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A PROMISING HYPOXIA-INDUCIBLE SUICIDE GENE THERAPY STRATEGY FOR PROSTATE CANCER

A Thesis submitted for the degree of
Doctor of Philosophy
by
Laure Marignol Bsc. Msc.

Trinity College,
University of Dublin January 2008
To my family
DECLARATION

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

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Laure Marignol

January 2008
Gene therapy targeted to hypoxic tumour cells may allow selective killing of malignant cells. The induction of gene expression under hypoxic conditions is governed by the activation of hypoxia-inducible factor 1 and its subsequent binding to hypoxia response elements. The HREs of a number of oxygen-responsive genes, including vascular endothelial growth factor (VEGF) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were cloned upstream of the cytosine deaminase (CD) gene. These constructs will drive the expression of this prodrug activation enzyme, which converts inactive 5-fluorocytosine (5-FC) to active 5-fluorouracil (5-FU), allowing selective killing of vector containing cells. 5-FU is also a radiosensitising agent, so specific expression of this agent in prostate cancer cells can also potentiate radiotherapy approaches in prostate cancer.

Expression vectors were constructed which contain arrangements of 5 or 8 copies of the VEGF (pH5VCD) or GAPDH (pH8GCD) HREs cassettes. These constructs were transfected into 5 prostate cancer cell lines (DU145, 22Rv1, LnCaP, PC3, RC58/T) and exposed to oxygen concentrations of 0.5% (pO₂<2mmHg) for 48 hours. Western blot analysis of protein extracts from these cells indicated hypoxic CD induction levels of 8.8- and 4-fold with pH5VCD and pH8GCD, respectively. No expression was observed in aerobic cells, confirming the specificity of the approach.

Transfected cells exposed to hypoxia for a 48h period showed a significant decrease in cell number and associated cell death (proliferation assay, p = 0.02) following a 4 days aerobic 5-FC treatment at the clinically relevant dose of 1mM, when compared to untransfected as well as transfected aerobic controls. These results correlated with a 3-fold increase apoptosis levels on day three of 5-FC treatment (Annexin V assay) in both 22RV1 and DU145 transfectants.

The combination with clinically relevant radiation doses (2Gy) on day one of 5-FC treatment resulted in significant reduction of the surviving fraction of
pH5VCD transfected cells (clonogenic assay) compared to either hypoxia or radiation alone, at all 5-FC concentrations tested. The addition of ionising radiation was associated with a therapeutic ratio of 2.8 (p = 0.0017) and 1.9 (p = 0.0236) in pH5VCD 22Rv1 transfectants treated with 0.1 and 1mM 5-FC, respectively. The surviving fraction of pH5VCD DU145 transfectants was similarly reduced by a factor of 1.3 (p = 0.2709) and 2.5 (p = 0.0005). There was no significant benefit of the combined protocol in pHSGCD transfectants. The fractionated delivery of the same total dose had a protective effect on the sensitivity of either transfectants to the combined treatment.

HREs of GADPH origin were also found to be radiation responsive (1.2-fold). This data suggests that targeting hypoxia using a gene therapy approach has demonstrated efficacy in selective killing of prostate cancer cells and could be used in combination with ionising radiation.

Finally, in order to ensure stable expression of the transgene a series of replication deficient adenoviral vectors have been developed and will be tested in this suicide gene therapy approach. The future inclusion of the radiation-responsive tissue plasminogen activator (t-PA) promoter upstream of the expression cassette has potential for confining gene expression to the radiation field, further enhancing the safety and allowing local administration of this gene therapy approach.
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**PUBLICATIONS**


PRESENTATIONS

- The European society of Gene therapy annual meeting in 2004 and 2005
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- The Irish Society of Human Genetics annual meeting 2004, 2005 and 2007
- The AACR “Innovations in prostate cancer” annual meeting 2006
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- Early investigator award, Association for Radiation Research (2007)
- Student travel award, International Conference for Radiation Research (2007)
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CHAPTER ONE: INTRODUCTION
1.1 PROSTATE CANCER

1.1.1 INCIDENCE

Carcinoma of the prostate is a disease of older men and the second leading cause of cancer-related mortality in the Western World. While treatment of advanced disease is currently palliative, the management of early lesions is controversial (Gray and Sims 2006). The mortality rate varies worldwide (Kamangar, Dores et al. 2006) and could be explained by variability in screening schemes and treatment pattern, but has also been linked to dietary differences and living environment (Divisi, Di Tommaso et al. 2006; Kabir and Clancy 2006; O’Malley and Taneja 2006). In Ireland, prostate cancer affects 1 in 10 men diagnosed with cancer (1900 new cases/year) and is the cause of the third highest mortality rate behind lung and colorectal cancer. It is estimated that by 2020, the number of cases will have increased by 275% (National Cancer Registry Ireland, 2006).

Although prostate cancer has been shown to follow Mendelian autosomal dominant inheritance in predisposed families (Carter, Beaty et al. 1992), there is a strong deterministic dependence between prostate cancer incidence and age, with a cut-off at 50 years of age (Leibovitz, Baumoehl et al. 2004).

1.1.2 DISEASE PROGRESSION

The first lesion to be observed in prostate cancer is called prostatic intraepithelial neoplasia (PIN). It is also referred to as a latent microscopic lesion because progression to a later stage of the disease is very slow and might never occur in the patient’s lifetime. While PIN has the cytologic characteristics of prostate cancer, the basal cell layer is present, which distinguishes it from an invasive lesion. PIN is usually classified into two categories, high grade PIN (HGPIN) and low grade PIN (LGPIN). The clinical importance of this distinction is that, when detected on prostate needle biopsy, HGPIN is usually associated with invasive prostate cancer in approximatively
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80% of cases, whereas LGPIN is associated with invasive prostate cancer only about 20% of the time (Brawer, Bigler et al. 1991; Aboseif, Shinohara et al. 1995).

Destruction of the basal layer and local invasion of the disease occurs following exposure to mitogenic promoting agents such as hormones and growth factors. Normal prostate cell development is strongly regulated by androgens (i.e. testosterone). In the absence of the hormone, androgen-dependent cells undergo apoptosis and help maintain a balance between cell loss and cell production. Through clonal selection, transformed cells eventually become immune to androgen deprivation and grow quicker in androgen-rich environment.

Progression of the disease is governed by increased gene instability and susceptibility to additional mutations. Mutation of the tumour-suppressor gene p53, involved in cell cycle regulation and apoptosis, has been linked to late-stage hormone-resistant tumours in up to 80% of specimen tested (Meyers, Gumerlock et al. 1998; Schlechte, Lenk et al. 1998). Furthermore, metastatic prostate cancer has been linked to the loss of invasion suppressor gene E-cadherin, whose expression correlated with Gleason grades of the tumour studied (Umbas, Schalken et al. 1992). Stimulation of angiogenic factor production and local secretion of collagenase enzymes, in synergy with local growth factors (i.e. bFGF) and antimetastatic nm23 protein, also have been found to participate in tumour spread (Stahl, Leone et al. 1991; Leone, Flatow et al. 1993).

1.1.3 Detection and Screening

Because the disease remains asymptomatic for a long period of time, prostate cancer detection is difficult. Obstructive or irritative voiding symptoms can result from local growth of the tumour into the urethra or the bladder, and the
presence of symptoms indeed suggests locally advanced or metastatic disease. Bone pain or impingement on the spinal cord is invariably associated with metastatic disease. Systematic prostate biopsy is the most commonly employed technique used in detecting prostate cancer. Biopsies are usually obtained under TRUS guidance. Algorithms combining PSA and Gleason score are useful in predicting extra capsular extension and risk of relapse following radical prostatectomy (Ravery, Boccon-Gibod et al. 1994; Peller, Young et al. 1995; Huland 1996).

Evaluation and management of prostate cancer patients through serum tumour markers has always been and remains a challenge. While the measurement of acid phosphatase was formerly widely used, it soon became apparent that its lack of sensitivity for early-stage disease compromised its usefulness as a diagnostic tool (Gutman 1968; Whitesel, Donohue et al. 1984; Burnett, Chan et al. 1992). Later discovery of the prostate specific antigen (PSA) gave physicians and scientist new hope (Papsidero, Wang et al. 1980). However, even though PSA is still used today as a diagnostic tool for staging and disease progression, several issues, such as the lack of international standards (Stamey 1997), have been raised over time. The measurement of gross PSA levels are now being replaced by normalized values over time (PSA velocity, PSA doubling time) (Carter, Morrell et al. 1992), prostate volume (PSA density) (Benson, Whang et al. 1992), age dependence and molecular state (complex PSA) (Catalona, Partin et al. 1998) in an attempt to increasing specificity and credibility of the technique.

1.2 CONVENTIONAL MANAGEMENT OF PROSTATE CANCER

1.2.1 MANAGEMENT OF LOCALIZED DISEASE

1.2.1.1 Controversy in the choice of treatment options

As a result of increased opportunistic PSA screening, the proportion of men diagnosed with advanced disease has decreased, while young men are
increasingly being diagnosed with localised disease (Moul, Wu et al. 2002). Consequently, stratifying the risk of disease progression is becoming crucial to treatment decision-making. Indeed, due to the nature of the side effects and co-morbidity (e.g. impotence, incontinence) associated with each treatment option, appropriate management for prostate cancer is complex and controversial. Because of the age migration of the disease, clinicians are indeed now confronted with a new balance between the risk and the benefit of treatment because of the likelihood of longer survival and the risk of decreased quality of life over an extended period of time.

Most clinicians would now agree that patients at low risk are suitable candidates for treatment initially with either watchful waiting or local treatment by a single modality. On the other end, multimodality approaches appear to result in better outcome in patients with intermediate risk disease. Current staging systems however seem limited in the estimation of the risk of disease progression in patients with localized disease. (Moul 2004; Roach, Weinberg et al. 2006; Thompson 2006). Magnetic resonance imaging (MRI) and proton magnetic resonance spectroscopy (1H MRSI) have already been proposed as an important tool for more precise stratification of patients in clinical trials as well as improved patient monitoring (Hricak 2005). The detection of epigenetic changes may also influence prognosis in the future (Dobosy, Roberts et al. 2007).

Conventional treatment options for men diagnosed with localised prostate cancer include active surveillance, androgen ablation, radical surgery and various forms of radiation therapy.

1.2.1.2 Radical prostatectomy

Radical prostatectomy is still the most recommended treatment for patients with clinically localised diseased with a life expectancy of at least 10 years.
However, the procedure is frequently associated with a permanently decreased quality of life (e.g. impotence and incontinence) which may be unacceptable for young men (Walsh 2000). The advantage of surgery however remains the possibility to acquire detailed histopathological information, which may help guide further adjuvant treatment. The risk of relapse, associated with increased PSA, is however around 30% and low dose radiation therapy of the tumour bed is subsequently often necessary (Zincke, Oesterling et al. 1994). Patients with a PSA doubling time (PSADT) of less than 3 month have been associated with a 20-fold increased risk of death of the disease (D’Amico, Moul et al. 2003; Zhou, Chen et al. 2005). The prognostic value of PSA failure following radical prostatectomy is however controversial and novel prognostic indicators may be needed. In this context, the intensity of androgen and epidermal growth factor receptor immunostaining in the primary tumour specimen has already been proposed (Schafer, Funke et al. 2006).

1.2.1.3 Radiation therapy

Radiation therapy has been shown to be an active modality in the management of local and regional prostate cancer. Long-term results are however dependent on stage (Lagerveld, Laguna et al. 2003) and potential side effects also include incontinence and impotence. Determining the risk of failure after radiation therapy is very difficult, because the prostate remains in place. Age, pre-treatment PSA velocity, Gleason score and PSADT all seem to be predictive of survival (Sandler, Dunn et al. 2000; D’Amico, Renshaw et al. 2005; Potters, Morgenstern et al. 2005) but up to 100 definitions of failure have been recently reported (Kuban, Thames et al. 2005). In a recent study, a PSADT in excess of 3 month was associated with mortality rates at 5 years of 15% for biopsy Gleason score 8 or greater and 4% for biopsy score 7 or less (Zhou, Chen et al. 2005). Pre-treatment estimation of the risk of recurrence is particularly important in the case of radiation therapy because surgery within the radiation field is
subsequently difficult, although salvage radical prostatectomy after radiation therapy failure may have a role in selected individuals.

The improvement in technology over the years has helped to change the concept of radiotherapy from palliation of advanced prostate cancer to a curative approach for localised disease. External beam radiotherapy (EBRT) is now widely accepted as a curative treatment modality for localised prostate cancer (Mangar, Huddart et al. 2005). In addition, the development of transrectal ultrasound, sophisticated computer planning and dosimetry have contributed to the resurgence of prostate brachytherapy as a viable option for the treatment of localised prostate cancer. Brachytherapy permits conformal radiotherapy and dose escalation, and offers the convenience of a single-day outpatient procedure. The reported biochemical, clinical tumour control and potency preservation rates with brachytherapy appear also as effective as both EBRT and surgery (Potters, Torre et al. 2001; Machtens, Baumann et al. 2006).

Despite improvements in delivery techniques, radiation therapy is associated with limited outcome in prostate cancer. Because post-radiation studies have showed a high incidence of positive prostate biopsies in patients treated with standard doses (70 Gy or less) (Babaian, Kojima et al. 1995), dose escalation has been proposed. Its impact on clinical outcome however remains inconclusive (Pollack, Zagars et al. 2000; Pollack, Zagars et al. 2002; Pickles and Pollack 2006). Moreover, controversy exists as to the increased risk of radiation-induced second malignancies in surrounding normal tissues exposed to low radiation doses associated with novel delivery techniques such as intensity-modulated radiotherapy (Hall and Wuu 2003; Kry, Salehpour et al. 2005; Smith, Heron et al. 2006). Altered fractionation schedules have also been proposed to try and maximize tumour control, while reducing early and late toxicity (Thames, Withers et al. 1982). Although theoretically this approach has potential in the prostate, where the difference between the $\alpha/\beta$ ratio of normal and tumour tissues is large, these protocols are so far still of limited efficacy.
(Nieder, Andratschke et al. 2004; Antognoni, Corvo et al. 2005). Moreover, it now appears that the tumour characteristics, such as reoxygenation rate, may dictate the number of fractions that can be used in radiobiologically optimized fractionation protocols (Ruggieri 2004).

1.2.1.4 Hormonal therapy

Since prostate tumours are dependent on testosterone, reduction of the testosterone level is often very effective in preventing further growth and spread of the disease. Androgen deprivation may be achieved through reduction of the luteinising hormone using pituitary down-regulators or gonadotrophin releasing hormone analogues (GnRH analogues), such as Zoladex® and Prostap® or with the administration of androgen-blocking agents (such as Drogenil® and Casodex®), which prevent testosterone binding to the androgen receptor of prostate cells. Hormonal therapy as a monotherapy is not curative but may lower the PSA levels, delay disease progression and reduce disease morbidity (Byar 1972; Walsh 1997). At some point however (18 - 24 month) hormone therapy fails in most men and the disease becomes hormone-refractory. As a result, hormonal therapy is increasingly used in combined protocols (described in section 1.3). A number of variables, such as increased serum alkaline phosphatase, bone pain and decreased serum testosterone have been associated with poor probability of prolonged response to hormonal therapy (Cho, Di Blasio et al. 2003; Gandhok, Looney et al. 2005).

1.2.2 MANAGEMENT OF ADVANCED DISEASE

Currently available therapies for advanced prostate cancer remain essentially palliative and are necessary to prevent the risk of complications, such as spinal cord compression (Kantoff 2005).

Because around 75% of metastatic prostate cancers are hormone sensitive, androgen ablation has been the most commonly used frontline therapy for the
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treatment of disseminated prostate cancer for the past six decades as a means
to reduce bone pain and improve quality of life. The average time for response
to androgen deprivation is about 18 months and survival after second-line
treatment varies from 6 to 10 months (Small and Vogelzang 1997). Prostate
tumours indeed eventually relapse to a hormone-refractory state that no longer
responds to conventional therapies and becomes lethal (Gittes 1991). Novel
strategies targeted to the androgen receptor may in the future prevent disease
progression and treat hormone-refractory disease (Reddy, Barrack et al. 2006).

Chemotherapy is often used as a palliative treatment of prostate cancers that
are resistant to hormonal treatments. Drugs that may be used to treat prostate
cancer include docetaxel, paclitaxel, mitoxantrone and epirubicin (Calabro and
Sternberg 2007).

Radiotherapy is the main treatment for non-diffuse painful bone metastases.
These occur in about 85% of progressive, hormone-resistant cancers. Short,
localized external beam irradiations (20 Gy in five fractions over one week or,
more often, 30 Gy in 10 fractions over two weeks) bring partial or total pain
relief in 80% of patients with negligible morbidity (Dearnaley, Bayly et al. 1992;
Di Lorenzo, Autorino et al. 2003). Systemic delivery of biphosphonates or
radionuclides (eg. strontium 89) may also be administrated (Hamdy and
Papapoulos 2001; Bauman, Charette et al. 2005).

1.2.3 IMAGING AS A TOOL TO IMPROVED DIAGNOSIS

While transrectal ultrasound (Mohler, Gregory et al.) is used to guide biopsies,
after histological confirmation of cancerous lesions, MR imaging with an
endorectal coil (endoMRI), with or without the addition of dynamic contrast-
enhanced agents, has been proposed to offer promise for local staging. It has
been estimated that the predicting accuracy of endoMRI is in the region of 50 to
80% (Quinn, Franzini et al. 1994; Jager, Severens et al. 2000). However, due to
the occurrence of blinding post-biopsy haemorrhage, MRI may only be used 4 to 8 weeks post-biopsy and the technique is too insensitive to be used as a primary diagnostic tool (White, Hricak et al. 1995; Ikonen, Kivisaari et al. 2001). Nonetheless, by revealing the anatomical location of prostate tumours, MRI can aid in staging and reveal a road-map for surgery or radiation treatment.

On the other end, MR spectroscopy imaging (MRSI) provides metabolic information about prostatic tissue by displaying the relative concentrations of chemical compounds within contiguous small volumes of interest. Three-dimensional proton MRSI metabolic mapping of the entire gland is possible and displays information on concentration of citrate and choline. Because high levels of cellular activity results in decreased citrate levels and increased choline concentration, the choline to citrate ratio can be used to detect malignant lesions in the prostate volume (Kurhanewicz, Vigneron et al. 1995; Kurhanewicz, Vigneron et al. 1996). More importantly, the ratio has been shown to correlate with Gleason grade (Zakian, Sircar et al. 2005). Unfortunately, the literature has shown a wide range (50-92%) in the accuracy of local staging by MRI and the routine use of the technique remains controversial (Engelbrecht, Jager et al. 2002). However MRI has the potential to assist planning of treatment delivery as well as predict the risk of morbidity of surgery (Coakley, Eberhardt et al. 2002; Coakley, Eberhardt et al. 2002; Hricak, Wang et al. 2004) and radiation therapy (Roach, Faillace-Akazawa et al. 1996; Debois, Oyen et al. 1999).

One of the most promising new technique in regards to personalised therapy is positron emission topography (PET) because the wide range of radiotracers available provides insight into the biological behaviour of tumours rather that their morphological appearance (Powles, Murray et al. 2007). However, far from the impact that these techniques may have in the future management of prostate cancer, the development of radiotracers has already provided valuable
information as to the nature of prostate tumours that may be exploited for the design of novel therapeutics, and/or explain the limitations encountered by current techniques.

Several PET tracers have been developed for imaging of cell proliferation (e.g. Fluorine-18 labelled 3-deoxy-3-[F-18]-fluorothymine (FTL); [11-C]-choline), glucose metabolism (e.g. [F-18]-fluorodeoxyglucose (FDG)), tissue perfusion and hypoxia (e.g. [F-18-misonidazole (FMISO)).

Imaging studies have for instance shown that FGD uptake is poor, suggesting that most prostate tumours do not display increased glucose metabolism, which can be associated with reduced metabolic activity. Interestingly however, patients with high FDG uptake appear to have significantly poorer prognosis. These patients may therefore benefit from mitochondrion-targeted strategies. Co-administration of the anti-tumour drug ionidamine, which induces the mitochondrial apoptotic pathways, blocks cell proliferation and cell progression through the cycle with anti-cancer drugs such as cisplatin, may thus potentate chemosensitisation (De Cesare, Pratesi et al. 1998). Moreover, glycolysis inhibition strategies, for instance through knockdown of adenine nucleotide translocase-2, an enzyme involved in ATP production, may also increase sensitisation of these prostate tumours (Le Bras, Borgne-Sanchez et al. 2006).

Despite poor FDG uptake, prostate carcinomas have been associated with increased KI-67 expression as well as enhanced [C]-acetate and choline uptake which correlated with tumour aggressiveness and could be used to detect recurrence (Kotzerke, Volkmer et al. 2002; Fricke, Machtens et al. 2003; Breeuwsma, Pruim et al. 2005).

Finally, imaging of androgen receptor expression and the use of labelled dihydrotestosterone, showed that the androgen receptor is overexpressed in androgen-independent metastatic disease (Larson, Morris et al. 2004), which
suggest that novel strategies, targeted to the androgen receptor may help control advanced disease. In addition to classic endocrine therapy by surgical and/or chemical castration, new strategies have been developed to inhibit the AR directly through anti-androgens, selective AR modulators, naturally occurring AR inhibitors, neutralizing antibodies and dominant-negative peptides (for review, see (Taplin 2007)).

Because post-treatment biopsies are not current practice in patients presenting with recurrence, the development of powerful imaging techniques may be the only possibility to gain biological information on treated tumours. Indeed, epigenetic studies may be developed to improve the diagnostic and prognosis evaluation of TRUS biopsies and radical prostatectomy specimen, but the genetic changes induced by hormonal deprivation and radiation therapy are poorly studied, due to the lack of tissue samples available.

1.2.4 THE LIMITED ROLE OF ANTI-ANGIOGENIC TREATMENTS

As well as permitting tumour growth and expansion and despite being poorly efficient, the tumour vasculature indirectly protects tumours from complete eradication due to its unstructured nature, leaving tumour foci beyond the diffusion limit of chemotherapeutic drugs and protected from damaging ionising radiation. The vasculature of prostate tumours has been shown to be increasingly disorganised as the disease progressed (Ozawa, Yao et al. 2005). Nonetheless, because tumour blood vessels offer unique and specific markers, such as VEGF, many targeted cancer therapies have been developed to deprive cancer cells from nutrients and prevent tumour expansion through vascular destruction. Indeed destruction and remodalisation of the tumour vasculature has been observed by interfering with VEGF signalling (Jain, Safabakhsh et al. 1998).
While vascular destruction is effective at inducing tumour necrosis, the effect of anti-angiogenic therapies seems to be transient. Loss of endothelial cells is not necessarily accompanied by simultaneous loss of pericytes and surrounding basement membrane, which together can result in regrowth of tumour vessels (Baluk, Hashizume et al. 2005). Upon completion of the protocol, the tumour vasculature has indeed been shown to rapidly regain its pre-treatment state (Mancuso, Davis et al. 2006). However, a potential benefit of anti-angiogenic therapies is their ability to normalise the tumour vasculature. Due to the restoration of a more normal basal membrane and better coverage of perivascular cells, the vessels of this normalized vasculature appear better organised, less leaky, less dilated, less distorted and participate in improved tumour oxygenation, leading to enhanced efficacy of drug absorption and radiation treatment (Tong, Boucher et al. 2004; Winkler, Kozin et al. 2004).

Despite their ability to gain access to tumour cells via leaky tumour vessels, antibodies and other macromolecular therapeutics transport has also been found to beneficial by the inhibition of VEGF signalling (Nakahara, Norberg et al. 2006). It thus appears that the concomitant delivery of anti-VEGF treatment with novel therapeutic may be an important strategic approach. Unfortunately, even after the tumour vasculature has been normalised, the therapeutic agent will face the challenge of passing through the endothelial barrier to reach tumour tissue itself. Vascular permeability may thus also need to be improved, for instance with the administration of pro-inflammatory cytokines and vasoactive agents (Curnis, Sacchi et al. 2002). On the other hand, sustained or aggressive anti-angiogenic regimens may eventually prune away these vessels, resulting in a vasculature that is both resistant to further treatment and inadequate for delivery of drugs or oxygen (Jain 2001).
1.3 MULTIMODAL THERAPY REGIMES

1.3.1 ROLE AND OBJECTIVES

Because single modality treatments offer at best a 5-year biochemical disease-free survival of only 50% of patients with high-risk prostate cancer, multimodal therapy regimes have come under study. The presence of micrometastasis at the time of diagnosis and treatment has been proposed as a hypothesis to the failure rates of current treatment options for localised disease and is supported by the reported 37% rising of serum PSA of recurrent prostate cancer patients that underwent radical prostatectomy (Zietman, Edelstein et al. 1994). To improve survival results of patients with locally advanced disease, multimodality approaches that combine systemic therapy with local therapy are increasingly being studied. Early systemic treatment may be given prior to local therapy...
(neoadjuvant hormonal therapy) to reduce recurrence risk in patients with suspected micrometastasis as well as reduce tumour volume to improve the probability of tumour control. The addition of systemic therapy following local treatment (adjuvant) has also been proposed to help prevent recurrence (Glode 2006).

1.3.2 CURRENT REGIMES

Although the administration of pre-operative hormonal therapy has been associated with reduced positive surgical margins in a number of clinical trials, this modality has not translated into increased disease-free survival. The rationale for neoadjuvant therapies was however confirmed when chemotherapy was used. Overall survival was indeed increased when docetaxel was administrated prior to surgery in a number of clinical trials. These results are thus encouraging as to the potential advantage of developing novel neoadjuvant strategies (Pendleton, Pisters et al. 2007).

One of the most potent multi-modality approaches to date is the discovery of the clinical benefit of adding androgen suppression therapy to external-beam radiation therapy. The additive effect has been reported in multiple randomised trials in several subsets of prostate cancer, recently reviewed by Lee et al. (Lee 2006). As a result, combining hormonal therapy with radiation therapy has become the standard of care for men with high-risk prostate cancers. Although the mechanisms are still unclear, the clinical impact of hormonal therapy has been seen in biochemical control, local control, distant metastases, disease-specific survival, and overall survival.

The potential of adjuvant chemotherapy prior to radiation therapy has also been investigated but so far has been associated with dose-limiting toxicity (Zelefsky, Kelly et al. 2000; Hanks, Pajak et al. 2003; Kumar, Perrotti et al. 2004; Ryan, Zelefsky et al. 2004).
1.3.3 THE POTENTIAL OF GENE THERAPY

The results obtained with multimodality strategies suggest that novel therapies may be developed for concomitant use with conventional treatment regimen. The fact that the prostate is an accessory organ, not required for potency or urinary continence as well as its easy accessibility make it an ideal target for gene therapy strategies. Furthermore, the presence of a large number of prostate-unique promoters and candidate antigens promises to facilitate the design of prostate-specific gene therapy (Nelson, Clegg et al. 2000).

Despite the numerous problems that novel therapeutic strategies face, gene therapy has the potential to improve current therapeutic outcomes. As opposed to silencing strategies (e.g. siRNA, Zinc finger proteins), which require the targeting of a single gene or protein, and novel chemotherapeutic drugs, whose efficacy may be limited by poor delivery and toxicity, gene therapy offers a potentially more flexible approach for the treatment of prostate tumours.

Cancer gene therapy in its purest form is the replacement, with a correct copy, of a gene whose mutation initiates or significantly alters the malignant phenotype. The goal of tumour suppressor gene therapy includes the induction of cell death and the production of changes in cell growth, behaviour, invasiveness, or metastatic ability. However tumour-suppressor gene therapy is still limited by the large number of target genes clearly known to induce or maintain malignancy.

Immuno-modulatory strategies focus on the stimulation of the patient's own immune system to combat prostate tumour growth (Kaminski, Summers et al. 2003). Prostate cancer is a great candidate for this form of therapy because of the large number of potentially unique antigens it has to offer as targets. Prostate cancer cells, as with other tumour cells, are poor antigen presenters with defects in the MHC class I expression in as high as 85% of primary and 100% of metastatic tumours (Blades, Keating et al. 1995). Immuno-modulatory gene therapy strategies for prostate cancer include the therapeutic delivery of cytokines and the transfer of tumour-associated antigens like PSA or PSMA.
The use of tumour-associated antigens like PSA or PSMA as a way of inducing cancer cells directed immunologic responses is now well under development. Direct transduction and expression of tumour-associated antigens has been shown to enhance the efficacy of viral vaccines in pulmonary metastases with a potentially further increased effect if co-transfected with cytokines (Bronte, Tsung et al. 1995; Bronte, Carroll et al. 1997). DNA vaccines have also been developed. A PSA-based DNA vaccine has been reported to induce a strong humoral response in PSA-positive tumour cells (Kim, Trivedi et al. 1998). Furthermore, polyvalent cancer vaccines targeting the entire antigenic spectrum on tumour cells are being investigated in dendritic cells (Heiser, Maurice et al. 2001) and could be expected to be adapted to prostate cancer in the future. The weakened immune system of cancer patients is however a limiting factor to immuno-modulatory strategies.

Cytokines that have been used in clinical trials include IL-2 and IL-12. IL-2 is the most effective antitumour cytokine used in clinical practice. IL-2 gene therapy both proved safe and effective in phase I clinical trials, with a reported decrease in serum PSA levels on day 1 in 67% of the patients enrolled (Belldegrun, Tso et al. 2001; Pantuck and Belldegrun 2001; Nasu, Ebara et al. 2004). Following encouraging results in mouse model (Nasu, Bangma et al. 1999; Nasu, Bangma et al. 2001; Nasu, Ebara et al. 2004), a replication-defective human IL-12 transducing adenoviral vector is to be tested in a phase I clinical trial in patients who have failed radiation therapy or have metastatic prostate cancer (Baylor College of Medicine). In the future, it has been suggested that NK cell-mediated, anti-tumour effects of IL-15 could provide a potential rationale for gene therapy of prostate cancer (Suzuki, Nakazato et al. 2001).

Suicide gene therapy (SGT), on the other hand is a flexible approach, which can merge both exploitation of tumour characteristics and the benefit of chemotherapy. SGT is defined as the transduction of a gene that converts a non-toxic form of a drug (pro-drug) into a toxic substance. Two such systems
have been extensively studied: the E. coli cytosine deaminase gene that transforms 5-Fluorocytosine into the chemotherapeutic 5-Fluorouracil (Austin and Huber 1993), and the herpes simplex virus thymidine kinase gene that phosphorylates Ganciclovir, converting it to a nucleoside analogue that inhibits DNA synthesis (Moolten 1986). Despite a significant bystander effect in which more cells are affected than are actually transfected (Vile, Nelson et al. 1994; Trinh, Austin et al. 1995; Mesnil, Piccoli et al. 1996), those two systems are limited by delivery specificity and transfection efficiency.

The success of any systemic targeted cancer therapy ultimately relies on the ability of the agent to reach target in sufficient concentration. Because tumour vasculature is responsible for the systemic drug delivery, the impact of previous therapeutic intervention on the tumour vasculature is an important factor as to the efficacy of the adjuvant administration of the novel therapeutics. This is especially relevant since anti-cancer agents are more than likely to enter clinical trials in patients with tumours resistant to current treatment modalities, whose histology and vasculature has more than likely been damaged. In this context, gene therapy for prostate cancer may be at a slight advantage because systemic delivery may be avoided due to the location of the prostate, and the development of viral vectors may ensure dissemination of the therapy.

1.4 OXIDATIVE STRESS AND TUMOUR HYPOXIA

1.4.1 THE CENTRAL ROLE OF THE TUMOUR VASCULATURE

A common downfall of new therapeutics for prostate cancer is their targeted delivery, which depends on adequate blood supply. Tumours, like normal tissues, rely on the vasculature to exist. The blood vessel network is therefore a central point of tumour biology and an important structure to consider for cancer treatment. Evidence has connected tumour aggressiveness and poor patient survival with microvessel density (MVD) (Figure 1-1), although structurally and
functionally defective, in many human malignancies including prostate cancer (Bostwick and Iczkowski 1998; Pallares, Rojo et al. 2006).

Figure 1-2 Microvessel density (MVD) in prostate cancer. (A) and (B) CD34 immunostaining of radical prostatectomy specimen shows disparity in MVD. (C) Box-Whisker plots of MVD determined by DCE-MRI as the mean vascular area per measure field (MVAF) in areas of cancer and normal peripheral zone. Adapted from Bono et al. 2002 and Schlemmer et al. 2004.

MVD has also been recently proposed as a molecular marker for the identification of HGPIN lesions more likely to progress (Sinha, Quast et al. 2004) and for the improvement of the prognostic stratification of patients with moderately differentiated prostatic adenocarcinoma after radical prostatectomy (Halvorsen, Haukaas et al. 2000; Bono, Celato et al. 2002). While immunostaining for the endothelial antigen CD34 of prostate cancer specimen is a commonly used technique for scoring of MVD (Bono, Celato et al. 2002), contrast-enhanced dynamic MR imaging for prostate cancer has been proposed
as a an imaging tool to provide important information about individual MVD in prostate cancer patients prior to surgery and facilitate assessment of individual prognosis (Schlemmer, Merkle et al. 2004).

Closer study has revealed that neovascularisation, triggered as a result of the release of pro-angiogenic factors by tumour, stromal and inflammatory cells, is unevenly distributed within the expanding tumour mass, showing regions of extreme and sparse MVD. The mean number of MVD is indeed estimated to be four to ten times greater at the invading tumour edge than in the inner tumour areas (Giatromanolaki, Koukourakis et al. 2000; Koukourakis, Giatromanolaki et al. 2000). Further research is however needed to understand the molecular mechanisms causing this abnormal vascular architecture (Dvorak 2003).

An important feature of tumour vasculature is the increased proliferation rate of its endothelial cells by up to 200 times compared to that of normal tissues, which may account for the anti-angiogenic properties of therapeutics targeted to dividing cell populations (Hobson and Denekamp 1984; Denekamp 1990). Moreover, tumour blood vessels are thin-walled, with abnormal branching and blind endings (Jain 1988). Their endothelial lining is incomplete, showing fenestrations and loss of intercellular junctions (Roberts, Delaat et al. 1998). The basement membrane is also often incomplete or absent, and associated with paucity of smooth muscle cells and pericytes (Steinberg, Konerding et al. 1990; Kakolyris, Giatromanolaki et al. 1999). Perfusion is also relatively ineffective, due to arteriovenous shunts, resulting in up to 30% of the total blood flow in tumours to bypass the exchange system of capillaries (Wheeler, Ziessman et al. 1986; Vaupel, Kallinowski et al. 1989). Solid (mechanical) stress generated by proliferating tumour cells also compresses vessels in tumours (Padera, Stoll et al. 2004). Tumour micro-circulation thus suffers from impaired, multi-directional and intermittent blood flow, impaired interstitial fluid drainage, increased interstitial fluid pressure and increased vascular permeability (Sivridis, Giatromanolaki et al. 2003).
1.4.2 OXIDATIVE STRESS

Oxidative stress is defined as the imbalanced redox state in which pro-oxidants overwhelm antioxidant capacity, resulting in increased production of Reactive Oxygen Species (ROS). Most cell types have been shown to elicit a small oxidative burst generating low concentrations of ROS when they are stimulated by cytokines, growth factors and hormones (Thannickal and Fanburg 2000). Growing evidence suggests that ROS within cells act as second messengers in intracellular signaling cascades and can play critical roles in controlling cell survival (Shackelford, Kaufmann et al. 2000; Storz 2005; Shen and Liu 2006).

The biological effect of ROS is dependent upon their intracellular concentration. The relationship appears to be associated with a toxic threshold, above which ROS may cause molecular damage and trigger both stress and adaptative responses. When ROS concentration reaches high levels, damage becomes the overriding result and triggers cell death. In cancer cells, reduced antioxidant defence may participate in increased ROS concentration that is high enough to trigger the pro-survival adaptive response but low enough to protect the cell from inevitable cell death. Moreover, the ROS-adaptative response protects against the accumulation of damage in tissues from renewed exposure to ROS bursts (Feinendegen 2002; Lehnert and Iyer 2002; Stadtman and Levine 2002). Preliminary exposure of lymphocytes, ovarian carcinoma and myeloma cells with low doses was shown to increase their resistance to subsequent exposure to high doses of radiation, in a time dependent manner (Filippovich, Sorokina et al. 1998). ROS concentration is thus a critical parameter in determining cellular fate.

Exposure of cells to a variety of stressors induces compensatory activations of multiple intracellular signalling pathways such as mitogen-activated protein kinase (MAPK) (Sun and Oberley 1996) and phosphatidylinositol-3-kinase (PI3K) (Zhan and Han 2004). Other pathways activated by stressors such as ionising radiation and hypoxia include those downstream of death receptors,
including pro-caspases, anti-apoptotic proteins and the transcription factor NFκB (Chresta, Masters et al. 1996; Gilbert and Knox 1997; Romashkova and Makarov 1999; Rugo and Schiestl 2004).

An important molecule in these stress-related molecular pathways is hypoxia-inducible factor 1 (HIF-1). HIF-1 functions as a heterodimer consisting of two basic-helix-loop-helix proteins, HIF-1α and HIF-1β. HIF-1 is activated through post-translational stabilisation of HIF-1α, which is degraded by the proteasome in aerobic conditions (Blancher and Harris 1998). Under hypoxic conditions, the prolyl hydrolase is inactive and ubiquitination is inhibited. HIF-1α is no longer degraded resulting in extended life time (30 min) and its accumulation in the cytoplasm. HIF-1α is translocated from the cytoplasm to the nucleus where it interacts with HIF-1β to form the heterodimer HIF-1.

As the effect of oxygen deprivation on cellular response is progressively understood, its impact is proving greater than expected. Cellular respiration and the production of ATP, already altered in cancer cells, is inevitably one of the first to suffer from oxygen deprivation (Figure 1-3). Physiologically produced by oxidative phosphorylation in normal cells, the ATP generated in cancer cells tend to result from increased aerobic glycolysis (Warburg effect), due to accumulated defects in the mitochondrial genome (Brandon, Baldi et al. 2006). In response to reduced oxygen tension, cells were shown to durably switch to glycolytic metabolism (Figure 1-3A). As a result of inefficient oxidative respiration, reactive oxygen species (ROS) are formed. ROS were shown to participate to the stabilisation of HIF-1α by hypoxia through activation of the Akt pathway and direct action of oncogenes such as Ras and Scr (Pelicano, Martin et al. 2006).

Increased HIF-1α levels result in up regulation of genes involved in glycolysis, enhancing anaerobic ATP production (Figure 1-3B). Moreover, the accumulation of succinate and fumarate in the cytosol, due to acquired
mutations of mitochondrial enzymes (succinate dehydrogenase, fumarate hydratase) was shown to inhibit the activity of prolyl hydrolases, participating in the stabilization of HIF-1α, further feeding this positive feedback loop (Brandon, Baldi et al. 2006; King, Selak et al. 2006). Finally, ROS may also be involved in hypoxia-induced resistance by inhibiting mitochondria-induced apoptosis through down regulation of the mitochondrial outer membrane permeabilization (MOMT) process (Kroemer 2006; Moll, Marchenko et al. 2006) (Figure 1-3C).

Loss of HIF-1 activity has been shown to inhibit growth of mouse xenografts, suggesting HIF-1 as an attractive tumour-specific target (Semenza 2002). Kung and colleagues designed a polypeptide with the ability to disrupt the binding of HIF-1α to its transcriptional co-activators p300/CREB and thereby inhibit hypoxia-induced transcription. Infection of tumour cells with this polypeptide resulted in a significant reduction in the growth of these cells when transplanted into nude mice (Kung, Wang et al. 2000). Compounds such as vincristine and paclitaxel have been shown to inhibit HIF-1 at the transcriptional level and consequently reduce tumour growth and vascularity (Mabjeesh, Escuin et al. 2003). Reduction of HIF-1 protein levels through proteosomal degradation, can be achieved through the action of the heat shock protein 90 inhibitor geldanamycin (Sun, Kanwar et al. 2001; Mabjeesh, Post et al. 2002). Direct injection of antisense constructs to HIF-1α have also been shown to have the potential to eradicate a small transplanted thymic lymphoma and to increase tumour-directed immunotherapy efficacy (Sun, Kanwar et al. 2001). Finally, an interesting approach would be to screen for compounds that are preferentially toxic to HIF-1α expressing cells.
Figure 1-3 Schematic representation of the effect of hypoxia on mitochondrial function. (A) The adaptation of cancer cells to hypoxia favors glycolysis over oxidative phosphorylation for ATP production. (B) Acquired mutations in mitochondrial enzymes may result in the accumulation of fumarate and succinate, participating in HIF-1α accumulation. (C) Inefficient respiration induces the formation of reactive oxygen species (ROS), stabilizing HIF-1α and inhibiting mitochondria-mediated apoptosis. ROS production may be inhibited by nitric oxide.

1.5 TARGETING TUMOUR HYPOXIA

1.5.1 OVERCOMING TUMOUR HYPOXIA

Among the methods suggested to overcome the problem of hypoxia are treatment in hyperbaric oxygen chambers, chemical (hypoxic) sensitizers and the introduction of high linear energy transfer radiation, such as neutrons and heavy ions, which have a reduced oxygen enhancement ratio (Fricke, Machtens et al.).

A first attempt to overcome the problem of hypoxia in radiotherapy was to expose patients to radiation while sealed in chambers filled with pure oxygen.
raised to a pressure of 3 atmospheres in the hope of achieving increased oxygen levels in the blood and hence the tumour. The clinical trials that were performed involved small numbers of patients and were difficult to interpret because unconventional fractionation schemes were used. That is, a few large fractions were used because of the time and effort involved in the technical procedures. Furthermore serious risk of fire and claustrophobia of patients made the technique difficult. Although most clinical trials weren’t as successful as planned due to the inevitable presence of acute hypoxia, the technique has been shown to increase local control and survival in patients with carcinoma of the cervix and advanced head & neck cancer (Sealy 1991; Haffty, Hurley et al. 1999) and reduce radiation therapy morbidity in prostate cancer (Mayer, Klemen et al. 2001).

The notion of improving tumour oxygenation by breathing 100% oxygen rather than air has been revived in recent years. Oxygen-induced vasoconstriction may be avoided if 5% carbon dioxide is added to the oxygen, a mixture called carbogen. Several clinical trials conducted by the NCI were looking at the combination of Accelerated Radiotherapy with Carbogen and Nicotinamide Radiosensitisation in glioblastomas (Miralbell, Mornex et al. 1999), head and neck cancer (Saunders, Hoskin et al. 1997; Bernier, Denekamp et al. 2000), laryngeal cancer (Kaanders, Pop et al. 1998) and small cells lung cancer (Bernier, Denekamp et al. 1999). While the protocol was feasible in all cancer types, preliminary results were encouraging in laryngeal cancer only.

Spurred largely by the efforts of radiation chemists, a search was under way in the early 1960s for compounds that mimic oxygen in their ability to sensitise biologic materials to the effects of x-rays. The first candidate compound that appeared to satisfy these criteria was misonidazole. It was very effective in vitro but unfortunately proved to be too toxic to normal tissues in vivo. It was followed by two less toxic compounds (etanidazole, nimorazole) but at the price of reduced effectiveness. The use of misonidazole in radiotherapy patients proved
to produce a small yet significant improvement in local tumour control in head and neck cancer. But overall, clinical trials proved to be disappointing and misonidazoles were progressively abandoned (Saunders, Anderson et al. 1982; Urtasun, Feldstein et al. 1982; Irie, Sakurai et al. 1984; van den Bogaert, van der Schueren et al. 1986; Overgaard 1994).

Studies on the effect of oxygen on cell death following exposure to neutrons revealed a loss of the protective effect of reduced oxygen levels. Combined with the fact that neutrons are biologically more effective killer of cancer cells than more frequently used radiation techniques, neutron therapy is conceptually an attractive alternative (Laramore 1997; Noel, Feuvret et al. 2003). Neutrons have an uncertain place in the treatment of a number of specific human cancers, particularly salivary gland tumours (Hummel, Buchholz et al. 1990), malignant melanomas (Kabalka, Nichols et al. 2003), soft tissue sarcomas (Glaholm and Harmer 1988) and advanced prostate cancer (Lindsley, Cho et al. 1998). While there is evidence in the literature of the potential of hadron therapy (HT), controversy exists in regards to its expansion as a major treatment modality. HT for prostate cancer has been limited by the availability of appropriate treatment facilities but nonetheless, encouraging results have been obtained (Rossi, Slater et al. 1998; Lodge, Pijls-Johannesma et al. 2007).

1.5.2 EXPLOITING TUMOUR HYPOXIA

Rather than designing therapeutic strategies to overcome hypoxic resistance, a number of groups have chosen to exploit the hypoxic nature of tumours to gain a therapeutic advantage. The molecules involved in the hypoxia-induction of genes have been studied in the hope of selectively controlling their expression as part of therapeutic strategies.

Systemic cytotoxic anticancer drugs rely primarily for their therapeutic effect on cytokinetic differences between cancer and normal cells. One approach aimed
at improving the selectivity of tumour cell killing by such compounds is the use of less toxic prodrug forms that can be selectively activated in tumour tissue. Hypoxic activation is based on the fact that the prodrug is a substrate for intracellular one-electron reductases (i.e. cytochrome P450 reductase). The reductase reacts with the molecule to add one electron. The resulting free radical reacts with oxygen if present to form superoxide and regenerates the original compound whereas, in the absence of oxygen, free radicals accumulate in the cell and confer its toxicity.

The first agents that have been used as selective hypoxic cells killers were quinine-containing alkylating agents (i.e. mitomycin C) and nitroaromatic compounds (i.e. misonidazole, RB6145). They were however surpassed when Brown and Lee discovered tirapazamine over 20 years ago (Brown 1993). This compound has the advantage of larger and more selective hypoxic cell toxicity. When combined with radiation treatment, the cytotoxicity conferred by each treatment modality was complementary to one another, resulting in enhanced local tumour effects (Shibata, Shibamoto et al. 1996; Zhang and Stevens 1998). Tirapazamine also acts in synergy with the anti-cancer drug cisplatin: a phase III clinical trial of the combination of the two treatments in small-cell lung cancer resulted in doubling of the overall response rate and significant increase in survival (von Pawel, von Roemeling et al. 2000). Similar results were reported in a phase II head and neck cancer study (Rischin, Peters et al. 2005). A phase III clinical trial is under way. The only drawback to this combined therapy remains dose-limiting neutropenia associated with the treatment.

Improved hypoxia-activated prodrugs are now being developed to reduce toxicity. Areas of development include the linkage DNA-targeting units to moieties known to damage DNA in hypoxic cells (i.e. NLCQ-1), the generation of prodrugs that can release more stable cytotoxins on reduction to generate a bystander effect, while being responsive to very low levels of oxygen to protect naturally hypoxic normal tissues such as the bone marrow, the retina and the
liver. An example of such newly developed prodrug is SN23862, a nitrogen mustard analog of CB1954. This prodrug, also metabolized by bacterial Nitroreductase, already has generated exciting preclinical data (Palmer, van Zijl et al. 1995). Other classes of prodrug being considered include nitrobenzyl phosphoraminade mustards, nitroheterocyclic methylquaternary salts, cobalt (III) complexes and indoloquinones (Ahn and Brown 2007).

Non-pathogenic, obligate anaerobic bacteria of the genus Clostridium have been used experimentally as anticancer agents because of their selective growth in the hypoxic regions of solid tumours after systemic application. Lemmon et al. used genetically engineered clostridia as tumour-specific vectors for the delivery of the gene for an E. coli nitroreductase known to activate the non-toxic prodrug CB1954 to a toxic anticancer drug. Nitroreductase produced by these clostridia enhanced the killing of tumour cells in vitro by CB1954, by a factor of 22. Tumours harbouring clostridia spores that did not possess the E. coli nitroreductase gene were devoid of nitroreductase activity. Most importantly, E. coli nitroreductase protein was not found in a large survey of normal mouse tissues following intravenous injection of nitroreductase containing clostridia, strongly suggesting that obligate anaerobic bacteria such as clostridia can be utilized as highly specific gene delivery vectors for cancer therapy (Lemmon, van Zijl et al. 1997).

1.6 RATIONALE FOR THE DEVELOPMENT OF A NOVEL HYPOXIA-TARGETED STRATEGY FOR PROSTATE CANCER

1.6.1 EVIDENCE OF TUMOUR HYPOXIA IN PROSTATE TUMOURS

When in the 1990s, an oxygen electrode became commercially available; it became possible for investigators to measure oxygen levels in human tumours. In prostate tumours, pO₂ measurements showed that increasing levels of hypoxia correlated significantly with increasing clinical stage and patient age (Movsas, Chapman et al. 1999; Movsas, Chapman et al. 2000). The oxygen
tension is thought to be reduced up to 7-fold due to poorly organized and functionally unsound outgrown tumour vasculature (Vaupel, Kelleher et al. 2001; Vaupel 2004).

