., 2012, Smith et al., 2009), and head and neck (Skvortsov et al., 2011, Svendsen et al., 2011, Feng et al., 2010, Tribius et al., 2001, Riva et al., 1995). Pathways associated with cell growth, survival and motility were most commonly deregulated in radioresistant cells which suggests that a number of proteins involved in these processes have the potential to act as clinical biomarkers of the radioresistant phenotype. For example, decreased expression of the 26S proteasome was suggested as a potential predictive biomarker of RT in breast cancer and laryngeal tumours (Smith et al., 2009), while Skorvortka et al reported that a panel of 45 proteins were similarity modulated in radiation resistant pharynx and tongue cell lines and found the Ras-related C3 botulinum toxin substrate 1 (RAC1) protein to be a putative biomarker of head and neck squamous cell carcinoma (HNSCC) radioresistance (Skvortsov et al., 2011). In CaP specifically, Skvortsova et al identified a number of proteins involved in survival, cell motility and metastasis deregulated in DU145, LNCAP and PC3 radioresistant isogenic sublines compared to controls (Skvortsova et al., 2008). Zhang et al showed that increased expression of nuclear factor erythroid-2–related factor 2 (Nrf2) via loss-of-function mutations in its inhibitor Kelch-like ECH-associated protein 1 (Keap1) was associated with increased radioresistance in the DU145 cell line and may act as a potential target for re-sensitisation of cancer cells to radiation (Zhang et al., 2010a).

1.4.4 CANCER STEM CELLS

The CSC hypothesis postulates the existence of a subpopulation of immortal tumour initiating cells capable of self-renewal and pluripotency which are responsible for tumour initiation and development, metastasis and recurrence following treatment failure (Reya et al., 2001). The number of cancer stem cells per tumour has been associated with reduced tumour control following radiation exposure in animal models (Baumann M et al., 2009) and increased radioresistance of cancer stem cells compared to non-tumorogenic cells has been suggested (Printz, 2012, Pajonk et al., 2010, Vlashi et al., 2009, Lagadec et al., 2010). Following each dose fraction, it has been proposed, that non-stem cancer cells die and cancer stem cells potentially increase in number, participating in accelerated repopulation (Gao et al., 2013). Multiple studies have made the connection between this stem like population and pathways involved in the radioresponse which are often identified as deregulated in radioresistant cells. For example, the isolation of cells expressing surface markers indicative of stemness has been associated with increased clonogenic survival and more efficient repair of DNA damage following irradiation (Bao et al., 2006). In thyroid cancer, the survival of CD133+ cells following radionuclide therapy were proposed as a major cause for treatment failure (Ke et al., 2013). PKH26+ stem-cell like nasopharyngeal carcinoma cells displayed increased clonogenicity, sphere formation and resistance to radiotherapy through overexpression of c-MYC resulting in increased expression of the Checkpoint kinase 1 (Chk1) and Checkpoint kinase 2 (Chk2) cell cycle checkpoint proteins and activation of the DNA damage response (DDR) (Wang et al., 2013). Similarly Chk1 knockdowns increased radiosensitivity of prostate (Wang et al., 2012) and glioma (Wu et al., 2012a) cancer stem cells. In glioma-initiating cells, failure of irradiated cells to arrest at the synthetic-phase (S-phase) cell cycle entry checkpoint was associated with increased homologous recombination and increased radioresistance (Lim et al., 2012). Finally, cancer stem

cells may also achieve enhanced ROS defence through high levels of free radical scavengers (Diehn et al., 2009, Phillips et al., 2006). This connection between this CSC subpopulation and the radioresponse has resulted in the identification of potential new biomarkers of RT outcome in a number of cancers.

Increased expression of the CSC surface marker CD44 in HNSCC correlates with local tumour control following radiotherapy (de Jong et al., 2010). In glioblastoma, cells expressing CD133 , a marker of both neural and brain cancer stem cells, show increased survival following ionising radiation when compared to those tumour cells which are CD133 negative (Bao et al., 2006). Combinations of stem cell markers have also been used to isolate cells with increased resistance to radiation, $CD24^{-/low}/CD44^{+}$ breast cancer cells were identified as having decreased radiosensitivity along with lower levels of ROS production and decreased H2AX phosphorylation following irradiation compared to controls (Phillips et al., 2006), Sahlberg et al found that $CD44^{high}/CD133^{high}$ expressing cells were associated with increased radioresistance in colon cancer cell lines (Sahlberg et al., 2014) and chemo-radioresistant pancreatic sublines showed increased expression of stem cell markers Oct4, ABCG2, CD24 and CD133 along with increase tumorigenicity (Du et al., 2011). The successful isolation of radioresistant subpopulations of tumour cells by stem cell markers present the opportunity for prediction of patient outcome and to help in the management and design of personalized interventional strategies for patients.

1.4.5 TUMOUR MICROENVIRONMENT

The tumour microenvironment (TME) is an essential mediator of tumour cell regulation, proliferation and metastasis (Gatenby and Gillies, 2008). This complex network of interacting processes involved in metabolism (Cairns et al., 2011), immune response (Coussens and Werb, 2002,

Whiteside, 2008) and angiogenesis (Watnick, 2012) results in an ideal environment for cancer cells to progress and survive and plays an important role in therapeutic resistance. Exposure to ionising radiation is known to affect the TME and adds to its neoplastic potential and radioresistance through initiation of the inflammatory response (Barker et al., 2015). Inflammation is the process by which immune cells are drawn to sites of injury or infection resulting in tissue recovery. Normal acute inflammation is self-limiting while chronic inflammation is dysfunctional (Coussens and Werb, 2002) and leads to DNA damage and mutagenesis (Maeda and Akaike, 1998) resulting in the proliferation of cells with potentially augmented cell survival programs. Chronic inflammation contributes to a microenvironment which enables neoplastic promotion through the induction of growth factors and proangiogenic signalling, and the facilitation of genomic instability (Shacter and Weitzman, 2002). While biomarkers associated with the inflammatory response have yet to be identified for use in monitoring radioresistance of cancer cells, the induction of the inflammatory response following exposure to radiation has potential for exploitation in the search for markers of RT failure.

As tumour size increases cells become progressively more hypoxic resulting from a lack of access to adequate vasculature. While induction of angiogenesis, via the angiogenic switch (Hanahan and Folkman, 1996) and proangiogenic signalling (Watnick, 2012), results in the formation of new vasculature (neovascularisation), these vessels are defective resulting in erratic blood flow to tumours and a hypoxic microenvironment. Hypoxia is a well-established contributor to the radioresistant phenotype (Bush et al., 1978) and an indicator of RT outcome (Gray et al., 1953). The dynamic nature of the TME results in areas of both acute (due to fluctuations in oxygen perfusion through poor vasculature) and chronic (out growing blood supply) (Bristow and Hill, 2008, Harris, 2002) hypoxia making not just treatment with RT

difficult (Gray et al., 1953) but also significantly alters drug action (Shannon et al., 2003) depending on the oxygen status of the cells.

The detection of hypoxic regions in tumours, and with it radioresistance, is not only an exploitable target for increasing tumour radiosensitivity but can also help guide treatment, and aid in the discovery of biomarkers of the radioresistant phenotype. There is no current definitive marker of hypoxia in use clinically due to heterogeneous nature of tumour oxygenation and the complex nature of tumour blood supply and oxygen consumption in cells (Tatum, 2006), (Le and Courter, 2008). Methods for measuring hypoxia include direct approaches such as needle electrodes to measure oxygen partial pressure (pO2) (Kallinowski et al., 1990) and imaging (^{19}F- magnetic resonance imaging (MRI), blood oxygen level-dependent MRI and electron paramagnetic resonance imaging) (Tatum, 2006). As wells as indirect methods which rely on injectable molecular markers of oxygen binding like 2-nitroimidazole analogues (Chapman, 1991); pimonidazole (Raleigh et al., 1998), [2-(2-nitro-1H-imidazol-1-yl)-N-(2, 2, 3, 3, 3-pentafluoropropyl) a cet amide] (EF-5) (Koch et al., 1995, Hodgkiss et al., 1997) and 7(-)[4'-(2-nitroimidazol-l-yl)-butyl]-theophylline (NITP) (Hodgkiss et al., 1995) or endogenous markers as surrogates for hypoxia e.g, hypoxia-inducible factor-1-α (HIF1α) (Semenza et al., 1994) and its downstream targets Vascular endothelial growth factor (VEG-F) (Forsythe et al., 1996), glucose transporter 1 (Glut 1) (Ebert et al., 1995), carbonic anhydrase IX (CA9) (Wykoff et al., 2000) and erythropoietin (Hammond et al., 2014). While no gold standard marker of hypoxia currently exists (Tatum, 2006) both direct and indirect methods of measuring hypoxia show clinical promise and have demonstrated their utility as prognostic markers of survival and treatment outcome (Bussink et al., 2003). The use of hypoxia as a model of the radioresistant phenotype can guide in the identification of novel biomarkers of the radioresistant phenotype.

The low oxygen environment also selects for the glycolytic pathway, a reliance on which continues even after reoxygenation. This process results in acidification of the TME and with it an altered metabolic state, this is known as the Warburg effect (Warburg, 1925, Warburg, 1956). The acidotic TME has been suggested to select for cancer cells with increased resistance to chemotherapy (Fais et al., 2014) and increased invasive (Gatenby and Gillies, 2004) and metastatic potential (Brizel et al., 2001, Walenta et al., 2000). The increased uptake of glucose associated with this TME feature can be measured using the glucose analogue tracer fluorine 18 (F-18) fluorodeoxyglucose (FdG) positron-emission tomography (PET) (Som et al., 1980). This method in conjunction with other diagnostic tools is currently in use clinically for staging and continued monitoring of a number of tumour types during treatment (Kelloff et al., 2005, Antoch et al., 2004). Changes in metabolism show potential for exploitation not just in treatment management but also in the search for biomarkers of the radioresistant phenotype. For example Lnam-Lennon et al found dysregulated mitochondrial function and bioenergetics is associated with the radioresistant phenotype in an isogenic oesophageal cell line model which was further supported in patient samples of poor responders (Lynam-Lennon et al., 2014). This shows the potential utility of the altered metabolic state for predicting treatment outcome in patient undergoing RT.

The stressed state associated with the TME also results in the induction of autophagy (White, 2012), a lysosomal degradation pathway for recycling intracellular components (Mizushima, 2005). While this mechanism has both tumour suppressive and promoting properties (White, 2012), stimulation of autophagy under stressed conditions such as hypoxia (Tracy et al., 2007, Azad et al., 2008, Zhang et al., 2008a) and acidosis (Marino et al., 2012, Wojtkowiak et al., 2012) is pro-tumorigenic. Increased induction of autophagy under these conditions results in the breakdown of intracellular organelles and the production

and release of substrates which support survival in the nutrient poor TME and removes damaging ROS during periods of acute hypoxia (Zhang et al., 2008a, Rouschop et al., 2009) thus preventing cell death and promoting tumour survival. Increased autophagy has been implicated in radioresistant phenotype in multiple cancers including glioblastoma (Ito et al., 2005), lung (Zhang et al., 2016), breast (Han et al., 2014) and prostate (Liao et al., 2015) and has shown potential as a therapeutic target in the modulation of treatment efficacy in both RT (Apel et al., 2008) and chemotherapy (Yang et al., 2011).

The influence of radiation exposure on the tumour microenvironment is an increasingly studied area and monitoring changes in the microenvironment presents a valuable tool for prediction of tumour radiosensitivity and RT outcome as well as a target for increasing RT efficacy (Barker et al., 2015). Investigation of underlying molecular changes associated with the TME of radio- sensitive and insensitive samples in combination with functional imaging may aid in the discovery of novel biomarkers associated with the radioresistant phenotype (Dewhirst and Chi, 2013).

1.5 miRNA AS BIOMARKERS OF RADIATION RESISTANCE

Micro Ribonucleic Acids (miRNAs/miRs) play a significant role in both normal and pathogenic processes (Kloosterman and Plasterk, 2006, Sayed and Abdellatif, 2011), and have shown considerable potential as biomarkers of a variety of disease including diabetes (Chen et al., 2008), cardiovascular disease (Fichtlscherer et al., 2010, Wang et al., 2010), hepatic related disease (Zhang et al., 2010b, Li et al., 2010) and neurodegenerative diseases (Cogswell et al., 2008) as well as cancer (Calin and Croce, 2006). These endogenous small non-coding molecules consist of ~22 nucleotides in their mature form- cleaved from

a 70-100 nucleotide hairpin pre-miR precursor. miRNA negatively regulate gene expression through a number of process' including cleavage of target mRNA and posttranscriptional suppression of translation (Bartel, 2004, Bartel, 2009, Ambros, 2004).

miRNA are known to play a prominent role in a number of fundamental biological processes such as developmental timing (Lee et al., 1993, Reinhart et al., 2000), stem cell regulation (Hatfield et al., 2005, Houbaviy et al., 2003), cell differentiation (Esau et al., 2004, Chen et al., 2006, Yi et al., 2008, Makeyev et al., 2007), and proliferation (Hayashita et al., 2005, Johnson et al., 2007, Corney et al., 2007). miRNA also play a vital role in cellular processes including cell cycle (Bueno and Malumbres, 2011), DDR (Hu and Gatti, 2010, d'Adda di Fagagna, 2014) and cell death (Jovanovic and Hengartner, 2006) all of which are modulated in response to radiation.

miRNA have similarly been shown to contribute to the deregulation of cellular processes in cancer. Changes in the expression levels of miRNA were first identified in B-cell chronic lymphocytic leukaemia (Calin et al., 2002) and have now been associated with a number of different cancers including prostate cancer (Ozen et al., 2008, Schaefer et al., 2010). The contribution made to oncogenesis by miRNA is varied (Calin and Croce, 2006, Zhang et al., 2007) including the oncogenic promotion of cancer as seen with the miR-17-92 cluster in lung cancer and lymphoma (Hayashita et al., 2005, He et al., 2005b) while also known to function as tumour suppressors inhibiting cancer via the regulation of oncogenes e.g. miRNA Let-7 regulation of RAS (Johnson et al., 2005).

Radiation induced modulation of miRNA is well established in the literature in both in vitro (Ishii and Saito, 2006, Simone et al., 2009, Josson et al., 2008) and in vivo (Koturbash et al., 2008, Ilnytskyy et al., 2008) models. miRNA have been widely reported for their potential as

diagnostic and prognostic markers of both tumorigenicity and treatment outcome (Yanaihara et al., 2006, Kosaka et al., 2010, Lu et al., 2005) as well as for their role in oncogenesis. A number of different studies have found deregulated miRNAs associated with radioresistance in a variety of cancers (Metheetrairut and Slack, 2013, Czochor and Glazer, 2014). miRNA-based biomarkers present additional advantages. First, they are relatively stable in tissue and biofluids even after standard sample processing when compared to mRNA (Hall et al., 2012, Zhang et al., 2008c), whose loss of integrity following fixation and long term storage is a limiting factor in their utility (von Ahlfen et al., 2007). Second, they can be rapidly quantified by standard molecular techniques including array based technologies and real-time reverse transcription polymerase chain reaction (q-RTPCR).

While the underlying mechanisms for radioresistance in CaP remain poorly understood there is a growing body of evidence showing that miRNAs play an important role in this pathology (Wen et al., 2014). Their ability to control radiobiological relevant cellular processes strengthens their potential as novel biomarkers of response to radiation therapy and could guide patients with nonresponsive tumours towards other treatment options, thus preventing unnecessary exposure to the risks associated with RT.

1.6 AIMS AND OBJECTIVES

In light of the high rate of biochemical recurrence in patients undergoing RT for CaP the need for clinical biomarkers of treatment outcome is clear. The development of isogenic radiation resistant cell line models allow one to investigate mechanisms and biomarkers of acquired radioresistance in cancer cells identified following fractionated exposure to RT without the confounding effects associated with using lines with different intrinsic sensitivities due to different cell origins. miRNA expression profiling presents an excellent opportunity for novel biomarker discovery for RT treatment response in prostate cancer due to; their abundance, tissue specificity, ease of detection in samples and the opportunity for manipulation of these molecules in cell lines to aid in the detection of downstream targets.

The specific aims of this thesis include:

1. To generate a radiation resistant cell line model of prostate cancer using a clinically relevant dose of fractionated radiation. (Chapter 3)

2. To validate and characterise the radioresistant phenotype of the isogenic cell line model using known mechanism of the radioresponse. (Chapter 4)

3. To identify a list of candidate miRNA biomarkers of the RT failure using three models of radiation resistance: Hypoxia, Isogenic model of acquired resistance and cancer stem cells. (Chapter 5)

4. To select and validate miRNAs commonly deregulated in the radioresistant models in cell lines. (Chapter 5)

5. To use in-silico prediction tools to generate a list of predicted targets of miRNA selected for further investigation. (Chapter 6)

6. To transfect the radiation resistant isogenic cell line with the selected miRNA and investigate its impact on the radiosensitivity of the model. (Chapter 6)

7. And finally, to validate the predicted targets mRNA expression levels in the transfected radiation resistant cell line. (Chapter 6)

CHAPTER 2:

MATERIALS AND METHODS

See appendix 1 for manufacturer's details.

2.1 CELL CULTURE

2.1.1 CELL LINES

The human prostate cell lines: two malignant 22Rv1 and Du145, one normal PWR1e and one benign BPH1 were obtained from American Type Culture Collection (Table 2-1). All purchased cell lines were authenticated using short tandem repeat profiling prior to use (DDC medical). 22Rv1, Du145 and BPH-1 cells were maintained as monolayers in complete growth media containing RPMI cell culture medium, l-glutamine (Lonza) with 10% fetal bovine serum (gibco) and 1% penicillin-streptomycin (Lonza) for complete growth medium. The PWR1e cell line was grown in kerationocyte serum free media (Gibco) supplemented with bovine pituitary extract (Gibco), epidermal growth factor (Gibco) and 1% penicillin-streptomycin to make complete growth media.

The cells were maintained in a forma steri cycle CO_2 incubator (Thermo Scientific) at 37°C in 95% humidified air containing 5% CO_2 and subcultured once to twice weekly to maintain exponential growth unless otherwise stated. Cells were grown in T25cm^2 (SPL Life Sciences), T75cm^2 (SPL Life Sciences), T175cm^2 (SPL Life Sciences) or 6 well plates (SPL Life Sciences)

Table 2-1: Prostate Cancer Cell lines

Cell Line	Source	Morphology	Features	Reference
22Rv1	Human prostate carcinoma epithelial cell line derived from a xenograft that was serially propagated in mice after castration-induced regression and relapse of the parental, androgen-dependent CWR22 xenograft.	Epithelial	AR positive PSA positive	(Sramkoski et al., 1999)
DU145	Brain metastases from 62 year old Caucasian adult male with prostate carcinoma	Epithelial	AR negative PSA negative P53 mutant	(Stone et al., 1978) (Isaacs et al., 1991)
PWR-1E	Normal prostate from 67 year old Caucasian male	Epithelial	AR positive PSA positive p53 positive	(Webber et al., 1996)
BPH-1	Prostate epithelial cells from a 68-year-old man with benign prostate hyperplasia	Epithelial	AR negative PSA negative P53 positive	(Hayward et al., 1995)

All data sourced from the American Type Culture Collection
Abbreviations: AR: androgen receptor; PSA: prostate specific antigen

2.1.2 SUBCULTURING OF CELL LINES

All cell culture was performed aseptically in a Telestar Bio-II-A laminar flow hood. Cells were checked daily using inverted phase contrast Optika B380 series microscope (Optika). Cells were subcultured when growth reached 70-80% confluency in order to maintain exponential growth. Growth media was decanted from flasks and adherent cells washed with 8ml of pre-warmed at 37°C phosphate buffered saline (PBS) (Lonza) to remove dead cells and remaining growth media, cells were then harvested using 1ml ($T25cm^2$), 2ml ($T75cm^2$) or 5ml ($T175cm^2$) of trypsin-ethylenediamiane tetra acetic acid (trypsin-EDTA) (Lonza) and incubated at 37°C for 5-10 min until all cells had detached from the flask. The trypsin was then deactivated using 4ml ($T25cm^2$), 8ml ($T75cm^2$) or 20ml ($T175cm^2$) of complete growth media. Cell suspensions were transferred to 30 ml sterile universal tubes (Sparks) and pelleted by centrifugation (Thermo Scientific) at 1300 x rpm for 3min at room temperature. Following centrifugation the supernatant was then decanted and the cells resuspended in 1ml complete media. Cells were then seeded and maintained in 10ml ($T25cm^2$ flask), 15ml ($T75cm^2$ flask) or 30ml ($T175cm^2$ flask) complete growth media at 37°C in 95% humidified air containing 5% CO_2.

2.1.2.1 DOUBLET FORMATION

Cells were trypsinised using trypsin-EDTA in order to reduce the number of doublets. Resuspension using P20 pipette tips was used to separate cell clumps. The 22Rv1 cell line is prone to increased doublet formation compared to other cell lines. Particular care must be taken to insure that cells are in single cell suspension prior to use in assays.

2.1.3 FROZEN CELL LINE STOCKS

2.1.3.1 PREPARATION OF FROZEN CELL LINE STOCKS

Cell stocks were prepared from early passage cells (where possible) in their exponential phase. Cells were washed, trypsinised, pelleted and the supernatant decanted as previously described. The cells were then resuspended in 3ml (T75cm^2 flask) of CellBanker 2 (Amsbio) cryopreservation media and 1ml aliquots were transferred to sterile 2ml screw cap tubes (Sarstedt) and placed at -80°C for long term storage.

2.1.3.2 REVIVAL OF FROZEN CELL LINE STOCKS

Cell aliquots were removed from -80°C and thawed rapidly; the cell suspension was immediately transferred to a sterile tube containing 9ml complete growth media and centrifuged in a Centre-GPs centrifuge (Thermo scientific) at 1300 x rpm for 3 min at room temperature. Supernatant was decanted and the pellet resuspended in 1ml complete media. Cells were then seeded into T25cm^2 flasks containing 10ml appropriate complete media (section: 2.1.1). Cells were allowed adhere overnight and dead cells removed the next day by removing media, washing with PBS and replacing with fresh complete growth media. Cells were maintained at 37°C in 95% humidified air containing 5% CO_2 and transferred to T75cm flasks once 70-80% confluency was reached. Cells were then subcultured as previously described.

2.1.4 CELL COUNTING

Cells were counted using brightline haemocytometer (Hausser scientific). Cell viability was assessed using trypan blue (Sigma-Aldrich) exclusion assay. Following harvesting 10µl of cells in growth media was added to 190µl trypan blue and vortexed briefly. 10µl of the trypan blue cell suspension was then added to the counting chamber of the haemocytometer. Viable live cells which exclude trypan blue and remain unstained were counted and dead cells which had absorbed trypan blue due to disruption in their membranes were disregarded. Viable cells were counted in each of the four corners and the central square of the grid and an average calculated. The following equation was used to calculate cells/ml:

$$\left(\frac{C1 + C2 + C3 + C4 + C5}{5}\right) \times (dilution\ factor) = X \times 10^4\ Cells/ml$$

2.1.5 MYCOPLASMA TESTING OF CELL LINES

Cell lines were tested monthly in the department for mycoplasma contamination using the polymerase chain reaction (PCR)-based method as previously described in nature protocols (Young et al., 2010b). Briefly 1ml culture medium was collected from 70-80% confluent cells. The supernatant was centrifuged for 1min at 2000 x rpm to pellet cell debris. The PCR reaction was combined as per Table 2-2 below

Table 2-2: Mycoplasma PCR reaction

Reagent	Volume (µl)
Green GoTaq mastermix*	25
Primers $^{\Psi}$ (forward$^{\Delta}$ and reverse$^{\phi}$)	1
Nuclease-free H_2Os	22
Growth media supernatant	1
Total per reaction	50

* Promega
$^{\Psi}$ Sigma-Aldrich
$^{\Delta}$ Forward: 5' to 3' - TGCACCATCTGTCACTCTGTTAACCTC
$^{\phi}$ Reverse: 5' to 3' - GGGAGCAAACAGGATTAGATACCCT

Samples were placed in the PTC-100 thermocycler (MJ Research) under the conditions in Table 2-3.

Table 2-3: Mycoplasma testing thermocyler programme

	Step 1	Step 2	Step 3	Step 4	Step 5
Temperature (°C)	95	94	55	72	72
Time	5 min	20 min	30 s	1 min	10 min

Following reaction completion the PCR was run on a 2% agarose gel with an expected band size of 270bp.

2.2 CELL TREATMENTS

2.2.1 IRRADIATION PARAMETERS

Unless otherwise stated all cells were irradiated as monolayers in 6-well plates, T25cm flasks and T75cm flasks at room temperature using an RS225 cabinet irradiator (Gulmay Medical) according to the dose rate and times below (Table 2-4).

Table 2-4: X-strahl RS225 dose rate and exposure times

Dose (Gy)	Rate	Time (min:sec)
0	-	-
2	200kV, 15mA	0:38
4	200kV, 15mA	1:15
6	200kV, 15mA	1:54
8	200kV, 15mA	2.31
10	200kV, 15mA	3:08

2.2.2 ESTABLISHMENT OF ISOGENIC RADIATION RESISTANCE CELL LINES

Cells were grown to approximately 70-80% confluency in vented T75cm^2 culture flask and irradiated with 2Gy x-rays. Cells were allowed to recover before being trypsinised and subcultured. This protocol was repeated to a total cumulative dose of 60Gy. Un-irradiated cells were grown in parallel to the isogenic cell lines resulting in age matched control (AMC) cell lines. Cells stocks were frozen in cell banker 2 after each 10Gy cumulative dose (approx. every five weeks).

2.2.3 HYPOXIA TREATMENT

Hypoxia (0.5% O_2, pO_2 < 2mmHg) was achieved by exposing cells in a 1000 *in vivo* hypoxic chamber (BioTrace) to a mixture of nitrogen, CO_2 (5%) and compressed air to achieve a 0.5% oxygen concentration.

Cells grown as monolayers in T75cm^2 flasks or 6-well plates at room temperature were placed into the chamber for 24 h or 48 h according to the experiment parameters. Following incubation cells were harvested for treatment in the chamber to prevent re-oxygenation.

2.3 CLONOGENIC CELL SURVIVAL ASSAY

Cell survival was evaluated using a standard colony forming assay. Clonogenic cell survival assays were carried out after each 10Gy cumulative dose in order to assess radiosensitivity of the isogenic cell lines.

Briefly, cells in exponential growth phase (approx. 70% confluent) were harvested by trypsinisation and counted in order to obtain a single cell suspension of 1x10^5 cells/ml. Cells were seeded into six well plates at a density of 1x10^3 - 1x10^4 cells/well and allowed to adhere to plates overnight in the incubator at 37°C in 95% humidified air containing 5% CO_2. Cells were then irradiated using increasing doses of radiation from 2-10Gy in 2Gy increments respective of the cell density and controls mock irradiated before being incubated for 7-10 days under the conditions previously described. Following incubation colonies were then fixed and stained using 0.05% crystal violet (Sigma-Aldrich) in 70% methanol solution (Fisher Chemical). Plates were dried overnight and colonies containing > 50 cells counted using the ColCount instrument (Oxford Optronix Ltd). Plating efficiency (PE) for each cell line (Table 2-5) was calculated using the formula:

$$PE = \frac{no.colonies}{no.cells\ seeded}$$

Table 2-5: Cell line Plating efficency

Cell Line	PE
BPH-1	0.17
DU145	0.22
PWR-1E	0.12
22Rv1	0.16
AMC-22Rv1	0.17
RR-22Rv1	0.23

Surviving Fraction was then calculated using the formula:

$$SF = \frac{no.colonies}{no.cells\ seeded\ x\ PE}$$

The mean and SEM was calculated for each dose (2-10Gy).

2.3.1 CLONOGENIC SURVIVAL OF TRANSFECTED CELLS

Cells transfected in 6-well plate were grown for 24 h prior to treatment with mock or 6Gy irradiation. Cells were then returned to the incubator for 1 h before harvesting and counting. The mock irradiated controls were seeded with $1x10^3$ and the 6Gy plates seeded with 6×10^3 cells per well. 2ml complete media was then added to each well and incubated under normal conditions. The plates were monitored for clone formation, stained and counted as above. Survival fraction as described above.

2.4 CELL LINE TRANSFECTIONS

2.4.1 TRANSIENT TRANSFECTION

*mir*Vana™ miRNA Mimics were purchased from Ambion®. A reverse transfection procedure was used to transfect 22Rv1 radiation resistant cells with hsa-miR-4284, hsa-miR-1 positive control and a negative scrambled control. The 5 nmol dried miRNA mimic was resuspended to a stock solution concentration of 100µM in nuclease free water (H_2O) (Sigma-Aldrich). A working solution of 10uM (10 pmol/µl) in nuclease free H_2O was aliquoted for immediate use and the remainder aliquoted and stored at -20°C for future use.

All cell transfections were carried out in 6-well plates. The cells were harvested and counted as previously described and a cell suspension of 1 x 10^6 cells/ml was made. Lipofectamine RNAi max transfection reagent (Ambion) was diluted in Opti-MEM® Medium (Ambion) in a ratio of 9µl:150µl per well. 3 µl of the miRNA mimic or scramble was mixed with 150 µl Opti-MEM and combined with the diluted transfection reagent and incubated for 5 mins at room temperature. 250µl of the miRNA/lipid complex and 2.5ml Opti-MEM was added per well in duplicate resulting in 25pmol miRNA/well and 7.5µl Lipofectamine RNAi max. Controls were grown without mimics/scramble and transfection reagents. 2.5 X 10^5 cells were seeded in each well and returned to the incubator for 24 h. Transfection was verified using q-RTPCR as described in section 2.8.

2.5 FLOW CYTOMETRY

2.5.1 PREPARATION OF CELLS

Cells harvested and counted as previously described. Cells were seeded in T25cm^2 flasks at a density of 5 X 10^5 (WT) and 3 X 10^5 cells/ml (RR and AMC) and allowed adhere overnight. They were then treated as necessary and harvested using trypsin.

2.5.2 APOPTOSIS ASSAY

Apoptotic cells were detected using Annexin V-FITC/PI staining and flow cytometry (Vermes et al., 1995). Cells seeded the previous day as described above were used in this assay and media was replaced prior to treatment to remove dead cells. Following exposure to 0, 2 or 4Gy x-rays cells were incubated for 6 h before being trypsinised and combined with supernatant containing non-adherent cells. Cells were centrifuged at room temperature for 3 min at 1300 rpm, washed twice with PBS and resuspended in ice cold binding buffer (0.1M hepes (Sigma-Aldrich), 1.4M sodium chloride (NaCL) (Fisher Chemical), 25mM calcium chloride ($CaCL_2$), pH 7.4). Cells were stained with 3µl Annexin V-FITC (IQ Products) and incubated at 4°C for 30 mins in the dark. The Cells were washed in 2ml ice cold binding buffer and resuspended in 500µl binding buffer and Propidium Iodide (PI) (0.05µg/ml) (Sigma-Aldrich). Controls containing no annexin V and no PI were included for analysis. Cell were analysed using CyAnTM ADP Analyser (Beckman Coulter).

2.5.3 CELL CYCLE ANALYSIS

Cell cycle analysis was performed using PI staining and flow cytometry (Krishan, 1975). Cells were seeded as previously described. Cells were irradiated with 2Gy and 4Gy x-ray radiation and control flasks were

mock irradiated. Cells were then harvested at 4 h, 8 h, 12 h, 24 h and 30 h post irradiation, cells were harvested using trypsin-EDTA, transferred to 5ml reaction tubes (Grenier Bio-One) and washed with PBS. The supernatant was decanted after spinning at 180g X 3min and the pellet was fixed and permeabilised by drop-wise addition of 4ml ice cold 70% ethanol (EtOH) (Sigma-Aldrich) while vortexing to avoid formation of cell aggregates. Cells were fixed for a minimum of 2 h at 4°C. Fixed cells were centrifuged at 180g x 3min and the supernatant decanted, samples were washed with PBS and centrifuged as before. Each sample was then resuspended in 0.5ml Triton X-100 (0.1% v/v in PBS) (Sigma-Aldrich) containing PI (0.02mg/ml) and RNase A (0.2mg/ml) (Sigma-Aldrich), unstained controls were resuspended 0.5ml PBS. Samples were incubated at 37°C for 30 min and analysed immediately using CyAnTM ADP Analyser.

2.5.4 SENESCENCE

β-Galactosisdase activity was measured by flow cytometry as previously reported (Noppe et al., 2009). Cells were seeded in 6 well plates at a density of 1-2 x 10^5 cells/ well and allowed adhere overnight then irradiated at 2Gy, 4Gy or 8Gy and returned to the incubator along with mock irradiated controls for 24 h. Cells were washed with PBS and incubated for 1 h at 37°C, 5% CO_2 with bafilomycin A1 (Sigma-Aldrich) supplemented growth media in order to adjust lysosomal pH to pH6. 2mM 5-Dodecanoylaminofluorescein di-β-D-galactopyranoside (C_{12}FDG) (Sigma-Aldrich) was added and samples were incubated for 1 h before analysis alongside controls. Cells were then trypsinised and resuspended in 1ml PBS and analysed immediately using CyAnTM ADP Analyser.

2.5.5 REACTIVE OXYGEN SPECIES MEASUREMENT

Intracellular oxygen species were detected using Di(Acetoxymethyl Ester) (6-Carboxy-2',7'-Dichlorodihydrofluorescein Diacetate) (CM-H_2DCFDA) (Life technologies) and flow cytometry (Eruslanov and Kusmartsev, 2009). Exponentially growing cells were collected by trypsinisation. Samples were incubated for 30 min in the dark at 37°C, 5% CO2, 95% humidified air in PBS containing 10μM CM-H2DCFDA, with controls incubated in PBS. After incubation cells were pelleted at 180 x g for 3 min and the supernatant decanted. Samples were resuspended in PBS and irradiated with 2Gy or 4Gy x-rays, controls were mock irradiated and placed directly on ice. PI (1mg/ml) was added to the samples except appropriate controls. Samples were analysed immediately using CyAnTM ADP Analyser

2.5.6 DATA ANALYSIS

All flow cytometry data was analysed using Flowjo software (FlowJo LLC) and all statistical analysis carried out using Graphpad Prism (version 6 for mac OS X and windows 7, Graphpad software.)

2.6 CATALASE ASSAY

Catalase activity was measured using a qualitative visual method which determines the enzymes presences in the cells using hydrogen peroxide (H_2O_2) as previously reported (Iwase et al., 2013). The addition of $H_2O_2$to catalase results in the generation of H_2O and O2. This was then captured and quantified by the addition of detergent to cell pellets. Foam formation was measured and compared to a standard curve generated using the measurement of foam from known units of catalase. Catalase powder (Sigma-Aldrich) was dissolved in 100-μL ddH_2O for each concentration of catalase standards (0-200units). Cells harvested as previously described were resuspended in Pyrex tubes

(Corning) in varying concentrations (10^5–1.5^7 cells) in 100µl of PBS prior to mock or 4 Gy irradiation. 100µl of 1% Triton X-100 and 100µl undiluted H_2O_2 (30%) (Sigma-Aldrich) was added to samples, mixed thoroughly and incubated at room temperature for 15 min before the height of O_2-forming foam was measured with a ruler. Catalase concentration was calculated using the standard curve.

2.7 ALKALINE COMET ASSAY

The alkaline comet assay (Singh et al., 1988) was used to determine levels of DNA damage and repair capability after irradiation. Other than cell culture all aspects of the experiment were performed under reduced light to prevent additional DNA damage.

2.7.1 SAMPLE/SLIDE PREPARATION

Cells were harvested and counted as previously described, 170µl low melting point agar (0.6% in PBS) (Sigma-Aldrich) was added to aliquots of 4 x 10^4 cells and 80µl of the cell/agar suspension was layered onto slides pre-coated with normal melting point agar (1% in dd$H_2$0) (Sigma-Aldrich), a cover slip was then placed on top and the process repeated to form two gels on each slide, the gels were then allowed to solidify on ice and the cover slips removed. All slides were made in duplicate with two gels per slide resulting in 4 gels per treatment.

2.7.2 SAMPLE TREATMENT AND LYSIS

Cell slides were irradiated in the dark on ice as previously described (with either 2Gy-10Gy (dose response study) or 8Gy (repair study) and unirradiated controls were mock irradiated. In order to investigate the repair capabilities of the cell lines slides were incubated at 37°C, 5%

CO_2, 95% humidified air in RPMI media containing 20% FBS and 5% pen/strep for 5 mins, 15 mins, 30 mins or 50 mins. Control slides were placed directly into lysis buffer. Slides were placed immediately into ice cold lysis buffer (100mM NA_2EDTA (Sigma-Aldrich), 2.5M NaCL, 10mM Tris-HCL(Sigma-Aldrich), pH 10 with 1% Triton X added immediately before use) following irradiation (dose response) or incubation (repair study) and lysed overnight at 4°C.

2.7.3 ELECTROPHORESIS AND NEUTRALISATION

Following lysis slides were drained and transferred to a horizontal electrophoresis tank which was surrounded with ice. Ice cold alkali electrophoresis buffer (300mM NaOH (Sigma-Aldrich), 1mM Na_2EDTA, pH13 (Sigma-Aldrich)) was added to the tank and the slides left to incubate for 20 minutes to allow for DNA unwinding. Electrophoresis was carried out at 30V and 30mA for 20 mins in the dark. Slides were removed from the tank, 1ml of neutralization buffer (0.4M Tris-HCL, pH7.5 (Sigma-Aldrich)) was added to each slide and left for 20 mins. Slides were then washed twice by covering with ice-cold ddH_20 for 10 minutes and transferred to the incubator to dry for 2 hours at 37°C.

2.7.4 STAINING

The gels were rehydrated with ddH_20 for 30 mins before each slide was flooded with 1ml PI (2.5µg/ml) and incubated at room temperature for 15-20 mins. The slides were rinsed by covering with ice-cold ddH_20 for 20 mins and placed in the incubator overnight to dry.

2.7.5 COMET IMAGE SCORING AND ANALYSIS

Comets were visualised and measured using Komet 5.0 software (Kinetics imaging software ltd, Brombourough UK) and fluorescent

microscopy at a magnification of 20X. 50 comets scored/ gel (Price et al., 2000) in order to give a representative result of the cell population.

The % tail DNA was selected as the parameter that best reflected DNA damage. % tail DNA was calculated from three (dose response) or four (repair study) independent experiments containing duplicate measures and presented as mean ± SEM (standard error mean).

2.8 MOLECULAR BIOLOGY

2.8.1 PREPARATION OF RNA CELL EXTRACTS

Cells grown to ~70-80% confluency as previously described were harvested by cell scraper rather than trypsinisation to ensure the integrity of the RNA sample. Briefly, the old media was removed and 5ml (T25cm^2) or 10ml (T75cm^2) of ice cold PBS added to the flask, cells were then scrapped form the surface of the flask using cell scrapers (Sarstedt) and the cell suspension decanted to a 30ml sterile tube, the cells were centrifuged at 1300rpm for 3 mins and immediately placed on ice. The supernatant was discarded, the pellet washed in 1ml ice cold PBS and transferred to a 1.5ml tubes (Sarstedt) tube and centrifuged in a bench top 5415 R microcentrifuge (eppendorf) at 500 x g for 5 minutes. The PBS was removed by vacuum and the dry pellet snap frozen on dry ice and stored at -80°C until extraction.

2.8.2 RNA ISOLATION

2.8.2.1 TRIAZOL RNA EXTRACTION

Cells were harvested as previously described and pellets were stored at -80°C until extraction. In the fumehood the frozen pellet was resuspended in 1ml TRI Reagent (Ambion) and left to homogenate at room temperature for 5 min. 100µl 1-bromo-2 chloropropane (BCP)

(Sigma-Aldrich) was added and the samples were mixed by inversion for 15 seconds (s) before incubation at room temperature for 15 min. Samples were then centrifuged at 4°C, 12,000 X g for 1 min. The upper aqueous phase was transferred to 1.5ml non-stick RNase-free tubes (Ambion). The RNA was precipitated using 1 ml isopropanol (Sigma-Aldrich) and shaken vigorously by inversion and left to homogenate at room temperature for 10 min. The samples were then centrifuged at 12,000 X g for 10 mins at 4°C and the supernatant discarded. The pellet was washed with 1 ml 75% EtOH in nuclease free H_2O and centrifuged at 7,500 x g for 5 mins at 4°C, the supernatant was discarded and the pellet air dried for 5 mins on the bench. The RNA was finally resuspended in 50µl nuclease free H_2O and incubated at 55°C for 10 mins to insure it fully dissolved. The quantity of RNA was assessed using the Qubit Fluorometer 1.0 (Invitrogen) as per manufactures instructions.

2.8.2.2 miRCURY™ RNA ISOLATION KIT

For the purpose of the Exiqon miRNA Array the miRCURY™ RNA Isolation Kit (Exiqon) was used to extract small RNAs from cell line samples as per the manufacturer's instructions. Briefly, 1-3 x 10^6 cells were collected and stored as previously described until processing. Centrifuge and buffers from this point forward used at room temperature. Pellets were lysed directly from frozen, 350µl lysing solution was added per pellet and vortexed for 15s until fully dissolved. 200 µl 100% EtOH was added to the lysate and vortexed for 10s. 600 µl of lysate was applied to a spin column and centrifuged for 1 min at 3,500 x g and the flow-through discarded. The column was washed three times with 400µl of wash solution and centrifuged for 1 min at 14,000 x g and the flow-through discarded. The column was dried by centrifugation for 2 min at 14,000 x g. The column containing the RNA was placed in an elution tube (Exiqon) and 50µl of elution buffer added, samples were centrifuged for 2 mins at 200 x g, followed by 1 mins at

14,000 x g and the elution containing the RNA retained. The RNA was quantified using the Qubit® Fluorometer 1.0 and stored at -80°C before shipping to Exiqon on dry ice.

2.8.3 RNA QUANTIFICATION

Total RNA samples were kept on ice throughout RNA quantification. The Qubit® Fluorometer 1.0 and Qubit® RNA BR assay (Invitrogen) was used to evaluate RNA concentration in all RNA samples as per the manufactures instructions. Briefly, a working solution was made by combining the Qubit® RNA buffer and RNA reagent at a ratio of 200:1, two standards were made by combining 190µl RNA reagent with 10µl of standard A and standard B. 2µl or 5µl of the RNA sample was then combined with 198µl or 195µl of working solution and vortexed with the standard's and incubated at room temperature for 2 mins. The fluorometer was then calibrated using the standards and the RNA concentration of each sample calculated.

2.8.4 cDNA SYNTHESIS

2.8.4.1 mRNA REVERSE TRANSCRIPTION

To synthesise complementary DNA (cDNA) from total RNA containing messenger RNA (mRNA) reverse transcription PCR (RTPCR) was performed. The high-capacity cDNA reverse transcription kit (Applied Biosystems) was used and all RNA samples and experimental reagents were thawed and stored on ice for the duration of the experiment. Following thawing, reagents were mixed briefly and centrifuged and RNA sample concentration was adjusted to 2ng/µl in nuclease free H_2O. The MultiScribe Reverse Transcriptase reagent was removed from -20°C storage and thawed immediately before use to prevent degradation. All reagents except the template RNA were combined in a master mix according to Table 2-6.

Table 2-6: High-capacity cDNA reverse transcription reaction mix

Reagent	Volume (µl)
10x RT buffer	2
10x RT Primers	2
25x dNTP Mix	0.8
MultiScribe Reverse Transcriptase	1
Nuclease-Free H_2O	4.2
20ng Total RNA	10
Total per reaction	20

10 µl of the mastermix was added to a strip tube (Ambion) and 10ul of template total RNA combined resulting in a 20ul reaction. The samples were mixed by pipetting and centrifuged before being placed in a PTC-100 thermocycler using the programme outlined below (Table 2-7). Following reaction completion the cDNA was stored at -20°C until needed.

Table 2-7: mRNA cDNA synthesis thermocycler programme

	Step 1	Step 2	Step 3	Step 4
Temperature (°C)	25	37	85	4
Time (min)	10	120	5	∞

Note: H_2O control replacing the RNA with nuclease free H_2O and a no reverse transcriptase (noRT) control replacing the reverse transcriptase enzyme with nuclease free H_2O were also included.

2.8.4.2 miRNA UNIVERSAL CDNA SYNTHESIS KIT II

First strand cDNA synthesis for miRNA was completed using the Universal cDNA Synthesis Kit II from Exiqon. RNA sample concentration was adjusted to 5ng/µl in nuclease free H_2O. All RNA samples and kit reagents were thawed and stored on ice for the duration of the experiment. Following thawing reagents were mixed and briefly centrifuged. The enzyme mixture was removed from -20°C

storage and thawed immediately before use to prevent degradation. A mastermix containing all reagents except the RNA template was combined according to Table 2-8.

Table 2-8: Universal cDNA synthesis kit II reaction mix

Reagent	Volume (µl)
5x Reaction buffer	2
Enzyme mix	1
Nuclease-free H_2O	5
Template total RNA	2
Total per reaction	10

8 µl of mastermix was added to a strip tube and 2 µl of total RNA template added resulting in a 10ul reaction; this was mixed gently by pipetting, centrifuged and placed in a PTC-100 thermocycler under the conditions in the Table 2-9. Following reaction completion the cDNA was stored at -20°C until needed.

Table 2-9: miRNA cDNA synthesis thermocyler programme

	Step 1	Step 2	Step 4
Temperature (°C)	42	95	4
Time (min)	60	5	∞

Note: H_2O control replacing the RNA sample with nuclease free H_2O and a noRT control replacing the enzyme mix with nuclease free H_2O were also included.

2.8.5 QUANTITATIVE RT-PCR

Real-time PCR (q-PCR) was used to compare expression levels of mRNAs/miRNAs in cell line samples. All experiments had a minimum of 3 technical and biological replicates. Data initially analysed using the Expression Suite Software 1.0.3 (Life Technologies). Further to this the ΔΔCT method was used to calculate relative change in expression.

2.8.5.1 GENE EXPRESSION

The TaqMan® Gene Expression Master Mix (Applied Biosystems) was used for all gene expression experiments. cDNA samples (synthesised as per section 2.8.4.1) and primers (Table 2-14) were thawed and stored on ice for the duration of their use. The TaqMan Mastermix and primers were kept protected from light throughout the experimental procedure. A mastermix minus the cDNA was prepared as per Table 2-10 for each gene being investigated.

Table 2-10: TaqMan reaction mix

Reagent	Volume (µl)
TaqMan buffer	5
Primer	0.5
Nuclease-free H_2Os	3.5
cDNA	1
Total per reaction	10

9ul of master mix was added to each well of a 96 or 384 well q-PCR plate (Applied Biosystems) and 1 µl of cDNA was added. The plate was covered with an optical adhesive film (Applied Biosystems) and centrifuged briefly to remove air bubbles. Q-PCR was performed using the ABI 7500 (Applied Biosystems) q-PCR system according to Table 2-11.

Table 2-11: Gene expression q-PCR amplification

			40 cycles	
	Step 1	**Step 2**	**Step 3**	
Temperature (°C)	50	95	95	60
Time	2 min	10 min	15 s	1 min

Note: A H_2O control containing nuclease free H_2O instead of cDNA was included in the gene expression analysis. The noRT and H_2O controls

from the cDNA synthesis reaction were also included to control for contamination during the cDNA synthesis step.

2.8.5.2 miRNA EXPRESSION

miRNA expression studies were completed using the ExiLENT SYBR® Green mastermix (Exiqon). cDNA samples (synthesised as per section 2.8.4.2) and primers (Table 2-15) were thawed and stored on ice for the duration of their use. Mastermix and primers were kept protected from light throughout the experimental procedure. The cDNA was diluted 1:80 immediately prior to use using nuclease free H_2O and the remains of the diluted stock discarded after use. A master mix minus the cDNA template was prepared as per Table 2-12 for each miRNA being investigated.

Table 2-12: ExiLENT SYBR® green reaction mix

Reagent	Volume (µl)
ExiLENT SYBR® green buffer	5
Primer	1
cDNA	4
Total per reaction	10

6ul of master mix was added to each well of a 96 or 384 well q-PCR plate and 4 µl of cDNA was added. The plate was covered with optical adhesive film and centrifuged briefly to remove air bubbles. q-PCR was performed using the ABI 7500 q-PCR system according to Table 2-13.

Table 2-13: miRNA q-PCR Amplification

		45 cycles	
	Step 1	**Step 2**	
Temperature (°C)	95	95	60
Time	10 min	10 s	1 min

Note: A H_2O control containing nuclease free H_2O instead of cDNA was included in the miRNA expression analysis. The noRT and H_2O controls from the cDNA synthesis reaction were also included to control for contamination during the cDNA synthesis step.

