ROLE OF p53 AND NER PROTEINS IN BER OF
METHYLENE BLUE + VISIBLE LIGHT INDUCED DNA DAMAGE

THE ROLE OF THE p53 AND NUCLEOTIDE EXCISION REPAIR PROTEINS IN
THE BASE EXCISION REPAIR OF
METHYLENE BLUE PLUS VISIBLE LIGHT INDUCED DNA DAMAGE

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ABSTRACT

The nucleotide excision repair pathway (NER) has been shown to efficiently remove bulky base lesions from the DNA, including those induced by solar light. It has been suggested that the NER pathway may be involved also in removing smaller oxidative base lesions from the DNA. Oxidative damage in the cell is caused by cellular aerobic respiration, with base damage to the nucleotides of the DNA being the most biologically relevant. One of the most common oxidative base lesions in the genome is the 7,8-dihydro-8-oxoguanine (8-oxoG). This lesion is pre-mutagenic since it can base pair with equal efficiency to the correct cytosine base, or the incorrect adenine base during DNA replication. Oxidative damage, including 8-oxoG, is repaired primarily by the base excision repair (BER) pathway, which is a multi-step, multi-protein pathway similar to NER. One key protein involved in both BER and NER is the p53 protein, which can act as a transcription factor and protein regulator to influence DNA repair. We have used a recombinant non-replicating human adenovirus, Ad5HCMVlacZ, which expresses the β-galactosidase (β-gal) reporter gene, to examine the role of several NER proteins and the p53 protein in the BER of oxidative damage in human cells. Methylene blue (MB) acts as a photosenstizer, and after irradiation by visible light (VL) produces reactive oxygen species that cause 8-OxoG in the DNA. By infecting several normal, NER deficient and p53 deficient - tumor, primary and transformed fibroblast cell lines with a MB+VL-treated Ad5HCMVlacZ reporter construct, we were able to determine the host cell reactivation (HCR) of the oxidatively damaged reporter. Results indicate that the HCR of the MB+VL-treated reporter and the expression of p53 are enhanced by UVC

pretreatment in normal human fibroblasts, suggesting that p53 may be involved in inducible BER. In addition, increased expression of p53 facilitated by pre-infection of normal cells with p53 expressing Ad5p53wt similarly enhanced HCR in the normal fibroblasts, giving further evidence that increased expression of p53 alone enhances BER. In contrast, although UVC pretreatment of p53 compromised cells resulted in enhanced HCR, the enhanced HCR did not correlate with enhanced p53 expression, suggesting that enhancement in BER can result from both p53 dependent and p53 independent mechanisms. We report also that HCR of the MB+VL-treated reporter gene was substantially reduced in SV40-transformed XP-C cells, with little or no reduction in SV40-transformed XPA, XPD, XPF, XPG and CSB cells, suggesting a role for the XPC protein in the BER of MB+VL-induced DNA damage. In particular, the XPC protein appears to be involved in both the constitutive and inducible aspects of BER, as the HCR of the MB + VL-treated reporter was reduced in 3 UVC pretreated as well as untreated XP-C primary human fibroblast strains. In addition, pre-infection of cells with Ad5p53wt, resulted in an enhanced HCR of normal but not XP-C deficient fibroblasts, consistent with a p53 dependent involvement of the XPC protein in BER of MB+VL-treated DNA. Additional studies were also conducted to determine the cell sensitivity of normal and NER deficient SV40-transformed cell lines to MB and MB + VL. The results show that MB alone and MB+VL are toxic to cells, and that cells deficient in NER are not more sensitive to MB or MB+VL compared to NER proficient normal cells. In fact the NER deficient cell lines were more resistant to MB alone compared to NER proficient normal cells. In particular, although the SV40-transformed XP-C cell line showed a

significant reduction in HCR of the MB-VL-treated reporter gene, suggesting a deficiency in the repair of MB+VL-induced DNA damage, the SV40-transformed XP-C cells were not more sensitive to MB or MB+VL. This suggests that the toxicity of human cells to MB and MB+VL results primarily from damage to cellular components other than DNA such as membrane structures including the mitochondria and lysozomes as has been reported for other photosensitizers.

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LIST OF ABBREVIATIONS

6-4 PP	6,4 Photoproduct
8-OxoG	7,8-dihydro-8-oxoguanine
AdCA17	Ad5HCMV*LacZ*
AdCA18	Ad5HCMV*Luc*
APE	Apurinic Endonuclease
AP-Site	Apurinic or Apyrimidinic nucleotide
BER	Base Excision Repair
CPD	Cyclobutane Pyrimidine Dimmer
CPRG	Chlorophenolred ß-D-galactopyranoside
CS	Cockayne Syndrome
D_{37}	Dose required to give a surviving fraction of 0.37
DNA	Deoxyribonucleic Acid
ETC	Electron Transport Chain
GGR	Global Genome Repair
HCMV-IE	Human Cytomegalovirus Immediate Early Promoter
HCR	Host Cell Reactiviation
HDM2	Human Double Minute 2 Protein
LFS	Li-Fraumeni syndrome
MB	Methylene Blue
MOI	Multiplicity of Infection
NER	Nucleotide Excision Repair
PBS	Phosphate Buffered Saline
PFU	Plaque Forming Units
Pol-ß	Polymerase Beta
RNAPII	RNA Polymerase II
ROS	Reactive Oxygen Species
SV40	Simian Virus 40
Tag	Large T-Antigen
TBS	Tris-Buffered Saline
TCR	Transcription Coupled Repair
Tg	Thymine Glycol
TTD	Trichotiodystrophy
UVR	Ultraviolet Radiation
VL	Visible Light
XP	Xeroderma Pigmentosum
a-MEM	Alpha Essential Medium
ß-gal	Beta-Galactosidase

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Chapter 1
Introduction

1. Cancer and Aging

1.a. _Oxidative damage in perspective_

Our environment is ever changing. In fact, at one time the atmosphere as we know it did not exist. It is widely accepted that the ambient environment years ago was anoxic, and gradual changes brought us to our now oxygen rich surroundings. Living organisms evolved with this change, adapting to their surroundings to be able to exploit and also to defend themselves against the abundance of oxygen that was becoming available. Although our cellular respiration is more efficient in an aerobic environment, this sword is indeed double sided. The atomic structure of an oxygen molecule is in itself benign, however upon excitation it has the potential to cause severe damage to our cells. It has been reported that approximately 10^5 oxidative lesions are generated in the human genome every day (Fraga et al, 1990). Mutations resulting from these lesions can lead to pathophysiologies such as neurodegeneration, aging and cancer (as reviewed by Guyton and Kensler, 1993, Beckman and Ames, 1998).

The leading model in carcinogenesis defines three stages: initiation, promotion and progression (as reviewed by Pitot et al, 1981 and Weinstein et al, 1984). Initiation is characterized by an alteration of the DNA sequence by oxidative or other types of damage. As these mutations accumulate, they have the potential to cause alteration in genes that control the regulation of cell proliferation. Mutations in these genes can lead to the activation of proto-oncogenes and the inactivation of tumor suppressor genes,

resulting in the next stage of carcinogenesis, promotion. Promotion involves the proliferation of the mutated cells due to the loss of tumor suppressor activity or gain in oncogene activity. This constant proliferation signal allows the cell to undergo repeated replication events, causing the cell to accumulate more genetic damage. At this point the cell mass enters the third and final stage of carcinogenesis, progression, where the cell's phenotype is transformed into a malignant form. A key indicator that oxidative damage is involved in this process is the fact that most cancers have elevated levels of oxidative damage (Malins and R Haimanot, 1991).

Aging is another aspect of cellular physiology in which oxidative damage plays a role (Beckman and Ames, 1998). One possible cause of aging is due to the shortening of telomeres, which are rich in bases that are preferentially susceptible to oxidative damage (Von Zglinicki et al, 1995). If oxidative lesions and the ensuing mutations are induced in these telomeres, they are shortened twice as fast, leading to a more rapid aging (Von Zglinicki et al, 1995)

2. DNA Damage

2.a. *Oxidative Damage*

Oxidative damage is that type of damage induced in a cell due to the toxicity of oxygen. As mammalian cells use oxygen for their respiration, the lesions caused by oxidative stress are of great importance, and it is the removal of these lesions that helps prevent carcinogenesis.

2.a.i. *Reactive Oxygen Species, and their cellular endogenous sources*

An excited form of molecular oxygen (O_2) can have a toxic effect on cellular components, such as proteins, lipids and DNA (Esterbauer et al, 1990). Molecular oxygen is unique in that it has two unpaired electrons in its outermost orbital, which in most circumstances would make the molecule extremely reactive. However, the atomic structure of the molecule allows the two unpaired electrons to possess the same spin, termed triplet state, which makes the molecule relatively stable (as reviewed by Epe, 1991). The stability of the O_2 is afforded by its distinct triplet state, which allows it only to react with other molecules of a similar state. The toxicity of the molecule occurs when it is excited by the addition of electrons or energy (as reviewed by Miller et al, 1990), which opens the door for the creation of reactive oxygen species (ROS). The most important endogenous source of ROS created by the addition of an electron is the electron transport chain (ETC) (Hanukoglu et al, 1993). By introducing an electron into the outer shell of the molecule, the stable parallel spins of the unpaired electrons are lost, and the molecule becomes very reactive. (As Reviewed by Miller et al, 1990). The ETC is a series of electron transfers which liberates usable energy for the cell. Although the ETC is very efficient, electrons do "leak" from it, interacting with oxygen to create the most common ROS, the superoxide radical ($O_2^{\bullet-}$) (Chance et al, 1979). Superoxide is created in the mitochondria, the power house of the cell, and most of its damage is induced in the mitochondrial DNA. Superoxide has the ability to directly damage the nuclear DNA, however the cell has engineered a mechanism to dismantle it before it can do so by superoxide dismutation (as reviewed by Slupphaug et al, 2003). Dismutation of

superoxide is enhanced by superoxide dismutase, and is the initiation of a multi-step pathway designed to remove it by reducing it to water. The product of dismutation is another ROS, hydrogen peroxide, (H_2O_2). The damaging potential of hydrogen peroxide is limited compared to superoxide, however it is further dismantled enzymatically by catalase to form water (H_2O) (Henle and Linn, 1997). Hydrogen peroxide, however, can follow an alternative pathway that leads to one of the most damaging ROS in the cellular system. If H_2O_2 is in the presence of transition metals such as copper and iron, it can be reduced to the hydroxyl radical ($OH^{\cdot}$), whose reactivity shadows that of superoxide and H_2O_2 (as reviewed in Loft and Poulsen, 1996, Bont et al, 2004)

The previous cascade of O_2 excitation involves the addition of an electron to the oxygen molecule creating radicals. Another way that the oxygen molecule can be excited is by the addition of energy. The major ROS that is a result of this mechanism is singlet oxygen ($^1O_2$) (Patterson et al, 1990). In contrast to the radical ROS which have an unpaired electron, the singlet oxygen molecule is similar in electron composition to the O_2 molecule. The extra energy however, causes the spins of the two unpaired electrons to become opposite. This makes the singlet oxygen molecule far more reactive than the regular oxygen molecule (Moan et al, 1979). The endogenous generation of singlet oxygen occurs primarily by the visible light excitation of photosensitizers found in the cell. The excited photosensitizer is able to transfer its energy to the O_2 molecule, switching its spins and creating singlet oxygen (as reviewed by Epe, 1991). These cascades are summarized in figure 1.

2.a.ii. *Types of oxidative damage*

Of the above ROS, the most biologically important are the hydroxyl radical and the singlet oxygen species which are able to cause damage in the bases of nuclear DNA. As superoxide is a relatively reactive and therefore short lived species, it generally exerts most of its influence within the confines of the mitochondria in which it is created (Pryor 1986). The hydroxyl radical however is able to travel outside of the mitochondria because of the less reactive hydrogen peroxide intermediate from which it is formed (as reviewed by Cadet et al, 1999.) Similarly singlet oxygen has a long half life (Patterson et al, 1990), and additionally specifically targets bases in the DNA (Cadet and Teoule, 1978). Of the sites within the cell susceptible to ROS damage, proteins and lipids are of less consequence than DNA as they are constantly generated and destroyed. The DNA however is not replaced, and therefore the integrity of the code is essential. There are many sites in the DNA which can be damaged, as an example, the sugar phosphate backbone can be broken leading to chromosomal rearrangements (Inoue and Kawanishi, 1987). The most significant damage caused by ROS in the DNA is damage to the bases in the nucleotides (Steenken and Jovanovic, 1997), which is mostly facilitated by electron or energy transfers. Damage induced by the hydroxyl radical preferentially oxidizes purines, especially guanines (Steenken and Jovanovic, 1997). It also has the capacity to oxidize the pyrimidines and create strand breaks, as well as abasic sites in the DNA (as reviewed by Valko et al, 2004). Singlet oxygen, which is not a radical, follows an oxidation mechanism distinct from the hydroxyl radical and is specific for creating predominantly oxidized purines, specifically guanines (Epe, 1988). Of the twenty known

oxidatively damaged bases, the most prevalent in the genome are the oxidized pyrimidine thymine glycol (Tg), and the oxidized purine 7,8-dihydro-8-oxoguanine (8-OxoG) (as reviewed by Slupphaug et al, 2003), which are derived from thymine and guanine bases respectively, shown in figure 2. Oxidative base damage caused by ROS can be induced in bases already incorporated in the DNA, or in bases of the cellular nucleotide triphosphate pool. If induced in the nucleotide triphosphate pool, these damaged bases can then be incorporated in the DNA during replication (as reviewed by Slaupphaug et al, 2003).

2.a.iii. *Genetic consequences of oxidative damage*

There are three consequences that oxidative base damage can have on the genetic stability of the cell: it can cause cell death, mutation, or have no effect at all (Reviewed by Wallace, 2002). If the base damage causes distortion of the DNA helix such that the DNA polymerase is stalled at the site of damage, the damage is considered to be lethal. If the cell cannot replicate its DNA, it cannot possibly divide. If, however, the oxidative base damage is non-helix distorting, and is able to pair with a base with which it usually does not pair, the oxidative damage can lead to mutation. If the damaged base still retains its ability to base pair correctly, the damage is said to have no effect. Of the two most common oxidized bases, the thymine glycol is highly helix-distorting and is lethal to the cell (Kao et al, 1993). Although it still retains its ability to base pair with an adenine base, the lethality of Tg is caused by the stalling of the DNA polymerase immediately after this pairing (Id et al, 1985). The oxidized guanine, 8-OxoG, in

contrast, is not as helix-distorting as Tg, and the DNA polymerase is thought to be able to continue with its synthesis in spite of this lesion (Wood et al, 1990). Similarly, the 8-oxoG has been shown to be a weak block in transcription, as RNA polymerases II was stalled at these lesions only 5% of the time (Larsen et al, 2004).

8-OxoGs are classified as pre-mutagenic lesions, as they are not mutations themselves but can lead to mutations (Duarte et al, 1999). The guanine base under normal circumstances base pairs exclusively with a cytosine base (Shown in figure 3a). When guanine is oxidized to 8-OxoG in the DNA, the modified base is able to pair with adenine or cytosine with equal efficiency during replication (Shown in figure 3b). If an 8-OxoG:Adenine base pair exists in the DNA, the next replication event will result in a Thymine: Adenine base pair. Thus, induction of 8-OxoG in the DNA can cause a G:C to a T:A conversion. (Shown in figure 3c). It is also of interest to note that if an 8-Oxo-GTP is incorporated from the nucleotide pool during replication, it can base pair with an adenine or a cytosine. If this occurs, it can cause a G:C → T:A, or a T:A → G:C conversion (review of the premutagenic nature of 8-OxoG; see Slupphaug et al, 2003 and Wallace, 2002).

2.a.iv. *Primary defense against Reactive Oxygen Species*

The cell has primary defense mechanisms to prevent the actions of ROS and to prevent the cytotoxic and genotoxic effects of the damage that they induce (as reviewed by Friedberg et al, 1995). There exist many enzymes that catalytically degrade ROS. For example, superoxide and hydrogen peroxide are degraded by superoxide dismutase and

catalase respectively. There also exist endogenous scavengers for singlet oxygen such as melatonin that detoxify the cell of singlet oxygen (as reviewed by Reiter, 1998). Organic substances which when ingested have antioxidant effects also exist. For example, antioxidants Vitamins A, E and C act in "mopping up" ROS before they can damage the bases (as reviewed by Valko et al, 2004). If these mechanisms fail and damage is induced in the nucleotide triphosphate pool, the cell also has mechanisms that can degrade oxidatively-damaged triphosphates into monophosphates (Sakumi et al, 1993). These monophosphates cannot be incorporated into the DNA, and thus cannot cause mutation. If, however, all of these mechanisms fail, there exists another mechanism called base excision repair that can repair oxidative damage induced in DNA bases (Slupphaug et al, 2003).

2.b.   *In-vitro Creation of DNA Damage*

2.b.i. *Methylene Blue + Visible Light Creates Oxidative Base Damage*

There are several ways available to increase the amount of ROS within the cellular environment. However, to study oxidative base damage in the DNA, it is prudent to use an *in vitro* system that is specific for causing such lesions in the DNA. As singlet oxygen has the specificity for causing base damage to guanine residues (Epe, 1988), one way to create singlet oxygen in the laboratory is to use methylene blue (MB) irradiated with visible light (VL) in an oxygen setting (Floyd et al, 1989).

Methylene blue (MB) is a dye that is normally found in its ground state. Upon being irradiated by visible light (VL), MB enters an energetically excited singlet state,

where its lone pair of electrons posses an anti-parallel spin. In an attempt to decay from this excited state, the molecule rearranges to its triplet state, where the electron spins are parallel. It is now in a form that is able to react with the triplet state of O_2. The triplet state MB can now transfer its remaining excited state energy to oxygen creating singlet oxygen, and itself returns to its ground state, shown in figure 4. It is the triplet state to triplet state reaction specificity that ensures that singlet oxygen is almost exclusively created (as reviewed by Floyd et al, 2004). This specificity of singlet oxygen creation by MB + VL has been shown also by the use of singlet oxygen scavengers. The damaging effect of MB + VL is greatly diminished when $^1O_2$ scavengers are administered after treatment, but not when other ROS scavengers are used (Floyd et al, 1989). It is for this reason that many groups use MB + VL to induce oxidative base damage, especially to guanines (Runger et al, 1995, Wong and Floyd, 1994). Methylene blue is incubated with cells or DNA to allow for the close proximity of DNA and MB. Upon excitation of MB with VL, MB in turn excites 0_2 to singlet oxygen which can then cause base damage in the DNA (Wong and Floyd, 1994).

2.c.   *Other types of DNA Damage*

2.c.i.   *Ultraviolet Radiation.*

Although oxidative base damage is one of the most biologically relevant lesions formed in DNA, other lesions found in the DNA are those induced by the ultraviolet radiation (UVR) component of solar radiation. Oxidative damage, especially that caused by the 8-OxoG, is a relatively small lesion (Wood et al, 1990), as it involves the damage

to a base of one nucleotide. UVR induced damage, on the other hand, creates bulkier damage which involves the dimerization of two adjacent pyrimidine nucleotides (Friedberg et al, 1995). In contrast to oxidative base damage, bulky damage is highly distorting to the DNA helix (Wang and Taylor, 1991), and as such cells cope with it in a different manner (see section on Nucleotide excision repair). There are two major lesions induced by UVR: the cyclobutane pyrimidine dimer (CPD) and the 6,4 photoproduct (6,4-PP), which differ in the covalent bonds that dimerize adjacent pyrimidines. (Sinah and Harris, 2002).

UVR can be divided into 3 categories: UV-A, UV-B, and UV-C, based on their wavelength and energy (de Laat et al, 1996).

Radiation Spectrum	Wavelength (nm)	
X-rays	< 100	
UV-C	100 – 290	E
UV-B	290 – 320	N E R G Y
UV-A	320 – 400	
Visible Light	> 400	
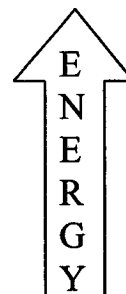
The damage spectra associated with UVR varies with these categories as well. The higher-energy UV-C and UV-B radiations are able to cause direct damage in the DNA, whereas lower-energy UV-A excites endogenous photosensitizers, resulting in more oxidative damage (as reviewed by Sinah et al, 2002). UV-B and UV-C create CPDs and 6,4-PP, while UV-B is also able to induce oxidative lesions. UV-A on the other hand creates significantly more oxidative lesions than CPDs. (Kielbassa et al, 1997). UV-C

induced damage is the easiest to create in the laboratory setting because many germicidal UV lamps emit UV-C. (UVR reviewed by Friedberg et al, 1995)

3.   Cellular Mechanisms that Repair DNA Damage

The constant generation of DNA damage either by solar radiation or ROS can have extremely detrimental effects on the genome. It is for this reason that repair pathways have evolved to facilitate removal of this damage. The Nucleotide Excision Repair (NER) pathway facilitates the removal of bulky adducts formed by UVR, while the Base Excision Repair (BER) pathway removes smaller ROS-induced damage. Each of these will be discussed.

3.a.   *Nucleotide Excision Repair*

NER is an evolutionary conserved pathway that removes bulky, DNA helix-distorting lesions from the genome.   NER's efficiency in removal of these adducts increases as the helix distorting abilities of the lesion increases. The two most prominent lesions recognized by this pathway are the CPDs and the 6,4-PP (Friedberg et al, 1995). The NER pathway can be broken down into 5 main steps: lesion recognition, incision of the DNA backbone upstream and downstream from the lesion, removal of the lesion containing oligonucleotide, resynthesis of the removed segment using the complementary strand as a template, and ligating the newly synthesized strand with the existing strand (reviewed by Hanawalt, 2002).

NER can be divided in to two distinct pathways, global genome repair (GGR) and transcription coupled repair (TCR) (reviewed by Hanawalt, P.C., 2002). GGR refers to repair of bulky damage that has been induced throughout the genome in all introns and exons. TCR refers to the repair of damage in transcribed strands of active genes, and occurs at a rate much faster than GGR (Bohr et al, 1985, Mellon et al, 1986). It is beneficial for cells to be able to quickly remove damage from DNA segments that are being transcribed; this ensures that the resulting proteins are functional. The urgency in repairing the lesions induced in the remainder of the DNA is of secondary importance as these genes are not active, and there is no immediate threat of producing damaged proteins. The main differences between GGR and TCR are found in the recognition step of the damage (Sugasawa et al, 1998). In GGR, a heterodimer, XPC-HHR23B, is responsible for the initial recognition of the bulky lesion in the DNA (reviewed by Friedberg, 2001). Another protein has also been implicated in the recognition of lesions in GGR which is another heterodimer of DDB1-DDB2, otherwise known as XPE (Hwang et al, 1999). The XPE protein is thought to recognize DNA lesions and to cause further distortion in the DNA helix to facilitate recognition by XPC-HHR23B (as reviewed by Tang and Chu, 2002). In contrast, the recognition step of TCR is a stalled RNA polymerase II (RNAPII) (Mellon and Hanawalt, 1989), which effectively allows for the preferential repair of transcribed strands seen in TCR. During transcription, the RNAPII is able to detect damage in the nucleotides that it is transcribing. If it comes across a bulky adduct such as a CPD, it will become stalled at that site. A stalled RNAPII is then able to attract other proteins such as CSA and CSB that facilitate the

mobilization of the remaining NER machinery (reviewed by Hanawalt, 2002). After recognition by XPC-HHR23B in GGR, and RNAPII – (CSA+CSB) in TCR, the remaining steps are the same for both sub-pathways of NER.   The XPA and RPA proteins are recruited to the site of DNA damage to assist in DNA unwinding around the area of damage. Unwinding of the DNA during this step is also facilitated by the XPB and XPD domains of the TFIIH protein complex, which possesses intrinsic helicase activity. Following the unwinding of the DNA in the vicinity of the damage, the oligonucleotide containing the lesion is removed from the DNA. This is accomplished by the XPG and XPF-ERCC1 proteins that incise the DNA 3' and 5' to the damage respectively. The endonuclease activities of these proteins allows them to remove an oligonucleotide of about 24 -32 nucleotides in length from the damaged strand of the DNA. Once the DNA oligonucleotide is removed, DNA polymerases d and e fill in the gap created by the endonucleases, using the undamaged strand of the DNA as a template. The final step in the NER pathway is the ligation of the newly synthesized strand with the existing strand by DNA ligase 1. Figure 5 depicts these proteins as they apply to the NER pathway. (NER is reviewed by Friedberg et al, 1995, Cleaver, J.E., 1999, and Hanawalt, 2002)

3.a.i. *Deficiencies in Nucleotide Excision Repair*

The importance of the NER pathway in genomic stability is evident in those individuals who have a deficiency in one or more of the proteins discussed above. A mutation in the genes encoding any of the XPA-XPG proteins can lead to a disorder

called Xeroderma Pigmentosum (XP) (reviewed in Lehmann et al, 2003, Cleaver et al, 1999). There are seven patient groups associated with deficient NER ranging from XPA-XPG, one for every XP protein involved in NER. It is important to understand that the mutation in the XP gene affected differs within individuals of that group. For example, all XPC patients do not have the same mutation in their XPC gene. These different mutations, however, all lead to a deficiency in NER. There also exist within the population of seemingly normal individuals who have polymorphisms in certain XP genes. Although these patients have XP genes that vary in genetic make-up compared to the rest of the population, they still do not exhibit NER deficiencies or XP symptoms (Blankenburg et al, 1995).

The clinical symptoms of XP include extreme sun sensitivity and a thousand times increase in the incidence of skin cancer (Kraemer, 1997). XP individuals of different groups show different severities of these clinical symptoms. For example, XPA is involved in both steps of NER: TCR and GGR, and is also responsible for recruitment of other NER proteins (reviewed in Hanawalt, 2002). It follows that XPA individuals with a defective XPA protein will show severe XP clinical symptoms. There exist another set of clinical symptoms that have been associated with a mutation in the XPD and potentially XPB proteins called Trichotiodystrophy (TTD). Individuals with this disorder exhibit mental retardation, unusual facial features, photosenstitivity and brittle hair (reviewed by Lehmann, 2003). Table 1 shows the relative severities of clinical symptoms of the XP patients, along with their roles in NER.

Another disorder which stems from a defect in NER is the Cockayne Syndrome (CS), where patients have a mutated CSA or CSB protein (reviewed in Lehmann et al, 2003, Cleaver et al, 1999). There are two groups of CS: CSA and CSB, depending on which protein is mutated. Although these individuals show increased sun sensitivity, they do not have the corresponding higher incidence of cancer. These individuals are, however, more likely to suffer from neurodegeneration caused by neuronal atrophy and demyelination (reviewed in Boer and Hoeijmakers, 2000). Table 1 shows the relative severity of clinical symptoms in these CS patients, along with their roles in NER. It is interesting to note that CS patients show neuronal death caused by unrepaired DNA damage, even though neurons are not exposed to solar light. Similarly, some XPB, XPD and XPG patients show this neurodegeneration as well. It has been suggested that the DNA damage induced in neurons *in vivo* is not caused by CPDs from UV light, but by oxidative damage induced by endogenous ROS (Reardon et al, 1997, and reviewed by Boer and Hoejimakers et al, 2000). It is therefore very probable that the CSA, CSB, XPB, XPD and XPG proteins my not only be involved in NER, but also in other oxidative repair mechanisms. Table 1 also shows the potential role for these proteins in the Base Excision Repair pathway, which is known to repair oxidative damage, and will be discussed next.

3.b. *Base Excision Repair*

The Base Excision Repair (BER) pathway is responsible for the removal and repair of oxidatively damaged bases in the DNA (as reviewed by Slupphaug et al, 2003,

Evans et al, 2004).   Although this pathway has not been studied in as much detail as the

NER pathway, the basic mechanism has been elucidated.   There are four distinct steps in

BER: detection and removal of the damaged base, nicking and removal of the DNA

backbone upstream and downstream of the lesion, filling in the gap created by the

previous step using the opposite strand as a template, and finally ligation of the new DNA

strand to the old one (reviewed in Slupphaug et al, 2003, Dizdaroglu, 2005).   Upon the

induction of an oxidative lesion such as the 8-OxoG in the DNA, the BER pathway is

initiated.   Detection of the base damage is facilitated by a group of enzymes called

glycosylases (reviewed by Krokan et al, 1997).   There are many different types of

glycosylases, some of which are able to recognize specific oxidative lesions, and others

that can recognize a broad range of oxidative lesions.   The 8-OxoG, for example, is

recognized by the hOGG1 glycosylase which has specificity for this type of lesion when

it is base pared with cytosine and thymine, whereas hOGG2 recognizes 8-OxoG when it

is paired with adenine or guanine (Hazra et al, 2001). Upon detection of the lesion, the

glycosylase enzymatically removes the base from the DNA, generating a nucleotide in

the DNA which lacks its nitrogenous base, called an AP site.   Prior to the glycosylase

departing from the DNA strand it may also nick the DNA 3' to the AP-Site. If this is the

case, the glycosylase is said to have a bifunctional activity because it performs two

hydrolytic functions.   Once the glycosylase activity has been completed, APE1, an

endonuclease, is recruited to the scene where it nicks the DNA on the 5' side of the AP

site.   This allows for the removal of the abasic nucleotide from the DNA.   The gap

created by APE1 is then filled by DNA polymerase ß (Pol- ß) and is joined together with

the existing DNA by ligases. The above mechanism results in the removal of only the oxidatively damaged nucleotide, and is termed short patch BER. This is shown in figure 6a. Alternatively, the oxidative lesion can be removed by long patch BER that removes a chain of 2-8 nucleotides that also contains the damaged nucleotide. Whether long patch or short patch repair will predominant is dictated by the functionality of the glycosylase. Bifunction glycosylases tend to direct the repair via the short patch repair, where as monofunctional glycosylases, those able only to excise the damaged base, shuttle the process through long patch repair. As 8-OxoG is removed by hOGG1 and hOGG2 (Rykhlevskaya and Kuznetsova, 2000), which are both bifunctional glycosylases, any discussion of BER from now will refer to short patch BER. (BER pathway is reviewed in Evans et al, 2004, and Slauphag et al, 2003)

3.b.i. *Existence of sub-pathways in BER*

Like NER, BER can be split into 2 distinct pathways: GGR and TCR. That is, it has been shown that oxidative base damage is preferentially repaired at a faster rate in the transcribed strand of active genes compared to non transcribed strands (Le Page et al, 2000, Cooper et al, 1997). Intriguingly, the proteins that facilitate TCR in NER are similar to those which are hypothesized to function in TCR of BER. It has been shown that an 8-OxoG lesion in the DNA can cause the stalling of the RNAPII as in NER (Lepage et al, 2000, Pastoriza et al, 2003). Whether it is actually distortion caused by the 8-OxoG, or the 8-OxoG associated with detection proteins, is yet to be determined. It has been hypothesized by Lepage et al, 2001, that once the RNAPII is stalled, it recruits CSB

18

which in turn recruits XPG and TFIIH (Lepage et al, 2000). The assembly of these three proteins further recruits the hOGG1 protein and the BER pathway then continues as described in figure 6a. It is therefore the stalled RNAPII that signals the existence of damage in the transcribed gene, and facilitates its rapid detection and repair. This sequence of events is described in figure 6b (Lepage et al, 2000). The rate of removal in the remainder of the genome is thought to proceed slower because it lacks the early detection mechanism afforded by the stalled RNAPII.

As yet, there are no known exclusive BER deficient syndromes (Lepage et al, 2000). However, with the involvement of CSB and XPG in the TCR of 8-OxoG, it indicates potential overlap of the NER and BER pathways.

4.   The role of p53

The p53 protein has been described as the "traffic cop at the cross roads of DNA repair" (Sengupta et al, 2005), as it is involved in many cellular processes that protects the integrity of the DNA (reviewed by Adimoolam and Ford, 2003). The protein p53 is the most commonly mutated gene in human cancers (Hollstein et al, 1991), emphasizing its role in the prevention of this disease. There are three major roles that p53 plays in the cell: it is able to induce cell cycle arrest after DNA damage to allow for repair, it can aid in DNA repair, and if the damage is too extensive it can induce apoptosis (reviewed in Adimoolam et al, 2003, Sengupta et al, 2005 and Ford, 2005).

