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# Exposure to reduced oxygenation following radiation exposure enhances the survival of prostate cancer cells in vitro: implications for radiation therapy delivery

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**A thesis submitted for the degree of Doctor in Medicine**

**(M.D.)**

**2009-2010**



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# Summary

**Introduction:** Hypoxia is a common feature of prostate tumours; hypoxic cells are more radioresistance compared to oxygenated cells. Hypoxic radioresistance has also been shown to be related to the duration of hypoxia prior to irradiation; as such treatments methods of targeting hypoxia before irradiation as a way of treating cancer were investigated. To date the effect of post-irradiation hypoxia on radioresistance and the cellular response to irradiation has been poorly studied. We propose that prostate cancer cells that are hypoxic after irradiation have a survival advantage over those that are fully oxygenated. We propose that this survival advantage is mediated by post-irradiation hypoxia induced changes to the cellular response, particularly cell cycle, DNA repair, cell death and senescence. Finally we propose to investigate the role of the transcription factor HIF-1α in post irradiation hypoxic radioresistance.

**Methods:** 22Rv1, DU145 and PC3 prostate cancer cell lines were used. Cells were exposed to 0.5% $O_2$ or a HIF-1α inducer $CoCl_2$ and irradiated (0-10 Gy). Cells were placed back into either an aerobic environment or hypoxia/$CoCl_2$ for 24 hrs. Post-irradiation hypoxic radioresistance and impact of DNA repair was determined by clonogenic assay. The effect of post-irradiation hypoxia on cell cycle and apoptosis was determined by flow cytometry. Impact of hypoxia on cellular senescence was determined by histochemical stain. HIF-1α, CHK1, CHK2, MRE11 and p53 expression was demonstrated by western blot.

**Results:** Post-irradiation hypoxic recovery increased the radioresistance of 22RV1 ($p<0.0001$), DU145 ($p=0.0003$) and PC3 ($p<0.0001$) prostate cancer cells compared to oxygenated cells. This survival advantage was correlated with G2/M cell cycle arrest, absent DNA repair, reduced apoptosis and a reduction cellular senescence. $CoCl_2$ treatment increased radioresistance similarly to cells exposed to hypoxia ($p=0.0121$). The effects of hypoxia on the cellular response to irradiation and decreased expression of CHK2, MRE1, p53 and $p16^{ink4a}$ proteins correlated with HIF-1α expression.

**Conclusion:** We sought to evaluate the effect of post-irradiation hypoxia on the radioresistance of prostate cancer cells and to determine if this effect is due to HIF-1α mediated altered cellular response to irradiation. We concluded that post-irradiation hypoxia confers a survival advantage compared to cells that are oxygenated after irradiation. This survival advantage may be mediated by HIF-1α inducing a cell cycle arrest in G2/M, reducing apoptosis after irradiation and the percentage of cells in the senescent state. The clinical implications are that this data suggests that patients may benefit from the continuation of anti-hypoxia treatments post irradiation. Furthermore it supports the introduction of anti-HIF-1α compounds in the management of prostate cancer patients receiving radiotherapy.

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# Presentations and publications

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Radioresponse of prostate cancer cells to oxygen levels above anoxia.

Hennessey D[13]., Coffey M[2]., Lynch TH[3]., Hollywood D[12]., Marignol L[12].

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# List of abbreviations

| | |
|---|---|
| Irish National Cancer Registry | (INCR) |
| Prostate specific antigen | (PSA) |
| Radical prostatectomy | (RP) |
| External beam radiation therapy | (EBRT) |
| Oxygen Enhancement Ratio | (OER) |
| Single strand breaks | (SSBs) |
| Double strand breaks | (DSBs) |
| 3D conformal radiotherapy | (3DCRT) |
| Intensity modulated radiotherapy | (IMRT) |
| Antero-posterior | (AP) |
| Postero-anterior | (PA) |
| Grays | (Gy) |
| Permanent implant | (PI) |
| Radical prostatectomy | (RP) |
| Radiotherapy | (RT) |
| Blood vessel | (BV) |
| Oxygen | ($O_2$) |
| Deoxyribonucleic acid | (DNA) |
| Reactive oxygen species | (ROS) |

| | |
|---|---|
| Ataxia-telangectasia-mutated | (ATM) |
| ATM and Rad3-related | (ATR) |
| Homologous recombination | (HR) |
| Hypoxia inducible factor-1 | (HIF-1) |
| Aryl hydrocarbon nuclear translocation | (ARNT) |
| von Hippel-Lindau protein | (pVHL) |
| Hypoxic response elements | (HRE) |
| Vascular endothelial growth factor | (VEGF) |
| Helix-loop-helix | (HLH) |
| Trans-activation domains | (TAD) |
| Oxygen-dependent degradation domain | (ODD) |
| Apoptotic protease activating factor 1 | (Apaf-1) |
| Accelerated radiotherapy combined with carbogen and nicotinamide | (ARCON) |
| Linear energy transfer | (LET) |
| Foetal calf serum | (FCS) |
| Degrees Celsius | ($^{o}$C) |
| Phosphate buffered saline | (PBS) |
| Revolutions per minute | (RPM) |
| Polymerase chain reaction | (PCR) |
| Cobalt chloride | ($CoCl_{2}$) |
| Radio-immunoprecipitation assay | (RIPA) |

| | |
|---|---|
| Tris-buffered saline | (TBS) |
| Sodium dodecyl sulfate | (SDS) |
| Bovine Serum Albumi | (BSA) |
| Horseradish peroxidase | (HRP) |
| Polyvinylidene diflouride | (PVDF) |
| Propidium iodide | (PI) |
| Fluorescence-activated cell sorting | (FACS) |
| 2',7'-dichlorodihydrofluorescein | (DCFH) |
| 2',7'-dichlorofluorescein | (DCF) |
| Glutathione | (GSH) |
| β-galactosidase | (SA-βGal) |
| Analysis of variance | (ANOVA) |

# Chapter 1

# Introduction

# 1 Introduction

## 1.1 Clinical Management of Prostate Cancer

Prostate cancer is the most common cause of non-cutaneous cancer in European men and is the third most common cause of male cancer deaths (Ferlay et al., 2007). In Ireland, the National Cancer Registry (NCRI) reported 2720 cases of prostate cancer and 543 deaths due to prostate cancer in 2007 (NCRI, 2010). Prostate cancer also represents the highest annual increase of incidence over a range of cancers between 1994 and 2004, (Figure 1.1/1.2). Similarly worldwide there has been rise in the incidence of prostate cancer over the past 20 years (Hsing et al., 2000). It is predicted that there will be a 275% increase in the incidence of prostate cancer by 2020 in Ireland.

The global increases in incidence of prostate cancer is due to improved patient awareness, increased prostate specific antigen (PSA) screening and the introduction of fast track prostate cancer clinics. The amount of men being diagnosed with advanced disease is decreasing while the incidence of younger men being diagnosed with localised disease is increasing (Moul et al., 2002) (Marignol et al., 2008). In most cases, the prostate cancer is relatively slow-growing, typically taking years for the disease to be detectable, and even longer for it to spread beyond the prostate. The majority of these men will remain symptom free and will often die of a cause unrelated to their prostate cancer. However, a small percentage of patients experience more rapidly growing, aggressive forms of prostate cancer that is ultimately fatal. Unfortunately, it is difficult to determine which prostate cancers will grow slowly and

which will grow more aggressively, thus complicating treatment decisions. This makes the management of prostate cancer difficult, complex and controversial (D'Amico et al., 2006).

Disease stage, grade and patient preference are the predominant factors in determining patient treatment. Consequently, determining the risk of disease progression is becoming crucial in treatment decision making. Several classification systems have been created to predict outcome. The D'Amico classification stratifies patients based on stage, grade, and PSA. Its major downside is that the 3-level system does not account for multiple adverse parameters in stratifying patients (D'Amico et al., 2006). Other systems exist such as the Partin tables or Kattan nomograms to predict disease recurrence (Partin et al., 1997) (Graefen et al., 2002). Data obtained from these stratifying systems classifies patients into to low; intermediate or high risk prostate cancers (D'Amico et al., 2006). Low risk patients can be suitable managed by active surveillance or treatment by a single modality. Intermediate or high risk prostate cancers on the other hand may require multimodal treatments. However when determining the treatment of choice for a patients with prostate cancer, several other factors must be taken into consideration such age, general state of health, life expectancy, insight, as well as individual patient preference. Furthermore when developing a treatment plan, it is important that the doctor and patient exchange information and discus the advantages and disadvantages of any possible treatment, before proceeding.

Established treatments for prostate cancer include active surveillance, radical prostatectomy (RP), external beam radiation therapy (EBRT) and brachytherapy (Grosu et al., 2005, Cesaretti et al., 2007). Other modalities such as cryotherapy are not recommended as there is no long term outcome data available. Hormonal therapy and chemotherapy are used as adjuncts to the above proven therapeutic options or can be used solely in advanced or metastatic prostate cancer.

## 1.2 Radiotherapy for Prostate Cancer

Radiation therapy can be used to treat all stages of prostate cancer and is commonly used in the treatment of patients who decide against or are not suitable for surgical intervention. About one-quarter of men with prostate cancer choose conventional external beam radiation therapy (EBRT) as a primary treatment option (Potosky et al., 2004).

Radiotherapy for prostate tumours can be performed in different ways: conventional, 3D conformal radiotherapy (3DCRT) and intensity modulated (IMRT). Conventional radiotherapy is the oldest technique and the only method which has follow-up results over 10 years after treatment. Conventional external beam radiation therapy EBRT is typically delivered using a 4-field technique. The 4 fields (antero-posterior [AP], postero-anterior [PA], left lateral, right lateral) are designed to include the prostate, seminal vesicles, and regional lymphatics. This is delivered to patients in a schedule of 5 fractions per week of 2 grays (Gy), up to 33 to 39 and a total of 66 to 78 Gy in 7 to 8 weeks (Fowler, 2005). In 3DCRT, the profile of each radiation beam is shaped

to fit the profile of the prostate gland using a multileaf collimator and a variable number of beams. The conformation of the beam to the shape of the gland reduces the relative toxicity of radiation to the surrounding normal tissues. 3DCRT has essentially replaced conventional (EBRT) in the management of early-stage prostate cancer. However no data is available for patients treated for over 5 years. Intensity Modulated Radiation Therapy (IMRT) is the most recent technique which should result in, through the use of special software and instruments, a better sparing of healthy tissues surrounding the prostate and in prostate irradiation with very high doses. Preliminary results are very interesting, even though the treatment is much more complex to perform and therefore entails potential margin of error higher than for the above techniques. This method has only been recently introduced so follow-up is even shorter than for conformal 3D therapy.

## 1.3 Radiation Induced Damage and the Cellular Response

Radiotherapy utilises the detrimental effects ionizing radiation to damage the DNA of cells. This occurs by direct interaction with a critical target in the cell or indirectly by the formation of reactive oxygen species and free radicals (Figure 1.1). When the effect of ionizing radiation is enhanced due to the presence of oxygen, this is known as the Oxygen Enhancement Ratio (OER).Ionizing radiation induces a variety of deoxyribonucleic acid (DNA) lesions that include DNA single strand breaks (SSBs); DNA double strand breaks (DSBs) and DNA base alterations. DNA DSBs are the most deleterious of DNA lesions produced by irradiation.

In response to DNA damage, cells have evolved groups of proteins that function in signalling networks that sense this damage. Firstly there is an cell cycle arrest, which allows time for DNA damage sensing proteins to activate DNA repair pathways (Su, 2006). The cell will make an attempt to repair the damage, failure to repair DNA damage, or mis-repair, can again result in cell death and apoptosis or large-scale chromosome changes that enhance genome instability and increase the malignant potential of the cancer cells.

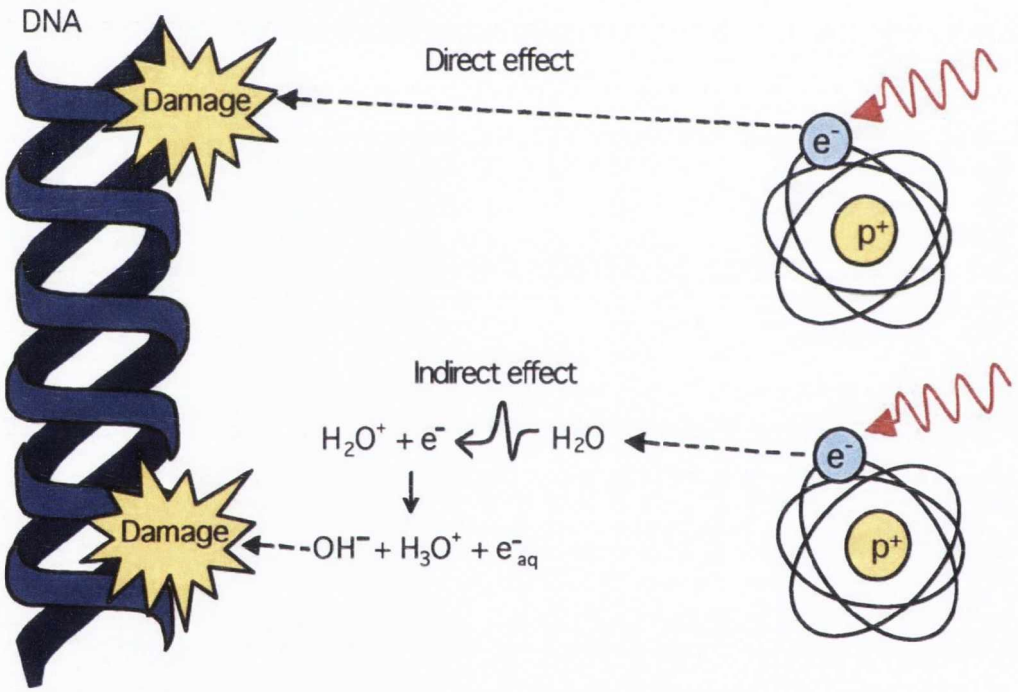



**Figure 1.1: Interaction of ionizing radiation with DNA**

Ionizing radiation damages DNA by two methods; though direct effects on DNA or indirectly though the generation of free radicals which interact with DNA to produce damage. Adapted from: (Morgan and Sowa, 2005).

## 1.4 DNA Damage Induced Cell Cycle Arrest

The cell cycle is the series of events that takes place in a cell leading to its division and duplication. In eukaryotic cells the cell cycle consists for phases, gap $(G)_1$, synthesis (S), $G_2$ and mitosis (M) and one phase outside the cell cycle, $G_0$. In the $G_1$ phase the cell increases in size and starts synthesizing RNA (transcription) and proteins (translation). In the subsequent S phase, DNA is replicated to produce an exact copy of the genome for the daughter cells. During $G_2$, the cell will grow and make extra proteins to ensure that the two viable daughter cells can be formed. The induction of DNA damage in dividing cells results in the activation of cell cycle checkpoints (Figure 1.2). These cell cycle checkpoints halt the proliferation of the cell in the cell cycle in order to allow DNA damage repair mechanisms fix the DNA damage, avoiding incorrect genetic information from being passed on to progeny. If the repair process fails, the cell cycle can be blocked permanently, leading to a senescent state of the cell or alternatively cell death by apoptosis. These mechanisms prevent potential harmful cells from dividing ensuring that no mutations are inherited by the next generation of cells.

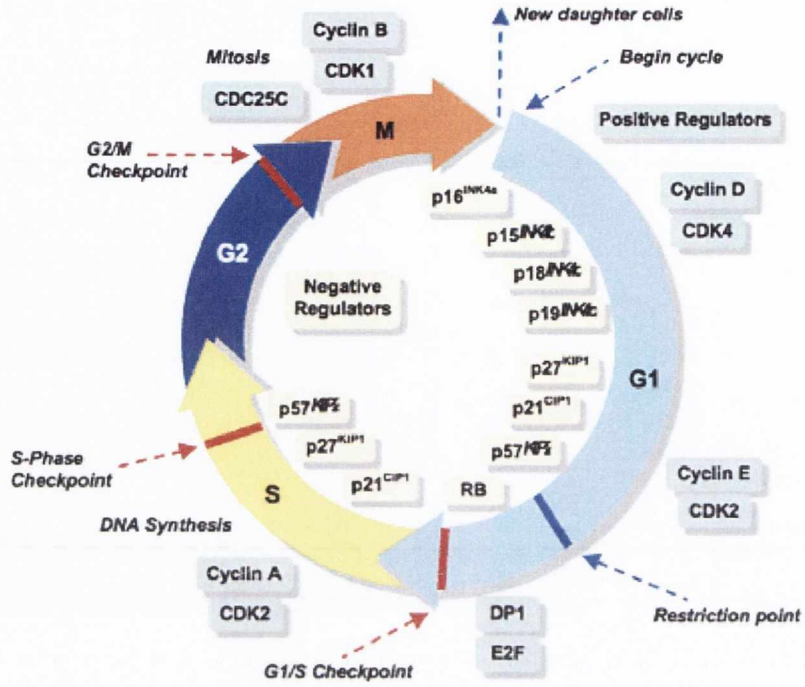



**Figure 1.2: The cell cycle; phases, regulators and checkpoints**

Human cell cycle phases and regulatory proteins involved in cellular replication. In response to irradiation, cell cycle regulatory proteins CHK 1 and CHK2 regulatory proteins initiate a cell cycle arrest at the G2/M checkpoint to allow DNA repair to occur. Adapted from: (Kong et al., 2003).

## 1.5 Repair of Radiation Induced DNA Damage

Under normoxic conditions, DNA DSBs are sensed by the MRE11-RAD50-NBS1 (MRN) complex. The MRN complex senses DNA damage it can activate and recruit ataxia telangiectasia mutated (ATM), DNA-dependent protein kinases and catalytic subunit kinases to the area. Subsequently, a number of other DNA damage sensing proteins and mediators of DNA damage are recruited to repair the DNA DSB. Such as checkpoint proteins and DNA DSB repair proteins involved in homologous recombination (HR) and non-homologous end joining (NHEJ) (Figure 1.2).

Human DNA DSBs are usually repaired through two main pathways: homologous recombination (HR) and non-homologous end joining (NHEJ) (Weterings and van Gent, 2004) (Helleday et al., 2007). The NHEJ repair pathway can be used within any phase of the cell cycle and can be error-prone. The HR pathway is preferentially active in the S and $G_2$ phases of the cell cycle when a homologous sister chromosome or chromatid is available for direct base-pairing to effect error-free repair of a DNA DSB. In studies of normoxic cells, the inhibition of the HR and NHEJ repair pathways has been linked to increased genetic instability and carcinogenesis (Collis et al., 2005). Therefore, maintaining error-free HR under normoxic and hypoxic conditions could be important to preserve genomic stability to prevent a mutated phenotype. (Chan et al., 2008).

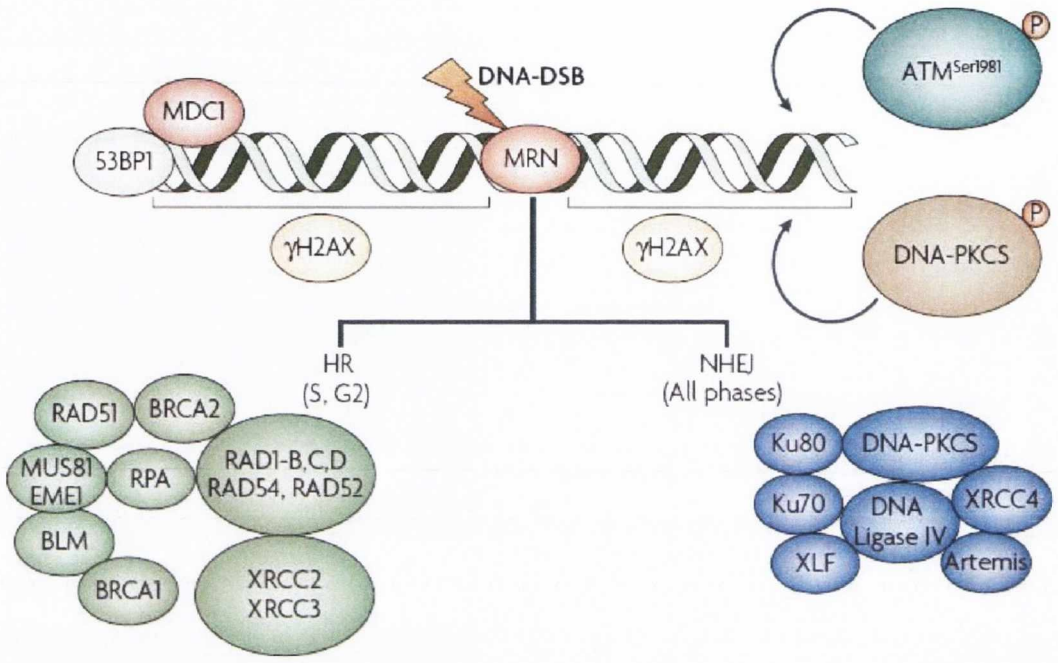



**Figure 1.3: DNA double-strand breaks sensing and repair pathways**

Cellular response to DNA DSB induced by irradiation. DNA DSB is sensed by the MRE11–RAD50–NBS1 (MRN) complex. This leads to activation and recruitment of the ATM and DNA-dependent protein kinases. Subsequently, a number of DNA damage sensing proteins are recruited to the area and repair can begin. Adapted from: (Bristow and Hill, 2008).

## 1.6 Radiotherapy Delivery and Schedule for Prostate Cancer

The most recent studies show that the dose factor in radiotherapy plays an essential role in deciding the probability of patients being cured. The differences between the various radiotherapy techniques are in the dose they can release at prostate level and their ability to spare the surrounding structures. The total dose is fractionated (spread out over time) for several important reasons. Fractionation allows normal cells time to recover, while tumour cells are generally less efficient in repair between fractions. Fractionation also allows tumour cells that were in a relatively radio-resistant phase of the cell cycle during one treatment to cycle into a sensitive phase of the cycle before the next fraction is given.

## 1.7 Clinical Outcome of Radiotherapy for Prostate Cancer

Pollack *et* al reported that up 40% of high risk prostate cancer patients may fail radical radiotherapy for prostate cancer (Pollack et al., 2003). Recent case series demonstrated that disease free survival following radiotherapy for grade I disease was 90%, grade II 76% and grade III 53% (D'Amico et al., 2000). Retrospective analyses have demonstrated that overall survival and disease-free survival outcomes are similar between RP and radiotherapy, with eight-year biochemical relapse-free survival rates for RP versus radiotherapy of 72% and 70%, respectively (Kupelian et al., 2002) (D'Amico et al., 2003). For patients with high grade disease radiotherapy has superior disease free survival compared to RP (Figure 1.4)

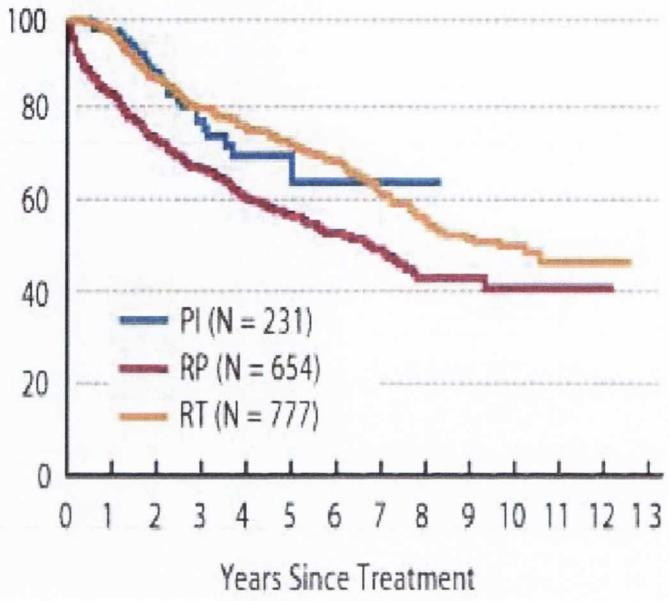



**Figure 1.4: Disease free survival for high grade prostate cancer**

Percentage biochemical relapse free survival for patients with high risk prostate cancer by treatment type. PI=permanent implant, RP= radical prostatectomy, RT=radiotherapy. N=1,662. Adapted from: (Greene FL, 2002).

## 1.8 Tumour Hypoxia and Cancer Biology

Hypoxic areas exist in solid tumours due to structural and functional abnormality of the tumour vasculature. Unlike normal blood vessels, tumour blood vessels are chaotic, irregular and leaky leading to uneven delivery of blood and oxygen to regions in the tumour (Figure 1.5). Hypoxia in tumours can be acute or chronic, acute hypoxia followed by re-oxygenation in tumours arises because of functional changes in vascular stability and dynamic fluctuations in the blood flow (Figure 1.6). Chronic anoxia is due to increasingly long oxygen ($O_2$) carrying erythrocyte transit times and an irregular distribution of tumour vessels that leads to limited $O_2$ diffusion within the tumour. $O_2$ tension in normal tissues is approximately 7% whereas in solid tumours $O_2$ tension is approximately 1.5% (Vaupel et al., 2001) (Vaupel et al., 2004). As well as hypoxia, the solid tumour microenvironment is characterized by acidic pH and nutrient deprivation. Collectively these conditions make the tumour microenvironment a unique setting for tumour progression and provide an environment where adaptive changes are more likely to occur. The presence of a hypoxic tumour environment correlates with increased invasiveness metastasis and resistance to radiation and chemotherapy (Vaupel et al., 2004) (Marignol et al., 2008). Hypoxia is also an independent prognostic indicator of poor clinical outcome for patients with cancer (O'Lorcain and Comber, 2007) (Cairns et al., 2003)

The presence of hypoxic regions in prostate cancer is widely documented in the literature, both through the use of physical measurements ($pO_2$) probes (Movsas et al., 1999), imaging modalities (positron emission tomography, magnetic resonance imaging) (Krohn et al., 2008) and the identification of specific biomarkers (Le and

Courter, 2008). However one important caveat to the relevance of hypoxia in the pathogenesis in prostate cancer is that, using a polarographic electrode, no significant difference was found between the oxygen tension of normal prostatic tissue and foci of prostate cancer. This is in contrast to the oxygenation status of other malignancies (Parker et al., 2004). As such it appears that the prostate is hypoxic, whether benign or cancerous. The existence if a hypoxic microenvironment within a tumour might be the crucial factor in evoking a hypoxia response and subsequent development of a phenotype that leads to chemo and radioresistance. Adding credence to this hypothesis are studies which demonstrate hypoxia associated protein expression in prostate cancer tissue but not in benign normal tissue (Vergis et al., 2008)

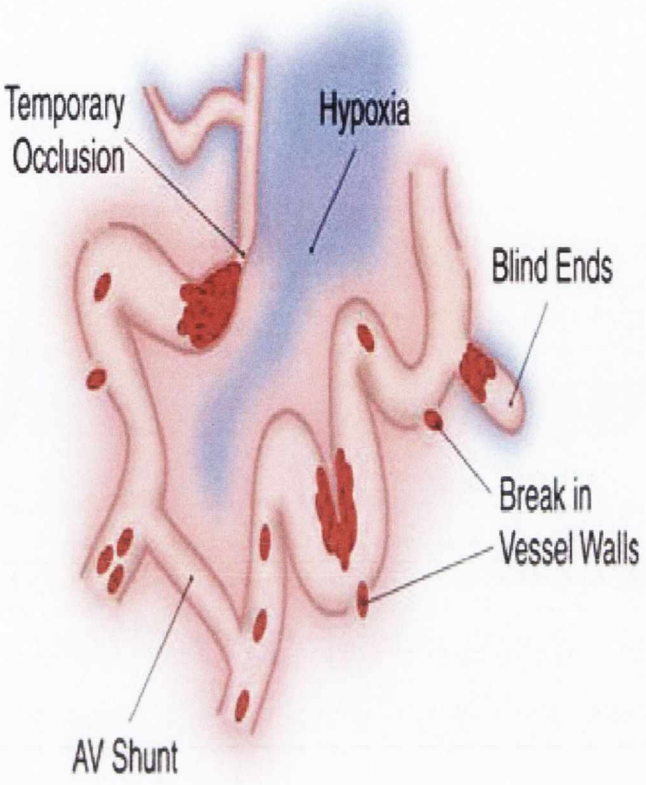



**Figure 1.5: Tumour blood vessels are chaotic, irregular and leaky**

The abnormal vascular architecture in a solid tumour can lead to the development of areas chronic and acute hypoxia. Adapted from: (Brown and Giaccia, 1998).

