THE ROLE OF P53 AND HYPOXIA IN NUCLEOTIDE EXCISION REPAIR

THE ROLE OF P53 AND HYPOXIA IN NUCLEOTIDE EXCISION REPAIR

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Abstract

The nucleotide excision repair (NER) pathway is essential for repair of UV-induced bulky DNA lesions. NER is divided into two subpathways: global genome repair (GGR) and transcription-coupled repair (TCR). UVC radiation has been shown to result in the formation of bulky DNA lesions, which are removed by NER. Previous published reports have shown a role for the p53 tumour suppressor protein in GGR and TCR, but the involvement of p53 in TCR has been controversial. In addition, it has also been suggested that hypoxia affects NER and expression of p53. In the present work, the role of p53, hypoxia and HIF-1α in NER was investigated.

It was determined that p53 overexpression in primary human fibroblasts resulted in up-regulation of both the GGR and TCR subpathways of a UV-damaged reporter gene. Pre-treatment of cells with low UVC-fluence and p53 overexpression also induced an up-regulation of GGR and TCR. These results are consistent with a p53-dependent up-regulation of TCR and GGR of the UVC-damaged reporter gene, as well with a UV-inducible TCR and GGR that is dependent on p53 expression prior to UV treatment.

Hypoxia coupled to low pH induced a transient up-regulation of p53 expression and NER in human primary normal fibroblasts and a concomitant decrease in UVC sensitivity. In contrast, in tumour cells hypoxia coupled to low pH resulted in a delayed, but not absent up-regulation of NER, which was p53-independent and did not result in a decrease in UVC sensitivity. We report here that it is the early transient p53-dependent up-regulation induced by hypoxia coupled to acidosis in human primary normal

fibroblasts that may play a significant role in cellular UVC sensitivity. These data suggest a different cellular NER response to hypoxia compared to hypoxia coupled to low pH. The NER response to hypoxia and hypoxia coupled with acidosis was also different in primary cells when compared to tumour-derived cells.

It was demonstrated that expression of dominant-negative HIF-1α in rat prostate tumour cells results in a reduction in host cell reactivation (HCR) of a UV-damaged reporter gene when compared to that in wild-type HIF-1α cells under normoxic conditions suggesting that basal HIF-1α expression may play an important role in NER. In addition we showed that hypoxia induced an up-regulation of NER in human primary normal fibroblasts that was delayed, but not absent in TCR-deficient CSB cells, suggesting a role for hypoxia in up-regulation of the GGR pathway of NER of a UV-damaged reporter gene. In contrast, HIF-1α-overexpression under conditions of hypoxia resulted in a down-regulation of NER in normal fibroblasts, which was delayed, but not absent in CSB fibroblasts. These results suggest that HIF-1α and CSB are involved in a hypoxia-induced NER response.

This work provides further evidence that both GGR and TCR are p53-dependent. In addition, this study provides evidence that hypoxia and hypoxia coupled to acidosis can up-regulate NER in both primary and tumour cells, and that HIF-1α and the CSB protein play an important role in a hypoxia-induced NER response.

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List of Abbreviations

α-MEM	α-minimum essential media
β-gal	β-galactosidase
Ad	adenovirus
ARNT	Per-Aryl Hydrocarbon Receptor Nuclear Translocator
ATP	adenosine triphosphate
BER	base excision repair
BRCA1	breast cancer 1 gene
CBP (CREB)	cAMP-response element-binding protein
CPD	cyclobutane pyrimidine dimer
CPRG	chlorophenol red-β-D-galactopyranoside
CS	Cockayne Syndrome
CSA	CS group A
CSB	CS group B
D_{37}	dose that gives a survival fraction of 0.37 (e-1)
D_{50}	does that gives a survival fraction of 0.5
DDB1	damaged DNA binding protein 1
DDB2 (also known as XPE)	damaged DNA binding protein 2
DNA	deoxyribonucleic acid
DSB	double-strand break
E1	early 1
ERCC	excision repair cross-complementing
FBS	fetal bovine serum
GGR	global genome repair
HCMV	human cytomegalovirus
HCR	host cell reactivation
HIF-1α	hypoxia-inducible factor 1 alpha
HIF-1β	hypoxia-inducible factor 1 beta
Hhr23b	human homologue of Rad23 B
HR	homologous recombination
HRE	hypoxia-response element
HSER	heat shock-enhanced reactivation
IE	immediate early
LFS	Li-Fraumeni syndrome
lacZ	gene that encodes for the enzyme β-galactosidase
MCMV	murine cytomegalovirus
MDM2	murine double-minute 2

MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MMR	mismatch repair
MOI	multiplicity of infection
NER	nucleotide excision repair
NHEJ	non-homologous end-joining
ODD	oxygen-dependent degradation domain
PBS	phosphate-buffered saline
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
pfu	plaque-forming unit
6-4PP	pyrimidine (6-4) pyrimidone dimer
RNAPII	ribonucleic acid
RNAPII	RNA polymerase II
RPA	replication protein A
SE	standard error
SSB	single-stranded break
SV40	simian virus 40
TAD	transactivation domain
TCR	transcription-coupled repair
TFIIH	transcription factor-II H
TTD	trichothiodystrophy
UV	ultraviolet
UV-DDB	UV-damaged DNA binding protein
UVER	UV-enhanced reactivation
VEGF	vascular endothelial growth factor
VHL	von Hippel-Lindau tumour suppressor protein
XP	xeroderma pigmentosum
XPA-XPG	XP group A-G

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CHAPTER 1

Introduction

1.0 UV Damage, cancer and DNA repair

1.1 Overview

Understanding tumour progression in its complexity has been the unfailing task of many researchers. The cell is constantly faced with protecting its contents from exogenous insults and endogenous metabolic factors. Each day on average the cell must cope with more than 10^4 lesions caused by spontaneous decay, cellular metabolism and stalled DNA-replication forks (1). Additionally, humans are exposed to environmental mutagens such as tobacco smoke and UV light, adding further damage to the genome and threatening its stability. It is not surprising that there is a strong link between the cell's capacity to repair damage and developing cancer. Unrepaired damage can lead to mutagenesis and all tumour cells contain mutations that will render them unstable and cause them to grow uncontrollably. However, complex DNA repair systems are in place to counteract the damage imposed on the genome.

1.2 UV radiation and damage

The leading environmental factor responsible for serious DNA damage and increasing the risk of developing skin cancer is chronic exposure to sunlight. Squamous cell carcinomas (SCC), melanomas (CMM) and non-melanoma skin cancers (basal cell carcinoma; BCC) are some of the most frequent cancers induced primarily by exposure to the sun (reviewed in (2,3)) and their prevalence is on the rise (reviewed in (4)). The sunlight's electromagnetic radiation spectrum is composed of ultraviolet (UV) light, visible light and infrared (reviewed in (5)). UV radiation forms 45% of the total light spectrum and is

divided into three distinct sections: UVC (200-280 nm), UVB (280-320 nm) and UVA (320-400 nm) as illustrated in Figure 1.1. The ozone layer absorbs UVC and most of the UVB light preventing their reach to the earth's surface (6). However, the current depletion of the ozone layer increases the amount of UVB reaching the planet (7). A major effect of UVB exposure on the skin arises through direct damage to the nucleic acids. In contrast, damage from UVA exposure is predominantly indirect through the formation and action of reactive oxygen species on the DNA (8).

Although UVC is completely absorbed by the ozone layer and therefore has no environmental relevance, there are several significant reasons for the frequent use by researchers of germicidal lamps, which emit light at the wavelength corresponding to the UVC spectrum (predominantly 254 nm) (reviewed in (9)). First, the maximum absorbance of DNA is at 260 nm and second, since UVC germicidal lamps emit at 254 nm wavelength, it minimizes damage to proteins, which do not efficiently absorb light at that particular wavelength (9). UVC is also of higher intensity and therefore produces lesions more efficiently (10) and produces the same type of lesions that the other, longer, wavelengths of UV radiation produce (11). UV radiation induces two types of lesions: the cyclobutane pyrimidine dimer (CPD) and the pyrimidine (6-4) pyrimidone dimer (6-4PP), distinguished by the position and the number of bonds formed as shown in Figure 1.2. CPDs form through two bonds and the helical distortion is milder (7-9°) than the 6-4PPs. On the other hand, 6-4PPs result through the formation of one bond, which causes a 44° DNA bending (12).

1.3 UV-induced cancer

UV-induced DNA lesions, if not properly repaired, will lead to mutations in cells of the epidermal layer leading to cancer. Mutations in the tumour suppressor gene, p53, have been detected in human non-melanoma skin cancers such as BCC and SCC and have been reported by several investigators (13-16). Studies have shown that p53 mutations were present in 90 % of the SCCs and 50 % of the BCCs examined (17,18) as well as in xeroderma pigmentosum (XP) patients (19,20). BCC and SCC develop primarily on areas that were exposed to the sun with increased risk to individuals that have sensitive skin, burn easily and therefore do not tan frequently (6,21,22). The most prevalent type of cancer in North America is BCC, but it rarely becomes metastatic or invasive, while SCC is not as common, it can metastasize (23,24). Cutaneous melanomas (CMM), on the other hand, are malignant and the incidence and mortality rates in the last decade have risen (25). A direct relationship between UV exposure and CMM incidence remains controversial, but has been suggested by several studies (26-28).

1.4 The role of DNA repair in cancer prevention and treatment

The extent of damage from normal cellular processes and environmentally-incurred sources would destroy cells if it wasn't for their ability to repair it. XP patients, along with the numerous studies showing that consistent unrepaired or misrepaired DNA damage leads to genomic instability, demonstrate the important contribution of cellular DNA repair pathways to preventing induction of cancer. Hence, cancer prevention depends on the equilibrium between the magnitude of DNA damage and the capacity of

error-free DNA repair pathways. If one side of the equilibrium is overwhelmed, the consequences can be cancer induction.

The main therapeutic method used to treat cancer is by causing DNA damage in tumour cells by chemotherapy and radiotherapy. Cells derived from patients with defects in the homologous recombination repair pathway were shown to be less resistant to ionizing radiation than repair-proficient cells (29). Greater benefit can result from tailoring treatments by using DNA repair pathway deficiencies in tumour cells to our advantage. For instance, tumour cells with a certain deficient DNA repair pathway can be made vulnerable by targeting the alternate proficient DNA repair pathways which they rely on. In contrast, surrounding normal tissue cells would be less sensitive to DNA damaging agents because they would still possess a proficient DNA repair pathway that is deficient in tumour cells (reviewed in (30)). This would allow for a more specialized treatment plan in which tumour cells are the target and normal cells are spared. Therefore, not only does DNA repair play a crucial role in cancer prevention by maintaining genomic integrity, but in the event of cancer induction it can be a key determinant in developing more specialized cancer therapies.

2.0 Cisplatin

Cisplatin (cis-diamminedichloroplatinum (II)) is a chemical that can induce DNA damage. This particular cross-linking agent was the first platinum compound permitted for cancer treatments despite its toxicity to the kidneys and the gastrointestinal tract (31,32). Cisplatin binds to the DNA forming an adduct, resulting in either intrastrand or

interstrand cross-links, which in turn can cause a distortion and unwinding of the helix structure as shown in Figure 1.3A (33). Cisplatin is relatively stable and is activated inside the cells due to the low chloride conditions of less than 100 mM concentration (34). The exact mechanism of how cisplatin binds to DNA is believed to start with the attack of the electronegative N7 guanine atom on the platinum resulting in a chloride being released. Subsequently, platinum binds to guanine and in some rare cases to adenine, forming DNA adducts (35). Metastatic testicular cancer treated with cisplatin is one of the most successful therapies with greater than ninety percent of the patients being cured (36). Cisplatin sensitivity has been detected in testicular carcinoma cell lines when compared to other cell lines and interestingly these cell lines showed a deficient NER due to low expression levels of the DNA repair proteins ERCC1 and xeroderma pigmentosum complementation group F (37).

Although cisplatin therapy for the aforementioned cancers is efficient, the toxicity of the drug poses a real clinical problem for patients. This led to the use of another platinum-based chemical, carboplatin (cis-diammine-[1,1-cyclobutanedicarboxylato]) (reviewed in (38)). The only difference between carboplatin and cisplatin is the leaving group, which is cyclobutanedicarboxylate for carboplatin instead of chloride as shown in Figure 1.3B (39). Carboplatin proved to lack nephrotoxicity and is an equally efficient antitumour drug. Randomized clinical trials have shown that the use of cisplatin and carboplatin displayed comparable survival rates for ovarian cancer patients (40).

Another limitation to cisplatin became evident soon after its approved use in clinical trials, that of some tumours becoming resistant to cisplatin treatment. Therefore,

it is important to investigate the role of repair mechanisms in removing cisplatin-induced DNA adduction, in order to understand how tumour cells become resistant during the course of cisplatin therapy. Studies suggest that since the sensitivity of testicular cancer cells is due to a deficiency in DNA repair (37), cisplatin-resistant cancer cells may survive due to increased repair of lesions. Johnson *et al.* (1994) and colleagues showed that cisplatin resistant cell lines exhibited increased removal of cisplatin-induced DNA adducts when compared to the parental cell line (41). Since nucleotide excision repair (NER) is the main mechanism of repair of cisplatin-induced DNA damage it is not surprising that studies have found a correlation between cisplatin resistance and increased NER activity in human ovarian cancer cells (42) and several other cell lines (43). On the other hand, loss of MMR gene, *hMLH1* in human colon tumour cell line renders cells more resistant to cisplatin-based treatment when compared to MMR-proficient cells (44), indicating that loss of repair function can also contribute to tumour resistance to chemotherapy. These studies underline the important role that DNA repair plays in cancer prevention and finding more specific cancer therapy methods.

3.0 Nucleotide excision repair pathways

3.1 DNA repair pathways

There are four main DNA repair pathways that are important in maintaining genomic stability in the cell: base excision repair (BER), mismatch repair (MMR), double-strand break repair such as homologous recombination (HR) and nonhomologous end joining (NHEJ), and nucleotide excision repair (NER). BER is responsible for

repairing insults that result in oxidative damage to bases or causes DNA single and double-stranded breaks (45). MMR handles base-base mismatches, which disrupt the hydrogen bonding and hence distort the helical structure, but essentially are not DNA lesions. The most lethal DNA insults include double-strand DNA breaks (DSB) and two pathways, HR and NHEJ are used by the cell to repair these lesions. A cell using one pathway over another to repair a double strand break is dependent on what type of breaks or in what part of the cell cycle the break occurred (46,47). NER is known to be a pathway that can handle a broad variety of lesions by sensing the distortion they cause in the DNA helical structure. Once it recognizes the type of damage, it makes incisions on both sides of the damaged strand and it excises a short 25-30 nucleotide long patch containing the lesion. The gap will then be filled by DNA polymerases using the undamaged strand as a template and ligation occurs (45). Therefore, NER is composed of five steps that are common among all organisms. This kind of repair mechanism can therefore work efficiently with only one common set of proteins that can handle different types of lesions. The lesions repaired by NER are most commonly a result of UV radiation causing DNA dimers, or chemicals, which will covalently bind to DNA resulting in a bulky DNA adduct. Specifically, NER repairs CPDs and 6-4PPs, which are caused by UV radiation.

3.2 Nucleotide excision repair

Excision repair was discovered roughly 45 years ago when studies showed that bacteria such as *Escherichia coli* after UV exposure could remove small fragments of

DNA containing UV-induced damage (48,49). Even though studies on repair done in *E. coli* were instrumental in elucidating the mechanism and overall steps of NER, there is little homology between the two NER in prokaryotes and NER in eukaryotes, despite being a conserved repair mechanism (reviewed in (50,51)). There are many eukaryotic NER proteins that do not correspond to a functional counterpart in prokaryotic repair and the mechanism of mammalian NER is far more complex than that of *E.coli* (reviewed in (50,52)).

It was James Cleaver who recognized the connection between a deficiency in DNA repair and UV-sensitivity (53) that ultimately leads to tumourigenesis. James Cleaver used xeroderma pigmentosum cell lines to show that UV-sensitivity is related to deficiency in NER in these patients (53). These results supported the notion that DNA repair played a pivotal role in the maintenance of genomic stability and preventing neoplastic transformation. The significance of NER is demonstrated by the existence of patients with autosomal recessive syndromes characterized by different deficiencies in NER, which include xeroderma pigmentosum (XP), Cockayne syndrome (CS) and trichothiodystropy (TTD). These syndromes are the result of a deficient protein involved in NER and therefore the outcome is a variety of serious clinical consequences including photosensitivity, neurodevelopmental abnormalities, ocular anomalies, decreased fertility and skin cancer in XP patients (54-56).

NER is divided into two pathways: global genome repair (GGR) and transcription-coupled repair (TCR). GGR eliminates lesions from the entire genome including from the non-coding region of the genome, the non-transcribed strand of active

genes and from silent genes. On the other hand, TCR is responsible for repairing damage with higher priority from the transcribed strand of active genes. The only difference between the two NER pathways is the way the lesion is recognized. The stalled RNA polymerase II acts as a signal in TCR for recognizing the presence of a damaged site (57), while GGR uses a complex composed of three subunits, XPC, HR23B and centrin 2, which identifies the helical distortion and recruits the repair machinery (58). A representation of these two subpathways is shown in Figure 1.4.

Early reports showed that RNA synthesis is temporarily blocked after UV-irradiation and that the recovery of RNA synthesis occurs faster in normal cells compared to that in CS and some XP cells (59). The slower recovery of RNA synthesis in CS cells was found to result from a deficiency in TCR of DNA lesions from the transcribed strand of active genes, rendering the cells sensitive to UV radiation (60). Increased efficiency of repair of the transcribed strand of active genes when compared to a sequence in a non-coding region downstream was first shown in Chinese hamster ovary cells (60). Subsequently, a preferential repair of the transcribed strand of active genes was also shown in human cells (61), in *E. coli* (62) and in *Saccharomyces cerevisiae* (63,64).

3.3 Global genome repair

The XPC-HR23B-centrin2 complex is the first component of the repair machinery arriving at the site of damage and necessary for recruiting subsequent repair proteins to the damaged site (65-67) (see Table 1.1). The 125 kDa XPC protein exhibits DNA

binding activity with a preference for damaged DNA (68). Uchida *et al.* (2002) demonstrated that the carboxyl terminal domain plays an important role in recruiting TFIIH to the lesion site (69). A more recent report shows that XPC binds opposite the lesion to the single-stranded DNA of the undamaged strand (70), suggesting a counterintuitive method of recognizing lesions.

In whole cell extracts, XPC is mostly found bound to HR23B, a 58 kDa protein (71), which is one of the two human homologues of the Rad23 protein involved in NER in yeast. Both HR23A and HR23B have been found to bind to XPC (58,72), although HR23B is most commonly associated with XPC in a complex. The exact function of HR23B is not entirely known, although it was shown that in vitro HR23B can stimulate XPC activity (73). The two human homologues of the yeast Rad23 have an N-terminal ubiquitin-like and two ubiquitin-associated domains that are indispensible to NER (74), implicating them in degradation (75). The third protein found within the XPC-HR23B complex is centrin 2, a protein that was shown to help the XPC-HR23 complex stability (58) and improve NER activity (76).

The XPC-HR23B complex is able to detect a variety of lesions, but in some instances, such as with CPDs, its recognition ability is reduced (77). CPDs cause a relatively mild distortion in the double helix compared to 6-4PPs (12), making it harder for the XPC-HR23B complex to recognize them. It is in enabling the XPC-HR23B complex to recognize the modest CPD damage that the UV-damaged DNA-binding complex (UV-DDB, also known as DDB) plays an important role. The heterodimeric UV-DDB complex is composed of a 127 kDa protein subunit (p127/DDB1) and a 48 kDa

subunit (p48/DDB2) (78). Mutations in the DDB2 subunit of the UV-DDB complex result in a clinical phenotype observed in xeroderma pigmentosum group E patients (79). XPE patients show mild sensitivity to UV-radiation and are at high risk of getting skin cancers (80). Despite this, the XPE protein is not necessary for NER *in vitro* (81), but the clinical phenotype of XPE patients underlines its importance *in vivo*. It was shown that UV-DDB binds to CPDs, 6-4PPs and other lesions that lead to excision of CPDs and therefore it has been suggested that as a consequence, the XPC complex can then be recruited to the site of damage (82). This supports the hypothesis that UV-DDB complex promotes recruitment of the XPC-HR23B complex to the site of lesion and its ability to recognize lesions that otherwise would not have been easily identified by the XPC complex (83).

The next step in the NER assembly machinery is the opening of the DNA around the lesion, which starts with transcription factor IIH (TFIIH). TFIIH is composed of ten protein subunits including XPB, XPD, p62, p52, p34, p44, TTD-A, cyclin H, cdk7 and MAT1 (84-88) and it is known for its role in transcription initiation of RNA polymerase II (RNAPII) (89), NER (90) and cell cycle regulation (91). XPB and XPD display ATPase and helicase activities with XPB unwinding the DNA in the 3' to 5' direction and XPD performing the same function in the opposite direction (92-94).

The three TFIIH subunits, cyclin H, cdk7 and MAT1, form a complex known as cyclin-activated kinase (CAK) and this in turn can phosphorylate the C-terminal domain of the large subunit of RNAPII facilitating its entrance in transcription elongation (95). The other five subunits (p62, p52, p44, p34 and TTD-A) form a ring-shaped structure

with XPB and XPD, to which the CAK complex attaches (96). It is thought that the five subunits stabilize the complex further and can activate and inhibit each other, but more specific functions have yet to be established (85,97). Overall, the TFIIH complex is indispensable to NER as it ultimately facilitates a 20 to 30 nucleotide-long opening surrounding the damaged site. For example, it has been shown that an opening of the helix around the lesion was absent when the TFIIH complex contained mutated XPB and XPD in cell extracts (98).

Two other important proteins involved in both pathways of NER are XPA and replication protein A (RPA). XPA binds to TFIIH (99) and RPA (100), and it is a DNA-binding protein showing preference for damaged DNA (101). The exact role of XPA is still to be determined, but it has been shown to displace the XPC complex from the repair machinery (102) and may be involved in identification of the lesion-containing strand (68). RPA is a single-strand DNA-binding protein, which attaches to the undamaged DNA (103) in order to stabilize single-stranded DNA intermediates and eliminate secondary structures. XPA and RPA are crucial proteins for the assembly of the NER pre-incision complex, but their exact roles are not fully elucidated. After the arrival of XPA and RPA, the following step involves the endonucleases XPG and XPF, which are responsible for the incision of the damaged strand. XPG performs the cut at the 3' end of the lesion (104) and XPF-ERCC1 is responsible for the incision on the 5' end (105). The XPF-ERCC1 incision step is dependent on the presence of XPG (106) and it is the XPF protein of the XPF-ERCC1 complex that conducts the endonuclease function (107). Once

the damaged fragment is removed, the DNA synthesis machinery starts the synthesis of the new fragment.

3.4 Transcription-coupled repair

TCR and GGR proceed through the same modes of action, by recruiting TFIIH and subsequently XPA and RPA. The XPC-HR23B complex is thought to be displaced at this point, and with the arrival of XPA and RPA an opening around the lesion allows the incision proteins, XPG and XPF-ERCC1 to enter (reviewed in (108)). As mentioned earlier, the difference between these two NER pathways is the mode of damage recognition. Since lesions on the transcribed strand block transcription (109), it is the stalled RNA polymerase that acts as a signal in TCR (57) for the repair machinery to assemble instead of the XPC-HR23B complex of the GGR pathway. TCR is a more specialized pathway, exhibiting rapid and preferential removal of lesions from the transcribed strand of active genes. TCR not only has to recognize the stalled RNA polymerase at the lesion, but it also needs to recruit the repair proteins to facilitate repair of the damage and subsequently restart the transcription machinery. It is no surprise that mutations in the proteins involved in TCR lead to the Cockayne syndrome (CS), a condition in which patients suffer from photosensitivity, neurological dysfunction and growth retardation.

The mutations causing CS are found in genes responsible for encoding the proteins CSA and CSB. Cells from CSA and CSB patients cannot repair lesions from the transcribed strand even though they are still able to remove DNA adducts from the entire

genome. The specific steps after a stalled RNA polymerase caused by a lesion and the involvement of CSA and CSB are not clearly elucidated. However, it is thought that CSB may recruit NER proteins (110), chromatin remodelers (111) and the CSA-E3—ubiquitin ligase complex to the stalled RNAPII (110,112,113). CSB is a 169 kDa protein with an ATPase domain composed of seven conserved helicase motifs (114) and is a member of the SWI/SNF family of ATP-dependent chromatin remodellers (115). CSB is similar to the SWI/SNF family members as it exhibits DNA-binding activity and ATPase activity, but not helicase functions (116).

A mutation in the ATPase motif of CSB resulted in impaired RNA synthesis recovery and inhibition of repair, indicating the importance of the ATPase motif for normal function of CSB in vivo (117). Studies conducted by van den Boom and colleagues (2004) have demonstrated that CSB can interact with the transcription machinery, a function that was prolonged upon DNA damage, supporting the model that CSB travels with the RNAPII from initiation and binds to it at the necessary times (118). Another report has shown that CSB cannot promote elongation past a stalled RNAPII at a damaged site, but with the addition of one nucleotide, CSB can activate a stalled RNAPII for transcription elongation (119). Therefore, the role of CSB is critical in TCR and mutations in the CSB gene are the leading cause for the CS phenotype, but the exact role of CSB still needs to be discovered. In contrast, CSA, although it interacts with CSB in vitro (120), is dispensable in recruiting NER proteins to the stalled RNAPII. CSA was shown to interact with TFIIH (120) and XAB2 (XPA binding protein 2) (121), a protein

important in pre-mRNA splicing and transcription, and known to interact with XPA (122).

4.0 Repair-deficient syndromes

4.1 Overview

Mutations in the genes encoding proteins of the NER pathways can result in three main autosomal recessive syndromes that have been identified as xeroderma pigmentosum (XP), Cockayne syndrome (CS), and trichothiodystrophy (TTD). The genes involved in encoding NER proteins have been identified and characterized through studies on both human and rodent cell lines and their protein products have been named with respect to their specific complementation group. For the XP syndrome seven complementation groups have been described and are classified as XPA to XPG, plus a variant form of the disease, XPV. The latter is a hereditary disease that results from a deficiency in the DNA polymerase eta and as a consequence XPV cells have to use more error-prone polymerases causing mutations to build up during DNA replication (123). There are two complementation groups that have been characterized to CS, known as CSA and CSB, and mutations in their genes affect TCR, but not GGR. Mutations in three genes, XPB, XPD and TTDA have been associated with TTD. Nine patients have been found to show pathology characteristic of both XP and CS with mutations in XPB (XPB/CS), XPD (XPD/CS) and XPG (XPG/CS) (124). The degree of clinical variation in all of the XP/CS patients is vast and suggests that different mutations in these genes can

cause specific phenotypes. Clinical pathology of combined XP/TTD has been described in two patients (125) underlying the diversity of phenotypes caused by XPD mutations.

4.2 Xeroderma pigmentosum

Xeroderma pigmentosum (XP) is a hereditary disease associated with extreme sensitivity to sunlight that causes skin atrophy, pigmentation abnormalities and approximately 1000-fold increased risk of skin cancer, including basal and squamous cell carcinomas and melanomas (reviewed in (126)). The frequency of XP is 1 in 250,000 individuals from the Western population and 1 in 40,000 individuals from Japan and North Africa (45). The onset of the syndrome occurs around two years of age when extreme sunburn is observed (127) and cancer has been detected as early as 8 years old, reducing the lifespan significantly, by as much as 30 years (127). Interestingly, among the different NER-deficient syndromes, only the XP patients exhibit a higher incidence of skin cancer. It has also been observed that XP patients are at greater risk of developing internal cancers, especially of the lung or gastro-intestinal tract (128). These clinical outcomes reflect the importance of NER in handling insults from air pollutants and food carcinogens (reviewed in (129)).

In addition, neurological anomalies are observed in 20% of XP patients, which can become prominent as early as childhood and are due to neuronal degeneration (130). The severity of the disease depends on the type of mutation and the impact that it has on NER. For example, patients with mutations in the XPA gene, which disrupts both NER pathways, display more severe neurological symptoms when compared to XPC patients,

which have a deficient GGR, but not TCR. In addition, milder NER defects, such as those seen in XPC and XPF patients, do not tend to cause any neurological dysfunction until late adulthood (52) or none at all in XPE individuals (79). Cases of severe neurological anomalies could be the result of neuronal death brought on by deficient DNA repair of lesions induced endogenously in the nerve cell (131). XPA, XPB, XPD and XPG individuals show severe clinical phenotypes due to the extent of NER affected since those proteins are involved in the common steps for both TCR and GGR.

4.3 Cockayne syndrome

Cockayne syndrome (CS) is the second disorder characterized by UV sensitivity. CS is a rare neurodegenerative disorder, which was first observed and described in 1936 by Edward Alfred Cockayne (132). Afflicted patients, aside from cutaneous photosensitivity also exhibit severe neurological abnormalities due to degeneration of white matter, progressive growth failure, microcephaly, systemic growth failure, retinal pigmentary degeneration, cataracts and tooth decay. The clinical manifestations observed in CS patients are resonant with traits of normal aging. Prenatal growth failure has been detected in CS and the first symptoms of CS can be visible as early as one year of age (55). The average life expectancy of CS patients is approximately 12.5 years due to the progressive multi-organ deterioration making the patients susceptible to respiratory infections causing death (55). In comparison to XP, CS patients do not have an increased risk of developing skin cancer.

CS results from mutations in either the CSA or CSB gene and therefore CS patients lack a proficient TCR. In contrast to XP patients, CS patients are not predisposed to cancer. In addition, the neurological and developmental abnormalities observed in CS patients cannot be entirely explained by TCR deficiency in NER. The discovery that in cell-free extracts a small proportion of RNAPII associates with CSB (133), suggesting a non-essential role for CSB in transcription, has led investigators to propose that the CS phenotype may be linked to moderate impairment in transcription. In addition, when $CSB^{-/-}$ knockout mice, which display milder neurological dysfunctions (134) compared to human CS, are crossed with XPC or XPA knockout mice that do not exhibit any neurological anomalies (135,136), the resulting crossed mice display combined symptoms that include neurological problems.

4.4 Trichothiodystrophy

Trichothiodystrophy (TTD) is the third hereditary disease characterized by brittle hair due to a deficiency in sulfur-rich proteins (137) and scaling of the skin (56). This genetic disorder can also cause decreased fertility, short stature, microcephaly, increased photosensitivity and intellectual impairment (138). Although all TTD patients show sparse, brittle hair, other clinical phenotypes range in severity and only approximately half of the patients display photosensitivity (138). The photosensitive TTD cases are due to a deficiency in NER caused by mutations in three genes coding for protein subunits in the TFIIH complex: XPB (139), XPD (140) and TTDA (84). The most common mutations in TTD patients are found in the XPD gene with the most mutations causing

disruptions in the C-terminal region of the protein (141). Since interaction of another TFIIH subunit, p44 with XPD occurs at the C-terminal region in order to activate the XPD helicase activity, mutations in that region eliminate XPD stimulation (142). Despite the photosensitivity that occurs, no malignancies have been detected in TTD patients (56).

4.5 Involvement of nucleotide excision repair proteins in base excision repair

An interesting aspect of some NER repair proteins is their involvement in other DNA repair pathways such as BER. Some reports have suggested that the proteins RPA and XPG also have an important role in repair of oxidative damage through BER (143-145). For example, fractionation experiments using human whole cell extracts revealed that repair of AP sites was greater in the presence of both RPA and the proliferating cell nuclear antigen (PCNA), a critical component for BER of AP sites, than when the proteins were added separately (143). These data suggested a role for RPA in PCNA-dependent BER of AP sites. Klungland and colleagues (1999) reported that XPG was able to stimulate activity of a BER protein, hNth1, and may enhance the ability of hNth1 to bind to damaged DNA (144). Moreover, other studies have also implicated XPG in the BER pathway (146,147). Another link between NER proteins and BER was demonstrated through the discovery of an interaction between the BER protein, responsible for excision of DNA lesions such as N-methylpurines, MPG (also known as 3-Methyladenine-DNA Glycosylase), and hHR23 proteins (148).

The hHR23 protein binds to XPC to form the XPC-hHR23B complex essential for proficient GGR (58,73). In addition, interaction of the XPC-hHR23B complex with thymine DNA glycosylase important for inducing repair of guanine-thymine mismatches through BER has been reported (149). Several researchers have suggested an involvement of CSB in BER (145,150-152) as repair of 8-oxoguanine was reduced in CSB mutant cells (145,152). Furthermore, it was demonstrated that CSB can associate with BER proteins including poly (ADP-ribose) polymerase 1 (PARP-1) (153) and apurinic/apyrimidinic (AP) endonuclease (APE1) (154).

The multi-functional aspect of these proteins can provide an explanation for the differences in clinical phenotypes observed in NER disorders. For instance, patients with defects in TCR proteins such as CSA and CSB and patients with XPG mutations manifest numerous and complex clinical symptoms (55,146). Deficiency in BER results in an increase in oxidative damage, which has been suggested to contribute to premature aging (155). Since CS patients exhibit premature aging, and oxidative damage was detected in brain cells of individuals with NER-deficient disorders (156), then this could support an involvement of CSB in BER. The emergence of XP/TTD and XP/CS patients that show combined clinical phenotypes (reviewed in (108)) underlines the complexity of NER protein functions and illustrates the importance of characterizing the specific roles of NER proteins as they are involved in different DNA repair pathways.

5.0 The p53 tumour suppressor protein

5.1 Overview

Tumour suppressor proteins play a significant role in maintaining normal cellular growth and differentiation. Mutations in tumour suppressor genes can prevent the protein product from regulating cellular functions and can therefore result in tumour growth. One of the tumour suppressor proteins is p53, a protein that is altered or inactivated in over 50% of cancers, including non-melanoma and melanoma skin cancers (reviewed in (2)). The p53 tumour suppressor protein plays a key role in maintaining genomic stability through cell cycle arrest, apoptosis and its involvement in DNA repair pathways such as NER.

The approximate half-life of p53 is between 20 and 30 minutes, but once stimulated by stress, its expression can increase significantly (157). The 53 kDa protein is activated by phosphorylation of its residues consequently preventing ubiquitylation by HDM2, the human counterpart of the mouse double-minute 2 (MDM2). HDM2, an E3 ubiquitin ligase, negatively regulates p53 by binding to it and targeting it for degradation (158). Reports have demonstrated that UV radiation phosphorylates p53 at several residues such as Ser15, Ser20, Ser33, Ser37, Ser46 and Ser392 (159-162).

During normal cell functions p53 is found at low levels, but accumulates in the cell in response to stress such as UV radiation (163), and as a transcription factor, regulates the expression of many different genes (reviewed in (2)). Expression of p53 also increases in response to ionizing radiation (164), heat shock (165) and hypoxia (166-170). A link between p53 and NER was identified when studies reported that p53 can

bind to different subunits of the TFIIH complex, including XPB and XPD, as well as the CSB protein involved in TCR (171-173).

5.2 The role of p53 in global genome repair

Support for the involvement of p53 in repair of UV damage by NER came from studies showing that cells lacking functional p53 displayed less repair of UV-induced DNA damage (174-176). Smith *et al.* (1995) reported reduced repair of UV-induced DNA damage in human colon carcinoma RKO cells, which have inactivated p53 function due to the human papillomavirus E6 or a dominant-negative p53 transgene (174). The same year, Ford and Hanawalt (1995) showed that cells homozygous for mutant p53 displayed a decrease in repair of UV-induced CPDs from the overall global DNA when compared to the heterozygous for p53 mutation Li-Fraumeni skin fibroblasts or normal cells, but TCR of CPDs was not affected (175). Experiments using a p53 homozygous mutant cell line transfected with a tetracycline regulated wild type p53 cDNA demonstrated the specificity of the effect of p53 on GGR (177). It was shown that regulated wild type p53 expression can restore normal repair of CPDs and 6-4 PPs of the global DNA, but did not affect TCR of CPDs (177). Reduced GGR was also reported in p53 mutant transgenic mice (178) and p53 null mice (179). These results support a role for p53 in GGR and further studies show that p53 modulates regulation of GGR through transactivation of the *p48-XPE* (83) and *XPC* (180) genes. A p53 DNA binding region was discovered in the promoter segment of the *DDB2* gene coding for XPE, which further strengthened the model of the p53 role in GGR (181). Furthermore, it has been

reported that UV radiation stimulated XPC mRNA and protein expression levels in p53 wild type cells, but not in p53-deficient cells (180).

5.3 The role of p53 in transcription-coupled repair

Although it is now well accepted that GGR is p53-dependent, the role of p53 in TCR has been rather controversial. However, compelling evidence is accumulating suggesting that p53 can play a role in regulating the repair of UV-induced DNA damage from the transcribed strand of active genes, through an effect on TCR. DNA damage in actively transcribed genes results in inhibition of RNA synthesis and so to study how this can affect the levels of p53 expression, Yamaizumi and Sugano (1994) used α-amanitin, an inhibitor of transcription in normal and XP cells (182). They found that α-amanitin treatment resulted in increased p53 expression in both normal and XP cells suggesting that nuclear p53 accumulation can result from UV-induced DNA damage in active genes (182). Further evidence for a role of p53 in TCR was demonstrated by Wang *et al.* (1995), who reported that p53 can bind to CSB, the protein important for proficient repair of the transcribed strand of active genes (172).

Previously, our laboratory demonstrated that adenovirus DNA synthesis in UV-irradiated human GGR-deficient XPC fibroblasts was similar to levels observed in human normal fibroblasts, but not similar to levels found in XPA cells, CSB cells, SV40-transformed fibroblasts, Li-Fraumeni cells (LFS) heterozygous or homozygous for mutant p53 (183). These results suggested that TCR may be involved in the cellular capacity to sustain DNA synthesis after UV-irradiation and that it is p53-dependent. In

addition, our laboratory has shown that heat shock-enhanced reactivation (HSER) and UV-enhanced reactivation (UVER) stimulated host cell reactivation (HCR) of UV-damaged reporter gene in normal fibroblasts but not in Li-Fraumeni cells lacking functional p53 (184). Since it has previously been demonstrated that HSER and UVER rely on TCR (185,186), the results suggest that p53 mutant cells lack the capacity to induce TCR through UV or HS. A similar response was reported in murine fibroblasts treated with UVB radiation (187).

Further studies employing a sensitive ligation-mediated PCR assay in order to quantify the repair of UVB-induced DNA damage, showed that p53-deficient LFS cells, human lung fibroblasts with inactivated p53 by expressing the human papillomavirus (HPV) showed less removal of UVB-induced CPDs from both the transcribed and non-transcribed p53 loci when compared to normal cells (188). However, another study showed that the role of p53 in TCR might be wavelength dependent as ligation-mediated PCR showed a reduced removal of UVC-induced CPDs in the non-transcribed strand of the c-jun and hprt loci in p53-deficient cells, but not in the transcribed strand (189). Therefore, further studies will help in clarifying the role of p53 in TCR.

6.0 The tumour microenvironment

6.1 Hypoxia

The heterogeneous microenvironment of the tumour affects the tumour's invasiveness, metastasis potential, radioresistance and risk of recurrence. The greatly dynamic tumour microenvironment consists of cell populations with differences in

cellular metabolism and genomic instability that are exposed to differing levels of pH, oxygen and nutrient amount. Low oxygen conditions have been shown to have a tremendous effect on the tumour resistance to radiotherapy and chemotherapy resulting in more aggressive tumours (190-192). The oxygen levels of mammalian tissues vary from 2% to 9%, while hypoxia is considered as less than 2% and anoxia at less than 0.02% (193). The extent of hypoxia or anoxia experienced by tumour cells depends on the distance from the blood supply and the rates of cellular oxygen consumption in the tissue. In addition to tumour progression, hypoxia is associated with several other physiological conditions including stroke, inflammation and tissue ischemia.

Ample studies have demonstrated that hypoxic cells are part of many human tumours (194-197) and can drastically modify the outcome of radiation treatment (190,198-200) and response to chemotherapeutic agents (201,202). Hypoxia has been a determining factor in several different human tumours such as head and neck (203), cervix as well as tumours of the soft tissue (204). For example, tumour oxygenation pretreatment in patients with squamous cell carcinoma of head and neck was highly correlated with a positive radiation response (203). These studies delineate the significance and relevance of studying the impact of the hypoxic tumour microenvironment and its molecular mechanisms that lead to tumour resistance of conventional treatment methods.

With the understanding of the effect of hypoxic conditions on the growth of tumours came the discovery that hypoxia correlates with, and contributes to genetic instability and metastasis (205-207). In addition, hypoxia has been associated with DNA

damage (208,209), increased mutagenesis (207,210,211), altered DNA repair pathway functions (211-213), angiogenesis (214), DNA replication (205,206) and decreased protein synthesis (215). Although severe and extended hypoxia can result in cell death, transient hypoxia is not deleterious to the cell and in fact cells have evolved adaptive responses to a short supply of oxygen that is crucial for cell survival. Increased glycolysis, angiogenesis and erythropoiesis are some of these essential adaptive metabolic changes that occur in response to hypoxia (reviewed in (216)). The physiological reactions also vary depending on whether the cell is exposed to acute or chronic hypoxia. For instance, during acute hypoxia cells switch from aerobic to anaerobic respiration in order to minimize oxidative stress, while erythropoiesis and angiogenesis are important during chronic hypoxia, which promote improved transportation of oxygen to tissues (reviewed in (216)). Unfortunately, these adaptive responses are at the root of tumour progression and determine patient prognosis.

Since hypoxia is perceived as a stress by the cell and therefore is believed to cause DNA damage and impair DNA repair pathways, it is evident that studies focusing on understanding how hypoxia affects DNA repair pathways may prove essential for developing more specific treatment methods. There have been several studies suggesting that hypoxia decreases both NER and MMR through regulation of specific DNA repair genes (211,212). Hypoxia induces a reduction in expression of *MLH1* (212) and *MSH2* genes (217), which both encode proteins involved in MMR. It was also found that low oxygen levels can down-regulate expression of some DNA double-strand break repair genes, such as *RAD51,* in both normal and tumour cells (213,218) and *BRCA1* in tumour

cells (219). Hypoxia resulted in decreased Ku70/80 expression in cervical tumours, a protein important in the NHEJ repair pathway (191). Decreased repair of UV-induced DNA damage in plasmids was reported in cells exposed to hypoxia and low pH conditions for 24 hours when compared to control cells, suggesting an impaired NER due to hypoxia and acidosis treatment (211).

6.2 Acidosis

The microenvironment of the tumour is not solely characterized by hypoxia, but is also accompanied by a decrease in pH (220) due to the switch from aerobic to anaerobic respiration. This change from respiration through glucose consumption to increased glycolysis results in proton production from lactic acid accumulation and ATP hydrolysis (221). During one cycle of anaerobic respiration and ATP hydrolysis, 2 protons are released leading to a lowering of physiological pH (222). However, compared to hypoxia, much less attention has been devoted to the effects of acidosis on genetic instability and neoplasia development. Acidosis plays an important role in maneuvering tumour response to therapy. An acidic microenvironment can affect protein and enzyme structure and function, as well as synthesis of macromolecules, and transport of drugs, which may result in mutagenesis (reviewed in (223)). It has been reported that acidosis can influence several DNA polymerases by changing their fidelity during DNA synthesis (224,225). In terms of relevance to cancer, it was demonstrated that acidosis augments the metastatic capacity of tumour cells (226-228). The low pH of the microenvironment has also been linked to radio- and chemoresistance, although the effect is not as dramatic as with

hypoxia (222). Therefore, acidosis plays an important role in maneuvering tumour response to therapy.