There are several ways that p53 fulfills its role in the cell; p53 can detect DNA damage, it can bind to other proteins, such as repair proteins, and regulate them, or it can

physically bind to the DNA and act as a transcription factor (reviewed by Sengupta and Harris, 2005). When damage is induced in the DNA, the p53 protein is modified through phosphorylation events that serve to activate p53, and prolong its half life within the cell (Latonen, L., et al, 2001, reviewed by Oren et al, 1999). Once activated, p53 can bind to the promoter regions of more than one hundred genes, regulating their transcription (Zhao et al, 2000). Some of the genes that are transcriptionally activated by p53 are cell cycle check point genes, genes involved in the induction of apoptosis, and genes important for DNA repair (as reviewed by Sengupta et al, 2005).

4.a.   *The Role of p53 in Nucleotide Excision Repair*

Human cells that do not have properly functioning p53 are unable to efficiently remove UV-induced lesions such as CPDs, potentially implicating p53 in NER (Ford and Hanawalt, 1995). After investigation, it was found that p53 has a role in the regulation of GGR of NER, however its role in TCR of NER is controversial (Ford and Hanawalt, 1995). The primary role of p53 upon being activated following DNA damage is to act as a transcription factor (Zhao et al, 2000), and it is with this specificity that p53 is involved in the regulation of GGR. The activated p53 protein binds to the promoter regions of two genes specifically involved in GGR, the XPC (Adimoolam and Ford, 2002) gene and a subunit of the XPE gene (Hwang et al, 1999), and upregulates their expression. As both the XPE and XPC proteins are involved in the lesion recognition step of NER, the role of p53 in GGR is suspected to predominate in this step. Another role that p53 plays in NER is to regulate the function of proteins by physically interacting with them (reviewed in

Adimoolam, 2003). The p53 protein is able to bind to the XPB and XPD domains of TFIIH and regulate their helicase activity (Wang et al, 1995). In so doing, p53 expedites the unwinding of the DNA near the UV-induced damage, allowing easier access for the rest of the repair machinery to the site of damage (Wang et al, 1995). Figure 7 shows the role of p53 in NER (Sengupta et al, 2005).

Although the role of p53 in GGR is established, its role in TCR of NER is still controversial. It would be reasonable to expect that p53 has a role in TCR as p53 has been shown to regulate XPD and XPB (Wang et al, 1995). However, it is possible that there is domain specificity for these proteins in TCR and GGR. Other than this, there is little evidence of p53 enhancing TCR of UVC damage, although there have been reports of p53 enhancement for TCR of CPDs following UVB damage (Therrien et al, 1999). The reason for this difference could be the fact that UVB radiation causes CPDs as well as oxidative damage, where as UVC is specific for CPDs.

4.b.   *The effect of UVC on p53 expression*

Irradiation of cells with UVC induces CPD lesions in the DNA of cells, which initiates the NER pathway. This DNA damage causes a concommittal increase in the amount of p53 by prolonging its life in the cell (reviewed by Oren, 1999). The p53 protein is a known transcription factor for many genes and involved in many processes. One of the upregulated transcriptional targets of p53 is a protein called the Human Double Minute 2 Protein (HDM2) (Barak et al, 1993). This protein facilitates the degradation of p53 by marking it for degradation via the ubiquitin-proteosome pathway,

creating an autoregulatory loop for p53 (Solomini et al, 2002), which is shown in figure 8a. UV-induced DNA damage, along with invoking DNA repair, also initiates a cascade of kinases that ultimately causes the N-terminal phosphorylation of p53 (Latonen, et al, 2001). This phosphorylation ultimately inhibits ubiquitination of p53 by preventing HDM2 from binding to it, leading to the accumulation of stable p53 within the cell (Sheih et al, 1997, Siliciano et al, 1997), shown in figure 8b. This effect of increasing the amount of p53 within the cellular environment is a dose-dependent phenomenon, with too high doses of UVC invoking the p53 induced apoptosis of the cell (Latonen et al, 2001). It was found that low doses of UVC, in the range of 10 J/m^2, actually lead to quick accumulation of p53 which induced cell cycle arrest and DNA repair (Latonen, et al, 2001).

4.c.   _Li Fraumeni Syndrome_

In the late 1970s, two researchers by the names of Frederick Li and Joseph Fraumeni noticed that there existed individuals in the population who had a higher incidences of sarcoma cancers at an earlier age than normal (Li and Fraumeni, 1969). This came to be known as Li-Fraumeni syndrome (LFS), and is characterized by a predisposition to several different types of cancers, from breast to pancreatic. The cellular physiology was determined to be an autosomal dominant germline mutation in one allele of the p53 gene (Li and Fraumeni, 1969), and to date, approximately 250 such mutations have been found (Varley, 2003). The implications of an aberrant p53 protein

are obvious in light of the above discussion. In brief, these individuals have an impaired capacity to detect and repair DNA damage (reviewed in Malkin, 2001).

In the laboratory setting, primary cells have a defined life span, at which point they stop growing and begin to senesce. There are several different strategies to transform a cell so that it can grow for an undefined length of time, so called immortalization. LFS cells undergo such a transformation after an extended period of cell culturing, though the mechanism of immortalization is not fully understood (Bischoff et al, 1990). In two specific cell lines, immortalization results in either the loss or mutation of the remaining wild type p53 allele, resulting in a cell with no functional p53 (Gollahon, L.S. et al, 1998). It is important to note that the cells may still express the p53 protein, but the protein itself is mutated.

4.d.   *Simian Virus 40 and Papilloma virus transformation*

Other *in vitro* mechanisms for reducing the amount of p53 in a cell have also been established. One potential way to reduce the amount of functional p53 in a cell is to infect it with either the Simian 40 virus (SV40) (Lane and Crawford, 1979), or the Papilloma Virus (reviewed by Mantovani and Banks, 1999). Upon infection with these viruses, a plethora of gene products are expressed. For the SV40 virus, the important protein expressed is the large T antigen (TAg), whereas for the papilloma virus the E6 protein is of interest. Once expressed in the host cell, these proteins bind to and abrogate endogenous p53, causing it to lose its tumor suppressor functions, and to be degraded by the proteosomes (as reviewed by Pipas, 2001). The SV40 large T antigen also binds and

abrogates the pRb protein in cells, which causes the cell to enter the cell cycle. This entry into the cell cycle and the loss of tumour suppressor activity due to abrogated p53 in SV40 transformed cells is sufficient enough to cause these cells to become immortalized (as reviewed by Pipas, 2001).

Although it has been considered that all the p53 in cells infected with SV40 or Papilloma viruses are abrogated or degraded, this is not the case. It has been shown that SV40-transformed cells still posses functional p53 that is unbound by the large T antigen (O'Neil, et al, 1997) In addition, some SV40 cell lines had p53 present in higher amounts than untransformed counterparts (Sladek et al, 2000), sometimes up to two hundred times as much. Other groups have also reported that the accumulation of p53 after DNA damage such as UV is still intact after SV40 and Papilloma transformation (Hess, 1997). This suggests that even though the p53 status of these cells is diminished, they still retain some p53 functions.

5.   The Role of NER and p53 proteins in the BER Pathway

The existence of XP individuals who show CS type phenotypes has uncovered a possible link between NER proteins and the BER pathway (Reardon et al, 1997). These so called XP/CS individuals all have neurodegeneration resulting from neuronal atrophy. As neurons are not exposed to solar light, lack of repair of CPDs due to deficiencies in NER cannot be the reason for this neuronal atrophy. It has therefore been proposed that oxidative lesions are the lesions that remain unrepaired in these cells, thus suggesting that

NER proteins may play a role in the repair of oxidative damage (as reviewed by Hoejimakers, 2000).

Role of NER proteins in BER

For many years research mostly focused on the repair of UV-induced damage, however as more evidence points to the carcinogenic tendencies of oxidative damage, research is now shifting to investigate the repair of such damage. Cooper et al have shown that the TCR of Tg is normal in patients from XPA, XPF, and XPG, but not from individuals from XPG/CS (Cooper et al, 1997). It has been shown that the XPG/CS individuals express a truncated XPG protein, while the XPG individuals have a protein that harbours a point mutation (Nouspikel et al, 1997). This suggests that different domains of the XPG protein are involved in NER compared to BER, and a point mutation may be sufficient to incapacitate NER, but not BER (Cooper et al, 1997). Leadon and Cooper also found that the CSA and CSB patients were deficient in TCR of oxidative damage (Tg) induced by ? radiation, however their capacity for GGR was unaffected (Leadon and Cooper, 1993). This suggests a role for the CSA and CSB proteins in TCR, but not GGR of oxidative damage (Cooper et al, 1997). In addition, it has been shown by Le Page et al that along with CSB and XPG, the XPD and XPB proteins are also involved in the TCR of oxidative damage ( ) induced by x-ray irradiation, while only the XPG protein is involved in GGR. It was shown that classical XPG, XPD and XPB individuals had normal 8-oxoG repair abilities, but it was the XP/CS phenotypes that were deficient in this repair (Le Page et al, 2000). The roles of XPG and CSB have been well

documented (Cooper et al, 1997, Le Page et al, 2000), but there is also evidence for a role of the XPC protein in the repair of oxidative damage. It has been shown by Runger et al that three out of four XPC cell lines used were deficient in the repair of MB + VL induced DNA damage (Runger et al, 1995). The cell lines used were all derived from classical XP patients, as the XP/CS phenotype is not seen in XPC individuals. Shimizu et al added to this finding by reporting that XPC physically interacts with a DNA glycosylase that recognizes oxidative damage in the DNA (Shimizu et al, 2003). The binding of XPC to the glycosylase also increases its activity which suggests a role for XPC in the recognition step of BER. A number of other groups have also indicated that XP proteins play a role in BER. These roles include: the existence of a secondary pathway for GGR of oxidative damage that involves the CSB protein (Osterod, et al, 2002, Tuo et al, 2003); the possible involvement of the XPG protein from pseudo-classical XP in BER (Francis, et al, 2000); and the prospective involvement of the XPF-ERCC1 protein in the BER of 8-OxoG (Hsia, K.T., et al, 2003). It is extremely interesting to note that the potential roles these proteins may play in BER are very similar to their roles in NER, further emphasizing some overlap of the two repair pathways.

5.b.   *Role of p53 in BER*

One role of p53 in NER is its role as a transcription factor which facilitates the expression of proteins required for NER (Zhao et al, 2000). It has been shown that BER capacity was better in cells with normal rather than mutant p53 (Offer, H. et al, 2002). As for the mechanism in BER, p53 appears to have a more important role in protein -

protein interactions than transcription (reviewed by Sengupta et al, 1995).   The p53

protein has been shown to physically interact with two major proteins in the initiation

step of the BER pathway, the hOGG1 and APE1 proteins (Achanta, et al, 2002).

Functionally, p53 was also shown to increase the rate at which these two proteins

removed 8-OxoG from the DNA (Achanta, et al, 2004).   The p53 protein has also been

shown to interact with other glycosylases, such as the 3-methyladenine DNA glycosylase

(Zurer et al, 2004). The role of p53 in the gap-filling step of BER has also been shown,

where p53 modulates the activity of Pol-ß by stabilizing it and helping it bind to the DNA

(Seo et al, 2002).   Although p53 appeared not to act as a transcription factor for

polymerase ß (Seo et al, 2002), this does not mean that the transcription activation

function of p53 is not involved in BER.   In NER, activated p53 acts as a transcription

factor for the XPC gene. As XPC may also be involved in recognizing oxidative damage

(Shimizu et al, 2003), its activity in BER may depend on the transcriptional activity of

p53 as well. Figure 9 shows the potential roles of p53 in the BER pathway. It has also

been shown that oxidative stress can upregulate Pol-ß levels, as well as those of p53,

indicating a possible link between the two (Cabelof et al, 2002). As the research in this

area progresses, more links between p53 and BER may be discovered.

6.   Use of Recombinant Adenoviruses as probes for DNA Repair

6.a.i *Recombinant Adenoviruses Vectors*

One of the most promising possibilities for cancer treatment is the use of gene

therapy. Gene therapy uses viruses to introduce genes into the cell, and the cell then uses

its own transcription and translation machinery to express the genes (Stewart et al, 1999). In this way, the virus is used as a vector to introduce new, potentially cancer fighting genes into the cell. Adenoviruses are ideal for this situation as they are able to infect mammalian cells with great efficiency (Stewart et al, 1999). The adenovirus genome is made up of many genes that help the virus replicate in mammalian cells, and the gene of interest in making recombinant adenoviruses is the E1 gene (Bett *et al*, 1994). If the E1 gene is deleted from the virus, the virus can still infect cells, however it will be unable to replicate itself. This loss of replication activity is important in gene therapy. If viral constructs were able to replicate, treatment in patients would lead to viral infections (reviewed by Lai et al, 2002). In introducing the new gene into the virus, a few considerations must be undertaken. Firstly, the location of the new gene must be decided. In the case of non-replicating adenoviruses, the location that is used is the site of the deleted E1 gene (Addison et al, 1997). The next consideration is initially how to make the cell transcribe the new gene, and secondly, transcribe it at a high level. This is accomplished by incorporating a promoter into the virus that will induce the host cell to express the gene. The most commonly used promoter is one derived from the human cytomegalovirus called the Immediate Early (HCMV-IE) promoter. The HCMV-IE promoter has been shown to induce high levels of gene expression in mammalian cells (Boshart et al, 1985). A diagrammatic view of a recombinant adenovirus and its genome is shown in figure 10.

Some of the recombinant vectors that have been created are ones that have genes that are easily detected once infected in cells. For example, to test the HCMV-IE

capacity to induce expression, the *E-Coli* ß-galactosidase (ß-gal) gene has been incorporated into the adenovirus, creating the AdHCMV*lacz* (AdCA17) recombinant virus (Addison et al, 1997). This gene, when expressed in cells, is easily detected as the protein it encodes can cause a colour change when the appropriate substrate is added. This color change ability is not usually present in cells. Another easily incorporated gene in the adenovirus is the firefly luciferase gene (AdCA18), which can be assayed in a similar manner as the ß-gal gene product (Gainer et al, 1996). Other recombinant viruses more pertinent to gene therapy encode the p53 virus, also under the control of the HCMV-IE gene (Bacchetti and Graham, 1993). In this case, when the cells are infected with the recombinant virus, high levels of p53 are expressed in the cell.

6.a.ii *Host Cell Reactivation of Recombinant Adenoviruses as probes for DNA repair ability*.

Methods for determining DNA repair capacity of cells include damaging the DNA by treating whole cells with DNA damaging agents, and assaying for their repair ability (as reviewed by Friedberg et al, 1995). Treating whole cells however also damages other parts of the cell, and activates DNA repair processes (Francis and Rainbow, 2000). It is therefore difficult to elucidate if DNA repair is being assayed, and if the repair responsible is constitutive or inducible (Francis and Rainbow, 1999).

Another method for determining cellular repair capacity is by introducing damaged reporter genes into cells. Cells are then investigated for repair of this damage by their ability to reactivate the reporter gene and express the protein it encodes, the so

called host cell reactivation (HCR) assay (reviewed in Rainbow et al, 1995). One popularly used DNA damaging agent is UV-irradiation, which creates CPDs in the template strand of the reporter gene stalling the progression of RNAPII (Donahue et al, 1994). Gene expression from the reporter gene can be eliminated by a single CPD, and as such reporter activity can only occur if transcription blocking lesions are removed from the transcribed strand by the host cell. One advantage to using a damaged reporter gene constructs is that the cells and the reporter gene can be treated separately, allowing for the investigation of constitutive and inducible repair (reviewed in Rainbow et al, 1995).

HCR assays can use DNA-damaged plasmid borne reporter constructs that are transfected into cells (Runger et al, 1995, Ganesan and Hanawalt, 1994), however, cellular uptake of exogenous DNA is low (Hoeijmakers et al, 1987, Canaani et al, 1986), and the plasmid transfection process is thought to initiate DNA repair mechanisms (Renzing and Lane, 1995). This may influence results of DNA repair experiments. Another popular reporter construct used is the recombinant non-replicating adenovirus reporter (Lee et al, 2004, Francis and Rainbow, 2000). The theory behind HCR of this construct is the same; however cells are infected with the recombinant adenovirus, such as the AdCA17 which expresses ß-gal (Addison et al, 1997). The AdCA17 is treated with a DNA damaging agent, and then infected into the cell of interest. Expression of the ß-gal protein is only expected to occur if the host cell in which it is infected is able to repair or bypass the damage that has been caused in the reporter gene. The recombinant adenovirus HCR method is the one that we have employed in our assays, whose protocol has been reported previously (Francis and Rainbow, 1999).

The use of a recombinant adenovirus has been used previously to show that the HCR of a UV-damaged reporter gene is reduced in several XP and CS cell strains compared to normal (McKay and Rainbow, 1997, Francis and Rainbow, 1999). As UV-induced damage is thought to be repaired by NER, these results are consistent with the role of XP and CS proteins in NER. Similarly, plasmid borne reporter genes have reported decreased HCR for expression of a MB + VL damaged reporter gene in NER-deficient cells XPC cells, suggesting a role for this protein in the BER of MB + VL induced damage.

7.   Project Introduction

Several groups have suggested a role of p53 and NER proteins in BER. Reports also suggest that BER is inducible. It was therefore of interest to investigate a role for p53 and NER proteins in the repair of oxidative DNA damage induced by MB + VL. MB + VL produces predominantly 8-OxoG, which can be repaired by the BER pathway. This project has examined HCR of a MB + VL damaged reporter gene in several NER deficient cells, as well as cells with compromised p53 status. By assaying HCR ability of this MB + VL damaged reporter in non-UVC treated cells, we examined the role of p53 and several NER proteins in constitutive BER. We also examined the HCR of the MB + VL damaged reporter in cells pre-treated with UVC, and were able to examine the inducibility of BER, as well as the role of p53 and several NER proteins in inducible BER.

The NER deficient cell lines included primary as well as SV40 transformed human fibroblasts from groups: XPA, XPC, XPD, XPG, XPF, and CSB. The p53 compromised cells included SV40 and papilloma transformed cells, as well as spontaneously immortalized LFS cells and tumor SKOV-3 cells. The reporter construct used was the recombinant adenovirus vector expressing the ß-gal gene (AdCA17).

$$^1O_2 \qquad\qquad OH^\bullet$$

Energy transfer from
excited cellular
photosensitizers

$Cu^{2+}, Fe^{2+} \longrightarrow$

$$O_2 \longrightarrow O_2^{\bullet-} \longrightarrow H_2O_2 \longrightarrow H_2O$$

Transfer of an e^-
from the electron
transport chain

Degradation by
Superoxide
Dismutase

Degradation
by Catalase
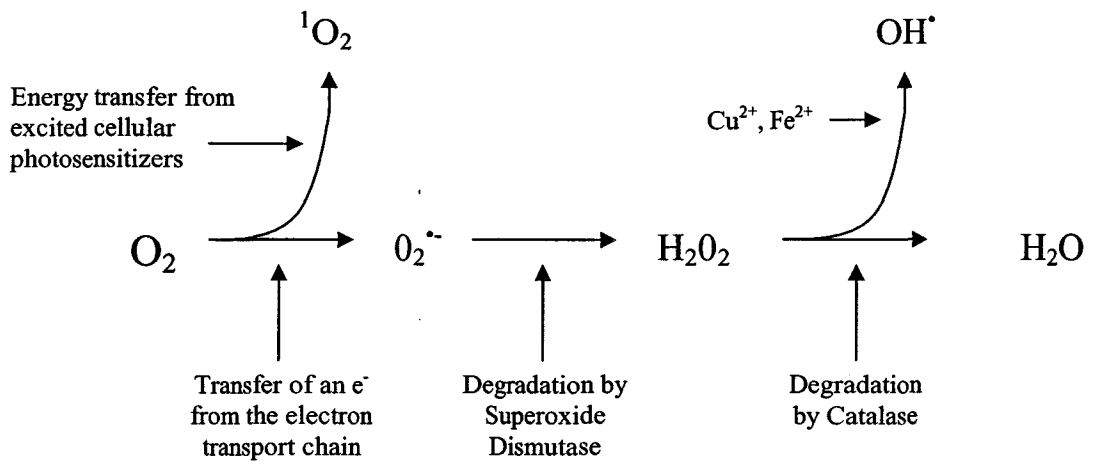
Figure 1: The various excitation cascades of molecular oxygen via electron transfer of energy transfer, creating the most common Reactive Oxygen Species

Thymine glycol   8-oxoguanine
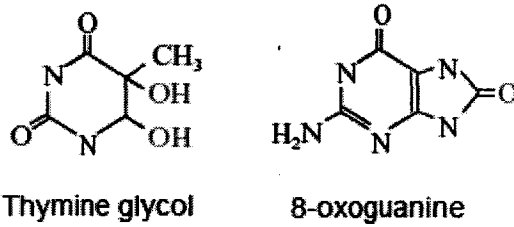
Figure 2: The most prominent oxidized purine
and pyrimidine bases in the DNA (Adapted from
Slupphaug et al, 2004)

A

B

Guanine : Cytosine Base Pair

8-OxoG : Cytosine Base Pair

8-OxoG :Adenine Base Pair

C

8-OxoGTP
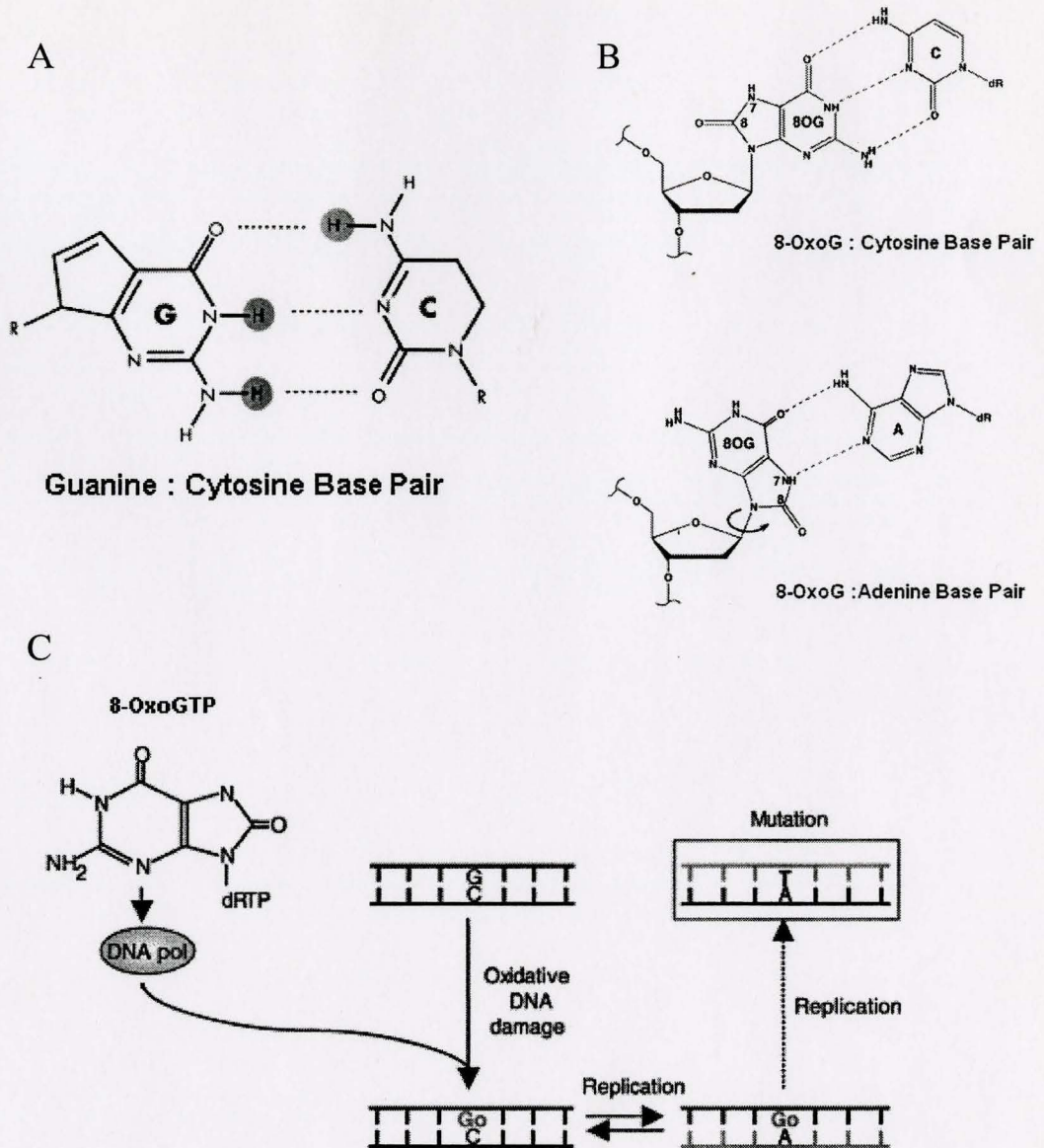
Figure 3: *A*. Guanine base paired with a cytosine base. *B*. A 7,8-dihydro-8-oxoguanine showing its base pairing abilities with cytosine and adenine. (Adapted from Krahn et al, 2003). *C*. The mutagenic potential of 8-OxoG after several rounds of replication. After the first round of replication, 8-OxoG is base paired with Adenine, following the next round of replication the original G:C base pair has been turned in to a T:A base pair (Adapted from Slupphaug et al, 2003)
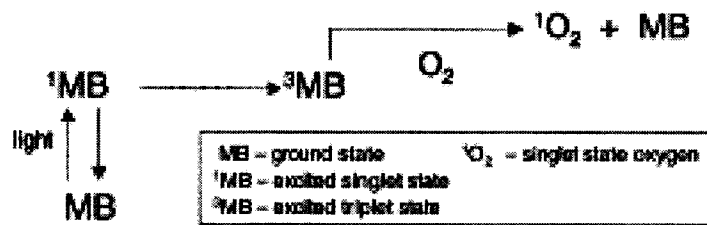
Figure 4: Basic Photochemistry of Methylene Blue and Visible   Light.
.dapted from Floyd et al. 2004)
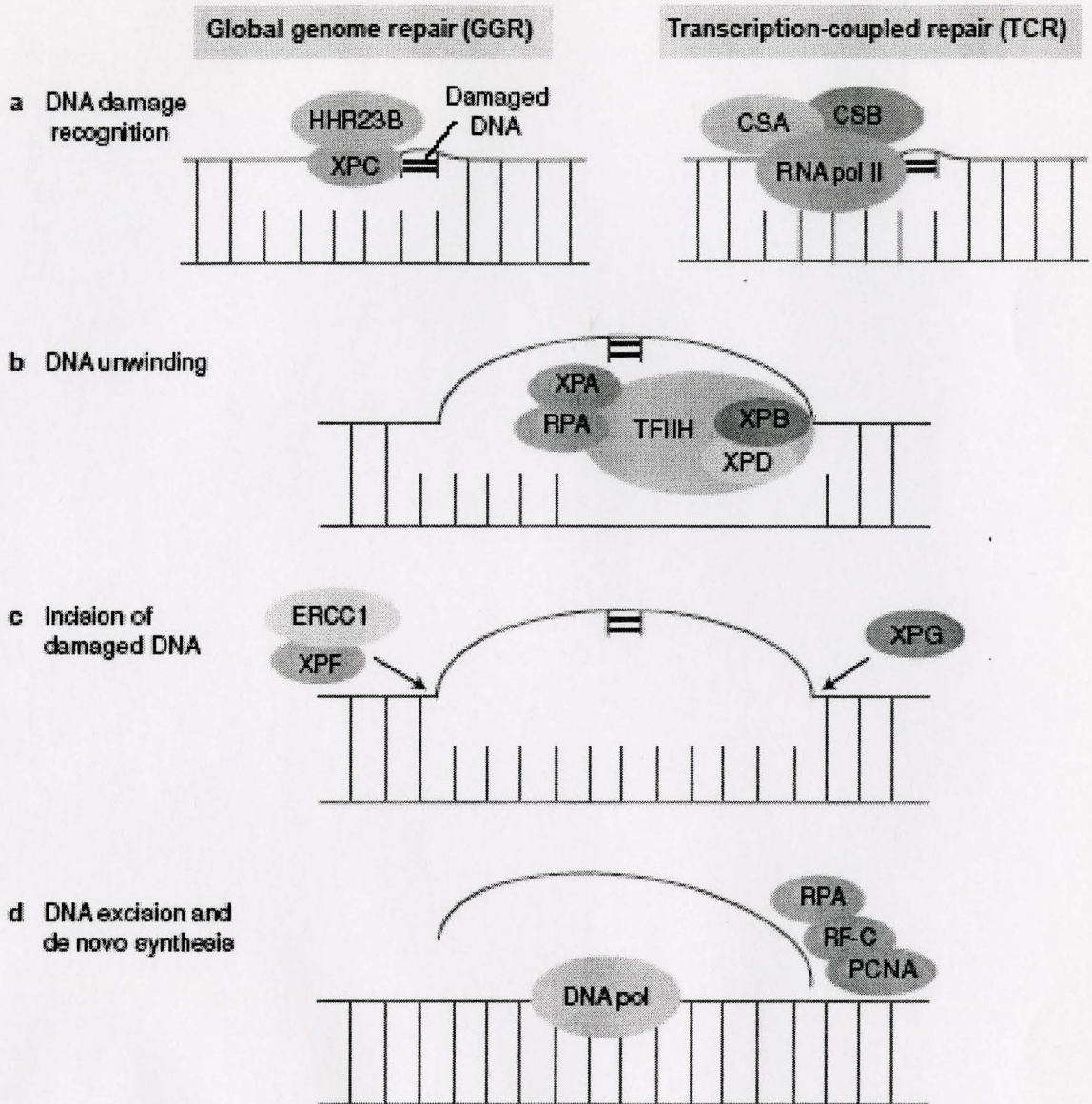
Figure 5: The proteins involved in mammalian nucleotide excision repair pathway. Recognition occurs by XPC-HHR23B in TCR, and by a stalled RNA Polymerase II that recruits CSA and CSB in GGR. XPA, XPB and XPD are recruited next and unwind the DNA in the vicinity of the damage using their helicase activity. Incision of DNA strand that contains the damage is next by XPF and XPG, and finally DNA polymerase fills in the gap. DNA ligase follows and joins the new strand with the old. (Matsumura et. al, 2002)

Table 1: Review of the XP and CS syndromes, and their involvement in NER and severity of clinical symptoms (Adapted from Francis and Rainbow, 2000, Hoeijimakers et al. 2000).