In addition, HIF-1α overexpression, evidenced by increased immunostaining, has been reported in a variety of human cancers and their metastases, including prostate cancer. (Zhong, De Marzo et al. 1999; Lekas, Lazaris et al. 2006). The presence of hypoxic regions in prostate tumours has also been confirmed with positron emission tomography of \[^{18}\text{F}\] fluoro-misonidazole (Rasey, Koh et al. 1996; O'Donoghue, Zanzonico et al. 2005).

Moreover, the incorporation of new hypoxia measurements from prostate cancer into the modelling of local tumour control probability (LTCP) following radiation therapy has suggested that it is the radioresistant hypoxic tumour cells that govern the overall response rate of prostate cancer to current therapies (Nahum, Movsas et al. 2003)

1.6.2 HYPOXIA MAY PARTICIPATE TO DISEASE PROGRESSION

Prostate cancer carcinogenesis and disease progression are complex processes strongly influenced by local microenvironment. Chronic exposure of initiated prostate cancer cells to inflammation as well as hormonal and oxidative stress are thought to contribute to the induction of DNA damage and genomic instability, participating in the progression of the disease. The adaptative potential of prostate cancer cells to modulated environment leads to rapid progression to a highly aggressive and treatment resistant phase of the disease, associated with poor prognosis (Petrylak 1999; Petrylak 2005).

The potential role of tumour hypoxia in disease progression is not well documented. Integrative molecular concept modelling of prostate cancer progression resulted in the discovery of influential set of genes supporting this hypothesis (Tomlins, Mehra et al. 2007). The study highlighted a strong role of
ETS family members. Although the involvement of these factors in prostate cancer progression is likely to be through their role in androgen signalling, ETS factors have been associated with the regulation of vascular inflammation and remodelling (Oettgen 2006) as well as hypoxia-inducible factor-2 target gene selection (Aprelikova, Wood et al. 2006) and tumour angiogenesis (Huang, Brown et al. 2006). The study also highlighted an inverse relationship between the expression of genes involved in glutathione metabolism and disease progression. Glutathione plays important roles in antioxidant defence and therefore impaired function may be evidence of cellular adaptation to hypoxia. Physiologic levels of androgens are also capable of increasing oxidative stress. Androgens alter intracellular glutathione levels and the activity of certain detoxification enzymes, such as gamma-glutamyl transpeptidase, that are important for maintenance of the cellular prooxidant-antioxidant balance (Ripple, Henry et al. 1997). Glutathione levels reduction has been a basis for novel apoptosis-inducing therapeutics (Meurette, Lefeuvre-Orfila et al. 2005).

The role of the PI3K-Akt pathway in prostate cancer progression, androgen independence and apoptosis resistance has been recently proposed (Edwards, Krishna et al. 2003; Li, Ittmann et al. 2005). This pathway is stimulated by a variety of signals, including hypoxia (Alvarez-Tejado, Naranjo-Suarez et al. 2001), and regulates a wide spectrum of substrates involved in cell survival and proliferation. Akt activation in prostate cancer was associated with increased calveolin-1 expression, resulting in reduced apoptosis sensitivity (Mouraviev, Li et al. 2002; Li, Ren et al. 2003). Akt indeed plays a central role in anti-apoptotic pathways. Phosphorylation of BAD and BAX by Akt results, respectively, in restoration of Bcl-XI's anti-apoptotic function (Datta, Dudek et al. 1997) and inhibition of mitochondria-mediated apoptosis (Yamaguchi and Wang 2001). BAD was recently proposed as a convergence point of several antiapoptotic signaling pathways in prostate cells (Sastry, Smith et al. 2006).
Bax and Bak have been shown to link the TRAIL death receptor pathway to the mitochondrial apoptosis signaling cascade upon DNA damage by ionising radiation in prostate cancer cells (Wendt, von Haefen et al. 2005). Ark further abrogates the mitochondrial pathway through phosphorylation of pro-caspase 9 (Cardone, Roy et al. 1998) and increased Bcl-2 expression (Wilson, Mochon et al. 1996). Resistance to the inhibition of PI3K-mediated response has been reported in response to long-term androgen ablation (Pfeil, Eder et al. 2004), and correlated with loss of PTEN (Li, Yen et al. 1997; Hermans, van Alewijk et al. 2004). Ark activation is a positive regulator of both hypoxic and radiation responses, though activation of CREB and stabilisation of HIF-1α and NFkB (Chrivia, Kwok et al. 1993; Romashkova and Makarov 1999; Jiang, Jiang et al. 2001). Moreover the PI3K pathways has been implicated in both chemo- and radio-resistance of prostate cancer cells (Lee, Steelman et al. 2004; Zhan and Han 2004; McCubrey, Steelman et al. 2006).

Growing evidence has linked prostate cancer carcinogenesis with early loss of cellular protection to oxidative damage, participating to increased DNA damage and genetic instability. Cancer cells may as a result be more vulnerable to the epigenetic effect of hypoxia; supporting the hypothesis that poor oxygenation may be an important factor in disease progression. Loss of genes involved in cellular protection against oxidative damage, such as the androgen-regulated prostate-specific homeobox Nkx3.1, when combined with other genetic factors such as loss of PTEN and glutathione S- transferase Pi (GSTPI) have already been proposed to participate in disease progression (Asatiani, Huang et al. 2005; Ouyang, DeWeese et al. 2005). As the carcinogenesis process unfolds, selected cells may therefore become less efficient at reducing the production of oxidative damage but at the same time more efficient at preventing associated DNA damage, for example through upregulation of enzymes involved in the reduction of oxidizing products such as superoxide dismutases and catalases (Trzeciak, Nyaga et al. 2004). These phenotypic changes may thus
progressively produce subclones with survival advantage to unfavourable environments.

1.6.3 HYPOXIA MAY BE THERAPEUTICALLY INDUCED

Recent evidence suggests that hypoxia/reoxygenation cycles are not only occurring during cancer development through tumour growth and subsequent regional angiogenesis but may also be therapeutically induced. It was indeed proposed that vasculature destruction and androgen deprivation may result in the development of transient acute hypoxia in treated tumours, potentially selecting for a more aggressive phenotype of surviving cells (Mabjeesh, Willard et al. 2003; Mohler, Gregory et al. 2004; Boddy, Fox et al. 2005; Park, Kim et al. 2006). If correct, this theory could have a huge impact on the design of future treatment protocols.

An important feature of tumour vasculature is the increased percentage of dividing endothelial cells in response to unbalanced angiogenic factors (Thorpe 2004). The preferential killing potential of ionising radiation towards mitogenically active cells thus results in its antiangiogenic effect. The cooperation between the anti-VEGF effect of hormonal deprivation and vascular destruction may therefore account to the efficacy of combined treatments. In response to radiation injury, endothelial cells were however shown to increase VEGF expression, resulting in both stimulation of revascularization and reduced radiosensitivity (Gupta, Jaskowiak et al. 2002). Interestingly, increased tumour hypoxia was associated with increased VEGF expression in prostate cancer carcinoma (Movsas, Chapman et al. 1999; Cvetkovic, Movsas et al. 2001) and the hypoxic prostate/muscle pO₂ ratio was shown to predict biochemical failure in patients with prostate cancer (Movsas, Chapman et al. 2002).

Disease progression and tumour recurrence may also be treatment induced by prolonged androgen-deprivation therapies. Worryingly, Casodex ®, commonly
used in hormonal therapy of prostate cancer, has already been found to induce hypoxia-related gene expression in vitro (Rothermund, Gopalakrishnan et al. 2005), and hypoxia was recently reported to increase the activity of the androgen receptor and sensitise tumour cells to low androgen levels (Park, Kim et al. 2006). Hypoxia directed therapies, previously considered of potential benefit in resistant advanced disease, may thus now also have their place early in the management of prostate cancer.

1.6.4 Hypoxia participates to increased chemoresistance

In prostate tumours, the transition from androgen-dependence to androgen-independence indeed appears as a key event in regards to chemoresistance. If long-term androgen deprivation is to induce transient acute hypoxia as recently proposed, the effect of oxidative stress and ROS on known mechanisms of resistance and disease progression may be worth investigating.

Cancer therapeutic agents can be absorbed and distributed to various tissues by penetrating into membrane bound compartments. Blocking cell adhesion molecules was reported to increase the sensitivity of cancer cells towards cytotoxins and inhibit tumour growth (Shain and Dalton 2001). Interestingly, the induction of cell adhesion molecules of the ADAMs transmembrane protein family by ROS via p38 mitogen-activated protein kinase has been associated with prostate carcinogenesis and therapeutic resistance (Fischer, Hart et al. 2004) (Figure 1-4A). Increased ADAM9 expression for instance was associated with androgen-independence progression in prostate cancer cells and prostatic tissue (Sung, Kubo et al. 2006). In response to toxic insult, both normal and cancer cells try to limit the intracellular accumulation of the agent by upregulating the detoxifying mechanism. It has indeed been demonstrated that the main cause of acquired chemoresistance, known as "multiple drug resistance" (MDR), by cancer cells, is their ability to efflux toxins at an accelerated rate through overexpression of certain transport proteins / efflux
pumps. One of the largest families of transporters consists of the ATP-binding cassette proteins (ABC), whose most clinically relevant member is the P-glycoprotein (Pgp).

Figure 1-4 Potential mechanisms of hypoxia-induced chemoresistance. (A) The induction of ADAMs adhesion proteins may participate to chemoresistance through inhibition of apoptosis. (B) The activity of ABC transporters has been shown to be activated in response to hypoxia, extracellular acidity, drug exposure and cytokines. (C) Pgp activity is increased in response to HIF-1α and ROS but inhibited by reduced ATP concentrations. (D) Activation of PI3K-Akt by hypoxia and/or cytokines participates in drug resistance through increased MRP-1 expression levels. (E) PIM-1 participates to chemoresistance through inhibition of apoptosis and stimulation of proliferation. (F) COX-2 participates to hypoxic resistance through inhibition of p53 activity.

Pgp expression has not been extensively evaluated in prostate cancer because chemotherapy is not the main treatment modality. However a retrospective study on archival material reported a positive regulation between staining intensity for Pgp and Gleason grade as well as PSA levels and presence of
metastasis (Bhangal, Halford et al. 2000). Moreover, patients with metastatic disease have been reported to express high levels of MRD1 whereas patients with localized disease did not (Izbicka, Dalton et al. 1998). Upregulation of the MDR-1 gene could interestingly be associated with increased mRNA expression of macrophage inhibitory factor (MIF), DNA binding protein inhibitor 1 (ID-1) and GSTPI in chemoresistant prostate cancer cells (Yu, Lin et al. 2006). Pgp levels were also found higher in androgen-independent prostate cancer cell lines PC3 and DU145 than in androgen-sensitive LnCaP cells (Fojo, Ueda et al. 1987; Theyer, Schirmbock et al. 1993).

Pgp expression has been shown to be regulated by cytokines (i.e. IL6, TNF-α), growth factors (Yang, Sullivan et al. 1997; Lee and Piquette-Miller 2003), androgens and oxidative stress (Figure 1-4B). ROS appear to act on Pgp both at the levels of gene expression and protein stability (Wartenberg, Gronczynska et al. 2005). Interestingly, Pgp expression has been recently reported to be regulated by HIF-1α (Wartenberg, Gronczynska et al. 2005). Overexpression of Pgp indeed correlated to hypoxia in central regions of three-dimensional tissues of multicellular prostate tumour spheroids (Wartenberg, Gronczynska et al. 2005; Wartenberg, Hoffmann et al. 2005). Moreover, the activity of Pgp increased in response to extracellular acidity induced by hypoxia, due to the accumulation of high levels of lactate and low glucose concentration associated with anaerobic glycolysis (Thews, Gassner et al. 2006). The higher efflux rate under acidic conditions and hypoxia-induced ROS may thus lead to a lower concentration of chemotherapeutic drugs within the cells (Figure 1-4C). Elevated Pgp could therefore act as a “double-edge sword” decreasing both androgen and intracellular anti-androgen concentration. This protein may therefore trigger a cellular response to androgen-deprivation early in hormonal therapy and as a result participate to the selection of an adaptative phenotype, upregulate cell survival response and encourage androgen independence.
The involvement of Pgp in drug resistance may however be more complex than expected. Downregulation through hypermethylation of the MDR-1 gene promoter was observed in 54.8% of radical prostatectomy specimen and correlated with worse clinico-pathological features (Enokida, Shiina et al. 2004). CpG methylation of MDR1 has thus been proposed as a frequent event involved in prostate cancer disease progression. According to these findings, it may be proposed that in some patients, hypermethylation of MDR1, correlating with low detection of the Pgp protein, participates in tumour progression early in the development of the disease by stimulating cellular proliferation whereas in other groups of patients hormone withdrawal and associated hypoxia may increase Pgp activity and inhibit associated apoptosis to enhance drug resistance.

The multidrug resistance protein family (MRP) is another class of drug pumps that have been reported to be responsible for acquired multidrug resistance in solid tumours. MPR-1 overexpression was detected in prostate cancer cell lines (Zalcberg, Hu et al. 2000) as well as in both normal prostatic tissue (37%) and prostatic adenocarcinoma (84%) (Sullivan, Amenta et al. 1998). Interestingly, the activation of the phosphatidylinositol 3'-kinase (PI3K) signal transduction cascade lead to increased MRP-1 expression levels (Lee, Steelman et al. 2004). The PI3K pathway, heavily involved in cellular proliferation and survival, is known to be activated by hypoxia (Alvarez-Tejada, Naranjo-Suarez et al. 2001; Jiang, Jiang et al. 2001; Zhong, Zheng et al. 2004). While PTEN mutation, observed in 60% of prostate cancer patients (Vlietstra, van Alewijk et al. 1998), may largely contribute to the heightened levels of PI3K-signaling activity reported during prolonged androgen-ablation therapy (Murillo, Huang et al. 2001; Pfeil, Eder et al. 2004), the link with hypoxia remains interesting. It may be thus be postulated that induction of PI3K by hypoxia results in upregulation of MDR-1 and concomitant HIF-1α stabilization, triggering survival response and increasing chemoresistance (Figure 1-4D). In this study the authors proposed that inhibition of PI3K activity with concomitant administration
of chemotoxic compounds may prove beneficial in preventing the development of drug resistance in patients with hormone-refractory prostate cancer. However it may also be evidence of the potential advantage in the targeting of hypoxia during androgen therapy.

1.6.5 HIF-1A OVEREXPRESSION MAY BE EXPLOITED

Because HIF-1 activity has been shown to be increased by ionising radiation, hypoxia and androgens; (Park, Kim et al. 2006; Pinthus, Bryskin et al. 2007), this protein emerges as an attractive target to novel prostate cancer therapies. Exploiting overexpression of HIF-1α, known to occur in prostate cancer, and use this protein to drive transgene expression to oxygen deprived cells in a gene therapy setting may improve the therapeutic outcome of both early and advanced disease.

The development of an effective gene delivery system to the site of therapeutic significance has proven to be the major hurdle to the advancement of gene therapy. Viruses have developed highly effective methods of entering cells and thus have been recognised to be excellent, natural candidates for transferring genes or other therapeutic agent into cancerous cells. However, while both virus-type vectors and non-viral vectors have been developed, the latter have not proved to be as effective as viral-based vectors in experimental systems and clinical applications (Frochlitiz 2000). Furthermore, the use of diffusible gene products allows for the treatment to reach untransfected cells. This bystander effect, an advantage in balancing poor transfection efficiencies, could however become a problem for unwanted gene expression outside the target volume. Therefore the addition of transcriptional control, in a tissue-, tumour-, or environment-specific manner is necessary.

A number of genes promoters, such as PSA, bcl-2, human kallikrein 3 and prostate-specific membrane antigen (PMSA) have been used for prostate-
specific gene therapy (Foley, Lawler et al. 2004). For instance, PSA promoter-driven adenoviral (AV) suicide gene therapy with HSVtk and nitroreductase genes has been shown to be effective in prostate, but not non-prostate, cell lines in vitro and in vivo (Gotoh, Ko et al. 1998; Latham, Searle et al. 2000). A disadvantage to tumour- and tissue-specific gene therapy is the resulting constitutive expression in the target tissue and the relative weakness of existing promoters such that they direct only low level transcription of their transgenes. A more attractive option is the development of inducible systems in which the transcription of exogenous genes introduced into cells can be controlled by administration of inducing or repressing agents. Many aspects of tumour microenvironment are unique and can be targeted. Promoters that are responsive to tumour microenvironment have been developed as an ingenious attempt to target gene expression to the tumour volume. The two approaches addressed in this thesis are hypoxia- and radiation-inducible systems.

In the view of the emergence of oxidative stress and tumour hypoxia as a factor to treatment failure, this thesis aims to design hypoxia-responsive promoters to be cloned upstream of the bacterial cytosine deaminase gene. Developing a radiation-responsive system seems to be the most ambitious answer to controlling gene expression and will be addressed in Chapter 8. Radiation-mediated gene therapies combine the destructive power of irradiation with intracellular drug delivery, suggesting an intelligent solution to tumour eradication. While numerous authors have documented the potential of radiogenic therapy (Marignol et al., 2007- In press), radiation-responsive promoters remain to be developed for prostate cancer gene therapy.

Hypoxia-targeted gene therapy may be used for radiosensitisation strategies, potentially leading to reduction of both dose per fraction and total dose to improve local control and reduce toxicity. In this context, while chemotherapy has thus far been modestly effective for prostate cancer (Tester, Ackler et al. 2006), novel combination of gene therapy and chemotherapy, as offered by
suicide gene therapy systems, may display synergistic improvements in efficacy. The HSV-tk/GCC gene-directed enzyme prodrug therapy (GDEPT) system has been shown to prolong median PSADT in a recent phase I clinical trial (Nasu, Saika et al. 2007) in patients with recurrent tumours following hormonal therapies. Combination with ionising radiation resulted in a dramatic systemic effect in mice associated with a marked increased in CD4-Tcell infiltration (Chhikara, Huang et al. 2001).

Because 5-fluorouracil has been associated with mild radiosensitising properties (Smalley, Kimler et al. 1991), the CD/5-FC GDEPT system appears a better strategy for the development of a novel suicide gene therapy strategy to be potentially administrated in a multi-modal therapeutic protocol. The enzyme cytosine deaminase (CD) can be found in certain bacteria and fungi but not in mammalian cells. CD catalyses the hydrolytic deamination of cytosine to uracil. It can therefore convert the prodrug 5-fluorocytosine (5-FC) to its active form 5-fluorouracil (5-FU). The CD gene used for gene-directed enzyme prodrug therapy (GDEPT) has been cloned from E.Coli (Austin et Huber, 1993). It has been shown to enhance mammalian cell sensitivity to 5-FC by up to 2000 fold (Ge et al., 1997). The bystander effect, initially described by Moolten et al (1986), is another important feature of this strategy. A hypoxia-inducible CD/5-FC gene therapy approach may thus have an application in overcoming the drug-and radiation-resistant hypoxic population in prostate tumours.

Once the experimental conditions are proved to be non-toxic or have limited toxicity to wild type cells, the potential of these vectors to induce enzyme production sufficient to selectively sensitisise prostate cancer cells to 5-FC treatment in response to a variety of hypoxic exposure scenarios was evaluated. The radiosensitising potential of the therapy was investigated. Finally, potential limitations of the technique, such as targetability, monitoring and delivery, are addressed. This research hopes to provide sufficient in vitro
data to justify further development of this hypoxia-targeted strategy towards improved therapeutic outcome of conventional therapies.
CHAPTER TWO: MATERIALS AND METHODS
2.1 MOLECULAR BIOLOGY

2.1.1 REHYDRATION OF LYOPHILISED OLIGONUCLEOTIDES

Oligonucleotides (MWG, Germany) were resuspended in 1xTE to a final concentration of 5pmol/μL. 1X TE was added to the lyophilised oligos and let to incubate on ice for 10 min before being vortexed. Aliquots were taken and stored at -20°C.

2.1.2 ANNEALING REACTION

30 pmol of each complementary oligos were added to 10X T4 DNA ligase buffer (Sigma-Aldrich, UK) and distilled water to a total volume of 24.5 μL. The reactions were placed on a thermocycler. The annealing program consisted of 2 min at 95°C followed by a cool down to 25°C in 45 min.

2.1.3 PHOSPHORYLATION REACTION

Annealed oligos mixes were combined and 10 units of T4 polynucleotide kinase (PNK) (Sigma-Aldrich, UK) were added. The phosphorylation reaction was incubated at 37°C for 30 min, followed by heat activation of PNK at 65°C for 20 min on a thermocycler.

2.1.4 LIGATION REACTIONS

The DNA Ligation Kit <Mighty Mix > is a premix reagent, including T4 DNA Ligase and an optimized buffer system that allows very rapid DNA ligation reactions. Ligation mixes contained equal volumes of DNA and Mighty Ligation Mix (Takara, UK) and were incubated at 25°C for 5 min on a thermocycler.

2.1.5 RESTRICTION DIGEST

Plasmid DNA was mixed with 10X enzyme buffer, BSA (1mg/μL), appropriate number of units of the restriction enzyme and distilled water to the desired total
volume. Sigma, UK supplied all restriction enzymes. The reaction was incubated at 37°C for 3 hours.

2.1.6 BLUNT-ENDING REACTION

The plasmid DNA was mixed with 10mM dNTPs, 10 units of T4 DNA polymerase, 1X T4 DNA polymerase buffer and distilled water to a total volume of 60 µL. The mix was incubated at room temperature for 10 min. The reaction mix was cleaned using Wizard® SV DNA clean up kit (Promega, UK).

2.1.7 DEPHOSPHORYLATION REACTION

The plasmid DNA was mixed with 10mM dNTPs, 10 units CIAP, 1X CIAP and distilled water to a total volume of 60 µL. The mix was incubated at 37°C for 1h. A further 10 units of CIAP were added 30 min into the incubation.

2.1.8 DNA GEL ELECTROPHORESIS

Low melting point agarose was melted into 1X TAE buffer before addition of ethidium bromide. The gel was poured in a gel rig (Biosciences, UK) and left to set for 30 min. Samples were loaded and run at 100V for 30 – 45 min.

2.1.9 DNA GEL PURIFICATION

The reaction was loaded on a low melting temperature agarose gel, along with a DNA step ladder (Sigma, UK). The gel was run at 100V for 30 min. Bands of the correct size were cut out under UV light using a razor blade and weighed. The DNA was cleaned up by centrifugation using Wizard® SV DNA clean up kit (Promega, UK), according to manufacturer’s instructions, to an elution volume of 50 µL.
2.1.10 Estimation of DNA Concentration

Estimation of the DNA concentration of samples was done by fluorospectroscopy. 1µL of DNA was loaded on a spectrophotometer (Nanodrop 100, Biosciences, UK). Absorbance at 390nm was measured and the DNA concentration estimated.

2.2 Cloning Protocols

2.2.1 Preparation of Chloramphenicol

0.4g of chloramphenicol (Sigma, UK) was dissolved in 20mL ethanol to obtain a solution at 20 µg/µL. Aliquots were taken and stored at -20°C.

2.2.2 Preparation of Agarose Plates

Agar was dissolved in deionised water (40g/L) and autoclaved, before being equilibrated to a temperature of 60°C in a water bath. Chloramphenicol was added to the warm agar solution at a concentration of 20 µg/µL. The solution was poured in petri dishes and allowed to settle. The plates were stored upside down at 4°C.

2.2.3 Preparation of LB Broth Culture Media

Luria broth base (Sigma, UK) was dissolved in deionised water, autoclaved and stored at 4°C. Just before use, chloramphenicol was added at a concentration of 20 µg/µL.

2.2.4 Ligation Reactions

2.2.4.1 Insert and vector ligation

Three vector: insert ratio were used: 1:1, 1:5 and 1:10.
Each ligation reaction contained 50ng of vector and the appropriate amount of insert to satisfy the defined ratios. The formula used to calculate \( x \), the amount of insert to be used was: 

\[
X = 50 \times \frac{\text{size of vector}}{\text{size of insert}}
\]

Ligation mixes were prepared according to section 2.1.4. The reactions were stored at -20°C.

2.2.4.2 Blunt-ending ligation

The ligation reactions contained 50ng of vector 1 \( \mu \text{L} \) 10X T4 ligase buffer, 0.5 \( \mu \text{L} \) T4 ligase and distilled water to a total volume of 10 \( \mu \text{L} \), in the presence or absence of 1mM PEG.

2.2.4.3 E.coli transformation

The protocol used was that of manufacturer’s instructions and were similar for each JM109 (Promega, UK), SURE® (Stratagene, Ireland) and TOP10® (Invitrogen, UK) competent cells. The ligation reaction was added to 50\( \mu \text{L} \) of competent cells. Control reactions contained the supplied ampicillin-resistant supercoiled DNA or no DNA. Once the reactions were prepared, cells were let to recover on ice for 10 min. The reactions were then plunged into a 42°C water bath for 45-50s. Cells were then incubated on ice for 2 min. Following the addition of cold SOC media (Sigma, UK) (4°C), the reactions were left to shake in an orbital shaker at 37°C, 150 rpm for 1 hour. The mixture was then centrifuged at 4000rpm for 2 min and half the supernatant was removed. The cells were resuspended in the remaining volume and en plated onto the appropriate warm antibiotic resistant agar plate.

The plates were incubated at 37°C for 12-16 hours, upside down.
2.2.5 SCREENING OF CLONES

2.2.5.1 Culture of clones

A single colony was inoculated into 5mL of chloramphenicol treated LB broth and left to incubate overnight at 37°C, shaking at 150 rpm. The next day, culture was spined down at 12,000 rpm, at 4°C. The supernatant was removed and the pellet dried in a speedvac at high heat for 5 min. DNA was extracted by centrifugation using Quiagen Spin Miniprep kit (Quiagen, UK).

2.2.5.2 Digestion of clones

1μL of each plasmid prep was digested with the appropriate enzyme at 37°C for 3 hours. 1 μL was run on a 1% agarose minigel with the appropriate undigested control DNA, 50 kb ladder. The size of the bands was estimated and correlated to the expected correct sizes.

2.2.5.3 Preparation of glycerol stocks

0.85 mL broth culture of the correct clones was mixed with 0.15 mL glycerol in a labelled cryovial, vortexed and stored immediately at -80°C.

2.2.6 PREPARATION OF HIGH GRADE PLASMID DNA

2.2.6.1 Inoculation onto agar plate

Using a sterile plastic loop dipped into the glycerol stock of the plasmid to be cultured, the cells were plated onto a chloramphenicol-resistant agar plate. The plate was then incubated at 37°C, up side down.

2.2.6.2 Starter culture

As single colony was inoculated into 3 mL of LB broth media (Sigma, UK), supplemented with chloramphenicol (0.17mg/mL), and incubated at 37°C for 8 hours, shaking at 250 rpm to ensure oxygen flow.
2.2.6.3 Overnight culture

100 µL of the starter culture was inoculated into 100mL of LB broth media supplemented with chloramphenicol (0.17mg/mL). The culture was incubated at 37°C overnight, shaking at 250 rpm, to ensure oxygen flow.

2.2.6.4 Endotoxin removal

Endotoxin-free plasmid DNA was obtained using Macheley-Nagel Maxi prep kit, according to manufacturer's instruction. Briefly, 100mL of broth culture media was centrifuged at 6000 rpm for 10 min. The resulting pellet was resuspended and lysed using the buffer provided by the kit. The lysate was filtered and loaded onto the provided nucleobond columns by gravity flow. Following several washes, the DNA was eluted and precipitated with isopropanol. Following centrifugation at 15000 rpm for 10 min at 4°C, the precipitate was resuspended in ethanol and re-centrifuged at room temperature. The pellet obtained was air dried for 20 min and resuspended into endotoxin-free water.

2.2.6.5 Plasmid concentration

The plasmid preparation was digested with HindIII and run on agarose mini-gel to check on the content. The plasmid concentration was determined by fluorescence measurement on a Nanodrop.

2.3 Sequencing

Sets of primers were designed to start ~ 30 bp from the cloning junction and commercially produced by MWG, UK. The sequences of the primers used are shown on Appendix 10.1.

Each mix contained 600ng of the plasmid, 2pmol of the relevant primer, 4µL of BigDye (BD Bioscience, UK) and deionised water to a total volume of 20 µL. The reactions were run on a thermocycler on a set program (94°C for 30s, 96°C
for 10s, 50°C for 10s, 60°C for 4 min, 24 times) for the dye to bind to the DNA. The sequencing reactions were then cleaned to remove residual dye, using Quiagen DyeEx column kit (Quiagen, UK). The recovered clean reaction was dried in a speedy vac at medium heat until dry. The dry pellets were resuspended in 10 μL. HiDi formamide and loaded on a 96-well sequencing plate. The plate was run on a sequencer (3100ABI) using a 36-cm array.

2.4 Cell Culture

2.4.1 Cell Lines

The transition to androgen-independence is an important event in prostate cancer in regards to treatment resistance. Both a primary androgen sensitive (22Rv1) and a metastatic androgen-resistant (DU145) cell line were thus chosen throughout the development of this strategy.

To confirm targetability of the technique to a range of prostate cancer phenotypes, PC-3 and LnCap cells were also used. These two commercially available cell lines are well-established models of metastatic disease. However, PC-3 cells may represent an exception to common phenotypes in regards to its HIF-1α amplification status and this cell line was not used beyond initial Western Blotting studies (section 5.3.1). Due to the technical difficulty to growing LnCap cells in vitro, this cell line could not be used in apoptosis and clonogenic studies.

In an attempt to strengthen our model of primary disease, a novel cell line, RC58/T, derived from a radical prostatectomy sample of a Caucasian male, was acquired from Prof. Rhim, Center for Prostate Disease, Bethesda, USA. However, little information is currently available regarding its phenotype and our results could only be analysed with great difficulty. These cells were thus not used in both apoptosis and clonogenic studies.
The androgen-responsiveness status of the cell lines is shown on Table 2-2.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Androgen-dependent</th>
</tr>
</thead>
<tbody>
<tr>
<td>LnCaP</td>
<td>Yes</td>
</tr>
<tr>
<td>22Rv1</td>
<td>Yes</td>
</tr>
<tr>
<td>DU145</td>
<td>No</td>
</tr>
<tr>
<td>PC3</td>
<td>No</td>
</tr>
<tr>
<td>RC58/T</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Table 2-1 Androgen sensitivity status of prostate cancer cells, as described in the literature.

This panel of prostate cancer cell lines was also used to investigate the potential role of p53 in the response to treatment. The published p53 status of the cell lines is shown on Table 2-2.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Loss of heterozygosity</th>
<th>p53</th>
<th>p53 IHC*</th>
</tr>
</thead>
<tbody>
<tr>
<td>LnCaP</td>
<td>No</td>
<td>Wild type</td>
<td>~ 10%</td>
</tr>
<tr>
<td>DU145</td>
<td>No</td>
<td>Mutant</td>
<td>&gt; 90%</td>
</tr>
<tr>
<td>22Rv1</td>
<td>No</td>
<td>Mutant</td>
<td>~ 10%</td>
</tr>
<tr>
<td>PC3</td>
<td>Yes</td>
<td>Null</td>
<td>Negative</td>
</tr>
<tr>
<td>RC58/T</td>
<td>Not determined</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2-2 TP53 characterisation of prostate cancer cell lines, adapted from (van Bokhoven, Varella-Garcia et al. 2003). *IHC, Immunohistochemical staining

Finally, PWR-1E and RPWE-1 cells were used as models for normal prostate tissue.

Human DU145, PC-3, LnCaP and 22Rv1 prostate cancer cells were routinely maintained in RPMI 1640 medium (Gibco, UK) supplemented with 10% foetal calf serum (Globepharm, UK) and 1% streptomycin-penicillin (Gibco, UK).
Human RC58/T prostate cancer cells, along with human normal prostate cells PWR-IE and RPWE-1, were routinely maintained in Keratinocyte SFM medium (Gibco, UK) supplemented with Bovine Pituitary Extract, recombinant Epidermal Growth Factor and 1% streptomycin-penicillin (Gibco, UK). The cells were grown as monolayers in tissue culture flasks at 37°C and passed every 3-4 days to maintain exponential growth.

2.4.2 Mycoplasma Testing of the Cell Lines

The cell lines were tested for mycoplasma every three month using Mycoalert® mycoplasma detection kit (Cambrex, UK). Briefly, working solutions were prepared by addition of Buffer to both Reagent and Substrate. A 2 ml media sample from the cells was collected from cultured for several days in antibiotic-free media. This sample was centrifuged for 5 min to remove cell debris, and then a 100 μL aliquot was placed in a 96-well plate or cuvette. To begin the assay, 100 μL of MycoAlert® Reagent was added, and the samples were incubated for 5 min. A reading of the cells was taken with a luminometer (Reading A) and then 100 μL of the MycoAlert® Substrate was added. After 10 min incubation, the luminescence was determined again (Reading B). A ratio was then calculated (Reading A/Reading B). A value below 1 was considered negative. Representative results for the cell lines used are presented in Appendix 10.2.

2.4.3 Plating of Cell Lines

80% confluent cell culture flasks were trypsinised (5mL) for 5-10 min. The cells in suspension were centrifuged for 3 min at 1300 rpm following addition of complete media for trypsin inactivation. The pellet was resuspended in 3mL complete media. 10 μL of resuspended cells were added to 90 μL of Ethidium Bromide and counted using a hematocytometer. The cells were allowed to seed overnight. Generally, the cells were plated at a density of 10^6 cell/well and 2.5x10^4 cells/well in 6-well and 96-well plates, respectively.
2.4.4 Transfection

2.4.4.1 With Gene porter 2

PC-3, DU145, RC58/T, PWR-IE and RPWE-1 cells were transfected with GenePorter 2 (Gene therapy systems, US). The plasmid DNA was diluted with the DNA diluent provided by the manufacturer at a 1:25 ratio. Gene porter 2 was diluted in serum free RPMI 1640 media at a 1:5 ratio. The two solutions were combined and incubated at room temperature for 15 min. The mixture was added to the cells together with 1mL of serum and antibiotic-free RPMI 1640 media and incubated at 37°C. After 3 hours, the medium containing the DNA/liposome complexes was replaced with normal growth medium.

2.4.4.2 With Transfast

22Rv1 and LnCaP cells were transfected with Transfast (Promega, UK). The plasmid DNA was diluted in pre-warmed RPMI 1640 medium (serum-free and antibiotic-free) and vortexed. Transfast was vortexed and added to the diluted DNA. The mixes were vortexed and incubated at room temperature for 15 min before addition to the cells. After 3 hours, the transfection medium was replaced with complete medium.

2.4.4.3 Transfection efficiency

Transfection efficiency was estimated by co-transfecting controls with the Green Fluorescent Protein (GFP) using the pS721IREeGFP plasmid provided by Dr. Southgate (Manchester, UK) and manually scoring the proportion of cells expressing GFP under fluorescent light. Transfections efficiencies were 40±5% in 22Rv1 cells, 38±5% in DU145 cells, 35±7% in LnCap cells, 45±2% in RC58/T cells, 37±5% in RPWE-1 cells and 41±5% in PWR1E cells. The difference is transfection efficiencies were not statistically significant (p=0.08) and therefore do not account for the differences observed in the response of our transfectants.
2.4.5 Preparation of 5-Fluorouracil

5-fluorouracil (Sigma-Aldrich, UK) was prepared in sterile complete RPMI 1640 medium to a concentration of 100 mM/μL. The drug was dissolved at 70°C for 1 hour. Aliquots were taken over liquid nitrogen fumes to reduce air oxygen content and ensure stability of the prodrug. The aliquots were stored at 4°C. 5-FC was diluted in complete RPMI 1640 medium to 0.1, 1, 5 and 10 mM/μL and added to the cells.

2.4.6 Preparation of 5-Fluorocytosine

5-fluorocytosine was prepared at a concentration of 100 μM. 0.387 mg of 5-FC was dissolved in 30mL complete RMPI medium at 70°C for 1 hour. Aliquots were prepared over liquid nitrogen to reduce oxygen content and reduce oxidation of the molecule. The aliquots were stored at 4°C. Serial dilutions in complete media of the stock 5-FC solution were used to obtain the desired 5-FC concentration.

2.5 Hypoxic and Radiation Exposure

2.5.1 Hypoxic Conditions

Hypoxic conditions were achieved in a 1000 in vivo hypoxic chamber (BioTrace, UK). The cells were exposed to a mixture of nitrogen, CO₂ (5%) and compressed air to the desired concentration in oxygen (0.5%).

2.5.2 PO₂ Measurement

pO₂ was monitored with an oxygen probe (OxyLab pO₂TM, Oxford Optronix, UK). Cellular pO₂ was measured in each cell lines plated at several densities over long hypoxic exposure as an attempt to evaluate the time at which cells reached anoxia. DU145, PC3 and 22Rv1 cells were plated at a cell density of 1, 2.5 and 5x 10⁴ cells / well on 96-well plates and 0.5 and 1 x 10⁶ cells / well on 6-well plates.
The plates were exposed to atmospheric oxygen levels of 4, 2, 1 and 0.5% oxygen and measurements of cellular pO₂ were taken every hour over a period of 24 hours.

2.5.3 RADIATION EXPOSURE

Irradiation was delivered under aerobic conditions using a RS225 cell irradiator (Gulmay Medical, UK), at a dose rate of 3.25 Gy/min. The uniform delivery of ionising radiation to cells plated on culture dishes was ensured with the generation of broad radiation beam. The plates were irradiated at the centre of the beam, at a distance of 40cm and with minimized media volume, to ensure minimal loss of dose through backscatter with a beam produced by 200kV and 15mA. The dosimetry was done by a medical physicist, using both ionization chambers and thermoluminescent dosimeters (TLDs).

The radiosensitivity of the DU145 prostate cancer cell line observed here (section 4.5.2) was in concordance to that published. Such information for 22Rv1 cells remains, to your knowledge, unavailable in the current literature.

2.6 WESTERN BLOTTING

2.6.1 PROTEIN EXTRACTION

The wells were washed twice with cold PBS. 100 μL of Cold RIPA lysis buffer (Santa Cruz) supplemented with a protease inhibitor cocktail was then added to each well. The plate was incubated on ice for 20 min. The lysates were then centrifuged at 15,000rpm for 15 min. The supernatant was stored at -70°C.

2.6.2 BRADFORD ASSAY

The standard curve was obtained with serial dilutions of BSA at 0.1 mg/μL into 500 μL of distilled water (0 to 32mg/μL). 1 μL of each sample was diluted into 500 μL of distilled water. 500 μL of Bradford reagent (Sigma-Aldrich, UK) was
added. The absorbance was read at 595 nm on a plate reader. The equation of the standard curve was used to estimate the sample's protein concentration. The results of each individual Bradford assay associated with the Western blots results presented here will not be shown here but the assay was used prior to each individual protein gel electrophoresis.

2.6.3 PROTEIN ELECTROPHORESIS

The Separating (15%) and stacking gels were prepared according to protocol. 25μg of whole cell extracts in laemni buffer (Sigma, UK) were heated to 90°C for 10 min and then loaded onto the gel. A protein molecular weight marker was also added. The apparatus was immersed in running buffer and electrophoresed at 200V for 45 min. The separated proteins were then transferred onto Hybond-C Super nitrocellulose membrane (Amersham, UK) by electrophoresis 100 V for 1 h into transfer buffer with cooling. The composition of gels and buffers is shown in Appendix 10.3.

2.6.4 PROTEIN DETECTION

The membrane was blocked for 1 h by shaking in 5% blocking solution and then incubated with either mouse monoclonal anti-cytosine deaminase antibody (MTM laboratory, Germany) at a 1:1200 dilution, mouse monoclonal anti-β-actin antibody at a 1:10000 dilution or mouse monoclonal anti-GFP antibody (BD Bioscience, Oxford, UK) at a 1:1000 dilution. Antibodies were diluted in blocking solution. The membrane was incubated at room temperature with the antibody for 1 hour under gentle agitation. The membrane was then washed with T-TBS for 3 x 5 min. Horseradish peroxidase conjugated mouse secondary antibody (Amersham, UK) was diluted 1:7500 in blocking solution and incubated at room temperature with the membrane for 1 h under gentle agitation. The membrane was then washed with T-TBS for 3 x 5 min. Bound antibody was detected using Pierce Luminal kit (Pierce, UK). The Luminal / Enhancer solution was mixed to the stable peroxide solution at equal volume and was incubated with the
membrane for 5 min at room temperature. Excess substrate was removed. The membrane was exposed to a blue sensitive X-ray film (Kodak) for 5 min and developed. The composition of the buffers is shown in Appendix 10.3.

2.6.5 DENSITOMETRY

Developed blue sensitive X-ray films were scanned and analyzed using the TINA2.0 software. The intensity of the bands corresponding to the protein of interest (cytosine deaminase, green fluorescent protein) or β-actin was measured on the simultaneously scanned pictures. The intensity of the bands corresponding to the detected proteins was corrected for β-actin. The magnitude of protein induction levels was expressed as a multiple of the controls. When the controls were the absence of a band, a representative background densitometry value was used for normalisation. The densitometry results of three independent experiments were used to constructs induction charts.

2.7 CELL SURVIVAL ASSAY (MTT)

2.7.1 MTT REAGENT

The sensitivity of prostate cancer cell lines to 5-fluorocytosine (Sigma chemicals, UK) was assessed using 96-non radioactive MTT reagent (Promega, UK). According to manufacturer's instruction, 15 μL of the dye was added to each well. The plate was incubated at 37°C for 4 hours. Following the addition of the solubilisation solution (100 μL/well), the plate was further incubated (37°C) overnight. The absorbance at 595 nm was measured on a plate reader.

2.7.2 DETERMINATION OF STANDARD CURVE TO MTT REAGENT

22Rv1 and DU145 cells were plated onto a 96-well plate at cell densities ranging from 0 to 1x10⁵. Cells were allowed to attach to the culture vessel for 1
hour. The cells were incubated with the dye for 4 hour. The solubilisation solution was then added and left overnight. The absorbance at 595 nm was read on a plate reader. The results were plotted in Microsoft Excel and the optimum cell density for linear relationship between absorbance and cell number determined. The results are shown in Appendix 10.4.

2.7.3 MTT DESIGN

The protocol we used was that described by Schoemaker et al. 2006 (Shoemaker 2006). Human prostate cancer cells (2.5x10^4 cells/well) were seeded into 96-well plates and transfected as described in section 2.4.4. Twenty-four hours after transfection, the plates were placed in hypoxia (0.5% O2, pO2 < 2mmHg) for up to 48 hours. Using the MTT reagent, cell viability of a control plate was read (Tz) to represent measurement of the cell population at the time of drug addition. The cells were then incubated with 5-FC or 5-FU under normoxic or hypoxic condition for four days. In combined protocols, ionising radiation was delivered as either single or fractionated radiation dose. Cell viability in the treated plates was measured (Ti). The growth of untreated cells (C) was used as a control. The percentage growth of the treated cells was calculated as \[\frac{(Ti-Tz)}{(C-Tz)}\] x 100 if Ti > Tz, resulting in a positive value of % growth, associated with a growth inhibition effect of the treatment. If Ti < Tz % growth was calculated as \[\frac{(Ti-Tz)}{(Tz)}\] x 100, resulting in a negative value, associated with cytotoxicity.

2.8 APOPTOSIS ASSAYS

2.8.1 PREPARATION OF THE CELLS

DU145 and 22Rv1 cells (0.7 x 10^6 cells /well) were seeded into 6 well plates and transfected as described in section 2.4.4. At 48 hours after transfection, the cells were incubated under hypoxic conditions (0.5% O2) for 48 hours. At the end of the hypoxic induction period, 5-FC (0.1, 1 mM) was added. The plates were incubated either in normoxic or hypoxic conditions for up to three days.
2.8.2 LABELLING OF THE CELLS

At the end of 5-FC treatment, the cell culture media was conserved and transferred to FACs tubes. The wells were trypsinised. The cell suspension was transferred to the corresponding FACS tubes and pelleted (1300 rpm, 3 min). The pellets obtained were washed (1300rpm, 3 min) in Annexin V binding buffer (Biosource, Nivelles, Belgium), which was diluted from 10X stock in PBS (Gibco, UK). Each sample was resuspended in binding buffer (100μL) and Annexin V-FITC (IQ Products, The Netherlands) (3μL) was added to all samples, except appropriate controls, to which only binding buffer (103μL) was added. All samples were vortexed, and incubated on ice in the dark for 10 min. All tubes were kept on ice from this point on in order to preserve the FITC tag on the Annexin V. The cells were again pelleted (1600rpm, 5 min), and washed (1600rpm, 5 min) in binding buffer. The cells were resuspended in binding buffer, and analyzed by FACS analysis, as described in section 2.5.2. Immediately before analysis, propidium iodide (125ng/ml, diluted in binding buffer) was added to all tubes except the appropriate controls.

2.8.3 ANALYSIS OF THE DATA

The effect of drug treatment on the level of apoptosis in sample, prepared as described in section 8.2, was assessed using a FACSCalibur flow cytometer (Becton Dickinson, New Jersey, USA). Analysis of data was carried out using CellQuest software (Becton Dickinson). The percentage of cells in each quadrants was automatically calculated. The lower left quadrant was representative the amount of live cells, while cells undergoing apoptosis were progressively shifted to the lower right quadrant (early apoptosis) and up to the top right quadrant (late apoptosis). Dead and necrotic cells were accounted for in the top left quadrant. The sum of the percentage of cells in each early and late, or total apoptosis, was used an endpoint in our analysis.
2.9 Clonogenic assays

DU145 and 22Rv1 cells \((10^6 \text{ cells/well})\) were seeded into 6 well plates and transfected as above. At 24 hours after transfection, cells were trypsinised and reseeded at 250 cells/well. The plates were exposed to hypoxia for 48 hours prior to a four-day 5-FC treatment \((0, 0.1, 1 \text{ mM})\) under aerobic conditions. The prodrug was then removed and fresh media added. Two weeks later, the plates were stained \((70\% \text{ methanol, crystal violet, Sigma-Aldrich, UK})\) and colonies counted. The plating efficiency was calculated as the ratio of the number of colonies counted over the number of cells plated. The surviving fraction in the treated wells was subsequently calculated as the ratio of the number of clones counted over the number of cells plated corrected for plating efficiency. The plating efficiency in the clonogenic assays presented was consistently in the region of 75-80%.

2.10 Statistical analysis

Following completion of the post-graduate diploma in statistics course in college (First grade, 2006), analysis of the data was undertaken. All experiments were performed in triplicate unless otherwise stated and results are representative of all repeats. The data was analyzed assuming a Normal distribution by two sample t-tests and analysis of variance (ANOVA). The associated residual plots are not presented here but were thoroughly examined to confirm the distribution of the data approached a Normal distribution. A p-value of less than 0.05 was considered statistically significant.
CHAPTER 3: DESIGN AND PRODUCTION OF HYPOXIA-INDUCIBLE VECTORS
3.1 Introduction

During the past decade, the adaptation to hypoxia at a molecular level has been extensively studied. A large number of genes have been found to be up regulated by falling oxygen tension. Genes that are oxygen-regulated are involved in glucose homeostasis, angiogenesis, vascular permeability and inflammation (Maxwell, Dachs et al. 1997; Gleadle and Ratcliffe 1998). Such genes include VEGF and the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GADPH). The DNA regulatory elements involved in the regulation of these oxygen-responsive genes include the specific binding and trans-activation by various inducible phosphorylation-dependent and/or redox sensitive transcription factors, including Hypoxia Inducible Factor 1 (HIF-1), Activator Protein 1 (AP1), Nuclear Factor λB (NFλB), p53 and the Heat Shock Transcription Factor. However, HIF-1 only has been shown to be specifically oxygen-responsive.

The role of HIF-1 has been discussed in section 1.4.2. HIF-1 is activated through post-translational stabilisation of HIF-1α and is degraded by the proteasomes in aerobic conditions (Blancher et al., 1998). Under hypoxic conditions, the prolyl hydrolase is inactive and the ubiquitination is inhibited (Figure 3-1). HIF-1α is no longer degraded resulting in its accumulation in the cytoplasm. HIF-1α is translocated from the cytoplasm to the nucleus where it interacts with HIF-1β to form the heterodimer HIF-1. HIF-1 then specially binds to hypoxia-responsive elements to induce gene expression (Chresta, Masters et al.).
Chapter 3

HIF-1α/HRE system.

Under hypoxic conditions, the prolyl hydrolase (pVHL), resulting in inhibition of HIF-1α proteasomal degradation. HIF-1α interacts with HIF-1β and triggers gene expression. Figure published in Marignol et al. (2005).