2.8.6 GENE EXPRESSION PRIMERS

All primers were designed by and purchased from Integrated DNA Technologies (IDT).

Table 2-14: mRNA primers

Gene Symbol	Name	Code*
ACT-B	Actin, Beta	39a.22214847
ADAMTS6	ADAM Metallopeptidase With Thrombospondin Type 1 Motif, 6	58.1295675
ATM	Ataxia Telangiectasia Mutated	56a.2596352
ATR	Ataxia telangiectasia And Rad3-Related Protein	56a.39957055
AURKB	Aurora Kinase B	58.2455113
CCNA1	Cyclin A1	58.2546264
CDK17	Cyclin-Dependent Kinase 17	58.2684258
CHEK1	Checkpoint Kinase 1	58.3518318
CHEK2	Checkpoint kinase 2	58.896697
E2F3	E2F Transcription factor 3	58.2282712
H2AFX	H2A Histone Family, Member X	58.23045743
LMTK2	Lemur Tyrosine Kinase 2	58.21022175
PARP1	Poly (ADP-Ribose) Polymerase 1	56a.112995
RAD51	RAD51	58.22760602
RASGEF1A	RasGEF Domain Family, Member 1A	58.3776983
RLIM	Ring Finger Protein, LIM Domain Interacting	58.14464001
TRPC4AP	Transient Receptor Potential Cation Channel, Subfamily C, Member 4	58.3073408
TWF1	Twinfilin Actin Binding Protein 1	18764315
UBFD1	Ubiquitin Family Domain Containing 1	58.366342

* Product code prefix for all primers- hs.

2.8.7 MIRNA EXPRESSION PRIMERS

All miRNA primers were designed by and purchased from Exiqon.

Table 2-15: miRNA primers

miR Name	Target Sequence	Code
hsa-miR-185-5p	UGGAGAGAAAGGCAGUUCCUGA	206037
hsa-miR-200a-3p	UAACACUGUCUGGUAACGAUGU	204707
hsa-miR-210	CUGUGCGUGUGACAGCGGCUGA	204333
hsa-miR-302a-3p	UAAGUGCUUCCAUGUUUUGGUGA	206059
hsa-miR-340-5p	UUAUAAAGCAAUGAGACUGAUU	206068
hsa-miR-423-5p	UGAGGGGCAGAGAGCGAGACUUU	205624
hsa-miR-4284	GGGCUCACAUCACCCCAU*	552044$^{\Psi}$
hsa-miR-574-3p	CACGCUCAUGCACACACCCACA	206011

* Custom designed by Exiqon $^{\Psi}$ Design I.D

2.8.8 CALCULATION OF GENE EXPRESSION

The ΔΔCT method was used to calculate fold change in gene expression as described by Livak and Schmittgen (Livak and Schmittgen, 2001)

2.8.9 EXIQON MICROARRAY

The miRNA microarray experiment was conducted by Exiqon Services, Denmark. Briefly, the quality of the total RNA was verified by an Agilent 2100 Bioanalyzer profile. 750ng of total RNA from both sample and reference was labelled with Hy3™ and Hy5™ fluorescent label, respectively, using the miRCURY LNA™ microRNA Hi-Power Labelling Kit, Hy3™/Hy5™ (Exiqon, Denmark). The Hy3™-labelled samples and a Hy5™-labelled reference RNA sample were mixed pair-wise and

hybridized to the miRCURY LNA™ microRNA Array 7th Gen (Exiqon), which contains capture probes targeting all human microRNAs registered in the miRBASE 18.0. The hybridization was performed according to the miRCURY LNA™ microRNA Array Instruction manual using a Tecan HS4800™ hybridization station (Tecan, Austria). After hybridization the microarray slides were scanned and stored in an ozone free environment (ozone level below 2.0 ppb) in order to prevent potential bleaching of the fluorescent dyes. The miRCURY LNA™ microRNA array slides were scanned using the Agilent G2565BA Microarray Scanner System (Agilent Technologies, Inc., USA) and image analysis was carried out using the ImaGene® 9 (miRCURY LNA™ microRNA Array Analysis Software, Exiqon, Denmark). The quantified signals were background corrected (Normexp with offset value 10, see (Ritchie et al., 2007)) and normalized using the global Lowess (LOcally WEighted Scatterplot Smoothing) regression algorithm.

2.9 miRNA TARGET PREDICTION

The process of selecting the prediction software and using it to predict targets of miRNA was conducted by our collaborator Dr Simon Wong at the Irish Centre for High-End Computing (ICHEC) using the Pictar and MirTarget2 programs. Briefly, the programmes assigned target genes scores, the higher the score the stronger the prediction. The results of the two programs were amalgamated and genes which were predicted to be targets of the miRNA under investigation by both methods had their scores combined. These genes were then ranked according to combined scores and seven of the top ranked genes selected for further validation in the transfected cell line.

2.10 PROTEIN EXTRACTION AND WESTERN BLOTTING

2.10.1 PREPARATION OF TOTAL PROTEIN EXTRACTS

Cells in exponential growth phase (approx. 70% confluent) were harvested by trypsinisation and washed twice with PBS before protein extraction.

2.10.2 TOTAL PROTEIN EXTRACTION

Proteins were extracted from cells using either RIPA lysis buffer (Santacruz) containing protease inhibitor cocktail (Santacruz), sodium orthovanadate (Santacruz) and phenylmethane sulfonyl fluoride (PMSF) (Santacruz). Cells were incubated in the buffer for 20 min and vortexed every 5 min before being snap frozen and stored at -80°C until needed. Cells pellets frozen in RIPA lysis buffer as described above were thawed on ice and vortexed for 30 seconds before centrifugation at 12,000 x g for 20 minutes. The supernatant containing the protein extract was decanted into new tubes and protein concentration determined using either NanoDrop (Thermo Scientific) or bicinchoninic acid assay (BSA) (Pierce). Protein extracts were then stored at -80°C.

2.10.3 NUCLEAR AND CYTOPLASMIC EXTRACTION

Proteins were extracted from cells using the NE-PER Nuclear and Cytoplasmic Extraction kit (Thermo Scientific) according to the manufactures instructions. Ice cold CER1 reagent (100µl/10µl packed cell volume) was added the cell pellet and resuspended by vortexing then incubated on ice for 10 min. Ice-cold CERII reagent was then added (5.5µl/10µl packed cell volume) and vortexed for 5s at the highest setting. Samples were incubated on ice for 1 min followed by vortexing for 5s and centrifugation for 5 min at 16,000 x g. The

supernatant containing the cytoplasmic extract was transferred to a pre-chilled tube and placed on ice. The insoluble pellet was resuspended in ice-cold NER reagent and vortexed on the highest setting for 15s every 10 min to a total of 40 min. Samples were centrifuged at 16,000 x g for 10 mins and the supernatant transferred to a new pre-chilled tube. Protein concentration was calculated for samples before being stored at -80°C

2.10.4 BSA ASSAY

The BSA assay was used to calculate protein content. The microplate procedure was used according to the manufacturers instructions. Albumin protein standards were prepared for the range of 20-2,000µg/ml, and 25µl of each standard, protein sample and a blank sample was added in triplicate to a 96 well plate. 200µl of the BCA working solution containing 50:1 BCA reagent A:B was combined with each sample and mixed thoroughly on a plate shaker for 30s. The plate was covered and incubated for 30 mins at 37°C, the plate was then cooled to room temperature and the absorbance at 562nm measured using VersaMax microplate reader (Molecular Devices). A standard curve was generated using the protein standards and the protein concentration of each sample calculated.

2.10.5 GEL PREPARATION

For western blot analysis a 12% resolving gel was mixed according to Table 2-16 and poured into gel casting cell (Bio-Rad) and 500µl H_2O added to prevent the gel surface drying out. Following polymerisation of the resolving gel the H_2O was decanted and the stacking gel (Table 2-16) was added followed by a 12 well comb (Bio-Rad) on top and allowed to solidify. Gels were wrapped in damp paper to prevent drying out and stored at 4°C overnight. Prior to loading protein samples gels

were removed from casting cells holders and placed on the electrode assembly (BioRad) which was placed into the gel tank.

Table 2-16: Gel Preparation

Reagent	12% Resolving Gel	Stacking Gel
ddH_2O	3.4ml	3.05ml
Acrylamide Ψ	4ml	667µl
1.5M Tris, pH 8.8 *	2.5ml	----------
0.5M Tris, pH 6.8 *	----------	1.25ml
10% Sodium Dodecyl Sulfate (SDS) Δ	100µL	50µl
10% Ammonium Persulfate (AMS)Φ	75µl	50µl
TemedΦ	10µl	5µl

Ψ Sigma-Aldrich * BioRad
Δ Gibco Φ Fisher Chemical

2.10.6 SAMPLE PREPARATION

Protein samples were thawed and stored on ice. Samples were then diluted to 20µg with nuclease free H_2O and combined with equal parts 2X running dye. Sample were incubated at 95°C for 5 min and placed on ice immediately to cool. A positive control for the protein of interest was also included.

2.10.7 PROTEIN ELECTROPHORESIS

Running buffer (10X) was made according to Table 2-17 and diluted to 1X in 1 litre ddH_2O prior to use. The inner and outer chambers of the electrode assembly were filled with the running buffer, the gels placed in the module and the combs withdrawn from the loading gel. 2µl EZ run protein marker (Fisher BioReagents) was used as a molecular weight

standard for comparison to samples detected following western blot. The protein ladder was pipetted into the first well followed by the protein samples and allowed settle. The electrode assembly was connected to a power supply and a current of 200 V for ~1 h was sufficient for migration of the lower molecular weight proteins to the end of the gel.

Table 2-17: 10X Running Buffer

Reagent	Quantity (g)
Tris	30.3
Glycine *	144
SDS *	10
Make to 1 litre with ddH_2O	

***** Fisher Chemical

2.10.8 MEMBRANE TRANSFER

Following electrophoresis, the gel was removed from the glass spacer and the staking gel discarded. The gel containing the protein was placed in a dish containing ice-cold transfer buffer (Table 2-18). The black colour side of the opened gel transfer cassette was immersed in transfer buffer and a pre-soaked fiber pad and filter paper placed on top followed by the protein gel and an activated Immobilon-P PVDF transfer membrane (Merck Millipore) (activated by submerging in 100% methanol for 10 seconds then washed in ddH2O) , more presoaked filter paper and a final wet fiber pad were placed on top, all bubbles were removed and the cassette closed and placed in the transfer module which was filled with pre-chilled transfer buffer. A current of 100V was maintained for 1 hour for the protein transfer.

Table 2-18: Transfer Buffer

Reagent	Volume
Tris	3.03g
Glycine	14.4g
methanol	200ml
Make to 1 litre with ddH_2O	

2.10.9 PROTEIN DETECTION

Following transfer the membrane containing the protein was blocked for 1 h using 5% milk (2.5g milk powder (Sigma-Aldrich), 50 ml PBS containing 0.05% tween (PBST) (Sigma-Aldrich)). The membrane was then washed three times in PBST for 5 mins on a roller. Protein expression was detected using a variety of antibodies (see Table 2-19 for antibodies and dilutions). Antibodies were diluted in 5% milk. The membranes were incubated at 4°C overnight in the dark with 4ml of diluted primary antibody on a roller to insure uniform binding. Cytoplasmic and nuclear specific antibodies were used as loading controls to confirm correct protein extraction and that the same quantity of protein was added to each lane. Following incubation the primary antibody was removed and the membrane washed in PBST as described above. The membranes were then incubated with 4ml of the appropriate horseradish peroxidase (HRP) conjugated secondary antibody against each primary antibody at room temperature for 1 h on a roller. The membrane was then washed as before and incubated with 3ml SuperSignal™ West Pico Chemiluminescent Substrate (1.5ml enhancer solution + 1.5ml stable peroxide solution) (Thermo Scientific) per membrane for 5 minutes in the dark. Proteins were detected using Fuji RX-N medical film (FujiFilm). Following protein detection membranes were washed, air dried and stored at 4°C.

Table 2-19: Protein Antibodies

Name	Description	Dilution	manufacturer	Code
Primary antibodies				
YB-1	Rabbit polyclonal	1:2000	Abcam	ab12148
Lamin B1	Rabbit polyclonal	1:1500	Genetex	GTX103292
Vinculin	Rabbit polyclonal	1:1500	Genetex	GTX113294
Secondary antibodies				
anti-mouse	Goat anti mouse igG-HRP	1:2000	SantaCruz	sc-2005
anti-rabbit	Goat anti rabbit igG-HRP	1:2000	SantaCruz	sc-2030

2.11 STATISTICAL ANALYSIS

All statistical analysis was carried out using either prism (version 6 for mac OS X and windows 7, Graphpad software La Jolla California USA, www.graphpad.com), Microsoft Excel 2011 (for Mac OS X) or Microsoft Excel 2010 (Window 7) or with the Komet data analysis macro for excel (Kinetic imaging ltd.).

Two way comparisons were calculated using unpaired two tailed Students t-tests. For larger comparisons ordinary One-way ANOVA were utilised. Single sample t-tests were used to assess changes in gene and miRNA expression following q-RTPCR with a comparative mean of 1 assumed. Data is presented as the mean of at least 3 independent replicates ± SD (standard deviation) or SEM (standard error mean) as stated in results and were considered to be statistically significant where $p \leq 0.05$.

CHAPTER 3: GENERATION OF A RADIATION RESISTANT ISOGENIC PROSTATE CANCER CELL LINE MODEL

3.1 INTRODUCTION

Radiation resistant isogenic cell lines, which are sublines produced through the repeated exposure of cells to fractionated radiation (McDermott et al., 2014) have been generated for a number of human cancer lines including both solid tumours: Ovarian (De Pooter et al., 1991), Neuroblastoma (Russel et al., 1995), Pancreatic (Lee et al., 1999), Lung (Henness et al., 2002, Lee et al., 2010, Mihatsch et al., 2011, Wei et al., 2008), oesophageal (Fukuda et al., 2004, Lynam-Lennon et al., 2010, Xie et al., 2009), prostate (Skvortsova et al., 2008) (Kyjacova et al., 2015), liver (Kuwahara et al., 2009), breast (Mihatsch et al., 2011, Smith et al., 2009, Wang et al., 2005), skin (de Llobet et al., 2013), oral (Lee et al., 2013) cancer, the Human T-cell leukaemia cell line CCRF-CEM (Harveie et al., 1997) and promyelocytic leukemia HL-60 cell line(Hazawa et al., 2012). These models have highlighted some mechanisms for the survival advantage of the generated sublines and allow for comparison of sensitive and resistant phenotypes without confounding factors due to difference in origin.

The use of appropriate control cell lines for the investigation of the underlying mechanisms of the radioresistant phenotype is essential to differentiate age-related from radiation-related phenotypic modifications. First, a *parent* cell line described as an age matched subline (Lynam-Lennon et al., 2010, Skvortsova et al., 2008, Fukuda et al., 2004, Wang et al., 2005, Russel et al., 1995, Lee et al., 2010, Mihatsch et al., 2011, Xie et al., 2009, Kuwahara et al., 2009) is necessary. This subline, grown over the same overall treatment time as the irradiated line, can control for age associated changes in radiosensitivity in the isogenic model. Although not always clearly described (Wei et al., 2008, De Pooter et al., 1991, Lee et al., 1999, Harveie et al., 1997, Henness et al., 2002, Smith et al., 2009, de Llobet et al., 2013), the inclusion of these controls is common in the literature. Second, the inclusion of the original, wild type line is necessary to account for abnormalities in

karyotype and changes in global gene expression patterns due to age which could additionally affect radiation response (Ruutu et al., 2004, Wenger et al., 2004).

Loss of colony forming ability is one of the most observable outcomes of exposing radiosensitive cells to ionising radiation, therefore the clonogenic cell survival assay is considered the gold-standard method for assessing the radiosensitivity of cells. This assay measures the fraction of irradiated cells that retain the ability to produce an expanding colony of new progeny (Franken et al., 2006). Measuring clonogenic cell survival in response to increasing radiation doses i.e. 2, 4, 6, 8, 10 Gy, in the proposed radioresistant isogenic and control cell lines has become the method of choice for classification (Franken et al., 2006) and to grade the level of resistance of each subline.

While loss of clonogenic reproductive capability through irreparable or misrepaired DNA (primarily DSB (Vignard et al., 2013)) resulting in a combination of lethal mutations and chromosomal aberrations (Jackson and Bartek, 2009) is known to be the primary mechanism for cell death after treatment with ionising radiation, activation of other cell death mechanisms such as apoptosis (Dewey et al., 1995) and senescence (Sabin and Anderson, 2011) in response to treatment is also well established. The role of the apoptotic cell death pathway in radioresistance is contentious with conflicting results presented in the literature (Lynam-Lennon et al., 2010, Wei et al., 2008, Xie et al., 2009, Russel et al., 1995). Investigation of changes in apoptosis levels in the cell lines may suggest a role for it in the evolution of the isogenic cell line from a sensitive to resistant phenotype. Cellular senescence, a state of stable exit from the cell cycle which is metabolically active, is another potential, apoptosis independent, mechanism of irreversible growth arrest that may explain the differences in cell survival in the isogenic model which may not be explained by alterations in programed cell death as measured by Annexin V. Senescence which results in

irreversible growth arrest can be initiated by age (Hayflick, 1965) due to telomere shortening (Harley et al., 1990) or by cellular injury/stress which results in a premature form of senescence (Lloyd, 2002). Reactive oxygen species, which are known critical mediators of DNA damage response to radiation, have been associated with the senescent cell state (Forsyth et al., 2003, Parrinello et al., 2003, von Zglinicki et al., 1995) therefore presenting senescence as an attractive potential mediator of the radioresistant phenotype.

Cell cycle position and progression are known to affect the radiation sensitivity of cells (Pawlik and Keyomarsi, 2004). Analysis of phase distribution using PI in conjunction with flow cytometry is commonly used to evaluate changes in the radioresistant phenotype of isogenic models (de Llobet et al., 2013, Lee et al., 2010, Kuwahara et al., 2009, Wei et al., 2008, Fukuda et al., 2004, Russel et al., 1995, Lynam-Lennon et al., 2010) with varying results. Some authors reported no significant correlation between cell cycle distribution and the radiation resistant phenotype of the isogenic cell lines (Lynam-Lennon et al., 2010, de Llobet et al., 2013, Fukuda et al., 2004), while others report that the number of radioresistant cells in the Gap2/Mitosis (G2/M) and the sub-Gap 1 (G1) phase were significantly less than that of the parent cell line (Lee et al., 2010) or that the resistant sublines showed higher percentages of cells in the more radioresistant S-phase (Wei et al., 2008, Kuwahara et al., 2009).

The selection of sub clones with survival advantage throughout fractionated radiation exposure is likely the result of multiple mechanisms, and validation of acquired radioresistance should rely on a combination of cellular assays. There appears however to be no consensus on how to accurately and consistently define the radioresistant phenotype of the isogenic subline. While clonogenic cell survival assays are generally used, additional characterisation methodologies are necessary.

3.2 AIMS AND OBJECTIVES

The aim of this chapter was to generate an isogenic model of radioresistant prostate cancer and evaluate the oncogenic properties and clinical relevance of the resulting sublines.

Specific objectives:

- To generate radiation resistant isogenic CaP cell lines (22Rv1, DU145) in response to weekly 2Gy fractionated irradiation.

- To generate a cumulative dose-series for the modification of clonogenic survival during the emergence of a radioresistant subline.

- To determine the stability of the radioresistant phenotype in isogenic cell line.

- To validate the radioresistant phenotype using a combination of cellular assays.

3.3 RESULTS

3.3.1 CLONOGENIC SURVIVAL OF A PANEL OF PROSTATE CELL LINES FOLLOWING TREATMENT WITH IONISING RADIATION

Four prostate cell lines, one normal PWR-1E, one benign BPH1 (benign prostate hyperplasia) and two cancer lines DU145 (brain metastasis) and 22Rv1 (primary tumour) were investigated for their tolerance to radiation (Figure 3-1) using the clonogenic cell survival assay, a colony formation assay which measures a cells reproductive capacity following treatment with radiation (2-10Gy) (Franken et al., 2006). The cell lines show a range of responses to radiation, with the normal prostate and benign cell lines PWR-1E and BPH1 exhibiting the highest proliferative potential following treatment with an area under the curve (AUC) of 3.376 and 3.401 respectively compared to DU145 2.79 and 22Rv1 with 2.30 demonstrating significant differences in radiation sensitivity.

The 22Rv1 cell line was significantly more sensitive to treatment than the benign BPH1 (ANOVA-2Gy: $p=0.0029$, 4Gy: $p<0.0001$, 6Gy:$p<0.0001$, 8Gy:$p=0.0057$) and normal PWR-1E (ANOVA-2Gy:$p= 0.0232$, 4Gy:$p < 0.0001$, 6Gy:$p= 0.0005$, 8Gy: $p=0.0339$) cell lines at all doses: except 10Gy (BPH1: $p=0.1137$, PWR-1E: $p=0.4510$) as well as DU145 at 4Gy (ANOVA-$p=0.0021$). DU145 similarly showed an increased sensitivity to radiation compared to BPH1 at higher doses (ANOVA-6Gy:$p= 0.0054$, 8Gy:$p= 0.0408$) while only significantly different to PWR-1E at 6Gy (ANOVA-$p=0.0165$).

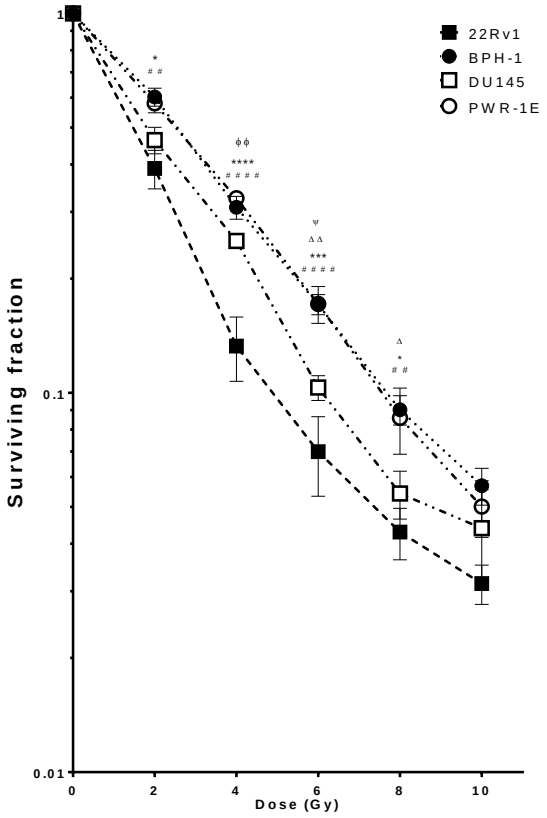

Figure 3-1: Radiosensitivity of four prostate cell lines

Cell line survival after radiation 2-10Gy. Data mean ± SEM from at least four independent experiments; statistical analysis performed using one-way ANOVA with Tukey multiple comparison correction. * refers to significant differences between 22Rv1 and PWR-1E, # differences between 22Rv1 and BPH1, ψ differences between DU145 and PWR-1E, ϕ differences between 22Rv1 and DU125 and Δ refers to differences between DU145 and BPH1. *P≤0.05, ** P≤0.01, ***P≤0.001, **** P≤0.0001

3.3.2 FRACTIONATED IRRADIATION SELECTS FOR 22RV1 CELLS WITH INCREASED REPRODUCTIVE POTENTIAL FOLLOWING RADIATION EXPOSURE

Wild type 22Rv1 (WT-22Rv1) were either exposed to 2Gy fractionated radiation to a cumulative dose of 60Gy (radiation resistant (RR-22Rv1)) (Figure 3-2) or mock irradiated (age matched control (AMC-22Rv1)). Protracted exposure to x-rays resulted in the selection of isogenic subline with a radioresistant like phenotype when compared to WT-

22Rv1 cell line. Protracted radiation exposure also selected for 22Rv1 cells with decreased population doubling time compared to the WT-22Rv1 (~40hrs) (Sramkoski et al., 1999) and AMC-22Rv1, the exact doubling time was not calculated for the newly generated RR-22Rv1 cell line.

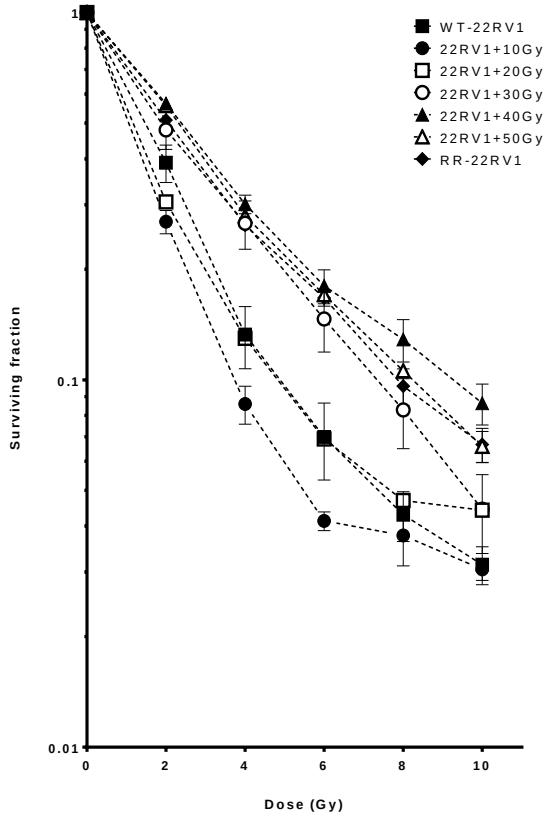

Figure 3-2: Generation of the RR isogenic 22Rv1 cell line

The 22Rv1 cell line was irradiated weekly with 2Gy X-radiation. Clonogenic cell survival assays were conducted after every 10Gy cumulative dose to monitor changes in radiation resistance. Data points are the mean ± SEM of at least 3 individual replicates.

Proliferative potential following increasing radiation doses (2-10Gy) was measured in RR-22Rv1, AMC-22Rv1 and WT-22Rv1 using clonogenic assays to generate radiation survival curves (Figure 3-3). The surviving fraction of RR-22Rv1 cells was significantly higher than that of the WT-

22Rv1 cell line at all doses tested. The area under the curve increased from 2.3 (WT-22Rv1) to 3.14 (RR-22Rv1). The AMC-22Rv1 subline exhibited an intermediate area under the curve of 2.7 and significantly reduced clonogenic survival compared to that of RR-22Rv1 cells at all doses tested, except 2Gy.

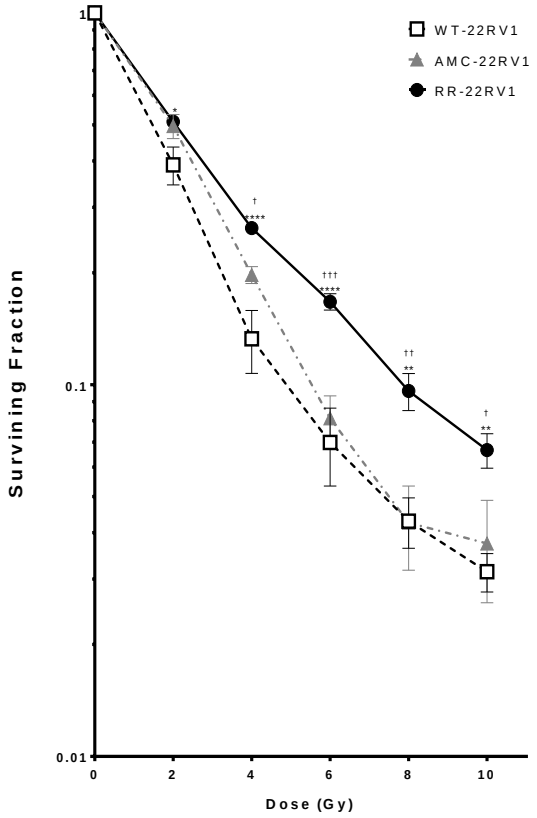

Figure 3-3: Clonogenic survival of isogenic 22Rv1 subline and controls after X-irradiation

Surviving fraction, after 60Gy cumulative dose of x-irradiation, of the radiation resistant RR-22Rv1 isogenic cell line model compared with WT-22Rv1 and AMC-22Rv1 controls measured by clonogenic cell survival assay after x-radiation with 2-10Gy. Points are means ± SEM of at least five individual experiments. * refers to significant differences between RR-22Rv1 and WT-22Rv1 cell lines, † refers to significant differences between RR and AMC cell lines. *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001

3.3.3 INCREASED REPRODUCTIVE POTENTIAL IS SUSTAINED FOLLOWING CESSATION OF TREATMENT

The increased reproductive capacity of the RR-22Rv1 cell line was confirmed by clonogenic assay following one month without exposure to radiation. There was no significant change in radiosensitivity of the RR-22Rv1 cell line after cessation of treatment (RR-22Rv1+1M) compared to RR-22Rv1. The RR-22Rv1+1M remained significantly more resistant than the WT-22Rv1 at all doses (ANOVA-2Gy: $p=0.0014$, 4Gy: $p<0.0001$, 6Gy: $p<0.0001$, 8Gy: $p=0.0005$, 10Gy: $p=0.0414$) (Figure 3-4).

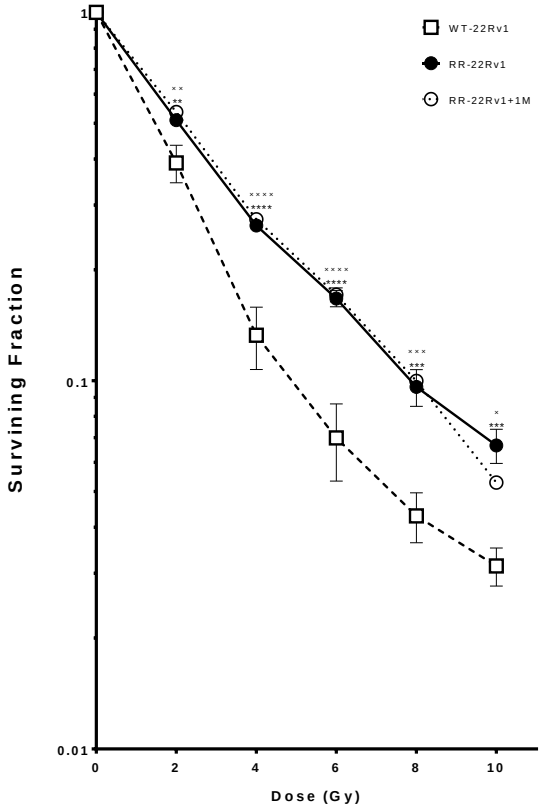

Figure 3-4: RR-22Rv1 isogenic cell line stability

Clonogenic cell survival, following X-radiation with 2-10Gy, of the radiation resistant isogenic cell line after one month with no treatment (RR-22Rv1+1M) compared to RR-22Rv1 cell line and the WT-22Rv1 control. Points are measured as means ± SEM of at least five individual experiments. * refers to significant differences between RR-22Rv1 and WT-22Rv1 cell lines, × refers to significant differences between RR-22Rv1+1m and WT-22Rv1 cell lines. *P≤0.05, ** P≤0.01, ***P≤0.001, ****P≤0.0001

3.3.4 TREATMENT WITH 2GY FRACTIONATED IRRADIATION DOES NOT SELECT FOR A MORE RADIORESISTANT PHENOTYPE IN THE DU145 CELL LINE

DU145 cells were treated with 2Gy fractionated radiation to a cumulative dose of 60Gy, survival fraction measured by clonogenic assay showed that this protocol failed to select for a radioresistant phenotype when compared to the untreated control (Figure 3-5)

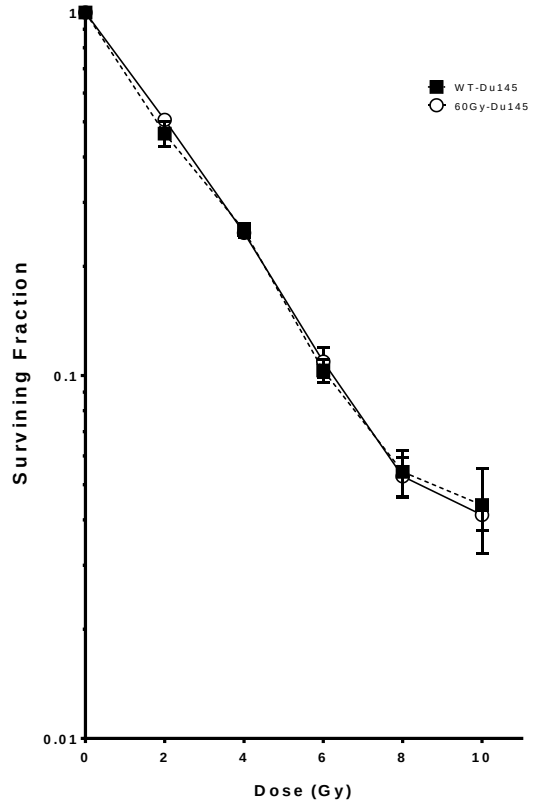

Figure 3-5: Du145 cells irradiated to a cumulative dose of 60Gy

Surviving fraction of Du145 cells after 60Gy cumulative dose of X-radiation (60Gy-Du145) and unirradiated control (WT-Du145) cell lines. Survival of irradiated cells measured by clonogenic cell survival assay following treatment with 2-10Gy . Points are measured as means ± SEM of at least five individual experiments.

3.3.5 FRACTIONATED RADIATION EXPOSURE SELECTS FOR 22RV1 CELLS WITH REDUCED SENSITIVITY TO RADIATION-INDUCED APOPTOTIC CELL DEATH BUT NOT CELLULAR SENESCENCE

Apoptotic cell death following exposure to radiation was quantified in all three lines using annexin V-FITC/PI. Cells were incubated for six hours following irradiation and initial response to treatment measured (Figure 3-6). The number of cells in early apoptosis was significantly reduced in RR-22Rv1 when compared to WT-22Rv1 and AMC-22Rv1 at both doses. The baseline numbers of apoptotic cells were similar across the three cell lines (ANOVA, p=0.77). Following exposure to radiation (2, 4Gy), the number of apoptotic cells increased in WT-22Rv1 (2Gy: 3.58 ± 0.15 to 6.50 ± 0.65 , p= 0.024; 4Gy: 3.58 ± 0.15 to 7.5 ± 0.83, p=0.03) and AMC-22Rv1 (2Gy: 4.59 ± 0.67 to 4.34 ± 1.17, p=0.86; 4Gy: 4.59 ± 0.67 to 8.22 ± 0.95, p=0.03) but not in RR-22Rv1 cells (2Gy: 3.79 ± 1.05 to 2.51 ± 0.77, p=0.38; 4Gy: 3.79 ± 1.05 to 2.25 ± 0.7, p=0.28).

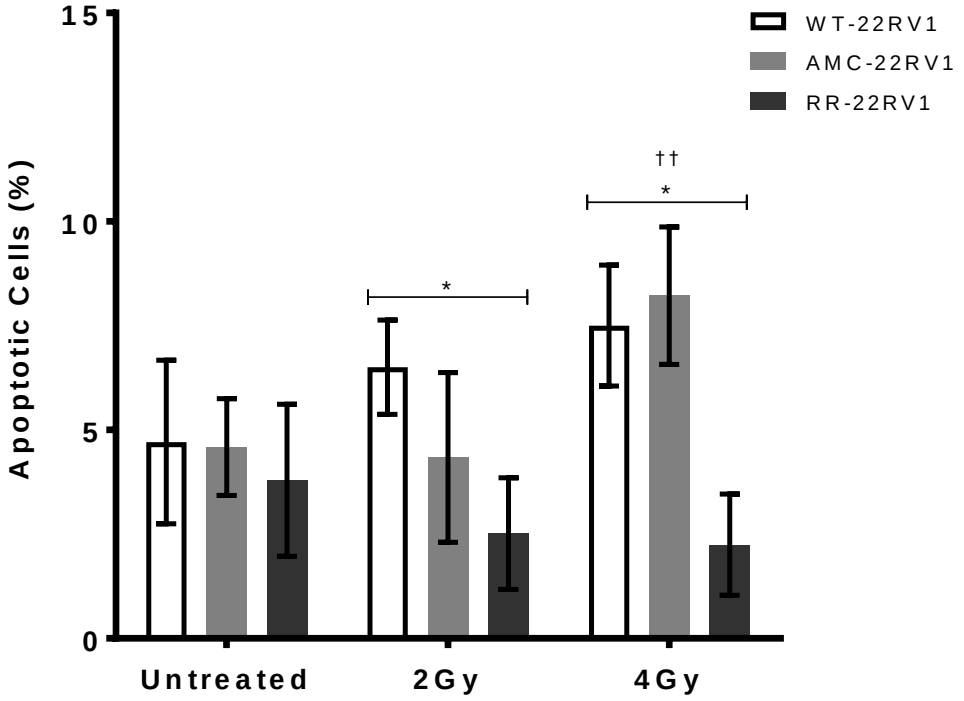

Figure 3-6 Radiation induced apoptosis in RR-22rv1, WT-22Rv1 and AMC-22Rv1 cell lines

Radiation induced apoptosis in WT-22Rv1, RR-22Rv1 and AMC-22Rv1 six hours post treatment with 2Gy and 4Gy x-irradiation. Data is mean ± SD. Significance based on one-way ANOVA with Tukey correction. * refers to significant differences between RR-22Rv1 and WT-22Rv1 control cells, † refers to significant differences between RR-22Rv1 and AMC-22Rv1 cells. *P≤0.05, ** P≤0.01 . n=3

The levels of senescence-associated β-galactosidase (SA-β-Gal) were next measured by flow cytometry (Noppe et al., 2009) (Figure 3-7). A trend towards elevated β-Gal was seen in untreated RR-22Rv1 cells, when compared to AMC-22Rv1 and WT-22Rv1 cells though statistical analysis revealed this was not significant (ANOVA, p=0.14). A similar non-significant trend was observed in irradiated cell lines (ANOVA, 2Gy, p=0.18; 4Gy=0.17, 8Gy p=0.06). Radiation exposure appeared to increase senescence levels within each of the three lines, but the

differences did not reach statistical significance (ANOVA, WT-22Rv1 p= 0.32, AMC-22Rv1, p=0.22, RR-22Rv1, p=0.56).

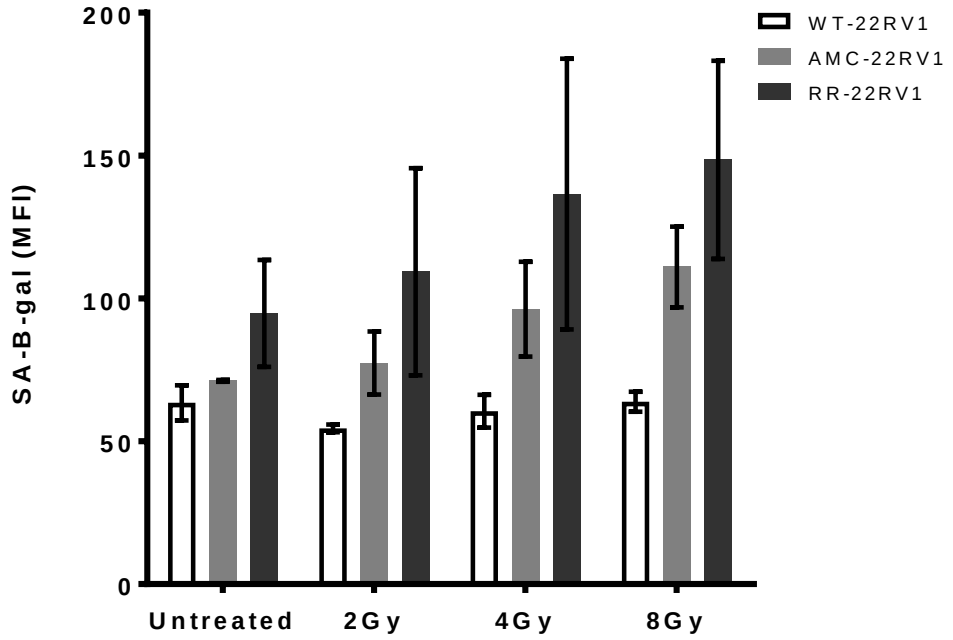

Figure 3-7: Cellular senescence as measured with β-galactosidase

SA-*β*-gal activity measured with C_{12}FDG and flow cytometry in WT-22Rv1, AMC-22Rv1 and RR-22Rv1 lines, with and without treatment of 2Gy, 4Gy and 8Gy of ionising radiation. Data is mean ± SD.. n=2

3.3.6 INCREASED REPRODUCTIVE POTENTIAL FOLLOWING RADIATION EXPOSURE IS ASSOCIATED WITH ENRICHMENT IN S-PHASE CELL POPULATION FOLLOWING RADIATION EXPOSURE

Cell cycle distribution and progression are known to affect the radiosensitivity of cells. PI staining and flow cytometry were used to characterise the cell cycle distribution in all three cell lines at five time points following 2Gy and mock irradiation.

The % of untreated cells in the S-phase was significantly higher in RR-22Rv1 (43.3±1.3%) than in WT-22Rv1 (20.9 ± 3.3%, p=0.0007) but not AMC-22Rv1 (34.6±5.1%, p=0.11) (Figure 3-8). This pattern was maintained following irradiation (2Gy) (ANOVA, p=0.002). Radiation exposure did not increase the percentage of S-phase cells within all three lines (4hrs: WT-22Rv1, p=0.42; AMC-22Rv1, p=0.76; RR-22Rv1, p=0.99)

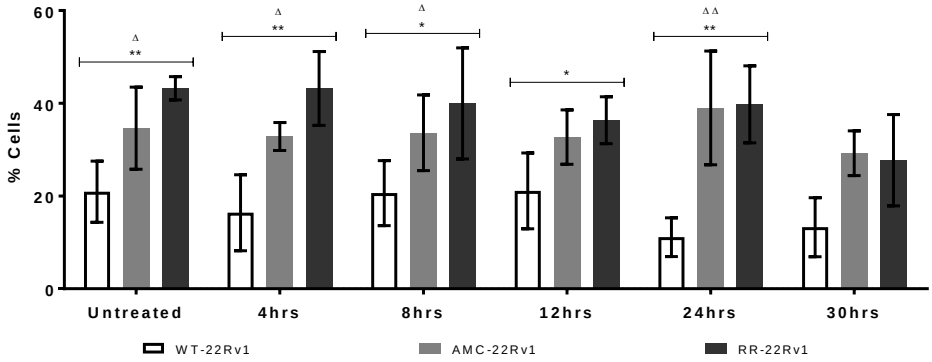

Figure 3-8: Time series analysis of S-phase cell cycle distribution between WT-22Rv1, RR-22Rv1 and AMC-22Rv1 cells

Cell cycle analysis, of WT-22Rv1, RR-22Rv1 and AMC-22Rv1 cells untreated and at five time points post irradiation with 2Gy, was measured using PI staining and flow cytometry. Results are means ± SD of 4 individual replicates significance is calculated using one-way ANOVA with Tukey correction. * refers to significant differences in the RR-22Rv1 cell line vs. WT-22Rv1 control. Δ refers to significant differences between AMC-22Rv1 and WT-22Rv1 control cells. * P≤0.05, ** P≤0.01

Additionally, WT-22Rv1 had a significantly higher percentage of cells in the G1 phase at 4hrs (ANOVA, P=0.007), 8hrs (P=0.03), 24hrs (P=0.013) and 30hrs (P=0.02) post treatment (Figure 3-9) compared to the other cell lines. A significant percentage of WT-22Rv1 cells were also present in the Sub G1 (data not shown) phase at 8 hours (WT: 11.25 ± 2.344, RR: 3.545 ± 0.8862) and 24 hours (WT: 10.28% ± 1.132, RR: 4.700% ± 0.7971) which may be explained by an increase in cell death in this cell line.

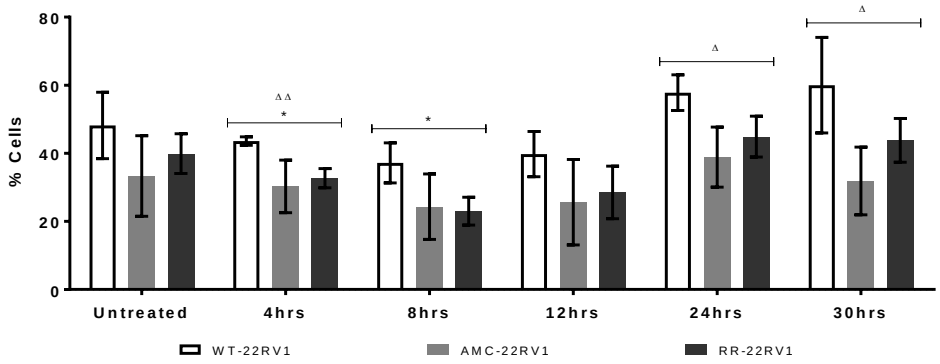

Figure 3-9: Time series analysis of G1-phase cell cycle distribution between WT-22Rv1, RR-22Rv1 and AMC-22Rv1 cells

Cell cycle analysis, of WT-22Rv1, RR-22Rv1 and AMC-22Rv1 cells untreated and at five time points post irradiation with 2Gy, measured using PI staining and flow cytometry. Results are means ± SD of 4 individual replicates significance is calculated using one-way ANOVA with Tukey correction. * refers to significant differences in the RR-22Rv1 cell line vs. WT-22Rv1 control. Δ refers to significant differences between AMC-22Rv1 and WT-22Rv1 control cells. * P≤0.05, ** P≤0.01

3.4 DISCUSSION

Radioresistant models have been derived from a variety of cancer cell lines including the prostate. Isogenic models of LNCaP, PC-3 and DU145 cells were generated through 2Gy daily exposure over 5 consecutive days and associated with a dose modifying factor of 1.6, 1.5 and 1.5 respectively at 0.1% survival (Skvortsova et al., 2008). The radiation-surviving cell population of DU145, PC-3, LNCaP and 22Rv1 cells following exposure to 35 doses of 2Gy was isolated to examine effects of radiation induced stress on phenotype heterogeneity (Kyjacova et al., 2015), and neuroendocrine differentiation (Deng et al., 2008).

Initial differences in radiosensitivities of prostate and prostate cancer cell lines were established using the clonogenic cell survival assay. The non-cancer cell lines PWR-1E and BPH1 demonstrated the least radiosensitive phenotype of the cell lines investigated. The 22Rv1 and DU145 lines showed significantly less resistance to radiation at various doses though 22Rv1 appeared to be the most sensitive of the four lines with the lowest AUC.

Isogenic models have been generated through exposure to a variety of fractionation schedules with mean total doses within a 40-80Gy range and overall treatment times varying from 5 days to 6 years (McDermott et al., 2014). A number of limitations prevent the reproduction of clinical radiotherapy delivery under experimental conditions such as cell ageing and the necessity for recovery periods (McDermott et al., 2014). In this study, 22Rv1 and DU145 cells were exposed to repeated 2Gy-dose fractions and allowed to recover to a set confluence of ~70%-80% between fractions to a total dose of 60Gy. The DU145 cell line though treated identically to the 22Rv1 line failed to become radioresistant after persistent treatment with 2Gy fractionated radiation. This suggests that differences in the intrinsic radiosensitivities of cells may play a role in

the establishment of a radiation resistant phenotype. An adjustment in the treatment protocol used in this study may be necessary to establish a radioresistant isogenic model of this cell line.

The cumulative exposure of 22Rv1 cells to 60Gy-fractionated radiation resulted in the generation of a subline with a significantly increased clonogenic survival potential following radiation treatment, when compared to wild type and mock irradiated, aged-matched cells; this suggests a substantial shift in the radiosensitivity of the isogenic RR-22Rv1 cell line to a relatively resistant phenotype. While this shift was significant compared to the WT-22Rv1 control at all doses tested (2-10Gy) this was not true of comparison to the AMC-22Rv1 at 2Gy. AMC-22Rv1 was also not significantly different to the WT-22Rv1 though showed a trend towards resistance which was further confirmed by cell cycle analysis at 2Gy and suggests that cell age may play a role in the shift from the sensitive to resistant phenotype.