Group	Severity Of Symptoms ¥	NER			BER		Function
		6-4 PP	CPD		TCR	GGR	
			TCR	GGR			
XP-A	Severe	-	-	-	+/-	+	damage recognition interacts with TFIIH, XP-F
XP-B	Severe	-	-	-	+	+	3'→5' helicase in TFIIH promoter clearance
XP-C	Intermediate	-	+	-	+	+	damage recognition (GGR)
XP-D	Severe	-	-	-	+	+	5'→3' helicase in TFIIH promoter clearance
XP-E	Mild	slow	+	-	+	+	damage recognition (GGR)
XP-F	Intermediate	-	-	-	+	+	5' incision endonuclease damage recognition?
XP-G	Severe	-	-	-	+/-	+	3' incision endonuclease open complex stabilizer
CS-A	Intermediate	+	-	+	-	+	RNA synthesis recovery
CS-B	Intermediate	+	-	+	-	+	RNA synthesis recovery Transcript elongation

+ = no evidence of repair defect   ? = has not been examined
- = significantly impaired repair capacity   +/- = inconclusive results
¥ Symptoms include sun sensitivity, increase cancer risk, and neurodegeneration.
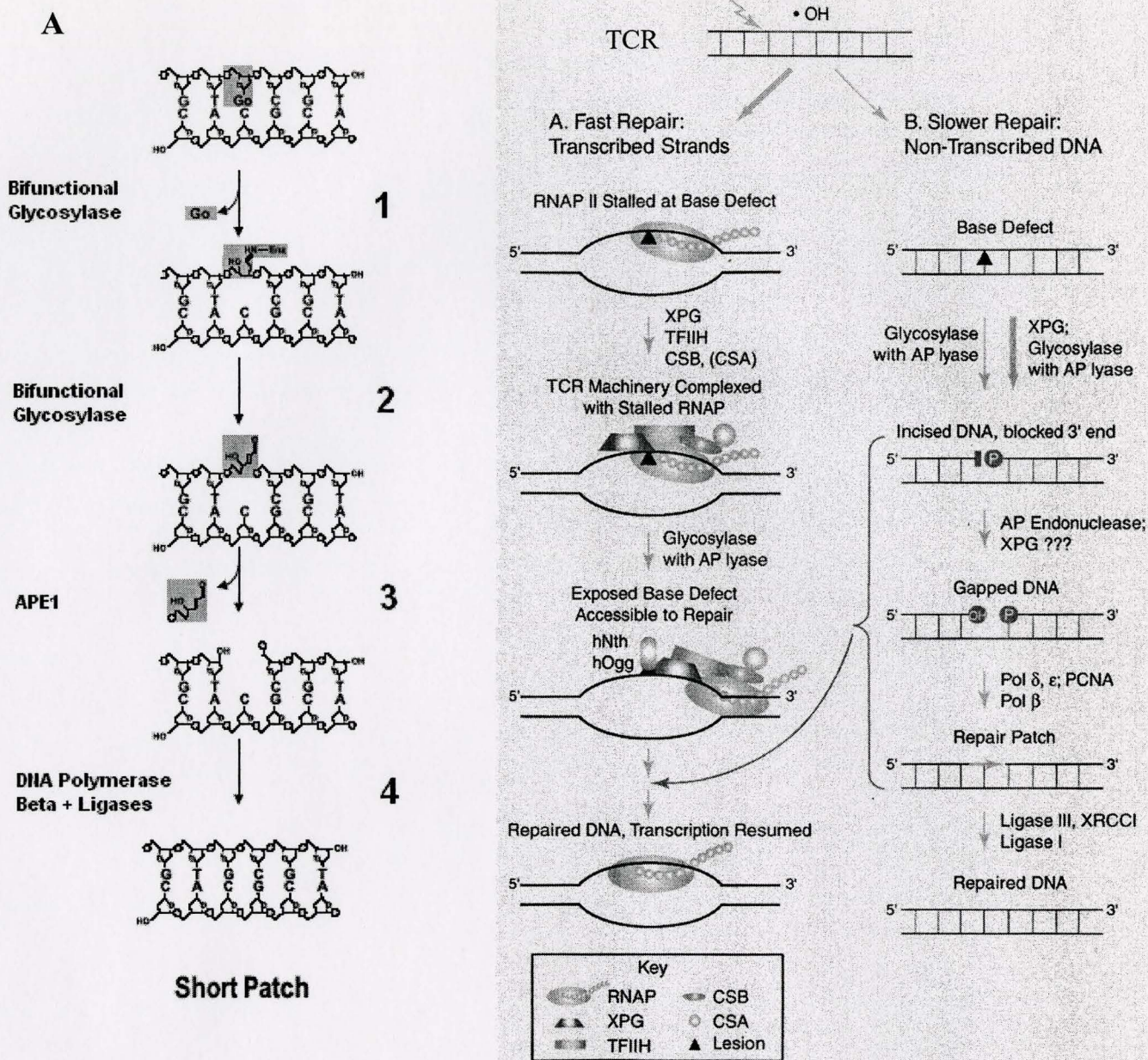
Figure 6:   **A.** The short patch Base Excision Repair pathway for 8-OxoG base damage. Detection and removal of the damaged base is facilitated by a bifunctional glycoslyase that also nicks the DNA 3' to the AP-Site. AP Endonuclease 1 then nicks the DNA 5' to the damage effectively freeing the nucleotide form the strand. Polymerase ß fills in the gap, and DNA ligase attaches the new nucleotide to the strand. **B.** The two sub pathways of BER: TCR and GGR. TCR occurs at a faster rated due to the stalling of RNAPII, which recruites CSB, XPG, TFIIH. This complex then recruits the bifunctional glycoslyase. The remainder of the steps are the same as A. (Adapted from A, Slupphaug et al, 2003, B, Lepage et al, 2000)
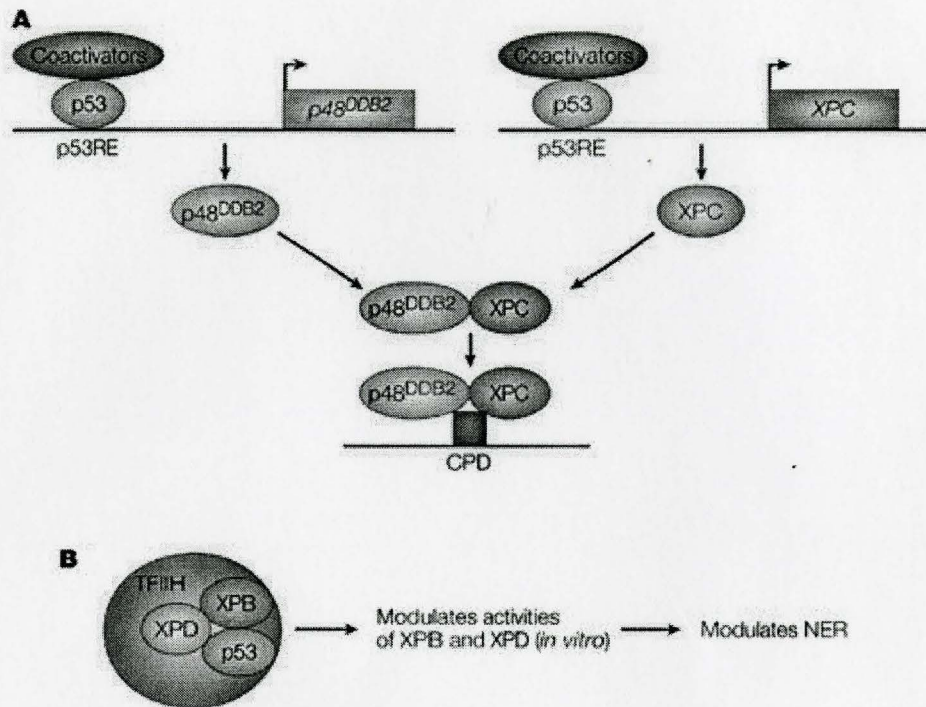
Figure 7:   The role of p53 in NER. **A.** The role of p53 in GGR of NER, where p53 acts as a transcription factor for the XPC and a subunit of XPE protiens. **B.** The regulatory role of p53 in helicase activity of the XPB and XPD proteins, facilitating NER. (Adapted from Sengupta et al. 2005)
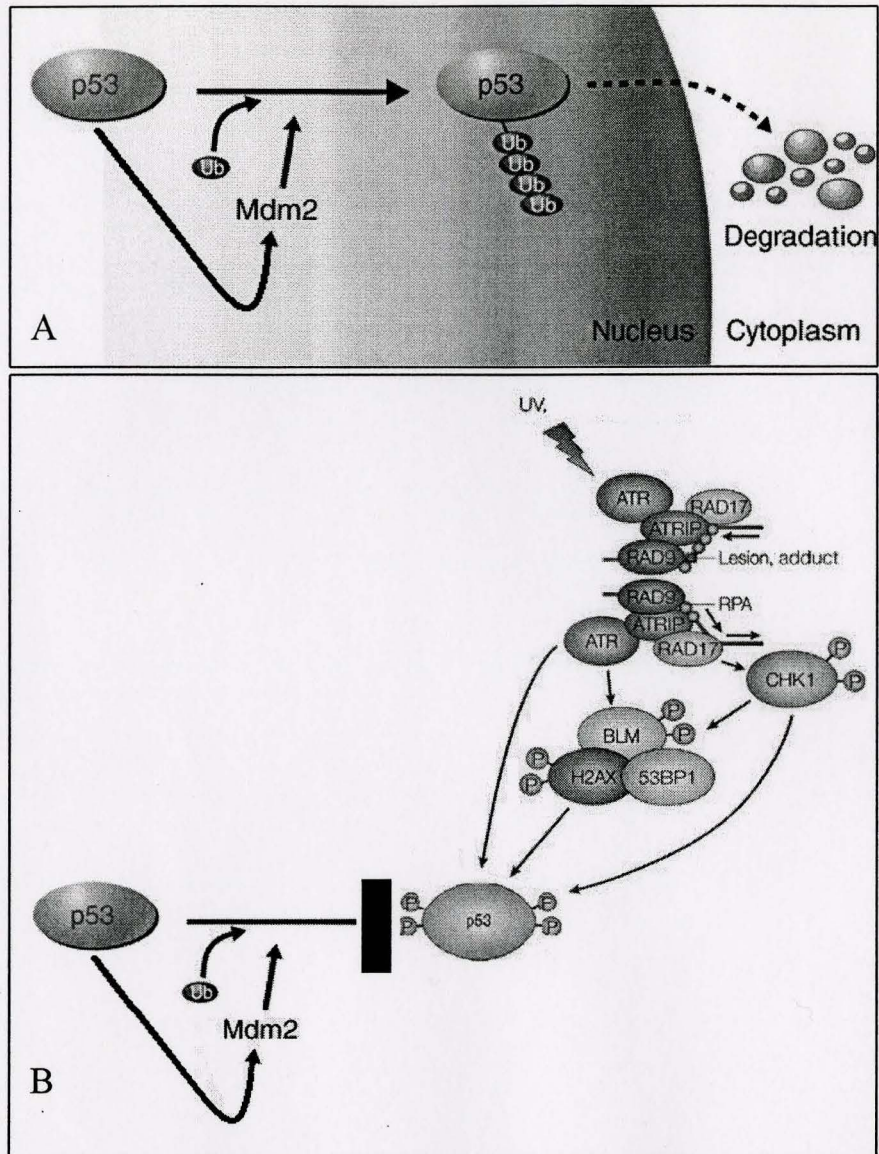
Figure 8: **A**. The autoregulation loop of p53. p53 causes the upregulation of transcription of the Mdm2 protein (HDM2 in humans) that causes the ubiquitin mediated degradation of p53. In this way the cell can maintain the p53 protein level. **B.** Upon UV DNA damage, a cascade of kinase activity leads to the phosphorylation of p53, the HDM2 facilitated degradation of p53 is then inhibited, leading to an accumulation of active p53 in the cell. (Adapted from Sengupta et al, 2005 & Salomoni et al. 2002)
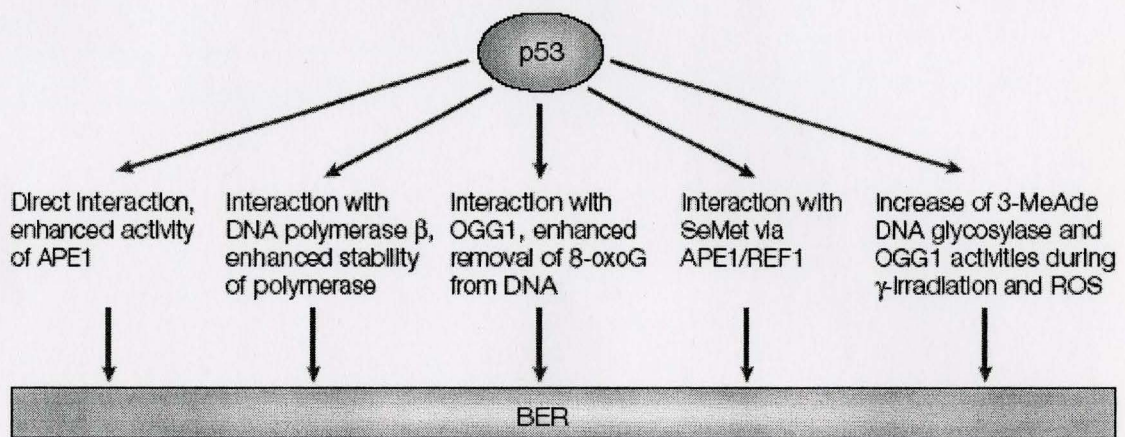
Figure 9: The several roles p53 can play in Base Excision Repair. p53's role in BER appears to be mostly in protein-protein regulation, however it does possess a transcription factor role that may be involved as well. (Adapted from Sengupta et al, 2005)
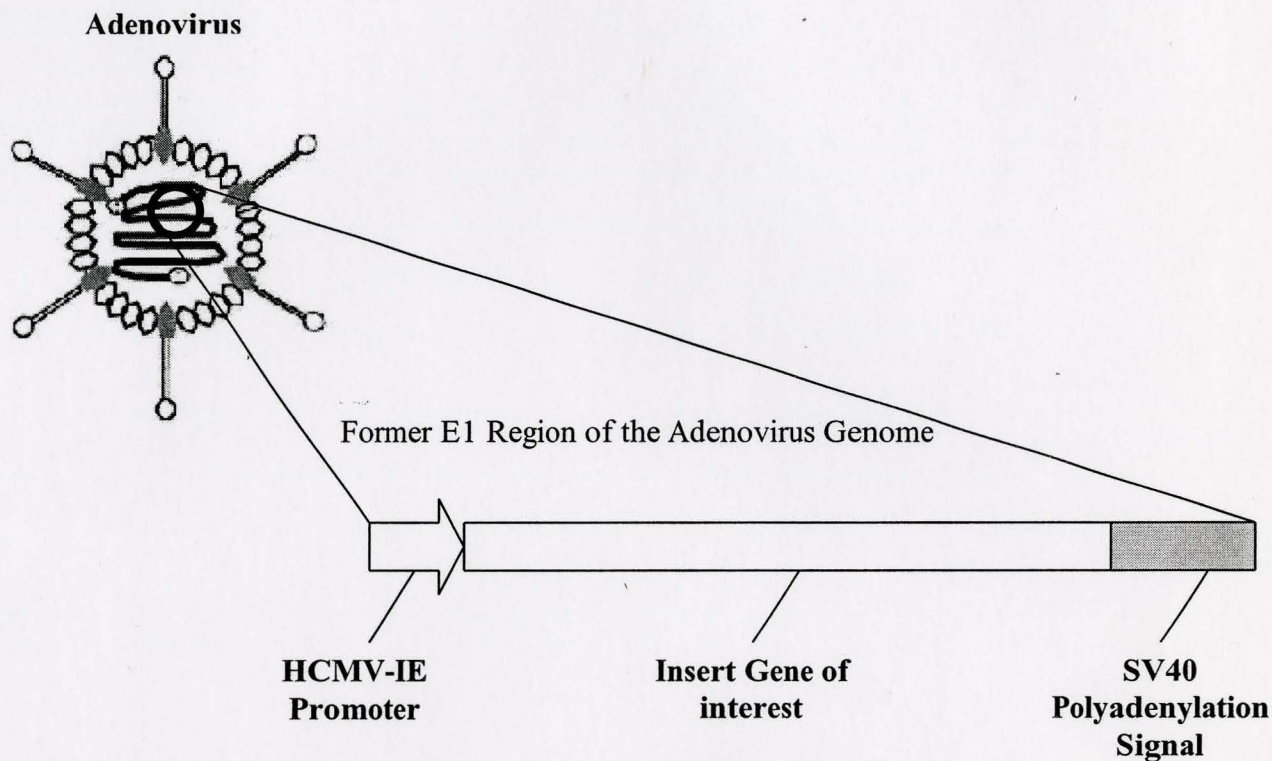
Figure 10 :   A diagrammatic view of a recombinant adenovirus. By replacing the E1 gene with the promoter and the gene of interest, the virus becomes unable to propagate, however infection and expression of the gene are unaffected. (Adapted from Addison et al, 1997)

Chapter 2:

Inducible base excision repair of methylene blue plus visible light damaged DNA in human cells by both p53 dependent and p53 independent mechanisms

Chapter 2

Inducible base excision repair of methylene blue plus visible light damaged DNA in human cells by both p53 dependent and p53 independent mechanisms

Abstract:

Methylene blue (MB) acts as a photosensitizer and after excitation by visible light (VL) produces reactive oxygen species that result in oxidative damage to DNA. (MB+VL) produces predominantly 8-hydroxyguanine as well as other single base modifications in DNA that are repaired by base excision repair (BER). We have used a recombinant non-replicating human adenovirus, Ad5HCMV*lacz*, which expresses the β-galactosidase (β-gal) reporter gene, to examine the role of the p53 tumor suppressor in constitutive and inducible BER of (MB+VL)-damaged DNA in human cells. Host cell reactivation (HCR) of β-gal activity for MB+VL-treated Ad5HCMV*lacz* was examined in non-treated and pre-UVC-treated normal human fibroblasts and several transformed and tumor cell lines with compromised p53 function. The normal human fibroblast strains were GM9503, GM38A, GM969 and NF. The cell lines with compromised p53 were two SV40-transformed cell lines (GM637, and AG02804), two spontaneously immortalized Li-Fraumeni syndrome cell lines (LFS 087 and LFS 041), normal human fibroblasts expressing the human papilloma virus E6 protein (NFE6) and a human tumor cell line (SKOV-3). Constitutive HCR of the MB+VL-treated reporter gene in untreated cells did not correlate with wild type p53 expression levels, suggesting that factors other than p53 expression levels can influence constitutive BER of the reporter gene.

Surprisingly, HCR was actually greater in some p53-compromised cells compared to normal human fibroblasts. In particular both LFS087 and GM637 showed greater HCR levels compared to most of the normal fibroblast strains in untreated cells. Pre-UVC-treatment of the normal fibroblast strains resulted in an enhanced HCR of the MB+VL-treated reporter gene and a concomitant increase in the expression of p53, suggesting that p53 may be involved in inducible BER in normal human fibroblasts. In addition, increased expression of p53 by pre-infection of cells with Ad5p53wt (which expresses wild-type p53) also resulted in an increased HCR in the normal fibroblasts giving evidence that increased expression of p53 alone enhances BER in normal human fibroblasts. In contrast to the results for the normal fibroblasts, p53 expression did not correlate with HCR values for the p53 compromised cells in either untreated or UVC-pretreated cells. In particular, the SKOV-3, LFS 087 and NF-E6 show no upregulation of p53 expression following UVC, and yet these cells show significant enhancement of HCR following UVC pretreatment. These results indicate that BER of MB+VL-damaged DNA is inducible in human cells by pre-UVC-treatment and that the enhancement in BER can result from both p53 dependent and p53 independent mechanisms.

Introduction:

Reactive oxygen species (ROS) are constantly generated in our cells due to aerobic respiration and it has been estimated that 10^5 damaging events occurs in every cell, every day as a result of ROS (Fraga et al, 1990). This damage can be directed towards many components of the cell, including the lipid membrane, proteins, and most

importantly the DNA, which can lead to carcinogenesis (as reviewed by Kawanishi et al, 2001). The main damage in DNA resulting from ROS is the oxidation of the purine and pyrimidine bases and oxidation of guanine residues is the most common giving rise to 7,8-dihydro-8-oxoguanine (8-OxoG) (Burrows et al, 1998, Neta et al, 1988, Wang et al, 1998). The mutagenic effect of this lesion is very well established, as it can pair efficiently with cytosine or guanine residues (Duarte et al, 1999). Therefore, if an 8-OxoG is established in the genome, it can potentially cause a G:C to a T:A transversion initiating the first step of carcinogenesis (as reviewed by Kovacic et al, 2001). Generation of this oxidative lesion *in vivo* is facilitated by the hydroxyl radical (OH$^\bullet$) as well as the singlet oxygen molecule ($^1O_2$) (Epe, 1991), which is created as a byproduct of aerobic respiration and the electron transport chain (Hanukoglu et al, 1993).

One of the common *in vitro* inducers of singlet oxygen mediated DNA damage is visible light (VL) activated methylene blue (MB) (Epe et al, 1993). MB is one of the few molecules which, when irradiated with VL, is able to excite molecular oxygen to singlet oxygen (Floyd et al, 2004). MB + VL is known to create predominantly 8-OxoG in the DNA of cell free extracts (Floyd et al, 1989) as well as in whole cells and viruses (Tuite et al, 2004).

8-OxoG lesions are repaired primarily by the base excision repair (BER) pathway, which is initiated by glycosylases. Glycosylases are a group of proteins that can recognize and excise oxidized bases in the DNA. The hOGG1 is one of the few glycosylases that are specific for recognizing 8-OxoG. hOGG1 also posseses an endonuclease activity that can "nick" the DNA 3' to the damaged site. Removal of the

oxidized base results in the creation of an apurinic nucleotide (AP-Site), which is recognized by an apurinic endonuclease (APE). APE1 is able to "nick" the DNA 5' to the damaged site and effectively remove the abasic nucleotide from the DNA. At this point DNA Polymerase ß (Pol-ß) fills in the gap created by APE1, and DNA ligase connects the old and new strands (for a review of BER see Slupphaug et al, 2003 and Dianov et al, 2001).

The p53 protein is a tumor suppressor responsible for the regulation of genes to prevent uncontrolled cell growth (Farmer et al, 1992 and Ginsberg et al, 1991). Besides p53's role in transcription it is also known to interact with the DNA, and with proteins that are involved with DNA repair (reviewed by Ford, 2005). In BER, p53 plays more of protein modulating role, where it physically and functionally interacts with hOGG1 and APE1 (Achanta et al, 2002). p53 has also been shown to interact with Pol-ß, stabilizing it and facilitating its attachment to DNA (Seo et al, 2002).

Nucleotide Excision Repair (NER) is a repair pathway responsible for the removal of bulky adducts from the DNA such as those created from exposure to solar radiation. (For review of NER and its subpathways see: Hoeijmakers et al, 2000, Balagee et al, 1999). It has been reported previously that the HCR of a UV-treated reporter is inducible by UVC (Francis et al, 1999) suggesting that NER is also inducible. The mechanism of this enhancement is the thought to be the activation of p53 and the response that p53 invokes (McKay et al, 1997). Findings that p53 and activated p53 accumulate in the cell following low dose UVC irradiation (Latonen et al, 2001) correlates well with a p53 involvement in enhancing HCR.

In the present study, we have sought to investigate the role of the p53 in BER. In particular, the inducibility of BER following UVC pretreatment, and the role of p53 in this induction was investigated. We have employed a recombinant adenovirus AdHCMV*Lacz* (AdCA17), which is a non-replicating virus that expresses the β-galactosidase (β-gal) reporter gene under the control of the human cytomegalovirus (HCMV) immediate early promoter (Rainbow et al, 2000). We have carried out host cell reactivation (HCR) assays by treating the AdCA17 virus with MB + VL, inducing predominantly 8-OxoGs in the viral genome (Epe, 1991). Cell lines of interest were infected with the treated probe and assayed for β-gal activity. Proper expression of β-galactosidase is only expected to occur if the host cell is able to repair the damage induced by the MB + VL in the β-gal reporter gene. In this way, HCR of the reporter gene indicates the BER ability of the infected cell. This HCR assay was used to investigate the BER abilities of various normal and p53 deficient cells. Results suggest that p53 is involved in the BER pathway. To investigate the role of p53 in the induction of BER, the HCR assay was performed in UVC treated and untreated cells. Results suggest that BER is UVC-inducible, and that p53 is involved in this induction. To further elucidate the role of p53 in this inducible response we performed HCR of MB + VL treated AdCA17 in normal cells that had been pre-infected with p53 expressing Ad5p53wt. These results indicate that even though p53 expression enhances HCR of the MB + VL damaged reporter, the enhancement due to pre-UVC-treatment was greater. This suggests that other factors besides p53 may be induced following UVC treatment.

Materials and Methods:

Cell lines and virus constructs:

The repair-proficient primary human cell lines: GM 9503, GM 38A, GM 969 along with the SV40 transformed normal GM637, were obtained from National Institute of General Medical Sciences (NIGMS) (Camden, NJ). The other SV40 transformed normal AG02804D was obtained from The National Institute on Aging (NIA) (Camden, NJ). A normal neonatal foreskin (NF) fibroblast strain (established by Dr. D. A. Galloway, Fred. Hutchinson Cancer Research Centre, Seattle, WA), and the E6-expressing transformants of this normal strain were all obtained from Dr. B. C. McKay, Centre for Cancer Therapeutics, Ottawa Regional Cancer Centre, Ottawa, Ontario, Canada. The immortalized LFS cells LFS 041, and LFS 087 were generously provided by Dr. Micheal A. Tainsky, Barbara Ann Darmanos Cancer Institute, Wayne State University, 110 East Warren Ave., Detroit, MI. Human ovarian cancer SKOV-3 cells were obtained from the American Tissue Culture Collection cell repository (Rockville, Md). Passage numbers, an indication of cell line age, of primary human fibroblasts are shown in the table below.

Cell Line	Group	Range of passage numbers
GM9503	Normal	18-22
GM969	Normal	18-22
GM38A	Normal	18-22
NF	Normal	26-29

All cell cultures were grown at 37°C in a humidified incubator in 5% CO_2 and cultured in Eagle's a-minimal essential media (a-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, Grand Island, NY). Media for the E6 transformants were supplemented with 250 μg/mL geneticin (G418; Sigma–Aldrich Canada, Oakville, Ontario, Canada).

The recombinant Adenovirus Ad5HCMV*lacz* (AdCA17), Ad5HCM*luc* (AdCA18) (Addison et al, 1997) and Ad5p53wt (Bachetti and Graham, 1993), were obtained from Dr. F.L. Graham, McMaster University. These vectors have had the E1 region deleted and are therefore unable to replicate in most mammalian cells. Ad5HCMV*lacz* carries the bacterial *lacz* gene; Ad5HCMV*luc* carries the firefly luciferase gene, while Ad5p53wt carries the wild-type p53 gene, all of which are under the control of the human cytomegalovirus (HCMV) immediate early (IE) promoter. The virus was propagated, collected, and titred as described previously (Prevec et al, 1991). It was shown, by western blot analysis, that infection with Ad5p53wt increased cellular

expression of p53 compared to infection with Ad5HCMV*luc* or no infection at all (appendix 1).

Methylene Blue and Visible Light:

Methylene Blue (Methylthionine chloride Trihydrate) was prepared by taking 0.02g of MB (Sigma Chemicals) in powder form, and dissolving it in 20ml of PBS at 37°C to make a concentration of 1000µg/ml. The solution was filter sterilized and aliquots were frozen at -20 °C in the dark. When needed, frozen aliquots were thawed in the dark and diluted appropriately for use. The Visible Light (VL) irradiation of cells and virus occurred under a General Electric 1000 watt halogen lamp (GE R1000). A platform was constructed at 83 cm below the bulb. Virus was irradiated on ice using a magnetic stir plate at distance of 70 cm from the bulb. The effect of MB + VL and VL alone was investigated, and it was shown that VL alone had a minimal effect compared to the effect of MB + VL (appendix 2).

HCR of MB+VL treated AdHCMV*lacz* virus in cells:

Seeding

Flasks containing confluent monolayers of each cell line were trypsinized using 2X trypsin/EDTA and re-suspended in supplemented a-MEM, and counted with a haemocytometer. The cells were diluted appropriately with supplemented a-MEM so that SV40 transformed, LFS, and SKOV-3 cells were seeded at a density of 3.8×10^4 cells/well, and primary human fibroblasts were seeded at a density of 1.9×10^4 cells/well

in 96-well plates (Falcon, Franklin Lakes, NJ). Cells were left to incubate for 24 hours. Incubation of cell cultures was at 37°C in a humidified incubator in 5% CO_2 unless otherwise stated.

Pretreatment with Ad5p53wt

If cells were to receive pre-infection with Ad5p53wt, the following procedure was followed. The seeding media was aspirated from the 96-well plate. The virus was diluted appropriately in unsupplemented and cells were infected at a multiplicity of infection (MOI) of 200 plaque forming units (PFU) per cell in an infecting volume of 40 μl per well. Infection proceeded for 90 minutes at which point the cells were re-fed with complete a-MEM, and allowed to incubate for 24 hours. The control virus used in these experiments was the Ad5HCMV*luc*, infected in the same manner.

MB+VL treatment of the virus

Viral suspensions were prepared and irradiated by suspending 80 μl AdHCMVlacz virus in 3.6ml of phosphate buffered saline (PBS) in 35 mm petri dishes on ice. The MB solution was then added to the petri dishes to create the appropriate concentration of MB. With continuous stirring, the virus suspension was irradiated (or mock irradiated) with visible light, for various amount of time. After each time point, 400μls of irradiated virus prep was removed from the Petri dishes and diluted appropriately in 2ml of un-supplemented a-MEM on ice. The newly diluted irradiated viral suspensions were vigorously vortexed to ensure homogeneity, and were used to infect cells within 20 minutes.

Pretreatment of cells with UVC

If cells were to be pretreated with UVC immediately before infection of the prepared virus, the following procedure would be followed. The overlaying media was aspirated from the 96-well plate, and 40 µl of warmed PBS was added to each well. The source of UVC was a General Electric germicidal lamp (model G8T5) emitting predominantly at 254 nm. The stage was set at a distance that facilitated a fluence of 1 J/m^2/s, and the cells were irradiated for 25 seconds. Immediately after treatment, the cells were infected as follows.

Viral infection

The overlaying media was aspirated from one horizontal row of the 96-well plate, and the cells were immediately infected with 40 µl of the appropriate irradiated virus. This was repeated for each row of the 96-well plate with the appropriate virus sample. The infection was allowed to carry on for 90 minutes at which point the 160 µl of complete a-MEM was added to each well to stop the infection, and the cells were allowed to incubate at 37 °C for 44-48 hours prior to harvesting. It was determined that the 40 + time point of harvesting cells was optimal for assessing repair proficiencies between cell lines (appendix 3).

Harvesting and Scoring

Incubation media was aspirated and 60µl of Chlorophenolred ß-D-galactopyranoside (CPRG; prepared in 0.01% Triton X-100, 1mM MgCl$_2$ 100 mM phosphate buffer at pH 8.3; Boehringer-Mannheim, Indianapolis, IN) was added to the infected cells. To assay for the expression of the ß-galactosidase levels from the *lacz* gene, optical density

readings were taken using a 96-well plate reader (Bio-Tek Instruments EL340 Bio Kinetics Reader) at several time intervals at 570 nm. When CPRG is in the presence of the *Lacz* gene product, ß-galactosidase, the CPRG turns from a yellow colour to a purple colour. The optical density (OD) of wells infected with untreated virus was plotted as a function of time. Plates with maximum OD readings just under the saturation plateau were analyzed in further detail. For plotting the β-gal survival curves, the average background level of ß-galactosidase activity was subtracted from each averaged point from the measurements taken from a minimum of quadruplicate wells.

Western Blot Analysis of p53 Expression:

All cells were seeded in 100 mm cell culture dishes (Falcon, Franklin Lakes, NJ) at a density that would achieve monolayer. Between 18-24 hours after seeding, media was replaced with 5.9 ml of PBS and then UVC irradiated at 1 J/m^2/s for 25 seconds or left untreated and then re-fed with the complete a-MEM. The cells were then allowed to incubate for 24 hours at which point they were scraped off the dishes into centrifuge tubes, and centrifuged for 10 minutes at 1000 rpm. The cells were then re-suspended in 10 ml PBS, and re-centrifuged for 10 minutes at 1000 rpm. The cells were suspended in lysis buffer (50 mM Tris, 150 mM NaCl, 1% NP40) containing protease inhibitors. After centrifugation (1 min at 13000 rpm), the supernatants were isolated and protein concentrations were measured in duplicate using the Bio-Rad protein reagent in a Bradford assay (Bio-Rad, Richmond, CA). Protein aliquots were prepared and 30-40 µg of each protein sample was loaded and separated by SDS-PAGE (10%). Proteins were

then transferred onto a nitrocellulose membrane and blocked overnight at 4 $^{\circ}$C in 10% skim milk in TBS with 0.05% Tween 20. Blots were then probed with a mouse monoclonal antibody to p53 conjugated to HRP (Santa Cruz Biotechnology, Santa Cruz, CA). Protein loading was verified by monitoring actin levels in each lane. After the addition of ECL staining reagent (Western Lightning Chemiluminescence Reagent, PerkinElmer Life Sciences), blots were visualized by exposure to Kodak X-Omat AR film.

Pooling data over many experiments:

The D_{37} value of a survival curve is the dose, in this case VL exposure, required to reduce the ß-gal activity of a cell line to 37% of the non treated control of that same cell line. D_{37} was used as a measure of the cellular HCR ability, such that a cell line with a higher average D_{37}, has a better ability to repair MB + VL induced damage.