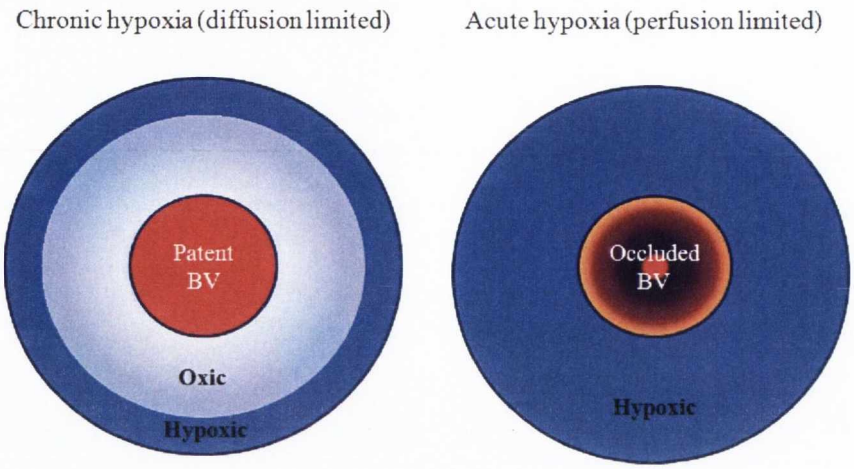



**Figure 1.6: Types of hypoxia in a solid tumour**

Areas chronic and acute hypoxia exists in tumours. Chronically hypoxic cells are found at increasing distance from tumour blood vessels are exposed to gradually decreasing levels of $O_2$ and increasing cellular hypoxia. Acute hypoxia is characterised by a sudden and complete reduction in blood glow leading to hypoxia. Necrotic cells are found at the limit if oxygen diffusion. BV; blood vessel.

## 1.9 Hypoxia Impairs the Efficacy of Radiotherapy and Increases Radioresistance

For fifty years, tumour hypoxia has been associated with radioresistance and a poor clinical outcome. Hypoxic tumour cells can be as much as two to three times more resistant to radiation damage than those in a normal $O_2$ environment. It is proposed that hypoxic cells were resistant to radiation therapy because of a lack of $O_2$ as a source of radiation induced reactive oxygen species (ROS) and free radical induced indirect DNA damage (Thomlinson and Gray, 1955). The mechanism proposed for the enhancement of radiation damage by oxygen is described as the $O_2$ fixation hypothesis. When radiation interacts with biological material, free radicals (R) are produced, most commonly through the ionisation of water. These compounds can break chemical bonds and produce chemical changes and initiate a chain of events that results in biological damage, indirectly on DNA. If oxygen is present, the free radicals react to produce ($RO_2$), this forms (ROOH) in the target molecule which changes the chemical composition of the radiation induced damage, and furthermore ROOH chemically fixes the damage. In hypoxic conditions, free radicals can react with hydrogen ions restoring them to the original form, thus the damage is not chemically fixed and repair the radiation induced damage can occur.

Initially it was demonstrated that hypoxia was shown to induce radioresistance at the time of irradiation. Subsequently it was reported that hypoxic is related to the duration of hypoxia and degree of hypoxia prior to irradiation. For example, acutely anoxic tumour cells irradiated immediately after re-oxygenation are radiosensitive when compared with cells irradiated under acute hypoxia alone (Koritzinsky et al.,

2006). While irradiated chronically hypoxic cells can acquire increased radiosensitivity when compared with irradiated cells exposed to acute hypoxia (Figure 1.7).

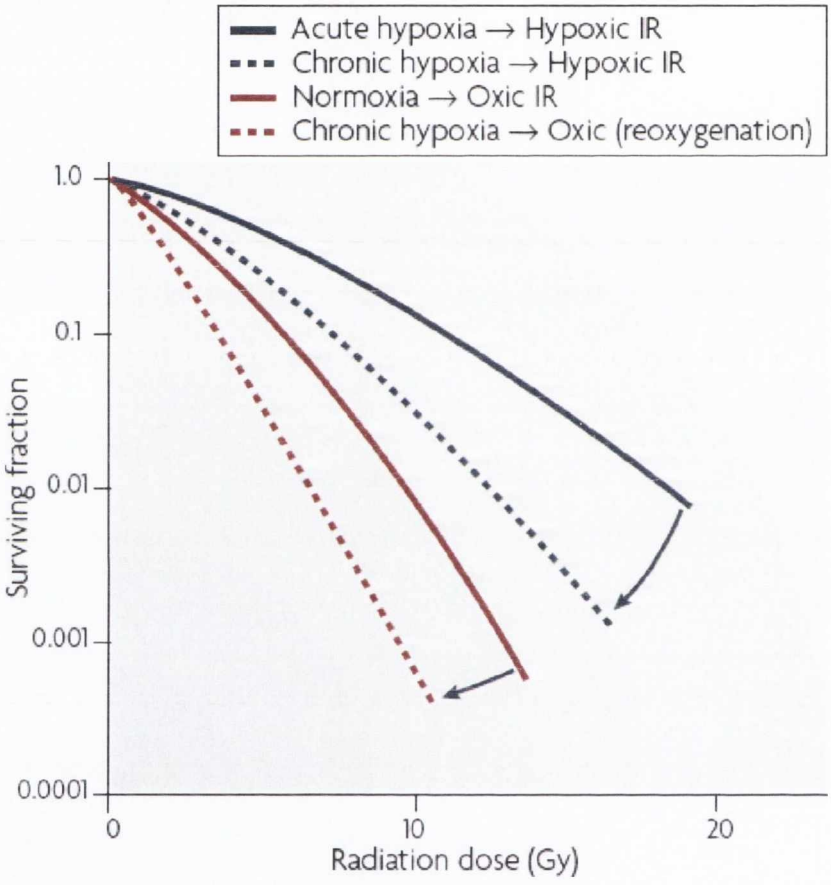



**Figure 1.7: Radioresponse of acute and chronically hypoxic cancer**

The graph shows an example of how chronically hypoxic tumour cells can have differential sensitivity to DNA damage (ionizing radiation (IR)) when compared with acutely hypoxic cells. Adapted from: (Chan et al., 2008).

However, less extreme hypoxic conditions provide tumour cells with a survival advantage. This is known as the hypoxic response, where at low oxygen levels tumour cells adapt to the hypoxic environment and attain survival characteristics. It is now clear that hypoxia leads to increased radioresistance for a variety of reasons other than the lack of oxygen derived free radicals. Hypoxic cells are less proliferative then aerobic cells, they have reduced or lost sensitivity to p53-mediated apoptosis (Tannock, 1968) (Teicher et al., 1981). Hypoxia also up-regulates transcriptional activity of specific genes involved in tumour resistance (Graeber et al., 1996) (Wartenberg et al., 2003) (Brown and Wilson, 2004). Activation of these genes can select for a malignant phenotype and are associated with increased mutation rates (Yuan and Glazer, 1998) (Vaupel et al., 2004) (Graeber et al., 1996). Hypoxia also leads to genetic adaptations that increase invasiveness and metastatic behaviour of malignant cells. Leading to increased angiogenesis and tumour invasion (Pennacchietti et al., 2003) (Subarsky and Hill, 2003).

Hypoxia also appears to contribute to reduced recognition of DNA DSB (Hammond and Giaccia, 2004) and may also down-regulate DSB repair by preferentially preventing homologous recombination (HR) (Chan et al., 2008) (Meng et al., 2005); (Sprong et al., 2006). In addition, radiobiological studies have suggested that hypoxia increases the $\alpha/\beta$ ratio of prostate cancer from <3 Gy (Brenner and Hall, 1999) to 8.5 Gy (Valdagni et al., 2005). The clinical implication is that this ratio determines the sensitivity if a particular type of cell to alterations in radiation fraction size (Barendsen, 1982). Cells with a high $\alpha/\beta$ are not very sensitive to alterations in faction size or dose rate (Fowler, 1989). However slowly proliferating cells would

have high capacity for repair of sub-lethal damage at low doses and low fraction (Fowler et al., 2006). Thus it has been proposed that logically it would be best to treat with a small number of large doses or hypofractionation.

### 1.10 The Cellular Response to a Hypoxic Environment

Hypoxia inducible factor-1 (HIF-1) is the major transcriptional regulator of the cellular response to hypoxia. This transcription factor is responsible for activating a number of genes in hypoxia resulting in cellular adaptation to low oxygen conditions (Dery et al., 2005). The HIF-1 family of transcription factors is made up of 3 alpha subunits (HIF-1α, HIF-2α, HIF-3α) and 1 beta subunit (HIF-1β) (Semenza and Wang, 1992). These heterodimers are basic helix-loop helix transcription factors that regulate the transcription of over 60 target genes allowing cellular adaptation to hypoxic environments (Vaupel et al., 2004). Even though there are similarities between the subunits, significant differences in oxygen sensitivity and trans-activation clearly suggest that HIF-1α is the primary functional protein of the HIF-1 complex. HIF-1β (also known as aryl hydrocarbon nuclear translocation, ARNT) (Wang et al., 1995) is strictly a nuclear protein that is independent of oxygen tension and readily found in cells. In contrast, HIF-1α is cytoplasmic protein virtually undetectable in normal oxygen levels even though it is constitutively expressed. Therefore transcription and translation of HIF-1α is continuous and unchanged regardless of oxygen tension levels but, HIF-1α in normoxic cells is rapidly degraded by an ubiquitin-proteasome system (Figure 1.8).

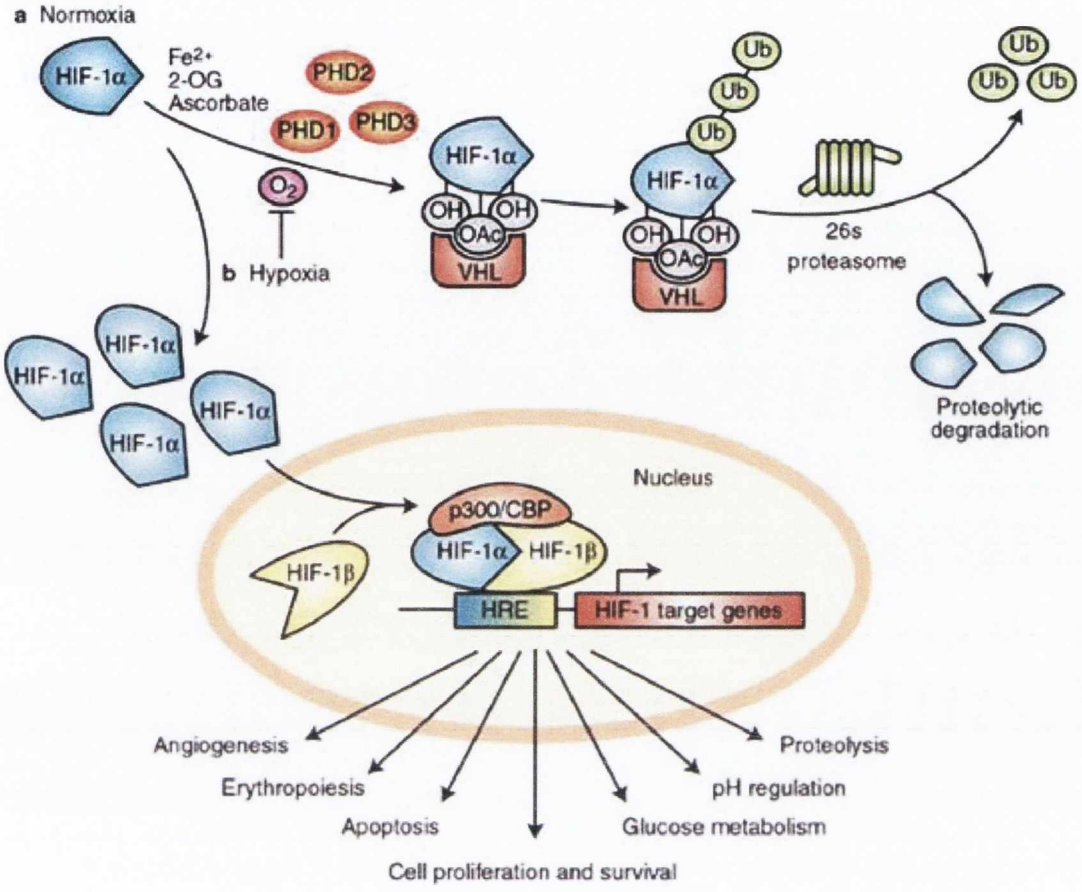



**Figure 1.8: HIF-1α Regulation by proline hydroxylation**

Normally, under normoxic conditions HIF-1α is hydroxylated and bonds to pVHL resulting in ubiquitination and proteasomal degradation. However under hypoxic conditions the un-hydroxylated HIF-1α is exempt from degradation and interacts with HIF-1α to form the stable HIF-1 complex. Adapted from: (Carroll and Ashcroft, 2006).

In normoxia, HIF-1α is hydroxylated by oxygen dependent proline hydroxylases (PHD). Only HIF-1α containing modified prolyl sites are then recognized by von Hippel-Lindau protein (pVHL) (the product of the von Hippel–Lindau tumour suppressor gene) and the recognition site of the E3 ubiquitylation ligase complex. This promotes ubiquitylation of HIF-1α targeting it for proteasomal degradation (Krieg et al., 2000) (Figure 1-8). As HIF-1α's is rapidly degraded in normoxia, it is an extremely labile protein with a half life of less than 5 mins (Dery et al., 2005). However in hypoxia, HIF-1α levels significantly increase as proteasomal degradation is inhibited due to the inability of pVHL to bind to HIF-1α. This results in HIF-1 accumulation and translocation to the nucleus where HIF-1α heterodimerizes with HIF-1α subunits producing an active HIF-1 complex (Dery et al., 2005) (Vaupel et al., 2004). Therefore oxygen is the rate limiting step in HIF-1α stabilization. HIF-1α degradation can also be inhibited by some metals, which are known as hypoxic mimicking agents. This includes Cobalt Chloride, nickel chloride and desferrioxamine. These metals are reported to induce HIF-1α expression by binding to and blocking HIF-1α pVHL binding and thereby HIF-1α stability (Kanaya and Kamitani, 2003).

The activation of HIF-1α then allows interaction with specific hypoxic response elements (HRE's). These HRE's are found in the promoter regions of a variety of target genes which ultimately leads to activation and transcription of these genes to produce a variety of proteins/products that promote cell survival under hypoxic conditions (Semenza, 2002).

To date over 60 genes have been discovered to be induced by HIF-1α. These genes encode for products such as erythropoietin, vascular endothelial growth factor (VEGF), a variety of glycolytic enzymes, transferrin and other proteins essential for cellular homeostasis (Semenza, 2003b).Over-expression of HIF-1 has been associated with increased patient mortality in several cancer types including breast, stomach, cervical, endometrial and ovarian cancers (Quintero et al., 2004). Furthermore the over expression of HIF-1 in patients with oropharyngeal cancer is associated with increased risk of failure to achieve complete remission after radiation therapy (Aebersold et al., 2001) . However the identification of hypoxic regions within tumours has not, to date, altered the clinical management of patients. HIF-1α has been proposed as a marker for radiosensitivity (Moeller and Dewhirst, 2004) (Aebersold et al., 2001), but to date the role of ionizing radiation on the expression patterns of HIF-1α and its downstream genes remains to be elucidated in prostate cancer.

### 1.11 The Role of HIF-1 in Tumour Resistance and Progression

HIF-1α is a critical transcription factor that is responsible for activating a number of genes in hypoxia ultimately resulting in cellular adaptation to low $O_2$ conditions. In neoplastic conditions these genes can give a survival advantage via the following mechanisms.

### 1.11.1 HIF-1 Increases Angiogenesis

The activation HIF-1α due to hypoxia results in the production of mediators of angiogenesis, which include vascular endothelial growth factor (VEGF) (Semenza, 2003b) (Marignol et al., 2008) (Lekas et al., 2006). VEGF is a potent stimulator of angiogenesis, which is essential for proliferation and migration of vascular endothelial cells. Almost all cells in the human body express VEGF in response to hypoxia and VEGF activity is regulated at a variety of levels including gene transcription, translation and protein excretion (Semenza, 2003a). There has been substantial published evidence both experimental and clinical that HIF-1α is paramount to this angiogenic process (Goonewardene et al., 2002) (Maxwell and Ratcliffe, 2002). In addition other vascular related gene products produced via the HIF-1α pathway also mediate angiogenesis via the regulation of blood flow and vascular tone and may also potentially influence tumour blood flow (Vaupel et al., 2004). Further evidence also links HIF-1α expression of VEGF in hypoxia mediated apoptosis. Hypoxic states which result in an amplification of normal HIF-1α dependent responses via loss of p53 function contributes to the angiogenic switch during tumour genesis, therefore facilitating tumour angiogenesis (Ravi et al., 2000).

### 1.11.2 HIF-1 Affects Apoptosis

Apoptosis is a deliberate form of programmed cell death that involves an orchestrated series of biochemical events leading to characteristic cell morphology and death. Apoptosis is regulated by a cascade of proteins called caspases, which are the

apoptosis executor proteins (Greijer and van der Wall, 2004). An important regulator of apoptosis after DNA damage is p53 protein accumulation. After DNA damage, p53 can induce proteins (Bax/Bak), which regulate the release of cytochrome C from the mitochondria, which initiate the caspase cascade leading to apoptosis. The induction of apoptosis by hypoxia, cytochrome C is released into the cytoplasm. Cytochrome C binds to the apoptotic protease activating factor 1 (Apaf-1). Apaf-1 activates caspase 9, which in turn cleaves caspases 3, 6 leading to cell death. In addition to intrinsic apoptotic pathways, extrinsic pathways have been identified that can initiate and execute the cell death process (Greijer and van der Wall, 2004).

There are several reports that indicate that HIF-1α stabilizes p53 resulting in hypoxia induced p53 dependent apoptosis (Hammond and Giaccia, 2005). Both *in vitro* and *in vivo* studies show this stabilization occurs via HIF-1α directly binding to p53 ubiquitin ligase which normally is involved in p53 degradation (Chen et al., 2003). However, the HIF-1α p53 relationship is complex and there is considerable confusion to the exact nature of the interaction between these two proteins. For example, HIF-1α may not only induce, but may also prevent apoptosis by inducing the expression of the anti-apoptotic protein IAP-2 (Greijer and van der Wall, 2004) and studies show that cell with high amounts of HIF-1α are more resistant to hypoxia induced apoptosis (Costa et al., 2001).It is possible that prolonged hypoxia may initiate apoptosis, yet acute or mild hypoxic stress may result in cellular adaptation and survival. Even though HIF-1α plays a key role in the regulation of hypoxia, differences in its effect may be due to varying oxygen levels and exposure times and

further research is required to elucidate its role in regulation of apoptosis (Figure 1.9) (Hammond and Giaccia, 2004).

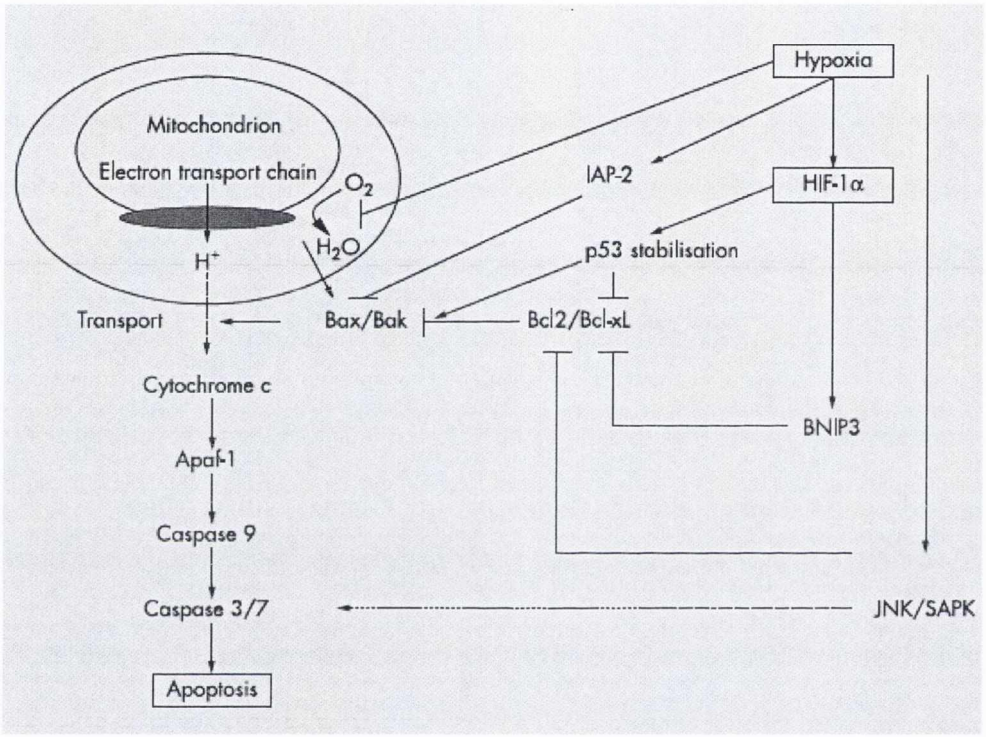



**Figure 1.9: Signalling pathways induced by hypoxia resulting in apoptosis**

Schematic representation of signalling pathways induced by hypoxia leading to apoptosis, Apaf-1, apoptotic protease activating factor-1; BNIP3, BCL-2/adenovirus; HIF-1, hypoxia inducible factor 1; IAP-2, inhibitor of apoptosis protein 2; JNK, c-Jun NH2 terminal kinase; SAPK, stress activated protein kinase. Adapted from: (Greijer and van der Wall, 2004).

## 1.12 Clinical Potential: Anti-hypoxia Agents for the treatment of Prostate Cancer

Clinical studies and modelling studies have demonstrated that cellular hypoxia, tumour cell proliferation rates and DNA repair capacity collectively modulate tumour control probability in response to radiation therapy (Marignol et al., 2008, Chapman, 2003, Nahum et al., 2003). The discovery that tumour hypoxia had an adverse effect on radiotherapy, lead to research into methods of targeting hypoxia as a way of treating cancer. Much research has been devoted to overcoming this problem including; accelerated radiotherapy combined with carbogen and nicotinamide (ARCON) (Kaanders et al., 2002), high pressure oxygen tanks, hyperbaric oxygen, hyper-oxic gas, blood substitutes and transfusions, erythropoietin and hyperthermia. Targeting hypoxic tumour cells with pro-drugs, activated by hypoxia such as tirapazamine, a hypoxic cytotoxin has been investigated (Lee et al., 1998). As have hypoxic cell radiosensitiser such as misonidazole and metronidazole (Acharya, 1994). There is also interest in the fact that high-linear energy transfer (LET) particles such as carbon or neon ions may have an anti-tumour effect which is less dependent of tumour oxygen because these particles act mostly via direct damage. The targeting of HIF-1 and associated pathways by small molecule inhibitors of HIF, HIF-1 DNA inhibitors and ribonucleic acid (RNAi) HIF-1α plasmids have been successful in vitro and in vivo (Greenberger et al., 2008). Although these agents might have activity as single agents, the evidence that HIF-1α contributes to chemo- and radioresistance suggests that hey could be of more value in combination with the established treatments (Greenberger et al., 2008).

It is now apparent that hypoxia plays a more important role than was previously considered, it can be concluded that hypoxia is an important component in the radiosensitivity in prostate cancer (Wang et al., 2005). In this project, we propose to determine if post irradiation hypoxic recovery affects the radioresistance of a number of prostate cancer cell lines. In addition we propose to determine the role HIF-1 plays in promoting cell survival in hypoxia within prostate cancer, by investigating the effects of HIF-1 on cell cycle, apoptosis and death.

# Chapter 2

# Aims and Objectives

# 2 Aims and Objectives

## 2.1 Aim 1: Determine whether prostate cancer cells that are hypoxic after irradiation show increased survival compared to fully oxygenated cells

**Hypothesis 1:** Prostate cancer cells that are hypoxic after irradiation have a survival advantage over those that are fully oxygenated.

**Objective 1:**

We initially proposed to evaluate the effect of post-irradiation hypoxia on the radioresistance of prostate cancer cell lines *in vitro* using clonogenic assays. We hoped to demonstrate that post-irradiation hypoxic exposure increase radioresistance and identify that hypoxic recovery after irradiation is an important determinant of cellular radioresistance.

**Significance:**

Current anti-hypoxia treatments such as ARCON, hypoxic cell radiosensitisers and hypoxic pro-drugs have been used to reduce radioresistance induced by pre-irradiation hypoxia to improve increases radiation induced cell death. To date these treatments have only been moderately successfully, we hope to demonstrate that post-irradiation hypoxia is an important determinant of radioresistance.

## 2.2 Aim 2: Examine whether this survival advantage correlates with modified cellular responses induced by hypoxia (reduced ROS, cell cycle, DNA repair, apoptosis and senescence)

**Hypothesis 2:** Post-irradiation hypoxia modifies the cellular response to DNA damage, increasing the cell cycle arrest, affecting DNA repair, reducing apoptosis and altering the percentage of senescent cancer cells.

**Objective 2:** We first evaluated the reactive oxygen species generated by aerobic and hypoxic irradiation (Flow cytometry, GSH luminance assay) to determine baseline ROS levels. We next examined the effect of hypoxia on the cell cycle. This was performed by Propidium iodide (PI) staining of mitotic bodies and were analysed by flow cytometry for position in the cell cycle. We then examined if hypoxic radioresistance was due to DNA repair, this was performed by split dose experiments and clonogenic assay. We further aimed to correlate this with expression of MRE11 protein (Western blot). Finally we aimed to determine the effect of hypoxia post-irradiation on apoptosis (Flow cytometry and western blot) and cellular senescence (SA-β-Gal activity and western blot).

**Significance:** Experimental data suggests that hypoxia can influence the cellular response to irradiation. Post-irradiation hypoxia may increase radioresistance and malignant potential by altering the cell cycle, disrupting DNA repair and by reducing apoptosis. Furthermore the effects of Post-irradiation hypoxia on cellular senescence are yet to be determined.

## 2.3 Aim 3: Investigate whether this survival advantage is mediated by HIF-1α

**Hypothesis 3:** HIF-1α expression is a significant factor in hypoxia induced radioresistance.

**Objective 3:** We proposed to evaluate the effect of post-irradiation hypoxia on HIF-1α protein expression by western blot. We further aimed to investigate the effects of a HIF-1α inducer, $CoCl_2$ on radioresistance. Finally we will examine the effect of HIF-1α on cell cycle arrest, cell cycle check point, DNA repair, apoptosis and cellular senescence.

**Significance:** The effect of HIF-1 on additional parts of the cell cycle, DNA repair, apoptosis and senescence may provide information on potential therapeutic targets and support the introduction of anti-HIF-1α compounds in the management of radiotherapy prostate cancer patients.