A wild type low passage control and age matched control (derived from the wild type and mock irradiated) were selected for use in this study. As previously suggested (McDermott et al., 2014), the question of genetic alterations due to the age of the cell lines, rather than radiation induced genetic instability, as a result of long overall treatment times needs to be controlled for. It cannot be confirmed that this age related shift in radiosensitivity was seen by other groups investigating radioresistance using isogenic cell line models as it appears that only either a low passage *or* the age matched control and the mutant isogenic line are compared in order to investigate the underlying changes in the newly acquired radioresistant sublines. While this allows for comparison of radiosensitivity between the lines and takes into account genetic aberrations due to age that may be influencing the radiation resistance of the new line, the inclusion of the original WT-22Rv1 low passage cell line exposes the role of age in radiosensitivity. High cell line passage number results in the augmentation of a variety

of cellular mechanisms including apoptosis (Pronsato et al., 2013), cell survival and gene expression (Chang-Liu and Woloschak, 1997), morphological and physical development (Briske-Anderson et al., 1997), membrane transport (Yu et al., 1997, Sambuy et al., 2005) as well as androgen sensitivity in the prostate cancer cell line LNCaP (Langeler et al., 1993, Esquenet et al., 1997, Lin et al., 2003) and so age effects should be considered. Therefore the change in radiosensitivity reported in the AMC-22Rv1 line when compared to the WT-22Rv1 line can be expected, but is under investigated.

Following the generation of the isogenic cell line the stability of the phenotype was confirmed one month after cessation of treatment. The increase in colony forming ability seen in the RR-22Rv1 line is one of the most commonly reported consequence of protracted fractionated radiation exposure (de Llobet et al., 2013, Mihatsch et al., 2011, Lynam-Lennon et al., 2010, Lee et al., 1999, Smith et al., 2009, Xie et al., 2009, Skvortsova et al., 2008, Fukuda et al., 2004, Russel et al., 1995, Henness et al., 2002) and is often associated with a modification of cell cycle distribution which was therefore next investigated.

It is well established that cell cycle phase affects the radiation sensitivity of cells. Cells in S-phase, particularly the latter end of the S-phase, are less sensitive to radiation than cells in the G1 phase with those cells in the G2/M phase being the most radiosensitive (Sinclair and Morton, 1966). It has previously been shown that a higher proportion of cells are found in the S-phase in radioresistant samples, while samples with cells in the G2/M phase show significantly higher sensitivity to radiation (Quiet et al., 1991, Tell et al., 1998). These results are consistent with this, the RR-22Rv1 line was enriched in S-phase cells, when compared to the WT-22Rv1 control with and without treatment with 2Gy. Enrichment in this radioresistant cell cycle phase has been reported in other radioresistant cell models (Wei et al., 2008, Kuwahara et al., 2009) and in prostate cancer specimens, a larger S-phase fraction has

been associated with more aggressive tumours (Abdel-Wahab et al., 2003, Nemoto et al., 1990) and reduced local tumour control probability following radiotherapy (Centeno et al., 1994). This suggests a role for cell cycle distribution in the radioresistance of the new cell line not only following treatment but also in un-stimulated cells, this change in distribution from the WT-22Rv1 cell line may play a critical role in prostate cancer progression to a radiation resistant phenotype. The AMC-22Rv1 cell line shared this pattern of cell cycle distribution when compared to WT-22Rv1 cells which may explain the initial increase in radioresistance of the AMC-22Rv1 as depicted in the clonogenic analysis at 2Gy and suggests a potential role for cell cycle distribution in the AMC-22Rv1's age associated ability to overcome the initial damage done at low doses.

The G1 phase is known to be moderately radiosensitive (Sinclair and Morton, 1966) therefore the increase of WT-22Rv1 cells in this phase may explain further differences between this cell line's resistance patterns, particularly in the time just after treatment resulting in less ability to cope with the initial radiological insult to the cells while differences in the S-phase of the cells suggest a role for cell cycle distribution in the RR-22Rv1 (and the AMC-22Rv1 to a lesser extent) radiation resistant phenotype, potentially due to less initial damage forming after irradiation. However no significant G2/M arrest was seen in any of the cell lines (data not shown) at any time point following treatment suggesting the protective mechanism of this checkpoint (DiPaola, 2002) is not playing a role in the differences between the phenotypes of our cell lines.

The role of apoptosis in radioresistance is controversial with the general consensus suggesting that alterations in the induction of apoptosis are not a likely cause for the radiation resistant phenotype. This was shown by Lynam-Lennon et al through the observation that there is no significant difference in apoptosis or even necrosis between the

radiosensitive parent cell line or the radioresistant subline OE33R up to 48 h post irradiation (Lynam-Lennon et al., 2010). Wei et al, also investigated differences in apoptotic cell death, using DNA content and Hoechst 33258 staining, in the radioresistant D6-R lung cancer subline compared to the sensitive control and found no differences between the two lines (Wei et al., 2008). However, this is not always the case, Russel et al found that two radiation resistant human neuroblastoma lines showed significantly less apoptotic cells following treatment with 5Gy (Russel et al., 1995) compared to IMR 32 WT line and Xie et al also found that there was a significant decrease in the percentage of apoptotic cells after 12Gy irradiation in the EC109/R oesophageal carcinoma subline compared to the parent line (Xie et al., 2009).

The data presented here shows that while no differences were seen in levels of apoptotic cell death between the radiation resistant line and the controls prior to treatment, the RR-22Rv1 alone showed a significantly lower percentage of cells undergoing early apoptosis 6 h post irradiation than the WT-22Rv1 control at both 2Gy and 4Gy as well as the AMC-22Rv1 at 4Gy. This may suggest that the WT-22Rv1 and AMC-22Rv1 control cells are undergoing a fatal event due to catastrophic damage to the DNA immediately after treatment with radiation resulting in their elimination from the proliferative cell population rather than relying on a loss of clonogenic ability post mitosis reducing the number of cells available for clonogenic growth. Though as Russell et al proposed this difference in apoptotic cell populations may potentially be accounted for by differences in the rate of clearance of these dead cells between the lines (Russel et al., 1995). The prognostic potential of apoptosis sensitivity in prostate radiotherapy patients has been proposed (Szostak and Kyprianou, 2000) but remains unconfirmed. Nonetheless, the pharmacologic radio-sensitisation of prostate cancer cells is often achieved through induction of apoptosis (Rae et al., 2015). Further evaluation of the underlying mechanisms for this reduced susceptibility to radiation-induced apoptosis is required.

The induction of permanent growth arrest through senescence was proposed as an alternative mechanism in the therapeutic response to therapy in cancer cells with deregulated apoptotic signalling (Schmitt et al., 2002) and the induction of senescence in response to elevated DNA damage has been associated with increased therapeutic sensitivity (Ewald et al., 2010). In prostate cancer patients, increased expression of β-galactosidase (GLB1) was associated with a reduced risk of recurrence (Wagner et al., 2015). A proportion of surviving DU145 and PC3 cells exposed to 35 fractions of 2Gy developed a senescent-like morphology associated with elevated mRNA expression of key senescence markers (Kyjacova et al., 2015). However the RR-22Rv1 cells failed to show statistically significant evidence for a possible reduction in the induction of therapeutic senescence. Analysis of β-galactosidase activity would not detect other permanent growth arrests such as the induction of neuroendocrine differentiation (NED).

Finally, while a clinically relevant total dose of 70-80Gy was not attained in the isogenic cell line due to the compounding factors cited above, the resulting RR-22Rv1 line was significantly more resistant to radiation than hypoxic WT-22Rv1 cells. Hypoxia is a known mediator of radiation sensitivity in cells (Wouters and Brown, 1997, Harris, 2002) and evaluations of the volume of prostate tumours suggest that 20% is exposed to less than 5 mmHg (0.7%) oxygen and prostate tumours are therefore considered hypoxic in nature (Chopra et al., 2009). Further enhancement of the radioresistance of RR-22Rv1 cells by hypoxia was small; suggesting that levels of radioresistance may have peaked in these cells and thus this isogenic cell line is a good model of the radioresistant phenotype in the 22Rv1 cell line. Of the four commonly used prostate cancer cell lines, only this one may be representative of primary disease (Sramkoski et al., 1999).

Results from this chapter are contained in an article published in Scientific reports

"Fractionated radiation exposure amplifies the radioresistant nature of prostate cancer cells"

McDermott N, Meunier A, Mooney B, Nortey G., Hernandez C, Hurley S, Lynam-Lennon N, Barsoom SH, Bowman KJ, Marples B, Jones GDD, Marignol L. Scientific Reports. October 2016; 6, 34796

CHAPTER 4:
CHARACTERISATION OF A RADIATION RESISTANT ISOGENIC CELL LINE MODEL

4.1 INTRODUCTION

Irradiation of cells results in a number of different types of DNA lesions; damage to single bases, or breaks in the DNA either in the form of SSBs or DSBs (Joiner and van der Kogel, 2008). Efficient DDR and DNA repair mechanisms are therefore essential to cell survival. Perverse activation of the DDR has been implicated in radiation resistance in a number of studies. Alterations in checkpoint response (Bao et al., 2006, Piao et al., 2012, Lundholm et al., 2013, Hazawa et al., 2012) and increased DNA repair capacity (Bao et al., 2006, Piao et al., 2012, Lundholm et al., 2013, Lynam-Lennon et al., 2010, Kuwahara et al., 2009, de Llobet et al., 2013, Wei et al., 2008) (Skvortsova et al., 2008) (Gerweck et al., 2006, Hazawa et al., 2012) have also been associated with radioresistance in cells and may account for the phenotype in our cell line.

The DDR involves a multi-faceted system of repair pathways. Following exposure to radiation DNA is subject to an array of different types of damage including single base damage, SSBs and DSBs, different repair pathways are then activated depending on the form of damage inflicted (Jackson and Bartek, 2009). DSBs are recognised as the most lethal form of radiation induced DNA damage (Olive, 1998) and result in the activation of both the non-homologous end-joining (NHEJ) (Lieber et al., 2003) and homologous recombination (HR) (Liang et al., 1998) repair pathways. Cell cycle checkpoints are activated following exposure to radiation to block cell cycle progression and allow for repair of damaged DNA (Iliakis et al., 2003). The G1, S and G2 checkpoints are all mediated by either ATM or Ataxia telangiectasia And Rad3-Related Protein (ATR) (Zhou and Elledge, 2000) and failure in checkpoint response may result in propagation of cells with pro-resistance mutations and the evasion of radiation mediated cell death.

ROS are critical mediators of radiation induced cell death. Exposure to ionising radiation results in the generation of ROS, mainly superoxide's, hydroxyl radicals and H_2O_2 (Riley, 1994) which disrupt membrane bi-layers, induce protein crosslinking (Wolff and Dean, 1986, Willson et al., 1985) as well oxidising amino acids, mitochondrial DNA and nuclear DNA resulting in cellular injury (Slater, 1988, Gilbert et al., 1984, Riley, 1994). Accumulation of ROS was investigated by a number of groups as a possible characteristic of radiation resistance. Lee et al found a significant decrease in ROS production in the radiation resistant H460R non small cell lung cancer line compared to sensitive controls (Lee et al., 2010), yet no difference in ROS accumulation was identfied by Lynam-Lennon et al in either the sensitive or resistant OE33 oesophogeal adenocarcinoma cell line model (Lynam-Lennon et al., 2010). This suggests that though ROS does play a role in the radioresistant phenotype of some cells another mechanism of oxidative stress may also be responsible for resistance in other models.

Expression of antioxidant enzymes are upregulated in response to oxidative stress following treatment with radiation (Riley, 1994). Variation in the amount of ROS scanvenging activity has also been explored as a potential mode of resistance in cancer cells (Lynam-Lennon et al., 2010, McDonald et al., 2010, Lee et al., 2004, Giménez-Bonafé et al., 2004). McDonald et al reported that activation of the NF-E2 related factor 2 - antioxidant DNA response element (Nrf2-ARE) pathway which is triggered in response to oxidative stress (Nguyen et al., 2009) and results in increased expression of antioxidants γ-glutamylcysteine synthetase (γ-GCS), glutathione peroxidase, heme oxygenase-1 (HO-1), catalase, and NAD(P)H:quinone oxidoreductase-1 (NQO-1), is also increased in response to ionising radiation in a dose dependant manner (McDonald et al., 2010). It has also been shown that down regulation of Nrf2 in cells (McDonald et al., 2010, Singh et al., 2010, Zhang et al., 2010a, Jayakumar et al., 2014, Shibata et al., 2011) and mice (McDonald et al., 2010) results in increased radiosensitivity in

comparison to controls and that constitutive activation of the pathway confers radioresistance (Singh et al., 2010).

While oxidative stress is known to causes DNA damage and cell death following treatment, ROS are also essential participants in cell signalling pathways involved in regulation of normal cellular functions including proliferation, differentiation, apoptosis and immune and inflammatory responses (Thannickal and Fanburg, 2000, Finkel, 1998). High endogenous levels of ROS are linked to increased cell survival, proliferation and motility, and anchorage independent growth in cancer through the modulation of intracellular signalling cascades (Storz, 2005, Liou and Storz, 2010). Increased basal levels of ROS have also been implicated in radioresistance in prostate cancer cells (Pinthus et al., 2007).

The ability of cancer cells exposed to fractionated radiation to manipulate the interrelationship between the formation of DNA damage and oxidative stress likely participates in their survival advantage following radiation exposure. Examination of this interplay has thus potential to inform the identification of key radiobiological features of prostate tumours. Investigation of the underlying molecular mechanisms of the radioresponse and its dysregulation during the switch from a sensitive to resistant phenotype as well as analysis of the eight identified hallmarks of cancer following irradiation presents an opportunity to truly define the mechanisms involved in the development of the radioresistant tumour population.

4.2 AIMS AND OBJECTIVES

The aim of this chapter was to characterise the radiation resistant isogenic model using known mechanisms of the radioresponse.

Specific objectives:

- To evaluate the formation of DNA damage and the repair capacity of the isogenic cell line model compared to control lines using the alkaline comet assay (ACA).

- To investigate dysregulation of DDR genes in the RR-22Rv1 cell line relative to WT- and AMC-22Rv1 controls.

- To determine the role of oxidative stress in the radioresistant phenotype of the isogenic subline.

4.3 RESULTS

4.3.1 FRACTIONATED RADIATION EXPOSURE SELECTS FOR 22RV1 CELLS WITH DECREASED LEVELS OF INDUCED DNA DAMAGE AND INCREASED DNA REPAIR CAPACITY

The alkaline comet assay (ACA) was used to quantify immediate levels of induced DNA damage (measured as % Tail DNA) in the three cell lines following irradiation (2-10Gy) (Figure 3A). The level of initial DNA damage was significantly lower in RR-22Rv1 cells when compared to both AMC-22Rv1 and WT-22Rv1 in unirradiated cells and was sustained across all doses tested (ANOVA, $p<0.0001$). DNA damage formation increased exponentially with increasing radiation dose in all three lines. At 10Gy, the absolute increase in % Tail DNA compared to unirradiated control was highest at 48.5% in WT-22Rv1 (61.6 ± 0.7% - 13.3 ± 0.55% = 48.3%), 30.6% in AMC-22Rv1 (40.9 ± 0.6% - 10.3 ± 0.36% = 30.6%) and lowest at 25.47% in RR-22Rv1 (32.5 ± 0.44% - 7.03 ± 0.32% = 25.47%).

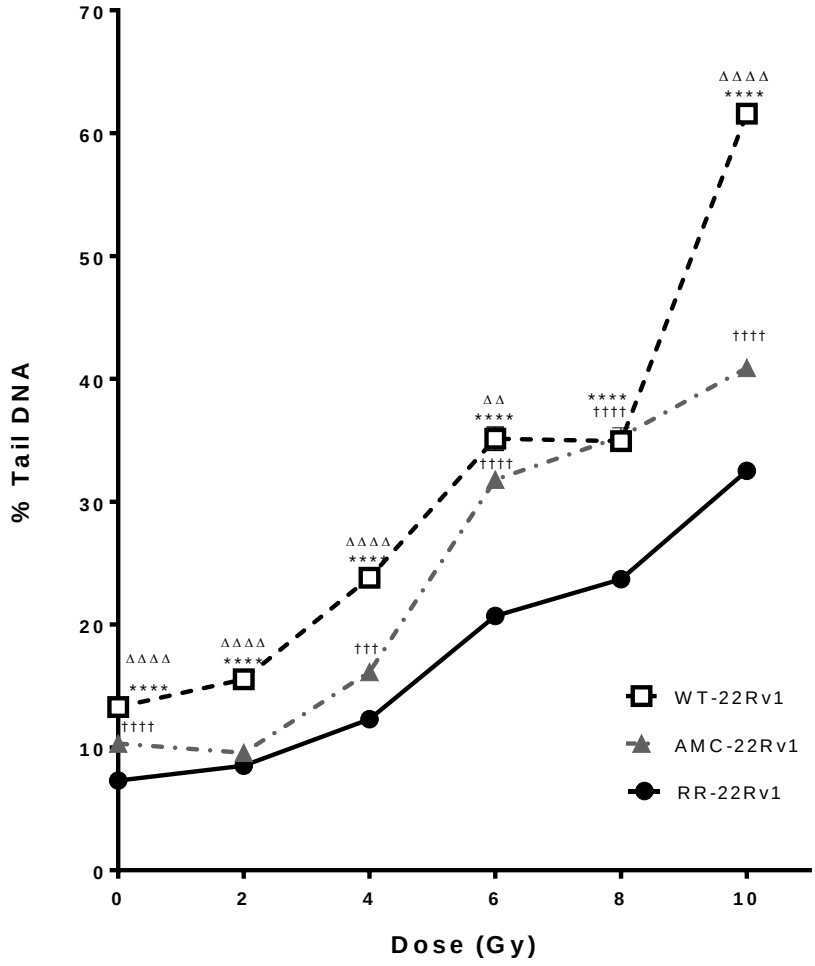

Figure 4-1: DNA damage formation following x-irradiatipon in WT-22Rv1, AMC-22Rv1 and RR-22Rv1 cell lines as measured by ACA.

DNA damage formation (% Tail DNA) in response to treatment with a range of radiation doses (2-10Gy). Data points are mean ± SEM of n=3 independent experiments. All results calculated using % tail DNA for each comet, 400 comets scored on two slides per replicate. * refers to significant differences in the RR-22Rv1 cell line vs WT control, † refers to significant differences between RR-22Rv1 and AMC cell lines and Δ refers to significant differences between AMC-22Rv1 and WT-22rv1 control cells. ** P≤0.01, *** P≤0.001,**** P≤0.0001

A strong correlation between the % tail DNA and clonogenic survival was identified in all three lines (WT-22Rv1, r=-0.94, p=0.01; AMC-22Rv1, r=-0.94, p=0.01; RR-22Rv1, r=-1, p=0.0028) (Figure 4-2).

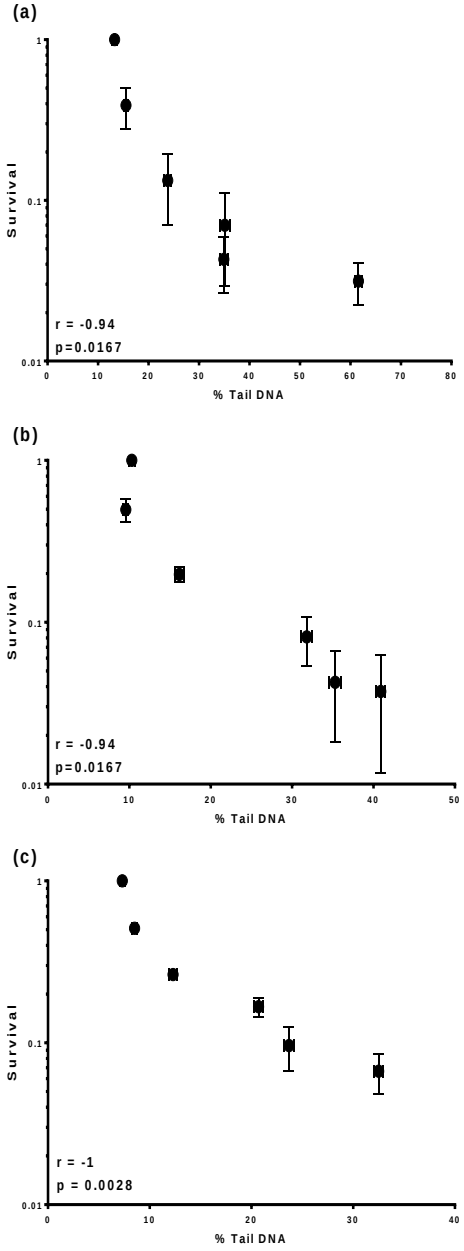

Figure 4-2: Correlation between clonogenic survival and % tail DNA in RR-22Rv1 cells.

Spearman correlation was used to analyse relationship between % tail DNA and cell survival as calculated using clonogenic cell survival assay (a) WT-22Rv1, (b) AMC-22Rv1, (c) RR-22Rv1. . Mean ± SD of at least 3 indepentant experiments.

The ACA was next used to assess the extent of DNA damage repair for up to 50 min following 8Gy exposure (Figure 4-3). The DNA damage

levels decreased rapidly within the first 15 min in all three lines. The reduction was greatest in RR-22Rv1 (2.37-fold), when compared to AMC-22Rv1 (1.81-fold) and WT-22Rv1 (1.3-fold) ($p<0.0001$). For RR-22Rv1, beyond this time point, the % amount of DNA damage continued to decrease, albeit at a slower pace. Fifty minutes post exposure, the initial % tail DNA was reduced 3.9-fold in RR-22Rv1 cells ($p<0.0001$) compared to 2.3- ($p<0.0001$) and 1.12-fold ($p=0.005$) in AMC-22Rv1 and WT-22Rv1 cells respectively.

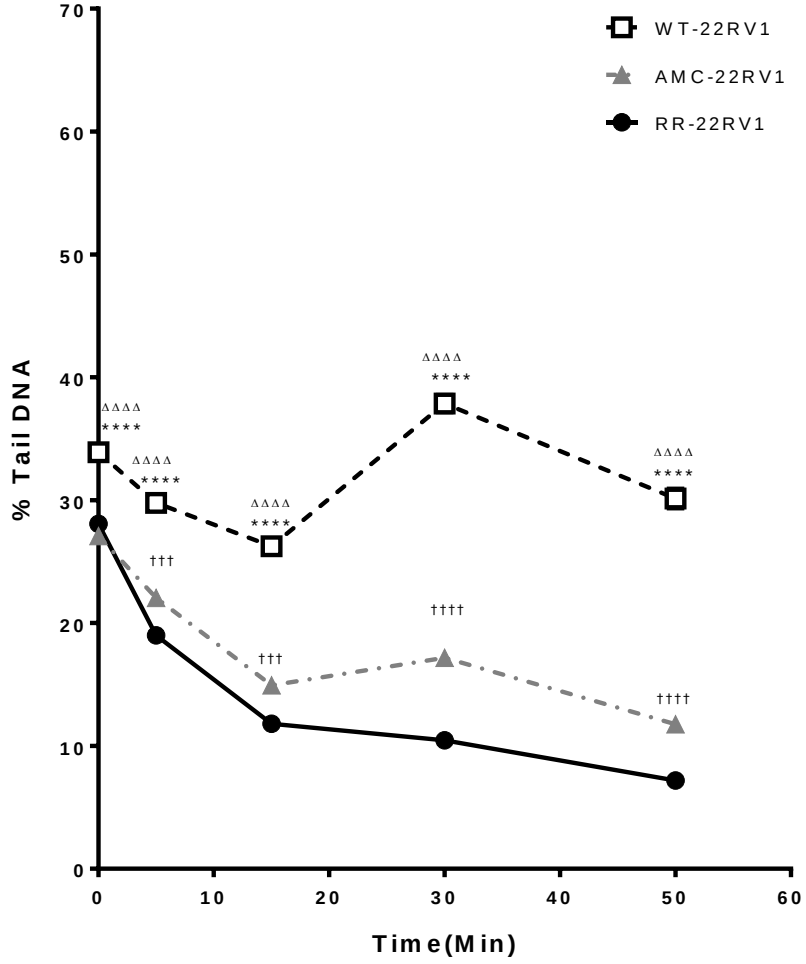

Figure 4-3: Time series analysis of DNA repair in WT-22Rv1, AMC-22Rv1 and RR-22Rv1 cell lines

Initial damage (% Tail DNA) at 0 mins and repair (% Tail DNA remaining) up to 50 mins post irradiation with 8Gy as measured by . n=5 independent experiments. Results are mean ± SEM of % tail DNA for each comet, with 400 comets scored on two slides per replicate. * refers to significant differences in the RR-22Rv1 cell line vs WT-22Rv1 control, † refers to significant differences between RR-22Rv1 and AMC-22Rv1 cell lines and Δ refers to significant differences between AMC-22Rv1 and WT-22Rv1 control cells. ** P≤0.01, *** P≤0.001, **** P≤0.0001

Photographic depiction of DNA damage (Figure 4-4a) and DNA repair (Figure 4-4b) in WT-22Rv1, AMC-22Rv1 and RR-22Rv1 cell lines following treatment with 0-10Gy and 8Gy respectively. The best representative pictures were selected to show tail formation on cells.

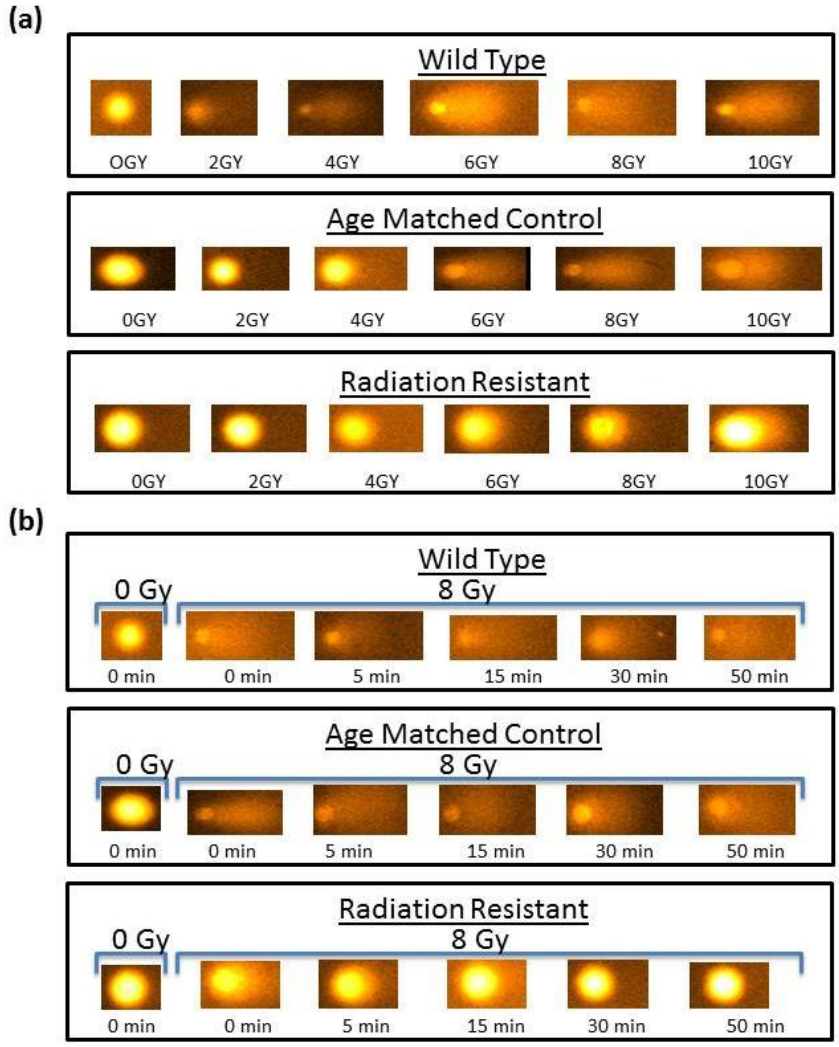

Figure 4-4: Comet Assay

Photographic representation of single cell (a) comet tail formation following treatment with Radiation (2-10Gy) and (b) DNA damage repair capacity represented in the shrinking of the comet tail up to 50 minutes post irradiation with 8Gy.

4.3.2 DNA DAMAGE AND REPAIR GENES

To investigate changes in expression of DDR genes which may be associated with the radioresistant phenotype seen in the RR-22Rv1 cell line q-RTPCR was performed using a panel of ten genes previously associated with the radioresponse of cancer cells. RNA extracted from RR-22Rv1, AMC-22Rv1 and WT-22Rv1 cell line samples was quantified by Qubit fluormeter and analysed by q-RTPCR. The expression profile of the RR-22Rv1 cell line was compared to the AMC-22Rv1 and WT-22Rv1 control lines, the control lines were also compared to each other with and without treatment (4Gy, 4 h incubation). The ΔΔCT method was used to calculate relative quantification (RQ) values (fold change) for each sample compared to the appropriate control. β-actin was selected as the endogenous control for this experiment.

4.3.2.1 COMPARISON OF ENDOGENOUS LEVELS OF DNA DAMAGE AND REPAIR GENES IN 22RV1 ISOGENIC CELL LINE AND CONTROLS

To determine whether endogenous levels of our panel of 10 DDR genes were differentially expressed in our cell lines the RR-22Rv1 was compared to the WT-22Rv1 and AMC-22Rv1 controls.

q-RTPCR results reported *CCNA1* as undetermined in WT-22Rv1. This suggests the gene is either not expressed or expressed at very low levels. Comparatively CCNA1 was found to be expressed in both RR-22Rv1 and AMC-22Rv1 cells (Figure 4-5). No significant difference in expression was identified between the two cell lines (p= 0.3586). Exposure to 4Gy x-irradiation did not result in any change in expression of this gene (AMC: p= 0.5724, RR: p= 0.3169)

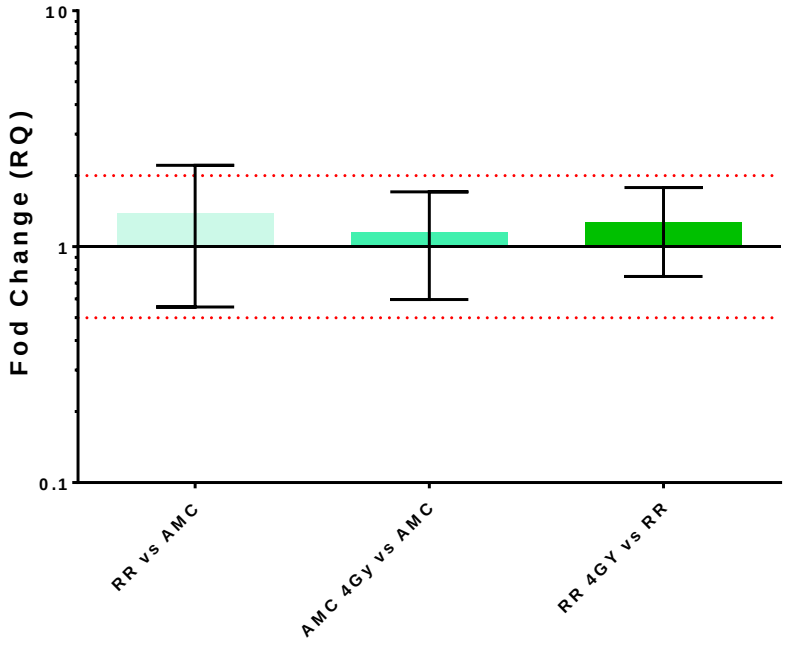

Figure 4-5: *CCNA1* gene expression

Differential gene expression of a panel of *CCNA1* gene expression in the RR-22Rv1 and AMC-22Rv1 sublines with and without exposure to 4Gy. Single sample t-tests were used for statistical analysis with theoretical mean of 1 being set for comparison. Data is plotted as Fold Change (log_{10} scale) ± SD. Red dotted lines represent threshold of 2 fold change.

Evaluation of the RR-22Rv1 relative to AMC-22Rv1 identified three significantly differentially expressed genes (Figure 4-6). Two of these, *CHEK1* and *RAD51*, were down regulated in the RR-22Rv1 compared to the AMC-22Rv1 cell line. *ATM* was the only gene identified as significantly up-regulated. None of these genes met the threshold of 2-fold change in expression suggesting changes in expression may be very small or possibly accounted for by variation in replicate samples rather than actual variation gene expression.

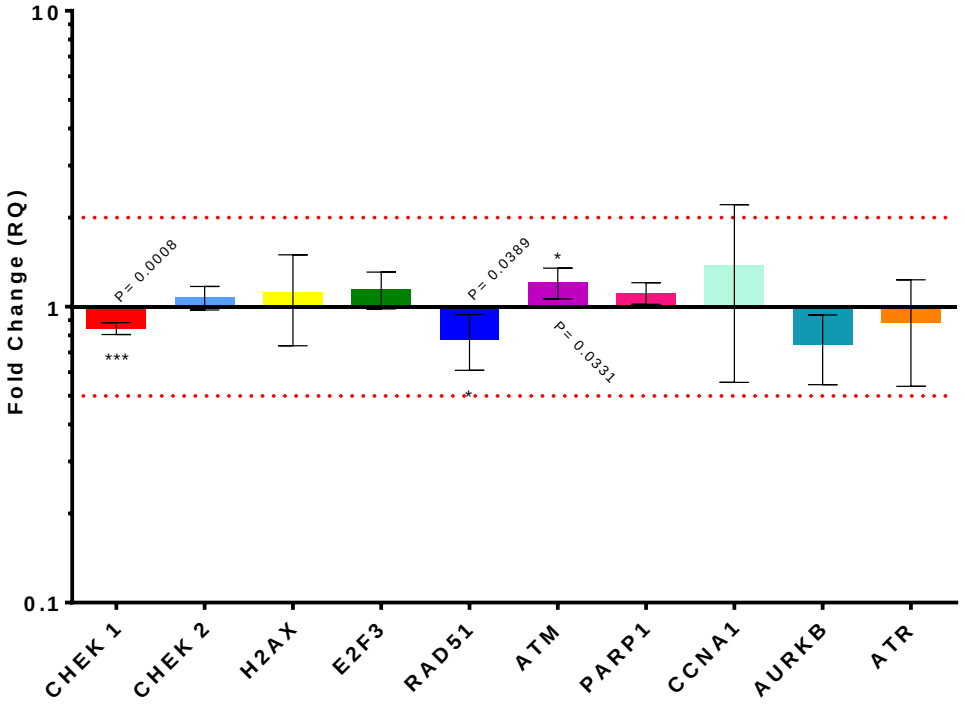

Figure 4-6: q-RTPCR results for a panel of DNA damage and repair genes in the RR-22Rv1 cell line relative to AMC-22Rv1.

Differential gene expression of a panel of DNA damage and repair genes in the RR-22Rv1 cell line compared to the AMC-22Rv1 control. Single sample t-tests were used for statistical analysis with theoretical mean of 1 being set for comparison. Data is plotted as Fold Change (log_{10} scale) ± SD. Red dotted lines represent threshold of a fold change of 2.

Six of the ten genes were identified as highly significantly down regulated in the RR-22Rv1 relative to WT-22Rv1 (P<0.0001): *CHEK1, CHEK2, H2AFX, RAD51, PARP 1* and *ATR* (Figure 4-7).

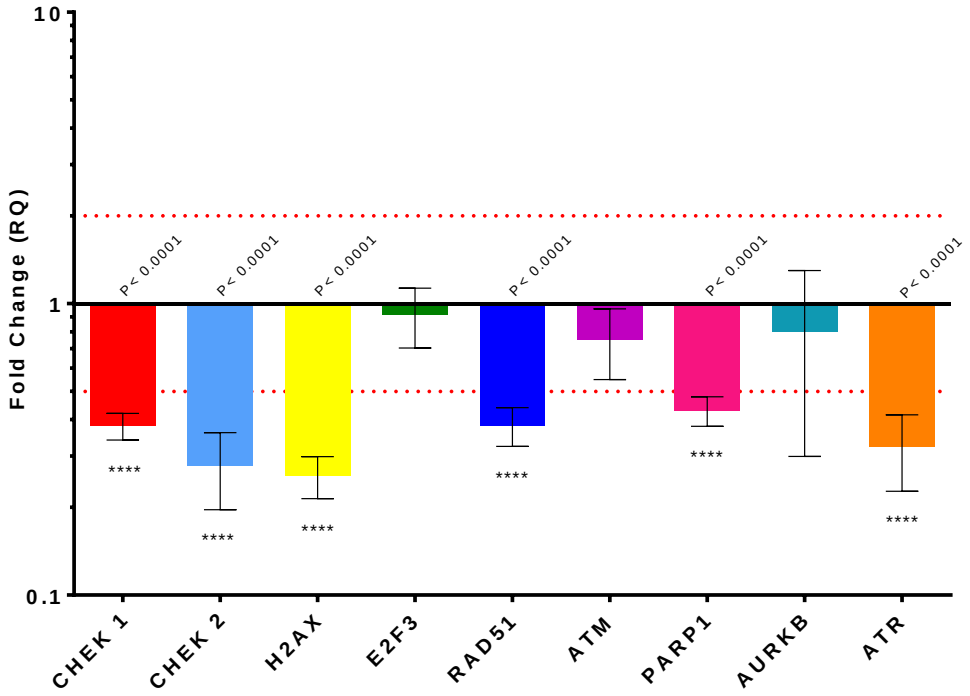

Figure 4-7: q-RTPCR results for a panel of DDR genes in the RR-22Rv1 relative to WT-22Rv1 control

Differential gene expression of a panel of DDR genes in the RR-22Rv1 cell line compared to the WT-22Rv1 control. Single sample t-tests were used for statistical analysis with theoretical mean of 1 set for comparison. Data is plotted as Fold Change (log_{10} scale) ± SD. Red dotted lines represent threshold of a fold change of 2.

Seven significantly down regulated genes were identified in AMC-22Rv1 relative to WT-22Rv1 (P≤0.001): *CHEK1, CHEK2, H2AFX, RAD51, ATM, PARP 1* and *ATR* (Figure 4-8). While *ATM* was found to be differentially expressed compared to the control expression did not meet the 2 fold change threshold. The six remaining genes were similarly down regulated in the RR-22Rv1 line.

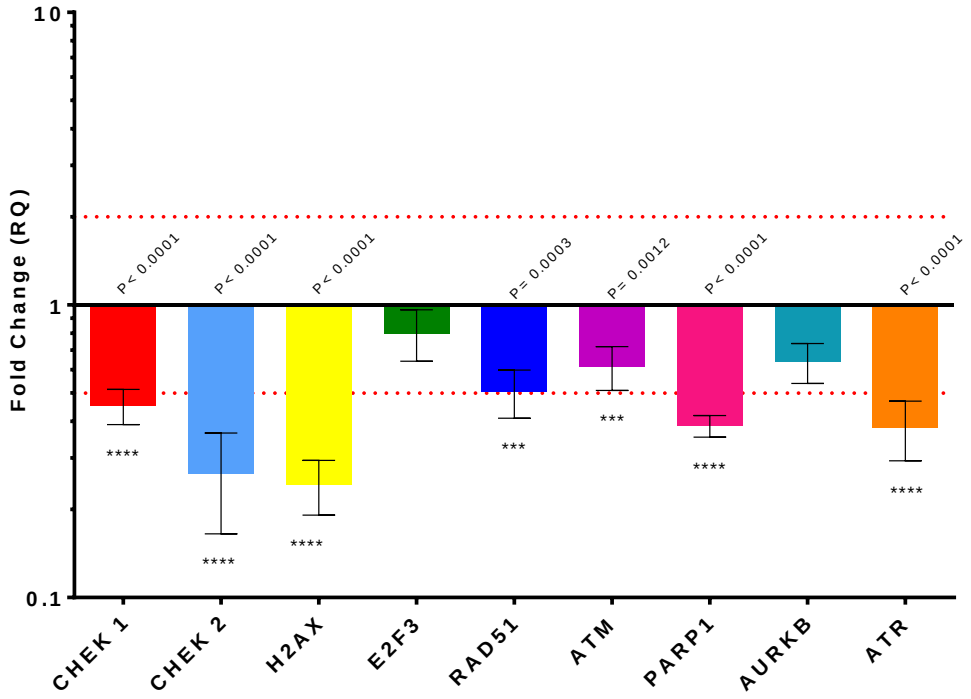

Figure 4-8: q-RTPCR results for a panel of DNA damage and repair genes in the AMC-22Rv1 relative to WT-22Rv1.

Differential gene expression of a panel of DNA damage and repair genes in the AMC-22Rv1 cell line compared to WT-22Rv1. Single sample t-tests were used for statistical analysis with theoretical mean of 1 being set for comparison. Data is plotted as Fold Change (log_{10} scale) ± SD. Red dotted lines represent threshold of a fold change of 2.

4.3.2.2 IONISING RADIATION DOES NOT INDUCE CHANGES IN DDR GENE EXPRESSION IN THE ISOGENIC CELL LINE MODEL

Cell line samples were exposed to 4Gy x-rays and incubated under normal conditions for 4 h and controls were mock irradiated and incubated under the same conditions. Treated samples (WT-22Rv1-4Gy, AMC-22Rv1-4Gy and RR-22Rv1-4Gy) and controls (WT-22Rv1, AMC-22Rv1 and RR-22Rv1) were analysed to investigate the gene response to radiation induced DNA damage within each cell lines. Exposure to ionising radiation did not result in significant changes in gene expression beyond the 2 fold threshold in the three cell lines though a number of genes were significantly differentially expressed .

Three genes were significantly altered in the WT-22Rv1 cell line following exposure to ionising radiation (Figure 4-9a). *H2AFX* was down regulated (p= 0.0051) compared to the untreated control while both *RAD51* (p= 0.0196) and *PARP1* (p= 0.0024) showed increased expression following treatment. No overlap was seen between the wild type and the other cell lines. Up-regulation of *CHEK1* (AMC: p= 0.0049, RR: p= 0.0060) and *E2F3* (AMC: p= 0.0095, RR: p= 0.0049) in response to treatment was common to both the AMC-22Rv1 (Figure 4-9b) and RR-22Rv1 (Figure 4-9c) cell lines.

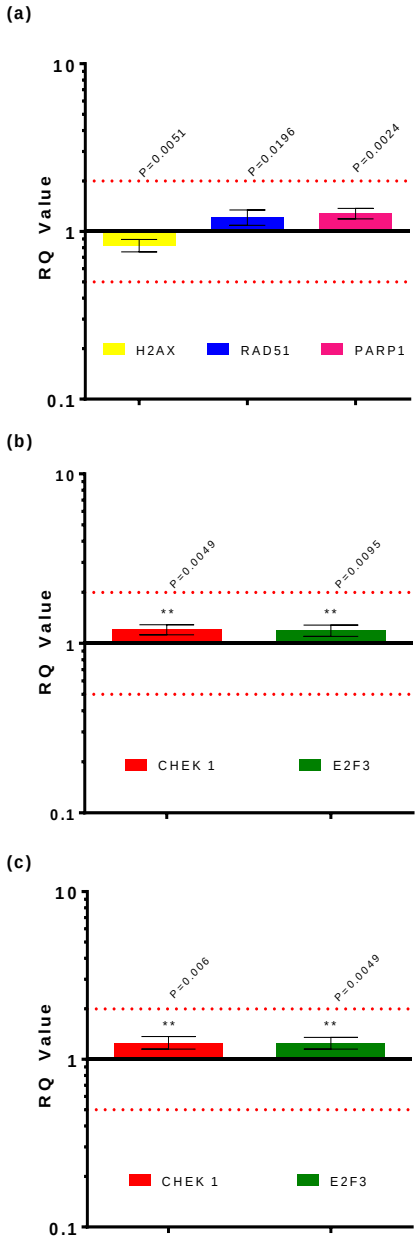

Figure 4-9: The effect of 4Gy x-rays on cell DDR genes in WT-22Rv1, AMC-22Rv1 and RR-22Rv1 cell line

Radiation induced gene expression changes in (a) WT-22Rv1-4Gy vs. WT-22Rv1, (b) AMC-22Rv1-4Gy vs. AMC-22Rv1, (c) RR-22Rv1-4Gy vs. RR-22Rv1. Single sample t-tests used for statistical analysis with theoretical mean of 1 set for comparison. Data is plotted as Fold Change (log_{10} scale) ± SD of five individual sample replicates. Red dotted lines represent threshold of fold change of 2.

RR-22Rv1-4Gy was also compared to the WT-22Rv1-4Gy and AMC AMC-22Rv1-4GY controls to investigate differences between the cell lines following treatment. Controls were also compared to each other.

Treatment with radiation induced significant changes in *RAD51* gene expression in the AMC-22Rv1-4Gy vs. WT-22Rv1-4Gy compared to their untreated equivalent (p=0.0098) (Figure 4-10a). No significant effect was seen in the RR-22Rv1-4Gy vs. WT-22Rv1-4GY following treatment when compared to untreated samples was found (P=0.8788), but comparison of the RR-22Rv1 line to the AMC did show a significant difference (P=0.0251) in *RAD51* expression following exposure to 4Gy X-irradiation (Figure 4-10a).

PARP1 expression was also altered between cell lines following exposure to 4Gy x-rays. Analysis of treated and untreated RR-22Rv1 relative to WT-22Rv1 samples showed significantly less expression of this gene in the RR-22Rv1-4Gy samples (P=0.0048) (Figure 4-10b). This significant decrease in expression was also common to the AMC-22Rv1 vs. WT-22Rv1 following treatment (p=0.0016) (Figure 4-10b). No significant change was seen between the treated and untreated RR-22Rv1 relative AMC-22Rv1 (P= 0.6112).

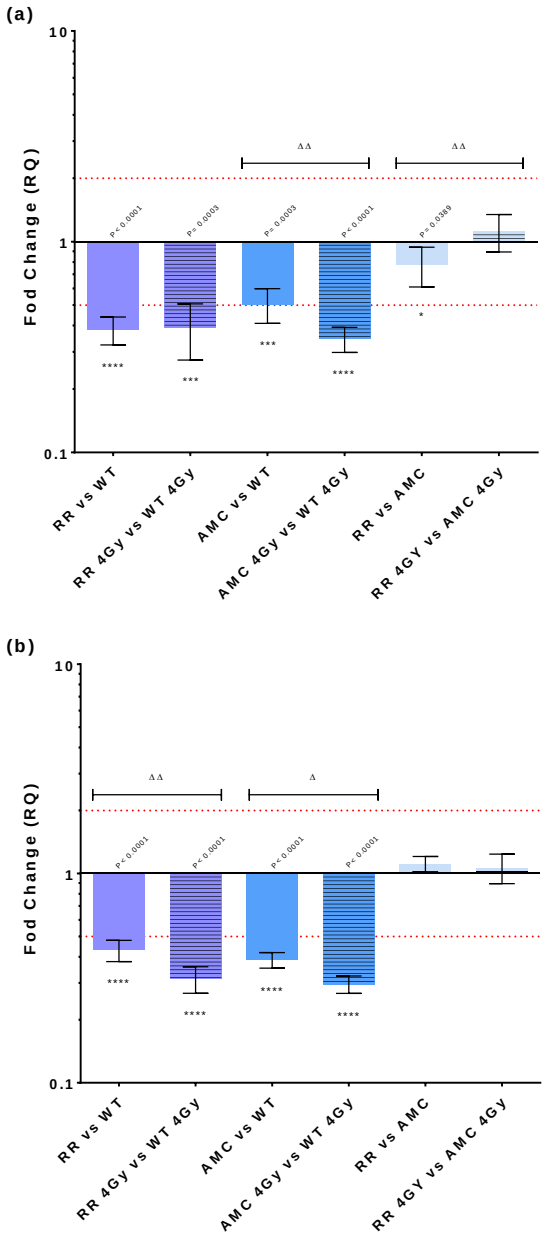

Figure 4-10: Differential gene expression of DDR genes in the RR-22Rv1, AMC-22Rv1 and WT-22Rv1 cell line with and without exposure to 4Gy x-rays

Changes in gene expression of (a) *RAD51* and (b) *PARP1* following treatment with 4Gy. Data is plotted as Fold Change (log_{10} scale) ± SD of five individual replicates. * refers to significant differences between sample and control calculated using single sample t-tests with theoretical mean of 1 set for comparison. Δ refers to significant changes between irradiated sample relatives to controls and unirradiated samples relative to controls as calculated using student t-test. Red dotted lines represent threshold of fold change of 2.

4.3.3 OXIDATIVE STRESS: ROS

The levels of ROS in the live and dead cell subpopulations of irradiated (2Gy, 4Gy) and unirradiated cells were measured using CM-H_2DCFDA staining analysed by flow cytometry. No significant difference was detected in the levels of ROS between the cell lines at all doses when measured in the total population (data not shown). However when the live and dying/dead cell populations were analysed separately (using PI uptake) a significant increase in levels of ROS generation was identified in the RR-22Rv1 cell line and the relatively resistant AMC-22Rv1 cell line compared to the WT-22Rv1 control.