6.3 Role of p53 in hypoxia and acidosis

Hypoxic and acidic stress regulates expression of certain genes and causes accumulation of specific enzymes and growth factors (reviewed in (216)). Some of these genes encode protein products such as the tumour suppressor protein p53, hypoxia inducible factor 1 alpha (HIF-1α) and vascular endothelial growth factor (VEGF). It was demonstrated that p53 expression increases in response to hypoxic treatment (166-170) as well as to hypoxia accompanied by acidosis (229), resulting in enhancement of its DNA binding and transactivation abilities. The expression of MDM2, the p53 regulator, decreases under hypoxic stress, accounting for the p53 increase (166). Interestingly, hypoxia does not induce the same genes that are normally activated by p53 as a result of DNA damage (170,230), suggesting the involvement of a different p53-dependent mechanism under oxygen deprivation conditions. This hypothesis is supported by results whereby caffeine has been shown to abolish p53 stabilization caused by DNA-damaging agents, but could not prevent p53 accumulation induced by hypoxic stress (231).

However, other studies have suggested that hypoxia does not change p53 expression until oxygen concentrations reach anoxic levels (169,170) and that hypoxia might have a transrepression effect on p53 target genes such as *p21* (170,230). Hypoxia for four hours (1% oxygen) resulted in increased transcription of HIF-1α in several different cell lines, but did not simultaneously up-regulate p53 (232). Similar results were

obtained with human HepG2 and HeLa cells exposed to hypoxic conditions for five hours (233). In addition, hypoxia-induced p53-dependent apoptosis was not significant until oxygen concentrations reached at or below 0.02% (234). Therefore, it is thought that hypoxia/anoxia, although it might induce p53 accumulation, it may not have transactivation capacity, but may still be able to activate apoptotic pathways (170).

It has also been suggested that hypoxia coupled to acidosis is able to induce p53 expression. Anoxic conditions accompanied by low pH resulted in p53 accumulation in a variety of tested tumour cell lines (235). Similar results were reported with transformed mouse embryo fibroblasts (229). The cyclin-dependent kinase inhibitor, p21/WAF1 was induced by acidic conditions in human glioblastoma cells in a p53-dependent manner (236). These results make it apparent that the involvement of p53 in the cellular response to hypoxic as well as acidic stress is complex.

Several factors may be involved in determining whether p53 accumulation in response to hypoxic stress occurs including the degree of oxygen deprivation and whether it is accompanied by acidosis, as well as the cell type. It is possible that low oxygen conditions may exert an indirect effect on p53 expression, which may explain why studies did not consistently observe p53 stabilization. Several reports have determined that hypoxia can induce cellular DNA damage including DNA strand breaks and oxidative damage such as 8-oxoguanine (209,237,238). It is therefore possible that the degree of damage caused by hypoxia and the repair capacity of the respective cell line used in studies, may determine whether p53 accumulation is detected. The significant role of p53 in DNA repair is well established (83,175,177,184,189,239,240). Therefore

studying the effects of hypoxia as well as hypoxia in conjunction with acidosis on DNA repair and p53 expression may help elucidate the mechanism of p53 dependent regulation of DNA repair following hypoxia and hypoxia in conjunction with acidosis.

7.0 Hypoxia-inducible factor 1

7.1 Overview

Low oxygen levels activate hypoxia-inducible factor 1 (HIF-1), a prominent transcription factor. HIF-1 is important for cells in order to adapt quickly to changes in oxygen levels with minimum tissue damage. It is responsible for regulating expression of various target genes, which encode proteins involved in functions vital for responding to low oxygen conditions such as proteins for angiogenesis, erythropoiesis, glucose transport, metabolism, and proliferation. Virtually undetected in normoxic conditions due to its highly regulated mechanism, HIF-1 expression increases exponentially, reaching its half maximal point between 1.5% and 2% oxygen and the maximal point at 0.5% oxygen (241).

7.2 HIF-1 regulation

Through biochemical studies it has been discovered that HIF-1 is a heterodimer consisting of HIF-1α (120 kDa) and HIF-1β protein subunits (242). Association of both of these subunits with the major groove of the DNA helix has been reported (243). HIF-1α contains an oxygen-dependent degradation domain (ODD) and two transactivation domains (TAD) that are involved in regulating HIF-1α target genes (242). In contrast to

HIF-1α, which was identified as a novel protein, HIF-1β was earlier described as a Per-Aryl Hydrocarbon Receptor Nuclear Translocator (ARNT) protein that is constitutively expressed irrespective of oxygen level fluctuations (reviewed in (193)).

HIF-1α regulation is dependent upon three hydroxylases that modify two proline and one asparaginyl residues (244,245). Under normoxic conditions, hydroxylation carried out by the proline hydroxylases in the ODD domain generates a binding site for the von Hippel-Lindau tumour suppressor (VHL) protein, a substrate recognition component for an E3 ubiquitin ligase complex targeting HIF-1α for degradation (see Figure 1.5) (244). Hydroxylation of the asparaginyl residue prevents interaction of the co-activator p300/CBP with the HIF-1α transactivation domain (245). When oxygen levels are low, the hydroxylases are inactivated and as a consequence HIF-1α and HIF-1β subunits are able to bind to each other and interact with the p300/CBP co-activator (244,245). Then, HIF-1α binds to the hypoxia-response element (HRE) on target genes to facilitate transcription (see Figure 1.5) (reviewed in (246)).

7.3 The role of HIF-1α in cancer

Increased HIF-1α expression under low oxygen conditions is an adaptive response essential for preventing tissue damage during ischemia and tissue recovery, as it induces a shift from aerobic to anaerobic energy metabolism and activates angiogenesis at sites of vascular impairment (247). However, tumour cells have various mechanisms through which they can benefit from HIF-1α-dependent adaptive responses. As a result, several studies have reported enhanced survival of tumour cells with high expression of HIF-1α.

Human malignant tumours exhibit overexpression of HIF-1α (248), which is correlated with poor survival in patients with cancers of the cervix (249), breast (250), ovaries (251) and endometrium (252). Therefore, HIF-1α can be used as a treatment target for these types of tumours through a variety of ways that include promoting its degradation, preventing its stabilization or inhibiting its targets such as VEGF, a growth factor responsible for promoting angiogenesis (reviewed in (253)).

7.4 The link between HIF-1α, CSB and p53

Although HIF-1α research has revealed the involvement of HIF-1α in many different molecular pathways and the numerous different genes that it affects, only recently has a link between HIF-1α and the nucleotide excision repair protein, CSB been suggested (254). Filippi *et al* (2008) reported two putative hypoxia response element sequences in the promoter region of CSB for HIF-1α binding and also found that CSB mutant cells showed deficient up-regulation of the HIF-1α target gene, VEGF compared to normal human fibroblasts under hypoxic conditions (254). Furthermore, immunoprecipitation experiments for p53 in MCF7 cells revealed the presence of HIF-1α from hypoxic treated cells (255). Subsequent studies suggested an indirect interaction between HIF-1α and p53 through a possible down-regulation of MDM2 (256), while others reported a direct association of the two transcription factors (257,258). These later studies found two p53-binding sites in the ODD domain of HIF-1α, strongly supporting a direct interaction between HIF-1α and p53.

Several reports have presented data that supports the current proposed model of a competitive relationship between HIF-1 and p53 (254,259,260). The protein p300 is a co-activator of both HIF-1 and p53, and it is thought that the two transcription factors compete for binding to this co-activator for transcriptional activity (260,261). The binding of p300 to p53 prevents Mdm2-depedent p53 degradation and therefore is crucial for p53 stabilization (262). It has also been shown that p300 is involved in regulation of HIF-1 transcriptional activity (263,264).

Another protein that has been recently implicated in this antagonistic interaction is the NER protein CSB (254). Previously it has been reported that CSB binds to p53 (172) and recent data suggests that p300 and CSB may be competing for p53 binding (254). It has been suggested that p53 shows preferential binding to CSB (254) and for this reason, this interaction disrupts p300-mediated p53 activation and therefore prevents p53-dependent apoptosis directing the cell towards survival (reviewed in (265)). In the case of CSB mutated cells, p300 binds with p53 leading to its activation and inhibiting HIF-1-dependent responses. This model is summarized in Figure 1.6. These reports suggest a possible link between p53, HIF-1α and CSB that is important for proficient NER. Since both CSB and p53 play critical roles in NER, it is not unlikely that HIF-1α may be involved in DNA repair of UV damage.

8.0 Host cell reactivation assay

8.1 Overview

The host cell reactivation (HCR) assay is a technique used to assess the capacity of cellular NER to repair damage in the DNA of the introduced reporter gene. The HCR assay employs a shuttle vector carrying a reporter gene that is exposed to a DNA-damaging agent, which is then introduced into the cells of interest (266). This allows for measurement of the relative expression of the reporter gene following its introduction into the host cell. Expression of the damaged reporter gene is thought to arise from the host cells' reactivation of the reporter gene resulting from the action of cellular DNA repair mechanisms on the exogenously introduced DNA (reviewed in (266,267)).

In addition to the relative swiftness of the assay, and reproducibility of results (268), there are several other advantages in using this technique. First, the cellular NER capacity can be determined by measuring the extent of repair of the introduced damaged reporter gene. This method facilitates the assessment of cellular constitutive and inducible DNA repair simultaneously, allowing comparison between the two. Assessment of DNA repair in untreated cells is known as measuring constitutive (non-inducible) cellular DNA repair. Most often DNA repair levels after induced DNA damage are assessed by treating cells with a DNA damaging agent and in turn imposing stress on cells. The type of DNA repair measured by such experimental techniques is known as inducible repair. Therefore, this assay allows the experimenter to compare constitutive DNA repair to inducible DNA repair, which is in contrast to most other experimental techniques used to evaluate NER. Other techniques involve damaging the cells in some

form, and thus compromising constitutive DNA repair and, in reality, assessing inducible NER without assessing constitutive NER (reviewed in (269)). Since the shuttle vector is being damaged with an agent and not the cells themselves, and the assay relies on the cellular DNA repair pathway for repairing the damage, the results of this technique are designed to reflect *in vivo* repair (270). Another advantage of the HCR assay is that a fixed time is given to the cells for repair to occur comparable to *in vivo* cellular NER.

8.2 Adenoviruses as shuttle vectors

Different shuttle vectors can be used for an HCR assay such as plasmids (268) or viruses (267,269,271). Adenoviruses (Ad) are very efficient vectors for introducing foreign DNA into mammalian cells (272) and their usefulness in a clinical setting such as their application in gene therapy for cancer has been widely recognized (reviewed in (273)). Ad is a non-enveloped virus containing its double-stranded 35kb DNA in an icosahedral protein capsid (274). Their usefulness comes from their ability to introduce their DNA into cells, the ease that their genome can be manipulated and their ability to infect many different types of cells (275).

The expression of the transgene in cells infected with Ad depends on the promoter driving its expression with the cytomegalovirus (CMV) immediate early (IE) promoter being the most frequently employed due to its great expression capacity (271). The adenovirus used in the HCR assays has the early-1 (E1) region of the genome deleted, which is otherwise necessary for transcriptional activation of its genome (276) and thus cannot replicate in most mammalian cells. It is in this region that the gene of interest can

be inserted (272). The HCR assay used in these studies employs a non-replicating form of adenovirus expressing the *Escherichia coli* reporter gene known as the lacZ β-galactosidase (β-gal) reporter gene (reviewed in (276)) under the control of the human CMV promoter (HCMV). In contrast to some transfection techniques with plasmids, it has been demonstrated that Ad infection does not induce a stress response in the cell (277).

9.0 Project introduction

This thesis describes the results of experiments designed to investigate the role of p53 and hypoxia in NER. The role of p53 in the nucleotide excision repair (NER) subpathway known as global genome repair (GGR) is well established and hardly debated, whereas the involvement of p53 in transcription-coupled repair (TCR) has been controversial. In Chapter 3 of this thesis we show that p53 overexpression results in enhanced HCR of the UV-damaged reporter gene in normal, TCR-deficient CSB and GGR-deficient XPC human fibroblasts. We show also that UV pretreatment of cells infected with the p53 overexpressing adenovirus resulted in an increased HCR of the β-gal activity in normal, CSB and XPC human fibroblasts. Through this work we are able to demonstrate that p53 plays an important role not only in GGR but also in TCR and that this subpathway can operate through a p53-dependent mechanism.

An effect of hypoxia on DNA repair has been suggested by several studies (211-213). However the mechanism of hypoxia induced alterations in NER and the role of p53 in such alterations are far from clear. The hypoxic and acidic microenvironment of the

tumour has been shown to diminish NER of mouse transformed and human tumours cells (211). Chapter 4 describes the effect of hypoxia and hypoxia accompanied by acidosis on HCR of UV-damaged reporter gene of human normal primary and tumour cell lines, as well as on the expression of p53 and cell survival after UV treatment. Data is presented showing that hypoxia and low pH conditions resulted in an early and transient enhancement in HCR of a UV-damaged reporter gene in human primary normal fibroblasts, which correlates with increased cell viability and is p53-dependent. In contrast, tumour cells displayed delayed enhanced HCR of β-gal expression, which did not correlate with increased cell viability and was not p53-dependent. Hypoxia induced a different effect on cellular UVC sensitivity than did hypoxia accompanied by acidosis.

Our studies of the effects of hypoxia on NER were also extended in Chapter 5 of this thesis. We investigated the role of HIF-1α in repair of a UV-damaged reporter gene under both normoxic and hypoxic conditions. We used normal and CSB-deficient human primary fibroblasts, which were infected with the Ad overexpressing wild-type HIF-1α and two rat prostate cancer cell lines, one cell line being wild-type for HIF-1α and the second cell line being dominant-negative (DN) for HIF-1α, to investigate the role of HIF-1α on HCR of β-gal expression. Murine tumour cells dominant-negative for HIF-1α showed a decrease in HCR of UV-damaged reporter gene when compared to wild-type cells under normoxic conditions, but this differential repair did not translate into a differential response following UVC treatment among the two cell lines under normoxic conditions. We demonstrate that hypoxia alone resulted in an enhaced HCR of UV-damaged reporter gene in normal and is delayed in CSB-deficient fibroblasts. In contrast,

overexpression of HIF-1α resulted in an early decrease in HCR of UV-damaged reporter gene in normal fibroblasts and this decrease was delayed in CSB-deficient fibroblasts that had been exposed to hypoxic conditions. These results suggest that HIF-1 and CSB play a role in the regulation of NER under hypoxic conditions.

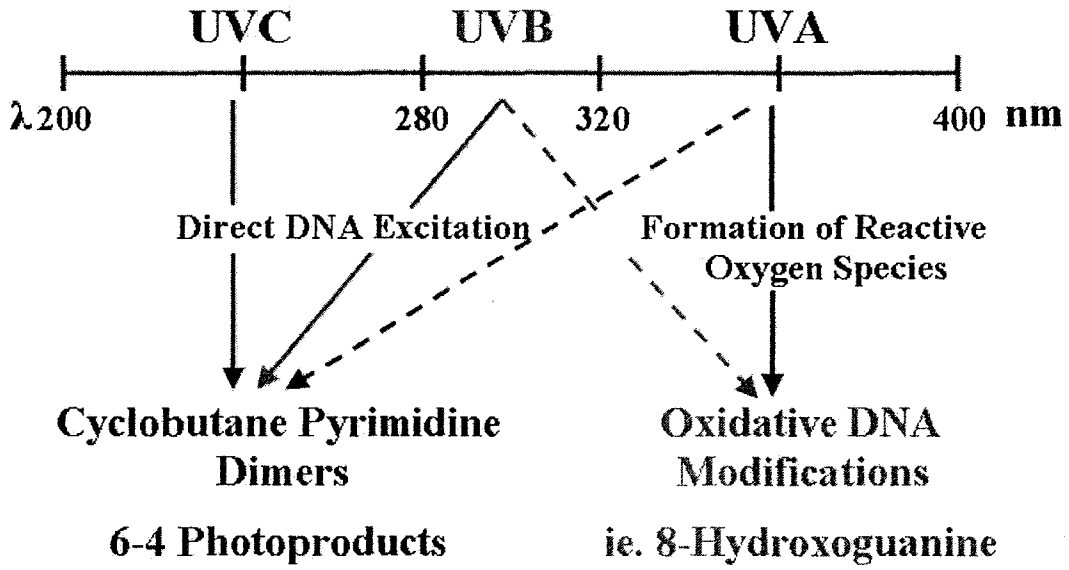

Figure 1.1. Ultraviolet radiation spectrum. The UV spectrum and the resulting DNA modifications that arise through either direct DNA excitation or formation of reactive oxygen species. (Adapted from Kielbassa *et al.*, *Carcinogenesis.* 1997; 18: 811-816.)

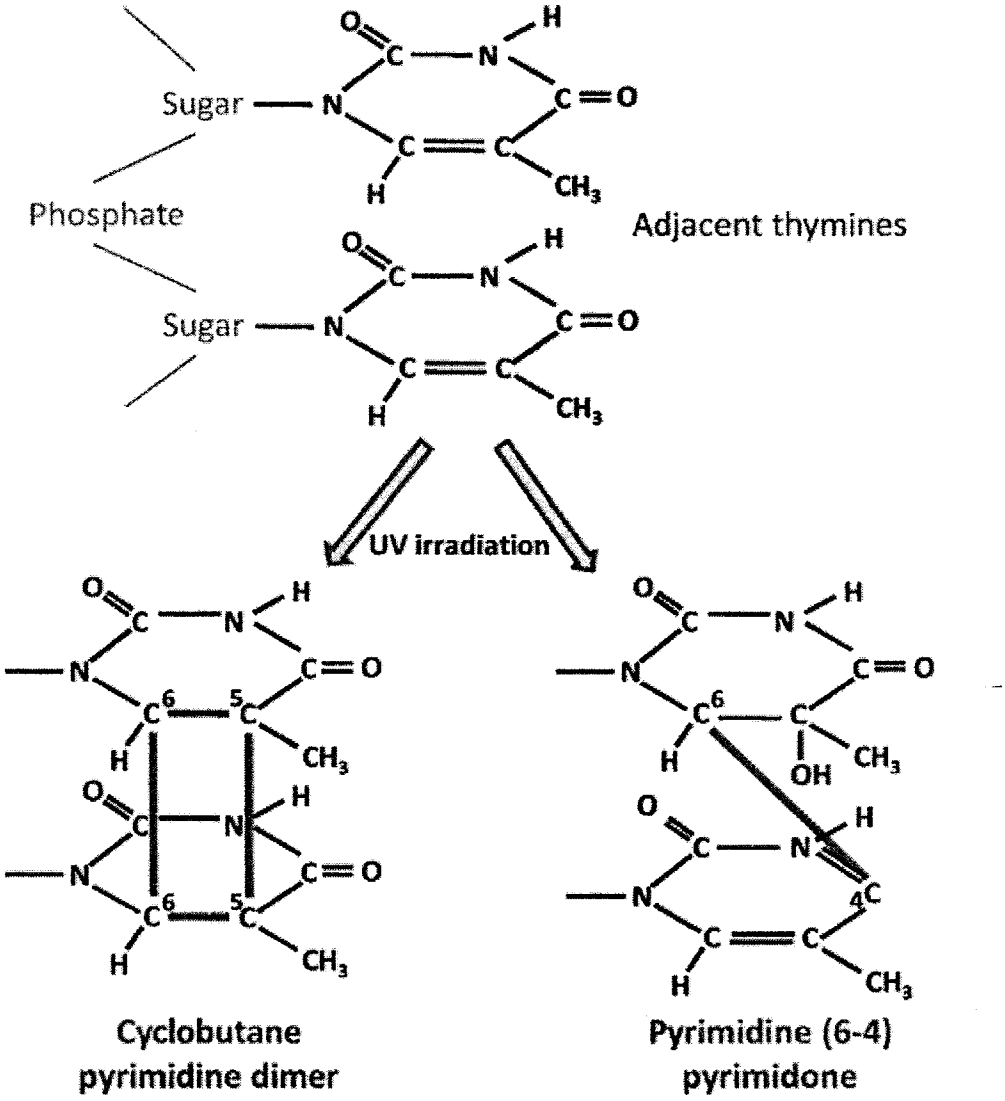

Figure 1.2. UV-induced lesions. Chemical structures that result from UV irradiation: cyclobutane pyrimidine dimer (CPD) and pyrimidine (6-4) pyrimidone (6-4PP). (Adapted from Batista et al., Mutat. Res. 2009; 681: 197-208.)

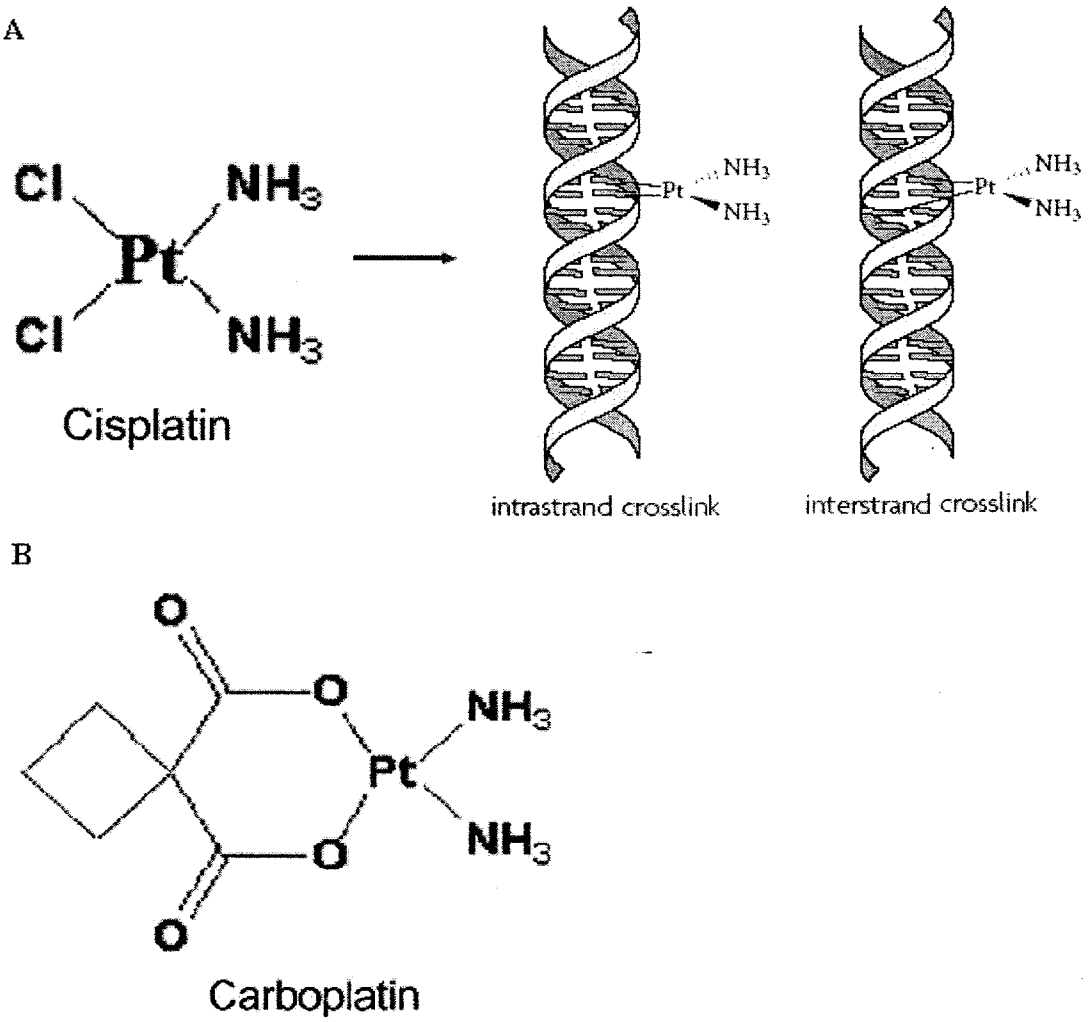

Figure 1.3. Cisplatin and Carboplatin. Diagram of cisplatin and carboplatin, two common chemotherapeutic agents used in treating cancer. A.Cisplatin and the adducts it produces. B. Carboplatin structure. (Adapted from Kelland, *Nat. Rev. Cancer.* 2007; 7(8): 573-584.)

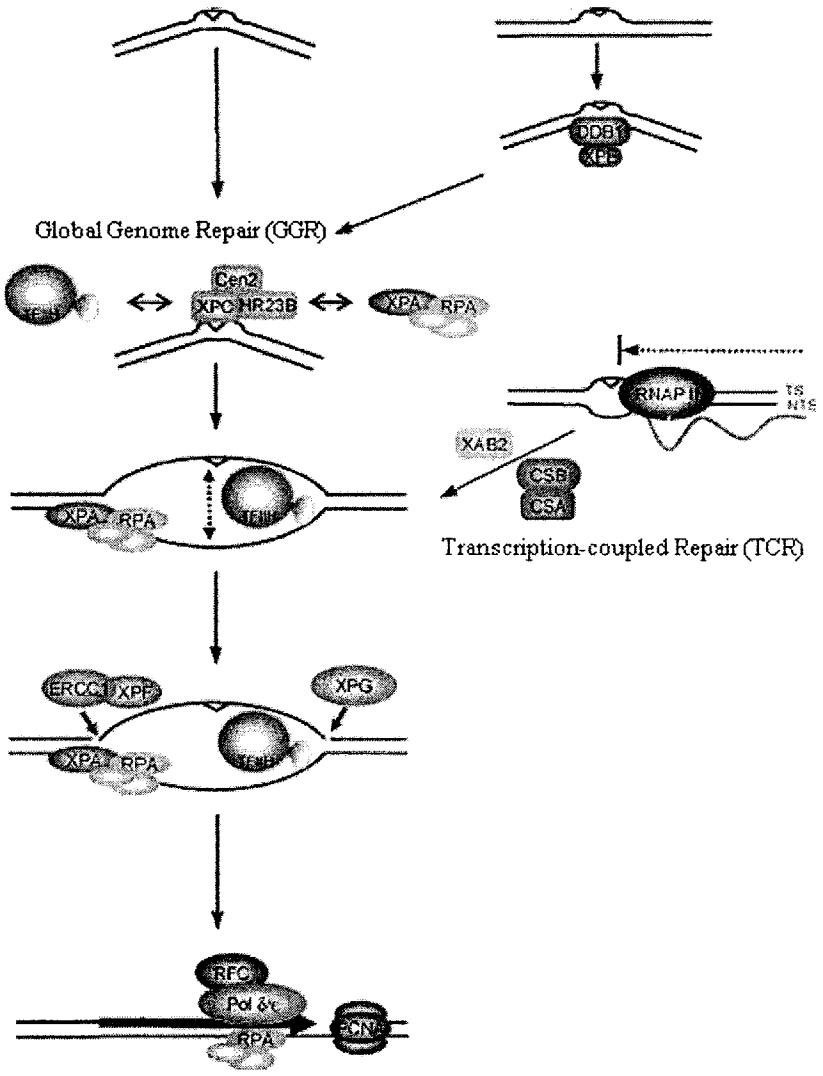

Figure 1.4. Nucleotide excition repair subpathways. Schematic of the two subpathways of nucleotide excision repair (NER): global genome repair (GGR) and transcription-coupled repair (TCR). (Adapted from Nouspikel, *DNA Repair.* 2008; 7(7): 1155-1167.)

Table 1.1. Functions of NER genes. The nucleotide excision repair genes and the functions of their protein products in global genome repair and transcription-coupled repair. (Adapted from Hanawalt and Spivak, *Nat. Rev. Mol. Cell Biol.* 2008; 9: 958-970.)

Human Gene	Role in DNA Repair
GGR genes	
XPE (also known as DDB2)	Lesion recognition
DDB1	Lesion recognition; Forms a complex with DDB2
XPC	Lesion recognition; Opens DNA
RAD23B	Lesion recognition; Forms a complex with XPC
Centrin-2	Lesion recognition; Forms a complex with XPC
GGR and TCR genes	
XPB	3' → 5' helicase activity, ATPase (TFIIH subunit)
XPD	5' → 3' helicase activity, ATPase (TFIIH subunit)
XPA	Lesion verification; Stabilizaes pre-incision complex for GGR and TCR
RPA	ssDNA binding; Binds to XPA
XPF	Structure-specific endonuclease (5' incision)
ERCC1	Forms a complex with XPF
XPG	Structure-specific endonuclease (3' incision)
PCNA	DNA replication sliding clamp; Contains docking sites for DNA pol
TCR genes	
CSA	Ubiquitin-ligase complex
CSB	TCR coupling factor and chromatin remodeling
XAB2	Transcription factor; Link between XPA and RNAPII

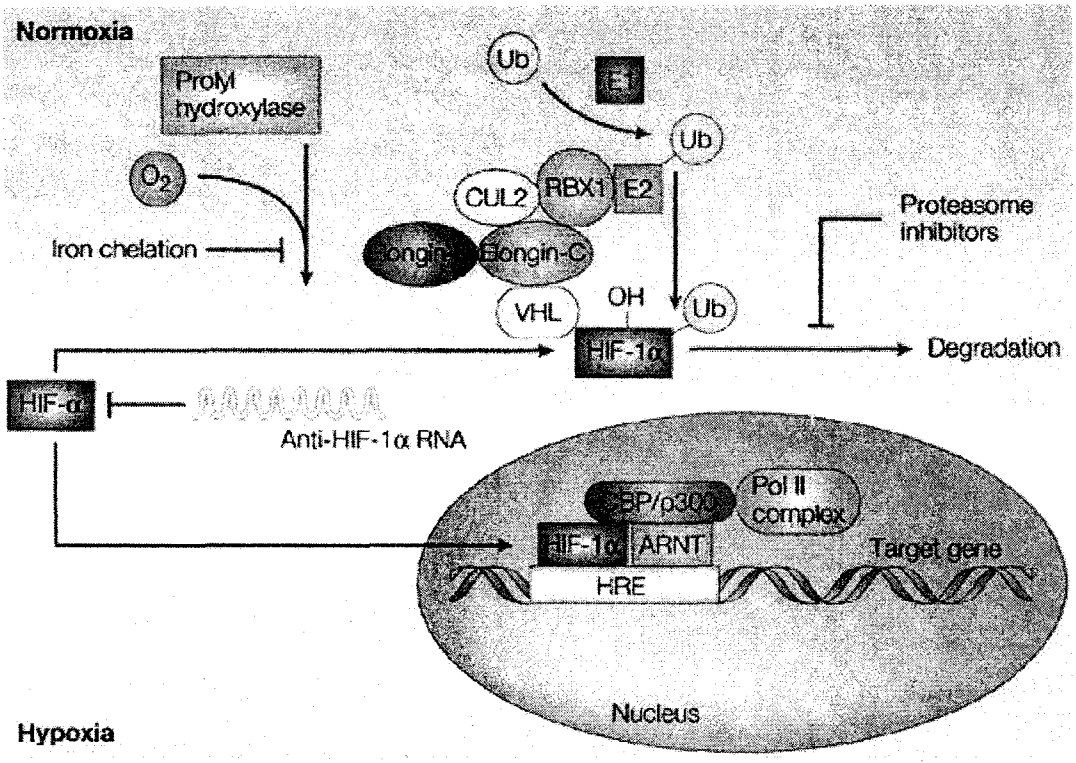

Figure 1.5. Regulation of HIF-1 by oxygen levels. Under normoxic conditions the prolyl enzymes hydroxylate residues allowing the tumour suppressor protein von Hippel-Lindau (VHL) to bind to HIF-1α, which acts as a substrate site for the E3 ubiquitin ligase targeting it for degradation. Under hypoxic conditions the hydroxylases are inactived and HIF-1α is able to heterodimerize with HIF-1β allowing the complex to bind to the HRE on target genes. (Adapted from Harris, *Nat. Rev. Cancer*. 2002; 1(2):38-47.)

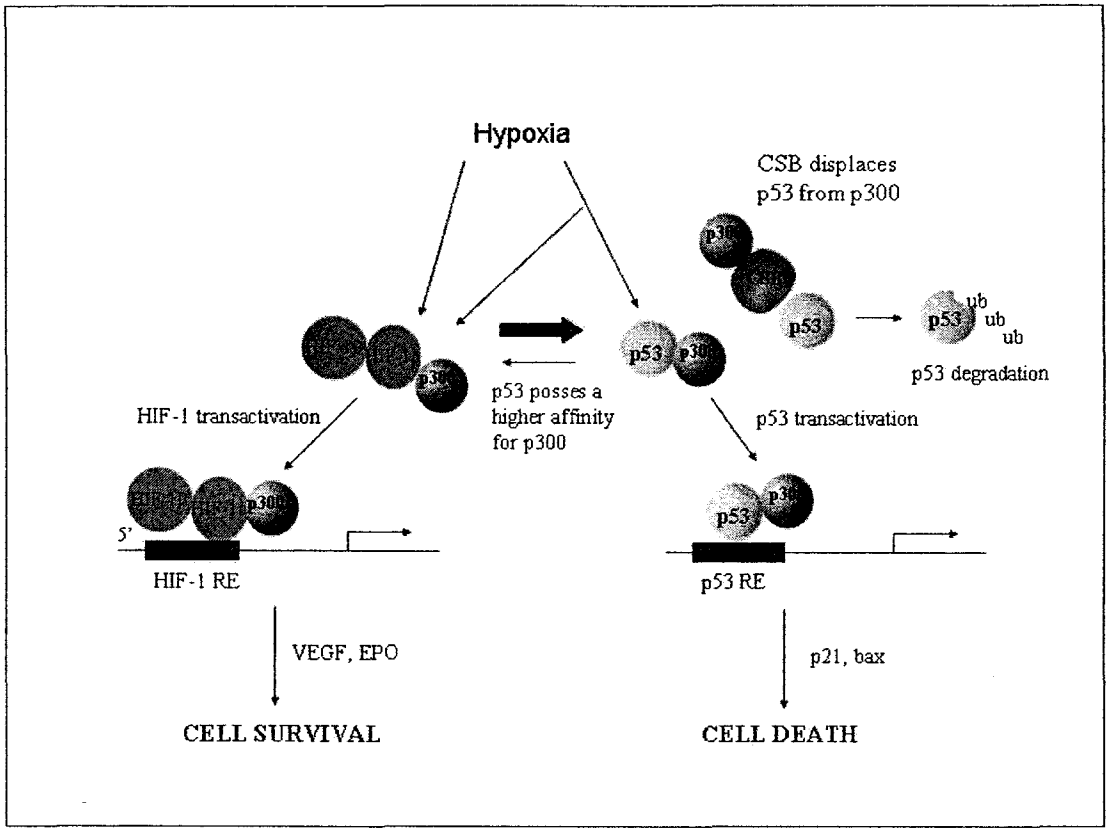

Figure 1.6. Hypoxia-induced HIF-1 and p53 effects and their expression regulation due to p300 and CSB competition. Stabilization of p53 can induce transcription of genes involved in cell cycle arrest or apoptosis. HIF-1 accumulation may result in cell death hindrance by inducing transcription of genes such as VEGF, by binding to responsive elements (RE) on the target genes and thus promoting adaptation to hypoxic stress. Some researchers have found evidence that suggests p53 may compete with HIF-1 for p300 binding. Furthermore, CSB by interacting with p53 may promote release of p300 from p53 and commit the cell towards cell survival pathways. Lack of CSB would cause p53 binding to p300 and subsequent p53 activation. (Adapted from Frontini and Proietti-De-Santis, *Cell Cycle*. 2009; 8:693-696.)

CHAPTER 2

Materials and Methods

1.0 Cell strains and cell lines

Primary human fibroblast cell strains and SV40-transformed human fibroblasts cell lines were obtained from the National Institute of General Medical Sciences (NIGMS) Human Genetic Cell Repository (Coriell Institute for Medical Research, Camden, NJ). The various human cell strains and cell lines obtained from NIGMS are shown in Table 1. The colon carcinoma HCT116p53$^{+/+}$ and HCT116p53$^{-/-}$ cells were obtained from Dr. B. Vogelstein, John Hopkins University School of Medicine, Baltimore, Maryland. The 3340WT cells are a mouse fibroblast cell line carrying in its genome 15 copies of the λsupFG1 shuttle vector DNA (278) and Rcneo cells are a RKO colorectal carcinoma cell line that has been transfected with the pCMVneo plasmid (279). The 3340WT and Rcneo cells were obtained from Dr. P.M. Glazer, Yale University School of Medicine, New Haven, Conneticut. MATLyLu is a rat prostate cancer cell line that is wild-type for HIF-1α and is derived from Dunning R3327 rat prostatic tumour subline (280). MDN2 is a stable clone obtained by transfection of MATLyLu cells with the pCEP4/dominant-negative HIF-1α (DNHIF-1α) plasmid, as described previously (192). MATLyLu (MLL) and MDN2 cells were obtained from Dr. Gurmit Singh, Juravinski Cancer Centre, McMaster University, Hamilton, Ontario.

Table 2.1. Primary and SV40-transformed fibroblast cells obtained from NIGMS.

NIGMS Designation	*Individual*	*Repair Phenotype*
GM9503s		Normal
GM969		Normal
IMR90	I90-19	Normal
GM739	CS1AN	CSB
GM5509	XP12BE	XPA
GM677	XP2BE	XPC
GM637 (SV40)		Normal
CSBSV40	CS1AN	CSB
XPCSV40	XP4PA	XPC
XPASV40	XP20OS	XPA
GM4420DXPA (SV40)	XP12BE	XPA
GM04312XPA (SV40)	XP20S	XPA

2.0 Media

The human fibroblasts and the colon carcinoma HCT116p53$^{+/+}$ and HCT116p53$^{-/-}$ cells were cultured in Eagle's α-MEM supplemented with 10 % fetal bovine serum and antimycotic/antibiotic (100 μg/ml penicillin, 100μg/ml streptomycin and 250 ng/ml amphotericin B, Gibco BRL). The mouse 3340WT and human Rcneo cell lines were grown in DMEM supplemented with 10% fetal bovine serum and antimycotic/antibiotic. (100 μg/ml penicillin, 100 μg/ml streptomycin and 250 ng/ml amphotericin B, Gibco BRL). The rat prostate cancer cell lines, MATLyLu and MDN2 were grown in RPMI 1640 media (Lonza) supplemented with 10 % fetal bovine serum, 10 mM HEPES (Gibco) and antimycotic/antibiotic (100 μg/ml penicillin, 100 μg/ml streptomycin and 250 ng/ml amphotericin B, Gibco BRL) or 0.2 mg/ml hygromycin (Roche) for MATLyLu and MDN2, respectively.

3.0 Cell culture

All cell cultures were grown as monolayers in growth medium in a humidified incubator kept at 5% CO_2 and 37 °C. Cells were passaged by rinsing the cells with phosphate buffered saline (PBS:140 mM NaCl, 2.5 mM KCl, 10 mM Na_2HPO_4 and 1.75 mM KH_2PO_4) followed by treatment with 0.1 % or 0.25 % trypsin (Gibco BRL). Cells were then suspended in growth media and an appropriate number of cells aliquoted into a fresh 75 cm^2 flask (Corning Incorporated, Corning, NY, catalogue #430641).

4.0 Recombinant adenovirus constructs

The AdCA17*lacZ* (272) recombinant adenovirus contains the *lacZ* gene under the control of the human cytomegalovirus immediate early (HCMV-IE) promoter (-299 to +72 relative to the transcription start site) inserted into the deleted E1 region of the adenovirus genome in the left-to-right orientation. Deletion of the E1 region of the genome renders the adenovirus unable to replicate in most mammalian cells. The recombinant adenovirus AdCA18*luc* (276) and Ad5p53wt (281) express the luciferase reporter gene and the wild-type p53 gene, respectively. The luciferase and p53 genes are under the control of the HCMV-IE promoter inserted into the E1 deleted region in the left-to-right orientation of the adenovirus genome. The recombinant adenoviruses were obtained from Dr. F.L. Graham, McMaster University, Hamilton, Ontario. The viruses were propagated, collected and titred as described previously (282). The recombinant adenovirus Ad*HIF-1α* contains the wild-type *HIF-1α* gene under the control of the HCMV-IE promoter into the E1 deleted region in the right-to-left orientation and was obtained from Applied Biological Materials Inc. (Richmond, BC, Canada; catalogue #: 000019A). The virus was propagated, collected and titred by Dr. F.L. Graham's laboratory, as previously described (282) and the stock virus preps contained 2.18×10^{11} plaque-forming units (pfu)/ml.

5.0 UV-irradiation of virus

The virus was suspended in 1.8 ml of cold PBS and was irradiated in 35 mm dishes (Falcon, Lincoln Park, NJ, catalogue #3001) on ice with continuous stirring using

General Electric germicidal lamp (model G8T5) emitting predominantly at a wavelength of 254 nm with an incident fluence rate of 2 $J/m^2/s$ as determined using a J-255 shortwave UV meter (Ultraviolet Products, San Gabriel, CA). Aliquots of 200 µl were removed following each exposure to the virus and diluted appropriately with unsupplemented media. In the case of simultaneous infection of AdCA17*lacZ* with either AdCA18*luc* or Ad*HIF-1α*, 100 µl aliquots of AdCA17*lacZ* were removed following each exposure to the virus and diluted appropriately with unsupplemented media, which also contained the appropriate multiplicity of infection (MOI) of AdCA18*luc* or Ad*HIF-1α*.

6.0 Cisplatin

The cisplatin used in this work was purchased as a solution of 1 mg/ml (Mayne Pharma Inc., Montreal, QC, Canada). For each experiment a fresh 2 mM stock solution of cisplatin in low chloride phosphate buffered saline (PBS) (4 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH2PO4) was prepared.

7.0 Cisplatin treatment of virus

Cisplatin reactivity is dependent on low chloride ion concentration and thus, the virus was treated with cisplatin in a suspension with 50 mM concentration of chloride ion. This suspension was prepared by using a ratio of low-chloride PBS (7 mM Cl^-) and serum-free α-MEM (140 mM Cl^-). Virus was suspended in 1.8 ml of cold 50mM Cl^- PBS and α-MEM solution and aliquots of 200 µl virus were removed and added to 20 µl of appropriately diluted cisplatin. Virus was treated with cisplatin for 12 hours at 37 °C and incubation was stopped with an appropriate amount of serum-free α-MEM to give a

suitable concentration of virus for infection. Then cells' media was aspirated from wells and were infected with 40 µl volume of virus at an MOI of 20-40 pfu/cell. Cells were incubated at 37 °C for 90 minutes, after which cells were overlaid with 160 µl of warm supplemented α-MEM and incubated at 37 °C in either a normoxic or hypoxic incubator for various lengths of time to allow for reporter gene expression. For these experiments cells were incubated in the hypoxic incubator for either 24 hours before infection only, 24 hours before and 40 hours after virus infection or 40 hours after infection only.

8.0 UV-irradiation of cells

Cells were seeded in 96 well plates (Falcon, Lincoln Park, NJ, catalogue #3072) at 1.5 -2 x 10^4 cell density. Cells monolayers were pre-infected with either AdCA18 or Adp53wt 24 hours after seeding, then media was aspirated from the wells and the monolayer was overlaid with 40 µl of warm PBS and UV-irradiated (or mock-irradiated) using a General Electric germicidal lamp (model G8T5) which emits predominantly at a wavelength of 254 nm with an incident fluence rate of 1 J/m^2/s. For UVC pretreatment experiments without pre-infection have been described previously (private communications from Adrian Rybak). Briefly, after 24 hours of incubation, the media was aspirated from each well, and 40 µl of warmed PBS was overlayed on the cells. Cells were UV-irradiated (or mock irradiated) at a fluence of 20 J/m^2 using a General Electric germicidal bulb (emitting predominantly at 254 nm) at a fluence rate of 1 W/m^2. UVC exposure of 20 J/m^2 was corrected for irradiation in 96-well plates (12 J/m^2) as previously reported (183).

9.0 Hypoxia

Hypoxic culture conditions were established by using an automatic O_2/CO_2 incubator (Thermo Electron Corporation, Marietta, Ohio) equipped with oxygen and carbon dioxide sensors, and connected to N_2 and CO_2 gas cylinders. The O_2 tension was set at 1 % and was maintained at this level throughout the entire length of the experiment. The O_2 (1%) and CO_2 (5%) readings were confirmed by use of a fyrite gas analyzer (Bachrach, Pittsburg, PA).