The actual D_{37} values of each cell line was determined from curves of each experiment and expressed as a ratio of the actual D_{37} value of an arbitrarily chosen normal. This is the relative D_{37} value compared to a normal. The relative D_{37} of the chosen normal in each experiment would therefore always be 1, and those cell lines with an actual D_{37} higher than the actual D_{37} of the chosen normal would have a relative D_{37} greater than 1. The relative D_{37} values for each cell line were then averaged over many experiments and expressed with its % standard error.

Results:

Constitutive HCR of the MB + VL–treated reporter gene in normal fibroblasts and p53 compromised cells

In order to determine the role of p53 in constitutive BER of MB + VL-induced DNA damage, we examined HCR of an MB+VL-treated reporter gene in normal human fibroblasts as well as in human cells with compromised p53. The normal human fibroblast strains were GM9503, GM38A, GM969 and NF.   The cell lines with compromised p53 were GM637, AG02804, NFE6, LFS 087, LFS 041 and SKOV-3. The SV40 transformed cell lines GM637 and AG02804D (Kohli et al, 1999), and the papilloma E6 transformed cell line NF-E6 (Meitz et al, 1992), have their p53 abrogated by the SV40 large T antigen or E6 protein respectively. The Li-Fraumeni syndrome, LFS 087, cells express mutant p53, while the LFS 041 and human ovarian cancer SKOV-3 cells express no p53 at all (Gollahon et al, 1998; Modisett et al, 2001).

Cells were infected at a multiplicity of infection (MOI) of 20 pfu/cell with Ad5HCMVlacz (AdCA17) virus that had been treated with 40 µg/ml MB and irradiated or mock-irradiated with VL. The infected cells were subsequently assayed for ß-gal activity 44-48 hours post infection. Representative survival curves of ß-gal activity for MB + VL-treated AdCA17 are shown for the normal fibroblasts in figure 1. It can be seen that HCR of MB+VL-treated AdCA17 was similar for the 4 different normal strains tested. Representative survival curves for ß-gal activity of MB +VL treated AdCA17 are shown for the various p53 compromised cell lines in figure 2. It can be seen that compared to the normal fibroblast strains, the HCR of MB+VL-treated AdCA17 was

somewhat greater in GM637, LFS087, LFS041 and NFE6 cells, but not in AG02804D and SKOV-3 cells. The VL exposure in seconds required to reduce β-gal activity to 37% of that for non VL-exposed virus (D_{37}) was used as a measure of HCR. Mean D_{37} values from multiple experiments are shown in Table 1. It can be seen that the mean D_{37} values were greater than that for the normal fibroblast strains in GM637, AG02804D, LFS087 and LF041 cells, but not in NFE6 and SKOV-3 cells. The difference was only significant from the average D_{37} of all normals in the GM637 cell line. In order to account for the variation in D_{37} values in individual experiments we also calculated the D_{37} value for each of the various fibroblast strains and human cell lines relative to the D_{37} value of the normal GM9503 strain obtained in individual experiments. The D_{37} values relative to the GM9503 strain for each cell line were then averaged over many experiments. The average relative D_{37} values for each cell line compared to GM9503 are shown in figure 3 and Table 2. For the p53 compromised cell lines the HCR value was greater than GM9503 in NFE6, GM637, LFS 087 and LFS 041 but not in AG02804D and SKOV-3 cells. However, there were also differences in HCR values of the normal strains, and none of the p53 compromised cell lines had HCR values significantly different when compared to all of the normal strains tested.

Induced HCR of the MB + VL treated reporter gene

Latonen et al, 2001 have shown that exposure of cells to low doses of UVC results in the accumulation of p53 (Latonen et al, 2001), which is thought to be caused by the stalling of RNAPII due to UVC-induced DNA lesions on the transcribed strand of

active genes (Ljungman et al, 1999). Pre-treatment of normal human fibroblasts with low

UVC fluences results in an enhanced HCR of a UVC-damaged reporter (Francis et al,

2000, McKay et al, 1997). The UVC-enhanced HCR was absent in LFS cells indicating a

requirement for wild type p53 in the enhanced HCR (McKay et al, 1997) and suggesting

that pretreatment of normal human fibroblasts leads to a p53 dependent upregulation of

the NER pathway. There is also evidence for an involvement of p53 in BER (as reviewed

by Sengupta and Harris, 2005). It was therefore considered of interest to examine HCR of

the MB+VL treated reporter gene in pre-UVC-treated cells in order to determine if prior

UVC pretreatment of cells can enhance the BER of an oxidatively damaged reporter

construct. Representative survival curves of ß-gal activity for MB + VL treated AdCA17

in pre-UVC-treated compared to non-treated cells are shown for the normal fibroblast

strains in figure 4 and for the p53 compromised cells in figure 5. It can be seen that pre-

UVC-treatment of all the normal fibroblast strains results in a substantially enhanced

HCR of the MB + VL-treated reporter suggesting that BER of MB + VL-induced

oxidative damage is inducible in normal human cells. In contrast, the enhanced HCR in

pre-UVC-treated p53 compromised cells was generally less than that obtained in the

normal fibroblasts or absent. The relative D_{37} value obtained in UVC-pretreated cells

compared to that in non-treated cells was determined for each experiment and the mean

relative D_{37} values for several experiments are shown in figure 6 and Table 3. It can be

seen that 3 of the 4 normal cell strains (GM9503, GM969 and GM38A), show a

significant enhancement of HCR following low dose UVC pretreatment to cells and 3 of

the 6 p53 compromised cell lines (NF-E6, LFS087 and SKOV-3), similarly showed a

significant enhancement following UVC treatment to cells. The remaining 3 cell lines (GM637, AG02804D and LFS 041) also showed an increased HCR in pre-UVC-treated cells, although the enhancement was not significant.

HCR of the MB + VL treated reporter gene in pre-UVC-treated cells

The activity of p53 in untreated cells becomes activated in the presence of genomic DNA damage (as reviewed by Oren, 1997). UVC-induced DNA damage is known to increase the transcription of DNA repair genes such as p53 (as reviewed by Bender et al, 1997, Latonen et al, 2001), and to activate p53 via post translational modifications (as reviewed by Oren, 1997). Therefore assaying the constitutive repair of a MB + VL-damaged reporter in untreated cells may not give us an accurate depiction of the total involvement of p53 in BER. It was therefore of interest to compare HCR of the MB + VL-treated reporter gene in the p53-compromised cells compared to normal fibroblasts for cells pretreated with UVC. Representative survival curves of ß-gal activity for MB + VL-treated AdCA17 in UVC pretreated normal fibroblasts are shown in figure 7, whereas figure 8 shows representative curves for ß-gal activity for MB + VL-treated AdCA17 in the UVC-pretreated p53 compromised cell lines. Mean D_{37} values for β-gal survival in pre-UVC-treated cells are shown in Table 4. Mean D_{37} values for each cell line relative to that for the GM9503 normal fibroblast strain obtained in individual experiments are shown in figure 9 and Table 5. It can be seen that HCR of the MB + VL-treated reporter gene was similar in the 4 different pre-UVC-treated normal fibroblasts. In contrast to the results for constitutive HCR in non-treated cells, HCR of the MB + VL-

treated reporter gene in pre-UVC-treated cells was similar or less in the p53 compromised cells compared to that in the normal strains. In particular for the p53 compromised cell lines, the LFS 041 line was significantly less proficient in HCR compared to GM9503, while the LFS 087 line showed a significantly reduced HCR compared to all of the normal strains tested. Although the other p53-compromised cells also showed a reduced HCR compared to GM9503 cells, this reduction was not significant.

Effect of low dose UVC treatment on p53 expression levels in normal and p53 compromised cells

To confirm that UVC pretreatment of cells increased the p53 expression levels, Western blot analysis was performed in untreated and UVC-treated cells. Cells were grown in 100 mm tissue culture dishes, and then irradiated (or mock irradiated) with 25 J/m^2 UVC. The cells were harvested 24 hours later, and lysates were created. Protein was separated by SDS PAGE and transferred to blot paper. Blots were then probed for p53. Representative Western blots are shown in figure 10. It can be seen that basal p53 levels in the untreated human fibroblasts (GM9503, GM969 and NF) are extremely low or undetectable and all show upregulation of p53 expression following UVC treatment. NF-E6, LFS 041 and SKOV-3 cells showed extremely low or undetectable p53 protein expression in untreated and UVC-pre-treated cells as reported by others (McKay et al, 2001, Gollahon et al, 1998, and Johnson et al, 1991). High expression levels of p53 were detected in both untreated and UVC-pre-treated LFS 087 cells. High p53 expression

levels have been reported previously for LFS087 cells (Gollahon et al, 1998) and is consistent with the accumulation of p53 in cells with mutant p53 (Anker et al, 1993). Both SV40-transformed cell lines, GM637 and AG02804D, also showed high p53 expression levels in both untreated and UVC-pre-treated cells with no p53 upregulation following UVC. This is consistent with previous reports showing that abrogation of p53 by SV40 Tag results in accumulation of p53 (O'Neil et al, 1997).

A comparison of the HCR values in untreated cells (figure 3 and Table 2) and UVC-treated cells (figure 9 and Table 5) with the p53 expression levels shown in figure10 indicated that increased p53 expression in the pre-UVC-treated cells correlated with enhanced HCR of the MB + VL-treated reporter gene in the pre-UVC-treated normal human fibroblast strains. This suggested some involvement of p53 in the inducible BER of the MB + VL-damaged reporter gene in normal human fibroblasts. In contrast, p53 expression did not correlate with HCR values for the p53 compromised cells in either untreated or UVC-pretreated cells. In particular, the SKOV-3, LFS 087 and NF-E6 show no upregulation of p53 expression following UVC, and yet these cells show significant enhancement of HCR following UVC pretreatment (figure 6).

HCR of the MB + VL-treated reporter gene in human fibroblasts pre-infected with Ad5p53wt virus

The correlation of enhanced p53 expression and enhanced HCR of the MB + VL-treated reporter gene in UVC-pre-treated normal human fibroblasts suggested some involvement of p53 in the enhanced HCR response in normal human fibroblasts.

However, UVC is known to up regulate not only p53 but also many p53 dependent and p53 independent genes (as reviewed by Bender et al, 1997). It was therefore considered of interest to examine whether the increased expression of p53 alone could enhance HCR of the MB + VL-treated reporter gene. HCR of the MB + VL-treated reporter gene was examined in fibroblasts that have been pre-infected with Ad5p53wt or a control virus, Ad5HCMV*luc* (AdCA18). Ad5p53wt (Bachetti and Graham 1993) expresses wild type p53 in cells, while the control AdCA18 expresses the luciferase protein in a similar E1 deleted recombinant adenovirus construct (Addison et al, 1997). Normal human fibroblasts were infected with Ad5p53wt or AdCA18 at an MOÎ of 200, and 24 hours later infected with MB + VL-treated AdCA17 and assayed for ß-gal activity 44-48 hours later. Representative survival curves of ß-gal activity for MB + VL-treated AdCA17 in normal fibroblasts pre-infected with Ad5p53wt or AdCA18 are shown in figure 11. Mean D_{37} values for the β-gal survival curves obtained from several experiments are shown in table 6. In addition, the relative D_{37} value in cells pre-infected with Ad5p53wt compared to cells pre-infected with AdCA18 were determined for individual experiments and the mean relative D_{37} values for several experiments are given in figure 12 and table 7. It can be seen that pre-infection of cells with Ad5p53wt resulted in an enhanced HCR compared to pre-infection of cells with AdCA18 indicating that increased expression of p53 alone can enhance HCR.

Since some functions of p53 are known to require a UV-induced post translation modification to be activated (Oren, 1997) we also examined HCR of the MB + VL-treated reporter gene in fibroblasts that have been pre-infected with Ad5p53wt or a

control virus, Ad5HCMV*luc* (AdCA18), and subsequently pre-treated with 25 J/m^2 UVC before infection with MB + VL treated AdCA17. In this way the cells were allowed to express increased amounts of wild type p53 protein from the Ad5p53wt genome that was subsequently activated by the UVC pretreatment cells. Representative survival curves of ß-gal activity for MB + VL-treated AdCA17 in normal fibroblasts pre-infected with Ad5p53wt or AdCA18 and pre-treated with UVC are shown in figure 13. Mean D_{37} values for the β-gal survival curves obtained from several experiments are shown in table 6. In addition, the relative D_{37} value in pre-UVC-treated cells pre-infected with Ad5p53wt compared to cells pre-infected with AdCA18 were determined for individual experiments and the mean relative D_{37} values for several experiments are given in figure 14 and Table 7. It can be seen that pre-infection of pre-UVC-treated cells with Ad5p53wt resulted in an enhanced HCR compared to pre-infection of pre-UVC-treated cells with AdCA18 giving further evidence that increased expression of p53 alone can enhance HCR.

As it has been shown that UVC alone (Table 3) and p53 alone (Table 6 and Table 7) enhanced HCR compared to the non-treated counterparts, it was of value to compare the relative enhancements seen due to UVC, p53, and UVC + p53 treatment. Relative D_{37} values in Ad5p53wt infected (top segment), UVC treated (middle segment), and Ad5p53wt infected + UVC treatment (bottom segment) compared to cells pre-infected with AdCA18 only are shown in Table 8. It can be seen that Ad5p53wt infection resulted in an average enhancement of about 25%, UVC pre-treatment resulted in a 37% enhancement, and Ad5p53wt + UVC treatment resulted in a 44% increase. The greater

enhancement in HCR of Ad5p53wt + UVC treatment compared to UVC pretreatment alone gives further support for the role of p53 in BER of MB + VL damaged DNA in normal human fibroblasts.

Discussion:

A role for p53 has been implicated in several aspects of the BER pathway. p53 has been reported to regulate BER in G1 (Offer et al, 2001, Offer et al 2002) and DNA Pol-β, the enzyme involved in BER repair synthesis *in vitro*, is affected by the presence or absence of p53, even when added as a recombinant protein suggesting a direct role for p53 in BER (Zhou et al, 2001). Achanta et al have shown that p53 physically associates with the hOGG1 glycosylase and APE1 proteins which are involved in the sensing and excision steps of BER (Achanta et al, 2002). A functional interaction of p53 and the glycosylase involved in recognizing 3-methyladenine oxidative damage has also been shown (Zurer et al, 2004). The p53 protein may also modulate the transcription and activity of Pol-ß, as some p53 mutant cells have very low levels of this enzyme (Seo et al, 2002). In addition, the pre-exposure of human cells with low "priming" doses of ionizing radiation leads to an enhanced removal of thymine glycols after higher doses (Le et al, 1998) providing evidence for an inducible BER response in human cells.

Constitutive HCR of the MB + VL-induced reporter gene in untreated cells

In the present work we report that constitutive HCR of the MB + VL-treated reporter gene did not display a good correlation with wild type p53 expression levels,

suggesting that factors other than p53 expression levels influence constitutive BER of the reporter gene in untreated cells. Surprisingly HCR was actually greater in some p53-compromised cells compared to normal human fibroblasts. In particular both LFS087 and GM637 showed greater HCR levels compared to most of the normal fibroblast in untreated cells. One explanation for this may be the high constitutive levels of p53 protein expression in these cells (figure 10). It has been reported that abrogation of p53 by the SV40 large T antigen or expression of mutant p53 by a cell causes p53 to accumulate (Anker et al, 1993, O'Neil et al, 1997), which explains these high constitutive levels. In addition, it has been shown that SV40 transformed cells have functional p53 that is not bound by the large T antigen (O'Neil et al, 1997), and even p53 that is bound, is capable of performing some of its transcription factor role (Sheppard, et al, 1999). p53 has five functional protein domains (as reviewed by Ford 2005), and it is reasonable to assume that a mutated p53 could still posses many of its functions if the mutation is not severe and affects only one domain if any. Thus it is possible that accumulated p53 mutated, abrogated or otherwise, could contribute to the greater constitutive proficiency of BER in LFS087 and GM637 cells compared to normal fibroblasts. Even the p53 compromised SKOV-3 and LFS041 cells, which express no p53 protein, did not show a significant reduction in HCR compared to the normal fibroblasts suggesting that p53 is not important in the constitutive BER levels of these cells.

Inducible HCR of the MB + VL–treated reporter gene

We have previously reported that pre-UVC-treatment of normal human fibroblasts results in enhanced HCR of a UVC-damaged reporter gene due to an inducible NER response (Francis and Rainbow 1999). The UVC-enhanced HCR was absent in LFS cells expressing mutant p53 suggesting that pre-UVC-treatment of cells leads to a p53 dependent upregulation of the NER pathway (McKay et al 1997). UVC causes CPDs and 6-4PPs that are blocks to transcription and it is the stalling of RNAPII at these UVC-induced lesions which is thought to trigger the activation of p53 (Ljungman et al, 1999, McKay et al, 1998). Pastoriza et al 2003, have suggested that the 8-OxoG lesion also constitutes a block to transcription (Pastoriza et al, 2003), and thus p53 could be upregulated in cells exposed to oxidizing agents which induce 8-OxoG. In the present work we have initiated the upregulation of p53 by pre-UVC-exposure of cells. We show that pre-UVC-treatment of the normal fibroblast strains results in an enhanced HCR of the MB + VL-treated reporter gene and a concomitant increase in the expression of p53. It can be seen that GM9503, GM969 and GM38A show a significant enhancement of HCR (approximately 50%) following low dose UVC pretreatment to cells and NF fibroblasts show an HCR enhancement of approximately 25%, which was not as significant (figure 6 and Table 3). The lower extent of enhancement of the NF cells could be attributed to several factors. It has been shown that the p53 response is attenuated in cells that are grown to a high density (Bar et al, 2004), and cells that are of high passage number (Seluanov et al, 2001). As the NF cells were of considerably higher passage number than the other normal fibroblast strains used, it is possible that the p53

response is attenuated in the NF fibroblasts. Notwithstanding, enhancement of HCR correlated with upregulation of wild type p53 in the normal human fibroblasts, suggesting that p53 may be involved in the enhancement of BER in normal human fibroblasts. Pre-infection of normal fibroblasts with Ad5p53wt also resulted in an increased HCR of the MB + VL-treated reporter gene giving further evidence that increased expression of p53 alone enhances BER in normal human fibroblasts.

In contrast to the results for the normal fibroblasts, p53 expression did not correlate with HCR values for the p53 compromised cells in either untreated or UVC-pretreated cells. In particular, the SKOV-3, LFS 087 and NF-E6 show no upregulation of p53 expression following UVC, and yet these cells show significant enhancement of HCR following UVC pretreatment. This indicates that BER of the MB + VL-treated reporter gene can be induced by pre-UVC-treatment of cells through both p53 independent and p53 dependent mechanisms. It has been suggested that DNA damage up regulates Pol-ß as well as p53 expression (Cabelof et al, 2002). It is therefore possible that the induced BER of the MB + VL-treated reporter gene in pre-UVC-treated cells with compromised p53 results, in part at least, from an increased expression of Pol- ß.

HCR of the MB + VL-treated reporter gene in pre-UVC-treated cells

In contrast to the results of constitutive HCR in untreated cells (figure 3), HCR values were generally greater in pre-UVC-treated normal human fibroblasts compared to pre-UVC-treated p53-compromised cells. The greater HCR values in the pre-UVC-treated normal fibroblasts may result from greater inducible BER levels due to both p53

dependent and p53 independent mechanisms compared to only p53 independent mechanisms in the p53 compromised cells.

The results of the present report indicate that BER of MB + VL-damaged DNA is inducible in human cells and that enhancement in BER can result from both p53 dependent and p53 independent mechanisms.

Figure 1: HCR of β-galactosidase activity for MB+VL-treated Ad5HCMV*lacz* virus in normal human fibroblast cells. Cells were infected at an MOI of 40 pfu/cell with untreated or [20 µg/ml of MB + VL]-treated Ad5HCMV*lacz* and subsequently harvested 44-48 hours after infection. Results shown are for a representative experiment and include GM9503 (■), GM 38A (●), NF (▲), and GM 969 (▼).   Each point is the average ± SE of triplicate determinations, done in one experiment. Each cell line was included with GM9503 in different experiments.

Visible Light Exposure to MB treated virus (s)
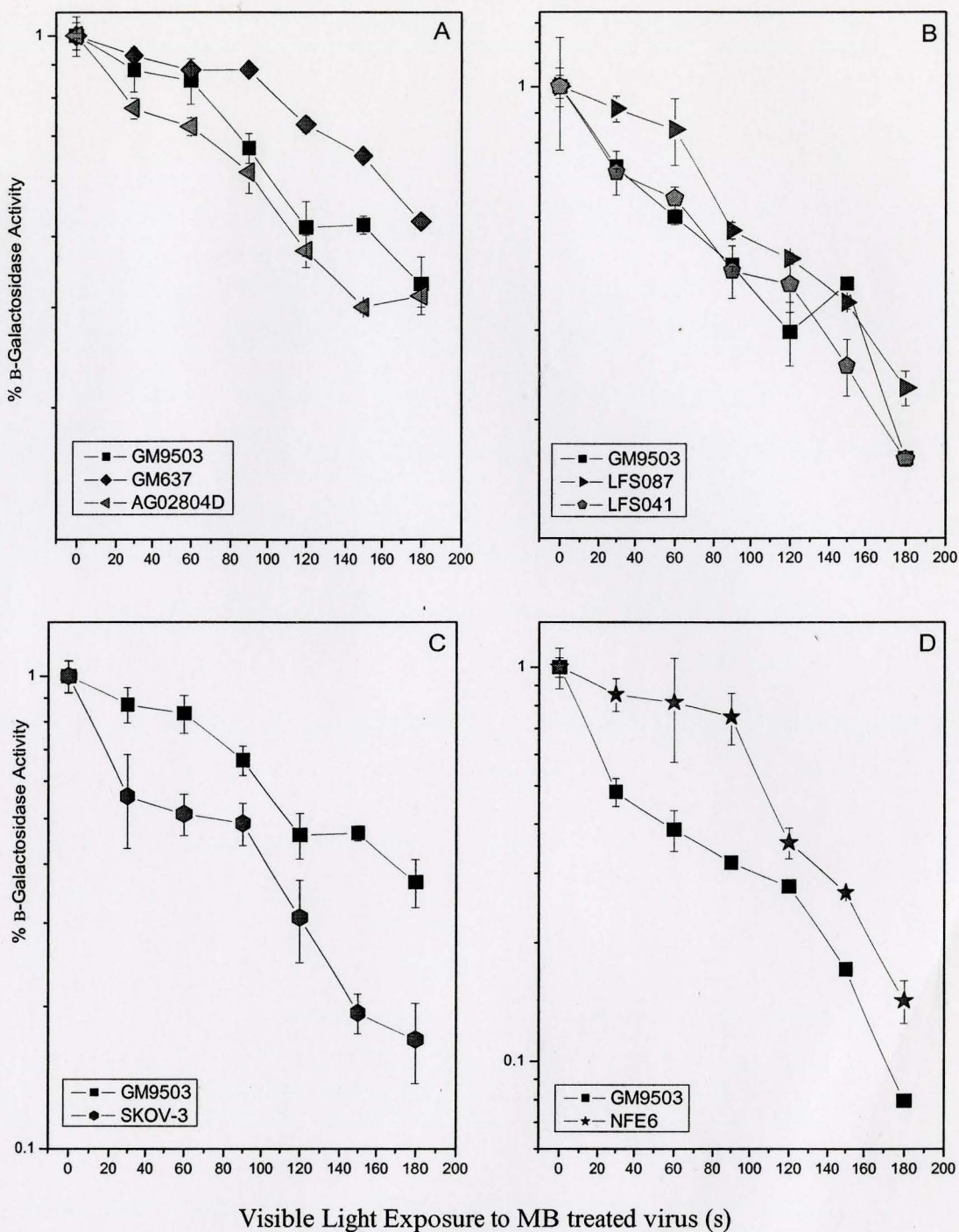
Figure 2: HCR of ß-galactosidase activity for MB+VL-treated Ad5HCMV*lacz* virus in p53 compromised cells. Cells were infected at a MOI of 40 pfu/cell with untreated or [20 µg/ml of MB + VL]-treated Ad5HCMV*lacz* and subsequently harvested 44-48 hours after infection. Results shown are for a representative experiment and include in panel **A:** GM9503 (■), p53 abrogated SV40 transformed cells GM 637F (◆) and AG02804D (◀). Panel **B:** GM9503 (■), p53 mutant and p53 null Li-Fraumeni Syndrome cells LFS087 (▶) and LFS041(◆). Panel C: GM9503 (■), p53 null ovarian cancer SKOV-3 cells (●). Panel D: GM9503 (■), p53 abrogated papilloma transformed NF-E6 cells (★). Each point is the average ± SE of triplicate determinations, done in one experiment. Cells in each panel were assayed in the same experiment.

Table 1. HCR capability for β-galactosidase activity of [20μg/ml MB + VL]-treated Ad5HCMV*lacz* virus as measured by the actual D_{37} values in normal and p53 compromised cell lines.

Cell Line	Group	Type of Cell	Average† Actual D_{37}	% Standard Error	# of Experiments	Average Actual values significantly ‡ Different Compared to Average of all normal cell lines	(P-Value)
GM9503	Normal	Fibroblast	97.742	8.124	17	-	-
GM38A	Normal	Fibroblast	93.056	7.936	5	-	-
GM969c	Normal	Fibroblast	72.322	13.726	4	-	-
NF	Normal	Fibroblast	90.22	9.369	6	-	-
Average Normal	Normal	Fibroblast	92.422	5.143	32	-	-
NF E6 §	p53 abrogated	NeoNatal Foreskin	82.762	4.264	5	No	0.471
GM637 ¥	p53 abrogated	Immortalized Fibroblast	133.337	18.289	4	Yes	0.014
AG02804D ¥	p53 abrogated	Immortalized Fibroblast	104.9	28.441	3	No	0.505
LFS087	LiFraumeni-p53 mutant	Immortalized Fibroblast	112.386	17.175	6	No	0.159
LFS041	LiFraumeni-p53 null	Immortalized Fibroblast	110.553	14.093	3	No	0.305
SKOV-3	p53 null	Ovarian Cancer Cells	97.81	14.34	2	No	0.799

† Mean visible light exposure to reduce reporter expression to 37% of that observed in the unexposed reporter construct in a given cell line then averaged over all experiments done under same conditions.
‡ significance tested by the two sampled independent t-test (p<0.05).
Transformation of cell lines is facilitated by, papilloma virus (§) and SV40 virus (¥).
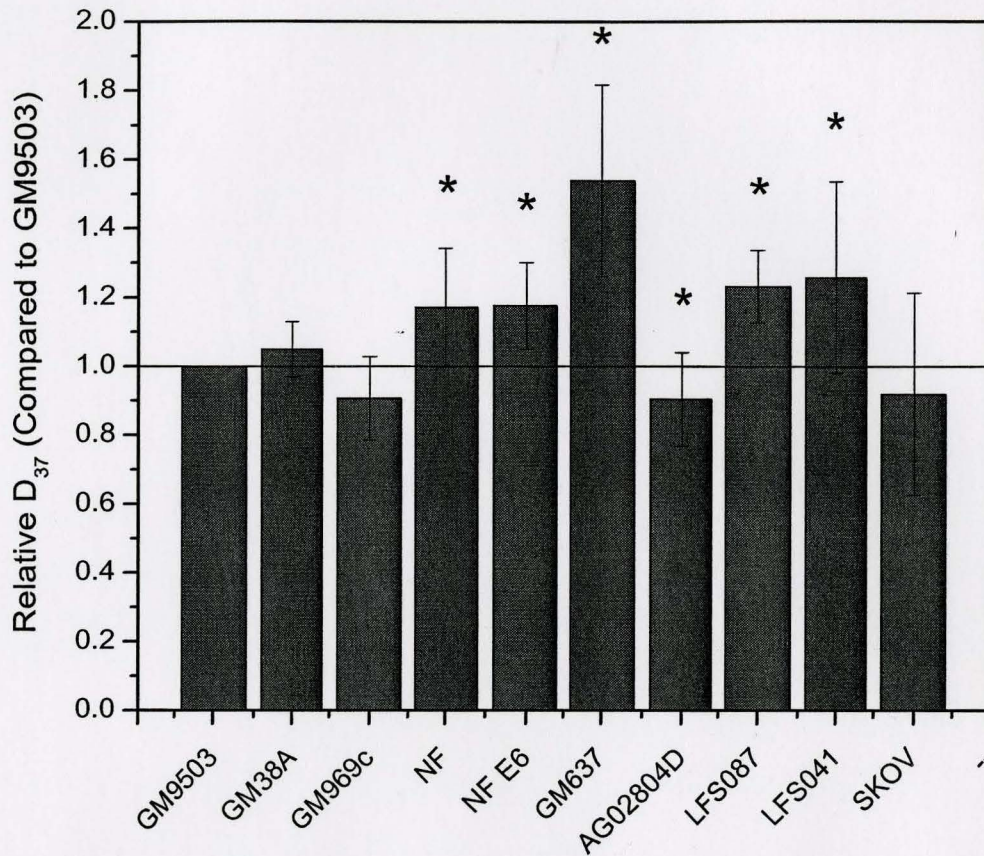
Figure 3: Relative D_{37} values obtained from HCR assays for ß-gal activity in normal and p53 compromised fibroblasts and transformed cells. Cells were infected at an MOI of 40 pfu/cell with untreated or [20 µg/ml of MB + VL]-treated Ad5HCMV*lacz* and subsequently harvested 44-48 hours after infection. Results shown are the average relative $D_{37} \pm$ SE for each cell line shown as a ratio of the GM9503 of 2 – 18 independent experiments. Relative D_{37} values significantly different than the normal GM9503 by the 2 sample independent t-test (p<0.05) are indicated (*).

Table 2.   HCR capability for β-galactosidase activity of [20μg/ml MB + VL]-treated Ad5HCMV*lacz* virus as measured by relative D_{37} values in normal and p53 compromised cell lines compared to the GM9503 Normal.

Cell Line	Group	Type of Cell	Average† Relative D_{37}	% Standard Error	# of Experiments	Average Relative values significantly ‡ Different Compared to GM9503	(P value)
GM9503	Normal	Fibroblast	1.000	-	18	-	-
GM38A	Normal	Fibroblast	1.049	0.0805	5	No	0.229
GM969c	Normal	Fibroblast	0.907	0.1209	4	No	0.087
NF	Normal	Fibroblast	1.170	0.1718	4	Yes	0.031
NF E6 §	p53 abrogated	NeoNatal Foreskin	1.175	0.1252	3	Yes	0.001
GM637 ¥	p53 abrogated	Immortalized Fibroblast	1.538	0.2787	3	Yes	0.000
AG02804D¥	p53 abrogated	Immortalized Fibroblast	0.904	0.1360	2	Yes	0.011
LFS087	Li Fraumeni -p53 mutant	Immortalized Fibroblast	1.232	0.1058	4	Yes	0.000
LFS041	Li Fraumeni -p53 null	Immortalized Fibroblast	1.258	0.2779	3	Yes	0.016
SKOV-3	p53 null	Ovarian Cancer Cells	0.919	0.2940	2	No	0.279

† Calculated by taking D_{37} [20μg/ml MB+VL exposed virus] for experimental cell line / D_{37} [20μg/ml MB+VL exposed virus] for GM9503 normal fibroblast within individual experiments then averaged over all experiments done under same conditions.

‡ significance tested by the two sampled independent t-test (p<0.05).

Transformation of cell lines is facilitated by, papilloma virus (§) and SV40 virus (¥).

Visible Light Exposure to MB treated virus (s)

Figure 4: HCR of ß-galactosidase activity for MB+VL-treated Ad5HCMV*lacz* virus in UVC pretreated (solid shapes) and non pretreated (outlined shapes) normal fibroblasts. Cells were either irradiated with 25 J/m^2 of UVC or mock irradiated then infected at a MOI of 40 pfu/cell with untreated or [20 µg/ml of MB + VL]-treated Ad5HCMV*lacz* and subsequently harvested 44-48 hours after infection. Each point is the average ± SE of triplicate determinations, done in one experiment. Cells in each panel were assayed in the same experiment.