In the live cells (Figure 4-11a), the levels of ROS appeared elevated in RR-22Rv1 and AMC-22Rv1 cells but were not significantly different between the three cell lines for the unirradiated (ANOVA, p=0.092) and the irradiated cells (ANOVA, 2Gy, p=0.07; 4Gy, p=0.16). Similarly, exposure to radiation did not significantly change the amounts of ROS within each of the three lines (ANOVA, WT-22Rv1, p=0.12; AMC-22Rv1, p=0.74, RR-22Rv1, p=0.68).

In the dead/dying subpopulation of cells (Figure 4-11b), the measures of ROS were significantly different between the three cell lines in the unirradiated (ANOVA, p=0.003) and irradiated cells (ANOVA, 2Gy, p=0.001; 4Gy, p=0.002) and highest in the AMC-22Rv1 and RR-22Rv1 cells. However, exposure to radiation did not significantly change the levels of ROS within all three lines (ANOVA, WT-22Rv1, p=0.92; AMC-22Rv1, p=0.09, RR-22Rv1, p=0.10).

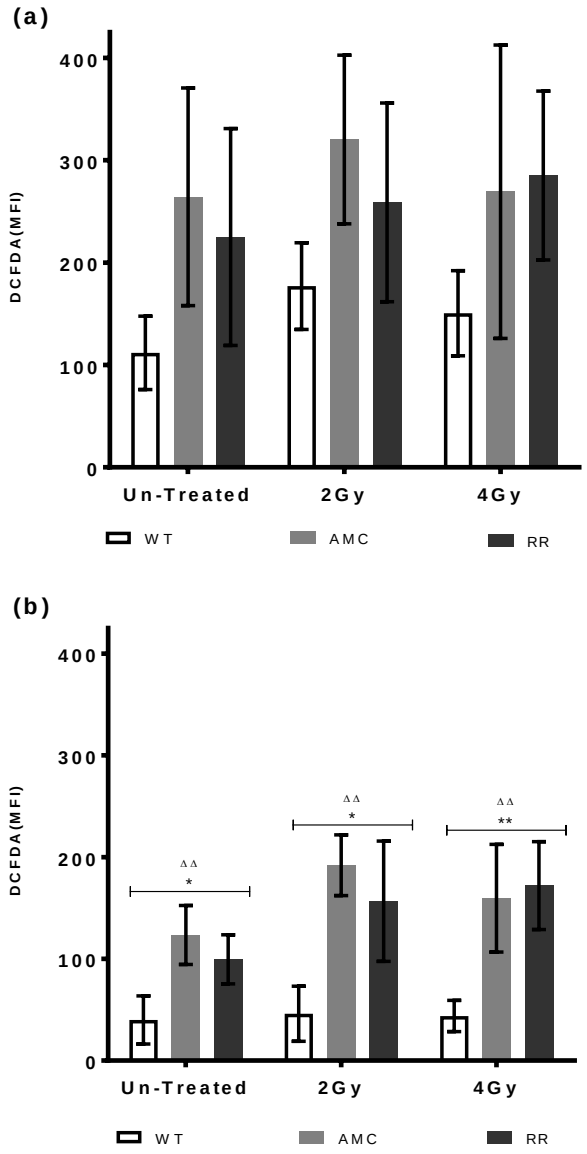

Figure 4-11: Levels of reactive oxygen species in RR-22Rv1 cell line and controls with and without treatment with radiation.

Levels of ROS in cells separated, using PI, into (a) live and (b) dying/dead populations, following treatment with radiation (2/4Gy) compared to untreated controls as measured by Median Fluorescence Intensity (MFI) of DCFDA. Mean ± SD for 4 experiments. * refers to significant differences in the RR-22Rv1 vs. WT-22Rv1 control. Δ refers to significant differences between AMC-22Rv1 and WT-22Rv1 control cells.

4.3.4 CATALASE ACTIVITY

Catalase activity was measured in irradiated (4Gy) and unirradiated cells (Figure 4-12 and Figure 4-13). At a cell number of $5x10^6$, catalase activity was significantly different between the three cell lines in unirradiated (ANOVA, p=0.01) and irradiated cells (ANOVA, p=0.01). Subgroup comparisons indicate that catalase activity is significantly lower in AMC-22Rv1 cells, when compared to WT-22Rv1 under both conditions. In response to radiation exposure, catalase activity was not statistically different in WT-22Rv1 (p=0.5), AMC-22Rv1 (p=0.85) and RR-22Rv1 (p=0.78), when compared to unirradiated controls.

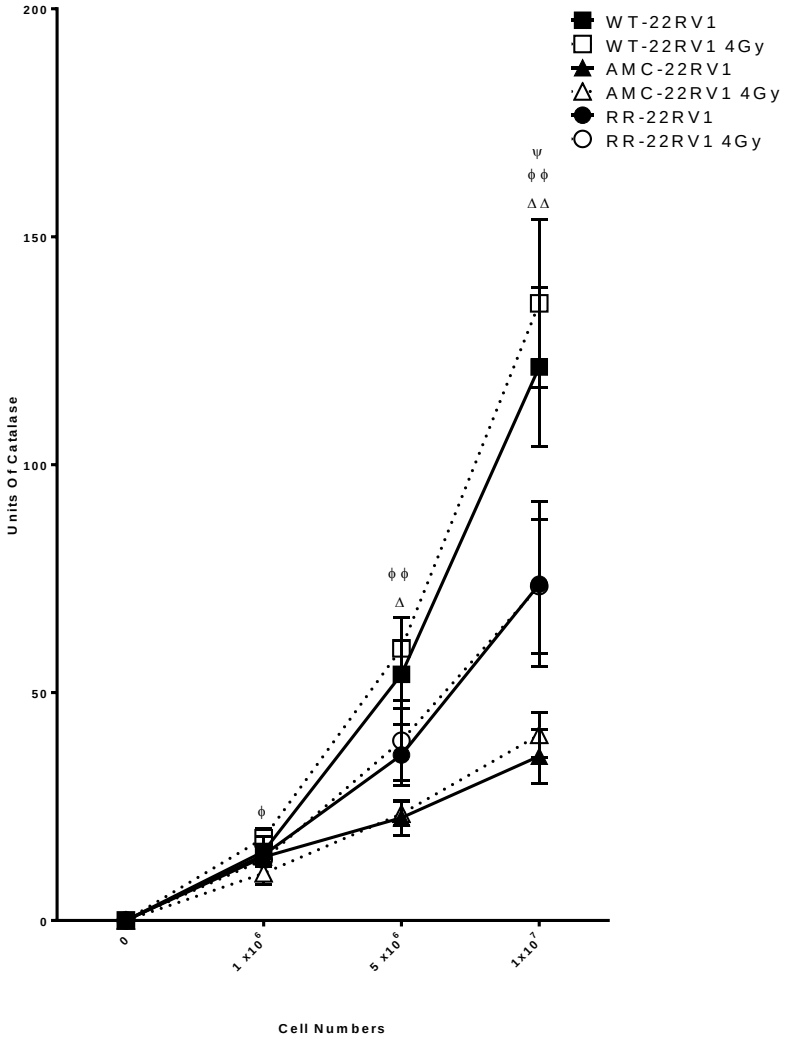

Figure 4-12: Catalase activity following exposure to ionising radiation in WT-22Rv1, AMC-22Rv1 and RR-22Rv1 cells

Catalase activity with and without treatment of 4Gy. Points mean of 4 independent experiments ± SEM. Δ refers to significant differences between AMC-22Rv1 and WT-22Rv1 control cells. * refers to significant differences in the treated RR-22Rv1-4Gy vs WT-22Rv1-4Gy and Φ refers to differences between AMC-22Rv1-4Gy and WT-22Rv1-4Gy treated line. *P≤0.05, ** P≤0.01

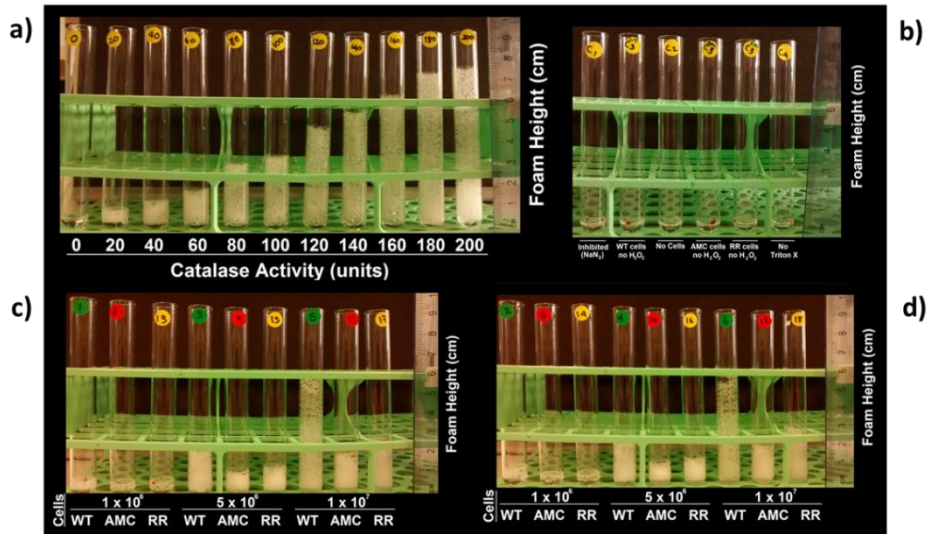

Figure 4-13: Representative images of catalase assay

Photographic representation of samples used in the assay showing foam development as a result of catalase activity. a) Calibration curve for known units of catalase (0-200 units), b) Assay sample controls, c) Catalase activity in un-stimulated cells and d) catalase activity following treatment with 4Gy X-irradiation. Foam measured 15 minutes following completion of reaction. Height of O_2-forming foam measured using a ruler and compared to calibration curve to calculate catalase content.

4.4 DISCUSSION

Formation of DNA damage is the key event for cell killing by ionizing radiation (Dugle et al., 1976) and a cell's ability to repair such DNA damage is crucial to determining a cell's fate following treatment (Bristow et al., 2007). The ACA is a highly sensitive method assessing DNA damage induction and DNA repair capacity of cells in response to clinically relevant doses of radiation which evaluates damage based on the % tail DNA per cell (Singh et al., 1988, Singh et al., 1994, Müller et al., 1994). This technique has been used previously in the evaluation of radiosensitivity in vitro with comet formation shown to correlate with intrinsic radiosensitivity of cancer cells (Moneef et al., 2003, Dunne et al., 2003, McKeown et al., 2003, Calini et al., 2002).

Analysis of % Tail DNA immediately and up to 50 min after radiation exposure indicated that RR-22Rv1 cells were significantly less sensitive to initial DNA damage induction than WT-22Rv1 and AMC-22Rv1 cells and repaired damage more effectively. This characteristic of radioresistant cells was documented in other isogenic cell models (McDermott et al., 2014). In prostate cancer patients receiving androgen deprivation therapy, the induction of reduced DNA repair capacity was proposed as a possible underlying mechanism for the improved response of these patients to subsequent radiotherapy treatment (Tarish et al., 2015). The % tail DNA damage peaked at 30min post-irradiation in WT-22Rv1 cells but not in AMC-22Rv1 or RR-22Rv1 cells. The mechanistic interpretation of this peak should be further investigated. It may reflect induction of DNA damage by secondary radiation-induced reactive oxygen species, such as those released by the mitochondria (Yamamori et al., 2012).

The RR-22Rv1 cell lines increased capacity for DNA damage repair following exposure to radiation led the preliminary investigation of endogenous and radiation induced gene expression of a panel of ten

DDR genes. The *CCNA1* gene was not expressed in the WT-22Rv1 cell line but was expressed in both the RR-22Rv1 and AMC-22Rv1. *CCNA1* is a p53-induced gene (Rivera et al., 2006) involved in cell cycle regulation (Yang et al., 1999), deficiency in it has been previously associated with increased radiosensitivity (Müller-Tidow et al., 2004). Muller-Tidow et al found that cyclin A1 was consistently induced in the duodenum carcinoma HuTu80 and colon carcinoma LoVo cell lines in response to ionising radiation and confirmed this in an in vivo mouse model (Müller-Tidow et al., 2004). Following this, experiments using cyclin $A1^{-/-}$ mice bone marrow irradiated with 1Gy revealed that deficient cells produced less colonies than wild type, the comet assay further confirmed this effect as DSBs were more common in the cyclin $A1^{-/-}$ mice MEF cells than the cyclin $A1^{+/+}$ following radiation exposure (Müller-Tidow et al., 2004). The induction of *CCNA1* gene expression in our cell lines presents it as a potential mediator of the radioresistant phenotype.

Evaluation of baseline gene expression revealed that six DDR genes were commonly down regulated in the RR-22Rv1 and AMC-22Rv1 cell lines relative to the wild type control. The products of these genes are involved in cell cycle progression (*CHEK1* (Liu et al., 2000, Takai et al., 2000, Feijoo et al., 2001) and *CHEK2* (Matsuoka et al., 1998, Bartek et al., 2001)), DNA damage detection (*H2AFX* (Rogakou et al., 1999), ATR), homologous recombination repair (*CHEK1* (Sørensen et al., 2005) CHEK2 (Huang et al., 2008) , *RAD51* (Vispé et al., 1998, Sung et al., 2003), *PARP1* (Schultz et al., 2003)) and base excision repair (*PARP1* (De Murcia et al., 1997) (Dantzer et al., 1999)). Genomic instability as a result of dysregulation of DDR pathways are commonly reported in cancer. CHK2 down-regulation in particular has been associated with radioresistance in multiple studies (Zhang et al., 2004, Hirao et al., 2000, Takai et al., 2002). It is down regulated in non-small cell lung cancer cell lines and patient samples (Zhang et al., 2004), these cells are also more resistant to DNA damage induced apoptosis

and closely mimic the chemoresistant and radioresistant phenotype of the disease seen clinically (Zhang et al., 2004). This radioresistant phenotype was also demonstrated by Hirao et al who showed Chk2$^{-/-}$ mouse thymocytes were 68% (24 h) and 40%(24 h) more viable following exposure to 4Gy γ-irradiation compared to wild type controls (Hirao et al., 2000). They were also more resistant to apoptosis induced by double strand breaks but not single strand breaks (Hirao et al., 2000). Chk2$^{-/-}$ deficient mice showed increased resistance to ionising radiation with a 50-70% reduction in P53 stabilisation compared to WT (Takai et al., 2002). This suggests a potential role for CHK2 in the radioresistant phenotype of our cell lines but down regulation of CHK2 needs to be confirmed on the protein level.

The down regulation of genes involved in the HR pathway and the expression *CCNA1*, which has previously been shown to play a significant role in activation of NHEJ (Müller-Tidow et al., 2004), suggests a potential switch to another repair pathway in the isogenic cell line, though further investigation is needed to confirm this hypothesis. This dependence of cells on one pathway over another shows potential for exploitation by synthetic lethality (Ashworth, 2008). Parp-1 inhibitors are currently in use in conjunction with radiation and chemo- therapy (Rouleau et al., 2010, Ratnam and Low, 2007, Lord and Ashworth, 2008) (Calabrese et al., 2004, Lapidus et al., 2005, Tentori et al., 2003, Albert et al., 2007, Senra et al., 2011, Morgan and Lawrence, 2015) resulting in increased sensitivity to both treatment modalities. While sublines generated in this study are resistant to radiation, data generated in our lab has shown that the RR-22Rv1 and AMC-22Rv1 lines have an increased sensitivity to the chemo therapeutic docetaxel when compared to WT-22Rv1. The down regulation of *PARP1* gene expression may be playing a role in the phenotype and this relationship should be investigated further.

The ability of prostate cancer cells to manage oxidative stress plays an important role in cell signalling and their response to therapeutic injury (Gupta-Elera et al., 2012). In particular, reduced susceptibility to ROS-induced cellular damage and efficient repair of radiation-induced DNA damage was proposed as an underlying mechanism to the observed radioresistance of prostate cancer stem cells (Jayakumar et al., 2014) and lower ROS basal levels have been associated with increased Nfr2 levels and radiosensitivity in prostate cancer cells (Liu et al., 2015, Pinthus et al., 2007). Basal ROS levels were lower in WT-22Rv1, than in AMC-22Rv1 and RR-22Rv1 cells. This reduction was associated with increased catalase activity in WT-22Rv1 cells. ROS levels were elevated in dying RR-22Rv1 and AMC-22Rv1 cells which may suggest that cells with high ROS levels are preferentially eliminated in this cell population or reflects the likely release of ROS in dying cells. The overall trend (though not significant) towards increased basal levels of ROS seen in the RR-22Rv1 compared to the radiosensitive WT-22Rv1 line, suggests a potential role for higher endogenous ROS in the radioresistant and hyper-oncogenic phenotype of our RR-22Rv1 cell line.

The decrease in catalase, an antioxidant enzyme which facilitates the decomposition of H_2O_2 to water and oxygen, in the isogenic RR-22Rv1 cell line and the relatively resistant AMC-22Rv1 may be influencing the DNA damage repair response associated with the radioresistant phenotype. This characteristic was also seen in lung cancer cell lines in which inhibition of catalase followed by treatment with H_2O_2 resulted in a significant increase in Apurinic/apyrimidinic endonuclease 1-redox effector factor 1 (APE1-ReF-1) (involved in base excision repair) expression and was linked to increased DNA damage repair in tumour regions of NSCLC patient samples (Yoo et al., 2008). Western blot analysis of catalase is needed to further confirm its decreased expression in the cell lines.

Chronic exposure of prostate cancer cell line 22Rv1 to fractionated ionising radiation resulted in the generation of a subline with an enhanced tolerance to radiotoxicity. The induction of the radioresistant phenotype seen in this isogenic model is clearly the result of changes to a combination of the mechanisms which underpin the cellular response to radiation exposure rather than alteration of one specific mechanism. Further evaluation of these mechanisms in the context of the radioresistant phenotype is needed and may lead to new targets for re-sensitising primary prostate cancer cells to radiotherapy.

Results from this chapter are contained in an article published in Scientific reports

"Fractionated radiation exposure amplifies the radioresistant nature of prostate cancer cells"

McDermott N, Meunier A, Mooney B, Nortey G., Hernandez C, Hurley S, Lynam-Lennon N, Barsoom SH, Bowman KJ, Marples B, Jones GDD, Marignol L. Scientific Reports. October 2016; 6, 34796

Chapter 5: miRNA MICROARRAY ANALYSIS OF A PANEL OF RADIATION RESISTANT CELL LINES

5.1 INTRODUCTION

While the underlying mechanisms for radioresistance remain poorly understood there is a growing body of evidence showing that microRNAs play an important role in CaP and its radioresistant phenotype (Wen et al., 2014). Expression profiling studies using cell lines and patient samples have shown widespread deregulation of miRNA in prostate cancer (Volinia et al., 2006, Lu et al., 2005, Porkka et al., 2007, Mattie et al., 2006, Ambs et al., 2008, Jiang et al., 2005). For instance, Lichner et al evaluated the miRNA signature of formalin fixed paraffin-embedded prostate tumours resulting in a signature of 25 differentially expressed miRs (including miR-148a, miR-1274b, miR-135a, miR-141, miR-19a, miR-19b, miR-374b, miR-26b, miR-20b, miR-374a, miR-29c, miR-151–5p, miR-174b, miR-196b, miR-26a, miR-331-3p, miR-193a, miR-365, miR-125a and miR-125b) a combination of which had prognostic value for the discrimination between high risk and low risk of biochemical failure in 105 radical prostatectomy samples (Lichner et al., 2013) emphasising the prognostic value of miRNAs in prostate cancer .

Profiling of the cell lines LNCaP and C4-2 by Josson et al, 4 hours post treatment with 6Gy radiation confirmed that several miRNA, miR-521, miR-196a, miR-133b, miR-487 , miR-122a, miR-372 miR-145, miR-143 miR-34c, miR-372 and miR520c in particular, were significantly modified in prostate cancer (Josson et al., 2008, Li et al., 2011). Over expression of miR-521 specifically resulted in significantly decreased cell viability in response to radiation in LNCaP cells, while treatment with a miR-521 inhibitor further confirmed the influence on this miR on the radiosensitivity of this cell line with a significant induction of radioresistance following treatment (Josson et al., 2008).

Global expression profiling using microarrays allow researchers to investigate the expression of thousands miRNA, simultaneously across

multiple samples. While this ability to investigate the expression of several thousand miRNA in multiple samples concurrently is invaluable the reliability of the results must be considered. Therefore candidate miRNA must be validated using q-RTPCR to confirm expression levels in samples. The selection of appropriate endogenous controls (ECs) for normalisation of the q-RTPCR data is vital and the importance of reliable ECs is well documented in the literature (Vandesompele et al., 2002, de Kok et al., 2005). In miRNA research a number of RNA species have been used as ECs in order to correct for non-biological sample to sample differences, these include; the ribosomal RNAs (rRNA) 5S (Takamizawa et al., 2004) and 18S (Kulshreshtha et al., 2007) and the small nuclear RNA (snRNA) U6 (Choong et al., 2007) as well as several miRNAs themselves (Genovesi et al., 2012, Davoren et al., 2008). In a study aimed at identifying suitable reference controls Peltier et al identified 5S rRNA and U6 snRNA as the least stably expressed RNA species as evaluated by q-RTPCR in 13 human tissues when compared to miRNA (Peltier and Latham, 2008), other studies investigating reliable ECs for miRNA expression profiling have also suggested miRNA as the most suitable candidate ECs (Davoren et al., 2008, Genovesi et al., 2012). The general consensus across studies investigating ECs not just for miRNA but gene expression as a whole is that a combination of at least two ECs should be used and validated in samples where possible prior to expression profiling by q-RTPCR.

In order to generate a list of candidate miRNAs which may be involved in the radioresistant phenotype in cancer miRNA expression profiling of cell lines with differing radiosensitivities and their molecular response to radiation exposure is needed. While isogenic models are of use in this context, the use of additional models of resistance has the potential to help identify miRNAs truly associated with the radiation resistant phenotype. It is well established that hypoxia in solid tumours results in a phenotype which is significantly more resistant to RT (Gray et al., 1953), with the difference in sensitively (the oxygen enhancement ratio)

of hypoxic cells normally in the range of 2.5-3 times the dose needed to produce the same level of cell killing in aerobic cells (Brown, 1999). Therefore cells grown under hypoxic conditions present a valuable model for studying radiosensitivity in cells. CSCs have long been hypothesised as a source of RT treatment failure in cancer (Wicha et al., 2006), potentially through changes in the DNA damage response via alteration of stem cell pathways such as Wnt/B-catenin signalling (Chen et al., 2007, Woodward et al., 2007) and Notch (Phillips et al., 2006) as well as others (Rich, 2007). Selection of the CSC subpopulation is difficult but cells capable of clonogenic reproduction or clonogenic cells may be a potential proxy for CSC's.

5.2 AIMS AND OBJECTIVES

The aim of this chapter was to analyse a panel of cell line samples using the Exiqon miRCURY LNA microRNA Array (7th Generation). The samples used were derived from the WT-22Rv1, AMC-22Rv1, RR-22Rv1 cell lines including samples treated with 5Gy x-irradiation, 48 h hypoxia (H48) and clonogenic cells (CLN) as well as untreated controls. The DU145 cell line and sublines were also profiled but due to a lack of significant change in radiosensitivity was discarded from analysis.

Specific objectives:

- To generate a candidate list of miRNA which may be associated with the radioresistant phenotype using global miRNA expression profiling.

- To analyse data generated from the miRNA microarray.

- To define selection criteria for miRNA to be validated using q-RTPCR.

- To independently analyse each model relative to its control, including analysis of the age matched control compared to the WT.

- To cross reference the three models with each other.

- To validate the expression profile of selected candidate miRNA in the cell lines.

5.3 RESULTS

5.3.1 EXIQON miRNA MICROARRAY ANALYSIS

Exiqon confirmed that the expression profiling, carried out according to the workflow in Figure 5-1, was successful and that a subset of the total miRNAs included on the array were differentially expressed in the samples we provided for analysis. The initial analysis of the array was carried out by Exiqon using R/bioconductor statistical software package. This analysis was carried out by Exiqon according to Table 5-1 below. A number of differentially expressed miRNAs were identified under these conditions.

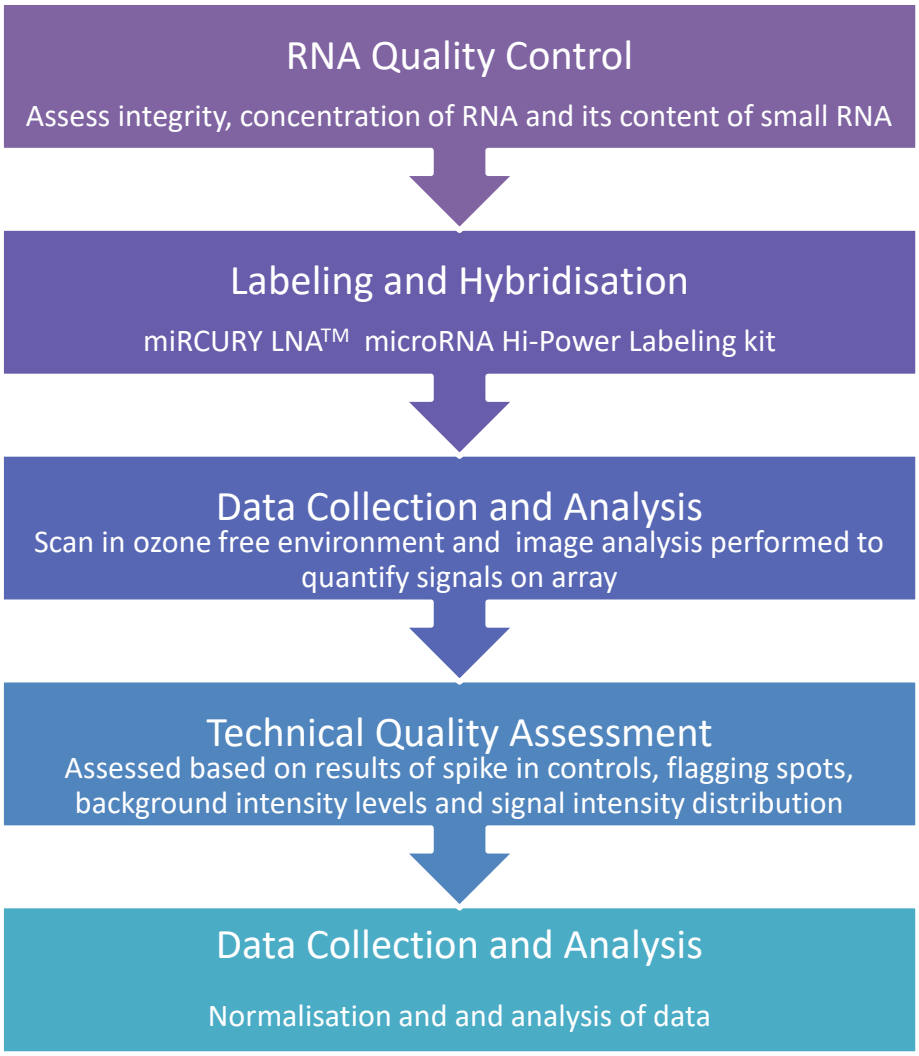

Figure 5-1: Exiqon microarray workflow

The five step workflow used by Exiqon for the miRNA profiling in the cell line samples. Adapted from Exiqon project summary report.

Table 5-1: miRNA microarray guidelines

Sample	Control	Analysis: Identification of differentially expressed miRs
S1. H48[§]-22Rv1	C1.WT[Ψ]-22Rv1	S1 vs. C1
S2. CLN[¶]-22Rv1		S2 vs. C1
		S3 vs. C1
S3. RR[ϕ]-22Rv1		S3 vs. C2
S4.5Gy[Δ]-WT-22Rv1	C2.AMC[¥] 22Rv1	S4 vs. C1
S.5 5Gy-AMC-22Rv1		S5 vs. C2
		C2 vs. C1

[§]Cells grown in Hypoxia for 48 hours
[¶]Clonogenic cells
[ϕ]Radiation resistant cells
[Ψ]Wild type cells
[¥]Age matched control cells
[Δ] cells irradiated with 5Gy x-rays and incubated for 5 hours before harvesting

5.3.1.1 SAMPLE RNA QUALITY CONTROL

Quality control was performed by Exiqon using an Agilent 200 bioanalyzer and the nanodrop to measure concentration of protein contamination and contamination by buffer components or organic compounds. The RNA samples provided for analysis were deemed of high enough quality to proceed with the array protocol.

5.3.1.2 QUALITY CONTROL AND ASSESSMENT

Fifty two separate spike in controls were added in different concentrations to both the HY3^{TM} and HY5^{TM}. High correlation (R^2>0.95) of the signal intensities was found across all the slides for

both labelling channels indicating that labelling and hybridisation reactions were successful.

5.3.1.3 BACKGROUND CORRECTION AND DATA NORMALIZATION

Background correction and normalization was performed successfully by Exiqon using the normexp and Lowess normalisation method respectively.

5.3.1.4 PRESENT CALLS AND THRESHOLD FILTERING

Exiqon calculated the background threshold for individual microarray slides as 1.2 times the 25th percentile of overall signal intensity, miRs with intensities above this threshold in less than 20% of the samples were removed from the data set resulting in 1525 probes being discarded and reported that the number of present calls obtained were in the expected range and comparable for all the samples.

5.3.1.5 UNSUPERVISED ANALYSIS

Principle component analysis was performed on all samples. The top 50 miRs with the largest variation were used to cluster samples based on variation resulting in the separation of samples based on biological differences. A two way hierarchical clustering heat map, also included in the analysis, was generated using the complete linkage method combined with euclidean distance measure. All samples were included in this analysis along with the top 50 miRNA with the highest standard deviation. The samples were seen to generally cluster based on cell line (22Rv1 and DU145) but also on condition (RR, AMC, WT, H48 etc…).

5.3.1.6 SUPERVISED ANALYSIS

Moderated t-statistics were used to calculate p-values for the gene expression analysis and corrected using the Benjamini and Hochberg

multiple testing adjustment method (Benjamini and Hochberg, 1995) resulting in adjusted-p-values (adj.p.value). This resulted in the discovery of 175 of differentially expressed genes between cell lines and treatments. All primary statistical analysis was carried out by Exiqon.

5.3.2 DIFFERENTIAL EXPRESSION OF MIRNA IN CELL LINE SAMPLES

The search for candidate miRNA associated with the radioresistant phenotype was conducted using three models of radiation resistance; hypoxia, the radiation resistant isogenic model and clonogenic cells (Figure 5-2) in the primary CaP cell line 22Rv1. Global modulation in response to treatment with radiation in the 22Rv1 cell line was also investigated in order to elucidate miRNA which may play an important role in prostate cancer's response to radiation therapy (Figure 5-2).

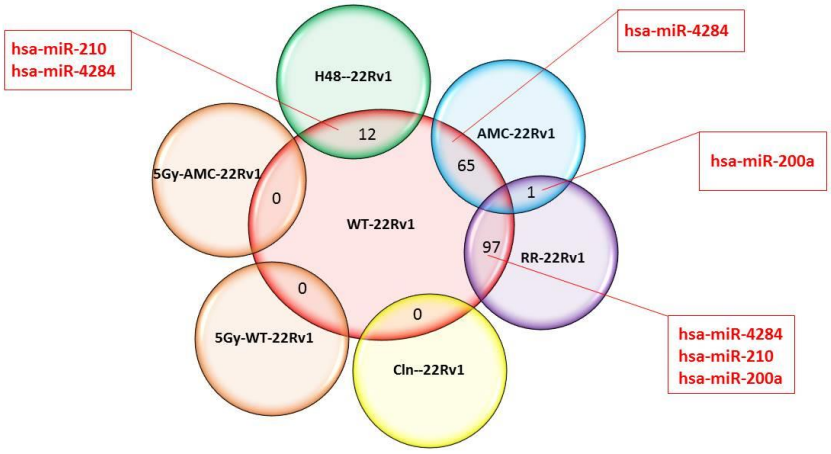

Figure 5-2: Model comparisons for miRNA expression profiling

Modulated miRNA in six 22Rv1 cell line derivatives relative to the untreated WT-22Rv1 control.

Volcano plots were generated for each of the comparisons analysed to enable quick visual identification of miRs which display large magnitude changes that are also statistically significant under each condition. miRNAs were selected for validation using q-RTPCR based on significant changes between the models and control lines. An adj.p.value of <0.05 was selected as statistically significant and the fold change expression levels calculated and represented in the $\log_{10}$ scale with a cut off value of -0.5>logFC>0.5

5.3.2.1 EXPOSURE TO 5GY X-IRRADIATION DOES NOT MODULATE MIRNAS IN THE 22RV1 PROSTATE CANCER CELL LINE

WT-22Rv1 and AMC-22Rv1 cells were exposed to 5Gy x-rays and incubated for 5 h. Following this global miRNA expression levels were measured and no significant change in expression was identified between the treated cells and control in ether line (Figure 5-3).

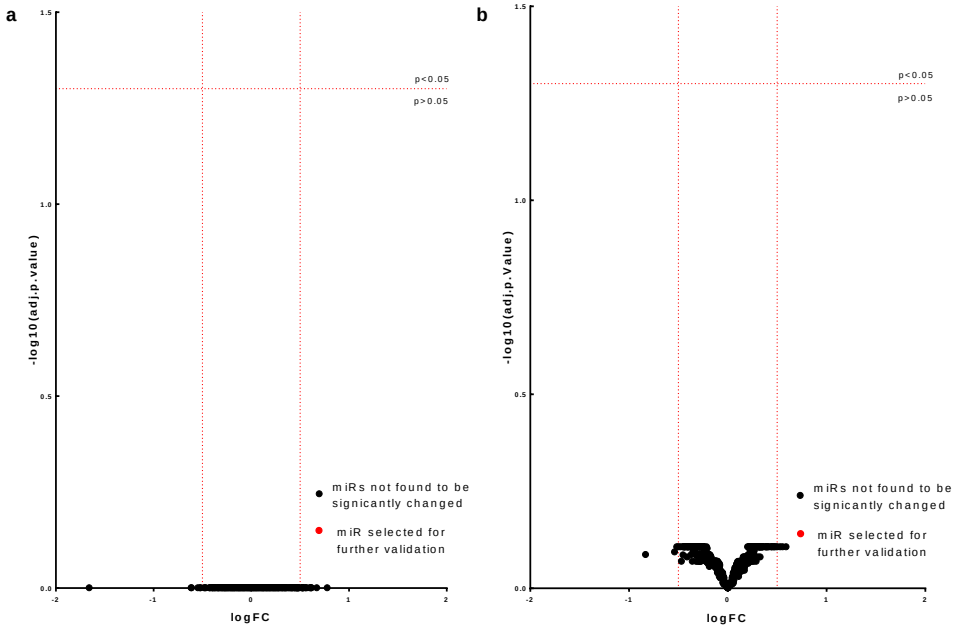

Figure 5-3: Volcano plot of miRNA expression in radiation treated cell lines

Volcano plot represeting the fold change in the expression of significantly altered miRNA in a) WT-22Rv1 and b) AMC-22Rv1 cells treated with 5Gy x-rays and incubated for 5 hours, samples were analysed for changes in global miRNA expression compared to untreated controls. Red dotted lines represent cut offs of statistical significance (P<0.05) and fold change (-0.5>FC>1.5). The negative log of the adj.p.value (base 10) is plotted on the y-axis and the logFC on the x-axis.

5.3.3 SELECTION OF RADIORESISTANT CELLS BY FRACTIONATED RADIATION EXPOSURE RESULTS IN THE MODULATION OF 22RV1 MIRNA PROFILE

Analysis of the RR-22Rv1 vs. WT-22Rv1 data presented 97 differentially expressed miRNAs with adj.p<0.05 with 51 found to be up regulated and 46 down regulated (Figure 5-4).

Table 5-2 lists the top ten miRs identified as differentially expressed between the radiation resistant cell line model and the wild type untreated control line. In order to select candidate miRNAs to be validated with q-RTPCR these were cross referenced to miRs found to be differentially expressed between RR-22Rv1 vs. AMC-22Rv1 and AMC-22Rv1 vs. WT-22Rv1.

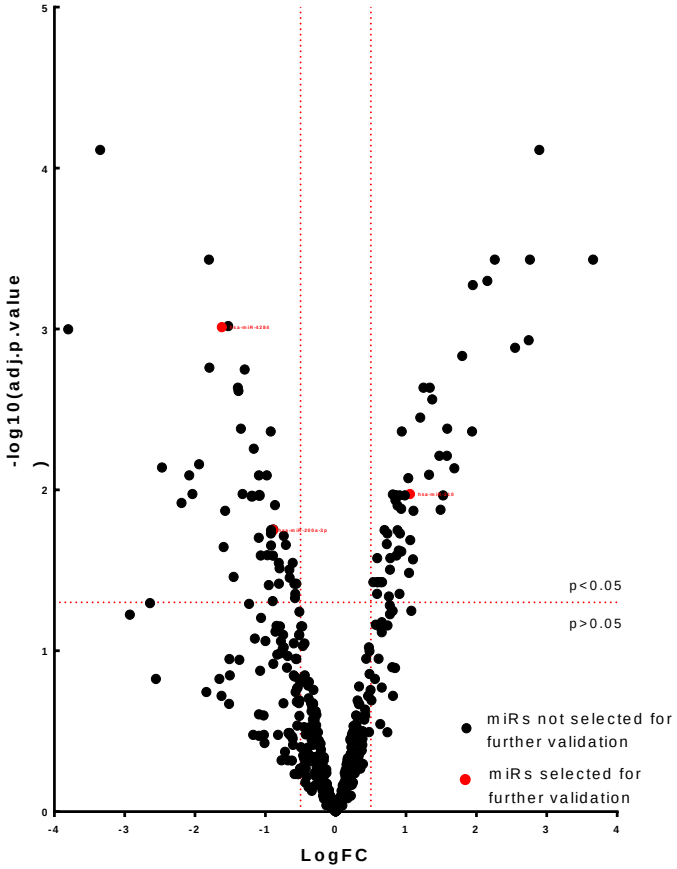

Figure 5-4: Volcano plot of miRNA expression between RR-22Rv1 and WT-22Rv1 cell lines

Volcano plot depicting the fold change in the expression of significantly altered miRNA in the RR-22Rv1 cell line compared to the WT-22Rv1 control. A total of 97 miRNA were identified as differentially expressed in the RR-22Rv1 cell line compared to WT-22Rv1 control. miRs selected for further validation are denoted in red. Red dotted lines represent cut offs of statistical significance ($p<0.05$) and fold change ($-0.5>FC>1.5$). The negative log of the adj.p.value (base 10) is plotted on the y-axis and the logFC on the x-axis.

Table 5-2: The top 10 differentially expressed miR's ranked according to adj.p.value in RR-22Rv1 cell line relative to. WT-22Rv1 control

	Fold Change	Adj.p.value
hsa-miR-130a-3p	2.894	7.72×10^{-5}
hsa-miR-141-3p	-3.349	7.72×10^{-5}
hsa-miRPlus-A1086	3.658	3.71×10^{-4}
hsa-miR-3607-3p	-1.802	3.71×10^{-4}
hsa-miR-222-3p	2.760	3.71×10^{-4}
hsa-miR-29a-3p	2.261	3.71×10^{-4}
hsa-miR-4521	2.155	5.03×10^{-4}
hsa-miR-221-3p	1.948	5.34×10^{-4}
hsa-miR-3607-5p	-1.529	9.6×10^{-4}
hsa-miR-4284	-1.620	9.74×10^{-4}

5.3.3.1 PROTRACTED GROWTH OF 22RV1 CELLS RESULTS IN A miRNA PROFILE SIMILAR TO THAT OF THE RADIORESISTANT SUBLINE

Expression analysis of RR-22Rv1 relative to the. AMC-22Rv1 control (Figure 5-5) identified one differentially expressed miRNA hsa-miR-200a-3p (miR-200a) (Table 5-3). No other miRs were significantly differentially expressed between RR-22Rv1 and AMC-22Rv1 when using adj.p.values.

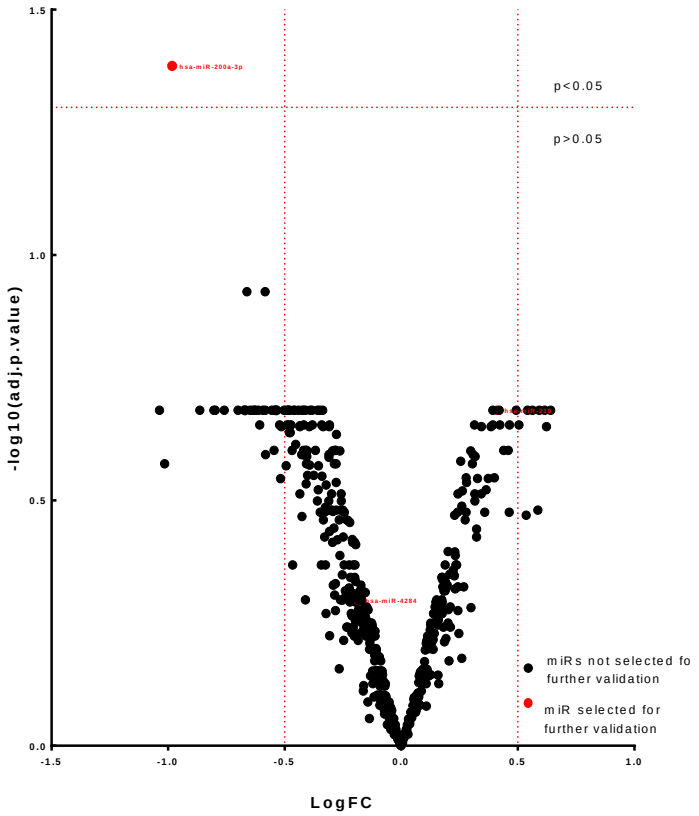

Figure 5-5: Volcano plot of miRNA expression between RR-22Rv1 and AMC-22Rv1 cell lines

Volcano plot showing the fold change in the expression of significantly altered miRNA in the RR-22Rv1 cell line relative to the AMC-22Rv1 control. Microarray analysis identified one differentially expressed miR.. miRs selected for further validation are denoted in red. Red dotted lines represent cut offs of statistical significance (P<0.05) and fold change (-0.5>FC>1.5). The negative log of the adj.p.value (base 10) is plotted on the y-axis and the logFC on the x-axis

Table 5-3: The top miRs differentially expressed according to adj.p.value in RR-22Rv1 cell line vs. AMC-22Rv1 control

	Fold Change	Adj.p.value
hsa-miR-200a-3p	-0.983	4.12×10^{-2}

5.3.3.2 PROTRACTED GROWTH RESULTS IN THE MODULATION OF 22RV1 miRNA PROFILE

Sixty five differentially expressed miRs were revealed between the two control cell lines 23 down regulated and 42 up regulated as visualised in the volcano plot generated from the expression data (Figure 5-6). The top ten differentially expressed miRNA are listed in Table 5-4.

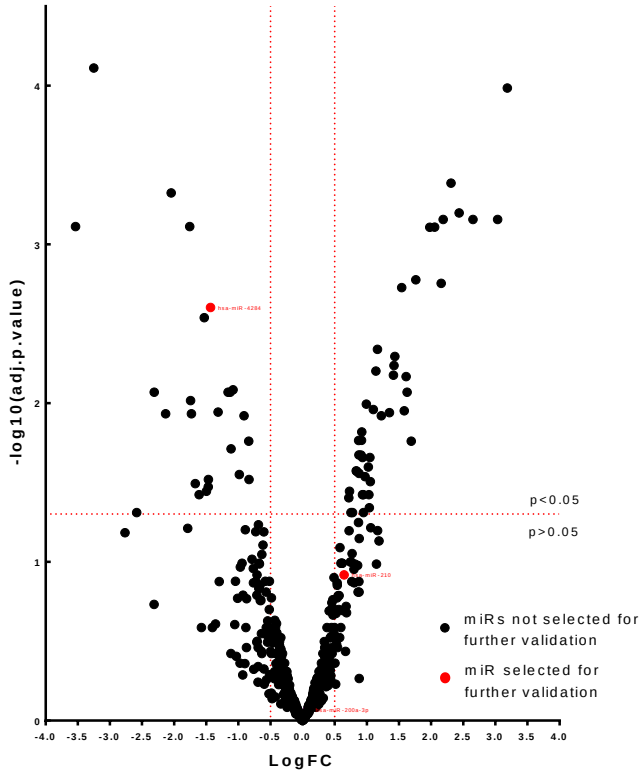

Figure 5-6: Volcano plot of miRNA expression between AMC-22Rv1 and WT-22Rv1 cell line samples

A volcano plot representing the fold change in the expression of significantly altered miRNA in the AMC-22Rv1 cell line compared to the WT-22Rv1 control. Microarray analysis identified 65 differentially expressed miRs. miRs selected for further validation are denoted in red. Red dotted lines represent cut offs of statistical significance (P<0.05) and fold change (-0.5>FC>1.5). The negative log of the adj.p.value (base 10) is plotted on the y-axis and the logFC on the x-axis.

Table 5-4: List of top 10 differentially expressed miRs according to adj.p.value in AMC-22Rv1 control vs. WT-22Rv1 control

	Fold Change	Adj.p.value
hsa-miR-141-3p	-3.252	7.75×10^{-5}
hsa-miR-130a-3p	3.187	1.04×10^{-4}
hsa-miR-29a-3p	2.311	4.11×10^{-4}
hsa-miR-3607-3p	-2.046	4.75×10^{-4}
hsa-miR-222-3p	2.437	6.34×10^{-4}
hsa-miR-31-5p	2.651	6.98×10^{-4}
hsa-miRPlus-A1086	3.035	6.98×10^{-4}
hsa-miR-4521	2.189	6.98×10^{-4}
hsa-miR-200c-3p	-3.536	7.72×10^{-4}
hsa-miR-3607-5p	-1.758	7.72×10^{-4}

5.3.3.3 miRNAS ARE MODULATED FOLLOWING TREATMENT WITH HYPOXIA

Twelve microRNAs were identified as differentially expressed between WT and H48 with adj.p≤0.05 (Figure 5-7). The miR with the highest fold change was hsa-miR-210 (miR-210) with a logFC of 3.711 and adj.p<0.005. This miR is known to be differentially expressed under hypoxic conditions. Only one of the twelve miRs is significantly down regulated- hsa-miR-4284 (miR-4284) with a logFC of -1.090 and adj.p=0.0434. The top differentially expressed miRs are listed in Table 5-5.

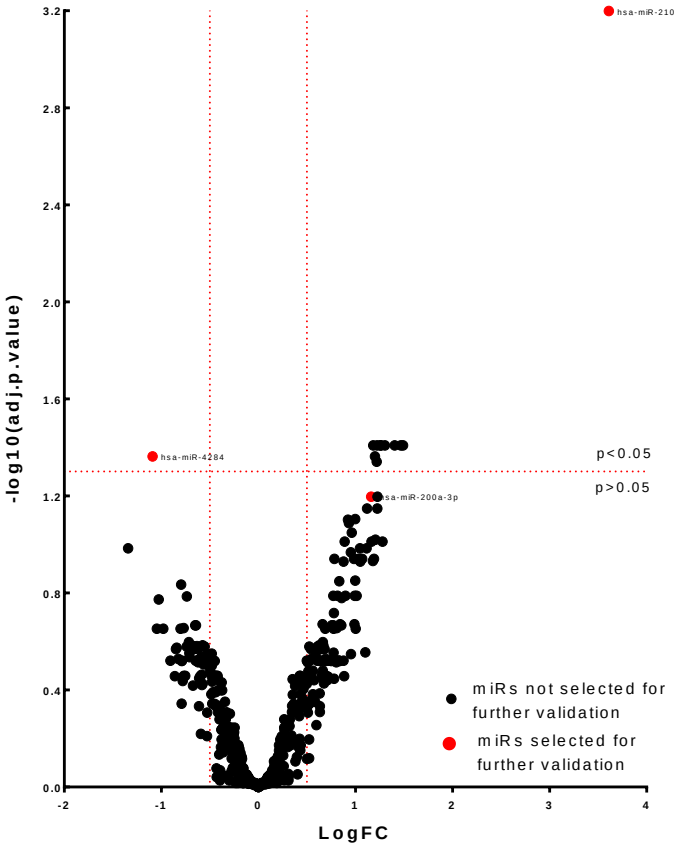

Figure 5-7: Volcano plot of miRNA expression between H48-22Rv1 and WT-22Rv1 cell lines

Volcano plot depicting the fold change in the expression of significantly altered miRNA in the H48-22Rv1 cell line relative to the WT-22Rv1 control. The H48-22Rv1 cell line was generated through exposure to 0.05% O_2 for 48 hours. Microarray analysis identified 12 differentially expressed miRs. miRs selected for further validation are denoted in red. Red dotted lines represent cut offs of statistical significance (P<0.05) and fold change (-0.5>FC>1.5). The negative log of the adj.p.value (base 10) is plotted on the y-axis and the logFC on the x-axis.