HREs are enhancers elements containing the consensus core sequence 5’-(A/G)CGT(G/C)(G/C)-3’, localised at varying positions and orientations of the coding region of several hypoxia-regulated genes. Positions and orientations of several examples of HREs are shown on Figure 3-2. Arrangements of these HREs sequences were shown to be utilisable to achieve hypoxia-inducible transgene expression in a gene therapy setting (for review, see (Marignol, Lawler et al. 2005)).
In contrast to the cell-restricted pattern of Epo expression, many different primary and cultured cell types respond to hypoxia by increasing VEGF gene expression (Gleadle, Ebert et al. 1995). Similarities between the oxygen-sensing mechanisms regulating the expression of VEGF and Epo have been reported (Goldberg and Schneider 1994). To determine whether the 5'-flanking region of the VEGF gene could mediate transcriptional responses to cellular hypoxia, Forsythe et al. constructed a reporter plasmid in which VEGF 5'-flanking sequences were fused to the luciferase coding sequences (Forsythe, Jiang et al. 1996). Transfected hepatoma cells expressed the luciferase gene with a 9-fold increase after hypoxic treatment (1% O₂). The ability of the reporter to respond to hypoxia has been investigated using a series of deletion mutants of the 5'-flanking sequences. As a result, the study delineated a 47-bp region between -985 and -939 that was sufficient for transcriptional activation of the SV40 basal promoter in hypoxic Hep3B cells.
Shibata et al. (2000) further tested the potential of the selective expression mediated by putative HREs from the human VEGF gene. They assessed the extent of hypoxia inducibility by a variety of DNA constructs using the luciferase assay in order to select the most appropriate vector for cancer gene therapy. They first replaced the SV40 promoter sequence in the pGL3 vector with a 385-bp fragment of the human VEGF 5'-flanking region. This construct, when transfected in murine tumour cell lines, showed marked increases in luciferase activity in response to hypoxia. To gain more hypoxia responsiveness, they inserted 5 concamerized copies of the HRE sequence upstream of the 385-bp fragment and observed an increase in luciferase activity (13 to 16-fold) under hypoxic conditions even though this sequence did not include a minimal promoter, TATA or CAAT box. The inclusion in the construct of the 32-bp of a minimal E1b fragment containing a typical TATA box resulted in further increased activity (50-fold) in human fibrosarcoma HT1080 cells.

Although the HRE/E1b minimal promoter appears to be the best construct for the induction of genes by hypoxia, induction levels obtained by this construct are considerably lower (100-fold) than that of a CMV promoter driven vector and may be insufficient to be used for genetically directed enzyme prodrug therapy. Shibata et al. (1998) therefore conducted further studies with the goal of establishing hypoxia-inducible vectors with higher expression of therapeutic genes. Two constructs were produced: one using the EF-1 alpha promoter and one using a 60-bp minimal promoter containing a TATA sequence derived from human CMV IE promoter. Each promoter was attached to 5 HREs and tested for hypoxia-induced gene expression. Undetectable induction was observed with the EF-1 alpha promoter whereas more than 500-fold induction could be detected with the CMV IE promoter after exposure to 0.02% O2 for 18 hours, which is similar to that of the full-length CMV promoter construct. This study thus proposes the 5HRE/CMV construct as the most promising vector for hypoxia-inducible gene therapy.
The glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GADPH) is also induced by hypoxia in endothelial cells. An evaluation of the GADPH promoter resulted in the delineation of six consensus HIF-1 binding sites (5'-RCGTG-3') (Lu, Gu et al. 2002). Two of these sites are in the sense orientation, and four in the antisense orientation. Transfections of constructs containing various fragment sizes showed that three of these sites (-989 to -985; -957 to -953; -340 to -336) are unlikely to represent functional HREs since their deletion did not abrogate hypoxic gene activation in either prostate cancer cells (Semenza, Jiang et al. 1996) or human endothelial cells (Graven, Yu et al. 1999). Graven et al. transiently transfected endothelial cells, fibroblasts and smooth muscle cells using constructs which contained various portions of the GADPH promoter linked to a chloramphenical acetyl transferase (CAT) reporter gene. The initial construct, containing a 509-nucleotide fragment of the human GADPH gene corresponding to nucleotides -487 to +21 in relation to the transcription start site, resulted in a 5.4-fold increase in CAT activity in response to hypoxia. The region of the gene coding for hypoxic response could later be restricted to a 157-nucleotide fragment corresponding to nucleotides -267 to -110. A computer search for known regulatory sequences present in this fragment showed the presence of a HRE between bases -126 and -119. They linked a 62-bp fragment containing this element to the SV40 promoter in a CAT vector and could report a 6-fold increase in CAT activity during hypoxia.
Within the GAPDH promoter, Lu et al. (2002) isolated the region spanning nucleotides -217 to -203 containing two HIF-1 consensus sites arranged as inverted repeats and separated by five nucleotides (Figure 3-4). One or two copies of a 30-bp sequence containing this novel HRE were linked to the minimal TATA promoter of the tissue transglutaminase gene and inserted into a luciferase reporter vector. Transfection with one copy resulted in a 1.9-fold increase in luciferase activity in response to hypoxia, whereas transfection of 2 copies resulted in a 4-fold induction. This strongly suggested that this sequence is also a functional HRE.

Figure 3-4 Localisation of the HRE within the GAPDH promoter. Sites 1, 2 and 3 are unlikely to be functional whereas sites 4, 5 and 6 represent functional HREs described by Graven et al. and Lu et al. Figure published in Marignol et al. (2005).

The number of copies of the HRE sequence isolated from the Epo gene was shown to influence the levels of gene expression induction. Under anoxia, increasing HRE copy number to 3 or 6 resulted in a 4-fold increase in basal gene expression compared to that observed in oxic conditions (Ruan et al., 2001). However the use of constructs containing 9 copies resulted in higher (27-fold) gene expression levels.
A similar pattern was reported for HRE isolated in the VEGF promoter (Shibata, Akiyama et al. 1998). However it was observed that a saturation effect can occur for constructs containing more than 5 copies. HRE copies numbers ≤ 3 were used in constructs for the PGK1/GADPH sequences. A slight increase in gene expression was observed between constructs containing 3 copies when compared with constructs containing a single HRE copy. Therefore, increasing HRE copy number appears to be a valuable option for promoting enhanced gene expression.

Oxygen concentration in human tumours is heterogeneous and ranges from 5mmHg (0.7% O₂) in glioblastoma (Rampling et al. 1994), to 15mmHg (2.1% O₂) in head and neck carcinoma (Terris et al., 1994). The mean oxygen level of a particular tumour is much lower than its normal tissue of origin. Induction
levels were demonstrated to increase with decreasing oxygen tensions in all HRE tested (Figure 3-6). Since the mean oxygen level of a particular tumour is much lower than its normal tissue of origin, it appears that the oxygen gradient in tumours could also participate to restricted localisation of transgene gene expression.

Figure 3-6 Representation of the effect of the oxygen status on gene expression (Fold induction calculated relatively to anoxia).
Adapted from Shibata et al., Ruan et al., Dachs et al. Figure published in Marignol et al. (2005).

HT1080 human fibrosarcoma cells transfected with a plasmid containing the cytosine deaminase gene whose expression was driven by a hypoxia-responsive promoter were more sensitive to 5-FC after anoxia than the untransfected parental cell line. Furthermore, transfectants exposed to anoxia for 16 hours were 5.4 more sensitive to 5-FC than transfectants kept in air and untransfected cells, demonstrating that oxygen-regulated production of cytosine deaminase could confer hypoxia-inducible sensitivity to 5-FC (Dachs, Patterson et al. 1997).
3.2 AIMS AND METHODS

While Dachs et al. exploited PGK-1 HRE sequences, following the review of hypoxia-inducible gene therapy vectors produced to date, the HRE sequences of the VEGF and GAPDH were selected to design a panel of hypoxia-responsive promoters. This is the first exploitation of GAPDH HREs for gene therapy.

The promoters were cloned upstream of the suicide gene cytosine deaminase in a previously developed construct, pCMV-CD1:10, whose CMV promoter and enhancer sequence will be removed. The new vectors were transformed into E. coli competent cells. The DNA extracted from the resulting clones were screened by restriction digest and confirmed by sequencing.

An HRE-null plasmid was constructed for later use as a negative control to hypoxia selectivity.

3.3 DESIGN AND PRODUCTION OF THE HYPOXIA-INDUCIBLE PROMOTERS

In this study, the promoters had to be synthetically produced. The structure of the hypoxia-inducible promoters was based on the 5HRE/CMV construct described by Shibata et al. (1998) (Figure 3-7) In this vector, each promoter consisted of 5 tandem HRE repeats linked to a fragment of the human cytomegalovirus immediate early gene minimal promoter encompassing the sequence between −53 and +7 of the original promoter as described by Boshart et al. (1985). The presence of this segment is essential for promoter activity since it provides the TATA box (position -29) required for transcription.
The HRE sequences used were that of VEGF and GAPDH, as published by Shibata and Lui, respectively (Table 3-1). On this basis, the sequence of 3 hypoxia-responsive promoters was designed: H5V contained 5 repeats of the VEGF HRE sequence, H6G- and H9G contained 6 or 9 repeats of the GAPDH HRE (Figure 3-8). The sequence of the two corresponding DNA strands was generated.

Figure 3-8 Schematic representation of the structure of H5V, H6G and H9G.

Generally, each promoter consisted in X tandem repeats of the HRE sequences (VEGF, V or GAPGH, G) followed by 60bp of the CMV promoter sequence.
Table 3-1 HRE and CMV sequences used to construct hypoxia-inducible promoters.

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>VEGF</strong></td>
<td>Shibata et al., 2000</td>
</tr>
<tr>
<td>5' CCACAGTGACATACGTGGGCTCCAACA GGTCCCTCTT -3'</td>
<td></td>
</tr>
<tr>
<td><strong>GAPDH</strong></td>
<td>Liu et al., 1995</td>
</tr>
<tr>
<td>5' GCCCACACGCTCGGTGCGTCGCCCAG TTGAAC -3'</td>
<td></td>
</tr>
<tr>
<td>60bp CMV</td>
<td>Boshart et al., 1985</td>
</tr>
<tr>
<td>5' GGTAGGCGTGTACGGTGGGAGGTCT ATATAAGCAGAGCTCGTTTAGTGAACC GTCAGATC -3'</td>
<td></td>
</tr>
</tbody>
</table>

The mammalian expression vector pALTMAX was previously modified to contain the full CMV enhancer and promoter sequence (1-1096bp), the cytosine deaminase (CD) gene sequence (1-1528bp, including EcoRI (GAATTC) and BamHI (GGATCC) sites) and the PBluescript sequence (725-744, including the NotI site). The resulting vector was named pCMV-CD. For the CD gene no longer to be under the control of the CMV promoter, this promoter sequence is to be replaced by the designed hypoxia-inducible promoter insert sequences. To facilitate the cloning of the promoter sequences in place of CMV, these inserts were designed to have cohesive ends. Because the CMV sequence is to be extracted by a double BglII and Xhol restriction digest, the sequence coding for the restriction sites of these enzymes was therefore added at the 5' and 3' ends, respectively. In addition, to facilitate cloning procedures, the Apal and HindIII recognition sites were inserted tandemly between the end of HRE...
repeats and the start of the CMV fragment. The final structure of the inserts is illustrated on Figure 3-9. The full sequences can be found in Appendix 10.5.

![Figure 3-9 Schematic representation of the structure of the DNA sequences coding for H5V, H6V and H9G. Cohesive ends encompassing the restriction sites of BgIII and XhoI were designed to facilitate the insertion of the promoters into the cloning vector. The addition of the HindIII and Apal restriction sites will facilitate the cloning procedures.](image)

The designed double-stranded DNA sequences were separated into a set of 12 oligonucleotides for commercial synthesis. Because of limitations in the length of the oligonucleotides that can be commercially synthesised, each double stranded promoter sequence had to be separated into 2 sets of complementary oligonucleotides (<130bp) for H5V and H6G and into 3 sets for H9G. Due the presence of repetitive sequences, special attention was needed in the design of the oligos to reduce the probability of unspecific annealing and ligation. 6-9bp overhangs between sets of oligos were thus carefully selected. The sequence of the 12 oligos can be found in appendix 10.5. Their spatial arrangement is represented on Figure 3-10.
Figure 3-10 Each promoter was divided into sets of overhanging complementary oligos. Schematic representation of the designed oligonucleotides to form the full promoter sequences.

3.4 REPLACEMENT OF THE CMV PROMOTER WITH HYPOXIA-INDUCIBLE PROMOTERS

To remove the CMV promoter sequence (1096bp), pCMV-CD was digested with both BglII and XhoI. The band corresponding to the opened cloning vector, pCD, was gel isolated. A representative DNA electrophoresis gel is shown on Figure 3-11.

Once each oligonucleotide was phosphorylated, complementary pairs were annealed and ligated to the appropriate set to construct the full promoter. The ligation products were run on a gel and the fragment of correct insert size was gel purified. Representative DNA electrophoresis gels are represented on Figure 3-12. Unfortunately, H6G could not be gel isolated in sufficient quantity despite several attempts and construction of this insert had to be abandoned.
Figure 3-11 Preparation of the CD-containing cloning vector.

(A) Map of the initial vector pCMV-CD1:10. (B) Simulated DNA electrophoresis of the double BglII/XhoI restriction digest of pCMV-CD1:10. (C) Representative picture of a DNA electrophoresis of the digestion mix prior to gel extraction of the opened cloning vector pCD.
Finally, promoters and cloning vector were ligated. The resulting clones were initially screened with a HindIII digest, followed by DNA sequencing.

Ligation of H5VCD into pCD generated a vector called pH5VCD. Digestion of pH5VCD with HindIII results in 2 bands at 2.85 and 3.3kb. Through recombination events, two derived plasmids (pH2VCD and pH6VCD) were generated, which contained 2 and 6 copies of the VEGF HRE enhancer, respectively. Representative DNA electrophoresis gels are presented on Figure 3-13 A,B.

The GAPDH-derived plasmid could not be generated as originally planned but a construct, pH8GCD, which contains a concamer of 8 copies of the GAPDH HRE, was generated through recombination events. Digestion of this vector
with HindIII results in 2 bands at 2.85 and 3.41kb. The results are shown on Figure 3-13 C,D.

Figure 3-13 Generation of pH5VCD and pH8GCD. (A) Map of the designed vector pH5VCD (B) Simulated and obtained HindIII restriction digests DNA electrophoresis gel, for screening of clones. Through recombination events, pH2VCD and pH6VCD were generated. (C) Map of the GAPDH-derived vector produced by recombination event, pH8GCD. (D) DNA electrophoresis gel of simulated and obtained HindIII digest of clones.

3.5 Generation of HRE-deficient control plasmid

To create pH0CD, a construct containing the -53 to +7bp sequence of human cytomegalovirus minimal promoter to drive the cytosine deaminase gene, the HRE tandem repeats of pH5VCD were removed by BglII/Apal digest, resulting in a band at 6009bp, which was gel isolated (Figure 3-14A). The digested plasmid was blunt-ended (Figure 3-14B) prior to self-ligation. The resulting clones were screened with HindIII, producing two bands at 3.1 and 2.85Kb (Figure 3-14D). DNA sequencing confirmed correct removal of the HREs.
Figure 3-14 Generation of an HRE-null plasmid, pHOCD. 
(A) The HREs were removed from pH5VCD with a BglII and Apal double restriction digest. Following blunt-ending (B) pHOCD clones were screened with HindIII (C, D). Representative DNA gels electrophoresis are shown here.

3.6 CONCLUSION

HREs have successfully been developed into synthetic promoters and used to control transgene expression in a hypoxia-responsive fashion in the past. While the HRE of the VEGF and Epo gene promoter have been widely exploited, GAPDH HREs were the only ones shown to be inducible in prostate tumours. In this chapter, these results were exploited to design and produce a panel of hypoxia-inducible promoters to be used for suicide gene therapy of prostate cancer. The approach was based on the vector described by Shibata et al.

Initially, three hypoxia-responsive promoters, derived from the GAPDH and VEGF HRE sequences, were designed, according to a structure described by Shibata et al, consisting of HRE tandem repeats linked to a 60bp fragment of
the CMV promoter sequence (section 3.3). This schema was subsequently modified with the inclusion of restriction enzymes recognition sites to facilitate cloning. Finally, the double-stranded promoter sequences were divided into 12 overlapping oligonucleotides and were synthesised commercially. Construction of the full-length promoter sequences required the correct annealing and ligation of the complementary oligos. The promoters were subsequently inserted upstream of the suicide gene cytosine deaminase (CD) in previously developed plasmid whose CMV promoter and enhancer sequence was extracted (section 3.4).

Four hypoxia-responsive and an HRE-null gene therapy vectors were successfully constructed. Hypoxia-responsiveness was ensured in three VEGF-derived vectors by 2, 5 or 6 concamer HRE sequences linked to the 60bp CMV fragment. These vectors were named pH2VCD, pH5VCD and pH6VCD, respectively. A unique GAPDH-derived vector was generated, containing 8 copies of the HRE sequences. A summary of the vectors produced is shown on Figure 3-15 and the maps are shown in Section 11.
Figure 3-15 Schematic representation of the hypoxia-inducible vectors generated.
CHAPTER FOUR: EVALUATING SENSITIVITY OF PROSTATE CANCER CELL LINES TO HYPOXIA, PRODRUG AND RADIATION EXPOSURE
4.1 INTRODUCTION

It is a common misconception that tumours consist of central hypoxic cores surrounded by better-oxygenated cells (Ljungkvist, Bussink et al. 2007). The spatial disorganisation and variability in oxygenation gradient make it very difficult to target hypoxia specifically and develop adequate hypoxic condition exposures for the development of hypoxia-targeted strategies.

The response of cells to hypoxic stress is influenced by the duration of hypoxic exposure. Because tumour cells proliferate near blood vessels, it is estimated that a functional vessel can support 5-10 layers around it, suggesting that it may take up to 100 hours for cells to reach the outermost layer (Hirst, Denekamp et al. 1982). Therefore, the cells located further away from blood vessels are expected to be hypoxic for long periods of time. The life time of hypoxic cells has indeed been estimated to range from one to up to 10 days (Durand and Aquino-Parsons 2001; Ljungkvist, Bussink et al. 2005). This hypoxic turnover rate may affect the responses to different treatment modalities. Indeed, radiation was shown to increase the oxygenation status and lengthen their lifetime (Durand and Aquino-Parsons 2006; Ljungkvist, Bussink et al. 2006).

Mutation of the tumour-suppressor gene p53, involved in cell cycle regulation and apoptosis, has been linked to late-stage hormone-resistant tumours in up to 80% of specimens tested (Chapter 1). Loss of p53 was also associated with reduced susceptibility to hypoxia-induced apoptosis (Graeber, Osmanian et al. 1996).

The sensitivity of cells to ionising radiation is dependent on the radiation dose, the intrinsic capacity to repair DNA damage, the doubling time and a number of environmental conditions. Prostate tumours are slowing growing and therefore likely to be relatively radiation resistant. In addition, hypoxia was shown to
increase cell cycle times and participate to an arrest in the radiation resistant S-phase in p53 mutant cells. Because androgen independence is thought to be a turning point for treatment resistance, novel strategies for prostate tumour need to be tested in both androgen-resistant and androgen-independent cell lines. The sensitivity of the models chosen to both hypoxia and radiation exposures, which may differ due to underlying phenotypic differences, had thus to be evaluated prior to transfections studies.

4.2 AIMS AND OBJECTIVES

In this chapter the sensitivity of androgen sensitive (22Rv1) and androgen-resistant (DU145) prostate cancer cell lines to hypoxia, prodrug and radiation exposure was evaluated.

1. Initially, the effect of decreasing oxygen levels in the growth environment on the oxygen tension of the cells over time was measured using oxygen probes, to determine a working oxygen concentration.

2. The effect of artificially-simulated hypoxia, 5-FC and their combination on the growth of both reoxygenated and hypoxic cell lines was measured with MTT assays. The associated apoptotic fraction was evaluated with an apoptosis assay (Annexin V-PI).

3. Finally, the sensitivity of the cells to the delivery of clinically relevant radiation doses alone or following hypoxic exposure was measured with clonogenic assays.

4.3 EFFECT OF AN HYPOXIC ENVIRONMENT ON WILD TYPE PROSTATE CANCER CELLS

4.3.1 OXYGEN CONSUMPTION AND HYPOXIC ENVIRONMENT

Because oxygen tension levels have been shown to modulate HRE activity, a reproducible pO₂ is an important prerequisite for future hypoxic experiments
Oxygen consumption and pO$_2$ have been shown to be modulated by cell density (Chrastina 2003) and environmental oxygen concentration in vitro (Braems and Jensen 1991). Indeed, an increase in cell density inevitably results in enhanced oxygen consumption, reducing time necessary to reach hypoxia.

The aim of this part of the study was thus to evaluate the effect of cell density on pO$_2$ as well as the length of hypoxic exposure required for the cells to reach a pO$_2$ close to 0, when exposed to decreasing atmospheric oxygen concentrations. Prostate cancer cell lines (DU145, 22Rv1) (see 2.4.1) plated at several cell densities (0.5-2.5x10$^4$) were exposed to atmospheric oxygen concentrations of 0.5, 1, 2 and 4% for up to 24 hours. pO$_2$ was monitored every hours. The results are shown on Figure 4-1 and Figure 4-2.

In cells exposed to an atmospheric oxygen concentration of 4%, pO$_2$ varied dramatically across the panel of cell densities tested. The variability was progressively reduced during hypoxic exposure and as the oxygen level in the environment was lowered. Cell density no longer had an influence on pO$_2$ at an atmospheric oxygen concentration of 1% and 0.5%. At 4% oxygen, it took over 24 hours for the cells to reach anoxia (data not shown). This was reduced to 5 hours at 1% and 1h at 0.5% (Figure 4-2).

Finally, the pO$_2$ in media only was monitored during exposure to a 1% oxygen environment. In cell-free wells, it took 24 hours for the pO$_2$ to equilibrate to the surrounding environment (Figure 4-3).

To minimise the impact of oxygen concentration and cell density on pO$_2$, a working oxygen concentration of 0.5% (pO$_2$< 2mmHg) was chosen for future hypoxic experiments.
Figure 4-1 $pO_2$ varies with cell density and time.

DU145 and 22Rv1 wild type prostate cancer cells were plated at a range of densities and exposed to a hypoxic environment. $pO_2$ was monitored every hour. The decline over time of $pO_2$ values associated with exposure to an hypoxic environment of 4% and 2% oxygen is represented here. The $pO_2$ of DU145 cells exposed to 4% and 2% oxygen are presented on graph (A) and (C), respectively. The response of 22Rv1 is shown on (B) and (D). Bars, SE; n=2.
Figure 4-2 At low oxygen concentration, pO₂ variability across the panel of cell density tested was reduced. The time course of pO₂ values decline in response to exposure to a 1% oxygen hypoxia environment is represented for DU145 (A) and 22Rv1 (B) cells, respectively. (C) and (D) plots represent the response of DU145 and 22Rv1 cells for a 0.5% hypoxic environment. Bars, SE; n=2.
4.3.2 Increased apoptosis levels correlated with a growth inhibition effect in chronically hypoxic cells.

The effect of hypoxia on apoptosis levels of wild type prostate cancer cells was initially evaluated by flow cytometry (Annexin-V-PI). 22Rv1 and DU145 cells were grown in either an aerobic or hypoxic environment for 4 hours, 48 hours and 5 days. The cells were then harvested prior to labelling with Annexin V-PI and total (early + late) apoptosis levels in the cell population were estimated. The results are shown on Figure 4-4.

Aerobic controls were associated with 13% apoptosis in both cell lines. A 4h hypoxic exposure (0.5% O₂, pO₂ < 2mmHg) did not have a significant effect on the proportion of apoptotic cells in the population. At the end of a 48 hours hypoxic exposure, the total number of apoptotic cells was 15.6 and 14%
in 22Rv1 and DU145, respectively, which was comparable to that of aerobic-grown controls. An extension of the hypoxic exposure to 5 days significantly increased the total number of cells in apoptosis to 20 and 22%, in 22Rv1 (p = 0.002) and DU145 (p = 0.034), respectively. The associated Hypoxic Enhancement Ratios, the ratio of the apoptotic fraction of hypoxic to oxic cells, were minimal at 1.5 and 1.6 in 22Rv1 and DU145, respectively.

![Figure 4-4](image)

**Figure 4-4** Apoptosis levels were significantly increased in chronically hypoxic cells (5 days). (A) Representative quadrant distribution of chronically hypoxic 22Rv1 cells labelled with Annexin V-PI for apoptosis detection. (B) Quantified Total (early+ late) apoptosis (%) in 22Rv1 cells exposed to air, 4 - 48 hours or 5 days hypoxia. The experiment was reproduced in DU145 cells. The results are represented in (C) and (D). Bars, SE; n=3; *p<0.05.

To evaluate whether this increase in apoptosis levels in chronically hypoxic cells (5 days) resulted in a cytotoxic effect, the absorbance of the cell culture media was monitored. Wild type and transfected prostate cancer cells (22Rv1, DU145) were initially conditioned in hypoxia for 48 hours prior to either
reoxygenation or further hypoxic exposure (4 days). Using a MTT reagent, the absorbance at 595 nm was read at each time point. An increase in absorbance between the end of the 48h hypoxic exposure (day 0) and the end of the growth period (day 4) can indeed be correlated with an increase in cell number. As a result, the growth potential of the cells can be estimated. In this setting, a decrease in absorbance would be associated with a decrease in cell number and cytotoxicity. The results are shown on Figure 4-5 and Figure 4-6.

In 22Rv1 and DU145 cells, the increase in the absorbance readings, correlating with an increase in cell number was evident, between the end of the hypoxic induction period and the end of a 4-day growth period. The difference was statistically significant (p<0.05) regardless of the growth condition. An analysis of variance of absorbance of the cells after each exposure condition was carried out in 22Rv1 and DU145 cells. The null hypothesis was that there is no difference in the absorbance between each growth conditions. In both cell lines, there was sufficient evidence to reject the null hypothesis, with associated p values of 0.000.

In both cell lines, hypoxia had however a significant growth inhibition effect associated with significant reduction of the absorbance on day 4 of culture (p = 0.04 and p = 0.0024 in 22Rv1 and DU145 cells, respectively).
Figure 4-5 Hypoxia has a significant growth inhibition effect in 22Rv1 cells. The 595 nm absorbance following the addition of the MTT reagent was measured at the end of hypoxic conditionment (48h) and following either reoxygenation or further hypoxic exposure (4 days). The ANOVA table and the result of the multiple comparisons (paired t-tests) are also presented. Bars, SE; n=3; *p<0.05.
Figure 4-6 Hypoxia has a significant growth inhibition effect on DU145 cells. The 595 nm absorbance following the addition of the MTT reagent was measured at the end of hypoxic conditionment (48h) and following either reoxygenation or further hypoxic exposure (4 days). The ANOVA table and the result of the multiple comparisons (paired t-tests) are also presented. Bars, SE; n=3; *p<0.05.

4.4 5-FC DID NOT ENHANCE HYPOXIA-INDUCED GROWTH INHIBITION DESPITE INCREASED APOPTOSIS INDUCTION

5-fluorocytosine is an antifungal agent known to be relatively nontoxic at clinically relevant concentrations (1mM). Chemical treatment may however be associated with cellular stress and its combination with a hypoxic environment may be associated with increased cytotoxic effect.

Initially the effect of 5-FC on apoptosis levels was evaluated in wild type prostate cancer cells exposed to hypoxia for 48 hours and treated with 0.1mM (22Rv1) and 1mM (DU145) for 3 days under aerobic or hypoxic conditions. The results are shown on Figure 4-7.
Figure 4-7 Hypoxic 5-FC exposure resulted in significantly increased apoptosis levels, when compared to reoxygenated controls.

(A) Representative quadrant distribution of wild type 22Rv1 cells treated with 0.1mM 5-FC in hypoxia and labelled with Annexin V-PI for apoptosis detection. (B) Quantitative representation of apoptosis assays in 5-FC treated and 5-FC naïve reoxygenated and hypoxic 22Rv1 cells. The experiment was repeated in DU145 cells, using a 5-FC concentration of 1mM. The results are represented in (C) and quantified in (D). Bars, SE; n=3; UT = untreated; ns = non significant; *p<0.05.

An analysis of variance of the proportion of apoptotic cells was carried out in 22Rv1 and DU145 cells to see whether there was a treatment effect and/or a treatment condition effect. The null hypothesis was that there is no difference in the percentage of apoptotic cells between each condition. In both 22Rv1 and DU145 cells, there was sufficient evidence to reject the null hypothesis of no treatment (p = 0.000 and 0.026, respectively) and no condition effect (p =0.000).
Figure 4-8 ANOVA tables for the simultaneous analysis of the 22Rv1 and DU145 database. The null hypothesis tested was that there was no 5-FC treatment, no condition (hypoxia, reoxygenation) effect and no interaction between the two. There was in both cases sufficient evidence reject the null hypothesis.

In both cell lines the percentage of apoptotic cells was not statistically increased in cells 5-FC treated in air, when compared with non-treated aerobic cells (p = 0.1072 and p = 0.056 in 22Rv1 and DU145 cells, respectively).

The difference in the percentage of apoptotic cells in cells 5-FC treated in hypoxia compared to non-treated hypoxic cells was significant in DU145 cells (p = 0.0175) but not in 22Rv1 cells (p = 0.0630) (Figure 4-7). Both cell lines were significantly more sensitive to 5-FC in hypoxia than in air (p = 0.0164 and p = 0.0039 in 22Rv1 and DU145 cells, respectively). The increased sensitivity in hypoxia correlated with Hypoxic Enhancement Ratios of 1.38 and 1.74 for 22Rv1 and DU145 cells, respectively.
The effect of 5-FC on cell growth was subsequently evaluated in wild type prostate cancer cells exposed to hypoxia for 48 hours and treated with either 0.1mM (22Rv1) or 1mM (DU145) for four days in aerobic or hypoxic conditions. The data is shown on Figure 4-9 and Figure 4-10.

In both cell lines, an increase in absorbance and hence an increase in cell number was evident in both treatment conditions, between the end of the hypoxic period and the end of 5-FC treatment. 5-FC was therefore not found to be cytotoxic either in air or in hypoxia. An analysis of variance of the effect of the treatment on % growth was carried out in 22Rv1 and DU145. The null hypothesis was that there is no difference in the percentage growth of cells treated in air or in hypoxia. In both 22Rv1 and DU145 cells, there was sufficient evidence to reject the null hypothesis of no treatment effect (p = 0.000).

In 22Rv1 cells, the absorbance was significantly increased between the start and the end of 5-FC treatment in hypoxia (p = 0.0023) and in air (p = 0.0155). The difference between aerobic and hypoxic 5-FC treatment was also statistically significant (p = 0.0174). However, 5-FC did not increase previously described hypoxia-induced growth inhibition.
Figure 4-9 Hypoxia-induced a significant growth inhibition effect, associated with a significant reduction in absorbance, was not enhanced by 5-FC in wild type 22Rv1 cells. The 595 nm absorbance of the cell culture media supplemented with MTT reagent of hypoxic (48h, 6days) and reoxygenated 22Rv1 cells was measured. Bars, SE; n=3, *p<0.05.

In DU145 cells, the increase in absorbance was also significant both in hypoxia (p = 0.0410) and in air (p<0.001). 5-FC induced a growth inhibition effect in hypoxia, with the absorbance in hypoxia being significantly reduced compared with cells treated in air (p = 0.0114). The effect was not enhanced compared to untreated controls.
Figure 4-10 Similarly to the previous figure, hypoxia-induced significant growth inhibition was not enhanced by 5-FC in DU145 cells.

4.5 EFFECT OF IONISING RADIATION ON WILD TYPE PROSTATE CANCER CELLS

4.5.1 IONISING RADIATION DID NOT HAVE A GROWTH INHIBITION EFFECT IN REOXYGENATED CELLS.

The effect of the delivery of single (2Gy) and fractionated (2 x 1Gy) doses on the growth of reoxygenated wild type prostate cancer cells (22Rv1, DU145) was evaluated with an MTT assay. The cells were conditioned in hypoxia for 48 hours prior to reoxygenation. Ionising radiation was delivered on Day 1 for the single protocols and Day 1 and 2 in the fractionated protocol. Growth was monitored through the measurement of absorbance prior and at the end of a 4 day reoxygenation period. The results are shown on Figure 4-11 and Figure 4-12.
The delivery of ionising radiation as single (2Gy) or fractionated (2 x 1Gy) doses did not significantly reduce the absorbance of MTT-reagent supplemented cell culture media in 22Rv1 cells (ANOVA, \( p = 0.315 \)).

Figure 4-11 The absorbance at 595 nm was measured in cells exposed to hypoxia alone or in combination to subsequent aerobic administration of single or fractionated radiation doses. The associated analysis of variance table is presented. Bars, SE; \( N = 3 \); ns, non significant.

An analysis of variance of absorbance of the cells after each exposure condition was carried out in 22Rv1. There wasn’t sufficient evidence to reject the null hypothesis that there is no radiation effect (\( p = 0.315 \)).

In DU145 cells, the addition of radiation doses also did not significantly reduce the absorbance of wild type cells, compared to unirradiated controls (ANOVA, \( p = 0.169 \)).
Figure 4-12 Ionising radiation did not have a growth inhibitory effect in DU145. The absorbance (595 nm) of the cell culture media supplemented with MTT reagent was not significantly reduced (two sample t test, p = 0.1039). Bars, SE; N = 3

4.5.2 Effect of Ionising Radiation on the Reproductive Potential of Wild Type Prostate Cancer Cells

Because the effect of radiation exposure on cell viability may be delayed in time and as a result may not be detected with MTT assays, the reproductive potential of wild type prostate cancer cells (22Rv1, DU145) was assessed using clonogenic assays.

Initially, the radiosensitivity of the cell lines was evaluated by exposing the cell to a single radiation dose of 2 Gy. The effect of protocols involving the combination of hypoxia with ionising radiation was then assessed. In this setting, the cells were exposed to hypoxia for 48hrs and reoxygenated. Ionising radiation then was administered in air either as a single 2Gy dose or as two 1Gy fractions. The colonies were counted 15 days later. The number of
colonies counted in cells treated with hypoxia alone was used as a control. The results are shown on Figure 4-13 and Figure 4-14.

In 22Rv1 cells, a radiation dose of 2Gy significantly reduced the number of colonies compared hypoxic cells \( (p = 0.0254) \). The combination of hypoxia with ionising radiation further reduced the number of colonies compared to hypoxic controls. The difference was statistically significant when a single dose fraction was added \( (p = 0.0229) \), but not when the total dose was split in two equal fractions \( (p = 0.0641) \). The combination of hypoxia with ionising radiation however did not amplify the effect of radiation alone in both protocols \( (p = 0.8512 \) and \( p = 0.3295 \) for single and split doses, respectively). Furthermore, there was no benefit of split doses over single doses, as the difference was not statistically significant \( (p = 0.3581) \).
Figure 4-13 The number of colonies was significantly reduced in 22Rv1 cells treated with radiation alone (2Gy) or with hypoxia and ionising radiation combination protocols, compared to hypoxic controls.

Columns, number of colonies counted in clonogenic assays; Bars,SE; two samples t-tests; ns = non significant. *p < 0.05; n = 3

DU145 cells were not more sensitive to a 2Gy dose fraction than to a hypoxic exposure (p = 0.1242). Combination protocols however significantly reduced the number of colonies (p = 0.0397 and p = 0.0002 for single and fractionated doses, respectively) compared to hypoxic controls, with no superiority of either protocol (p = 0.2420) (Figure 4-14).

Importantly, the combination of hypoxia with fractionated radiation resulted in significant loss of colonies, compared to radiation alone (p = 0.0097), which was not the case when hypoxia was combined with a single radiation dose (p = 0.2020) (Figure 4-14). Finally, the response of the cell lines was not significantly different across the four treatment protocols tested (Figure 4-15).
Figure 4-14 The number of colonies counted in DU145 cells was significantly reduced in cells treated with the combined protocols but not with radiation alone, compared to the hypoxic controls.
Figure 4-15 The difference in the number of clones counted in the cell lines tested was not statistically significant in response to all four treatment protocols.

4.6 DISCUSSION

To objectively assess the potential of the gene therapy technique being developed here, the effect of the experimental conditions used on wild type prostate cancer cell lines was thoroughly examined and the analysis is presented in this chapter.

Creating an artificial, stable, and reproducible hypoxic growth environment proved to be a technical challenge. While hypoxic conditions may be reproduced by placing cells attached to oxygen impermeable dishes in sealed containers gassed with a set mixture of oxygen, nitrogen and carbon dioxide, an air-tight specifically designed hypoxic chamber was used here (section 2.5.1). Despite manufacturer's specification, an artificial environment composed of 0.1% oxygen was technically difficult to produce. Moreover, as cell density and oxygen levels had an impact on cellular $pO_2$, extensive analysis of the artificial
environment on pO₂ was undertaken in 22Rv1 and DU145 prostate cancer cells. While the level of oxygen made available to the cells would have had an impact on cell growth, achievement of cellular anoxia was considered here as the best end-point to guarantee reproducibility of experimental conditions. Moreover, defining a hypoxic environment that would allow for long exposure without a strong growth inhibition effect or cytotoxicity in vitro was essential if the gene therapy strategy was to be tested under clinically relevant conditions.

An environment composed of 0.5% oxygen and 5% CO₂ was initially found to be technically stable. Under these conditions, prostate cancer cells reached anoxia, associated with a pO₂ value lower than 1mmHg, within an hour regardless of cell density and cell type. The effect of such dramatic hypoxic exposure on cellular growth was subsequently measured. Cytotoxicity, associated with reduced cell number, was not evident at the end of the longest (5 days) hypoxic exposure tested. Hypoxia however did induce significant growth inhibition, which correlated with significantly increased apoptosis levels, compared to reoxygenated controls.

Simultaneous prodrug exposure had no effect on both the growth and the apoptosis levels of reoxygenated cells. The combination of prolonged hypoxia and 5-FC exposure however resulted in significantly increased apoptosis levels in both cell lines but did not translate into a cytotoxic effect, highlighting limitations in the sensitivity of MTT assays. Wild type hypoxic cells were thus more sensitive to the prodrug in hypoxia than in air, which would result in an important advantage to the gene therapy technique being examined in the studies described later in this thesis.

The radiosensitivity of DU145 and 22Rv1 cells is poorly documented. Noneless, our results were in concordance to previously published data (Bromfield, Meng et al. 2003), where available. The delivery of ionising radiation to hypoxia-naive and reoxygenated cells did not result in a significant reduction in absorbance -
hence cell number - in our MTT studies but the implications of this results may be limited by the time frame of the assay. Indeed, the effect of ionising radiation may not have peaked within the 4-day end-point of the experiment.

Because radiation-induced cell death is mainly mitotic, clonogenic assays are a more suitable technique to fully assess radiosensitivity. Clonogenic survival was significantly reduced in response to irradiation, compared to hypoxia, in both cell lines. Hypoxic conditioning did not enhance radiation resistance in both cell lines. In fact, DU145 cells appeared more sensitive to the effect of ionising radiation following hypoxic exposure. The radiosensitivity of the reoxygenated cell lines appeared enhanced in response to fractionated radiation doses. While fractionation was used as an attempt to generate clinically-relevant data, the efficacy of fractionation is best assessed in vitro and these results can only be interpreted with caution.

Finally, the response of wild type androgen-sensitive and androgen-resistant cells was not significantly different across the conditions tested. This detailed analysis of the effect of treatment conditions on wild type prostate cancer cells has showed that if the gene therapy strategy developed here is to induce cytotoxicity and reduce survival in vitro, the effect observed should not be biased.
CHAPTER FIVE: HYPOXIA-INDUCED CYTOSINE DEAMINASE DETECTION IN TRANSFECTED PROSTATE CANCER CELLS
5.1 INTRODUCTION

While hypoxia activates a variety of cellular messengers, Hypoxia-Inducible Factor-1 (HIF-1) is the only DNA regulatory element truly regulated by oxygen. This protein thus plays a central role in the induction of HRE containing promoters. It is the binding of this heterodimer to hypoxia response elements located in the promoter region of those genes, along with a variety of transcription factors (e.g. p300/CBP, STAT3) that indeed dictates the hypoxia-induced cellular response (Chresta, Masters et al. 1996) (Figure 5-1A). HIF-1α cytoplasmic levels are controlled post-transcriptionally by ubiquitination and interaction with the von Hippel-Lindau tumour suppressor protein (pVHL) for degradation by the 26S proteasome. The recognition of HIF-1α by pVHL follows hydroxylation at the proline residues 402 and 577 and is mediated by prolyl hydroxylase domain-containing (PHDs) proteins (Figure 5-1.B). The activity of PHDs requires several co-factors such as O₂, iron, ascorbate and 2-oxoglutarate. The inhibition of PHDs enzymatic activity by poor oxygen availability is the primary event modulating hypoxia-stimulated molecular response and constitutes the basis of hypoxia-selective transgene expression.

While the conditional induction of the promoters is dependent on the stabilisation of HIF-1α in hypoxia, its magnitude may be influenced by the amount of HIF-1α protein available to the cell. Upon stabilisation, several stress-responsive signalling pathways were shown to influence the cellular concentration and interaction affinity of HIF-1α (Figure 5-1.C). A number of genes involved in the control of growth signalling, cell survival and invasion, for example the Harvey rat sarcomal viral oncogene homology/extra cellular signal-regulated kinase (RAS/Raf/MEK) and Protein kinase B / phosphatidyl-inositol-3-kinase (PI3K/AKT/PKB) pathways, were found to be induced directly or indirectly by hypoxia (Jiang, Rue et al. 1996; Jiang, Zheng et al. 1997; Eliceiri, Paul et al. 1999; Alvarez-Tejado, Naranjo-Suarez et al. 2001; Jiang, Jiang et al. 2001; Chun, Lee et al. 2003; Zhong, Zheng et al. 2004). Activation of PI3K is
mediated in poorly oxygenated tissue in response to increased activity of protein tyrosine kinases such as c-Src, resulting in increased HIF-1α stability and VEGF gene expression in both tumour and normal cells under hypoxia (Namiki, Brogi et al. 1995; Ellis, Staley et al. 1998). In addition, free oxygen-free radicals generated in oxygen-deprived environments generate catechol estrogens (De Cesare, Pratesi et al.) capable of producing ROS. These in turn have been shown to induce HIF-1α and VEGF expression, probably through activation of PI3K, in LnCaP and DU145 prostate cancer cells (Gao, Jiang et al. 2002; Kaelin 2005; Muzandu, Shaban et al. 2005). Moreover, research in prostate cancer has identified the role of androgens in HIF-1α stabilisation (Mabjeesh, Willard et al. 2003).

Reduced oxygen levels thus influence HIF-mediated cellular responses both directly through inhibition of PHDs and indirectly through the formation of ROS and stimulation of cellular pathways, suggesting that cellular responses to hypoxic stress may be maintained over time. This indirect stabilization of HIF-1α could be an advantage for hypoxia-responsive gene therapy as it may participate to prolonged transgene expression in chronically hypoxic transfectants. Furthermore, the generation of ROS by hypoxia, when combined with radiation-induced free radicals, may contribute to increased DNA damage and potentiate therapeutic outcome. Finally, the Phosphatase and tensin homolog (Thompson and Optenberg) gene product has been reported to inhibit the PI3K pathway, participating in a reduction in HIF-1α cytoplasmic levels. Loss of PTEN has been associated with higher HIF-1α activation response to hypoxia (Zundel, Schindler et al. 2000).
Figure 5-1 Schematic representation of the HIF/HRE system.

(A) Upon stabilisation, HIF-1α is translocated into the nucleus, binds to HREs with a cluster of transcription factors such as STAT3, HIF-1β and p300/CRB and triggers gene expression. HRE/HIF interaction may be prevented by p53 and the Factor Inhibiting HIF-1 (FIH-1). (B) Stabilization of HIF-1α occurs in response to hypoxia by inhibition of oxygen dependent proteosomal degradation mediated by the Von Hippel-Lindau protein (pVHL) by prolyl hydrolases (PHDs). Normoxic stabilization of HIF-1α may be the result of pVHL mutations, inhibition of PHDs or mutations/amplification of the HIF-1α gene. (C) HIF-1α protein levels are regulated by several signalling pathways (i.e. PI3K and RAS) and reactive oxygen species. Increased HIF-1α cytoplasmic levels translate into enhanced transcription of HRE-regulated genes and constitute the rationale to hypoxia-responsive gene therapy. (D) Hypoxia increases the constitutive levels of HIF-2α, enhancing hypoxia-related gene expression. (E) Hypoxic induction of transcriptionally active wild-type p53 is achieved as a result of the stabilization of p53 by its association with HIF-1alpha. (F) Hypoxia increases the activity of the androgen receptor.
Disruption of the HIF-1α network, alongside increased level of constitutive HIF-2α in hypoxia (Hu, Wang et al. 2003; Carroll and Ashcroft 2006), results in increased gene expression (Figure 5-1D), which contribute to the selection of a more aggressive phenotype. Of interest is the role of p53 on the HIF-1α balance. The induction of p53 in response to oxidative stress has been linked with HIF-1α stabilisation (An, Kanekal et al. 1998) and repressed HIF-mediated gene transcription (Blagosklonny, An et al. 1998). Loss of p53 in tumour cells resulted in enhanced HIF-1α levels as well as amplified HIF-1-dependent transcriptional activation of the VEGF gene in response to hypoxia (Ravi, Mookerjee et al. 2000).

HIF-1α has been identified as a potential molecular target by several approaches but merging the potential of suicide gene therapy with oxygen sensitive promoters is a novel exploitation of the hypoxic nature of prostate tumours. The oxygen-dependent response of cells is a complex process and involves the consideration of several scenarios to ensure hypoxia-directed gene therapy is able to target a range of potentially hypoxia-adapted aggressive phenotypes. The screening of a variety of prostate cancer cell lines is thus crucial. Moreover, the unsteady nature of the hypoxic compartment involves multiple time factors that may influence the activity levels of the promoters. Although hypoxia is known to inhibit HIF-1α degradation, the time frame in which this occurs could dictate the efficacy of this HRE-driven suicide approach on acutely, chronically and reoxygenated hypoxic cells.

5.2 Aim and Methods

In this chapter cytosine deaminase (CD) was used as a reporter gene for the hypoxic induction of the designed synthetic promoters. Inducibility of the expression cassettes was assessed by Western Blot analysis. The intensity of the CD bands was measured by densitometry. While this technique does not provide accurate information on CD levels, the ratio of hypoxic to oxic density may be used to quantify magnitude of the vector induction. A ratio of unity was
associated with the absence of CD protein detection. This chapter therefore provides information on hypoxia-induced CD protein production.

The study, composed of three phases, involved the comparison of HRE sequences of different origin (VEGF, GAPDH) in their ability to drive the production of detectable CD protein levels in response to a range of hypoxic treatments.

1. The hypoxia selectivity of pH5VCD and pH8GCD was first tested in four established (22Rv1, DU145, LnCaP, PC3) and one newly developed (RC58/T) prostate cancer cell lines grown either in aerobic or hypoxic conditions.

2. Information on the modulation in the HRE response was then gathered in regards to HRE levels. The effect of HRE copy number was monitored in hypoxic 22Rv1 and DU145 cells transfected with pH2VCD, pH5VCD and pH6VCD.

3. Finally, the kinetics of the hypoxic response of pH5VCD and pH8GCD were extensively studied in 22Rv1 and DU145 transfectants.

The aim of this study was to evaluate the technical efficacy and the targetability of each HRE enhancer sequences (VEGF, GAPDH) in response to diverse hypoxia scenarios, with the ultimate objective of selecting the strongest candidates for the success of this gene therapy strategy.
5.3 Hypoxic selectivity of the promoters

5.3.1 Hypoxia selectively induces cytosine deaminase gene expression in four prostate cancer cell lines

To determine whether employing the HIF-1/HRE system could be used to selectively induce transgene expression in hypoxic conditions, the ability of human VEGF and GAPDH HREs to initiate efficient transcription in human prostate cancer cell lines was tested. After establishing transient transfectants of one primary (22Rv1) and three metastatic (DU145, PC3, LnCaP) prostate cancer cell lines with pH5VCD and pH8GCD, the cells were made hypoxic for 48 hours and the expression of the prodrug activation enzyme was examined. The results are shown on Figure 5-2. The CD protein levels of cells that were maintained under oxic conditions served as a control.

CD gene expression in cells transfected with a plasmid containing the CMV promoter, pCMV-CD, was measured as a positive control (panel 1). As a negative control, the ability of an HRE-null promoter (pHOCD) to induce CD expression levels was also determined (panel 2). Both plasmids produced efficient CD expression regardless of the oxygenation conditions. The addition of either HRE enhancer sequence in the expression cassette resulted in loss of aerobic CD expression in all four-cell lines (panels 3 and 4), indicating that hypoxia-induced transgene expression was mediated through HREs.
Figure 5-2 Hypoxic selectivity of HREs was in a panel of commercially available prostate cancer cell lines. Cytosine deaminase was detected by Western Blot analysis of total protein lysates of normoxic and hypoxia-treated (48 hours) transfectants. Representative immunoblots for CD and β-actin are presented. The induction of cytosine deaminase placed under the control of the CMV promoter in positive controls (panel 1) and an HRE-null promoter as a negative control to HREs hypoxic selectivity (panel 2). Cytosine deaminase was not detected in normoxic prostate cancer cells transfected with either HRE-containing vector (panel 3 and 4).