10.0 Low pH

Regular DMEM or α-MEM was supplemented with 25 mM HEPES and 25 mM 4-morpholinepropanesulfonic acid (Sigma, ST. Louis, MO) to acidify the medium. The medium was adjusted to pH 6.5 with 1 N HCl. The pH of the acidic media after hypoxic treatment was measured to be between pH 6.5 and 7, the pH of the media after hypoxic treatment was approximately 7.5, and the normoxic media was measured to be between pH 7.5 and 8.

11.0 Host cell reactivation assay

Fibroblasts were seeded in 96-well plates (Falcon, Lincoln Park, NJ, catalogue #3072) at a density of 1.5 - 2.0x10^4 cells per well in supplemented α-MEM and incubated in a 5% CO_2 humidified incubator at 37 °C for 12-24 hours to allow for cells to adhere to the wells prior to infection. In order to examine the effects of enhanced p53 expression on HCR of the UV-damaged reporter gene the media was aspirated from the wells following

12-24 hours incubation and the confluent cell monolayer was infected with the viral vectors Ad5p53wt or AdCA18*luc* in a 40 µl volume at an MOI of 150 pfu/cell in cold phosphate buffered saline (PBS: 150 mM NaCl, 2.5 mM KCl, 10 mM Na_2HPO_4, 1.75 mM KH_2PO_4 (pH 7.4)). Cells were overlaid with 160 µl of warm supplemented α-MEM following a 90 minute incubation period at 37 °C in the incubator. Further incubation followed for approximately 24 hours after the first viral infection. The media was then aspirated from the wells and for some experiments the monolayer was overlaid with 40 µl of warm PBS and UV-irradiated (or mock-irradiated) using a General Electric germicidal lamp (model G8T5), which emits predominantly at a wavelength of 254 nm with an incident fluence rate of 1 $J/m^2/s$. UV exposures used for UV-irradiation in 96-well plates were determined as previously reported (183). After treatment (or mock-treatment) with UV, cells were infected with either non-irradiated or UV-irradiated AdCA17*lacZ* in a 40 µl volume at an MOI of 40-160 pfu/cell. Cells were incubated at 37 °C for 90 minutes, after which time cells were overlaid with 160-200 µl of warm supplemented α-MEM and incubated at 37 °C in a normoxic incubator and for studying the effect of hypoxia, cells were placed in the hypoxic incubator for various lengths of time to allow for reporter gene expression.

12.0 Quantitation of β-gal activity

Fibroblasts were harvested at various time points following infection with AdCA17*lacZ*. The infected cell monolayer was incubated with 60 µl per well of 1mM chlorophenol red β-D-galactopyranoside (CPRG; Boehringer–Mannheim, Indianapolis,

IN) in 0.01% Triton X-100, 1 mM $MgCl_2$, and 100 mM phosphate buffer at pH 8.3. Light absorbance at 570 nm (A_{570}) was determined several times following the addition of the β-gal substrate using a 96-well plate reader (Labsystems Multiscan MCC/340 and/or Bio-Tek Instruments EL340 Bio Kinetics Reader).

13.0 MTT reduction assay for cell viability following UV exposure

Cells were seeded in 96-well plates (Falcon, Lincoln Park, NJ, catalogue #3072) at a density of 2.0 – 2.5 $x10^3$ cells/well for primary cells and 1.25 x 10^3 cells/well for tumour cells. Cells were seeded 6-12 hours prior to being treated using a General Electric germicidal lamp (model G8T5) at a wavelength of predominantly 254 nm with an incident fluence rate of 1 $J/m^2/s$ (J-255 shortwave UV meter, Ultraviolet Products, San Gabriel, CA) in PBS. After UVC treatment, cells were overlayed with warm α-MEM supplemented media or with low pH 6.5 warm α-MEM supplemented media and incubated in either a normoxic or hypoxic incubator for 24, 30 or 40 hours. After the allotted time interval, the low pH media was removed from each well and replaced with fresh media. All plates were subsequently incubated in a normoxic incubator. Cell viability was quantified 4-6 days after UVC treatment using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma catalog no. M2128) assay as previously described (283). The absorbance of each well was measured at 570 nm light using a 96-well plate reader (Labsystems Multiscan MCC/340 and/or Bio-Tek Instruments EL340 Bio Kinetics Reader) and the percentage viability was calculated.

14.0 Western blotting

For experiments testing whether Ad5p53wt infection induces p53 overexpression, confluent monolayers of cells in 6-well plates (Falcon, Lincoln Park, NJ, catalogue #3046) were infected with Ad5p53wt or AdCA18*luc* at an MOI of 150 plaque-forming units (pfu)/cell or mock-infected. Cells were then collected following 48 hours incubation using a cell scraper and suspended in lysis buffer [50 mM Tris, 150 mM NaCl, 1% NP40, 10% protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), pH 8.0] and kept on ice for 60 minutes. For hypoxic experiments, confluent monolayers of cells in 6-well plates were collected after different time exposures to hypoxia or hypoxia and low pH conditions. The lysates were cleared by centrifugation at 13,000 g for 1-2 minutes and the protein concentration was determined by the Bradford microassay procedure. Samples were resolved over a 10 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), for 1 hour at 100 V (8% SDS-PAGE when probing for CSB) and transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech) (100 V for 1 hour. Blots were blocked with 20 % skim milk in 1X TBST (Tris-buffered saline with Tween 20) for a minimum of 1 hour. The primary antibodies used were: anti-p53 (Santa Cruz Biotechnology Inc., Santa Cruz, CA), anti-XPC (C-terminal; Sigma-Aldrich Inc., Saint Louis, MO), anti-HIF-1α (BD Biosciences, San Jose, CA), and anti-beta-actin (Sigma–Aldrich Inc., Saint Louis, MO). Membranes were probed with the primary antibody for 1 hour at room temperature or overnight at 4 °C. Subsequently, the blots were stripped using a specific buffer (100 mM 2-mercaptoethanol, 62.5 mM Tris-HCl, 2% SDS, Ph 6.7) for 30 minutes at 65°C and re-probed with monoclonal anti-β-actin

(Sigma-Aldrich Inc.). The data were analysed using a Kodak Digital Science Image Station 440 CFand protein levels were determined relative to actin levels. Specific antibody-labeled proteins were detected using enhanced chemiluminescence detection according to manufacturer's instructions (Western Lighting, Perkin–Elmer Life Sciences).

15.0 Total RNA isolation and RT-PCR

Confluent monolayers of MATLyLu and MDN2 rat prostate tumour cells in 60 x 15 mm Petri dishes were treated with either normoxic or hypoxic conditions for 5 hours. RNA was isolated using the RNeasy Plus mini kit (QIAGEN, Valencia CA) following the manual's protocol and then RNA was quantified in an Eppendorf Biophotometer. 1 μg of RNA was treated with DNase I (Invitrogen) to remove genomic DNA contamination. cDNA synthesis was performed by using random primers (100 ng) and the Superscript II First-Strand Synthesis (Invitrogen) using 1 μg of total DNase-treated RNA. A control that was not reverse transcribed was used to confirm a lack of DNA contamination.

PCR was performed using a Stratagene MX3000P machine (La Jolla, CA) and using the following VEGF and lamin primers: VEGF 5'-ACC AGC GCA GCT ATT GCC GT-3' (forward) and 5'-CAC CGC CTT GGC TTG TCA CA-3' (reverse); lamin 5'-GCA TGT ACA TAG AAG GAG CTA-3' (forward) and 5'-CAT GCA TAT TCC TGG TAC TCA T-3'(reverse). Primers were designed using GeneFisher and were synthesized by MOBIX (McMaster University, ON). A typical 50 ul reaction in a 0.2 ml thin-walled PCR tube contained 2 μl of DNA and 48 μl of PCR master mix (5 μl 10 x PCR buffer (Invitrogen), 1 μl of each of the appropriately diluted primer volume, 10 mM

dNTP mix (Invitrogen), 50 mM $MgCl_2$, 0.4 µl Taq DNA polymerase (Invitrogen) and water (to a volume of 50 µl per reaction including cDNA template). The thermal cycler program used for VEGF consisted of a 2 minute initial polymerase activation and denaturation step at 94°C, 30 seconds at 94°C, 45 seconds annealing step at 55°C, 45 seconds at 72°C, and then 35 reaction cycles of 30 seconds at 94°C, followed by a 10 minute elongation step at 72°C. RT-PCR products were separated on by electropgoresis on a 2% agarose gel and the ethidium bromide-stained DNA bands were visualized using an ultraviolet lightbox (Alpha Innotech Corporation, San Leandro CA)

16.0 Clonogenic survival assay

Cells were seeded in 6-well plates (Falcon, Lincoln Park, NJ, catalogue #3046) at a density of 400 cells/well in 1 ml supplemented RPMI 1640 media. Following a 6-12 hour incubation period at 37 °C in a 5% CO_2 incubator, the media was aspirated from the wells and 1 ml of warm PBS was added to the cells prior to UVC irradiation. Cells were either irradiated or mock irradiated with UVC of increasing fluences at a fluence rate of 1 $J/m^2/s$. Following UVC-irradiation, the PBS was aspirated and 1 ml of RPMI 1640 media, which was supplemented with 10 % fetal bovine serum and 10 mM HEPES (Gibco), but lacked antibiotics, was added to each well and the cells were incubated for 12 hours in either a normoxic or hypoxic incubator. After the specified treatment, media was aspirated and the cells were overlayed with 1 ml fresh media that did not contain antibiotics. The plates were further incubated for 4 to 5 days from the time the cells were

UVC-irradiated in a humidified 37 °C and 5% CO_2 incubator in order to provide sufficient time for surviving cells to form colonies.

At the end of the incubation period, media was aspirated from each well and the cells were overlayed with approximately 1 ml of crystal violet solution (63% absolute ethanol, 27% H_2O, 10% methanol, 5g/l crystal violet) to stain the colonies for a period of 30 minutes. Cells were de-stained by submerging the plates in water to remove the crystal violet solution and let to dry. Colonies that contained more than 32 cells were counted using a VWR hand tally counter and a clonogenic survival curve was plotted by expressing clonogenic survival as a ratio of the number of colonies in the treated cells compared to the control-treated cells.

CHAPTER 3

Increased expression of p53 enhances transcription-coupled repair and global genomre repair of a UVC-damaged reporter gene in human cells

Diana Dregoesc, Adrian P. Rybak, Andrew J. Rainbow
DNA Repair 6(5): 588-601

Preface

Previous work from our lab has shown that UV-irradiated primary normal and XPC-deficient fibroblasts exhibit a similar capacity to support Ad synthesis, but not XPA and CSB cells (183). A diminished capacity to sustain Ad synthesis after UV irradiation was also detected in SV40-transformed human fibroblasts, heterozygous p53 mutant Li-Fraumeni syndrome (LFS) fibroblasts, and immortalized mutant p53 LFS cells suggesting a p53-dependent TCR role in Ad synthesis after UV damage. Although the role of p53 in global genome repair (GGR) is well established (83,174,175,284), the involvement of p53 in transcription-coupled repair (TCR) is less characterized. To determine if p53 plays an important role in TCR we employed the HCR assay using a recombinant non-replicating adenovirus expressing *lacZ* under the control of the human cytomegalovirus immediate early promoter. It is reported here that overexpression of p53 in primary fibroblasts resulted in enhanced HCR of the UV-damaged reporter gene in normal, CSB and XPC fibroblasts at 12 hours post-infection suggesting that p53 is important in both TCR and GGR of a UVC-damaged reporter gene in human cells.

This chapter consists of of the following article reprinted in its published format: Diana Dregoesc, Adrian P. Rybak and Andrew J. Rainbow. **Increased expression of p53 enhances transcription-coupled repair and global genome repair of a UVC-damaged reporter gene in human cells.** (2007). *DNA Repair* 6(5):588-601. This article is reprinted with permission from Elsevier Science, Amsterdam, The Netherlands. I performed all of the experiments that generated the p53 overexpression data with and without UVC pre-treatment, as well as the p53 expression western blots. I also assembled

the results, generated the figures, wrote the first draft of the manuscript. Adrian P. Rybak conducted the HCRs of the UV-damaged reporter gene in UVC-pre-treated primary human fibroblasts. Dr. Andrew J. Rainbow made revisions to the manuscript and provided significant intellectual direction and assistance.

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Increased expression of p53 enhances transcription-coupled repair and global genomic repair of a UVC-damaged reporter gene in human cells

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ABSTRACT

Ultraviolet (UV) light-induced DNA damage is repaired by nucleotide excision repair, which is divided into two sub-pathways: global genome repair (GGR) and transcription-coupled repair (TCR). While it is well established that the GGR pathway is dependent on the p53 tumour suppressor protein in human cells, both p53-dependent and p53-independent pathways have been reported for TCR. In the present work, we investigated the role of p53 in both GGR and TCR of a UVC-damaged reporter gene in human fibroblasts. We employed a non-replicating recombinant human adenovirus, AdCA17*lacZ*, that can efficiently infect human fibroblasts and express the β-galactosidase (β-gal) reporter gene under the control of the human cytomegalovirus promoter. We examined host cell reactivation (HCR) of β-gal expression for the UVC-treated reporter construct in normal fibroblasts and in xeroderma pigmentosum (XP) and Cockayne syndrome (CS) fibroblasts deficient in GGR, TCR, or both. HCR was examined in fibroblasts that had been pre-infected with Ad5p53wt, which expresses wild-type p53, or a control adenovirus, AdCA18*luc*, which expresses the luciferase gene. We show that increased expression of p53 results in enhanced HCR of the UVC-damaged reporter gene in both untreated and UVC-treated cells for normal, CS-B (TCR-deficient), and XP-C (GGR-deficient), but not XP-A (TCR- and GGR-deficient) fibroblasts. These results indicate an involvement of p53 in both TCR and GGR of the UV-damaged reporter gene in human cells.

1. Introduction

The primary DNA lesions induced by far UV light (UVC) are the cyclobutane pyrimidine dimer (CPD) and the 6-4 photoproduct (6-4 PP), both of which are repaired by the nucleotide excision repair (NER) pathway in human cells (reviewed in Ref. [1]). NER has two sub-pathways: (1) transcription-coupled repair (TCR) which is dedicated to the removal of damage from the transcribed strand of active genes by a process coupled to RNA polymerase II, and (2) global genome repair (GGR) which removes DNA damage from the entire genome, including the non-transcribed strand and the transcribed strand of active genes (reviewed in Ref. [2]).

The genetic disorders Cockayne syndrome (CS) and xeroderma pigmentosum (XP) are characterized by acute sunlight sensitivity and XP patients are also predisposed to developing skin cancer. Cells from patients with XP and CS exhibit various deficiencies in the repair of UV-induced DNA damage. XP has seven NER-deficient complementation groups (XP-A to XP-G), which are all deficient in both GGR and TCR, except for the

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XP-C complementation group, which is deficient in GGR but proficient in TCR [1,3,4]. In contrast, CS has two complementation groups, CS-A and CS-B that are both deficient in TCR but proficient in GGR [5].

The *p53* tumor suppressor gene plays a central role in the cellular response mechanisms to various stresses such as UV-irradiation, chemical DNA-damaging agents, and hypoxia (reviewed in Refs. [6–8]). Mutations in *p53* have been shown to increase the risk of cancer [9,10]. When a cell is confronted with a stress, p53 accumulates in the cell in order to protect it against potential DNA damage. In response to DNA-damaging agents, p53 arrests the cell cycle which provides time for DNA repair prior to cell division [11]. In addition there is evidence that p53 and p53-regulated gene products contribute directly to NER of UV-induced DNA damage in mammalian cells [12–14].

The mechanism by which p53 up-regulates GGR is, in part at least, through the up-regulation of several genes involved in GGR. These genes include the GADD45 gene [14,15], the DDB2 gene, which is mutated in XP-E cells [16–19], and the XPC gene [18,20–22]. UV-induced, p53-dependent up-regulation of the GADD45, XPE and XPC proteins requires the removal of UV-induced lesions from the transcribed strand of these genes and is therefore also dependent on TCR [23]. In contrast, both p53-dependent and p53-independent pathways have been reported for TCR in human cellular DNA. Some reports indicate a substantial involvement of p53 in TCR of UV-induced CPDs [24–27] whereas others indicate little or no involvement of p53 in TCR [12,28–31].

We have used a non-replicating recombinant adenovirus that can efficiently infect human fibroblasts and express the β-galactosidase (β-gal) reporter gene, to examine both constitutive and inducible repair of UV-damaged DNA in human cells [32,33]. We have reported previously that host cell reactivation (HCR) of reporter gene activity for UV-damaged DNA is significantly reduced in all NER-deficient XP (groups A to G) and CS (groups A and B) cell strains tested, relative to that in normal diploid human fibroblasts [34,35]. Using a quantitative polymerase chain reaction (PCR) technique we have shown a significant removal of photolesions from the recombinant adenovirus based reporter gene after infection of normal human fibroblasts, and a significantly diminished lesion removal following infection of XP-C, XP-A and CS-B cells [36]. Taken together, these results indicate that the removal of photolesions from the UV-damaged reporter gene involves both TCR and GGR and leads to β-gal expression of the UV-damaged reporter gene. In addition we have reported that pre-treatment of normal human fibroblasts with UV fluences prior to infection with UV-irradiated Ad5HCMVsplacZ results in enhanced HCR of the UV-damaged reporter gene [34,35]. Using a quantitative PCR technique we have reported that prior UV-irradiation of normal human fibroblasts results in an enhanced rate of removal of photolesions from the recombinant adenovirus encoded reporter gene [36]. We have therefore suggested that the enhanced HCR for expression of the UV-damaged reporter gene reflects the presence of one or more inducible DNA repair pathways in human cells. Since both TCR and GGR contribute to the removal of photolesions in the UV-damaged reporter gene in untreated normal human fibroblasts, the enhanced HCR in pre-UV-irradiated cells could result from an up-regulation of TCR and/or GGR in the transcribed strand of the reporter gene. In addition, the UV-enhanced HCR response was absent in Li-Fraumeni syndrome (LFS) cells expressing mutant p53 [37] suggesting that pre-UV-treatment of cells leads to a p53-dependent up-regulation of the NER pathway. The enhanced HCR was detected in normal, XP-C and CS-B cells, although the up-regulation was delayed in CS-B cells [35]. The delay in enhanced HCR observed in CS-B cells is consistent with a p53- and TCR-dependent up-regulation of GGR reported for cellular DNA. The mechanism for the UV-enhanced HCR in XP-C cells is less clear and could result from p53-dependent and/or p53-independent upregulation of TCR. Therefore, we examined the involvement of p53 in TCR and GGR of the recombinant adenovirus encoded reporter gene in human cells.

In the present work we have examined the effect of increased expression of p53 on HCR of a UVC-damaged reporter gene in normal, XP-C, XP-A and CS-B human fibroblasts. Increased p53 expression was obtained by infecting cells with Ad5p53wt, a replication-deficient recombinant adenovirus encoding the wild-type p53 gene, prior to the HCR assay. We show that increased expression of p53 results in enhanced HCR of the UVC-damaged reporter gene in both untreated and UVC-treated normal, XP-C, CS-B but not XP-A fibroblasts, indicating an involvement of p53 in both TCR and GGR of the UV-damaged reporter gene in human cells.

2. Materials and methods

2.1. Cells and cell culture

The repair-proficient primary human cell line, GM09503 and the repair-deficient primary human cell lines, CS1AN (GM 00739; CS-B), XP2BE (GM00677; XP-C) and XP12BE (GM05509; XP-A) were obtained from NIGMS Human Genetic Cell Repository (Coriell Institute for Medical Research, Camden, NJ). All cell cultures were grown in a humidified incubator kept at 5% CO_2 and 37 °C, and cultured in Eagle's α-MEM supplemented with 10% fetal bovine serum and antimycotic/antibiotic (100 μg/ml penicillin, 100 μg/ml streptomycin and 250 ng/ml amphotericin B, Gibco BRL).

2.2. Virus

The recombinant adenovirus AdCA17*lacZ* [38] contains the *lacZ* gene under the control of the human cytomegalovirus immediate early (HCMV-IE) promoter (−299 to +72 relative to transcription start site) inserted into the deleted E1 region of the adenovirus genome in the left to right orientation. Deletion of the E1 region of the genome renders the adenovirus unable to replicate in most mammalian cells. The recombinant adenoviruses AdCA18*luc* [39] and Ad5p53wt [40] express the luciferase reporter gene and the wild-type p53 gene, respectively. The luciferase and p53 genes are under the control of the HCMV-IE promoter inserted into the E1 deleted region in the left-to-right orientation of the adenovirus genome. The adenovirus recombinants were obtained from Dr. F.L. Graham, McMaster University, Hamilton, Ontario, Canada. The viruses were propagated, collected and titred as described

previously [41]. Infection of cells with E1 deleted recombinant adenovirus vectors expressing the wild-type p53 gene under the control of the human cytomegalovirus promoter have been reported previously to result in upregulation of p53 responsive genes such as p21 [42,43].

2.3. *UV-irradiation of virus*

UV-irradiation of virus has been described previously [44]. Virus was suspended in 1.8 ml of cold PBS and was irradiated in 35 mm dishes on ice with continuous stirring using General Electric germicidal lamp (model G8T5) at a wavelength of 254 nm predominantly with an incident fluence rate of 2 J/m^2/s (J-255 shortwave UV meter, Ultraviolet Products, San Gabriel, CA). Aliquots of 200 μl were removed for each exposure to the virus and diluted appropriately with unsupplemented media. Under these conditions the induction of CPDs in the transcribed strand of the adenovirus encoded lacZ gene was approximately 3.3×10^{-6} CPD/nucleotide/J/m^2 as reported previously [36].

2.4. *Host cell reactivation of the reporter gene*

Fibroblasts were seeded in 96 well plates (Falcon, Lincoln Park, NJ) at a density of 2.0×10^4 cells per well in supplemented α-MEM and incubated for 12–24 h in a 5% CO_2 humidified incubator at 37 °C. The method used to examine the effects of pre-UV-exposure on HCR of the UV-damaged reporter gene has been described previously [34].

In order to examine the effects of enhanced p53 expression on HCR of the UV-damaged reporter gene the media was aspirated from the wells and the confluent cell monolayer was infected with the viral vectors Ad5p53wt or AdCA18luc in a 40 μl volume at a multiplicity of infection (MOI) of 150 plaque forming units (pfu) per cell in cold phosphate buffered saline (PBS: 150 mM NaCl, 2.5 mM KCl, 10 mM Na_2HPO_4, 1.75 mM KH_2PO_4 (pH 7.4)). Cells were overlaid with 160 μl of warm supplemented α-MEM following a 90 min incubation period at 37 °C in the incubator. Further incubation followed for approximately 24 h after the first viral infection. The media was then aspirated from the wells and the monolayer was overlaid with 40 μl of warm phosphate buffered saline and UV-irradiated (or mock-irradiated) using a General Electric germicidal lamp (model G8T5) which emits predominantly at a wavelength of 254 nm with an incident fluence rate of 1 J/m^2/s. UV exposures used for UV-irradiation in 96-well plates were determined as previously reported [45]. After treatment (or mock-treatment) with UV, cells were infected with either unirradiated or UV-irradiated AdCA17*lacZ* in a 40 μl volume at an MOI of 40–160 pfu/cell. Cells were incubated at 37 °C for 90 min, after which time cells were overlaid with 160 μl of warm supplemented α-MEM and incubated at 37 °C for 12, 24 or 40 h to allow for reporter gene expression.

2.5. *Quantitation of β-gal activity*

Fibroblasts were harvested at 12, 24 or 40 h following the infection with AdCA17*lacZ*. The infected cell monolayer was incubated with 60 μl per well of 1 mM chlorophenol red β-D-galactopyranoside (CPRG; Boehringer–Mannheim, Indianapolis, IN) in 0.01% Triton X-100, 1 mM $MgCl_2$, and 100 mM phosphate buffer at pH 8.3. Light absorbance at 570 nm (A_{570}) was determined several times following the addition of β-gal substrate using a 96-well plate reader (Labsystems Multiscan MCC/340 and/or Bio-Tek Instruments EL340 Bio Kinetics Reader). Background readings of β-gal activity for uninfected cells were determined in all experiments and only readings for AdCA17*lacZ* infected cells that were twice background were included in the β-gal survival curves. In a few experiments when β-gal activity was scored at 12 h after infection, survival of β-gal activity following UV to AdCA17*lacZ* of less than 37% could not be determined directly from the survival curve even at the highest UV fluences given to the virus. This was due to the reduced amount of β-gal synthesized by 12 h compared to 24 and 40 h at the MOI of AdCA17*lacZ* employed. In these instances the β-gal survival curve was fitted to the linear quadratic function $\ln(\text{surviving fraction}) = -(\alpha X + \beta X^2 + \gamma)$ and the D_{37} value of the curve obtained by extrapolation as reported previously [34].

2.6. *Western blotting*

Confluent monolayers of cells in 6-well plates were infected with Ad5p53wt or AdCA18luc at an MOI of 150 plaque-forming units (pfu)/cell or mock-infected. Forty-eight hours later infected and mock-infected cells were scraped and suspended in lysis buffer [50 mM Tris, 150 mM NaCl, 1% NP40, 10% protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), pH 8.0] and kept on ice for 60 min. The lysate was cleared by centrifugation at 13,000 × *g* for 2 min and the protein concentration was determined by the Bradford microassay procedure. Samples were resolved over a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis for 1 h at 100 V and transferred to nitrocellulose membrane (Amersham Pharmacia Biotech) (100 V for 1 h). Blots were blocked with 20% skim milk in 1× TBST (Tris-buffered saline with Tween 20) for a minimum of 1 h. Membranes were incubated with horseradish peroxidase (HRP)-conjugated mouse monoclonal anti-p53 (Santa Cruz Biotechnology Inc., Santa Cruz, CA) for 1 h at room temperature. Specific antibody labeled p53 protein was detected using enhanced chemoluminence (ECL) detection according to manufacture's instructions (Western Lighting, Perkin-Elmer Life Sciences). Subsequently, the blots were stripped using a specific buffer (100 mM 2-mercaptoethanol, 62.5 mM Tris–HCl, 2% SDS, pH 6.7) for 1 h at 65 °C and re-probed with anti-beta-actin monoclonal antibody (Sigma–Aldrich Inc.). The data were analysed using a Kodak Digital Science Image Station 440 CF and p53 protein levels were determined relative to actin levels.

3. Results

3.1. *Pre-treatment of cells with low UVC fluence enhances HCR of β-galactosidase expression for UVC-irradiated AdCA17lacZ in normal, XP-C and CS-B, but not XP-A fibroblasts*

AdCA17*lacZ* and Ad5HCMVsp1*lacZ* adenoviruses both express the lacZ reporter gene under the control of the human

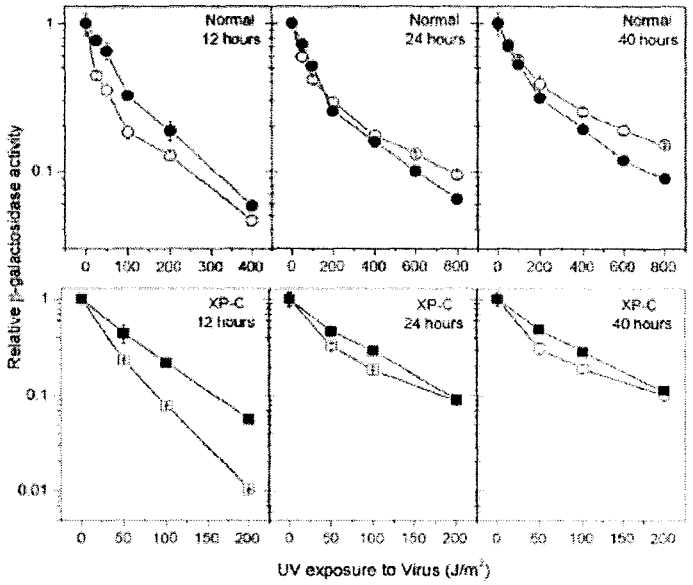

Fig. 1 – Effects on pre-UV-treatment of cells on HCR of UV-damaged AdCA17*lacZ* in normal and XP-C primary human fibroblasts. Results of typical experiments are shown for normal (●, ○) and XP-C (■, □) fibroblasts. Closed symbols represent pre-UV-treated cells, open symbols represent non-treated cells. The time between infection and scoring for β-gal activity is given in the figure. Each point is the average of 3 replicates; error bars represent one standard error.

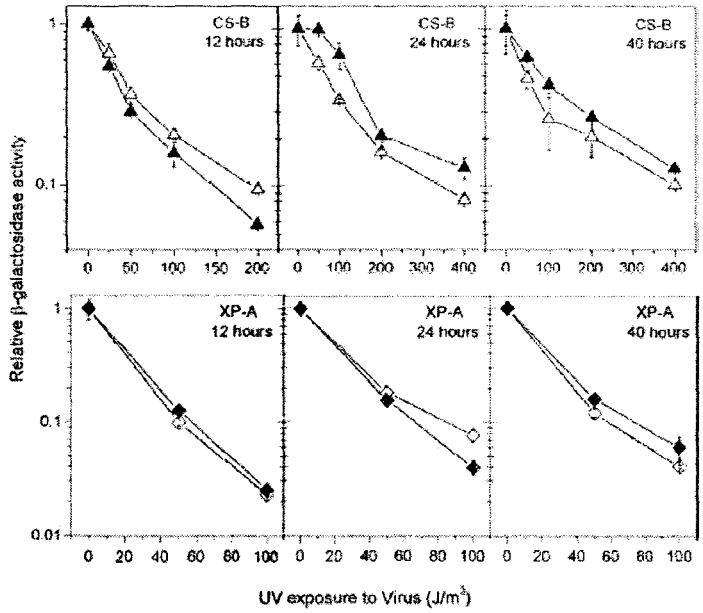

Fig. 2 – Effects on pre-UV-treatment of cells on HCR of UV-damaged AdCA17*lacZ* in CS-B and XP-A primary human fibroblasts. Results are shown for CS-B (▲, △) and XP-A (◆, ◇). Closed symbols represent pre-UV-treated cells, open symbols represent non-treated cells. The time between infection and scoring for β-gal activity is given in the figure. Each point is the average of three replicates; error bars represent one standard error.

cytomegalovirus (HCMV) inserted in the deleted E1 region of the viral genome. The orientation of the *lacZ* reporter gene in AdHCMVsp1*lacZ* is right-to-left, whereas the orientation in AdCA17*lacZ* is left-to-right. It has been reported that adenovirus encoded reporter gene cassettes in the left-to-right orientation express 7-fold higher levels of β-gal than those with inserts in the right-to-left orientation [39]. We have reported previously that pre-UV-treatment of normal, XP-C and CS-B cells resulted in enhanced HCR for β-gal expression of a UV-damaged reporter gene encoded in Ad5HCMVsp1*lacZ*, although the enhanced HCR was delayed in CS-B cells [35]. In the present work, we have used the recombinant adenovirus AdCA17*lacZ* to examine HCR of a UVC-damaged reporter gene. Since the orientation of the *lacZ* gene within the viral genome might influence HCR, it was considered important to first examine the effects of pretreatment of cells with low UV fluences on HCR for β-gal expression for the UV-damaged reporter gene using the AdCA17*lacZ* virus. Normal, CS and XP fibroblasts were pre-treated with a low UV fluence (12 J/m^2) and subsequently infected with either UV-irradiated or non-irradiated AdCA17*lacZ* and scored for β-gal activity at 12, 24 and 40 h after infection. Typical results for relative β-gal activity of the UV-damaged reporter gene in untreated and UV-treated fibroblasts are shown in Figs. 1 and 2. It can be seen that enhanced HCR of UV-damaged β-gal reporter gene is detected in normal and XP-C deficient fibroblasts that were pre-treated with UV at 12 h post-infection (Fig. 1, left panels), but not in UV-irradiated CS-B or XP-A deficient fibroblasts (Fig. 2, left panels). Pretreatment of XP-C and CS-B deficient fibroblasts with UV resulted in enhanced HCR of the UV-damaged reporter gene at later times of β-gal activity scoring, but not in normal or XP-A deficient fibroblasts (Figs. 1 and 2, middle and right panels). The UV fluence required to reduce the β-gal activity to 37% of that for non-irradiated virus (D_{37}) was extrapolated from the relative β-gal activity curves and used as a measure of HCR. The D_{37} values for survival of β-gal activity of UV-damaged reporter gene were combined from multiple experiments and the average D_{37} values in UV-irradiated compared to non-irradiated cells are shown as a function of time between virus infection and β-gal scoring in Fig. 3. In addition, the relative D_{37} value in UV-irradiated compared to that in non-irradiated cells was obtained for individual experiments and the average relative D_{37} values are shown in Table 1. It can be seen that HCR is reduced in untreated XP-A, XP-C and CS-B compared to normal fibroblasts (Figs. 1–3). In addition, pre-UV-exposure to cells resulted in enhanced HCR for normal, XP-C and CS-B, but not XP-A, and the HCR enhancement in CS-B was delayed, consistent with previously published results using the AdHCMVsp1*lacZ* virus [35]. These results indicate that pre-UVC-exposure to cells results in up-regulation of both TCR and GGR in the UVC-damaged reporter gene.

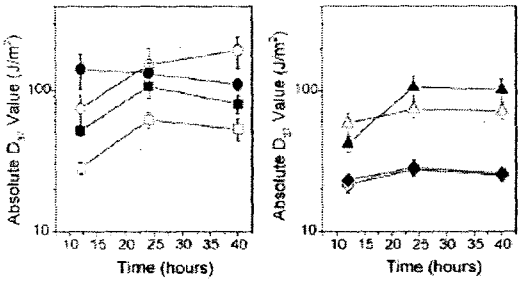

Fig. 3 – UV-enhanced HCR was observed in normal, XP-C and CS-B fibroblasts but not in XP-A fibroblasts. Results show the mean D_{37} ± S.E. value for β-gal expression of UV-irradiated AdCA17*lacZ* in pre-UV-treated compared to that in non-treated cells for normal (●, ○), XP-C (■, □), CS-B (▲, △) and XP-A (◆, ◇) fibroblasts. Closed symbols represent pre-UV-treated cells; open symbols represent non-treated cells. Each data point is the average ± S.E. of several independent experiments.

3.2. *Pre-infection of cells with Ad5p53wt enhances HCR of β-galactosidase expression for UVC-irradiated AdCA17lacZ in normal, CS-B and XP-C but not XP-A fibroblasts*

Exposures in the 2–20 J/m^2 range result in several fold accumulation of p53 in both normal and NER-deficient cells detectable as early as 6 h after UV and this increased accumulation of p53 is still evident at 24 h after UV exposure ([23,46–48] and data not shown). The up-regulation of both TCR and GGR in the UV-damaged reporter gene described in Section 3.1 could therefore result from the increased accumulation of p53 due to UV exposure. To examine the possible role of

Table 1 – Relative D_{37} values for β-gal activity of UV-irradiated AdCA17*lacZ* in pre-UV-treated (12 J/m^2) compared to untreated cells

Cell strain	Relative D_{37}, 12 h	P value	Relative D_{37}, 24 h	P value	Relative D_{37}, 40 h	P value
Normal	1.59 ± 0.15* (12)	5.79×10^{-4}	1.05 ± 0.14 (12)	0.694	0.82 ± 0.13 (12)	0.182
CS-B	0.78 ± 0.08* (5)	0.031	1.38 ± 0.16* (6)	0.042	1.44 ± 0.23 (6)	0.085
XP-C	1.85 ± 0.16* (4)	0.002	1.75 ± 0.19* (3)	1.64×10^{-4}	1.53 ± 0.11* (4)	0.002
XP-A	1.12 ± 0.10 (5)	0.236	1.03 ± 0.13 (3)	0.815	1.03 ± 0.06 (3)	0.587

Pooled results are shown for expression of β-gal activity at 12, 24 or 40 h post-infection and are represented as mean relative D_{37} ± S.E. UV-enhanced HCR (relative D_{37}) of the UV-damaged reporter gene activity is observed early in normal and TCR-proficient fibroblasts XP-C, is delayed in TCR-deficient CS-B fibroblasts and absent in XP-A fibroblasts. # is the number of independent determinants, each performed with three replicates.

* Significantly >1 by an independent t-test (P value <0.05).

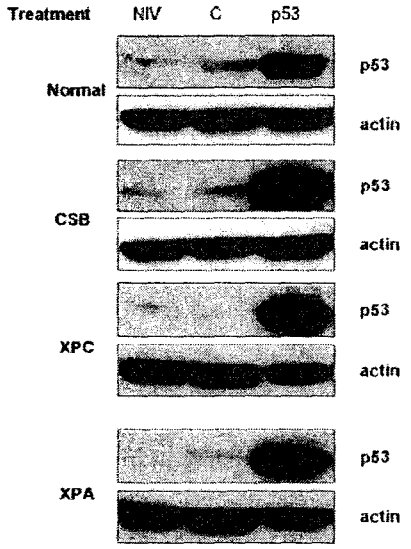

Fig. 4 – Increased p53 expression upon Ad5p53wt virus infection in human fibroblasts. Confluent normal, CS-B, XP-C and XP-A were either mock-infected (NIV-no virus infection), infected with a control virus (AdCA18luc) or infected with the Ad5p53wt virus. Equal amounts of complete cell lysates were run on 10% SDS-PAGE gel, blotted on a nitrocellulose filter and examined for the expression levels of p53 using anti-p53 antibody. The amount of actin was determined by using anti-actin antibody to obtain the sample loading control.

p53 in enhanced HCR, we examined HCR of UV-irradiated AdCA17*lacZ* in human normal, CS and XP fibroblasts, which were pre-infected with either Ad5p53wt or a control virus, AdCA18*luc*. The p53 expression levels were determined in the NER-proficient and deficient fibroblasts and are shown in Fig. 4. It can be seen that the p53 expression levels were increased in all cell lines when cells were pre-infected with Ad5p53wt compared to AdCA18*luc*-pre-infected cells and scored at 40 h after infection. Similar results were obtained when infected cells were scored at 24 h after infection (data not shown), such that increased p53 levels were present in all the different fibroblast strains at the time of infection with AdCA17*lacZ* used in the HCR experiments.

Cells were infected with Ad5p53wt or AdCA18*luc*. Twenty-four hours later cells were infected with untreated or UV-treated AdCA17lacZ and subsequently scored for β-gal activity at 12, 24 and 40 h after infection. Typical survival curves for β-gal activity in Ad5p53wt-pre-infected normal, CS-B, XP-C and XP-A deficient fibroblasts compared to AdCA18*luc*-pre-infected (control virus) fibroblasts are shown in Figs. 5 and 6. It can be seen that there is an increase in HCR of UV-damaged β-gal reporter gene in Ad5p53wt-pre-infected normal, CS-B and XP-C deficient fibroblasts, but not in XP-A deficient fibroblasts when compared to AdCA18*luc*-pre-infected fibroblasts at 12 h after virus infection (Figs. 5 and 6, left panels). In addition there was an enhancement in HCR of the UV-damaged β-gal in Ad5p53wt-pre-infected CS-B deficient fibroblasts at 24 and 40 h after virus infection, but not in normal, XP-C, or XP-A deficient fibroblasts (Figs. 5 and 6, middle and right panels). The D_{37} values for survival of β-gal activity of the UV-damaged reporter gene were combined from multiple experiments and the average D_{37} values in Ad5p53wt-pre-infected compared to AdCA18*luc*-pre-infected cells are shown as a function of time between virus infection and β-gal scoring in Fig. 7. In addition, the relative D_{37} value in Ad5p53wt-pre-infected compared to AdCA18luc-pre-infected cells was obtained for individual experiments and the average relative D_{37} values are shown in Table 2. These results indicate that increased expression of p53 results in up-regulation of both TCR and GGR of the UVC-damaged reporter gene.

3.3. *Pre-infection of cells with Ad5p53wt results in enhanced expression of the β-gal reporter gene for undamaged AdCA17lacZ in normal, CS-B, XP-C and XP-A fibroblasts*

We have reported previously that pretreatment of human fibroblasts with UV results in an increased expression of the recombinant adenovirus encoded reporter gene. Up regulation in expression of the undamaged reporter gene due to pre-UV-treatment of cells occurs at lower UV fluences and to higher levels in TCR deficient compared to normal and GGR-deficient human fibroblasts [49,50]. Since a stalled transcription complex due to transcription blocking UV-induced photolesions is thought to be a signal for the recruitment of the DNA incision complex and subsequent removal and repair of the lesion [51], increased transcription might be expected to lead to an enhanced rate of repair of photolesions by TCR. It was therefore considered of interest to examine expression of the undamaged AdCA17*lacZ* reporter gene in cells that had been pre-infected with Ad5p53wt compared to cells pre-infected with the control virus AdCA18. The result shown in Table 3 indicate that increased expression of p53, due to pre-infection of cells with Ad5p53wt, results in an upregulation in expression of the undamaged reporter gene by about 2-fold in both NER-deficient and NER proficient human fibroblasts.

3.4. *Enhanced HCR of β-gal expression for UVC-irradiated AdCA17lacZ due to pre-UVC-treatment of cells is greater in cells pre-infected with Ad5p53wt in normal, CS-B and XP-C but not XP-A fibroblasts*

UV treatment of normal human cells results in increased p53 expression (reviewed in Ref. [52]) as well as several post-translational modifications, including phosphorylation, which can activate p53 function [53,54]. Since we have shown that increased expression of p53 alone or pre-UV-irradiation of cells resulted in enhanced HCR of the UV-damaged reporter gene, we also examined the combined effects of both increased p53 expression and pre-UVC-treatment of cells on HCR of the UVC-damaged reporter gene. Cells were pre-infected with either Ad5p53wt or AdCA18*luc* virus and pre-treated with low UVC-fluence prior to infection with UVC-treated AdCA17*lacZ*. Representative results for β-gal activity of the UV-damaged reporter gene in UV-irradiated cells that

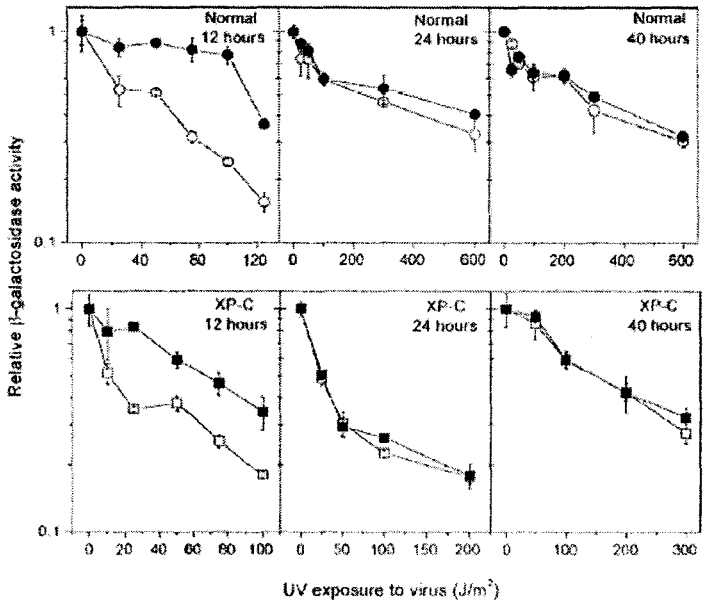

Fig. 5 – Pre-infection with Ad5p53wt virus results in enhanced HCR of β-gal activity for UV-irradiated AdCA17*lacZ* in normal and XP-C primary human fibroblasts. Results of typical experiments are shown for normal (●, ○) and XP-C (■, □) fibroblasts. Closed symbols represent pre-Ad5p53wt-infected cells, open symbols represent pre-AdCA18*luc*-infected cells. The time between infection and scoring for β-gal activity is given in the figure. Each point is the average of 3 replicates; error bars represent one standard error.