Table 5-5: List of top 10 differentially expressed miRs according to adj.p.value in H48-22Rv1 vs. WT-22Rv1

	Fold Change	Adj.p.v alue
hsa-miR-210	3.611	6.32×10^{-4}
hsa-miR-27a-3p	1.490	3.19×10^{-2}
hsa-miR-23a-3p	1.468	3.19×10^{-2}
hsa-miR-19a-3p	1.261	3.19×10^{-2}
hsa-miR-29c-3p	1.224	3.19×10^{-2}
hsa-miR-29a-3p	1.183	3.19×10^{-2}
hsa-miR-141-3p	1.260	3.19×10^{-2}
hsa-miR-30b-5p	1.404	3.19×10^{-2}
hsa-miR-23b-3p	1.303	3.19×10^{-2}
hsa-miR-19b-3p	1.201	4.34×10^{-2}
hsa-miR-4284	-1.090	4.34×10^{-2}

5.3.4 CLONOGENIC CELLS FAIL TO SHOW MIRNA MODULATION RELATIVE TO THE WT-22RV1 CONTROL

No miRNA were identified as differentially expressed between the clonogenic cells (Cln-22Rv1) and the WT-22Rv1 samples (Figure 5-8).

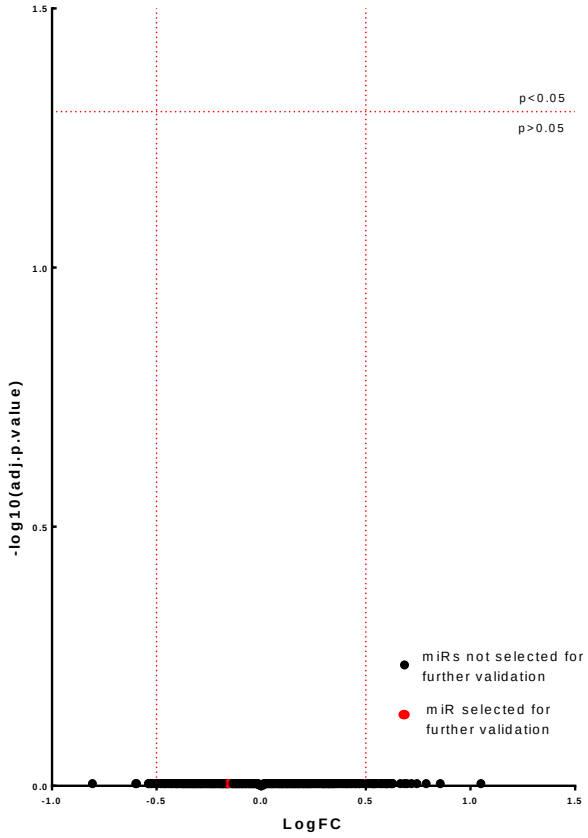

Figure 5-8: Volcano plot of miRNA expression between Cln-22Rv1 and WT-22Rv1 cell line samples

A volcano plot showing the fold change in the expression of significantly altered miRNA in the Cln-22Rv1 cell line compared to the WT-22Rv1 control. no miRNA were identified as differentially expressed following microarray profiling. Red dotted lines represent cut offs of statistical significance (P<0.05) and fold change (-0.5>FC>1.5). The negative log of the adj.p.value (base 10) is plotted on the y-axis and the logFC on the x-axis.

5.3.4.1 IDENTIFICATION OF DIFFERENTIALLY EXPRESSED miRNA IN TWO MODELS OF RADIATION RESISTANCE

Following individual analysis of each model and control differentially expressed miRNA were cross-referenced between the three cell line models (Figure 5-9). With no differentially expressed miRNAs between the clonogenic model and control we selected to remove this model from the analysis. Following this eleven miRNA were identified as

deregulated in both the hypoxic H48-WT-22Rv1 and isogenic RR-22Rv1 radioresistant models.

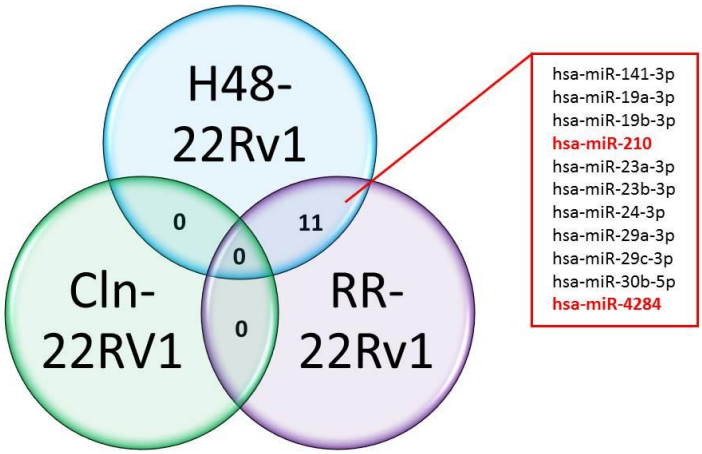

Figure 5-9: Differentially expressed miRNA identified in two models of radiation resistance

Eleven miRNA showed similar differential expression between the H48-22Rv1 and RR-22Rv1 cell lines relative to the WT-22Rv1 control.

Of the eleven shared miRNA five were disregarded due to different patterns of expression in the samples, i.e. up vs. down regulation (Figure 5-10). Of the remaining six miRNAs only one miRNA miR-4284 was down regulated in both models with a logFC of -1.62 and -1.09 in RR and H48 respectively.miR-210 was the most highly expressed of all the miRNAs combined though not the highest in the RR model.

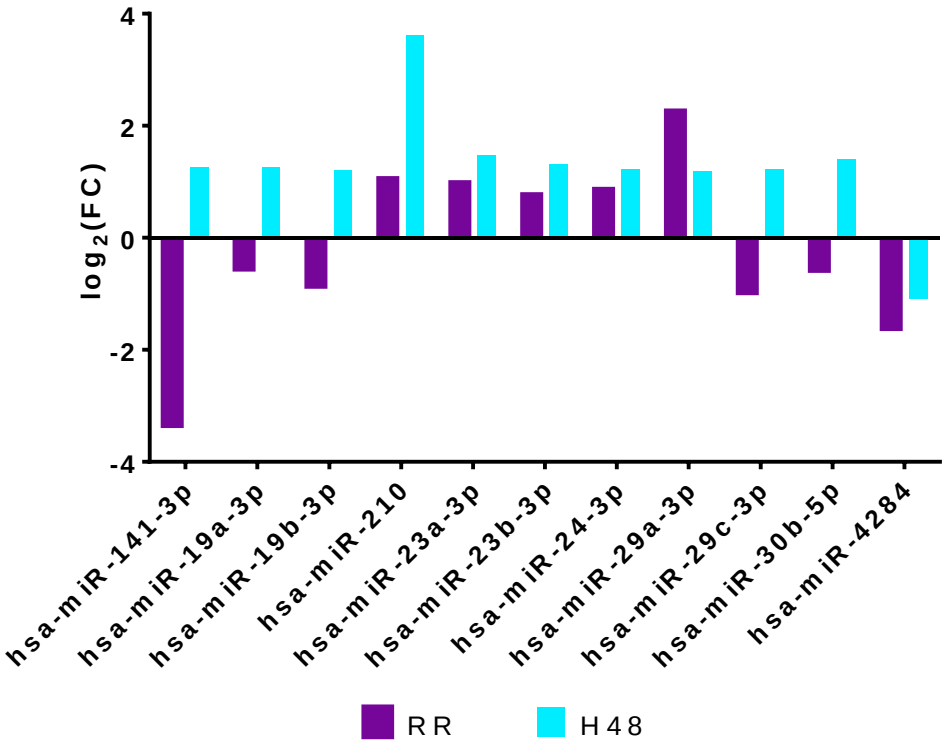

Figure 5-10: Deregulated miRNAs common to both the hypoxic and isogenic radiation resistant cell line models.

Expression of deregulated miRNA common to RR-22Rv1 cell line and H48-22Rv1.Analysed relative to the WT control.. Data is plotted as Fold Change (log2).

5.3.5 SELECTION AND VALIDATION OF A PANEL OF miRNAS AS POTENTIAL BIOMARKERS OF THE RADIORESISTANT PHENOTYPE.

miRNAs were selected from the microarray data for validation with q-RTPCR based on a combination of three criteria: (1) differential expression under each condition, (2) analogues expression in the models of radiation resistance and (3) previous association with treatment response as reported in the literature.

This resulted in the selection of three miRNA; miR-200a-3p, miR-210 and miR-4284. These three miRs can be seen in red on the volcano

plots generated from the microarray data (Figure 5-4, Figure 5-5, Figure 5-6, Figure 5-7), they have been highlighted in the model comparisons (Figure 5-2, Figure 5-9) and Table 5-6 presents the fold change, statistical significance and rank according to the array data for each miR selected for further analysis.

Table 5-6: Differentially expressed miRNAs selected for validation by q-RTPCR

	RR vs.WT			RR vs. AMC			AMC vs.WT			H48 vs. WT		
Name	**logFC**	**adj-p-value**	**Array Rank**	**logFC**	**adj.p-value**	**Array Rank**	**logFC**	**adj-p-value**	**Array Rank**	**logFC**	**adj-p-value**	**Array Rank**
miR-200a-3p	-0.896	**0.0178**	60	-0.983	**0.0412**	1	0.087	0.853	461	1.162	0.0636	13
miR-210	1.056	**0.0106**	39	0.410	0.207	19	0.646	0.121	103	3.611	**0.0006**	1
miR-4284	-1.620	**0.00097**	10	-0.186	0.504	230	-1.434	**0.0025**	16	-1.09	**0.0434**	11

LogFC, adj.p.value and array ranking for miRNA selected for further validation

In red are data showing significant differences (i.e. adj.p.<0.05)

5.3.6 q-RTPCR VALIDATION OF hsa-miR-200a-3p, hsa-miR-210 AND hsa-miR-4284 EXPRESSION IN CELL LINES

RNA extracted from RR-22Rv1, AMC-22Rv1 and WT-22Rv1 cell line samples, along with samples exposed to 48 hours treatment with 0.5% oxygen (H48), were quantified by Qubit flourometer and analysed by q-RTPCR. The three miRNAs selected for validation were detected in all cell lines as were the ECs. The expression profiles (under hypoxic and normoxic conditions) of the RR-22Rv1 cell line were compared to the AMC-22Rv1 and WT-22Rv1 control lines, the control lines were also compared to each other as were the normoxic and hypoxic samples.

5.3.6.1 SELECTION OF ENDOGENOUS CONTROL miRNAS

Prior to validation of our candidate miRNA two miRs were selected as ECs from five potentials; miR-185-5p, miR-302a-3p, miR-340-5p, miR423-5p and miR-574-3p, these were initially suggested by Exiqon from the array data based on stable expression (low stability value) and high signal intensity in the samples (Table 5-7).

Table 5-7: Normfinder results for miRNA suggested as endogenous controls by Exiqon based on microarray data

	Stability Value	Minimum Signal	Average Signal
hsa-miR-185-5p	0.219	6.883	7.380
hsa-miR-574-3p	0.228	7.240	7.693
hsa-miR-340-5p	0.235	7.004	7.513
hsa-miR-302a-3p	0.236	7.718	8.249
hsa-miR-423-5p	0.246	8.324	8.930

q-RTPCR results indicated that miR-302a-3p was not present in all samples and was therefore rejected as a candidate control. Pairwise variation of the combined four remaining candidate miRNA was

calculated using the expression suit software resulting in a stability score for each miR (Table 5-8). Based on these values miR-574-3p and miR-423-5p were proposed as the best ECs for the validation study. Further analysis of the stability scores comparing each miRNA in pairs confirmed this with miRNA hsa-miR-574-3p + hsa-miR-423-5p showing the lowest score of 0.535 followed by hsa-miR-185-5p + hsa-miR-423-5p with a stability value of 0.691. We then interrogated each individual sample replicate to confirm uniform expression of our three candidate miRNA controls. This resulted in the selection of hsa-miR-423-5p (miR-423) and hsa-miR-185-5p (miR-185) (highlighted in red in Table 5-8) as endogenous controls for the study due to their combined stability score and steady similar expression pattern in the samples tested.

Table 5-8: Stability values of candidate miRNA endogenous controls validated using qRT-PCR

	Stability Value*
hsa-miR-185-5p	1.251
hsa-miR-574-3p	0.974
hsa-miR-340-5p	1.777
hsa-miR-302a-3p	-
hsa-miR-423-5p	0.969
	Stability Value$^{\varphi}$
hsa-miR-185-5p + hsa-miR-340-5p	2.163
hsa-miR-185-5p + hsa-miR-574-3p	0.899
hsa-miR-185-5p + hsa-miR-423-5p	**0.691**
hsa-miR-574-3p + hsa-miR-423-5p	0.535
hsa-miR-574-3p + hsa-miR-340-5p	1.487
hsa-miR-423-5p + hsa-miR-340-5p	1.681

*Score is pairwise variation or each miRNA is compared to all other candidates

$^{\phi}$Combined pairwise comparison stability score for each miRNA

Samples in red were selected as endogenous control miRNA for validation study

5.3.6.2 miR-200a

Validation (Figure 5-11a) in the cell line samples showed that expression of miR-200a-3p was not in fact down regulated in the RR-22Rv1 cell line compared to both control lines as suggested in the array and presented in Table 5-9. While a significant down regulation was seen when compared to the AMC the opposite was true of comparison with the WT-22Rv1 control. A significant up regulation of miR-200a-3p was also seen when the AMC-22Rv1 was compared to the WT-22rv1 control. Following treatment with hypoxia the significant down regulation of miR-200a-3p continued to be seen in the H48 RR-22Rv1 vs. H48 AMC-22Rv1 samples while no significant difference was discernable between the H48 RR-22Rv1 vs. H48 WT-22Rv1.

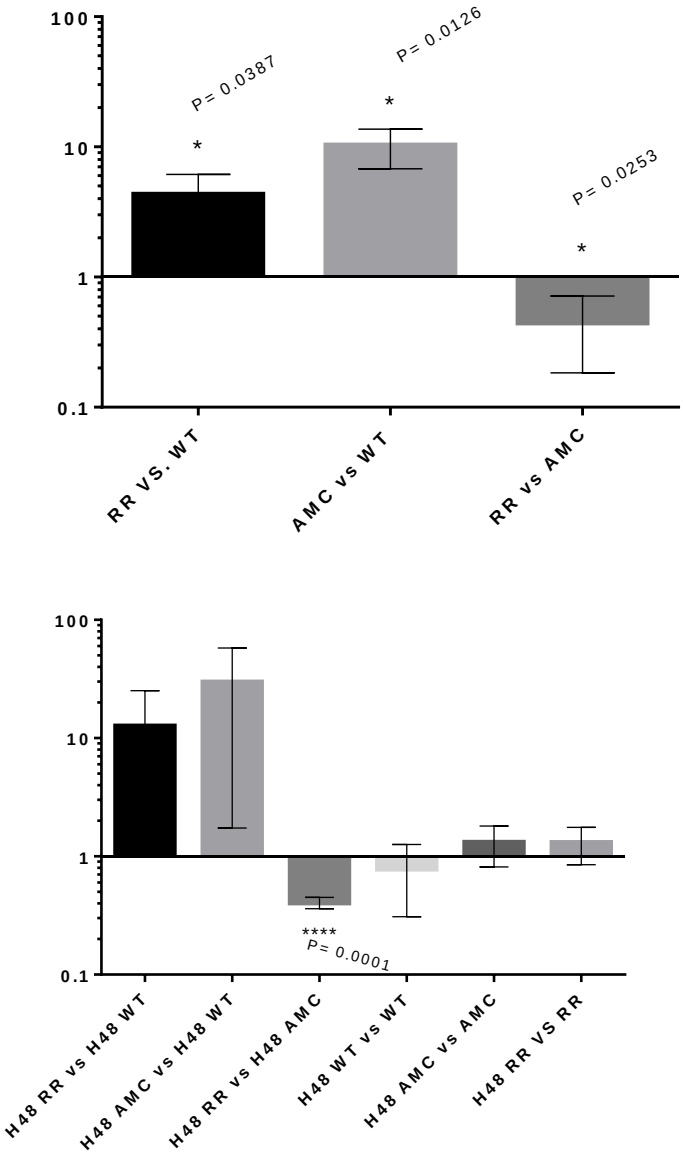

Figure 5-11: q-RTPCR results for miR-200a-3p under normoxic conditions and hypoxic conditions

a) Differential expression of miR-200a-3p under normoxic conditions and b) 48 h hypoxia. Single sample t-tests were used for statistical analysis with theoretical mean of 1 set for comparison. Data is plotted as mean fold change (log_{10} scale) ± SD.

Table 5-9: q-RTPCR validation of miR-200a-3p under normoxic and hypoxic conditions

	Mean Fold Change	Std-Dev	P.value*
RR vs. WT	4.287	1.864	**0.0387**
AMC vs. WT	10.23	3.431	**0.0126**
RR vs AMC	0.448	0.265	**0.0253**
H48 RR vs. H48 WT	12.59	12.6	0.1631
H48 AMC vs. H48 WT	29.74	28.01	0.1324
H48 RR vs. H48 AMC	0.4052	0.04383	**0.0001**
H48 WT vs. WT	0.7839	0.4756	0.4304
H48 AMC vs AMC	1.308	0.4938	0.3006
H48 RR vs. RR	1.305	0.4532	0.2709

$^{\phi}$Mean fold change calculated using Δ ΔCT method

* Statistically significant p.values highlighted in red

5.3.6.3 miR-210

miR-210 showed significantly increased expression (Figure 5-12a) in the RR-22Rv1 cell line compared to both the WT-22Rv1 and AMC-22Rv1 cell lines. This increase in expression (Figure 5-12b) was also present under hypoxic conditions when compared to their untreated controls (Table 5-10). The H48 RR-22Rv1 and H48 AMC-22Rv1 cell lines were also significantly differentially expressed compared to hypoxic WT-22Rv1 samples though no change in expression was found between H48 RR-22Rv1 and H48 AMC-22Rv1 under hypoxic conditions (Table 5-10).

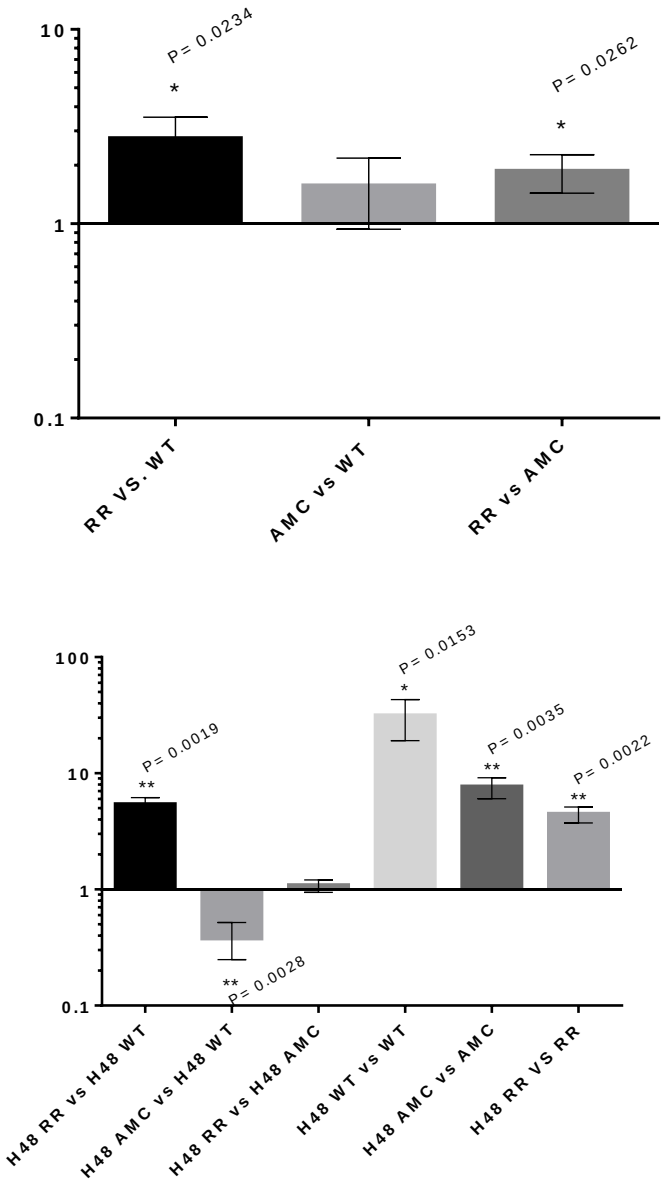

Figure 5-12: q-RTPCR results for miR 210 under normoxic and hypoxic conditions

a) Differential expression of miR-210 under normoxic conditions and b) 48 hours hypoxia. Single sample t-tests were used for statistical analysis with theoretical mean of 1 being set for comparison. Data is plotted as mean fold change (log_{10} scale) ± SD.

Table 5-10: q-RTPCR validation of miR-210 under normoxic and hypoxic conditions.

	Mean Fold Change$^{\phi}$	Std-Dev	P.value*
RR vs. WT	2.727	0.8072	**0.0234**
AMC vs. WT	1.557	0.6207	0.1707
RR vs AMC	1.850	0.4144	**0.0262**
H48 RR vs. H48 WT	5.343	0.8355	**0.0019**
H48 AMC vs. H48 WT	0.3838	0.1352	**0.0028**
H48 RR vs. H48 AMC	1.075	0.1333	0.3429
H48 WT vs. WT	31.12	12.02	**0.0153**
H48 AMC vs AMC	7.596	1.563	**0.0035**
H48 RR vs. RR	4.429	0.6936	**0.0022**

$^{\phi}$Mean fold change calculated using Δ ΔCT method

* Statistically significant p.values highlighted in red

5.3.6.4 miR-4284

miR-4284 was confirmed to be highly significantly down regulated in the RR-22Rv1 and AMC-22Rv1 cell lines compared to WT-22Rv1 control (Figure 5-13a) as was suggested by the array data. Hypoxia was also seen to further decrease its expression in the H48 RR-22Rv1 and H48 WT-22Rv1 cell lines when compared to untreated samples and in the H48 RR-22Rv1 and H48 AMC-22Rv1 samples compared to H48 WT-22Rv1. No significant difference in expression was identified between the radiation resistant isogenic model and the age matched control or their hypoxic counterparts (Figure 5-13b). Changes in expression between samples and controls are presented in Table 5-11.

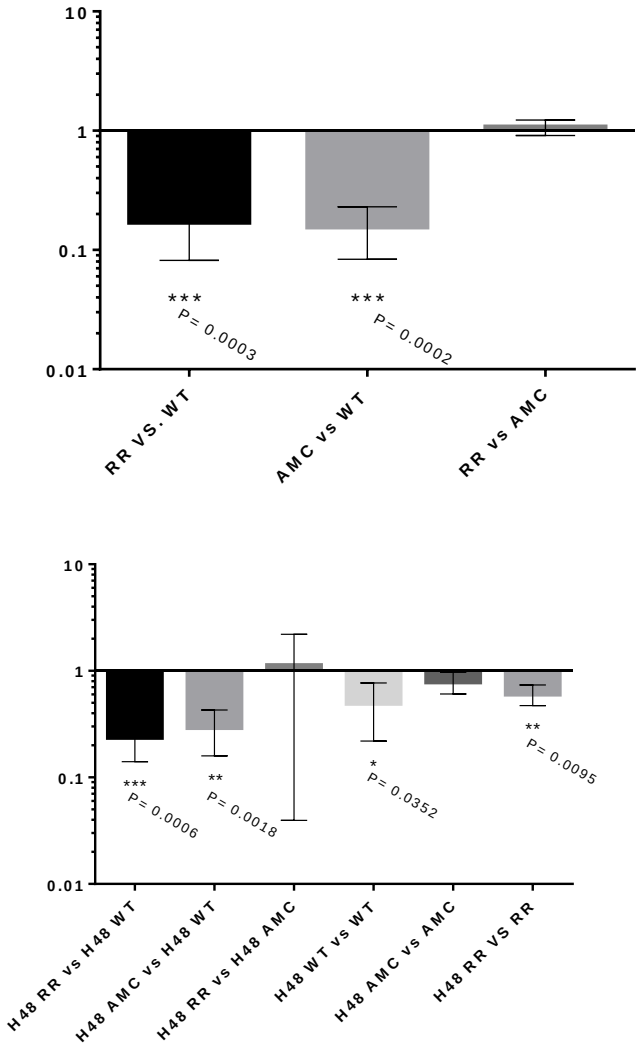

Figure 5-13: q-RTPCR results for miR-4284 under normoxic and hypoxic conditions

a) Differential expression of miR-4284 under normoxic conditions and b) 48 hours hypoxia. Single sample t-tests were used for statistical analysis with theoretical mean of 1 being set for comparison. Data is plotted as mean fold change (log_{10} scale) ± SD.

Table 5-11: qRTPCR validation of miR-4284 under normoxic and hypoxic conditions

	Mean Fold Change$^{\phi}$	Std-Dev	P.value*
RR vs. WT	0.1715	0.0899	**0.0003**
AMC vs. WT	0.1568	0.0734	**0.0002**
RR vs AMC	1.071	0.1596	0.4404
H48 RR vs. H48 WT	0.2376	0.09736	**0.0006**
H48 AMC vs. H48 WT	0.2938	0.1346	**0.0018**
H48 RR vs. H48 AMC	1.12	1.081	0.8384
H48 WT vs. WT	0.4952	0.2756	**0.0352**
H48 AMC vs AMC	0.7881	0.1814	0.1017
H48 RR vs. RR	0.6042	0.133	**0.0095**

$^{\phi}$Mean fold change calculated using Δ ΔCT method

* Statistically significant p.values highlighted in red

5.4 DISCUSSION

To investigate the role of miRNAs in the radioresistant phenotype of CaP, the global miRNA expression pattern of the isogenic model, wild type and age matched controls along with hypoxic and clonal models of radiation resistance was assessed. This identified three candidate miRNAs using the Exiqon miRCURY LNA™ microRNA Array service.

While microarrays are useful tools for researcher their results must be validated due to the lack of reliability in the findings obtained following data analysis (Chuaqui et al., 2002). The identification of differentially expressed miRs between samples and conditions is done using statistical testing, as large numbers of miRs are being tested simultaneously in each array it is of vital importance that a method of multiple testing adjustment is used to control for the false discovery rate (FDR) a type I error. This is the expected proportion of positively selected miRs as differentially expressed when they are in fact not. Therefore methods of adjustment are needed to ensure adequate statistical power for analysis of the microarray. To decrease the overall number of tests being carried out on the data set, therefore increasing the power of the analysis, filtering methods were employed. This resulted in the reduction of the number of probes eligible for further analysis. There are multiple methods available to do this including filtering by variation, MAS detection call and signal intensity (Hackstadt and Hess, 2009). Exiqon selected filtration by signal intensity which removes those probes with a signal close to a designated level by setting a threshold below which miRs are removed from the data set. In this instance threshold filtering resulted in the removal of 1525 probes and found that the obtained number of present calls, i.e. the number of miRs above background threshold, was within the expected range and similar for all samples which also suggests comparable sample quality.

Following unsupervised analysis, which showed that samples tended to cluster within triplicates, a series of moderated t-tests were performed on the data. The p-values from these tests were then corrected using the Benjamini-Hochberg method (Benjamini and Hochberg, 1995), which further controls for the FDR. Using the data generated by the Exiqon microarray 175 statistically significant differentially expressed miRNA were identifed across all 22Rv1 cell lines and conditions, 104 up regulated and 71 down regulated, with a range of logFC from -3.8 to 3.66.

To investigate the role of miRNAs in the radioresponse of prostate cancer expression profiling was performed on the primary prostate cancer cell line WT-22Rv1 and the AMC-22Rv1 subline derived from it following exposure to 5Gy x-irradiation. Though modulation of miRNA in response to radiation treatment has previously been reported in a number of normal (Simone et al., 2009, Vincenti et al., 2011, Wagner-Ecker et al., 2010, Chaudhry et al., 2012, Nikiforova et al., 2011) and cancer cell lines (Niemoeller et al., 2011, Chen et al., 2010, Chaudhry et al., 2010, Shin et al., 2009, Arora et al., 2011, Mueller et al., 2013, Weidhaas et al., 2007) including in the prostate cancer cell lines LNCaP (Li et al., 2011, Josson et al., 2008) and C4-2 (Josson et al., 2008) no significant variation of miRNA expression was identified in either sample following 5 h incubation. While treatment protocols vary from study to study with no consensus on dose or incubation, time course experiments and dose response studies of miRNA alteration which have explored the ideal conditions under which miRNA profiling should be performed confirm that miRNA expression does fluctuate depending on these variables (Chaudhry et al., 2010, Simone et al., 2009) suggesting modification of our treatment protocol may result in the identification of differentially expressed miRNA in the WT-22Rv1 and AMC-22Rv1 cell lines and should be further investigated.

Two candidate biomarkers of treatment response were initially identified from assessment of the individual models and their controls: miR-210 (Grosso et al., 2013) and miR-200a-3p (as part of the miR-200 cluster)(Lin et al., 2013, Shi et al., 2013, Cortez et al., 2014), both of which have previously been associated with radiosensitivity in cancer cells. Following this, differentially expressed miRNA identified in this original analysis were cross-compared between the models for overlapping miRNA which may be involved in radiation resistance. However the analysis performed by Exiqon revealed that the clonogenic samples showed no statistical difference to the WT-22Rv1 control. We selected to use these cells as a potential proxy for CSCs due to the small size and difficulty of isolating the CSC subpopulation in our cell line. It is well established that only a small percentage of cells within a tumour are capable of clonogenic proliferation in vitro (Park et al., 1971) and in vivo (Bruce and Van Der Gaag, 1963), isolation of CSCs from a number of cancers including; blood (Dick, 1997, Caceres-Cortes et al., 1994), pancreatic (Hermann et al., 2007, Li et al., 2007), head and neck (Prince et al., 2007), brain (Singh et al., 2004, Yuan et al., 2004), breast (Al-Hajj et al., 2003), Ewings sarcoma (Suva et al., 2009), liver (Yang et al., 2008), bladder (Chan et al., 2009), ovarian(Zhang et al., 2008b), colon (Ricci-Vitiani et al., 2007, O'Brien et al., 2007) and CaP (Patrawala et al., 2006, Hurt et al., 2008) have shown that this small subpopulation of cells are enriched for this ability to form tumours when transplanted into mice where other cells could not. We therefore predicted that by selecting for the cells which retain this proliferative capability we would also select for the rare subpopulation of CSCs within our cell line. Unfortunately this does not appear to be the case, this may be due to a number of reasons; the cells selected by low density plating did not contain the CSC subpopulation or the presence of non CSCs in much greater numbers within the selected population of cells masked the CSC subpopulation. The clonogenic model was removed from our analysis and we selected to move forward with the

isogenic RR-22Rv1 and hypoxia treated H48-22Rv1 cell lines as models of the phenotype.

Cross-referencing of these models resulted in the identification of six candidate miRNA which are similarly expressed in both lines. miR-210 was further endorsed as a candidate biomarker of treatment response as it was highly significantly up regulated in both the RR-22Rv1 and H48-22Rv1 models. Of the 5 remaining candidate miRNA only one was down regulated in both lines, miR-4284. Additionally this miRNA was down deregulated in the AMC-22Rv1 cell line as well. As previously discussed while this cell line is significantly less sensitive to radiation than the isogenic and hypoxic cells, a trend towards increased resistance was seen in the clonogenic cell survival assay. This cell line consequently could model age related acquisition of increased radioresistance in the cells. Taken in this context the parallel down regulation of this miRNA in the AMC-22Rv1 cell line as seen in the isogenic and hypoxic models may suggest a role for miR-4284 in the radioresistant phenotype. This miRNA also presented a novel marker of the radiation resistance as it has not to our knowledge been associated with prostate cancer or treatment failure. We therefore selected miR-200a-3p, miR-210 and miR-4284 for further investigation.

miR-200a was selected following interrogation of microarray data: it was the only miR significantly differentially expressed between the RR-22Rv1 cell line and both controls and therefore an obvious candidate for validation. This miRNA is a member of the miR-200 family (miR-200a/c/b, miR-140 and miR-429), a number of which have been suggested as potential biomarkers for detecting cancer (Madhavan et al., 2012, Cheng et al., 2011, Park et al., 2009), as well as being associated with disease outcome in various cancers (Cheng et al., 2011, Madhavan et al., 2012, Tejero et al., 2014, Li et al., 2015, Valladares-Ayerbes et al., 2012, Xu et al., 2012, Hu et al., 2009, Torres et al., 2013, Pecot et al., 2013). Down regulation of miR-200a

specifically has been associated with poor treatment outcome in prostate cancer following prostatectomy (Barron et al., 2012). This family of microRNAs have also been associated with tumour progression in a number of cancers (Feng et al., 2014) including prostate (Williams et al., 2013) through mechanisms such as promotion of metastases, as they are known to play a significant role in the epithelial to mesenchymal transition (EMT), (Park et al., 2008, Gregory et al., 2008, Korpal et al., 2008) (Korpal et al., 2011) (Dykxhoorn et al., 2009) and angiogenesis, (Pecot et al., 2013). They have also been implicated in treatment failure including chemoresistance (Manavalan et al., 2013, Manavalan et al., 2011, Liu et al., 2014, Muralidhar and Barbolina, 2015) (Brozovic et al., 2015, Senfter et al., 2015) and radiation sensitivity (Shi et al., 2013, Lin et al., 2013) with overexpression of miR-200c found to increase cellular radiosensitivity in lung cancer (Cortez et al., 2014). Previous association of this miR with tumorigenesis and its prognostic potential in other cancers made it an attractive candidate for further validation with q-RTPCR.

miRNAs are known modulators of the hypoxic response (Zhang et al., 2009) with multiple miRs found to be deregulated in response to oxygen deprivation (Kulshreshtha et al., 2007). The up-regulation of miR-210 following exposure to hypoxic conditions in both malignant and normal cells is well-established in the literature (Kulshreshtha et al., 2007, Zhang et al., 2009, Huang et al., 2010). It has also been associated with poor prognosis in multiple cancers including; Breast (Zhang et al., 2009), melanoma (Zhang et al., 2009) colorectal (Qu et al., 2014) and non-small cell lung cancer (Osugi et al., 2015). In CaP, its serum levels, alongside those of miR-220c, miR-141, miR-200a and miR-375, may be indicative of metastatic disease (Haldrup et al., 2014, Cheng et al., 2013). Knockdown of miR-210 in human hepatoma xenograft enhanced the anti-tumour effects of radiotherapy (Yang et al., 2013) and may prevent the development of radiation enteropathy (Hamama et al., 2014). But treatment with the LNA miR-210 inhibitor did not enhance

the clonogenic survival of PC3 CaP cells following irradiation (Quero et al., 2011) We selected to validate the findings of the array that miR-210 is up-regulated not only in response to hypoxic conditions in the 22Rv1 cell line but also in the 22Rv1 sublines compared to the wild type control under normoxic and hypoxic conditions.

miR-4284 is not well characterised in the literature and has not previously been directly associated with radioresistance in prostate cancer, its deregulation in our models therefore presents it as an attractive candidate for a novel marker of RT treatment outcome.

While all three miRNAs were found to be detectable using q-RTPCR in the cell line samples our results further emphasise the importance of array validation as, even though miR-210 and miR-4284 were confirmed to be differentially expressed (up and down regulated respectively) in the RR-22Rv1 and hypoxic samples as was suggested by the microarray data, miR-200a-3p showed a different expression pattern in contradiction to the microarray. Though differential expression was still significant between the isogenic cell line and controls, expression of miR-200a-3p was found to be up-regulated in both the RR-22Rv1 and AMC-22Rv1 compared to wild type. Although all candidate miRNA validated successfully we selected to further investigate miR-4284 due to its novel nature in prostate cancer and validate its effect on the radioresistant phenotype.

Chapter 6:

VALIDATION OF hsa-miR-4284 AS A MARKER OF RADIORESISTANCE IN PROSTATE CANCER

6.1 INTRODUCTION

Deregulation of miR-4284 has been reported in multiple cell types (Zhang et al., 2012, Abu-Halima et al., 2013, Xie et al., 2014, Shi et al., 2015, Qin et al., 2016, Chang et al., 2016, Stuopelytė et al., 2016, Xiong et al., 2013) including prostate cancer (Hessvik et al., 2012, Stuopelytė et al., 2016, Munari et al., 2014, Wang et al., 2015) but very few have validated and explored the functional role of this miRNA further (Li et al., 2013, Wang et al., 2014, Yang et al., 2014, Koukos et al., 2015, He et al., 2015).

Increased expression of miR-4284 in exosomes has been associated with IFN-α-induced antiviral activity in hepatitis B virus (Li et al., 2013). It was suggested as potential novel biomarker of early diagnosis of Arteriosclerosis obliterans (He et al., 2015) and implicated along with increased c-Jun N-terminal kinase / stress-activated protein kinase (JNK/SAPK) signalling in the anti-tumour activity of the Berbamine derivative BBMD3 in cancer stem cell like glioblastoma cells (Yang et al., 2014). Down regulation has been associated with clear cell papillary renal cell carcinoma and paediatric ulcerative colitis and validated as targeting the C-X-C motif chemokine 5 (CXCL5) mRNA in this disease (Koukos et al., 2015). miR-4284 was additionally significantly down regulated in the kidney cell line HK-2 following treatment with calcium oxalate monohydrate, a major component in kidney stones, and involvement of its targets in cell death was predicted (Wang et al., 2014)..

This miRNA has also previously been shown to be bound by the cold shock domain protein Y-box binding protein 1 (YB-1) (Belian et al., 2010) an oncoprotein with multiple biological functions involved in regulation of transcription, translation and DNA repair along with pre-mRNA transcription and splicing, regulation of mRNA stability and translation and mRNA packaging (Eliseeva et al., 2011). Dysregulation

of YB-1 protein expression is widely reported in cancer (Kohno et al., 2003) including prostate (Giménez-Bonafé et al., 2004). Elevated levels of YB-1 are associated with poor outcome (Shibahara et al., 2001, Gessner et al., 2004, Janz et al., 2002, Oda et al., 2003, Kamura et al., 1999, Wu et al., 2012b), chemoresistance (Bargou et al., 1997, Ohga et al., 1996, Guay et al., 2008, Janz et al., 2002, Yahata et al., 2002), increased metastasis and invasion (Wu et al., 2012b, Evdokimova et al., 2009, Khan et al., 2014) through promotion of EMT (Evdokimova et al., 2009, Khan et al., 2014), and has been reported in high risk sarcomas as a critical regulator of HIF1α (El-Naggar et al., 2015); dysregulation of which is seen in radiation resistant tumours (Moeller et al., 2004). High cytoplasmic levels of this protein in patients' pre-treatment biopsies were also correlated with reoccurrence following radiation therapy in nasopharyngeal cancer (Tay et al., 2008). The relationship between YB-1 and miR-4284 along with the association of its expression with prostate cancer reoccurrence, recommended (in conjunction with MTA1) by Sheridan as a predictor of prostate cancer reoccurrence (Sheridan et al., 2015), plus its correlation with radioresistance make it an attractive candidate for further investigation as a possible target and biomarker of treatment response.

The validation of candidate miRNA can be broken down into two steps. First, the manipulation of endogenous expression levels of miRs. This has become a standard tool in miRNA research with multiple protocols for both stable and transient transfection available commercially. We selected to re-express miR-4284 in the RR-22Rv1 cell line using Ambion® *mir*Vana™ miRNA Mimics. These mimics are small chemically modified double stranded RNAs which mimic endogenous miRNA, following transfection increased expression of the miRNA is confirmed using q-RTPCR; this method results in transient expression of the desired miRNA. Cell line samples with increased expression of miR-4284 can then be assayed for changes in radiosensitivity and used in the investigation of downstream targets.

Second, is the validation of miRNA targets. Individual miRNAs can have hundreds of targets, making their identification challenging (Thomas et al., 2010). While miRNA may only account for ~1% of genes, >60% of protein coding genes show evidence of selective pressure to maintain pairing with miRNA in humans (Friedman et al., 2009). Predicting what these target genes are has become easier with advancement in high-throughput in-silico prediction methods but experimental validation is still vital (Rajewsky, 2006). These prediction tools use data generated from experimental methods like q-RTPCR to build models which can then be used to predict targets for miRNA which have no experimental data available (Fan and Kurgan, 2015).

The PicTar software (Grün et al., 2005, Anders et al., 2011) uses a heuristic model while MirTarget2 (Wang and El Naqa, 2008) used an empirical machine learning model based on support vector machines and a training dataset. A 2015 study by Fan et al reviewed 38 miRNA target predictors and evaluated seven prediction tools using four benchmark data sets to look at miRNA:mRNA duplexes, target genes and proteins (Fan and Kurgan, 2015). While they did not show the overall highest predictive value PicTar and MirTarget2 were identified by the authors as displaying the highest specificity combined with lowest number of incorrectly predicted functional genes/duplexes and was suggested as suitable for researchers looking for a small subset of accurate predictions for functional duplexes and genes (Fan and Kurgan, 2015).

6.2 AIMS AND OBJECTIVES

The aim of this chapter was to investigate the influence of miR-4284 on the radiosensitivity of the RR-22Rv1 isogenic cell line and to validate a panel of predicted downstream targets following over expression of miR-4284.

Specific objectives:

- To transfect the radiation resistant isogenic model with miR-4284 using *mir*Vana™ miRNA Mimics.

- To investigate the impact of miR-4284 over expression on the radiosensitivity of the model.

- To select a panel of predicted gene targets of miR-4284 using in-silico methods.

- To validate the predicted targets mRNA expression levels in the transfected radiation resistant cell line using q-RTPCR.

6.3 RESULTS

6.3.1 CELL LINE TRANSFECTION

The RR-22Rv1 cell line was transfected with miR-4284 using the Ambion® *mir*Vana™ miRNA Mimics. q-RTPCR analysis showed that miR-4284 was successfully over expressed relative to the un-transfected RR-22Rv1 cell line (p=0.0004) (Figure 6-1). The transfection with a negative control scrambled mimic also resulted in a significant increase in miR-4284 expression compared to the un-transfected control (P=0.008) suggesting that the transfection machinery itself is having an effect on miRNA expression. The increase in miR-4284 expression following transfection was significantly higher than the increase in expression in cells transfected with the negative control ($p<0.003$) (Figure 6-1).

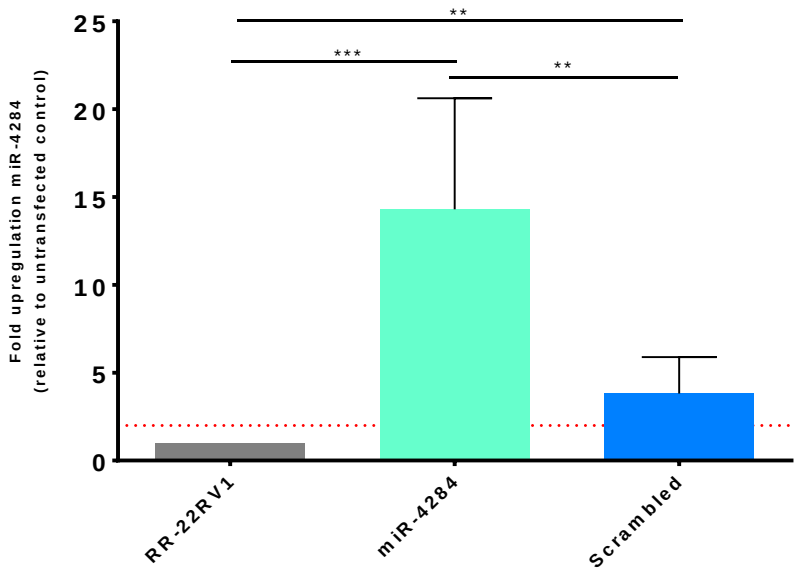

Figure 6-1: miR-4284 expression following transfection in RR-22Rv1 cell line

q-RTPCR results following isogenic cell line transfection with Ambion® *mir*Vana™ miRNA Mimics. RR-22Rv1, RR-22Rv1-miR-4284 transfected and RR-22Rv1 scrambled negative control cells. *t*-tests were used for statistical analysis . Data is plotted as Fold Change (RQ value) ± SD. *≤0.05 **, P≤0.01, *** P≤0.001. Red dotted line indicates 2-fold increase/decrease threshold expression.

While the transfection was successful, its efficiency varied between replicates (T1-6) (Figure 6-2a), suggesting the protocol should be modified to attain more stable transfection efficiency in the cells. The three replicates with the largest fold increase in miR-4284 expression (T2, T4 and T6) were selected for further evaluation. To assess the biological effectiveness of our transfection protocol, we monitored changes in levels of *TWF1 (PTK9)* gene expression a known target of hsa-miR-1 (miR-1). Transfection with the *mir*Vana™ miR-1 Mimic in these samples resulted in significant down regulation of the *TWF1* gene in our cell line (P<0.0001) (Figure 6-2b).

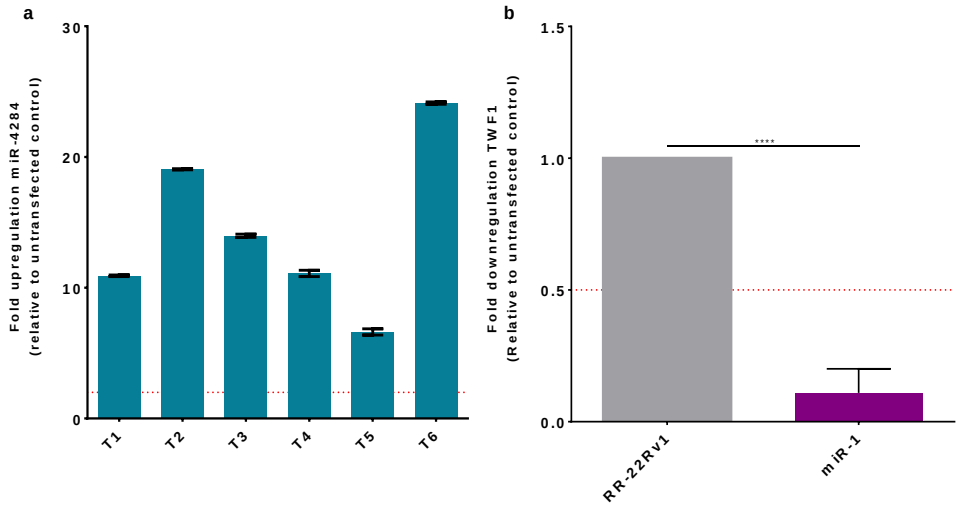

Figure 6-2: miR-4284 and *TWF1* expression following transfection in therr22rv1 cell line.

q-RTPCR results following isogenic cell line transfection with Ambion® *mir*Vana™ miRNA Mimics. a) Variation in expression of miR-4284 in individual replicates following transfection. Data is plotted as individual replicate fold change ± SD(CT) relative to the un-transfected control. b) Down regulation of TWF1 gene expression following transfection with miR-1 positive control. Data is plotted as Fold Change (RQ value) ± SD. P≤0.0001. Red dotted line indicates 2-fold increase/decrease threshold expression.

Clonogenic survival was used to assess changes in radiosensitivity in the RR-22Rv1 cell line 24 h following transfection (Figure 6-3). Irradiation with 6Gy resulted in significant reduction in clonogenic capacity in all cells assayed ($P<0.0001$). Increased expression of miR-4284 did not result in a significant change in radiosensitivity in the transfected RR-22Rv1 cell line following exposure to 6Gy, similar survival was reported in the negative (Scrambled) and positive (miR-1) transfected cells. No difference in survival was identified between the transfected cells and the un-transfected RR-22Rv1 control.