The increase in CD detection levels in hypoxic samples was compared to that of oxic transfectants to measure the magnitude of promoter induction in hypoxia. The induction levels ranged from 1.1 to 8 among cell lines (Figure 5-3). From this analysis, the activity of pH5VCD appeared enhanced by up to 70% in 22Rv1 cells, when compared to that of LnCaP transfectants. Within the same cell line, the HRE enhancer sequence used in the expression cassette also influenced hypoxic CD, with a difference of up to 50% in 22Rv1 cells.
The intensity of the CD protein bands obtained in hypoxic samples (Figure 5-2) were corrected for that of β-actin and expressed as a multiple of that associated with aerobic lysates. The hypoxic induction levels of pH5VCD and pH8GCD varied across the panel of four prostate cancer cell lines tested. A HRE sequence effect was also evident in 22Rv1 cells, with pH5VCD inducing higher expression levels than pH8GCD, under identical hypoxic conditions. Bars, SE; *p<0.05; ns, non significant; N=3

The general variability of hypoxic induction levels among prostate cancer cell lines was more pronounced in pH5VCD than in pH8GCD transfectants. pH5VCD driven CD expression levels appeared significantly weaker in LnCaP cells (2.5-fold) than in either DU145 (5-fold) or 22Rv1 (7.7-fold) cells (p = 0.002). 22Rv1 cells showed significantly increased CD levels (p = 0.04) compared to DU145 cells.

While densitometry is often used as a method of quantification, the result needs being interpreted with caution, because the repetitive blots were not exposed simultaneously, which may induce errors in densitometry measurements.
According to the pictures of the blot, CD protein levels did consistently appear strongest in 22Rv1 HRE transfectants, compared to either DU145 and LnCap cells. This cell line was thus used as a reference in downstream studies.

5.3.2 Hypoxia selectivity was lost in novel RC58/T prostate cancer cell line

*In vitro* models of localised prostate cancer are currently limited, with 22Rv1 cells being the closest representation. Therefore, in addition to commercially available prostate cancer cell lines, the inducibility of the vectors was tested in a newly developed cell line, RC58/T. These immortalised cells originate from a radical prostatectomy biopsy obtained from a 52-year old Caucasian male and might represent a novel model for early stage disease. RC58/T pH5VCD and pH8GCD transfectants were grown in either aerobic or hypoxic conditions for a period of 48 hours prior to total protein extraction. The results are shown on Figure 5-4.

CD expression was strongly induced (3-fold) in the transfected cells, regardless of the growth environment. Treatment effects were statistically tested with an analysis of variance. There wasn’t sufficient data to reject the null hypothesis that all fold inductions were the same and it was concluded that there was no plasmid effect ($p = 0.576$), no condition effect ($p = 0.94$) and no interaction effect between the plasmid used and the exposure condition ($p = 0.596$).
Figure 5-4 Hypoxic response of RC58/T prostate cancer cells transfected with pH5VCD and pH8GCD. Representative CD immunoblots are represented. The associated β-actin immunoblots (used for normalisation) are also presented. Cytosine deaminase was detected in both normoxic and hypoxic lysates (48 hours). The magnitude of induction levels was not statistically increased in hypoxia ($p = 0.94$) and was not dependent on the plasmid used ($p = 0.596$). Bars, SE; ns, non significant. N=3

5.4 Effect of HRE Copy Number on Hypoxia-Induced CD Expression

To investigate the influence of HRE copy number, the induction ratio of hypoxic to oxic gene expression in plasmids containing 2X, 5X of 6XHREs was calculated. 22Rv1 cells were transiently transfected and exposed to a variety of hypoxic conditions (8h, 16h, 24h, 48h hypoxia). Basal expression in oxic cells was used as a control to hypoxic inducibility. The results are shown in Figure 5-6 and Figure 5-6.
Generally, CD expression levels were significantly increased with either 5X or 6X copy number compared to 2X (p<0.05). While 2X resulted in weak expression levels (less than 5 fold) at all time points tested, the benefit of increased HRE copy number was accentuated with the length of hypoxic exposure. When the hypoxic induction in pH2VCD transfectants was used a reference, the response in pH5VCD was enhanced by a factor of 2.25 (8h), 1.77 (16h), 4.15 (24h) and 6.8 (48h). The difference between pH2VCD and pH6VCD (7.2) was statistically significant at 48h only (p = 0.01).

Figure 5-5 Effect of HRE copy number on hypoxic induction of CD. 22Rv1 cells transfected with pH2VCD, pH5VCD and pH6VCD were exposed to (A) 8, 16, 24hrs hypoxia; (B) 24hrs hypoxia followed by 2hrs reoxygenation, and (C) 48 hrs hypoxia. Cytosine deaminase was detected by Western Blot analysis of total protein lysates transfectants. Representative immunoblots for CD and β-actin are presented.
Figure 5-6 Quantitative analysis of western blots in 22Rv1 transfectants.
22Rv1 cells were transfected with vectors containing either 2X, 5X or 6X HREs (VEGF) in the expression cassette. Cytosine deaminase was detected in normoxic and hypoxic lysates (8, 16, 24 and 48 hours). The increase in the CD protein was quantified by densitometry of the Western blot films. Increasing the number of HRE copy from 2 to 5 or 6 increased CD expression levels by up to 80% at 48 hours hypoxia. Bars, SE; *p<0.05; ns, non significant; N=3.

5.4.1 COMPARISON OF pH5VCD AND pH6VCD

To determine whether pH6VCD was superior to pH5VCD, we analyzed CD levels under several hypoxic exposure conditions in 22Rv1 and DU145 transfectants. Transiently transfected cells were made hypoxic for up to 48 hours. CD protein levels were measure at several time points (8, 16, 24 and 48h). The effect of reoxygenation (24h) was also tested. The Western blot results were shown on Figure 5-5. The quantitative analyse is presented in Figure 5-7 and Figure 5-8.
Hypoxic treatment (16, 24 hours) resulted in higher induction of pH6VCD (8.3- and 6.9-fold) than pH5VCD (5.7- and 6.2-fold) but the difference was not statistically significant (p<0.05, two-sample t-test). Prolonged hypoxia (48 hours) resulted in very similar promoter induction levels (7.75-fold and 7.5-fold, respectively). In reoxygenated cells, the difference between 5X and 6X was not statistically significant in both 22Rv1 (p = 0.4, two-sample t test) and DU145 (p = 0.08, two-sample t test).

Figure 5-7 Comparison of the hypoxic response of pH5VCD and pH6VCD in The hypoxic fold induction levels associated with each vector were compared at each time point but no superiority of pH6VCD over pH5VCD was evident. Bars, SE; *p<0.05; ns, non significant; N=3
Figure 5-8 DU145 cells transfected with either pH5VCD or pH6VCD were reoxygenated for 24 hours following hypoxic treatment. The increase in CD protein levels measured in the lysates was compared to that of the associated normoxic control. No superiority of pH6VCD over pH5VCD was evident in DU145 transfectants. Bars, SE; ns, non significant; N=3

5.5 KINETICS OF THE HYPOXIC HRE RESPONSE

5.5.1 KINETICS HRE-DRIVEN CD PRODUCTION IN HYPOXIA

To determine the time frame in which cytosine deaminase expression was induced under hypoxic conditions, CD protein levels in hypoxic 22Rv1 transfectants (pH5VCD, pH8GCD) was monitored at 8, 16, 24 or 48 hours time points.

The time of onset of the hypoxic response appeared dependent on the HRE enhancer sequence present in the promoters (Figure 5-9). pH5VCD induction was relatively quickly detected (16 hours) compared to pH8GCD (48 hours)
Figure 5-9 Time course of the hypoxic induction of HREs in 22Rv1 cells. The cells were transfected with either pH5VCD or pH8GCD and exposed to hypoxic conditions for up to 48 hours. Representative immunoblots are presented. The time of onset of protein expression was estimated by taking lysates throughout hypoxic treatment. The CD protein was relatively quickly detected in pH5VCD transfectants (16 hours). pH8GCD did not produce CD under the hypoxic exposures tested shorter than 48 hours.

22Rv1 cells transfected with pH5VCD showed a steady increase in CD expression levels (Figure 5-10). Hypoxic induction ratios increased by 70% every 8 hours for the first 24 hours. Twenty-four hours later, the levels had increased by a further 30%, suggesting a reduction in promoter activity over time.

No CD protein was detected in the first 24 hours in cells transfected with pH8GCD but the increase was rapid thereafter with a 500% increment at 48h.

These results clearly demonstrate a difference in the dynamics of the translation of HRE-directed transgene expression.
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Figure 5-10 Quantitative analysis of time course experiments in 22Rv1 transfectants. The cells were transfected with pHSVCD (A,C) and pHSGCD (B,D). The production of CD by the two vectors under hypoxic conditions was monitored over time.

5.5.2 KINETICS OF HRE-DRIVEN CD EXPRESSION IN REOXYGENATED CELLS

Due to the unstable nature of tumour hypoxia, the effect of reoxygenation on the HRE response was also evaluated. Reoxygenation may be relevant in the clinical tumour response and therefore the activity of the vectors in this situation is of interest. To test whether reoxygenated cells could be targeted by hypoxia-inducible strategies, 22Rv1 and DU145 cells transfected with either pH5VCD or pH8GCD were reoxygenated for up to 4 days following hypoxic treatment (48h). CD expression levels were monitored every 24 hours. The results are shown on Figure 5-11.

While CD production increased rapidly with time in hypoxic pH5VCD 22RV1 transfectants, CD levels decreased progressively during the reoxygenation period and were significantly reduced on day four (p = 0.0058, 2 sample t test).
Figure 5-11 Kinetics of CD protein levels in transfected 22Rv1 cells. The cells were transfected with pH5VCD (A) and pH8GCD (B) and reoxygenated following hypoxic treatment. CD protein levels were monitored daily for four days. Representative CD and β-actin immunoblots are represented. Bars, SE; N=3.

In pH8GCD transfectants, CD levels had significantly increased on day one of reoxygenation (p = 0.0094) but decreased progressively over the next three days. At the end of the reoxygenation period CD induction levels were not significantly reduced compared to that measured at the end of hypoxic treatment (p = 0.0767).

Despite the fact that the initial response of pH5VCD to hypoxia appeared to be almost twice as strong as pH8GCD (8-fold versus 5-fold, p = 0.006), at the end of reoxygenation, CD induction levels in pH8GCD transfectants were not significantly higher than that of pH5VCD (p = 0.1212).
In DU145 cells, CD expression was lost in both transfectants on day one of reoxygenation. Kinetics curves were thus not constructed (Figure 5-12).

![Figure 5-12](image)

Figure 5-12 The experiment was repeated in DU145 cells. Lysates were taken at the end of hypoxic treatment (48 hours) and on Day 1 of the reoxygenation period. (A) Quantitative analysis of western blots. The CD response of aerobic controls was used to estimate hypoxic induction levels. (B) Representative CD and β-actin immunoblots of hypoxic and reoxygenated DU145 transfectants.

5.5.3 EFFECT OF LONG TERM HYPOXIA ON HRE-DRIVEN CD EXPRESSION

To determine whether the activity of the promoters could be sustained in a chronically hypoxic environment, 22Rv1 and DU145 cells transfected with either pH5VCD or pH8GCD were grown in hypoxia for 5 days prior to total protein extraction. CD gene expression in cells transfected with a plasmid containing the CMV promoter, pCMV-CD, was measured as a positive control. The results are shown on Figure 5-13. While expression was not sustained on day 5 of hypoxic treatment in DU145 transfectants, CD hypoxic levels in 22Rv1 cells
remained strong (5- and 2-fold in pH5VCD and pH8GCD transfectants, respectively). When compared to that obtained at 48h, CD amounts were significantly reduced in pH8GCD ($p = 0.0474$) but not in pH5VCD transfected cells ($p = 0.721$).

![Figure 5-13 Chronic hypoxic response of HREs.](image)

(A) Representative CD and β-actin immunoblots of 22Rv1 hypoxic transfectants and associated densitometry analysis. The CD response of aerobic controls was used to estimate hypoxic induction levels. (B) Kinetics of pH5VCD in hypoxic 22Rv1 transfectants. (C) Representative CD and β-actin immunoblot of DU145 hypoxic transfectants. (D) Kinetics of pH8GCD in hypoxic 22Rv1 transfectants. Bars, SE; N=3.

5.5.4 DELAYED HRE-DRIVEN CD PRODUCTION FOLLOWING ACUTE HYPOXIA

Although CD was not detected early in the time course experiment (Figure 5-9), the possibility that the HIF-1α machinery was not yet activated in that time frame was tested. The effect of short hypoxic exposures on HREs was thus further investigated by allowing for reoxygenation prior to harvest. 22Rv1 cells
transfected with either pH5VCD or pH8GCD were thus acutely exposed to hypoxia for 4 hours and reoxygenated for 24 hours. CD gene expression in cells transfected with a plasmid containing the CMV promoter, pCMV-CD, was measured as a positive control. The results are shown on Figure 5-14. Acute hypoxic treatment (4 hours) was found sufficient to induce detectable levels of CD protein (1.35- and 2-fold induction in pH5VCD and pH8GCD transfectants, respectively), providing time was allowed between exposure and harvest.

Figure 5-14 HRE response in acutely hypoxic 22Rv1 transfectants.

To test the possibility that the translation of hypoxia-induced transgene expression protein synthesis is delayed, 22Rv1 transfectants were allowed to recover from acute hypoxic treatment for 24 hours prior to harvest. CMV-dependent CD production was used as a positive control. Representative CD and β-actin immunoblots are presented with the associated quantitative analysis. The CD response of aerobic controls was used to estimate hypoxic induction levels.
5.5.5 TIME FRAME OF THE DELAYED INDUCTION OF CD PRODUCTION

To test whether the time of onset of the delayed response was similar during longer hypoxic exposures and reoxygenation, the induction of the promoters in hypoxic (24 hours) and reoxygenated transfectants was compared. The results are shown on Figure 5-15.

While the response of pH5VCD was significantly enhanced by the prolongation of hypoxic exposure (p = 0.05), the addition of a reoxygenation period did not further enhance protein amounts. These results suggest that by 24 hour into hypoxic treatment, HIF-1α mediated gene expression has been triggered and translation is complete. Interestingly, the protein levels after a 24h reoxygenation period were similar after either 24 or 48h hypoxia, despite the fact that CD induction was stronger at 48 than at 24 hours (see 5.5.1). The inhibiting effect of reoxygenation on promoter activity may thus be independent of prior stimulation.
Figure 5-15 To estimate the time scale of hypoxia-mediated protein synthesis, the CD induction levels estimated in 22Rv1 transfectants (pH5VCD, A; pH8GCD, B) in response to a variety of hypoxic treatment protocols were compared. Bars, SE; *p<0.05; N=3.

In pH8GCD transfectants, the magnitude of the response was not influenced by prolongation of hypoxic treatment from 4 to 24 hours prior to reoxygenation (p = 0.138). These results suggest that HIF-1α-mediated response in this case is fully triggered in the first four hours of exposure but not translated into protein synthesis until a later time. Since CD protein was not detected in lysates taken at 24h into hypoxic exposure, but present in reoxygenated samples and in lysates taken at 48h hypoxia, the delay in translation following HIF-1α induction of the machinery may be estimated to be in the region of 24 hours. Moreover, expression levels at 48h hypoxia were significantly greater than that of reoxygenated samples. Therefore, while additional hypoxic time does not appear to change the time of onset of the response, it may amplify its magnitude.
5.6 Selection of Strongest Candidate for This Gene Therapy Strategy

While the HRE copy number effect was studied in VEGF-derived promoters (see 5.4) and pH5VCD emerged as the optimal in terms of hypoxia inducibility, the kinetics of induction of this vector were found to differ from that of pH8GCD. The potential of each promoter in inducing strong CD expression levels were finally compared across the conditions and the panel of cell lines tested. The results are shown in Figure 5-16, Figure 5-17 and Figure 5-18.

The induction levels achieved with the plasmids were comparable in DU145, PC3 and RC58/T transfectants but pH8GCD activity was significantly reduced in both 22Rv1 and LnCaP cells, at 48 hours hypoxia (Figure 5-16). The response of both plasmids under hypoxic conditions was extensively studied in 22Rv1 cells. The difference in the hypoxic inductions was statistically significant at all hypoxic time points tested (Figure 5-17). In reoxygenated samples, the response of pH8GCD was significantly enhanced in lysates taken on Day 4 of reoxygenation, following hypoxic treatment (48 hours). pH5VCD produced greater protein amounts than pH8GCD in transfectants allowed to recover in air from hypoxic shock (4 and 24 hours).
Figure 5-16 In this summary plot, the induction levels of CD protein production associated with pH5VCD in response to hypoxic treatment (48h) were compared to that of pH8CD in five transfectant cell lines. Bars, SE; *p<0.05; ns, non significant; N=3.
Figure 5-17 The response of pH5VCD and pH8GCD 22Rv1 transfectants was extensively studied. pH8GCD was superior to pH5VCD only in lysates taken on Day 4 of reoxygenation, following hypoxic treatment (48 hours). *p<0.05

In DU145 cells, the hypoxic inducibility of all plasmids was reduced compared to 22Rv1 cells (Figure 5-18). Induction of the plasmids was lost in acute and long-term hypoxia, suggesting the response in this cell line to be transient. At 48h hypoxia, the induction of each vector was similar (5-fold). The response of pH5VCD was strongly attenuated compared to that observed in 22Rv1 transfectants, but was similar in either pH8GCD transfectants. Reoxygenation of hypoxic cells (24 hours), resulted in potentially stronger induction of pH8GCD than pH5VCD in DU145 cells but the difference was not statistically significant (p = 0.9).

If we consider DU145 and 22Rv1 cells to be a model of a metastatic and primary disease, respectively, these results suggest that either plasmid could
be used to target primary and metastatic prostate cancer but pH5VCD may be most suitable for the targeting of primary tumours.

![Graph showing hypoxic fold induction for different plasmids](image)

Figure 5-18 The response of the vector in DU145 cells was compared across the protocols tested. The difference in hypoxic protein induction mediated by the plasmids in response to reoxygenation and hypoxic exposure (48h) was not statistically significant.

5.7 Discussion

Plasmids constructed with gene fragments of hypoxic responsive genes, may be useful for inducing specific transgene expression in hypoxic cells. A series of gene therapy plasmid vectors containing arrangements of HREs have been proposed in the literature for the targeting of solid tumours (for review, see (Marignol, Lawler et al. 2005). In this study, the cytosine deaminase suicide gene was placed under the control of a panel of promoters designed to contain different HRE copy number isolated from the VEGF and GAPDGH gene (Chapter 3). Hypoxic selectivity was tested using a HRE-null plasmid, pH0CD, while a CMV-driven plasmid was used as a positive control. The ability of the
designed vectors to induce detectable levels of transgene product in response to hypoxic treatment was extensively tested in this chapter, using Western Blots analysis for the CD protein. The magnitude of CD induction was estimated by densitometry. While widely used, this method remains limited by the variability in the blots due to the difficulty in achieving consistent film exposures. This interpretation of Western blotting studies must thus be taken with caution.

Transient transfectants (pHSVCD, pH8GCD) were specifically induced in response to hypoxic treatment (48h) in one primary (22Rv1) and three metastatic (DU145, PC3 and LnCaP) prostate cancer cell lines. However, although Western Blotting revealed no CD expression in air from vectors encoding HREs, there is a possibility levels of expression were below detection sensitivity. Moreover, the androgen responsiveness of the cells did not appear to influence the induction of the vectors. These results therefore suggest that the vectors could be used for the treatment of both early and advanced disease. This conditional expression, shown to be directly controlled by HREs (section 5.3.1) is an essential requirement for a hypoxia-inducible gene therapy strategy, because strong CD expression will be required in hypoxic tumour tissue but should not be present in normoxic normal cells.

Increased expression of HIF-1α has been associated with increased growth rate and metastatic potential of rat prostate cancer cell lines (Zhong, Agani et al. 1998) and reported in a majority of prostate tumours (Zhong, De Marzo et al. 1999). In our study, variability in the response of the vectors among cell lines was evident and potentially reflects the complexity of HIF-1α stabilisation and translocation process (Figure 5-1). Analysis of the phenotype of the cell lines used confirmed the inhibitory effect of both wild type and mutated p53 expression on the HIF-1α mediated machinery. Cells known to express low levels of mutated p53 (22Rv1) showed highest HRE response and were thus used as the model of choice for further study of the vectors. While mutation of p53 is a frequent event in prostate cancer (Meyers, Gumerlock et al. 1998),
p53-null PC3 transfectants surprisingly exhibited poor CD levels, despite the fact that, unlike other common prostate cancer cell lines, these cells were found to overexpress the HIF-1α protein even in an aerobic growth environment. (Saramaki, Savinainen et al. 2001; Zhong, Hanrahan et al. 2001; Hermans, van Alewijk et al. 2004). CD expression was high in both controls, associating the presence of HREs to the lack of responsiveness of the plasmids. HIF-1β has been shown to be expressed in both oxic and hypoxic prostate cancer cells, including PC3, excluding the possibility of inhibited HIF-1 formation (Zhong et al., 2001). The weakened response may thus be due to poor HRE binding affinity. PC3 cells indeed lack the STAT3 transcription factor (Yuan, Guan et al. 2005), which binds to HIF-1 to induce gene expression. Loss of STAT3 may thus have contributed to reduced HREs responsiveness.

The levels of CD expression achieved with VEGF-derived constructs correlated with increasing number of HRE repeats in both 22Rv1 and DU145 cells (section 5.4). pH2VCD resulted in weaker CD expression than pH5VCD. The difference in CD expression levels between pH5VCD and pH6VCD was however not significant at all conditions tested and pH5VCD only was selected for more extensive analysis. The comparison in the potential of either VEGF or GAPDH HRE sequences pointed towards the superiority of pH5VCD over pH8GCD in terms of CD production. A crucial difference was seen in time course experiments (section 5.5.1), with pH5VCD driving CD expression more rapidly (16 hours) than pH8GCD (48 hours) in 22Rv1 cells. Induction ratios followed a hyperbolic increase in pH5VCD transfectants, to reach 8-fold at 48 hours, whereas with pH8GCD, CD levels were increased 5-fold compared to aerobic controls at this time point, following an exponential relationship with time. Unfortunately, there are few studies looking at the time course of HIF-1α expression in prostate cancer cells in the literature. One isolated study reported differential time of onset in the induction of the protein in hypoxic PC3 (8 hours) and LnCaP (24 hours) cells. These results suggest there are differences in the
dynamics of HRE-driven responses among cell lines (Coffey, Morrissey et al. 2005).

Selective hypoxic induction was lost in the newly developed RC58/T prostate cancer cell line, suggesting inhibition of HIF-1α ubiquitination in normoxia (section 5.3.2). RC58/T cells have been poorly characterised and it may only be speculated as to the cause of aerobic CD production. Degradation of HIF-1α in the presence of oxygen is a multi-step process involving a variety of factors, whose alteration may lead to constitutive expression. A possible explanation to ubiquitination inhibition would be mutation, methylation or loss of the VHL tumour suppressor gene. Cells lacking pVHL indeed express HIF-1α constitutively (Krieg, Haas et al. 2000). Mutations of this gene have been extensively studied in renal cell carcinoma (Kim and Kaelin 2004) but are poorly documented in prostate cancer. pVHL seems to be mutated in approximately 50% in renal clear-cell carcinoma, but only 11% in colorectal cancer. Hypermethylation of the pVHL has been found to be a rare event in genitor-urinary cancers (Chung, Hong et al. 2001). The pVHL gene was mapped by linkage analysis to the short arm of chromosome 3 in renal cell carcinoma (Seizinger, Rouleau et al. 1988). Chromosome 3 is reportedly lost in the karyotype of RC58/T cells (Yasunaga, Nakamura et al. 2001). Loss of pVHL may thus account for constitutive HIF-1α expression and subsequent HRE-driven gene expression. Other disruptions of the HIF-1α protein include mutations of the ODD domain and gene amplification. Polymorphisms in proline 582 of HIF-1α gene have been associated with increased normoxic HIF-1α expression levels and a more aggressive phenotype (Fu, Choi et al. 2005). Experiments in our laboratory involving a large panel of prostate cell lines revealed that the P582S mutation was only detected in RPWE-1 normal prostate cells prostate cancer cells (unpublished data).

Finally, the HIF-1α gene has been shown to be duplicated in prostate tumours representing BPH, androgen-dependent primary tumours and lymph-node
metastasis as well as locally recurrent hormone-refractory prostate carcinomas. It may be postulated that this gene amplification may reflect the requirement for several HRE copy number to improve transgene expression. The gene copy number was increased to four in DU145 and LnCaP cells and to six in PC-3. The level of amplification of the HIF-1α gene observed in PC3 cells was however not commonly found in prostate tumours, suggesting this cell line as an exception (Saramaki, Savinainen et al. 2001). Neither HIF-1α mutation nor gene amplification thus does explain the results obtained with RC58/T and loss of pVHL appears the most plausible cause.

Reoxygenation has been shown to result in rapid degradation of the HIF-1α protein due to increased activity of PHDs. Under hypoxic conditions, the activity of PHDs in glioma cells has been shown to be limited by the lack of oxygen. In contrast, transcription of genes coding for PHDs was increased for PHD activity to be sufficiently high to stop hypoxia-induced signalling in freshly reoxygenated cells (D'Angelo, Duplan et al. 2003). Degradation of HIF-1α during reoxygenation has also been confirmed through real-time imaging (Liu, Qu et al. 2005). If HIF-1/HRE interaction is compulsory for the induction of transgene expression, loss of HIF-1α upon reoxygenation would be expected to result in undetectable transgene product in associated lysates. In the present study however, reoxygenation following hypoxic treatment (48h) induced different kinetics in transfected cells. CD protein levels were sustained in reoxygenated 22Rv1 transfectants but lost in DU145 cells (section 5.5.2). In 22Rv1 cells, reoxygenation led to a progressive decrease in CD induction levels over time in pH5VCD transfected cells, whereas pH8GCD transgene expression significantly increased in the first twenty-four hours but reduced thereafter. The results suggest that, while hypoxia-mediated gene expression might be prevented upon reoxygenation, translation of gene products is sustained. Indeed, studies in neuroblastoma cells showed the hypoxic phenotype to be conserved for up to twenty four hours (Holmquist, Jogi et al. 2005).
The time frame in the synthesis of CD in response to hypoxia was thus further investigated. Transfectants were allowed aerobic recovery following hypoxic shock prior to lyses and Western blot analysis. In this scenario, CD was detected in acutely hypoxic 22Rv1 cells (4 hours), whereas no protein was detected immediately after hypoxic treatment (section 5.5.4). Prolongation of hypoxic exposure to 24 hours resulted in the increased induction levels of the same magnitude in pH5VCD transfectants, regardless of reoxygenation. This delayed response was more pronounced in pH8GCD transfectants. These results suggest that HIF-1α mediated transgene expression is triggered early into the hypoxic exposure but that protein synthesis may not occur until later (section 5.5.5). There is evidence in the literature that overall mRNA translation is severely but reversibly inhibited during both acute and chronic hypoxic exposures (Koritzinsky, Seigneuric et al. 2005). Protein synthesis indeed costs large amount of cellular ATP. In response to reduced ATP availability due to lack of oxygen, hypoxic cells are forced to limit its consumption. This inhibition is thus thought to serve to the maintenance of energy homeostasis. Gene-specific changes to mRNA translation efficiency may thus contribute to hypoxia-induced differential gene expression and could explain the delay in CD protein synthesis observed in this study. Sustained transgene expression is a real advantage to the technique, as it would ensure efficient targeting of chronically hypoxic cells that may reoxygenate during treatment due to tumour shrinkage. Long-term hypoxia resulted in reduced but sustained CD induction levels in 22Rv1 but not in DU145 transfectants (section 5.5.3). Attenuated Akt phosphorylation and subsequent glycogen synthetase kinase 3β or forkhead transcription factor FOXO4 activation have both been associated with decreased HIF-1α protein amount in prolonged hypoxic exposure (Berra, Richard et al. 2001; Berra, Benizri et al. 2003; Mottet, Dumont et al. 2003; Tang and Lasky 2003). Moreover p53 accumulation under conditions of severe/prolonged hypoxia may result in progressive competition with HIF-1α for p300 interaction and contribute to the repression of HIF-1α transcriptional activity (Ravi, Mookerjee et al. 2000; Schmid, Zhou et al. 2004). These
phenomenons may explain the loss of CD induction in chronically hypoxic DU145 cells. P53 mutated 22Rv1 were shown to express low levels of the protein, and were associated with strong HRE response. However, accumulation of the protein in hypoxia may progressively contribute to the observed reduction in CD levels.

In conclusion, the use of the HIF-1/HRE complex to drive transgene expression in response to hypoxic treatment was successfully achieved in five prostate cancer cells. VEGF and GAPDH HRE sequences arrangements were both induced in acutely and chronically hypoxic primary and metastatic prostate cancer cell lines. Overall comparison of the inducibility of the vectors however suggests pH5VCD as the strongest candidate for this hypoxia-directed suicide gene therapy approach.
CHAPTER SIX: INHIBITION OF HYPOXIA-INDUCED CHEMORESISTANCE IN TRANSFECTED PROSTATE CANCER CELLS
6.1 INTRODUCTION

Several mechanisms have been proposed for the emergence of hormone resistance in prostate cancers. The similarities in the signaling pathways involved with hypoxia-induced responses are interesting (Figure 6-1). Activation of the androgen receptor has indeed been shown to induce both RAS and PI3K (Culig and Bartsch 2006), whose effect on HIF-1α was previously discussed in Chapter 5 (Figure 6-1A). Anti-androgens, such as Flutamide, act by binding to the androgen receptor to antagonize sex hormones in the tumour. However, these compounds also have an affinity to pituitary and hypothalamus receptors, stimulating the testes in oversecretion of androgens, limiting total ablation of the hormone. The effect of this background production of androgens on therapy outcome may be greater than expected. Recurrent prostate cancers are indeed associated with increased expression, stability and translocation of the androgen receptor, making tumour cells more sensitized (up to four times) to the growth-promoting effect of dehydrotestosterone (DHT) (Bakin, Gloell et al. 2003). In addition, long-term hormonal deprivation may select for prostate cancer cells with higher 5α-reductase activity, which can produce higher intracellular DHT from adrenal androgen. Intracellular DHT, interestingly, was shown to stabilize HIF-1α, further strengthening the hypoxic response (Mabjeesh, Willard et al. 2003). Therefore the activity and sensitivity of the receptor, shown to be increased by hypoxia (Park, Kim et al. 2006), when combined with increased expression, reported in up to 30% of tumours (Feldman and Feldman 2001), may trigger the selection of adapted phenotypes.

The correlation between hypoxia and androgen resistance however does not stop there. Many co-activators have been identified as enhancing the ligand-induced transcriptional activity of the androgen receptor and participating to the androgen response. The most characterized of these cofactors is the steroid receptor (SCR) family. Expression levels of SCR-1 were enhanced in higher-grade prostate cancer with poor response to endocrine therapy and in recurrent
tumours. Upregulation of SCR-1 and SCR-2 confers increased sensitivity to low androgen concentration and may contribute to tumour regrowth (Fujimoto, Mizokami et al. 2001; Gregory, He et al. 2001). Not surprisingly, SCR-1 also acts as a coregulator of the HIF-1α mediated response in conjunction with yet another mutual actor, p300/CREB (Aarnisalo, Palvimo et al. 1998; Ruas, Poellinger et al. 2005). Activation of the MAPK pathway and transcription factors such as STAT3 in response to growth factor and/or cytokines were also reported to be involved in the development of androgen- and hypoxia-mediated resistance (Figure 6-1B) (Culig, Hobisch et al. 1994; Chen, Wang et al. 2000; Niu, Wright et al. 2002; Wei, Le et al. 2003). Finally, mutations of p53 and the PTEN tumour suppressor gene were associated with both hypoxic and androgen-independent phenotypes (Li, Yen et al. 1997; Koivisto and Rantala 1999; Zundel, Schindler et al. 2000).
The serine/threonine kinase Pim-1 proto-oncogene is regulated via the STAT factors by Interleukin-6 (IL6) in prostate cancer cells and involved in IL-6 induced ligand-independent activation of the androgen receptor (Kim, Jiang et al. 2004). The Pim-1 protein has been implicated in the positive regulation of the cell cycle (Liang, Hittelman et al. 1996) and the attenuation of androgen-dependent transcription (Thompson, Peltola et al. 2003). Increasing evidence suggests that Pim-1 may play a role in survival signaling and have anti-apoptotic properties (Figure 6-1C). Pim-1 was indeed shown to inactivate BAD by phosphorylation and enhance Bcl-2 anti-apoptotic activity, thus promoting cell survival (Aho, Sandholm et al. 2004). Overexpression of this protein was
associated with high Gleason score (Valdman, Fang et al. 2004) and as a result Pim-1 has recently emerged as a potential diagnostic marker in prostate cancer (Dhanasekaran, Barrette et al. 2001). Unsurprisingly, this protein is tightly related to hypoxia- and androgen- responsive regulators, such as p53. The tumour suppressor gene p53 is involved in the stabilisation of HIF-1α (An, Kanekal et al. 1998; Blagosklonny, An et al. 1998; Chen, Li et al. 2003) and the regulation of STATs factors (Zhong, Zheng et al. 2004), which are also positive modulators of HIF-1α availability in response to hypoxia. Mutations of p53 may thus be associated with increased HIF-1α transcriptional activity and Pim-1 overexpression through inhibition of p53-induced repression of STAT3. Enhanced expression of Pim-1, leading to increased proliferation and reduced apoptotic response may thus be a key factor of the androgen-independent progression of the disease.

Novel therapeutics targeting the hypoxic compartments of tumours may thus not only improve the therapeutic ratio where conventional treatments fail but have their place as preventive treatments or in early diagnosis. While numerous strategies have been developed to downregulate HIF-1α (Kimbro and Simons 2006; Melillo 2006), their effectiveness may be compromised by the concomitant activation of other molecules (i.e. Pim-1) involved in androgen-, inflammation- and hypoxia-responsive cellular responses. One possible therapeutic strategy is to exploit HIF-1α enhanced expression to target a suicide gene.

6.2 AIMS AND OBJECTIVES

The HIF-1α/HRE complex was successfully used to selectively induce cytosine deaminase expression in four hypoxic prostate cancer cells (Chapter 5). Out of a panel of four HRE-directed vectors, pH5VCD and pH8GCD were found the most potent but were associated with differential kinetics. The ability of cytosine deaminase produced by these two vectors to sensitize these cells to clinically
relevant prodrug concentrations (1mM) was studied in this chapter. The protocols tested were composed of a hypoxic induction period (HIP) known to be sufficient to result in acceptable CD levels followed by a drug treatment period of up to four days. The effects of the experimental conditions and exposure to 5-FC on wild-type cells have been discussed in chapter 3.

Sensitisation was initially assessed using a proliferation assay (MTT), which allows for the simultaneous screening of a range of drug concentrations as well as the compensation for cell growth during the length of the experiment. Because the MTT assay is based on the fact that proliferating cells are more metabolically active, this assay is only suitable for the determination of cell viability, proliferation and factor-mediated toxicity (Cory, Owen et al. 1991). The potential of the technique can be extended further by using the NCI protocol (section 2.7.3), where an extra reading is taken prior to the start of the treatment period to fully assess the growth of the control and treated cells. The % growth was calculated as described in section 2.7.3. While growth inhibition was associated with a positive % growth, a negative value can be correlated with reduced cell number and cytotoxicity. Sensitisation was compared using estimates of the EC\textsubscript{50} and EC\textsubscript{50}, concentrations of drug needed to achieve 50% growth inhibition and 50% cytotoxicity, respectively.

Limitations as to the information that may be gathered from an MTT assay include underestimation of cellular damage, detection of cell death only at later stage of apoptosis, and overestimation of reduced metabolic activity. The cytotoxic effect of the strategy was thus subsequently confirmed with clonogenic assays and studied for apoptosis induction using an Annexin V-Propidium Iodine assay for flow cytometry. This assay was first used to study the time-scale and secondly as a mean to quantify the cytotoxic response.
1. Previously detected CD protein levels following hypoxic induction of the vectors were initially correlated to 5-FC induced sensitization in 22Rv1 cells using MTT and clonogenic assays.

2. While likely to be effective in reoxygenated tumour cells, the success of this strategy may however suffer from the chemoresistance known to be induced by long-term hypoxia. The sensitivity of aerobic and hypoxic 22Rv1 and DU145 wild type cells to increasing 5-FU concentrations (0 – 50μM) was thus measured to assess potential occurrence of hypoxia-induced chemoresistance.

3. Thirdly, the cytotoxic effect of aerobic and hypoxic prodrug treatment on reoxygenated and hypoxic transfected cells was measured and quantified. The magnitude of the cytotoxic response, expressed as fold induction in apoptosis levels, was finally compared according to oxygenation status and length of HIP.

6.3 HRE-DRIVEN CD EXPRESSION CORRELATED WITH 5-FC SENSITIVITY

6.3.1 AEROBIC PROSTATE CANCER CELLS WERE NOT SENSITISED TO 5-FC

To examine whether the expression of the CD gene could confer increased sensitivity of prostate cancer cells to 5-FC, cytotoxicity/growth inhibition was assessed in 22Rv1 cells with an MTT assay. Western blot analysis of aerobic transfectants confirmed strong expression in cells transfected with the positive control pCMV-CD plasmid and loss of induction in cells transiently transfected with HRE-driven plasmids (Chapter 5, section 5.3.1). Aerobic transfected 22Rv1 cells were grown for 24 hours in air prior to exposure to a range of 5-FC concentrations, in air, for 4 days. The results are shown on Figure 6-2.

The positive control showed strong cell killing effects in normoxia, demonstrating the ability of the cytosine deaminase protein to sensitise prostate
cancer cells to 5-FC. These transfectants exhibited a strong growth inhibition at 0.1 mM (EC$_{50}$ = 0.078 mM) and a cytotoxic effect at 1 mM (EC$_{50}$ = 3.41 mM).

The viability of cells transfected with either HRE vector was excellent under aerobic conditions, showing that 5-FC was non-toxic in the absence of prodrug activation enzyme.

![Figure 6-2 The presence of the prodrug activation enzyme is essential for 5-FC directed sensitisation of prostate cancer cells. 22Rv1 cells were transfected with either control (pCMV-CD) or test (pHSVCD, pHSGCD) vectors. The transfectant were exposed to increasing prodrug concentrations for four days in normoxic conditions. Viability was assessed with an MTT assay and % growth calculated. Bars, SE; N=2.](image)

6.3.2 INCREASED CD PROTEIN LEVELS RESULTED IN INCREASED 5-FC SENSITIVITY

Western Blots revealed marked increase in CD protein levels in hypoxic pH5VCD transfected 22Rv1 cells following lengthening of the HIP from 24 to 48
hours (Chapter 5, Section 5.5.1). It was thus investigated whether this would translate into increased killing effect of the vectors. Although CD could not be detected at 24 hours hypoxia, CD synthesis was found to be delayed and would occur during prodrug treatment. 22Rv1 cells transfected with pH8GCD were therefore also assayed.

In the first set of experiments (Protocol 1), transfected 22Rv1 cells were grown for 24 hours in a hypoxic environment prior to a 4-days aerobic 5-FC treatment (0 - 5 mM). Cells transfected with pCMV-CD were used as a positive control. The results are shown on Figure 6-3.

![Figure 6-3](image)

**Figure 6-3** Both hypoxia-responsive vectors induced strong growth inhibition effect at a clinically relevant prodrug concentration (1mM). 22RV1 cells were transfected with either control or test plasmids. Following an HIP of 24 hours, sufficient to induce detectable amounts of prodrug activation enzyme, the transfectants were exposed to aerobic 5-FC treatment for 4 days (Protocol 1). Viability was assessed with an MTT assay. The positive control pCMV-CD showed a strong killing effect at all concentrations tested. Bars, SE; N=4.
pCMV-CD transfectants were dramatically sensitised to 5-FC (EC\textsubscript{50} = 0.04 μM). Cytotoxicity was evident at a prodrug concentration of 0.1 mM. A 10-fold increase in 5-FC concentration did not further improve sensitivity. The effect was however more pronounced at 5 mM (EC\textsubscript{50} = 3.14 mM).

Cytotoxicity was achieved at 5-FC concentrations higher than 1 mM and the EC\textsubscript{50} was beyond the concentrations tested for both HRE-driven plasmids. There was no superiority of either HRE enhancer sequence at either 5-FC concentration (p = 0.140). The EC\textsubscript{50} of pH5VCD and pH8GCD was increased by a factor 1.8 and 1.5, respectively, compared to that of pCMV-CD.

The experiment was repeated with a HIP increased to 48 hours (Protocol 2), known to result in increased protein levels (Chapter 5). The results are shown on Figure 6-4 and Table 6-1. The extension of hypoxic exposure had a dramatic effect on both growth inhibition and cytotoxic effects in all transfectants, with strong killing effect evident at 1 mM. There was no advantage in increasing hypoxic exposure in terms of growth inhibition in cells transfected with pCMV-CD. However, the cytotoxic effect was improved by a factor of 5. In cells transfected with HRE vectors, EC\textsubscript{50} was improved by a factor of 1.75 and 2 in cells transfected with pH5VCD and pH8GCD, respectively. EC\textsubscript{50}s dramatically increased by a factor of up to 5. There was again no apparent superiority of either HRE enhancer sequence, with p values of 0.639 and 0.638 at 0.1 and 1 mM 5-FC concentrations, respectively. Moreover, the levels of cytotoxicity achieved with HREs were similar to that of pCMV-CD.
Figure 6-4 Increased CD levels, associated with long HIP (Chapter 5) translated with enhanced cytotoxic effect of prodrug treatment. 22Rv1 cells were transfected with a panel of constructs. The effect of longer HIP (48 hours) on CD/5-FC sensitising effect was tested with an MTT assay. Bars, SE; N=5.

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Table 6-1 The magnitude of sensitisation achieved with protocols 1 and 2 was compared by calculating the ratios of EC\textsubscript{50s} and EC\textsubscript{50s}. Increasing HIP to 48 hours resulted in up to 2- and 5-fold increase of growth inhibition and cytotoxicity, respectively.
These experiments showed that while prodrug sensitisation requires CD expression, the magnitude of the treatment effect was dependent on the amount of prodrug activation enzyme available to the cells. Maximum protein levels, detected in response to an HIP of 48 hours indeed resulted in enhanced sensitisation, even at prodrug concentrations 10-fold lower than clinically relevant. More importantly, HREs were able to induce as much a killing effect as the CMV promoter, which is the considered the gold standard in gene therapy.

As a result, protocol 2 was referred to as Optimal Protocol (OP) in the following studies.

6.3.3 THE HRE/CD/5-FC SYSTEM REDUCED THE SURVIVING FRACTION OF REOXYGENATED PROSTATE CANCER CELLS

Because MTT assays measure metabolic activity as opposed to cell death, the killing effect of each plasmid was confirmed using clonogenic assays. Wild type and transfected 22Rv1 and DU145 were chronically exposed to hypoxia prior to aerobic prodrug exposure (0.1, 1, 4 days). While the surviving fraction of aerobic cells remained close to 100%, reoxygenated wild type cells appeared sensitised to 5-FC (p=0.029 at 1mM in 22Rv1 cells). Transfection with either plasmid significantly reduced 22Rv1 and DU145 cell survival in response to 5-FC exposure (p<0.001, ANOVA). At the clinically-relevant 5-FC concentration of 1mM, the surviving fraction of transfectants was reduced to 42 ± 8% (pH5VCD) and 32 ± 7% (pH8GCD) in 22Rv1 cells; to 28 ± 4% (pH5VCD) and 30 ± 8% (pH8GCD) in DU145 cells.
Figure 6-5 Clonogenic survival in response to 5-FC treatment of (A) 22Rv1 and DU145 (B) cells. The cell lines were transfected with pH5VCD- (○), pH8GCD (○), exposed to hypoxia (48hrs) prior to a 4 days 5-FC treatment. The response of wild type aerobic (*) and reoxygenated (△) cells was used as a control. Error bars, SEM; n=3.

6.3.4 THE KILLING EFFECT OF THE VECTORS WAS MAXIMAL IN 22Rv1

To further investigate the effect of detected CD protein amounts on the ability of the vectors to sensitize cells to 5-FC, the killing effect of each plasmid was tested and compared across a panel of prostate cancer cell lines. Transfectants
were exposed to hypoxia for 48 hours prior to aerobic prodrug exposure (OP). The results are shown on Figure 6-6 and Figure 6-7. (The % growth of LnCaP and RC58/T untransfected controls are shown in Appendix 10.7, on Figure 10-1 and Figure 10-2).

The first observation made was that within the same cell line, the sensitisation achieved with each vector was not statistically significant different, at all 5-FC concentrations tested (p<0.05). Secondly, low 5-FC concentrations (0.1 mM) were sufficient to induce strong growth inhibition in all transfectants tested. Finally, the androgen-independent cell line DU145 appears among the most resistant cells. Overall, both vectors induced the highest sensitisation in 22Rv1 cells, with 50% cytotoxicity achieved at the commonly used prodrug concentration of 1 mM.
Figure 6-6 Induction of CD in (Chapter 5) translated with enhanced cytotoxic effect of prodrug treatment in a panel of prostate cancer cell lines. 22Rv1, RC58/T, LnCap and DU145 cells were transfected with pH5VCD. The cells were exposed to an HIP of 48 hours prior to a 4-days 5-FC treatment (0, 0.1, 1mM) under aerobic conditions.
6.4 Magnitude of Prodrug Sensitisation

6.4.1 5-FC Induces Apoptosis on Day Three of Treatment

Considering the strong response of the plasmids in 22Rv1 at low 5-FC concentrations and the intermediate sensitisation of DU145 cells, the effect of 0.1mM and 1mM 5-FC on apoptosis levels was studied in each cell line, respectively. The time frame of apoptosis induction in response to OP was initially tested to determine when the magnitude of the treatment effect should be measured. The passage of cells from early to late apoptosis was visualised with an Annexin V-PI assay on the flow cytometer. The images are shown on Figure 6-8 and Figure 6-9.
In both cell lines, the number of apoptotic cells increased progressively and moved from the lower right corner (early apoptosis) to the upper right corner (late apoptosis) over time. The levels of apoptosis in androgen-independent DU145 cells were reduced compared to that of androgen-sensitive 22Rv1 cells. On Day 3 of treatment, up to 60% of the cells were undergoing apoptosis. The effect of 5-FC treatment was thus quantified on Day 3 of treatment in subsequent experiments.

Figure 6-8 A daily increase in the number of apoptosis cells was evident as prodrug treatment went on. 22Rv1 cells were transfected with either pH5VCD or pH8GCD and treated with 0.1mM 5-FC, according to OP. Apoptosis induction was monitored daily using an Annexin V-PI assay and flow cytometry. Representative results are shown here.
Figure 6-9 The fraction of apoptotic cells was dramatically increased (56%) on Day 3 of prodrug exposure. DU145 cells transfected with the test plasmids were treated with 1mM 5-FC according to OP. The levels of apoptosis induction detected daily using an Annexin V-PI assay and flow cytometry were reduced compared to 22Rv1 cells. Representative results are shown here.

6.4.2 QUANTIFICATION OF 5-FC INDUCED APOPTOSIS IN HRE TRANSFECTIONTS

Apoptosis levels were quantified and compared to that achieved with the positive control pCMV-CD in 22Rv1 and DU145 transfectants treated according to OP with 0.1mM and 1mM 5-FC, respectively.

In 22Rv1 cells, the average total number of cells in apoptosis was 54%, 57% and 52.5% in pCMV-CD, pH5VCD and pH8GCD transfectants, respectively (Figure 6-10). The increase was statistically significant, compared to wild type (untransfected) treated cells, with associated p values of 0.0221 (pCMV-CD), 0.0073 (pH5VCD) and 0.008 (pH8GCD).
These apoptosis levels corresponded to a 2.15-, 2.62- and 2.1-fold increase, compared to treated wild type cells (Figure 6-11). An analysis of variance of the fold induction of apoptosis by each plasmid was carried out. The p value associated with this analysis was 0.455; there was not sufficient evidence to prove that the apoptosis induction levels varied with the plasmid used.