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# Chapter 3

# Materials and Methods

# 3 Materials and Methods

## 3.1 Cell Culture

### 3.1.1 Cell Lines

In this study, three human prostate cancer cell lines (22Rv1, DU145 and PC3) were used for experimentation. All human prostate cancer cell lines were obtained from the American Type Culture Collection (Teddington, Middlesex, UK). 22RV1 is a human prostate carcinoma cell line derived from a xenograft that was serially propagated in mice after castration-induced regression and relapse if the parental, androgen dependent CWR22 xenograft (Sramkoski et al., 1999). 22Rv1 prostate cancer cells are hormone sensitive and do not express PSA (Prostate Specific Antigen). In this study 22Rv1 cells represent a primary prostate carcinoma. DU145 is a metastatic human prostate cancer cell line that was established from a brain metastasis (Stone et al., 1978). DU145 are not hormone sensitive and do not express PSA. DU145 are considered to have a moderate metastatic potential. PC3 human prostate cancer cell lines were originally derived from an advanced androgen independent bone metastasis. PC3 cells have a low testosterone-5-alpha reductase activity and express PSA. PC3 cells do not express p53 or p63 (Alimirah et al., 2006).

### 3.1.2 Cell Culture and Growth Conditions

All cell lines were cultured in RPMI 1640 plus glutamax (Gibco, Paisley, UK) supplemented with 10% foetal calf serum (FCS) (Globepharm, Guildford, UK), 50 μ/ml penicillin and 5 μ/ml streptomycin (Gibco, Paisley, UK). Cell cultures were

grown in T75 flasks (Nunc, Roskilde, Denmark) in an incubator (Thermo Fischer Scientific, Ohio, USA) at 37 degrees Celsius (°C) and 5% $CO_2$. All culture handling was undertaken within a Class II laminar flow cabinet (Medical Air Technology, Oldham, UK). Cell lines were passaged weekly as described below and fed once weekly by replacing the culture medium with fresh media.

### 3.1.3 Cell Culture Maintenance

Media was removed from the T75 flasks containing the human prostate cancer cell lines. Cells were subsequently washed with phosphate buffered saline (PBS) (Gibco, Paisley, UK). This was then discarded and 5 ml of fresh trypsin (Gibco, Paisley, UK) was added to the T75 flasks. The flash was then placed in an incubator for 5 mins at 37°C. Next the tryspin was neutralized with 5ml of fresh media. This solution was then transferred to a 20 ml sterile tube (Sterilin, Caerphilly, UK). The tube was placed in a Centra GP8R centrifuge (Therma IEC, MA, USA) and spun at 1300 revolutions per minute (RPM) for 3 mins. All the supernatant was discarded and the pellet was re-suspended in 5 ml of fresh media. Cells were subsequently transferred to a new T75 flash containing fresh media. The 22RV1 cell line was routinely passaged at a 1:6 ratio, where as DU145 and 22RV1 cell lines were split at ratio of 1:10. After 2 months of cell passage, cell lines were discarded and new cell lines were prepared from frozen samples

### 3.1.4 Cell Counting

After re-suspension of the pellet on 5 ml fresh media, 10 µl of suspension was added to 90 µl of trypan blue solution 0.4% (Sigma-Aldrich, Poole, UK), 10 µl of cell/trypan blue solution was then loaded onto wach side of an improved Neubauer haemacytometer (Hawksley, Lancing, UK) with a 22 mm x 40 mm cover-slip (Chance Propper, Warley, UK). Cells in the central 25 squares of each chamber were counted under fluorescence using a Dialux 20EB microscope (Lietz, Germany). The number of cell per chamber was averaged over the 2 chambers and the cell concentration was calculated as:

$$N = \frac{5 \, x \, 10^5}{average \, x \, 10^5} \, ml$$

### 3.1.5 Clonogenic Assays

Cells were trypsinised and counted as outlined previously. The cells of appropriate cell lines were plated into 6-well plates (Sarstedt, Wexford, Ireland0, 1000-5000 cells/well were prepared for aerobic control plates, whereas 2000-10,000 cells/well were plated for hypoxic conditions. The plates were labelled un-irradiated controls or aerobic/hypoxic plate's followed by aerobic or hypoxic recovery. They plates were agitated after addition of the cells to ensure uniform seeding. Cells were left to seed overnight at 37°C.

The following day, aerobic control plates received radiation exposure. Cells for hypoxic exposure were transferred into the hypoxic chamber for the required duration of hypoxia and then irradiated. Selected cells were placed into hypoxia for 24 hrs recovery. All cells were subsequently transferred back into aerobic incubator at 37 degrees Celsius and 5% $CO_2$. Approximately one to two weeks later depending on cell line, the plates were removed from the incubator and the media was removed. A staining solution made up of crystal violet (Signa-Aldrich, Poole, UK) and 70% ethanol was added to each well. The plates were then rinsed with di-ionized water and left to air dry. Colonies were counted using Colcount™ (Oxford Optronix, Oxford, UK). The plating efficiency in the aerobic controls and the surviving fraction in hypoxic cells were determined as follows.

$$\text{Platting efficency}(\%) = \frac{\text{number of clones}}{\text{number of cells plated}} \times 100$$

$$\text{Surviving fraction} = \frac{\text{number of clones}}{\text{number of cells plated x platting efficency}} \times 100$$

## 3.2 Mycoplasma Testing

All cell lines were routinely tested for Mycoplasma infection, testing was performed every 3 months using the Mycoplasma polymerase chain reaction (PCR) ELISA kit (Roche, West Sussex, UK) (Appendix B).

### 3.3 Hypoxic Exposure and Protocol

Cellular hypoxia was achieved by incubating human prostate cancer cells in a 1000 in vivo hypoxic chamber (Bio Trace, Bracknell, UK) to 0.5% $O_2$ (pO2 < 2mmHq). Hypoxia was maintained by exposing cells to a mixture of nitrogen, $CO_2$ (5%) and compressed air to achieve a 0.5% oxygen concentration. The $pO_2$ levels were monitored by an $O_2$ probe (OxyLab pO2TM, Oxford Optronix, Abingdon, UK). For hypoxic experiments, cells of appropriate cell lines were plated according to clonogenic protocol. Cells were then exposed to 0.5% $O_2$ for 4, 24, 48 and 72 hrs. At the selected time point plates were removed from the hypoxic chamber and underwent irradiation according to protocol. Immediately post-irradiation the cells were either returned to the hypoxic chamber for 24 hrs or returned directly to an incubator. Following post-irradiation hypoxic recovery for 24 hrs the cells were transferred to an aerobic chamber for 1 to 2 weeks to allow colony formation.

### 3.4 Simulated Hypoxia treatment with $CoCl_2$

$CoCl_2$, induces HIF-1α expression by binding to the PAS domain resulting in blockage of HIF-1α pVHL binding and thereby HIF-1α stability (Kanaya and Kamitani, 2003). To simulate hypoxic exposure and HIF-1α expression, cells were exposed to a 100 μM solution of $CoCl_2$ for 4 hrs and irradiated. Cells were then washed and transferred to new media immediately or 24 hrs later to simulate post-irradiation hypoxic recovery. Cells were transferred to an aerobic chamber for 1 to 2 weeks to allow colony formation and were analysed by clonogenic assays.

## 3.5 Irradiation Parameters

Prostate cancer cells were exposed to aerobic and hypoxic exposure as describe in section 3.3, the plated cells were irradiated. Single plates were placed in a RS225 cell irradiator (Gulmay Medical, Camberley, UK). Radiation was delivered at a dose rate of 3.25 Gy/min. Cells of all 3 human prostate cancer cell lines received doses of 2,4,6,8,10 Gy or 2 Gy x 2, separated by specific time intervals according to protocol. Cells that were exposed to aerobic conditions prior to irradiation were irradiated in aerobic conditions; correspondingly hypoxic cells were irradiated in hypoxia. Following irradiation cells were returned to incubators to allow colony formation. For all experiments un-irradiated controls were maintained for comparison.

## 3.6 Preparation of Samples for Western Blot

### 3.6.1 Extraction of Protein from Samples

Prostate cancer cell lines, 22Rv1 and Du1245 were grown to 80% confluence in T75 cell culture flasks and were exposed to hypoxic conditions and irradiation as described in sections 3.2 and 3.3. Cells were harvested by washing in ice-cold PBS (5ml) and scraping with a cell scraper. This solution was then centrifuged at (3 min, 1500 RPM, 4°C). The pellet was re-suspended in cold radio-immunoprecipitation assay (RIPA) lysis buffer supplemented with a protease inhibitor cocktail (Santa Cruz, UK). The composition of the RIPA buffer was a follows: tris-buffered saline (TBS), 1% Nonidet P-40, 0.5% sodium desoxycholate, 0.1% sodium dodecyl sulfate (SDS), and 0.004% sodium azide, which was supplemented with 10 µl/ml

phenylmetanesulfonyl fluoride (PMSF), 10ul/ml sodium orthovanadate and 20 µl/ml protease inhibitor cocktail (Appendix A). Following 10min incubation on ice, the lysates were centrifuged at 4°C for 20 min (15,000 rpm), and the supernants (comprising total cell lysates) were stored at -70°C until required.

### 3.6.2 Determination of Protein Concentration: The Bradford assay

The amount of protein present in both prostate cancer cell line lysates was determined by a Bradford assay. A standard solution of Bovine Serum Albumin (BSA) (Sigma-Aldrich, Poole, UK), ranging in concentrations from 0 – 32 µg/ml was used. Samples were prepared using 500 µl of $dH_20$ and 1 µl of lysate extract. 500 µl of Bradford reagent (Sigma-Aldrich) was added to each standard and sample. 200 µl of solution was transferred to a 96 well plate (Nunc, Roskilde, Denmark). The 96 well plates were placed into a SpectraFlour Plus flourometer (Tecan, Dorset, UK) and the mean absorbance was calculated at 595 nm. A Microsoft Excel spreadsheet (Microsoft) was used to compare µg/ml BSA against the absorbance of the standards. The protein concentration (µg/ml) of each lysate sample was interpolated from the standard curve constructed from the BSA solution absorbencies.

### 3.6.3 Preparation of Samples for Western Blotting

Aliquotes of each treated cell lysate sample (volume calculated from total cell lysate protein concentrations to give equal amount of protein (~25-50 µg) in each sample) were added to an appropriate volume of $H_20$ to give final equal volumes for each

sample. There were prepared for SDS-polyacrylamide gel electrophoresis by mixing with an equal volume of 2X buffer which consisted of: 0.5 M Tris-HCl pH 6.8 (1.25 ml), H20 (3.35 ml), glycerol (2.5 ml, 10% SDS (2 ml), 0.5% bromophenol blue(0.2 ml) and b-mercaptoethanol (50 µl/950 µl sample buffer) (Appendix A). Samples were boiled at 95°C for 5 min, plus centrifuged and stored on ice until required.

### 3.7 Western Blotting

Western blotting is a commonly used method for detecting the presence of a specific protein on a gel in the presence of a mixture of other proteins. It utilizes the concept of a complementarity between a probe and a target molecule, with the target molecule being the protein of interest, while the probe is a known antibody to the protein which is labelled appropriately, e.g. horseradish peroxidase (HRP). Western blotting is essentially a quantification process, which can be used to provide information about the presence and quantity of a protein in a sample.

#### 3.7.1 SDS-polyacrylamide Gel Electrophoresis

The proteins contained in cell lysate samples, as prepared in section 3.4.3 were separated on 12% SDS-polyacrylamide gels, using the Lamelli buffering system (Lamelli reference), and a Mini-PROTEAN 3 Electrophoresis System (Bio-Rad, Hertfordshire, UK). The resolving gel was prepared as follows; H20 (3.4 ml), 30% Bis-Acrylamide (4 ml), 1.5 M Tris-HCL pH 8.8 (2.5 ml), and 10% SDS (100 µl) were mixed, and degassed by sonication in a water bath for 10 seconds. 10%

ammonium persulfate (APS) (50 µl freshly prepared), and N,N,N,N'-tetramethylethylenediamine (TEMED) (10 ul) were added to polymerise the gel. A 4% stacking gel was prepared as follows H20 (3.05 ml), 30% Bis-Acrylamide (665 µl), 0.5 M Tris-HCL pH 6.8 (1.25 ml), and 10% SDS (50 µl) were mixed and degassed using a Branson 200 ultrasonic water bath (Branson, Wethersfield, CT, USA). 10% APS (2 5µl, freshly prepared), and TEMED (10 ul) were then added. The running buffer consisted of Tris-HCl (0.025 M), glycine (0.192 M), and SDS (0.2%). Cell lysate samples (20ul/well was loaded onto the gel, along with pre-stained protein marker (Precision Plus ProteinTM Standars, Bio-Rad). Gels were electrophoresed at 200 V for 1 hour, or until the dye front had reached the end of the gel.

### 3.7.2 Protein Transfer

Following SDS-polyacrylamide gel electrophoresis as describe in section 3.5.1, the gel was equilibrated, by soaking for 20 min, in transfer buffer (Tris (3.03 g), glycine (14.4 g), methanol (200ml), made up to 1 L with $H_20$. Four sheets of filter paper and a polyvinylidene diflouride (PVDF) membrane, pre-soaked in methanol, and washed in $H_20$ for 5 min (Amersham Bioscience, Buckinghamshire, UK); along with the transfer fibre pads were soaked in transfer buffer for 10 min. The gel and PVDF membrane were arranged in a Mini Trans-Blot Cell (Bio-Rad, Hertfordshire, UK) gel holder cassette, between the filter paper (2 sheets on each side) and fibre pads as per the manufacturer's instructions. The resolving proteins on the gel were transferred to

the PVDF membrane at 100 V for 60 min. The membrane was air dried, and stored at 4°C between two sheets of filter paper, and wrapped in cling film.

### 3.7.3 Immunodectection

The PVDF membranes, to which the protein gels had been transferred, and were stored at 4°C, as described in section 3.5.1, were soaked in 100% methanol for 10 seconds, then washed in $H_20$ for 5 min. The membrane was then blocked by incubation at room temperature, for 60 min in blocking solution which consisted of 5% (W/V) Marvel in PBS containing 0.1% (v/v) Tween (PBS-T). The membranes were then washed 3 times (5 min per wash) in PBS-T, and incubated, on a rolled, with one of the following primary antibodies, which were dilutes as stated:

- rabbit anti-HIF-1a (Cell signaling technology, Massachusetts, USA), diluted 1:500 in blocking solution.
- mouse anti-VEGF (Abcam, Cambridge, UK) diluted 1:1000 in blocking solution.
- mouse anti-MRE11 (Santa Cruz, Biotechnology), diluted diluted 1:500 in blocking solution.
- rabbit anti-p53 (Cell signaling technology, Massachusetts, USA), diluted 1:1000 in blocking solution.
- rabbit anti-CHK1 (Cell signaling technology, Massachusetts, USA), diluted 1:1000 in blocking solution.

- rabbit anti-CHK2 (Cell signaling technology, Massachusetts, USA), diluted 1:1000 in blocking solution.
- rabbit anti-$p16^{ink4a}$(Cell signaling technology, Massachusetts, USA), diluted 1:1000 in blocking solution.
- mouse anti-B-actin, diluted 1:5000 in blocking solution.

For HIF-1a, VEGF, MRE11, p53, chk1, chk2 and $p16^{ink4a}$, PVDF membranes were incubated overnight at 4°C in primary antibody. For B-actin, blots were incubated in primary antibody for 1 hour at room temperature. All membranes were then washed 3 times (5min per wash) in PBS-T, and incubated for 60 min on a roller, in secondary antibody solution, which consisted of anti-mouse or anti-rabbit HRP-labelled antibody (DakoCytomation) diluted 1:5000 in blocking solution. The membranes were washed 4 times (5min per wash) in PBS-T, and incubated with SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, Illinois, USA) according to the manufacturer's instructions. The membranes were then placed between acetate sheets, and exposed to X-ray film (AGFA, Middlesex, UK) for the appropriate amount of time. This was then developed using an AGFA Curix 60 developer.

### 3.7.4 Membrane Re-Probing

Where possible, PVDF membranes were re-probe to detect proteins of significantly different sizes. Following completion of the first immuno-detection procedure, the membrane was incubated in blocking solution, before the process was performed as described using a different antibody.

## 3.8 Flow cytometry

### 3.8.1 Cell Cycle Analysis

22Rv1 and Du145 cells, which had been isolated as described in section 3.1, and exposed to hypoxia for the required period of time as described in section 3.2. Were treated with irradiation as described in section 3.3, were transferred to fluorescence-activated cell sorting (FACS) tubes (Beckton Dickinson, New Jersey, USA). Cell pellets (1600 RPM, 5 min) were re-suspended in PBS (1ml). Cells were permeabilised and fixed by adding cold 70% ethanol.

Ethanol (2.5 ml) drop-wise to each sample while vortexing gently. Cells were incubated at room temperature for 30 min, harvested (1300 RPM, 3 min), and stained by re-suspending in freshly prepared staining solution containing stock PI (30 µl) (Molecular Probes, Oregon, USA, 1 mg/ml), PUREGENE®RNase A (4 mg/ml) (10 µl) (Gentra Systems Inc., Minneapolis, USA) and PBS (460 ul). Samples were incubated at 37°C for 30 min, then were immediately analysed on a FACS Calibur software cytometer (Becton Dickinson) and data analysis was performed using CellQuest software (Becton Dickinson). PI fluorescence was measured on FL2 channel on the flow cytometer (536 nm, 488 nm laser) excitation, and 623 nm emission), with a along the Fl2 axis representative increase in the DNA content of the cell population.

### 3.8.2 Annexine V/Propidium Iodide Staining

22Rv1 and DU145 cells, which had been isolated as described in section 3.1, and exposed to hypoxia for the required period of time as described in section 3.2. Were treated with irradiation as described in section 3.3, were transferred to FACS and harvested (1600 RPM, 5 min). Cell pellets were washed in Annexine V Binding buffer (500 ul) (Abcam Plc), annexine V fluorescein isothiocyanate (FITC) (5 µl) and PI (5 µl) was added to each sample, except appropriate controls to which only binding buffer was added. Samples were vortexed, and incubated on ice, in the dark, for 15-20 min. All tubes were kept on ice from this point on in order to preserve the FITC tag on the Annexine V. The flouresence in each sample was determined by flow cytometry analysis using a FACS Calibur flow cytometer. Analysis of data was performed using CellQuest software. The total population of apoptotic cells was defined as those showing an increase in flouresence along the FL1 (positive for AnnexineV/FITC staining) and FL2 (positive for PI staining) axes. These cells appear in either the upper or lower quadrants of the Cell Quest output.

## 3.9 Detection of Reactive Oxygen Species

Intracellular ROS levels were measured by flow cytometry with 5-(-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester (CM-$H_2$DCFDA) (Invitrogen, Carlsbad, CA, USA), a cell permeant, non- fluorescent molecule. CM-$H_2$DCFDA is hydrolyzed by intracellular esterases to the non-permeable 2',7'-dichlorodihydrofluorescein (DCFH), which is then oxidised to the strongly

fluorescent 2',7'-dichlorofluorescein (DCF). The intensity of this fluorescence is proportional to the level of cellular oxidative stress. Cells were also stained with PI to allow for the detection of dead cells, thus ensuring that only the fluorescence of viable cells was measured.

22Rv1 and DU145 cells were treated as described in section 3.2 and 3.3. Cells in the exponential growth phase were harvested by trypsinisation as previously described (section 3.1), and pelleted by centrifugation at 1300 RPM for 3 min. Cells were re-suspended in PBS at a concentration of $1 \times 10^5$ cells (per sample) in 5 mL falcon tubes (BD Biosciences). Lyophilised CM-$H_2$DCFDA probe was re-suspended in 86.54 µL dimethyl sulfoxide (DMSO) to make a 1 M stock solution. Cells were loaded with 10µM CM-$H_2$DCFDA (diluted in PBS) except for appropriate controls, and incubated for 30 min at 37°C in 5% $CO_2$/95% humidified air, in the dark. Control samples were incubated in PBS. Cells were pelleted by centrifugation at 1300 RPM × g for 3 min, and re-suspended in 0.5 mL PBS. A volume of 5 µL PI solution (1 mg/mL) was then added to all samples, except appropriate controls. Appropriate samples were then irradiated with 2 Gy of radiation (as described in section 2.4), and all samples were placed on ice in the dark. Instrumental controls were set up in parallel with the samples, in order to define the boundaries of each population during analysis on the flow cytometer. These included unstained controls, single stained cells with CM-$H_2$DCFDA only, single stained cells with PI only, and a positive control. Positive controls were treated with 100 µM $H_2O_2$ for 30 min. Fluorescence was analysed immediately using a $CyAn_{ADP}$ flow cytometer. A minimum of 10,000

events, in a live gate were collected. Mean fluorescence intensity was used as a measure of ROS, using Summit v4.3 software.

## 3.10 Estimation of Reactive Oxygen Species by Glutathione Detection

### 3.10.1 Glutathione Assay

Glutathione (GSH), a nonprotein thiol, is an antioxidant found in eukaryotic cells (Sies, 1999). Reactive chemical species can cause a drop in GSH levels either by oxidation or reaction with the thiol group. A change in GSH levels is important for assessing toxicological responses and can promote oxidative stress, potentially leading to apoptosis and cell death (Townsend et al., 2003). Glutathione Assay is ideal for many applications, including the measurement of cellular glutathione levels as an indicator of cell viability or oxidative stress.

22Rv1 and DU145 cells, which had been isolated as described in section 3.1, were plated onto a 96 well plate (Nunc) and exposed to hypoxia for the required period of time as described in section 3.2. The cells were treated with irradiation as described in section 3.3. Immediately after the treatment the media was removed from each well of the 96 well plates and replaced with GSH-Glo™ Reaction Buffer (Promega Corp, Madison, WI, USA). This made by adding Luciferin-NT substrate (100 µl) (Promega) and Glutathione S-Transferase (100 µl) (Promega) to GSH-Glo™ Reaction Buffer (10ml). The 96 cell plate was gently mixed on a plate shaker and left to incubate at room temperature for 30 min. Reconstituted Luciferin Detection Reagent (100 µl) was then added to each well of the 96 well plate and again allowed

to incubate at room temperature for 15 min. Luminescence was read by transferring the 96 well plate to a SpectraFlour Plus flourometer (Tecan, Dorset, UK) and quantifying sample luminescence.

The net GSH-dependent luminescence of each sample was calculated by subtracting the average luminescence of the negative control reactions (reactions without GSH) from that of GSH-containing reactions. Changes from the average net signal for total GSH activity to the net signals for reactions with test compound reflect the effect of the compound on the GSH levels, and changes in the luminescent signal will typically be seen as a decrease. Since reactive chemical species often cause a drop in GSH levels either by oxidation or reaction with the thiol group.

### 3.11 Detection of Cellular Senescence

Cellular senescence represents an arrested state in which the cells remain viable, but not stimulated to divide by serum or passage in culture. Senescent cells display increase of cell size, senescence-associated expression of β-galactosidase (SA-β-Gal) activity, and altered patterns of gene expression. SA-β-gal is a hypothetical hydrolase enzyme that catalyzes the hydrolysis of β-galactosides into monosaccharides only in senescent cells and is not found in pre-senescent, quiescent or immortal cells. The Senescence detection kit (BioVision, Mountain View, Ca, USA) is designed to histochemically detect SA-β-Gal activity in cultured cells and tissue sections, as a measure of cellular senescence.

22Rv1 and DU145 cells, which had been isolated as described in section 3.1, were plated onto a 12 well plate (Nunc, Roskilde, Denmark) and exposed to hypoxia for the required period of time as described in section 3.2. The cells were treated with irradiation as described in section 3.3 and placed into hypoxic or aerobic recovery for 24hrs. The next day the media was removed from each well of the 12 well plates. The cells were washed once with 1X PBS (Gibco) and 500 µl of Fixative solution (BioVsion) was added to each well and let incubate for 10min at room temperature. Cells were washed two further times with 1X PBS (Gibco) the 500 µl Staining Solution Mix was added to each well. This was comprised of 470 µl Staining solution (BioVsion), 5 µl Staining supplement (BioVsion) and 25 µl of 20 mg/ml X-gal in DMSO (Signa-Aldrich, Poole, UK). The cells were covered and incubated overnight at 37°C. The following morning the cells were observed under a microscope for the development of a blue colour (marker of SA-β-Gal activity) and cell morphology at 200X total magnification. A minimum of 200 cells was scored at random according to SA-βgal staining (positive or negative). 3 wells were analysed per experiment and experiments were performed in triplicate, therefore for each experiment a mean of 600 hundred cells was counted. Digital images were taken with an Olympus CKX41 microscope (Olympus, Hamburg, Germany) and were processed with DP Controller version 1.2.1.108 software (Olympus, Hamburg, Germany). For long term storage of the stained plates, the Staining Solution Mix (Biovision) was removed and the cells were over-layed with 70% glycerol and stored at 4°C.

### 3.12 Statistical Analysis

All experiments were performed in triplicate unless otherwise stated. Difference in surviving fraction was compared using non-parametric t-tests or an analysis of variance (ANOVA). A p-value of $<0.05$ was considered statically significant. Data are represented as mean ± standard error of the mean.

# Chapter 4

# Results

# 4 Results

## 4.1 Impact of Hypoxic Recovery Post-Irradiation on Radioresistance

### 4.1.1 Post-Irradiation hypoxia Increases the Surviving Fraction of Prostate Cancer cells

The impact of post irradiation hypoxic recovery on the radioresistance of aerobic or hypoxic (4 hrs) 22Rv1, DU145 and PC3 cells exposed to a single dose of radiation (4 Gy) and allowed to recover in aerobic or hypoxic conditions for 24 hrs was determined. Survival was measured using clonogenic assays and clonogenic survival was determined by calculations shown in section 3.2.5 (Figure 4.1). The response of aerobic cells and cells reoxygenated following irradiation were used as a control.

The baseline radioresistance of aerobic prostate cancer cells was different for each cell line. 22Rv1 cells were the most radiosensitive cell line, DU145 showed increased radioresistance compared to 22Rv1 cells (*t* test p=0.0041). PC3 cells were the most radioresistant cell line after aerobic irradiation (*t* test p=0.0009). Hypoxia increased the radioresistance of the three cell lines significantly. DU145 cells are the most radioresistance cell line after hypoxic irradiation and hypoxic recovery (*t* test p< 0.0001) (Figure 4.2). Hypoxia followed by aerobic irradiation increased the surviving fraction of 22Rv1 (*t* test p=0.0400), DU145 (*t* test p=0.0067) and PC3 cells (*t* test p=0.0012). Post irradiation hypoxic recovery of aerobic cells increased the surviving fraction of 22Rv1 (*t* test p=0.0017), DU145 (*t* test p=0.0002) and PC3 cells (*t* test p=0.0022).