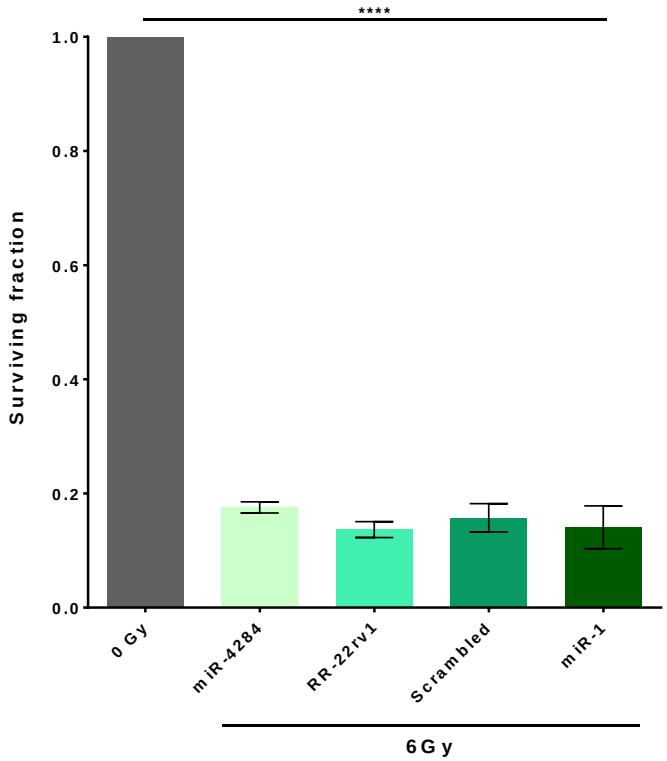

Figure 6-3: Survival fraction of RR-22Rv1 transfected and control cells following exposure to 6Gy.

Cell line survival after exposure to 6Gy x-rays as measured by clonogenic assay. Data mean ± SEM from at least three independent replicates; statistical analysis performed using one-way ANOVA with Tukey multiple comparison correction. **** P≤0.0001

6.3.2 TARGET PREDICTION

Target prediction was carried out in collaboration with Dr Simon Wong at ICHEC who selected to use the Pictar and MirTarget2 programs to identify potential targets of miR-4284.

These programmes assign target genes a score, the higher the score the stronger they are predicted to be a target. The results of the two programs were then amalgamated and genes which were predicted to be targets of miR-4284 by both methods had their scores combined resulting in 30 potential miR-4284 target genes. These genes were then ranked according to combined scores (Table 6-1). The top 6 targets

were selected based on their aggregate score for validation in the transfected cell line. *RASGEF1A* was also selected for validation due to the role Guanine Nucleotide Exchange Factors (GEFS) play in activating the RAS family of proteins which are frequently deregulated in cancer.

Table 6-1: Predicted gene targets of hsa-miR-4284

Symbol	Pictar Score	MirTarget Score	Aggregate Score*
RLIM^	38	99	137
UBFD1	39	82	121
TRPC4AP	23	95	118
LMTK2	27	89	116
CDK17	21	95	116
ADAMTS6	26	89	115
ZMYM3	20	94	114
ZNRF3	24	89	113
NFAT5	30	81	111
ZNF292	13	91	104
RASGEF1A	14	90	104
ERC2	17	85	102
ZDHHC9	29	73	102
UHMK1	37	63	100
XPO1	5	94	99
TBL1XR1	9	90	99
ELP5	14	83	97
FIZ1	14	82	96
FAM134B	5	91	96
LRRC15	25	56	81
PHF2	20	57	77
BACH2	22	54	76
NAV2	19	57	76
SH2B1	10	62	72
CELF5	18	54	72
N4BP2L2	6	65	71
AHDC1	3	66	69
ARHGAP32	13	54	67
HK1	9	56	65
TEX2	11	50	61

* Predicted gene targets are ranked by the aggregate score of the two prediction programs

^Genes in red were selected for validation in the miR-4284 transfected cell line

6.3.2.1 VALIDATION OF PREDICTED TARGETS IN miR-4284 TRANSFECTED CELLS

To study the effect of over-expression of miR-4284 on the gene expression of the seven predicted targets total RNA was isolated from cells 24 h after transfection and q-RTPCR analysis conducted. Combined analysis of the top three transfected cell line replicates did not result in significant differential expression of any of the target genes, though results did show that *ADAMST6* is not expressed in either the transfected or control RR-22Rv1 cells. The transfected cells were then evaluated by individual replicate. The results of this analysis indicate that over expression of miR-4284 results in a dose dependant change in target expression (Figure 6-4). The higher miR-4284 expression the more down regulated the gene while in some cases lower expression of miR-4284 resulted in increased expression of the target gene.

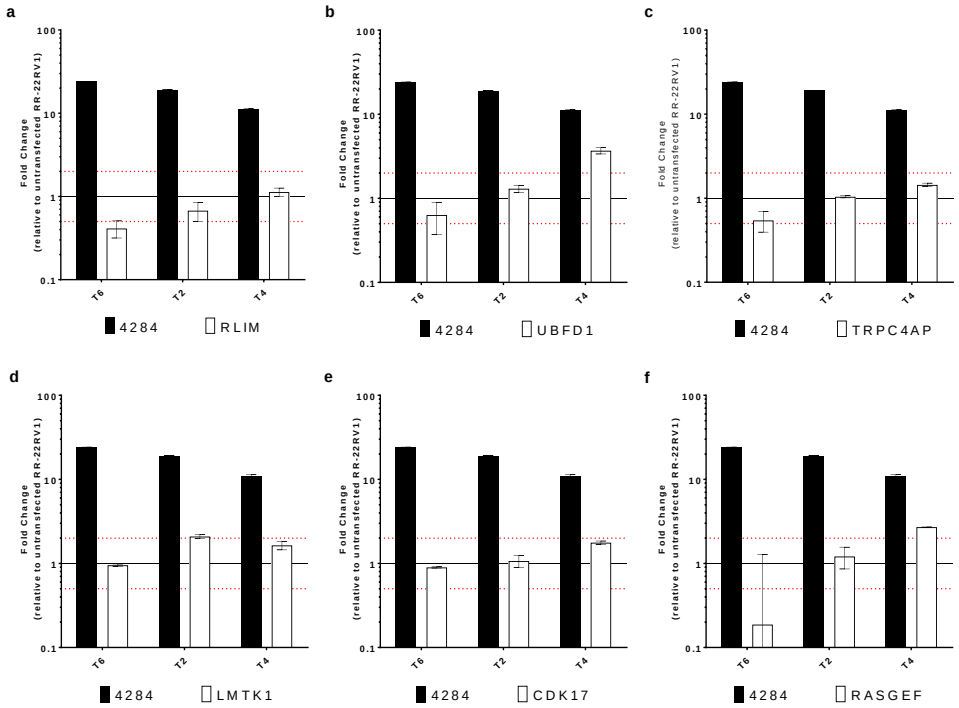

Figure 6-4: Expression of predicted miR-4284 target genes

Gene expression results of predicted targets following miR-4284 transfection in the isogenic cell line. a) *RLIM* ,b) *UBFD1*, c) *TRPC4AP*, d) *LMTK1*, e) *CDK17*,and f) *RASGEF*. Fold Change (Log 10 scale) ± SD of CT relative to un-transfected controls

Analysis using Pearson correlation identified a strong inverse relationship between expression of miR-4284 and target gene expression for *RLIM* (r^2= 0.9994, p=0.015) and *RASGEF* (r^2= 0.9995, p=0.0135) suggesting interaction between these genes and miR-4284 expression (Figure 6-5).

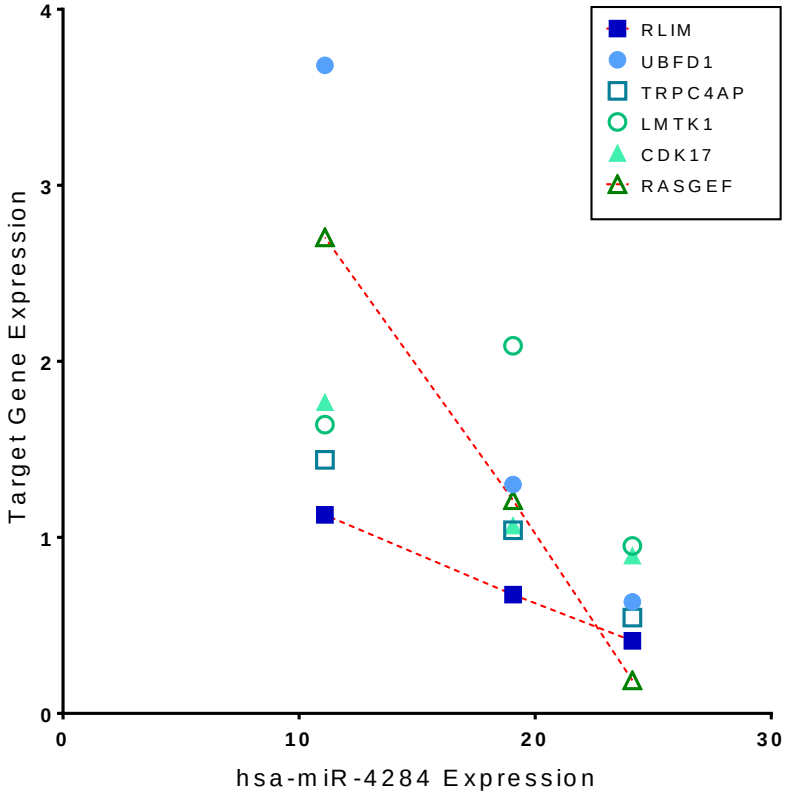

Figure 6-5: Correlation between miR-4284 expression and target expression

Pearson correlation depicting the relationship between miR-4284 expression and target gene expression in individual sample replicates.

6.3.3 *YBX1* GENE EXPRESSION

YBX1 gene expression was analysed in the isogenic RR-22Rv1 cell line and controls at baseline and following exposure to 4Gy ionising radiation. q-RTPCR analysis of *YBX1* did not identify significant differential expression of the gene in the cell lines (Figure 6-6). A trend towards increased expression of the gene was seen in the RR-22Rv1 and AMC-22Rv1 cell lines compared to the WT-22Rv1 control following treatment with 4Gy, though the change in expression was below 2-fold.

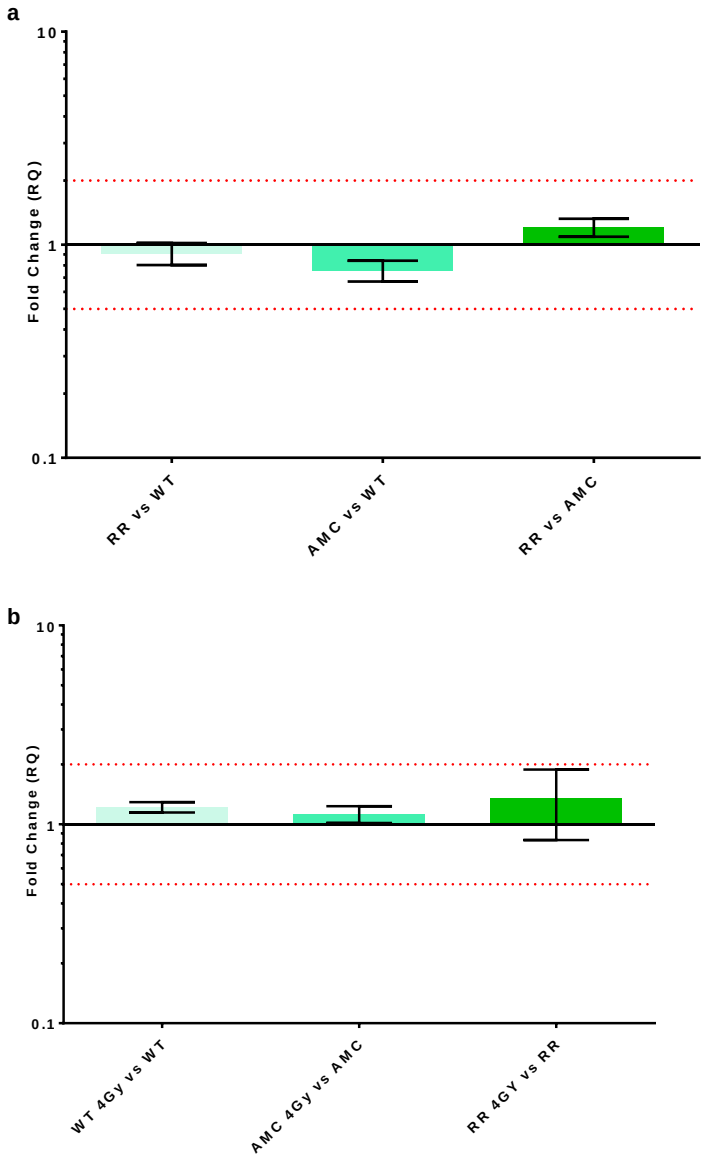

Figure 6-6: *YBX1* gene expression

Differential gene expression of a panel YBX1 in WT-22Rv1, AMC-22Rv1 and RR-22Rv1. Single sample t-tests were used for statistical analysis with theoretical mean of 1 being set for comparison. Data is plotted as Fold Change ± SD. Red dotted lines represent threshold of 2 fold change.

6.3.4 YB-1 PROTEIN EXPRESSION

The YB-1 protein is a 36 kilodalton (kDA) protein which displays an electrophoretic mobility of ~47 kDA with the cleaved form migrating at ~36 kDA. Western blot analysis revealed that while there is no difference in the expression of the full length YB-1 protein between cell lines, no cleaved YB-1 is present in the nucleus of radiosensitive WT-22Rv1 cells yet a band is visible at 36kDA in the relatively resistant AMC-22Rv1 sample with an even stronger band corresponding to cleaved YB-1 in highly resistant RR-22Rv1 (Figure 6-7). Higher expression levels of full length YB-1 were seen in the nuclear than the cycloplasmic protein fraction in all samples. No change was identified in protein expression following exposure to 4Gy x-irradiation.

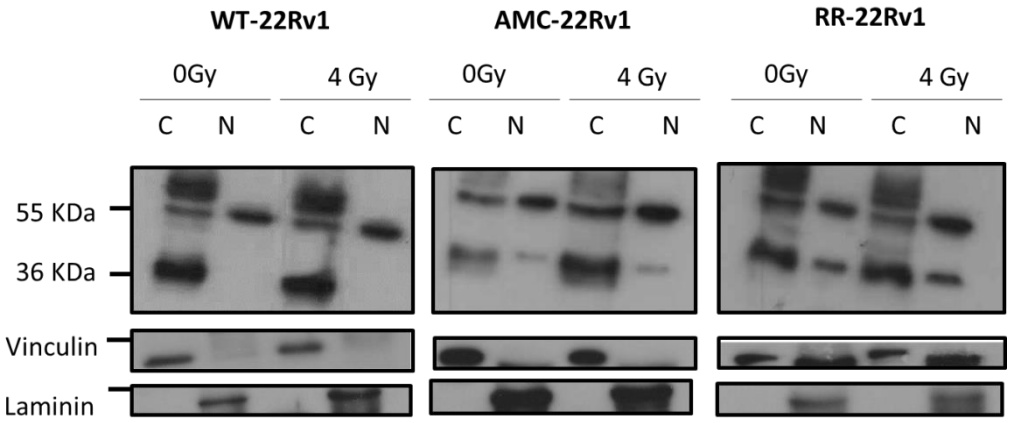

Figure 6-7: YB-1 protein expression

Representative immunoblots of the YB-1 protein in the cytoplasmic (C) and nuclear (N) fraction of unirradiated and 4Gy irradiated WT-22Rv1, AMC-22Rv1 and RR-22Rv1 cells. Vinculin and Laminin used as controls.

6.4 DISCUSSION

The aberrant expression of miRNAs in cancer presents not only new targets for treatment but also have potential as biomarkers for early detection and treatment response in patients. We identified a number of dysregulated miRNAs in the radioresistant 22Rv1 isogenic cell line as possible markers of radiation therapy failure in primary prostate cancer. Following validation of these miRs in our cell lines we selected to move forward with miR-4284 as a candidate marker of radiation treatment response. Transient over expression of miR-4284 was successfully achieved using Ambion® *mir*Vana™ miRNA Mimics. Our protocol resulted in varied levels of increased expression of miR-4284 in our cell line samples and will need to be adapted for future studies. Further investigation into the effect the transfection machinery has on miR expression is also necessary. While transfection with the scrambled control mimic produced increased expression of miR-4284 its effect on miR-4284 function needs to be validated once a target gene has been identified and validated for this miRNA.

Though overexpression of miR-4284 in the RR-22Rv1 cell line did not result in re-sensitisation of the cells to ionising radiation and is not itself a target for re-sensitising tumours to radiotherapy, investigation of its downstream predicted targets may present new therapeutic targets for radiation resistant prostate cancer while also helping to elucidate the underlying mechanism of radiotherapy resistance in this disease.

miRNAs are complex biological molecules with multiple mRNA targets (Lewis et al., 2005), individual miRNA have also been shown to play conflicting roles of both tumour suppressors and oncogenes in different tissues (He et al., 2005b, Hossain et al., 2006, Xiang and Wu, 2010, Pekarsky and Croce, 2010). The complicated nature of their mode of action has given rise to a hypothesis of a dose or threshold dependent mechanism of miRNA associated gene regulation (Levine et al., 2007,

Mukherji et al., 2011, Shu et al., 2012). It has been suggested that miRNA select and regulate their targets based on their own level of expression as well as that of its target mRNA, allowing them to modulate their role in the cell depending on different cellular conditions (Mukherji et al., 2011, Shu et al., 2012). Measuring miRNA-target interaction on a single cell basis Mukherji et al. found that miRNA regulation establishes threshold levels for mRNA under which gene expression is repressed (Mukherji et al., 2011). Experiments with a cMYC-3′UTR luciferase reporter construct found that much a higher dose of let-7a—7f cluster miRNA plasmid needs to be transfected to affect cMYC activity than for down regulation of a *DICER* 3′UTR luciferase reporter in the same cells (Shu et al., 2012). This dose effect on target selection was also seen using the miR-17-92 cluster which needed a much high level of plasmid transfection (0.03ug/well) to down regulate *DICER* than the let-7a—7f cluster (Shu et al., 2012). In this study the level of miR-4284 expression showed a significant inverse correlation with expression of the predicted target genes *RLIM* and *RASGEF* in a dose dependent manner suggesting a threshold for miR-4284 mediated repression of these targets in the model.

Increasing concentrations of miRNA have not only shown to differentially select targets but also that targets can be regulated contrarily depending on the concentration of miRNA transfected (Shu et al., 2012). The increase in expression of the target genes in replicates with less miR-4284 expression may be explained by this dose dependent mechanism. Using a luciferase reporter system it was shown by Shu et al. that at low dose (0.0003ug/well) let-7a—7f cluster plasmid transfection resulted in down regulation of *DICER* 3′UTR luciferase reporter but at a higher dose (0.3ug/well) an increase in a luciferase reporter activity was seen (Shu et al., 2012). The suggestion that not only sequence homology but also the endogenous concentration of a target mRNA transcripts is important for miRNA target selection (Shu et al., 2012) and regulation (Shu et al., 2012, Mukherji et al., 2011) mean

that further study of not just the dose effect of miR-4284 but also investigation of the mRNA levels of the targets is needed in this model to fully understand the interaction between the miRNA and its potential targets.

The protein coded for by the *RLIM* gene is a RING H2 zinc finger protein which acts as a negative co-regulator, via the recruitment of the Sin3A/histone deacetylase co-repressor complex, for LIM homeodomain transcription factors (Bach et al., 1999) and is involved in modulation of telomere length (Her and Chung, 2009) and an activator of random X chromosome inactivation (Jonkers et al., 2009). RLIM has also been implicated as both a positive (Johnsen et al., 2009, Huang et al., 2011) and negative regulator (Chen et al., 2014) of process' involved in oncogenisis. It has been associated with enhanced estrogen receptor transcriptional activity in breast cancer and along with LIM cofactor CLIM has been suggested as a therapeutic target for ERα-positive breast tumours (Johnsen et al., 2009) and was found to up-regulate TGF-β signalling and TGF-β-driven migration in osteosarcoma U2OS cells (Huang et al., 2011). It has also been associated with the oncoprotein stathmin in osteosarcoma on which it acts as a negative regulator and shows potential as a novel therapeutic target of the disease (Chen et al., 2014). Further investigation of both the relationship between RLIM protein expression and miR-4284 as well as its function in the primary prostate cancer cell line 22Rv1 and the radioresistant subline is necessary.

The *RASGEF1A* gene encodes the guanine nucleotide exchange factor Ras-GEF domain-containing family member 1A protein. This protein shows specificity for the RAP2 (Yaman et al., 2009), KRAS, HRAS and NRAS (Ura et al., 2006) members of the RAS superfamily. GEF proteins modulate RAS by promoting the release of GDP bound to it (inactive form) allowing for the association of GTP and activation of RAS and stimulation of downstream signalling pathways (Quilliam et al.,

2002). Increased expression of RASGEF1A has been associated with enhanced motility and cell growth in intrahepatic cholangiocarcinoma and has been suggested as a therapeutic target in this disease (Ura et al., 2006). Members of the RAS family of small GTPases have long been associated with oncogenesis (Bos, 1989, Malumbres and Barbacid, 2003, Downward, 2003, Pylayeva-Gupta et al., 2011) and radiation therapy resistance (Kasid et al., 1989, Hagan et al., 2000, Gupta et al., 2001, Ling and Endlich, 1989, Miller et al., 1993, McKenna et al., 1990, Bernhard et al., 1998, Bernhard et al., 2000) suggesting the dysregulation of this gene in the RR-22Rv1 cell line may play a role in the genesis of the radioresistant phenotype seen in our model. Ura et al found the expression of RASGEF1A is essential for the growth of COS7 cancer cells with cells transfected with siRNA against RASGEF exhibiting growth retardation (Ura et al., 2006). The effect of inhibition of RASGEF1A on proliferation may explain the differences identified in the growth of the WT-22Rv1 cell line which grows comparatively slower than the RR-22Rv1 and AMC-22Rv1 lines. COS7-RASGEF1A cells expressing exogenous RASGEF1A were also found to migrate faster than control cells using the wound healing assay and showed increased migration using the matrigel invasion assay further implicating this protein in cellular migration of cancer cells (Ura et al., 2006).

Studies in our lab using the wound healing assay have shown that the RR-22Rv1 cell line shows increased migratory properties compared to the WT-22Rv1 control (unplublished data generated by Gabriella Nortey) and RASGEF expression may be playing a role in this phenotype. Further evaluation of RASGEF1A expression on both the gene and protein level in basal and miR-4284 transfected cells is needed to confirm this gene as a bona fide target of miR-4284 and to fully elucidate the role of this molecule in the radioresistant phenotype of prostate cancer. Confirmed dysregulation of this gene in prostate cancer could result in a novel therapeutic target for control of the disease.

YB-1 has previously been associated with tumourgenicity (Rubinstein et al., 2002, Schittek et al., 2007, Janz et al., 2002, Faury et al., 2007) and chemotherapy resistance (Bargou et al., 1997, Ohga et al., 1996, Guay et al., 2008, Janz et al., 2002, Yahata et al., 2002) in a multitude of cancers resulting in high levels of nuclear YB-1 being identified as predictor of poor prognosis (Shibahara et al., 2001, Gessner et al., 2004, Janz et al., 2002, Oda et al., 2003, Kamura et al., 1999). Yet to our knowledge evidence of a link to radioresistance in prostate cancer has yet to be identified. No significant difference was seen in the gene expression of *YBX1* but western blot analysis revealed that full length YB-1 expression was stronger in the nucleus than the cytoplasm consistent with other studies of cancer cell lines (Yahata et al., 2002). This needs to be further investigated as studies in our lab have shown differential resistance to the anti-mitotic chemotherapeutic docetaxel in these lines (McDermott et al, 2016 *under review*) and nuclear accumulation of YB-1 has also been associated with cisplatin resistance in cell lines (Yahata et al., 2002).

While there was no significant difference in the expression of the full length protein between samples a difference in the expression of the cleaved form was identified between the WT-22Rv1 radiosensitive cells and the radioresistant and relatively resistant RR-22Rv1 and AMC-22Rv1 cells respectively. It has been suggested that the truncated 36 kDA form of YB-1 may in fact be Heterogenouus nuclear ribonucleaoprotein A1(hnRNP A1) (Cohen et al., 2010), a RNA binding protein involved in RNA processing (Krecic and Swanson, 1999), this may still be a strong target as a diagnostic tool for patients destined to fail radiation therapy treatment. In order to confirm the identity of the protein further analysis is needed. The use of different antibodies for both the cleaved and full length form of the protein and perhaps siRNA knockdown of each protein and western blot analysis may help elucidate the true nature of this band. This finding still presents a novel opportunity not just for prediction of treatment response in prostate

cancer but also a potential target for re-sensitisation of prostate cells to radiotherapy.

Chapter 7: DISCUSSION

7.1 INTRODUCTION

The cumulative lifetime risk of developing cancer for Irish men is 33.6% and according to 2012 statistics compiled by the national cancer registry 31.5% of these cancer diagnosis are prostate cancer (National Cancer Registry Ireland, 2012). The prevalence of this disease presents a significant burden on the public health sector which is further increased as a result of disease recurrence following treatment failure. Owing to the high incidence of CaP, practice improvements in both detection and treatment are important issues that need to be addressed. This thesis aimed to answer this challenge through the elucidation of new biomarkers of radioresistance in prostate cancer and through the discovery of new actionable targets for the re-sensitisation of prostate cancer tumours following the shift from a sensitive to resistant phenotype.

7.2 THE GROWING DEMAND FOR CANCER BIOMARKERS

Recent years have seen a dramatic increase in the identification of biomarkers of cancer. These molecular markers have the potential to be used in the diagnosis, prognosis and treatment of cancer in order to obtain the optimal clinical management of the disease. Biomarkers are commonly used in the risk assessment of patients, e.g. the breast cancer 1 gene (*BRCA1*) in breast and ovarian cancer (Easton et al., 1995, Hall et al., 1990), as well as in screening and early detection programmes as seen with the regular use of PSA in CaP (Catalona et al., 1994) and human papillomavirus (HPV) in cervical cancer (Ferenczy and Franco, 2001). The clinical use of diagnostic and prognostic biomarkers allow for a much more refined understanding of the disease once it has been confirmed. This can be seen with the frequent use of the Philadelphia chromosomal translocation in the diagnosis of chronic

myelogenous leukaemia (CML) which allows for the sub classification of the cancer (Sweet et al., 2013). Another example is P53 status which is commonly used for prognostic evaluation in multiple cancers because the presence of mutations in this protein can indicate a particularity aggressive form of the disease (Goldstein et al., 2011). Cancer biomarkers also play a vital role in treatment management with KRAS mutations used to predict response to therapy in colorectal cancer (Allegra et al., 2009), HER2 status as a biomarker of Trastuzumab utility in breast (Piccart-Gebhart et al., 2005, Romond et al., 2005) and gastric cancers (Bang et al., 2010) and the presence of oestrogen receptor α (ERα) in the selection of Tamoxifen treatment for breast cancer patients (Fisher et al., 1989).

The ultimate goal of cancer treatment is the total eradication of tumours, though biomarkers of treatment outcome as described above are a vital aspect of this, markers capable of predicting metastatic disease or those tumours likely to recur following treatment are also needed. While predictive biomarkers of metastasis such as the MammaPrint test in breast cancer (Van't Veer et al., 2002) have shown some clinical success (Buyse et al., 2006, Drukker et al., 2013, Cardoso et al., 2008), no definitive marker of recurrence following RT has been successfully brought to the clinic. The success of biomarkers in other cancer sites demonstrates their potential for alleviating the clinical burden of CaP on both patients and clinicians through improved diagnosis and treatment outcome resulting in truly personalised management of the disease.

7.3 FORECASTING TREATMENT OUTCOMES

There is a 50/50 chance for patients presenting in the clinic with locally confined primary prostate cancer to undergo radiation therapy vs. radical prostatectomy, yet no biomarker of treatment response is currently available to help guide therapeutic decisions for those patients who remain at high risk of RT failure in spite of optimal clinical

management and treatment. The discovery of new biomarkers of treatment outcome and the elucidation of new therapeutic targets to increase the efficacy of current radiation therapy strategies will assist the treatment decision making process.

Sensitive and specific markers for both accurate early detection and treatment outcome are needed to aid in the effective management of cancer and miRNAs have proven to be successful candidates for biomarker discovery in a multitude of cancers. Abnormal expression of miRNA have long been associated with disease specific profiles in numerous cancers (Lu et al., 2005, Volinia et al., 2006) and allow for clustering of tumours into different cell lineages (Lu et al., 2005). miRNA signatures have been found to successfully differentiate patient tumour samples from controls in a number of cancers including: B cell chronic lymphocytic leukaemias (CLL) (Calin et al., 2004), hepatocellular carcinoma (Murakami et al., 2006), papillary thyroid carcinoma (He et al., 2005a) CaP (Porkka et al., 2007), lung (Yanaihara et al., 2006, Xing et al., 2010, Boeri et al., 2011), breast (Iorio et al., 2005, Heneghan et al., 2010, Kodahl et al., 2014) and colon cancers (Schetter et al., 2008, Balaguer et al., 2011), along with many others. Metastatic specific (Hur et al., 2015, Nam et al., 2015) as well as prognostic signatures (Yanaihara et al., 2006, Villaruz et al., 2015, Boeri et al., 2011, Landi et al., 2010, Patnaik et al., 2010, Schetter et al., 2008, Calin et al., 2005, Lichner et al., 2013, Raponi et al., 2009, Yu et al., 2008, Kelly et al., 2015) have also shown promise in predicting patient outcomes.

In this thesis the role of miRNA expression profiles in radiation sensitive primary prostate cancer cell line 22Rv1 and the newly derived radioresistant subline RR-22Rv1 was investigated. Using the Exiqon microarray service it was demonstrated that prolonged treatment with fractionated 2Gy ionising radiation to a cumulative dose of 60Gy results in the differential expression of 97 miRs relative to the untreated control.

This work also identified cell line age as playing a potential role in the transition from a radiation sensitive to radiation resistant phenotype. Indeed, the age matched AMC-22Rv1 control subline demonstrated a marginal increase in resistance to x-irradiation at 4Gy but not at higher doses following prolonged growth. Though it displayed differential expression of 65 miRs relative to the young WT-22Rv1 control only one miRNA was identified as differentially expressed between the AMC-22Rv1 and the RR-22Rv1 cell lines. But validation with q-RTPCR reported this result as a false positive. Further validation of these two expression profiles in other radioresistant prostate cancer cell lines and patient samples is needed to identify a true miRNA profile capable of discriminating between treatment sensitive and resistant cancer. The elucidation of such a signature will indicate the most appropriate treatment of prostate cancer patients.

7.4 TOWARDS A BETTER UNDERSTANDING OF RADIORESISTANCE

The study of pathways associated with radioresistance has been facilitated through our increased ability to model the impact of a course of RT on cancer cells. The generation of isogenic radiation resistant cell lines allows for the modelling of clinically relevant acquired resistance and provide opportunity for the identification of mechanisms crucial for the switch from the sensitive to resistant form of the disease. While miRNA signatures have been used to discriminate between tumour and normal samples, the role of individual miRNAs in tumorigenesis has been highlighted by the identification of miRs which are important in the regulation of pathways vital to cancer progression. The dysregulation of numerous miRNA has been associated with increased oncogenesis (Jansson and Lund, 2012) through the alteration of multiple pathways involved in: proliferation, cell cycle, cell death, angiogenesis and treatment response. The repeated exposure of CaP cells to 2Gy

fractionated ionising radiation selected for super-oncogenic 22Rv1-cells with increased proliferative potential following radiation exposure but increased sensitivity to Docetaxel. These cells were enriched in S-phase cells, less susceptible to DNA damage and radiation-induced apoptosis and have acquired enhanced migration potential. The selection of these super-oncogenic cancer cells during fractionated radiation therapy may have implications in the development and administration of future targeted therapy in conjunction with RT.

The characterisation of the RR-22Rv1 subline identified strong evidence of the dysregulation of DDR pathways in the switch from a sensitive to resistant phenotype. A number of chemotherapeutics are presently used in combination with RT to aid in the re-sensitisation of radioresistant cells (Kvols, 2005, Bischoff et al., 2009) and the evidence of deregulation of the DDR pathways identified in our cell lines could help guide selection of these chemotherapeutics for optimal sensitisation of resistant prostate cancer cells through synthetic lethality. Further investigation of deregulated genes implicated in this preliminary gene expression study is necessary to truly identify pathways which are vulnerable to modulation. This presents a number of potential areas for therapeutic intervention for the re-sensitisation of prostate cancer cells to RT in addition to biomarker discovery.

7.5 miR-4284: A POTENTIAL KEY MIRNA IN RADIORESISTANT PROSTATE CANCER

The diagnostic and prognostic clinical potential of miRNAs is highly significant. Biomarkers of therapeutic outcome will allow for truly personalised care of patients undergoing cancer therapy and ensure that they are receiving the best possible clinical management of their disease through a combination of early detection initially and following recurrence, treatment monitoring and potentially through the use of

novel direct targeted miRNA therapeutics (Nana-Sinkam and Croce, 2013). A number of individual miRNAs have emerged as candidate clinical targets in cancer and are currently in various stages of development and pre-clinical trial (Wahid et al., 2010, Li and Rana, 2014). The first miRNA therapy, for hepatitis C virus (HCV) infection, targeting miR-122 Mirravirsen is now in a phase II clinical trial and shows very promising results with significant decreases and in some cases undetectable levels of serum HCV RNA in patients following treatment (Lindow and Kauppinen, 2012). In cancer, miR-34 in particular has shown great promise for clinical application, this miRNA is commonly deregulated in lung, ovarian, CLL and colorectal cancers, and is currently under investigation in a phase I clinical trial under the name MRX34 by Mirna Therapeutics (Bouchie, 2013). The success of miRNA research in cancer shows the importance of these small non-coding molecules not just for monitoring of treatment outcomes and targets but also for patient stratification based on personal genetic profiles which will allow for the best possible clinical management of the disease.

This thesis identified a number of miRs involved in the radioresistant phenotype of CaP cells, but miR-4284 in particular emerged as a potential key to this pathology. miR-4284 was down regulated in the significantly resistant RR-22Rv1 subline as well hypoxic cells and the AMC-22Rv1. This miRNA has not been previously associated as a biomarker of prostate cancer recurrence following RT treatment and though re-expression of this miR did not result in radiosensitisation, it still represents an exciting avenue of research potential. The fact that individual miRNAs may have multiple targets (Thomas et al., 2010) and that these may be dose dependant (Levine et al., 2007, Mukherji et al., 2011, Shu et al., 2012) suggests that although augmentation of miR-4284 did not directly affect sensitivity to radiation further investigation of its downstream predicted interactions may present new therapeutic

targets and aid in elucidation of the underlying mechanism of RT resistance in this disease.

To further explore the utility of miR-4284 and its targets as biomarkers of treatment outcome, validation in patient samples is necessary. The retrospective evaluation of miR-4284 expression in a cohort of FFPE tissue specimens of patients diagnosed with localised Gleason 8-10 prostate carcinoma subsequently treated with radical RT combined with androgen deprivation therapy is warranted. It is anticipated that the expression level of miR-4284 is significantly different between patients with evidence of biochemical failure compared to patients in stable biochemical remission. Determination of the sensitivity, specificity, positive/negative predictive value and accuracy of the miR in the detection of risk of failure and/or biochemical failure will be a prerequisite for the development of a new companion diagnostic test. This test may ultimately provide clinicians with a powerful tool for the development of alternative follow-up and treatment protocols, and in addition highlight new areas for targeted drug development that will potentially improve treatment efficacy and patient survival.

7.6 TOWARDS MORE PERSONALISED TREATMENT

While the majority of clinical and preclinical trials involving the application of miRNA revolve around their utility as biomarkers rather than treatment targets (Nana-Sinkam and Croce, 2013), a number of methodologies exist for therapeutic targeting of miRNA. The three principle approaches currently used in the inhibition of these small non coding molecules are: expression vectors as miRNA sponges (Ebert et al., 2007), small molecule inhibitors (Gumireddy et al., 2008) (Young et al., 2010a) and antisense oligonucleotides (Li and Rana, 2014). While these methods show promise for the clinical silencing of miRNA fewer strategies are available for the re-expression of down regulated miRNA. Currently the most successful strategy for the reintroduction of miRNA

to cells is through the use of synthetic miRNA mimics as reported in the MRX34 trial which uses a liposome based delivery system (Bouchie, 2013). Other methods for the delivery of miRNA to target cancer cells may include intramural delivery (Ibrahim et al., 2011) though this is limited by the placement of the tumour, viral vectors (Xie et al., 2012), lipid based delivery systems (Trang et al., 2011) and polymer nanoparticle systems (Davis et al., 2010, Davis and Shin, 2008).

This work identified a number of likely targets of miR-4284 which have potential for therapeutic manipulation. YB-1 dysregulation has been previously associated with tumour progression in CaP (Giménez-Bonafé et al., 2004) and while no direct small molecule inhibitor currently exists for this target a number of other strategies have been utilised in the investigation of this protein for therapeutic intervention. Molecular decoys to sequester the protein (Lasham et al., 2003) or compete with it for phosphorylation (Law et al., 2010) have shown success in the lab as have siRNAs to target YB-1 on the gene expression level (Schittek et al., 2007, Zhu et al., 2012). Inhibition of kinases involved in signal transduction pathways associated with YB-1 activation also show promise for therapeutic intervention (Evdokimova et al., 2006, To et al., 2007, Stratford et al., 2008, Shiota et al., 2014). For example, Shiota et al, 2014 reported treatment with a ribosomal S6 kinase (RSK) inhibitor in CaP cell lines lead to complete suppression of YB-1 and resulted in the increased efficacy of Enzalutamide in LNCaP and C4-2 prostate cells (Shiota et al., 2014).

The YB-1 inhibitor fisetin, a dietary flavonoid found in fruits and vegetables, has shown success as a potent inhibitor of oncogenic progression in CaP through interaction with the cold shock domain of YB-1 resulting in inhibition of this oncoprotein through reduced phosphorylation at Ser^{102} (Khan et al., 2008, Khan et al., 2014) . A number of small molecule inhibitors also currently exist for the targeting of RASGEFs (Evelyn et al., 2014, Spiegel et al., 2014, Airoldi et al.,

2007). Although none have been reported for RLIM inhibition, Krämer et al reported reduced levels of RLIM protein following treatment with the antifungal antibiotic/histone deacetylase inhibitor trichostatin A (Krämer et al., 2003) and a number of studies have successfully used siRNA to down regulate its expression (Her and Chung, 2009, Jiao et al., 2013). Modulation of these predicted target proteins may help elucidate their involvement in the radioresistant phenotype.

Finally, evaluation of the prognostic value of these targets is also important. Increased levels of truncated nuclear YB-1 expression (or hnRNP A1 expression) identified in the radioresistant cell line may correlate to decreased resistance to RT/increased sensitivity to Docetaxel. Further evaluation of this profile is necessary to discern the utility of this as a potential prognostic tool in CaP. If validated it would have positive implications in the clinic for the selection of treatment modality for patients. Within the context of the RTOG 0521 clinical trial (https://clinicaltrials.gov/show/NCT00288080) which reports that adjuvant chemotherapy (Docetaxel and prednisone) combined with androgen suppression and RT in high risk CaP resulted in an increased overall survival after four years (Sandler et al., 2015), a diagnostic test which evaluates both radiosensitivity and chemosensitivity has the potential to revolutionise treatment decision making for these patients. As a result an invention disclosure form relating this finding was filed (ref. LM02-610) and is currently under evaluation.

7.7 SUMMARY AND CONCLUSION

This thesis aimed to identify novel biomarkers in CaP. The generation of a radioresistant model of primary CaP (Chapter 3) created a novel tool to assist future investigations of this phenotype. The validation of this model (Chapter 4) has also identified key mechanisms for future investigation of radiation resistance in CaP. miRNA profiling of the model (Chapter 5) selected miR-4284 as a candidate biomarker of the

radiation resistant phenotype for future validation in a cohort of clinical specimens currently being collected in a number of hospital sites. This thesis further explored the functional role of this candidate biomarker (Chapter 6) and identified a two associated potential targets: *RLIM* and *RASGEFA1* as well as a potential key regulatory protein (YB-1). Further evaluation of these sets of interrelated markers may pioneer the development of personalised radiation oncology solutions for CaP patients.

Chapter 8: APPENDIX

8.1 MANUFACTURERS DETAILS

Product	Manufacturer	Address
Agilent G2565BA Microarry Scanner	Agilent Technologies, Inc.	California, United State Of America (USA)
Mirna Mimic	Ambion	Dublin, Ireland
TriReagent,	Ambion	Dublin, Ireland
Rnase Free Tubes 1.5ml	Ambion	Dublin, Ireland
Cellbanker 2	Ams Biotechnology	Oxfordshire, United Kingdom (Uk)
High-Capacity Cdna Revers Transcription Kit,	Applied Biosystems	Dublin, Ireland
ABI 7500 Q-PCR System	Applied Biosystems	Dublin, Ireland
PCR Strip Tubes	Axygen-Corning	California, USA
Cyantm ADP Analyser	Beckman-Coulter	Galway, Ireland
0.5m Tris 6.8	Biorad	California, USA
1.5 Tris, Ph 8.8	Biorad	California, USA
Western Blot Module	Biorad	California, USA
1000 *In Vivo* Hypoxic Chamber	Biotrace	Bracknell, UK
Vacuum	Charles Austen Pumps	Surrey, UK
Clean Air Biohood		
Pyrex Tubes	Corning	Flintshire, UK
15/50ml Tubes	Corning	Flintshire, UK
Bench Top 5415 R Microcentrifuge	Eppendorf	Hertfordshire, UK
1.5ml Tubes	Eppendorf	Hertfordshire, UK
Mircurytm RNA Isolation Kit	Exiqon	Vedbaek , Denmark
Mirna Primers,	Exiqon	Vedbaek , Denmark
Mircury LNA™ Microrna Array 7th Generation	Exiqon	Vedbaek , Denmark
Imagene® 9	Exiqon	Vedbaek , Denmark

Product	Manufacturer	Address
Mircury LNA™ Microrna Hi-Power Labelling Kit, Hy3™/Hy5™	Exiqon	Vedbaek , Denmark
EZ Run Protein Ladder	Fisher Bioreagents	Dublin, Ireland
10% Aps	Fisher Chemical	Dublin, Ireland
Glycine	Fisher Chemical	Dublin, Ireland
Methanol	Fisher Chemical	Dublin, Ireland
Temed	Fisher Chemical	Dublin, Ireland
Tris	Fisher Chemical	Dublin, Ireland
Filter Paper	Fisherbrand	Dublin, Ireland
Flowjo	Flowjo LLC	Oregon, USA
Fuxi-Film Super RX-N Medical X-Rat Film	Fuji Film	Dublin Ireland
Optimem Media,	Gibco	Dublin, Ireland
10% Sds	Gibco	Dublin, Ireland
Fbs	Gibco	Dublin, Ireland
Prism Software	Graphpad Prism	California, USA Www.Graphpad.Com
Reaction Tubes 5ml	Grenier Bio-One	Frickenhausen, Germany
RS225 Cabinet Irradiator	Gulmay Mediacal Ltd	Surrey, UK
Haemocytometer	Hausser Scientific	Pensilvania, USA
Mrna Primers	Integrated Dna Technologies	Leuven, Belgium
Lipofectamine Rnaimax	Invitrogen	Dublin, Ireland
Qubit Fluorometer	Invitrogen	Dublin, Ireland
Annexin V	IQ Products	Groningen, Netherlands
Komet 5.0 Software	Kinetics Imaging Software Ltd	Brombourough UK
Cm-H2dcfda	Life Technologies	Dublin, Ireland
Tripsin	Lonza	Castleford, UK
Penstrep	Lonza	Castleford, UK
Rpmi	Lonza	Castleford, UK
Pbs	Lonza	Castleford, UK

Product	Manufacturer	Address
Immuobilon-P Transfer Membrane	Merck Millipore	Cork, Ireland
PTC-100 Thermocycler-	Mj Research Inc	Massachusetts, USA
Versamax Microplate Reader	Molecular Devices	Berckshire, UK
Microscope-Optika	Optika	Bergamo, Italy
Col Count	Oxford Optronix	Oxford UK
Bca Protein Assay Kit	Pierce	Dublin, Ireland
RIPA Lysis Buffer	Santa Cruz Biotechnology, Inc.	Dallas, Texas, Usa
Ripa Lysis Buffer	Santa Cruz Biotechnology, Inc.	Dallas, Texas, Usa
Cell Scrappers	Sarstedt Ltd	Wexford Ireland
Trypan Blue	Sigma-Aldrich	'Wiclow Ireland
Tween	Sigma-Aldrich	'Wiclow Ireland
Acrylamide	Sigma-Aldrich	'Wiclow Ireland
Running Dye Laemmlii 2X	Sigma-Aldrich	Wiclow Ireland
Trireagent	Sigma-Aldrich	Wiclow Ireland
Hepes	Sigma-Aldrich	Wiclow Ireland
Nacl	Sigma-Aldrich	Wiclow Ireland
Cacl2	Sigma-Aldrich	Wiclow Ireland
Triton-X	Sigma-Aldrich	Wiclow Ireland
Ethanol	Sigma-Aldrich	Wiclow Ireland
Bafilomycin A1	Sigma-Aldrich	Wiclow Ireland
C12fdg, Rnase A	Sigma-Aldrich	Wiclow Ireland
Catalase	Sigma-Aldrich	Wiclow Ireland
Tryapan Blue	Sigma-Aldrich	Wiclow Ireland
Bcp	Sigma-Aldrich	Wiclow Ireland
Na2edta	Sigma-Aldrich	Wiclow Ireland
Tris-HCL	Sigma-Aldrich	Wiclow Ireland
Naoh	Sigma-Aldrich	Wiclow Ireland
Cystal Violet	Sigma-Aldrich	Wiclow Ireland
Milk	Sigma-Aldrich	Wiclow Ireland
Forma Steri Cycle CO2 Incubator	Thermo Scientfic	Dublin, Ireland
Centrifuge-Centra Gp8	Thermo Scientific	Dublin, Ireland

Product	Manufacturer	Address
Nanodrop,	Thermo Scientific	Dublin, Ireland
NE-PER Nuclear And Cytoplasmic Extraction Kit	Thermo Scientific	Dublin, Ireland
Super Signal West-Pico	Thermoscientfic	Dublin, Ireland

Chapter 9: BIBLIOGRAPHY

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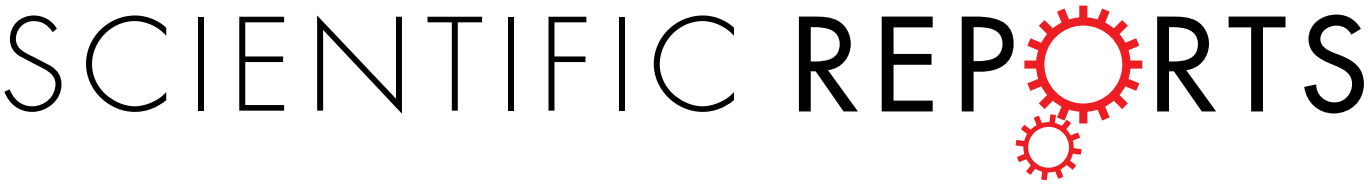

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Fractionated radiation exposure amplifies the radioresistant nature of prostate cancer cells

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The risk of recurrence following radiation therapy remains high for a significant number of prostate cancer patients. The development of *in vitro* isogenic models of radioresistance through exposure to fractionated radiation is an increasingly used approach to investigate the mechanisms of radioresistance in cancer cells and help guide improvements in radiotherapy standards. We treated 22Rv1 prostate cancer cells with fractionated 2 Gy radiation to a cumulative total dose of 60 Gy. This process selected for 22Rv1-cells with increased clonogenic survival following subsequent radiation exposure but increased sensitivity to Docetaxel. This RR-22Rv1 cell line was enriched in S-phase cells, less susceptible to DNA damage, radiation-induced apoptosis and acquired enhanced migration potential, when compared to wild type and aged matched control 22Rv1 cells. The selection of radioresistant cancer cells during fractionated radiation therapy may have implications in the development and administration of future targeted therapy in conjunction with radiation therapy.

Following a prostate cancer diagnosis, approximately 50 percent of men will receive radiation therapy. Patients with PSA >20 ng/ml or biopsy Gleason score 8–10 or T2-3N0M0 localised prostate carcinoma are recognised as high risk[1]. The optimal management of these patients remains unclear. Randomized control trials recommend the combination of external beam radiotherapy with androgen deprivation therapy to improve overall survival[2], but recurrence rates in these patients remain high and are associated with a limited chance of cure[3]. The characterisation of the radiobiological properties of prostate tumours, increasingly related to the eight cancer hallsmarks[4], is essential to guide the evaluation of current as well as novel therapeutic options. It may also provide a means to select patients most likely to benefit from these strategies.