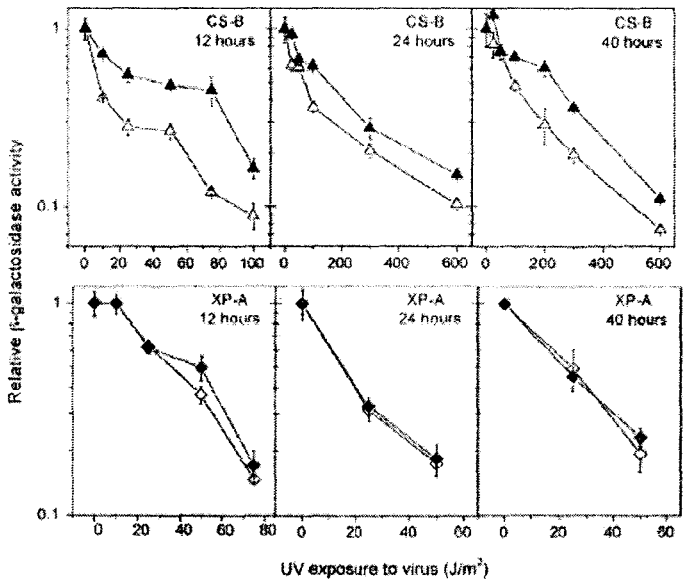

Fig. 6 – Pre-infection with Ad5p53wt virus results in enhanced HCR of β-gal activity for UV-irradiated AdCA17*lacZ* in CS-B, but not XP-A primary human fibroblasts. Results are shown for CS-B (▲, △) and XP-A (◆, ◇). Closed symbols represent pre-Ad5p53wt-infected cells; open symbols represent pre-AdCA18*luc*-infected cells. The time between infection and scoring for β-gal activity is given in the figure. Each point is the average of 3 replicates; error bars represent one standard error.

Table 2 – Relative D_{37} values for β-gal activity of UV-irradiated AdCA17*lacZ* in Ad5p53wt-pre-infected compared to that in AdCA18-pre-infected cells

Cell strain	Relative HCR, 12 h	P value	Relative HCR, 24 h	P value	Relative HCR, 40 h	P value
Normal	1.58 ± 0.17* (6)	0.007	1.18 ± 0.14 (14)	0.205	1.11 ± 0.25 (5)	0.658
CS-B	1.94 ± 0.35* (7)	0.022	1.70 ± 0.19* (13)	0.001	3.40 ± 1.21 (7)	0.070
XP-C	2.04 ± 0.48* (8)	0.049	1.07 ± 0.13 (12)	0.571	0.97 ± 0.11 (4)	0.839
XP-A	1.11 ± 0.21 (7)	0.607	1.00 ± 0.12 (10)	0.987	1.13 ± 0.16 (6)	0.419

Pooled results are given for expression of β-gal activity at 12, 24 and 40 h post-infection and are represented as mean relative D_{37} ± S.E. # is the number of independent determinants each performed with three replicates.

* Significantly >1 by an independent *t*-test (*P* value <0.05).

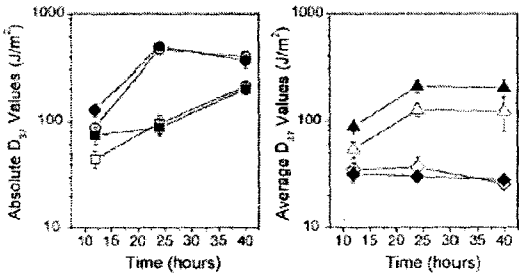

Fig. 7 – Pre-infection with Ad5p53wt virus results in enhanced HCR of β-gal activity for UV-irradiated AdCA17*lacZ* in normal, XP-C and CS-B, but not XP-A cells. Results show the mean D_{37} ± S.E. value for β-gal expression of UV-irradiated AdCA17*lacZ* in pre-Ad5p53wt-infected compared to that in pre-AdCA18*luc*-infected cells for normal (●, ○), XP-C (■, □), CS-B (▲, △) and XP-A (◆, ◇) fibroblasts. Closed symbols represent pre-UV-treated cells; open symbols represent non-treated cells. Each data point is the average ± S.E. of several independent experiments.

had been pre-infected with either Ad5p53wt or AdCA18*luc* virus are shown in Figs. 8 and 9. The D_{37} values for survival of β-gal activity of the UV-damaged reporter gene were combined from multiple experiments and the average D_{37} values in Ad5p53wt-infected compared to AdCA18*luc*-infected UV-irradiated cells are shown as a function of time between virus infection and β-gal scoring in Fig. 10. In addition, the relative D_{37} value in Ad5p53wt-infected and pre-UVC-treated compared to AdCA18*luc*-infected and pre-UVC-treated cells was obtained for individual experiments and the average relative D_{37} values are shown in Table 4. It can be seen that prior UVC-irradiation (12 J/m^2) of cells resulted in a significant enhancement in HCR of the UV-irradiated reporter gene in Ad5p53wt-pre-infected when compared to AdCA18*luc*-pre-infected normal fibroblasts when scored for β-gal activity at 12 and 40 h post-infection (Fig. 8, top left panel; Fig. 10, left panel; Table 4). In addition, prior UVC-irradiation of XP-C cells resulted in a significant enhancement in HCR of the UV-irradiated reporter gene in Ad5p53wt-pre-infected when compared to AdCA18*luc*-pre-infected normal fibroblasts when scored for β-gal activity at 24 and 40 h post-infection (Fig. 8, bottom middle and right panels; Fig. 10, left panel; Table 4). Prior UVC-irradiation of CS-B cells resulted in an enhancement in HCR of the UV-irradiated reporter gene in Ad5p53wt-pre-infected when compared to AdCA18*luc*-pre-infected cells when scored for β-gal activity at 12 and 24 h post-infection (Fig. 9, top left panel; Fig. 10, right panel). Although the enhancement in CS-B cells was not significant when considering the 12 and 24 h after infection data separately, it was close to significance for the 24 h time ($P = 0.05$) and pooling data for the 12 and 24 h time points resulted in a significant enhancement in HCR ($P = 0.023$) (Table 4). In contrast, no enhancement in HCR was detected in XP-A fibroblasts. This indicates that increased expression of p53 results in up-regulation of both TCR and GGR of the UVC-damaged reporter gene following treatment of human cells with low UV fluence.

4. Discussion

4.1. *Pre-UVC-treatment of cells results in enhanced TCR and GGR of the UVC-damaged reporter gene*

We have reported previously that pre-UV-treatment of normal, XP-C and CS-B cells resulted in enhanced HCR for

Table 3 – Relative expression for β-gal activity of undamaged AdCA17lacZ in Ad5p53wt-pre-infected compared to that in AdCA18-pre-infected cells

Cell strain	Relative expression, 12 h	P value	Relative expression, 24 h	P value	Relative expression, 40 h	P value
Normal	2.00 ± 0.28* (6)	0.005	1.57 ± 0.20* (15)	0.007	2.48 ± 0.43 (6)	0.006
CS-B	1.44 ± 0.38 (6)	0.274	1.77 ± 0.29* (12)	0.014	2.63 ± 0.38* (7)	0.001
XP-C	1.61 ± 0.42 (7)	0.169	1.91 ± 0.27* (12)	0.002	2.10 ± 0.29* (4)	0.009
XP-A	1.35 ± 0.44 (6)	0.446	2.02 ± 0.24* (10)	5.16×10^{-4}	2.04 ± 0.33* (6)	0.010

Pooled results are given for expression of β-gal activity at 12, 24 and 40 h post-infection and are represented as mean relative expression ± S.E. # is the number of independent determinants each performed with three replicates.

* Significantly >1 by an independent *t*-test (*P* value <0.05).

Table 4 - Relative D_{37} values for β-gal activity of UV-irradiated AdCA17*lacZ* in UV-irradiated and Ad5p53wt-pre-infected compared to that in UV-irradiated and AdCA18-pre-infected cells

Cell strain	Relative HCR, 12 h	P value	Relative HCR, 24 h	P value	Relative HCR, 40 h	P value
Normal	1.90 ± 0.34* (4)	0.040	1.03 ± 0.03 (7)	0.262	1.58 ± 0.18* (3)	0.019
CS-B	1.67 ± 0.50 (4)	0.23	1.50 ± 0.23 (6)	0.058	1.08 ± 0.15 (5)	0.575
XP-C	0.97 ± 0.16 (5)	0.885	1.86 ± 0.29* (8)	0.009	1.83 ± 0.31* (4)	0.036
XP-A	0.86 ± 0.19 (4)	0.519	1.10 ± 0.13 (7)	0.463	1.32 ± 0.15 (4)	0.135

Results are given for each time point. Pooled results are given for expression of β-gal activity at 12, 24 and 40 h post-infection and are represented as mean relative D_{37} ± S.E. # is the number of independent determinants each performed with three replicates.

* Significantly >1 by an independent t-test (*P* value <0.05).

β-gal expression of a UV-damaged reporter gene encoded in Ad5HCMVsp1*lacZ*, although the enhanced HCR was delayed in CS-B cells [35]. The orientation of the *lacZ* reporter gene in AdHCMVsp1*lacZ* is right-to-left, whereas the orientation in AdCA17*lacZ* is left-to-right. In addition it has been reported that adenovirus encoded reporter gene cassettes in the left-to-right orientation express higher levels of the reporter gene than those with inserts in the right-to-left orientation [39]. We therefore first examined whether the different orientation of the lacZ gene and increased β-gal expression in AdCA17*lacZ* compared to AdHCMVsp1*lacZ* influenced our ability to detect HCR and UV-enhanced HCR of the adenovirus encoded β-gal reporter gene in human fibroblasts. In the present work, we report that HCR of the UVC-damaged reporter gene in AdCA17*lacZ* is reduced in XP-C, CS-B and XP-A cells compared to that in normal human fibroblasts, consistent with our previous reports using AdHCMVsp1*lacZ*, indicating that both TCR and GGR are involved in repair of UVC-induced lesions in the transcribed strand of the reporter gene [34,36]. In addition, we show here that pre-UV-treatment of normal, XP-C and CS-B, but not XP-A cells resulted in enhanced HCR for β-gal expression of the UV-damaged reporter gene encoded in AdCA17*lacZ* and the enhanced HCR in CS-B cells was also delayed compared to that detected in normal and XP-C cells. These results indicate that the gene orientation within the deleted E1 region of the recombinant adenovirus does not affect our ability to detect HCR and UV-enhanced HCR of the UV-damaged reporter gene in human fibroblasts. The increased HCR detected in normal, GGR-deficient XP-C and TCR-deficient CS-B, but not TCR- and GGR-deficient XP-A cells reported here indicates that

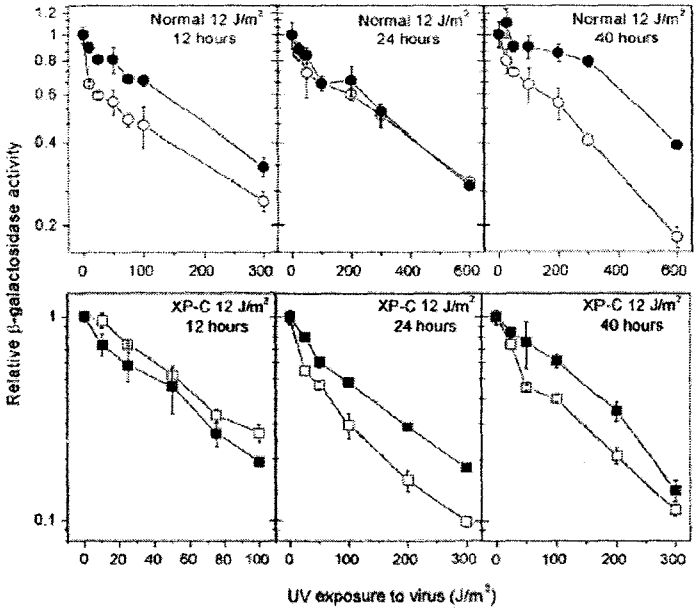

Fig. 8 – Pre-UV-irradiation of cells results in enhanced HCR of β-gal activity for UV-irradiated AdCA17*lacZ* in pre-Ad5p53wt-infected normal and XP-C fibroblasts. Results of typical experiments are shown for normal (●, ○) and XP-C (■, □) fibroblasts. Closed symbols represent pre-Ad5p53wt-infected cells; open symbols represent pre-AdCA18luc-infected cells. The time between infection and scoring for β-gal activity is given in the figure. Each point is the average of 3 replicates; error bars represent one standard error.

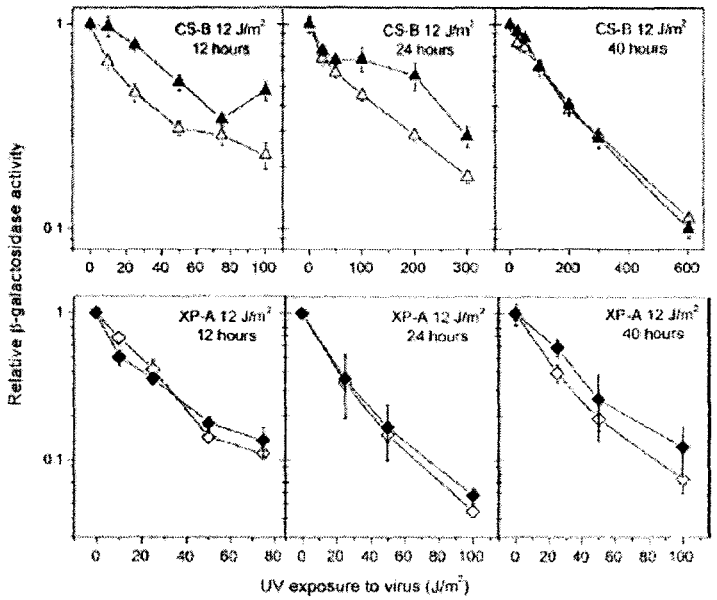

Fig. 9 – Pre-UV-irradiation of cells results in enhanced HCR of β-gal activity for UV-irradiated AdCA17*lacZ* in pre-Ad5p53wt-infected CS-B but not XP-A fibroblasts. Results are shown for CS-B (▲, △) and XP-A (◆, ◇). Closed symbols represent pre-Ad5p53wt-infected cells; open symbols represent pre-AdCA18*luc*-infected cells. The time between infection and scoring for β-gal activity is given in the figure. Each point is the average of 3 replicates; error bars represent one standard error.

pre-UVC-treatment of cells with low UVC fluence resulted in an up-regulation of both TCR and GGR of the UVC-damaged reporter gene in human cells. UV-induced TCR [55] and GGR [2] have also been reported for mammalian cell DNA.

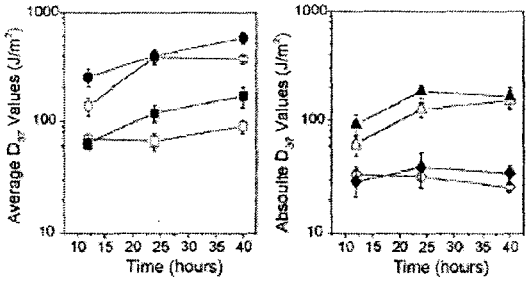

Fig. 10 – Pre-UV-irradiation of cells results in enhanced HCR of β-gal activity for UV-irradiated AdCA17*lacZ* in pre-Ad5p53wt-infected normal, XP-C and CS-B but not XP-A fibroblasts. Results show the mean D_{37} ± S.E. value for β-gal expression of UV-irradiated AdCA17*lacZ* in pre-Ad5p53wt-infected and pre-UV-treated compared to that in pre-AdCA18*luc*-infected and pre-UV-treated cells for normal (●, ○), XP-C (■, □), CS-B (▲, △) and XP-A (◆, ◇) fibroblasts. Closed symbols represent pre-UV-treated cells; open symbols represent non-treated cells. Each data point is the average ± S.E. of several independent experiments.

P53 has been shown to regulate expression of genes that are involved in the GGR pathway such as DDB2, the gene coding for the p48 protein product [16], and XPC [21]. In response to UV-induced DNA damage specifically, it is known that XP-C is recruited to the DNA damage site [48] and that p48 binds to pyrimidine dimers formed in the DNA [57,58]. Both genes are regulated in a p53-dependent manner and p53 expression determines the recruitment of these genes at the damage site [16,56]. However, activation of these genes by p53 accumulation does not contribute to accumulation of XPC and p48 products unless the genes are lesion-free [23]. Therefore, GGR-proficient CS-B cells do not accumulate p53-dependent protein products as efficiently as TCR-proficient cells [23]. Consequently, it was not surprising to observe that TCR-deficient UV-irradiated CS-B fibroblasts showed a delay in enhanced HCR of the UV-treated reporter gene [35]. The delayed UV-enhanced HCR of the UV-damaged reporter gene in TCR-deficient CS-B cells is consistent with a p53- and TCR-dependent upregulation of GGR in human cells.

4.2. *Increased expression of p53 results in enhanced HCR of the UV-damaged reporter gene in normal, XP-C and CS-B, but not XP-A fibroblasts*

Pre-infection of normal, CS-B and XP-C but not XP-A fibroblasts with Ad5p53wt resulted in enhanced HCR of the UV-damaged reporter gene compared to cells infected with

the control AdCA18luc virus (Figs. 5 and 6). These results indicate that increased expression of p53 up-regulates both GGR and TCR in the UV-damaged reporter gene. Increased expression of p53 in CS-B fibroblasts resulted in elevated HCR of the reporter gene detected at 12, 24 and 40 h post-infection. The p53-dependent up-regulation of HCR in CS-B cells is consistent with a p53-dependent up-regulation of GGR due to increased expression of the p53-dependent XPE-p48 [16] and XPC proteins [21]. In contrast to the delay in enhanced HCR in pre-UV-treated CS-B compared to pre-UV-treated normal and XP-C cells, increased expression of p53 following infection of cells with Ad5p53wt did not result in a delay in enhanced of HCR in CS-B cells. Since pre-infection of cells with Ad5p53wt resulted in increased expression of p53 but no UV-induced photolesions in the p48 or XPC genes, the lack of delay in enhanced HCR in CS-B cells pre-infected with Ad5p53wt, is consistent with our hypothesis that the delay in HCR observed in UV-irradiated CS-B cells is due to lack of TCR of lesions in the p48 and XP-C genes.

Increased expression of p53 in normal and XP-C fibroblasts resulted in elevated HCR of the reporter gene detected at 12 but not 24 or 40 h after infection. This indicated that increased expression of p53 alone resulted in an increased rate of repair of the UV-damaged reporter gene in normal and XP-C cells. TCR is the sub-pathway of NER dedicated to the removal of UV-induced DNA damage from the transcribed strand of active genes [59,60]. The increased rate of repair in pre-Ad5p53wt infected XP-C cells is consistent with a p53-dependent up-regulation of TCR in the transcribed strand of the UVC-damaged reporter gene. P53-induced enhancement in HCR of the UV-damaged reporter gene was not observed in XP-A cells at any of the tested time points after infection. Since XP-A fibroblasts are deficient in both GGR and TCR, this result supports our conclusion that the p53-dependent enhancement in HCR observed in XP-C cells is due to a p53-dependent enhancement in TCR of the UV-damaged reporter gene.

Previous research reports have suggested a role for p53 in TCR of UV-damaged DNA in human cells [25,26,30,61]. Wang et al. demonstrated that the XP-B and XP-D proteins employed in GGR and TCR interacts with p53 [30]. In addition, Wang et al. demonstrated that p53 binds to CS-B suggesting a role for p53 in the TCR pathway [30]. Direct evidence for a role of p53 in both TCR and GGR came from Therrien et al. who used a sensitive ligation-mediated PCR technique to demonstrate a repair deficiency of UVB-induced CPDs in both non-transcribed and transcribed strands of the p53 and/or c-jun loci in LFS cells (mutant p53) and human fibroblasts expressing the human papilloma virus (HPV) E6 oncoprotein, which abrogates p53 [25]. Furthermore, Mathonnet et al. reported that p53-deficient human lymphoblastoid cells displayed decreased repair of UVB-induced CPDs in both the non-transcribed and transcribed strand of *c-jun* and *hprt* gene loci when compared to the control wild-type p53-expressing cells, whereas the repair of UVC-induced CPDs was reduced only in the non-transcribed strand of the targeted genes [26]. This finding suggested a role for p53 in the removal of CPDs by TCR that is dependent upon the UV-wavelength incident on the cell. The wavelength dependence may result from the difference in the quantity and spectrum of photolesions induced in cellular DNA following UVB compared to UVC-irradiation and/or due to differences in the signalling pathways induced by UVB compared to UVC exposure. More recently, Mathonnet et al. have reported that expression of the hepatitis B virus protein (HBX) (which binds and functionally inactivates p53) in human TK6 cells results in reduced removal of CPDs along both the transcribed and non-transcribed strand of the c-jun gene following exposure to either UVB or UVC, giving further evidence of p53-dependent as well as p53-independent TCR in human cells [27].

We report here that increased expression of p53 due to infection with Ad5p53wt results in an up-regulation in expression of the undamaged reporter gene by about 2-fold in both NER-deficient and NER-proficient cells. In contrast we have reported that up-regulation in the expression of the undamaged reporter gene due to pre-UV-treatment of cells occurs at lower UV fluences and to higher levels in TCR-deficient compared to normal and GGR-deficient fibroblasts [49,50]. The latter results suggest that unrepaired UV-induced DNA damage in active genes triggers increased reporter activity from constructs driven by CMV promoters in human fibroblasts as reported previously. This suggests also that the mechanism leading to increased expression of the undamaged reporter due to pre-UV-exposure of cells is different from that due to increased expression of p53 due to pre-infection of cells with Ad5p53wt. The increased expression of the undamaged reporter gene due to pre-infection with Ad5p53wt is surprising since we found no consensus sequences of transcription factor binding sites for p53 in the human cytomegalovirus immediate early promoter controlling the p53 gene in Ad5p53wt [62]. However, the CMV promoter does contain transcription factor binding sites for NF-κB, AP1, CRE/ATF and SP1 [62]. Since p53 can activate NF-κB [63,64] it is possible that the increased expression of the undamaged reporter gene results from p53-dependent up-regulation of NF-κB.

Since a stalled transcription complex due transcription blocking UV-induced photolesions is thought to be a signal for the recruitment of DNA incision complex and subsequent removal and repair of the lesion [51], increased transcription might be expected to lead to an enhanced rate of repair of lesions by TCR. It is therefore possible that the enhanced expression of the reporter gene in normal and XP-C cells contributes, in part at least, to an increased rate of repair of lesions in the reporter gene by the TCR pathway. This is consistent with reports of increased repair of UV-induced lesions in the metallothionein gene of Chinese hamster ovary cells that has been induced to transcribe compared to the same gene in the uninduced state [65]. In addition, Huang et al. show that wild-type p53 mediated enhancement in the transcription of a plasmid encoded reporter gene directs a resistance of the transcribed DNA to UV inactivation and reactivates the reporter gene (increases HCR of the UV-damaged reporter gene) in rodent cells [66]. Furthermore some single point substitution mutants of p53 that maintain near normal ability to activate transcription did not display increased HCR of the UV-damaged reporter gene, whereas other mutants with reduced transcriptional activity retained an increased HCR of the UV-damaged reporter gene. This suggested that although HCR of the UV-damaged reporter gene is transcriptionally dependent, these two activities are genetically distinct.

4.3. *Increased expression of p53 together with prior-UV-treatment of cells results in enhanced HCR of the UV-damaged reporter gene in normal, XP-C and CS-B, but not XP-A fibroblasts*

The p53 protein exists at low basal levels in a latent state in normal, unstressed cells. In response to a cellular stress, p53 levels increase in the cell due to its decreased degradation or increased stabilization. Mdm2 (murine double minute 2, also known as Hdm in humans) regulates the level of p53 protein available after a cellular stress (as reviewed in Ref. [52]). Briefly, Mdm2 protein controls p53 levels by binding directly to p53 and inhibiting its gene expression activities [67], or by adding ubiquitin to promote p53 degradation [68]. The stabilization of p53 is achieved by inhibiting Mdm2 binding to p53, which is controlled through p53 phosphorylation. There are several post-translational modifications that can activate p53, including phosphorylation (reviewed in Ref. [54]). There is evidence that p53 phosphorylation occurs on serine residues 15, 20, 33, 37 and Thr18, which inhibits Mdm2 binding to p53 [69–71] and can be achieved by UV-irradiation [53]. In addition, it has been shown that at low UV fluence only Ser33 and Ser37 were maximally phosphorylated, while Ser15 was phosphorylated when higher fluences were used [53]. This indicates that the p53 changes undergone at low UV fluences are only a subset of the modifications possible.

UV damage causes both rapid and delayed expression of genes. For example p53 phosphorylation occurs within 10–30 min of UV exposure at different sites [72,73]. In addition to rapid activation of transcription factors by UV, p53 is also activated by persistent DNA damage in active genes. This process is believed to activate delayed responsive genes [74]. Therefore, it appears that the p53-phosphorylated sites in response to persistent DNA damage are different when compared to those sites phosphorylated by the immediate response. This difference in the p53-phosphorylation pattern likely plays a role in how and through which NER subpathway(s) p53 responds to DNA damage in non-irradiated compared to UV-irradiated cells and in TCR-deficient compared to TCR-proficient cells.

The p53 protein can exert both protective and apoptosis-enhancing functions after exposure to DNA-damaging agents. In addition to its role as an activator and repressor of transcription, p53 may also be able to participate directly in NER and other cellular pathways independent of its ability to act as a transcription factor [30,61,75,76]. P53 can regulate the level of p53 responsive genes in unstressed cells [16,77] suggesting that p53 may regulate cellular processes without being activated by DNA damage and therefore also be biologically important for NER and other cellular pathways prior to UV exposure. Using a stable human cell line expressing a temperature sensitive p53, McKay et al. reported that functional p53 expression for 12 or more hours before UV-irradiation protected cells from apoptosis and enhanced the recovery of RNA synthesis even when functional p53 expression was maintained after irradiation [78]. It has been reported that the recovery of mRNA synthesis after the induction of RNA polymerase II-blocking lesions by UV light is greatly affected by the TCR capacity of cells [61,79,80]. The enhanced recovery of RNA synthesis in cells expressing functional p53 before UV irradiation is consistent with the repair of UV-induced cellular DNA lesions by TCR that is dependent on the level of p53 prior to UV exposure.

In the present work, we show that pre-Ad5p53wt-infection together with UVC-irradiation of normal, XP-C and CS-B, but not XP-A fibroblasts results in enhanced HCR of the UV-damaged reporter gene when compared to pre-AdCA18*luc*-infection together with UVC-irradiation of cells. The difference in the time course of the enhanced HCR in the XP-C and CS-B may reflect a difference in the contribution of transcription-dependent as opposed to transcription-independent functions of p53 to TCR and GGR. Notwithstanding the differences in the time course of the enhanced HCR among the normal, XP-C and CS-B cells, these results indicate the presence of UV-inducible TCR and GGR in the UV-damaged reporter gene that are dependent, in part at least, on the level of p53 prior to UV exposure. These results show that several aspects of the NER pathway acting on human cellular DNA are reflected in the host-mediated repair of a recombinant adenovirus-encoded and exogenously introduced reporter gene.

Conflict of interest

None.

Acknowledgement

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CHAPTER 4

Differential effects of hypoxia and acidosis on p53 expression, repair of UVC-damaged DNA and viability after UVC in normal and tumor-derived human cells

Diana Dregoesc and Andrew J. Rainbow
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Preface

In Chapter 3, a role for the p53 tumour suppressor protein in TCR and GGR of UVC-damaged reporter gene was suggested (285). Previous reports have shown that p53 can be up-regulated by the microenvironment of the tumour such as with conditions of hypoxia and hypoxia accompanied by acidosis (166-170), and that hypoxia, as well as hypoxia coupled to low pH, diminishes NER of a UV-damaged plasmid (211). Therefore, it was of interest to assess the role of p53 and hypoxia in the repair of a UV-damaged reporter gene and consequently the effect on NER.

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Differential effects of hypoxia and acidosis on p53 expression, repair of UVC-damaged DNA and viability after UVC in normal and tumor-derived human cells

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ABSTRACT

Hypoxia and low pH are commonly associated with the tumor microenvironment. We have examined the effects of hypoxia alone (HA) and hypoxia coupled to low pH (HApH) on p53 expression, nucleotide excision repair (NER) and cellular sensitivity to UVC in normal human fibroblasts and human tumor cells. p53 expression was measured using Western blotting, NER using host cell reactivation (HCR) of a UV-damaged reporter gene and cell sensitivity using the MTT assay. HApH resulted in a transient increase in p53 expression in normal fibroblasts at 6 h and in tumor cells at 6–18 h. In normal fibroblasts HApH resulted in a transient increase in HCR at early times (12–24 h) and a concomitant decrease in UVC sensitivity. In contrast, for the tumor-derived cells, the increased HCR of the UVC-treated reporter gene was delayed (36–40 h) and UVC sensitivity increased or remained the same after HApH treatment. These results suggest that early upregulation of p53 and increased repair of UV-damaged DNA after HApH treatment is required for increased cell viability after UVC. HA treatment alone also resulted in a transient increase in HCR of the UVC-damaged reporter gene at early times (12–24 h) in normal fibroblasts and a delayed increase (36–40 h) in the tumor-derived cells. However, the enhanced p53 expression was less or even absent for treatment with HA alone, and HA had no significant affect on cell viability after UVC for any of the cell lines. These results indicate a different cellular response following HApH compared to HA alone.

1. Introduction

Maintenance of cellular aerobic respiration requires a stable supply of oxygen. However, when cells are exposed to an imbalance in oxygen supply and consumption they are forced to adapt to oxygen deprivation [1]. Hypoxia, low pH and nutrient deprivation are common features of the tumor microenvironment [2]. This microenvironment heterogeneity has been shown to cause genetic instability [3,4], and has been linked to tumor progression [5]. More recent research has focused on the effects of hypoxia and acidity on the DNA repair pathways in human cells [6,7].

One of the early consequences of hypoxic stress is a drop in ATP levels in the cell [8], causing the cell to undergo G1 cell cycle arrest [9]. The switch from aerobic to anaerobic metabolism causes a decrease in cellular pH levels as a result of increased glucose consumption and lactic acid production. Cellular, non-physiological pH levels have been shown to affect the structure and function of proteins such as DNA polymerases [10]. Studies suggest that the genetic instability due to low pH and hypoxia is the additive result of increased DNA damage, defective DNA repair and enhanced mutagenesis [3,4,6,7,11]. Hypoxia is known to cause DNA lesions due to increased production of reactive oxygen species (ROS). For example, it has been found that 8-Oxoguanine can be formed in the cell under hypoxic stress, which can cause C:G to A:T transversions [12]. In addition, hypoxia has also been associated with increased endonuclease activity, resulting in DNA strand breaks [13]. Therefore, accumulating evidence suggests that DNA repair is affected by hypoxic stress.

Recent studies have reported diminished DNA repair and increased mutagenesis in mammalian cells under hypoxic and low pH (HApH) conditions [3,6,7]. Yuan et al. found a reduction in reactivation of a UV-damaged plasmid in a mouse fibroblast line and a human colorectal carcinoma cell line under conditions of HApH when compared to normoxic cells, suggesting that nucleotide excision repair (NER) was diminished under HApH conditions in these cells [6]. NER repairs UV-induced DNA damage and other bulky DNA adducts, such as cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts that are caused by UVC-irradiation [14].

In addition to reports on the effects of hypoxia on DNA repair pathways there are also reports on the effects of hypoxia on gene

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expression [3,4,15–17]. Growing evidence suggests that the p53 tumor suppressor protein plays a role in the response pathway of cellular stresses including hypoxia [9,17–22]. Hammond et al. showed that in RKO colorectal carcinoma cells p53 is only stabilized in anoxic conditions (0.02%), but not in hypoxic conditions (2%) for several treatment times between 6 and 24 h [23]. However, Chandel et al. showed that 1.5% hypoxia upregulated p53 in IMR90 lung fibroblasts after 8 h [18]. The extent of p53 involvement under such stresses could have significant implications for its involvement in DNA repair under hypoxic, as well as hypoxic coupled to acidosis conditions due to its known role in NER [24–26].

The role of DNA repair has resurfaced as a crucial factor in understanding differential tumor radiosensitivity and chemosensitivity and how it can be used as a tool for novel cancer therapies. It has been reported that DNA repair deficient cells under hypoxic conditions display an increased sensitivity to irradiation when compared to DNA repair proficient cells [27,28]. In contrast, several studies have reported that loss of mismatch repair (MMR) renders cells resistant to platinum-containing drugs [7,29,30]. Hypoxic CHO cells showed a greater survival after X-ray irradiation when compared to aerobic cells [11] and chronic hypoxia can increase tumor cells' sensitivity when compared to cells exposed to acute hypoxia prior to irradiation [31].

In the present work we have examined the effect of hypoxia alone (HA) and HApH on p53 expression, NER and viability following UVC exposure in human primary fibroblasts and some tumor-derived cell lines. We show that HApH resulted in a transient increase in p53 expression in both normal fibroblasts and tumor-derived cells. In normal fibroblasts HApH resulted in a transient increase in host cell reactivation (HCR) of a UVC-damaged reporter gene at early times and a concomitant decrease in UVC sensitivity. In contrast, for the tumor-derived cells, including HCT116p53$^{-/-}$, a tumor-derived cell line having no detectable p53 expression, there was a delayed increased HCR of the UVC-treated reporter gene and UVC sensitivity increased or remained the same after HApH treatment. These results suggest that early upregulation of p53 and early increased repair of UV-damaged DNA after HApH treatment is required for increased cell viability after UVC. HA treatment alone also resulted in a transient increase in HCR of the UVC-damaged reporter gene in normal fibroblasts and a delayed increase in the tumor-derived cells, including HCT116p53$^{-/-}$ cells. Thus the delayed upregulation of repair in the tumor cells appears to be p53 independent and does not lead to increased cell viability after UVC. The enhanced p53 expression was less or even absent for treatment with HA alone, and HA had no significant affect on cell viability after UVC for any of the cell lines. Our results indicate a different cellular response following HApH compared to HA alone.

2. Materials and methods

2.1. Cells and cell culture

Normal primary fibroblasts strains (GM9503, IMR90, GM969) were obtained from NIGMS Human Genetic Cell Repository (Coriell Institute for Medical Research, Camden, NJ), and the colon carcinoma HCT116p53$^{+/+}$ and HCT116p53$^{-/-}$ cells were obtained from Dr. B. Vogelstein, John Hopkins University School of Medicine, Baltimore, Maryland. All cell cultures were grown in a humidified incubator kept at 5% CO_2 and 37 °C, and cultured in Eagle's α-MEM supplemented with 10% fetal bovine serum and antimycotic/antibiotic (100 μg/ml penicillin, 100 μg/ml streptomycin and 250 ng/ml amphotericin B, Gibco BRL). 3340WT is a mouse fibroblast cell line carrying in its genome 15 copies of the λsupFG1 shuttle vector DNA [32] and Rcneo is a RKO colorectal carcinoma cell line that is transfected with pCMVneo plasmid [33]. The cell lines were obtained from Dr. P.M. Glazer, Yale University School of Medicine, New Haven, Connecticut. The cells were grown in DMEM supplemented with 10% fetal bovine serum and antimycotic/antibiotic (100 μg/ml penicillin, 100 μg/ml streptomycin and 250 ng/ml amphotericin B, Gibco BRL).

2.2. Virus

The recombinant non-replicating adenovirus, AdHCMV*lacZ* (AdCA17*lacZ*) was obtained from Dr. F.L. Graham, McMaster University, Hamilton, Ontario. The adenovirus, AdCA17*lacZ* contains the *lacZ* gene under the control of the HCMV-IE promoter (−299 to +72 relative to transcription start site) inserted into the E1 deleted region. The E1 region deletion of the genome renders the adenovirus unable to replicate in most mammalian cells. The viral vector was propagated, collected and titred as described previously [34].

2.3. UV irradiation of virus

UV irradiation of virus has been described previously [35]. Virus was suspended in 1.8 ml of cold PBS and was irradiated in 35 mm dishes on ice with continuous stirring using General Electric germicidal lamp (model G8T5) at a wavelength of 254 nm predominantly with an incident fluence rate of 2 J/m^2/s (J-255 shortwave UV meter, Ultraviolet Products, San Gabriel, CA). Aliquots of 200 μl were removed for each exposure to the virus and diluted appropriately with unsupplemented media.

2.4. Low pH medium

Regular DMEM or α-MEM was supplemented with 25 mM HEPES and 25 mM 4-morpholinepropanesulfonic acid (Sigma, St. Louis, MO) to acidify the medium. The medium was adjusted to pH 6.5 with 1 N HCl. The pH of the acidic media after hypoxic treatment was measured to be between pH 6.5 and 7, the pH of the media after hypoxic treatment was approximately 7.5, and the normoxic media was measured to be between pH 7.5 and 8.

2.5. Hypoxia

Hypoxic culture conditions were established by using an automatic O_2/CO_2 incubator (Thermo Electron Corporation, Marietta, OH) equipped with oxygen and carbon dioxide sensors, and connected to N_2 and CO_2 gas cylinders. The O_2 tension was set at 1% and was maintained at this level through the entire length of the experiment. The O_2 (1%) and CO_2 (5%) readings were confirmed by use of a fyrite gas analyzer (Bachrach, Pittsburg, PA).

2.6. Host cell reactivation assay

Host cell reactivation assays were performed as previously described [36]. Confluent cell monolayer was infected with either unirradiated or UV-irradiated AdCA17*lacZ* at various times in a 40 μl volume at an MOI of 80 pfu/cell. Cells were incubated at 37 °C for 90 min, after which the media was removed and cells were overlaid with 200 μl of either warm supplemented normal pH or low pH (6.5) DMEM or α-MEM and incubated under standard conditions or in a hypoxic incubator for various time periods to allow for reporter gene expression. For quantitation of β-galactosidase (β-gal) activity, the cells layer was incubated with 60 μl of 1 mM chlorophenol red β-D-galactopyranoside (CPRG; Boehringer–Mannheim, Indianapolis, IN) per well in 0.01% Triton X-100, 1 mM $MgCl_2$, and 100 mM phosphate buffer at pH 8.3. Absorbance at 570 nm light (A_{570}) was determined several times following the addition of the β-gal substrate using a 96-well plate reader (Labsystems Multiscan MCC/340 and/or Bio-Tek Instruments EL340 Bio Kinetics Reader).

2.7. Western blotting

Western blots were performed as described previously [36]. The primary antibodies used were: anti-p53 (Santa Cruz Biotechnology Inc., Santa Cruz, CA), anti-XPC (C-terminal; Sigma–Aldrich Inc., Saint Louis, MO), anti-Hif-1 alpha (BD Biosciences, San Jose, CA), and anti-beta-actin (Sigma–Aldrich Inc., Saint Louis, MO). Cell lysates were prepared as described previously [36]. Specific antibody was detected using enhanced chemoluminence (ECL) detection according to manufacturer's instructions (Western Lighting, Perkin–Elmer Life Sciences). The data were analysed using a Kodak Digital Science Image Station 440 CF and protein levels were determined relative to actin levels. The band intensities corresponding to p53 both treated and non-treated samples were digitized by using a densitometry program and then were further normalized to the intensity of the β-actin bands.

2.8. MTT reduction assay for cell viability

Cells seeded 6–12 h prior were treated using A General Electric germicidal lamp (model G8T5) at a wavelength of predominantly 254 nm with an incident fluence rate of 1 J/m^2/s (J-255 shortwave UV meter, Ultraviolet Products, San Gabriel, CA) in PBS. After UVC treatment, cells were overlayed with warm α-MEM supplemented media or with low pH 6.5 warm α-MEM supplemented media and incubated in either a normoxic or hypoxic incubator for 24, 30 or 40 h. After the times indicated, the low pH media was removed and fresh media was added and the plates from the hypoxic incubator were incubated in a normoxic incubator. Cell viability was quantified after 4–6 days of UVC treatment using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma catalog no. M2128) assay as previously described [37]. The absorbance of each well was measured at 570 nm light using a 96-well plate reader (Labsystems Multiscan MCC/340 and/or Bio-Tek Instruments EL340 Bio Kinetics Reader) and the percentage viability was calculated.

2.9. Statistical analysis

Statistical analysis of the relative D_{37} values was performed using a one sample two-tailed *t*-test. Statistical analysis of the relative Western blot band intensities obtained using a densitometry program was performed using a one sample one-tailed *t*-test. Statistical analysis of the relative D_{50} values was also performed using a one sample two-tailed *t*-test for all cell lines except for HCT116p53$^{-/-}$ at the HApH treatment, for which a one sample one-tailed *t*-test was used. For the human primary fibroblasts, GM9503, under HApH treatment after 30 and 40 h, greater than D_{50} values were obtained and the statistical analysis was performed on the greater than relative D_{50} values using a one sample two-tailed *t*-test.

3. Results

3.1. Hypoxia and hypoxia accompanied by acidosis resulted in early enhanced host cell reactivation of a UV-damaged reporter gene in primary human fibroblasts

Previously, we have reported that overexpression of p53 in primary human fibroblasts resulted in an enhanced HCR of a UVC-damaged reporter gene [36]. Since it has been reported that treatment of cells with hypoxia can alter the expression of p53 [16,18,22], it was considered of interest to examine how HA and HApH affect HCR of the UV-damaged reporter gene in normal human primary fibroblasts and tumor cells. Hypoxic response was confirmed by detection of Hif-1 alpha in primary human fibroblasts (GM9503 and IMR90) at 2 and 3 h after hypoxia and hypoxia accompanied by low pH treatment but not after normoxic conditions (data not shown). Representative graphs for the relative β-gal activity of the UV-damaged reporter gene in normoxic, HA and HApH treated cells for 24 and 30 h are shown in Fig. 1A for GM9503. The UVC fluence required to reduce β-gal activity to 37% of that for non-irradiated virus (D_{37}) was utilized as a measure of HCR. The D_{37} values for survival of β-gal activity of the UV-damaged reporter gene were calculated and used to measure the relative D_{37} value in HA compared to normoxic and HApH compared to normoxic cells for early and late time points as shown in Table 1A. A transient enhancement in HCR was observed in normal skin fibroblasts exposed to HA and HApH at the early time points tested between 12 and 24 h. In contrast, when β-gal activity was measured in these cells at later times between 30 and 40 h of HA and HApH, it was found that HCR was decreased in these cells and this decrease was significant in HApH treated cells.

Similarly, IMR90 fibroblasts exhibited an early, but transient enhancement in HCR when incubated in HA and HApH conditions for 12 h. Representative graphs for relative β-gal activity of the UV-treated reporter gene for IMR90 cells incubated in normoxic, HA and HApH conditions for 12 and 24 h are given in Fig. 1B and the D_{37} values are shown in Table 1A. HCR was enhanced when β-gal was scored at 12 h after HA and HApH treatment, but showed a significant decrease when scored at later time points (18–30 h). In summary, HA and HApH treatment of both primary fibroblasts resulted in an early and transient enhancement of HCR.

3.2. Hypoxia and hypoxia accompanied by acidosis induced a delayed enhancement in host cell reactivation of a UV-damaged reporter gene in tumor-derived cells

Representative graphs for the relative β-gal activity of the UV-damaged reporter gene in Rcneo, HCT116p53$^{+/+}$ and HCT116p53$^{-/-}$ cells scored at 24 or 30 and 40 h after treatment with normoxia, HA and HApH are shown in Fig. 2A–C. Pooled results of the relative D_{37} values in HA and HApH compared to normoxic conditions for a number of individual experiments are given in Table 1B. In agreement with published data [6], we observed a significantly reduced HCR in Rcneo (Fig. 2A, left panel) and 3340WT (data not shown) cells for 24 h treatment under HApH. Pooled results for Rcneo cells showed a reduced HCR when cells were incubated between 24 and 30 h in HA and HApH (Table 1B). In contrast, when the tumor-derived cells were incubated under HA and HApH for 36 and 40 h, an enhanced HCR was observed that was delayed compared to that observed in normal human fibroblasts

In order to determine whether p53 status influences the hypoxia enhanced HCR, we also examined the effects of HA and HApH in HCT116p53$^{+/+}$ and HCT116p53$^{-/-}$ cells. Results are summarized in Fig. 2B and C and Table 1B. Similar to the Rcneo cell's response, HCR was significantly reduced in both HCT116p53$^{+/+}$ and HCT116p53$^{-/-}$ cells at the early time points between 24 and 30 h and 24 h of HA and HApH treatment, respectively. However, when HA and HApH continued for 36–40 h in HCT116p53$^{+/+}$, and for 30–40 h in HCT116p53$^{-/-}$ cells, an enhanced HCR was detected in both cell lines irrespective of p53 status. This suggests that both HA and HApH result in a delayed enhancement of NER in HCT116 cells at 36–40 h after treatment through a p53 independent mechanism.