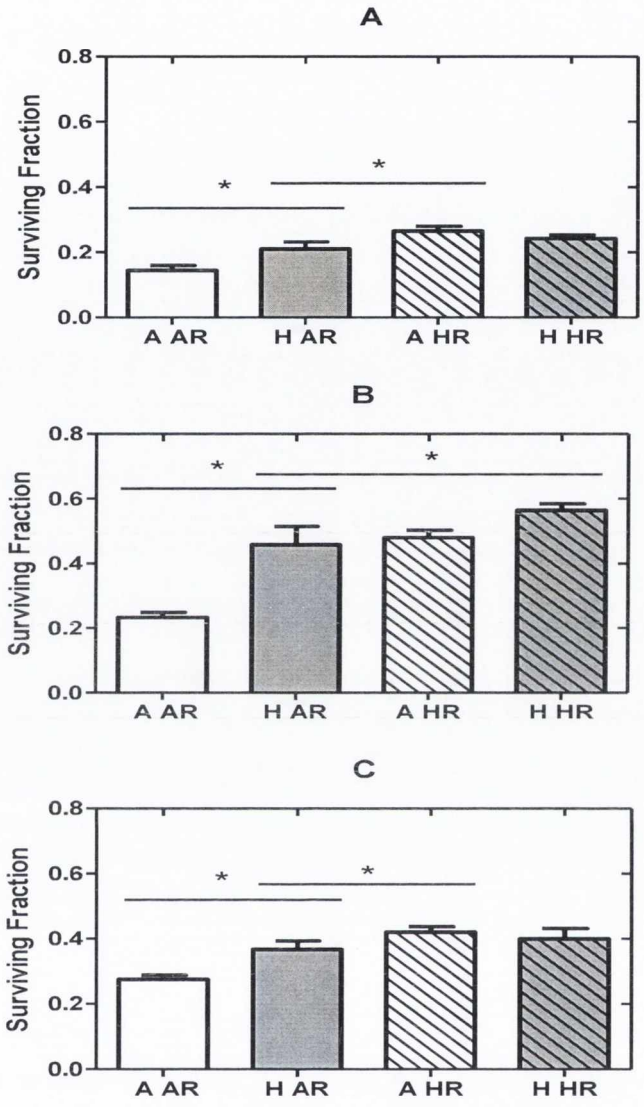



**Figure 10: Post-irradiation hypoxia increases the surviving fraction**

Surviving fraction of (A) 22Rv1, (B) DU145 and (C) PC3 cells exposed to the delivery of single dose fractions (4 Gy) and allowed to recover in aerobic or hypoxic conditions for 24 hrs. A AR=Aerobic irradiation, aerobic recovery, H AR= Hypoxic irradiation, aerobic recovery, A HR = Aerobic irradiation, hypoxic recovery, H HR = Hypoxic irradiation, hypoxic recovery. N=3; Mean ± SEM; *$p<0.05$.

## 4.2 Post-Irradiation Hypoxia Increases the Radioresistance across Large Dose Range

The effect of post-irradiation hypoxia on the radioresistance of prostate cancer cells was determined. Aerobic or hypoxic (4 hrs) 22Rv1, DU145 and PC3 cells were exposed to the delivery of single dose fractions (0-10 Gy) and allowed to recover in aerobic or hypoxic conditions for 24 hrs. Survival was measured using clonogenic assays and radiation survival curves were generated (Figure 4.2). The response of aerobic cells and cells reoxygenated following irradiation were used as a control.

The pattern of radioresistance was similar between all three cell lines. Oxygenated 22Rv1, DU145 and PC3 cells were the most radiosensitive, while hypoxic cells that were placed in hypoxic recovery for 24 hrs were the most radioresistant (22Rv1 (*t* test $p<0.0001$), Du145, (*t* test $p<0.0001$), PC3 (*t* test $p<0.0001$) (Figure 4.1). Pre-irradiation hypoxia did increase the radioresistance of DU145 cells (*t* test $p=0.0053$) but did not increase radioresistance of 22Rv1 (*t test*, $p=0.2034$) and PC3 cell (*t* test $p=0.4000$) (10 Gy). Post-irradiation hypoxic recovery of aerobic and hypoxic prostate cancer cells increased the radioresistance significantly in all three cell lines compared to irradiated aerobic controls, (22Rv1 (*t* test $p<0.0001$), Du145, (*t* test $p=0.0003$), PC3 (*t* test $p=0.0073$).

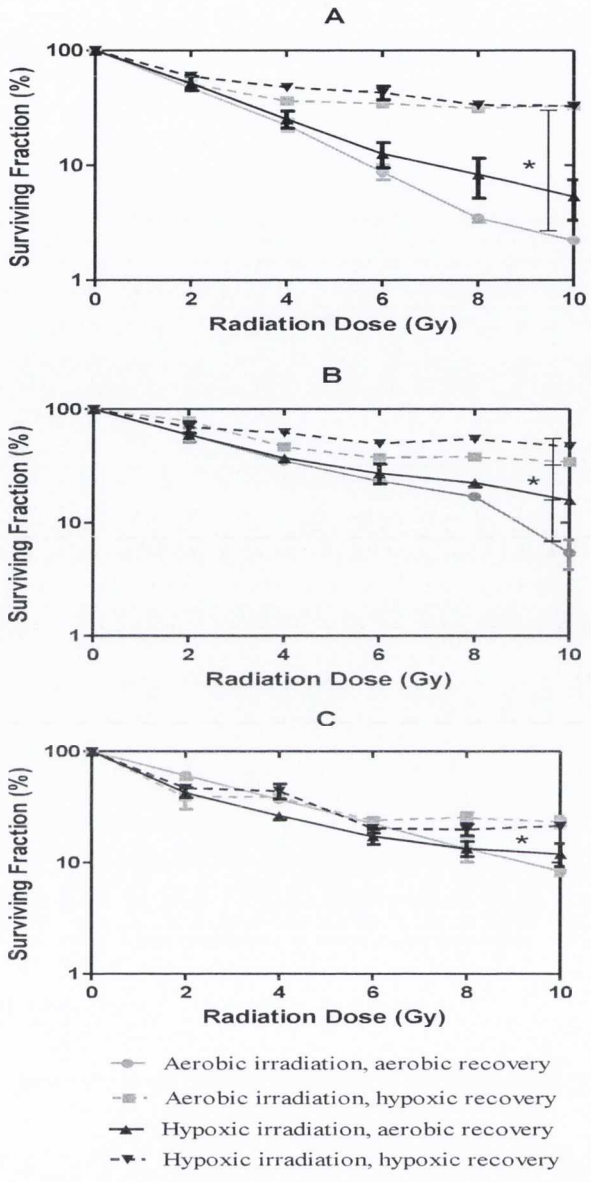



**Figure 11: Cell survival curve demonstrating post-irradiation hypoxic radioresistance**

Surviving fraction of (A) 22Rv1, (B) DU145 and (C) PC3 cells exposed to the delivery of single dose fractions (0-10 Gy) and allowed to recover in aerobic or hypoxic conditions for 24 hrs. N=3; Mean ± SEM; *$p<0.05$.

## 4.3 Post-Irradiation Hypoxic Recovery Decreases Radiosensitivity of Chronically Hypoxic Cells

The effect of post-irradiation hypoxia on the radioresistance of chronically hypoxic prostate cancer cells was determined. 22Rv1, DU145 and PC3 cells were exposed to hypoxia (24, 48, 72 hrs) prior to irradiation with (4 Gy) and allowed to recover in hypoxic conditions for 24 hrs. Survival was measured using clonogenic assays and clonogenic survival was determined by calculations shown in section 3.2.5 (Figure 4.3). The response of aerobic cells and cells reoxygenated following irradiation were used as a control.

Chronic hypoxia exposure (24, 48, 72 hrs) prior to irradiation did not increases the surviving fraction of cells following post-irradiation hypoxic recovery, compared to cells that were in hypoxia for just 4 hrs prior to irradiation, 22Rv1 (ANOVA, $p= 0.2865$), DU145 (ANOVA, $p= 0.1675$) and PC3 (ANOVA, $p= 0.1926$). Post-irradiation hypoxic recovery also negated the increased radiosensitivity that has been reported with chronically hypoxic cells (Koritzinsky et al., 2006).

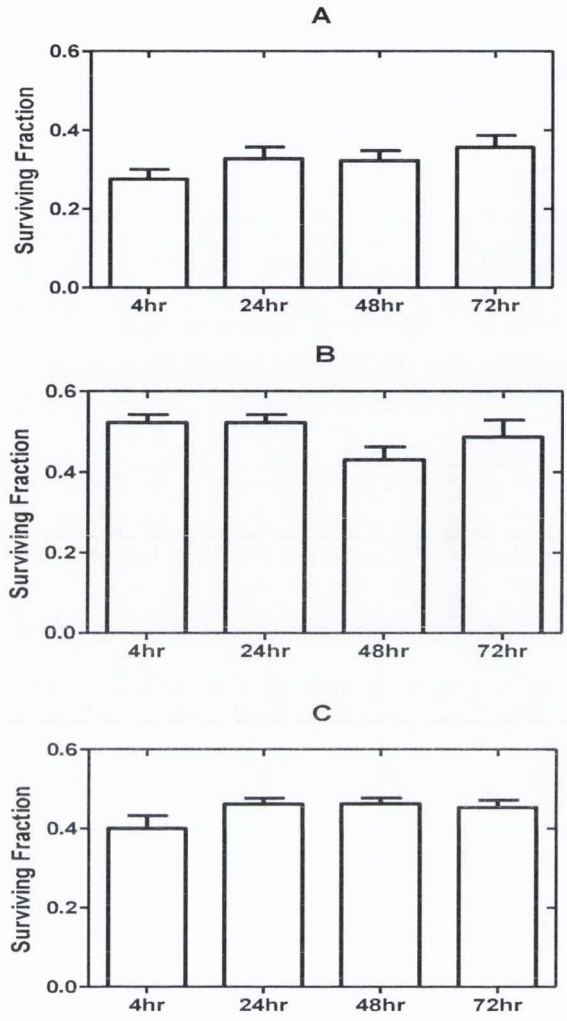



**Figure 12: Chronic pre-irradiation hypoxia does not increase the radioprotective effect of post-irradiation hypoxic recovery**

Surviving fraction of hypoxic (4, 24, 48, 72 hrs) (A) 22Rv1, (B) DU145 and (C) PC3 cells exposed to the delivery of single dose fractions (4 Gy) and allowed to recover in hypoxic conditions for 24 hrs. N=3; Mean ± SEM.

## 4.4 The Impact of a Modified Cellular Responses on Post-Irradiation hypoxic Survival Advantage

### 4.4.1 Impact of Hypoxia on Reactive Oxygen Species Levels

The effect of hypoxia of intracellular levels of ROS was determined. Aerobic or hypoxic (4 hrs) 22Rv1 and DU145 cells were exposed to the delivery of single dose of radiation (4 Gy) and were immediately analysed for ROS levels. Median intracellular ROS levels were detected by flow cytometry with a fluorescent probe (Figure 4.4). The response of aerobic cells and hypoxic cells were used as controls.

There was a significant difference in the total amount of ROS generated between 22Rv1 and DU145 controls. Mean baseline ROS levels in 22Rv1 cells was 687.6±125.3, DU145 cells were significantly lower 294.0 ±52.65 (*t* test, p= 0.0007). Irradiation with 4 Gy increased the median ROS levels in both cell lines significantly, 22Rv1 (*t* test, p=0.0179), DU145 (*t* test, p< 0.0001) in hypoxia (4 hrs) prior to irradiation also increased the amount of intracellular ROS 22Rv1 (*t* test, p=0.0021) and DU145 (*t* test, p< 0.0034). Interestingly this was only significantly less compared to aerobic irradiation in DU145 cells (*t* test, p=0.0147) and not 22Rv1 cells (*t* test, p=0.1163). Hypoxic exposure prior to irradiation significantly lead to increased intracellular ROS levels compared to controls in DU145 cells (*t* test, p < 0.0001) but not 22Rv1 cells (*t* test, p=0.0586).

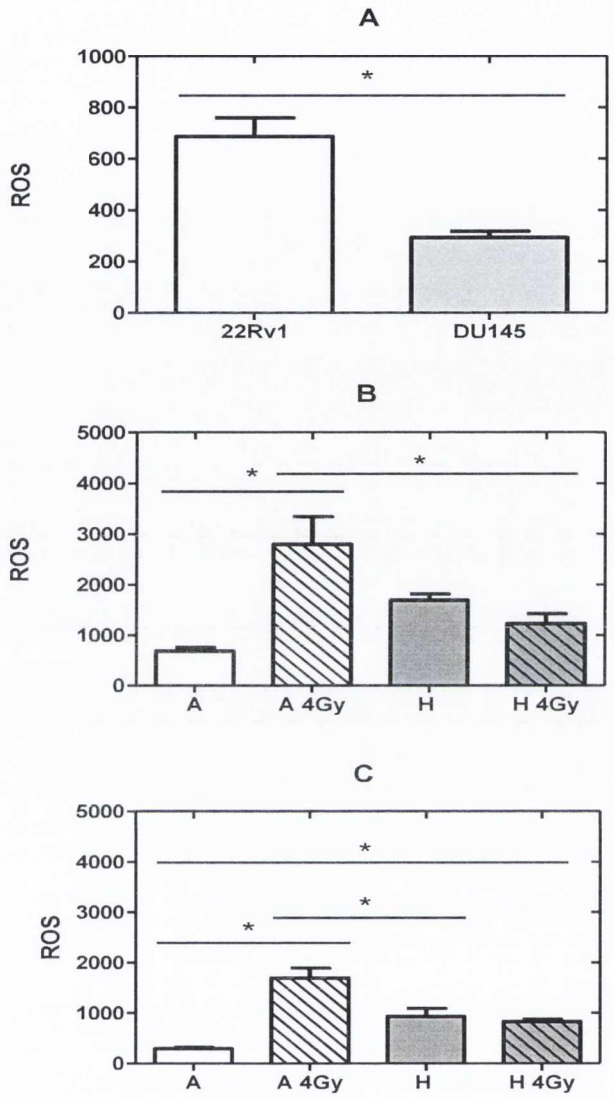



**Figure 13: Hypoxia decreases reactive oxygen species level**

The median ROS levels of aerobic or hypoxic (4 hrs) prostate cancer cells. (A) Comparison of baseline ROS for 22Rv1 and DU145 cells. (B) 22Rv1, (C) DU145 cells exposed to the delivery of single dose fractions (4 Gy). A=Aerobic, A 4 Gy=Aerobic irradiation, H=hypoxia, H 4 Gy=Hypoxia irradiation. N=3; Mean ± SEM; *$p<0.5$.

### 4.4.2 Impact of Hypoxia on Glutathione level

The effect of hypoxia on intracellular GSH levels was determined. Aerobic and hypoxic (4 hrs) 22Rv1 and DU145 cells were exposed to the delivery of single dose of radiation (4 Gy) and were immediately analysed for GSH levels. Intracellular GSH levels were detected by GSH-dependent luminescence (Figure 4.5). The response of aerobic cells and hypoxic cells were used as controls.

Irradiation with 4 Gy did not decrease the intracellular GSH levels of 22Rv1 compared to controls (*t* test $p= 0.1592$), irradiation did decrease the intracellular GSH levels in DU145 cells ($p= 0.0020$). Hypoxia significantly decreased the intracellular levels of GSH in both cell lines, 22Rv1 ($p= 0.0026$), DU145 (*t* test, $p < 0.0001$). Irradiation after hypoxia (4 hrs) only decreased this level further in 22Rv1 cells (*t* test, $p = 0.0148$) and not DU145 (*t* test, $p = 0.3590$). Baseline GSH levels were significantly higher in DU145 cells compared to 22Rv1 cells (*t* test, $p < 0.0001$)

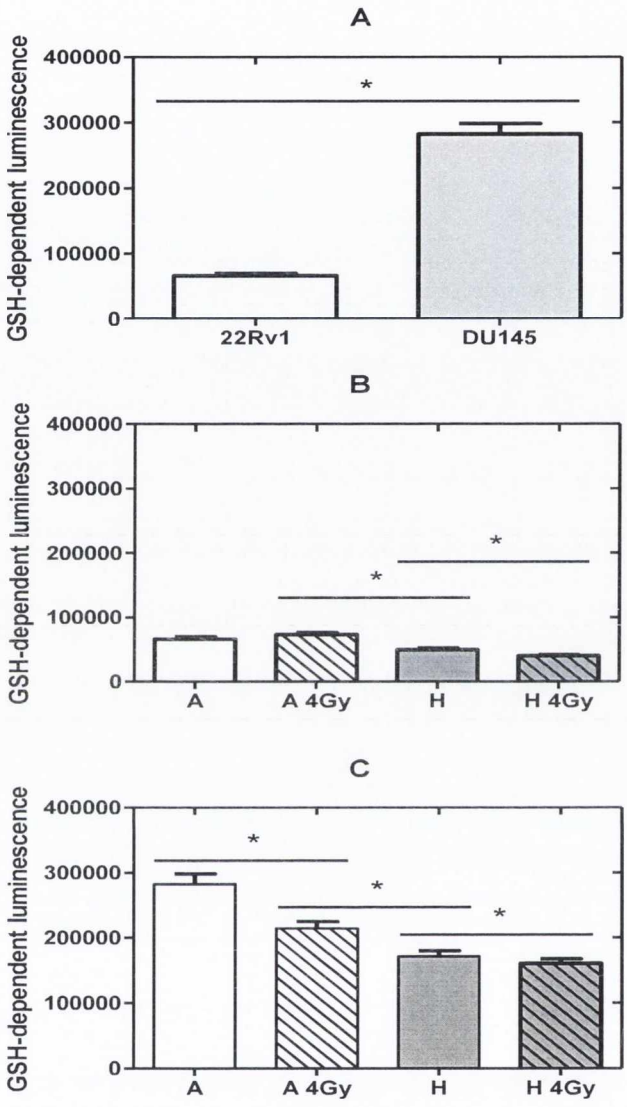



**Figure 14: Hypoxia decreases the intracellular GSH level**

The intracellular GSH levels of aerobic and hypoxic (4 hrs) prostate cancer cells. (A) Comparison of baseline GSH levels or 22Rv1 and DU145 cells. (B) 22Rv1, (C) DU145 cells exposed to the delivery of single dose fractions (4 Gy). (A=Aerobic, A 4Gy=Aerobic irradiation, H=hypoxia, H 4 Gy=Hypoxia irradiation. N=3; Mean ± SEM; *$p<0.5$.

## 4.5 The Role of HIF-1α in Radioresistance

The effect of hypoxia of HIF-1α protein expression was determined and correlated with VEGF protein expression. Aerobic and hypoxic (4 hrs) 22Rv1 and DU145 cells were exposed to the delivery of single dose of radiation (4 Gy) and allowed to recover in aerobic or hypoxic conditions for 24 hrs. Protein collection was then performed. HIF-1α and VEGF protein expression was then determined by Western blot analysis (Figure 4.6). The response of aerobic cells and hypoxic cells were used as controls.

HIF-1α protein expression was not demonstrated in aerobic samples of 22Rv1 and DU145 cells. Hypoxia (4 hrs) increased HIF-1α protein expression in both cell lines. Hypoxic 22Rv1 and DU145 cells that were irradiated also demonstrate HIF-1α protein expression. However cells that were placed into hypoxia post irradiation had the greatest levels of HIF-1α protein expression (Figure 4.6). The expression of HIF-1α was comparable between both cell lines. To confirm the effect of hypoxia VEGF protein expression was evaluated. VEGF protein expression was evident in both cell lines in all conditions. The expression of VEGF protein appears to greatest in cells that were incubated in hypoxia, specifically 22Rv1 and DU145 cells that were placed in post irradiation hypoxic recovery. Both cell lines responded the same regarding VEGF expression.

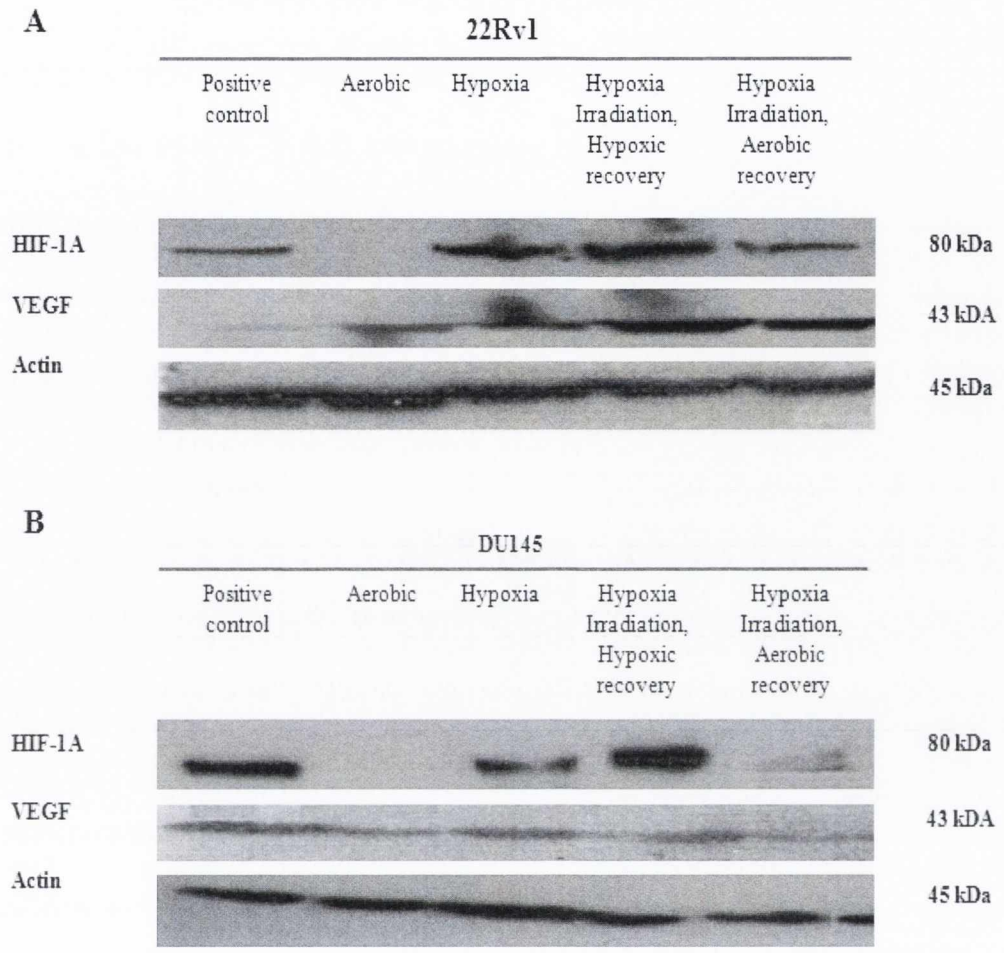



**Figure 15: Post-irradiation hypoxia increases HIF-1α and VEGF expression**

Representative HIF-1α, VEGF and β-actin immunoblots of aerobic and hypoxic (4 hrs) (A) 22Rv1 and (B) DU145 cells that were exposed to the delivery of single dose of radiation (4 Gy) and allowed to recover in aerobic or hypoxic conditions for 24 hrs.

### 4.6 HIF-1α Expression is Associated with a Survival Advantage

To evaluate the role of HIF-1α in radioresistance, prostate cancer cells were treated with $CoCl_2$. Aerobic and $CoCl_2$ treated media (4 hrs) 22Rv1, DU145 and PC3 cells were exposed to a single dose of radiation (4 Gy) and allowed to recover in aerobic or $CoCl_2$ treated media for 24 hrs. Survival was measured using clonogenic assays and clonogenic survival was determined by calculations shown in section 3.2.5 (Figure 4.7). The response of aerobic cells and cells reoxygenated following irradiation were used as a control.

Cells treated with $CoCl_2$ had increased radioresistance compared to aerobic controls in 22Rv1 (*t* test, p = 0.0287), DU145 (*t* test, p = 0.0002) and PC3 cells (*t* test, p = 0.0310) (Figure 4.6). Cells allowed to recover in $CoCl_2$ for 24 hrs post irradiation again had increased radioresistance compared to aerobic controls 22Rv1 (*t* test, p =0.0121), DU145 (*t* test, p = 0.0010) and PC3 (*t* test, p = 0.0163). Post-irradiation $CoCl_2$ treatment did not increase the radioresistance further compared to cells treated with $CoCl_2$ prior to irradiation alone, 22Rv1 (*t* test, p =0.2180), DU145 (*t* test, p =0.3754), PC3 (*t* test, p =0.8421).

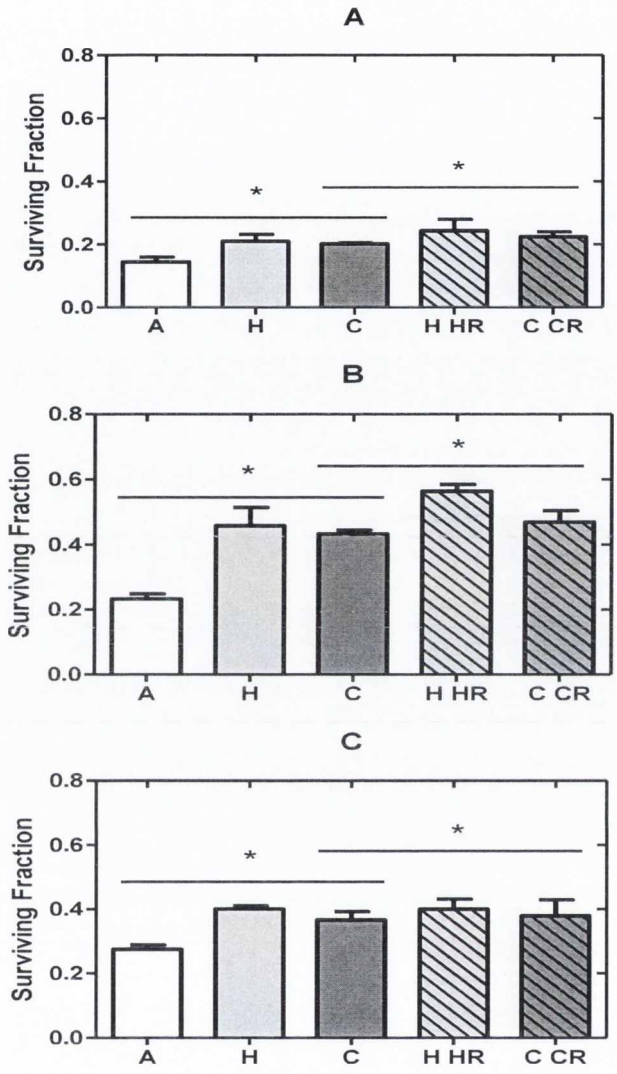



**Figure 16: CoCl2 treatment increases the radioresistance**

Surviving fraction of aerobic and $CoCl_2$ treated (A) 22Rv1, (B) DU145 and (C) PC3 cells exposed to the delivery of single dose fractions (4 Gy) and allowed to recover in aerobic and $CoCl_2$ treated media for 24 hrs. A=Aerobic irradiation, aerobic recovery, H = Hypoxic irradiation, aerobic recovery, A HR = Aerobic irradiation, hypoxic recovery, H HR = Hypoxic irradiation, hypoxic recovery. N=3; Mean ± SEM; *$p<0.5$.

## 4.7 $CoCl_2$ Increases HIF-1α Protein Expression

The effect of $CoCl_2$ treatment on HIF-1α protein expression was determined and correlated with VEGF protein expression. Aerobic and $CoCl_2$ treated (4 hrs) 22Rv1 and DU145 cells were exposed to the delivery of single dose of radiation (4 Gy) and allowed to recover in aerobic or $CoCl_2$ for 24 hrs. Protein collection was then performed. HIF-1α and VEGF protein expression was then determined by Western blot analysis (Figure 4.9). The response of positive controls, aerobic cells and hypoxic cells were used as controls.

HIF-1α protein expression was not demonstrated in aerobic samples of 22Rv1 and DU145 cells. $CoCl_2$ treatment (4 hrs) increases HIF-1α protein expression in both cell lines. $CoCl_2$ treated 22Rv1 and DU145 cells that were irradiated also demonstrate HIF-1α protein expression. However cells that were placed into $CoCl_2$ treated media post irradiation had the greatest levels of HIF-1α protein expression (Figure 4.8).The expression of HIF-1α was comparable between both cell lines. To confirm the effect of hypoxia VEGF protein expression was evaluated. VEGF protein expression was evident in both cell lines in all conditions. The expression of VEGF protein appears larger in cells that were incubated with $CoCl_2$, specifically 22Rv1 and DU145 cells that were placed in $CoCl_2$ for post irradiation recovery. Both cell lines responded the same regarding VEGF expression. These results are similar to cells exposed to hypoxia regarding HIF-1α and VEGF protein expression.