Figure 6-10 The total number of apoptotic cells in transfectants (~75%) was significantly increased when compared to the wild type controls (~25%). The apoptotic fraction of transfected 22Rv1 cells treated with 0.1mM 5-FC according to OP was estimated on Day 3 of treatment. Bars, SE; N=4, *p<0.05
Figure 6-11 Up to a 3-fold increased in apoptotic cells was induced in transfected 22Rv1 cells treated according to OP.
(A) The apoptotic fraction estimated in 22Rv1 transfectants with an Annexin V-PI assay was corrected for that of treated parental controls to calculate apoptosis fold induction levels. (B) A one-way analysis of variance of the data concluded that fold induction levels were not statistically different ($p = 0.455$) across the vectors tested. Bars, SE; $N=4$, $^{*}p<0.05$

In androgen-resistant DU145 cells, the effect was reduced by a factor of up to 2, with an average total number of apoptotic cells of 30% (pCMV-CD), 35% (pHSVCD) and 25% (pHSGCD) in transfectants (Figure 6-12) in response of 1mM 5-FC treatment. The increase in apoptosis between each transfectants and treated wild type cells was also significant, with $p$ values of 0.0122 (pCMV-CD), 0.0228 (pHSVCD) and 0.0065 (pHSGCD).
Figure 6-12 Androgen-independent DU145 were transfected with a control and the two test vectors prior to treatment with 1mM 5-FC according to OP. On Day 3, the apoptotic fraction in each cell group was estimated using an Annexin V-PI assay. The apoptotic fraction was significantly higher in transfected cells (~25%) than in wild type controls (15%). Bars, SE; N=4, *p<0.05, ns, non significant.

The induction of apoptosis in each transfectant was of 2.6-, 3- and 2.5-fold compared to the treated control (Figure 6-13). An analysis of variance of the apoptosis induction in each plasmid was associated with a p value of 0.802, concluding that all three plasmid induced similar induction levels of apoptosis in DU145 cells.
Figure 6-13 The apoptotic fraction estimated in DU145 transfectant was expressed as a multiple of that of parental controls.

(A) Associated apoptosis induction levels in the region of 3-fold. (B) There was no significant difference (p = 0.802) between the fold inductions of apoptosis achieved with each plasmid tested, according to a one-way analysis of variance of the data. Bar, SE; N=3.

3.4.3 EFFECT OF HIP ON APOPTOSIS INDUCTION LEVELS

Since cytosine deaminase was detected in reoxygenated 22Rv1 transfectants after an HIP of 4h (Chapter 5, section 5.5.4), the effect of 5-FC treatment on acutely hypoxic cells was also studied. CD protein levels detected were however low (1.2-fold) compared to that of chronically hypoxic cells (5 to 8-fold); therefore a prodrug concentration of 1mM was used to treat acutely hypoxic cells. The levels of apoptosis quantified on day three of treatment in transfected cells were compared to that measured in treated wild type cells. The results are shown on Figure 6-14.
Figure 6-14 Apoptotic fraction in acutely hypoxic 22rv1 transfectants. 22Rv1 cells were transfected with pCMV-CD, pH5VCd or pH8GCD. The transfectants were acutely exposed to hypoxia for 4 hours prior to aerobic prodrug (1mM) treatment. (A) On Day 3, the apoptotic fraction was estimated using an Annexin V-PI assay. The apoptosis levels measured in transfected cells were expressed as a multiple of that measured in treated wild type cells. (B) There was no statistical difference in the apoptosis fold induction levels (~2-fold) associated with the plasmids tested (ANOVA). Bars, SE; N=4

Prodrug treatment resulted in a significant enhancement in the total number of apoptotic cells consistent with a 1.8-, 1.5 and 1.8-fold increase, when compared with treated wild type cells. An analysis of variance was carried out. The associated p value of 0.242 suggested that all three plasmid had the same apoptosis induction potential.

These results show that HRE-driven cytosine deaminase could target both acutely and chronically hypoxic cells and induce similar 5-FC sensitivity to that of a CMV-driven strategy.
Since increased amounts of CD protein detected in Western blots were previously found to result in increased prodrug sensitisation (section 6.3.2), the effect of HIP (acute, chronic) on apoptosis induction levels was analyzed (Figure 6-15).

Figure 6-15 Effect of HIP on apoptosis induction levels.
The length of the HIP, previously shown to increase CD protein levels (chapter 5), significantly influenced apoptosis induction levels in pH5VCD transfectants ($p = 0.0194$). This effect was not seen in cells transfected with pCMV-CD ($p = 0.234$) and pH8GCD ($p = 0.3499$). Apoptosis fold induction represent the ratio of total apoptosis levels in transfected to wild type cells. Bars, SE; $N=4$, *$p<0.05$

In 22Rv1 cells transfected with pCMV-CD and ph8GCD, apoptosis levels were increased by a factor of 2 in both acutely and chronically hypoxic cells. The difference was not statistically significant ($p = 0.2340$ and $p = 0.3499$, respectively). Chronically hypoxic pH5VCD transfectants were significantly more sensitised to 5-FC than acutely hypoxic cells ($p = 0.01$), despite the fact that the 5-FC concentration used in acutely hypoxic cells was 10-fold higher.
These results suggest that HRE-driven cytosine deaminase has the potential to preferentially sensitise chronically hypoxic prostate cancer cells that may reoxygenate.

6.5 INHIBITION OF HYPOXIA-INDUCED CHEMORESISTANCE

6.5.1 INCREASE RESISTANCE OF HYPOXIC CELLS TO 5-FLUOROURACIL

Hypoxia was reported to increase the resistance of cells to chemotherapeutic drugs in the literature (Tomida and Tsuruo 1999; Shannon, Bouchier-Hayes et al. 2003). To test whether this resistance occurred in vitro, the sensitivity of wild type 22Rv1 and DU145 cells to 5-FU in hypoxia was compared to that of aerobic cells. Wild type cells were exposed to a range of 5-FU concentrations (0, 1, 5, 10, 50 μM) in aerobic and hypoxic conditions for four days. The sensitivity of the cell lines was assessed with an MTT assay. The results are shown on Figure 6-16 and Figure 6-17.

Even with such a short treatment period, hypoxic 22Rv1 cells were found to be significantly more resistant to 5-FU than aerobic cells (p = 0.0233) across the range of concentrations tested. The concentration required to achieve a 50% cytotoxicity effect (EC_{50}) was increased by a significant factor of 11 in hypoxia.

Hypoxic DU145 cells were significantly more resistant to 5-FU than aerobic cells at low concentrations. The difference was not found to be statistically significant beyond concentrations of 10 μM. The hypoxic enhancement ratio for cytotoxicity was not as large as in 22Rv1 cells, but resistance was still increased by a factor of 5.

This simple study was irrevocable proof that hypoxia rapidly induces a state of increased resistance that may be difficult to abolish using novel strategies. Interestingly, androgen independent DU145 cells appeared more resistant to
intermediate 5-FU concentrations in hypoxia than androgen-sensitive 22Rv1 cells \( (p = 0.04) \).

Figure 6-16 22Rv1 wild type cells were treated with a range of 5-FU concentrations in normoxic or hypoxic conditions for 4 days. The viability of the cells was assessed using an MTT assay and % growth calculated. Bars, SE; \( N=3 \), *\( p<0.05 \).
Figure 6-17 DU145 wild type cells were incubated in the presence of 5-FU (1-10μM) in either aerobic or hypoxic conditions for 4 days. The viability of the cells was assessed with an MTT assay and % growth was calculated.

Bars, SE; N=3, *p<0.05.

6.5.2 LOSS OF HYPOXIA-INDUCED CHEMORESISTANCE IN HYPOXIC HRE TRANSFECTANTS

To test whether hypoxia-induced chemoresistance occurred in transfected cells, the killing effect and apoptosis levels of the vectors were measured in cells exposed to the prodrug in hypoxia.

The killing ability of the vectors in hypoxia was initially compared in 22Rv1 and DU145 cells exposed to hypoxia for 48 hours prior to a four-day exposure to 5-FC (0.1, 1, 5 mM) in hypoxic conditions (OP_Hypoxia). The ratios of hypoxic to oxic EC values, or Hypoxic Enhancement Ratio (HER) were calculated for both 50% growth inhibition (HER_{50}) and 50% cytotoxicity (HER_{50}) effect. The results in 22Rv1 cells are shown on Figure 6-18 and Table 6-2.
The cytotoxic effect was dramatic and evident for all transfectants at 0.1 mM and 50% cytotoxicity was achieved with 1 mM 5-FC, where a 5-FU concentration of 39 μM (EC_{50}) would have been necessary to achieve the same effect.

![Graph showing the viability of cells transfected with various vectors treated with 5-FC.](image)

Figure 6-18 22Rv1 cells were transfected with a panel of vectors and treated with increasing prodrug concentrations according to OP_{hypoxia} The viability of the cells was assessed with an MTT assay and % growth was calculated.

Bars, SE; N=3, *p<0.05.

The sensitivity induced by the positive control pCMV-CD was not influenced by hypoxia (HERs = 1). Surprisingly, cells transfected with either HRE plasmids were more sensitive to 5-FC in hypoxia than in air, with associated HER_{50} values of 3.75 (pH5VCD) and 8.8 (pH8GCD). No significant superiority of either HRE enhancer sequence was evident, with p values of 0.626 and 0.11 at 0.1 and 1 mM 5-FC, respectively.
Table 6-2 The Hypoxic Enhancement Ratio (HER) associated with sensitivity of transfected 22Rv1 to OP or OP<sub>hypoxia</sub> were calculated in terms of both growth inhibition (HER<sub>50</sub>) and cytotoxicity (HER<sub>50</sub>).

This hypoxia-induced sensitivity to the prodrug was further investigated with an apoptosis assay. While the total fraction of apoptotic cells was increased in hypoxia by a factor of 1.5 (Figure 6-19), when corrected for the controls, the magnitude of apoptosis induction was not statistically increased by hypoxia (Figure 6-20).
Figure 6-19 22Rv1 wild type and transfected cells were treated according to either OP or OP_Hypoxia. The associated apoptosis fractions were plotted for comparison. Cells receiving hypoxic prodrug treatment consistently presented apoptosis levels ~1.5 times higher than in normoxic conditions. Bars, SE; N=3
Figure 6-20 22Rv1 wild type and transfected cells were treated according to either OP or OP hypoxia. When relative to wild type controls, the induction of apoptosis by 5-FC was not statistically increased in hypoxia. Bars, SE; N=3.

The hypoxic prodrug sensitivity of 22Rv1 transfectants was also tested in acutely hypoxic cells (Figure 6-21). Transfectants were made hypoxic for 4 hours prior to 5-FC treatment (1 mM). The induction of apoptosis in hypoxia, relative to that measured in treated untransfected controls was interestingly not significantly different than that measured in air, despite the fact that during hypoxic prodrug exposure CD protein levels would have dramatically increased.
Figure 6-21 The effect of hypoxic prodrug treatment on apoptosis induction levels acutely hypoxic 22RV1 transfectants was compared. The magnitude of apoptosis induction mediated by the vectors in response to hypoxic prodrug treatment was not statistically increased. Bars, SE; N=3.

In DU145 cells, the experiments lead to similar results. The increased sensitisation of HRE transfectants indicated in MTT assays (Figure 6-22, Table 6-3) was initially confirmed with a significant increase in the total fraction of apoptotic cells in response to hypoxic prodrug exposure (1mM). However, when relative to that detected in the treated wild type controls, the hypoxic to normoxic difference in fold increase in apoptosis was not statistically significant (p>0.05).
Figure 6-22 DU145 cells were transfected with both HRE vectors and treated according to $OP_{hypoxia}$. The viability of the cells was assessed with an MTT assay and % growth was calculated. Bars, SE; N=3, *p<0.05.

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Table 6-3 The Hypoxic enhancement ratio associated with sensitivity of transfected DU145 to $OP$ or $OP_{hypoxia}$ were calculated in terms of both growth inhibition (HER$_{50}$) and cytotoxicity (HER$_{50}$. )
Finally, although CD could not be detected in Western Blot analysis of acutely hypoxic DU145 transfectants, CD protein levels would have dramatically increased over the course of hypoxic prodrug treatment. The sensitising effect was however found to be delayed and had to be measured on day 5. Apoptosis levels were still significantly increased from 20% to 52%, 60% and 70% in pCMV-CD (p = 0.00463), pH5VCD (p = 0.016) and pH8GCD (p = 0.0129) transfectants, respectively. This corresponded to a 2.8-, 3.1- and 3.5-fold increase, when compared to wild type treated cells. An analysis of variance of the apoptosis induction levels was performed and again concluded that all three plasmid had the same apoptosis induction potential (p = 0.245).
Figure 6-24 Apoptosis induction in acutely hypoxic DU145 transfectants.

DU145 cells were transfected with pCMV-CD, pH5VCD or pH8GCD. Following acute hypoxia (4 hours), the transfectants were treated with 1 mM 5-FC in hypoxia. On day 5, the apoptotic fraction was estimated using an Annexin V-PI assay and expressed as a multiple of that of treated wild type controls (fold induction levels). (A) Up to a three fold increase in apoptosis could be recorded in acutely hypoxic DU145, in response to hypoxic prodrug treatment (1mM). (B) The difference between the plasmids was not statistically significant (ANOVA). Bars, SE; N=3.

6.5.3 LOSS OF INTER-CELL LINE VARIATION IN THE KILLING EFFECT OF THE VECTORS IN HYPOXIA

To test whether prodrug sensitisation was still statistically increased in 22Rv1 cells compared to other prostate cancer cell lines, MTT experiments were reproduced in hypoxia DU145, LnCaP and RC58/T cells. The results are shown on Figure 6-25 and Figure 6-26. (The % growth of LnCaP and RC58/T untransfected controls are shown in Appendix 10.7, on Figure 10-1and Figure 10-2).
Interestingly, compared to that in air (section 6.3.3), in response to hypoxic prodrug treatment, the magnitude of killing effects of the plasmids was similar across the panel of cell lines tested.

![Graph showing % growth of a panel of prostate cancer cell lines transfected with pH5VCD and treated with increasing prodrug concentrations according to OP\textsubscript{Hypoxia}. Bars, SE; n=3.](image)

*Figure 6-25 % growth of a panel of prostate cancer cell lines transfected with pH5VCD and treated with increasing prodrug concentrations according to OP\textsubscript{Hypoxia}. Bars, SE; n=3*
Figure 6-26 % growth of a panel of prostate cancer cell lines were transfected with pHSGCD and treated according to OP\textsubscript{Hypoxia}. Bars, SE; n=3.

6.5.4 EFFECT OF HIP ON HYPOXIC PRODRUG SENSITIVITY

Hypoxia-inducible cytosine deaminase was shown to potentate apoptosis in both chronically and acutely transfectants treated with 5-FC in air. The magnitude of the apoptosis induction was found to be higher in chronic hypoxia than in acutely hypoxic cells (section 6.4.3). Since long-term hypoxia would increase CD expression levels, the effect of HIP on the induction of apoptosis in hypoxia was analyzed.

In 22Rv1 cells transfected with pCMV-CD, 5-FC induced apoptosis was increased by a factor of 2 in both acutely and chronically hypoxic cells. The difference was not statistically significant (p = 0.1495). Chronically hypoxic pH5VCD transfectants were significantly more sensitised to 5-FC than acutely hypoxic cells (p = 0.0194), despite the fact that the 5-FC concentration used in acutely hypoxic cells was 10-fold higher. The difference in the apoptosis fold
induction levels in pH8GCD transfectants was also statistically significant ($p = 0.0485$).

Figure 6-27 The magnitude of apoptosis induction of each vector associated with hypoxic prodrug treatment following either acute or chronic HIP was compared. The response of pCMV-CD and pH8GCD transfectants to hypoxic prodrug exposure was not influence by prior hypoxic status. Chronically hypoxic cells however appear to be preferentially targeted by both pH5VCD ($p = 0.0194$) and pH8GCD ($p = 0.0485$).

6.6 DISCUSSION

The interplay between the cellular responses to androgen, cytokines and oxidative stress is known to be tightly interrelated to ensure the growth and survival of cancer cells (Liotta and Kohn 2001). It thus becomes apparent that manipulation of one factor will influence the others, triggering adaptation and resistance. In this chapter, sensitisation of both androgen-independent (DU145) and androgen-sensitive (22Rv1) cell lines was extensively studied.
The limitations of MTT assays were addressed in the testing of pH5VCD and pH8GCD. 5-FC exposure has been shown to result in decreased metabolic activity and transient S-phase arrest after 96 hours (4 days) of treatment in prostate cancer cells (Corban-Wilhelm, Ehemann et al. 2004). Although a 5-FC treatment period of 4 days was chosen, the cytotoxic effect observed was confirmed with an apoptosis assay on flow cytometry. This technique was confirmed by Corban-Wilhem et al. as appropriate as it matched results obtained with acridine orange staining.

While MTT experiments did not result in apparent difference between the cell lines in response to aerobic 5-FU treatment, hypoxic DU145 cells appeared significantly more resistant than 22Rv1 to the drug at intermediate concentrations. Moreover, apoptosis experiments showed reduced total apoptosis levels (up to 2-fold) in both wild type and transfected DU145 cells, compared to 22Rv1 cells. These results confirmed increased chemoresistance of androgen-resistant cells associated with reduced apoptosis induction.

Transfection of prostate cancer cells with a suicide gene was demonstrated in this study to chemosensitise hypoxic and reoxygenated cells. The synthesis of the prodrug activation enzyme cytosine deaminase, evidenced in Chapter 5, was successfully related to the induction of growth inhibition and cytotoxicity through the induction of apoptosis in four prostate cancer cell lines in response to both hypoxic and aerobic prodrug treatment. 5-FC toxicity was completely dependent on the presence of the prodrug activation enzyme (section 6.3.1). The amount of CD available clearly correlated with prodrug sensitivity. The length of the HIP indeed dramatically influenced the response of reoxygenated HRE transfected cells to prodrug exposure. Increasing HIP from 24 to 48 hours in 22Rv1 transfectants was associated with significantly enhanced enzyme levels and resulted in up to a 5-fold decrease of EC_{50} values (section 6.3.2) in response to aerobic 5-FC treatment. The severe growth inhibition effect seen at 0.1 mM in these cells correlated with a significant increase in apoptosis levels,
when compared to treated wild type cells (section 6.4.2). CD transduction with either control or tested plasmids, resulted in a response of similar magnitude. Apoptosis levels in the cells population had indeed increased by an average of 2-fold in response to prodrug treatment. In acutely hypoxic transfectants, associated with reduced CD levels, the treatment resulted in significantly reduced apoptosis induction levels in pH5VCD transfectants. The controls, whose CD levels did not increase with HIP, were equally sensitive to the treatment.

Reproduction of the protocol across a panel of chronically hypoxic prostate cancer cell lines resulted in various levels of sensitisation (section 6.3.3). Growth arrest was achieved in all transfectants at the clinically relevant prodrug concentration of 1mM, regardless of the vector used. The response was however significantly increased in 22Rv1 cells and associated with a strong cytotoxic effect. Apart from the fact that CD protein levels were found to be detected at significantly higher levels in this cell line previously, the difference may also be due to reduced apoptosis potential in the other cell lines. Apoptosis induction was thus also analyzed in 22Rv1 and DU145 cells. In either transfectants, the number of apoptosis cells appeared maximal at Day three of prodrug treatment. The levels of apoptosis measured in DU145 cells were consistently reduced by up to a factor of 2, when compared to that of 22Rv1 cells exposed to the same treatment conditions.

The chemoresistance of advanced tumours may indeed also be due to either inhibition or reduction of the sensitivity of apoptosis pathways (Figure 6-28). The induction of apoptosis by cytotoxic drugs may be a deterministic effect, in the sense that cancer cells may be able to tolerate higher amounts of DNA damage before activating and completing the signaling cascade that leads to cell death. An important factor in this balance is the anti-apoptotic gene Bcl-2, involved in mitochondria-induced apoptosis. The near absence of this protein in testicular cells was indeed shown to predispose these cells to undergo
apoptosis even after a small amount of DNA damage (Chresta, Masters et al. 1996). Bcl-2 is positively involved in androgen-, inflammation- and hypoxia-dependent responses, via the Pim-1 protein (Figure 6-1). The 44kDa isoform of this protein was shown to disrupt p53 binding to the tyrosine kinase Etk and confer resistance to chemotherapeutic drugs in prostate cancer cells (Xie, Xu et al. 2006). Loss of p53 activity in tumour cells is indeed associated with reduced cellular apoptosis potential in response to hypoxia (Graeber, Osmanian et al. 1996). Cyclooxygenasase-2 (COX-2) overexpression, associated with prostate carcinogenesis (Fujita, Koshida et al. 2002), and targeted by novel therapeutics (Pruthi and Wallen 2005) was recently reported to participate to hypoxic resistance through inhibition of p53 activity (Liu, Kirschenbaum et al. 2005) (Figure 1-4F and Figure 6-28). Interestingly, independently of the hypoxia involvement, the administration of COX-2, PI3K/Akt and matrix metalloproteinase (MMP)-9 inhibitors during androgen deprivation were recently hypothesized to prevent the transition to androgen-independence (Miyamoto, Altwaijri et al. 2005).

In addition, translocation of the proapoptotic protein Bax to the mitochondria is suppressed during hypoxia as a result of increased concentrations of the inhibitor or apoptosis protein 2 (IAP2) by the hypoxia-induced transcription factor nuclear factor kB (NF-kB), activated by the PI3K/Akt pathway (Alvarez-Tejado, Naranjo-Suarez et al. 2001; Dong, Wang et al. 2003). In the cytosol, HIF-1α may have anti-apoptotic properties since cells with high amounts of HIF-1α are more resistant to hypoxia induced apoptosis (Unruh, Ressel et al. 2003). Finally, intracellular levels of the anti-apoptotic glycoprotein clusterin in androgen-independent PC3 cells were shown to participate in chemoresistance (Miyake, Hara et al. 2003). Although the effect of hypoxia on clusterin levels in prostate cancer cells is not reported, this glycoprotein is interestingly regulated by androgens and overexpressed in response to androgen deprivation (Cochrane, Wang et al. 2007). Moreover clusterin was proposed as a secreted marker for HIF-independent pVHL function in familial pheochromocytoma (type
2C VHL disease) (Nakamura, Abreu-e-Lima et al. 2006). These findings are again in favor of the possibility that hypoxia may be involved in the transition to androgen independence.

Because reoxygenation of chronically hypoxic cells may be very slow, the preferential sensitisation of long-term hypoxic cells should be a key feature of hypoxia-directed strategies, which has not really been addressed in previously developed HRE-directed gene therapy approaches. The sensitisation of transfectants to hypoxic prodrug treatment was thus evaluated. The sensitivity of transfectants was unexpectedly increased in hypoxia in all four cell lines tested (section 6.5.3). Cytotoxicity was evident at both 0.1 and 1 mM and associated with increased apoptosis levels in 22Rv1 and DU145 cells, respectively. When corrected for the treated controls, apoptosis induction levels were however of the same magnitude than that measured in reoxygenated transfectants (section 6.5.2), despite evident and strong hypoxic resistance to 5-FU (section 6.5.1), in both cell lines tested. These results suggest that increased sensitivity in hypoxia is not due to increased potency of the vectors.

Although an important advantage of the strategy, this inhibition of hypoxia-induced chemoresistance is difficult to explain. Increased prodrug sensitivity of wild type cells in hypoxia was already reported in chapter 4. It may however be postulated that reduced drug efflux pumps activity in hypoxia due to low ATP availability may have contributed to increased intracellular levels of CD-produced 5-FU in transfected cells, thus sensitising the cell to lower prodrug concentrations (MTT assays). This explanation must however be considered with caution. Although compelling data exist indicating an important role for Pgp in determining efficacy to chemotherapy, there are a large number of other ATP-binding cassette (ABC) transporters, whose relevance to clinical multiple drug resistance is still unknown. In this case it may be important to note the known role of ABCC5 and ABCC11 in conferring resistance to 5-FU (Pratt, Shepard et al. 2005; Oguri, Bessho et al. 2007). Specific expression patterns in
prostate cancer have not been published. In addition, the activity of thymidylate synthetase (TS), the target enzyme of 5-FU, was shown to be modulated under prolonged oxygen deprivation (Ehrnrooth, von der Maase et al. 1999). The effect of hypoxia on TS however appeared to be dependent on cell type and could result in both up- and down-regulation of this gene. Upregulation of TS in tumours was associated with poorer response to 5-FU (Horikoshi, Danenberg et al. 1992; Johnston, Lenz et al. 1995). In prostate cancer specimen, TS expression levels correlated with differentiated histology (Miyoshi, Uemura et al. 2005). P53 was also reported to inhibit TS promoter activity. This inhibition was reduced with the use of a mutated form of the p53 gene (Lee, Chen et al. 1997). Both 22Rv1 and DU145 cells would thus be intrinsically resistant to 5-FU and if TS downregulation was to occur in hypoxia, the effect would be seen in both wild type and transfected cells, which was not the case. Interestingly, p53 wild type LnCaP cells were not among the most sensitive cells. Finally, TS enzyme levels have been associated with cellular proliferation and the non-cycling nature of hypoxic cells may also protect them from 5-FU (Pestalozzi, McGinn et al. 1995).

Inhibition of hypoxia-induced chemoresistance by the CD/5-FC system may be nonetheless be explained by the apoptosis pathways involved (Figure 6-28). While the cellular pharmacology of 5-FC has been extensively studied, little indeed is know about the mechanisms of how suicide gene approaches actually kill cancer cells. In contrast to extracellular-response of the death receptor, the CD/5-FC system was recently shown to induce apoptosis intracellularly through the mitochondrial pathway, which is more prominent for apoptosis induced upon DNA damage. In a recent study involving glioma cells, Prodrug treatment was indeed associated with decline of the anti-apoptotic Bcl-2 protein levels and mitochondrial membrane depolymerization (Fischer, Steffens et al. 2005).

Interestingly, cancer cells are often resistant to mitochondrial apoptosis, a phenomenon accentuated in hypoxic cells through upregulation of Bcl-2 and
ROS formation (Kroemer 2006). It thus seems that the CD/5-FC system may be able to re-sensitise hypoxic cells to the mitochondrial apoptosis pathway. This hypothesis would thus account for the higher levels of apoptosis induced in wild type and transfected prostate cancer cells in response to both aerobic and hypoxic prodrug exposure.

Figure 6-28 Simplified representation of extrinsic and intrinsic apoptotic pathways. Increased chemoresistance in hypoxia may be due to inhibition of mitochondria-induced apoptosis through increased Bcl-2 levels and increased IAPs levels. Moreover loss or inhibition of p53 by Pim-1 and COX-2 participates with HIF-1α to the inhibition of apoptosis. The CD/5-FC system may restore mitochondrial apoptosis inhibition by downregulating Bcl-2. Adapted from Sigma-Aldrich.

In conclusion, the CD/5-FC system can be used to selectively sensitise HIF-1α expressing prostate cancer cells using hypoxia-inducible promoters. The levels of sensitisation correlate with the amount of cytosine deaminase available to the cell, with no background toxicity associated with prodrug exposure in both normoxic wild type and transfected cells. The technical difficulty of inducing
sufficient transgene protein associated with gene therapy strategies was overcome in this approach. The treatment effect in cells transfected with the test plasmids (pHSVCD, pH8GCD) was indeed consistently similar to that obtained with the control vector. While androgen-independent cells appeared more resistant to the technique, apoptosis induction levels were of the same magnitude as that measured in androgen-dependent cells. Hypoxia rapidly induced strong chemoresistance in wild type cells treated with 5-FU. The associated hypoxic enhancement ratio was of 11- and 5-fold in 22Rv1 and DU145, respectively was abolished when the cells were transfected with either vector. Chronically hypoxic transfectants were even sensitized to lower prodrug concentrations in hypoxia, regardless of androgen-dependence. The associated magnitude of apoptosis induction was similar to that obtained with the control vector. This suicide gene therapy strategy has thus the potential to preferentially sensitize the most therapeutically resistant cell population in prostate tumours, and may improve the therapeutic ratio of conventional treatment modalities.
CHAPTER SEVEN: THE CD/5-FC SYSTEM SENSITISES REOXYGENATED PROSTATE CANCER CELLS TO IONISING RADIATION
7.1 INTRODUCTION

Ionising radiation is widely used as an alternative to invasive surgery early in the therapy of prostate cancer. In recent years, radiotherapy departments have seen a large improvement in the equipment and treatment planning techniques, (i.e conformal radiotherapy and intensity-modulated radiation therapy), translating into improved targeting of dose delivery to the tumour bed whilst sparing the surrounding normal tissues. The success rate of radiation therapy is however limited. As a result, recent radiological and radiobiological research has focused on the analysis of molecular mechanisms of radioresistance and the potential for molecular enhancement of radiation effect.

The interaction of ionising radiation with biological matter results in direct, indirect, subsequent and non-targeted effects (Bystander effect). Incoming electron tracks may indeed induce damage directly or via the production of reactive oxygen species (ROS) through interaction with cellular water. It is estimated that up to 70% of all damage occurs as a result of these indirect effects. Damage to DNA has long been considered the major initiator of cellular responses to ionising radiation. Recently, damage to other cellular components (i.e. cell membrane, mitochondria) has been also proposed to initiate or modify stress signalling in response to ionising radiation exposure (subsequent effects). The cellular response to ionising radiation involves complex signalling pathways, which activate cell cycle checkpoints, DNA repair and apoptosis. Radiation-induced alterations in cellular RNA and protein levels may be seen to occur in two distinct phases (for review see (Chastel, Jiricny et al. 2004). The earliest nuclear targets, which are induced in the absence of de novo protein synthesis, are referred to as the immediate-early genes. These family of genes includes mainly transcription factors, such as cFOS, s-JUN, CREB, AP-1, NFkB and ERG-1, whose gene products may participate to subsequent events by binding to specific promoter elements of late response genes. These
downstream genes encode cytokines (i.e. IL6) and growth factors (i.e. TNF-α, TGF-β) involved in the rescue of the tissue from radiation-induced damage.

Ionising radiation induces a large variety of DNA lesions, but double strand breaks (DSB) are considered to be the most critical change (Kumar, Coltas et al. 2007). Although cells can adapt to low levels of irreparable damage, one DSB can be sufficient to kill a cell if located in an essential gene or if it triggers apoptosis (Rich, Allen et al. 2000). In response to DNA damage, the induction of sensors proteins such as RAD1, RAD9 and RAD17 triggers the Ataxia Telangiectasia mutated (ATM) gene to phosphorylate some target proteins, such as CHK1/CHK2 and p53 (Niu, Wright et al. 2002; Bakkenist and Kastan 2003; Pollycove and Feinendegen 2003). Stabilisation of p53 through inhibition of MDM2 binding by ATM, is a determinant event in regards to cell survival. Induction of p53 triggers G1 arrest through the activation of p21\textsuperscript{waf1} (Grimberg, Coleman et al. 2005), and G2 arrest through its downstream effector proteins 14-3-3σ and GADD45 (Hermeking, Lengauer et al. 1997; Wang, Zhan et al. 1999) to allow for DNA repair. Moreover, p53 appears to be linked to both principal DNA repair pathways, non-homologous and homologous recombination, through interaction with RAD51 and BCRA1 (Sturzbecher, Donzelmann et al. 1996; Jongmans, Vuillaume et al. 1998). While ionising radiation may delay the cell cycle, it will not stop it and damaged cells eventually will undergo the mitotic process. The main cause of radiation-induced cell kill is indeed due to mitotic cell death through blockage of the mitotic process by structural chromosomal damage (i.e. dicentrics, centric rings) formed as a result of unrepaired DNA breaks. Slowly proliferating tissues, such as prostate tumours, are thus intrinsically radiation resistant and associated with a longer radioresponse.

Basic radiobiological research has highlighted over the years a number of factors other that mitotic activity influencing cellular response to ionising radiation. Of all modifying factors, oxygenation is the most important. Hypoxic
cells are up to three times more resistant to x-rays than normoxic cells. Oxygen interacts with radiation-induced free radicals to form stable RO$_2$ molecules, preventing repair and compromising survival (Oxygen fixation hypothesis). The Oxygen Enhancement Ratio (Fricke, Machtens et al.) is relatively small at the daily dose fractions clinically used (2Gy). Hypoxic radioresistance may thus not be explained by the oxygen fixation hypothesis but through the molecular mechanisms involved in the stress response. At the low daily doses routinely used in radiation therapy delivery, the induction of DSB may indeed be limited and apoptosis induction may become critical to therapeutic success. Cells with apoptosis-resistant phenotypes were indeed associated with a broad radiation survival curve (Zhivotovsky, Joseph et al. 1999). The induction of apoptosis in response to cellular stress may also contribute to radiobiological effects at low doses and participate to the dose-dependent low-dose hypersensitivity effect recently described (Robson, Joiner et al. 1999). Moreover, the high OER described at intermediate radiation doses may be conserved at low doses, through hypoxia-induced apoptosis resistance.

The molecular pathways induced in response to ionising radiation exposure are in some ways similar to that triggered by hypoxia. Normoxic HIF-1α stabilisation in response to ionising radiation exposure may therefore be triggered. Exposure of cells to ionising radiation slows the rate of degradation of substrates through the proteasome. Because the 26S proteasome degrades most short-lived cellular proteins, such as HIF-1α and p53, changes in its activity might significantly, and selectively, alter the life span of many signaling proteins and play a role in promoting the biological consequences of radiation exposure, such as cell cycle arrest, DNA repair, and apoptosis (McBride, Iwamoto et al. 2003; Pervan, Iwamoto et al. 2005). Moreover the induction of p53, RAS and the PI3K pathway by ionising radiation (Dent, Yacoub et al. 2003; Pollycove and Feinendegen 2003; Zhan and Han 2004), as well as radiation-induced ROS may induce normoxic HIF-1α stabilisation or increase HIF-1α levels in hypoxia. The induction of HIF-1α by radiation is poorly documented. In a recent study,
the combination of hypoxic treatment (16 hours, 1% oxygen) with a radiation dose of 5Gy resulted in higher HIF protein levels in HCT116 colorectal cells, whereas in DU145 cells HIF-2α appeared more responsive than HIF-1α to the treatment conditions used (Chadderton, Cowen et al. 2005), showing that induction is possible.

While the hypoxic inducibility of HRE sequences has been widely studied, their radio-enhancibility remains poorly documented. Transfection with a HRE directed plasmid resulted in the amplification of transgene expression in all the cell lines tested by Chadderton et al. but radiation alone had little effect. However, HREs of PGK-1 and VEGF origin were reported to be induced in response to radiation (5Gy) in MCF-7 breast carcinoma cells (2.4 and 1.7 fold, respectively) and T24 bladder carcinoma (1.7 and 1.3 fold, respectively) (Greco, Marples et al. 2002). These results suggest the induction of HREs by ionising radiation to be cell-type specific. Nonetheless, activation of HIF-1α and HRE induction remains possible in response to radiation injury. Although radiation-responsiveness would be a bonus to the strategy, targeting hypoxic cells with the CD/5-FC system may nonetheless improve the radiosensitivity of oxic, reoxygenated and hypoxic tumour cell populations.

7.2 AIMS AND METHODS

Ionising radiation has the potential to be used in combination with the HRE/CD/5-FC system to either sustain CD expression levels or improve prodrug treatment outcome. The effect of the experimental conditions on wild type cells was discussed in chapter 4 and was thus not mentioned here.

1. In the first part of the study, ionising radiation was used as an inducer of gene expression. The response of HREs to ionising radiation stimulation was assessed with Western Blots in three prostate cancer cell lines (DU145,
22Rv1, LnCaP). Transfectants were challenged with single doses of 1 and 2 Gy and harvested twenty-four hours later.

2. In the second part of the study, ionising radiation was used during 5-FC treatment to exploit the radiosensitising effect of 5-FU and improve treatment efficiency. Because the S phase is radiation resistant, it would have been expected for the addition of ionising radiation during 5-FC treatment to result in underestimation of the additive effect. While MTT assays were initially used, because in colony-forming assays, survival is assessed up to two weeks after treatment, this assay appeared as the most appropriate for the estimation of sensitivity.

- The effect of addition of a single dose of 2Gy during aerobic prodrug treatment was initially tested in combination with the Optimal Protocol (OP) described in Chapter 6. Following a hypoxic induction period of 48 hours, 22Rv1 and DU145 transfectants were treated with 5-FC (0.1, 1, 5mM) for 4 days. Ionising radiation was delivered on day 1 of prodrug treatment.

- The effect of the same total radiation dose delivered as two equal fractions twenty-four hours apart on survival was finally studied.

**7.3 Effect of radiation alone on HRE-transfectants**

**7.3.1 HRES were radiation responsive**

The potential of using ionising radiation to induce cytosine deaminase gene expression was evaluated in 22Rv1, DU145 and LnCaP cells. Transient transfectants were administered single radiation doses of 1Gy or 2Gy twenty-four hours prior to harvest. The cytosine deaminase protein was detected with Western Blotting. Levels of induction were compared to that of unirradiated controls. The results are shown on Figure 7-1.
The induction of HREs was sequence and cell-type specific. Radiation alone had no effect on pH5VCD mediated cytosine deaminase expression in all cell lines tested. DU145 transfectants (pH5VCD or pH8GCD) did not respond to ionising radiation exposure.

CD synthesis was induced following single radiation doses in both 22Rv1 and LnCaP cells transfected with pH8GCD. In 22Rv1 pH8GCD transfectants, induction levels were relatively weak at 1.2- and 1.1-fold after 1Gy and 2Gy, respectively. The response of LnCaP pH8GCD transfectants was increased with radiation dose. CD expression was enhanced by 1.6- and 2.2-fold in response to 1Gy and 2Gy, respectively.

![Figure 7-1](image)

**Figure 7-1** The effect of radiation alone on HREs induction was tested in a panel of prostate cancer cell lines (DU145, 22Rv1, LnCaP).

(A) Representative GFP and β-actin immunoblots. (B) Quantitative analysis of the radiation response of the vectors in 22Rv1 and LnCaP transfectants. Bars, SE; N=3.
7.3.2 HRE-MEDIATED CD EXPRESSION RESULTED IN REDUCED SURVIVING FRACTION IN 5-FC TREATED PH8GCD 22RV1 TRANSFECTANTS.

In order to test whether CD protein levels detected in pH8GCD transfected 22Rv1 cells in response to a single 2Gy dose fraction would be sufficient to sensitize cells to 5-FC, the surviving fraction of the transfectants was evaluated with clonogenic assays.

At 24 hours after transfection, the transfectants received a single radiation dose of 2Gy. Twenty-four hours later, the cells were treated with 5-FC for 4 days. Clonogenic survival was measured two weeks later and the surviving fraction was calculated. The results are shown on Figure 7-2.

While both wild type and pH5V transfectants were insensitive to 5-FC, the delivery of a single 2Gy fraction was sufficient to reduce the surviving fraction of PH8GCD transfectants to 14±3%, in response to 1 mM 5-FC exposure.
Figure 7-2 Transfection with pHSGCD and treatment with 5-FC (4-days) enhanced the radiosensitivity (2Gy) of 22Rv1 cells. Bars, SE; N=3; *p<0.05.

7.4 MODULATION OF PRODRUG SENSITIVITY BY SINGLE DOSES OF IONISING RADIATION

7.4.1 IONISING RADIATION SENSITISED 22Rv1 TRANSFECTANTS TO LOWER 5-FC CONCENTRATIONS

The effect of the addition of ionising radiation during 5-FC treatment was initially evaluated in 22Rv1 transfectants using a MTT assay. Following a hypoxic induction period of 48 hours, 22Rv1 transfectants were incubated in air with increasing prodrug concentrations (0.1, 1 and 5mM) for 4 days. Ionising radiation in the form of a single 2 Gy fraction was added on day 1 of 5-FC treatment. The benefit of the combined protocol was evaluated with the calculation of the Radiation Therapeutic Ratio, ratio of the % growth of combined to single treatment protocol for each prodrug concentration. The results are shown on Figure 7-3.
The combination protocol dramatically increased the 5-FC sensitivity of both transfectants. In both 22Rv1 transfectants, the difference in % growth was statistically significant at a 5-FC concentration of 0.1mM. The associated Radiation Therapeutic Ratios were 1.7 and 2.1 in pH5VCD and pH8GCD transfectants, respectively. While no enhancement was observed at 1mM, the difference may have been below the limits of sensitivity of the assay.

Since the difference in protocols is most evident at 0.1mM, their effect on % growth was compared with a balanced analysis of variance (ANOVA). The null hypothesis initially tested was that the % growth is not influenced by the plasmid and the treatment protocol used (oxic 5-FC, hypoxic 5-FC, and addition of radiation). The ANOVA table is shown on Table 7-3. The p values associated with the analysis suggest there is (1) no plasmid effect ($p = 0.117$), (2) a treatment effect ($p = 0.019$) and (3) no association between plasmid and treatment ($p = 0.856$). From this analysis it may be concluded that there are no superiority of pH5VCD over pH8GCD, that the choice of one plasmid does not influence the protocol used and that one of the treatments used has a significant impact of % growth.
Figure 7-3 The delivery of a single radiation dose (2Gy) reduced the sensitivity of both pH5VCD (A) and pH8GCD (B) 22Rv1 transfectants. The calculated % growth of the reoxygenated transfectant cell lines 22Rv1 in response to 5-FC treatment (4 days) is presented. % growth was significantly reduced in cells treated with 0.1 mM 5-FC. Bars, SE; N=3; *p<0.05.
22Rv1 ANOVA table (MTT)

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Analysis of Variance for % growth

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$S = 25.6645 \quad R^2 = 54.50\% \quad R^2(adj) = 35.54\%$

Table 7-1 Two factors were tested in this ANOVA table. The plasmid effect has two levels: pHSVCD or pHGGCD.

The effect of three treatments was tested: hypoxic 5-FC, oxic 5-FC and radiation. The null hypothesis was that (1) the choice of plasmid does not influence the % growth; (2) there is no difference between the % growth associated with hypoxic, oxic or radiation associated prodrug exposure and (3) there is no interaction between the two effects tested.

The analysis was repeated excluding the oxic 5-FC data set to further study the positive treatment effect observed previously. The ANOVA table is shown on Table 7-2. The associated p values suggest that there was still no plasmid effect ($p = 0.109$). There was not sufficient evidence to reject the null hypothesis that the difference in the % growth associated with hypoxic 5-FC and radiation is not statistically significant ($p = 0.532$). There was finally no interaction between plasmid and protocols. The poor R-Sq associated with this analysis could be due to the relatively small datasets used.
Table 7-2 In this ANOVA table the simultaneous effect of plasmids and treatments (hypoxic 5-FC, radiation) was studied.
The null hypothesis was (1) there difference in the % growth associated with each plasmid is not statistically significant; (2) there is no difference in the % growth associated with radiation and hypoxic 5-FC is not statistically significant and (3) there is no interaction between the two factors tested.

These results suggest that through the addition of a single 2Gy dose fraction, the sensitivity of 22Rv1 cells transfected with either pH5VCD or pH8GCD to low prodrug concentrations can be significantly increased. Moreover, the levels of cytotoxicity were comparable to that previously achieved with hypoxic 5-FC treatment.

7.4.2 IONISING RADIATION (SINGLE DOSE) SIGNIFICANTLY DECREASED THE SURVIVING FRACTION OF REOXYGENATED PROSTATE CANCER CELLS

To assess the potential of combining ionising radiation with hypoxia-inducible cytosine deaminase, the reproductive potential of 22Rv1 and DU145 wild type and transfected cells was tested with clonogenic assays. The cells were
chronically preconditioned in hypoxia and reoxygenated in the presence of 5-FC (0.1, 1 mM) for four days. A single dose of ionising radiation (2 Gy) was delivered on Day 1 of prodrug treatment. The results are shown on Figure 7-4 and Figure 7-5. The response of unirradiated reoxygenate cells, discussed in section 6.3.3 (Figure 6-5), is presented as a control.

Despite high levels of survival (65% ± 10%), both wild type cell lines were more sensitive to the combined treatment than with either modality alone, but the HRE/CD/5-FC further enhanced the radiosensitivity of reoxygenated 22Rv1 and DU145 cells. In pH5VCD transduced 22Rv1 cells, radiosensitivity was reduced from 70±3% to 25±6% (0.1 mM, p=0.01) and 12±6% (1 mM, p<0.001), respectively. The radiosensitivity of pH8VCD transfectants was also significantly reduced, compared to wild type cells (26±5% at 0.1 mM, p=0.02; 13±5% at 1 mM, p<0.001) (Figure 7-4). In pH5VCD DU145 transfected cells, the SF2 was decreased from 67±2% to 25±6% at 0.1 mM (p=0.009) and 9±3% at 1 mM 5-FC (p=0.0001). pH8GCD transfectants were further sensitised to the combined treatment by a factor of 2.5 at 0.1 mM (p=0.009) and 2 at 1 mM (p=0.02) (Figure 7-5).
Figure 7-4. Effect of combined treatment protocol on the radiosensitivity of 22Rv1 transfectants. (A) The addition of a 2Gy during prodrug treatment significantly decreased the surviving fraction of pH5VCD transfectants at all 5-FC concentrations tested. (B) pH8GCD transfectants were more resistant to the combined treatment. Bars, SE; N=3, *p<0.05.
Figure 7-5 Effect of combined treatment protocol on the surviving fraction of (A) pH5VCD and (B) pH8GCD DU145 transfectants. Bars, SE; N=3, *p<0.05.

The data was statistically analyzed with an ANOVA table to simultaneously test the effect of the cell lines and transfection on the surviving fraction. The results are show on Table 7-3. The p value associated with the ANOVA table lead to the conclusion that there was a transfection effect (p = 0.000) but no cell line effect (p = 0.709). Moreover, there was no evidence of an interaction between
cell line and transfection (p = 0.503). The associated R-sq table is relatively high (82.34%), showing that the model suited the data set.

<table>
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Analysis of Variance for SF

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S = 9.67299 R^2 = 82.34% R^2(adj) = 78.66%

Table 7-3 22Rv1 clonogenic assays ANOVA table.
The null hypothesis tested were that, at 1mM 5-FC), (1) there was no difference between the surviving fraction associated with each cell line; (2) there was no difference between untransfected and transfected cells and (3) there was no interactions between the two factors tested.

Next, the data was analyzed with an ANOVA table to simultaneously test the effect of the cell lines, the test plasmids and the treatments (hypoxia, combination) on the surviving fraction. The p values associated with the analysis suggested a treatment (p = 0.000) but no plasmid (p=0.479), no cell line effect (p=0516) nor interactions between the factors tested (p=0.696). The R-sq value was relatively acceptable.
Table 7-4 Clonogenic assays ANOVA table (1mM 5-FC).

The null hypothesis tested were that, in response to 1mM 5-FC, (1) there was no difference between the surviving fraction associated with the cell line; (2) there was no difference between the surviving fraction associated with each plasmid; (3) there was no difference between combined and hypoxia alone treatments and (3) there was no interactions between the factors tested.

There was sufficient evidence to conclude that both HRE/CD/5-FC systems have the ability to enhance the radiosensitivity of reoxygenated prostate cancer cells.

7.5 MODULATION OF PRODRUG SENSITIVITY WITH FRACTIONATED RADIATION DOSES

7.5.1 SENSITISATION COULD BE ACHIEVED WITH FRACTIONATED DOSES

Since ionising radiation is routinely delivered in multiple dose fractions, the effect of splitting the total dose (2Gy) in two equal daily fractions on the sensitivity of 22Rv1 transfectants was evaluated with an MTT assay.
Following a hypoxic induction period of 48 hours, 22Rv1 transfected cells were treated with 5-FC (0, 0.1 and 1mM) for four days. Dose fractions of 1Gy were delivered at a twenty-four hours interval on day 1 and 2 of prodrug treatment. The results are shown on Figure 7-6.

Figure 7-6 % growth of 22Rv1 transfectants exposed to single or fractionated radiation doses during prodrug treatment.

Delivering the total dose in equal daily dose fractions did not enhance the sensitivity of either 22Rv1 transfectants. The data set was analyzed in an ANOVA table (Table 7-5).

The levels of cytotoxicity measured in the fractionation protocols were similar to that of single doses and hypoxic prodrug treatment ($p = 0.576$), regardless of the plasmid used ($p = 0.459$). There was no interaction between the factors tested ($p = 0.086$).
Table 7-5 ANOVA table of 22Rv1 MTT results.

The null hypotheses tested were that there was no difference between the % growth associated with (1) the plasmid used and (2) the use of either fractionated, single doses and hypoxic prodrug treatment. (3) the possibility of an interaction between the factors was tested.

7.5.2 Fractionation could be used to reduce the surviving fraction of transfectants

The experiment was repeated in 22Rv1 and DU145 cells using clonogenic assays to fully assess the effect of fractionation on HRE-mediated sensitivity. The split dose recovery factor, commonly used in radiobiological studies, was calculated as the ratio of the surviving fractions of each combined protocol at a particular 5-FC concentration. The results are shown on Figure 7-7 and Figure 7-8.