3.3. Accumulation of p53 under hypoxic and hypoxic/acidic stress is time dependent in normal primary human fibroblasts and several tumor-derived cells

Previously published data have shown that early hypoxic stress can upregulate p53 accumulation [16,18,22], therefore we sought to assess the levels of p53 protein in primary human fibroblasts

Table 1
Effects of hypoxia or hypoxia and low pH on HCR of the UVC-damaged reporter gene and cell survival after UVC.

Cell line	Time (h)	Hypoxia		Hypoxia and pH6.5		Time	Hypoxia		Hypoxia and pH6.5	
		Rel D_{37} early (n)	P	Rel D_{37} early (n)	P		Rel D_{37} late (n)	P	Rel D_{37} Late (n)	P
(A)										
GM9503	12–24	2,03 ± 0,38 (22)	**0,011***	2,15 ± 0,54 (20)	**0,047***	30–40	0,86 ± 0,10 (8)	0,259	0,58 ± 0,12 (4)	**0,040§**
IMR90	12	1,75 ± 0,69 (7)	0,320	1,66 ± 0,74 (7)	0,409	18–30	0,8 ± 0,08 (14)	**0,036§**	0,82 ± 0,16 (15)	0,163
(B)										
Rcneo	24–30	0,79 ± 0,14 (8)	0,189	0,67 ± 0,12 (8)	**0,035§**	36–40	1,61 ± 0,24 (7)	**0,044***	1,24 ± 0,21 (6)	0,301
HCT116p53$^{+/+}$	24–30	0,78 ± 0,06 (8)	**0,01§**	0,52 ± 0,05 (8)	**$1,66 \times 10^{-4}$§**	36–40	1,61 ± 0,17 (8)	**0,008***	1,92 ± 0,61 (8)	0,174
HCT116p53$^{-/-}$	24	0,76 ± 0,06 (5)	**0,016§**	0,49 ± 0,07 (5)	**0,001§**	30–40	1,72 ± 0,22 (11)	**0,008***	1,77 ± 0,30 (11)	**0,031***

Cell line	Time (h)	Hypoxia		Hypoxia and pH6.5	
		Rel D_{50} (n)	P	Rel D_{50} (n)	P
(C)					
GM9503	24	1,03 ± 0,10 (3)	0,751	1,68 ± 0,04 (3)	**0,004***
	30	1,12 ± 0,11 (9)	0,088	<1,54 ± 0,05 (9)	**<0,001***
	40	1,08 ± 0,05 (3)	0,315	<1,77 ± 0,07 (3)	**<0,009***
Rcneo	24	1,08 ± 0,21 (5)	0,723	1,16 ± 0,26 (5)	0,576
	30	0,83 ± 0,21 (5)	0,497	1,22 ± 0,32 (5)	0,534
	40	1,21 ± 0,21 (5)	0,376	1,16 ± 0,23 (5)	0,514
HCT116p53$^{+/+}$	24	1,05 ± 0,06 (5)	0,496	0,85 ± 0,11 (4)	0,319
	30	1,10 ± 0,16 (5)	0,572	0,88 ± 0,04 (5)	**0,025****
	40	1,09 ± 0,16 (5)	0,521	0,88 ± 0,13 (4)	0,463
HCT116p53$^{-/-}$	24	1,08 ± 0,12 (5)	0,552	0,86 ± 0,10 (5)	0,283
	30	1,02 ± 0,07 (4)	0,786	0,82 ± 0,07 (5)	**0,033****
	40	0,92 ± 0,18 (5)	0,698	0,97 ± 0,05 (4)	0,72

Relative D_{37} ratios ± standard error of HCR in hypoxia or hypoxia with acidosis (pH 6.5) compared to aerobic treated (A) human primary skin (GM9503), lung (IMR90) fibroblast cells, (B) colorectal carcinoma Rcneo cells, colon carcinoma HCT116 p53$^{+/+}$ and HCT116p53$^{-/-}$ cells of β-galactosidase expression of UV-irradiated AdCA17/*lacZ*. Cells were kept in aerobic, hypoxia or hypoxia with acidosis (pH 6.5) after AdCA17/*lacZ* infection for several different treatment times. (C) Relative D_{50} survival ratios ± standard error of hypoxia or hypoxia and low pH compared to aerobic treated human primary fibroblasts (GM9503), colorectal carcinoma (Rcneo) cells, colon carcinoma HCT116p53$^{+/+}$ and HCT116p53$^{-/-}$ cells. Cells were kept in aerobic, hypoxia or hypoxia with acidosis (pH 6.5) after UV treatment for 24, 30 or 40 h. Shows pooled results for (n) experiments as indicated for each cell line. Bold values indicate *significantly > 1 by an independent two-tailed t-test (P value < 0.05), § significantly < 1 by an independent two-tailed t-test (P value < 0.05) or **significantly < 1 by an independent one-tailed t-test (P value < 0.05).

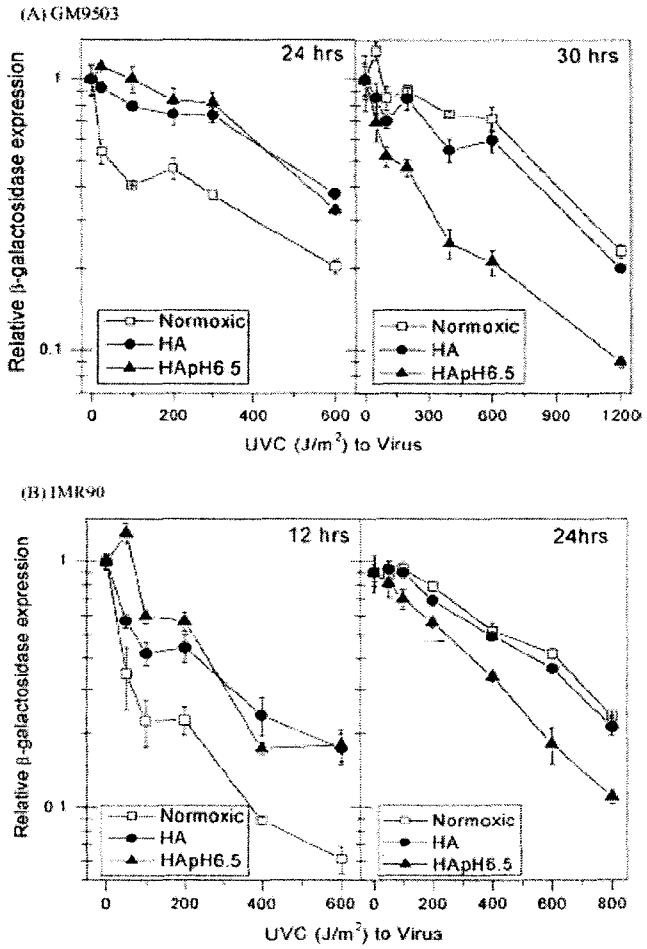

Fig. 1. Results of typical experiments representing (A) normal human primary skin (GM9503) and (B) lung fibroblasts (IMR90) under standard normoxic culture conditions (□), HA (●) or HApH6.5 (▲) conditions. Cells were infected with either non-irradiated or UV-irradiated AdCA17*lacZ*, followed by incubation under either normoxic, HA or HApH conditions for 12, 24 and/or 30 h, after which scoring for β-galactosidase was performed. Each point is the average of three replicates; error bars represent one standard error.

and tumor-derived cells at different time points up to 24 h of treatment. Results of representative Western blots are shown in Figs. 3A, B and 4A–C and the ratio of p53 expression in cells incubated under HA and HApH compared to normoxia are shown in Figs. 3C and 4D. Results indicate that in the GM9503 skin fibroblasts, p53 expression increased by 3 h of HA treatment, peaked at 12 h and subsequently decreased significantly by 24 h. HApH conditions caused an increase in p53 levels as early as 3 h, significantly peaking by 6 h in GM9503 primary human fibroblasts, and with further exposure to HApH causing a reduction in p53 protein levels at 18 h.

Similarly, significant p53 accumulation was detected in HApH treated IMR90 lung cells after 6 h of treatment. However, the level of enhancement in p53 expression in IMR90 cells due to HApH was lower than the enhancement detected in GM9503 fibroblasts. In addition, when IMR90 cells were incubated in HA alone, p53 was downregulated by 12 h.

Since the XPC gene, which is involved in the global genomic repair (GGR) pathway of NER, has been reported to be upregulated in a p53-dependent manner [38], we sought also to determine the expression levels of the XPC protein under HA and HApH conditions in GM9503 fibroblasts. Using Western blotting, we found that compared to cells treated under normoxia, there was no significant increase in XPC expression following up to 40 h of HA or HApH treatment (data not shown). In contrast, and as previously described [38], UV irradiation (15 J/m²) resulted in increased expression of XPC in GM9503 cells at 40 h post irradiation (data not shown).

Treatment with HA alone resulted in a downregulation of p53 in HCT116p53$^{+/+}$ and Rneo cells by 3 and 12 h, respectively. As expected, there was no p53 expression detected in the p53 deficient cell line, HCT116p53$^{-/-}$, at any time points tested. In contrast, HApH induced p53 accumulation in HCT116p53$^{+/+}$ cells at 12, 18 and 24 h of treatment. Pooling results for 12, 18 and 24 h indicated a significant increase in p53 expression following HApH treatment in

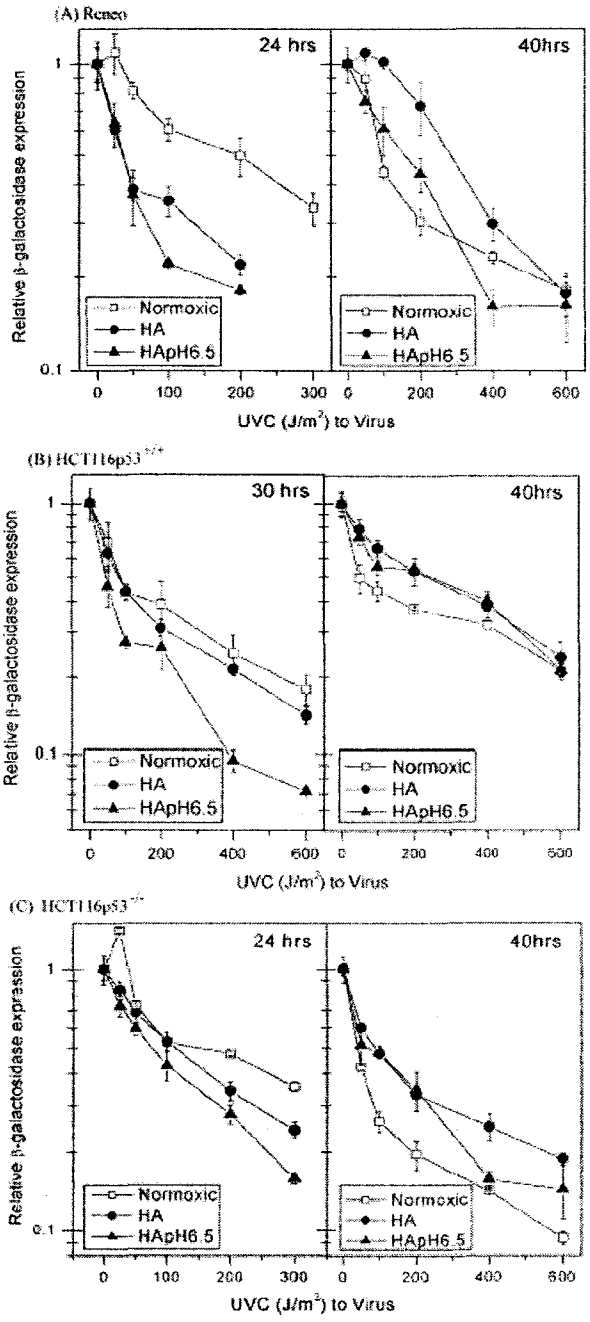

Fig. 2. Results of typical experiments representing (A) colorectal carcinoma cell line (Rcneo), (B) colon carcinoma cells HCT116 p53$^{+/+}$ and (C) HCT116p53$^{-/-}$ cells under standard normoxic culture conditions (□), HA (●) or HApH6.5 (▲) conditions. Cells were infected with either non-irradiated or UV-irradiated AdCA17*lacZ*, followed by incubation under either normoxic, HA or HApH conditions for 24, 30 and 40 h, after which scoring for β-galactosidase was performed. Each point is the average of three replicates; error bars represent one standard error.

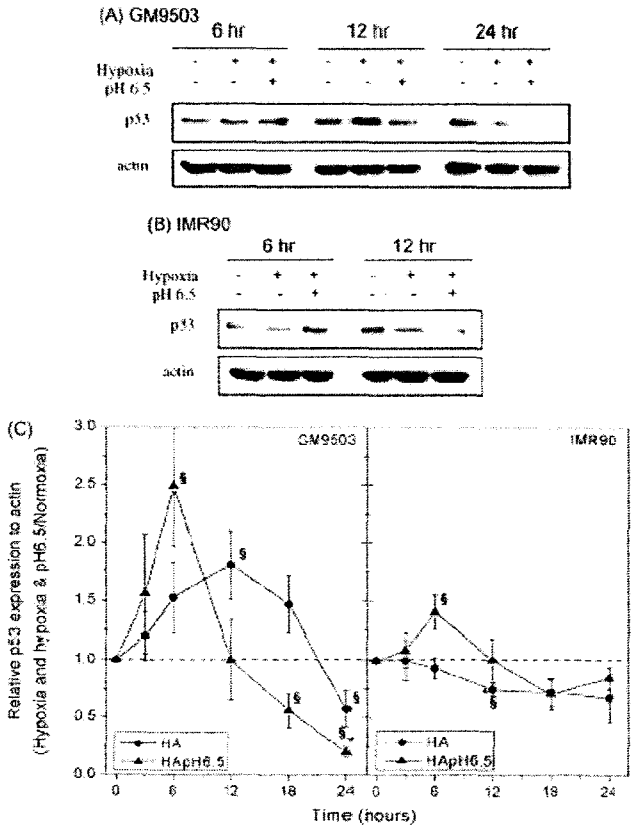

Fig. 3. Expression of the p53 tumor suppressor protein under hypoxic and hypoxic/acidic stress. Western blot analysis of p53 protein levels in (A) GM9503 and (B) IMR90 cells harvested at the indicated time after treatment of hypoxia and hypoxia coupled to pH6.5. (C) Quantitation of p53 expression in HA (●) and HApH6.5 (▲) treated GM9503 and IMR90 primary human fibroblasts at 3, 6, 12, 18 and 24 h compared to normoxic cells. Band intensities were approximated using standard densitometry. The protein estimation was normalized to actin levels and is based on three to five experiments.

HCT116p53$^{+/+}$ cells (mean increase = 1.47 ± 0.21; P = 0.027 as determined by a one-tailed t-test). HApH also induced p53 accumulation in Rcneo cells at 6, 12 and 18 h of treatment. However, this increase was not significant when data for 6, 12 and 18 h were pooled (mean increase = 1.93 ± 0.57; P = 0.07 as determined by a one-tailed t-test).

3.4. Differential cell growth and viability of primary cells and tumor cells after UVC treatment and under hypoxia and low pH conditions

Although we found that DNA repair can be increased due to HA and HApH in both primary and tumor-derived cells, we thought it beneficial to investigate whether this finding reflects cell growth and viability of cells after UV exposure. Representative graphs for the effects of HA and HApH on cell growth following exposure to UVC using the MTT assay are shown in Fig. 5. The UVC fluence required to reduce cell growth to 50% of that for non-treated cells (D_{50}) was utilized as a measure of cell growth and viability. The D_{50} values were calculated and used to measure the relative D_{50} value in HA compared to normoxic cells and HApH compared to normoxic cells for treatment times of 24, 30 and 40 h (Table 1C).

The relative D_{50} values in human primary fibroblasts indicated no significant effect was induced in HA treated cells when compared to normoxic cells for any of the treatment times tested (Fig. 5A). However, the relative D_{50} values were significantly higher (P < 0.05) in HApH treated fibroblasts compared to normoxic fibroblasts for all the treatment times tested. Similarly, as shown in Fig. 5A (Panel 3), a significant increase in cell growth and viability after UV exposure was also found in fibroblasts exposed to low pH levels without HA. The average relative D_{50} value for 30 h treatment with low pH alone compared to treatment under aerobic conditions for six independent experiments was 1.43 ± 0.15 (P = 0.034), similar to the relative D_{50} value for 30 h treatment with HApH compared to treatment under aerobic conditions (1.54 ± 0.05) as shown in Table 1C.

In contrast, HApH reduced cell viability after UV exposure in both HCT116p53$^{+/+}$ and HCT116p53$^{-/-}$ cells indicating a reduction in cell growth after UV irrespective of p53 status (Fig. 5C and D). Although there was a significant decrease in cell growth following UV exposure in both of these cell lines after 30 h of HApH treatment (Table 1C), cell growth under HA alone was indistinguishable from normoxic conditions in both cell lines. Furthermore, there was no significant difference in cell growth after UV exposure in Rcneo cells after HA or HApH for any of the treatment times tested (Fig. 5B).

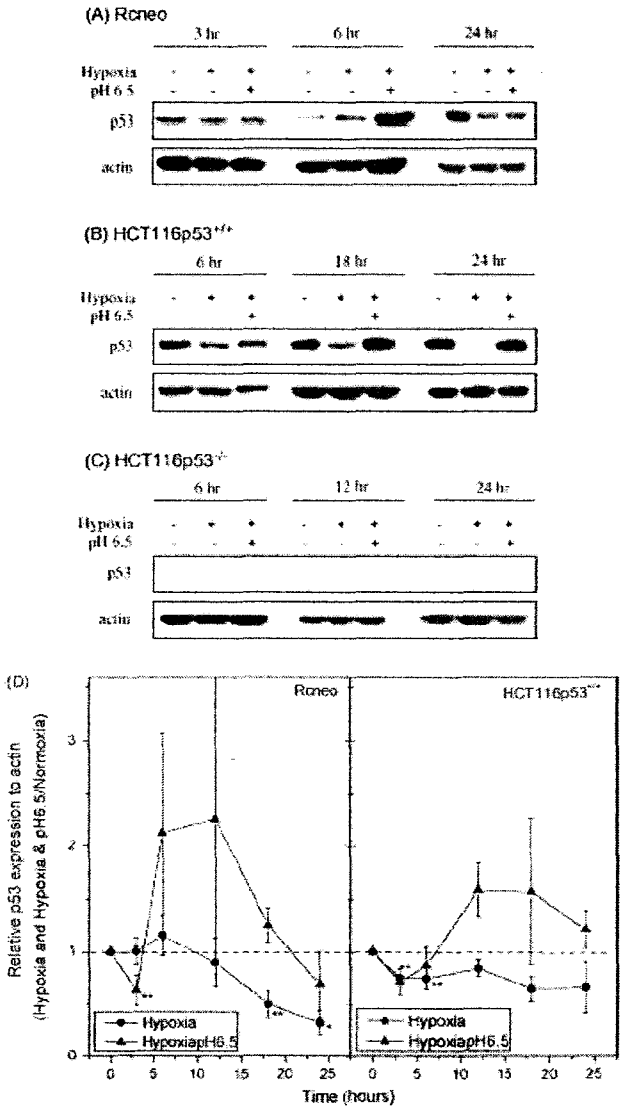

Fig. 4. Expression of the p53 tumor suppressor protein under hypoxic and hypoxic coupled to acidic stress. Western blot analysis of p53 protein levels in (A) Rcneo, (B) HCT116 p53$^{+/+}$ (C) HCT116p53$^{-/-}$ cells harvested at the indicated time after treatment of hypoxia and hypoxia coupled to pH6.5. (D) Quantitation of p53 expression in HA (●) and HApH6.5 (▲) treated Rcneo, HCT116 p53$^{+/+}$ and HCT116p53$^{-/-}$ at 3, 6, 12, 18 and 24 h compared to normoxic cells. Band intensities were approximated using standard densitometry. The protein estimation was normalized to actin levels and is based on three to four experiments.

4. Discussion

4.1. Hypoxia and low pH stress induces a transient increase in p53 and NER in primary human cells

The main focus of this study was to investigate the effects of HA and HApH on NER of UV-induced DNA damage and UV sensitivity in human cells. When GM9503 primary human fibroblasts were treated with HA and HApH, a significant enhancement of HCR of the UVC-damaged reporter gene was detected at 12–24 h of treatment, which was absent at later time points of 30–40 h. Previously we have shown that pretreatment of NER-proficient GM9503 normal human fibroblasts with UV results in a transient p53-dependent increase in HCR of a UVC-damaged reporter gene as early as 12 h after infection that is absent in cells scored 40 h after infection [36,39–41]. The current data indicates that HA and HApH can also induce a transient upregulation of HCR in normal human fibroblasts.

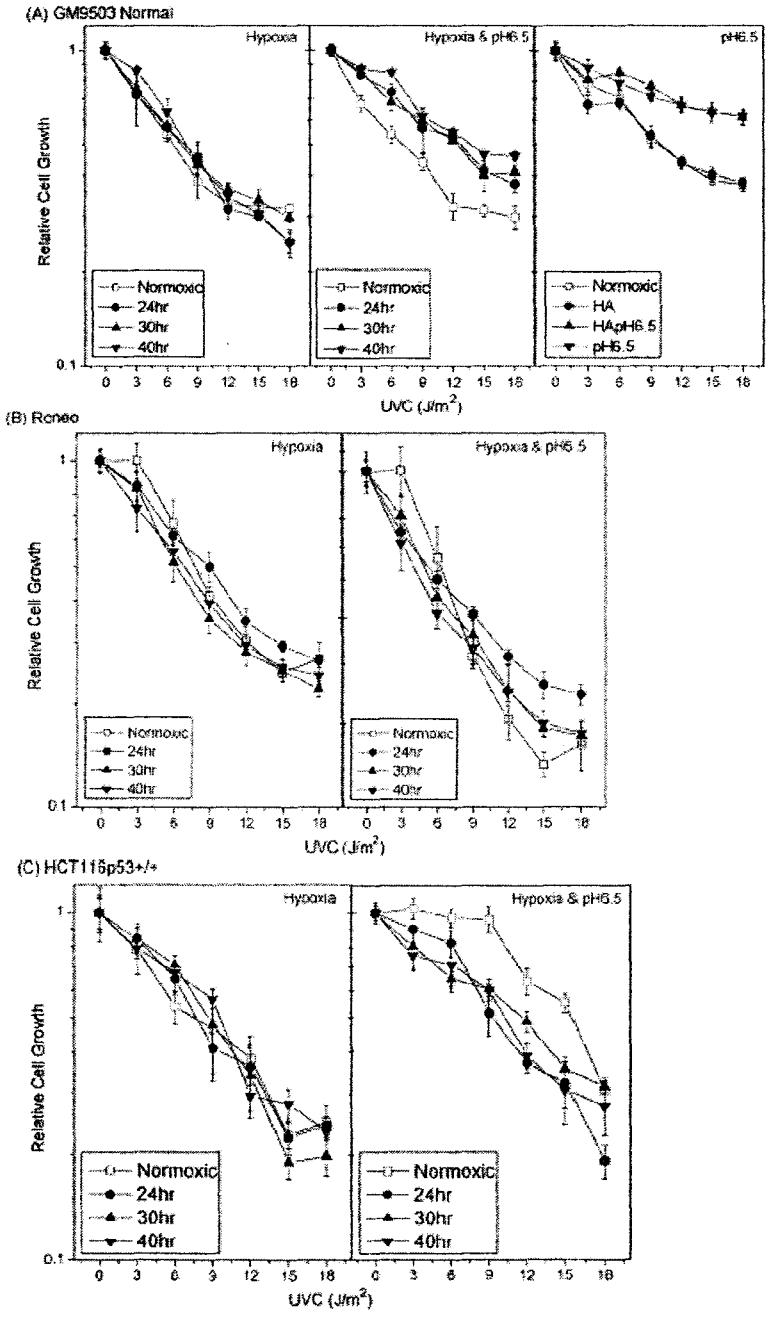

Fig. 5. Differential susceptibility of (A; Panels 1 and 2) human primary skin (GM9503), (B) colorectal carcinoma cell line (Rcneo), (C) colon carcinoma HCT116 $p53^{+/+}$ and (D) $HCT116p53^{-/-}$ cells, to treatment of hypoxia and hypoxia accompanied by low pH following UVC treatment. The cells were treated with the indicated fluence of UVC and then either incubated in normoxic (□), hypoxic or hypoxic with acidosis conditions for 24 h (●), 30 h (▲) or 40 h (▼). (A; Panel 3) GM969 normal human primary fibroblasts were treated with either normoxia (□), HA (●), HApH6.5 (▲) or pH6.5 alone (▼) for 30 h following UVC treatment. Each point is the average of three replicates; error bars represent one standard error.

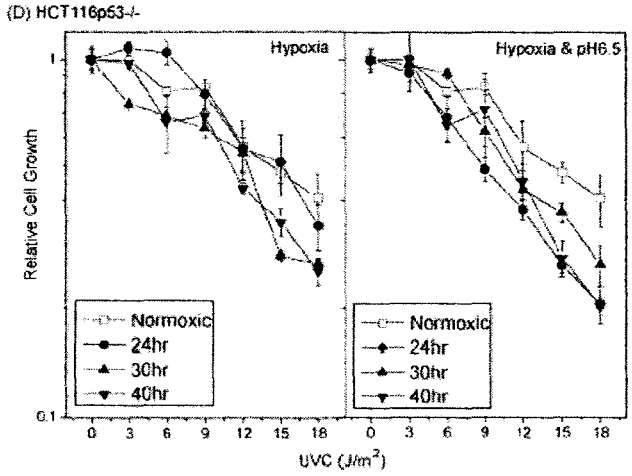

Fig. 5. (Continued).

We have reported also that increased expression of p53 alone can upregulate HCR of the UVC-damaged reporter gene in normal human fibroblasts through an enhancement of both the transcription-coupled repair (TCR) and the global genomic repair pathway of NER [36]. There is evidence that stabilization of p53 triggers increased NER through both transcription dependent [42,43] and transcription-independent mechanisms [44]. The current results are therefore consistent with a transient upregulation of NER due to HA and HApH detected at 12–24 h (Table 1) resulting from a prior early and transient upregulation of p53 in GM9503 fibroblasts (Fig. 3C). p53-dependent upregulation of the GGR XPC protein has been reported following UVC exposure of normal human fibroblasts [38]. In contrast, we were unable to detect an increase in the XPC protein following treatment of cells with HA or HApH (data not shown). We have reported previously that increased p53 expression alone upregulates HCR of the UVC-damaged reporter gene in XPC fibroblasts that are deficient in the GGR but proficient in the TCR pathway of NER [36]. It therefore appears likely that the upregulation in HCR in GM9503 cells following HA and HApH treatment results from a p53-dependent, but transcription-independent mechanism that enhances the TCR pathway of NER.

Although the enhanced HCR of β-gal activity was significant only in GM9503 fibroblasts for both HA and HApH, HCR in IMR90 cells followed a similar trend with a moderate enhancement in HCR following HA and HApH. This may be a consequence of the transient increase but lower p53 expression levels induced by HApH in IMR90 cells when compared to GM9503 fibroblasts. However, as seen in Fig. 3B and C, HA treatment of IMR90 cells resulted in a significant downregulation of p53 by 12 h, suggesting that the transient early enhancement in HCR might be owing to additional factors, such as existence of p53-independent hypoxia induced repair pathways. As reported by others, $p53^{+/+}$ E1a/Rad-transformed MEF mouse embryo cells showed a moderate increase in p53 expression after hypoxia and acidosis treatment for 1 and 2 h, but a downregulation of the tumor suppressor protein after hypoxia alone treatment [45]. Our results also suggest the presence of a differential DNA repair response of HA compared to HApH in IMR90 cells. It appears likely that different cell types behave differently to hypoxic stress. Since IMR90 cells are lung fibroblasts, they may be able to withstand much lower hypoxic levels, and p53-dependent DNA repair pathways may be activated only at anoxic levels or in conjunction with low pH in these cells. Zhang et al. reported that two tumor cell lines showed reduced p53 expression after hypoxia treatment, while p53 expression was induced in HT1080 human fibrosarcoma cells [46]. In contrast to the results reported here, Chandel et al. detected p53 accumulation in response to hypoxic stress (1.5% oxygen) for 8 h in normal lung fibroblasts, IMR90 [18]. However, it is not clear whether the IMR90 cells used in the Chandel et al. studies were a primary cell strain or an immortalized cell line [18].

4.2. Hypoxia induced a delayed, but not absent enhancement in HCR of the UV-damaged reporter gene in both p53 proficient and p53 deficient tumor-derived cells

The HCR of a UV-damaged plasmid encoded reporter gene was decreased in the 3340WT mouse fibroblast cell line and in the Rcneo human colon carcinoma cell line, when treated with HApH for 24 h [6]. In the present study we found also that Rcneo and $HCT116p53^{+/+}$ (Fig. 2A and B) and 3340WT cells (data not shown) showed a moderate decrease in HCR of a UV-damaged adenovirus encoded reporter gene when incubated under HA and a significant decrease under HApH for 24–30 h when compared to normoxic cells. However, our results indicate that at 36–40 h after HA and HApH treatment, DNA repair was enhanced in Rcneo and $HCT116p53^{+/+}$ cells suggesting that NER upregulation was delayed compared to that in primary human fibroblasts, but not absent in these cells. Since HA induced a decrease in p53 expression as early as 3 h in $HCT116p53^{+/+}$ and at 12 h in Rcneo cells, this suggests the presence of a p53-independent upregulation in the NER pathway in these tumor cells in response to hypoxia. In addition, our data showed no detectable p53 in the p53 deficient $HCT116p53^{-/-}$ cells even though HA induced a significant decrease in HCR of the UVC-damaged reporter gene at 24 h followed by a significant enhanced HCR of β-gal expression at 30–40 h when compared to normoxic cells, similar to $HCT116p53^{+/+}$ cells. This further supports the hypothesis that hypoxia results in an increased NER response that is delayed in the tumor-derived cells and occurs irrespective of p53 status.

A decrease in p53 expression has also been reported in human HepG2 hepatoma and HeLa cells following treatment with 1% O_2 for 5 h [47]. In contrast, hypoxia of 0.3% for 8–16 h induced p53 expression in $HCT116p53^{+/+}$ cells when compared to normoxic cells

[22] and Hammond et al. reported increased p53 accumulation in response to 0.02% oxygen, but not to 2% oxygen, suggesting the cellular response of p53 expression to hypoxia may depend on the level of oxygen concentration [23]. Furthermore, chronic exposure to hypoxia might have a different effect on p53 expression and NER compared to acute hypoxic stress and these effects may be p53-independent. It has been reported that the homologous recombination mediator, Rad51, was significantly reduced by chronic hypoxia (by 48 h) in several cancer cells, whereas acute hypoxia (0.5% for 24 h) only slightly downregulated expression of this gene [48]. The variability in p53 accumulation due to hypoxia may in part be attributed to the physiology of the cell as it has been reported that 8 h of hypoxic treatment induced significantly greater p53 accumulation in S-phase populations than in G1-phase cells [23].

4.3. Hypoxia and low pH treatment of tumor-derived cells resulted in an increased p53 expression

In contrast to the results for treatment of the tumor-derived Rcneo and HCT116p53$^{+/+}$ cells with HA, treatment with HApH induced an increase in p53 expression levels at 6–12 h when compared to normoxic conditions. Other reports show a similar differential effect of HA compared to HApH on p53 expression levels in tumor cells. Over a range of oxygen levels, from 1.5% to less than 0.02%, Pan et al. were unable to observe p53 accumulation in several tumor-derived cell lines [49]. Only after treatment with a combination of HA and acidosis/nutrient deprivation did some cells exhibit p53 induction [49]. Schmaltz et al. reported that hypoxia accompanied by low pH for 2 h caused upregulation of p53 expression in tumor-derived cells, but hypoxia alone decreased the levels of p53 when compared to control conditions [45]. The differential p53 response due to HA compared to HApH strengthens the hypothesis that hypoxic stress alone may affect NER pathways through a different mechanism than when coupled to low pH [6,45,50,51].

4.4. Enhanced cell viability following UV exposure under hypoxia and low pH conditions is dependent on early enhanced repair of UV-induced DNA damage, which is absent in tumor-derived cells

Hypoxic tumors are often found to be resistant to radiation and some anticancer drugs [52], while cells under hypoxia which were exposed to reoxygenation proved to be more sensitive to killing by radiation [31]. Since the results of the present study showed both primary and tumor-derived cells displayed increased repair of the UV-damaged reporter gene in HA and HApH conditions, it was important to determine whether the increased repair was reflected in improved cell growth after UV.

The microenvironment of a tumor cell is not solely characterized by hypoxia alone, but also by acidosis and nutrient deprivation due to the switch from aerobic respiration to anaerobic fermentation, resulting in an increased production in lactic acid [53]. The results of the MTT assay in primary and tumor-derived cells suggest several effects of the microenvironment on the viability of cells following UV exposure. HA treatment for any of the tested time points after UV irradiation did not significantly affect cell growth and viability for primary or tumor-derived cells. In contrast, HApH treatment after irradiation significantly affected cell growth in both human primary and tumor-derived cells. This is consistent with studies that reported low pH conditions or the synergistic effect of hypoxia and low pH influence cellular metabolism and cell survival, but not hypoxia alone [6,50,51].

We report here that HApH after UV irradiation of cells induced a different effect on cell viability in human primary fibroblasts compared to tumor-derived cells. The HApH induced early enhancement in HCR of β-gal activity in human primary fibroblasts correlates with an increase in cell viability of primary cells exposed to HApH after UV irradiation. Therefore, it is likely that early upregulation of NER by HApH in human normal primary fibroblasts is a requirement for increased cell viability and growth. This is in contrast to the observed delayed increase in HCR of the UV-damaged reporter gene in the tumor-derived cells following HA and HApH, which did not correlate with an increase in cell viability after UV irradiation. On the contrary, HApH for 30 h after UV irradiation resulted in a significant fall in cell viability, irrespective of p53 status. This result suggests that HCT116p53$^{+/+}$ and HCT116p53$^{-/-}$ cells possess defective DNA repair pathways or may experience downregulation of stress response genes, which may disrupt NER function and thus hinder these cells' ability to respond to DNA damage efficiently under HApH stress as early as primary cells. In accordance with other studies [31], this repair deficiency may be the determining factor whether cells will be able to survive and grow under HApH conditions, which on a clinical level may prove to be advantageous for radiotherapy and chemotherapy. Interestingly, Rcneo cells displayed no significant effect on cell viability under any of the conditions tested, indicating that tumor cells derived from different tissues may display different cell growth pattern in response to HA and HApH conditions.

The present data suggests that HApH but not HA induces an early and transient p53-dependent increase in NER in normal primary fibroblasts that leads to an increased viability of cells following UV exposure. We show also that treatment of normal human fibroblasts with low pH alone also leads to an increased viability of cells following UV exposure. Ohtsubo et al. have reported that low pH conditions alone result in increased p53 expression in wild-type p53 expressing human glioblastoma cells [54]. Taken together, these results suggest that it is an early and transient p53-dependent increase in NER due to the low pH conditions, rather than due to hypoxia, that leads to increased viability of primary human fibroblasts after exposure to UV following HApH treatment.

HApH treatment of the tumor-derived cells also resulted in an upregulation of p53 and enhanced DNA repair. However, the enhancement in DNA repair was delayed in the tumor cells compared to that in normal primary cells, was present irrespective of p53 status, and did not lead to an increased viability of cells after UV. Therefore, it appears likely that the increased NER response under HA and HApH in human primary cells is mechanistically different from that of tumor-derived cells. Schmaltz et al. demonstrated that oncogene-transformed mouse cells respond differently to hypoxia when compared to non-transformed cells, in that transformed cells exhibited apoptosis under hypoxia, while primary fibroblasts showed G_0/G_1 arrest, similarly to ionizing radiation [45]. Previous reports have shown that tumor cells treated with deferoxamine mesylate (DFX), which mimics hypoxia, lost transcription activity of stabilized p53 corresponding to cytoplasm p53 accumulation, while normal cells displayed partial activation [55]. This is further evidence that tumor cells may have other defects under hypoxic and hypoxic accompanied by acidosis stress including the inability to localize p53 to the nucleus.

The NER pathway plays a role in the survival of cells following treatment with several chemotherapeutic agents for cancer treatment including cisplatin. It appears likely that the acute lowering of the pH used in the present work is different from a chronic lowering of pH that would be expected to arise in a tumor and as such may limit our experimental approach. Notwithstanding, the difference in cell viability response between primary and tumor-derived cells following UVC exposure and conditions of hypoxia and low pH, further underlines the potential role that DNA repair plays in the response of tumors to radiation and chemotherapy. This is an indication of a potential therapeutic advantage which could be further tailored according to the DNA repair pathway efficiencies of the cell type and timing of hypoxic conditions (acute versus chronic)

as recent results suggest that chronic hypoxia may render a tumor more radiosensitive [31].

Conflict of interest statement

None declared.

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CHAPTER 5

The effect of HIF-1α on repair of UVC-induced DNA damage

1.0 Abstract

Hypoxia-inducible factor 1 alpha (HIF-1α) acts as a transcription factor that transactivates many genes involved in adaptation to hypoxic stress. HIF-1α accumulates in response to hypoxia, which has been suggested to induce DNA damage. Previously, it was shown that mouse embryonic fibroblasts deficient for HIF-1α showed an increase in sensitivity to chemotherapeutic agents and ionizing radiation, as well as a decrease in repair of a fragmented reporter gene when compared to HIF-1α wild-type cells. It has been suggest that the NER protein, CSB, accumulates under hypoxic conditions and is a target of HIF-1α. Therefore, it was of interest to investigate the role of HIF-1α in nucleotide excision repair (NER) of a UVC-damaged reporter gene. We employed a non-replicating recombinant human adenovirus, AdCA17*lacZ*, that can efficiently infect murine and human cells and expresses the β-galactosidase (β-gal) reporter gene in murine tumour cells exposed to normoxic, hypoxic, hypoxic coupled to low pH or low pH only conditions. We also examined cell sensitivity using the MTT and colony survival assays in murine tumour cells exposed to the same conditions. We determined that dominant-negative HIF-1α (MDN2) rat tumour cells showed a decrease in HCR of UVC-damaged reporter gene when compared to HIF-1α wild-type cells (MATLyLu) exposed to normoxic conditions for 12 hours, but not at 24 or 40 hours. Hypoxia resulted in reduced repair of the UVC-damaged reporter gene in MDN2 cells compared to MATLyLu at 12 hours for UVC fluences of 150 and 200 J/m^2, but not at later time points tested. Hypoxia coupled to low pH and low pH alone did not result in a significant difference in HCR of β-gal expression between the two cell lines. In addition, differential repair between the

two murine tumour cell lines detected at 12 hours after normoxic and hypoxic treatment did not result in a significant difference in cell growth or cell survival after UVC exposure. The effect of hypoxia on NER was also examined in primary human normal and CSB fibroblasts. Hypoxia after infection induced an enhancement in HCR of the UVC-damaged reporter gene in normal fibroblasts and this enhancement was delayed, but not absent in CSB fibroblasts. These results suggest that hypoxia up-regulates NER. In addition, by infecting cells with Ad*HIF-1α*, an adenovirus expressing wild-type HIF-1α, we examined the effect of overexpression of HIF-1α on HCR of a UVC-damaged reporter gene in normal and CSB-deficient primary fibroblasts under hypoxic conditions. HIF-1α overexpression resulted in a decrease in HCR of a UVC-damaged reporter gene in normal fibroblasts exposed to hypoxia at early time points (*i.e.* at 12 hours), and it was delayed but not absent in CSB fibroblasts. The data indicate that HIF-1α plays an important role in NER of a UVC-damaged reporter gene and that HIF-1α-dependent responses under hypoxic conditions may in part depend on CSB expression.

2.0 Introduction

The tumour microenvironment is characterized by hypoxia, acidosis and nutrient deprivation. Hypoxia induces responses in gene expression that result in physiological changes such as angiogenesis, erythropoiesis and enhanced glycolysis (reviewed in (216)). The principal protein in regulating cellular processes in response to low oxygen conditions is the hypoxia inducible factor 1 alpha (HIF-1α) (reviewed in (247)). HIF-1α is a protein that is degraded under normoxic conditions, but is stabilized by low oxygen conditions and transactivates over 200 genes (reviewed in (286)). Under normoxic conditions, HIF-1α is targeted for degradation through the von Hippel-Lindau (VHL) E3 ligase complex, whereas when oxygen levels decrease, HIF-1α binds to HIF-1β, forming a heterodimer that in turn binds to the hypoxia-response element (HRE) on target genes (reviewed in (193)).

One of the most prominent HIF-1α target genes is the vascular endothelial growth factor (VEGF) (reviewed in (247)), which is activated in many tumours and promotes angiogenesis (reviewed in (287)). HIF-1α stabilization was detected in many different cancers (248) and correlated with poor patient prognosis (288,289). Therefore, hypoxia is responsible for producing cellular changes such as increased glycolysis and angiogenesis due to HIF-1α up-regulation that in turn creates an optimal environment for tumour growth.

In addition to HIF-1α accumulation by hypoxic stress, p53 stabilization under low oxygen conditions was also been suggested by several studies (166-170), although there is conflicting evidence pertaining to the oxygen levels that can up-regulate p53 and

whether it is transcriptionally active (170) (reviewed in (290)). We have previously shown that hypoxia induces p53 accumulation in human primary cells, but not in human tumour cells unless accompanied by acidosis (291). These findings were consistent with other studies that showed p53 stabilization was not observed in tumour cells unless it was coupled with acidity and nutrient deprivation (235). Furthermore, it has been proposed that p53 accumulation is HIF-1α-dependent and that HIF-1α can co-immunoprecipitate with p53: an interaction that stabilizes p53 (255). The discovery of two HIF-1α sequence motifs that bind to the p53 DNA-binding site supports a direct interaction between HIF-1α and p53 (257). It has also been reported that *in vivo* MDM2 can augment an interaction between p53 and HIF-1α, suggesting an indirect interaction between these two transcription factors through MDM2 association (256). The study by Chen *et al.* (2003) hypothesized that p53 and HIF-1α interaction can result in HIF-1α degradation and this was supported by a study demonstrating that high p53 expression down-regulated HIF-1α accumulation (260). In addition, Schmid *et al.* (2004) showed that low p53 expression suppressed HIF-1α transactivation without affecting the protein levels by competing for binding to the transcriptional co-activator, p300 (260).

Recent evidence has also implicated the transcription coupled repair (TCR) protein, CSB, in the hypoxic response by suggesting that HIF-1α controls its expression (254). Filippi and colleagues (2008) reported that hypoxic stress (1% oxygen) results in increased CSB mRNA and protein expression in NER-proficient human primary cells as well as enhanced HIF-1α occupancy of the CSB promoter (254). In addition, hypoxic stress induced a decrease in VEGF mRNA expression in human primary CSB-deficient

fibroblasts compared to wild-type cells, suggesting a role for CSB in the HIF-1α pathway. It was also suggested that the p53 and HIF-1α shared transcriptional activator, p300, plays a major role in CSB-deficient cells as p300 overexpression was found to release the inhibition of HIF-1α activities on its target genes (254). It was proposed that the high p53 expression levels observed in CSB mutant fibroblasts following hypoxic treatment inhibit HIF-1α responses due to p53 competition for binding to p300 and, furthermore, that CSB and p300 compete for p53 binding, with CSB having a greater affinity (reviewed in (265)). As a consequence, less p53 is available to activate p53-dependent apoptotic pathways and therefore CSB plays a significant role in hypoxic conditions by committing a cell toward cell survival instead of programmed cell death.