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Visible Light Exposure to MB treated virus (s)

Figure 5: HCR of ß-galactosidase activity for MB+VL-treated Ad5HCMV*lacz* virus in UVC pretreated (solid shapes) and non pretreated (outlined shapes) p53 compromised cells. Cells were either treated with 25 J/m^2 of UVC or mock treated then infected at an MOI of 40 pfu/cell with untreated or [20 µg/ml of MB + VL]-treated Ad5HCMV*lacz* and subsequently harvested 44-48 hours after infection. Each point is the average ± SE of triplicate determinations, done in one experiment. Cells in each panel were assayed in the same experiment.
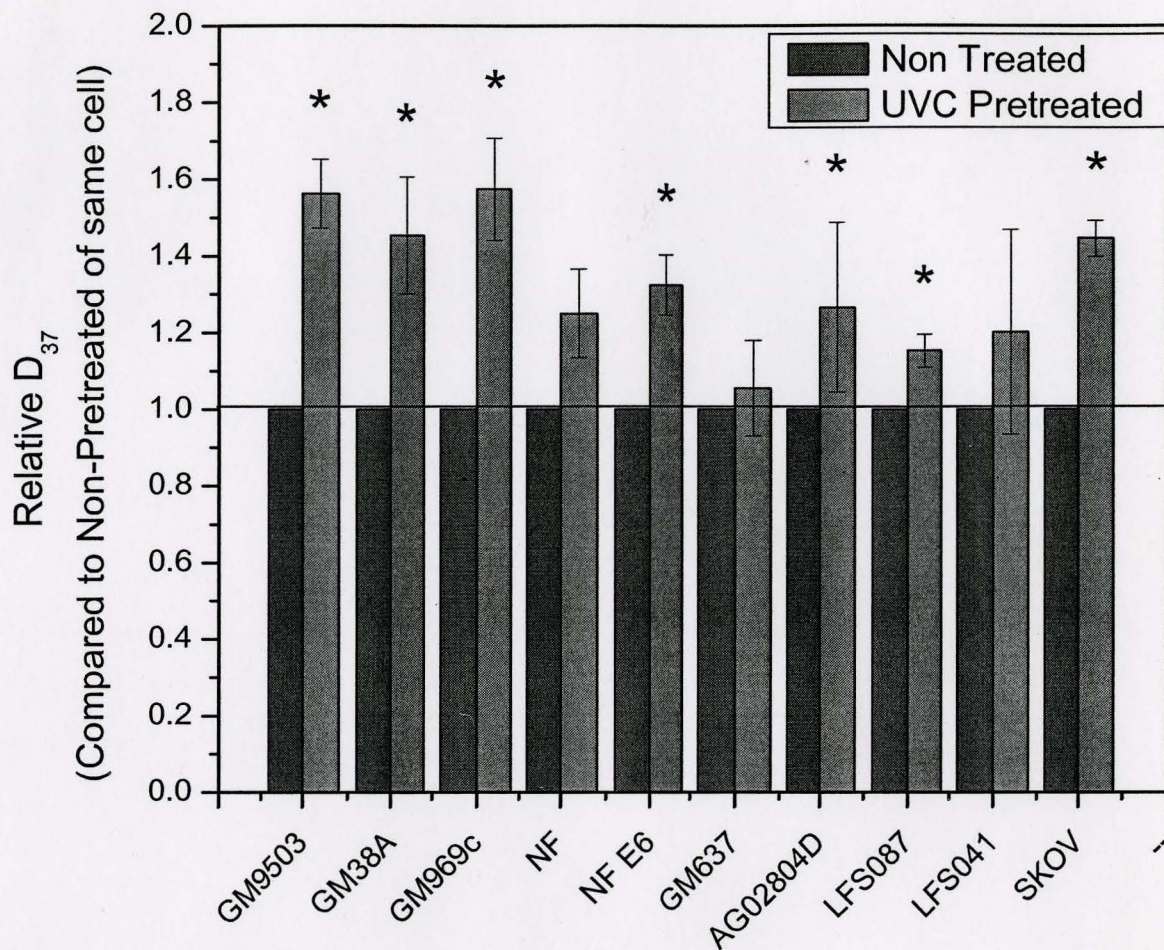
Figure 6: Relative D_{37} values obtained from HCR assays for ß-gal activity in normal and p53 compromised cells pretreated with or without UVC. Cells were treated with 25 J/m^2 of UVC or mock treated then infected at an MOI of 40 pfu/cell with untreated or [20 µg/ml of MB + VL]-treated Ad5HCMV*lacz* and subsequently harvested 44-48 hours after infection. Results shown are the average relative D_{37} ± SE for each pretreated cell line shown as a ratio of the same non-pretreated cell line. The data that populates each cell lines graph is derived from 2 – 15 independent experiments. Relative D_{37} values significantly increased compared to the non pretreated of that same cell line by the 2 sample independent t-test (p<0.05) are indicated (*).

Table 3.   HCR capability for β-galactosidase activity of [20μg/ml MB + VL] treated Ad5HCMV*lacz* virus as measured by the relative D_{37} values in UVC pretreated normal and p53 compromised cell lines compared to the non-pretreated of the same cell line.

Cell Line	Group	Type of Cell	Relative Average† D_{37} Non Pre / Non Pre	Pre / Non Pre	% S.E.	# of Experiments	Significantly Increased‡ Compared to Non Pretreated	(P-Value)
GM9503	Normal	Fibroblast	1	1.562	0.090	15	Yes	9.523E-07
GM38A	Normal	Fibroblast	1	1.454	0.152	4	Yes	0.024
GM969c	Normal	Fibroblast	1	1.574	0.133	4	Yes	0.005
NF	Normal	Fibroblast	1	1.250	0.115	6	No	0.055
NF E6 §	p53 abrogated	NeoNatal Foreskin	1	1.324	0.078	5	Yes	0.003
GM637 ¥	p53 abrogated	Immortalized Fibroblast	1	1.055	0.125	4	No	0.676
AG02804D	p53 abrogated	Immortalized Fibroblast	1	1.266	0.221	3	No	0.294
LFS087	p53 mutant	Immortalized Fibroblast	1	1.153	0.043	6	Yes	0.005
LFS041	p53 null	Immortalized Fibroblast	1	1.201	0.266	3	No	0.492
SKOV-3	p53 null	Ovarian Cancer Cells	1	1.445	0.047	2	Yes	0.011

† Calculated by taking D_{37} value of [20μg/ml MB+VL exposed virus] for pretreated cell line / D_{37} of [20μg/ml MB+VL exposed virus] for non pretreated same cell line within individual experiment, then averaged over all experiments under same conditions.

‡ significance tested by the two sampled independent t-test (p<0.05)

Transformation of cell lines is facilitated by, papilloma virus (§) and SV40 virus (¥).

Visible Light Exposure to MB treated Virus (s)

Figure 7: HCR of β-galactosidase activity for MB+VL-treated Ad5HCMV*lacz* virus in UVC pretreated normal human fibroblast cells. Cells were pretreated with 25 J/m^2 of UVC then infected at an MOI of 40 pfu/cell with untreated or [20 µg/ml of MB + VL]-treated Ad5HCMV*lacz* and subsequently harvested 44-48 hours after infection. Results shown are for a representative experiment and include GM9503 (■), GM 38A (●), NF (▲), and GM 969 (▼). Each point is the average ± SE of triplicate determinations, done in one experiment. Each cell

Visible Light Exposure to MB treated Virus (s)

Figure 8: HCR of ß-galactosidase activity for MB+VL-treated Ad5HCMV*lacz* virus in UVC pretreated normal and p53 compromised cells. Cells were pretreated with 25 J/m^2 UVC then infected at a MOI of 40 pfu/cell with untreated or [20 μg/ml of MB + VL]-treated Ad5HCMV*lacz* and subsequently harvested 44-48 hours after infection. Results shown are for a representative experiment and include in panel **A:** GM9503 (■), p53 abrogated SV40 transformed cells GM 637F (♦) and AG02804D (◄). Panel **B:** GM9503 (■), p53 mutant and p53 null Li-Fraumeni Syndrome cells LFS 087 (►) and LFS041(⬠). Panel C: GM9503 (■), p53 null ovarian cancer SKOV-3 cells (●). Panel D: GM9503 (■), p53 abrogated papilloma transformed NF-E6 cells (★). Each point is the average ± SE of triplicate determinations, done in one experiment. Cells in each panel were assayed in the same experiment.
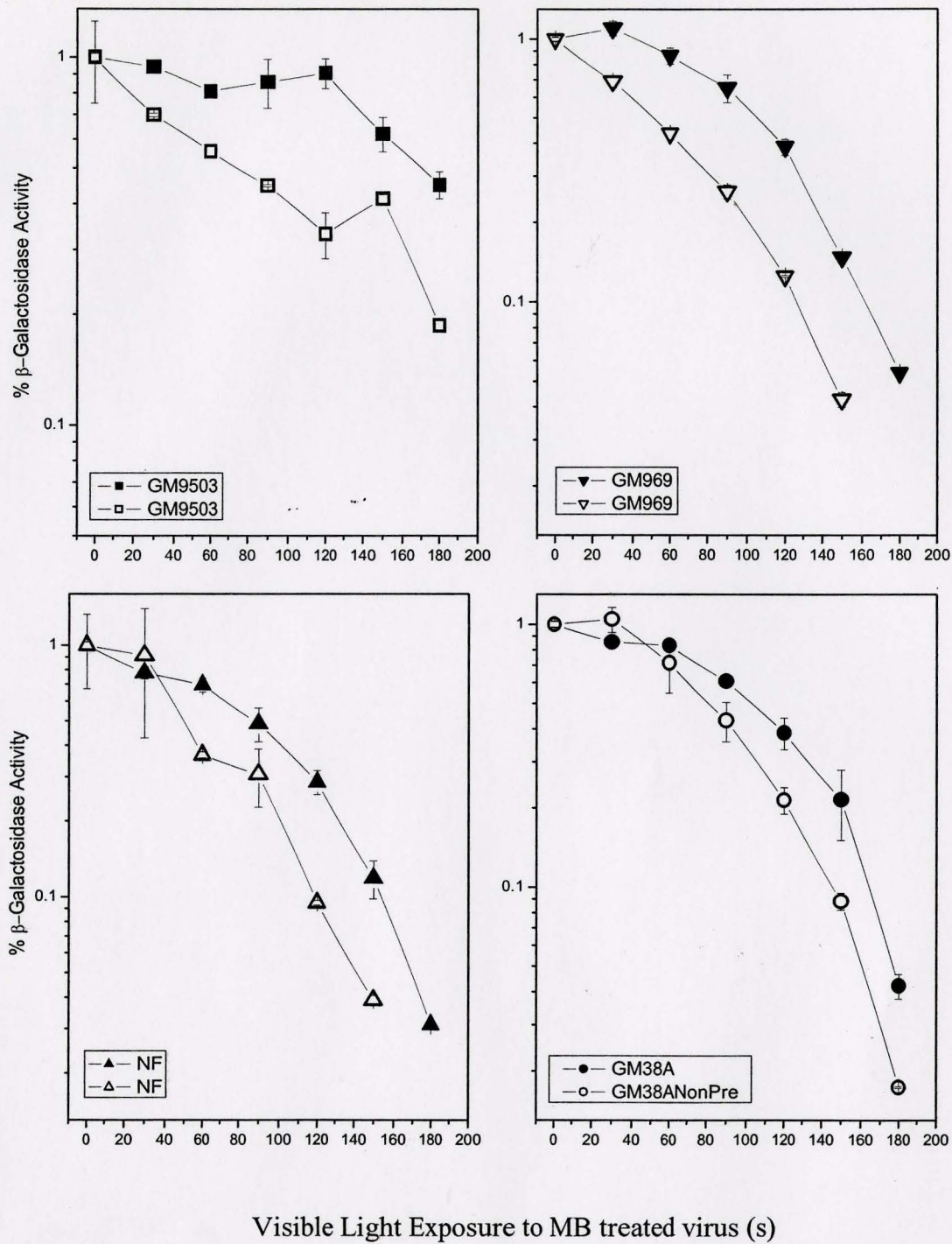
Table 4:   HCR capability for β-galactosidase activity of [20μg/ml MB + VL] treated Ad5HCMV*lacz* virus as measured by the actual D_{37} values in UVC treated normal and p53 compromised cell lines.

Cell Line	Group	Type of Cell	Average Actual D_{37}	% Standard Error	# of Experiments	Actual values significantly Different Compared to GM9503	(p-value)
GM9503	Normal	Fibroblast	138.00	13.47	14	-	-
GM38A	Normal	Fibroblast	123.41	5.88	4	No	0.50014
GM969c	Normal	Fibroblast	109.63	12.80	4	No	0.38361
NF	Normal	Fibroblast	108.30	5.32	6	No	0.27304
NF E6	Papilloma - p53 abrogated	NeoNatal Foreskin	110.21	9.64	5	No	0.33372
GM637FRain	SV40 - p53 abrogated	Immortalized Fibroblast	146.05	38.04	4	No	0.7419
AG02804D	SV40 - p53 abrogated	Immortalized Fibroblast	121.57	24.19	3	No	0.54747
LFS087	LiFraumeni -p53 mutant	Immortalized Fibroblast	127.18	16.64	6	No	0.45334
LFS041	LiFraumeni -p53 null	Immortalized Fibroblast	126.78	19.29	3	No	0.59089
SKOV-3	p53 null	Ovarian Cancer Cells	140.63	16.08	2	No	0.76764

† Mean visible light exposure to reduce reporter expression to 37% of that observed in the unexposed reporter construct in a given cell line then averaged over all experiments done under same conditions.

‡ significance tested by the two sampled independent t-test ($p<0.05$)

Transformation of cell lines is facilitated by, papilloma virus (§) and SV40 virus (¥)
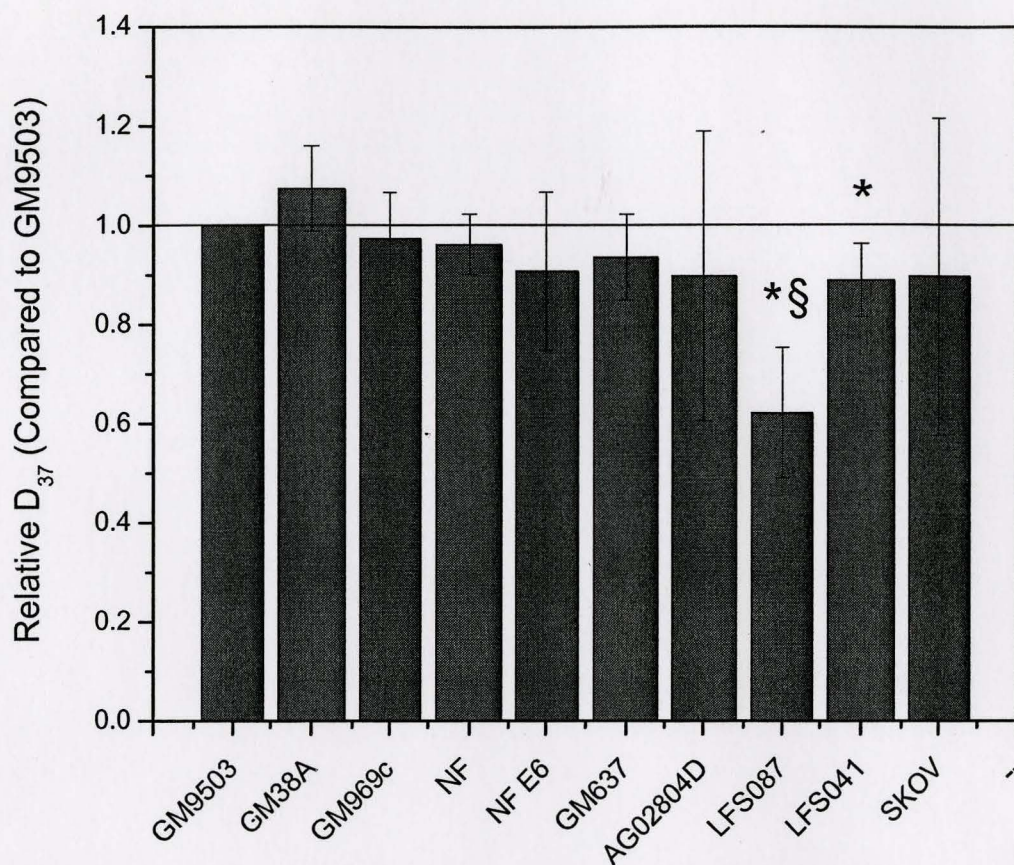
Figure 9: Relative D_{37} values obtained from HCR assays for ß-gal activity in UVC pretreated normal and p53 compromised cells. Cells were pretreated with 25 J/m^2 of UVC then infected at an MOI of 40 pfu/cell with untreated or [20 µg/ml of MB + VL]-treated Ad5HCMV*lacz* and subsequently harvested 44-48 hours after infection. Results shown are the average relative D_{37} ± SE for each cell line shown as ratio of the GM9503 of 2 – 15 independent experiments. Relative D_{37} values significantly reduced than the normal GM9503 by the 2 sample independent t-test (p<0.05) are indicated (*). Those relative D_{37} values similarly reduced compared to all of the normals are indicated (§).

Table 5:   HCR capability for β-galactosidase activity of [20µg/ml MB + VL] treated Ad5HCMV*lacz* virus as measured by relative D_{37} values in UVC pretreated normal and p53 compromised cell lines compared to the GM9503 Normal.

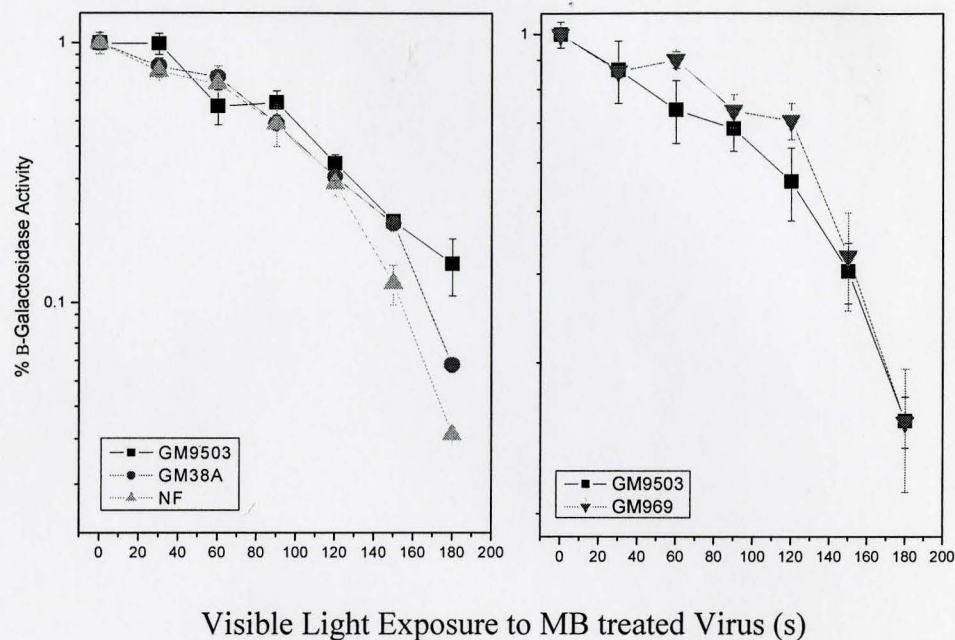| Cell Line	Group	Type of Cell	Average† Relative D_{37}	% Standard Error	# of Experiments	Relative values ‡ significantly Different Compared to GM9503	(P value)
GM9503	Normal	Fibroblast	1.000	0.000	15	-	-
GM38A	Normal	Fibroblast	1.075	0.086	4	No	0.094
GM969c	Normal	Fibroblast	0.974	0.094	4	No	0.572
NF	Normal	Fibroblast	0.962	0.062	4	No	0.225
NF E6 §	p53 abrogated	NeoNatal Foreskin	0.908	0.160	3	No	0.172
GM637 ¥	p53 abrogated	Immortalized Fibroblast	0.936	0.087	3	No	0.088
AG02804D ¥	p53 abrogated	Immortalized Fibroblast	0.898	0.292	2	No	0.241
LFS087	LiFraumeni -p53 mutant	Immortalized Fibroblast	0.622	0.132	6	Yes (§)	0.0002
LFS041	LiFraumeni -p53 null	Immortalized Fibroblast	0.890	0.073	3	Yes	0.0019
SKOV-3	p53 null	Ovarian Cancer Cells	0.896	0.320	2	No	0.275

† Calculated by taking D_{37} [20µg/ml MB+VL exposed virus] for experimental cell line / D_{37} [20µg/ml MB+VL exposed virus] for GM9503 normal fibroblasts within individual experiment then averaged over all experiments under same conditions.

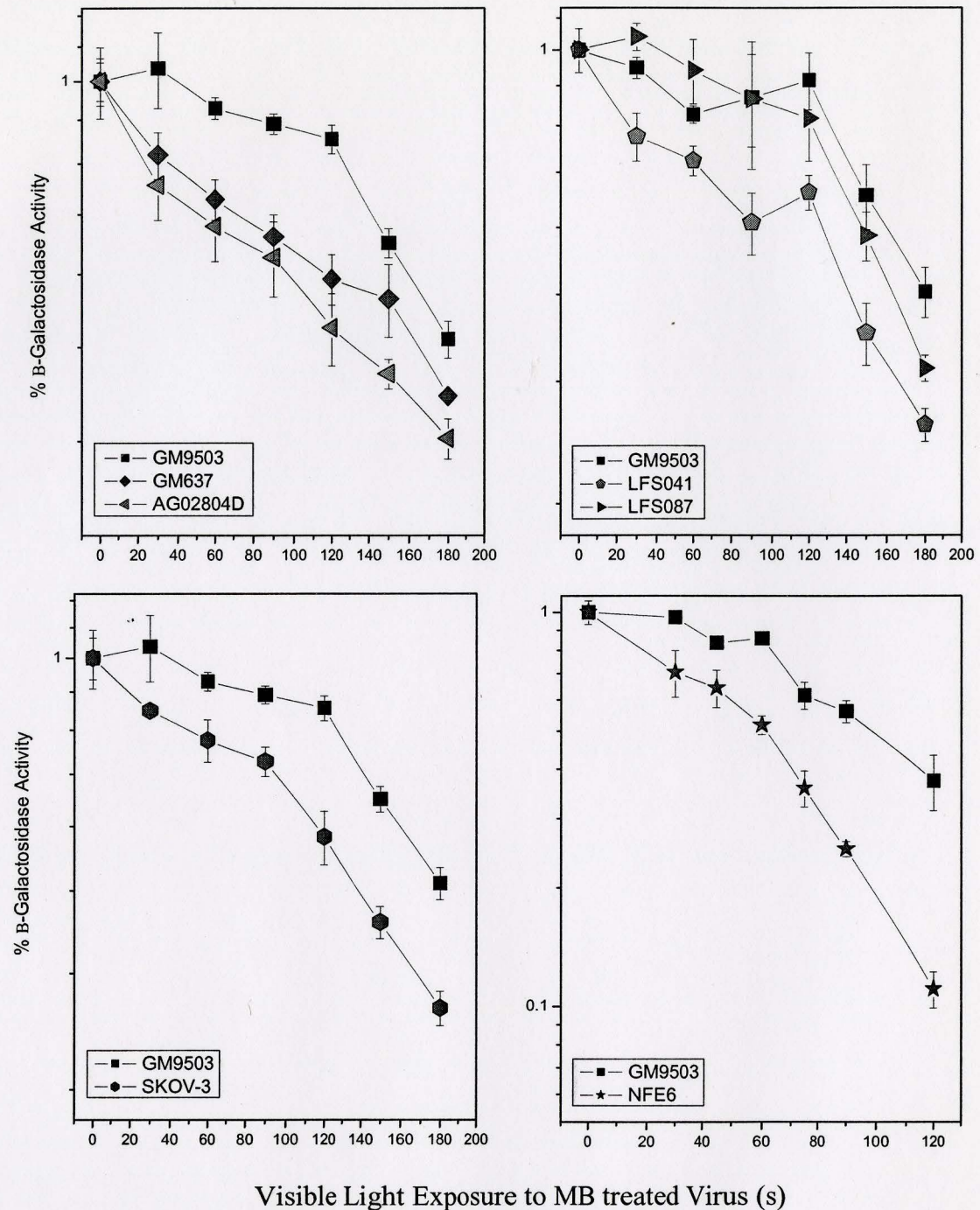‡ significance tested by the two sampled independent t-test (p<0.05).

§ significantly different compared to all the normals.

Transformation of cell lines is facilitated by, papilloma virus (§) and SV40 virus (¥).
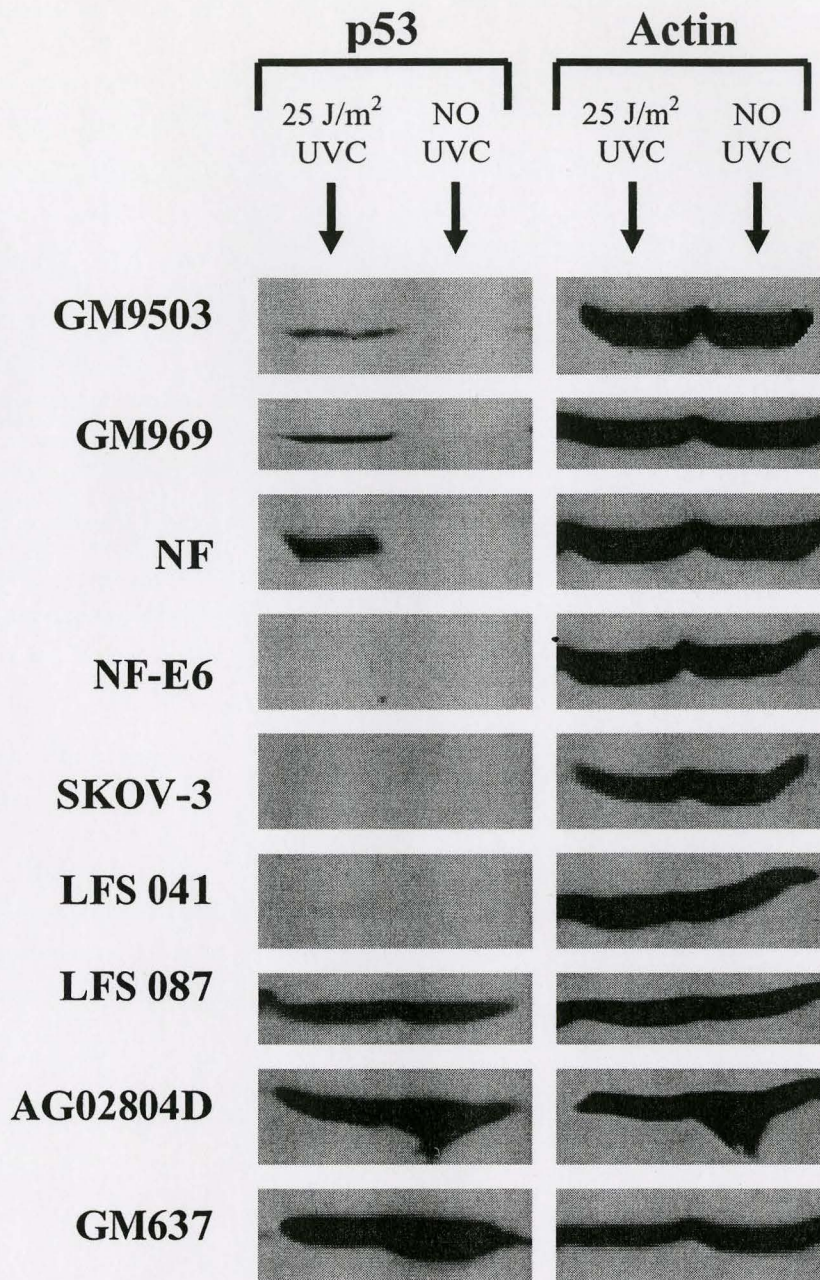
Figure 10: Western blot of p53 protein expression in normal GM9503, GM969, NF as well as p53 compromised NF-E6, SKOV-3, LFS 041, LFS 087, AG02804D, and GM637 cell lines. Cell lines were either pretreated with 25 J/m^2 of UVC or untreated, and lysates were collected 24 hours later. Blots was probed with anti-p53 antibody, stripped and re-probed with anti-actin to confirm equal loading. LFS 087, AG02804D, GM637 and all Actin blots were allowed to expose film from between 15 sec – 1 minute. p53 blots for GM9503, GM969, NF, NFE6, SKOV-3 and LFS 041 were allowed to expose film overnight.
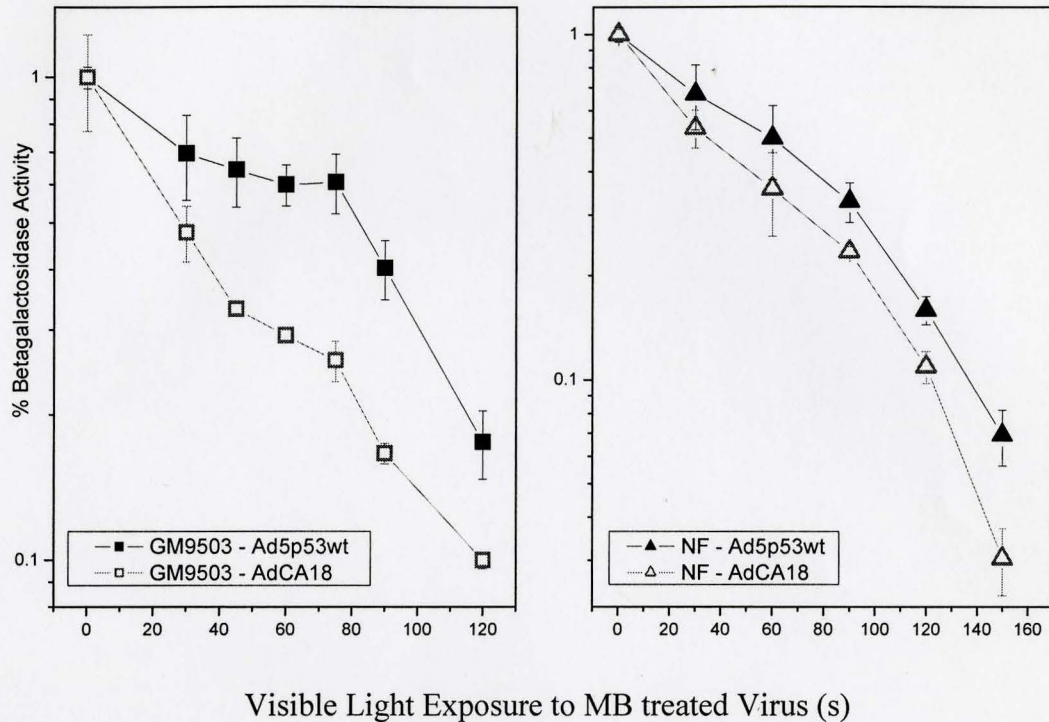
Visible Light Exposure to MB treated Virus (s)

Figure 11: HCR of β-galactosidase activity for MB+VL-treated Ad5HCMV*lacz* virus in normal human fibroblast cells pretreated with Ad5p53wt (solid shapes) or AdCA18 (outlined shapes). Cells were pre-infected with Ad5p53wt or AdCA18 at an MOI of 200 followed by infection at an MOI of 40 pfu/cell with untreated or [20 μg/ml of MB + VL]-treated Ad5HCMV*lacz* and subsequently harvested 44-48 hours after infection. Results shown are for a representative experiments and include GM9503 (■) and NF (▲). Each point is the average ± SE of triplicate determinations, done in one experiment. Cells in each panel were assayed in the same experiment.

Table 6 HCR capability for β-galactosidase activity of [20μg/ml MB + VL] treated Ad5HCMV*lacz* virus as measured by the actual D_{37} values in normal cell lines pre-infected with Ad5p53wt virus compared to the same cell lines pre-infected by the AdCA18 control.

Cell Line	Group	Average Actual D_{37} †			# of Experiments	Does p53 Preinfection result in a significant increase compared to CA18 Preinfection ‡	P(Value)
			p53	S.E.			
No UVC pretreatment following pre-infection							
GM9503	Normal		78.71	15.13	4	No	0.57
NF	Normal		63.38	7.58	2	No	0.33
GM969	Normal		104.66	18.23	2	No	0.87
25 J/m² UVC Pretreatment following pre-infection							
GM9503	Normal		95.65	14.263	4	No	0.80
NF	Normal		80.49	9.42	2	No	0.44
GM969	Normal		104.95	27.25	2	No	0.84

† Mean visible light exposure to reduce reporter expression to 37% of that observed in the untreated reporter construct in a given cell line then averaged over all experiments done under same conditions.