The radiosensitivity of pH5VCD and pH8GCD 22Rv1 and DU145 transfectants was not further increased in response to the fractionated protocol, compared to single-dose experiments.
Figure 7-7 The prodrug sensitivity of transfected 22Rv1 cells was increased with the addition of fractionated ionising radiation. The surviving curves of transfected with pH5VCD and pH8GCD are shown on plots (A) and (B), respectively. Bars, SE; n=3.
Figure 7-8 The prodrug sensitivity of transfected DU145 cells was increased with the addition of fractionated ionising radiation. The surviving curves of transfected with pH5VCD and pH8GCD are shown on plots (A) and (B), respectively. Bars, SE; n=3.

The data sets at 1mM 5-FC were simultaneously analyzed with an ANOVA table. The factors tested were the cell line, the plasmids and the treatment protocols (single and fractionated doses). The results are shown on Table 7-6.
The cell line used had no effect on the surviving fractions ($p = 0.128$). The cell line factor did not interact with the plasmids ($p = 0.405$). However, the effect to the treatments was dependent on the cell line used ($p = 0.015$). Plasmids and treatments did not have a significant effect on survival ($p = 0.645$ and $p = 0.738$, respectively). There was no evidence of an interaction between these two factors ($p = 0.023$). Finally, there was no interaction between all three factors tested ($p = 0.246$). While the analysis suggests an effect of the cell line use on radiosensitivity, further investigation would be required due to the relatively low R-Sq value (28.92%) associated with the ANOVA table.

**ANOVA table**

**Radiosensitivity reoxygenated cells**

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Analysis of Variance for SF

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$S = 7.48749$  $R-Sq = 28.92\%$  $R-Sq(adj) = 13.37\%$

Table 7-6 ANOVA table for the simultaneous analysis of the clonogenic assays data sets in response to 1mM 5-FC. The null hypotheses tested were that the difference in the surviving fractions associated with (1) the cell line used, (2) the plasmids used and (3) the treatment protocol used, was null. The interactions between the factors were also tested.
In conclusion, fractionation did not enhance the radiosensitivity of either reoxygenated transfectants, compared to the delivery of a single dose.

7.6 Discussion

The role of oxygen on tumour radiosensitivity has long been documented, with hypoxic cells commonly up to three times more resistant to the effect of ionising radiation than normoxic cells. The effect of extreme hypoxia on cellular response was dependent on the dose-rate and the length of hypoxic exposure (Hall, Bedford et al. 1966). The sensitivity of chronically hypoxic cells has been reported as being reduced by up to 30%, when compared to acutely hypoxic cells. The survival of cell lines to chronically hypoxic culture conditions was recently reported to depend on their p53 status. Cells carrying a mutated copy of the gene were indeed more resistant to oxygen deprivation, probably because hypoxia-induced p53-mediated apoptosis was no longer taking place. The p53 mutant cultures showed increasing amount of inactive S-phase cells under hypoxic conditions. Because of loss of the p53-regulated G1 checkpoint, cells enter S phase without delay or block. Although the S phase is the most radiosensitiant phase of the cell cycle, increased sensitivity in hypoxia may be due to the fraction of arrested cells unable to complete replication. In addition, breakdown of cellular metabolism in response to oxygen deprivation makes cells more vulnerable to radiation damage. Low cellular ATP concentration indeed impairs double-strand-breaks repair (Meng, Jalali et al. 2005) and may participate to increased radiosensitivity in long-term hypoxic cells. Overall, the difference in radiation sensitivity appears to be reduced when the hypoxic condition lasts for several days (Zolzer and Streffer 2002).

Transfection of prostate cancer cells with pHSVCD and pH8GCD resulted in dramatic sensitisation to hypoxic prodrug treatment, associated with apoptosis induction (Chapter 6). Transfection of DU145 and 22Rv1 cells with either pHSVCD or pH8GCD resulted in significant sensitisation and apoptosis
induction in response of both aerobic and hypoxic prodrug treatment following hypoxic exposure. The combination of ionising radiation and hypoxic prodrug treatment was experimentally difficult due to both the already dramatically decreased surviving fraction in the single modality protocol and the technical difficulty of delivering ionising radiation in hypoxia. Hypoxic combined protocols were thus not tested. In regards to the previous findings it seems however that long-term hypoxic cells may not be the principal targets of hypoxia-directed strategies.

As opposed to chronic hypoxia, oxygenation fluctuations have been shown to progressively select for tumour cells with defects in mitochondrial apoptotic pathways and Bcl-2 overexpression (Weinmann, Jendrossek et al. 2004). The association between increased Bcl-2 expression in prostatic tumours and poor therapeutic response to radiation therapy has been widely documented (Huang, Gandour-Edwards et al. 1998; Scherr, Vaughan et al. 1999). Bcl-2 immunohistochemical overexpression in radical prostatectomy specimens was positively associated with a high risk of biochemical failure in clinically localized prostate cancer (Revelos, Petraki et al. 2005). Increased Bcl-2 levels were shown to delay the chemotherapy-dependent potentiation of TRAIL-induced apoptosis in many cancer cells, through its interference with the release of cytochrome c (Rohn, Wagenknecht et al. 2001). Recent development on the effect of reoxygenation on radiation sensitivity highlighted the induction of resistance mechanisms in response to hypoxic stress. Intermittent hypoxia was recently reported to be more potent than chronic hypoxia at increasing HIF-1α activity. Moreover, cyclic hypoxic exposure had a protective effect on endothelial cells against the killing effect of ionising radiation (Martinive, Defresne et al. 2006). As a result, HIF-1α was proposed as a marker for radiosensitivity (Moeller and Dewhirst 2004).

HREs isolated from the VEGF gene promoter were previously reported to drive reporter gene expression in an *in vitro* setting, but the radiation dose delivered
was relatively high (5Gy) (Greco, Marples et al. 2002). The responsiveness of the synthetic promoter in prostate cancer cell lines may therefore also be dose-dependent. The CD induction levels in pH8GCD transfectants (1.2 and 2.2 at 1 and 2 Gy, respectively) were similar to that reported with HRE plasmids in the literature. Irradiation of tumours grown in mice stimulated a two-fold increase in HIF protein and activity levels, associated with an increase in the expression of downstream genes such as VEGF and HRE-regulated green fluorescent protein (Moeller and Dewhirst 2004). Despite documented evidence of VEGF upregulation in response to radiation injury, irradiation of pH5VCD transfectants did not result in detectable cytosine deaminase. Moeller et al. interestingly reported that irradiation of the tumour cells in vitro did not lead to activation of HIF-1 signalling. These results suggest that although transgene induction was not evidenced in pHSVCD transfectants, it may still occur in vivo. Nonetheless, the radiosensitivity of hypoxia-naïve 22Rv1 and DU145 cells treated with HRE/CD/5-FC gene therapy was significantly increased (section 7.3.2).

Reoxygenation appeared to be the stimulus responsible for post-irradiation HIF activation in vivo and for enhanced HIF signaling in vitro. CD protein levels were indeed sustained or enhanced in reoxygenated pH5VCD and pH8GCD transfectants (chapter 5). Hypoxia/reoxygenation leads to chronic oxidative stress, associated with constant formation of ROS and nitrogen species (NO). In irradiated tumours, Moeller et al. reported that pimonidazole binding, associated with HIF-1 activated tissue, correlated with increased tumour oxygenation. HIF-1 activation was indeed evident in tissue well oxygenated 48 hours after treatment. The excess production of reactive species in reoxygenated tissues was proposed as the underlying cause for sustained HIF-1 levels. While the role of ROS was previously discussed, nitric oxide has also been reported to be involved in the HIF-1 pathway in a dose-dependent manner. At low doses, NO can downregulate mitochondrial activity. At high doses it interferes with the function of prolyl hydrolases (Callapina, Zhou et al. 2005) and participates to the amplification of oxidative stress through up
regulation of catechol estrogen levels in prostate cancer cells (Muzandu, Shaban et al. 2005).

Due to poor availability of ATP, translation mechanism were recently shown to be downregulated under hypoxic conditions (Koritzinsky, Seigneuric et al. 2005). Hypoxic exposure may thus induce a state of constant struggle between hypoxia-regulated gene expression and hypoxia-regulated translational suppression. Untranslated transcripts may as a result be temporarily stored in stress granules (Kedersha and Anderson 2002; Kedersha, Stoecklin et al. 2005; Anderson and Kedersha 2006). Reoxygenation was proposed to result in release and translation of these HIF-regulated transcripts, enhancing survival and thus reducing radiation sensitivity (Moeller, Cao et al. 2004). Although evidenced in endothelial cells, this phenomenon may be present in tumour cells. Moreover, radiation-induced reoxygenation results in the secretion of radioprotective cytokines by endothelial cells, participating to decreased radioresponse of tumour cells. Therefore, therapeutics that tend to increase tumour oxygenation may also enhance downstream HIF-1 activity and negatively impact treatment outcome. Strategies targeting HIF-1α may thus be more effective. HIF-1α siRNA technologies were indeed shown to reduce hypoxia-driven cell survival and abrogate tumour growth (Jensen, Ragel et al. 2006; Mizuno, Nagao et al. 2006). The ability of HIF-1α to indicate radiobiologically relevant levels of tumour hypoxia however seems to be cell type specific (Vordermark, Katzer et al. 2004). HRE-driven gene therapy may emerge as a more effective tool for the targeting of hypoxia scenarios. Despite the occurrence of reoxygenation-driven resistance, combined strategies may help potentate treatment outcome.

Improvement in local tumour control and control of systemic disease progression are the new challenges of gene therapy. Increasing total radiation dose to the tumour bed is currently being investigated in many cancers with the hope that higher total radiation doses may result in improved rates of local
control (e.g. IMRT). The pairing of gene therapy with radiation therapy would present many advantages (Weichselbaum, Kufe et al. 2001). Ionising radiation and gene therapy can simultaneously be used as local therapy to kill tumour cells via independent cytotoxic actions minimizing the evolution of treatment-resistant tumour cells. In addition, gene therapy products can be chosen to act synergistically with ionising radiation further improving local tumour eradication. Toxicity to normal tissue could be reduced, as lower doses of both therapies would be required when used in combination. In this chapter, the delivery of ionising radiation during prodrug treatment was studied in pH5VCD and pH8GCD reoxygenated transfectants. The combined protocols sensitised 22Rv1 transfectants to lower prodrug concentrations, to the same extend as hypoxic 5-FC exposure (section 7.4.1). Ionising radiation may indeed act as a proteasome inhibitor (McBride, Iwamoto et al. 2003) and attenuate the P-glycoprotein drug efflux pump activity to the same extend as ATP-deprivation in hypoxia (Fujita, Washio et al. 2005). Increased radiosensitivity was confirmed by clonogenic assays (section 7.4.2), with associated surviving fractions, 1 mM5-FC, of ~10±3% in irradiated, compared to ~30±8% in unirradiated transfectants (section 6.3.3).

Although apoptosis is not the main biological effect of ionising radiation, apoptosis resistance has been correlated with radiation therapy failure and proposed as an effective marker for the radioreponse of prostate tumours (Zhivotovsky, Joseph et al. 1999; Szostak and Kyprianou 2000; Wang, Reed et al. 2004). Differential sensitivity to apoptosis may thus govern the radioreponse of cells. Androgen-independent DU145 cells were indeed previously found more resistant to apoptosis (chapter 6), which correlates here with reduced sensitivity to combined protocols.

Human prostate cancer cells undergo apoptosis in response to androgen ablation, chemotherapeutic agents and ionising radiation. The apoptotic process is a complex cascade with activators and inhibitors requiring the correct
balance of signals and stimuli to achieve the required effect. Apoptosis occurs through two interrelated extrinsic and intrinsic pathways that converge to a final common pathway involving the activation of a cascade of caspases (Okada and Mak 2004; Ghobrial, Witzig et al. 2005). Prostate cancer cells, both androgen-dependent and androgen-independent, contain intact cell death programs. The failure to initiate apoptosis is influenced by molecular alterations that block the apoptotic pathways at various levels. The effect of ionising radiation on apoptosis induction is diverse (Figure 7-9) and seems to mimics that of hypoxia. The biological effect of ionising radiation may thus either work in conjunction or against hypoxia-induced apoptosis resistance.

Figure 7-9 Effect of ionising radiation on apoptosis pathways. Although death following radiation exposure in mainly mitotic, resistance to radiation-induced apoptosis is associated with radiation therapy failure in prostate cancer. Radiation injury regenerates a state of struggle between pro- and anti-apoptosis signal inductions. Ionising radiation seems to work in synergy with hypoxic response toward therapeutic resistance.
The effect of radiation injury on signalling pathways is complex and involves the simulation of both survival and death responses. Radiation-induced apoptosis has been correlated with wild type p53 status in a variety of tissues. Activation of the ATM protein in response to DNA damage results in the stabilisation of p53 through the inhibition of mdm2 activity, leading to cell cycle arrest and DNA repair, or apoptosis. Down-regulation of ATM was associated with increased sensitivity to radiation-induced apoptosis in both p53 mutated and p53 wild type prostate cancer cells (Truman, Gueven et al. 2005). Wild type p53 was also correlated with both radio sensitive and radioresistant phenotypes (McIlwrath, Vasey et al. 1994; Servomaa, Kiuru et al. 1996; Meng, Jalali et al. 2005). In prostate cancer, patients with abnormal p53 immunoreactivity were associated with radiation failure (Rakozy, Grignon et al. 1999). However functional p53 was also reported to participate in increased survival of prostate cancer cells in response to fractionated irradiation (Marple, Greco et al. 2003) and as a result is proposed to be a cause of treatment failure. There results suggest the involvement of other actors in molecular radio responses.

The rates of protein degradation by proteasomes are modulated by radiation, hydrogen peroxide or cytokines. The inhibition of proteasomal activity in prostate cancer cells resulted in enhanced radiosensitivity (Warren, Grimes et al. 2006). Activation of NFkB in response to a large variety of stimuli including ionising radiation is associated with treatment resistance (Mayo and Baldwin 2000) and prostate cancer transition to androgen-independence (Chen and Sawyers 2002). NFkB is constitutively activated in DU145 cells and may account for their apoptosis resistance. This factor can indeed increase the expression of anti-apoptotic proteins, such as IAP2 and Bcl-2 and inhibit the ability of p53 to stimulate gene expression (Wang, Mayo et al. 1998; Wang, Guttridge et al. 1999). Moreover, NFkB competes with the androgen receptor for the transcriptional coactivator p300/CREB. Activation of NFkB is also mediated by PI3K and RAS (Romashkova and Makarov 1999), suggesting a cooperative effort of hypoxia and radiation towards therapeutic resistance.
Downregulation of NFkB activity has emerged as a novel therapeutic approach (Bharti and Aggarwal 2002). Activation of RAS, RAF-1, MAPK, p38 and PI3K pathways was also associated with radioresistance (Dent, Yacoub et al. 2003) and may account to hypoxia-induced surviving advantages through direct inhibition of apoptosis or via enhanced HIF-1α activity (McKenna and Muschel 2003; McKenna, Muschel et al. 2003). Although not documented, the PIM protein may also play a role in cellular radioresponse. Ionising radiation thus appears to work with hypoxia to enhance therapeutic resistance, through apoptosis inhibition. In fact, apoptosis induction was inhibited in irradiated human leukemia cells lines exposed to hypoxia after irradiation (Hamasu, Inanami et al. 2005). Strategies restoring the sensitivity of tumour cells to programmed cell death thus seem a required feature of novel combined treatments.

Dose fractionation, or split dose effect has important radiobiological effect on cellular radioresponse. The delivery of a radiation dose in two equal split fractions has opposite consequences on cell survival. Allowing for time in between fraction is indeed associated with increased survival due to repair of radiation damage. On the contrary, in split dose experiments, survival may be reduced due to the redistribution of cells through the cell cycle and restoration of drug sensitivity with down-regulation of Pgp (Ryu, Um et al. 2004). The delivery of radiation as two dose fractions on day 1 and 2 of prodrug treatment did not enhance the radiosensitivity of pHSV5CD transfectants in both 22Rv1 and DU145 cells, when compared to the delivery of a single dose (section 7.5.2). This effect may be due to an insufficient interfraction time interval for effective reassortment of the cells. Interestingly however, the response of pH8GCD was not enhanced by fractionation, despite being shown to mediate transgene expression following radiation exposure in 22Rv1 cells (section 7.3).

Taxanes are the only single chemotherapeutic agent to date proven to be efficacious in prolonging overall survival in patients with prostate cancer
Despite the apparent benefit of multimodal therapy, associating hormonal deprivation with radiation therapy, disease-free survival remains limited. Although apoptosis resistance has been targeted by several methods, bcl-2 overexpression remains an important resistance factor. A recent phase II clinical trial combining oblimersen sodium, a phosphorothioate antisense oligonucleotide directed to the bcl-2 mRNA, with docetaxel in patients with hormone-refractory prostate cancer demonstrated an encouraging antitumour activity (Tolcher, Chi et al. 2005). These results are encouraging for novel approaches to downregulating bcl-2.

The administration of HRE-driven suicide gene cytosine deaminase in this study to both hormone-responsive and hormone-naïve prostate cancer cells proved efficient at inducing sensitisation to both aerobic and hypoxic prodrug treatment following hypoxic treatment. The cytotoxic effect correlated with apoptosis induction. Exposure of transfectants to 5-FC has been shown to reduce bcl-2 levels (Fischer, Steffens et al. 2005) and proposed to resensitise cells to mitochondrial apoptosis. Reoxygenation was recently shown to participate in therapeutic resistance through up regulation of HIF-1α. In our study, the radiosensitivity of reoxygenated cells was however improved through transfection with both HRE/CD plasmids and subsequent exposure to 5-FC. While hypoxic cell sensitisers have to date translated to poor clinical efficacy, these gene therapy vectors may successfully help overcome the problem of hypoxia/reoxygenation in radiation therapy.
CHAPTER EIGHT: DEVELOPING MORE EFFICIENT APPROACHES TO EXPRESSING DRUG METABOLISING ENZYME IN PROSTATE CANCER CELLS
8.1 INTRODUCTION

While gene therapy appeared in the 1990s as a “miracle cure” to all genetic disease, has yet to translate into improve patient care. The major hurdle of gene therapy remains transcriptional control and detection of transgene expression but more importantly efficient non-toxic plasmid delivery. These issues were addressed in this chapter through

1) The evaluation of a radiation-responsive promoter,
2) The inclusion of the Green Fluorescent Protein (GFP) reporter gene in the plasmids,
3) The development of adenoviruses.

While hypoxia-selective transcriptional targeting was successfully achieved through the use of HREs in prostate cancer cells, ionising radiation may also be used to control gene expression (Mezhir, Smith et al. 2006). The induction of genes by ionising radiation implies that their promoters have elements that respond to ionising radiation. Despite the successful localization of these radiation-responsive sequences, the mechanism by which genes are induced by ionising radiation remains unclear. While certain genes seem to acquire radiation responsiveness through their p-53 binding sites, similarities in other genes suggest involvement of the SP-1 transcription factor as well as the Fos-jun heterodimer Activator Protein (AP)-1. Studies on the radio-enhancibility of the promoter suggest a bell-shaped relationship both in terms of dose response and gene expression levels. While this was to be expected for transient gene expression levels, it is somehow surprising, as one might expect a linear relationship with increasing radiation doses. The optimal dose needed to achieve acceptable gene expression levels appears to differ with the promoter studied but all were found to be responsive to the clinically used 2Gy fraction doses. The peak of gene expression also varied with the promoter, but all were under 12h. However, strong cell-type dependence was evident (for review, see (Marignol, Coffey et al. 2007)).
The early growth response gene promoter (Erg1) has been the most widely studied and successfully developed in gene therapy approaches. This promoter was used to drive the expression of the tumoricidal cytokine tumour necrosis factor (TNF-α) gene, as an attempt to improve tumour control (Mezhir, Smith et al. 2006). The Egr-1 promoter was also successfully developed into a suicide gene therapy strategies involving the herpes simplex virus thymidine kinases suicide gene (HSVtk) (Joki, Nakamura et al. 1995; Scott, Marples et al. 2000; Jorgensen, Katz et al. 2001). Analysis of the p21\(^{WAF-1/CIP}\) regulatory region and immunochemistry studies revealed that expression of the p21\(^{WAF-1/CIP}\) gene was also induced by radiation-induced DNA damage in human skin through three p-53 binding sites located upstream of the transcription start site. The radiation-inducibility of the WAF-1 promoter appears thus strongly dependent upon p53, which could be a limiting factor to its use in cancer gene therapy strategies. Nonetheless, WAF-1 driven radiation-induced gene expression has been achieved at clinically relevant radiation doses in endothelial cells (9.5-fold), in rat-tail segment artery (4.5-fold) (Worthington, Robson et al. 2000) and breast cancer cells (Nenoi, Daino et al. 2006).

Human tissue plasminogen activator (t-PA) is a serine protease that transforms plasminogen into plasmine. It is involved in the cellular response to DNA damage and thus is induced by UV- and ionising radiation (Miskin and Reich 1980). In radioresistant human melanoma cells t-PA was shown to be induced over 50-fold after x-irradiation (4.5Gy) (Boothman, Lee et al. 1994). This promoter was proposed as a candidate to radiation-induced gene therapy strategies (Boothman, Lee et al. 1994) but hasn’t been exploited to date. The ability of this gene promoter to drive transgene expression in response to ionising radiation was evaluated in this study.

Prostate tumours are slow growing (Schmid, McNeal et al. 1993), with only approximatively 5% of prostatic tumour cells in the S-phase at any one time,
which may account for their poor response to conventional treatments (Visakorpi, Kallioniemi et al. 1991). Since adenoviruses affect both dividing and non-dividing cells, these vectors are valuable to prostate cancer gene therapy. Adenoviruses are medium-sized DNA viruses associated with minor infections in humans. More than 50 serotypes have been identified, but most adenoviral vectors are derived from serotype 5 (Ad5) (Kochanek 1999).

Figure 8-1 Cellular integration of adenoviruses.
Adenoviral infection begins with the interaction of the knob portion of the adenoviral fiber with the cellular receptor CAR (Coxsackie and Adenovirus Receptor). Internalisation by endocytosis is subsequently triggered by interaction of the viral penton proteins with epithelial integrins. Upon escape from the early endosome, the virus is transported to the nucleus, where the adenoviral genome remains episomal. (Contreras, Smyth et al. 2004).

The adenoviral genome consists of a single, double stranded, linear DNA molecule that codes for 11 virion proteins. Tropism of adenoviruses is mediated through the widely distributed Coxsackie and Adenovirus Receptor (CAR) (Bergelson, Cunningham et al. 1997). Upon primary attachment, internalisation
is triggered by interaction of the viral penton proteins with epithelial integrins. The adenoviral genome is packaged in a non-enveloped icosahedral protein capsid that emanates fiber coat proteins. It eventually escapes the capsid and is transported to the nucleus, where it remains episomal (Figure 8-1). The fact that the adenoviral genome is non-integrating is a great advantage to gene therapy. As a result permanent virally induced genomic damage is of little concern.

The Ad5 adenoviral genome contains two origins for DNA replication in each terminal repeat (Inverted Terminal repeat, ITR) and consists early (E1-E4) and late genes, involved mainly in viral replication and packaging, respectively. The E3 gene product, although also involved in viral replication, was associated with an immuno-modulatory role (Verma and Somia 1997) (Figure 8-2).
Figure 8-2 Schematic representation of type 5 adenovirus.

Viral gene expression is sequential beginning with the immediate early genes E1-E4 and followed by the late genes L1-L5. E1, E2 and E4 are involved in viral replication and E3 gene products play an important immuno-suppressive role. Late gene products are involved in the production of capsid and assembly proteins. The viral chromosome contains two origins of DNA replication in each terminal repeat (inverted terminal repeat, ITR). Early gene products are involved in a variety of processes, such as initiation of the S-phase, inhibition of DNA repair and initiation of viral replication. (Adapted from (Verma and Somia 1997))

Adenoviral integration relies on the expression of CAR and integrins proteins, whose production levels may be affected by tumour hypoxia. While Ad5 binding and uptake by cancer or normal cells remained unaffected, viral replication was attenuated under hypoxic conditions, through downregulation of viral protein expression. (Pipiya, Sauthoff et al. 2005; Shen and Hermiston 2005). This was however abolished when the E1A gene was placed under the transcriptional control of hypoxia-response elements in a conditionally replicating adenoviral gene therapy strategy (Cho, Seong et al. 2004).
First-generation adenoviral vectors have been designed by replacing E1A gene, crucial for viral replication, with a recombinant construct (Bett, Haddara et al. 1994). In this system, viral genes are expressed at too low levels for effective replication and packaging of the virus. The ability of replication-deficient adenoviral vectors was shown to remain unaffected by the micro environment (Shen and Hermiston 2005). Therefore, despite the emergence of the clinical potential of oncolytic viruses (for review see (Chiocca 2002)), replication deficient vectors may be more suitable for this hypoxia-directed gene therapy.

Another limiting factor in the application and optimisation of gene therapy protocols is the inability to monitor the delivery and expression of therapeutic genes in target tissues. Non-invasive imaging techniques would indeed be of considerable value in both experimental and clinical gene therapy. A variety of reporter gene have been developed (for review see (Serganova and Blasberg 2005). Reporter systems based on the expression of fluorescence proteins are for instance widely used in cell culture and are becoming more widely applied in small animal studies (Welsh and Kay 1997; Choy, Choyke et al. 2003). Therefore in the present approach, the inclusion of a reporter gene in the expression cassette, such as the Green Fluorescent Protein (GFP), will allow for easy monitoring of gene transfer and improve the scope of the vectors.

### 8.2 AIMS AND OBJECTIVES

In this chapter, the problem of transcriptional control, detection of transgene expression and delivery of the technique was addressed.

1. The potential of the tissue Plasminogen activator (tPA) gene promoter to drive the expression the Green Fluorescent Protein (GFP) reporter gene in a radiation-responsive manner was initially tested in a panel of normal (PWR-IE, RPWE1) and tumour (DU145, 22Rv1, RC58/T) prostate cell lines. This construct, ptPA-eGFP, was kindly provided by Dr. Robson (Queen's
University Belfast, Northern Ireland). The sequence of the tPA promoter is shown in Appendix 10.9.

2. Detection of transfection was made possible with the inclusion of the GFP reporter gene downstream of CD in pCMV-CD, pH0CD, pH5VCD and pH8GCD. The sequence of the IREeGFP fragment is shown in Appendix 10.8.

3. Finally, these improved vectors were cloned into the adenoviral pAdLox vector for the future production of replication-deficient adenoviruses. Both pAdLox and pS72IREeGFP were kindly provided by Dr. Southgate (Paterson Institute, Manchester, UK).

8.3 Improving control of gene expression: Evaluation of a radiation-responsive promoter

To evaluate whether the t-PA promoter could be used to sensitise prostate cancer cells, the radioresponsiveness of the promoter was measured in normal (PWR-1E, RPWE-1) and malignant (DU145, 22Rv1, RC58/T) prostate cell lines. Initially, lysates of untransfected controls were taken as negative controls for background promoter activity in unirradiated transfected cell lines. A representative Western blot is presented on Figure 8-3A. Promoter activity was evident in all unirradiated transfected cell lines (Figure 8-3B).
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Figure 8-3 The tPA promoter was induced by ionising radiation. Promoter induction in normal prostate (PWR-IE, RPWE-1) and prostate cancer (DU145, 22Rv1, RC58/T) cell lines. (A) Representative immunoblot of untransfected cell lines (B) Immunoblots of p-tPA-eGFP transfectants exposed to radiation doses of 0, 1 or 2 Gy. The β-actin immunoblots (used for normalization) are also shown.

To estimate the magnitude of GFP induction, the intensity of the GFP bands was normalised to a representative background densitometry value, measured in blots of untransfected cells. The results are shown on Figure 8-4. Induction levels were estimated at: DU145 (2.45±0.15), 22Rv1 (2.48±0.27), RC58/T (4.35±0.35), PWR-IE (3.25±0.25) and RPWE1 (4.25±0.25) cells. Statistical analysis of promoter activity across the range of unirradiated transfected cell lines tested showed significantly inter-cell line variability (ANOVA, p<0.0001). Among the malignant cell lines, background GFP expression was significantly enhanced in RC58/T cells, compared to DU145 (p = 0.0019) and 22Rv1 (p = 0.0069) cells. Promoter activity also varied in normal cell lines and appeared significantly superior in RPWE-1 compared to PWR1E cells (p = 0.0082). t-PA
induction was generally higher in normal compared to malignant cell lines (p<0.05), except for RC58/T transfectants, whose background GFP levels were superior or equivalent to PWR1E (p = 0.13) and RPWE1 (p = 0.08) cells, respectively.

Figure 8-4 The tPA promoters induced GFP protein expression in transfected normal (PWR1E, RPWE1) and tumour (DU145, 22Rv1, RC58/T) prostate cell lines. Quantitative analysis of immunoblots for background t-PA promoter activity. Columns represent intensity of the GFP band measured in unirradiated transfectants normalized to representative background density value in untransfected controls. Bars, SE; *p<0.05; n=3.

Next, the cells were exposed to single radiation doses of 1 and 2 Gy at 24 h after pt-PA-eGFP transfection. Induction of t-PA was monitored 24 h post-irradiation by immunoblotting of the cell lysates to detect GFP. Representative Western blots for each of the five transfected cell lines are shown on Figure 8-3B. To estimate the magnitude of radiation induction of the promoter, the intensity of the GFP bands in irradiated transfected cells was normalised to that of the corresponding unirradiated, transfected cells. The results are shown on
Figure 8-5. GFP levels were not significantly enhanced in irradiated DU145 cells (p = 0.6 and p = 0.07 at 1 and 2Gy, respectively). In response to a 1Gy radiation dose, GFP protein levels were significantly enhanced (2-fold) in PWR1E cells only (p = 0.0474). Following a radiation dose of 2 Gy, GFP levels were significantly increased by 2.9±0.1 fold in 22Rv1 cells (p = 0.000), 2±0.2 fold in RC58/T cells (p = 0.01), 2.35±0.05 fold in PWR-1E cells (p = 0.029) and 1.85±0.05 fold in RPWE-1 cells (p = 0.04). The response was maximal in 22Rv1 cells (ANOVA, p<0.0001).

Figure 8-5 Radiation doses of 1 and 2Gy enhanced GFP protein levels. Quantitative analysis of immunoblots for radio-induction of the promoter in transfectant cell lines. Columns represent the fold increase in the intensity of the western blot bands associated with GFP detection in irradiated transfectants normalized to that of the corresponding unirradiated transfectant cell line. Bars, SE; n=3; *p<0.05, **p< 0.0001.
8.4 Improving Monitoring of Transfection: Inclusion of IREeGFP Sequence

Constitutive expression of the Green Fluorescent Protein (GFP) may be used for visual monitoring of transfection. Inclusion of the sequence coding for the internal ribosomal entry site (IREs) upstream of the GFP sequence is necessary for independent translation of protein synthesis.

The full IREeGFP sequence (1.3Kb) was isolated from a pS72IREeGFP plasmid with a BglII/BamHI double restriction digest (Figure 8-7.A). The IREeGFP sequence contains a HindIII site (at 1091bp), which was used for the screening of resulting clones (Figure 8-7B, C).

The sequence was cloned downstream of the CD gene at the BamHI site of pCMV-CD, pH0CD, pH5VCD and pH8GCD. The newly formed vectors were screened with HindIII digest, resulting in the formation of three fragments. Digestion with this enzymes produces 3.9-, 2.35- and 2.09-Kb fragments in pCMV-CD-GFP (Figure 8-7A); 3.16-, 2.35- and 1.82-Kb in pH0CD-GFP (Figure 8-7B); 3.3-, 2.35- and 1.82-Kb in pH5VCD-GFP (Figure 8-7C) and 3.42-, 2.35- and 1.82-Kb in pH8GCD-GFP (Figure 8-7D). The vectors were subsequently sequenced (for primers sequences, see Appendix 10.1).
Figure 8-6 IREeGFP fragment.

(A) DNA gel electrophoresis. The IREeGFP fragment (1.3Kb) was isolated from pS721IREeGFP through BamHI/BglII double restriction digest. (B) DNA gel electrophoresis. The fragment contains a HindIII site to be used for clone screening. Digestion of the IREeGFP fragment results in the production of two fragment of 1.1- and 0.2-Kb. (C) Map of the IREeGFP fragment highlighting important restriction sites.
Figure 8-7 Cloning of the IREeGFP sequence into gene therapy vectors. The IREeGFP fragment was cloned at the BamHI site of pCMV-CD, pH0CD, pH5VCD and pH8GCD. Clones were screened with HindIII. The simulated and obtained DNA gel electrophoresis associated with the HindIII digestion of pCMV-CD-GFP, pH0CD-GFP, pH5VCD-GFP and pH8GCD-GFP clones are presented on panels A, B, C and D, respectively.

The maps of the GFP-containing plasmids generated are presented in Sections 11.5 to 11.8.

8.5 IMPROVING GENE DELIVERY: ADENOVIRUSES CONSTRUCTION

To improve transfection efficiency, replication-deficient adenoviruses were chosen as appropriate vectors. In this protocol, construction of the E1-substituted adenoviruses relied on the recombination between a donor virus Ψ5 to supply the viral backbone and a shuttle virus, pAdLox, containing the expression cassette (Hardy, Kitamura et al. 1997). The pAdLox vector was initially digested with XhoI and EcoRI to remove the human cytomegalovirus
major immediate early promoter sequence (usually designated as hCMV) (Figure 8-8).

Figure 8-8 Preparation of the shuttle cloning vector.
(A) Map of the original pAdlox shuttle vector. ITR, inverted terminal repeat; Ψ, packaging site; CMV, cytomegalovirus; MCS, multiple cloning site; An, polyadenylation site. (B) Simulated DNA electrophoresis gel of a BglII/HindIII and a EcoRI/XhoI double restriction digests. BglII/HindIII digest produces 3.9- and 3.2-Kb fragments. EcoRI/XhoI digest results in 3.5- and 0.66-Kb fragments. (C) pAdLox was digested with EcoRI and XhoI to remove hCMV. The digestion mix was resolved by agarose gel electrophoresis. Digestion of pAdlox with these enzymes produces 0.7 and 3.5Kb fragments.

The cloning process is illustrated for pAd.CMV-CD-GFP on Figure 8-9 and Figure 8-10. The expression cassette, consisting of the CMV-CD-GFP sequences, was isolated from the vector with a BglII and NotI double restrictions digest. Digested with a BglII and NotI, resulting in the production of two fragments of 5.55- and 4.39-Kb (Figure 8-9C). The 3.9-Kb fragment, containing the expression cassette, was gel isolated and blunt-ended.
Figure 8-9 Extraction of the expression cassette of pCMV-CD-GFP.

(A) Map of pCMV-CD-GFP showing the position of selected restriction sites. (B) Simulated DNA electrophoresis gel of HindIII and BgIII/NotI restriction digests of pCMV-CD-GFP. HindIII has three restriction sites, resulting in 3.8-, 3.6- and 2.3-Kb fragments. BgIII/NotI double digest produces two fragments at 5.5- and 4.4-Kb. (C) DNA gel electrophoresis of BgIII/NotI digestion mix of pCMV-CD-GFP (both lanes). The 5.5-Kb, encompassing the expression cassette was gel isolated.

The CMV-CD-GFP cassette was inserted at the blunt-ended cloning site of the cloning shuttle vector. The resulting clones were screened with HindIII. This enzyme indeed has one restriction site in pAdloxs but two in pAd.CMV-CD-GFP, resulting in the production of two fragments at 3.7- and 1.87-Kb (Figure 8-10B,C). Correct orientation of the expression cassette was confirmed with a BgIII and HindIII double restriction digest, resulting in three fragments of 3.7-, 1.11- and 0.76-Kb (Figure 8-10C, D).
Figure 8-10 pAd.CMV-CD-GFP vector.
(A) Map of the vector highlighting the restriction enzymes used for screening. (B) Simulated DNA gel electrophoresis of pAd.CMV-CD-GFP digested with HindIII or HindIII/BglII. (C) DNA gel electrophoresis of HindIII digest. (D) DNA gel electrophoresis of HindIII/BglIII restriction digest, confirming correct orientation of the expression cassette.

The procedure is to be repeated with pH0CD-GFP, pH5VCD-GFP and pH8GCD-GFP. The maps are presented in Sections 11.9 o 11.11.

The method of production of the recombinant vectors is illustrated on Figure 8-11 and involves Cre/loxP recombination. Both ß5 and modified pAdlox are initially to be cotransfected in the Cre recombinase -expressing cell line CRE8. Initially, the enzyme will catalyse recombination between the two loxP sites in ß5, removing the packaging site from the virus (Figure 8-11A). Such a deletion renders the affected viral chromosome unusable for packaging but leaves all other viral functions intact. Recombination between ß5 and modified pAdlox will subsequently catalyse by Cre recombinase, leading to the transfer of the
recombinant genes into Ψ5 (Figure 8-11B). The resulting recombinant virus will carry a single loxP site and as a result has enhanced growth advantage over Ψ5. Over 90% of the Ψ5 molecules are indeed recombined in CRE8 cells (Hardy, Kitamura et al. 1997). In co-transfected CRE8 cells, this growth advantage generates virus stocks comprised predominantly of the recombinant virus. The virus may finally be harvested through lysis of transfected CRE8 cells.

Figure 8-11 Production of pAd-CMV-CD-GFP through Cre/lox recombination. (A) Cre recombinase catalyses recombination between the two loxP sites in Ψ5 and removes the packaging site (Ψ) from the virus. (B) Through recombination between the loxP site of pAd.CMV-CD-GFP and the remaining loxP site of Ψ-deleted Ψ5, the E1-subsituted virus carrying the CMV-CD-GFP expression cassette is produced. (Hardy, Kitamura et al. 1997)
8.6 DISCUSSION

Gene therapy targeted to the radiation field appears as an attractive approach for improving the outcome of conventional radiation therapy treatment. The technique has been proven to be selective and responsive to clinically relevant radiation doses both \textit{in vivo} and \textit{in vitro}. As well as acting as an externally controllable switch to therapeutic gene expression, radiation has been shown to facilitate the efficacy of gene therapy at several levels. Ionising radiation was reported to improve tumour blood flow and oxygenation as it has been shown to restore the NO-mediated vaso-relaxation property in tumour blood vessels, facilitating the delivery of gene therapy and associated prodrugs (Sonveaux, Frerart et al. 2007). Radiation was also reported to enhance the integration capacity of adenoviral vectors (Zhang and Stevens 1998), the oncolytic activity of viruses (Jorgensen, Katz et al. 2001; Chung, Advani et al. 2002; Lichtenstein, Toth et al. 2004) and the transfection efficiency of liposome-mediated gene delivery (Lu, Li et al. 2002). In addition, radiation could have immunostimulatory effects (Matzinger 1994; Truman, Gueven et al. 2005), suggesting that it could improve the efficacy of immunogenic therapies and the bystander effects of suicide gene therapy strategies. Radiogenic therapy could thus help overcome some limitations associated with gene therapy as well as improving local control of radiation-resistant tumours that have a high incidence of local failure.

To date, a number of neoadjuvant therapies have been used in high-risk localized prostate cancer patients (Gleason score 7, PSA > 10ng/mL) and proved feasible. Neoadjuvant gene therapy so far shows promise. For instance, administration of adenoviral p53 injection before prostatectomy resulted in biochemical-free disease in 46% of patients after a median follow-up of 42 month (Pisters, Pettaway et al. 2004). The combination of \textit{in situ} gene therapy with radiation therapy also showed promise (Tetzlaff, Teh et al. 2006).

The induction of early response by ionising radiation involves the expression of immediate early genes that code for transcription factors (i.e. \textit{Fos}, \textit{Jun}, AP-1, ...
NFkB) (Weichselbaum, Hallahan et al. 1994) as well as cytokines and growth factors (i.e. early growth factor-1 (Egr-1)) (Hallahan, Sukhatme et al. 1991). These genes can be seen as the first elements of the cascade of events leading to cellular response to radiation injury. The response of these genes to ionising radiation was shown to be variable and cell type-dependent (Hamdi, Kool et al. 2005). Activated immediate early genes interact with the promoter region of so-called late responding genes, coding for cytokines, involved in the inflammation process (i.e. Interleukin-6) (Beetz, Peter et al. 2000) and growth factors, involved in cell survival and tissue recovery (i.e. TNF-α (Blalock, Weinstein-Oppenheimer et al. 1999), TGF-B1 (Martin, Vozenin et al. 1997), PCGF-α (Witte, Fuks et al. 1989)). Many of the genes induced by ionising radiation are related to cellular fate following DNA damage. This includes genes involved in cell cycle checkpoints, DNA repair, cellular stress and apoptosis.

Despite being proposed as a candidate for radiation-induced gene therapy strategies, the tPA promoter has not yet been exploited (Boothman, Lee et al. 1994). In this study, the ability of this promoter to induce the expression of the GFP reporter gene in response to ionising radiation was evaluated in both normal (PWR-1E, RPWE1) and tumour (22Rv1, DU145, RC58/T) prostate cell lines. The GFP protein was unfortunately detected in all five unirradiated transfectant cell lines, with induction levels of up to 4-fold. The administration of a radiation dose of 1Gy significantly increased GFP protein levels in RWP1E normal prostate cells only. In response to a 2-Gy delivery, the protein was detected at higher levels in all transfected cell lines, except DU145. The response was maximal in 22Rv1 cells (section 8.3). These results suggest that, although leaky, this promoter may be used to enhance transgene expression in radiogenic protocols.

Replication-deficient adenoviruses emerged as the vector of choice for this hypoxia-inducible gene therapy strategy for several reasons, some of which were already exposed in section 8.1. In addition to these, the fact that
recombinant adenoviruses can be easily constructed and produced to very high titers, compared to other viral gene therapy systems, was an important factor in the decision process. The anatomic location of the prostate makes intra-tumoral injections of the adenoviral therapy possible, limiting antiviral immune reactions associated with systemic delivery. Moreover, the first adenoviral gene therapy strategy clinically tested in prostate cancer involved the herpes simplex virus thymidine kinase suicide gene (HSV-tk). The trial was the first to demonstrate safe anticancer activity of suicide gene therapy in patients with prostate cancer. (Herman, Adler et al. 1999). Finally, adenoviruses were shown to trigger radiosensitivity (Chen, DeWeese et al. 2001; Lupold and Rodriguez 2005). The adenovirus genome being linear double-stranded DNA, it indeed must evade recognition by the tricomponent DNA protein kinase complex (DNA-PK) to avoid self-ligation. Inhibition of the DNA-PK pathway sensitises cell to ionising radiation (Taccioli, Amatucci et al. 1998; Chernikova, Wells et al. 1999),

Following encouraging results of this liposome-delivered hypoxia-mediated suicide gene therapy approach in vitro, the vectors have been improved to facilitate future studies. Although the response of the tPA promoter proved disappointing in this ability to increase transcriptional control, more commonly used radiation-responsive promoters may still be exploited in this approach. The inclusion of the GFP reporter gene will facilitate monitoring of gene transfer in the future. Finally, the first steps towards adenoviral delivery have been made and will allow for further evaluation of the potential of the technique.
9.1 Introduction

Prostate cancer is progressively becoming a middle-age disease as screening programmes are increasingly leading to earlier detection. While the rationale for early diagnosis is improved prognosis, clinicians are now more than ever faced with difficult decision as to the best treatment options to offer their younger patients, due to the nature of associated side effects as well as the difficulty in the evaluation of the risk of disease progression. Because prostate tumours tend to be multifocal and histologically heterogeneous, tumour periphery is not easy to define and biopsies are difficult to grade. Indeed, estimates suggest that even with a threshold PSA value of 4.1 ng/mL, biopsy will miss 82% of cancers in men less than 60 years old and 65% of cancers in men 60 years of age and older (Kurhanewicz, Vigneron et al. 1996). Moreover, studies revealed that biopsy correctly predicted radical prostatectomy Gleason grade in less than 60% of cases (Cookson, Fleshner et al. 1997; Steinberg, Sauvageot et al. 1997).

The Consensus Statements of the College of American Pathologists defined three categories of prognostic factors in prostate cancer (Bostwick and Foster 1999): Category 1 includes factors that proved of prognostic importance in published clinical trials, such as PSA level, TNM staging and Gleason grading. Category two included promising factors extensively studied but not yet yielding sufficiently robust statistical benefit, such as tumour volume and DNA ploidy. Category three related to novel promising but not sufficiently documented factors including a wide variety of histopathologic and genetic markers, microvessel density and neuroendocrine differentiation.

For curative treatment, the identification of early tumour stage in asymptomatic individuals is crucial. Moreover to select patients for radical prostatectomy, accurate preoperative staging is important. While substantial effort is being invested into the discovery of novel markers of prostate cancer (Dobosy,
Roberts et al. 2007), the technical improvement of imaging technology progressively offers non-invasive diagnostic and staging tools. Medical teams involved in prostate cancer management indeed now suffer from an increasing pressure to the development of more minimally invasive curative techniques. As a result, novel treatment modalities may need to be geared toward a male “lumpectomy”, to match the results obtained in organ preservation approaches, such as for example in breast cancer.

The study of the effect of conventional therapies on the histology and molecular biology of prostate tissue is important to understand the emergence of resistance, determine a therapeutical success as well as for the development and delivery of novel complementary therapies. Because of changes in the histological appearance, grading of post-therapy specimen is difficult. It is indeed believed that the histological grading using the Gleason system for treated cancers may be inadequate. Most studies suggest an increase in Gleason score in treated tumours which may be an inaccurate representation of biologic behaviour (Helpap 1985; Crook, Robertson et al. 1994; Hellstrom, Ranepall et al. 1997; Crook, Malone et al. 2000; Bullock, Srigley et al. 2002).

Androgen deprivation therapies have been shown to cause atrophy of the prostatic epithelium as a result of apoptosis and reduced cell proliferation. Indeed a decrease in mitotic index, lower Ki-67, downregulated VEGF expression and mutant p53 expression seem to be associated with a favourable effect of antiandrogen treatment (Armas, Aprikian et al. 1994; Polito, Muzzonigro et al. 1996; Mazzucchelli, Montironi et al. 2000; Szende, Romics et al. 2001). Histologically, hormonal treatment results in a reduction of cancerous gland size and number. The glands also appear to be composed of almost completely degenerated cells (Tetu, Srigley et al. 1991).

Following radiation therapy, the prostate gland is usually small and hard. The degree of radiation-induced changes on prostate cancer tissue is variable
among patients. The neoplastic glands become fewer and smaller and the glandular pattern may be lost (Helpap 1985; Siders and Lee 1992).

As a result, novel strategies, as well as being compatible with the initial microenvironment of tumours, need to be realistically deliverable as a second line treatment. Finally, novel therapeutic approaches ideally should enhance the efficacy of current treatment without increasing morbidity.

9.2 Hypoxia-inducible gene therapy is specifically targeted.

As previously discussed, oxidative stress is an important feature of prostate tumours, whose signalling pathways are triggered by the formation of reactive oxygen species, in response to hypoxia, androgens and radiation. The molecular response to oxidative stress involves a variety of messenger and results in the activation of repair mechanisms, inhibition of apoptosis and enhanced resistance. An important molecule in these pathways is hypoxia-inducible factor 1 alpha, whose expression is enhanced in prostate cancer specimens. Because HIF-1α is restricted to cancerous lesions, the HIF-1/HRE system should ensure targeting of the CD gene where it is most needed within prostate tissue. In our study, HREs were found to be completely hypoxia selective in the four commercially available prostate cell lines tested (Chapter 5). Although selectivity appeared compromised in the novel RC58/T cell line, the technique will remain tumour-selective, since HIF-1α expression has not been reported in normal prostate tissue. HREs responsiveness has been shown to increase with decreasing oxygen tension (Dachs, Patterson et al. 1997; Ruan, Su et al. 2001), ensuring that transgene expression is maximal in highly hypoxic tissues.

The induction of p53 in response to oxidative stress has been linked with HIF-1α stabilisation (An, Kanekal et al. 1998) and repressed HIF-mediated gene transcription (Blagosklonny, An et al. 1998). CD expression levels indeed
appear weaker in p53 wild type LnCaP cells than in either DU145 or 22Rv1 p53 mutated cells (section 5.3.1). Cells characterised with low expression levels of mutated p53 (22Rv1) showed increased CD levels compared to cells associated with high levels of the protein (DU145). Finally, loss of p53 resulted in strong inhibition of HRE-dependent gene expression (see PC3 results).

Immunochemistry staining of radical prostatectomy specimens has shown that CD34, VEGF and HIF-1α staining reactions were significantly higher in prostate cancer than in the BPH group (Lekas, Lazaris et al. 2006). Staining was less frequently observed in stromal cells within the tumours and in normal tissue adjacent to tumour margins (Talks, Turley et al. 2000). HIF-1α expression was restricted to the prostate epithelium of malignant lesions and significantly correlated to the androgen receptor (Zhong, De Marzo et al. 1999; Boddy, Fox et al. 2005). Controlling of transgene expression with HIF-1α will thus ensure targeted delivery of the technique. More importantly, hypoxia may be used as a molecular switch to transgene expression, providing an extra level of control on the therapy. Moreover, 5-FC was found to be non-toxic to untransfected aerobic cells (chapter 4), with an EC₅₀ higher than 10mM. As a result, non-targeted toxicity should not be an issue.