Modifications in the radiobiological properties of tumours can take several forms. Most likely, it results in an increased capacity of irradiated clonogens to overcome the anti-proliferative effects of radiation, evidenced by a quantifiable change in the relationship between clonogenic survival and radiation dose[5]. This change can be attributed to the capacity for these cells to overcome the induction and repair radiation damage[6], ignore pro-apoptotic signals[7] and avoid the transition to a senescent state[8,9]. But other factors complicate this relationship. First, tumour microenvironmental factors and the tumour vasculature[10] may also reduce response to radiotherapy[11]. Second, rapidly accumulating evidence identifies the number of uncontrolled cancer stem cells following a radiotherapy regimen as a key to local tumour control probability[12–14].

Exposure of cancer cells to fractionated radiation schedules can select a cancer subpopulation with modified cell fate in response to subsequent radiation exposure and affect tumour control probablity[15]. This selection process is increasingly reproduced *in vitro* to investigate the molecular response of cancer cells and guide the development of novel biomarkers of radiotherapy failure (reviewed in[16]). Few of these isogenic models currently exist for prostate cancer.

This study aimed to generate and characterise an isogenic model of radioresistant prostate cancer. Of the four commonly used prostate cancer cell lines, only 22Rv1 cells may be representative of primary disease[17]. This

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non-metastatic prostate cancer cell line was exposed to a fractionated radiation protocol. The resulting subline was evaluated for modification in radiation response and oncogenic properties. Our data suggests that this newly established radioresistant model has the potential to support discovery of novel biomarkers predictive of radiotherapy success.

Results

Selection of radioresistant 22Rv1 cells by fractionated irradiation. Wild type 22Rv1 (WT-22Rv1) were either exposed to 2-Gy fractionated radiation to a cumulative dose of 60Gy (RR-22Rv1) or mock irradiated (age matched controls AMC-22Rv1). At the end of this process, the proliferative potential following increasing radiation doses (2–10Gy) was measured in RR-22Rv1, AMC-22Rv1 and WT-22Rv1 using clonogenic assays. The individual experiments were used to define an average survival curve, with the deviation defined by summing the variance for each individual experiment at the corresponding dose and taking the square root (Fig. 1A). The surviving fraction of RR-22Rv1 cells was significantly higher than that of the WT-22Rv1 cell line at all doses tested. This increase was sustained one month later (RR-22Rv1-1M). Each experimental repeat was fitted with a linear curve between 0–6 Gy. The slope was then calculated to assess the decrease in survival. Wild-type 22RV1 cell survival decreased 15.24%/Gy (±0.6%), age-matched control cell survival decreased 15.20%/Gy (±0.37%), and radiation resistant cell survival decreased 13.72%/Gy ± (0.38%). There was no significant difference in the survival of wild type and age-matched controls ($p > 0.05$), but there was a significant difference between wild type and radiation resistant ($p < 0.001$) and between age-matched control and radiation resistant ($p < 0.001$) cells. The selection of radioresistant 22Rv1 cells was further evidenced by an increase in the area under the curve of the radiation survival curve from 2.3 (WT-22Rv1) to 3.14 (RR-22Rv1 cells). Mock-irradiated, aged-matched controls (AMC-22Rv1) exhibited an intermediate area under the curve of 2.7 and significantly reduced clonogenic survival compared to that of RR-22Rv1 cells at all doses tested, but 2 Gy ($p > 0.05$).

Hypoxic response of selected radioresistant 22Rv1 cells. To evaluate whether the radioresistance of RR-22Rv1 could be further increased, the clonogenic survival of WT-22Rv1 and RR-22Rv1 was measured following exposure to 0.5% oxygen for 24 h prior to irradiation (Fig. 1B). Hypoxic treatment increased the survival of WT-22Rv1 cells by up to 4-fold following irradiation (ANOVA, $p < 0.0001$) with an oxygen enhancement ratio of ~2.2 at 50% survival. The clonogenic survival of RR-22Rv1 cells was significantly higher than that of hypoxic WT-22Rv1 cells over the 0–6 Gy dose range (mean difference in survival of 18% at 2 Gy ($p < 0.001$), 10% at 4 Gy ($p < 0.05$) and 11% at 6 Gy ($p < 0.05$)). Hypoxia further protected RR-22Rv1 from the effect of radiation with a significant increase in clonogenic survival at all doses tested with an oxygen enhancement ratio of ~1.2.

Sensitivity of selected radioresistant 22Rv1 cells to Docetaxel. The response of RR-22Rv1, AMC-22RV1 and WT-22RV1 cells to docetaxel was investigated using clonogenic assays (Fig. 1C). Clonogenic survivals following docetaxel treatment (0.01, 0.05, 0,1nM, 48 h) were significantly reduced in 22Rv1-RR and 22Rv1-AMC, when compared to 22Rv1-WT cells at all concentrations tested ($p < 0.005$).

S-phase cell cycle fraction of radioresistant 22Rv1 cells. Cells cycle distributions were next measured by PI staining and flow cytometry (Fig. 2A). The % of cells in the S-phase was significantly higher in RR-2Rv1 (43.2 ± 1.3%) than in WT-22Rv1 (20.9 ± 3.3%, $p = 0.0007$) but not AMC-22Rv1 (34.6 ± 5.1%, $p = 0.11$). This pattern was maintained following irradiation (2-Gy) (ANOVA, $p = 0.002$). Radiation exposure did not increase the % of S-phase cells in all three lines (WT-22Rv1, $p = 0.42$; AMC-22Rv1, $p = 0.76$; RR-22Rv1, $p = 0.99$). The % of cells in the G2 phase were not significant different in both untreated (ANOVA, $p = 0.23$) and irradiated cell lines (ANOVA, $p = 0.11$).

Apoptosis sensitivity of radioresistant 22Rv1 cells. Apoptotic cell death following exposure to radiation (2, 4 Gy) was quantified in all three lines using annexin V-FITC/PI staining and flow cytometry and compared to that of aged matched controls (Fig. 2B). The % of cells in early apoptosis was significantly reduced in RR-22Rv1 when compared to WT-22Rv1 and AMC-22Rv1 at both doses. The baseline % of apoptotic cells were similar across the three cell lines (ANOVA, $p = 0.77$). Following exposure to radiation (2, 4 Gy), the % of apoptotic cells increased in WT-22Rv1 (2 Gy: 3.58 ± 0.15 to 6.50 ± 0.65. $p = 0.024$; 4G y: 3.58 ± 0.15 to 7.5 ± 0.83, $p = 0.03$) and AMC-22Rv1 (2 Gy: 4.59 ± 0.67 to 4.34 ± 1.17, $p = 0.86$; 4 Gy: 4.59 ± 0.67 to 8.22 ± 0.95, $p = 0.03$) but not in RR-22Rv1 cells (2 Gy: 3.79 ± 1.05 to 2.51 ± 0.77, $p = 0.38$; 4 Gy: 3.79 ± 1.05 to 2.25 ± 0.7, $p = 0.28$).

Susceptibility to radiation-induced senescence of radioresistant 22Rv1 cells. The levels of senescence-associated β-galactosidase (SA-β-Gal) were next measured by flow cytometry[18] (Fig. 2C). A non-significant trend towards elevated β-galactosidase levels was seen in untreated RR-22Rv1 cells, when compared to AMC- and WT-22Rv1 cells (ANOVA, $p = 0.14$). A similar trend was observed in irradiated cell lines (ANOVA, 2 Gy, $p = 0.18$; 4 Gy $p = 0.17$, 8 Gy $p = 0.06$). Radiation exposure appeared to increase senescence levels within each of the three lines, but the differences did not reach statistical significance (ANOVA, WT-22Rv1 $p = 0.32$. AMC-22Rv1, $p = 0.22$. RR-22Rv1, $p = 0.56$).

DNA repair capacity of radioresistant 22Rv1 cells. The alkaline comet assay (ACA) was used to quantify immediate levels of induced DNA damage (% Tail DNA) in the three cell lines following irradiation (0–10 Gy) (Fig. 3A). The level of initial DNA damage was significantly lower in RR-22Rv1 cells when compared to both AMC-22Rv1 and WT-22Rv1 in unirradiated cells and sustained across all doses tested (ANOVA, $p < 0.0001$). Initial DNA damage formation increased exponentially with increasing radiation dose in all three lines. At 10 Gy, the absolute increase in % Tail DNA compared to unirradiated control was highest in 48.5 ± 0.9% in WT-22Rv1 (0 Gy: 13.3 ± 0.55% to 10 Gy 61.6 ± 0.7%) 30.6 ± 0.68% in AMC-22Rv1 (0 Gy: 10.3 ± 0.36% to 10 Gy:

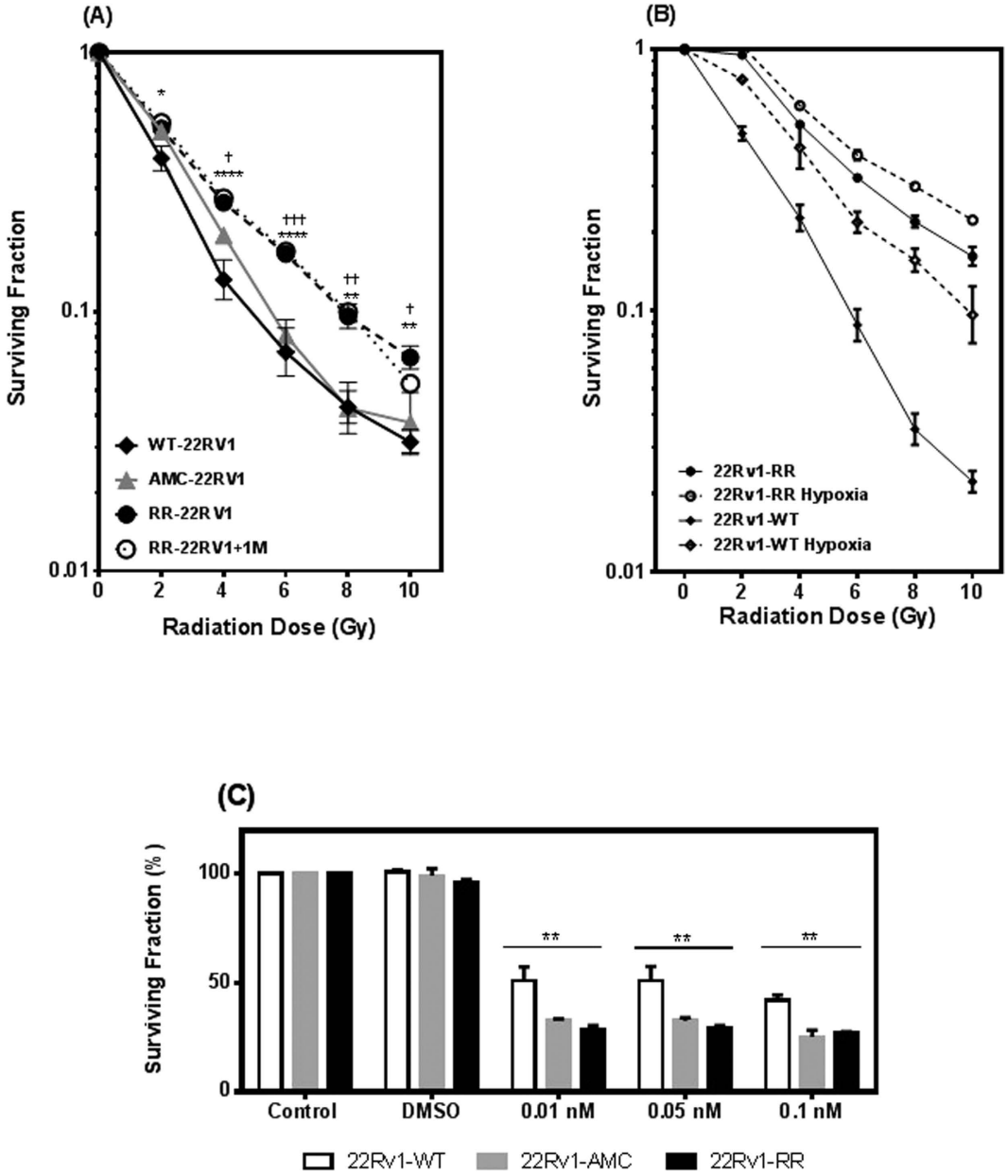

Figure 1. Generation of a stable isogenic radiation resistant prostate cancer cell line. (**A**) Surviving fraction, after 60Gy cumulative dose of X-radiation, of the radiation resistant (RR-22RV1) isogenic cell line model compared with 22RV1 wild type (WT-22RV1) control and 22RV1 age matched control (AMC-22RV1). The isogenic cell line shows consistent resistance to radiation after one month of growth with no treatment (RR-22RV1 + 1M). Survival of irradiated cells was measured by clonogenic cell survival assay after X-radiation with 0–10 Gy. Points are measured as means ± SEM of at least five individual experiments. (**B**) Surviving fraction of aerobic and hypoxic (0.5% O2, 24 hrs) WT-22Rv1 and RR-22Rv1 cells. (**C**) Clonogenic survival of 22RV1-WT, 2RRv1-AMC and 22RV1-RR cells treated with 0.01, 0.5 and 0.1 nM Docetaxel for 48 hours. Points are measured as means ± SEM of at least five individual experiments. Mean ± SEM, *refers to significant differences in the RR cell line vs WT control. †refers to significant differences between RR and AMC cells. Δ refers to significant differences between AMC and WT control cells. *$P \leq 0.05$, **$P \leq 0.01$.

40.9 ± 0.6%) and lowest in 25.47 ± 0.5% in RR-22Rv1 (0 Gy: 7.03 ± 0.32% to 10Gy: 32.5 ± 0.44%). A strong correlation between the % tail DNA and clonogenic survival was identified in all three lines (22Rv1-RR, $r = -0.79$, $p = 0.0028$; 22Rv1-AMC, $r = -0.94$, $p = 0.01$; 22Rv1-WT, $r = -0.94$, $p = 0.01$) (Fig. 3B). ACA was next used to assess the extent of DNA damage repair for up to 50 min following 8Gy exposure (Fig. 3C). The DNA damage levels decreased rapidly within the first 15 min in all three lines. The reduction was greatest in RR-22Rv1 (2.37-fold), when compared to AMC-22Rv1 (1.81-fold) and WT-22Rv1 (1.3-fold) ($p < 0.0001$). For RR-22Rv1, beyond this time point, the % amount of DNA damage continued to decrease, albeit at a slower pace. Fifty minutes

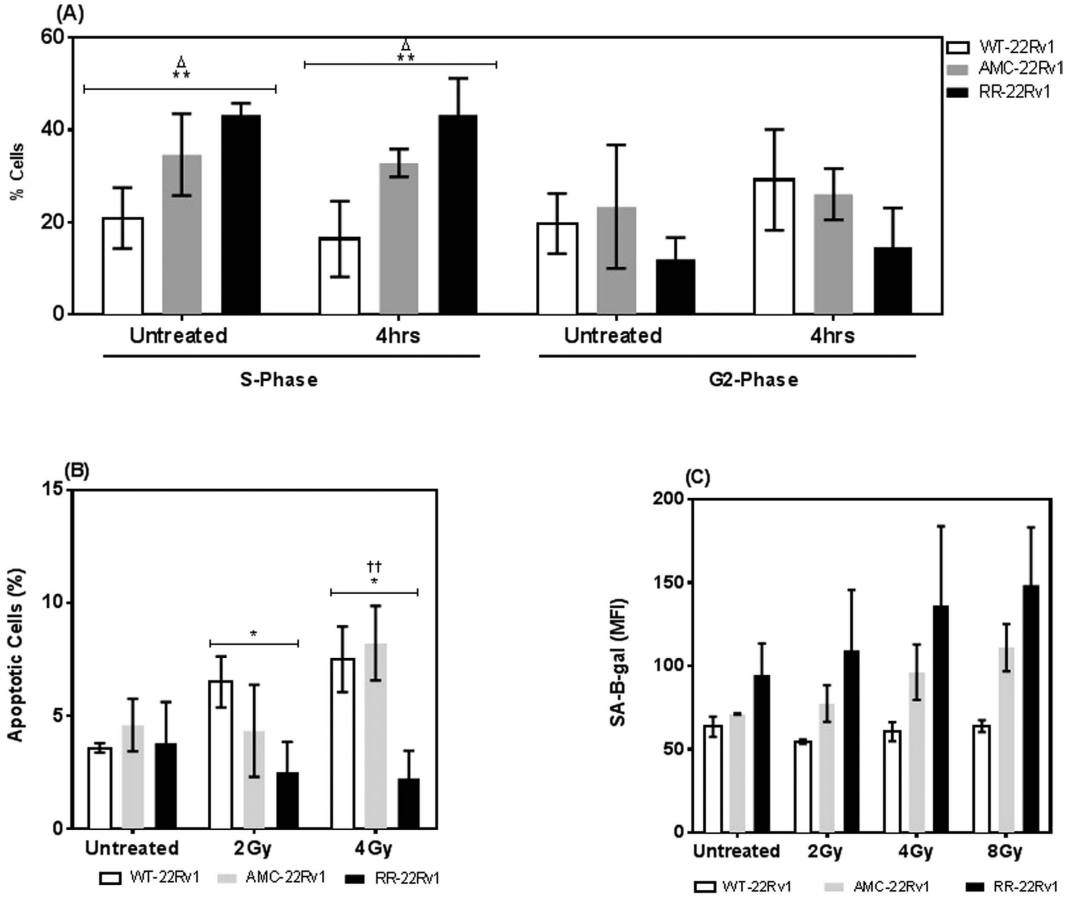

Figure 2. Fractionated radiation exposure selects for 22Rv1 cells with enriched S-phase fraction and apoptosis resistance. (**A**) Cell cycle analysis of WT-22RV1, RR-22RV1 and AMC-22RV1 cells untreated and four hours post irradiation with 2 Gy. (**B**) Radiation induced apoptosis measured using Annexin V and PI staining in WT-22RV1, RR-22RV1 and AMC-22RV1, 6 hours after treatment with 2 Gy and 4 Gy X-irradiation. (**C**) SA-β-gal activity measured with C_{12}FDG and flow cytometry, with and without treatment of 2 Gy, 4 Gy and 8 Gy of ionising radiation. n = 2 *refers to significant differences in the RR cell line vs WT control. †refers to significant differences between RR and AMC cells. Δrefers to significant differences between AMC and WT control cells. *$P \leq 0.05$, **$P \leq 0.01$.

post exposure, the initial % tail DNA was reduced 3.9-fold in RR-22Rv1 cells ($p < 0.0001$) compared to 2.3- ($p < 0.0001$) and 1.12-fold ($p = 0.005$) in AMC-22Rv1 and WT-22Rv1 cells, respectively.

ROS levels in radioresistant 22Rv1 cells. The levels of reactive oxygen species (ROS) in the live and dead cell subpopulations of irradiated (2 Gy, 4 Gy) and unirradiated cells were measured using CM-H_2DCFDA staining analysed by flow cytometry. In the live cells (Fig. 4A), the measures of ROS appeared elevated in RR-22Rv1 and AMC-22Rv1 cells but were not significantly different between the three cell lines for the unirradiated (ANOVA, $p = 0.092$) and the irradiated cells (ANOVA, 2 Gy, $p = 0.07$; 4 Gy, $p = 0.16$). Similarly, exposure to radiation did not significantly change the amounts of ROS within each of the three lines (ANOVA, WT-22Rv1, $p = 0.12$; AMC-22Rv1, $p = 0.74$, RR-22Rv1, $p = 0.68$). In the dead/dying subpopulation of cells (Fig. 4B), the measures of ROS were significantly different between the three cell lines in the unirradiated (ANOVA, $p = 0.003$) and irradiated cells (ANOVA, 2 Gy, $p = 0.001$; 4 Gy, $p = 0.002$) and highest in the AMC-22Rv1 and RR-22Rv1 cells. However, exposure to radiation did not significantly change the levels of ROS within all three lines (ANOVA, WT-22Rv1, $p = 0.92$; AMC-22Rv1, $p = 0.09$, RR-22Rv1, $p = 0.10$). Catalase activity was measured in irradiated (4 Gy) and unirradiated cells (Fig. 4C). At a cell number of 5×10^6, catalase activity was significantly different between the three cell lines in unirradiated (ANOVA, $p = 0.01$) and irradiated cells (ANOVA, $p = 0.01$). Subgroup comparisons indicate that catalase activity is significantly lower in AMC-22Rv1 cells, when compared to WT-22Rv1 under both conditions. In response to radiation exposure, catalase activity was not statistically different in WT-22Rv1 ($p = 0.5$), AMC-22Rv1 ($p = 0.85$) and RR-22Rv1 ($p = 0.78$), when compared to unirradiated controls. Finally, the radiation clonogenic survival curve of both WT-22Rv1 and RR-22-Rv1 cells was not modified following treatment with the anti-oxidant epigallocatechin-3-gallate (EGCG) (Fig. 4D).

Migration capacity of 22Rv1 cells. The CD44+ fraction was determined in each cell line by flow cytometry (Fig. 5A). The fraction of CD44+ cells was significantly reduced in the radioresistant RR-22RV1 (52%), when

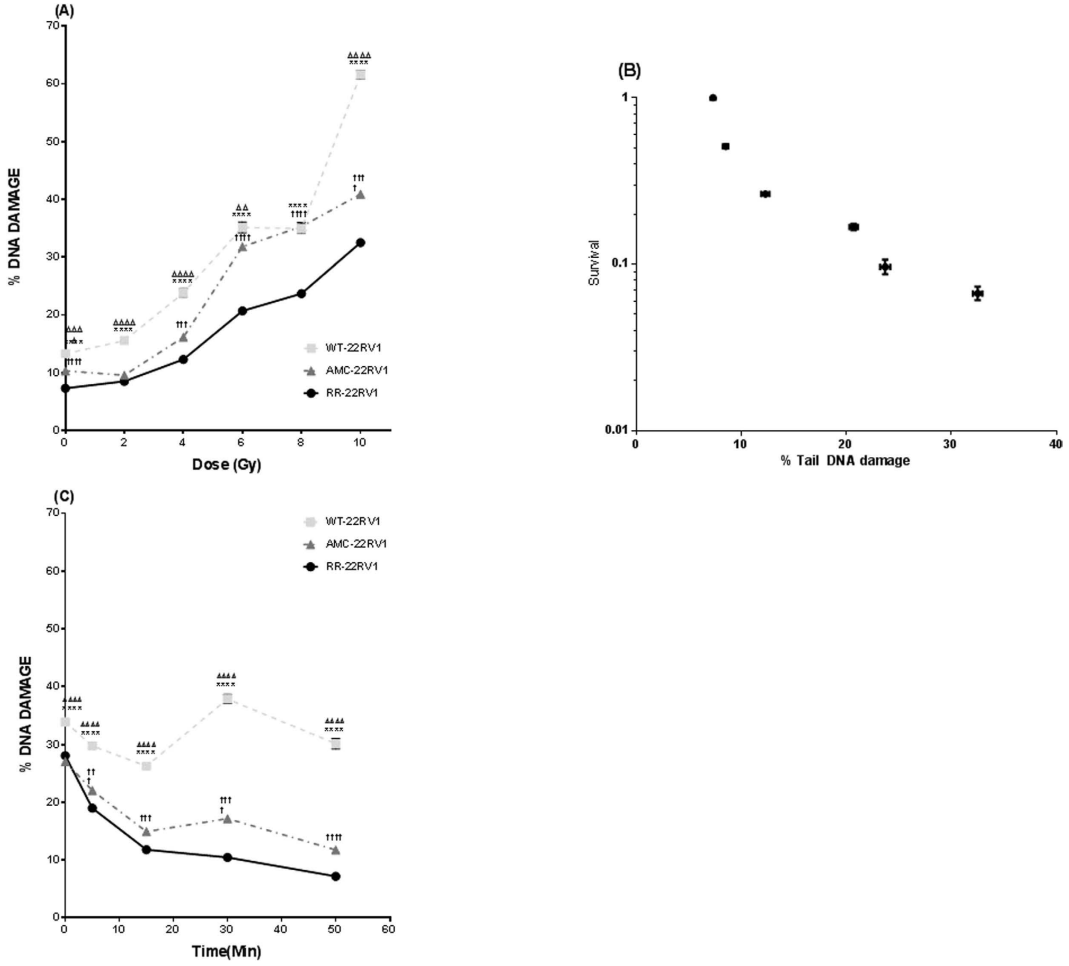

Figure 3. Increased proliferative potential following radiation exposure is associated with reduced levels of DNA damage. (**A**) DNA damage in response to treatment with a range of radiation doses (0–10 Gy) in each of the three cell lines as measured by alkaline comet assay. N = 3 independent experiments. (**B**) Correlation between clonogenic survival and % tail DNA damage in 22Rv1-RR cells. (**C**) DNA repair capabilities of the 22Rv1-RR, WT and AMC cell lines. Initial damage and repair up to 50 min post irradiation with 8 Gy is measured using the alkaline comet assay. n = 5 independent experiments. DNA damage was calculated using % tail DNA for each comet, 400 comets were scored on two slides/4 gels per replicate and SEM calculated. *refers to significant differences in the RR cell line vs WT control. †refers to significant differences between RR and AMC cells. Δ refers to significant differences between AMC and WT control cells. *$P \leq 0.05$, **$P \leq 0.01$.

compared to WT-22RV1(91%) and AMC-22RV1 cell populations (95%) (ANOVA, $p < 0.0001$). There was no significant difference between the fractions of CD44+ cells in the WT-22RV1 cell population when compared to the AMC-22RV1. A wound healing assay was performed on RR-22Rv1, AMC-22Rv1 and WT-22Rv1 cells to assess cellular motility (Fig. 5B)[19]. Over a 48 h period, wound healing was most pronounced in 22Rv1-RR, as evidenced with significantly decreased wound area, when compared to 22Rv1-WT cells ($p = 0.02$). This effect was prevented by treatment with the stem cell inhibitor salinomycin in all three lines (Fig. 5B).

Discussion

Isogenic models of radioresistance have been generated through exposure of cancer cell lines to a variety of fractionation schedules with total doses within a 40–60 Gy range[20–22] and overall treatment times varying from 5 days[23] to 6 years[16,24]. In prostate cancer, isogenic models of LncaP, PC-3 and DU145 cells were generated through 2 Gy daily exposure over 5 consecutive days and associated with a 1.6, 1.5 and 1.5 fold increase in the radiation dose needed to induce 0.1% survival (dose modifying factor), when compared to wild type cells[23]. The radiation-surviving cell population of DU145, PC-3, LnCaP and 22Rv1 cells following exposure to 35 doses of 2 Gy was isolated to examine effects on plasticity[25] and neuroendocrine differentiation[26]. A number of limitations prevent the reproduction of a clinical radiotherapy delivery under experimental conditions such as cell ageing and the necessity for recovery periods[16].

In this study, 22Rv1 cells were exposed to repeated 2 Gy-dose fractions and allowed to recover to a set confluence of ~70–80% in between fractions. The cumulative exposure of 22Rv1 cells to 60 Gy-fractionated radiation resulted in the generation of a sub-line with a significantly increased clonogenic survival potential following

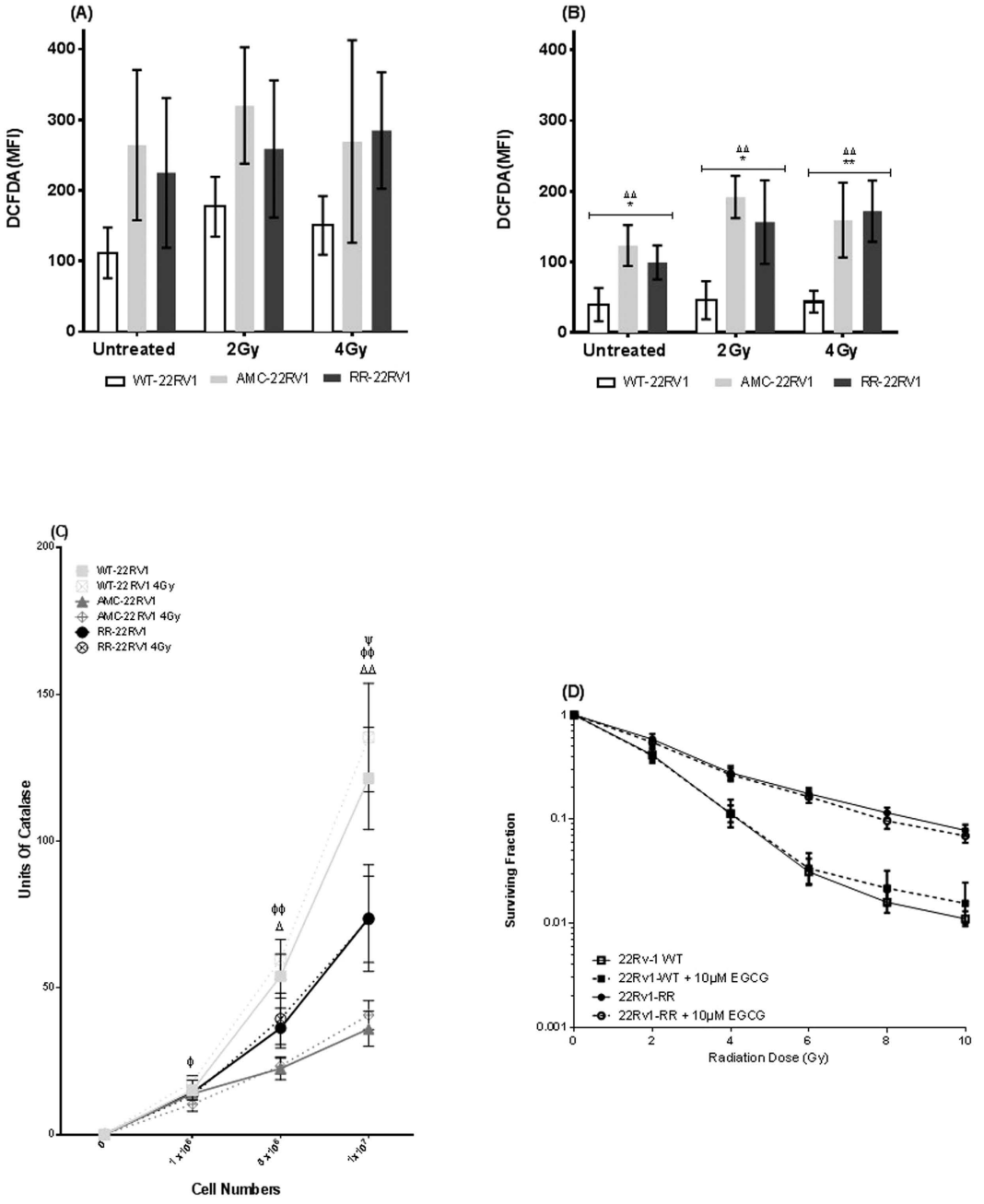

Figure 4. Fractionated radiation exposure selects for cells with high reactive oxygen species levels. (**A**) Reactive Oxygen species levels in the live cell population (isolated using PI) following treatment with radiation (2/4 Gy) compared to untreated controls as measured by Median Fluorescence Intensity (MFI) of DCFDA. Mean ± SD for 4 experiments. (**B**) Levels of reactive oxygen species in the measured in the dead/dying cell population (isolated using PI uptake) following treatment with radiation (2/4 Gy) compared to untreated controls as measured by Median Fluorescence Intensity (MFI) of DCFDA. Mean ± SD for 4 experiments. (**C**) Catalase activity of isogenic radiation resistant cell lines and controls before and after treatment with 4 Gy x-irradiation. Points mean of 4 independent experiments ± SEM. (**D**) Radiation clonogenic survival curve of 22Rv1-WT and 22Rv1-RR cells treated with 10 uM ECGC and untreated controls. Mean ± SD for 4 experiments. *refers to significant differences in the RR cell line vs WT control, †refers to significant differences between RR and AMC cell lines and Δ refers to significant differences between AMC and WT control cells. Φ differences between WT 4 Gy vs AMC 4 GY, ψ differences between WT 4 Gy and RR 4 Gy. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001.

radiation exposure, when compared to wild type and mock irradiated, aged-matched cells. Increased colony forming ability is one of the most commonly reported consequences of protracted fractionated radiation exposure and is often associated with a modification of cell cycle distribution[16,22]. The resulting RR-22Rv1 cell line was

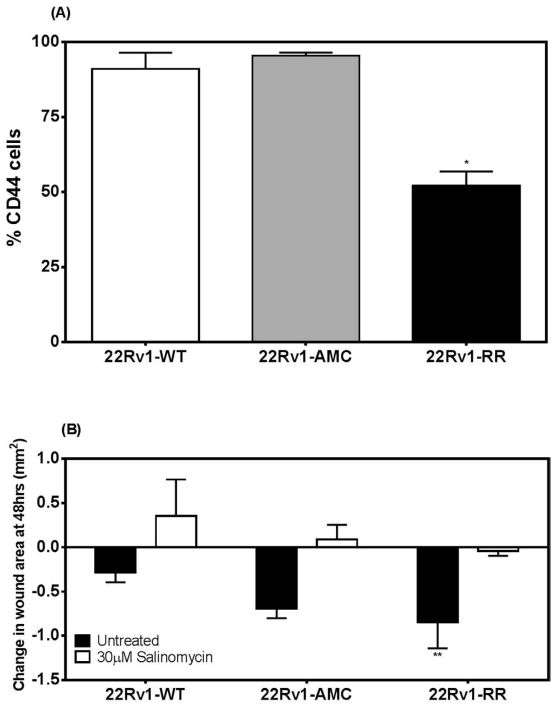

Figure 5. Exposure to fractionated radiation selects for 22Rv1 cells with reduced CD44 expression and enhanced migration capacity. (**A**) The %CD44 positive cells was determined by flow-cytometry from the fluorescence intensities measured in 22Rv1-RR, AMC, and WT cells stained with FITC-conjugated CD44 antibody (**B**) The change in wound area was measured 48 hours following injury in 22Rv1-WT, AMC and RR cells in the absence or presence of 30 μM salinomycin (24 hours). $n = 3$; $^*p < 0.05$.

enriched in S-phase cells, when compared to WT-22Rv1 cells. Enrichment in this radioresistant cell cycle phase has been reported in other radioresistant cell models[16]. In prostate cancer specimens, a larger S-phase fraction has been associated with more aggressive tumours[27,28] and reduced local tumour control probability following radiotherapy[29]. Further evaluation of the underlying mechanisms for the amplification of the S-phase cell population is warranted.

The microtubule targeting agent Docetaxel is the standard of care first line chemotherapeutic drug for the treatment of hormone refractory prostate cancer[30]. Its effect is however limited by the poorly understood development of taxane-refractory tumours[31]. RR-22Rv1 and AMC-22Rv1 cells were more sensitive to Docetaxel than WT-22Rv1, suggesting distinct, possibly age-related, mechanisms of resistance.

Evaluations of the volume of prostate tumours suggest that 20% is exposed to less than 5 mmHg (0.7%) oxygen and prostate tumours are therefore considered hypoxic in nature[32]. Clinical evidence and modelling studies have demonstrated that tumour cell proliferation rates, DNA repair capacity and cellular hypoxia collectively modulate tumour control probability in response to radiation therapy[33]. The resulting RR-22Rv1 line was significantly more resistant to radiation than hypoxic WT-22Rv1 cells. Further enhancement of the radioresistance of RR-22Rv1 cells by hypoxia was small, suggesting that levels of radioresistance may have peaked in these cells.

Formation of DNA damage is the key event for cell killing by ionizing radiation[34] and a cell's ability to repair such DNA damage is key to determining a cell's fate following irradiation[35]. Analysis of % Tail DNA immediately and up to 50 min after radiation exposure indicated that RR-22Rv1 cells were significantly less sensitive to initial damage induction than WT-22Rv1 and AMC-22Rv1 cells and repaired damage more effectively. This characteristic of radioresistant cells was documented in other isogenic cell models[16,36,37]. In prostate cancer patients receiving androgen deprivation therapy, the induction of reduced DNA repair capacity was proposed as a possible underlying mechanism for the improved response of these patients to subsequent radiotherapy treatment[38]. The % tail DNA damage peaked at 30 min post-irradiation in WT-22Rv1 cells but not in AMC-22Rv1 or RR-22Rv1 cells. The mechanistic interpretation of this peak should be further investigated. It may reflect induction of DNA damage by secondary radiation-induced reactive oxygen species, such as those released by the mitochondria[39].

Mitochondrial respiration is increasingly documented to contribute to tumor cell survival and proliferation[40]. Mitochondrial dysfunction has been associated with cellular fate following irradiation[41] and a direct role in radiation-induced G2/M arrest[39] and apoptosis induction has been proposed[42]. Following irradiation, WT-22Rv1 and AMC-22Rv1 cells were more susceptible to apoptosis induction than RR-22Rv1 cells. Modification in radiation-induced apoptosis sensitivity has been reported in some[43] but not other[21] isogenic models. The prognostic potential of apoptosis sensitivity in prostate radiotherapy patients has been proposed[44] but remains unconfirmed. Nonetheless, the pharmacologic radio sensitization of prostate cancer cells is often achieved through induction of apoptosis[45]. Further evaluation of the underlying mechanisms for this reduced susceptibility to radiation-induced apoptosis is required.

The induction of permanent growth arrest through senescence was proposed as an alternative mechanism in the therapeutic response to therapy in cancer cells with deregulated apoptotic signalling[46]. The induction of senescence in response to elevated DNA damage has been associated with increased therapeutic sensitivity[47]. In prostate cancer patients, increased expression of β-galactosidase (GLB1) was associated with a reduced risk of recurrence[48]. A proportion of surviving DU145 and PC3 cells exposed to 35 fractions of 2 Gy developed a senescent-like morphology associated with elevated mRNA expression of key senescence markers[25]. Our RR-22Rv1 cells however failed to show statistically significant evidence for a possible reduction in the induction of therapeutic senescence. Our analysis of β-galactosidase activity would not detect other permanent growth arrests such as the induction of neuroendocrine differentiation (NED). Therapeutic NED has also been identified as a cause of radioresistance that may be limited to CD44+ prostate cancer stem cells[49]. The evaluation of the stemness of our model is pending, but our preliminary analysis of CD44 expression suggests that exposure to fractionated radiation reduces the fraction of CD44-expressing 22Rv1 cells. Downregulation of CD44 was associated with increased radiosensitivity in DU145 and PC3 cells[50], but increased migration potential[51]. Wound closure was more efficient in CD44-deprived RR-22Rv1 than WT-22Rv1 or AMC-22Rv1 cells. This process was inhibited by salinomycin, an anti-coccidial drug increasingly tested for its potential anti-cancer and anti-cancer stem cell properties[52,53]. This sodium ionophore monensin similarly inhibited proliferation and migration of LnCaP cells[51]. Further investigation into the role of CD44 in the selection of a radioresistant subpopulation of cells is warranted.

The ability of prostate cancer cells to manage oxidative stress plays an important role in cell signalling and their response to therapeutic injury[54]. In particular, reduced susceptibility to ROS-induced cellular damage and efficient repair of radiation-induced DNA damage was proposed as an underlying mechanisms to the observed radioresistance of prostate cancer stem cells[55]. Lower ROS basal levels were associated with increased Nfr2 levels and radiosensitivity in prostate cancer cells[56]. Basal ROS levels were lower in WT-22Rv1, than in AMC-22Rv1 and RR-22Rv1 cells. This reduction was associated with increased catalase activity in WT-22Rv1 cells. ROS levels were elevated in dying RR-22Rv1 cells. This suggests that cells with high ROS levels are preferentially eliminated in this cell population or reflects the likely release of ROS in dying cells. However, the sensitivity of cells to the anti-oxidant epigallocatechin-3-gallate (EGCG), whose therapeutic potential in prostate cancer was proposed previously[57], remained unchanged in both WT- and RR-22Rv1 cells.

Conclusion

Exposure to fractionated radiation progressively selected for 22Rv1 cells with enhanced oncogenic properties protective against radiation injury and supportive of tumour invasion. The characterisation of the radioresistance of these cells using *in vivo* dose response assays is required to account for the likely effect of spontaneous (non-radiation) death rates[58] and the tumour microenvironment[59] on tumour control probability in a clinical setting[15]. This data must be considered within the context of one cell line, and the limitations of *in vitro* models, but the phenotyping modifications observed support the clinical relevance of this model to enable further study of the mechanisms of radioresistance in prostate cancer cells.

The progressive selection of radioresistant cells throughout a protracted radiation therapy protocol may have clinical implications. In the era of personalised medicine, concurrent targeted therapies may require careful timing and perhaps may be more effective when administered toward the end of radiotherapy delivery.

Materials and Methods

Cell lines and culture. The human prostate cancer cell line 22Rv1 was obtained from American Type Culture Collection. Cells were maintained as monolayers in RPMI cell culture medium containing l-glutamine (Lonza, Castleford, UK) with 10% foetal bovine serum (Gibco, Dublin, Ireland) and 1% pen/strep (Lonza). The cells were maintained at 37 °C in 95% humidified air containing 5% CO_2 and sub-cultured once to twice weekly to maintain exponential growth unless otherwise stated. Cells were grown to approximately 70–80% confluency in vented 75 cm^2 culture flasks prior to irradiation. Each irradiation consisted of 2 Gy X-rays dose (250 keV, 15 mA) using an RS225 cabinet irradiator (Gulmay Medical, Surrey, UK). This process was repeated weekly until the cells received to a total cumulative of 60 Gy. Mock irradiated cells were cultured in parallel as age-matched controls (AMC-22Rv1). Hypoxia (0.5% O_2, $pO_2 < 2$ mmHq) was achieved by exposing cells in a 1000 *in vivo* hypoxic chamber (BioTrace, Bracknell, UK) to a mixture of nitrogen, CO_2 (5%) and compressed air to achieve a 0.5% oxygen concentration.

Clonogenic Assay. Cell survival was evaluated using a standard colony forming assay. Cells in exponential growth phase (approx. 70% confluent) were harvested by trypsinisation (Lonza) and counted in order to obtain a single cell suspension of 1×10^6 cells/ml. Cells were seeded into six well plates at a density of $1 \times 10^3 - 1 \times 10^4$ cells/well and allowed to adhere to plates overnight in the incubator at 37 °C in 95% humidified air containing 5% CO_2. Cells were then irradiated (2, 4, 6, 8 or 10 Gy single doses) and incubated for 7–10 days under the conditions previously described. Following incubation, colonies were fixed and stained using 0.05% crystal violet in 70% methanol solution. Plates were dried overnight and colonies containing >50 cells counted using the ColCount instrument (Oxford Optronix Ltd, Oxford, UK). Surviving fraction was calculated as no. colonies/(no. cells seeded X PE). The platting efficiency PE was calculated using the no. colonies/no. cells seeded in the unirradiated cells.

Cell Cycle Analysis. Cell cycle analysis was performed using Propidium Iodide (PI) staining and flow cytometry as previously described[60]. Cells were harvested at 4 h, 8 h, 12 h, 24 h, 30 h post irradiation, using trypsin-EDTA(Lonza), and the pellet fixed and permeabilised by dropwise addition of 4 ml ice cold 70% EtOH (Sigma-Aldrich, Wicklow, Ireland). Cells were fixed for a minimum of 2 h at 4 °C. Fixed cells were centrifuged at

180 g × 3 min and the supernatant decanted, samples were then washed in PBS and centrifuged as before. Each sample was resuspended in 0.5 ml Triton X-100 (0.1% v/v in PBS)(Sigma-Aldrich) containing PI (0.02 mg/ml PI) (Sigma-Aldrich) and RNase A (0.2 mg/ml) (Sigma-Aldrich). Unstained fixed controls were resuspended in PBS. Samples were incubated at 37 °C for 30 min and analysed immediately using CyAn™ ADP Analyzer (Beckman Coulter, Clare, Ireland.). Cell cycle phase was determined using flowJo software (FlowJo LLC, Or, USA).

Apoptosis Assay. Apoptotic cells were detected using annexin V-FITC/PI staining and flow cytometry as previously described[61]. Following treatment with 0, 2 or 4 Gy of X-irradiation, cells were trypsinised and combined with supernatant containing non-adherent cells. Cell pellets were resuspended in ice cold 1X binding buffer (0.1M Hepes (Sigma-Aldrich) 1.4M NaCl (Sigma-Aldrich) and 25 mM $CaCl_2$, at pH 7.4 (Sigma-Aldrich). Cells were stained with 3 μl Annexin V-FITC (IQ Products, Groningen, Netherlands) and incubated at 4 °C. Cells were washed in ice cold PBS and resuspended in 1X binding buffer containing PI (0.05 μg/ml) (Invitrogen, Dublin, Ireland). Cell were analysed using the CyAn™ ADP Analyser (Beckman Coulter) and apoptotic cells were detected using the FlowJo software (FLowJo LLC, Or, USA).

Senescence. β-Galactosisdase activity was measured by flow cytometry as previously reported[18]. Cells were seeded in 6 well plates at a density of $1–2 \times 10^5$ cells/well and allowed adhere overnight prior to irradiation (2 Gy, 4 Gy or 8 Gy) and returned to the incubator along with mock irradiated controls for 24 h. Cells were washed with PBS and incubated for 1 h at 37 °C, 5% CO_2 with Bafilomycin A1 (Sigma-Aldrich) supplemented growth media to adjust lysosomal pH to pH6. 2 mM 5-Dodecanoylaminofluorescein di-β-D-galactopyranoside (C_{12}FDG) (Sigma-Aldrich) was added and samples were incubated for 1 h before analysis. Cells were then trypsinised and resuspended in 1 ml PBS and analysed immediately using a CyAn™ ADP Analyser (Beckman Coulter). Mean fluorescence intensity was calculated using FlowJo software.

Alkaline comet assay. A previously reported version of the alkaline comet assay (ACA)[62] adapted from the original protocol by Singh *et al.*[63] was used to determine levels of DNA damage and repair capability after irradiation. All reagents used were obtained from Sigma-Aldrich, Dorset, UK. Briefly, 4×10^4 cells resuspended in low melting point agar (0.6% in PBS) were layered onto agar-coated slides (1% in ddH_2O) prior to irradiation on ice and in the dark. Following this slides for the repair study were incubated at 37 °C, 5% CO_2, 95% humidified air in RPMI media containing 20% FBS and 5% pen/strep for up to 50 min. All slides were then incubated overnight in ice cold lysis buffer (100 mM NA_2EDTA, 2.5M NaCL, 10 mM Tris-HCL, pH 10 with 1% Triton X added immediately before use). Following electrophoresis (30 V, 30 mA, 20 min in 300 mM NaOH, 1 mM Na_2EDTA, pH 13), the slides were incubated in neutralization buffer (0.4M Tris-HCL, pH7.5), washed and allowed to dry for 2 h at 37 °C prior to PI staining (2.5 μg/ml). Comets were visualised and measured using Komet 5.0 software (Kinetics imaging software ltd, Brombourough UK) and fluorescent microscope at a magnification of 20X. 50 comets were scored/gel. The mean % tail DNA, calculated as the percentage of DNA present in the comet tail (measured as an intensity) compared to total comet intensity), was deduced from three (dose response) or four (repair study) independent experiments and presented as mean ± SEM.

Oxidative Stress. Intracellular oxygen species were detected using Di(Acetoxymethyl Ester) (6-Carboxy-2′,7′-Dichlorodihydrofluorescein Diacetate) (CM-H_2DCFDA)(Life Technologies, Dublin, Ireland) and flow cytometry. Exponentially growing cells were collected by trypsinisation. Samples were incubated for 30 min in the dark at 37 °C, 5% CO_2, 95% humidified air in PBS containing 10 μM CM-H_2DCFDA, with controls incubated in PBS. After incubation cells were pelleted at 180 × g for 3 min and the supernatant decanted. Samples were resuspended in PBS and irradiated with 2 Gy or 4 Gy X-rays, controls were mock irradiated and placed directly on ice. PI (1 mg/ml) was added to the samples except appropriate controls. Samples were analysed immediately using CyAn™ ADP Analyser (Beckman Coulter). Data was analysed using FlowJo software.

Catalase Activity. Catalase activity was measured using a visual method as previously reported[64]. Cells harvested as previously described were resuspended in pyrex tubes (Corning, Flintshire, UK) in varying concentrations (10^5–1.5^7 cells) in 100 μl of PBS (Lonza) prior to mock or 4-Gy irradiation. Catalase powder (Sigma-Aldrich) was dissolved in 100 μl distilled pure water to generate a calibration curve for catalase activity (0–200units). 100 μl1% Triton X-100 and 100 μl undiluted hydrogen peroxide (30%) were added to the solutions, mixed thoroughly and incubated at room temperature for 15 min before the height of O_2-forming foam was measured with a ruler.