‡ significance tested by the two sampled independent t-test (p<0.05).
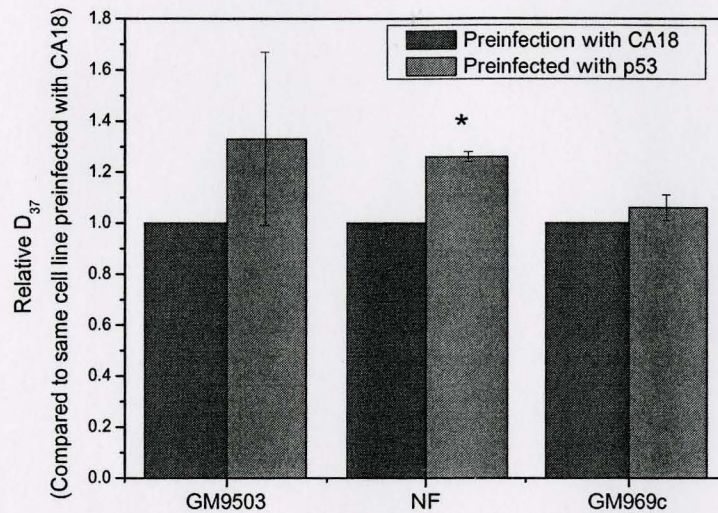
Figure 12: Relative D_{37} values obtained from HCR assays for ß-gal activity in cells pre-infected with Ad5p53wt or the AdCA18 control in normal fibroblasts. Cells were pre-infected at an MOI of 200 pfu/cell with Ad5HCMVp53wt or AdCA18. 24 hours later the same cells were infected at an MOI of 40 pfu/cell with untreated or [20 µg/ml of MB + VL]-treated Ad5HCMV*lacz* and subsequently harvested 44-48 hours after infection. Results shown are the average relative $D_{37} \pm$ SE for each cell line infected with p53, shown as a % increase, versus the control virus of 2 – 4 independent experiments. D_{37} values significantly increased compared to the control infected cells by the 2 sample independent t-test (p<0.05) are indicated (*).

Table 7:   HCR capability for β-galactosidase activity of [20μg/ml MB + VL]-treated Ad5HCMV*lacz* virus as measured by the relative D_{37} values in normal cell lines pre-infected with Ad5p53wt virus compared to the same cell lines pre-infected by the AdCA18 control.

Cell Line	Group	Relative Average D_{37} †			# of Experiments	Does p53 Preinfection ‡ result in a significant increase compared to CA18 pre-infection	P(Value)
		CA18	p53	S.E.			
No UVC pretreatment following pre-infection							
GM9503	Normal		1.33	0.34	4	No	0.37
NF	Normal		1.26	0.02	2	Yes	0.004
GM969	Normal		1.06	0.05	2	No	0.34
25 J/m² UVC Pretreatment following pre-infection							
GM9503	Normal		1.06	0.08	4	No	0.48
NF	Normal		1.13	0.11	2	No	0.37
GM969	Normal		1.09	0.005	2	Yes	0.003

†- Calculated by taking D_{37} [20μg/ml MB+VL exposed virus] for p53 infected cell line / D_{37} [20μg/ml MB+VL exposed virus] for same control infected cell line, then averaged over all experiments under same conditions.

‡ - significance tested by the two sampled independent t-test (p<0.05).

Visible Light Exposure to MB treated Virus (s)
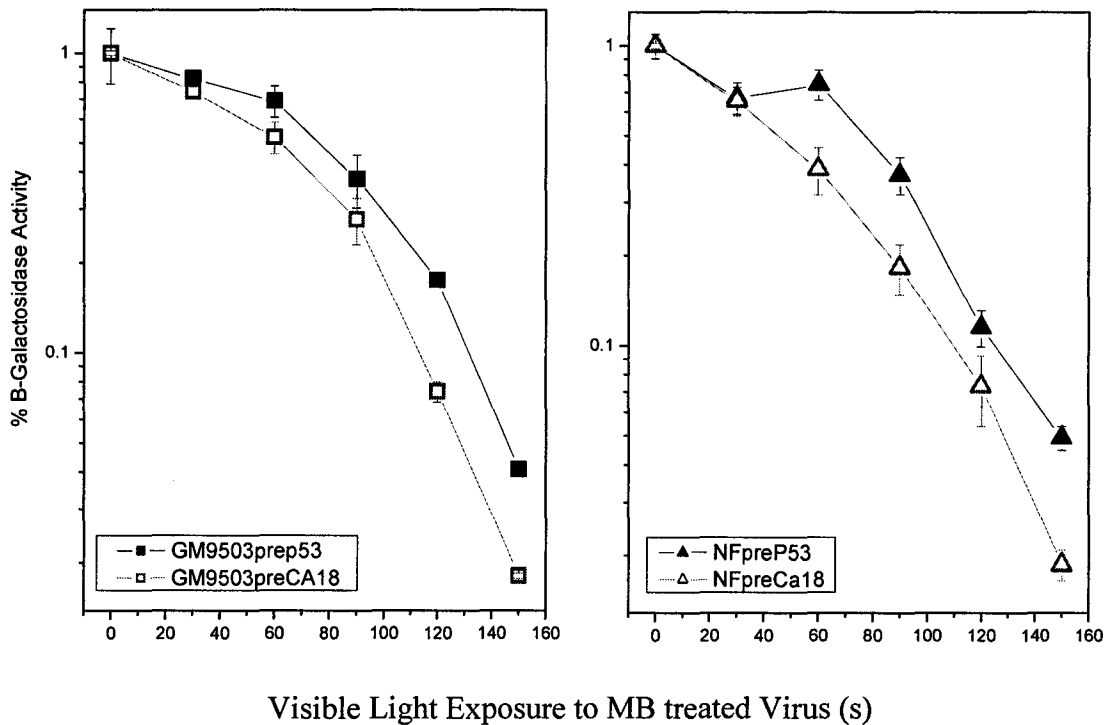
Figure 13: HCR of β-galactosidase activity for MB+VL-treated Ad5HCMV*lacz* virus in normal human fibroblast cells pretreated with Ad5p53wt (solid shapes) or AdCA18 (outlined shapes) followed by UVC pretreatment. Cells were pre-infected with Ad5p53wt or AdCA18 at an MOI of 200 followed by treatment with 25 J/m^2 of UVC then infection at an MOI of 40 pfu/cell with untreated or [20 μg/ml of MB + VL]-treated Ad5HCMV*lacz* and subsequently harvested 44-48 hours after infection. Results shown are for a representative experiments and include GM9503 (■) and NF (▲). Each point is the average ± SE of triplicate determinations, done in one experiment. Cells in each panel were assayed in the same experiment.
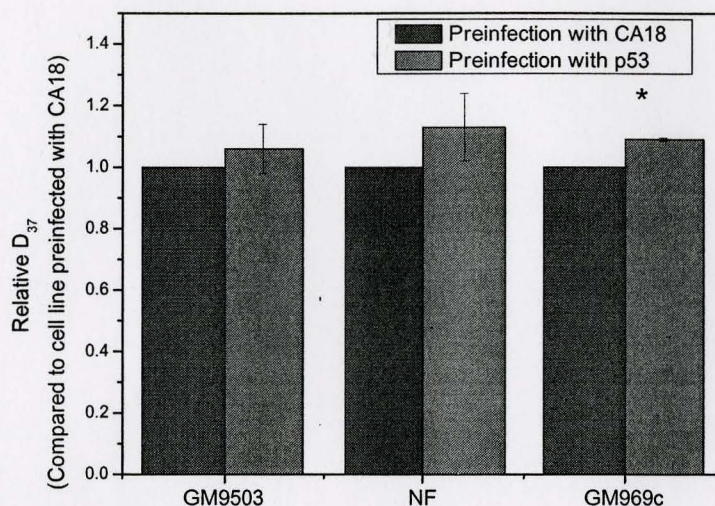
Figure 14: Relative D_{37} values obtained from HCR assays for ß-gal activity in cells pre-infected with Ad5p53wt or the AdCA18 control followed by 25 J/m^2 UVC pretreatment in normal fibroblasts. Cells were pre-infected at an MOI of 200 pfu/cell with Ad5HCMVp53wt or AdCA18 followed by treatment with 25 J/m^2 UVC then infected at an MOI of 40 pfu/cell with untreated or [20 µg/ml of MB + VL]-treated Ad5HCMV*lacz* and subsequently harvested 44-48 hours after infection. Results shown are the average relative $D_{37} \pm$ SE for each cell line infected with p53, shown as a % increase, versus the control virus of 2 – 4 independent experiments. D_{37} values significantly increased compared to the control infected cells by the 2 sample independent t-test (p<0.05) are indicated (*).

Table 8 - HCR capability for β-galactosidase activity of [20μg/ml MB + VL] treated Ad5HCMV*lacz* virus as measured by the relative D_{37} values in normal cell lines treated in different ways compared to the same non-UVC-treated cell lines pre-infected by the AdCA18 control.

No UVC pretreatment following pre-infection					
Cell Line	Group	Relative Average D_{37} †		# of Experiments	Does p53 pre-infection result in a significant increase compared to CA18 pre-infection ‡
		p53	S.E.		P(Value)
GM9503	Normal	1.33	0.34	4	No	0.370
NF	Normal	1.26	0.02	2	Yes	0.004
GM969	Normal	1.06	0.05	2	No	0.340
Average Normal	Normal	1.25	0.16	8	No	0.151

Effect of UVC pretreatment of CA18 infected cells					
Cell Line	Group	Relative Average D_{37} †		# of Experiments	Does UVC pretreatment result in a significant ‡ increase compared to non UVC treated CA18 pre-infected cells
		UVC pretreated CA18	S.E.		P(Value)
GM9503	Normal	1.547	0.341	4	No	0.160
NF	Normal	1.438	0.165	2	No	0.116
GM969	Normal	0.960	0.045	2	No	0.468
Average Normal	Normal	1.373	0.185	8	No	0.064

Effect of UVC pretreatment of p53 infected cells					
Cell Line	Group	Relative Average D_{37} †		# of Experiments	Does UVC pretreatment of p53 infected cells result in a significant increase compared to non UVC treated CA18 pre-infected cells ‡
		UVC pretreated p53	S.E.		P(Value)
GM9503	Normal	1.564	0.211	4	Yes	0.037
NF	Normal	1.602	0.025	2	Yes	0.002
GM969	Normal	1.045	0.045	2	No	0.423
Average Normal	Normal	1.443	0.131	8	Yes	0.004

§   the differently treated cell lines (No UVC, UVC, UVC + Ad5p53wt) are all compared to non-UVC-treated cells, infected with AdCA18.

†   Calculated by taking D_{37} [20μg/ml MB+VL exposed virus] for the differently treated cell lines / D_{37} [20μg/ml MB+VL exposed virus] of same cell lines infected with AdCA18 only, then averaged over all experiments under same conditions.

‡   significance tested by the two sampled independent t-test (p<0.05).

Appendix:

Appendix 1 – p53 expression resulting from Ad5p53wt and AdCA18 infection

Introduction:

To increase the intracellular levels of wild type p53, we infected various cell lines with recombinant adenovirus, Ad5p53wt (Bachetti and Graham, 1993), expressing the wild type p53 gene. As a control, we infected the same cell lines with another recombinant adenovirus construct, AdCA18 (Addison et al, 1997), expressing the firefly luciferase gene. The luciferase protein is not known to be involved in DNA repair, and as such serves as a proper control. It was, however, of interest to determine if the infection of Ad5p53wt did in fact increase the intracellular levels of p53, and what effect if any, did infection with AdCA18 have on p53 expression in the same cell lines.

Materials and Methods:

GM9503 cells were seeded in six well cell culture dishes (Falcon, Franklin Lakes, NJ) at a density that would achieve monolayer. Between 18-24 hours after seeding, media was aspirated, and cells were infected with Ad5p53wt or AdCA18 at an MOI of 200 in incomplete a-mem. The infection proceeded for 90 minutes at which point it was stopped with the addition of complete a-MEM. The cells were then allowed to incubate for 24 hours at which point they were scraped off the dishes into centrifuge tubes, and centrifuged for 10 minutes at 1000 rpm. The cells were then re-suspended in 10 ml PBS, and re-centrifuged for 10 minutes at 1000 rpm. The cells were suspended in lysis buffer (50 mM Tris, 150 mM NaCl, 1% NP40) containing protease inhibitors. After centrifugation (1 min at 13000 rpm), the supernatants were isolated and protein

concentrations were measured in duplicate using the Bio-Rad protein reagent in a Bradford assay (Bio-Rad, Richmond, CA). Protein aliquots were prepared and 30-40 µg of each protein sample was loaded and separated by SDS-PAGE (10%). Proteins were then transferred onto a nitrocellulose membrane and blocked overnight at 4 °C in 10% skim milk in TBS with 0.05% Tween 20. Blots were then probed with a mouse monoclonal antibody to p53 conjugated to HRP (Santa Cruz Biotechnology, Santa Cruz, CA). Protein loading was verified by monitoring actin levels in each lane. After the addition of ECL staining reagent (Western Lightning Chemiluminescence Reagent, PerkinElmer Life Sciences), blots were visualized by exposure to Kodak X-Omat AR film.

Results and Discussion

Western blots probing for p53 and actin expression for GM9503 cells infected with Ad5p53wt and AdCA18 are shown in figure A-1. It can be seen that the infection of cells with AdCA18 does not cause any appreciable p53 expression in cells, where the levels remain at the non-infected basal levels. In contrast, it can be seen that infection with Ad5p53wt virus does cause a substantial increase in the amount of p53 in the cell. This confirms that Ad5p53wt infection does increase p53 expression, while AdCA18 does not, and is therefore an appropriate control.
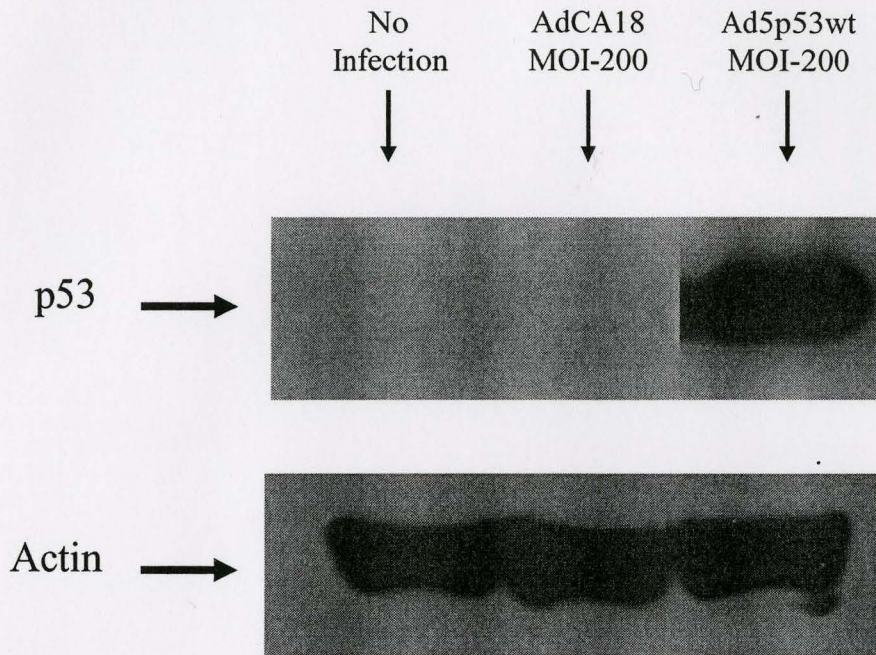
Figure A-1: Western blot of p53 protein expression in normal GM9503. Cells were either infected with Ad5p53wt, AdCA18 or not infected, and lysates were collected 24 hours later. Blots was probed with anti-p53 antibody, stripped and re-probed with anti-actin to confirm equal loading. The p53 blots were allowed to expose film overnight, while, the actin blots were allowed to expose film for 15 seconds (private communication from D. Dregoesc, McMaster University).

Appendix 2 – The HCR of the ß-galactosidase gene following damage induced by visible light only.

Introduction:

It was seen in Chapter 2 that methylene blue (MB) + visible light (VL) reduces expression of the ß-gal gene in a dose dependent manner. MB + VL is known to create predominantly 7,8-dihydro-8-oxoguanine (8-OxoG) (Floyd et al, 1989) which is repaired by the base excision repair pathway (reviewed by Slupphauge et al, 2003). VL on the other hand has been shown to create 8-OxoG, as well as cyclobutane pyrimidine dimers (CPDs) (Kielbassa et al, 1997) which are repaired by the nucleotide excision repair (NER) pathway (reviewed by Balagee et al, 1999). As our studies focus on the BER pathway, it was important to ensure that the damage induced by VL was minimal compared to the damage created by MB + VL. This was done by examining the HCR of a MB + VL damaged, and a VL only damaged reporter construct in various cell lines.

Materials and Methods:

The normal cell lines used were, GM637, AG02804D, while the NER deficient cell lines used were: XPCSV40, CSBSV40, GM4429DXPA. The experimental technique followed has been previously described in Chapter 2. In this case however, there was also a separate Petri dish created that contained no MB, and just PBS with AdCA17 virus. In this way the HCR of a VL and a MB + VL damaged reporter could be investigated in the same experiment.

Results and Discussion:

A representative curve for the HCR of ß-gal activity for VL and MB + VL damage reporter is shown in figure A-2. Although the cell lines showed a significant reduction due to VL treatment alone compared to the non visible light treated control, it can be seen from this figure, that the cell lines are substantially more proficient at repairing the VL alone damage compared to the MB + VL damage. This suggests that only a small percentage of the damage created by MB + VL is due to the VL component. If the damage due to MB + VL was predominantly due to VL, we would expect equal proficiencies in repair of the two types of damage. This figure also suggests that the creation of CPDs by VL is small, as we picked up no significant differences in repair in the NER deficient cell lines (XPC, CSB, XPA) compared to the normal cell lines. The substantial difference in repair between VL and MB + VL induced damage would then suggest that VL excitation of MB is much more efficient at creating 8-OxoG than VL alone.
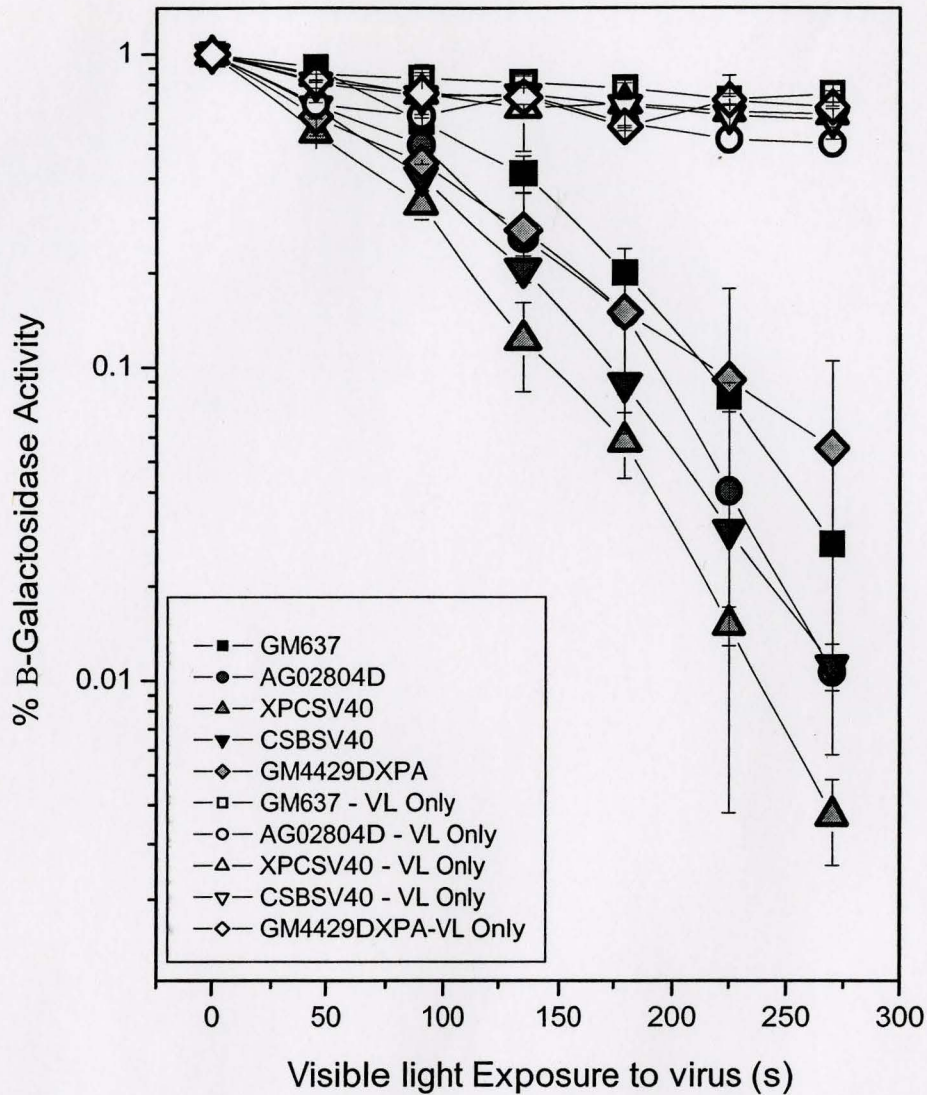
Figure A-2: HCR of ß-galactosidase activity for VL treated (Outlined shapes) and MB+VL-treated (Solid shapes) Ad5HCMV*lacz* virus in various SV40 transformed human fibroblasts. Cells were infected at an MOI of 20 pfu/cell with [0μg/ml of MB+ VL] or [20 μg/ml of MB + VL]-treated Ad5HCMV*lacz* and subsequently harvested 40-44 hours after infection. Each point is the average ± SE of 1-3 experiments each with triplicate determinations.

Appendix 3 – Optimal conditions for HCR of MB+VL damaged reporter appear to be Ad5HCMV*lacz* virus treated with 20 µg/ml MB, infected at an MOI of 20, harvested 40-44 hours post infection.

Introduction:

The host cell reactivation (HCR) assay tests the ability of a cell to repair a damaged reporter construct and subsequently express the protein encoded by that construct. The assay is made possible because the protein expressed is easily detectable (Addison et al, 1997), and thus detection of protein correlates to repair of the gene. It is therefore important to harvest the cells after allowing for the appropriate amount of repair. If given sufficient time, even deficient cell lines will show repair abilities which could be comparable with those of the normals, as deficient cell lines still possess some ability to repair the construct. At sufficiently early times after infection, all cell lines will show a similar low level of ß-gal expression, as none of the cells will have actually begun to repair the damage. It was therefore of interest to determine what amount of time after infection was appropriate to highlight any differences in repair.

Materials and Methods:

HCR assays were completed as described in Chapter 2, with the difference being in the amount of time given to cells to repair the damaged virus after infection. The two time points chosen were 24 hours, and 40 + hours after infection. These experiments were conducted for the SV40 transformed and primary human fibroblasts. The cell lines

used to highlight differences in repair were the normal GM637 and GM9503, and the

NER deficient XPCSV40 (XPC) and GM677 (XPC).

Results and Discussion:

Representative curves of HCR abilities in SV40 transformed, and primary human

fibroblasts are shown in figure A-3. It can be seen from these figures that a significant

difference in HCR ability is seen in both types of cells at the 40 + hour time point. In

contrast, the 24 hour time points do not show any significant differences between cell

lines, where the variation within cell lines is great. Consequently, all subsequent HCR

experiments were performed by examining ß-gal expression at greater than 40 hours after

infection with AdCA17.

Visible light exposure to MB treated Virus (s)
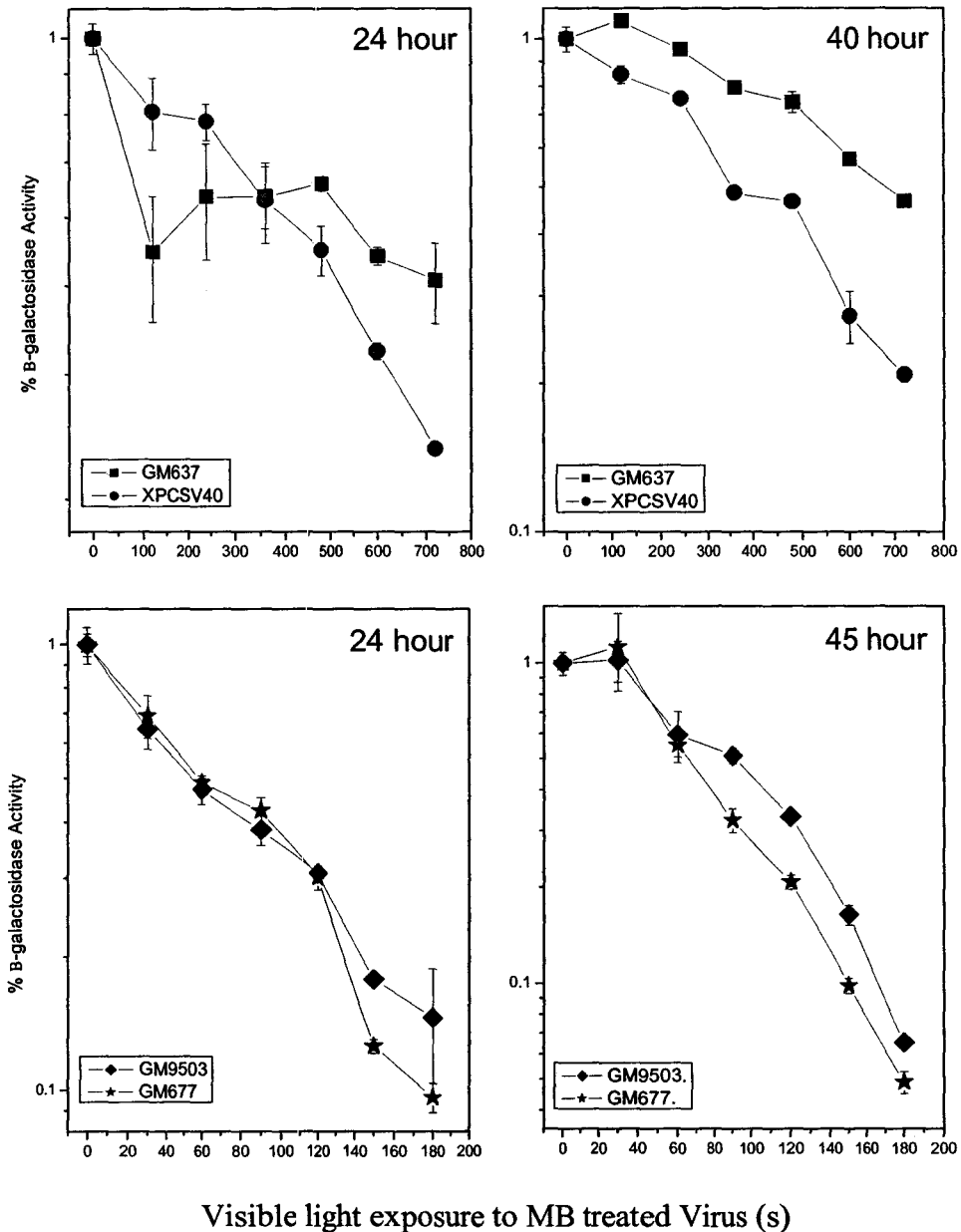
Figure A-3: HCR of ß-galactosidase activity for MB+VL-treated Ad5HCMV*lacz* virus in primary (bottom panels) and SV40 transformed (top panels) human fibroblasts at 24 (left panels) and 40 + (right panels) hours. Cells were infected with untreated or [20 µg/ml of MB + VL]-treated Ad5HCMV*lacz* and subsequently harvested 24 or 40 + hours after infection. Results shown are for a representative experiment and include in top panels: GM637 (■) and XPCSV40 (●). In bottom panels GM9503 (◆) and GM677 (★). Each point is the average ± SE of triplicate determinations, done in one experiment. Cells in each panel were assayed in the same experiment.

Chapter 3:

A p53 dependent role for the XPC protein in base excision repair of oxidative DNA damage induced by methylene blue plus visible light in human cells.

Chapter 3

A p53 dependent role for the XPC protein in base excision repair of oxidative DNA damage induced by methylene blue plus visible light in human cells.

Abstract:

Methylene blue (MB) acts as a photo-sensitizer and after excitation by visible light (VL) produces reactive oxygen species that result in oxidative damage to DNA. (MB+VL) produces predominantly 8-OxoG as well as other single base modifications in DNA that are repaired by base excision repair (BER). In contrast UVC produces predominantly cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4PPs) in DNA that are repaired by nucleotide excision repair (NER). AdCA17 is non-replicating recombinant human adenoviruses that can efficiently infect a variety of mammalian cell types and express the β-galactosidase (β-gal) reporter gene driven by the human cytomegalovirus immediate early promoter. We have examined HCR of β-gal activity for (MB+VL)-treated AdCA17 in several nucleotide excision repair (NER) proficient normal cells as well as several NER deficient human cells from patients with Xeoroderma Pigmentosum (XP) and Cockayne syndrome (CS). We have reported previously a substantial reduction in HCR for the UVC-treated reporter gene in CSA, CSB, XPA, XPB, XPC, XPD, XPE, XPF and XPG cells compared to normal cells, consistent with a substantial involvement of the *CS* and *XP* genes in the removal of CPDs and 6-4 PPs from the reporter gene by NER. In contrast, HCR for the MB+VL-treated reporter gene was substantially reduced only in XP-C-SV40 cells with only a small or no reduction in

SV40-transformed CSB, XPA, XPD, XPF and XPG cells compared to SV40-transformed normal cells. These results indicate a role for the *XPC* gene in BER of (MB+VL)-induced DNA damage. We also examined constitutive and inducible repair of MB+VL-induced DNA damage by examining HCR of β-gal activity for (MB+VL)-treated AdCA17 in untreated and pre-UVC-treated untransformed human fibroblasts. Results indicate that both constitutive and UVC-induced HCR of the MB+VL-treated reporter gene was reduced in three XP-C fibroblast strains compared to that in NER proficient normal fibroblasts giving further evidence for an involvement of the XPC protein in BER. In addition by pre-infecting cells with a recombinant adenovirus, Ad5p53, which results in increased p53 expression, we show that increased expression of p53 alone results in enhanced HCR of the MB+VL-treated reporter gene in normal but not XP-C fibroblasts, consistent with a p53 dependent involvement of the XPC protein in BER of MB+VL-treated DNA.

Introduction:

One of the consequences of aerobic respiration is oxidative damage. Although oxygen allows us to efficiently create energy, it also has the potential for being very toxic to our cells (Valko et al, 2004). The most biologically relevant site of oxidative damage is the DNA, where any damage to the integrity of the genetic code can lead to carcinogenesis (Klaunig et al, 2004). The site of oxidative damage in the DNA is predominantly the bases of nucleotides, in particular the guanine bases (Burrows et al, 1998, Neta et al, 1988), where the 7,8-dihydro-8-oxoguanine (8-OxoG) is the most

common oxidative lesion (reviewed by Slupphaug, 2003). The mutation potential of this lesion is very great as it can base pair with the correct cytosine base or the incorrect adenine base with equal efficiency (Duarte et al, 1999).

The major repair mechanism of oxidative DNA damage is the base excision repair (BER) pathway (reviewed in Slupphaug et al, 2003 and Dianov et al, 2001). BER is a multi-step, multi-protein repair pathway that involves the recognition, excision and replacement of nucleotides with oxidatively damaged bases. The oxidized base is recognized and excised by a group of proteins called glycosylases. There are many known human glycosylases, each with specificity for a specific set of oxidative base damage (Croteau and Bohr, 1997). The removal of the oxidized base initiates the action of AP-Endonuclease (APE), which excises the abasic nucleotide. Polymerases and ligases then fill in and ligate the gap in the DNA.