The advantage of the 5-FC/CD system is that 5-FC is an anti-fungal agent routinely used in the clinic. The pharmacology of this compound has thus been well studied, and as a result, if were delivered systemically in a gene therapy setting, the cytotoxic effect of the technique should be localised to the site of transduction. The usual dose for anti-fungal treatment is 12.5 to 37.5 milligrams (mg) per kilogram (kg) of body weight every six hours, with main side effects including nausea, vomiting and tiredness.

In prostate cancer, the 5-FC/CD system has been used in a small number of complex constructs. Transcriptional control was achieved using the prostate specific membrane antigen (PSMA) (O'Keefe, Uchida et al. 2000; Uchida,
O'Keefe et al. 2001), the cytomegalovirus promoter (CMV) promoter (Anello, Cohen et al. 2000; Yin, Fu et al. 2001; Yoshimura, Suzuki et al. 2001; Kato, Koshida et al. 2002; Zhang, Yin et al. 2002), the prostate specific antigen (PSA) (Yoshimura, Suzuki et al. 2001; Yoshimura, Ikegami et al. 2002) and the CAG promoter (Miyagi, Koshida et al. 2003). These strategies reported selectivity of the technique in the sense that non-transfected cells were not sensitised to 5-FC. However, gene therapy requires a second level of control for transgene expression. The use of PSA or PMSA proved successful. However, PSA is downregulated under conditions of androgen deprivation, which may limit the use of PSA-driven construct. PSMA is abundantly expressed in virtually 100% of prostate cancers and metastases, and upregulated in condition of androgen deprivation, suggesting an advantage to this type of constructs. Unfortunately, while PSMA is prostate specific, expression may be found in normal prostate tissue as well as primary and metastatic prostate cancer. As a result tumour-selectivity is not complete.

9.3 HRE-driven CD expression matches CMV-driven expression

Both VEGF and GAPDH-derived constructs were found to drive CD expression to similar levels than CMV, providing sufficient HRE copy numbers were used, in response to hypoxic exposure in both androgen-dependent (22Rv1) and androgen-sensitive (DU145) cells. While the CMV promoter appeared to induce constant CD protein levels, CD gene expression was triggered early in hypoxic conditions but translation was delayed. Importantly, similar to the CMV promoter, HREs were found able to sustain CD production over both a 4 days reoxygenation period and long-term hypoxic exposure in 22Rv1 cells. Few studies have investigated the impact of hypoxia on translation efficiency in hypoxia. Detectable CD protein levels in lysates of transfected cells exposed to hypoxia for 6 days are a positive factor for the strategy, since overall mRNA translation is known to be low during severe hypoxia, and associated with disruption of the unfolded protein response (UPR) system, decreased mTOR
activity and reduced ATP levels (see review, (Magagnin, Koritzinsky et al. 2006).

To improve the efficacy of the strategy, a number of groups have fused CD with the herpes simplex virus type 1 thymidine kinase (TK) sequence. When placed under the control of a human inducible heat shock protein 70 promotional sequence in a recombinant adenovirus, expression levels obtained were dependent on the multiplicity of infection used and the incubation time after heat shock in PC3 cells (Blackburn, Galoforo et al. 1998). A similar pattern was seen when CD/TK was placed under the control of the CMV promoter, strong expression was detected 24 hours following adenoviral infection. Expression levels increased over time and were maximal at 48 hours (Blackburn, Galoforo et al. 1999). In LnCaP cells, PMSA-driven activity of the luciferase has been shown to be increased 20-fold compared to controls, showing promise to this type of construct (O'Keefe, Uchida et al. 2000). Indeed the PSMA/CD system has been proposed as an alternative to androgen deprivation therapy, following encouraging results drawn from in vivo studies (Uchida, O'Keefe et al. 2001).

9.4 HYPOXIA-INDUCED CD EXPRESSION SENSITISED PROSTATE CANCER CELLS TO 5-FC

The combination of either vector described above with hypoxia has not been reported. In the present study, the EC_{50} of the transfected cell lines to 5-FC depended on the amount of CD available to the cell. Prolongation of the hypoxic induction period from 24 to 48 hours indeed resulted in the reduction of EC_{50} of 22Rv1 cells by factor of 2 in pH5VCD and pH8GCD transfectants, whereas EC_{50} of cells transfected with pCMV-CD remained constant (0.04mM). However, the cytotoxic effect of the treatment (EC_{50s}) was improved 5-fold in pCMV-CD and pH5VCD transfectant cell lines (EC_{50s} = 0.56 and 1, respectively) but remained constant in pH8GCD transfectants. At the clinically relevant 5-FC concentration of 1mM, sensitivity was evident in all four cell lines tested, with a
maximal effect in 22Rv1 cells. This prodrug concentration (1mM) was well below the 500 mM/L serum concentration obtained by usual dosage for antifungal treatment in human clinical used (Petersen, Demertzis et al. 1994). This is the first study of its kind being assessed in a panel of prostate cancer cell lines.

Data on the in vitro 5-FC sensitivity of prostate cell lines transfected with the CD gene is also limited. The in vitro EC$_{50}$ of cells to a 3-days 5-FC treatment was in the region of 1-5mM in response to PMSA-driven CD expression in LnCaP and C2-4 prostate cancer cells (O'Keefe, Uchida et al. 2000). In another study, CMV-driven CD expression was associated with a 41% cell viability ratio in response to a 7-days 5-FC treatment (100ng/mL) in LnCaP cells. Sensitivity was significantly reduced when CD was placed under the control of the PMSA promoter/enhancer (Yoshimura, Suzuki et al. 2001). In DU145 cells transfected with an adenoviral vector containing the CD gene driven by the CAG promoter, in vitro sensitivity to a 2-days 5-FC treatment were associated with an IC$_{50}$ of 0.88 mM. Sensitivity was increased 4-fold when the cells were co-infected with an adenovirus containing the E.coli uracil phosphoribosyltransferase (UPRT) gene, due to improved conversion capacity of cytosine deaminase (Miyagi, Koshida et al. 2003). When tested in vivo against RM1 prostate cancer in mice, AdCD/UPRT infection suppressed the aggressive growth of the tumour in response to daily systemic administration of 5-FC for a duration of 15 days, associated with a 1.8-fold increase in apoptosis levels compared to the controls (Khatri, Zhang et al. 2006). In vivo, mice treated with CD/5-FC gene therapy revealed tumour growth inhibition comparable to that obtained with 15mg/kg 5-FU. Moreover, treatment with CD/5-FC resulted in higher tumour but lower serum concentration of 5-FU than with systemic 5-FU chemotherapy.
9.5 5-FC INDUCED APOPTOSIS IN REOXYGENATED AND HYPOXIC CELLS

The CD/5-FC system has been shown to induce apoptosis both in vitro and in vivo in a variety of human cancer, including pancreatic (Zhang, Yuan et al. 2000; Eisold, Antolovic et al. 2006), colon (Ju, Tao et al. 2000; Richard, Duivenvoorden et al. 2007), glioma (Wang, Mayo et al. 1998; Kurozumi, Tamiya et al. 2004; Fischer, Steffens et al. 2005), breast (Brade, Szmitko et al. 2003), liver (Xie, Gilbert et al. 1999) and melanoma (Cao, Kuriyama et al. 1999). Unfortunately apoptosis induction was not always quantified. Otherwise, levels of apoptosis induction in response to 5-FC treatment were 34.5% (100 mM/L, 48 hours) (Zhang, Yuan et al. 2000) and 46.17% (50ug/mL, 48 hours)(Eisold, Antolovic et al. 2006) in pancreatic cell lines.

The efficacy of the CD/5-FC system has been improved by several methods, including co-transfection with the UTRP gene (Kambara, Tamiya et al. 2002; Miyagi, Koshida et al. 2003; Khatri, Zhang et al. 2006; Richard, Duivenvoorden et al. 2007), co-administration of a monoclonal antibody, TRA-8, that specifically binds to death receptor 5,(Kaliberov and Buchsbaum 2006), co-expression of procaspase-3 (Song, Kong et al. 2006) and fusion of CD with endostatin (Ou-Yang, Lan et al. 2006).

5-FC has been shown to induce apoptosis through downregulation of bcl2 in glioma cells, (Fischer, Steffens et al. 2005), which was confirmed in vivo in prostate cancer (Pandha, Martin et al. 1999). Bcl-2 is an anti-apoptotic mediator that has been associated with resistance to anti-cancer therapies and showed to play an important role in prostate cancer (for review, see (Chaudhary, Abel et al. 1999; Catz and Johnson 2003). Bcl-2 has been associated with the emergence of androgen-independent prostate cancer (McDonnell, Troncoso et al. 1992; Furuya, Krajewski et al. 1996; Krajewska, Krajewski et al. 1996). Furthermore, bcl-2 overexpression in response to androgen deprivation has been reported to contribute to increased apoptosis resistance (Lu, Tsai et al. 1999; Catz and Johnson 2001). More importantly, cells already overexpressing
bcl-2 could be selected by androgen ablation because of their natural ability to resist apoptosis.

As a result, a variety of therapeutic strategies against bcl-2 have been designed (for review, see (DiPaola and Aisner 1999)). Initially, bcl-2 was targeted by a hammerhead ribozyme directed against this protein (Dorai, Olsson et al. 1997). Down regulation of bcl-2 with an antisense phosphorothioate ODN resulted in significant prostate tumour growth in vitro and in vivo (Campbell, Dawson et al. 1998; Gleave, Tolcher et al. 1999; Gleave, Nelson et al. 2003) and has been the basis of various clinical trials. In prostate cancer patients, bcl-2 antisense treatment enhanced the sensitivity of prostate tumours to the chemotherapeutic drug docetaxel (Chi, Gleave et al. 2001). Similar results with paclitaxel were observed when clusterin was targeted (Miyake, Hara et al. 2003), suggesting that antisense oligonucleotides directed to anti-apoptotic agents could be used as enhancer of the effect of conventional chemotherapeutic drugs. Indirectly, blockade of NFkB, resulting in bcl-2 downregulation has been associated with suppression of angiogenesis, invasion and metastasis in mice (Huang, Pettaway et al. 2001).

Therefore, if the 5-FC/CD system acts on bcl-2, as proposed, this technique could have great potential in prostate cancer. Moreover, because bcl-2 has been shown to be activated by the hypoxia-responsive PI3K pathway, this strategy may be ideal for hypoxia targeting. In DU145 cells, overexpression of Bcl-2 has been shown to effectively prevent low extracellular pH-augmented TRAIL cytotoxicity (Lee, Song et al. 2004). Because hypoxia is associated with acidosis, bcl-2 overexpression may have contributed to the increased resistance of 22Rv1 and DU145 wild type cells to 5-FU in hypoxia reported in Chapter 6. In the present research, although bcl-2 levels were not measured, bcl-2 down regulation appears a likely event in the response of both wild type and transfected prostate cancer cells to hypoxic 5-FC treatment. Indeed, wild type cells were intrinsically more sensitive to 5-FC in hypoxia than in air.
(Chapter 4). This study is the first evidence of the effect of long-term hypoxia on 5-FC activity and sensitivity. Although this requires further investigation, it may be postulated that 5-FC exposure is able to reverse hypoxia-induced bcl-2 expression in untransfected cells, resulting in increased apoptosis levels, ultimately participating to the growth inhibition effect of hypoxia. In transfected cells, although apoptosis levels were increased in hypoxic cells, when compared to reoxygenated transfectants, induction levels were similar (3-fold) when corrected for the controls. It thus seems that the vectors are as potent in chronic as they are in acute hypoxia. The apoptotic fraction was generally increased in 22Rv1 cells (60-85%), compared to DU145 cells (30-50%), despite the fact that 22Rv1 cells were treated with a tenth of the 5-FC concentration used for DU145 cells. Indeed, an important aspect of hypoxic 5-FC treatment was the oversensitivity of the four cell lines tested to very low prodrug concentrations (0.1mM). This may have significant consequences for the therapeutic effect of the technique in vivo.

Despite poor diffusion of 5-FC and reduced transduction efficiency within prostate tissue, this therapy may still result in increased therapeutic sensitivity. Moreover, the 5-FC/CD system abolished hypoxia-induced chemoresistance in both 22Rv1 and DU145 cells at all 5-FC concentrations tested, proving this technique as a potent anti-hypoxic strategy.

Another important aspect of the HRE/CD system was its ability to be switched on by transient hypoxic exposure. Indeed, due to the incredibly unsteady nature of tumour hypoxia, acute hypoxia is usually deemed difficult to manage. This HRE/CD system may be able to sensitize tumour cells up to twenty-four hours after hypoxic exposure, an option impossible to achieve with HIF-1α silencing strategies due to the rapid degradation of the protein upon reoxygenation.
Chapter 9

9.6 Hypoxia-inducible CD/5-FC radiosensitises prostate cancer cells.

The surviving fraction of aerobic and reoxygenated 22Rv1 and DU145 cells treated with HRE/CD/5-FC gene therapy was significantly decreased by the addition of 2Gy, delivered either as single or split fractions. The additive effect of gene therapy and radiation was observed regardless of the vector used, despite the fact that radiation alone did not result in CD production in pH5VCD-transfected cells.

Although the HRE/CD strategy has proven successful for the targeting of tumour cells, radiation-controlled gene therapy may have the advantage of ubiquitous, steady and guaranteed transgene expression for the radiosensitisation of all sub-population of cells within the radiation field. The ability of tPA to induce the expression of the GFP reporter gene in response to ionising radiation was as a result evaluated in both normal and tumour prostate cell lines (Chapter 8). Despite significant radioresponse, tumour specificity was compromised and the benefit of this promoter for this gene therapy approach will need to be further evaluated.

The combination of the 5-FC/CD system with ionising radiation in the form of external beam or radioimmunotherapy has been studied previously in prostate cancer. The combination of adenovirus mediated cytosine deaminase plus 5-fluorocytosine and external radiation therapy resulted in synergistic activity to approximately 2 logs of cell kill at low doses of radiation in the mouse prostate cancer cell line RM-1 in vitro (Anello, Cohen et al. 2000). In this study, starting 5-fluorocytosine 24 hours before external radiation therapy resulted in the most profound killing, suggesting maintaining prodrug therapy during external radiation therapy is important. An additive antitumour effect was obtained when CD/5-FC therapy was combined with 1Gy irradiation in mice bearing xenograft of transfected LnCaP cells. The effect was enhanced with the administration of three 3Gy fractions but not when the technique was combined with radio labelled anti-PSA monoclonal antibodies due to insufficient accumulation to the
target tumours (Kato, Koshida et al. 2002). Enhanced radiosensitivity was also evident in PC3 cells transfected with a defective, recombinant adenovirus containing a fusion gene encoding the CD/TK fusion gene under the control of a cytomegalovirus promoter (Blackburn, Galoforo et al. 1999). The radiation enhancement ratio to achieve an isosurvival of 1% was 1.1 when 5-FC alone (10μg/mL, 24 hours) and radiation doses in excess of 5Gy were administered. The combination with GCV further increased the ratio to 1.4, showing the additive potential of the fusion gene.

Freytag et al investigated the most successful combination of CD, TK and ionising radiation. In 1999, the Baylor college of Medicine conducted the first in situ herpes simplex virus thymidine kinase (HSV-tk) gene therapy clinical trial for human prostate cancer (Herman, Adler et al. 1999). In that clinical trial, men with biochemical recurrence of localised prostate cancer after radiation therapy received a single injection of the adenoviral vector. A degree of toxicity was observed at high doses and there were preliminary indications of efficacy. Additional safety studies confirmed the safety of the viral dose (Shalev, Kadmon et al. 2000). Further analysis of the patients data revealed increased levels of activated CD8 T cells, which correlated with increased number of apoptotic cells, suggesting an important immune component in the response to this gene therapy protocol (Miles, Shalev et al. 2001). In 2002, an additional phase I/II clinical trial was started involving the combination of three to doses of HSV-tk with intensity modulated radiation therapy (Teh, Aguilar-Cordova et al. 2002). Intravenous ganciclovir was also replaced by its oral bio equivalent drug valacyclovir. Men in this trial were stratified in three groups as to their clinical stage (low, high and lymph-node metastases). Groups in the higher two stages of the disease also received hormonal therapy. Despite added toxicity, the study showed that combined radiotherapy, short-course hormonal therapy, and \emph{in situ} therapy provided good loco-regional control but inadequate systemic control in patients with positive pelvic lymph nodes. Longer term use of hormonal therapy in addition to gene therapy and radiotherapy has been now
adopted for this group of patients to maximize both loco-regional and systemic control (Satoh, Teh et al. 2004). Five-year follow-up showed that this strategy was associated with a lengthening of the PSA doubling time and delayed the projected onset of salvage therapy by an average of 2 years (Freytag, Stricker et al. 2007).

High bcl-2 expression and low bax expression have been correlated with the poor therapeutic response of prostate cancer to radiotherapy (Mackey, Borkowski et al. 1998). Therefore, the potential of 5-FC to downregulate this protein may be an important advantage if the strategy was to be combined with radiation therapy. Moreover, combination of prodrug treatment with ionising radiation has been shown to improve transfection efficiency and transgene integration (Stevens, Zeng et al. 1996), to maximise cell cycle effect by targeting difference phase than chemotherapy (Simons and Marshall 1998), to increase susceptibility to DNA damage, reduce DNA repair and amplify the bystander effect.

Other gene therapy strategies showing great potential for combination with radiation therapy are the HSV/Tk suicide gene system (Teh, Ayala et al. 2004) and adenoviral delivery of InterLeukin-3 (IL-3) (Oh, Chen et al. 2004). A study comparing the antitumour activity of CD and TK in the Dunning prostate adenocarcinoma cell line R3327 AT-1 reported the superiority of TK/GCV over CD/5-FC due to the low degree of activation (DOA) (Corban-Wilhelm, Ehemann et al. 2004) This statement however seems skewed by the way DOA was calculated. Indeed, DOA was defined as the ratio of IC50 to 5-FC or GCV in wild type versus transfected cells. This calculation did not account for the potential increased prodrug toxicity in wild type cells, which is not necessarily a downfall of the strategy. Finally, compared to the HRE/CD/5-FC system developed here, specific targeting of the vectors was not addressed in these approaches.
9.7 Coupling Molecular Imaging with Gene Therapy

The development of molecular imaging is particularly interesting for the gene therapy field because it can help monitor the targeting, efficacy and therapeutic progress of the therapy as well as guide its delivery. While 5-FC metabolism may be monitored with 19F-nuclear resonance spectroscopy (Corban-Wilhelm, Hull et al. 2002), optical imaging technology based on bioluminescent (e.g. luciferase genes) and fluorescent proteins (e.g. GFP) produced in the nature have been widely used as imaging reporters of transgene delivery. However, their application is limited in humans.

Recently, the Cancer Research Imaging Program of the National Cancer Institute workshop yielded recommendations in using hypoxia measurement to identify patients who would respond best to radiation therapy (Tatum, Kelloff et al. 2006). Hypoxia in prostate tumours has been detected using oxygen microelectrodes during surgery, PET and MRI. Studies have shown that the hypoxic fraction varies across tumours, and in response to therapeutic intervention (Rasey, Koh et al. 1996; McNab, Yung et al. 2004; Skov, Adomat et al. 2004). In the case of PET imaging, tumour uptake of positron-labelled choline and acetate has been shown to be closely related to the degree of oxygenation of tumour tissues. Uptake is also very poor in androgen-deprived tumours, suggesting that PET may underestimate tumour cell viability of the hypoxic compartment of prostate tumours (Hara, Bansal et al. 2006).

HREs have been used to direct GFP expression to the hypoxic regions in a human prostate cancer model using magnetic resonance and optical imaging. In this study, the vector used was that described by Shibata et al, and thus of similar structure as pH5VCD. In this PC-3 derived system, the GFP protein was detected in lysates of tumours at a minimum of 20 hours hypoxia (<1% oxygen). Hypoxic regions were consistently characterised by low vascular volume. Vessel density was also lower in GFP-expressing regions. Despite liposomal delivery of the vector, Raman et al. were able to achieve transgene expression
in regions of low vascular density and create a functional vascular MR map of the tumours studied (Raman, Artemov et al. 2006).

Dynamic maps of tumour oxygenation have also been generated in Dunning prostate adenocarcinoma using FREEDOM (Fluorocarbon Relaxometry Using Echo Planar Imaging for Dynamic Oxygen Mapping) in combination with hexafluorobenzene as reporter molecule (Zhao, Ran et al. 2003). The results of this imaging technique were successfully correlated with more traditional hypoxia detecting methods (immunochemistry, pO2 measurements). In this study, larger tumours were significantly less well oxygenated than small tumours. In addition to providing tumour baseline pO2, the FREEDOM technique appears to allow for the study of oxygen dynamics in response to interventions. This technique was able to identify two categories of hypoxic regions: those that reoxygenated within 24 hours and those that continued to show increase hypoxia at later times, versus those that were unresponsive. This type of imaging could have far-reaching consequences in the guiding and planning of radiation therapy.

It has been proposed that short hypoxic turnover may affect the outcome of hypoxic imaging modalities, because cells imaged as pre-treatment assessment may have died and be replaced by the time treatment commences. Indeed it is not uncommon for a 1-2 week interval to take place between imaging and treatment. It may be however argued that untreated chronically hypoxic cells are unlikely to reoxygenate within this time scale, because the reoxygenation process is mainly triggered by tumour shrinkage over the course of therapeutic intervention.

9.8 Future perspectives

In the view of both the need for novel therapeutics and the increasing evidence of the therapeutic role of tumour environment, the combination of hypoxia
targeting and suicide gene therapy appears a good strategy for the management of prostate cancer. While hypoxic targeted therapy as a single modality is unlikely to be effective on its own due to the complex patterns of oxygenation in human tumours and the influence on the known cellular pathways that respond to hypoxia, these strategies might provide the level of control that standard gene therapy lacks. The convergence of hypoxia, androgens and radiation toward oxidative stress signaling and HIF-1α upregulation further suggest the potential of HIF-1α regulation in a gene therapy approach. Finally, suicide gene therapy appears superior to corrective and immuno-modulatory strategies, because of the larger scale of molecular effect offered by localised chemotherapy. Hypoxia-inducible CD/5-FC gene therapy therefore merges the appeal of hypoxia-targeted therapies, the efficacy of chemotherapeutic agents and places itself at the service the radiation therapist for the amplification of therapeutic efficacy. Indeed, while 5-FC induced bcl-2 downregulation, 5-FU acts at the DNA level, maximising the damaging effect of ionising radiation.

This approach, using vectors derived from the VEGF and GAPDGH HRE sequences, was shown to be effective in vitro in a variety of prostate cell lines in response to a range of hypoxic scenarios and radiation dose delivery. This is the first time GAPDH HREs have been developed to drive a gene therapy system. Finally, the limiting factor of poor vector delivery was addressed with the development of replication-deficient adenoviruses and monitoring of therapeutic efficacy was facilitated with the inclusion the GFP gene. It must be noted however, that while the GFP protein is a very attractive way to follow vector integration, this reporter gene will not be suitable for in vivo imaging. Indeed, in vivo visualisation of GFP expression requires removal of the tissue. If gene therapy vectors are to be monitored in a clinic, the inclusion of an infrared or some other imaging system in the vector would be appropriate (Funovics, Weissleder et al. 2004).
In the context of in vitro testing, it may be beneficial to test the vectors in androgen-enriched and androgen-depleted media to further evaluate the potential of the technique in a situation of androgen-depletion, since it is now routinely administrated in prostate cancer patients. In the present experimental work, the cell lines were grown in the presence of low level androgen supplied by foetal calf serum. In an androgen-withdrawal situation, the difference in efficacy between androgen-sensitive and androgen-resistant cell lines may be more prominent. Another area of improvement would be the effect of successive exposure to acute hypoxia and low radiation dose on levels of CD protein. Because intermittent hypoxia was shown to result in the progressive accumulation of HIF-1α, treatment of cells subjected to cycles of hypoxia/reoxygenation with this gene therapy may be interesting. The enzymatic activity of CD under each experimental condition may also need to be evaluated, while a bcl-2 expression study may elucidate the mechanism for the cytotoxic action of the strategy.

Finally, because the tumour vasculature is an additional significant approach to tumour targeting, the effect of the HRE/CD system on vascular cells should be investigated for the potential anti-angiogenic effect of the strategy to be evaluated. Indeed, it may be postulated, that intra-prostatic injection of the vector would result in the transduction of both tumour and vascular cells. As a result, the strategy may be able to achieve where anti-VEGF therapies fail, and sensitise endothelial cells, pericytes and basal cells and participate to complete vascular wall destruction. It may indeed be hypothesised that targeting of the vasculature as opposed to tumour cells may be the future of gene therapy. Indeed, this would reduce the problem of delivery and may result in a tremendous anti-angiogenic effect, resulting in tumour shrinkage. Recent evidence has shown endothelial cells are exposed to intermittent hypoxia, resulting in HIF-1α accumulation (Martinive, Defresne et al. 2006; Dewhirst 2007). Activity of GAPDH in response to hypoxia has been discovered in
endothelial cells (Graven, Yu et al. 1999), suggesting pH8GCD may be an ideal candidate for vascular- and hypoxia- targeted suicide gene therapy.

In the next stage of its development, the adenoviral vectors are to be tested in an *in vivo* model for prostate cancer. Because most animals and non-human primates do not spontaneously develop prostate cancer, the few models available today are limited to a few species (rats, dog, humans) and their relevance must be considered with caution (for review, see (Lamb and Zhang 2005). Indeed significant anatomical, histological and metastatic potential differences exist between species in regards to the prostate gland. In addition, there are few human prostate cancer cell lines available, especially as a model of primary prostate tumour.

The Dunning R3327 rat model is the most commonly used. It is a well-differentiated, slow-growing tumour that spontaneously developed in an inbred Copenhagen rat (Lubaroff, Canfield et al. 1978). Sublines with various characteristics that mimic a wide range of aspects of human prostate cancer have been derived from this model. Over 600 papers using the Dunning model and its derivatives have been published. In addition, a variety of transgenic mouse models have been developed through genetic manipulation of pathways implicated in prostate cancer such as the TRAMP (transgenic adenocarcinoma model of the mouse prostate) model (Greenberg, DeMayo et al. 1994). Male TRAMP mice rapidly develop progressive prostate disease that mimics human histology and pathology and metastasises to distant organ. High-grade PIM or prostate cancer is usually evident by age 12 weeks with metastases occurring by week 30. This model also responds to androgen deprivation and has been used for chemoprevention studies (Bosland 1999). A limitation of these transgenic models however remains the rapid onset of the disease and the occurrence of neuroendocrine tumours, which are inexistent in the human malignancy (Abate-Shen and Shen 2002). Xenograft models in immuno-compromised mice (e.g. nude, SCID, NOD, SCID-NOD) are another alternative
In regards to the \textit{in vivo} evaluation of the HRE/CD/5-FC system, the xenograft model appears as the most appropriate because it may allow the evaluation of the therapy in a 3-dimensional model of the cell lines tested \textit{in vitro}. Indeed, 22Rv1, RC58/T, PC3, LnCaP and DU145 have all been shown to form tumours in nude mice. As a result direct correlation between \textit{in vitro} and \textit{in vivo} studies will be possible.

\section*{9.9 Potential Contribution of this Gene Therapy Approach to Improved Cancer Care}

Translational research has already proven the feasibility of gene therapy alone or in multi-modal approaches in prostate cancer (for review see (MacRae, Giannoudis et al. 2006). Early clinical outcome including biochemical and biopsy data related to the use of in situ gene therapy as an adjuvant to radiation therapy (GT/RT) is very promising. GT/RT dramatically reduced cell viability \textit{in vitro} and tumour growth \textit{in vivo}, with no significant worsening of the toxicities normally observed in single-modality approaches were identified in Phase I/II clinical studies. Enhancement of both local and systemic T-cell activation was noted with this combined approach suggesting anti-tumour immunity (Tetzlaff, Teh et al. 2006).

The optimal delivery of novel gene-based therapeutics is still a subject of debate. While viral vectors appear attractive, they proved of limited effectiveness because of the strong immune reaction they induce. An additional problem associated with adenoviruses is the poor expression of coxsackie adenoviral receptor (CAR) in prostate cancer, which may limit integration of the vectors. However, solutions exist for these problems and there is potential for the design of maximized adenoviruses, ranging from retargeting strategies to the inclusion of additional expression cassettes to reduce immune responses (for review, see (Essand 2005)).
Serum levels of osteopontin have been proposed as a marker of tumour hypoxic in Head and Neck cancer patients (Le, Sutphin et al. 2003). Studies in prostate cancer have correlated osteopontin levels with disease progression but the link with hypoxia may need to be investigated (Forootan, Foster et al. 2006). If confirmed, this marker could be used to identify patients that may benefit from hypoxia-directed therapies.

In a clinical setting, the HRE/CD system is unlikely to be effective as a single modality. Because delivery may be difficult, the strategy would probably be administered prior to or during current front-line therapy protocols, especially in radiation therapy patients. The advances in imaging technology may see this approach integrated within radiation therapy planning. One could imagine a situation where functional imaging would define hypoxic areas, guiding the localisation of vector injection as well as locally compensated dose delivery. In the view of the recent return of brachytherapy in prostate cancer, radioactive seeds encompassing a gene therapy vector loaded capsule could be placed at these strategic points. Implantation of seeds, although locally more invasive than external beam therapy would follow oral administration of 5-FC. This strategy would be both cost-effective and an attractive option for patients, as it may limit lifestyle change.

9.10 Conclusion

The research aims of this study were achieved in the sense that a novel strategy has been designed, developed, tested and improved. The potential of the CD/5-FC suicide gene therapy system has been maximised by exploiting the microenvironmental characteristics of prostate tumours. Specificity has been achieved and associated with acceptable transgene expression for sensitisation and apoptosis induction to occur in both androgen-sensitive and androgen-insensitive prostate cancer cells, in response of both acute and chronic hypoxia, as well as reoxygenation. Hypoxia-induced chemotherapeutic resistance was
abolished following transfection. Moreover, the biological effectiveness of clinically relevant radiation doses has been amplified. Finally, limitations of the strategy have been addressed. Monitoring and improved delivery have been implemented to facilitate future research.

The results presented in this thesis have indicated that a suicide gene therapy approach in prostate cancer has significant potential. Exploiting both hypoxia and ionising radiation independently or in combination represents a novel and potentially additive approach to maximise specific killing of prostate cancer cells. While the work presented here has focused on in vitro, we are looking forward to apply this approach utilising a highly efficient adenoviral delivery system in the appropriate animal model.
APPENDICES
10.1 PRIMER SEQUENCES

<table>
<thead>
<tr>
<th>Primer Sequence</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward (BglIII cloning junction)</td>
<td>5'- GGC CTT TTT ACG GTT CCT GG – 3'</td>
</tr>
<tr>
<td>Reverse (Xhol cloning junction)</td>
<td>5'- CTC GAA GCA TTA ACC CTC AC – 3'</td>
</tr>
<tr>
<td>Forward IRE sequence</td>
<td>5' – AAC AAC CGC GCA TTC CTC CAA – 3'</td>
</tr>
<tr>
<td>Forward GFP sequence</td>
<td>5' – TGG CTC TCC TCA AGC GTA TT – 3'</td>
</tr>
<tr>
<td>Forward end GFP sequence</td>
<td>5’ – AGA ACG GCA TCA AGG TGA AC – 3’</td>
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10.2 MYCOPLASMA TEST RESULTS

All prostate cell lines were tested for Mycoplasma following 14 days in culture in antibiotic-free media using the Micoalert® kit, as described in section 2.4.2.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Ratio</th>
<th>Result</th>
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<tbody>
<tr>
<td>22Rv1</td>
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<td>Negative</td>
</tr>
<tr>
<td>DU145</td>
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<tr>
<td>LnCaP</td>
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<td>PWR-IE</td>
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10.3 WESTERN BLOT GELS AND BUFFER COMPOSITION

10.3.1 ACRYLAMIDE GEL BUFFERS

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Composition</th>
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<tbody>
<tr>
<td>0.5M Tris HCl</td>
<td>0.5M Tris HCI</td>
</tr>
<tr>
<td></td>
<td>Tris 18g</td>
</tr>
<tr>
<td></td>
<td>dH₂O 300 mL</td>
</tr>
<tr>
<td></td>
<td>pH 6.8</td>
</tr>
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</table>
### Appendices

#### 1.5M Tris-HCl

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<td>pH</td>
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#### 10.3.2 15% RESOLVING GEL

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<tr>
<td>AdH₂O</td>
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</tr>
<tr>
<td>Acrylamide</td>
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</tr>
<tr>
<td>1.5M Tris, pH 8.8</td>
<td>1.82 mL</td>
</tr>
<tr>
<td>10% SDS</td>
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</tr>
<tr>
<td>10% APS</td>
<td>70 μL</td>
</tr>
<tr>
<td>TEMED</td>
<td>2.8 μL</td>
</tr>
</tbody>
</table>

#### 10.3.3 STACKING GEL

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<tr>
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<td>330 μL</td>
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<tr>
<td>0.5M Tris, pH 6.8</td>
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<tr>
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<td>20 μL</td>
</tr>
<tr>
<td>10% APS</td>
<td>20 μL</td>
</tr>
<tr>
<td>TEMED</td>
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</tr>
</tbody>
</table>

#### 10.3.4 RUNNING BUFFER (10X)

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<td>dH₂O</td>
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<td>Glycine</td>
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<tr>
<td>Methanol</td>
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<tr>
<td>dH₂O</td>
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Store at 4°C.

10.3.6 TWEEN PBS (T-PBS)

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<td>PBS tablet</td>
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<td>Tween</td>
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10.3.7 BLOCKING SOLUTION

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10.4 MTT STANDARD CURVES

To determine the optimal cell density to be used and the limits of the assay, a standard curve of absorbance reading over cell density was constructed for 22Rv1 and DU145 cells. Ideally the cell density used should be in the straight portion of the MTT standard curve to justify the use of proportionality properties in later analysis of absorbance readings. For 22Rv1 cells, a working cell density of 3x10⁴ was chosen. A linear relationship between absorbance and cell number per well was found to be correct for absorbance values within a 0.12 and 196 brackets. The lowest value absorbance can take in our experiments is thus 0.12. This value was subtracted to our MTT readings to remove
background and ensure that the minimum value calculated % growth value can take is 100%.

MTT standard curves

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<tr>
<td>DU145</td>
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<td>2.00600</td>
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10.5 Oligonucleotides and Insert Sequences

10.5.1 5xVEGF

A11 [1:130] (130 nt)
5'GATCTCCACAGTGCACTACGTGGGCTCCAAACAGGTCTCTTCCACAGTGCTACCGTGGGCTCAAACAGGTCTCTTCCACAGTGCTACCGTGGGCTCTCTTGGAGCCCACGTATGCACTGTGGAAGAGGACCCTGTTGGGAGACCACGCGACCTGTTGGGAGCCCACGTATGCACTGTGGAAAGGACCCTGTTGGGAGGCCACGTATGCACTGTGGAA 3'

B11 [5:136] (132 nt)
5'TGTTGGAGGCCACGTATGCACTGTGGGAGGACCTGTTGGGAGACCACGCGACCTGTTGGGAGCCCACGTATGCACTGTGGAAAGGACCCTGTTGGGAGGCCACGTATGCACTGTGG 3'
Appendices

3'AGGTGTACGTATGCAACCGAGGTTGTCCAGGAGAAGGTGTACGTATGCACCCGAGGTTGTCCAGGAGAAGGTGTACGTATGCACCCGAGGTT - 5'

A12 [131:253] (123 nt)
5'CCAACAGGGTCTCTTCCACAGTGCATACGTGGGCTCCAACAGGGTCCTCTTGGGCCCAAGCTTGGTAGGGGTGTACGGTGGGAGGTCTATATAAGCAGAGTCTCGTTTAGTGAACCGGTACAGATCC 3'

B12 [137:257] (121 nt)
5'TCGAGGATCTGACGGTTCACTAAACGAGCTCTGCTTATATAGACCTCCACCGTACACGCCTACCAGCTTGGGCCCAAGAGACCTGTTGGAGCCCAGGTATGCACTGTTGGAAGAGGACC-3'

3'CCAGGAGAAGGTGTACGTATGCAACCGAGGTTGTCCAGGAGAACCCCGGTTGCAACCACATCCGACATGCCACCCTCCAGATATATTGTCTCTGAGCAAATCACTTGGGCAGTCTAGGAGCT - 5'
### VEGF

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| 288 |
10.5.2 6xGAP

A21 [1:138] (138 nt)
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CAGGTCTCGGGTGCAGTCCGAGCTCGGTGCGTGCCCAGTTGACGCCCA
AGTTGAAACGCCACACGTGTCGTGCGTGCCCAGTTGAAACGCCCA
5'GATCTatactagatctGCCACACGCTCGGTGCGTGCCCAGTTGAAACGCCCA
AGTTGAAACGCCACACGTGTCGTGCGTGCCCAGTTGAAACGCCCA

A22 [139:275] (137 nt)
5'ACGCCACACGTGTGCTGGCCAGTTGAAAAGCCCA
CGGTGAGCTCGGTGCGTGCCCAGTTGAAACGCCCA
5'ACGCCACACGTGTGCTGGCCAGTTGAAACGCCCA
CGGTGAGCTCGGTGCGTGCCCAGTTGAAACGCCCA

B21 [5:144] (140 nt)
5'GGGCGTTCAACTGGGCACGCACCGAGCGTGTGGGCGTTCAACTGGGCACGCACCGAGCGTGTGGGC
GATCGGTGTGTGGCCAGTTGAAACGCCCA
5'GGGCGTTCAACTGGGCACGCACCGAGCGTGTGGGCGTTCAACTGGGCACGCACCGAGCGTGTGGGC
GATCGGTGTGTGGCCAGTTGAAACGCCCA

B22 [145:279] (135 nt)
5'TCGAGGATCTGACGGTTCACTAAACGAGCTCTGCTTATATAGCCCA
CCGGCTACAGCCTACAAAGCTCGTGTTGGGCCCGTCAACTGGCACGCACCGAG
5'TCGAGGATCTGACGGTTCACTAAACGAGCTCTGCTTATATAGCCCA
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289
Appendices

6GAP

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10.5.3 9xGAP

290
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CAGGTGCCTCGGTGCGTGCCCCAGTTGAACGCCACACGCTCGGTGCGTGCCC
AGTTGAACGCCACACGCTCGGTGCGTGCCCCAGTTGA-3'

A32 [139:258] 120 nt
5'ACGCCACACGCTCGGTGCGTGCCCCAGTTGAACGCCACACGCTCGGT
CGTGCCCCAGTTGAACGCCACACGCTCGGTGCGTGCCCCAGTTGAACGC
CCACACGCTCGGTGCGTGCCCAG-3'

A33 [259:368] 110 nt
5'TTGAACGCCACACGCTCGGTGCGTGCCCCAGTTGAACGGGCCGAAGCT
TGGTAGGCGTGTACCGGTGGGAGGTCTATATAAGCAGAGCTCGTTAGTG
ACCCTCAGATCC-3'

B31 = B21 [5:144] (140 nt)
3'ATATGATCTAGACCGGGTGTGCGAGCCACGACGGGTTCAACTTTCGGGT
GTGCGAGCCACGACGGGTTCAACTTTCGGGTGCGAGCCACGACGGG
TCAACTTTCGGGTGCGAGCCACGACGGGTTCAACTTTTCGGGTGCGAGCCACGAC

B32 [145:264] 120 nt
3'TGTGCGAGCCACGACGGGTTCAACTTTCGGGTGCGAGGGCCACGACGG
GTACACCACTTTCGGGTGTGCGAGCCACGACGGGTTCAACTTTTCGGG
CGAGCCACGACGGGTTCAACTTTCGGGTGCGAGCCACGACGGGTTCAACTTT

B33 [265:372] 108 nt
5'GTTCAACTCGCCCGACCGAGCGGCGTTCAACTTTGGCCCGCGAGCC
CAGCGAGCGGCGTTCAACTTTGGCCCGCGAGCCAGCGAGCGGCGTTCAACTT
CGAGCGGCGAGCCAGCGA-3'
3'CGGGTGTCGAGCCACGCAGGGGCTAACTTGCCCCTGGTGACACCATC
CGCACATGCCACCCCTCCAGATATATTCGTCTCGAGCAATCACTTGGCAGT
CTAGGAGCT -5'

5'TCGAGGATCTGACCGTTCACTAAACGAGCTCTGTTATATAGACCTCCCA
CCGTACACGCCTACCAAGCTTGGGCCCCTTTCAACCTGGGCACCGCACCAG
CGTGTGGGC - 3'
Appendices

**9GAP**

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151 AGCTCTGGATG CCGAACAAGG TTTAGTTATA CGGCCGTTTT TGGAGCCACA
201 TTATCCACTG GACACCCGAC GCACCACGCG GAACACAGGC ACAAACGACG
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301 TAAACCATG ACGATGTGAA ACAACGGCAG TAGCAGTTACG TGGAGAAGCC
351 GATGCAAGGG CGCAGGGATG ACGCGCGCG CAGCGGCGCG CAAGGGGGCC
401 CAGGGCTTGTG GATGTCCGAT GAGGGGAGCC GAGGAGGAGG GAGGAGGAGG
451 CAGGGTCTTTG GTTTGTTGAT AATGTTTATT ATTATGAGTT ATTTATGAGT
501 ATGTTTTTATG GATGTTTATT ATTATGAGTT ATTTATGAGT ATTTATGAGT
551 GAGGCGCCTGT GGTGCTTTTG AGGCGCGCGC GCTTGGGGCC GGGGGGCGCG
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10.7 ABSORBANCE OF UNTRANSFECTED LnCaP AND RC58/T CELLS

LnCaP and RC58/T wild type cells were exposed to hypoxia for 48 hours prior to 5-FC treatment (5mM) for 4 days under either normoxic (reoxygenated) or hypoxic conditions. Absorbance was measured at the end of initial hypoxic exposure and following 5-FC treatment. The results are shown on Figure 10-1 and Figure 10-2. In both cell lines, an increase in absorbance and hence an increase in cell number was evident in both treatment conditions between the end of the hypoxic period and the end of 5-FC treatment. Hypoxia however induced a significant growth inhibition effect. Neither hypoxia, 5-FC nor their combination was cytotoxic in both these cell lines.
Figure 10-1 Hypoxia induced a significant growth inhibition effect, associated with a significant reduction in absorbance in LnCaP cells exposed to hypoxia (48h) prior to a 4-days 5-FC (5mM) exposure under either normoxic (reoxygenated) or hypoxic conditions. Bars, SE; n=3; *p<0.05
Figure 10.2 Hypoxia induced a significant growth inhibition effect, associated with a significant reduction in absorbance in RC58/T cells exposed to hypoxia (48h) prior to a 4-days 5-FC (5mM) exposure under either normoxic (reoxygenated) or hypoxic conditions. Bars, SE; n=3; *p<0.05

10.8 IreeGFP SEQUENCE

AGATCTGCCC CTCTCCCTCC CCCCCCCTA ACGTTACTGG CGGAAGCCGC
TTGGAATAAG GCCGGTGTGC GTTTGTCTAT ATGTTATTTT CCACCATATT
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CGAGCATTCC TAGGGGTCTTT TCCCTCTCG CCAAGGAAT GCAAGGCTTG
TTGAATGTCG TGAAGGAAGC AGTTCCTCTG GAAGCTTCTT GAAGACAAAC
ACGTCTGTA GGGACCCCTT GCAGGCAGCG GAAACCCCCA CCTGGGCGACA
GGTGCCCTCTG CGGCAAAGG CACCTGATG AATACACCC TGCAAGGGCG
GCCAACCCTT AGTGCAACGT ATGTAGTTGG ATAGTTTGG AAAGAGTCAA
ATGGCTCTCC TCAAGCGAT TCAACAGGG GCTGAAGGAT GCCCAGGGA
TACCCATTG TATGGGATCT GATCTGGGAC TCTGGTACAC ATGCTTTACA
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297
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701 AGGCGGAGGG CGATGCCACC TGACCTGCTA TGCTACCTCG
751 ACCACGGGCA AGCTGGGGCG TCCCGCTGCA ACCCTGAGCT
801 CTACCGATTG CAGGTGCTTT GCCGCTTACC CGACACTATG AAGCAGCAC
851 ACTTCTTCAA GTCCGGCCATG CGGCAAGGCT AGCTGCAGCT
901 TTCTTCAAGG CAGCACCCCT CTAACAGATT CAGACGGCAG
951 AGGGCGAGGG CGATGCCACC TGACCTGCTA TGCTACCTCG
1001 AAGACCGGCG CATCCTGGGG GAATTACGTCA AGCTGCAGCT
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1101 ACCAGGCTA CAACCTGCCA GGCGACGGCC CCGTGCTGCT GCCCGACAAC
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1251 AGGGGCGAGG CGGGCGAGGG TTAATATATG AAGCCTCCAT CCTCACCATC
1301 GCCCGACCGA CCGCCCGACG GGGCGACGGG GCTGCTGCTG GCCCGACAAC

10.9 TPA PROMOTER SEQUENCE

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51 AAGTGCAACC ATTCGATGGA AAACCATCCT CTCAAGAGTT CAAATGAGAG
101 TCCTCATGAC CAGCATCCAC TGCTGAAGAG GTCTACCTCG
201 GCCACCCAGA CAGGGCCACA GCTGCTGCTG CTCATGGGAG GTCTACCTCG
301 AGCAAGGCTG CAGCTTCATA AAAATGTGAA AAACAGTTTG AAAGCAATGT
401 CAAAAACGAG TAGGTTCTTG TCAACTCTCA ACGAGGCTG CACGTAACC
501 GCCGTTTCTT TTTTACCTTT CATTTATATG GGGCCAAGGG GCTGCTGCTG
601 ACGAGTGATCA CATATGATTG AGATGCTTTG GCTGCTGCTG CTCATGGGAG
701 CAGTTACTGG CAGCAGCCGGA TTTTACCTTT TTTTTTACCT CTAACAGATG
801 GGCGTTTCTT TTTTACCTTT CATTTATATG GGGCCAAGGG GCTGCTGCTG
901 ACGAGTGATCA CATATGATTG AGATGCTTTG GCTGCTGCTG CTCATGGGAG
951 TTTCGGGAG CCACCTACTG CAGCCCCGGT CTTTACAAAG AAGGAAAGAG
Appendices

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1351 TCCCCCAGCT AATTTAAGCA GATGGCCGCT GTGCAATGCA AATTTAATTC
1401 AGCCTGGGCCC GAAGCCAGGA TGGGCTGTGC TGCTTCCACC GGGCAACTTC
1451 TCCCCCTGCC TTATAAAAAAC AGGCTGCTC CAGCTCCCTC ATGGCCCTGT
1501 CACTGAGCA TCCTCCGCAC ACACAGAAAC CCGCAAGGAA GGGCCACCC
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1801 TCCCGTACAA TCCACTCCAG AGGGAGATG TCTGTGGCA TTTGCATGCT
1851 ATGAGGATAG
VECTOR MAPS
11.1 pCMV-CD

![Diagram of pCMV-CD with restriction enzyme sites and nucleotide sequence information.](image)

**Restriction Enzyme Sites:**
- **BglII** - 1 - A'GATC_T
- **HindIII** - 762 - A'AGCT_T
- **XhoI** - 1097 - C'TCGA_G

**Vector Information:**
- pCMV-CD1:10
- 7037 bp

**Full length CMV**
11.2 pH5VCD

BgIII - 1 - A'GATC_T
HindIII - 188 - A'AGCT_T
XhoI - 254 - C'TCGA_G
EcoRI - 259 - G'AATT_C

HindIII - 3038 - A'AGCT_T

pH5VCD
6194 bp

BamHI - 1778 - G'GATC_C
11.3 pH8GCD
11.4 pH0CD

HindIII - 2 - A'AGCT_T

BamHI - 1592 - G'GATC_C

HindIII - 2852 - A'AGCT_T

pH0CD
6013 bp
11.5 pCMV-CD-GFP

Vector maps

pCMV-CD-GFP
8365 bp

BglII - 8365 - A'GATC_T

HindIII - 761 - A'AGCT_T

HindIII - 2851 - A'AGCT_T

HindIII - 5208 - A'AGCT_T

BamHI - 3943 - G'GATC_C
11.6 pH5VCD-GFP

pH5VCD-GFP
7522 bp
11.7 pH8GCD-GFP

![Diagram of pH8GCD-GFP vector map](image)

- **BglII** - 1 - A'GATC_T
- **HindIII** - 261 - A'AGCT_T
- **EcoRI** - 332 - G'AATT_C

**pH8GCD-GFP**

7595 bp

- **HindIII** - 4439 - A'AGCT_T
- **BamHI** - 3174 - G'GATC_C

**Vector maps**
11.8 pH0CD-GFP
11.9 \textit{pAdCMV-CD-GFP}

\begin{center}
\includegraphics[width=3in]{vector_map.png}
\end{center}

\textit{pAdLoxpCMV-CD-GF}

7514 bp

\textit{HindIII} - 762 - A'AGCT\_T

\textit{HindIII} - 2852 - A'AGCT\_T

\textit{BgIII} - 4217 - A'GATC\_T
11.10 pAd.H5VCD-GFP
11.11 pAd.H8GCD-GFP

pAd.H8GCD-GFP
6744 bp

HindIII - 260 - A'AGCT_T

HindIII - 2081 - A'AGCT_T

BgIII - 3446 - A'GATC_T
CreloxP - 3426 - ATAACTTCGTATAGCATACTATACGAGTAT
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Review

Achieving Hypoxia-Inducible Gene Expression in Tumors

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KEY WORDS
hypoxia, hypoxia response element, gene therapy

ABSTRACT

Hypoxia is an inevitable feature of solid tumors and a common cause of treatment failure. Hypoxia acts as a trigger to genetic instability, apoptosis and possibly metastases. The adaptive response to cellular hypoxia involves the modulation of the synthesis of multiple proteins controlling processes such as glucose homeostasis, angiogenesis, vascular permeability and inflammation. The hypoxia responsive element (HRE) sequences isolated from oxygen-responsive genes have been shown to selectively induce gene expression in response to hypoxia when placed upstream of a promoter. The levels of induced gene expression were dependent on the number of HRE copies and the oxygen tension. Hypoxia-mediated cancer gene therapy strategies may represent a promising mean to significantly improve the efficacy of standard radiation therapy and chemotherapy approaches.