We have previously demonstrated that p53 overexpression alone in human normal and CSB-deficient primary fibroblasts resulted in enhanced HCR of a UV-damaged reporter gene at 12 hours after infection (285). In addition, we have demonstrated that hypoxia, as well as hypoxia accompanied by low pH, resulted in a p53-dependent enhanced HCR of β-gal activity in normal fibroblasts between 12 and 24 hours after treatment.

Furthermore, Unruh *et al.* (2003) showed that mouse embryonic fibroblasts deficient for HIF-1α showed a decrease in repair of double-strand breaks in a reporter gene when compared to HIF-1α wild-type cells (292). HIF-1α mutant cells were also more susceptible to chemotherapeutic treatment and ionizing radiation when compared to wild-type HIF-1α cells (292). The roles of p53 and CSB in NER and their interaction is well known (172), and taken together with recent reports suggesting a role for HIF-1α in

DNA repair, and a connection between p53, HIF-1α and CSB, it was of interest to determine whether a link between HIF-1α, CSB and p53 exists in the DNA repair of a UV-damaged reporter gene under hypoxic conditions.

Here we have examined the role of HIF-1α, CSB and hypoxia on NER in human fibroblasts and two murine tumour cell lines. We have shown that rat tumour cells deficient in HIF-1α showed a decrease in HCR of the UV-damaged reporter gene under normoxic conditions at 12 hours after infection when compared to wild-type HIF-1α tumour cells. This differential repair between the wild-type rat prostate tumour cell line, MATLyLu, and the dominant-negative MDN2 tumour cell line did not correlate with cell survival following UVC treatment. These results suggest that HIF-1α may play a role in NER of a UVC-damaged reporter gene, but does not translate into a difference in UVC sensitivity or cell survival. In addition, we have determined that hypoxia induces an early and transient increase in HCR of a UVC-damaged reporter gene in human primary normal fibroblasts that is delayed, but not absent in CSB-deficient fibroblasts. Furthermore, HIF-1α overexpression under hypoxic conditions resulted in a reduction in HCR of β-gal expression in normal fibroblasts, which was delayed but not absent in CSB fibroblasts. These data further support a role for HIF-1α in NER and suggests that there is a link between HIF-1α and CSB that may affect NER response.

3.0 Materials and methods

3.1 Cells and cell culture

MATLyLu and MDN2 cells were obtained from Dr. Gurmit Singh, Juravinski Cancer Centre, McMaster University, Hamilton, Ontario. All cell cultures were grown in a humidified incubator maintained at 5% CO_2 and 37 °C, and cultured in RPMI 1640 media (Lonza) supplemented with 10 % fetal bovine serum, 0.2 mg/ml hygromycin (Roche) and 10 mM HEPES (Gibco). MATLyLu is a rat prostate cancer cell line that is wild-type for HIF-1α and is derived from Dunning R3327 rat prostatic tumour sublines (280). MDN2 is a stable clone obtained by transfection of MATLyLu cells with the pCEP4/dominant-negative HIF-1α (DNHIF-1α) plasmid, as described previously (192).

3.2 Recombinant adenovirus constructs

The AdCA17*lacZ* recombinant adenovirus (272) contains the *lacZ* gene under the control of the human cytomegalovirus immediate early (HCMV-IE) promoter (-299 to +72 relative to the transcription start site) inserted into the deleted E1 region of the adenovirus genome in the left-to-right orientation. Deletion of the E1 region of the genome renders the adenovirus unable to replicate in most mammalian cells. The recombinant adenovirus AdCA18*luc* (276) expresses the luciferase reporter gene under the control of the HCMV-IE promoter inserted into the E1 deleted region in the left-to-right orientation of the adenovirus genome. The recombinant adenoviruses were obtained from Dr. F.L. Graham, McMaster University, Hamilton, Ontario. The viruses were

propagated, collected and titred as described previously (282). The recombinant adenovirus Ad*HIF-1α* contains the wild-type *HIF-1α* gene under the control of the HCMV-IE promoter into the E1 deleted region in the right-to-left orientation and was obtained from Applied Biological Materials Inc. (Richmond, BC, Canada; catalogue #: 000019A). The virus was propagated, collected and titred by Dr. F.L. Graham's laboratory, as previously described (282) and the stock virus preps contained 2.18 x 10^{11} plaque-forming units (pfu)/ml.

3.3 UV-irradiation of virus

The virus was suspended in 1.8 ml of cold PBS and was irradiated in 35 mm dishes (Falcon, Lincoln Park, NJ, catalogue #3001) on ice with continuous stirring using General Electric germicidal lamp (model G8T5) emitting predominantly at a wavelength of 254 nm with an incident fluence rate of 2 J/m^2/s as determined using a J-255 shortwave UV meter (Ultraviolet Products, San Gabriel, CA). Aliquots of 200 µl were removed following each exposure to the virus and diluted appropriately with unsupplemented media. In the case of simultaneous infection of AdCA17*lacZ* with either AdCA18*luc* or Ad*HIF-1α*, 100 µl aliquots of AdCA17*lacZ* were removed following each exposure to the virus and diluted appropriately with unsupplemented media to a final multiplicity of infection (MOI) of 40-80 pfu/cell, which also contained the appropriate MOI of either AdCA18*luc* or Ad*HIF-1α*.

3.4 Hypoxia

Hypoxic culture conditions were established by using an automatic O_2/CO_2 incubator (Thermo Electron Corporation, Marietta, Ohio) equipped with oxygen and carbon dioxide sensors, and connected to N_2 and CO_2 gas cylinders. The O_2 tension was set at 1 % and was maintained at this level throughout the entire length of the experiment. The O_2 (1%) and CO_2 (5%) readings were confirmed by use of a fyrite gas analyzer (Bachrach, Pittsburg, PA).

3.5 Low pH

Regular RPMI 1640 or α-MEM was supplemented with 25 mM HEPES and 25 mM 4-morpholinepropanesulfonic acid (Sigma, ST. Louis, MO) to acidify the medium. The medium was adjusted to pH 6.5 with 1 N HCl.

3.6 Host cell reactivation assay

Fibroblasts and murine prostate tumour cells were seeded in 96-well plates (Falcon, Lincoln Park, NJ, catalogue #3072) at a density of 1.5 - 2.0 x 10^4 and 3.5 x 10^4 cells per well, respectively, in supplemented α-MEM or RPMI 1640 media and incubated in a 5% CO_2 humidified incubator at 37 °C for 12-24 hours to allow for cells to adhere to the wells prior to infection. Subsequently, the media was then aspirated from the wells and cells were infected with either non-irradiated or UV-irradiated AdCA17*lacZ* in a 40 µl volume at an MOI of 80 pfu/cell of the. For experiments with the human fibroblasts, after the media was aspirated, cells were infected with either non-irradiated or UV-

irradated AdCA17*lacZ* at an MOI of 40 pfu/cell and either AdCA18*luc* or Ad*HIF-1α* in a 40 µl volume at an MOI of 200 pfu/cell. Cells were incubated at 37 °C for 90 minutes, after which time virus was aspirated and cells were overlaid with 160 µl of warm supplemented RPMI 1640 with 10 mM HEPES (Gibco) and no antibiotics for the murine tumour cells lines or with supplemented α-MEM for the human fibroblasts. Cells were then incubated at 37 °C in either a normoxic or hypoxic incubator for 12, 24 or 40 hours.

3.7 Quantitation of β-gal activity

Fibroblasts and murine tumour cells were harvested at various time points following infection with AdCA17*lacZ*. The infected cell monolayer was incubated with 60 µl per well of 1mM chlorophenol red β-D-galactopyranoside (CPRG; Boehringer–Mannheim, Indianapolis, IN) in 0.01% Triton X-100, 1 mM $MgCl_2$, and 100 mM phosphate buffer at pH 8.3. Light absorbance at 570 nm (A_{570}) was determined several times following the addition of the β-gal substrate using a 96-well plate reader (Labsystems Multiscan MCC/340 and/or Bio-Tek Instruments EL340 Bio Kinetics Reader).

3.8 MTT reduction assay for cell viability following UV exposure

Cells were seeded in 96-well plates (Falcon, Lincoln Park, NJ, catalogue #3072) at a density of 2.0 – 2.5 x 10^3 cells/well for primary cells and 1.25 x 10^3 cells/well for murine tumour cells. Cells were seeded 6-12 hours prior to being treated using a General Electric germicidal lamp (model G8T5) at a wavelength of predominantly 254 nm with an incident fluence rate of 1 J/m^2/s (J-255 shortwave UV meter, Ultraviolet Products, San

Gabriel, CA) in PBS. After UVC treatment, cells were overlayed with either warm α-MEM or RPMI 1640 supplemented media without antibiotics as described in 3.6 and incubated in either a normoxic or hypoxic incubator for 12 hour. After the allotted time interval, the media was removed from each well and replaced with fresh media. All plates were subsequently incubated in a normoxic incubator. Cell viability was quantified 4-6 days after UVC treatment using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma catalog no. M2128) assay as previously described (283). The absorbance of each well was measured at 570 nm light using a 96-well plate reader (Labsystems Multiscan MCC/340 and/or Bio-Tek Instruments EL340 Bio Kinetics Reader) and the percentage viability was calculated.

3.9 Western blotting

For experiments testing whether Ad*HIF-1α* infection induces HIF-1α overexpression and establishing the appropriate MOI for use in subsequent experiments, confluent monolayers of cells in 6-well plates (Falcon, Lincoln Park, NJ, catalogue #3046) were infected with either Ad*HIF-1α* at an MOI of 50, 100 and 200 pfu/cell, AdCA18*luc* at an MOI of 200 pfu/cell or mock-infected and exposed to either normoxic or hypoxic conditions for 6 hours. Cells were then collected using a cell scraper and suspended in lysis buffer [50 mM Tris, 150 mM NaCl, 1% NP40, 10% protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), pH 8.0] and kept on ice for 60 minutes. The lysates were cleared by centrifugation at 13,000 g for 1 minute and the protein concentration was determined by the Bradford microassay procedure. Samples

were resolved over a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for 1.5 hour at 100 V (8% SDS-PAGE when probing for CSB) and transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech). Blots were blocked with 20 % skim milk in 1X TBST (Tris-buffered saline with Tween 20) for a minimum of 1 hour. The primary antibodies used were: anti-HIF-1α (BD Biosciences, San Jose, CA), anti-HIF-1α NB100-479SS (Novus Biologicals, Littleton, CO), anti-CSB A301-345A (Bethyl Laboratories, Montgomery, TX) and anti-beta-actin (Sigma–Aldrich Inc., Saint Louis, MO). Membranes were probed with the primary antibody for 1 hour at room temperature or overnight at 4 °C. The blots were stripped using a specific buffer (100 mM 2-mercaptoethanol, 62.5 mM Tris-HCl, 2% SDS, Ph 6.7) for 30 minutes at 65°C and re-probed with monoclonal anti-β-actin (Sigma-Aldrich Inc.). Specific antibody-labeled proteins were detected using enhanced chemiluminescence detection according to the manufacturer's instructions (Western Lighting, Perkin–Elmer Life Sciences).

3.10 Total RNA isolation and RT-PCR

Confluent monolayers of MATLyLu and MDN2 rat prostate tumour cells in 60 x 15 mm Petri dishes were treated with either normoxic or hypoxic conditions for 5 hours. RNA was isolated using the RNeasy Plus mini kit (QIAGEN, Valencia CA) following the manual's protocol and then RNA was quantified in an Eppendorf Biophotometer. 1 μg of RNA was treated with DNase I (Invitrogen) to remove genomic DNA contamination. cDNA synthesis was performed using random primers (100 ng) and the Superscript II

First-Strand Synthesis (Invitrogen) using 1 μg of total DNase-treated RNA. A control that was not reverse transcribed was used to confirm a lack of DNA contamination.

PCR was performed using a Stratagene MX3000P machine (La Jolla, CA) and using the following VEGF and lamin primers: VEGF 5'-ACC AGC GCA GCT ATT GCC GT-3' (forward) and 5'-CAC CGC CTT GGC TTG TCA CA-3' (reverse); lamin 5'-GCA TGT ACA TAG AAG GAG CTA-3' (forward) and 5'-CAT GCA TAT TCC TGG TAC TCA T-3'(reverse). Primers were designed using GeneFisher and were synthesized by MOBIX (McMaster University, ON). A typical 50 ul reaction in a 0.2 ml thin-walled PCR tube contained 2 μl of DNA and 48 μl of PCR master mix (5 μl 10 x PCR buffer (Invitrogen), 1 μl of each of the appropriately diluted primer volume, 10 mM dNTP mix (Invitrogen), 50 mM $MgCl_2$, 0.4 μl Taq DNA polymerase (Invitrogen) and water (to a volume of 50 μl per reaction including cDNA template). The thermal cycler program used for VEGF consisted of a 2 minute initial polymerase activation and denaturation step at 94°C, 30 seconds at 94°C, 45 seconds annealing step at 55°C, 45 seconds at 72°C, and then 35 reaction cycles of 30 seconds at 94°C, followed by a 10 minute elongation step at 72°C. RT-PCR products were separated on by electropgoresis on a 2% agarose gel and the ethidium bromide-stained DNA bands were visualized using an ultraviolet lightbox (Alpha Innotech Corporation, San Leandro CA).

3.11 Clonogenic survival assay

Cells were seeded in 6-well plates (Falcon, Lincoln Park, NJ, catalogue #3046) at a density of 400 cells/well in 1 ml of supplemented RPMI 1640 media. Following a 6-12

hour incubation period at 37 °C in a 5% CO_2 incubator, the media was aspirated from the wells and 1 ml of warm PBS was added to the cells prior to UVC irradiation. Cells were either irradiated or mock irradiated with UVC of increasing fluences at a fluence rate of 1 $J/m^2/s$. Following UVC-irradiation, the PBS was aspirated and 1 ml of RPMI 1640 media, which was supplemented with 10 % fetal bovine serum and 10 mM HEPES (Gibco), but lacked antibiotics, was added to each well and the cells were incubated for 12 hours in either a normoxic or hypoxic incubator. After the specified treatment, media was aspirated and the cells were overlayed with 1 ml fresh media that did not contain antibiotics. The plates were further incubated for 4 to 5 days from the time the cells were UVC-irradiated in a humidified 37 °C and 5% CO_2 incubator in order to provide sufficient time for surviving cells to form colonies.

At the end of the incubation period, media was aspirated from each well and the cells were overlayed with approximately 1 ml of crystal violet solution (63% absolute ethanol, 27% H_2O, 10% methanol, 5g/l crystal violet) to stain the colonies for a period of 30 minutes. Cells were de-stained by submerging the plates in water to remove the crystal violet solution and let to dry. Colonies that contained more than 32 cells were counted using a VWR hand tally counter and a clonogenic survival curve was plotted by expressing clonogenic survival as a ratio of the number of colonies in the treated cells compared to the control-treated cells.

4.0 Results

4.1 Rat tumour cells expressing a dominant-negative HIF-1α show reduced repair of a UVC-damaged reporter gene compared to rat tumour cells expressing only wild-type HIF-1α

Although the role of p53 in DNA repair and its involvement during hypoxic stress is well established, the link between p53 and HIF-1α in DNA repair is relatively unexplored (166,168,169). Several studies have suggested the possibility of cross-talk between HIF-1α and p53 (255-258) and there are conditions when p53 and HIF-1α behave in a competitive manner (260). Moreover, a possible link between HIF-1α and CSB in relation to DNA repair was recently suggested (254). It was therefore of interest to investigate the role of HIF-1α in DNA repair of a UV-damaged reporter gene under normoxia, hypoxia, low pH and a combination of hypoxia and low pH.

In order to determine the status of HIF-1α in the two rat prostate tumour cell lines, HIF-1α expression was confirmed in MATLyLu and MDN2 cells as shown in Figure 5.1, and the mRNA levels of VEGF were determined through RT-PCR in both cell lines as shown in Figure 5.2. It can be seen that hypoxia induces HIF-1α expression in both MATLyLu and MDN2 cells, but the mRNA levels of VEGF were only increased under hypoxic conditions in MATLyLu cells when compared to normoxic conditions, and not in MDN2 cells. These results are consistent with the data published by Alqawi and colleagues (2007) and indicate that the HIF-1-dependent up-regulation of VEGF is deficient in MDN2 cells (192).

Rat prostate cancer cells expressing wild-type HIF-1α (MATLyLu) and rat prostate cancer cells expressing a dominant negative HIF-1α (MDN2) were exposed to

conditions of normoxia, hypoxia, hypoxia accompanied by acidosis or acidosis alone after infection with UV-irradiated AdCA17*lacZ* for 12, 24 and 40 hours. Representative graphs for the relative β-gal activity of the UV-damaged reporter gene in MATLyLu and MDN2 cells after 12, 24 and 40 hours are shown in Figures 5.3, 5.4 and 5.5, respectively. The UVC fluence required to reduce β-gal activity to 37% of that for non-irradiated virus (D_{37}) was utilized as a measure of HCR and the relative D_{37} values are reported in Table 5.1. The absolute D_{37} values for survival of β-gal activity of the UV-damaged reporter gene were combined from multiple experiments and the average D_{37} values in MATLyLu compared to MDN2 cells are shown as a function of time between virus infection and β-gal scoring under normoxic and hypoxic conditions in Figure 5.6. The D_{37} values for survival of β-gal activity of the UV-damaged reporter gene were calculated and used to measure the relative D_{37} value in hypoxic, hypoxic accompanied by low pH and low pH conditions compared to normoxic treatment as shown in Tables 5.1-5.3.

It can be seen that the MDN2 cells expressing a dominant-negative *HIF-1α* gene show a significantly reduced HCR of the UV-damaged reporter gene compared to MATLyLu cells, which express the wild-type HIF-1α, when scored for β-gal expression 12 hours post-infection under normoxic conditions (Fig.5.3, top left panel; Fig. 5.6). Hypoxia and hypoxia accompanied by acidosis for 12 hours resulted in decreased HCR of β-gal activity in HIF-1α wild-type cells, but not in dominant-negative HIF-1α murine tumour cells. Despite the decrease in HCR of the β-gal expression under hypoxic conditions for 12 hours in the wild-type HIF-1α cells, when compared to the dominant-negative HIF-1α cells, pooled results for each UVC fluence point show a decrease in

HCR of the UV-damaged reporter gene in MDN2 cells compared to MATLyLu cells under conditions of hypoxia at the higher UV fluences of 150 ($p = 2.8 \times 10^{-5}$) and 200 J/m2 ($p = 0.0047$). In contrast, hypoxia accompanied by acidosis or acidosis only did not result in a significant difference in repair of the damaged reporter gene between the two cell lines at 12 hours as shown in Figure 5.3 (bottom left & right panel). Furthermore, there were no significant differences in repair of the damaged reporter gene between the two cell lines at 24 or 40 hours in any of the conditions tested as shown by the representative graphs in Figure 5.4 and 5.5 and the relative D_{37} values in Table 5.1. These results indicate that HIF-1α plays a role in NER of a UVC-damaged reporter gene.

It was of interest to determine whether the differential repair between the two HIF-1α wild-type and dominant-negative cell lines under normoxic conditions can be correlated with increased cell viability and cell survival after UV exposure in these aforementioned cell lines. Representative graphs of the effects of normoxia and hypoxia on cell growth and survival following exposure to UVC using the MTT and colony survival assay are shown in Figure 5.7 and 5.8, respectively. The UVC fluence required to reduce cell growth to 50% of that for non-treated cells (D_{50}) was utilized as a measure of cell survival. The absolute D_{50} values were calculated and used to measure the relative D_{50} value in hypoxia compared to normoxia following a treatment length of 12 hours (Tables 5.2 and 5.3). The absolute D_{50} values of the two cell lines after UVC exposure indicated no significant difference in survival under conditions of normoxia or hypoxia. In addition, the relative D_{50} values in the wild-type and dominant-negative murine prostate tumour cells indicated no significant effect was induced by 12 hours of hypoxia

treatment (Tables 5.2 and 5.3). However, hypoxia for 12 hours resulted in a small, but not significant increase in cell survival after UVC treatment in wild-type HIF-1α cells, but not in the dominant-negative HIF-1α cells. The present results indicate that the significant difference in DNA repair of a UVC-damaged reporter gene between the two tumour cell lines detected at 12 hours under normoxic conditions does not translate into a difference in cell survival for MDN2 compared to MATLyLu cells following exposure to UVC.

4.2 Hypoxia results in enhanced HCR of a UV-damaged reporter gene in primary human normal fibroblasts and is delayed, but not absent in CSB-deficient fibroblasts

In Chapter 4 we showed that hypoxic treatment for 12 or 24 hours results in an enhanced HCR of a UV-damaged reporter gene in primary normal fibroblasts (291). Recently published data showed that primary human CSB-deficient fibroblasts were unable to respond efficiently to hypoxic stress when compared to normal fibroblasts, implicating CSB in a HIF-1α-dependent response to hypoxia (254). In order to determine the role of the CSB protein in the enhancement of NER by hypoxic treatment, we have examined the effects of hypoxia on HCR of a UVC-damaged reporter gene in primary normal and CSB-deficient human fibroblasts.

Preliminary experiments were conducted to determine the optimal conditions to detect the effect of hypoxia on HCR of UV-damaged reporter gene can be detected. Representative graphs of HCR of a UV-damaged reporter gene under the different conditions of hypoxia for primary fibroblasts are shown in Appendix A, Figure A.1. The most significant effect of hypoxic treatment was observed when cells were exposed to

hypoxia after infection only, rather than prior to infection, or both before and after infection, and this type of treatment was used for subsequent experiments.

Representative graphs for the relative β-gal activity of the UV-damaged reporter gene in hypoxic treated cells for 24 and 40 hours are shown in Figure 5.9 for GM9503 and GM739CSB cells. The absolute D_{37} values for survival of β-gal activity of the UV-damaged reporter gene were combined from multiple experiments and the average D_{37} values in normal fibroblasts (GM9503) compared to CSB-deficient (GM739) cells are shown as a function of time between virus infection and β-gal scoring under hypoxic conditions in Figure 5.10. The D_{37} values for survival of β-gal activity of the UV-damaged reporter gene were calculated and used to measure the relative D_{37} value in hypoxic compared to normoxic cells for 12, 24 and 40 hours as shown in Table 5.4. It can be seen that hypoxia induces an enhancement in HCR of the UV-damaged reporter gene in primary normal fibroblasts as early as 12-24 hours and this enhancement reduces by 40 hours. In contrast, in CSB-deficient fibroblasts, significant enhancement in HCR of β-gal activity due to hypoxia was delayed and only detected at 40 hours after infection. These data indicated that hypoxia results in an early enhancement in HCR of β-gal expression in normal primary fibroblasts and is delayed, but not absent in CSB cells.

4.3 Overexpression of HIF-1α by infection with AdHIF-1α reduces HCR of a UVC-treated reporter gene in normal human fibroblasts and this reduction is delayed in CSB fibroblasts

Recent published data has reported that VEGF mRNA levels were lower in CSB-deficient human primary fibroblasts compared to normal fibroblasts under hypoxic

conditions, suggesting a link between HIF-1α and the CSB protein (254). To further examine the role of HIF-1α and its relationship to CSB in the repair of UV-induced DNA damage, we examined HCR of UV-irradiated AdCA17*lacZ* in human normal and CSB human primary fibroblasts, which were infected with either Ad*HIF-1α* or a control virus, AdCA18*luc*, and then exposed to either normoxic or hypoxic conditions.

The HIF-1α expression levels were determined for MOI values of 50, 100 and 200 pfu/cell in normal primary fibroblasts 12 hours after Ad*HIF-1α* infection under hypoxic conditions as shown in Appendix B, Figure B.1. Increased HIF-1α expression was readily detectable at a MOI of 200 pfu/cell and this MOI was used for subsequent experiments. HIF-1α expression under hypoxic conditions for 6 hours was also determined for infection with both the control virus and Ad*HIF-1α* at an MOI of 200 pfu/cell in normal and CSB-deficient primary human fibroblasts, as shown in Figure 5.11. It can be observed that Ad*HIF-1α* infection results in a significantly greater up-regulation of HIF-1α expression when compared to AdCA18*luc* infection in both cell lines.

Representative survival curves for β-gal activity of the UV-damaged reporter gene in Ad*HIF-1α*-infected normal and CSB-deficient fibroblasts compared to AdCA18*luc*-infected (control virus) fibroblasts after 12, 24 or 40 hours of hypoxic treatment are shown in Figures 5.12, 5.13 and 5.14, respectively. The D_{37} values for survival of β-gal activity of the UV-damaged reporter gene were combined from multiple experiments and the average D_{37} values in Ad*HIF-1α*-infected compared to AdCA18*luc*-infected cells are shown as a function of time between virus infection and β-gal scoring under hypoxic

conditions in Figure 5.15. The D_{37} values for survival of β-gal activity of the UV-damaged reporter gene were pooled from multiple experiments and the average relative D_{37} values in Ad*HIF-1α*-infected compared to AdCA18*luc*-infected cells after hypoxic treatment for the 12, 24 and 40 hours are shown in Table 5.5. It can be seen that Ad*HIF-1α*-infection resulted in a significant decrease in HCR of the UV-damaged reporter gene in normal fibroblasts exposed to hypoxic conditions for 12 hours, but this reduction was not observed in CSB-deficient fibroblasts until 40 hours post-infection. This indicated a delayed effect of HIF-1α overexpression on repair of the UVC-damaged reporter gene on CSB-deficient fibroblasts. In addition, there was no significant difference in HCR of β-gal activity in normal or CSB-deficient cells due to Ad*HIF-1α*-infection at 24 hours after hypoxic conditions.

The CSB expression levels were also examined to determine if the reduction in HCR of the UV-damaged reporter gene observed in normal fibroblasts after Ad*HIF-1α* infection exposed to hypoxic conditions for 6 hours could be correlated with CSB expression levels. As seen in Figure 5.11, the CSB expression levels in the GM9503 normal fibroblasts did not significantly change due to Ad*HIF-1α*-infection in cells that were exposed to hypoxic conditions for 6 hours. CSB expression was also determined after hypoxic exposure for 12 hours as shown in Appendix B, Figure B.2 (panel A). As expected, CSB protein in CSB-deficient human fibroblasts was not detected at any of the conditions tested. It has been reported recently that normal primary human fibroblasts show an up-regulation of the CSB protein following hypoxia treatment at 24 hours (254). In contrast, in the present work, although we show that hypoxia treatment of normal

human fibroblasts for 24 hours resulted in up-regulation of HIF-1α, we show no up-regulation of the CSB protein following hypoxia (Figure 5.11). We were also unable to detect up-regulation of CSB protein following 2, 4 (data not shown, private communications from Derrik Leach) and 12 hours of hypoxia treatment.

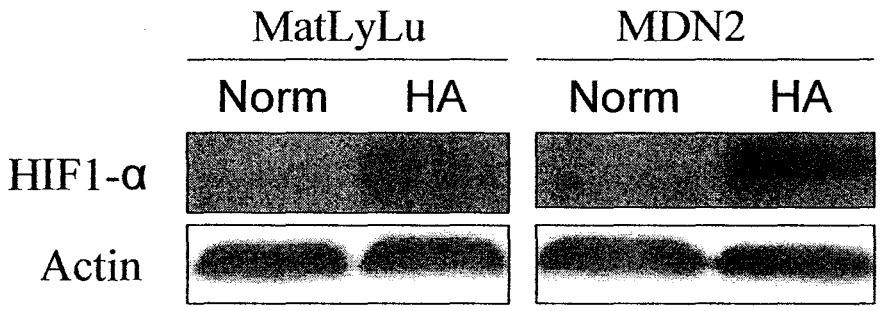


Figure 5.1. Expression of HIF-1α under normoxic (Norm) and hypoxic stress (HA). Western blot analysis of HIF-1α protein levels in rat prostate wild type HIF-1α (MATLyLu) and dominant-negative HIF-1α (MDN2) tumour cells harvested at 5 hours after treatment. Equal amounts of complete cell lysates were separated by 10% SDS-PAGE, blotted on a nitrocellulose membrane and examined for the expression levels of HIF-1α using anti-HIFα antibody. Actin serves as a loading control.

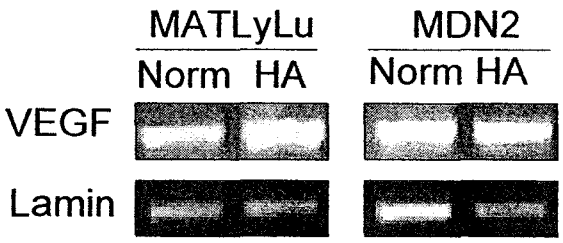


Figure 5.2. Effect of dominant-negative HIF-1α on VEGF mRNA levels under hypoxic conditions (1% O_2 for 5 hours). mRNA levels of VEGF and lamin by RT-PCR in MATLyLu and dominant-negative HIF-1α MDN2 rat prostate tumour cells. Equal amounts (1 μg) of mRNA were isolated from MATLyLu and MDN2 cells after 5 hours of exposure to either normoxic (Norm) or hypoxic (HA) conditions. The RT-PCR products were then separated on a 2% agarose gel by electrophoresis and the ethidium bromide-stained DNA bands were visualized using an ultraviolet lightbox. Lamin expression is shown for comparison.

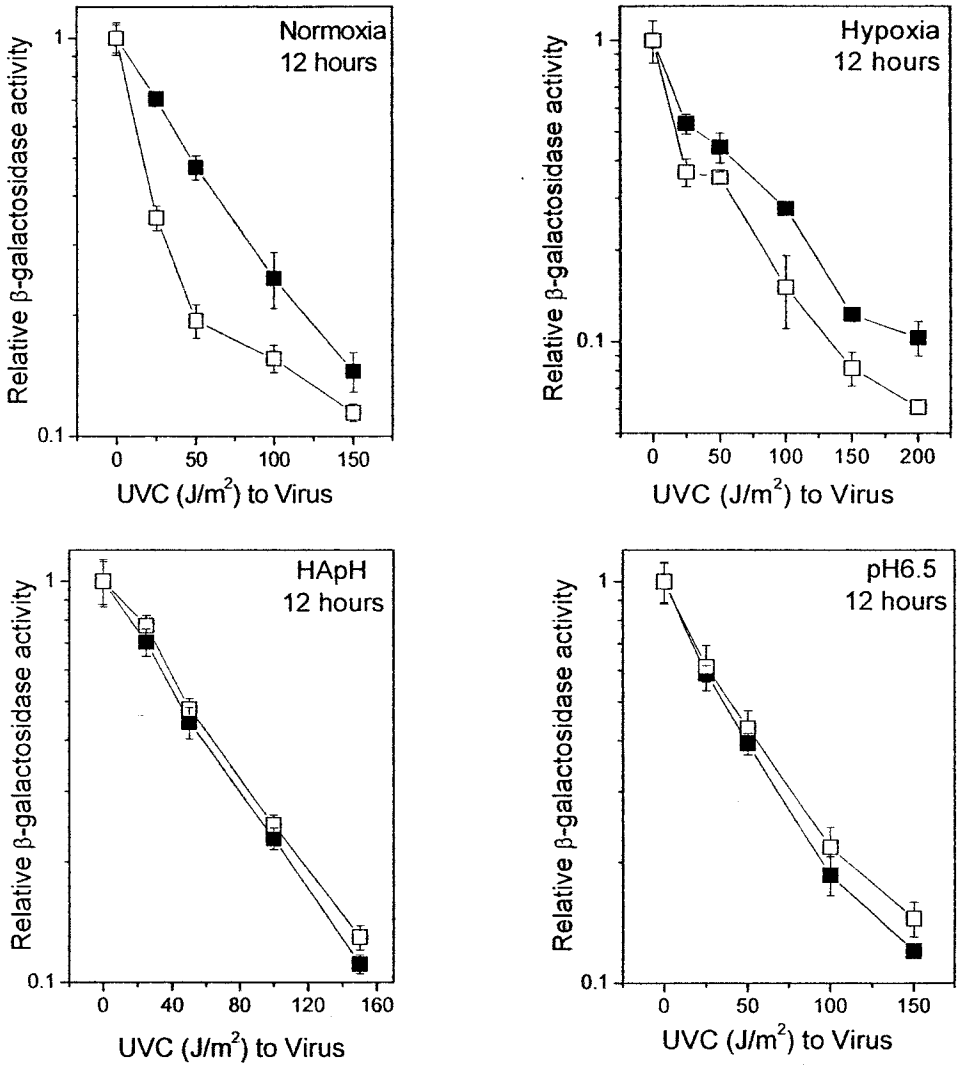


Figure 5.3. Representative results showing host cell reactivation of a UV-irradiated reporter gene in wild-type HIF-1α (MATLyLu, ■) and dominant-negative HIF-1α (MDN2, □) rat prostate tumour cells. Cells were infected with either non-irradiated or UV-irradiated AdCA17*lacZ*, followed by incubation in either normoxia, hypoxia (HA), hypoxia with acidosis (HApH) or acidosis (pH6.5) only for 12 hours, after which scoring for β-galactosidase was performed. Each point is the average of 3 replicates; error bars represent one standard error.

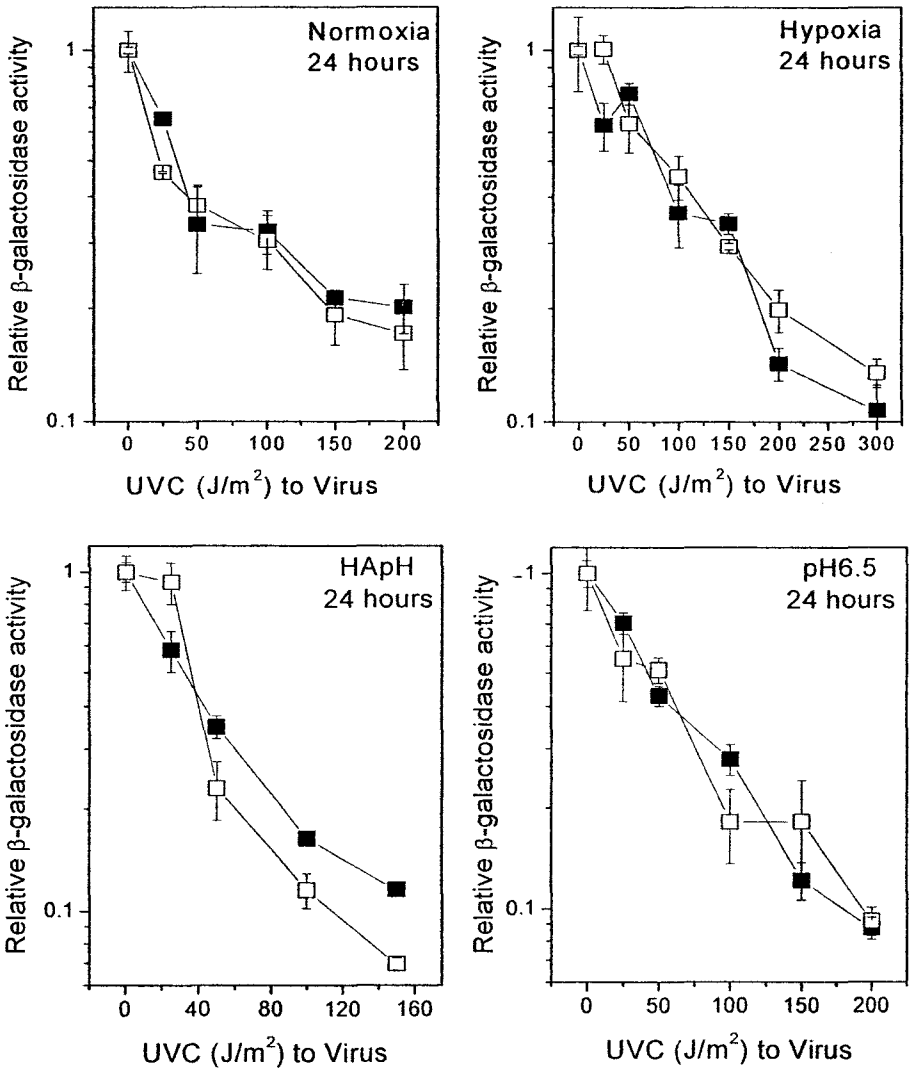


Figure 5.4. Representative results showing host cell reactivation of a UV-irradiated reporter gene in wild-type HIF-1α (MATLyLu, ■) and dominant-negative HIF-1α (MDN2, □) rat prostate tumour cells. Cells were infected with either non-irradiated or UV-irradiated AdCA17*lacZ*, followed by incubation in either normoxia, hypoxia (HA), hypoxia with acidosis (HApH) or acidosis (pH6.5) only for 24 hours, after which scoring for β-galactosidase was performed. Each point is the average of 3 replicates; error bars represent one standard error.

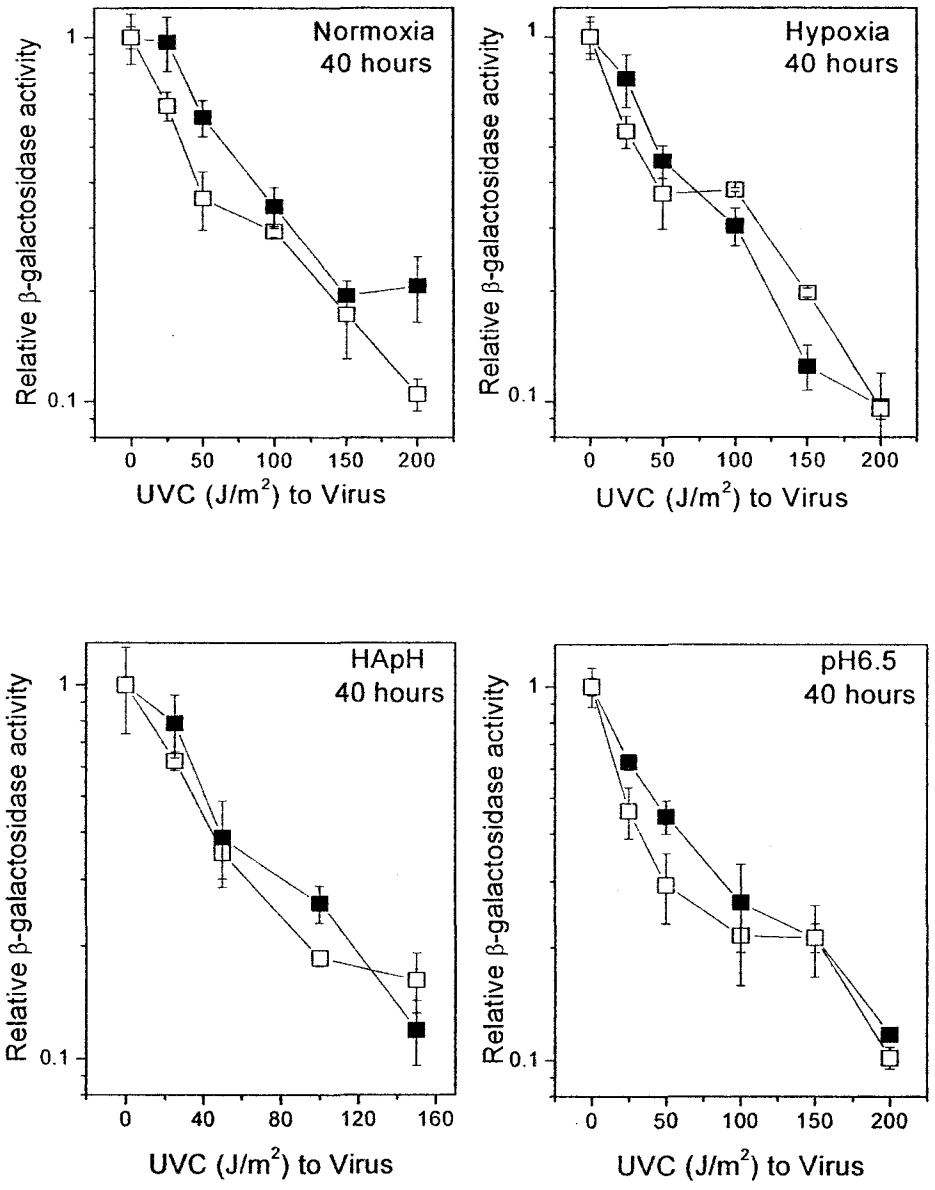


Figure 5.5. Representative results showing host cell reactivation of a UV-irradiated reporter gene in wild-type HIF-1α (MATLyLu, ■) and dominant-negative HIF-1α (MDN2, □) rat prostate tumour cells. Cells were infected with either non-irradiated or UV-irradiated AdCA17*lacZ*, followed by incubation in either normoxia, hypoxia (HA), hypoxia with acidosis (HApH) or acidosis (pH6.5) only for 40 hours, after which scoring for β-galactosidase was performed. Each point is the average of 3 replicates; error bars represent one standard error.

Table 5.1. Relative D_{37} ratios ± S.E. of HCR of a UV-damaged reporter gene in hypoxia, hypoxia coupled to acidosis or acidosis alone compared to normoxia-treated rat prostate wild type HIF-1α tumour cells (MATLyLu) and dominant-negative HIF-1α cells (MDN2). Cells were maintained in normoxia, hypoxia (HA), hypoxia with low pH (HApH) and low pH (pH6.5) conditions after AdCA17*lacZ* infection for (A) 12 hours, (B) 24 hours and (C) 40 hours. Number in brackets indicates number of experiments performed. *Significantly > 1 by an independent t-test (P value < 0.05); § Significantly < 1 by an independent t-test (P value < 0.05)

A.

Cell Line	Relative D_{37} Values		
	HA	HApH	pH6.5
MATLyLu	0.69 ± 0.07 p= **0.020§** (4)	0.70 ± 0.04 p= **0.006§** (4)	0.74 ± 0.11 p= 0.113 (4)
MDN2	1.12 ± 0.28 p= 0.691 (4)	1.74 ± 0.48 p= 0.218 (4)	2.45 ± 0.76 p= 0.151 (4)

B.

Cell Line	Relative D_{37} Values		
	HA	HApH	pH6.5
MATLyLu	1.20 ± 0.25 p= 0.439 (8)	1.15 ± 0.20 p= 0.482 (8)	0.95 ± 0.10 p= 0.654 (8)
MDN2	0.84 ± 0.11 p= 0.236 (8)	0.96 ± 0.19 p= 0.854 (8)	0.97 ± 0.14 p= 0.887 (8)

C.

Cell Line	Relative D_{37} Values		
	HA	HApH	pH6.5
MATLyLu	1.01 ± 0.18 p= 0.926 (3)	0.82 ± 0.21 p= 0.502 (3)	1.03 ± 0.49 p= 0.951 (3)
MDN2	1.10 ± 0.50 p= 0.852 (3)	0.93 ± 0.38 p= 0.873 (3)	1.21 ± 0.40 p= 0.653 (3)

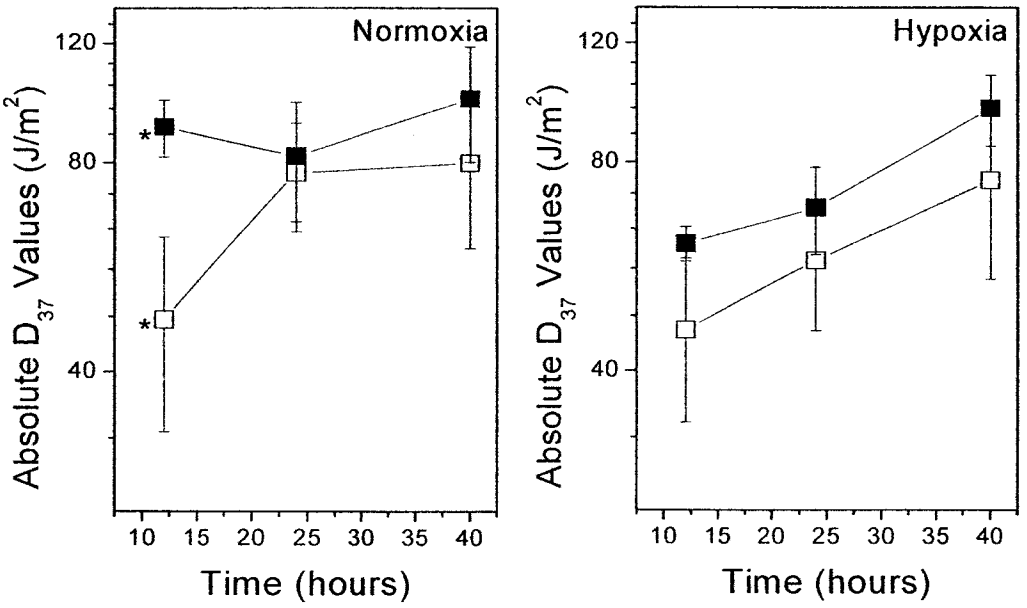


Figure 5.6. Dominant-negative HIF-1α rat tumour cells showed a reduction in HCR of β-gal activity for UV-irradiated AdCA17*lacZ* when compared to wild-type HIF-1α tumour cells. Results show the mean D_{37} ± S.E. value for β-gal expression of UV-irradiated AdCA17*lacZ* in dominant-negative HIF-1α (□) and wild-type HIF-1α (■) rat tumour cells after normoxic or hypoxic conditions. *Significant by a two sample independent t-test (P value < 0.05). Each data point is the average ±S.E. of several independent experiments.