Another repair mechanism that is important for the integrity of DNA is the nucleotide excision repair (NER). NER is responsible for the removal of bulky adducts from the DNA including those created by exposure to solar radiation. The NER pathway can be divided into two sub pathways: transcription coupled repair (TCR) and global genome repair (GGR). TCR is responsible for the rapid removal of bulky adducts from the transcribed strand of active genes, where as GGR removes these adducts from the non-transcribed strand of active genes as well as the rest of the genome at large. The two sub pathways differ only in the initial recognition step of NER. Detection of the bulky adduct is facilitated by the XPC and XPE proteins in GGR, where as the CSA and CSB proteins are responsible for this step in TCR. The XPA, XPB and XPD proteins are then

recruited to the site of damage and act to unwind the DNA in the vicinity of the damage. XPF and XPG then incise the DNA upstream and downstream from the damage, and an oligonucleotide containing the bulky adduct is removed. DNA polymerases then fill in the gap created and the new and old DNA are ligated together. (For a review of NER and its sub pathways see Hoeijmakers et al, 2000, Balagee et al, 1999). The HCR of UV-induced damage has been shown to be inducible (Francis et al, 2000, McKay et al, 1997), and this induction is thought to require p53 (McKay et al, 1997). The multi functional p53 protein is very important to DNA repair, where it can act as a transcription factor or a regulator of protein function (reviewed by Adimoolam and Ford, 2003). Its role in NER has predominantly been shown to be in GGR, where it facilitates the transcription of the XPC and the XPE proteins (Adimoolam and Ford, 2002, Hwang et al, 1999). For this reason, patients with germline mutations in the p53 protein, have lower abilities to perform NER (McKay et al, 1997).

Another group of patients with reduced NER are the Xeroderma pigmentosum individuals, who are extremely sensitive to the sun and have a 1000 times higher incidence of skin cancer (Hoeijmakers et al, 2000). Individuals with XP can be divided into 7 NER deficient complementation groups XPA through XPG. Similarly, Cockayne syndrome individuals also show a deficiency in NER, exhibiting sun sensitivity, neurodegeneration but no elevated risk of skin cancer and can be divided into two complementation groups, CSA and CSB. These 7 XP proteins and 2 CS proteins are needed in NER, and a mutation in any one can lead to the XP or CS syndrome

respectively. (XP and CS syndromes are discussed in Lehmann, 2003, and Friedberg et al, 1995).

It has been suggested that the accumulation of un-repaired oxidative damage results in neuronal cell death and that the neurodegeneration seen in all CS patients and some XP (so called XP/CS) patients results from an involvement of some of the XP and CS genes in the BER of oxidative DNA damage (Le Page et al, 2000, Reardon et al, 1997). Specifically, it has been shown that patients from classical XPA, XPD and XPG who do not show neurodegeneration are normal in the TCR of oxidative damage, whereas the XPG/CS, CSA and CSB patients have a deficiency in the repair of oxidative damage (Cooper et al, 1997). The mutations in XPG/CS patients lead to truncations in the protein, whereas those in classical XP generally have a point mutation (Cooper et al, 1997), suggesting that the XPG domain involved in NER is different from that involved in BER. Similarly, the XPD and XPB proteins are also involved in the TCR of oxidative damage (LePage et al, 2000). Classical XPG, XPD and XPB individuals had normal BER repair abilities, whereas patients with the XP/CS phenotypes showed a deficiency in the BER of oxidative damage. In addition it has been reported that the XPC interacts both physically and functionally with a glycosylase that recognizes oxidative damage suggesting a role for the XPC protein in BER (Shimizu et al, 2003). An involvement of XPC in some aspect of BER is supported by Runger et al, who report a reduced host cell reactivation of a MB+VL-treated plasmid in three out of four XPC lymphoblast cell lines tested. The fact that one of the XPC cell lines tested did not show a deficiency in HCR

suggests that different domains of XPC protein may be required for BER compared to NER.

Most of the previous studies designed to examine the role of the NER protein in BER have examined the repair of cellular DNA in cells treated with various oxidizing agents. Since the treatment of cells themselves with an oxidizing agent can result in an induced BER response for the removal of some oxidative lesions (Le et al, 1998, Weinfeld et al, 2001) most of the previous reports reflect the involvement of NER genes in the combined constitutive and inducible BER response of cells. We have reported previously the use of a non-replicating recombinant human adenovirus that expresses the β-galactosidase (β-gal) reporter gene to examine both constitutive and inducible repair of UVC-damaged DNA (Rainbow et al, 2000). In the present report we have used a similar non-replicating recombinant human adenovirus, Ad5HCMVlacz, to examine constitutive and inducible BER of methylene blue (MB) + visible light (VL) damaged DNA in human cells. VL irradiated MB is known to excite molecular oxygen to singlet oxygen (Floyd et al, 2004), which has been shown to create predominantly 7,8-dihydro-8-oxoguanine (8-OxoG) in the DNA (Epe et al, 1988). As MB + VL induces 8-OxoGs in the viral genome expression of β-galactosidase from the damaged reporter gene is expected to occur only if the host cell is able to repair these lesions by BER. Host cell reactivation (HCR) of β-gal activity for MB+VL-treated Ad5HCMVlacz was examined in non-treated and pre-UVC-treated NER proficient as well as NER deficient human cells. Results indicate that HCR of the MB+VL-treated reporter gene was reduced in XP-C compared to NER proficient normal cells giving evidence for an involvement of the XPC protein in BER of

MB+VL-damaged DNA. In addition using a recombinant adenovirus, Ad5p53, we show that increased expression of p53 alone results in enhanced HCR of the MB+VL-treated reporter gene in normal but not XP-C cells, consistent with a p53 dependent involvement of the XPC protein in BER of MB+VL-treated DNA.

Materials and Methods:

Cell lines and virus constructs.

The repair-proficient primary human cell lines: GM 9503, GM 969, and GM 38A, along with the NER deficient XP12BE (GM 5509; XP-A), XP2BE (GM 677; XP-C), XP1MI (GM 2096; XP-C), XP1BE (GM 10881; XP-C), CS1AN (GM 739; CS-B) and all the SV40 transformed normal GM637 (Normal), XPASV40 (XP20OS; XPA), GM4429DXPA (XP12BE; XPA), XPCSV40 (XP4PA; XPC), XPD (XP6BE; XPD), XPFSV40 (XP2Y0; XPF), XPG (XP3BR; XPG) and CSBSV40 (CS1AN; CSB) were obtained from NIGMS (Camden, NJ). The other SV40 transformed normal AG02804D was obtained from NIA (Camden, NJ). A normal neonatal foreskin (NF) fibroblast strain (established by Dr. D. A. Galloway, Fred Hutchinson Cancer Research Centre, Seattle, WA), was obtained from Dr. B. C. McKay, Centre for Cancer Therapeutics, Ottawa Regional Cancer Centre, Ottawa, Ontario, Canada.

All cell cultures were grown at 37°C in a humidified incubator in 5% CO_2 and cultured in Eagle's a-minimal essential media (a-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, Grand Island, NY).

The recombinant adenovirus Ad5HCMV*lacz* (AdCA17), Ad5HCM*luc* (AdCA18) (Addison et al, 1997) and Ad5p53wt (Bachetti and Graham 1993), were obtained from Dr. F.L. Graham (McMaster University). These vectors have had the E1 region deleted and are therefore unable to replicate in cells. Ad5HCMV*lacz* carries the bacterial *lacz* gene which is under the control of the human CMV-IE promoter. The virus was propagated, collected, and titred as described previously (Prevec et al, 1991). It was shown, by western blot analysis, that infection with Ad5p53wt increased cellular expression of p53 compared to infection with Ad5HCMV*luc* or no infection at all (appendix 1).

Methylene Blue and Visible Light.

Methylene Blue (Methylthionine chloride Trihydrate) was prepared by taking 0.02g of MB (Sigma Chemicals) in powder form, and dissolving it in 20ml of PBS at $37^{\circ}C$ to make a concentration of $1000\mu g/ml$. The solution was filter sterilized and aliquots were frozen at $-20\ ^{\circ}C$ in the dark. When needed, frozen aliquots were thawed in the dark and diluted appropriately for use. The Visible Light (VL) irradiation of cells and virus occurred under a General Electric 1000 watt halogen lamp (GE R1000). The stage was set at an 83 cm distance below the bulb. Irradiation of virus occurred at a distance of 70 cm from the bulb as a magnetic stir plate was used during the HCR experiments. The effect of MB + VL and VL alone was investigated, and it was shown that VL alone had a minimal effect compared to the effect of MB + VL (appendix 2).

HCR of MB+VL treated AdHCMVLacz virus in cells.

Seeding:

Flasks containing confluent cells of each cell line were trypsinized using 2X trypsin/EDTA and resuspended in supplemented a-MEM, and counted with a haemocytometer. The cells were diluted appropriately with supplemented a-MEM so that SV40 transformed, LFS, and SKOV-3 cells were seeded at a density of 3.8×10^4 cells/well, and primary human fibroblasts were seeded at a density of 1.9×10^4 cells/well in 96-well plates (Falcon, Franklin Lakes, NJ). Cells were left to incubate for 24 hours. (Incubation occurred in 37°C in a humidified incubator in 5% CO_2 unless otherwise stated).

Virus Preparation and Irradiation:

Viral suspensions were prepared and irradiated by suspending 80 μl AdHCMV*Lacz* virus in 3.6ml of Phosphate Buffered Saline (PBS) in 35 mm Petri dishes on ice. The MB solution was then added to the Petri dishes to create the appropriate concentration of MB. With continuous stirring, the virus suspensions were irradiated (or mock irradiated) with visible light, for various amount of time. After each time point, 400 μls of irradiated virus prep was removed from the Petri dishes and diluted appropriately in 2ml of unsupplemented a-MEM on ice. The newly diluted irradiated viral suspensions were vigorously vortexed to ensure homogeneity, and were used to infect cells within 20 minutes.

Pretreatment of cells with UVC:

If cells were to be pretreated with UVC immediately before infection of the prepared virus, the following procedure would be followed. The overlaying media was aspirated, and 40 µl of warmed PBS was added to each well. The source of UVC was a General Electric germicidal lamp (model G8T5) emitting predominantly at 254 nm. The stage was set at a distance that facilitated a fluence of 1 J/m^2/s, and the cells were irradiated for 25 seconds. Immediately after treatment, the cells were infected as follows.

Viral Infection:

The overlaying media was aspirated from one horizontal row of the 96 well plate, and the cells were immediately infected with 40 µl of the appropriate irradiated virus. This was repeated for each row of the 96-well plate with the appropriate virus sample. The infection was allowed to carry on for 90 minutes at which point 160 µl of complete a-MEM was added to each well to stop the infection, and the cells were allowed to incubate at 37 °C for 44-48 hours prior to harvesting. It was determined that the 40 + time point of harvesting cells was optimal for assessing repair proficiencies between cell lines (appendix 3).

Harvesting and Scoring:

Incubation media was aspirated and 60 µls of chlorophenolred ß-D-galactopyranoside (CPRG; prepared in 0.01% Triton X-100, 1 mM MgCl$_2$, 100 mM phosphate buffer at pH 8.3; Boehringer-Mannheim, Indianapolis, IN) was added to the infected cells. To assay for the expression of the ß-galactosidase levels from the *Lacz* gene, optical density readings were taken using a 96-well plate reader (Bio-Tek Instruments EL340 Bio

Kinetics Reader) at several time intervals at 570 nm. When CPRG is in the presence of the *Lacz* gene product, ß-galactosidase, the CPRG turns from a yellow colour to a purple colour. Average optical density of control wells vs. time was plotted in order to observe the saturation curve for the control wells. A point just under the saturation plateau was taken and analyzed in further detail. Survival curves were derived from this point. For plotting the survival curves, the average background level of ß-galactosidase activity was subtracted from each averaged point from the measurements taken from a minimum of triplicate wells.

Pooling data over many experiments:

The D_{37} value of a survival curve is the dose, in this case VL exposure, required to reduce the ß-gal activity of a cell line to 37% of the non treated control of that same cell line. D_{37} was used as a measure of the cellular HCR ability, such that a cell line with a higher average D_{37}, has a better ability to repair MB + VL induced damage.

The actual D_{37} values of each cell line was determined from curves of each experiment and expressed as a ratio of the actual D_{37} value of an arbitrarily chosen normal. This is the relative D_{37} value compared to a normal. The relative D_{37} of the chosen normal in each experiment would therefore always be 1, and those cell lines with an actual D_{37} higher than the actual D_{37} of the gold standard would have a relative D_{37} greater than 1. The Relative D_{37} values for each cell line were then averaged over many experiments and expressed with its % standard error.

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Results:

HCR of the MB+VL-treated reporter gene in normal and NER deficient SV40 transformed human fibroblasts.

The HCR of ß-gal activity for MB+VL treated virus was investigated in normal and NER deficient SV40 cell lines. Typical survival curves for ß-gal activity for MB+VL-treated AdCA17 virus in the various SV40 transformed cells are shown in figure 1. It can be seen that HCR of the MB+VL-treated reporter gene is reduced in the SV40-transformed NER deficient cell lines compared to the two normal SV40-transformed normal cell lines. The VL exposure in seconds required to reduce β-gal activity to 37% of that for non VL-exposed virus (D_{37}) was used as a measure of HCR and mean D_{37} values from multiple experiments are shown in Table 1. It can be seen that the mean D_{37} values were lower in the NER deficient cells compared to that for the two normal cell lines. In particular there was a significant reduction in HCR for the XP-C cell line compared to that of the normal cells lines, suggesting a substantial involvement of the XPC gene in BER of the MB+VL-treated reporter gene. In order to account for the variation in D_{37} values in individual experiments we also calculated the D_{37} value for each of the various NER deficient SV40-transformed cell lines relative to the D_{37} value of the SV40 transformed normal cell lines obtained in individual experiments. The D_{37} value of each NER deficient cell lines relative to each of the two normal cell lines were then averaged over many experiments. The average relative D_{37} values for each cell line compared to the normal line GM637 as well as the normal line AG02804 are shown in figure 2 and Table 2. It can be seen that the relative HCR values for the MB+VL-treated

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reporter gene were reduced in the NER deficient cell lines compared to that in the normal cell lines. All the NER deficient cell lines tested, except GM4429DXPA, showed a significant reduction in HCR compared to the GM637 normal, whereas only the XPCSV40 cell line showed a significantly reduced HCR compared to both the GM637 and the AG02804 normal. Previously published results for HCR of β-gal activity for the UVC-treated reporter gene in the same SV40-transformed normal and NER deficient cell lines are shown for comparison in Table 3. All the NER deficient cell lines show a marked deficiency in HCR of the UVC-treated reporter gene compared to that in the NER proficient normal strain and relative HCR values ranged from 0.067 for the XP-D to 0.53 for the CS-B cell line. In contrast the HCR deficiency of the MB+VL-treated reporter gene was much less with relative HCR values ranging from 0.56 for the XP-C lines to 0.82 for one of the XP-A cell lines.

HCR of the MB+VL-treated reporter gene in normal and NER deficient human fibroblasts.

The HCR results using the SV40-transformed cells suggested an involvement of the *XPC* gene in the BER of MB+VL-induced oxidative DNA damage. We were therefore interested in examining HCR of ß-gal activity for the MB+VL-treated reporter gene in normal and NER deficient human fibroblasts including three NER deficient strains from the XPC complementation group. Typical survival curves for ß-gal activity of the MB+VL-treated reporter gene in normal human fibroblasts are shown in figure 3. It can be seen that HCR of the MB+VL-treated reporter gene was similar for each of the

4 normal human fibroblast strains tested. Typical survival curves for ß-gal activity of the MB+VL-treated reporter gene in the various NER deficient human fibroblasts relative to that in the normal GM9503 strain are shown in figure 4. It can be seen that the XP-C strains GM10881, GM677 and GM2096, and the XP-A strain GM5509, but not the CS-B strain GM739, show a reduction in HCR compared to the GM9503 normal. Mean D_{37} values for β-gal survival in the various normal and NER deficient fibroblasts are shown in Table 4. Mean D_{37} values for each cell line relative to that for the GM9503 normal fibroblast strain obtained in individual experiments are shown in figure 5 and Table 5. It can be seen that the relative HCR values of the MB+VL-treated reporter gene were reduced in the 3 XP-C and the XP-A strain, but not the CS-B strain compared to that in the 4 normal strains.

Inducible HCR of MB + VL induced reporter damage in normal, and NER deficient cell lines.

We have reported previously (see chapter 2) that pre-UVC-treatment of human cells results in an enhanced HCR of the MB+VL-treated reporter gene indicating an inducible BER response for MB+VL-induced oxidative DNA damage in human cells. In addition, this induced response was shown to result from both p53 dependent as well as p53 independent pathways. It was therefore considered of interest to examine inducible HCR of the MB+VL-treated reporter gene in NER deficient fibroblasts, in particular XP-C fibroblasts. Representative survival curves for β-gal activity of the MB+VL-treated reporter gene in UVC pretreated compared to non-treated normal cells are shown in

figure 6. Representative survival curves for ß-gal activity of the MB+VL-treated reporter gene are shown for the NER deficient cell lines in figure 7. The relative D_{37} value obtained in UVC-pretreated cells compared to that in non-treated cells was determined for each experiment and the mean relative D_{37} values for several experiments are shown in figure 8 and Table 6.   It can be seen that there is enhancement in all 4 of the normal fibroblast strains, indicating that BER of MB + VL induced damage is inducible by UVC in normal cells (as reported in chapter 2).   It can be seen also that pre-UVC-treatment of cells resulted in enhanced HCR of the MB+VL-treated reporter gene in all the NER deficient fibroblast strains tested.

HCR of the MB + VL- treated reporter gene in pre-UVC-treated cells.

Representative survival curves of ß-gal activity for MB + VL-treated AdCA17 in UVC-pre-treated normal fibroblasts are shown in figure 9, whereas figure 10 shows representative curves for ß-gal activity for MB + VL-treated AdCA17 in the UVC-pre-treated NER deficient fibroblasts. Mean D_{37} values for β-gal survival in pre-UVC-treated cells are shown in Table 7 and mean D_{37} values for each cell line relative to that for the GM9503 normal fibroblast strain obtained in individual experiments are shown in figure 11 and Table 8. It can be seen that HCR of the MB + VL-treated reporter gene in pre-UVC-treated fibroblasts was similar in the four different normal fibroblasts. In contrast, the XP-C fibroblast strains (GM677, GM2096 and GM10881) as well as the XP-A deficient strain (GM5509) show a significantly reduced HCR compared to that in the

normal fibroblast strain GM9503. These results suggest that the XPC and XPA proteins play a role in BER of MB + VL damage in pre-UVC-treated fibroblasts.

HCR of the MB + VL-treated reporter gene in human fibroblasts pre-infected with Ad5p53wt virus.

We have reported previously (Chapter 2) that pre-UVC- treatment of human cells results in an enhanced HCR of the MB+VL-treated reporter gene through both p53 dependent and p53 independent mechanisms. In addition we have shown that increased expression of p53 alone leads to enhanced HCR in normal human fibroblasts. Recent reports indicate that the XPC and p48XPE proteins are inducible by pre-UVC-treatment of cells through a p53 dependent upregulation leading to an enhancement in the global genomic repair (GGR) pathway of NER (Fitch et al, 2003, Adimoolam and Ford, 2002). Thus the reduced HCR of the MB+VL-treated reporter gene in pre-UVC-treated XP-C compared to pre-UVC-treated normal human fibroblasts (figure 9 and Table 9) may result, in part at least, from the p53 dependent upregulation of the XPC protein in pre-UVC-treated normal human fibroblasts. It was therefore considered of interest to examine the effect of increased p53 expression alone on the HCR of the MB+VL-treated reporter gene in XP-C cells. HCR of the MB + VL-treated reporter gene was examined in fibroblasts that had been pre-infected with Ad5p53wt or a control virus, Ad5HCMV*luc* (AdCA18). Ad5p53wt (Bachetti and Graham 1993) expresses wild type p53 in cells, while the control AdCA18 expresses the luciferase protein in a similar E1 deleted recombinant adenovirus construct (Addison et al, 1997). Normal and XP-C fibroblasts

were infected with Ad5p53wt or AdCA18 at an MOI of 200, and 24 hours later infected with MB + VL-treated AdCA17 and assayed for ß-gal activity 44-48 hours later. Representative survival curves of ß-gal activity for MB + VL-treated AdCA17 in normal and XP-C fibroblasts pre-infected with Ad5p53wt or AdCA18 are shown in figure 12. Mean D_{37} values for the β-gal survival curves obtained from several experiments are shown in table 9. In addition, the relative D_{37} value in cells pre-infected with Ad5p53wt compared to cells pre-infected with AdCA18 were determined for individual experiments and the mean relative D_{37} values for several experiments are given in figure 13 and Table 10 (top segment). It can be seen that pre-infection of cells with Ad5p53wt resulted in an enhanced HCR compared to pre-infection of cells with AdCA18 in normal fibroblasts (as reported previously in Chapter 2), but not in XP-C fibroblasts indicating a p53 dependent upregulation of HCR in normal but not XP-C cells. In addition the combination of pre-infection with Ad5p53wt and pre-UVC-treatment of cells, table 10 (bottom segment), resulted in a significantly further upregulation of HCR in the normal (GM9503, NF and average normal) fibroblasts but not in XPC (GM677) fibroblasts, giving further evidence of p53 dependent upregulation of XPC in BER of a MB+VL-treated reporter gene.

Discussion:

NER is an evolutionary conserved DNA repair pathway that removes bulky DNA damage induced by solar irradiation, and requires the XP and CS proteins to function efficiently (for review of NER see Hoejimakers et al, 2000, and Balagee et al, 1999). A

deficiency in any one of these proteins can have significant clinical repercussions including sun sensitivity, increased cancer incidence and in some cases neurodegeneration (Lehman, 2003). It has been suggested by Reardon et al and others, that some of these clinical symptoms in XP and CS patients may result from an inability to repair less bulky oxidative damage such as the 8-OxoG by BER (Reardon et al, 1997 and reviewed by Hojimakers et al, 2000) and there is mounting evidence for an involvement of several of the XP and CS proteins in BER (Reardon et al, 1997, Osterod et al, 2002, and Ward et al, 2003).

HCR of the MB+VL-treated reporter gene in normal and NER deficient cells

We have reported previously a substantial reduction in HCR for the UVC-treated reporter gene in CSA, CSB, XPA, XPB, XPC, XPD, XPE, XPF and XPG cells compared to normal cells, consistent with a substantial involvement of the *CS* and *XP* genes in the removal of CPDs and 6-4PPs from the reporter gene by NER (Table 3; Francis et al, 2000, Francis et al, 1997). In contrast, HCR for the MB+VL-treated reporter gene was substantially reduced only in XP-C SV40 cells, with only a small or no reduction in SV40-transformed CSB, XPA, XPD, XPF and XPG cells compared to SV40-transformed normal cells. These differences in HCR values for the various XP and CS cells are thought to reflect the different spectrum of DNA lesions induced by UVC compared to MB+VL and indicate a significant role for the *XPC* gene in BER of (MB+VL)-induced DNA damage.

Infection and transformation by the SV40 virus results in the abrogation and inactivation of the p53 protein by the SV40 large T-antigen (Meitz et al, 1992, as reviewed by Pipas et al, 2001). The abrogation of p53 leads to the immortalization of these cells (Manos et al, 1984) as well as a deficiency DNA repair since p53 has been reported to play a role in several DNA repair processes (and reviewed by Sengupta et al, 2005). The results of figure 2 show a small, but significant reduction in HCR of the MB-VL-treated reporter gene in the SV40 transformed XP-A, XP-D, XP-F, XP-G and CS-B cells as well as in the AG02804D normal line when compared to that in the GM637F normal. The difference in HCR between the two normal cell lines GM637 and AG02804 could be due to the existence of polymorphisms in genes involved in DNA repair. It has been shown for example that certain polymorphisms in the XPC and XPD genes lead to different repair capacities for oxidative DNA damage (Qia et al, 2002). It is therefore possible that the AGO2804D normal has a polymorphism in one or more of its XP genes that reduce its BER capacity of MB+VL-induced DNA damage. Another more likely reason for the difference in HCR between the two SV40 transformed normal cell lines could be that BER is cell type specific, as the AG02804D cell line was derived from a lung fibroblast whereas the GM637 line was derived from a skin fibroblast. Previous reports have also suggested that the cellular capacity for the repair of oxidative DNA damage is cell-type specific (de Waard et al, 2003). Although the reduced HCR in the SV40 transformed XP-A, XP-D, XP-F and XP-G cells was not significant when compared to the AG02804 cells (Table 3) their small reduction in HCR compared to the GM637 cell line suggests some small involvement of the XPA, XPD, XPF, XPG and

CSB genes in the repair of MB+VL-induced DNA damage. This is consistent with the results of Reardon et al who show that although 8-OxoG lesions are generally removed by BER they can also be removed more slowly by the human NER system (as defined by the requirement for the entire set of excision repair proteins encoded by the XP genes) in cell free extracts *in vitro* (Reardon et al, 1997).

In contrast, the reduction in HCR for the XPCSV40 cell line was substantial compared to both the GM637 and AG02804 normal cell lines indicating a substantial involvement of the XPC protein in BER of MB+VL-induced oxidative DNA damage. In addition the three XP-C fibroblast strains showed a reduced HCR compared to thât in the normal fibroblast strains for both untreated as well as pre-UVC-treated fibroblasts, giving further evidence that the *XPC* gene is involved in BER of MB + VL induced damage. These findings are consistent with the results of Runger et al, who have examined HCR of a MB+VL-treated plasmid in untreated human lymphoblast cell lines (Runger et al, 1995). HCR of the MB+VL-treated plasmid was reduced compared to normal in three of for XP-C lympoblast cell lines suggesting the role of the XPC protein in BER is domain specific. The XPC lymphoblast cell line used by Runger et al showing normal HCR and the GM10081 fibroblast strain used by us showing reduced HCR were obtained from the same patient. This discrepancy may arise from a difference in sensitivity of the two assays or some cell type specific difference. Although the exact role of XPC in BER is yet to be determined, Shimizu et al have shown that the XPC protein interacts physically and structurally with a glycosylase involved in BER, facilitating the detection of thymine

glycols in the DNA (Shimizu et al, 2003). This suggests that XPC may play a role in the detection of oxidative damage, similar to its role in NER.

Runger et al also show no reduction in HCR of a MB+VL-treated reporter gene in XP-A lymphoblasts whereas we show reduced HCR in the XPASV40 cell line and the GM5509 XP-A fibroblast, but not the GM4429D SV40 transformed XP-A cell line. This may suggest a domain specific activity of XPA in BER which would help to reconcile differences in the literature concerning the role of XPA in BER (Waard et al, 2003, Leadon et al, 1993 and Runger et al, 1995).

There are several reports indicating a role for *CSB* in BER (Cooper et al, 1997, Waard et al, 2003, LePage et al, 2000, Dianov et al, 1999). In the present work we show a significant reduction in HCR of the MB+VL-treated reporter gene in the SV40-transformed CS-B fibroblasts compared to the normal GM637 line but not in the CS-B fibroblasts compared to that in normal fibroblasts. In addition XPB, XPD and XPG fibroblasts derived from XP/CS patients but not from classical XP patients also showed significant reductions in repair of oxidative damage (Cooper et al, 1999 and LePage et al, 2000). Since the SV40 transformed XPD and XPG deficient cell lines used in our present study were from classical XP and pseudo-classical patients respectively, we would expect these cells to harbor slight to no deficiency in BER of oxidative damage.

Inducible HCR of MB+VL-treated reporter gene in normal and NER deficient cell lines.

We have reported previously that pre-UVC-treatment of human cells results in an enhanced HCR of the MB+VL-treated reporter gene and the increased HCR was detected

in both normal and p53 compromised cells (see chapter 2). In addition, increased expression of p53 by pre-infection of cells with Ad5p53wt (which expresses wild type p53) also resulted in an increased HCR in the normal fibroblasts giving evidence that increased expression of p53 alone enhances BER in normal human fibroblasts. These results indicate that BER of MB+VL-damaged DNA is inducible in human cells by pre-UVC-treatment and that the enhancement in BER can result from both p53 dependent and p53 independent mechanisms. Increased BER capacity following pretreatment of cells with DNA damaging agents has also been reported previously by others (Tomasevec et al, 1998, Lan et al, 2003). It was therefore of interest to examine HCR of the MB+VL-treated reporter gene in pre-UVC-treated NER deficient fibroblasts. In the present work we show that pre-UVC-treatment of cells resulted in varying degrees of enhanced HCR of the MB+VL-treated reporter gene for both the NER proficient and NER deficient strains tested. Since UVC-treatment of cells results in a p53 dependent upregulation of the XPC protein (Adimoolam and Ford, 2002) and there is evidence that the XPC protein plays a role in BER, the upregulation of HCR in pre-UVC-treated normal human fibroblasts could result, in part at least, from a p53 dependent upregulation of XPC. In the present report we show that increased expression of p53 by pre-infection of cells with Ad5p53wt resulted in a significant increase in HCR for the normal fibroblasts but not for the XP-C fibroblasts, consistent with a p53 dependent involvement of the XPC protein in BER of MB+VL damaged DNA.

Visible Light Exposure to MB treated virus (s)
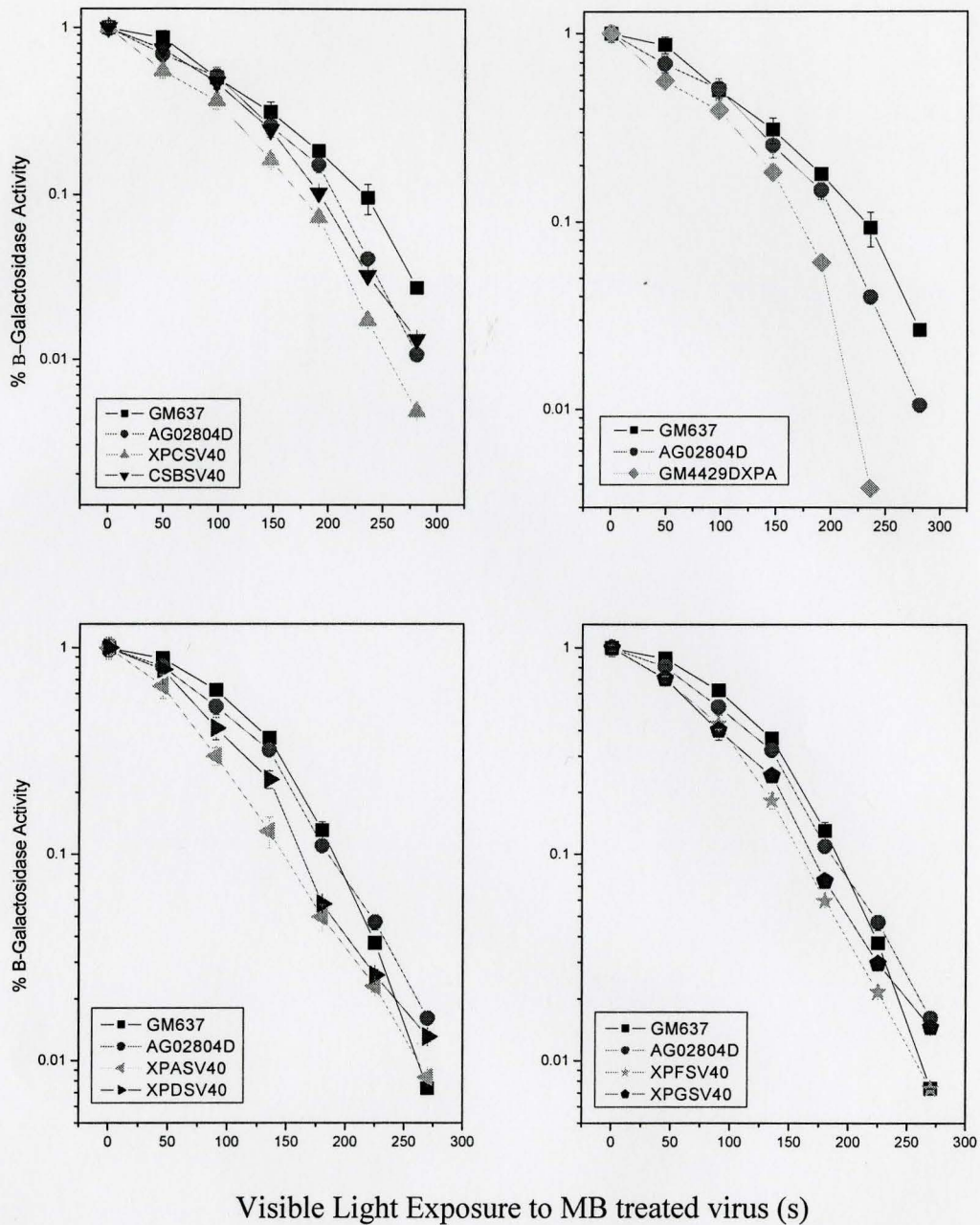
Figure 1: HCR of β-galactosidase activity for MB+VL-treated Ad5HCMV*lacz* virus in SV40 transformed human fibroblast cells. Cells were infected at an MOI of 20 pfu/cell with untreated or [20 µg/ml of MB + VL]-treated Ad5HCMV*lacz* and subsequently harvested 40-44 hours after infection. Results shown are for a representative experiment and include the following SV40 transformed fibroblasts: GM637 (■), AG02804D (●), XPDSV40 (▲), CSBSV40 (▼), GM4429DXPA(◆), XPASV40 (◀), XPDSV40 (▶), XPFSV40 (✳) and XPGSV40 (⬟). Each point is the average ± SE of triplicate determinations, done in one experiment. Cell lines shown in each panel were included with both normals, GM637 and AG02804D, in different experiments.