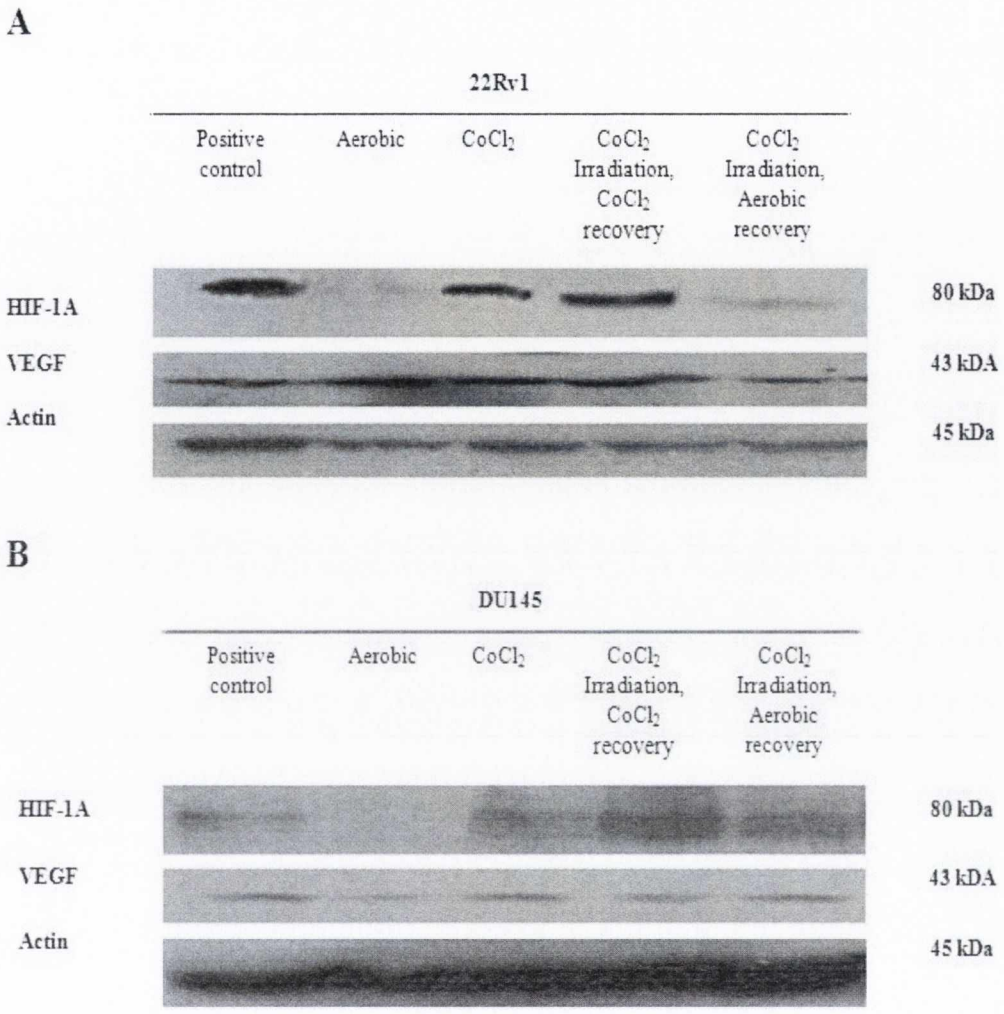



**Figure 17: CoCl2 treatment increases HIF-1α and VEGF expression**

Representative HIF-1α, VEGF and β-actin immunoblots of aerobic and $CoCl_2$ treated (4 hrs) (A) 22Rv1 and (B) DU145 cells that were exposed to the delivery of single dose of radiation (4 Gy) and allowed to recover in aerobic or $CoCl_2$ media for 24 hrs.

## 4.8 Impact of DNA Repair in Hypoxic Radioresistance

### 4.8.1 Split Dose Radiation Treatments do no Alter DNA repair

The impact of post irradiation hypoxic recovery on DNA repair was determined using split dose experiments. Aerobic and hypoxic (4 hrs) 22Rv1, DU145 and PC3 cells were exposed to the delivery of single dose fractions 4 Gy or 2 Gy x 2 separated by a 2 hr-time interval and allowed to recover in aerobic or hypoxic conditions for 24 hrs. Survival was measured using clonogenic assays and radiation survival curves were generated (Figure 4.9). The response of aerobic cells and cells reoxygenated following irradiation were used as a control.

The survival of aerobic cells was not increased in response to 2 Gy x 2 versus 4 Gy, 22Rv1 (*t* test, p = 0.8920), DU145 (*t* test, p = 0.7930) and PC3 (*t* test, p = 0.6359). Pre-irradiation hypoxia did not increase the surviving fraction of 22Rv1 (*t* test, p = 0.4834), DU145 (*t* test, p = 0.9434) and PC3 (*t* test, p = 0.3521). Post-irradiation hypoxic recovery of aerobically irradiated cells did not increase the surviving fraction of 22Rv1 (*t* test, p = 0.1358), DU145 (*t* test, p = 0.2851) and PC3 (*t* test, p = 0.0895) compared to a single dose of radiation. Post-irradiation hypoxic recovery of hypoxic cells did not increase the surviving fraction of 22Rv1 (*t* test, p = 0.6364), DU145 (*t* test, p = 0.4938) and PC3 (*t* test, p = 0.3093). Split doses of radiation did not alter the survival of 22Rv1 (2way ANOVA, p = 0.5878), DU145 (2way ANOVA, p = 0.3345) and PC3 (2way ANOVA, p = 0.1561).

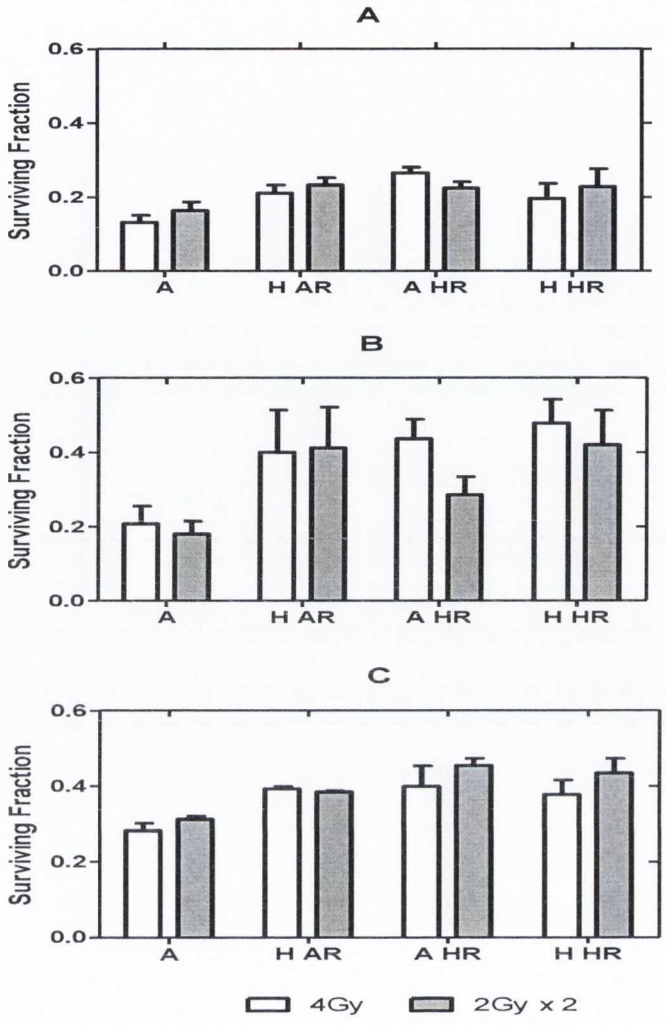



**Figure 18: Split doses of radiation do not increase radioresistance**

Surviving fraction of (A) 22Rv1, (B) DU145 and (C) PC3 cells exposed to dose fractions 4 Gy and 2 Gy x 2 separated by 2 hr time interval and allowed to recover in aerobic or hypoxic conditions for 24 hrs. A=Aerobic irradiation, aerobic recovery, H = Hypoxic irradiation, aerobic recovery, A HR = Aerobic irradiation, hypoxic recovery, H HR = Hypoxic irradiation, hypoxic recovery N=3; Mean ± SEM; *$p<0.05$.

### 4.8.2 Increased Inter-fraction Times does not Effect DNA Repair

To evaluate if DNA repair was occurring at increased time points, split dose experiments were performed with increased inter-fraction times. Aerobic and hypoxic (4 hrs) 22Rv1, DU145 and PC3 cells were exposed to a single dose fraction 4 Gy or 2 Gy x 2 separated by increasing inter-fraction time(s) (0, 0.5, 2, 4, 6, 8 hrs) and allowed to recover in aerobic or hypoxic conditions for 24 hrs. Survival was measured using clonogenic assays and radiation survival curves were generated (Figure 4.10). The response of aerobic cells and cells reoxygenated following irradiation were used as a control.

Increasing inter-fraction times did not increase the surviving fraction of aerobically irradiated cells, (*0.5 hr versus 8 hr*), 22Rv1 (*t* test, p = 0.4791), DU145 (*t* test, p = 0.6841), PC3 (*t* test, p = 0.9594). Increasing inter-fraction times also did not increase the surviving fraction of hypoxic cells (*0.5 hr versus 8 hr*), 22Rv1 (*t* test, p = 0.4548), DU145 (*t* test, p = 0.2449), PC3 (*t* test, p = 0.4589) (Figure 4.8). There was no increased survival across all times points of aerobic or hypoxic cells, 22Rv1 (ANOVA, p = 0.4554), DU145 (ANOVA, p = 0.4729) and PC3 (ANOVA, p = 0.1499).

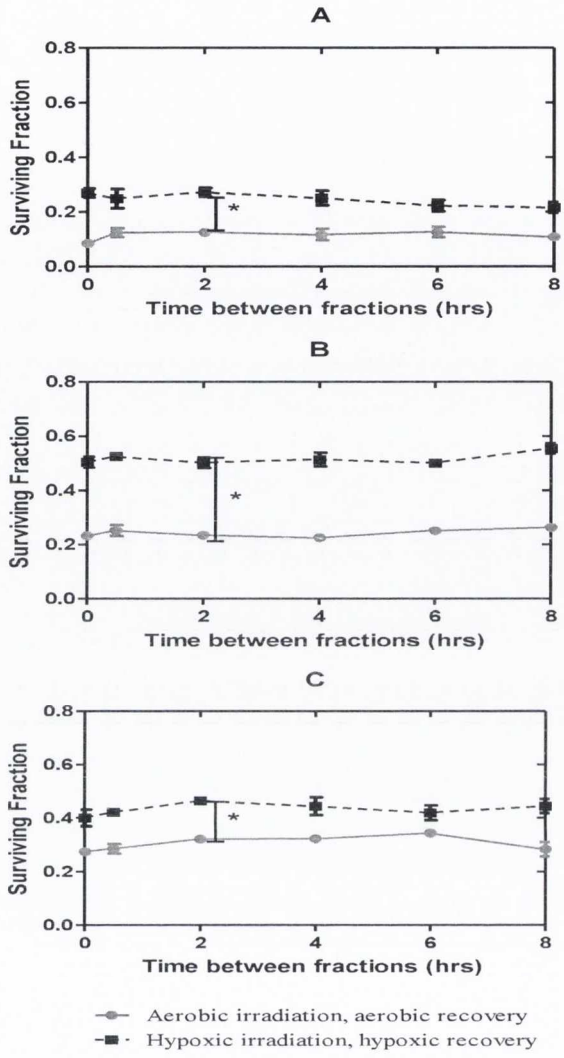



**Figure 19: Increased inter-fraction times demonstrate that no repair is occurring**

Surviving fraction of (A) 22Rv1, (B) DU145 and (C) PC3 cells exposed to dose fractions 4 Gy and 2 Gy x 2 separated by increasing time intervals (0, 0.5, 2, 4, 6, 8 hrs) and allowed to recover in aerobic or hypoxic conditions for 24 hrs. N=3; Mean ± SEM; *$p<0.05$.

### 4.8.3 Split Doses Treatments do not Alter HIF-1α Expression

The effect of split doses of radiation and hypoxia on HIF-1α protein expression was determined and correlated with VEGF protein expression. Aerobic and hypoxic (4 hrs) 22Rv1 and DU145 cells were exposed to the delivery of single (4 Gy) and split doses of radiation (2 Gy x 2 separated by 2 hrs) and allowed to recover in aerobic or hypoxic conditions for 24 hrs. Protein collection was then performed. HIF-1α and VEGF protein expression was then determined by Western blot analysis (Figure 4.12).The response of positive controls, aerobic cells and hypoxic cells were used as controls.

HIF-1α protein expression was not demonstrated in aerobic samples of 22Rv1 and DU145 cells. Hypoxia (4 hrs) induced HIF-1α protein expression in both cell lines. Hypoxic 22Rv1 and DU145 cells that were irradiated also demonstrated HIF-1α protein expression. Hypoxic recovery post-irradiation induced the greatest levels of HIF-1α protein expression in both cell lines. Split dose irradiation of 2 Gy x 2 separated by 2 hrs did not reduce HIF-1α protein expression in both cell lines (Figure 4.9). To confirm the effect of hypoxia, VEGF protein expression was evaluated. VEGF protein expression was evident in both cell lines in all conditions. The expression of VEGF protein appears to greatest in cells that were in hypoxic recovery post irradiation. Split dose irradiation of 2 Gy x 2 separated by 2 hrs does not reduce VEGF protein expression in both cell lines (Figure 4.11).

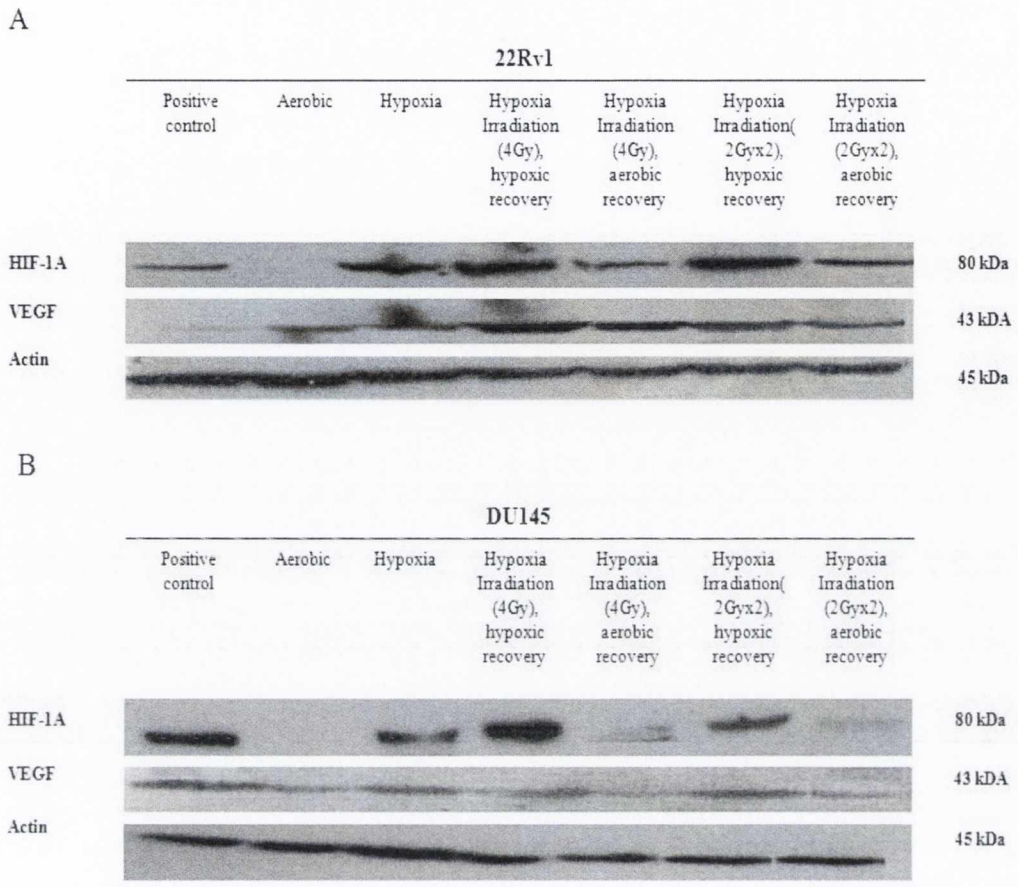



**Figure 20: HIF-1α and VEGF protein expression is not affected by split doses**

Representative HIF-1α, VEGF and β-actin immunoblots of aerobic and hypoxic (4 hrs) (A) 22Rv1 and (B) DU145 cells that were exposed to the delivery of single dose of radiation 4 Gy or a split dose 2 Gy x 2 separated by 2 hrs) and allowed to recover in aerobic or hypoxic conditions for 24hrs.

### 4.8.4 Hypoxia Decreases MRE11 Protein Expression

The effect of hypoxia of MRE11 protein expression was determined. Aerobic and hypoxic (4 hrs) 22Rv1 and DU145 cells were exposed to the delivery of single dose of radiation (4 Gy) and allowed to recover in aerobic or hypoxic conditions for 24 hrs. Protein collection was then performed. MRE11 protein expression was then determined by Western blot analysis (Figure 4.12). The response of positive controls, aerobic cells and hypoxic cells were used as controls.

The expression of MRE11 protein was not demonstrated in 22Rv1 cells, DU145 cells did express MRE11. Hypoxia decreased the expression of MRE11 in DU145 cells compared to aerobic control cells. MRE11 protein expression was increased in aerobically irradiated cells that were placed into aerobic conditions for recovery. MRE11 expression was also induced in cells that were placed into hypoxia for post-irradiation recovery. DU145 cells that were hypoxic prior to irradiation and placed in aerobic or hypoxic conditions for recovery had the lowest levels of MRE11 protein expression.

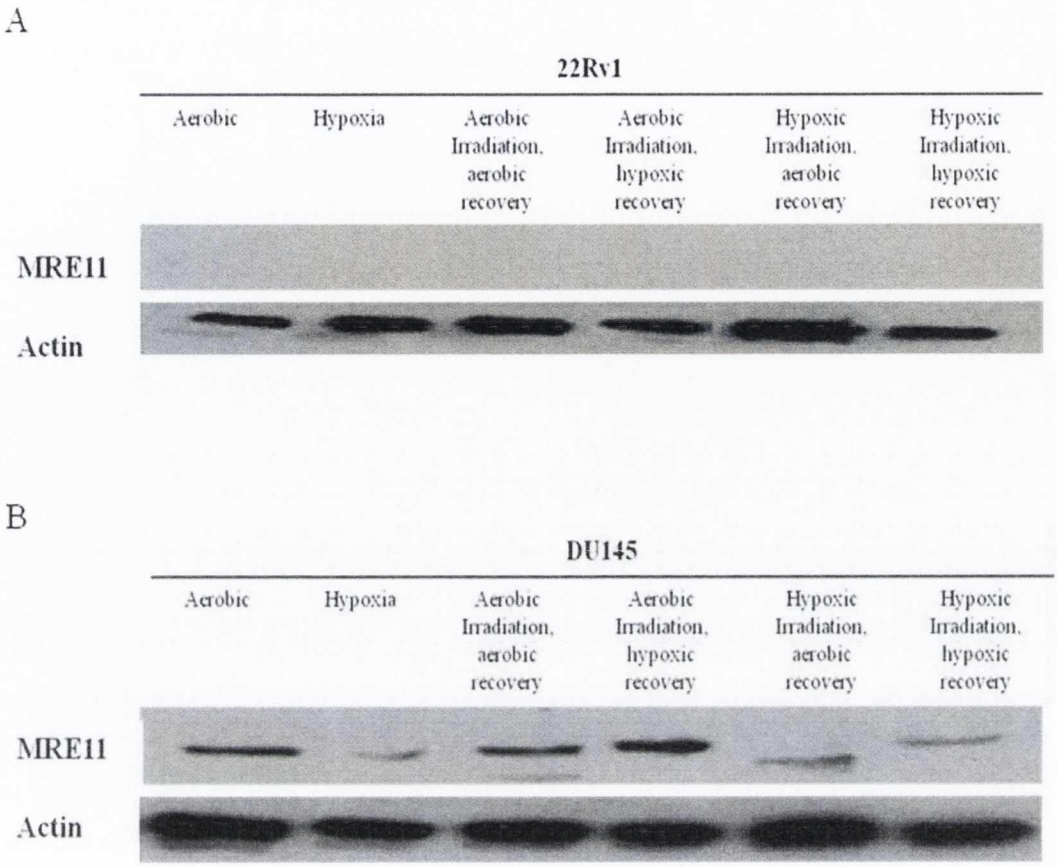



**Figure 21: Hypoxia decreases MRE11 protein expression**

Representative MRE11 and β-actin immunoblots of aerobic and hypoxic (4 hrs) (A) 22Rv1 and (B) DU145 cells that were exposed to the delivery of single dose of radiation 4 Gy and allowed to recover in aerobic or hypoxic conditions for 24 hrs.

### 4.8.5 HIF-1α can Decrease MRE11 expression

The effect of HIF-1α on MRE11 protein expression was determined. Aerobic, hypoxic and $CoCl_2$ treated (4 hrs) 22Rv1 and DU145 cells were exposed to the delivery of single dose of radiation (4 Gy) and allowed to recover in aerobic, hypoxic or $CoCl_2$ treated media for 24 hrs. Protein collection was then performed. MRE11 protein expression was then determined by Western blot analysis (Figure 4.13). The response of positive controls, aerobic cells and hypoxic cells were used as controls.

The expression of MRE11 was again not demonstrated in 22Rv1 cells, DU145 did express MRE11 protein. Hypoxic irradiation and post-irradiation hypoxic recovery decreased the expression of MRE11 protein. $CoCl_2$ exposure prior to irradiation and $CoCl_2$ for post-irradiation recovery also decreased the expression of MRE11.

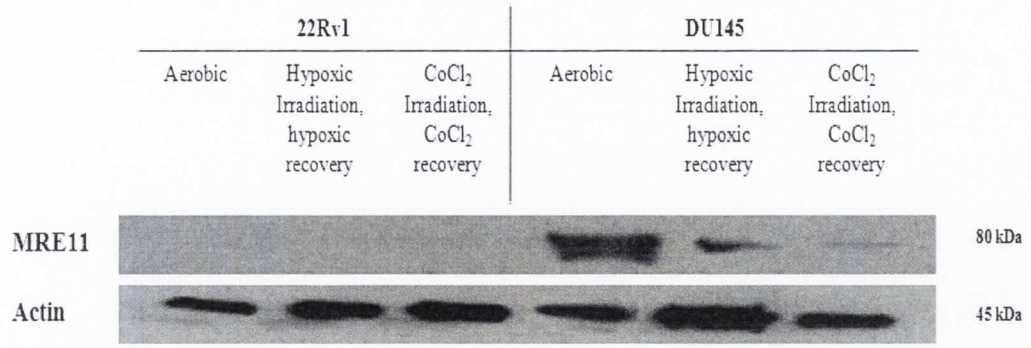



**Figure 22: HIF-1α decreases the expression of MRE11 protein expression**

Representative MRE11 and β-actin immunoblots of aerobic and $CoCl_2$ treated (4 hrs) 22Rv1 and DU145 cells that were exposed to the delivery of single dose of radiation 4 Gy and allowed to recover in aerobic, hypoxic or $CoCl_2$ treated media for 24 hrs.

## 4.9 Post-Irradiation Hypoxic Recovery Effects the Cell Cycle

The impact of post irradiation hypoxic recovery on cell cycle was determined. Aerobic and hypoxic (4 hrs) 22Rv1, DU145 cells were exposed to a single dose of radiation (4 Gy) and allowed to recover in aerobic or hypoxic conditions for 24 hrs. The presence of cell cycle arrest was determined using flow cytometry (Figure 4.14/4.15/4.16). The response of aerobic cells and cells reoxygenated following irradiation were used as a control.

Hypoxia does not affect the number of 22Rv1 cells in $G_1$, (1way ANOVA, p= 0.8229). Hypoxia decreases significantly the number of 22Rv1 cells in S phase (1way ANOVA, p= 0.0322). Hypoxia did not induce a $G_2$/M phase cell cycle arrest in 22Rv1 (1way ANOVA, p= 0.1849). However when compared to aerobic controls there were significantly more cells in $G_2$/M phase that were placed into post-irradiation hypoxic recovery 22Rv1, (*t* test p =0.0405) (Figure 4.15). DU145 cells responded differently, hypoxia induced a $G_1$ phase arrest (1way ANOVA, p= 0.0441), decreased the number of cells in S phase (1way ANOVA, p= 0.0006) and induced a $G_2$/M phase arrest (1way ANOVA, p= 0.0077) (Figure 4.14/4.15).

Compared to aerobic controls hypoxia significantly decreases the number of 22Rv1 cells in S phase (*t* test p =0. 0.0015) and increased the number of cells in $G_2$/M (*t* test p =0.0405). Hypoxia similarly decreased the number of DU145 cells in S phase (*t* test p = 0.0002) and increased the number of cells in $G_2$/M (*t* test p = 0.0331) (Figure 4.16)

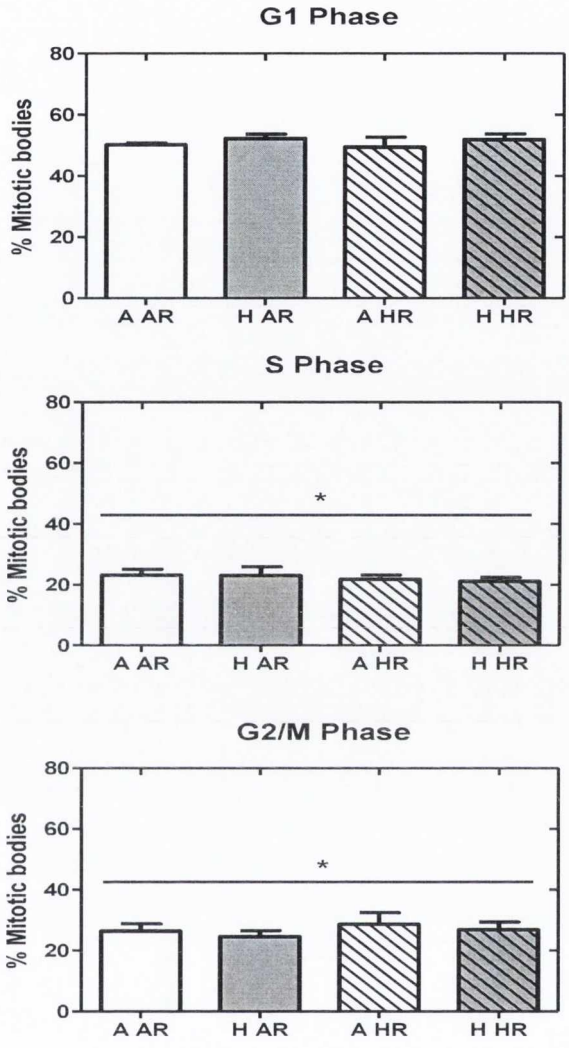



**Figure 23: Hypoxia causes a $G_2$/M arrest in 22Rv1 cells**

Cell cycle phase distribution of aerobic and hypoxic 22Rv1 cells exposed to the delivery of single dose fractions (4 Gy) and allowed to recover in aerobic or hypoxic conditions for 24 hrs. A AR=Aerobic irradiation, aerobic recovery, H AR= Hypoxic irradiation, aerobic recovery, A HR = Aerobic irradiation, hypoxic recovery, H HR = Hypoxic irradiation, hypoxic recovery. N=3; Mean ± SEM; *p<0

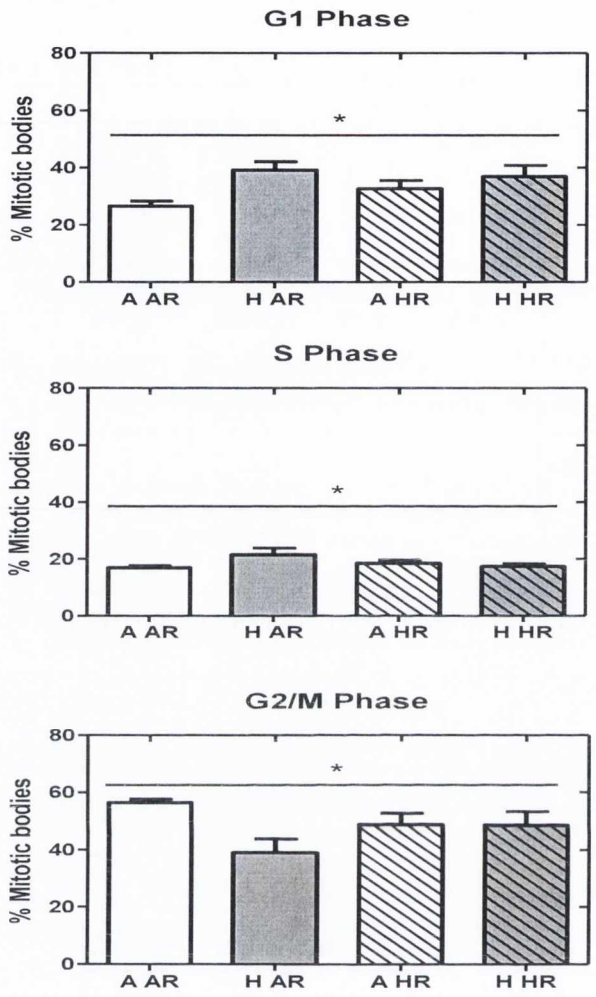



**Figure 24: Hypoxia causes a $G_2$/M arrest in DU145 cells**

Cell cycle phase distribution of aerobic and hypoxic DU145 cells exposed to the delivery of single dose fractions (4 Gy) and allowed to recover in aerobic or hypoxic conditions for 24 hrs. A AR=Aerobic irradiation, aerobic recovery, H AR= Hypoxic irradiation, aerobic recovery, A HR = Aerobic irradiation, hypoxic recovery, H HR = Hypoxic irradiation, hypoxic recovery. N=3; Mean ± SEM; *$p<0.5$.