INTRODUCTION

Since the first observation of regions of low oxygenation levels in tumors by Thomlinson and Gray, hypoxia in tumors has been well documented. Low cellular oxygen levels have been reported to induce gene instability, initiate apoptosis and stimulate a range of pro-angiogenic factors, suggesting hypoxia as a trigger to tumor metastasis. It is therefore associated with poor prognosis.

Rather than designing therapeutic strategies to overcome hypoxic resistance, a number of groups have chosen to exploit the hypoxic nature of tumors to gain a therapeutic advantage. This has led to the development of hypoxia-selective agents (i.e., bioreductive drugs) and hypoxia-directed gene therapies. Enzyme/prodrug systems have been developed as part of suicide gene therapy strategies: in this approach, transfected cells express a prodrug activation enzyme (i.e., cytosine deaminase), which converts a nontoxic prodrug into a toxic drug in the tumor. Crucial to this approach is to ensure that the prodrug is only active in the tumor. This can be achieved by placing the prodrug activation enzyme under the control of a tumor-specific promoter, e.g., the prostate specific antigen gene promoter in prostate cancer. A more attractive generic option might be to target gene expression to hypoxic cells by placing the prodrug activation enzyme under the control of a hypoxia-responsive promoter. Genes that are oxygen-regulated are involved in a number of cellular processes including glucose homeostasis, angiogenesis, vascular permeability and inflammation. Such genes include phosphoglycerate kinase 1 (PGK-1), glucose transporter 1 (Glut-1), lactate dehydrogenase A (LdhA), aldolase A (ALDA), glyceraldehydes-3-phosphate dehydrogenase (GAPDH), vascular endothelial growth factor (VEGF) and nitric oxide synthetase (iNOS). The only directly oxygen-responsive DNA regulatory element regulating the expression of oxygen-responsive genes has been defined as hypoxia inducible factor-1 (HIF-1). Inactive under normoxic conditions, reduced oxygen tension induces HIF-1 to bind to HREs found in the promoter of various oxygen-responsive genes. The HRE/HIF-1 regulation system is common to all human tissues tested to date and is deregulated in 70% of cancers, which makes HRE-mediated gene expression an interesting perspective for cancer treatment. Targeting hypoxia cells in tumors could indeed increase the therapeutic ratio of conventional cancer treatments by reducing their resistance to radiation therapies and chemotherapeutic drugs.

HYPOXIA INDUCIBLE FACTOR 1

HIF-1 functions as a heterodimer consisting of two basic-helix-loop-helix proteins, HIF-1α and HIF-1β. HIF-1 is activated through post-translational stabilisation of...
HIF-1α and is degraded by the proteasome in aerobic conditions. Under hypoxic conditions, the prolyl hydroxylase (pVHL) is inactive and ubiquitination is inhibited. HIF-1α is no longer degraded resulting in an extended life time (30min) and its accumulation in the cytoplasm. HIF-1α is translocated from the cytoplasm to the nucleus where it interacts with HIF-1β to form the heterodimer HIF-1. HIF-1 then specifically binds to HREs to induce gene expression (Fig. 1).

**HYPOXIA-RESPONSIVE ELEMENTS**

HREs are enhancers elements containing the consensus core sequence 5'-[(A/G)CGT(G/C)(G/C)-3', localized at varying positions and orientations of the coding region of several hypoxia-regulated genes (Fig. 2) including Epo, VEGF, Glut 1, and glycolytic enzymes such as PGK-1, LdhA, ALDA, and GAPDH.

HRE from the erythropoietin gene

Hypoxia-induced expression of Epo is regulated by both the rate of gene transcription and by post-transcriptional events. Transcriptional regulation is mediated by the action of several transacting factors interacting with the proximal promoter region and with the 3' untranslated region. The Epo 3' untranslated region contains a 50-bp HRE located approximately 120-bp 3' from the polyadenylation site (Fig. 3). The element can be functionally divided into three parts. One site (-3458 to -3465) binds the hypoxia-inducible factor (HIF-1), a second site (-3472 to -3477) is required for transactivation of the Epo gene mediated by HIF-1 while the last site (-3481 to -3500) is a binding site for factors such as the orphan receptors, hepatocyte nuclear factor 4 and EAR3/COUP-TF-1. Fibrosarcoma HT1080 cells transiently transfected with a construct containing this 150-bp fragment of the Epo gene had a luciferase activity 2-fold higher after exposure for 6 hours to 0.1% O₂. Ruan et al. transfected LacZ reporter plasmids containing 2–3 150-bp copies into U-87MG and U-251 MG-NCI human brain tumor cells and tested their ability to induce LacZ gene expression under anoxia. They reported a 12- and 4-fold increase in gene expression in U-87MG and 251 MG-NCI respectively when cells were exposed to anoxia for 16 hours after transfection as compared to aerobic controls. Such results suggest differential response to hypoxia exposure in different cell types.

**HRE from the phosphoglycerate kinase 1 gene**

Using transient transfection of human hepatoma and human cervical carcinoma cells, Firth et al. isolated cis-acting control sequences responsible for hypoxia-inducible expression in the 5' flanking region of the phosphoglycerate kinase 1 (PGK-1). They characterized an 18-bp element necessary for hypoxia-inducible expression, which is similar to the sequence within the Epo gene. The murine PGK-1 HRE promoter was inserted in an adenoviral vector and a panel of cancer cells lines were transfected. A low basal level of β-galactosidase transgene expression was observed, with levels of induction comparable to the full-length CMV promoter. Dachs et al further examined the murine HRE in combination with three different promoters and extended their work to a prodrg activation system. They made a series of constructs with three copies of this HRE linked to the PGK-1 promoter, the minimal thymidine kinase promoter or the interferon-stimulated 9–27 gene promoter, and driving the CD2-encoding gene. The production of this marker protein was monitored by fluorescent-activated cell sorting analysis. After 16 hrs in anoxia, they observed different levels of the CD2 marker protein depending on the promoter used. The 9–27 promoter induced the highest level of gene expression with a 7-fold increase as compared to controls in aerobic conditions. Furthermore, using a comet assay protocol, they could correlate the HRE-dependent marker gene expression with tumor hypoxia. The 9–27 promoter

**Figure 1.** Under hypoxic conditions, the prolyl hydroxylase (pVHL) is inactivated, resulting in inhibition of HIF-1α proteosomal degradation. HIF-1α interacts with HIF-1β to form HIF-1, and triggers gene expression.

**Figure 2.** HREs from the Epo, VEGF, PGK1, LdhA, ALDA and GAPDH gene. Hypoxia-related DNA signatures are indicated in bold. Functional HIF-1 binding sites (arrow) and sequences with > 4/5 match to the functionally essential Epo sequence 5'CAAGCAGG-3' (overline). Adapted from Semenza et al., 1996 and Iu et al., 2002.

**Figure 3.** Identification of a hypoxia-response element in the Epo 3'-untranslated region. Highlighted is the fragment used for HRE-directed gene expression. Adapted from Semenza et al., 1996.
Hypoxia Inducible Gene Expression in Tumors

A HRE sequence was then used to drive the expression of the cytosome deaminase (CD) gene that codes for an enzyme that transforms inactive 5-fluorocytosine (5-FC) to active 5-fluorouracil (5-FU). They reported a 6.8-fold increase in CD activity in cells exposed to anoxia for 16 hours as compared to control normoxic cells. The transfected cells were also more sensitive to 5-FC after anoxia whereas hypoxic andoxic cells were equally sensitive to 5-FU. When the transfected tumor cells were grown as xenografts in nude mice, expression of the CD gene was limited to areas adjacent to necrosis. These experiments demonstrate the activation of a well-defined pathway of hypoxic gene regulation within solid tumors. It also shows how this system may be used to develop hypoxia-mediated suicide gene therapy.

HRE from the vascular endothelial growth factor gene

In contrast to the cell-restricted pattern of Epo expression, many different primary and cultured cell types respond to hypoxia by increasing vascular endothelial growth factor (VEGF) gene expression. Similarities between the oxygen-sensing mechanisms regulating the expression of VEGF and Epo have been reported.

To determine whether the 5'-flanking region of the VEGF gene could mediate transcriptional responses to cellular hypoxia, Forsythe et al. constructed a reporter plasmid in which VEGF 5'-flanking sequences were fused to the luciferase coding sequences. Transfected hepatoma cells expressed the luciferase gene with a 9-fold increase after hypoxic treatment (1% O₂). The ability of the reporter to respond to hypoxia was investigated using a series of deletion mutants of the 5'-flanking sequences. As a result, the study delineated a 47-bp region between -985 and -939 (Fig. 4) that was sufficient for transcriptional activation of the SV40 basal promoter in hypoxic Hep3B cells.

Shibata et al. further tested the potential of the selective expression mediated by putative HREs from the human VEGF gene. They assessed...
the extent of hypoxia inducibility by a variety of DNA constructs using the luciferase assay in order to select the most appropriate vector for cancer gene therapy. They first replaced the SV40 promoter sequence in the pGL3 vector with a 385-bp fragment of the 5'-flanking region of the human VEGF gene. This construct, when transfected in murine tumor cell lines, showed marked increases in luciferase activity in response to hypoxia. To gain more hypoxia responsiveness, they inserted five copies of the HRE sequence upstream of the 385-bp fragment and observed an increase in luciferase activity (13 to 16-fold) under hypoxic conditions even though this sequence did not include a TATA or CAAT box. The inclusion in the construct of the 32-bp sequence did not include a sequence upstream of the 362

Although the HRE/E1b minimal promoter appears to be the best construct for the induction of genes by hypoxia, induction levels obtained by this construct are considerably lower (100-fold) than that of a CMV promoter driven vector and may be insufficient to be used for genetically directed enzyme prodrug therapy. Shibata et al. therefore conducted further studies with the goal of establishing hypoxia-inducible vectors with higher expression of therapeutic genes. Two constructs were produced: one using the EF-1 alpha promoter and one using a 60-bp minimal promoter containing a TATA sequence derived from the human CMV IE promoter. Each promoter was attached to HREs and tested for hypoxia-induced gene expression. Undetectable induction was observed with the EF-1 alpha promoter whereas more than 500-fold induction could be detected with the CMV IE promoter after exposure to 0.02% O2 for 18 hours, which is similar to that of the full-length CMV promoter construct. This study thus proposes the 5HRE/CMV construct as the most promising vector for hypoxia-inducible gene therapy.

Shibata et al. tested their construct with the bacterial nitroreductase (NTR)/CB1954 enzyme prodrug system. They successfully correlated in vitro hypoxic induction of the NTR protein with increased cell death. The in vivo efficacy of CB1954 conversion to its cytotoxic form was later investigated through growth delays experiments. Significant anti-tumor effects were achieved with intraperitoneal injections of CB1954 both in tumor that express NTR constitutively or with a hypoxia-inducible promoter. Respiration of 10% O2 further increased tumor hypoxia in vivo and enhanced anti-tumor effects were observed. These results further demonstrate that hypoxia-inducible vectors may be useful for tumor-selective gene therapy.

HRE from the glycolytic enzyme glycolaldehyde-3-phosphate dehydrogenase (GAPDH) is induced by hypoxia in endothelial cells. A valuation of the GAPDH promoter resulted in the delineation of six consensus HIF-1 binding sites (5'-RCGTCG-3'). Two of those are in the sense orientation, and four in the antisense orientation. Transfections of constructs containing various fragment sizes showed that three of those sites (-989 to -985; -957 to -953; -340 to -336) are unlikely to represent functional HREs (Fig. 5) since their deletion did not abrogate hypoxic gene activation in either prostate cancer cells or human endothelial cells. Graven et al. transiently transfected endothelial cells, fibroblasts and smooth muscle cells using constructs which contained various portions of the GAPDH promoter linked to a chloramphenical acetyl transferase (CAT) reporter gene. The initial construct, containing a 509-nucleotide fragment of the human GAPDH gene corresponding to nucleotides -487 to +21 in relation to the transcription start site, resulted in a 5.4-fold increase in CAT activity in response to hypoxia. The region of the gene coding for hypoxic response could later be restricted to a 157-nucleotide fragment corresponding to nucleotides -267 to -110. A computer search for known regulatory sequences present in this fragment showed the presence of a HRE between bases -126 and -119. They linked a 62-bp fragment containing this element to the SV40 promoter in a CAT vector and could report a 6-fold increase in CAT activity during hypoxia.

Lu et al. isolated the region spanning nucleotides -217 to -203 containing two HIF-1 consensus sites arranged as inverted repeats and separated by five nucleotides (Fig. 5). One or two copies of a 30-bp sequence containing this novel HRE were linked to the minimal TATA promoter of the tissue transglutaminase gene and inserted into a luciferase reporter vector. Transfection with one copy resulted in a 1.9-fold increase in luciferase activity in response to hypoxia, whereas transfection of two copies resulted in a 4-fold increase.
induction. This strongly suggested that this sequence is also a functional HRE.

**Effect of the HRE copy number.** The number of copies of the HRE sequence isolated from the Epo gene was shown to influence the levels of gene expression induction. Under anaerobic conditions, increasing HRE copy number to three or six resulted in a 4-fold increase in basal gene expression compared to that observed in aerobic conditions. However the use of constructs containing nine copies resulted in higher (27-fold) gene expression levels (Fig. 6).

A similar pattern was reported for HRE isolated in the VEGF promoter. However it was observed that a saturation effect can occur for constructs containing more than five copies. HRE copies numbers ≤3 were used in constructs for the PKG1/GADPH sequences. A slight increase in gene expression was observed when constructs contained three copies when compared with constructs containing a single HRE copy. Therefore, increasing HRE copy number appears to be a valuable option for promoting enhanced gene expression.

**Expression at intermediate oxygen levels.** Oxygen concentration in human tumors is heterogeneous and ranges from 5 mm Hg (0.7% O_2_) to 15 mm Hg (2.1% O_2_) in head and neck carcinoma. The mean oxygen level of a particular tumor is much lower than its normal tissue of origin. Induction levels were demonstrated to increase with decreasing oxygen tensions in all HRE tested (Fig. 7). Furthermore, responsiveness of HRE to intermediate levels of oxygen was reported to be dependent on HRE copy number, with a threshold of a minimum of 3 copies.

**HYPOXIA-REGULATED CANCER GENE THERAPY**

By inclusion of a HRE in the expression cassette, the expression of a therapeutic gene of interest is placed under the transcriptional regulation of HIF-1. HRE-mediated gene expression is thus potentially an ingenious strategy to confer tumor-selectivity to existing suicide gene-directed enzyme prodrug therapy strategies.

The enzyme/prodrug systems tested to date for HRE-mediated selective hypoxic expression strategies include bacterial cytosine deaminase/5-fluorocytosine in human fibrosarcoma HTIO80 cell, E. Coli nitroreductase/CB-1954 in human U-81MG and U-251 glioblastoma cells, HSV-TK/Ganciclovir in A11 lung carcinoma cells and human flavoprotein cytochrome cP450 reductase/RSU1069 in HT1080 cells.

However hypoxia-mediated proapoptotic gene expression has also been considered. Overexpression of BAX has been shown to sensitise human breast cancer cells to radiation-induced apoptosis and human glioma cells to apoptosis-inducing agents. Furthermore, HRE-mediated expression of the pro-apoptotic gene harakiki in breast cancer cells also resulted in the induction of cell death by hypoxia suggesting another novel strategy of breast cancer gene therapy.

Many other gene therapy approaches are being investigated as candidates for hypoxia-mediated cancer gene therapy and the number of successful strategies can be expected to extend in the near future.

**CONCLUSION**

In recent years, hypoxia targeting has made significant progress and as our understanding of the molecular basis of hypoxia-induced gene expression evolves, new possible targets have been defined.

**Hypoxia-mediated tumor targeting could therefore well become a feature of cancer treatment strategies in the near future. Hypoxia-mediated gene therapy would ideally complement current treatment strategies, particularly if employed as adjuvant therapy in radiation and/or chemotherapeutic protocols.**

**References**


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Review

Radiation to Control Transgene Expression in Tumors

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KEYWORDS
radiation, hypoxia, gene therapy

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ABSTRACT

Significant evidence has accumulated indicating that certain genes are induced by ionising radiation. An implication of this observation is that their promoter regions include radiation-responsive sequences. These sequences have been isolated in the promoter of several genes including Erg-1, p21/WAF1, GADD45α and p53. The mechanism by which radiation induces gene expression remains unclear but involves putative binding sites for selected transcription factors and/or p53. Consensus CCA[TT]GG sequences have been located in the Erg-1 promoter and are referred to as serum response elements or CArG elements. The tandem combination of CArG elements has been shown to improve gene expression levels, with a 9-copy motif conferring maximum inducibility. The response of these genes to ionising radiation appears to follow a sigmoid relationship with time and dose. Therapeutic induction of suicide genes and significant cytotoxicity can be achieved at clinically relevant x-rays doses both in vitro and in vivo but was found to be cell-type dependent. Radiation-inducible gene therapy can be potentially enhanced by exploiting hypoxia through the inclusion of hypoxia-response element motif in the expression cassette, the use of the anaerobic bacteria or the use of neutron irradiation. These results are encouraging and provide significant evidence that gene therapy targeted to the radiation field is a reasonably attractive therapeutic option and could help overcome hypoxic radioresistant tumors.

INTRODUCTION

The pairing of gene therapy with radiation therapy has the potential to provide significant advantage to other approaches alone. Ionising radiation and gene therapy can simultaneously be used as local therapy to kill tumor cells via independent cytotoxic actions, minimizing the evolution of treatment-resistant tumor cells. In addition, gene therapy products can be chosen to act synergistically with ionising radiation, further improving local tumor eradication. Toxicity to normal tissue could be reduced, as lower doses of both therapies would be required when used in combination. Several genes have thus been found to be activated following exposure to ionising radiation and have been recently (reviewed in ref. 1). The induction of early response by ionising radiation involves the expression of immediate early genes that code for transcription factors (e.g., Fos, Jun, AP-1, NFκB)2 as well as cytokines and growth factors (e.g., early growth factor-1 (Egr-1)).3 These genes can be seen as the first elements of a cascade of events leading to cellular response to radiation injury. The response of these genes to ionising radiation has been shown to be variable and cell type-dependent.4 Activated immediate early genes interact with the promoter region of so-called late responding genes, coding for cytokines, involved in the inflammation process (e.g., Interleukin-6)5 and growth factors, involved in cell survival and tissue recovery (e.g., TNFα,6 TGF-B1,7 PCGFα8). Finally, many of the genes induced by ionising radiation are related to cellular fate following DNA damage. This includes genes involved in cell cycle checkpoints, DNA repair, cellular stress and apoptosis.

RADIATION-RESPONSIVE SEQUENCES

WAF-1 gene promoter. The WAF1 promoter physiologically drives the expression of the p21 gene, whose expression is triggered by cellular stress. The p21CIP/WAF1 protein is involved, along with p27KIP1 and p57KIP2, in the inhibition of a variety of CDK/cyclin complexes as well DNA replication.3,10 p21CIP/WAF1 is also a key player of cell cycle checkpoints and can cause efficient cell cycle block, in a p53-dependent manner.11 Analysis of

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This promoter was proposed as a candidate for radiation-inducible genes, there could be some other undetected small factor involved. However, since SP-1 correlated with insignificant increase in CAT activity following addition of poly-clonal antibodies against the general transcription factor SP-1, show in both irradiated and unirradiated HeLa, this shift was not seen with anti-AP-2 antibodies. These results resulted in a shift in the bands in both irradiated and unirradiated plasmidine. It is involved in the cellular response to DNA damage and thus is induced by UV- and ionising radiation. Several studies have reported that the -118 bp/-59 bp region of the GADD45α gene is involved with the UV-response of the gene. Through extensive EMSA studies, Daino et al. revealed the existence of x-rays inducible factors that bind to the regulatory region of the GADD45α gene at 0.5–2 h after x-rays irradiation in the myeloblastic leukemia ML-1 cell line. X-rays-inducible HNF family, NF-At family and KLF family transcription factors were found to bind to the region near the p53 consensus recognition site (+1575 bp/-1594 bp), in the third intron of the GADD45α gene. These findings could explain the p53 dependence of GADD45α to radiation-induced expression.

**Tissue-type plasminogen activator gene.** Human tissue plasminogen activator (t-PA) is a serine protease that transforms plasminogen into plasmin. It is involved in the cellular response to DNA damage and thus is induced by UV- and ionising radiation. In radioreistant human melanoma cells t-PA was shown to be induced over 50-fold after x-irradiation (4.5 Gy). The entire t-PA promoter region was used in DNA band shift assays to determine X-ray induced transcription factor binding. Several x-rays selective protein-DNA binding sites appeared at specific loci within the t-PA promoter. The sequence responsible for increased CAT activity following exposure to UV-light or mitomycin was found to be located (-119/+169) in the 5'-flanking region of the gene. This sequence contains several putative binding sites for regulatory factors AP-2 (-89/-82 and +43/+50), NFKB like (+53/+58) and AP-2 like (+62/+69). Introduction of mutations in the AP-2 and NFKB binding sequences resulted in reduction of basal activity and UV-induced t-PA expression, whereas mutation of the AP-2 like sequence spared basal expression but resulted in complete loss of the UV response. This mutation however did not alter mitomycin-induction of t-PA, suggesting this AP-2 like sequence as a strong factor in the UV-induction of t-PA expression. This hypothesis has however proven unlikely when components of the region were tested through gel mobility assays. Addition of poly-clonal antibodies against the general transcription factor SP-1, shown to bind to the AP-2 like sequence in the human IL-2 and t-PA gene, resulted in a shift in the bands in both irradiated and unirradiated HeLa. This shift was not seen with anti-AP-2 antibodies. These results correlated with insignificant increase in CAT activity following addition of effectors of the AP-2 signaling pathway. These results would suggest SP-1 as the real UV-responsive factor. However, since SP-1 is a general transcription factor, involved in the expression of many genes, there could be some other undetected small factor involved. This promoter was proposed as a candidate for radiation-inducible gene therapy strategies but hasn't been exploited to date.

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**Early growth response gene promoter.** The early growth response gene promoter has been the most widely studied. This gene, also known as zif268, NGFI-A, Krox-24, TIS-8, encodes a nuclear phosphoprotein with a Cys2-His2 zinc-finger motif that is partially homologous to the corresponding domain in the Wilms tumor-susceptibility gene. The rapid and transient induction of Egr-1 expression in the transition of quiescent cells from the G0 to G1 phases of the cell cycle is regulated by efficient cis- and trans-regulatory mechanisms. Egr-1 is thus an immediate early gene that is induced during tissue injury and participates in signal transduction during cellular proliferation and differentiation.

Detailed functional analysis of the radiation-inducible Egr-1 gene demonstrated that the DNA sequences that drive the radiation-inducible response are located in the enhancer region of the promoter. These were identified as ten nucleotide motifs of the consensus sequence CC(A/T)GG, also known as CarG elements (Fig. 1). The human Egr-1 promoter contains at least four CarG elements.

When the chloramphenicol acetyltransferase (CAT) reporter gene was linked to the ERG1 promoter region extending from position -957 upstream of the transcription start site to position +248, a 4.1-fold increase in CAT activity was observed in irradiated human HL-525 myeloid leukaemia cells. The inducibility was lost if the -550 to -50 sequence was deleted, suggesting that this portion of the promoter is critical for x-rays inducibility. The testing of a series of ERG-1 deletion constructs demonstrated that the three 5' distal CarG boxes were essential to the radiation response.
The mechanism by which Erg-1 expression is induced by ionising radiation is still unclear. CARG elements were shown to regulate the expression of other immediate-early genes such as c-fos and members of the actin family, also called Serum Factor Elements, through the binding of specific accessory proteins such as Elk-1 and SAP-1. CARG elements are thus sometimes referred to as Serum Response Elements. The presence of the putative binding site sequences within the Erg-1 promoter also suggests potential involvement of the Sp-1 transcription factor and the Fos-jun heterodimer Activator Protein (AP)-1.\(^{16-28}\) Phosphorylation by mitogen-activated proteins (MAP) kinases, often triggered by cellular stresses such as radiation, was also reported to play a role in the responsiveness of the Erg-1 promoter.\(^{29}\)

Finally, the reactive oxygen species produced following cellular irradiation could also play a role as CARG elements have been shown to be responsive to hydrogen peroxide treatment.\(^{34}\)

**RADIO-ENHANCIBILITY OF THE PROMOTERS**

Although one would expect a linear relationship with increasing radiation doses, studies on the radio-enhanchibility of the responsive promoters suggest a sigmoid relationship both in terms of dose response and gene expression levels. The optimal dose needed to achieve acceptable gene expression levels appears to differ with the promoter studied but all were found to be responsive to the clinically relevant 2 Gy fraction doses. The peak of gene expression also varied with the promoter, but all were detected under 12 h.

**WAF-1 Promoter.** The inducibility of this promoter was assessed using the Green Fluorescent Protein (GFP) reporter gene. After a radiation dose of 4 Gy, GFP expression was induced in endothelial cells (9.5-fold) and in rat-tail segment artery (4.5-fold). This promoter activation reached a maximum within the radiation field at 8 hours after a priming dose of 4 Gy and lasted for 24 hours.\(^{30}\)

WAF-1 driven radiation-induced gene expression was thus achieved at clinically relevant radiation doses but no linear dose response relationship was reported. It was thus suggested that a conventional fractionation regimen of 2 Gy per fraction could be sufficient for treatment. Transduction of the WAF-1 promoter by rAAV vectors was more highly radiation-responsive than that transiently transfected by electroporation, with significant induction by low radiation doses down to 0.5 Gy.\(^{31}\)

**Erg-1 promoter.** Joki et al.\(^{32}\) have shown the radio-enhanchibility of the Erg-1 promoter in glioma cells by attaching the luciferase reporter gene to Egr-1 (EGR-Luc). The EGR-Luc construct showed a radiation response between 0 Gy and 20 Gy. Dose delivery of 20 Gy to transfected glioma cells resulted in a 12 hours increase in luciferase activity compared to nonirradiated transfected cells. At three hours post-irradiation, a maximal 9-fold increase in luciferase activity was reported. Luciferase activity was however similar to non irradiated transfected cells twenty-four hours after irradiation. It must also be noted that the Erg-1 response elements had a basal constitutive transcription rate in the absence of ionising radiation.

Introduction of the bovine growth hormone polyadenylation signal (BGH PA) as insulating sequence in adenoviral vectors however resulted in tighter tempo-spatial control of Erg-1-mediated gene expression.\(^{33}\)

**Synthetic promoters.** Marples et al.\(^{34}\) were the first to test whether the four CARG elements described in the wild-type Erg-1 gene could be induced by ionising radiation independently of the promoter sequence context. Shortening of the promoter sequence without loss of response is very attractive for gene therapy use if viral delivery is to be considered, due to the limitations in vector capacity to accommodate large inserts. The development of synthetic promoters is also a means of inhibiting non radiation-induced transcription factors and ensuring the response was selectively radiation-stimulated. The four CARG elements cassette (E4) has been cloned upstream of the GFP reporter gene to investigate its radio-inducibility in transfected human breast adenocarcinoma (MCF-7) as well as human glioma cells (U87-MG). Single doses of 5 Gy resulted in significantly higher GFP induction levels (0.3-fold) than wild type Erg-1. The synthetic promoter was shown to be induced by single radiation doses as low as 1 Gy, in both cell lines. Interestingly, the response reached a peak at 3 Gy, with single doses of 10 Gy resulting in GFP induction levels comparable to that obtained after 1–2 Gy. Fractionation of the radiation dose (5 x 1 Gy), separated by 12 h, resulted in the same response as a single dose of 1Gy.

Head and neck squamous cell carcinoma HLaC79 cells transfected with the E4/eGFP plasmid showed weak induction levels as well a high level background expression, suggesting a strong cell-type dependence and bringing into question its radiation-specificity. Indeed, when the E9 promoter was used to drive the iNOS gene, basal levels of iNOS increased progressively up to 8 hrs in transfected human microvascular endothelial cells (HMEC-1) and resulted in inhibition of the vascularator phenylphrine by 83%\(^{36}\) and a seven-day growth delay in transfected murine sarcoma (RIFI) in vivo.\(^{37}\)

Other promoters. The X-rays responsive elements of the GADD45α have been reported to be induced by radiation doses as low as 0.5 Gy with expression levels reaching a maximum 0.5 to 1 h after exposure.\(^{38}\) Following radiation doses of 4.5 Gy, t-PA activity in ML-1 cells reached a maximum activity 12 h after irradiation, and was very low after 24 hrs. Radio-induction appeared to be restricted to radiation doses between 1 and 8.5 Gy. Doses outside this window resulted in decreased activity levels.\(^{39}\) In HeLa cells, maximum CAT activity was reached after exposure to 20 J/m\(^2\) of UV-irradiation.\(^{40}\) CAT activity began to increase 6 h after UV exposure and reached its maximum 24 h after UV irradiation.

**IMPROVING THE RADIATION RESPONSE**

Optimized synthetic promoters. Deletions studies on the Erg-1 promoter resulted in a clear correlation between the number of CARG elements present in the promoter and its inducibility by serum and specific growth factors.\(^ {41}\) On this basis, Scott et al.\(^{32}\) tested the GFP inducibility by a panel of promoter containing either 4 (E4), \(6 (E6), 9 (E9)\) or \(12 (E12)\) copies of the CARG elements sequences in tandem. GFP expression levels increased with the number of CARG elements to reach maximal fold-induction levels of 2.74 in MCF-7 cells with the E9 promoter after a single radiation dose of 3 Gy. A saturation effect was reported above nine copies as the E12 promoter induced GFP levels similar to that achieved with E4 or E6.

A second class of CARG elements, while fitting the overall CC(A/T)\(_2\)GG consensus, were shown to differ slightly, resulting in variability in responsiveness. When a six copies tandem motif of two different CARG sequences (ns1 and ns2), found in the native Erg-1 gene promoter, were tested against the prototype CARG sequence, higher induction levels (2.96 -fold) following a single radiation dose of 3 Gy could be achieved.\(^{38}\) Increasing the number of this ns2 sequence from 6 (E6ns2) to 9 (E9ns2) copies further improved the radio-inducibility of the promoter and reduced basal GFP expression, when compared with the CMV, native Erg-1 and...
E4-driven constructs. The inclusion of spacer sequences (6, 10 or 18 nucleotides) within the tested promoters did not improve their radio-enhancibility. Neither did the addition of an extra SP-1 binding site upstream of the CArG sequences.38

The E9 and E9ns2 constructs were also found to be responsive to clinically relevant neutron doses (1.3 Gy) to a similar extent than that obtained after x-ray doses of 2 Gy, or even 4 Gy.39 These findings are of great interest as neutrons have a higher relative biological effectiveness than photons, making them more effective at cell killing. Neutrons also present the advantage of a reduced oxygen effect, making them more effective at treating hypoxic tumors.

Radiation-controlled Cre/Lox recombination system. Another mean of improving both levels and length of gene expression is through the use of the Cre/loxP recombination system. This approach, developed by Scott et al.,40 requires the design of two vectors. The 1st vector contained the Cre recombinase driven by a radiation responsive promoter. In the second, the GFP reporter gene and the CMV-IE cytomegalovirus immediate-early (CMV-IE) promoter and enhancer sequences are separated by a transcriptional silencer preventing gene expression. In this system, radiation-induced expression of the Cre recombinase results in the cleavage of the silencer at the Lox sites, permitting CMV-IE-driven GFP expression. This system was tested in human MCF-7 breast adenocarcinoma cells. Cells transfected with both plasmids and receiving a single dose of 5 Gy of 60 Co γ-rays showed a 40-fold amplification of the radiation signal compared to unirradiated cells. When the system was tested in a gene therapy setting, single exposure to radiation doses as low as 1 Gy were reported to be sufficient to induce the molecular switch and resulted in a growth inhibition comparable to that obtained after doses of 3 Gy without the switch.

RADIATION-INDUCED CANCER GENE THERAPY STRATEGIES

WAF-1 promoter. The WAF-1 promoter has been used to drive the expression of the inducible nitric oxide synthase (iNOS) gene as an attempt to improving the efficacy of conventional radiation therapy through the vasodilatation properties of nitric oxide. Endothelial cells and rat tail artery segments were transfected with the WAF1/iNOS construct.30 A five-fold induction of iNOS protein after a dose of 4 Gy was reported. Production of nitric oxide was proven, when artery segments, preconstricted with phenylephrin, showed a significant (65%) relaxation one hour after exposure to 4 Gy. Moreover, a clinically relevant schedule of p21((WAF1))-driven iNOS gene therapy resulted in significant sensitization of both p53 wild-type RIF-1 tumors and p53 mutant HT29 tumors to fractionated radiotherapy.41

MCF-7 cells transduced with WAF-1- driven HSVtk gene in an rAAV vector were significantly sensitized to repetitive treatment with low dose radiation (1 Gy) in the presence of the prodrug ganciclovir (GCV).31

Wild type Erg-1 gene promoter. Several authors have used the Erg1 promoter to drive the expression of the tumoricidal cytokine tumor necrosis factor (TNFα) gene. TNFα is a multifunctional cytokine with anticancer properties. It is however associated with severe systemic toxicity, preventing its routine clinical use. Localised, radiation-induced TNFα gene expression thus emerged as an attractive alternative to direct administration of the cytokine. Adenoviruses containing the Erg-1 driven TNFα were developed by several groups and tested in several cell lines as well as in preclinical studies. Human epidermoid carcinoma xenografts (SQ-20B), intratumorally infected with the adenovirus and treated by the combination of a fractionated radiation dose schedule of 40 Gy (5 Gy fractions twice a day for four days) showed increased tumor regression by up to 90% of control values, and 40% of vector and radiation alone.42 When the construct was delivered to Seg-1 human esophageal adenocarcinoma cells by an adenovirus (Ad.Egr.TNF111D), cells treated with radiation presented increased TNFα levels 72 hours later, compared with unirradiated controls. Erg-1TNFα was also developed into a second-generation replication-deficient adenoviral vector named TNErada, whose potential was recently (reviewed in ref. 43). The Egr-1 promoter was successfully developed into a suicide gene therapy strategy involving the CYP4B1/4-1pomeanol system25 and the herpes simplex virus thymidine kinases suicide gene (HSVtk).17,32 Transfected glioma cells were sensitized to the antiviral agent ganciclovir (GCV), a substrate for HSVtk, after exposure to a single radiation dose of 20 Gy. These results were confirmed in vivo.10 Adenoviral delivery of this vector to rat sarcoma R1H tumor cells, resulted in a surviving fraction to GCV of 17.3% following exposure to 6 Gy of x-rays irradiation.33

Synthetic promoters. Growth delay experiments in MCF-7 human adenocarcinoma cells transfected with the HSVtk placed under the control of the E4 promoter showed a dose-dependent increase in sensitivity to GCV (50 μM).34 Reduced surviving fractions were successively achieved when the E9 and E9ns2 promoter were used (65.7% and 61.95%, respectively), after single radiation dose of 3 Gy.34 In a nude mouse U87-MG glioblastoma xenograft model context, the E9-driven HSVtk construct resulted in a significant tumor regression delay (44.3 days) in irradiated GCV treated mice, compared to unirradiated GCV treated controls (33.4 days). Activation by neutron irradiation (1.3 Gy) resulted in an average of 63% growth inhibition in GCV treated transfect MCF-7 cells, even though the transfection efficiency was low (15–20%). This was higher than that obtained with a 2 Gy x-rays dose (48%) and similar to that obtained with the strong constitutive CMV-IE promoter.35 These promoters were also activated by exposure to the drugs cispalatin (5 and 50 μM) and doxorubicin (0.1 and 1 μM). After drug incubation, transfected MCF-7 cells were efficiently and selectively sensitised to GCV treatment. Growth inhibition of 65% and 67% were reported for each E9HSVtk and E9ns2HSVtk transfected cells, respectively, making the technique as efficient as that obtained with radiation.36

Cre/Lox molecular switch. The Cre/Lox molecular switch system has been tested in a gene therapy setting, using the HSVtk suicide gene.40 The MCF-7 cell line was first stably transfected with the radiation-inducible Cre/E4 plasmid. The cells were then transfected with the HSVtk containing vector. Through activation of the Cre/Lox molecular switch by radiation doses ranging from 0 to 5 Gy, sensitivity to GCV was considerably increased, with a growth inhibition at 2 Gy of 27%. This was considerably higher than irradiated Cre/E4 transfected cells and comparable to the levels of growth inhibition obtained when the HSVtk gene was placed under direct CMV-IE control.

COMBINATION WITH HYPOXIA

Clostridium-mediated gene therapy. Nonpathogenic, obligate anaerobic bacteria of the genus Clostridium have been used experimentally as anticancer agents because of their selective growth in the hypoxic regions of solid tumors after systemic application. It however was the possibility of controlling TNFα expression with radiation that lead to the study of bacterial radiation-responsive promoters of
genes involved in the SOC repair system, such as RecA. This gene was indeed found to be expressed in Clostridium within 15 min of hypoxic irradiation (2 Gy). When the promoter region of this gene was used to drive the expression of lacZ, significant increase of hypoxic irradiation (2 Gy). Expression levels reached a peak 3.5 h after irradiation (44%).

The success of targeted cancer therapy relies on the ability of the agent to reach the target in sufficient concentration with limited toxicity to surrounding tissues. Ionizing radiation has been shown some promise in a variety of cancer types (e.g., breast, rectal, lung, colon, skin) and phase II trials are now under way.

Only the Epo/E9 chimera showed greater EGFP expression levels (3-fold in T24 cells and 3.5-fold in MCF-7 cells) than with either responsive promoter alone. When used to drive the expression of the suicide gene herpes simplex virus thymidine kinase, GCV-mediated cell toxicity was efficient and selective following dual treatment, with surviving fraction comparable to that obtained with CMV in air. The combination of the Epo pentamer with the Egr-1 promoter resulted in enhanced hypoxic response to 1% O₂ when followed by a single 5 Gy fraction in only one of the five cell lines tested by Chadderton et al. 48

Radiation to Control Transgene Expression in Tumors

Table 1 Summary Table of the functional analysis of radiation-responsive promoters exploited to date

<table>
<thead>
<tr>
<th>Promoter</th>
<th>Radiation response elements</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>WAF-1</td>
<td>3x p53 binding sites</td>
<td>9, 11</td>
</tr>
<tr>
<td></td>
<td>1.3, 1.7 and 2.2kb upstream of 1st exon of human p21</td>
<td>30, 31, 41</td>
</tr>
<tr>
<td></td>
<td>WAF-1</td>
<td></td>
</tr>
<tr>
<td>GADD45α</td>
<td>Near p53 recognition site (+157bp/-1594bp)</td>
<td>12-14, 18</td>
</tr>
<tr>
<td></td>
<td>HPX</td>
<td>19,20,21</td>
</tr>
<tr>
<td></td>
<td>Putative site sequence in the 5' sequence of the gene</td>
<td></td>
</tr>
<tr>
<td></td>
<td>[-119/+169bp]</td>
<td></td>
</tr>
<tr>
<td>Erg-1 WT</td>
<td>4 - 5' distal CArG elements (550bp/-50bp)</td>
<td>10, 17, 23-25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>32, 33, 42, 43</td>
</tr>
<tr>
<td>Synthetic E4</td>
<td>4 CArG elements in tandem (prototype sequence: CCA×ATTGG)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>34</td>
</tr>
<tr>
<td>Synthetic E9</td>
<td>9 CArg elements in tandem (prototype sequence: CCA×ATTGG)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>35-37</td>
</tr>
<tr>
<td>Synthetic E9/n2</td>
<td>9 CArg elements in tandem (new sequence CCATATAAGG)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>38, 39</td>
</tr>
<tr>
<td>Cre/Lox system</td>
<td>WT Erg-1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>40</td>
</tr>
<tr>
<td>RecA</td>
<td>Not defined</td>
<td>44</td>
</tr>
</tbody>
</table>

The concept of gene therapy targeted to the radiation field has been proposed nearly 15 years ago 31 and has since been proven to be selective and responsive to clinically relevant radiation doses both in vivo and in vitro in a variety of cancer models. However to date, the potential of these results has not been translated into clinical efficacy and gene therapy still faces a number of issues, such as delivery, safety and selectivity. Nonetheless, the TNFerade adenoviral vector has shown some promise in a variety of cancer types (e.g., breast, rectal, lung, colon, skin) and phase II trials are now under way. 52, 53
concomitant administration of anti-VEGF, pro-inflammatory cytokines and vasoactive agents.\textsuperscript{55,56} Normalization of the vasculature by anti-angiogenic therapies indeed resulted in improved tumor oxygenation, enhanced drug absorption and increased radiosensitivity.\textsuperscript{75,76} Because the blood vessel network is responsible for systemic drug delivery, the impact of previous therapeutic intervention on the tumor vasculature is an important factor as to the efficacy of the adjuvant administration of novel therapeutics, especially since novel anti-cancer agents are more likely to enter clinical trials in patients with treatment-refractory tumors. In this context, it may be argued that the place of gene therapy in cancer treatment may be limited to a narrow therapeutic window. Nonetheless, there is now agreement that molecular approaches are unlikely to be used as a single treatment modality and novel strategies exploiting current treatment options in combination with molecular approaches have greater potential. It may indeed be postulated that the disappointing clinical results of gene therapy protocols may be due in part to the late administration of these strategies. Gene therapy is indeed often thought of as a "last-resort" treatment, when its true potential may be as a front-line complementary therapy.

As opposed to silencing strategies (e.g., siRNA, Zinc finger proteins) which require the targeting of a single gene or protein, and novel chemotherapeutic drugs, whose efficacy may be dose-limited, gene therapy promises to offer targeted therapeutic activity. The development of an effective gene delivery system to the site of therapeutic significance has however proven to be the major hurdle to the advancement of cancer gene therapy. While both virus-type vectors and nonviral vectors have been developed, the latter have not proved to be as effective as viral-based vectors in experimental systems and clinical applications.\textsuperscript{79} Mammalian cells (e.g., fibroblasts,\textsuperscript{60} dendritic cells\textsuperscript{51,62} and blood endothelial cells\textsuperscript{63}), have also been considered as vehicles for delivery of anti-cancer therapeutics. However, the desired efficacy of cell-based strategies in general has not yet been reached, and specificity needs to be improved considerably. Finally, oncolytic viruses are now emerging as safe and efficient delivery systems.\textsuperscript{64} Because radiation has been reported to enhance the infectivity capacity of adenoviral vectors,\textsuperscript{65} the oncolytic activity of viruses,\textsuperscript{66-68} the transfection efficiency of liposome-mediated gene delivery\textsuperscript{69} and hypothesised to have immunostimulatory effects,\textsuperscript{70,71} radiogenic therapy may address some of these limitations. Conditionally-replicating adenoviruses have recently emerged as powerful tools for cancer treatment.\textsuperscript{72} In the view of the recent success for HRE-controlled oncolytic adenoviruses,\textsuperscript{73} radiation-responsive promoters may become an attractive tool to control these vectors. While an ideal vector would ensure precise and efficient transgene delivery, it is unlikely that such vector could be designed to target the tumor volume uniformly due to the known disparities in the cellular level within cancer cells that form a tumor.\textsuperscript{74} Externally-controlled delivery systems on the other end, offer the possibility of biologically-independent control of therapy. While radiogenic toxicity in normal tissues in the radiation field may be difficult to avoid, unwanted transduction may be limited through re-targeting of viral vectors, through pseudo-typing of lent- and retro-viruses as well as genetic or chemical modification of the fibre knob domain of adenoviruses.\textsuperscript{75-77}Despite improvements in delivery techniques, radiation therapy is associated with limited outcome in many cancer types. While dose escalation has been proposed as an alternative, it has not delivered on its expectations and remains associated with increased side effects and limited outcome.\textsuperscript{78,79} Moreover, controversy exists as to the increased risk of radiation-induced second malignancies in surrounding normal tissues exposed to low radiation doses associated with novel delivery techniques such as intensity-modulated radiotherapy.\textsuperscript{80,82} As a result, a reduction of total dose and possibly dose per fraction through concomitant administration of novel therapeutics may have a significant clinical impact. Altered fractionation schedules have as a result also been proposed to try and maximize tumor control, while reducing early and late toxicity.\textsuperscript{83} Such strategies however are limited to tumors sites, such as the prostate, where the difference between the α/β ratio of normal and tumor tissues is large and so far these protocols are still of limited efficacy.\textsuperscript{84,85} Moreover, it now appears that the tumor characteristics, such as reoxygenation rate, may dictate the number of fractions that can be used in radiobiologically optimized fractionation protocols.\textsuperscript{86}

In the new era of improved imaging system, evaluation of tumor features is facilitated\textsuperscript{87} and monitoring of therapeutics delivery and biodistribution is progressively becoming possible.\textsuperscript{88,89} HREs have for instance been successfully used to direct GFP expression to hypoxic regions in a human prostate cancer model.\textsuperscript{90} Using magnetic resonance and optical imaging, the authors were able to create a functional MR map of the tumor studied. Dual hypoxia and radioreponsive promoters may thus have the advantage over more classical approaches as to the monitoring of vector distribution and identification of areas of resistance. Of all tumor features, hypoxia is progressively emerging as an important factor to be incorporated into theoretical modeling of tumor control probability (TCP).\textsuperscript{91} Dose escalation calculations for overcoming tumor hypoxia in prostate tumors based on clinical data have proposed escalation to 165 Gy (Cl: 153-186 Gy) (instead of 145 Gy) for 125I permanent implants and from 71 Gy to 88 Gy in 2 Gy fractions in order to achieve a TCP of 81% for the hypoxic tumor group.\textsuperscript{92} Such increase in total dose may however result in elevated toxicity and enhance morbidity. Temporal and spatial variations in the oxygen distribution, nonuniform cell density and cell proliferation during treatment have also been included in tumor modeling and shown that dose redistribution could impact on the TCP.\textsuperscript{93}

It must nonetheless be noted that radiation therapy failure is not necessarily dependent on the total dose but more likely due to biological characteristics of the tumors, such as low mitotic index, hypoxia and apoptosis resistance, that ionizing radiation alone may not be able to overcome. To address these issues, as opposed to novel therapeutics relying on systemic delivery (e.g., cytotoxic drugs), radiation sensitisers), presence of biological feature (e.g., hormones, cytokines, PSA), specific gene expression (e.g., VEGF), radiogenic therapy offers the flexibility of an externally controllable therapeutic option that can be tailored to specific tumor characteristics. In addition, while the strategies developed to date have been targeted to tumors, radiation-responsive systems could be designed for radioprotective gene therapy.\textsuperscript{94,95} As radiation therapy is progressively moving towards dose painting therapy,\textsuperscript{81,96,97} radiation-controllable vectors could easily be incorporated to target specific aspects of tumors and improve local control probability. In addition, direct administration of gene therapy vector along with seed implantation in brachytherapy protocols may be envisaged to facilitate targeted gene delivery and limit normal tissue toxicity from unspecific transduction.\textsuperscript{98}

**CONCLUSION**

Controversy as to the future of cancer gene therapy exists but the literature provides substantial evidence of its potential. While
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The scope of radiobiology will more than likely be extended to easily-accessible tumor sites (e.g., prostate, skin and breast) to limit systemic delivery and associated toxicity, there is sufficient evidence to suggest that radiogenic therapy will translate into improved patient care.

References


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