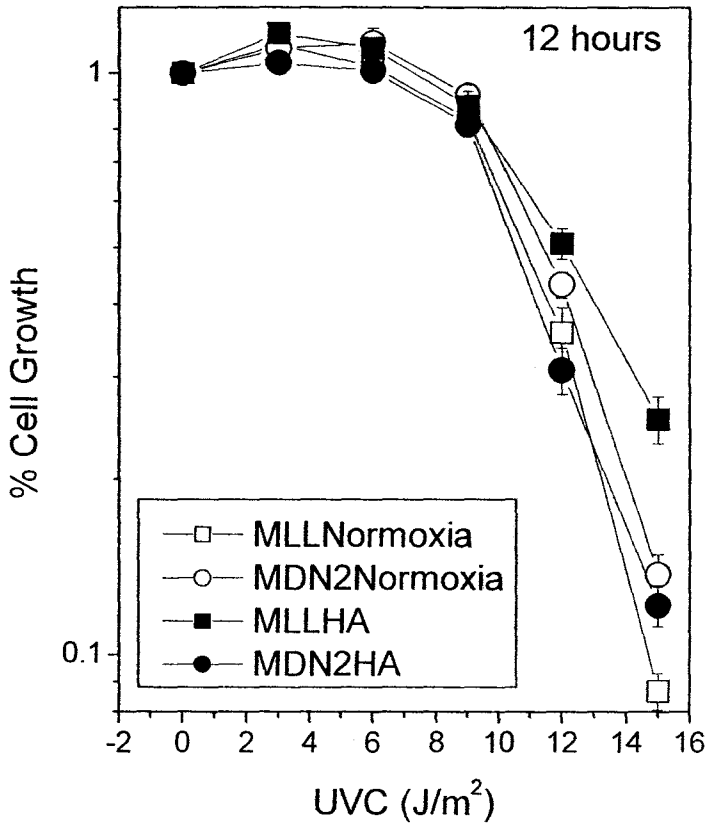


Figure 5.7. Susceptibility of rat wild-type HIF-1α (MATLyLu, MLL; ■, □) and dominant-negative HIF-1α (MDN2; ●, ○) prostate tumour cell lines to treatment of either normoxic or hypoxic conditions following UVC treatment. The cells were treated with the indicated fluence of UVC and incubated in normoxia (open symbols) or hypoxia (HA; closed symbols) for 12 hours. Each point is the average of 3 replicates; error bars represent one standard error.

Table 5.2. Absolute and relative D_{50} values ± S.E. for MTT assays in murine HIF-1α wild-type (MATLyLu) and dominant-negative HIF-1α (MDN2) prostate cancer cells. Relative D_{50} values of hypoxia-treated compared to normoxia-treated cells are shown. Cells were kept in normoxic or hypoxic conditions after UV treatment for 12 hours. Shows pooled results for three experiments. Number in brackets indicates number of experiments performed. *Significantly > 1 by an independent t-test (P value < 0.05) §Significantly < 1 by an independent t-test (P value < 0.05)

Cell Line	Absolute D_{50} Values		Relative D_{50} Values
	Normoxic	HA	HA
MATLyLu (MLL)	10.1 ± 1.0	10.8 ± 1.0	1.1 ± 0.03
	p= 0.948	p= 0.599	p= 0.169
MDN2	9.9 ± 1.7	10.1 ± 0.6	1.1 ± 0.15 p= 0.690

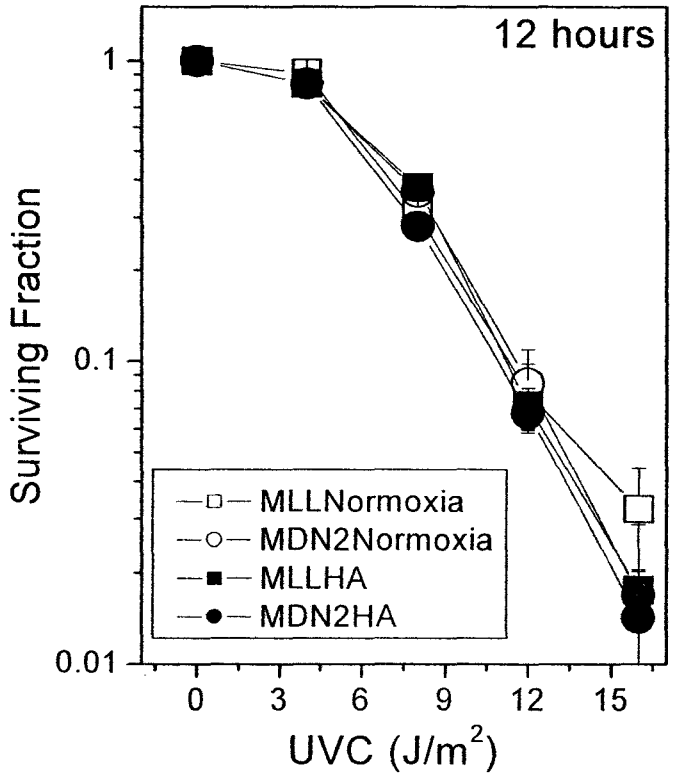


Figure 5.8. Clonogenic survival curves of murine wild-type HIF-1α (MATLyLu, MLL; ■, □) and dominant-negative HIF-1α (MDN2; ●, ○) rat prostate tumour cell lines exposed to either normoxic or hypoxic conditions following UVC treatment. The cells were treated with the indicated fluence of UVC and incubated in normoxia (open symbols) or hypoxia (HA; closed symbols) for 12 hours. Each data point is the average of 3 replicates; error bars represent one standard error.

Table 5.3. Absolute and relative D_{50} values ± S.E. for colony survival assays in murine HIF-1α wild-type (MATLyLu) and dominant-negative HIF-1α (MDN2) prostate cancer cells. Relative D_{50} values of hypoxia-treated compared to normoxia-treated cells are shown. Cells were kept in normoxic or hypoxic conditions after UV treatment for 12 hours. Shows pooled results for three experiments. *Significantly > 1 by an independent t-test (P value < 0.05) §Significantly < 1 by an independent t-test (P value < 0.05)

Cell Line	Absolute D_{50} Values		Relative D_{50} Values
	Normoxic	HA	HA
MATLyLu (MLL)	6.2 ± 0.2	6.7 ± 0.4	1.1 ± 0.01
	p= 0.878	p= 0.084	p= **0.047***
MDN2	6.1 ± 0.3	5.7 ± 0.1	0.94 ± 0.1 p= 0.540

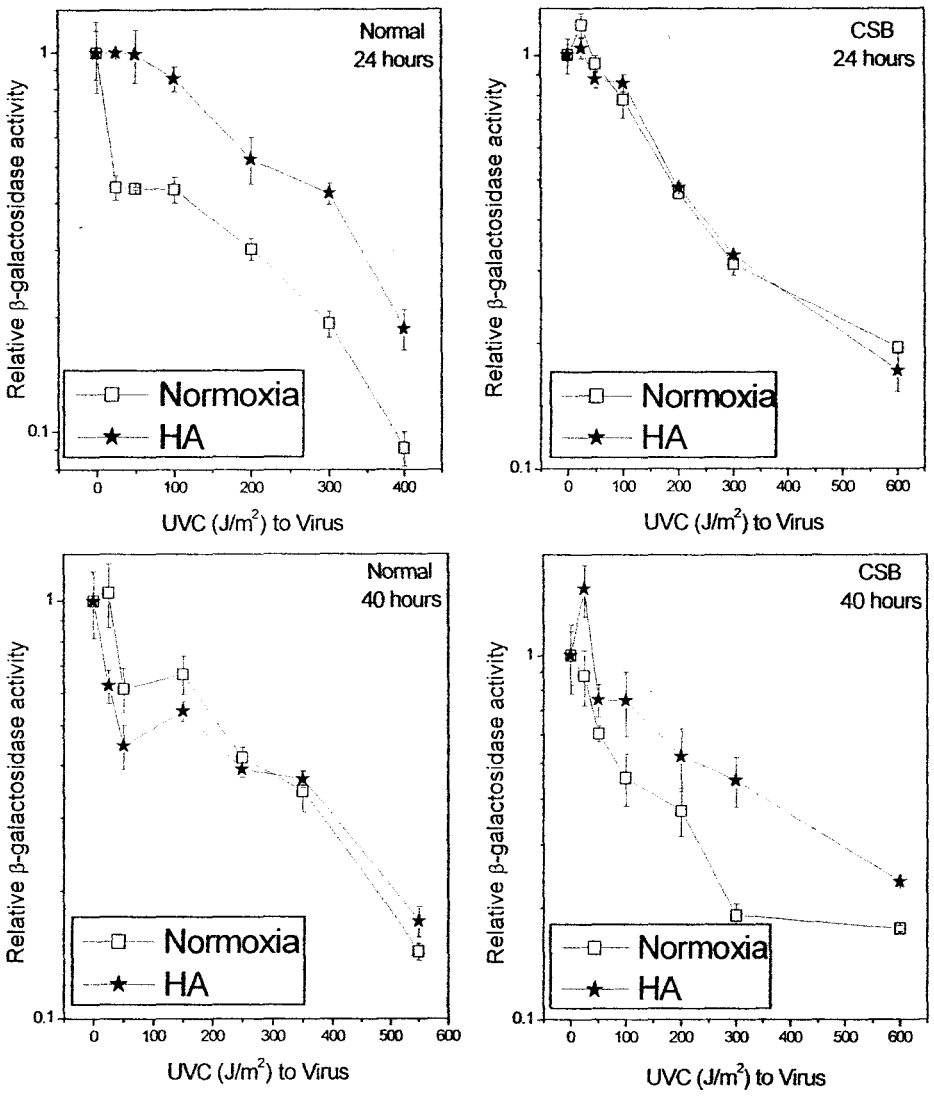


Figure 5.9. Representative results of experiments showing normal (GM9503) and CSB (GM739) primary fibroblasts under standard culture conditions (Normoxia, □) and hypoxia after infection (HA, ★) conditions. Cells were infected with either non-irradiated or UV-irradiated AdCA17*lacZ*, followed by incubation under either normoxic or hypoxic conditions for 40 hours, after which scoring for β-gal was performed. Each point is the average of 3 replicates; error bars represent one standard error.

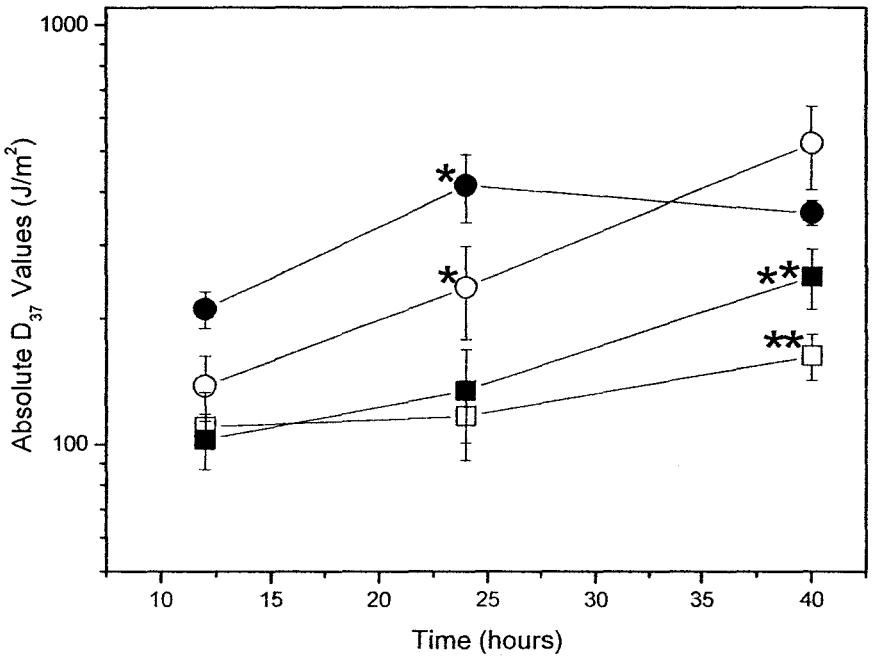


Figure 5.10. Hypoxic treatment results in enhanced HCR of β-gal expression for UV-irradated AdCA17*lacZ* in primary normal human fibroblasts (GM9503) and is delayed in CSB fibroblasts (GM739). Results show mean D_{37} ± S.E. for β-gal expression of UV-irradated AdCA17*lacZ* in hypoxic (●, ■) treated compared to normoxic (○, □) treated cells for normal (circles) and CSB (squares) primary human fibroblasts. Each data point is the average ± S.E. of several independent experiments. *Significant by a paired two sample t-test for absolute D_{37} values (P value < 0.05); **indicates significant difference for the relative D_{37} values (Significantly > 1 by an independent t-test (P values < 0.05)) as indicated in Table 5.4.

Table 5.4. Relative D_{37} ratios ± S.E. of HCR in hypoxia compared to aerobic treated human primary normal (GM9503) and CSB (GM739) fibroblast cells of β-galactosidase expression of UV-irradiated AdCA17*lacZ*. Cells were kept in aerobic or hypoxia after AdCA17*lacZ* infection for 12, 24 and 40 hours. Number in brackets indicates number of experiments performed. *Significantly > 1 by an independent t-test (P value < 0.05)

Cell Line	**Time (hours)**		
	12	24	40
GM9503	1.96 ± 0.7 p= 0.212 (9)	2.45 ± 0.6 p= **0.046*** (9)	0.8 ± 0.2 p= 0.450 (4)
GM739CSB	1.1 ± 0.3 p= 0.756 (4)	1.37 ± 0.5 p= 0.496 (6)	1.6 ± 0.2 p= **0.046*** (5)

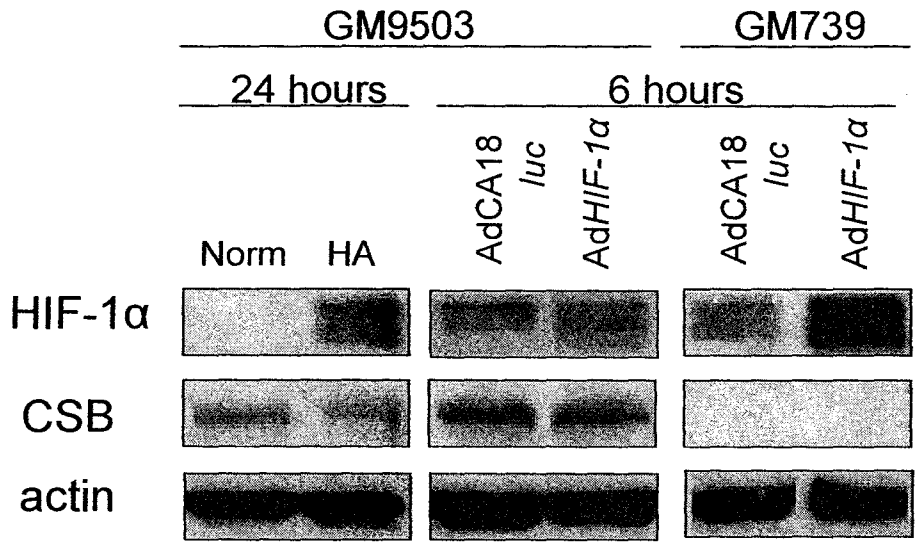


Figure 5.11. HIF-1α and CSB expression levels in primary normal and CSB-deficient fibroblasts 6 hours after Ad*HIF-1α* or AdCA18*luc* infection. Confluent human primary normal (GM09503) and CSB-deficient (GM739) fibroblasts were either infected with a control virus (AdCA18*luc*) or Ad*HIF-1α* at an MOI of 200 pfu/cell and subsequently incubated for 6 hours under hypoxic conditions. GM9503 normal primary fibroblasts were also exposed to either normoxic or hypoxic conditions for 24 hours without virus infection. Equal amounts of complete cell lysates were separated by 10% SDS-PAGE, blotted on a nitrocellulose membrane and examined for the expression levels of HIF-1α using anti-HIFα antibody and CSB using anti-CSB antibody. The amount of actin was determined by using anti-actin antibody to obtain the sample loading control.

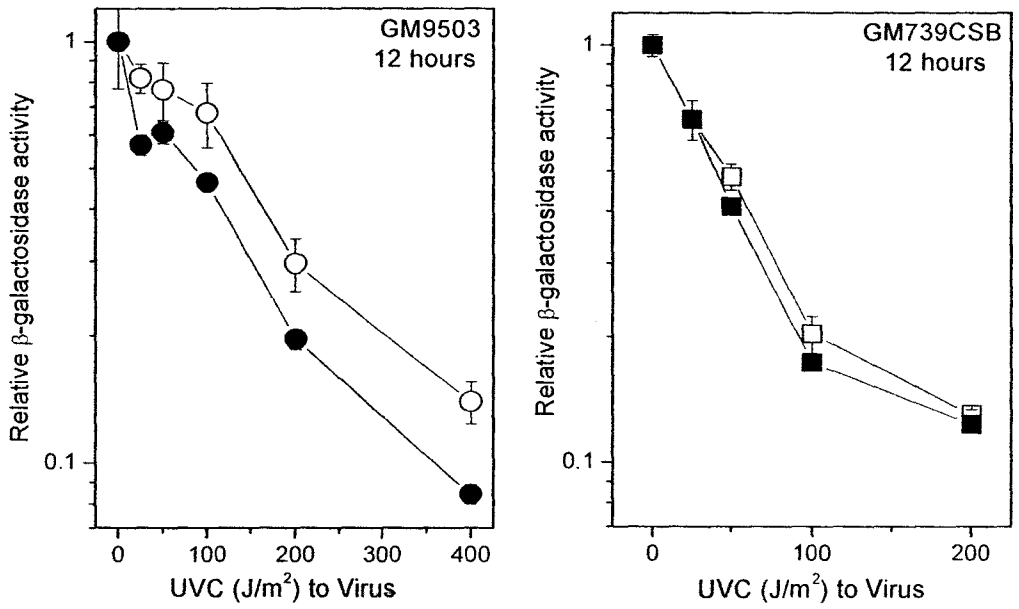


Figure 5.12. Infection with Ad*HIF-1α* virus results in reduced HCR of β-gal activity by UV-irradiated AdCA17*lacZ* in normal (circles), but not CSB primary human fibroblasts(squares) exposed to hypoxic conditions. Results of typical experiments are shown for normal (GM9503) and CSB fibroblasts (GM739). Closed symbols represent Ad*HIF-1α*-infected cells, open symbols represent AdCA18*luc*-infected cells. The time between infection and scoring for β-gal activity is 12 hours. Each point is the average of 3 replicates; error bars represent one standard error.

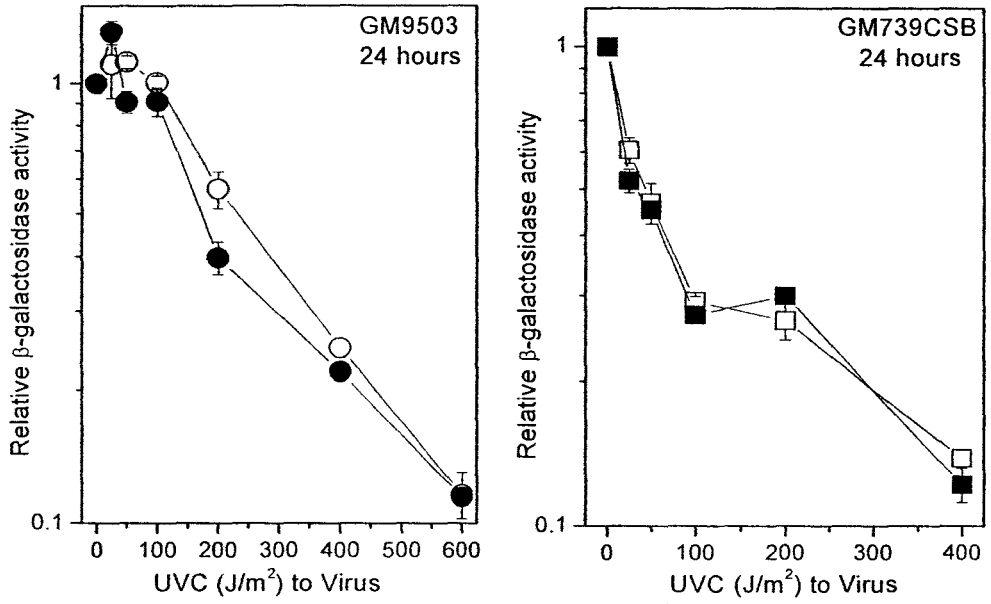


Figure 5.13. Infection with Ad*HIF-1α* virus in normal (circles) and CSB (squares) primary human fibroblasts exposed to hypoxic conditions. Results of typical experiments are shown for normal (GM9503) and CSB (GM739) fibroblasts. Closed symbols represent Ad*HIF-1α*-infected cells, open symbols represent AdCA18*luc*-infected cells. The time between infection and scoring for β-gal activity is 24 hours. Each point is the average of 3 replicates; error bars represent one standard error.

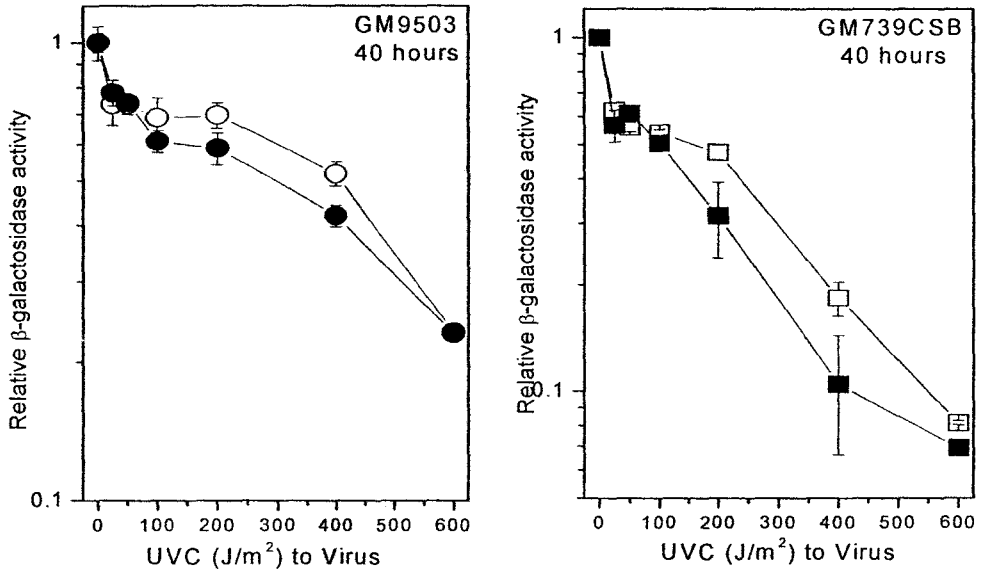


Figure 5.14. Infection with Ad*HIF-1α* virus in normal (circles) and CSB (squares) primary human fibroblasts exposed to hypoxic conditions. Results of typical experiments are shown for normal (GM9503) and CSB (GM739) fibroblasts. Closed symbols represent Ad*HIF-1α*-infected cells, open symbols represent AdCA18*luc*-infected cells. The time between infection and scoring for β-gal activity is 40 hours. Each point is the average of 3 replicates; error bars represent one standard error.

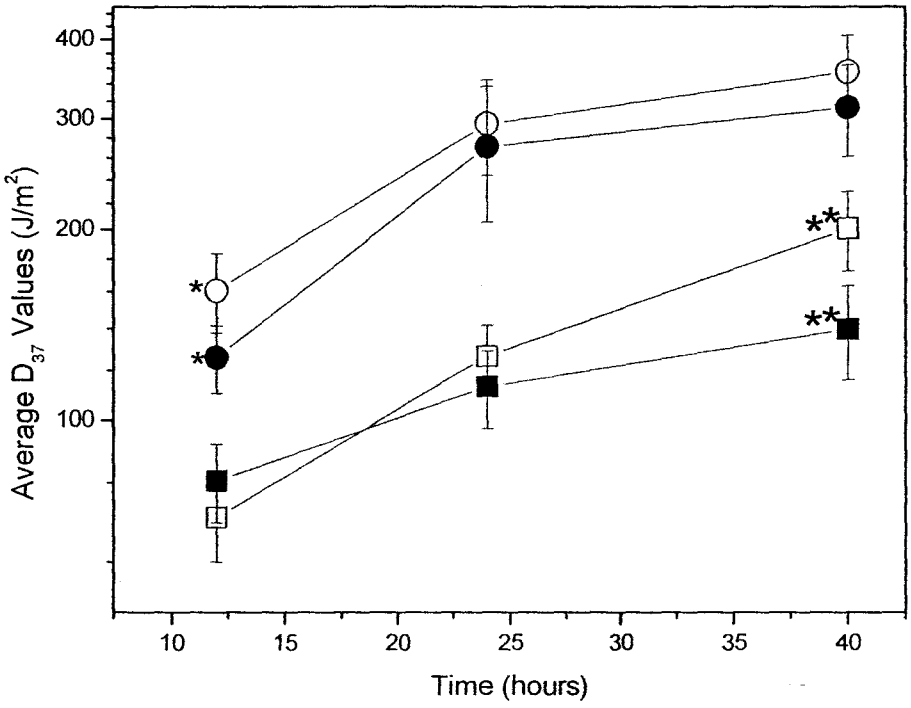


Figure 5.15. HIF-1α overexpression results in reduction of HCR of β-gal expression for UV-irradated AdCA17*lacZ* in human primary normal (GM9503) fibroblasts and is delayed in CSB (GM739) fibroblasts. Results show the mean D_{37} ± S.E. value for β-gal expression of UV-irradiated AdCA17*lacZ* in Ad*HIF-1α*-infected cells (●, ■) compared to that in AdCA18*luc*-infected cells (○, □) for normal (circles) and CSB (squares) fibroblasts after hypoxic exposure. Each data point is the average ± S.E. of several independent experiments. *Significant by a paired two sample t-test for absolute D_{37} values (P value < 0.05); **indicates significant difference for the relative D_{37} values (SignificantLY < 1 by an independent t-test & Wilcoxon Rank Sum Test (P values < 0.05)) as indicated in Table 5.7

Table 5.5. Relative D_{37} values for β-gal activity of UV-irradiated AdCA17*lacZ* in Ad*HIF-1α*-infected cells compared to that in AdCA18*luc*-infected cells for normal (GM9503) and CSB (GM739) fibroblasts. Pooled results are given for cells infected at 12, 24 and 40 hours of hypoxic conditions post-infection and are represented as mean relative D_{37} ± SE. Number in brackets indicates number of experiments performed. §Significantly < 1 by an independent *t*-test (*P* value < 0.05)

	Relative D_{37} Values for Ad*HIF-1α*/AdCA18*luc*		
Group	12 hrs	24 hrs	40 hrs
Normal GM9503	0.80 ± 0.06 p= **0.017§** (8)	0.87 ± 0.08 p= 0.200 (7)	0.87 ± 0.05 p= 0.110 (4)
CSB GM739	1.20 ± 0.11 p= 0.129 (9)	0.89 ± 0.05 p= 0.104 (7)	0.71 ± 0.09 p= **0.038§** (5)

5.0 Discussion

5.1 HIF-1α-dependent repair of a UVC-damaged reporter gene

HIF-1α is a master regulator of adaptive responses to low oxygen levels in mammalian cells and is responsible for regulating many different genes involved in angiogenesis, metastasis, glucose metabolism and numerous other functions (reviewed in (247)). The DNA-dependent protein kinase, which is known to be important for DNA double strand break repair has been suggested to directly associate with HIF-1α and possibly play a role in its phosphorylation (293). Previously, it was demonstrated that HIF-1α–deficient transformed mouse embryonic fibroblasts were more sensitive to carboplatin treatment and ionizing radiation when compared to wild-type HIF-1α cells (292). Unruh *et al.* (2003) found that double-strand breaks in a reporter gene were less efficiently repaired in HIF-1α-deficient cells compared to wild-type HIF-1α mouse fibroblasts (292).

Using two rat prostate tumour cell lines expressing either the HIF-1α wild-type or the dominant-negative *HIF-1α* gene, we demonstrate that HIF-1α dominant-negative tumour cells repair UV-damage less efficiently when compared to wild-type HIF-1α cells. HIF-1α expression was detected in both cell lines as expected since the plasmid containing the dominant-negative HIF-1α cDNA overexpresses its product, which inhibits wild-type HIF-1α functionally, but will not prevent its stabilization under hypoxic conditions (Figure 5.1). RT-PCR confirmed equal VEGF mRNA levels in dominant-negative HIF-1α cells exposed to hypoxic conditions compared to normoxic conditions, while VEGF mRNA levels were increased under hypoxic conditions in wild-

type HIF-1α cells when compared to standard culture conditions as shown in Figure 5.2. The dominant-negative HIF-1α prevents HIF-1α-dependent transactivation of its targets genes such as VEGF in the dominant-negative HIF-1α murine prostate tumour cells, but does not in the wild-type cells, which is in accordance to data published by Alqawi and colleagues (2007) (192).

This difference in repair of UVC-induced DNA damage in the reporter gene between the two cell lines was detectable and significant under normoxic conditions at 12 hours post-infection, but not at 24 or 40 hours after treatment as shown in Figure 5.3, 5.4 and 5.5. The reduction in repair of UV-induced DNA damage due to HIF-1α-deficiency is consistent with results obtained by Unruh and colleagues (2003) for ionizing radiation (292). Although HIF-1α is degraded quickly under normal physiological oxygen levels, the ability of wild-type HIF-1α tumour cells to repair a UV-damaged reporter gene more efficiently than the dominant-negative HIF-1α cells by 12 hours under normoxic conditions suggests that HIF-1α status alone may be significant for a proficient cellular DNA repair system in tumour cells. Taken together, our finding is the first indication that HIF-1α may include NER proteins as its target.

Hypoxia coupled to acidosis and acidosis alone did not have the same effect on the repair of the damaged reporter gene at any of the times tested in the rat prostate tumour cells, which is in line with previous results where hypoxia induced a different cellular response when compared to hypoxia and low pH conditions in tumour cells (291). Furthermore, hypoxia and hypoxia coupled to acidosis for 12 hours resulted in a decrease in HCR of β-gal activity in the wild-type, but not dominant-negative tumour

cells or at any other time points tested. These results are consistent with our previous published work, which showed that hypoxia as well as hypoxia accompanied by acidosis induced a decrease in HCR of UV-damaged reporter gene by 24 hours after treatment in human tumour cells (291). Hypoxia resulted in down-regulation of p53 in tumour cells, while hypoxia and low pH caused an increase in p53 expression and previous research, although strongly debated, indicates that p53 may regulate HIF-1α accumulation (259,294), and therefore hypoxia or hypoxia coupled to acidosis-induced p53 expression may target HIF-1α for degradation. Acidosis alone in wild-type HIF-1α tumour cells caused similar results to hypoxia and low pH conditions, confirming previous results that it is low pH rather than the synergistic effect of hypoxia and low pH affecting repair of a UV-damaged reporter gene (291).

Cell viability and colony survival assays results shown in Figure 5.7 and 5.8 did not reveal a differential effect between the two rat tumour cell lines exposed to hypoxic or normoxic conditions for 12 hours after UVC-irradiation. Abrogated HIF-1α did not influence cell growth or cell survival after UVC in the rat prostate tumour cells dominant-negative for HIF-1α. The differential DNA repair of a UV-damaged reporter gene among the two rat tumour cell lines under normoxic conditions did not correlate with diminished cell survival of the dominant-negative HIF-1α when compared to the wild-type cells. These results are in contrast to previous reports that have detected an increase in sensitivity of HIF-1α mutant transformed mouse embryonic fibroblasts to several mutagens, including ionizing radiation, when compared to wild-type cells (292). However, ionizing radiation causes types of DNA damage including DNA double strand

breaks, which are not repaired by NER and thus may involve response pathways that can affect cell survival differently when compared to UVC-induced DNA damage. We have shown that hypoxia for 12 hours did not render the wild-type or the dominant-negative HIF-1α rat prostate tumour cell lines more sensitive to UVC-irradiation. The cell growth and survival were comparable between the two cell lines and in line with our human tumour cell lines results, which were not sensitive to UVC-irradiation after exposure to hypoxia (291). We have shown that HIF-1α status may affect repair of a UV-damaged reporter gene, but that it does not translate into a difference in cell sensitivity to UVC-irradiation. HIF-1α involvement in hypoxic responses is multifaceted and further investigations are crucial in understanding HIF-1α involvement in DNA repair and those implications for cancer treatment.

5.2 Hypoxia induces early enhancement in HCR of a UV-damaged reporter gene in normal human fibroblast and is delayed in CSB-deficient cells

As shown in Table 5.6 and Figures 5.9 and 5.10, hypoxia after infection induced an enhancement in HCR of β-gal expression that occurs early in human normal primary fibroblasts and is delayed, but not absent in primary CSB-deficient fibroblasts. Since CSB fibroblasts have an impaired TCR and, as a result, are deficient in repairing DNA damage from the transcribed strand of active genes, this data suggests that the TCR subpathway of NER is important for repair of DNA damage in active genes following exposure to hypoxic conditions. This response is similar to a UV-inducible NER response.

UV-induced DNA damage in the transcribed strand of active genes in cells can stall RNAPII and it has been shown that RNA synthesis is blocked by one UV-induced lesion in a reporter gene (295). TCR removes lesions from the transcribed strand of active genes in mammalian cells and thus, TCR-deficient CSB cells cannot recover transcription elongation as efficiently as normal fibroblasts. Previously we have shown that UV-irradiation caused a transient increase in HCR in normal fibroblasts and it was delayed, but not absent in CSB fibroblasts (296). Similar results were obtained with Chinese hamster ovary cells (CHO) (297). In addition, UV-induced lesions that block transcription also activate p53 and p53-dependent responses (109,182,298). However, stabilization of protein products from p53-dependent genes is contingent on these genes not containing UV-induced lesions (299). For instance, previously our laboratory has shown that p53-dependent p21 up-regulation in UV-irradiated TCR-deficient cells was delayed (299). Therefore, TCR-deficient cells show a delay in recovery of RNA synthesis after UV exposure (59,109,182) and a delay in accumulation of p53-dependent protein products (299) when compared to TCR-proficient cells. These reports provide evidence for the presence of a TCR- and p53-dependent UV-inducible repair.

Since hypoxia is perceived by the cell as a stress (reviewed in (223)), it is possible that hypoxic treatment may activate stress response pathways inducing an enhancement of DNA repair, similar to how a cell reacts to UV damage. The delayed enhancement in HCR of a UV-damaged reporter gene in CSB-deficient primary fibroblasts following hypoxia is consistent with the hypothesis that hypoxia can cause cellular oxidative DNA damage. Reports have suggested that hypoxia may directly or indirectly induce DNA

damage, either by producing superoxide radicals through repeated cycles of hypoxia-reoxygenation (208) or by generating endonuclease activity, which promotes DNA strand breaks (209,300). Yuan *et al.* (2000) quantified mutation frequency in rat fibroblasts (3340WT) exposed to hypoxic and low pH conditions after UV-irradiation and found that the levels were augmented compared to normoxic conditions (211). Therefore, TCR-deficient CSB fibroblasts not only cannot repair transcription-blocking lesions as efficiently as TCR-proficient cells, but these cells need to first repair the damage from the genes involved in DNA repair.

We have demonstrated that hypoxia resulted in p53 stabilization, reaching a maximum at approximately 12 hours of treatment in normal primary fibroblasts, which correlated with a hypoxia-induced enhancement in HCR of a UV-damaged reporter gene in normal cells. These results are in accordance with numerous reports demonstrating that anaerobic conditions produce p53 stabilization (166,168,170). We have also shown in Figure A.2 that hypoxia for 40 hours after infection resulted in enhanced HCR of a UV-damaged reporter gene in SV40-transformed CSB cells. Since SV40-transformed fibroblasts have abrogated p53, hypoxia-induced enhancement in HCR of β-gal activity in primary and SV40-transformed CSB fibroblasts at 40 hours of hypoxia suggests a p53-independent NER response to anaerobic stress. However, T antigen expression levels under hypoxic conditions in CSB SV40-transformed fibroblasts were decreased compared to normoxic conditions as shown in Appendix B, Figure B.3. Although p53 expression levels remained relatively unchanged from normoxic compared to hypoxic conditions, attenuated T antigen levels under anaerobic conditions in CSB cells would

suggest that some p53 may be able to transactivate responsive genes essential for NER. Also, Tzang *et al.* (1999) reported that in CHO-K1 cells, which contain the p53 gene mutated at codon 211, UV-irradiation caused p53 accumulation and resulted in transactivation of p53-responsive genes (301). It was apparent that the missense mutation at codon 211 did not disrupt several functional activities of p53. Renton *et al.* (2003) discovered that caffeine, which is known to inhibit p53 accumulation in response to several different DNA damaging agents including UVC-irradiation in tumour cells (231,302), did not prevent p53 stabilization due to hypoxia. Therefore, it is possible that hypoxic conditions may not impair p53 function in SV40-transformed cells. It is evident that although hypoxia may exert similar DNA repair responses to UV, it may do so through different pathways, but that may still involve p53.

5.3 HIF-1α overexpression results in a diminished HCR of β-gal activity in human primary normal fibroblasts, but is delayed in CSB-deficient cells under hypoxic conditions

HIF-1α has been associated with DNA double strand break repair proteins such as DNA-dependent protein kinase (293), but it is only recently that HIF-1α has been connected to a NER protein, CSB (254). To examine a possible role of HIF-1α in NER and its connection to CSB, human primary normal and CSB-deficient fibroblasts were infected with either AdCA18*luc* (a control virus) or Ad*HIF-1α*, exposed to hypoxic conditions and assessed for repair of a UV-damaged reporter gene.

Here, we show that HIF-1α overexpression induces a decrease in HCR of β-gal expression in human NER-proficient fibroblasts, which is delayed but not absent in CSB-

deficient fibroblasts when exposed to hypoxia (Figures 5.9 and 5.10). These results suggest that HIF-1α indeed may play an important role in repair of UV-induced DNA damage of a reporter gene in human fibroblasts. The delay in CSB-deficient cells is consistent with a delay in up-regulation of HIF-1-dependent VEGF expression. Filippi *et al.* (2008) showed that CSB mutant cells exposed to hypoxia exhibited lower VEGF mRNA expression levels compared to CSB wild type cells at different times of treatment up to 16 hours, but not thereafter (254). It is possible that in CSB cells VEGF mRNA expression levels may reach similar levels to the ones in normal fibroblasts after 16 hours of hypoxic treatment, therefore CSB cells exhibit a delayed, but not absent HIF-1-dependent expression of target genes. Our results are not in contradiction with the conclusion made by Filippi and colleagues (2008) that the CSB protein may be involved in a HIF-1-dependent hypoxia response pathway (254).

In contrast to the study by Filippi *et al.* (2008) (254), we were unable to detect increased CSB expression after 24 hours of hypoxia or after Ad*HIF-1α* infection and different hypoxic treatment times in normal fibroblasts despite observed accumulation of HIF-1α under hypoxic conditions. However, the normal and CSB mutant cell lines used in the aforementioned report are different from the ones used in our study. Nevertheless, in addition to the role of CSB in TCR (133), CSB has been implicated in transcription (111,119), inucleosome remodeling (115) and BER of 8-oxoguanine (145,150-152). Since hypoxic conditions were shown to induce oxidative DNA damage (reviewed in (223)), it is not unlikely that CSB may play a crucial role in DNA repair in mammalian cells exposed to low oxygen conditions.

Filippi *et al.* (2008) proposed a model in which CSB and p300 compete for binding to p53, with CSB having a greater affinity to p53 (254). Under hypoxic conditions, HIF-1 expression increases, therefore CSB is up-regulated and binds to p53, which in turn inhibits p53-dependent transactivation activities including the ones that dedicate cells towards apoptosis or DNA repair (reviewed in (265)). In CSB mutant cells, the absence or inactivation of CSB allows p300 to bind to p53 leading to activation of p53-dependent genes and degradation of HIF-1α.

We have previously demonstrated that p53 overexpression leads to enhanced HCR of a UVC-damaged reporter gene in normal, CSB and XPC primary human fibroblasts, but not in XPA cells (291). In addition, hypoxia-induced increase in HCR of β-gal expression for a UVC-damaged reporter gene in normal and CSB fibroblasts may depend on the tumour suppressor p53. On the other hand, Ad*HIF-1α* infection diminished HCR of β-gal expression in both normal and CSB primary human fibroblasts after exposure to low oxygen conditions, indicating that HIF-1α overexpression can down-regulate NER (Figures 5.12, 5.14, and 5.15). If HIF-1α and p53 have antagonistic effects, the reduction in HCR of a UV-damaged reporter gene observed in Ad*HIF-1α*-infected normal and CSB fibroblasts exposed to hypoxia could be the result of inhibition of NER responses due to HIF-1α overexpression. Previously it has been reported that the expression level of a protein vital for DNA double strand break repair, NBS1 (or "nibrin") was attenuated by hypoxia in a HIF-1α-dependent way (303). It is possible that when the balance between p53 and HIF-1α expression levels is disrupted by HIF-1α overexpression, it can down-regulate genes important for NER of a UV-induced lesion.

Our results are consistent with a competition between HIF-1α and p53 that may play a role in DNA repair of a UVC-damaged reporter gene and involve the protein CSB.

It should also be noted that HIF-1α expression levels in AdCA18*luc*-infected normal and CSB fibroblasts were moderately reduced compared to non-infected cells exposed to hypoxia for 6 hours, suggesting that AdCA18*luc* infection may down-regulate HIF-1α expression. It is possible that AdCA18*luc*-induced HIF-1α down-regulation may contribute to a diminished HCR of a UV-damaged reporter gene in normal and CSB-deficient fibroblasts due to Ad*HIF-1α* under hypoxic conditions. However, it has been demonstrated that infection with a constructed adenovirus does not generate DNA damage-elicited responses (277). In addition, the relative D_{37} values of CSB (GM739) compared to normal fibroblasts (GM9503) for cells infected with AdCA18*luc* or Ad*HIF-1α* indicate a significant decrease in HCR of β-gal expression of CSB-deficient cells relative to normal cells, consistent with previous data published by our laboratory (269). Nevertheless, it would be of importance to assess whether the expression level of HIF-1α in AdCA18*luc*-infected cells is significant enough that it can influence repair of a UV-damaged reporter gene in normal and CSB fibroblasts.