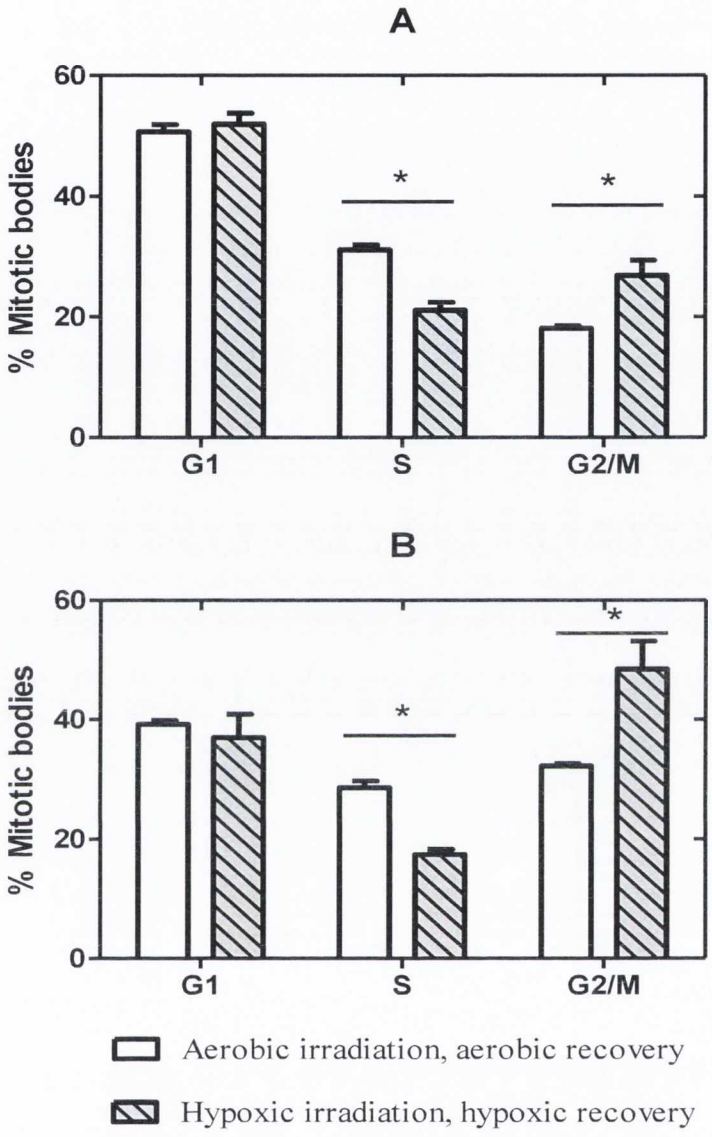



**Figure 25: Hypoxia induces $G_2$/M arrest and decreases cells in S phase**

Cell cycle phase distribution of hypoxic (A) 22Rv1 and (B) DU145 exposed to the delivery of single dose fractions (4 Gy) and allowed to recover in aerobic or hypoxic conditions for 24 hrs, compared to aerobic controls. N=3; Mean ± SEM; *p<0.5.

### 4.9.1 Post-Irradiation hypoxia can Decreases Expresion of Cell Cycle check Point Proteins

The effect of hypoxia and HIF-1α on CHK1 and CHK2 protein expression was determined. Aerobic, hypoxic and $CoCl_2$ treated (4 hrs) 22Rv1 and DU145 cells were exposed to the delivery of single dose of radiation (4 Gy) and allowed to recover in aerobic, hypoxic or $CoCl_2$ treated media for 24 hrs. Protein collection was then performed. CHK1 and CHK2 protein expression was then determined by Western blot analysis (Figure 4.17). The response of positive controls, aerobic cells and hypoxic cells were used as controls.

Expression of CHK1 and CHK2 proteins was comparable between 22Rv1 and DU145 cells. Hypoxia pre-irradiation and hypoxic recovery post-irradiation increased the the expression of CHK1 and CHK2 proteins in both cell lines. $CoCl_2$ treatment pre and post irradiation increases expression of CHK2 in both cell lines. $CoCl_2$ treatment pre and post irradiation decreased expression of CHK1 in both 22Rv1 and DU145 cells.

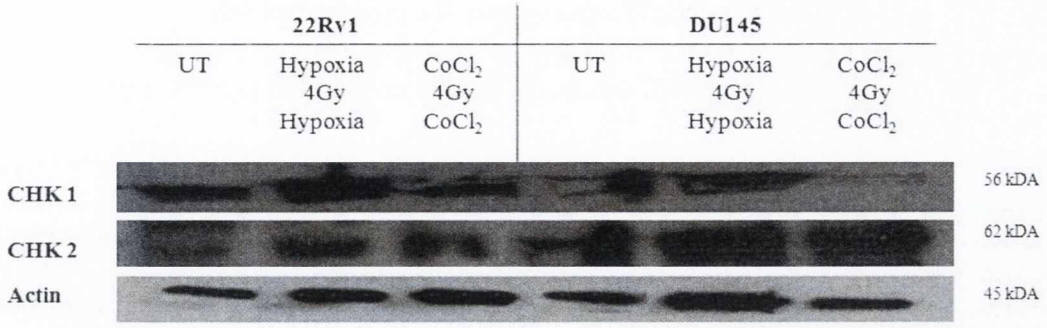



**Figure 26: Hypoxia and HIF-1α increases CDK 2 protein level**

Representative CHK1, CHK2 and β-actin immunoblots of aerobic, hypoxic and $CoCl_2$ treated (4 hrs) 22Rv1 and DU145 cells that were exposed to the delivery of single dose of radiation (4 Gy) and allowed to recover in aerobic, hypoxia or $CoCl_2$ treated media for 24 hrs.

### 4.10 Post-Irradiation Hypoxic Recovery can Reduce Apoptosis

The effect of hypoxia on apoptosis was determined. Aerobic or hypoxic (4 hrs) 22Rv1 and DU145 cells were exposed to the delivery of single dose of radiation (4 Gy) and allowed to recover in aerobic or hypoxic conditions for 24 hrs. Percentage apoptosis was determined by an Annexine V/Propidium Iodide Staining and FITC flow cytometry (Figure 4.19). The response of aerobic cells and hypoxic cells were used as controls.

Hypoxic (4 hrs) exposure prior to irradiation did not decreased apoptosis in 22Rv1 and DU145 cells compared to irradiated control cells, 22Rv1 cells (*t* test $p = 0.1548$), DU145 cells (*t* test $p = 0.0758$). Post-irradiation hypoxic recovery in both cell lines, 22Rv1 cells (*t* test $p = 0.0307$), DU145 cells (*t* test $p = 0.0320$) (Figure 4.18).

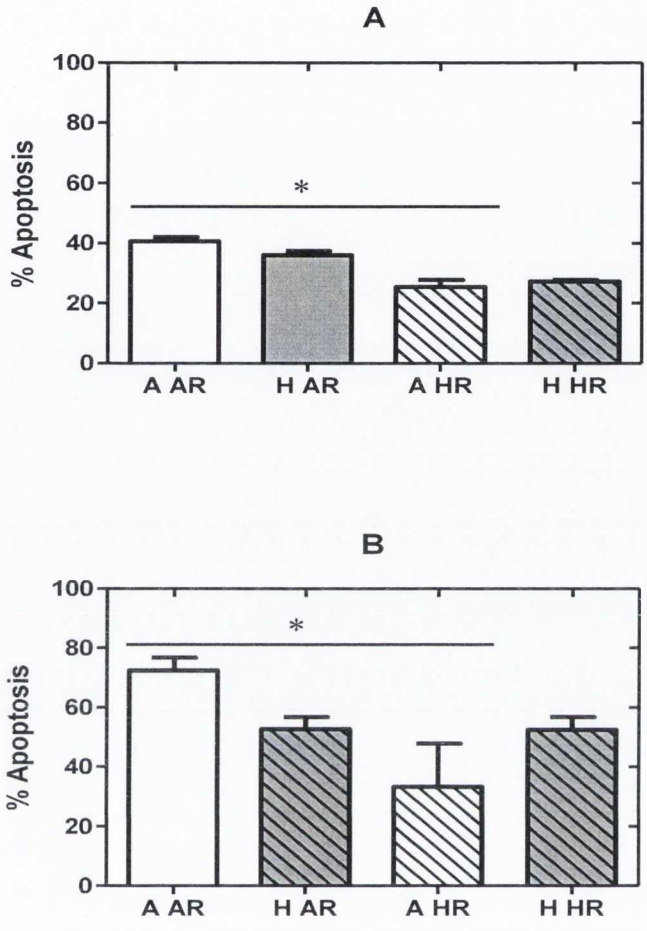



**Figure 27: Post-irradiation hypoxia decreases apoptosis**

Percentage apoptosis of aerobic or hypoxic (A) 22Rv1 and (B) DU145 cells exposed to the delivery of single dose fractions (4 Gy) and allowed to recover in aerobic or hypoxic conditions for 24 hrs. N=3; Mean ± SEM; *p<0.05. A AR=Aerobic irradiation, aerobic recovery, H AR= Hypoxic irradiation, aerobic recovery, A HR = Aerobic irradiation, hypoxic recovery, H HR = Hypoxic irradiation, hypoxic recovery. N=3; Mean ± SEM; *p<0.5.

### 4.10.1 Post-Irradiation Hypoxia can Decrease p53 Expression

The effect of hypoxia on p53 protein expression was determined. Aerobic and hypoxic (4 hrs) 22Rv1 and DU145 cells were exposed to the delivery of single dose of radiation (4 Gy) and allowed to recover in aerobic and hypoxic conditions for 24hrs. Protein collection was then performed. p53 protein expression was then determined by Western blot analysis (Figure 4.20). The response of positive controls, aerobic cells and hypoxic cells were used as controls.

22Rv1 cells (Figure 4.20 A) expressed p53 protein. Aerobic and hypoxic samples expressed minimal amounts of p53. Irradiation of aerobic samples increased p53 expression. Hypoxic exposure prior to, or post-irradiation hypoxic recovery increases p53 expression the most in 22Rv1 cells. Hypoxic exposure prior to irradiation and post irradiation hypoxic recovery reduced the expression of p53. DU145 cells (Figure 4.19 B) expressed p53 protein differently. Aerobic DU145 cells expressed p53, hypoxic DU145 cells did not. Irradiation increased the expression of p53, DU145 cells that were either hypoxic before or after irradiation expressed the largest amounts of p53 protein. Hypoxic exposure prior to irradiation and post irradiation hypoxic recovery reduced the expression of p53 in DU145 cells.

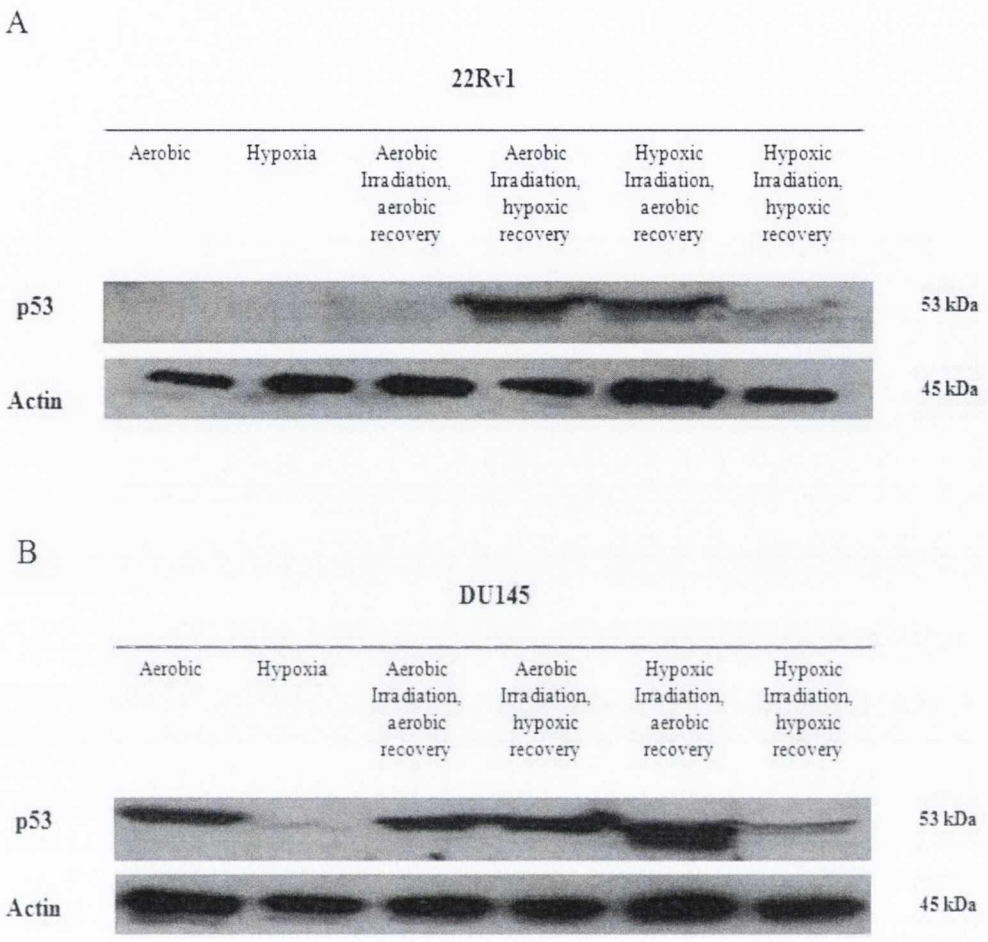



**Figure 28: Post-irradiation hypoxia recovery decreases expression of p53**

Representative p53 and β-actin immunoblots of aerobic and hypoxic (4 hrs) (A) 22Rv1 and (B) DU145 cells that were exposed to the delivery of single dose of radiation (4 Gy) and allowed to recover in aerobic or hypoxic conditions for 24 hrs.

### 4.10.2 HIF-1α Expression can Effect p53 expression

The effect of HIF-1α on p53 protein expression was determined. Aerobic, hypoxic and $CoCl_2$ treated (4 hrs) 22Rv1 and DU145 cells were exposed to the delivery of single dose of radiation (4 Gy) and allowed to recover in aerobic, hypoxic or $CoCl_2$ treated media for 24 hrs. Protein collection was then performed; p53 protein expression was then determined by Western blot analysis (Figure 4.20). The response of aerobic cells was used as controls.

Expression of p53 was not comparable between 22Rv1 and Du145 cells; p53 expression was greater in DU145 aerobic control cells compared to 22Rv1 aerobic control cells. In both cell lines hypoxic irradiation followed by hypoxic recovery for 24hrs reduced the expression of p53. This effect was more evident in DU145 cells than 22Rv1 cells. $CoCl_2$ treatment affected the expression of p53 in both cell lines. $CoCl_2$ reduced the expression of p53 in 22Rv1 cells, but significantly more in DU145 cells.

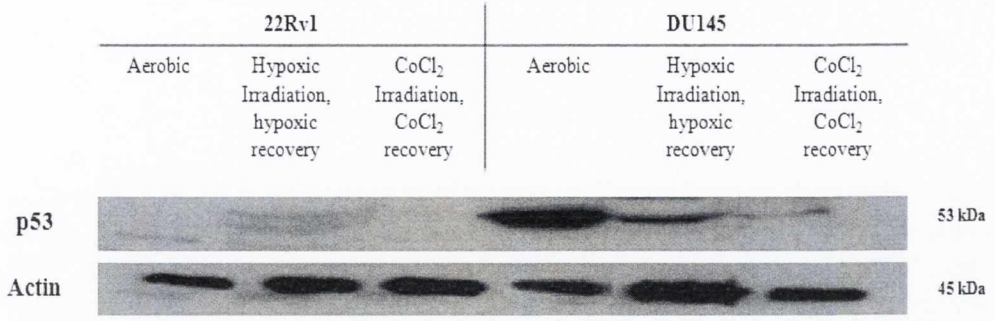



**Figure 29: HIF-1α decreases the expression of p53 protein expression**

Representative p53 and β-actin immunoblots of aerobic, hypoxic and $CoCl_2$ treated (4 hrs) 22Rv1 and DU145 cells that were exposed to the delivery of single dose of radiation (4 Gy) and allowed to recover in aerobic, hypoxic or $CoCl_2$ treated media for 24 hrs.

### 4.11 Hypoxia can Reduces Cellular Senescence

The effect of hypoxia on cellular senescence was determined. Aerobic and hypoxic (4 hrs) 22Rv1 and DU145 cells were exposed to the delivery of single dose of radiation (4 Gy) and allowed to recover in aerobic or hypoxic conditions for 24 hrs. The presence of senescent cells was determined by histochemically detection of β-galactosidase (SA-β-Gal) activity (Figure 4.22). The response of positive controls, aerobic cells and hypoxic cells were used as controls.

The amount of cellular senescence differed between each cell line. The percentage of DU145 cells in the senescent state was significantly lower than 22Rv1 cells Table 4.1 shows that mean baseline cellular senescence of 22Rv1 cells was 15.4±0.9% and DU145 cells was 8.7±2.4%. Oxygenated 22Rv1 and DU145 that were irradiated and allowed to recover in aerobic conditions demonstrated increased cellular senescence compared to controls. Percentage of irradiated senescent 22Rv1 cells was 24.9±2.1% and DU145 cells were 11.73±1.3%. Post-irradiation hypoxia reduced the percentage of cells in the senescent state in both cell lines compared to irradiated control cells. Percentage of 22Rv1 that were senescent was 9.314±1.0% and DU145 was 5.536±0.74. This was a statistically significant reduction in cellular senescence, 22Rv1 (*t* test p =0.0003), DU145 cells (*t* test p =0.0020) (Figure 4.21).

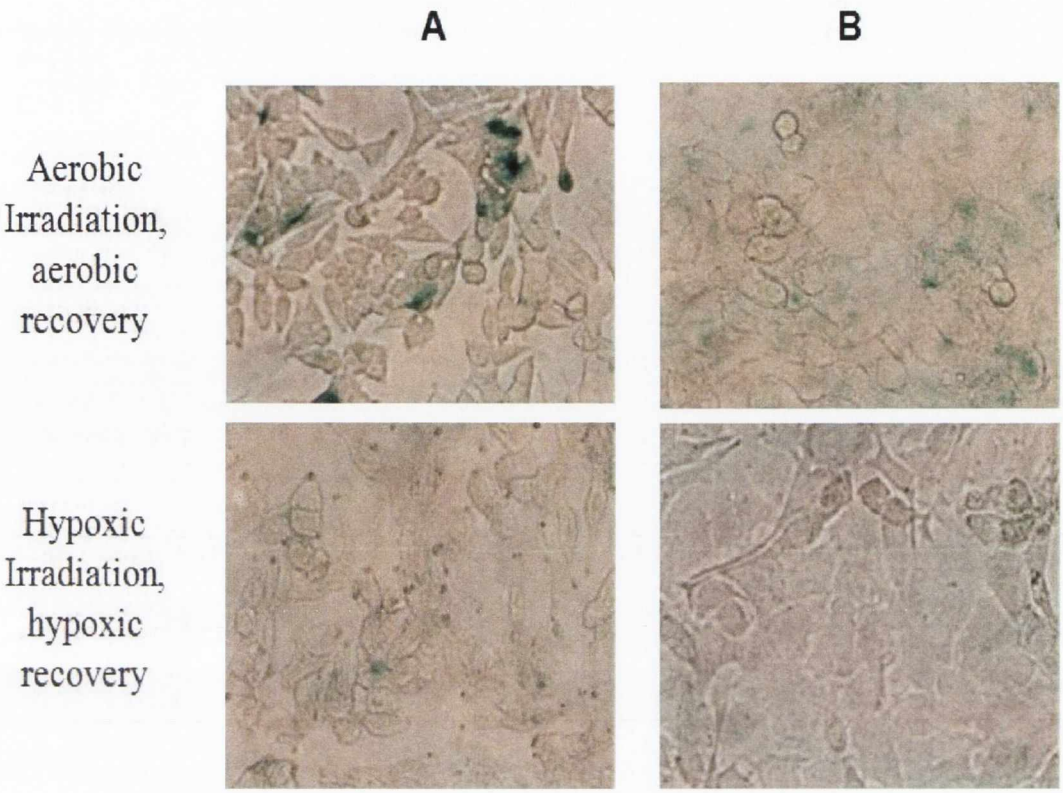



**Figure 30: Hypoxia reduces the amount of cells in the senescent state**

Representative histochemical stain for SA-β-Gal activity (green) in aerobic and hypoxic (4 hrs) (A) 22Rv1 and (B) DU145 cells that were exposed to the delivery of single dose of radiation (4 Gy) and allowed to recover in aerobic or hypoxic conditions for 24 hrs.

| | % cells expressing SA-β-Gal | | | |
|---|---|---|---|---|
| 22Rv1 | Repeat 1 | Repeat 2 | Repeat 3 | Mean |
| Control | 15.6 | 14.4 | 16.3 | 15.4±0.9 |
| A AR | 27.3 | 23.5 | 24.3 | 24.9±2.1 |
| H HR | 8.5 | 10.6 | 9.5 | 9.314±1.0 |
| DU145 | Repeat 1 | Repeat 2 | Repeat 3 | Mean |
| Control | 8.5 | 11.0 | 6.5 | 8.7±2.4 |
| A AR | 12.8 | 10.2 | 12.3 | 11.73±1.3 |
| H HR | 4.9 | 6.3 | 5.5 | 5.536±0.74 |

**Table 4.1: Table summarising SA-β-Gal expression of Figure 4.31**

22Rv1 and DU145 cells were observed under a microscope for the development of a green colour (marker of SA-β-Gal activity) at 200X magnification. A minimum of 200 cells was scored at random according to presence of SA-βgal staining. Table above represents percentage of cells that were senescent for each given condition. A AR = Aerobic irradiation, hypoxic recovery, H HR = Hypoxic irradiation, hypoxic recovery. Mean ± SEM.

### 4.11.1 Hypoxia and HIF-1α can reduce $p16^{ink4a}$ Expression

The effect of HIF-1α on $p16^{ink4a}$ protein expression was determined. Aerobic, hypoxic and $CoCl_2$ treated (4hrs) 22Rv1 and DU145 cells were exposed to the delivery of single dose of radiation (4 Gy) and allowed to recover in aerobic, hypoxic or $CoCl_2$ treated media for 24 hrs. Protein collection was then performed; $p16^{ink4a}$ protein expression was then determined by Western blot analysis (Figure 4.22). The response aerobic cells were used as controls.

Expression of $p16^{ink4a}$ was not comparable between 22Rv1 and Du145 cells; $p16^{ink4a}$ expression was greater in DU145 aerobic control cells compared to 22Rv1 aerobic control cells. In both cell lines hypoxic irradiation followed by hypoxic recovery for 24 hrs reduced the expression of $p16^{ink4a}$. This effect was more evident in DU145 cells than 22Rv1 cells. $CoCl_2$ treatment affected the expression of $p16^{ink4a}$ in both cell lines. $CoCl_2$ reduced the expression of $p16^{ink4a}$ in 22Rv1 cells, but significantly more in DU145 cells.

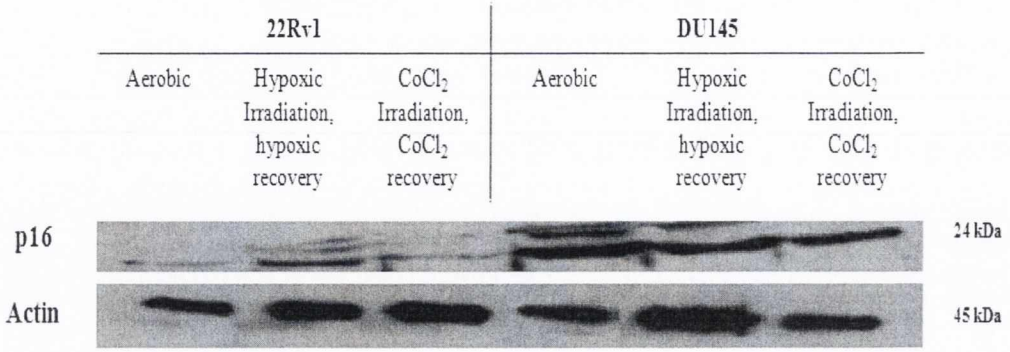



**Figure 31: Hypoxia decreases p16ink4a protein expression**

Representative $p16^{ink4a}$ and β-actin immunoblots of aerobic, hypoxic and $CoCl_2$ treated (4 hrs) 22Rv1 and DU145 cells that were exposed to the delivery of single dose of radiation (4 Gy) and allowed to recover in aerobic, hypoxic or $CoCl_2$ treated media for 24 hrs.

# Chapter 5

# Discussion

# 5 Discussion

## 5.1 Hypoxia in Prostate Cancer

It is clear that the incidence of prostate cancer is increasing; in Ireland by 2020 the number of cases is expected to reach 6,330. This rise is attributed to the widespread use of PSA as a screening tool. This increase in incidence is associated with the early detection of prostate cancer and the stage migration from advanced disease to localised and curable prostate cancer. It is now apparent that many of these cancers are biologically insignificant and a proportion of patients are being over treated. The current challenge facing clinicians and scientists is the identification of patients who will benefit from immediate treatment. The tumour microenvironment is a factor that determines tumour aggressiveness, metastatic potential and invasiveness (Vaupel et al., 2004) (Yuan and Glazer, 1998) (Pennacchietti et al., 2003) (Subarsky and Hill, 2003).