Migration Assay. A wound healing assay was performed with the Ibidi Wound Healing 6 Well Plate Assay Kit (Ibidi, GmbH, Martinsried, Germany). Cells were seeded into Ibidi insert wells at 500,000 cells/well with total volume 70 μl, allowed to adhere for 24 h and incubated in 30 μM salinomycin (Sigma-Aldrich) treated media for 24 h. After incubation, the insert was removed and images were taken every at 24 and 48 h. Image analysis and migration rate determination was performed using ImageJ Analysis software (Bethesda, USA).

CD44 Expression. Cell pellets were resuspended in staining buffer containing CD44 (FITC-conjugated mouse monoclonal anti-human, BD Pharminogen) and incubated for 20 min at 4 °C in the dark. Cells were washed and fluorescence intensity was measured using CyAn™ ADP Analyser (Beckman Coulter).

Docetaxel treatment. Cells were seeded into 6 well plates at a density of 750 cells/well for control and vehicle control (1X DMSO) and 3×10^3 cells/well for treated. Cells were treated with 0.01 nM, 0.05 nM, 0.1 nM, 0.25 nM, 0.5 nM and 1 nM of Docetaxel (Sigma Aldrich) for 48 h or left untreated as controls.

Statistical Analysis. All statistical analysis was carried out using Prism (version 6 for mac OS X and windows 7, Graphpad software La Jolla California USA, www.graphpad.com). The analysis of differences in the means of continuous data was performed with unpaired two tailed Student *t*-Tests, under the assumption that the data is Normally distributed. A One-way ANOVA with Bonferroni correction was used when the means of more than two groups were compared. A spearman correlation was used to quantify the non-linear relationship between the % tail DNA and clonogenic surviving fractions. All experiments were performed in triplicates unless otherwise stated. A p-value < 0.05 was considered statistically significant.

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Author Contributions

N.M.D. contributed to all aspects of the manuscript. A.M. all experimental aspects of the study. N.G. conducted the CD44 and cell migration assays. C.H. conducted the senescence study. S.H. generated the docetaxel data. K.B. and S.B. assisted with the comet assays. B.M. contributed to the ROS experiments. B.M. performed the survival curve data analysis. N.L. assisted with the generation of the isogenic cell line. G.D.D.J. assisted with the comet assay and preparation of the manuscript. L.M. contributed to all aspects of the manuscript. All authors reviewed the manuscript.

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Isogenic radiation resistant cell lines: Development and validation strategies

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Abstract

Purpose: **The comparison of cell lines with differing radiosensitivities and their molecular response to radiation exposure has been used in a number of human cancer models to study the molecular response to radiation. This review proposes to analyze and compare the protocols used by investigators for the development and validation of these isogenic models of radioresistance.**

Conclusion: **There is large variability in the strategies used to generate and validate isogenic models of radioresistance. Further characterization of these models is required.**

Keywords: Isogenic, cell lines, radiation

Introduction

The risk of tumour regrowth following radiation therapy remains high for a proportion of cancer patients, despite our ability to deliver high radiation doses to the tumour volume through the development of modern radiation oncology techniques such as three-dimensional conformal radiotherapy (3D-CRT), intensity modulated radiotherapy (IMRT) and volumetric modulated arc therapy (VMAT) (Smith et al. 2006, Otto 2008). Our understanding of the radiation response of cancer cells has led to the identification of key mechanisms of resistance and actionable drug targets (Begg et al. 2011, Ree and Hollywood 2013), but robust patient stratification algorithms and targeted therapies are still required.

The understanding of the molecular mechanisms underlying the radioresistant phenotype of tumours forms the basis for the identification of prognostic markers of radiotherapy treatment failure. This phenotype is likely the result of the tumour clinical features, intrinsic differences in cellular radiosensitivity relating to constitutive and/or radiation-induced activation of protective intracellular signalling pathways (altered DNA repair, altered cell cycle check point operation, altered growth, altered reactive oxygen species biology and altered induction of apoptosis) (Bristow et al. 2007, Skvortsova et al. 2008, Diehn et al. 2009), the tumour microenvironment (inflammation, hypoxia) (Bristow and Hill 2008) and the cancer stem cell population (Baumann et al. 2009). The comparison of cell lines with differing radiosensitivities and their molecular response to radiation exposure has been used in a number of cancer models to study the molecular response to radiation.

Radiation resistant isogenic cell lines have been generated for a number of human cancer lines including both solid tumours: Ovarian (De Pooter et al. 1991), Neuroblastoma (Russel et al. 1995), Pancreatic (Lee et al. 1999), Lung (Henness et al. 2004, Wei et al. 2008, Lee et al. 2010, Mihatsch et al. 2011), oesophageal (Fukuda et al. 2004, Xie et al. 2009, Lynam-Lennon et al. 2010), prostate (Skvortsova et al. 2008), liver (Kuwahara et al. 2009), breast (Wang et al. 2005, Smith et al. 2009, Mihatsch et al. 2011), skin (de Llobet et al. 2012), oral (Lee et al. 2013) cancer and the Human T-cell leukaemia cell line CCRF-CEM (Harveie et al. 1997). These models have highlighted some mechanisms for the survival advantage of the generated sub lines. The selection methods used however differ across studies, and comparative analysis across these models is as a result limited. This review proposes to analyze and compare the protocols used by investigators for the development and validation of these human isogenic models of radioresistance.

Induction of radioresistance in human cell lines

The selection of a radioresistant subline has been successfully achieved through the repeated exposure of parent cell lines to fractionated radiation treatment protocols of variable overall total dose, overall treatment time (days/months) and recovery time of the irradiated cells. The variations between protocols likely reflect the fundamental differences in cell biology which transverse the different cell lines used in these studies. While cell lines are known to lose some tissue specific up-regulation of genes throughout their lifetime there is still distinct differences between and even

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within lines isolated from the same tumours (Sandberg and Ernberg 2005). A number of carcinoma derived cell lines have been shown to divide into different hierarchical branches separated into those lines expressing genes which are characteristic of epithelial cells and those typically associated with stromal cells (Ross et al. 2000). Further to this Ross et al. showed that the cell lines included in their study (NCI60 cell lines) could be further separated based on cell line doubling time, expression of genes associated with cell line proliferation rate and drug metabolism (Ross et al. 2000). Therefore the mechanisms of radioresistance identified in isogenic models may be cell type-specific and have limited applicability in clinical practice. Nonetheless, analysis of endpoints measured in isogenic pairs generated to date suggests that these sublines share common survival advantages.

The question of genetic alterations due to the age of the cell lines, rather than radiation induced genetic instability as a result of long overall treatment times does not appear to have been answered in the literature. The use of the parent line as a control for the newly created isogenic lines in investigating the differences between acquired radiation resistance and radiosensitivity is common. The *parent line* can be described as an age matched control/wild type cell line grown (De Pooter et al. 1991, Harveie et al. 1997, Lee et al. 1999, Henness et al. 2002, Fukuda et al. 2004, Wang et al. 2005, Skvortsova et al. 2008, Wei et al. 2008, Kuwahara et al. 2009, Smith et al. 2009, Xie et al. 2009, Lee et al. 2010, de Llobet et al. 2012) or sham irradiated (Lynam-Lennon et al. 2010, Mihatsch et al. 2011) over the same overall treatment time as the irradiated line. It appears that only the age-matched control and the mutant isogenic line are compared in order to investigate the underlying changes in the newly acquired radioresistant subline. While this will allow for comparison of radiosensitivity between the lines and it will take into account genetic aberrations due to age which may be influencing radiation resistance of the new line, it would be necessary to analyze the original young cell line along with the age matched control and isogenic line in order to rule out other causes for radiation resistance in the isogenic cell line such as abnormalities in karyotype and changes in global gene expression patterns due to age (Ruutu et al. 2004, Wenger et al. 2004).

Total dose

Extreme variations in the total dose of radiation delivered were evident across the cell lines used, from 12 Gy in cycles of 3 Gy in the SK-BR-3 human breast cancer cell line (Mihatsch et al. 2011), to 85 Gy in varying cycles starting at 0.5 Gy and escalating to a dose of 3 Gy per fraction in the development of the radiation resistant subcell line in A431 Human Epidermoid Carcinoma line (de Llobet et al. 2012), and an extreme 120 Gy in the PANC-1/Rad-24 human pancretic cancer cell line (Lee et al. 1999). On average the total radiation dose ranged between 40 and 60 Gy (Russel et al. 1995, Henness et al. 2002, Fukuda et al. 2004, Wang et al. 2005, Wei et al. 2008, Smith et al. 2009, Lynam-Lennon et al. 2010, Lee et al. 2013). Though the reason for this variation may be intrinsic differences in the cell lines or preferred clinically relevant treatment protocols, a justification for the total dose used by authors was not provided.

Overall treatment time

The overall treatment time was highly variable, with some authors characterizing isogenic lines as radiation resistant after as little as five consecutive days of radiation (Skvortsova et al. 2008) and others extending the period of treatment as long as six years. Kuwahara et al. (2009) indeed exposed cells to 0.75 Gy of X-rays twice a day for over six years resulting in over 1600 Gy of ionizing radiation exposure. The average overall treatment time was 4–7 months (De Pooter et al. 1991, Lee et al. 1999, Henness et al. 2002, Fukuda et al. 2004, Wei et al. 2008, Lee et al. 2010, de Llobet et al. 2012), with some groups radiation regime extending treatment to a maximum of ~ 10 months (Harveie et al. 1997, Smith et al. 2009, Xie et al. 2009).

Recovery time

Fractionation schedules varied between studies, particularly in terms of the confluence at which the isogenic cell lines were irradiated and the period of recovery between radiation exposures. While some authors suggest the initial fraction merely be performed on sub-confluent (Russel et al. 1995) or exponentially growing lines (Harveie et al. 1997, Lee et al. 1999, Henness et al. 2004, Wei et al. 2008, Kuwahara et al. 2009) others suggest anything from cells 50–60% confluent (Fukuda et al. 2004, Xie et al. 2009, Lee et al. 2010, Lynam-Lennon et al. 2010) to irradiation 24 h after seeding of the cell line (Mihatsch et al. 2011), or do not address it (De Pooter et al. 1991, Wang et al. 2005, Skvortsova et al. 2008, Smith et al. 2009, de Llobet et al. 2012). There appears to be no overall prevailing approach for determining when to irradiate a cell line. Some cell lines received fractionated radiation over consecutive days without a significant amount of recovery time (Skvortsova et al. 2008, Kuwahara et al. 2009), while others were allowed to recover either to a set confluence of ~ 80–100% (Wei et al. 2008, Lee et al. 2010, Lynam-Lennon et al. 2010, Lee et al. 2013) or for a set amount of time between radiation treatment, usually 10–12 days. De Llobet et al. (2012) serially iradiated human epithelial carcinoma A431 cells daily over a period of seven months with treatment stopped only as necessary to allow for cells to recover. Nonetheless, across the literature it is generally agreed that a minimum period of ~ 12–14 days should be left between the final fraction of radiation and any experiments on the new subline, to allow the cells to recover.

Characterization of isogenic cell lines as radioresistant

The selection of sub clones with survival advantage throughout fractionated radiation exposure is likely the result of multiple mechanisms, and validation of acquired radioresistance should rely on a combination of cellular assays. There appears however to be no consensus on how to accurately and consistently define an isogenic subline as having a radiation resistant phenotype. While clonogenic

cell survival assays are generally used to establish induction of radiation resistance, this is not always the case. Additional characterization methodologies differ vastly across studies; from molecular techniques to investigate cell cycle, lactate dehydrogenase release, DNA damage and apoptosis as a marker of radiation resistance to protein and growth inhibition studies. Nonetheless, common traits are apparent across the models tested to date.

Radioresistant sublines demonstrate higher clonogenic survival, proliferative activity and motility than the parent line following radiation exposure

Clonogenic cell survival assays are considered the gold-standard method for assessing the radiosensitivity of cells, and measure the fraction of irradiated cells that retain the ability to form new progeny (Franken et al. 2006). Loss of colony-forming ability is one of the most observable outcomes of exposing radiosensitive cells to ionizing radiation, therefore a clonogenic cell survival assays in which the proposed radioresistant isogenic cell line is compared with the parent line in respect to increasing doses of irradiation, i.e., 2, 4, 6 8, 10 Gy, and the survival fraction used to grade the level of resistance and sensitivity in each line has become the method of choice for classification (Franken et al. 2006). This assay combined with other molecular techniques for the investigation of processes such as apoptosis, cell motility, cell doubling time, and cell cycle analysis are generally used in the characterization of these lines, to varying degrees (Table I).

Authors generally report that the newly created sublines had equal if not significantly greater clonogenic cell survival capacity than that of the parent cells (Russel et al. 1995, Lee et al. 1999, Henness et al. 2002, Fukuda et al. 2004, Skvortsova et al. 2008, Smith et al. 2009, Xie et al. 2009, Lynam-Lennon et al. 2010, Mihatsch et al. 2011, de Llobet et al. 2012). However, there is no universal use of a standard unit of measurement and a variety of radiobiological parameters are used. Some report clonogenic ability as number of colonies that have grown in comparison to a control (Lee et al. 2010) or by plating efficiency (Smith et al. 2009), others the survival fraction (SF) (Wang et al. 2005, Skvortsova et al. 2008, Xie et al. 2009), mean inactivation dose (Fukuda et al. 2004), alpha and beta ratio, doubling time (DT), the dose-modifying factor (DMF) (Wang et al. 2005, Skvortsova et al. 2008) or a combination of these (Russel et al. 1995, Lynam-Lennon et al. 2010, Mihatsch et al. 2011, de Llobet et al. 2012). Xei et al reported a ~ 7% difference in the surviving fraction at 4 Gy (SF4) between radioresistant EC109/R and EC109 human esophageal squamous carcinoma cell lines (Xie et al. 2009). The DT in this radiation resistant line was increased to ~ 29–45 h in comparison to ~ 21–34 h in the parental line. The DMF at 0.1 survival in newly established human prostate cancer LNCaP-IRR, PC3-IRR and Du145-IRR was 1.6, 1.5 and 1.5, respectively (Skvortsova et al. 2008). Lee et al. (2013) reported increased release of lactate dehydrogenase following irradiation of the radioresistant subline.

The selected sublines have increased proliferative capacity, when compared to the parent line. The 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay has been utilized in order to characterize isogenic sublines as radiation resistant (Mosmann 1983, Denizot and Lang 1986, Carmichael et al. 1987, Price and McMillan 1990). Cell proliferation is calculated post radiation treatment in both the parent cell line and the new subline. Henness et al. reported a 2-fold increase in cell proliferation after treatment with radiation in the selected subline in comparison to the parent (Henness et al. 2002). The human T cell leukemia CEMRR line was 1.5 time more resistant to radiation that the parent line with a 50% inhibitory radiation dose (ID50) of ~ 7.5 Gy and ~ 5 Gy, respectively (Harveie et al. 1997). On the other hand, Xie et al. (2009) did not observe significant proliferative difference between the radioresistant isogenic EC109R and EC109 human esophageal squamous cell carcinoma lines.

The tumourigenecity of parent and radioresistant cell lines did not appear to differ. Seven days after cell injection all animals exhibited tumour growth, with similar average tumour sizes for either A431-WT cells or A431-R human epidermoid carcinoma cells. Tumours grew exponentially as a function of time showing a similar pattern for both types of cells. Following irradiation (30 Gy in 2 weeks, 5 fractions of 3 Gy/week), the size of resistant tumours was however 1.8 times larger than that of tumours from parental cells (de Llobet et al. 2012).

Finally, the radioresistant sublines appear to possess an increased ability to migrate and heal wounds in a shorter period of time than the radiosensitive cells, which is proposed to be consistent with the more aggressive phenotype of the radiation resistant cell lines (Skvortsova et al. 2008, de Llobet et al. 2012).

Radioresistant sublines can have a modified cell cycle distribution

It is well established that cell cycle phase effects radiation sensitivity of cells (Pawlik and Keyomarsi 2004). Cell cycle analysis with propidium iodine staining in conjunction with flow cytometry to assess what proportion of cells and in which phase of the cell cycle irradiated lines have arrested, along with further proteomic analysis to determine how cell cycle checkpoints including mutations in genes for tumour protein 53 (P53) and 21 (P21) affect radiation resistance, are therefore common techniques used to investigate radioresistance.

Cells respond to ionizing radiation through the activation of cell cycle checkpoints (Pawlik and Keyomarsi 2004), leading to controlled temporary cell cycle arrest, allowing for repair of defects or the initiation of cell death (Pawlik and Keyomarsi 2004). Irradiated cells show an increased number of cells arrested in the G1, S and G2 cell cycle phases (Pawlik and Keyomarsi 2004). Cells in Synthetic (S) phase, particularly the latter end of the S phase, are less sensitive to radiation than cells in the G1 phase with those cells in the G2-M phase being the most radiosensitive (Sinclair and Morton 1966). Analysis of cell cycle distribution using propidium iodine staining in conjunction with flow cytometry was used by a number of authors to further characterize the generated radioresistant subline (Russel et al. 1995, Fukuda et al. 2004, Wei et al. 2008, Kuwahara et al. 2009, Lee et al. 2010, Lynam-Lennon et al. 2010, de Llobet et al. 2012),

Table I. Isogenic radioresistance models developed and validated to date.

Isogenic pair	Fractionation schedule	Overall treatment time	Inter-fraction recovery time	Clonogenic survival and proliferation (RR vs. P)	Cell cycle distribution (RR vs. P)	DNA damage repair capacity (RR vs. P)	Oxidative stress	Apoptosis	Reference
QLL1/RR SCC15/RR SCC25/RR (*Human Squamous cell carcinoma*)	2 Gy to total dose 60 Gy	–	Cells at 80% confluence	↑ (LDH assay)	–	–	–	–	Lee SY et al. (2013)
A431/A431-R (*Human Epidermoid Carcinoma*)	0.75–3 Gy per fraction up to a total dose of 85 Gy (*DR: 2.7 Gy/min*)	7 months	As required	↑	Non-relevant variation	↑	–	–	de Llobet et al. (2012)
A549-P/A549-R (Human *non-SCLC*) SK-BR-3-P/SK-BR-3-R (*Human Breast adenocarcinoma*)	4 Gy to total dose 16 Gy 3 Gy to total dose 12 Gy	–	10–12 days	↑	–	↑	–	–	Mihatsch et al. (2011)
OE33/OE33 R (*Human Esophogeal adenocarcinoma*)	2 Gy to total dose of 50 Gy	–	Cells at 90% confluence	↑	Slight ↑ in cells in G2/M	↑	ROS levels similar. ↓ in GSH levels post IRR in parent line compared to un-IRR controls.	Similar	Lynam-Lennon et al. (2010)
H460/H460R (*Human SCLC*)	2 Gy to total dose of 80 Gy (*DR: 0.5 Gy/min*)	5 months	Cells at 60% confluence	↑	Significant ↓ G2/M and sub-G1 phase	–	Significant ↓ intracellular ROS post IRR	–	Lee YS et al. (2010)
Ec109/EC109/R (Human *Esophageal squamous cell carcinoma*)	10 Gy to total of 80 Gy (*DR: 3 Gy/min*)	8 months	20 days	↑	–	–	–	↓ 4.43%	Xie et al. (2009)
HepG2/HepG2-8960-R (*Human hepatocellular carcinoma*)	0.5 Gy every 12 h, once subline formed 2, 3, 5 Gy (*DR: 1.01 Gy/min*)	2 Gy for 5 days/ 2, 3, 5 Gy for 3 days	Cells in exponential growth	↑	↑ S-G2/M-phase	↑	–	–	Kuwahara et al. (2009)
MCF7/MCF7RR MDA-MB-231/MDA-MB-231RR T47D/T47DRR (*Human Breast epithelial*)	2 Gy to total of 40 Gy	10 months	–	↑	–	–	–	–	Smith et al. (2009)
LNCap/LNCap-IRR PC3/PC3-IRR Du-145/Du-145-IRR (*Human Prostate carcinoma*)	2 Gy to total of 10 Gy (*DR: 1.8 Gy/min*)	5 consecutive days	–	↑	–	–	–	Change in expression of proteins associated with apoptosis	Skvortsova et al. (2008)
D6/D6-R (*Human Non-SCLC*)	6.5 Gy fractions to total of 52 Gy (*DR: 2 Gy/min*)	~3–4 months	10–14 days between fractions	↑	S_{phase} ↑ 15.6% G1 ↓ 13.1% G2-M ↓ 2.5%	↑	–	No variation	Wei et al. (2008)

(*Continued*)

Table I. (*Continued*)

Isogenic pair	Fractionation schedule	Overall treatment time	Inter-fraction recovery time	Clonogenic survival and proliferation (RR vs. P)	Cell cycle distribution (RR vs. P)	DNA damage repair capacity (RR vs. P)	Oxidative stress	Apoptosis	Reference
MCF7/ MCF+ FIR30/MCF+ FIR3 / MCF-FIS4 (*Human Breast adenocarcinoma*)	2 Gy to total dose of 60 Gy (*DR: 5 Gy/1.15 min*)	6 weeks	–	↑	–	–	PrxII selectively ↑ regulated	–	Wang et al. (2005)
TE-2/TE-2R TE-5 TE-9/TE-9R TE-13/TE-13R KYSE170/KYSE-170R KYSE180 (*Human Esophogeal adenocarcinoma*)	2 Gy to total of 60 Gy	5–7 months	Cells at ~ 50% confluency or 5–7 days between fractionations	(D_0) ↑	Non relevant variation	–	–	Change in expression in genes associated with apoptosis	Fukunda et al. (2004)
H69/H69/R38 (*Human SCLC*)	0.75 Gy to total of 37.5 Gy	8 months	Cells in exponential growth	↑	–	–	BSO treatment restored radiation sensitivity	↓ anti-apoptotic protein bcl-2	Henness et al. (2002)
PANC-1/PANC-1/RAD-17/ PANC-1/RAD-24/PANC-1/ RAD-20 AsPC-1/AsPC-1/RAD-13 AsPC-1/RAD-15 (*Human Pancreatic carcinoma*)	5 Gy fractionations to total: 85 Gy, 120 Gy, 100 Gy, 65 Gy, 75 Gy, respectively	–	Cells in exponential growth	↑	–	–	–	Non relevant variation	Lee JU et al. (1999)
CEM/CEMRR (*Human T-Cell Leukaemia*)	1.5 Gy to total of 75 Gy (*DR: 1.4 Gy/min*)	10 cycles, Cycle repeated every 3 weeks	Cells in exponential growth	↑	–	–	–	–	Harveie et al. (1997)
IMR 32/XIMR 32/Clone F (*Human Neuroblastoma*)	2 Gy to total of 30–60 Gy (*DR: 1 Gy/min*)	–	Sub-confluent cells	↑	Non-relevant variation	No variation	–	↓	Russel et al. (1995)
AovC-0 (COV413.B)/AOvC-IR/0.5/ AOvC-IR/1.0/AOvC-IR/1.5 (*Human Ovarian*)	0.5 Gy 1 Gy 1.5 Gy every 48 h (*DR: 1.47 Gy/min*)	6 months	–	(RF) ↑	–	–	–	–	De Pooter et al. (1991)

RR, Radiation resistant; P, Parent; IRR, Irradiated; DR, Dose rate; SCLC, Small cell lung cancer; ROS, Reactive oxygen species; RF, Resistance factor = ratio of the ID50 of treated cells over the ID50 of control cells, when RF > 1 change in sensitivity scored as resistant; D, mean inactivation dose; $D37/D_0$, parameter to indicate the amount of irradiation required to reduce the survival fraction to approximately 0.37 from the survival curve.

with conflicting results. Some authors report no significant correlation between cell cycle distribution and the radiation resistant phenotype of the isogenic cell lines (Fukuda et al. 2004, Lynam-Lennon et al. 2010, de Llobet et al. 2012), while others report that the number of radioresistant cells in the G2/M and the sub-G1 phase were significantly less than that of the parent cell line (Lee et al. 2010) or that the resistant sublines showed higher percentages of cells in the more radioresistant S phase (Wei et al. 2008, Kuwahara et al. 2009). While Wei et al. (2008) observed a more obvious G2 arrest and longer G1 recovery time in the radioresistant D6-R human non-small cell lung cancer subline post irradiation, Kuwahara et al. (2009) identified that the fraction of cells in the G2/M phase in the resistant HepG2-8960-R human hepatocellular carcinoma cell line was steadily high while the number of cells in this phase increased to the same level in the parent cell line only 12 hours post irradiation with 2 Gy.

Cell cycle analysis could however have been made difficult due to the polyploidy commonly observed in cancers cells (Davoli and de Lange 2011) that is linked to p53 inactivation (Aylon and Oren 2011). A 'tetraploidy checkpoint' that prevents cell-cycle progression after failed cytokinesis has been proposed but fails to explain the proliferative activity of polyploid cells (Ganem and Pellman 2007). Polyploid cells undergo apoptosis at substantially higher rates than diploids, and prevention of apoptosis leads to an increased number of polyploid cells (Castedo et al. 2006). Therefore the selection of potentially apoptosis-resistant cells through repeated radiation exposure may lead to a sub line enriched in polyploidy cells. Measuring the fraction of 8N or 4N cells by flow-cytometry should be performed (Krueger and Wilson 2011). Polyploid cells are thought to be resistant to DNA damaging agent, such as radiation (Illidge et al. 2000). Therapy-induced cellular senescence, a possible cause for tumour relapse, was associated with aberrant expression of cyclin-dependent-kinase-1 (Cdk1), leading to reduced apoptosis and formation of polyploid senescent cells in patients with locally advanced non-small cell lung cancer treated with neoadjuvant chemotherapy or chemoradiotherapy (Wang et al. 2013a). Analysis of aurora kinase activity in these isogenic models, whose inhibition is associated with increased polyploidy (Nair et al. 2012) and radiosensitivity (Tao et al. 2009, Sak et al. 2012) could be performed.

Radioresistant sublines have increased ability to repair DNA damage

Irradiation of cells results in a number of different types of DNA lesions; damage to single bases, or breaks in the DNA either in the form of single-strand breaks or double-strand breaks (Joiner and van der Kogel 2008). Efficient DNA damage response and DNA repair mechanisms are therefore essential to cell survival. These processes were studied at varying time points up to 48 h post irradiation.

A number of authors investigated the ability of their newly created radioresistant sublines to repair DNA lesions when compared to the wild type line (Russel et al. 1995, Lynam-Lennon et al. 2010, Mihatsch et al. 2011, de Llobet et al. 2012). Using Pulse field gel electrophoresis to study the residual post repair radiation damage induced DNA fragments retained in the two lines, De Llobet et al. (2012) observed an attenuation in residual DNA fragments for the resistant sublines, demonstrating an increased ability in radiation induced damage repair mechanisms in these cells in comparison to the wild type cell lines. Neutral filter elution was also used to examine induction and repair of DNA double-strand breaks (DSB)in both the wild type and resistant lines over a range of doses (0–8 Gy) but no significant difference in the induction of the DNA lesions or repair after 30 min was seen (Russel et al. 1995). Similarly, the dose-response relationship for micronuclei formation was indistinguishable between the three lines under investigation.

In order to examine the efficiency of DSB repair between wild-type radiosensitive cell line and radioresistant subline, formation of phosphorylated H2A histone family member X (Y-H2AX) foci is explored (MacPhail et al. 2003) using Y-H2AX foci formation assay and Hoescht staining (Lynam-Lennon et al. 2010, Mihatsch et al. 2011). The histone 2AX is known to be phosphorylated to Y-H2AX in reaction to DNA lesions induced by radiation damage (Rogakou et al. 1998, Bonner et al. 2008), but upon repair of these DSB Y-H2AX foci are eradicated (Banath et al. 2010), therefore measuring the number of foci remaining is indicative of the cells ability to repair DNA lesions. While the basal level of Y-H2AX was comparable in the two cell lines and irradiation induced a build-up of Y-H2AX foci in both, the radiosensitive cell line had less capacity for repairing double-strand breaks suggesting that an increased capacity for DSB repair in the radiation resistant subline has given the cell a significant survival advantage over the parent line (Lynam-Lennon et al. 2010, Mihatsch et al. 2011).

Finally Kuwahara et al. (2009) investigated induction of micronuclei in parent HepG2 and resistant HepG2-8960-R human hepatocellular carcinoma cells after 2 Gy/day for 5 days and showed that while there was a larger number of visible micronuclei in the radiation resistant cell line at basal levels there was a marked increase in the parental line over the radiation resistant line (which remained at basal levels) after radiation exposure, suggesting induction of more DNA damage than in the new subline.

Radioresistant sublines can show reduced oxidative stress after radiation exposure

Accumulation of reactive oxygen species (ROS) was investigated by a number of groups as a possible characteristic of radiation resistance as they are known to be critical mediators of the damage response to ionizing radiation (Lee et al. 2010, Lynam-Lennon et al. 2010). While Lee et al. (2010) reported a significant decrease in the induction of ROS production in radiation resistant compared to the parent line, Lynam-Lennon et al. (2010) investigated both the basal and radiation-induced levels of ROS in both the parent and radiation resistant subline and reported that basal ROS levels were comparable in OE33-P (wild type) and OE33 R (radioresistant) lines and that while there was an increase in ROS after irradiation with 2 Gy the level of increase was similar for parent and subline. This data suggests that an increase in the ROS level is not a marker of radiation resistance.

The lack of conclusive evidence for increased ROS as a marker of radiation resistance raised the question of whether an increase in cellular glutathione levels (GSH) could be a possible cause for radiation resistance in the new subline due to its protective role as an antioxidant. The enzyme was studied through the comparison of basal levels in controls which were unirradiated vs. irradiated (2 Gy) parent and radiation resistant lines. This showed that while the control samples had similar levels of GSH, 20 min post irradiation the level of GSH in the irradiated parent cell line was much lower than that of the radiation-resistant subline suggesting the newly produced subline had retained a greater ability of preserving glutathione levels (Lynam-Lennon et al. 2010). In comparison Henness et al, (2002) observed that while the level of cellular glutathione was not significantly different between parent and sub cell lines in the SCLC line, the reduction of glutathione with L-buthionine-sulfoximine (BSO) treatment radiation sensitivity could be restored to the human small cell lung cancer H69/R38 radiation-resistant subline which would also suggest involvement of glutathione in the radioresistant phenotype.

Radioresistant sublines can show reduced apoptosis induction following radiation exposure

Some authors investigated changes in radiation-induced apoptosis as a possible marker of radiation resistance using standard annexin V Fluorescein isothiocyanate (FITC) staining and flow cytometry. The general consensus is that alterations in the induction of apoptosis is not a likely cause for the radiation resistant phenotype. This was shown by Lynam-Lennon et al. (2010) through the observation that there is no significant difference in apoptosis or even necrosis between the radiosensitive parent cell line or the radioresistant subline up to 48 h post irradiation. In comparison Xie et al. (2009) investigated apoptosis after treatment with 12 Gy of radiation and reported a basal level of approx. 1–2% apoptosis in the control samples with a significant difference seen in the radiation resistant cell line vs. parent cell line of $6.81 \pm 0.78\%$ compared to $11.24 \pm 1.21\%$ 24 h post irradiation. This reduced rate of apoptosis in the radioresistant subline was proposed as evidence of acquired radio resistance (Xie et al. 2009). Russel et al. (1995) also observed highly significant differences in the levels of apoptosis after 5 Gy between the cell lines outside the basal levels with the apoptotic fraction higher in the parent line when compared to the resistant lines. Finally the FITC-tunnel method was used to detect DNA fragmentation in cells to investigate apoptosis verified with Hoechst staining. The number of stained cells positively increased with dose but the authors did not seem to investigate this in radiation resistant lines as well as the parent PANC-1 and AsPC-1 pancreatic cell lines. However, Western blotting showed that the basal level of the B-cell lymphoma 2 associated protein X/B-cell lymphoma 2 (Bax/Bcl-2) ratio reflected the radiosensitivity of these cell lines (Lee et al. 1999).

Future directions

This review highlights a need for standardization of protocols for the generation and validation of isogenic radioresistant cell line models. Furthermore, these similarities observed in the response of the radioresistant sublines isolated to date, and that of cancer stem cells suggest that the characterization of the stemness properties of the generated isogenic radioresistant sublines should be explored. Clinically-relevant dose fractions (2 Gy) should be used, delivered in cells at least 50% confluent, up to a clinically relevant total dose. Clonogenic survival in the first instance should be monitored at regular intervals throughout protracted fractionated radiation dose. An increase in survival in the irradiated cells, compared to mock-irradiated control, should be interpreted as a sign of acquired radioresistance. Once a large and statistically significant increase in clonogenic survival is observed, acquired radioresistance should be validated through the measure of other endpoints, such as DNA repair capacity, apoptosis resistance and cellular motility.

Mechanisms for radioresistance identified in these models

The isogenic models developed to date have formed the basis for the identification of mechanisms of radioresistance. Lee et al. (2010) identified 1,463 genes differentially expressed genes between the H420 isogenic lung cancer cell lines using the 22K Human Genome Array. Ingenuity Pathways Analysis linked these 'radioresistance' genes to cell death, cancer, cellular growth and proliferation, cell cycle and cellular movement. Reverse transcription polymerase chain reaction (RT-PCR) confirmed upregulation of early growth response protein 1 (EGR1), A-kinase anchor protein 12 (AKAP12), F-box and WD repeat domain containing 8 (FBXW8), Ras-related protein Rab-3C (RAB3C) and downregulation of tumour protein 53 inducible protein 3 (TP53I3), CCAAT/enhancer-binding protein gamma (CEBPG), cathepsin E (CTSE), and myelin protein zero (MPZ). Focusing on cell death, the tumour protein p53-inducible protein 3 (TP53I3) gene was more specifically studied. TP53I3 was expressed significantly more in the radiosensitive parental human small cell lung cancer line H460 than the newly derived radiation resistant H460R line following 10 Gy irradiation. In the parent line, TP5313 induction correlated with Poly-(ADP-ribose) polymerase (PARP) cleavage and induction of apoptosis following irradiation. This was further established using a recombinant adenovirus expressing green fluorescent protein (GFP)-fused TP53I3. While overexpression of TP53I3 led to decreased cell survival in both H460 and H460R cells, clonogenic survival after X-ray irradiation in TP53I3-overexpressed cells was significantly reduced in comparison to GFP-overexpressed cells in both cell lines (Lee et al. 2010). The results indicate that TP53I3 may confer sensitivity to ionizing radiation. Similarly, 19 upregulated and 28 downregulated genes common to radioresistant sublines were identified in oesophageal isogenic cancer cell lines. Upregulated genes were associated with apoptosis and inflammatory response (baculoviral IAP repeat containing 2 [BIRC2] and cytochrome c oxidase subunit II [COX-2]), DNA metabolism (5′-nucleotidase, ecto CD73), and cell growth (plasminogen activator, urokinase [PLAU]). Downregulated genes were associated with apoptosis (caspase 6, apoptosis-related cysteine peptidase

[CASP6]), cell adhesion (cadherin 1 [CDH1] and cadherin 3 [CDH3]), transcription (mixed-lineage leukemia 3 [MLL3]), and cell cycle (cyclin-dependent kinase 6 [CDK6]). Baculoviral IAP repeat containing 2 (BIRC2) mRNA levels were significantly higher in pre-treatment biopsy specimens of patients with poor response to radiation therapy, than in good responders, showing that analysis of isogenic models can lead to the identification of clinically relevant markers of radioresistance (Fukuda et al. 2004).

Using two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) analysis of the protein profiles of prostate cancer isogenic cell lines, 27 proteins were identified as altered in radioresistant cells, when compared to the parental lines (Skvortsova et al. 2008). These proteins appeared to share endothelial growth factor receptor (EGFR) and androgen receptor as common targets. Altered expression of nucleoside diphosphate kinase 1 (NME1), heat shock 70 kDa protein 8 (HSPA8), apurinic/apyrimidinic endonuclease 1 (APEX1), SERPINE1 mRNA binding protein 1 (SERBP1) and Ras-related protein Rab-11A (RAB11A) was validated by western blotting. APEX1 is involved in repair of DNA damage through modulation of multiple cellular transcription factors, including nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) (Raffoul et al. 2007). Treatment with small interfering RNA (siRNA) against APEX1 and subsequent irradiation resulted in a significant enhancement of radiation sensitivity in all three radioresistant sublines. Similarly, a range of complementary proteomic methods to analyze radioresistance in breast cancer cells (two-dimensional gel electrophoresis mass spectroscopy [2DE-MS], Isobaric tags for relative and absolute quantitation [iTRAQ], and matrix-assisted laser desorption/ionization [MALDI]) yielded a large number of altered proteins, but very few targets overlapped across approaches. A decrease in the expression of the glucose regulated protein (GRP78), chromosomes 3 (SMC3), triosephosphate isomerase 1 (TPI1) and protein 26S proteasome were validated by Western blotting in all radioresistant derivatives in comparison with their parents (Smith et al. 2009). Functional analysis of these targets in their ability to radiosensitize radioresistant cells however has not yet been reported. Finally, downregulation of peroxiredoxin II by siNRA following the identification of this protein as elevated in radioresistant MCF+ FIR3 compared to radiosensitive MCF+ FIS4 breast cancer cells following 2-D DIGE analysis, led to reduced clonogenic survival following irradiation, through its ability to eliminate the generation of reactive oxygen species (Fujii and Ikeda 2002). Downregulation of peroxiredoxin II also increased the radiosensitivity of glioma (Smith-Pearson et al. 2008) and head and neck cancer cell lines (Park et al. 2000), while peroxiredoxin IV protects head and neck carcinoma cells from radiation-induced apoptosis (Park et al. 2009).

Lee et al. (2013) used a combination of cDNA array and analysis and 2-D DIGE to identify markers of radioresistance in oral (QLL1) and tongue (SCC15 and SCC25) radioresistant isogenic cell lines. Overexpression of both mRNA and protein levels was only observed for two candidates: Non-metastatic cells 1 protein (NM23-H1) and proliferation associated 2G4 protein (PA2G4). NM23-H1 upregulation only was however confirmed by Western blotting in all three lines. The authors concluded that NM23-H1, which has a debatable role in head and neck cancer despite being associated with poor prognosis (Pavelic et al. 2000, Wang et al. 2004), may represent a novel predictor of radiation resistance. Nuclear localization of NM23-H1 was strongly associated with overall and recurrence-free survival in head and neck squamous cell carcinoma patients treated with radiation therapy (Kim et al. 2011).

Lynam-Lennon et al. (2010) used the Human MicroRNA Panel TaqMan Low Density array to profile global miRNA expression in their radioresistant isogenic model of oesophageal cancer. Dyregulation of 35 miRNAs was identified between the radioresistant and parent line, while irradiation with 2 Gy significantly altered the expression of 11 miRNAs. This study identified miR-31 as the only miRNA significantly differentially expressed between the two lines and in response to radiation. Overexpression led to altered expression of 13 DNA repair genes [PARP1, single-strand-selective monofunctional uracil-DNA glycosylase 1 (SMUG1), mutL homolog 1 (MLH1), RAD51L3, nucleotide excision repair homolog MMS19, ligase 3 (LIG3), excision repair cross-complementing rodent repair deficiency, complementation group 1 (ERCC1), nei endonuclease VIII-like 1 (NEIL1), cyclin-dependent kinase 7 (CDK7), damage-specific DNA binding protein 1 (DDB1), topoisomerase (DNA) III alpha (TOP3A), xeroderma pigmentosum, complementation group C (XPC) and replication protein A3 (RPA3)]. Transient transfection of synthetic pre-miR-31 precursor molecules into the miR-31 overexpressing radioresistant subline sensitized these cells to radiation. MiR-31 expression was also significantly reduced in patients demonstrating poor histomorphologic response to neoadjuvant chemoradiation, when compared to good responders (Lynam-Lennon et al. 2010).

De Llobet et al. used their A431 epidermoid carcinoma isogenic model to further study the epidermal growth factor (EGF) pathway following the observation that these cells lines have cellular traits linked to sustained proliferative cell signaling, and because, the A431 parental cells overexpress the endothelial growth factor receptor (EGFR). EGFR overexpression is associated with resistance to chemotherapy and radiotherapy (Bai et al. 2012) and many preclinical studies have targeted this pathway to increase radiosensitivity (Xu et al. 2011, Illum 2012, Kabolizadeh et al. 2012). A431-R cells presented higher baseline levels of phosphorylated EGF receptor, as well as the Protein Kinase B and extracellular signal-regulated kinases (ERK1/2) transducers linchpin proteins, which are involved in cell growth, mitogenesis, survival, DNA repair ability, and cell migration (Liang et al. 2003). In the parental line, ERK1/2 levels were increased following radiation exposure irrespectively of EGF presence, whereas this response was not observed in the radioresistant line. The level of vascular endothelial growth factor (VEGF) released following irradiation has been directly associated with tumour resistance to irradiation in a number of cell models (Brieger et al. 2007). Similarly, release of EGFR- and radiation- responsive VEGF in response to radiation (8 Gy) was more efficient in the A431-R cells (de Llobet et al. 2012). Similarly, Skvortsova

et al. (2008) identified increased phosphorylation of EGFR following EGF treatment in the radioresistant prostate cancer cell lines, when compared to parents. Future experiments testing the ability of EGRF inhibitors in reversing radioresistance in isogenic models are required.

MTT assays have been used to study cross resistance to DNA-damaging agents in the newly derived radiation resistant lines (De Pooter et al. 1991, Harveie et al. 1997, Henness et al. 2002, Wei et al. 2008, Xie et al. 2009, Lee et al. 2010). Evidence of cross-resistance in the isogenic cell lines to DNA damaging agents is conflicting. Xie et al. (2009) showed that while cisplatin resistance was increased in the radiation resistant line by 1.7-fold, the cells were all more sensitive to the other DNA damaging agents: 0.324-fold for 5-fluorouracil, 0.44-fold for Doxorubicin, 0.64-fold for Paclitaxel and 0.81-fold for Etoposide, in comparison to the parent line. Wei et al. (2008) also found that the isogenic D6R line was more resistant to Adriamycin and 5-fluorouracil with IC 50 of 0.63 μg/ml compared to 0.20 μg/ml in the parent line and 64.38 μg/ml compared to 16.67 μg/ml, respectively. Henness et al. (2002) reported a 1.7- to 14-fold increase in resistance to chemotherapeutic drugs in the small cell lung cancer cell line H69/R38: between 1.5 and 2.1 times more resistant to Daunorubicin, Idarubicin, Epirubicin, Doxorubicin and Etoposide with no difference in resistance to Cisplatinum, Paclitaxel or Chlorambucil. The evaluation for possible mechanisms of chemo-radiation cross resistance could be identified in these models.

These isogenic cell lines could provide a template for the testing of DNA damage repair inhibitors and further investigation of the DNA damage repair pathways associated with radioresistance. Compounds such as poly(ADP-ribose) polymerase (PARP)-1 inhibitors (Chalmers et al. 2010) should be tested for their ability to reverse radioresistance in the sublines. To evaluate the regulation of DNA-DSB repair through DNA-PKcs-dependent non-homologous end joining (NHEJ) in their isogenic model, Mihatsch et al. (2011) inhibited PI3K/Akt signaling (10 μM LY294002) prior to irradiation. Clonogenic survival of the radioresistant subline in the presence of the inhibitor was equivalent to that of the untreated parent line. Similarly, in the isogenic prostate cancer models, enhanced phosphorylation of the regulatory subunit (p85) of phosphatidylinositol 3-kinase (PI3K) was observed in all three IRR cell lines (Skvortsova et al. 2008). PI3K inhibition is increasingly reported as a means to increase the radiosensitivity of cancer cells (Dumont and Bischoff 2012, Kuger et al. 2013, Potiron et al. 2013), and isogenic models may provide a template for detailed mechanistic studies.

Radioresistant sublines or enriched stem cell-like population?

The survival of cancer stem cell population following radiation therapy has been proposed as the achilles' heel of tumour control (Krause et al. 2011, Nguyen et al. 2011): the number of cancer stem cells per tumour has been associated with reduced tumour control following radiation exposure in animal models (Baumann et al. 2009) and the radioresistance of cancer stem cells compared to non-tumorogenic cells has been suggested (Vlashi et al. 2009, Lagadec et al. 2010, Pajonk et al. 2010, Printz 2012). Following each dose fraction, it has been proposed that non-stem cancer cells die and cancer stem cells potentially increase in number, participating to accelerated repopulation (Gao et al. 2013). In irradiated prostate cancer lines, long-term recovery resulted in increased cancer stem cells properties of the recovered cell population (Cho et al. 2012). The progressive selection of surviving sublines during fractionated irradiation may thus be reflective of the survival of the stem cell population. De Llobet et al. (2012) investigated the expression of the cell surface antigen CD44, a putative stem cell marker functionally validated as a predictive biomarker of local control for early laryngeal cancer treated with radiotherapy but did not find differential expression of CD44 antigen in their A431 isogenic cell lines. Similarly, protein expression of the stem cell markers octamer-binding transcription factor 4 (Oct4), SRY-box containing gene 2 (Sox2), and aldehyde dehydrogenase 1 (ALDH1) was analyzed in two isogenic models (A549-R/A549-p; SK-BR-3-R/SK-BR-3-p). While Oct4 expression appeared approximately 2-fold increased in SK-BR-3-R as compared to SK-BR-3p cells, expression was similar in A549-R/A549-p cells, and differences in expression were not observed for Sox2 and ALDH1 in either models. Yet the radiosensitivity of ALDH1 + cells isolated from the radioresistant subline was significantly reduced when compared to that of ALDH1 + cells isolated from the parent line (Mihatsch et al. 2011). These cells were also more sensitive to ALDH1 inhibition. These results suggest that the relevance of ALDH1 for radioresistance needs to be further investigated. In oesophageal isogenic models, the expression of the stem cells markers β-catenin, Oct3/4, and β_1 integrin gene and protein levels were elevated in the radioresistant, when compared to the parent line (Zhang et al. 2008).

Like in the isogenic models, increased clonogenic survival and proliferative activity, efficient DNA repair systems and modified cell cycle regulation have been attributed to the radioresistance of cancer stem cells. Increase proliferative activity has been proposed to result from a shift from asymmetric to symmetric division of cancer stem cells following irradiation (Gao et al. 2013, Insinga et al. 2013). The isolation of cells expressing surface markers indicative of stemness has been associated with increased clonogenic survival and more efficient repair of DNA damage following irradiation (Bao et al. 2006). In thyroid cancer, the survival of CD133 + cells following radionuclide therapy was proposed as a major cause for treatment failure (Ke et al. 2013). PKH26 + stem-cell like nasopharyngeal carcinoma cells displayed increased clonogenicity, sphere formation and resistance to radiotherapy through overexpression of c-MYC resulting increased expression of the CHK1 and CHK2 cell cycle checkpoint proteins and activation of the DNA damage response (Wang et al. 2013b). Similarly CHK1 knockdowns increased radiosensitivity of prostate (Wang et al. 2012) and glioma (Wu et al. 2012) cancer stem cells. In glioma-initiating cells, failure of irradiated cells to arrest at the S-cell cycle phase entry checkpoint was associated with increased homologous recombination and increased radioresistance (Lim et al. 2012). Finally, cancer stem cells may achieve

enhanced ROS defence through high levels of free radical scavengers (Phillips et al. 2006, Diehn et al. 2009). Radiation exposure induced mitochondria damage in lung cancer cells, but not in its stem cell population (Xia et al. 2013). Treatment of human head and neck carcinoma SQ20B stem cells with dimethylfumarate, a glutathione-depleting agent, induced cell death following irradiation, supporting the hypothesis that glutathione levels are associated with radioresistance (Boivin et al. 2011).

Conclusion

Isogenic radioresistant cell lines represent attractive models for the determination of the molecular radiation response. The current laboratory procedures for analyzing these biological events may however not be readily transferable to a clinical setting. The need for the discovery of novel prognostic and predictive biomarkers of radioresponse is widely recognized. A number of possible candidates for markers have been investigated including changes in DNA damage response and cell cycle checkpoints, the development of micronuclei, apoptotic events and clonal cell survival. The potential of these models may however be underutilized and data to date suggests selection of a stem-cell like cell population. Future studies using isogenic radioresistant models should thus explore their stemness properties. These studies may lead to the identification of novel drug targets and the development of therapeutic agents that will increase tumour cell sensitivity to radiation therapy.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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