CHAPTER 6

Summary and Discussion

1.0 The role of p53 in TCR and GGR

The DNA repair pathways of normal human cells can be compromised by both endogenous (genetic) and exogenous factors. UV light-induced DNA damage is repaired by NER, which is divided into two sub-pathways: global genome repair (GGR) and transcription-coupled repair (TCR). The p53 tumour suppressor protein has many roles in cellular functions that protect the cell from uncontrolled proliferation, including a role in DNA repair. Previously it was shown that p53 regulates GGR (175,177,239,304,305), but its involvement in TCR has been strongly debated (183,184,188,189). The purpose of this study is to address the disparity in our present understanding of the role of p53 in response to DNA damage in the two sub-pathways of NER. We have employed a non-replicating recombinant adenovirus expressing the β-gal reporter gene under the control of the human cytomegalovirus promoter to examine the role of the p53 tumour suppressor protein in the repair of UV-induced DNA damage.

We show here that pre-infection of normal, CSB and XPC, but not XPA fibroblasts with Ad5p53wt resulted in enhanced HCR of the UV-damaged reporter gene compared to cells infected with the control AdCA18*luc* virus (285). These results indicate that increased expression of p53 up-regulates both GGR and TCR of the UV-damaged reporter gene. Overexpression of p53 in CSB fibroblasts resulted in an elevated host cell reactivation (HCR) of β-gal expression detected at 12, 24 and 40 hours post-infection. The p53-dependent up-regulation of HCR in CSB cells is consistent with a p53-dependent up-regulation of GGR due to increased expression of the p53-dependent

XPE-p48 (83) and/or XPC proteins (180). Increased expression of p53 following Ad5p53wt infection did not result in a delay in increased HCR of the reporter gene in CSB cells, which is in contrast to the observed delay in enhanced HCR in pre-UV-treated CSB compared to pre-UV-treated normal and XPC cells (285,296). Ad5p53wt-pre-infection of cells induced increased expression of p53, but did not result in UV-induced lesions in the *XPC* and *p48* genes and therefore the absence of delay in enhanced HCR of a UV-damaged reporter gene in Ad5p53wt-pre-infected CSB cells is consistent with our hypothesis that the delay in HCR detected in UV-irradiated CSB cells is due to the lack of TCR of lesions in the *XPC* and *p48* genes.

In addition, increased expression of p53 resulted in an enhancement in HCR of a UV-damaged reporter gene in normal and XPC fibroblasts at 12 hours, but not 24 or 40 hours post-infection. These results suggest that increased expression of p53 alone induced an increase in rate of repair of the UV-damaged reporter gene in normal and XPC fibroblasts. Since TCR is responsible for removal of lesions from the transcribed strand of active genes (61,306), the increased rate of repair in XPC cells pre-infected with Ad5p53wt is consistent with a p53-dependent up-regulation of TCR in the transcribed strand of a UV-damaged reporter gene.

These results demonstrate that increased expression of the p53 tumour suppressor protein enhanced HCR of the UV-damaged β-galactosidase gene in normal, CSB and XPC deficient fibroblasts, but not in XPA fibroblasts. These results strongly suggest that increased p53 expression results in an enhancement of both the TCR and GGR pathways of UV-induced DNA damage in human cells. However, due to the nature of the HCR

assay that uses UV-irradiated or non-irradiated virus to infect human cells, it remains unclear whether the repair of the adenovirus-encoded β-gal gene is a true reflection of the cellular DNA repair.

Previously, Boszko and Rainbow (1999) have used the PCR assay to examine HCR of a UV-damaged adenovirus-encoded reporter gene in order to determine whether repair of UV lesions in a reporter gene is reflective of DNA repair in cells (307). It was found that NER-deficient cells (CSB- and XPC-deficient fibroblasts) showed a diminished lesion removal capacity compared to normal cells, indicating that both TCR and GGR are involved in HCR for lesion removal. This suggests that repair of lesions in adenoviral DNA is similar to repair in cellular DNA. However, in those studies the relative contribution of GGR and TCR to removal of UV-induced lesions in the transcribed compared to the non-transcribed strand was not determined.

By conducting a more quantitative molecular method, it could be determined to what extent the repair of UV-induced DNA damage in an adenovirus-encoded reporter gene in human cells reflects repair in human cellular DNA. This assay, as previously described (306), quantifies lesions with the use of specific endonucleases such as T4 endonuclease V, which makes single-strand incisions in the DNA strands at sites of pyrimidine dimers. This technique facilitates an examination of the differences in repair between transcribed and non-transcribed regions of a UV-damaged recombinant adenovirus genome and in the transcribed strand compared to the non-transcribed strand of an actively transcribed adenovirus-encoded reporter gene in human cells. By employing this assay, one could examine removal of UV-induced lesions from the

recombinant adenovirus at the DNA level in both untreated and UV-treated cells. The results of such a technique would consolidate to what extent repair of a UV-damaged reporter gene reflects cellular DNA repair. In the event that repair of adenovirus DNA reflects repair of cellular DNA, then it can also be concluded that p53 expression affects both TCR and GGR in human cells.

The tumour suppressor protein p53 and its role in NER play an important part in circumventing mutagenic risks and development of cancer. Mutations in the *p53* gene are commonly found in squamous cell carcinomas (13,16), and basal cell carcinomas (14). In addition, cells neighboring basal cell carcinomas have been found to contain mutations in the *p53* gene (308) indicating that *p53* mutations can contribute to cancer development. Furthermore, development of nonmelanoma skin cancers have been linked to lifetime exposure to UV (309) and NER deficiencies (310,311). Taken together, genetic mutations in the *p53* gene and DNA repair deficiencies can greatly contribute to tumour development. Further studies in understanding the role of p53 in the regulation of DNA repair pathways, such as the TCR and GGR subpathways of NER, will prove to be important and relevant to understanding the effects of lifetime UV exposure on tumour development.

2.0 The role of p53 and hypoxia in NER

Previous reports have suggested that the exogenous environment of the cell, such as low oxygen and pH levels, can also influence DNA repair. We report here that hypoxia coupled to low pH conditions in normal fibroblasts results in a transient increase in HCR

of a UV-damaged reporter gene at early time points (12-24 hours) and a concomitant increase in cell viability following UV exposure. In contrast, in tumour-derived cells hypoxia coupled to acidosis results in a delayed (36-40 hours) enhancement in HCR of a UV-damaged reporter gene compared to normal fibroblasts. These results are not in contradiction with data reported by Yuan *et al.* (2000) who reported that hypoxia coupled to low pH results in a reduction in HCR of a UV-damaged plasmid in tumour cells at 24 hours (211), but did not investigate the effect of hypoxia accompanied by low pH at 36-40 hours. In the present work we show that hypoxia coupled to low pH also induced p53 expression at 6 hours in normal fibroblasts and between 6 and 18 hours in tumour-derived cells. In addition, in tumour-derived cells, hypoxia coupled to acidosis for 30 hours after UV-irradiation resulted in a significant reduction in cell viability, irrespective of p53 status.

It is evident that in normal fibroblasts, early up-regulation of NER after hypoxia coupled to low pH treatment is necessary for an enhancement in cell viability and growth. These results suggest that early up-regulation of p53 and increased repair of UV-damaged DNA after hypoxia coupled to acidosis treatment is required for increased cell viability after UVC exposure. However, in tumour-derived cells, although hypoxia coupled to acidosis up-regulated p53 expression and increased DNA repair, this enhancement in repair was delayed when compared to that in normal fibroblasts and did not result in an increase in cell viability after UV exposure. Thus, these results indicate that in normal primary fibroblasts, hypoxia coupled to low pH induces a different NER response than in tumour cells. Schmaltz *et al.*(1998) reported that mouse transformed cells exhibited

apoptosis under hypoxia, while primary fibroblasts showed G_0/G_1 arrest (229), similar to the effects of ionizing radiation and other chemotherapeutic agents (reviewed in (312)), demonstrating that oncogene-transformed cells respond differently to hypoxia when compared to non-transformed cells (reviewed in (229)). It appears likely that tumour cells and other transformed cells possess additional alterations that influence the DNA repair response under hypoxia and acidosis conditions.

Treatment with hypoxia alone also induced an enhancement in HCR of β-gal expression in normal fibroblasts that was delayed in tumour-derived cells. Hypoxia increased the expression of p53 in normal fibroblasts, reaching a peak at 12 hours of treatment. Previously we have reported that UV-irradiation of NER-proficient normal human fibroblasts results in a transient p53-dependent increase in HCR of a UV-damaged reporter gene as early as 12 hours after infection and is not present at 40 hours post-infection (184,267,285,296). The current data suggests that hypoxia can also induce a transient up-regulation of HCR in normal human fibroblasts. We have demonstrated that in normal human fibroblasts increased p53 expression induced enhancement of HCR of a UV-damaged reporter gene through up-regulation of both TCR and GGR (285) and here we show that hypoxia resulted in p53 accumulation in normal fibroblasts. Taken together, the hypoxia-induced enhancement in HCR of β-gal expression in normal human fibroblasts is consistent with a p53-dependent up-regulation of NER due to hypoxia.

On the other hand, in tumour-derived cells hypoxia resulted in a decrease of p53, and hypoxia did not affect cell viability after UVC treatment in normal primary fibroblasts or tumour-derived cells. Others also could not detect a hypoxia-induced p53

accumulation in several tumor-derived cell lines, unless hypoxia was coupled to acidosis or nutrient deprivation (229,235). These results suggest that hypoxia alone may affect NER through different mechanisms than when coupled to acidosis.

Although hypoxia-induced p53 expression has been reported by several researchers (166-170), other reports have suggested that hypoxic stress is insufficient for p53 up-regulation unless accompanied by acidosis (229,235,313), or that even if stabilized, p53 lacks transactivation capacities (230,314). Here we demonstrated that in primary human fibroblasts both hypoxia and hypoxia coupled to acidosis induced p53 accumulation, which correlated with enhanced repair, but that in tumour-derived cells, only hypoxia coupled to low pH induced p53 expression. Taken together, our data provide an explanation for the aforementioned disparity in results by demonstrating that the timing of hypoxia-induced p53 expression depends on cell type (primary versus tumour cells) and confirm that hypoxia coupled to low pH, but not hypoxia alone, causes increased p53 expression in primary and tumour cells.

3.0 The role of HIF-1α and hypoxia in NER

3.1 Role of HIF-1α in NER

Here, we present evidence that HIF-1α may play an important role in NER of a UV-damaged reporter gene. We showed that prostate rat tumour cells expressing dominant-negative HIF-1α exhibited reduced repair of a UV-damaged reporter gene when compared to that in wild-type HIF-1α cells. Unruh *et al.* (2003) have reported that HIF-1α-deficient mouse transformed fibroblasts repaired double-strand DNA breaks less

efficiently compared to wild-type HIF-1α cells and were more sensitive to treatment with chemotherapeutic agents and ionizing radiation (292). This susceptibility of HIF-1α-deficient cells to toxic agents was observed even under normoxic conditions, in spite of the fact that HIF-1α is not stable under normoxic conditions in HIF-1α wild-type mouse cells (292). It was also reported that even under normoxic conditions embryonic stem cells deficient for HIF-1α show reduced expression of HIF-1α target genes (315,316). Taken together, these results suggest that basal HIF-1α expression levels may be enough for activation of target genes, including genes involved in NER. In addition to the microenvironment of the tumour, it is evident that the genetic make-up of tumour cells, such as a HIF-1α status, may be a key determinant as to how tumour cells respond to chemo- and radiotherapy. If further investigations reveal that HIF-1 has a differential effect on DNA repair, which can translate into differential susceptibility to chemotherapeutics and/or radiotherapy then an anti-HIF-1 treatment method could be adopted.

3.2 Effects of hypoxia on TCR and GGR

We have reported that hypoxia results in up-regulation of NER in normal primary fibroblasts (291), although it was not clear whether this up-regulation in NER results from an up-regulation in TCR, GGR or both. A recent report by Filippi *et al.* (2008) indicates that the TCR protein, CSB, may be a target of HIF-1α (254), suggesting a role for HIF-1 in the TCR pathway of NER. It was therefore of interest to investigate HCR of

a UV-damaged reporter gene in normal and CSB-deficient fibroblasts exposed to hypoxic conditions.

We have detected a significant hypoxia-induced enhancement in host-mediated repair of the DNA damaged reporter gene in normal and in CSB fibroblasts, but not XPC or XPA cells. In CSB fibroblasts hypoxia induced a delayed, but not absent enhancement in HCR of a UV-damaged reporter gene when compared to normal fibroblasts. CSB fibroblasts have a deficient TCR and thus cannot repair DNA damage from the transcribed strand of active genes as efficiently as repair-proficient normal fibroblasts indicating that the TCR subpathway of NER is important for repair of DNA damage in active genes following exposure to hypoxic conditions. This is similar to a UV-inducible NER response that is delayed in CSB cells (296,299).

More specifically, in mammalian cells it was shown that UV-induced lesions in the transcribed strand of active genes block RNA synthesis in a reporter gene (295). UV-irradiation caused a transient increase in HCR of a UV-damaged reporter gene in normal fibroblasts and it was delayed, but not absent in CSB fibroblasts (296). This delay in the enhanced repair in CSB cells is thought to occur because TCR removes lesions from the transcribed strand of active genes, but CSB cells have a deficient TCR and therefore remove UV-induced lesions less efficiently than normal fibroblasts. Furthermore, transcription-blocking lesions also induce p53 stabilization, which induces expression of target genes (109,182,298), but expression of p53-dependent protein products requires the genes to be lesion-free (299). For example, p53-dependent p21 up-regulation was delayed in UV-irradiated TCR-deficient cells (299). Since TCR-deficient CSB cells

exhibited a delay in recovery of RNA synthesis (59,109,182) and a delay in the up-regulation of p53-dependent target genes (299) following UV exposure compared to TCR-proficient cells, these results indicate the existence of a TCR- and p53-dependent UV-inducible repair. Therefore, if hypoxia causes DNA damage as previously suggested (208,209,300), the delayed enhancement in HCR of β-gal expression in CSB fibroblasts is consistent with the presence of a hypoxia-inducible GGR in primary human fibroblasts that may also be TCR- and p53-dependent.

It was also shown that p53 can up-regulate several GGR genes including *GADD45* (174,317), *DDB2*, which encodes the XPE protein (83,181,284,318), and *XPC* (180,318-320). Therefore, we have investigated the expression levels of XPC under hypoxic and hypoxic coupled to acidosis conditions in normal human fibroblasts treated for as much as 40 hours, but could not detect any difference in expression when compared to normoxic conditions. These results suggest that enhanced HCR of a UV-damaged reporter gene in normal exposed to hypoxia or hypoxia coupled to low pH is not due to up-regulation of XPC. Although speculative, up-regulation of NER could be due to other genes involved in GGR, such as XPE, and thus further investigations would help elucidate the effect of hypoxia and/or hypoxia coupled to acidosis on NER genes.

3.3 Increased expression of exogenous HIF-1α results in a diminished HCR

In section 3.1 we reported that a compromised HIF-1 pathway in rat cells resulted in a reduced HCR of the UV-damaged reporter gene. In contrast, we show also that increasing the expression of exogenous HIF-1α resulted in a diminished HCR of the UV-

treated reporter gene in normal fibroblasts under hypoxic conditions and that this reduction in HCR was delayed, but not absent, in CSB-deficient fibroblasts. Taken together, these results indicate that the repair of UVC-induced DNA damage is dependent on HIF-1α expression levels in mammalian cells. A reduction in the HIF-1α-dependent pathway increases NER, whereas an increased expression of exogenous HIF-1α results in reduced NER.

There has been some evidence to support the hypothesis that p53 competes with HIF-1α for binding to the co-activator p300 (260). In addition, it was shown that CSB and p53 can physically interact (172), and that CSB may be a direct target of HIF-1α (254). At the same time, Filippi *et al.* (2008) also found that CSB competes with p300 for binding to p53, with CSB having a greater affinity for p53 compared to p300 (254). Therefore, in normal fibroblasts exposed to hypoxic conditions, HIF-1α accumulates and increases expression of target genes such as CSB, which binds to p53 and as a result prevents p300 from binding to p53, which in turn inhibits p53-dependent transactivation activities including the ones that dedicate cells towards apoptosis or DNA repair (refer to Figure 6.1) (reviewed in (265)). On the other hand, in CSB mutant cells exposed to hypoxia, p300 binds to p53 due to lack or deficiency of CSB, which results in expression of p53-dependent genes and degradation of HIF-1 (reviewed in (265)). Supporting evidence for this model came from experiments that showed CSB expression was increased in normal fibroblasts under hypoxic conditions, while expression of HIF-1α responsive genes was reduced in CSB mutant human fibroblasts compared to normal fibroblasts and was believed to be due to higher p53 levels in CSB cells compared to

normal cells (254). Filippi *et al.* (2008) also found that p53 depletion by siRNA in CSB mutant cells rescued accumulation of HIF-1α target genes, and increased p300 expression in CSB mutant cells abated suppression of HIF-1α-mediated responses (254).

Our current results show that HIF-1α overexpression under hypoxic conditions resulted in a reduction of NER for a UV-damaged reporter gene in both normal and CSB fibroblasts, whereas hypoxia alone resulted in an enhancement of NER. These results support the model of a competitive relationship between HIF-1α and p53 for binding to p300 that can also influence the outcome of repair of a UV-damaged reporter gene. It is possible that when HIF-1α is overexpressed in normal cells exposed to hypoxia, it can down-regulate genes involved in NER or up-regulate genes that inhibit NER, while the normal levels of HIF-1α induced by hypoxia may not be sufficient to prevent significant binding of p53 to p300, which would result in up-regulation of p53-dependent genes important in NER. Although we were unable to detect increased CSB expression under hypoxic conditions in normal fibroblasts as Filippi *et al.* (2008) have shown (254), the rest of their findings are not inconsistent with our results.

The current results suggest a role for HIF-1α regulation of NER of a UV-damaged reporter gene. We report here that increased expression of HIF-1α resulted in a reduction in HCR of β-gal expression in normal fibroblasts and this reduction was delayed, but not absent in CSB fibroblasts. The observed HIF-1α-dependent down-regulation of HCR in normal and CSB cells following hypoxic treatment are not inconsistent with data published by Filippi *et al.* (2008), who showed that the mRNA expression levels of VEGF were decreased in CSB mutant fibroblasts compared to normal cells following up

to 16 hours of hypoxic treatment (254), but later time points were not tested. Thus, the delay in down-regulation of NER in CSB fibroblasts under hypoxic conditions compared to normal fibroblasts could result from a delay in the expression level of one or more HIF-1α-dependent genes involved in NER. The delayed expression of the HIF-1α–dependent VEGF gene in CSB cells could result from the TCR deficiency in CSB cells. The hypoxia-induced DNA damage would take longer to be repaired from the VEGF gene in CSB compared to normal cells and CSB cells would therefore display delayed expression of the HIF-1-dependent VEGF gene. Up-regulation of HIF-1-dependent VEGF could also be delayed in CSB cells even under normoxic conditions due to oxidative DNA damage caused by the endogenous reactive oxygen species occurring during normal respiration.

A significant extension to this study would be to determine the mRNA expression levels of several different HIF-1α targets, such as p300 and VEGF, in normal and CSB-dedicient cells infected with Ad*HIF-1α* under hypoxic conditions and to compare the expression levels of these genes in cells infected with a control virus. Examining the p53 mRNA and protein expression levels in AdHIF1-α-infected normal and CSB fibroblasts after hypoxic treatment should reveal whether HIF-1α overexpression affects p53. This will help further elucidate the role of HIF-1, CSB and p53 in response to hypoxic stimuli.

Understanding the mechanistic interplay between p53, HIF-1 and p300 may prove to be significant for understanding the clinical phenotypes of patients with mutations in the *CSB* gene. CS patients experience photosensitivity, growth retardation, neurological abnormalities and premature aging, but do not exhibit an increased risk of developing

cancer despite reduced DNA repair capacity. Since DNA damage is not efficiently repaired by CS patients with mutations in the *CSB* gene, it is possible that a lack of functional CSB will target any potential tumour cells for p53-induced programmed cell death (see Figure 6.1). Therefore, p53-dependent apoptosis may prevent development of tumours, but this increase in cell death may contribute to the premature aging observed in CS patients as tissues must cope with an excess of apoptosis.

APPENDIX A

Effects of different hypoxic treatments on NER

Preliminary experiments were conducted to determine the optimal conditions for observing the effect of hypoxia on HCR of a UV-damaged reporter. Normal, CSB, XPC and XPA primary and SV40-transformed fibroblasts were infected with UVC-irradiated AdCA17*lacZ* and exposed to normoxic or hypoxic conditions either for 24 hours before infection only, 24 hours before and 40 hours after infection or just 40 hours after infection. Representative graphs of HCR of the UV-damaged reporter gene under the different conditions for both primary and SV40-transformed fibroblasts are shown in Figure A.1 and A.2. A more pronounced and significant enhancement in HCR of a UVC-treated reporter gene expressing β-gal expression was observed in the human primary CSB-deficient and CSB SV40-transformed fibroblasts under hypoxic conditions 40 hours after the infection.

The relative D_{37} values in Table A.1 also indicate that a significant increase in HCR was detected in CSB SV40-transformed at 40 hours after infection when cells were kept under hypoxic conditions 24 hours prior to infection. In addition, Table A.2 indicated that a significant enhancement in HCR of β-gal activity is observed when primary CSB fibroblasts were maintained under conditions of hypoxia before and after AdCA17*lacZ* infection, but a significant decrease in HCR of the UV-damaged reporter gene is detected in these cells when only exposed to hypoxia before infection. Furthermore, primary XPC-deficient fibroblasts exhibited a slight reduction in HCR of β-gal activity when only exposed to hypoxia before infection. Based on preliminary results, hypoxia after infection resulted in a more pronounced effect on HCR of a UV-damaged

reporter gene in both primary and SV40-transformed CSB-deficient fibroblasts and consequently hypoxia after infection only was used for all subsequent experiments.

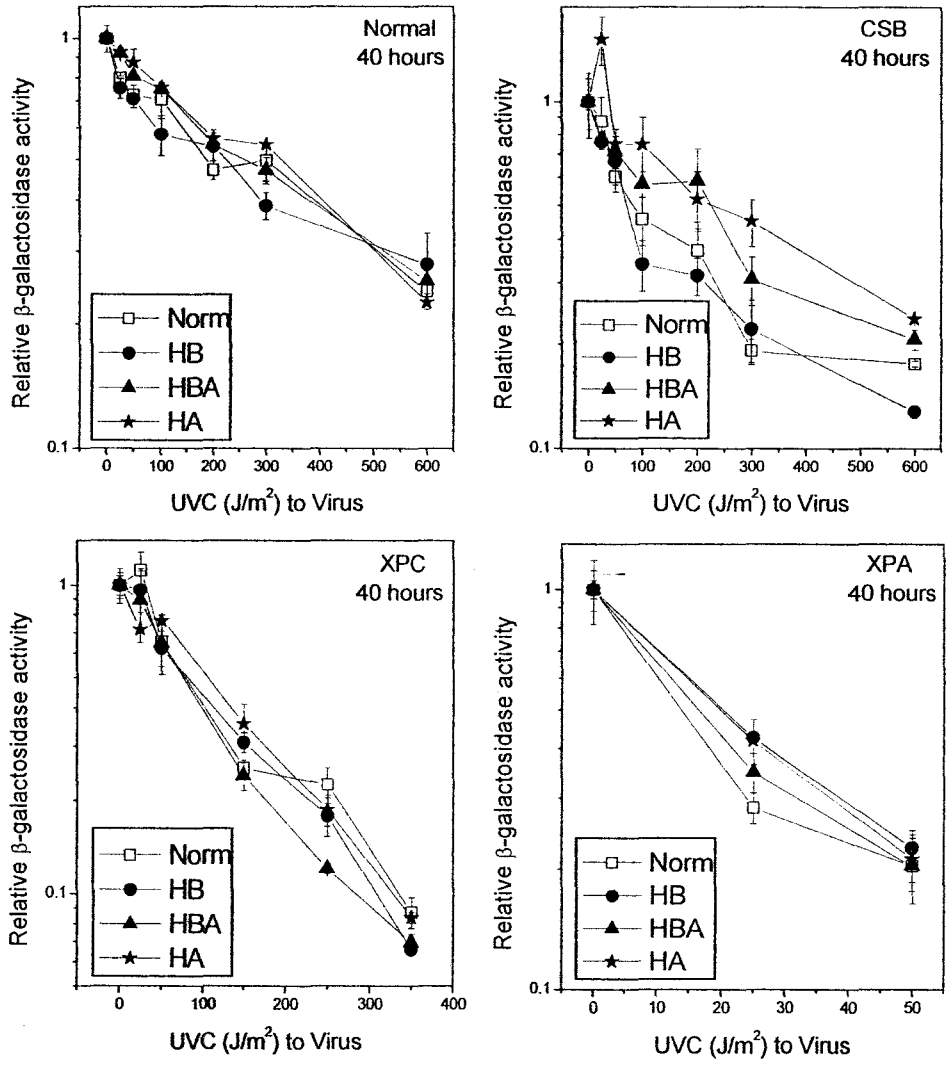

Normal
40 hours
CSB
40 hours
XPC
40 hours
XPA
40 hours
Relative β-galactosidase activity
UVC (J/m²) to Virus
Norm
HB
HBA
HA
1
0.1
0
100
200
300
400
500
600
10
20
30
40
50

Figure A.1. Representative results showing HCR of a UV-irradiated reporter gene in GM9503, GM739CSB, GM677XPC and GM5509XPA primary fibroblasts under standard culture conditions (Norm,□), hypoxia before infection (HB, ●), hypoxia before and after (HBA, ▲) and hypoxia after infection only (HA, ★). Cells were infected with either non-irradiated or UV-irradiated AdCA17*lacZ*, followed by incubation under either normoxic or hypoxic conditions before infection for 24 hours, hypoxic conditions before and after infection (total of 64 hours) and hypoxic conditions after infection only for 40 hours, after which scoring for β-gal was performed. Each point is the average of 3 replicates; error bars represent one standard error.

Table A.1 Relative D_{37} values of hypoxia before infection (HB), hypoxia before and after infection (HBA), and hypoxia after infection for 40 hours (HA) treatment in human primary normal (GM9503), CSB (GM739), XPC (GM677) and XPA (GM5509) fibroblasts of β-galactosidase expression of UV-irradiated AdCA17*lacZ*. *Significantly > 1 by an independent t-test (P value < 0.05), § Significantly < 1 by an independent t-test (P value < 0.05)

	Relative D_{37}								
Cell Lines	**N**	**HB/Oxic**	**P Value**	**N**	**HBA/Oxic**	**P Value**	**N**	**HA/Oxic**	**P Value**
GM9503	3	0.9 ± 0.2	0.613	4	1.0 ± 0.1	0.912	4	0.8 ± 0.2	0.45
GM739CSB	5	0.7 ± 0.09	**0.022 δ**	5	1.3 ± 0.1	**0.049***	5	1.6 ± 0.2	**0.046***
GM677XPC	3	1.1 ± 0.02	**0.004***	3	1.1 ± 0.2	0.597	4	1.2 ± 0.2	0.19
GM5509XPA	4	1.3 ± 0.2	0.260	3	1.2 ± 0.2	0.297	4	1.3 ± 0.2	0.158

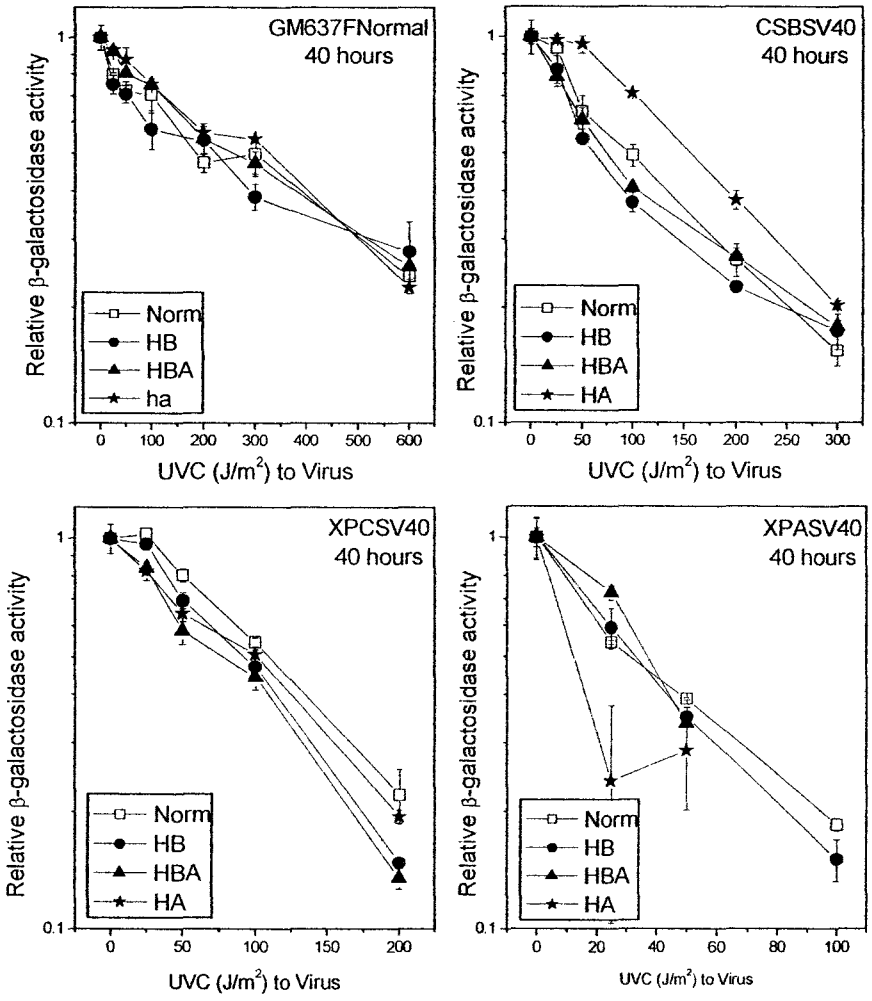


Figure A.2. Representative results showing HCR of a UV-damaged reporter gene in GM637F, CSBSV40, XPCSV40 and GM4429XPA SV40-transformed fibroblasts under standard culture conditions (Norm,□), hypoxia before infection (HB, ●), hypoxia before and after (HBA, ▲) and hypoxia after infection only (HA, ★). Cells were infected with either non-irradiated or UV-irradiated AdCA17*lacZ*, followed by incubation under either normoxic or hypoxic conditions before infection for 24 hours, hypoxic conditions before and after infection (total of 64 hours) and hypoxic conditions after infection only for 40 hours, after which scoring for β-gal was performed. Each point is the average of 3 replicates; error bars represent one standard error.

Table A.2. Relative D_{37} values ± S.E. of HCR of UV-damaged reporter gene in human SV40-transformed normal (GM637F), CSBSV40, XPCSV40, and XPASV40 (GM4429DXPA) fibroblasts exposed to hypoxia before infection, hypoxia before and after infection, and hypoxia after infection for 40 hours. *Significantly > 1 by an independent t-test (P value < 0.05)

Cell Line	N	Relative D_{37} Hypoxia before	P Value	N	Relative D_{37} Hypoxia before & after	P Value	N	Relative D_{37} Hypoxia after	P Value
GM637F	4	0.9 ± 0.08	0.13	4	1.2 ± 0.2	0.27	4	1.1 ± 0.3	0.7
CSBSV40	4	1.2 ± 0.08	**0.03***	4	1.1 ± 0.08	0.16	4	1.4 ± 0.1	**0.03***
XPCSV40	5	0.9 ± 0.05	0.06	4	1.2 ± 0.1	0.18	5	1.1 ± 0.2	0.507
GM4429DXPA	4	1.1 ± 0.1	0.42	3	1.3 ± 0.2	0.23	4	0.6 ± 0.2	0.096

APPENDIX B

Hypoxia effect on HIF-1α, CSB and T antigen expression

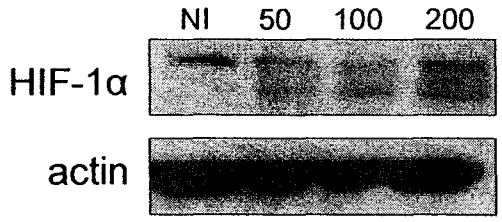
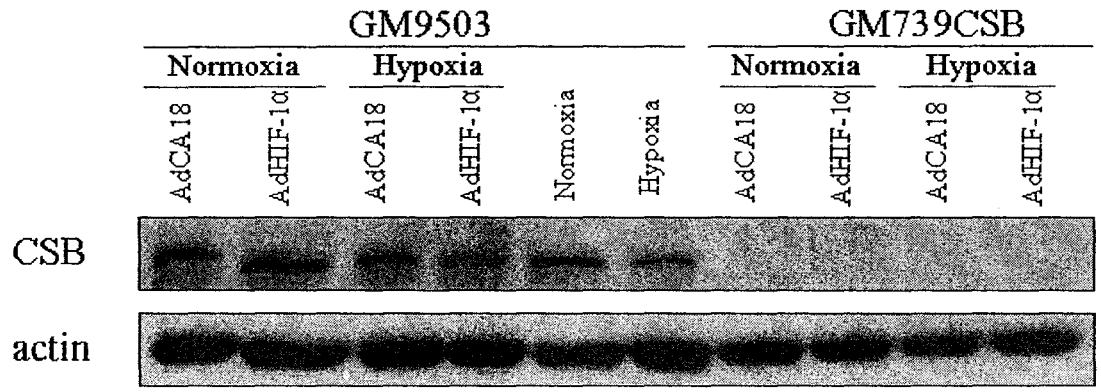


Figure B.1. HIF-1α expression upon Ad*HIF-1α* virus infection in human fibroblasts. Confluent normal (GM09503) were either infected with Ad*HIF-1α* at different MOI of 50, 100 and 200 pfu/cell or mock infected (NI) and exposed to hypoxic conditions for 6 hours. Equal amounts of complete cell lysates were run on 10% SDS-PAGE gel, blotted on a nitrocellulose filter and examined for the expression levels of HIF-1α using anti-HIF-1α antibody. The amount of actin was determined by using anti-actin antibody to obtain the sample loading control.

A.



B.

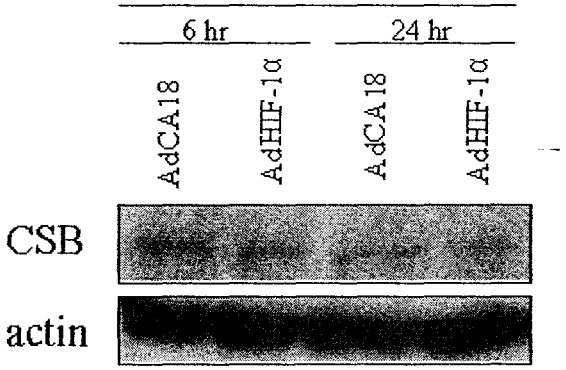


Figure B.2. CSB expression upon Ad*HIF-1α* virus infection in human fibroblasts. Confluent normal (GM09503) and CS-B (GM00739) were exposed (A) to either normoxic or hypoxic conditions for 12 hours or either infected with a control virus (AdCA18*luc*) or infected with the Ad*HIF-1α* virus and subsequently exposed to normoxic or hypoxic conditions for 12 hours. (B) Confluent normal (GM09503) were either infected with a control virus (AdCA18*luc*) or infected with the Ad*HIF-1α* virus and subsequently incubated for 6 or 24 hours under normoxic conditions. Equal amounts of complete cell lysates were run on 10% SDS-PAGE gel, blotted on a nitrocellulose filter and examined for the expression levels of CSB using anti-CSB antibody. The amount of actin was determined by using anti-actin antibody to obtain the sample loading control.

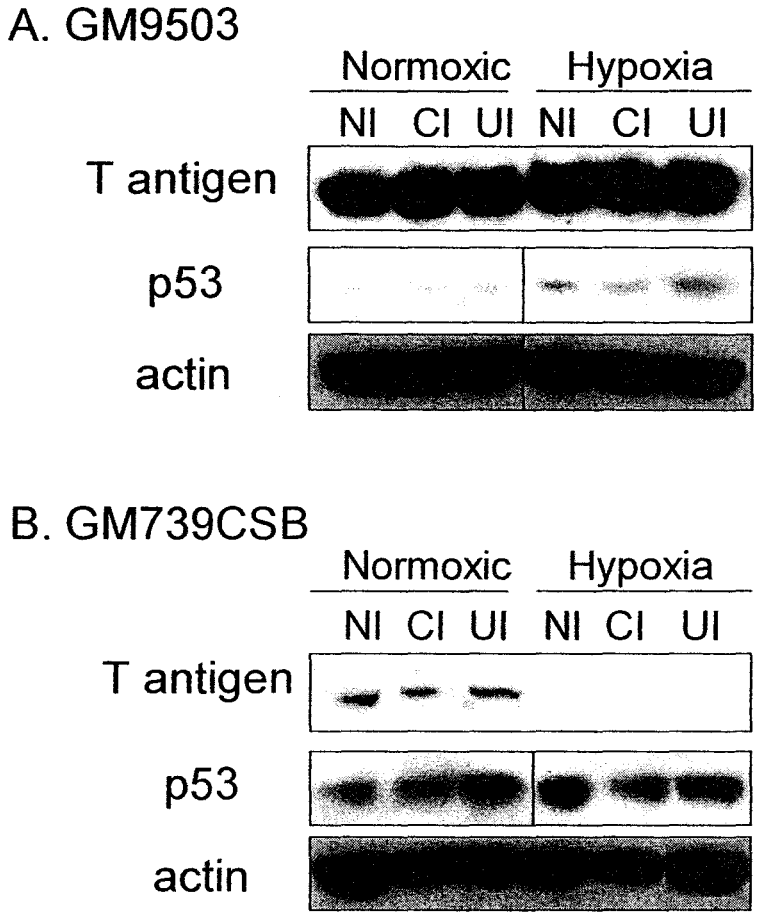


Figure B.3. Decreased T antigen expression in CSBSV40-transformed fibroblasts. Confluent normal (GM637F) (A) and CSBSV40-transformed fibroblasts (B) were either mock infected (N.I.) , infected with undamaged virus (C.I.) or infected with UV-damaged virus (U.I.) and exposed to either hypoxic or normoxic conditions. Equal amounts of complete cell lysates were run on 10% SDS-PAGE gel, blotted on a nitrocellulose filter and examined for the expression levels of T antigen using anti-T antigen antibody and p53 using anti-p53 antibody. The amount of actin was determined by using anti-actin antibody to obtain the sample loading control.

APPENDIX C

Effect of hypoxia on repair of cisplatin-induced DNA damage

Cisplatin is extensively used as an anti-cancer drug and causes inter-strand and intra-strand platinum-DNA crosslinks, which are deleterious to the cell if unrepaired. Two DNA repair pathways are responsible for repairing the lesions caused by cisplatin, NER and homologous recombination (HR) repair. Since the results presented in Chapter 5 implicate NER in response to UV-induced DNA damage of a reporter gene in human fibroblasts under hypoxic conditions, it was of interest to examine how hypoxia may affect repair of cisplatin-induced DNA damage. We examined the effect of hypoxia before and after infection on the HCR of the cisplatin-induced DNA damage in normal, CSB, XPC and XPA SV40-transformed fibroblasts 40 hours after infection. Representative graphs of HCR of the cisplatin-damaged reporter gene for primary human normal, CSB, XPC and XPA SV40-transformed fibroblasts after hypoxia exposure are shown in Figure C.1. Hypoxia treatment before and after infection did not induce a significant difference in any of the cell lines tested (Table C.1). The data suggests that NER of cisplatin-induced DNA damage is not significantly affected by hypoxia. However, hypoxia after infection only had the greatest effect on HCR of a UV-damaged reporter gene in human primary and SV40-transformed CSB cells and therefore further experiments using cisplatin as a DNA damaging agent on human fibroblasts exposed to hypoxic conditions after infection only may be an important area of future research.

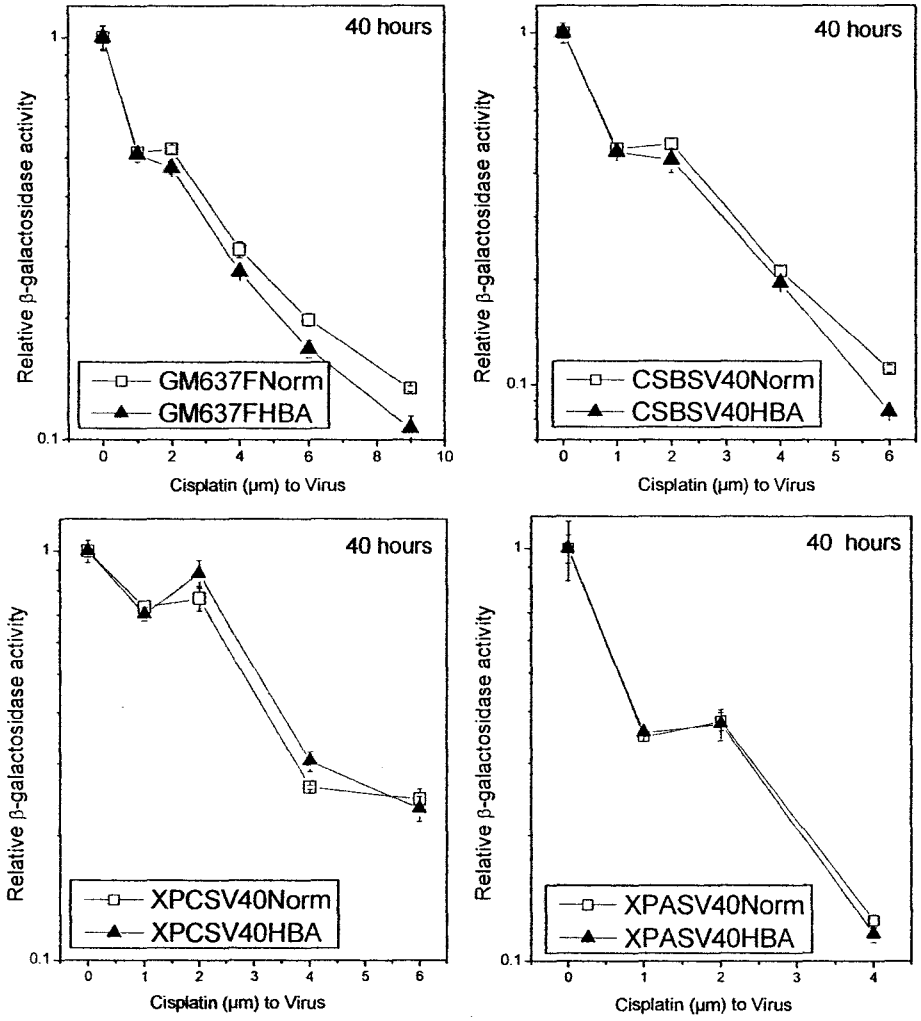


Figure C.1. Representative results showing HCR of a UV-damaged reporter gene in GM637F, CSBSV40, XPCSV40 and GM04312XPA SV40-transformed fibroblasts under standard culture conditions (Norm,□) and hypoxia before and after (HBA, ▲) infection conditions. Cells were infected with either non-treated or cisplatin-treated AdCA17*lacZ*, followed by incubation under either normoxic or hypoxic conditions before infection for 24 hours and after infection for 40 hours (total of 64 hours), after which scoring for β-gal was performed. Each point is the average of 3 replicates; error bars represent one standard error.

Table C.1. Relative D_{37} values ± S.E. of HCR of a UV-damaged reporter gene in human SV40-transformed normal (GM637F), CSBSV40, XPCSV40, and XPASV40 (GM04312XPA) fibroblasts exposed to hypoxia before (24 hours) and after infection (40 hours) (HBA) treated human SV40-transformed normal (GM637F), CSBSV40, XPCSV40, and XPASV40 (GM04312XPA).

Cell Lines	N	HBA	P Value
GM637F	4	0.9 ± 0.1	0.563
CSBSV40	3	0.9 ± 0.04	0.340
XPCSV40	2	1.3 ± 0.2	
GM04312XPA	4	1.2 ± 0.2	0.453

CHAPTER 7

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