Tumour hypoxia is increasingly recognised as major factor that influences apoptosis, (Tannock, 1968), is involved with the up regulation of genes that select for genetic adaptations that increase invasiveness and metastatic behaviour of malignant cells (Graeber et al., 1996) (Wartenberg et al., 2003). The main transcription factor responsible for this response is HIF-1. This transcription factor is responsible for the activation of a number of genes resulting in cellular adaptation to low oxygen conditions (Dery et al., 2005). HIF-1 is expressed in over 70% of all tumours and their metastases (Zhong et al., 1999). We proposed to investigate the effect of post-irradiation hypoxia and HIF-1α on radioresistance.

## 5.2 Post-Irradiation Hypoxic Recovery Increases Radioresistance of Prostate Cancer Cells *in-vitro*

Tumour hypoxia as a cause of radioresistance has been established for greater than 50 years (Thomlinson and Gray, 1955). Hypoxic radioresistance has been shown to be dependent on duration of hypoxic exposure prior to irradiation and the tumour repair capacity. Pre-irradiation hypoxic experiments have shown that acutely hypoxic tumour cells irradiated immediately after re-oxygenation are radiosensitive when compared with cells irradiated under acute hypoxia alone. Furthermore, irradiated chronically hypoxic cells (72 hrs) can acquire increased radiosensitivity when compared with irradiated cells exposed to acute hypoxia (4–24 hr) (Bristow and Hill, 2008) (Chan et al., 2008). Accordingly so far anti-hypoxia treatments have been administered prior to irradiation as this was thought to be the critical time for hypoxic radioresistance. The first objective was to assess the effect of post-irradiation hypoxic recovery on the radioresistance of primary and metastatic prostate cancer cell lines.

We confirmed that post-irradiation hypoxia increased the radioresistance of all three cell lines across a wide dose range. Prostate cancer cells that were placed in hypoxic recovery for 24 hrs post-irradiation had increased resistance compared to cells that were oxygenated post-irradiation (Figure 4.1/4.2). We noted that 22Rv1 cells were the mist radiosensitive cell line tested and that PC3 cells were the most radioresistant. Hypoxic recovery increased the radioresistance the most in DU145 cells (Figure 4.1/4.2).

Previous research has shown that irradiated chronically hypoxic cells (72 hr) have increased radiosensitivity when compared with irradiated cells exposed to acute hypoxia (4–24 hr). We next assessed the affect of post-irradiation hypoxic recovery on chronically hypoxic cells. We demonstrated that post-irradiation hypoxic recovery reverses the increases radiosensitivity reported in chronically hypoxic prostate cancer cells. Chronically hypoxic (24-72 hr) prostate cancer cells had similar radioresistance to cells that were hypoxic for four hours pre-irradiation (Figure 4.3). This data indicates that post-irradiation recovery is important in the development of radioresistance; furthermore the effect of hypoxic exposure during this period suggests that targeting hypoxia after irradiation may benefit patients with aggressive hypoxic prostate tumours.

### 5.3 Reactive oxygen species levels

ROS are chemically-reactive molecules delivered from oxygen; they are highly reactive due to the presence of unpaired valence shell electrons. It is proposed that hypoxic cells are more resistant to irradiation because of a lack of oxygen as a source of radiation induced radicals (Thomlinson and Gray, 1955). We next determined the effect of hypoxia on intracellular ROS levels in two prostate cancer cell lines. We further wanted to correlate this difference with intracellular Glutathione (GSH) levels. GSH is an antioxidant and it has been shown that ROS can cause a drop in GSH levels either by oxidation or reaction with the thiol group.

We first determined that there was a significant difference in the amount of ROS present between primary and metastatic prostate cancer cell lines. Baseline ROS levels in 22Rv1 cells were significantly higher than DU145 Cells. Irradiation with 4 Gy significantly increased the amount of ROS present in both cell lines compared to aerobic controls. Hypoxic exposure alone also increased the ROS present in both cell also (Figure 4.4). ROS levels after hypoxic irradiation were higher than baselines ROS levels, but were significantly lower than aerobic irradiation in DU145 cells only. GSH levels were significantly different between the two cell lines; DU145 cells had significantly more intracellular GSH than 22Rv1 cells. Irradiation decreased Cellular GSH in both cell lines, as did hypoxia and hypoxic irradiation (Figure 4.5).

This data suggests that hypoxic radioresistance could be in part due to different ROS levels and antioxidant levels between primary and metastatic cell lines. 22Rv1 cells a primary prostate cancer cell line have the highest baseline ROS levels and lowest GSH levels, an antioxidant. DU145 cells are more radioresistant, have lower ROS levels and higher GSH. Hypoxia is associated with increased radioresistance, in both cell lines hypoxia reduced the amount of ROS generated by irradiation, however this was associated with a reduction in GSH, an antioxidant that can bind ROS. If hypoxic reduction of ROS is responsible for observed increased radioresistance, this should be due to a failure of oxygen containing compounds to fix the DNA damage and thus allowing DNA repair to occur.

## 5.4 The Role of HIF-1α in Radioresistance

Cellular adaptations to hypoxia involve the coordinated expression of a large and diverse group of genes, many of which are transcriptionally regulated by HIF-1α. In hypoxia, HIF-1α levels significantly increase as proteasomal degradation is inhibited due to the inability of pVHL to bind to HIF-1α that allows the activation of over 60 genes (Semenza, 2003b). This affects many processes: including glycolysis, mitosis, apoptosis, and angiogenesis, all of which have been shown to influence radio-responsiveness (Moeller et al., 2007). As previous thought hypoxic radioresistance has been shown to be dependent on duration of hypoxic exposure prior to irradiation. Increased expression of HIF-1α in prostate cancer cells placed in to post-irradiation hypoxic recovery could be responsible for increased radioresistance.

We confirmed the expression of HIF-1α protein in hypoxic prostate cancer cell lines and correlated this with the expression of VEGF protein (Figure 4.6). Aerobic 22Rv1 and DU145 cells did not express HIF-1α this is due to the degradation of HIF-1 by the ubiquitin-proteasome system. Hypoxia exposure for 4 hrs prevented this degradation in both cell line and the expression of HIF-1α was demonstrated. Cell placed into post-irradiation hypoxic recovery for 24 hrs had the greatest expression of HIF-1α. Cells placed in an aerobic environment for recovery expressed HIF-1α but less than hypoxic cells. Previous studies investigated HIF-1 expression under long hypoxic incubation times (Mottet et al., 2003). Foley *et al.* found that HIF-1α protein expression in 22Rv1 and DU145 cell lines was no longer detectable after 48 hrs of hypoxic exposure. They concluded that maximum HIF-1α protein expression appeared to be between 4 and 8 hrs of exposure (Foley et al., 2009). In contrast our

data demonstrated that HIF-1α expression was greatest 24 post irradiation and 28 hrs post hypoxic exposure. This suggests that post-irradiation hypoxic recovery is important for the continued stabilisation of HIF-1α. One theory is that radiation alone increases HIF-1α levels; it has been shown that between 24 and 48 hrs after irradiation, HIF-1α activity in a tumour will increase approximately two-fold (Moeller et al., 2004). Interestingly, this phenomenon has only been demonstrated in vivo, as it relies on micro-environmental mechanisms that take place only in intact tumour tissue. We conclude that post-irradiation hypoxia increases the expression of HIF-1α protein expression in both primary and metastatic prostate cancer lines. Cells that were placed into $CoCl_2$ treated media post irradiation had the greatest levels of HIF-1α protein expression, similar to cells placed into hypoxia for post-irradiation recovery.

### 5.5 Impact of HIF-1α on Radioresistance

To simulate the effects HIF-1α has on cellular radioresistance. Three prostate cancer cell lines were incubated in $CoCl_2$ instead of hypoxia. $CoCl_2$ is known to induce HIF-1α expression by binding to the PAS domain, blockage of HIF-1α pVHL binding and degradation (Kanaya and Kamitani, 2003). We found that 22Rv1, DU145 and PC3 cells that were treated with $CoCl_2$ had increased radioresistance compared to aerobic controls (Figure 4.6). Cells treated with$CoCl_2$ for 24 hrs post irradiation again had increased radioresistance compared to aerobic controls. The expression of HIF-1α protein induced $CoCl_2$ was determined by Western blot (Figure 4.7). These results

suggest that HIF-1α stabilisation and the induction of downstream genes govern the radioresponse of prostate cancer cells (Figure 4.7).

## 5.6 DNA Repair does not Increase Hypoxic Radioresistance

Ionizing radiation, such as that used in radiotherapy, kills cells by producing DNA damage, particularly DNA DSB. The failure to repair DSBs, or mis-repair, can result in cell death or large-scale chromosome changes enhance genome instability. (Chaudhuri et al., 2007, Sheng et al., 2007) (Murnane, 2006). Therefore, maintaining error-free DNA repair under normoxic and hypoxic conditions could be important to preserve genomic stability to prevent a mutated phenotype (Chan et al., 2008). Human DNA DSBs are usually repaired through two main pathways: HR and non-homologous end joining (NHEJ) (Weterings and van Gent, 2004) (Helleday et al., 2007). These pathways vary in their protein components, their cell cycle specificity and their fidelity of repair. Under normoxic conditions, a DNA double-strand break (DNA DSB) is sensed by the MRN complex. This leads to activation and recruitment of the ATM and DNA-dependent protein kinase (DNA-PK) catalytic subunit (DNA-PKCS) kinases and phosphorylation of the histone variant H2AX (termed γH2AX) around the site of the break. Subsequently, a number of DNA damage sensing proteins (such as mediator of DNA damage checkpoint 1 (MDC1) and p53- binding protein 1 (53BP1)) and DNA DSB repair proteins involved in homologous recombination (HR) and non-homologous end joining (NHEJ) are recruited within the first 1–6 hrs of damage, recognition to repair the DNA DSB. The NHEJ repair pathway can be used within any phase of the cell cycle and can be error-prone. The

HR pathway is preferentially active in the S and $G_2$ phases of the cell cycle when a homologous sister chromosome or chromatid is available for direct base-pairing to effect error-free repair of a DNA DSB (Sandhu et al., 2000). In studies of normoxic cells, the inhibition of the HR and NHEJ repair pathways has been linked to increased genetic instability and carcinogenesis (Collis et al., 2005).

In this section we performed split dose radiation experiments to assess the effect of dose fractionation on prostate cancer cells. Dose fractionation in radiotherapy is thought to partially overcome intra-tumour hypoxia resistance because radioresistant hypoxic cells become reoxygenated and therefore re-sensitised between individual dose fractions. We found that split doses of 2 Gy separated by 2 hrs was not superior to a single dose of 4 Gy (Figure 4.9). We next examined the effect of extending the dose inter-fraction time. We did this to evaluate if DNA repair was occurring at increased time points and if DNA repair is responsible for increased radioresistance. We found that increasing the inter-dose fraction time up to 8 hrs had no impact on the surviving fraction of aerobic or hypoxic (Figure 4.10).

We next examined for the presence of MRE11 protein expression to assess for the presence of DNA repair proteins. MRE11is a Double-strand break (DSB) repair protein that in humans is encoded by the *MRE11A* gene (Petrini et al., 1995). MRE 11 protein is involved in homologous recombination, telomere length maintenance, and DNA DSB repair. By itself, the protein has 3' to 5' exonuclease activity and endonuclease activity. MRE11 forms the MRN complex with Rad50 and Nbs1. this complex is required for nonhomologous joining of DNA ends and possesses

increased single-stranded DNA endonuclease and 3' to 5' exonuclease activities. In conjunction with a DNA ligase, this protein promotes the joining of noncomplementary ends in vitro using short homologies near the ends of the DNA fragments. It also appears that as part of its end-processing of DSBs, short single-strand oligonucleotides are produced, which induce activation of ATM ultimately leading to activation of the DNA damage checkpoint (Jazayeri et al., 2008).

We found that 22Rv1 cells did not express MRE11 protein or expressed the protein in such small quantities that could not be quantifiable. DU145 control cells expressed MRE11 protein; hypoxia decreases the level of MRE11 protein compared to aerobic controls. Irradiation with 4 Gy increased the expression of MRE11; irradiation then hypoxic recovery for 24 hrs also increased the expression of MRE11. Interestingly Pre-irradiation hypoxic exposure and aerobic and hypoxic recovery decreased the expression of MRE11 protein. These results suggest that pre-irradiation hypoxia is a more important inhibitor of MRE11 protein expression and that irradiation as predicted will increase the level of the protein (Figure 4.10). We next investigated the effect on HIF-1α on MRE11 expression. PC3 and DU145 cells were incubated in both hypoxia and the selective HIF-1α stabiliser, $CoCl_2$. Expression of HIF-1α protein was done by Western blot analysis. We again found that 22Rv1 cells did not express MRE11. However both hypoxic and $CoCl_2$ treated DU145 cells had reduced levels of MRE11. This suggests that HIF-1α negatively regulates MRE11 protein expression and inhibits DNA repair.

Despite the importance of DNA repair in genomic stability, few papers studying the effect of hypoxia on the specific HR or NHEJ repair pathways in detail have been published. However our findings are in keeping with the published results. RAD51, a gene that is a member of the RAD51 protein family, is known to be involved in the HR and repair of DNA, has reduced expression during periods of hypoxia greater than 24 hrs both *in vitro* and *in vivo*. This observation was made across multiple cell histopathologies, but was independent of HIF-1α expression (Bindra et al., 2004). Hypoxia has also been shown to decrease the expression of NBS1 which is part of the MRN) complex that initially recognizes DNA DSBs, leading to the induction of γH2AX foci (which may be indicative of DNA DSBs). This down regulation of NBS1 occurs in a HIF-1α-dependent manner and requires threonine phosphorylation of the PASB (Per–ARNT–Sim B) domain in the HIF-1α protein (To et al., 2006).

Bindra and colleagues have also studied the effects of hypoxia on NHEJ and HR using a plasmid reporter system. They observed that the frequency of lesions repaired by HR was decreased with hypoxia; there were no decreases in NHEJ activity (Bindra et al., 2004). This result was confirmed using restriction-enzyme induced breaks and a flow-cytometric repair assay that measures high-fidelity HR. These multiple studies also confirmed that the decrease in HR activity was HIF-1α independent by studying the expression of HR proteins in cells isogenic for HIF-1α function and cells treated with the HIF-1α inducing agent, $CoCl_2$ (Bindra et al., 2004) (Meng et al., 2005). Together these data suggested that the effect on HR proteins is observed under conditions of low O2 levels and might not always be replicated with the use of chemicals that solely mimic hypoxic transcriptional responses. The data

concerning the effects of chronic hypoxia on NHEJ are mixed. Gene expression studies demonstrated down regulation of many mRNA species encoding proteins associated with NHEJ following chronic hypoxia (72 hrs of 0.2% $O_2$), but there was no change in protein expression (Meng et al., 2005). In addition to DNA DSB repair and NER, another important pathway that can be modified by hypoxia is the mismatch repair (MMR) pathway. MMR normally corrects DNA base pair mismatches during DNA replication and when defective gives rise to micro-satellite instability. Hypoxia appears to induce the down regulation of two MMR genes, *MLH1* and *MSH2*, leading to increased mutagenesis and di-nucleotide repeat instability; this was also associated with hypoxia-mediated alterations in histone deacetylation (Mihaylova et al., 2003) (Koshiji et al., 2005). Our results and the published literature suggest that hypoxia may influence DNA repair, by inhibiting HR pathway more than the NJEH pathway. This is possibly due to the hypoxia induced HIF-1α dependent decrease in expression of key components of the MRN complex; MRE11 and NBS1.

## 5.7 Post-Irradiation Hypoxia Effects the Cell Cycle in $G_2$/M

Cell cycle checkpoints are control mechanisms that ensure the fidelity of cell division in eukaryotic cells. These checkpoints verify whether the processes at each phase of the cell cycle have been accurately completed before progression into the next phase. The main checkpoints which control the cell division cycle are, $G_1$ (Restriction) Checkpoint and $G_2$ checkpoint: $G_1$ checkpoint is located at the end of the cell cycle's

$G_1$ phase, just before entry into S phase, making the key decision of whether the cell should divide, delay division, or enter a resting stage. Many cells stop at this stage and enter a resting state called $G_0$. The $G_1$ checkpoint is where eukaryotes typically arrest the cell cycle if environmental conditions make cell division impossible or if the cell passes into $G_0$ for an extended period. In animal cells, the $G_1$ phase checkpoint is also called the restriction point. The $G_2$ Checkpoint is located at the end of $G_2$ phase, triggering the start of the M phase (mitosis). In order for this checkpoint to be passed, the cell has to check a number of factors to ensure the cell are ready for mitosis. If this checkpoint is passed, the cell initiates the many molecular processes that signal the beginning of mitosis.

We found that the effect of hypoxia on prostate cancer cells was most pronounced when the cells were hypoxic prior to irradiation and placed in hypoxic recovery for 24 hrs. Both 22Rv1 and DU145 cells were found that there was a statistically significant decrease in the percentage of cells in S phase. Whereas there was an increase of prostate cancer cells in $G_2$/M (Figure 4.14). This suggests that hypoxic recovery post irradiation induced a $G_2$/M phase cell cycle arrest. These results correspond with published data that suggests that anoxia induces a $G_1$ and intra-S phase arrest, while re-oxygenation post hypoxia is associated with a $G_2$ phase arrest (Gibson et al., 2005) (Freiberg et al., 2006). These checkpoints were noted to be controlled by the WAF1-p53-CDK2, ATM-p53-CDK2 and ATR-CHK1 pathways. CHK1 is a serine/threonine-protein kinase that in humans is encoded by the *CHK1* gene. CHK1 kinase phosphorylates cdc25, an important phosphatase in cell cycle control, particularly for entry into mitosis (Chini and Chen, 2004). CHK2 is a

member of the cyclin-dependent kinase family of Ser/Thr protein kinases. When activated, the encoded protein is known to inhibit CDC25C phosphatase, preventing entry into mitosis, and has been shown to stabilize the tumor suppressor protein p53, leading to cell cycle arrest in $G_1$ (Chehab et al., 2000) (Lee et al., 2000).

We examined the protein CHK1 and CDK2 expression levels of hypoxic and $CoCl_2$ treated 22Rv1 and DU145 cells to examine for the effect of hypoxia on these checkpoint proteins and to determine if this was mediated by HIF-1α. We found that hypoxia increased the expression of CHK1 and CDK2 protein expression in 22Rv1 cells. CDK2 expression was HIF-1α dependent but CHK1 expression was reduced by HIF-1α. Similar results were seen in DU145 cells, were we found that hypoxia increased the expression of CHK1 and CDK2 protein expression, yet only CDK2 expression was HIF-1α dependent (Figure 4.15). Suggesting that the decrease in S phase seen was due to HIF-1α regulated reduced cell synthesis, but the $G_2$/M arrest could also be due to HIF-1α regulated CHK prevention of entry into mitosis.

However previous studies suggested that re-oxygenation produces ROS that generate DNA damage that elicits a CHK2-dependent $G_2$ checkpoint. Indeed, CHK2-deficient cells do not undergo $G_2$ arrest following anoxia and instead undergo apoptosis (Gibson et al., 2005) (Freiberg et al., 2006). By contrast, chronic hypoxia leading to cellular adaptation may not activate $G_1$ or S checkpoints and could potentially lead to accumulation of DNA replication errors or DNA breaks over time (Koritzinsky et al., 2006). However, whether it is the level of $O_2$, the duration of hypoxia or anoxia, or the re-oxygenation events that determines whether cell cycle progression is affected

within the complexity of a hypoxic solid tumour, this has not been adequately addressed *in vivo*. It remains to be determined whether cycling hypoxia or chronic hypoxia can be directly linked to tumour progression.

## 5.8 Radiation Induced Apoptosis is Reduced by Post-Irradiation Hypoxia

In spite of numerous publications that report the role of apoptosis in determining tumour response to radiation has been and remains poorly understood and controversial. One of the main controversies is the time course of apoptosis following irradiation. For apoptosis to contribute to a loss in clonogenic survival, the apoptosis must occur prior to the first post-irradiation mitosis or early apoptosis (Meyn et al., 2009). Apoptosis that occurs hours to days after the first mitosis is secondary to death of the cell following an aberrant mitotic event caused by radiation-induced chromosome abnormalities. This later mode of apoptosis is sometimes referred to as mitotic death, reproductive cell death, mitotic linked death or mitotic catastrophe. Since the same cell cannot die twice, apoptosis that occurs after the cell has been rendered non-clonogenic due to fragmentation of the nucleus via formation of multiple micro-nuclei cannot contribute to any additional loss of clonogenic cell survival. Furthermore, any modulation of secondary or late apoptosis observed due changes in the tumour microenvironment are not relevant to any observed changes in clonogenic survival (Endlich et al., 2000).

We found that the hypoxia did statistically reduced apoptosis in both 22Rv1 and DU145 cell lines. As the effect of hypoxia on later apoptosis cannot contribute to

increased clonogenic survival we will only focus on the effects of hypoxia on early apoptosis. As expected we found that irradiation of prostate cancer cells in an aerobic environment increased apoptosis. Hypoxic exposure decreased apoptosis in both cell lines; again it was 22Rv1 and DU145 cells that were hypoxic prior to irradiation and placed in hypoxic recovery for 24 hrs that had the lowest level of early apoptosis (Figure 4.16). We next examined the effect of hypoxia on p53 protein expression, because it is known that p53 can initiate apoptosis, if DNA damage proved to be irreparable. We again noted differences in p53 expression between the two cell lines. Control 22Rv1 cells did not express p53, but DU145 cells did, hypoxia thus had no effect on 22Rv1 p53 protein expression, but hypoxia did reduce the expression of p53 in DU145 cells. Irradiation did lead to the increased expression of p53 protein in both cell lines and this appeared to be the most apparent in 22Rv1 and DU145 cells that were either hypoxic only pre or post irradiation. Hypoxia before irradiation and hypoxic recovery for 24 hrs post irradiation decreases p53 protein expression in contrary to other variations of hypoxic exposure. We finally then assessed the effect of HIF-1 on p53 expression. We found that expression of p53 protein was reduced by $CoCl_2$ treatment suggesting that this is regulated by HIF-1.

In keeping with our results recent studies have shown that in hypoxia can increase resistance to apoptosis. Dong *et al* showed that cells treated with a strong apoptosis inducer, staurosporine, were less sensitive to apoptosis in severe hypoxia than when oxygen levels are normal (Dong et al., 2003). Resistance to apoptosis induced hypoxia cells occurs on at least two levels: in the mitochondria and in the cytosol. In the staurosporine treated cells, translocation of the pro-apoptotic protein Bax to the

mitochondria was suppressed by hypoxia. Accumulation of Bax in the mitochondria causes the release of cytochrome C into the cytosol, which was strongly reduced by hypoxia, prohibited the cascade that leads to cell death.

In contrast to our findings it was shown that that HIF-1α directly binds to the p53 ubiquitin ligase mdm2 both in vivo and in vitro, thereby stabilising p53 (Chen et al., 2003). However, another report showed a direct binding of p53 to the ODD domain of HIF-1α (Hansson et al., 2002) and that HIF-1α interacts with wild-type p53 but not with tumour derived mutant p53 (An et al., 1998). It is possible that this explains the difference we seen in HIF-1α in prostate cancer cells.

### 5.9 Cellular Senescence is Reduced by Hypoxia

Senescence is thought to be a tumour suppressive mechanism and an underlying cause of aging. Senescence represents an arrested state in which the cells remain viable, but not stimulated to divide by serum or passage in culture. Cellular senescence, a state of irreversible growth arrest, can be triggered by multiple mechanisms including telomere shortening, the epigenetic depression of the *INK4a/ARF* locus, and DNA damage. Together these mechanisms limit excessive or aberrant cellular proliferation, and so the state of senescence protects against the development of cancer. Recent evidence suggests that cellular senescence also may be involved in aging. Hypoxia has been demonstrated to decrease the proportion of cells in the senescent population of human HCT116 colon carcinoma cells, via a HIF-

1α mediated pathway and to increase chemotherapeutic resistance (Sullivan et al., 2008).

In this section we found that hypoxia decreased the proportion of cells in the senescent population of 22Rv1 and DU145 prostate cancer cells. This is demonstrated by histochemical staining of β-galactosidase (SA-β-Gal) (Figure 4.19). SA-β-Gal is present only in senescent cells and is not found in pre-senescent, quiescent or immortal cells. We further noted that SA-β-Gal expression was related to hypoxic exposure and that prostate cancer cells that were hypoxic prior to irradiation and placed in hypoxic recovery post irradiation had the least amount of SA-β-Gal expression (Figure 4.19). There was also a difference between the cell lines, 22Rv1 cells the primary prostate cancer cell line seemed to express more SA-β-Gal than the metastatic DU145 cells. It therefore must be noted that the most radioresistant cells of both cell lines were also the cells that had the least amount of its population in the senescent state.

We next determined the effect of hypoxia and HIF-1 on the expression of $p16^{ink4a}$. This is a tumour suppressor protein that is also known as Cyclin-dependent kinase inhibitor 2A, which in humans is encoded by the *CDKN2A* gene (Nobori et al., 1994). P16 plays an important role in regulating the cell cycle. Increased expression of the p16 gene associated with organisms aging is associated with reduced proliferation of stem cells. This reduction in the division and production of stem cells protects against cancer while increasing the risks associated with cellular senescence. We found that hypoxia or $CoCl_2$ treatment decreases the expression of $p16^{ink4a}$ in both cell lines. This

suggests that HIF-1α interacts with $p16^{ink4a}$ and prevents cells entering the senescent state. Again this is further evidence that HIF-1α increases the radioresistance of prostate cancer cell.

Evidence supporting this theory is that treatment of MDA-MB-231 cells with small interfering RNA targeting the α-subunit of HIF-1α, prevented hypoxia induced chemotherapeutic resistance. HIF-1α small interfering RNA also selectively abolished the hypoxia-induced changes in the senescent population, indicating that the increased survival was due to protection against drug-induced senescence. These results support a requirement for HIF-1α in the adaptations leading to drug resistance and reveal that decreased drug-induced and possibly radiation-induced senescence is also an important contributor to the development of hypoxia-induced resistance (Sullivan et al., 2008).

### 5.10 HIF-1α: the Future of Cancer care?

### 5.11 HIF-1α as a Prognostic Factor

The discovery of HIF-1α as a crucial player of the response of mammalian cells to hypoxia has changed our perspective on hypoxia as prognostic marker and therapeutic target. The identification of the small subset of patients with rapidly growing and aggressive prostate cancer that is ultimately fatal will be the current focus of prostate cancer management. Identification of these patients with high risk prostate cancer has been enhanced with the use of nomograms and risk stratification approaches. Such as D'Amico classification that stratifies men to low, intermediate,

or high risk based on stage, grade, and PSA. The downside of this normogram is that the 3-level system does not account for multiple adverse parameters in stratifying patients (D'Amico et al., 2006).

The incorporation of the presence of tumour hypoxia in to risk stratification systems has been suggested (Marignol et al., 2008). Tumour hypoxia is associated with a cancer that has increased malignant phenotype, invasiveness and reduced apoptosis (Yuan and Glazer, 1998) (Pennacchietti et al., 2003). The incorporation of tumour hypoxia into nomograms may benefit those patients that are in most need of multimodal treatment. HIF-1α could potentially be used as a prognostic marker for tumour hypoxia. Expression of HIF-1α in diagnostic biopsies from prostate cancer patients was correlated with poorer outcomes. It was found that increased staining of HIF-1α was associated with a shorter time to biochemical (PSA) relapse following radiotherapy (Vergis et al., 2008). The specificity of HIF-1α has also been studied, it was shown that that the immunohistochemical intensity for HIF-1α was significantly enhanced in 75% if prostate cancer specimens when compared to matched benign specimens (Foley et al., 2009). This suggests that the expression of intrinsic markers of tumour hypoxia such as HIF-1α could be an important predictor of radiotherapeutic, and possibly surgical, outcome in prostate cancer.

## 5.12 HIF-1α Targeted Therapies

In pre-clinical studies, inhibition of HIF-1α activity has marked effects on tumour growth (Semenza, 2002). There is also an increasing body of evidence to suggest that targeting and the inhibition of the transcription factor HIF-1α may be a viable therapeutic target. HIF-1α has several features that make it an attractive target for therapy. In addition to its involvement in tumour metabolism, angiogenesis and metastasis, all of which are hallmarks of cancer progression, HIF-1α is also unique for its expression and function in multiple cells of the tumour microenvironment, so that HIF-1α inhibition may truly represent a global strategy targeting the tumour microenvironment (Melillo, 2004). However it must be noted that targeting transcription factors for the development of cancer therapeutics is a conceptually attractive, yet is a challenging task and is perceived by many as an impractical strategy (Melillo, 2007).

Initial research demonstrated that the inhibition of trans-activation of HIF-1α target genes such as VEGF has an anti-tumour effect. Tumours infected with a polypeptide that disrupts the binding of HIF-1α to its transcriptional co-activators, markedly inhibiting hypoxia induced transcription reduced the growth of cancer cells transplanted into nude mice (Kung et al., 2000). These data led investigators to examine for other methods to inhibit HIF-1α. A second approach was to suppress HIF-1 protein levels either by destabilizing the protein or inhibiting its production. The heat shock protein geldanamycin reduced HIF-1α levels by promoting its $O_2$ and VHL-independent degradation (Mabjeesh et al., 2002). Targeting of HIF-1α by direct

injection of an anti-sense construct to HIF-1α has been shown to increase the efficacy of immunotherapy against larger tumours (Sun et al., 2001).

The current most promising therapy targeting HIF-1α is the small molecule inhibitors of HIF-1α. It has been reported that microtubule inhibitors such as 2-methoxyyestradiol, vincristine and paclitaxel reduce HIF-1α levels by inhibition of HIF-1α mRNA (Mabjeesh et al., 2003). These compounds reduce tumour growth and vascularity, but it is unknown whether this is an effect if reduced levels of HIF-1α or the direct effect of the microtubules. A second small molecule that has been reported to reduce HIF-1α levels and inhibit tumour growth is the soluble guanylyl cyclase stimulator YC-1. The anti-tumour and anti-angiogenic effects of YC-1 were attributed to a reduction in HIF-1α protein levels by an unknown post-translational effect (Yeo et al., 2003).Finally an ideal therapeutic targeting agent of HIF-1α would be to screen for compounds that are preferentially toxic to cells expressing HIF-1α. At present this is a theoretical possibility with no published data demonstrating its efficacy (Brown and Wilson, 2004).

## 5.13 Conclusion

In our hypothesis, we sought to evaluate the effect of post-irradiation hypoxic recovery on the radioresistance of prostate cancer cell lines. To examine whether this survival advantage correlates with modified cellular responses induced by hypoxia and investigate whether this survival advantage is mediated by HIF-1α. We concluded that prostate cancer cells that are hypoxic after irradiation gain a survival advantage compared to cells that were fully oxygenated after irradiation. We determined that this survival advantage was associated with induction of a $G_2$/M cell cycle arrest, was not associated with increased DNA repair, was associated with reduced induction of apoptosis and that hypoxia decreased the amount of senescent prostate cancer cells. Furthermore we demonstrated that these modified cellular responses could be mediated by HIF-1α. The clinical implications of this research, is that our findings suggest that the identification of hypoxia in patients will be an important part in treatment planning in the future and risk stratification. Those patients with hypoxic prostate tumours may benefit from the continuation anti-hypoxia treatments post irradiation. Finally our data supports the need for continued research into anti-HIF-1α compounds as an adjuvant therapy for prostate cancer patients receiving radiotherapy.

# Chapter 6

# Appendices

# 6 Appendices

## 6.1 Appendix A: Solutions

Table 1: Whole cell lysis buffer for protein extraction for Western blotting.

| Ingredient | Final concentration in lysis buffer | Amount |
|---|---|---|
| 500 mM TRIS, pH 7.5 | 50 mM | 1 mL |
| 1.5 M NaCl | 150 mM | 1 mL |
| 1005 IGEPAL | 1% | 100 µl |
| 10% SDS | 0.1% | 100 µl |
| 0.5 M EDTA | 1 mM | 20 µl |
| 0.06 M Sodium Orthovanadate | 600 µM | 100 ul |
| 500 mM Sodium Flourid | 50 mM | 1 mL |
| Complete Protease Inhibitor Tablet | | 1 Tablet |
| 0.2 M Phenylmethysulfonylflouride (PMSF) | 1 mM | 5 µL |
| Dithiothreitol | 1 mM | 2 µl |

Table 2: Solutions used for Western blotting protocols

| Solution | Ingredient |
|---|---|
| 0.5 M Tris HCL pH 6.8 | Tris 18g $dH_20$ 300 mls<br>(Adjust pH to 6.8 with HCL) |
| 1.5 M Tris HCL pH 8.8 | Tris 54.46 gg $dH_20$ 300 mls<br>(Adjust pH to 8.8 with HCL) |
| 10% SDS | SDS 20 g<br>$dH_20$ 200 mls |
| APS | Ammonium Persulfate 0.1G<br>$dH_20$ 1 mL (Fresh) |
| Running Buffer | Tris 30.3 g<br>Glycine 144.0 g<br>SDS 10 g<br>$dH_20$ 1000 ml |
| Transfer Buffer | Tris 3.03 g<br>Glycine 14.4 g<br>Methanol 200 ml<br>Store at 4°C |
| 0.05% Tween PBS | $dH_20$ 1000 ml<br>PBS Tablets x 2<br>Tween 500 µL |
| 5% Marvel in 0.05% Tween PBS | Marvel 5 g<br>0.05% Tween PBS |
| 5% BSA in 0.05% Tween PBS | BSA 0.5 g<br>0.05% Tween PBS |

Table 3: 12% Resolving Gel for SDS-PAGE

| Solution | Amount (2 Gels) |
|---|---|
| $dH_2O$ | 3.4 mL |
| 1.5 M Tris Buffer | 2.5 mL |
| 10% SDS | 100 µL |
| 30% Acrylamide | 4 mL |
| APS | 50 µL |
| TEMED | 10 µL |

Table 4: 10% Resolving Gel for SDS-PAGE

| Solution | Amount (2 Gels) |
|---|---|
| $dH_2O$ | 4.1 ml |
| 1.5 M Tris Buffer | 3.3 ml |
| 10% SDS | 2.5 ml |
| 30% Acrylamide | 100 µl |
| APS | 75 µl |
| TEMED | 30 ul |

Table 5: Stacking Gel for SDS-PAGE

| Solution | Amount (2 Gels) |
|---|---|
| $dH_2O$ | 3.05 ml |
| 1.5 M Tris Buffer | 0.65 ml |
| 10% SDS | 1.25 ml |
| 30% Acrylamide | 50 µl |
| APS | 50 µl |
| TEMED | 10 µl |

## 6.2 Appendix B: Mycoplasma test

All cell lines were tested for mycoplasma infections every three months. The cell lines used in this study consistently tested negative for mycoplasma contamination. Cell lies were grown in their own specific antibiotic-free media in T25 culture flasks for 2 passages prior to testing using a mycoplasma PCR ELISA kit (Roche, West Sussex, UK). 1 ml of cell culture supernant was removed and centrifuged at 200 x g for 10 mins at room temperature. The supernant was removed and centrifuged at 13,000 x g for 10 mins at 4°C to sediment mycoplasma.

The supernant was then discarded and any pellet formed was re-suspended in 10 µl sterile (autoclaved deionised, ultraviolet irradiation) and 10µl lysis solution (ELISA kit). 2 positive and negative controls were included in testing. Positive controls consisted of 10 µl of positive control DNA and 10 µL lysis buffer. Negative controls were made up of 10 µL of lysis solution. Both samples and controls were then incubated at 37°C for 1 hr. The 30 µL of neutralisation solution was added to each sample.

Next PCR reactions were performed using the cell extracts and controls. 10 µL was added to each 25 µL PCR mix and 15 µL sterile water. Amplification was carried out on PTC -1 thermocycler as follows.

| | |
|---|---|
| 1 | 95°C for 5 minutes |
| 2 | 39 cycles<br>(94°C for 30 seconds)<br>(62°C for 30 seconds)<br>(72°C for 30 seconds) |
| 3 | 72°C for 10 minutes |

Positive control PCR products were electrophoresed on a 1% agarose gel to ensure that the 520bp product has been successfully amplified before proceeding to the ELISA step. 40 μL denaturation reagent was then added to 10 μL PCR product and incubated for 10 minutes at room temperature. 450 μL hybridization reagent was added to each sample and mixed thoroughly. 200 μL of each sample was transferred into a 96 well plate (Sarstedt, Wexford, Ireland) which was sealed with adhesive foil and aluminium foil. The samples were shaken at 150 RPM at 37°C for 3 hrs in a Stuart Scientific S150 incubator (Premier Scientific, Belfast, UK). Next wells are washed 3 times with 250 μL washing buffer. 200 μL of room temperature anti-DIG-peroxidase was added to samples and plates were further shaken for 30 minutes at 150 RPM at room temperature. Wells are then washed 5 times with 250 μL with washing buffer. Next, 100 μL TMB (3,3',5'5-tetramethylbenzidine) is added to each well and again the sample are shaken at 150 RPM at room temperature for 20 mins. 100 μL of stop solution is then added.

Absorbance was measured with a spectraFlour Plus fluorometer (Tecan, Dorset, UK) at an absorbance of 450nm with a reference absorbance at 690 nm. A450-A690 was greater than 1.2 for positive controls and less than 0.25 for negative controls. For controls that fell outside this range the test was repeated. Samples were considered mycoplasma negative based on the following formula:

(A450-A690) – (mean A450-A690 of negative controls)

## 6.3 Appendix C: Figures

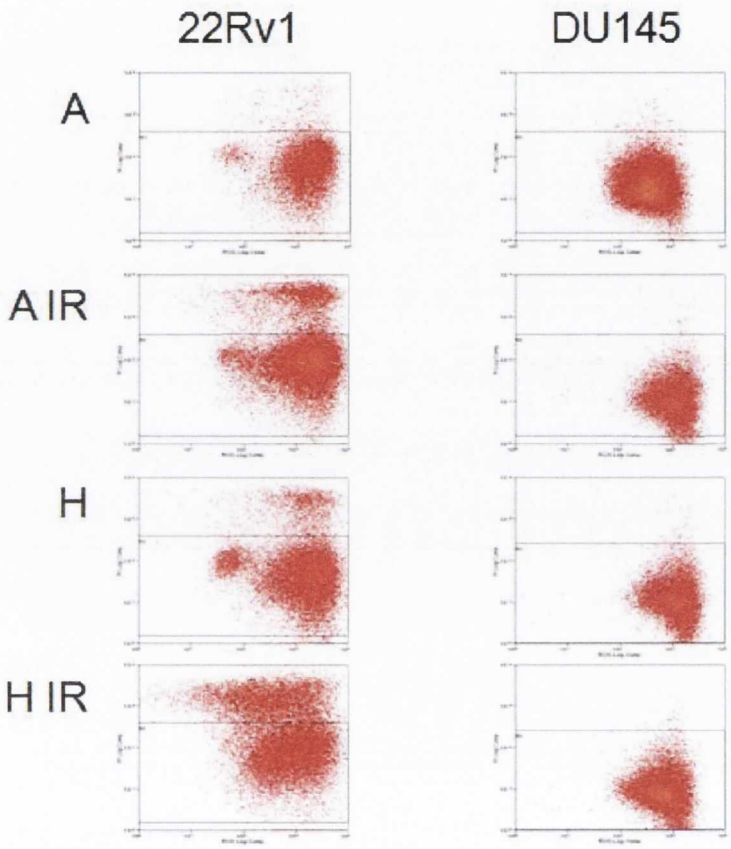



**Figure 32: Hypoxia decreases the Median ROS levels induced by irradiation**

Median ROS levels measured by flow cytometry with 5-(-6)-chloromethyl-2',7' dichlorodihydrofluorescein diacetate acetyl ester demonstrating variations in intensity with hypoxic exposure. A= Aerobic, A IR= Aerobic irradiation, H= Hypoxic, H IR= hypoxic irradiation

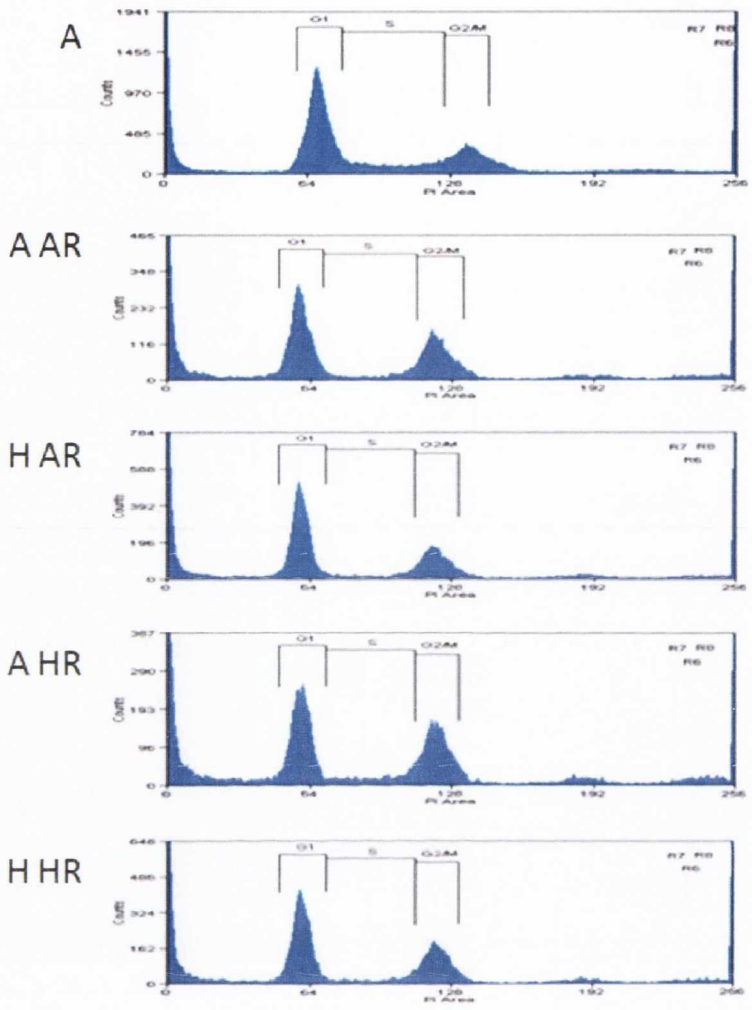



**Figure 33: Hypoxia leads to increase in $G_2$/M arrest in 22Rv1 Cells**

Flow cytometry images demonstrating PI staining of cell cycle G2/M arrest in 22Rv1 cells due to hypoxic exposure. A=Aerobic control; A AR=Aerobic irradiation, aerobic recovery; H AR= Hypoxic irradiation, aerobic recovery; A HR = Aerobic irradiation, hypoxic recovery; H HR = Hypoxic irradiation, hypoxic recovery

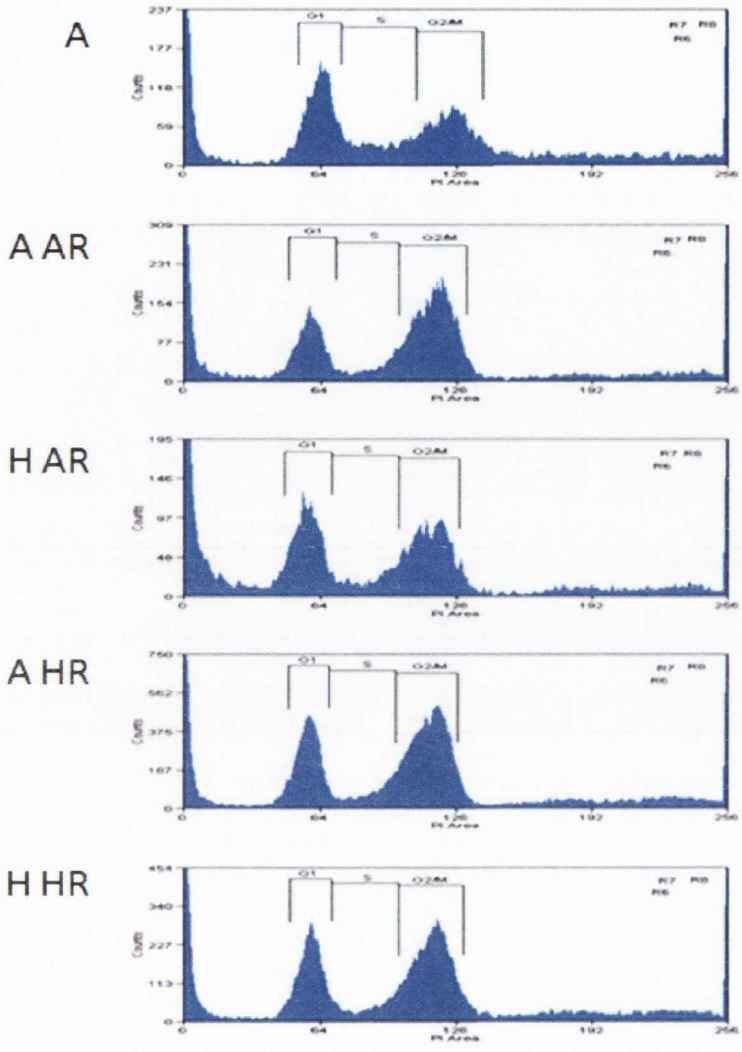



**Figure 34: Hypoxia leads to increase in $G_2$/M arrest in DU145 Cells**

Flow cytometry images demonstrating PI staining of cell cycle G2/M arrest in DU145 cells due to hypoxic exposure. A=Aerobic control; A AR=Aerobic irradiation, aerobic recovery; H AR= Hypoxic irradiation, aerobic recovery; A HR = Aerobic irradiation, hypoxic recovery; H HR = Hypoxic irradiation, hypoxic recovery.

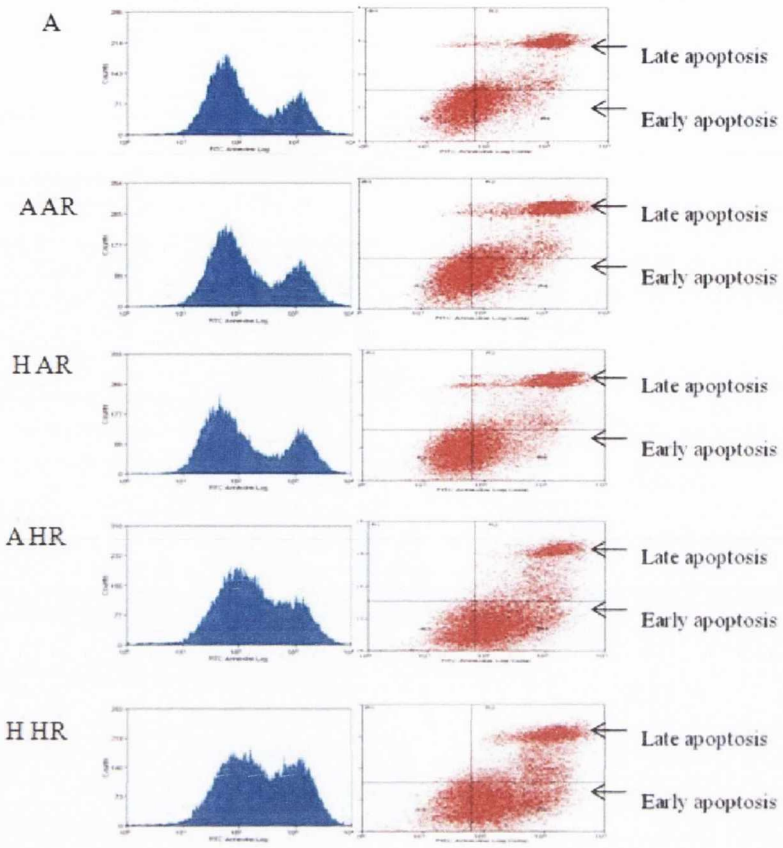



**Figure 35: Hypoxia decreases apoptosis induced in 22Rv1 cells**

Flow cytometry images demonstrating Annexine V/Propidium Iodide Staining apoptosis in 22Rv1 cellsA=Aerobic control; A AR=Aerobic irradiation, aerobic recovery; H AR= Hypoxic irradiation, aerobic recovery; A HR = Aerobic irradiation, hypoxic recovery; H HR = Hypoxic irradiation, hypoxic recovery.

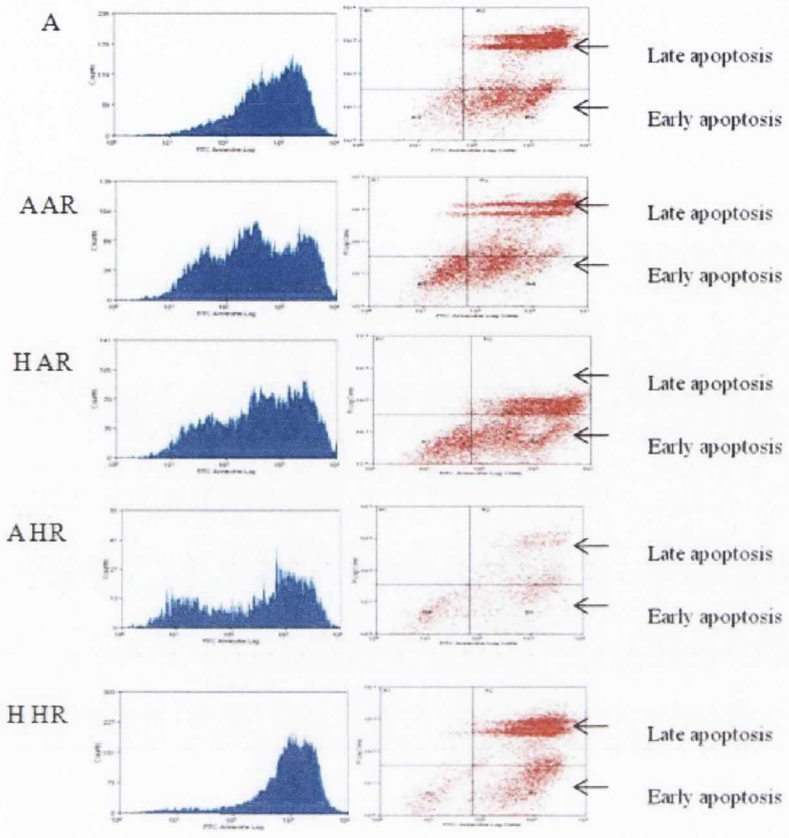



**Figure 36: Hypoxia decreases apoptosis induced in DU145 cells**

Flow cytometry images demonstrating Annexine V/Propidium Iodide Staining of apoptosis in DU145 cells A=Aerobic control; A AR=Aerobic irradiation, aerobic recovery; H AR= Hypoxic irradiation, aerobic recovery; A HR = Aerobic irradiation, hypoxic recovery; H HR = Hypoxic irradiation, hypoxic recovery

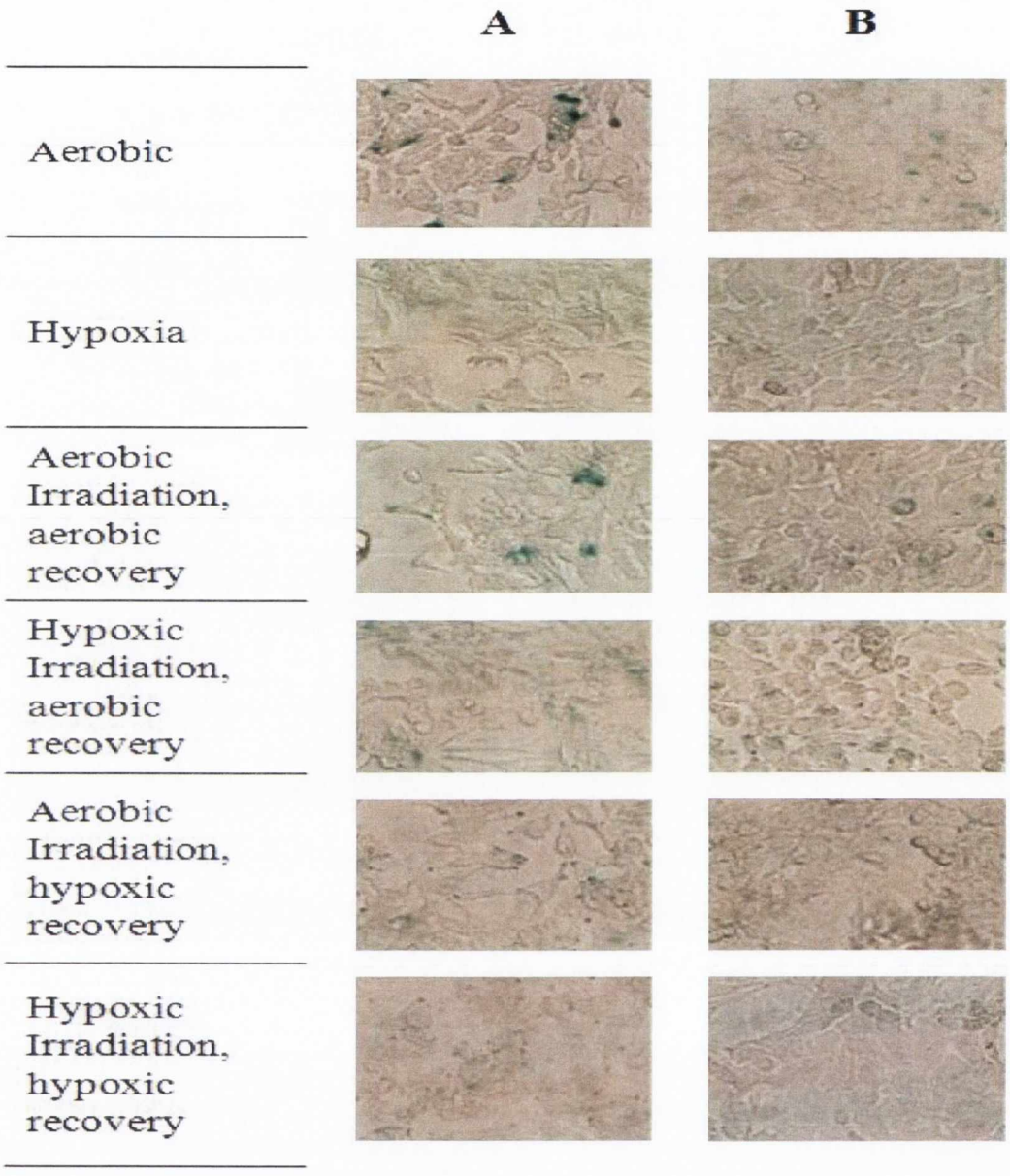



**Figure 37: Hypoxia reduces the amount of cells in the senescent state**

Representative histochemical stain for SA-β-Gal activity (green) of all aerobic and hypoxic conditions examined. Aerobic and hypoxic (4 hrs) (A) 22Rv1 and (B) DU145 cells that were exposed to the delivery of single dose of radiation (4Gy) and allowed to recover in aerobic or hypoxic conditions for 24 hrs.

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