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Table 1: HCR capability for β-galactosidase activity of [20μg/ml MB + VL] treated Ad5HCMV*lacz* virus as measured by the actual D_{37} values in SV40 transformed fibroblasts.

Cell Line	Group	Average† Relative D_{37}	% Standard Error	# of Experiments	Actual values Significantly Different Compared to GM637F‡		Relative values Significantly Different Compared to AG02804D‡	
					Significance	P-Value	Significance	P-Value
GM637	Normal	163.920	15.197	9	-	-	No	0.11216
AG02804D	Normal	134.288	7.268	8	No	0.11216	-	-
XPCSV40	XP-C	87.715	6.317	4	Yes	0.00822	Yes	0.00214
CSBSV40	CS-B	115.693	13.447	4	No	0.07825	No	0.20961
GM4429DXPA	XP-A	129.370	13.313	4	No	0.19131	No	0.72916
XPASV40	XP-A	113.102	13.677	5	Yes	0.04717	No	0.16028
XPDSV40	XPD	117.158	15.581	5	No	0.07093	No	0.28357
XPFSV40	XPF	115.532	15.470	5	No	0.06247	No	0.24082
XPGSV40	XPG	116.318	11.352	4	No	0.07727	No	0.19687

†- Mean visible light exposure to reduce reporter expression to 37% of that observed in the unexposed reporter construct in a given cell line then averaged over all experiments done under same conditions.

‡ - significance tested by the two sampled independent t-test (p<0.05).
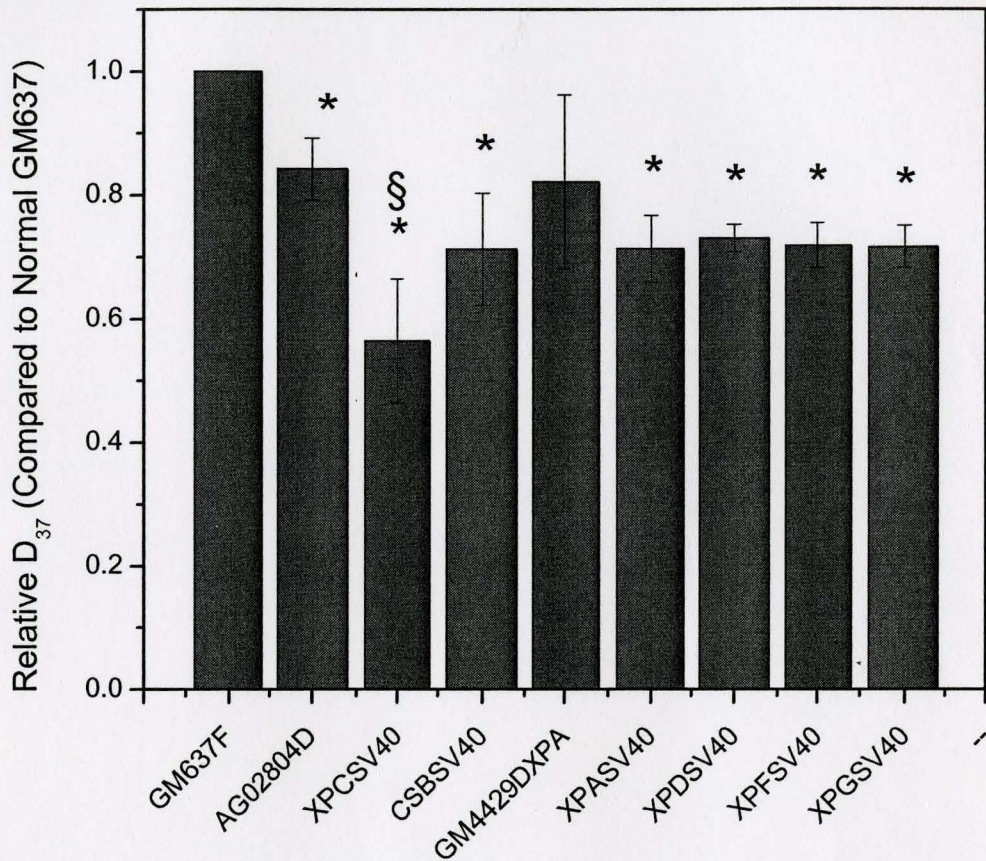
Figure 2: Relative D_{37} values obtained from HCR assays for ß-gal activity in SV40 transformed human fibroblasts. Cells were infected at an MOI of 20 pfu/cell with untreated or [20 µg/ml of MB + VL]-treated Ad5HCMV*lacz* and subsequently harvested 40-44 hours after infection. Results shown are the average relative $D_{37} \pm$ SE for each cell line shown as a ratio of the GM637 of between 4 – 9 independent experiments. Relative D_{37} values significantly decreased than the normal GM637 by the 2 sample independent t-test (p<0.05) are indicated (*), and D_{37} values similarly decreased compared to the normal AG02804D are indicated (§).

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Table 2:   HCR capability for β-galactosidase activity of [20μg/ml MB + VL] treated Ad5HCMV*lacz* virus as measured by relative D_{37} values in SV40 transformed human fibroblast cell lines compared to the GM637 Normal.

Cell Line	Group	Average † Relative D_{37}	% Standard Error	# of Experiments	Relative values Significantly Different Compared to GM637F‡		Relative values Significantly Different Compared to AG02804D‡	
					Significance	P-Value	Significance	P-Value
GM637F	Normal	1	0	9	-	-	Yes	0.00441
AG02804D	Normal	0.8425997	0.050083361	8	Yes	0.00441	-	-
XPCSV40	XPC	0.564222724	0.100307173	4	Yes	2.51E-05	Yes	0.01839
CSBSV40	CSB	0.712063331	0.090756627	4	Yes	0.000369	No	0.19827
GM4429DXPA	XPA	0.820947354	0.141072514	4	No	0.06816	No	0.85956
XPASV40	XPA	0.713558007	0.053568349	5	Yes	7.99E-06	No	0.11958
XPDSV40	XPD	0.730814566	0.021956307	5	Yes	9.06E-10	No	0.12139
XPFSV40	XPF	0.71910323	0.03641937	5	Yes	1.70E-07	No	0.10647
XPGSV40	XPG	0.716758975	0.03429734	4	Yes	4.49E-08	No	0.12937

† Calculated by taking D_{37} [20μg/ml MB+VL exposed virus] for experimental cell line / D_{37} [20μg/ml MB+VL exposed virus] for GM637 normal cell within individual experiments then averaged over all experiments done under same conditions

‡ Significance tested by the two sampled independent t-test (p<0.05).

Table 3 - HCR capability for β-galactosidase activity of [20µg/ml MB + VL]-treated and [UVC]-treated Ad5HCMV*lacz* virus as measured relative D_{37} values in SV40 transformed human fibroblast cell lines compared to Normal.

Cell Line ¶	Group	Average Relative D_{37} § [MB+VL] treated virus	Average Relative D_{37} ¥ [UVC] treated virus
GM637	Normal	1	1
AR5	Normal	ND	1
XPCSV40	XPC	0.564 ± 0.10	0.13 ± 0.04 ‡
CSBSV40	CSB	0.712 ± 0.09	0.53 ± 0.09 †
GM4429DXPA	XPA	0.821 ± 0.141	ND
XPASV40	XPA	0.714 ± 0.05	0.081 ± 0.05 ‡
XPDSV40	XPD	0.731 ± 0.02	0.067 ± 0.03 ‡
XPFSV40	XPF	0.719 ± 0.04	0.37± 0.13 ‡
XPGSV40	XPG	0.717 ± 0.03	0.10 ± 1.01 ‡

§ D_{37} [MB+VL exposed virus] for experimental cell line / D_{37} [MB+VL exposed] virus SV40-transformed GM637 within individual experiments.

¥ D_{37} [UVC exposed virus] for experimental cell line / D_{37} [UVC exposed virus] SV40-transformed AR5 (†) GM637 (‡) within individual experiments. (Francis et al, 2000).

¶   All cell lines were SV40 transformed.
ND - Not Determined.
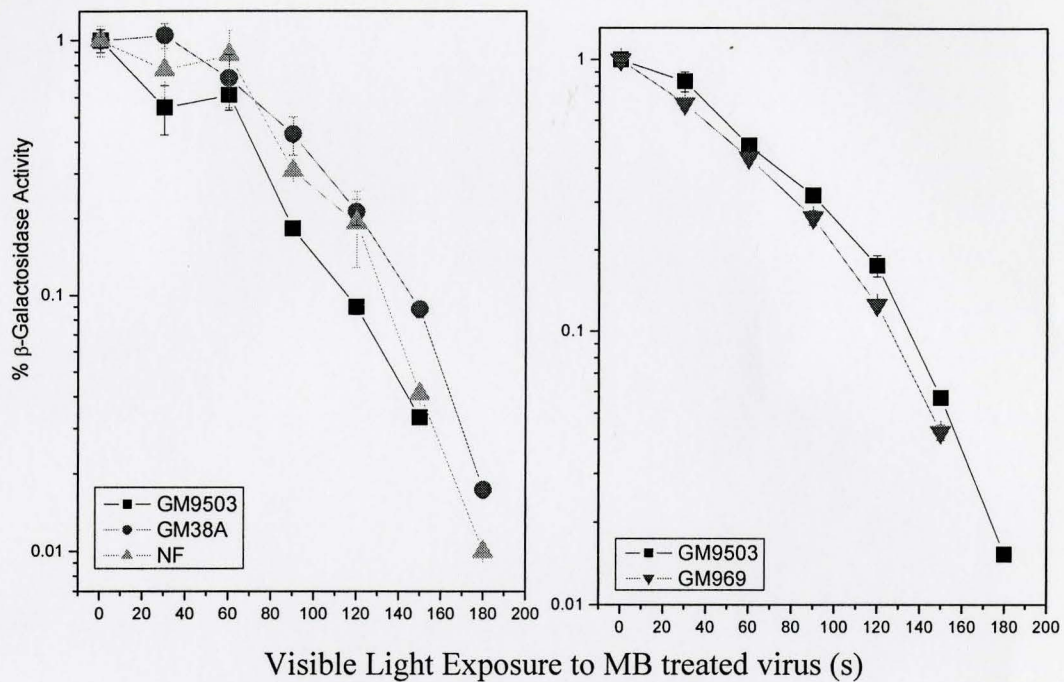
Figure 3: HCR of β-galactosidase activity for MB+VL-treated Ad5HCMV*lacz* virus in normal human fibroblast cells. Cells were infected at an MOI of 40 pfu/cell with untreated or [20 µg/ml of MB + VL]-treated Ad5HCMV*lacz* and subsequently harvested 44-48 hours after infection. Results shown are for a representative experiment and include GM9503 (■), GM38A (●), NF (▲), and GM969 (▼). Each point is the average ± SE of triplicate determinations, done in one experiment. Each cell line was included with GM9503 in different experiments.

Visible Light Exposure to MB treated Virus (s)
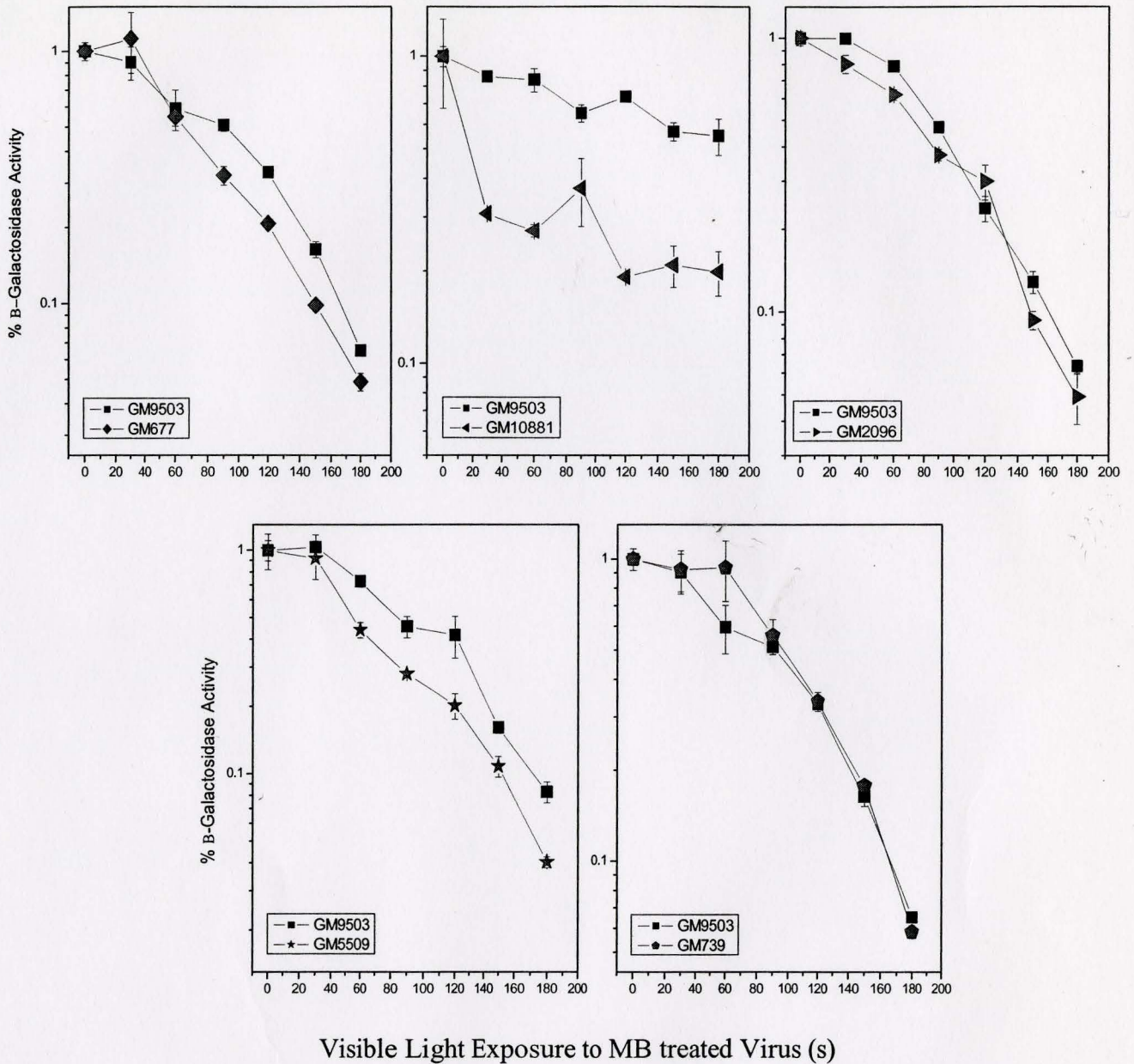
Figure 4: HCR of ß-galactosidase activity for MB+VL-treated Ad5HCMV*lacz* virus in NER deficient human fibroblasts. Cells were infected at a MOI of 40 pfu/cell with untreated or [20 µg/ml of MB + VL]-treated Ad5HCMV*lacz* and subsequently harvested 44-48 hours after infection. Results shown are for a representative experiment and include GM9503 (Normal) (■),XPC- GM677 (♦), XPC - GM10881 (◄), XPC-GM2096 (►), XPA-GM5509 (★), CSB-GM739 (⬢). Each point is the average ± SE of triplicate determinations, done in one experiment. Cells in each panel were assayed in the same experiment.

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Table 4: HCR capability for β-galactosidase activity of [20μg/ml MB + VL] treated Ad5HCMV*lacz* virus as measured by the actual D_{37} values in normal and NER deficient fibroblasts.

Cell Line	Group	Average[†] Actual D_{37}	% Standard Error	# of Experiments	Actual values significantly[‡] Different Compared to GM9503	
GM9503	Normal	97.743	8.124	17	-	-
GM969c	Normal	93.056	7.936	5	No	0.769
GM38A	Normal	72.323	13.727	4	No	0.177
NF	Normal	90.220	9.370	6	No	0.618
GM677	XPC	82.503	9.151	9	No	0.254
GM10881	XPC	76.015	7.765	2	No	0.385
GM2096	XPC	74.593	13.902	4	No	0.218
GM5509	XPA	79.285	13.939	10	No	0.230
GM739	CSB	109.115	9.288	8	No	0.408

† Mean visible light exposure to reduce reporter expression to 37% of that observed in the unexposed reporter construct in a given cell line then averaged over all experiments done under same conditions.

‡ Significance tested by the two sampled independent t-test ($p < 0.05$).
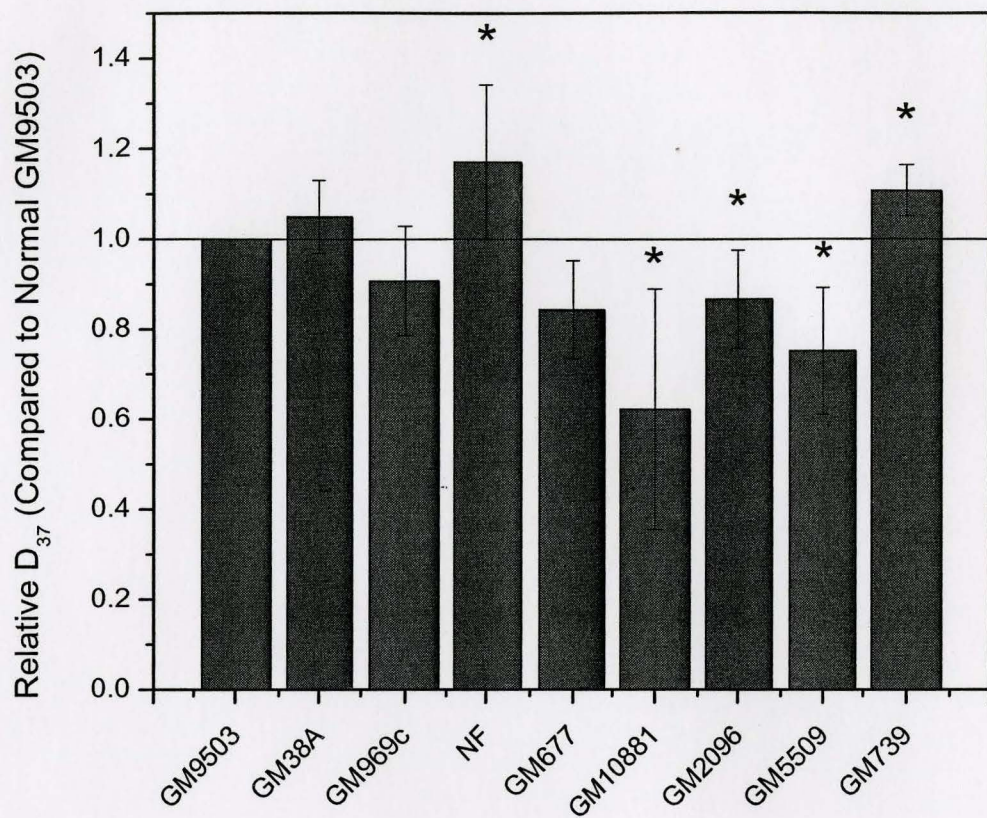
Figure 5: Relative D_{37} values obtained from HCR assays for ß-gal activity in normal and NER deficient human fibroblasts. Cells were infected at an MOI of 40 pfu/cell with untreated or [20 µg/ml of MB + VL]-treated Ad5HCMV*lacz* and subsequently harvested 44-48 hours after infection. Results shown are the average relative D_{37} ± SE for each cell line shown as a ratio of the GM9503 of 3 – 18 independent experiments. Relative D_{37} values significantly different than the normal GM9503 by the 2 sample independent t-test (p<0.05) are indicated (*).

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Table 5:   HCR capability for β-galactosidase activity of [20μg/ml MB + VL] treated Ad5HCMV*lacz* virus
as measured by relative D_{37} values in normal and NER deficient cell lines compared to the GM9503 normal.

Cell Line	Group	Average[†] Relative D_{37}	% Standard Error	# of Experiments	Relative values significantly[‡] Different Compared to GM9503	(P-Value)
GM9503	Normal	1	0	18	-	-
GM38A	Normal	1.049	0.081	5	No	0.229
GM969c	Normal	0.907	0.121	4	No	0.087
NF	Normal	1.170	0.172	4	Yes	0.031
GM677	XPC	0.843	0.109	10	No	0.060
GM10881	XPC	0.622	0.267	3	Yes	0.001
GM2096	XPC	0.867	0.108	5	Yes	0.021
GM5509	XPA	0.752	0.140	9	Yes	0.017
GM739	CSB	1.107	0.057	8	Yes	0.008

† calculated by taking D_{37} [20μg/ml MB+VL exposed virus] for experimental cell line / D_{37} [20μg/ml MB+VL exposed virus]
for GM9503 normal fibroblast within individual experiment then averaged over all experiments done under same conditions.
‡ - significance tested by the two sampled independent t-test (p<0.05).

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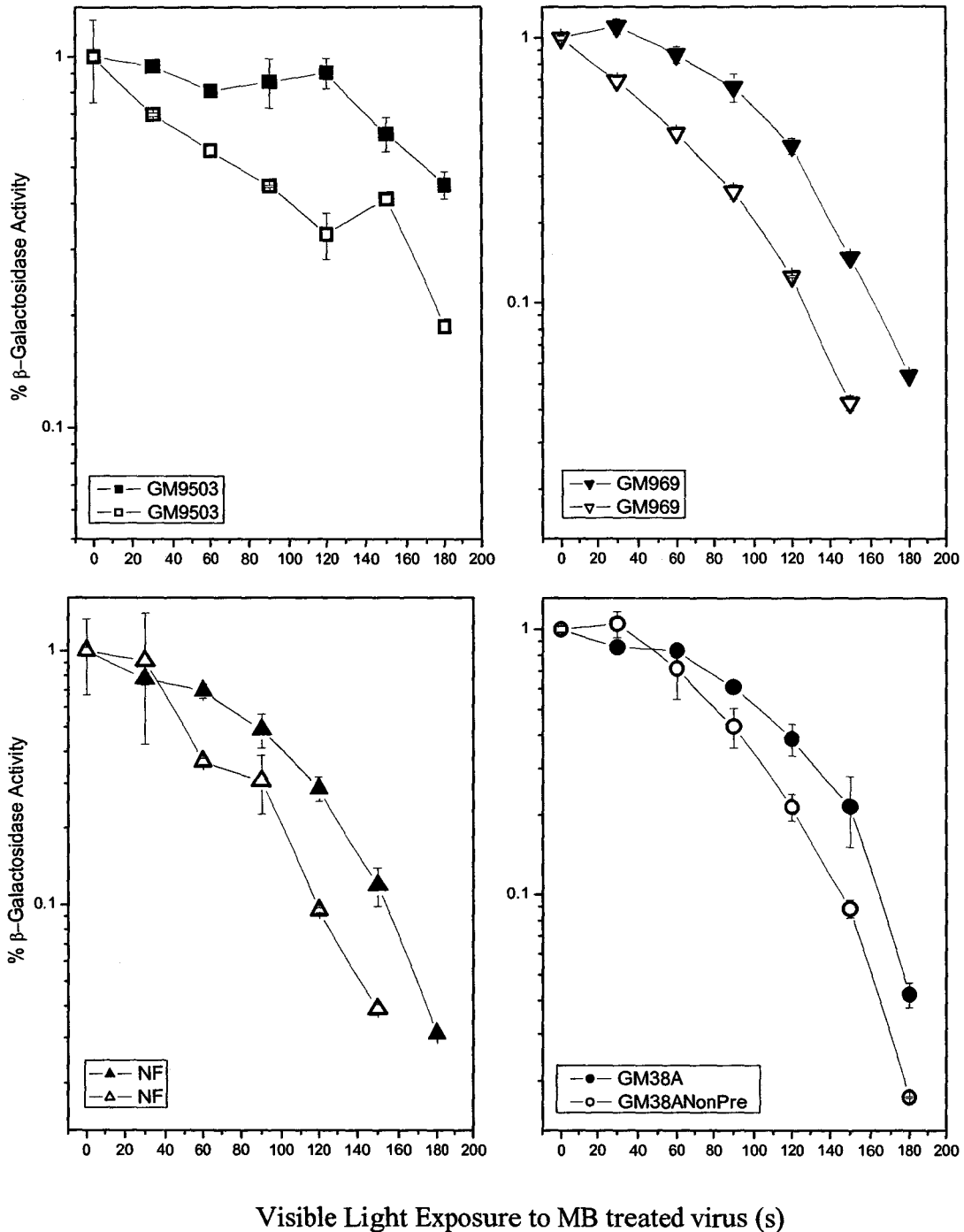
Visible Light Exposure to MB treated virus (s)

Figure 6: HCR of ß-galactosidase activity for MB+VL-treated Ad5HCMV*lacz* virus in UVC pretreated (solid shapes) and non pretreated (outlined shapes) normal fibroblasts. Cells were either irradiated with 25 J/m^2 of UVC or mock irradiated then infected at a MOI of 40 pfu/cell with untreated or [20 µg/ml of MB + VL]-treated Ad5HCMV*lacz* and subsequently harvested 44-48 hours after infection. Each point is the average ± SE of triplicate determinations, done in one experiment. Cells in each panel were assayed in the same experiment.

Visible Light Exposure to MB treated virus (s)

Figure 7: HCR of ß-galactosidase activity for MB+VL-treated Ad5HCMV*lacz* virus in UVC pretreated (solid shapes) and non pretreated (outlined shapes) NER deficient cells. Cells were either treated with 25 J/m^2 of UVC or mock treated then infected at an MOI of 40 pfu/cell with untreated or [20 µg/ml of MB + VL]-treated Ad5HCMV*lacz* and subsequently harvested 44-48 hours after infection. Each point is the average ± SE of triplicate determinations, done in one experiment. Cells in each panel were assayed in the same experiment.
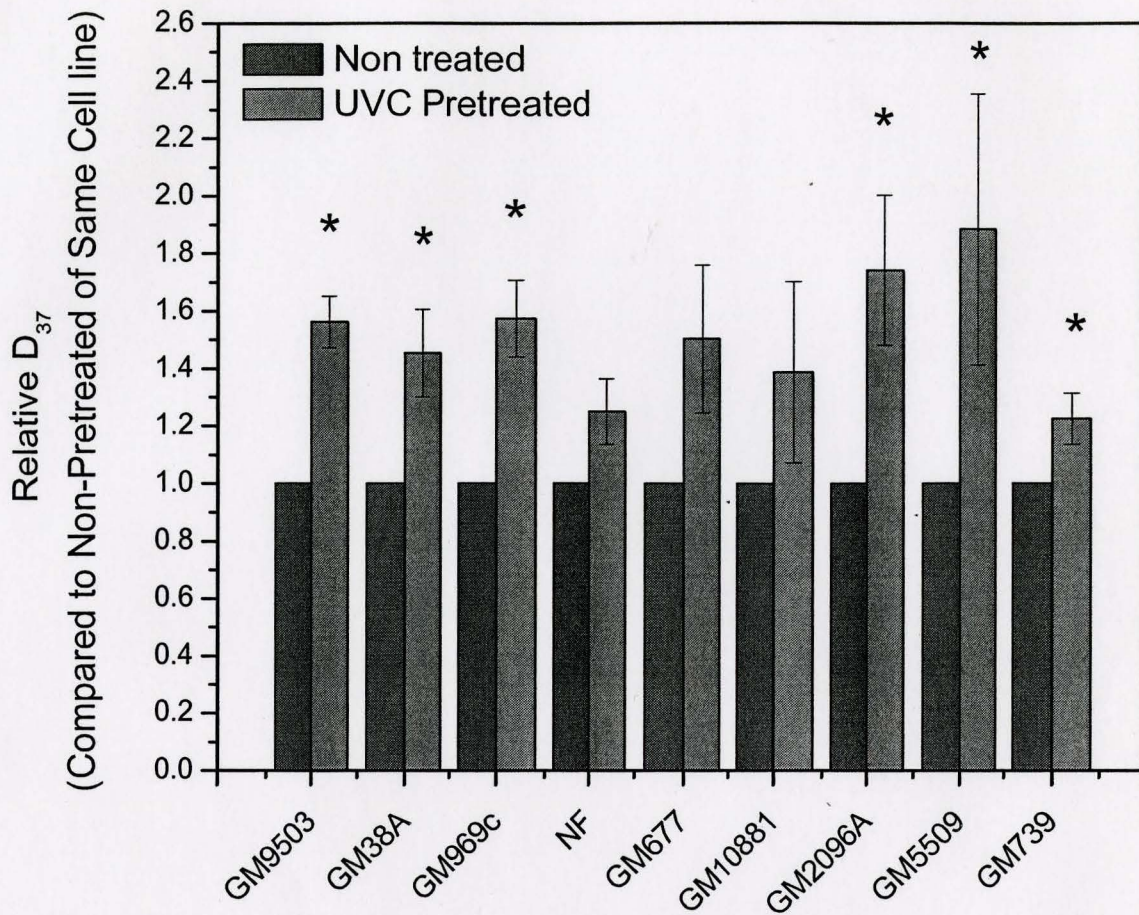
Figure 8: Relative D_{37} values obtained from HCR assays for ß-gal activity in normal and NER deficient pretreated with or without UVC. Cells were treated with 25 J/m^2 of UVC or mock treated then infected at an MOI of 40 pfu/cell with untreated or [20 µg/ml of MB + VL]-treated Ad5HCMV*lacz* and subsequently harvested 44-48 hours after infection. Results shown are the average relative D_{37} ± SE for each pretreated cell line shown as a ratio of the same non-pretreated cell line. The data that populates each cell line graph is derived from 3 – 15 independent experiments. Relative D_{37} values significantly increased compared to the non pretreated of that same cell line by the 2 sample independent t-test (p<0.05) are indicated (*).

Table 6.   HCR capability for β-galactosidase activity of [20μg/ml MB + VL] treated Ad5HCMV*lacz* virus as measured by the relative D_{37} values in UVC pretreated normal and NER deficient cell lines compared to the non-pretreated of the same cell line.

Cell Line	Group	Relative Average D_{37}†		% S.E.	# of Experiments	Significantly Increased‡ Compared to Non Pretreated (P-Value)	
		Non Pre/ Non Pre	Pre / Non Pre				
GM9503	Normal	1	1.562	0.090	15	Yes	9.52E-07
GM38A	Normal	1	1.454	0.152	4	Yes	0.024
GM969c	Normal	1	1.574	0.133	4	Yes	0.005
NF	Normal	1	1.250	0.115	6	No	0.055
GM677	XPC	1	1.503	0.257	7	No	0.075
GM10881	XPC	1	1.387	0.315	2	No	0.344
GM2096A	XPC	1	1.741	0.260	5	Yes	0.022
GM5509	XPA	1	1.883	0.471	9	Yes	0.021
GM739	CSB	1	1.224	0.089	6	Yes	0.030

† Calculated by taking D_{37} value of [20μg/ml MB+VL exposed virus] for pretreated cell line / D_{37} of [20μg/ml MB+VL exposed virus] for non pretreated same cell line within individual experiment, then averaged over all experiments under same conditions.
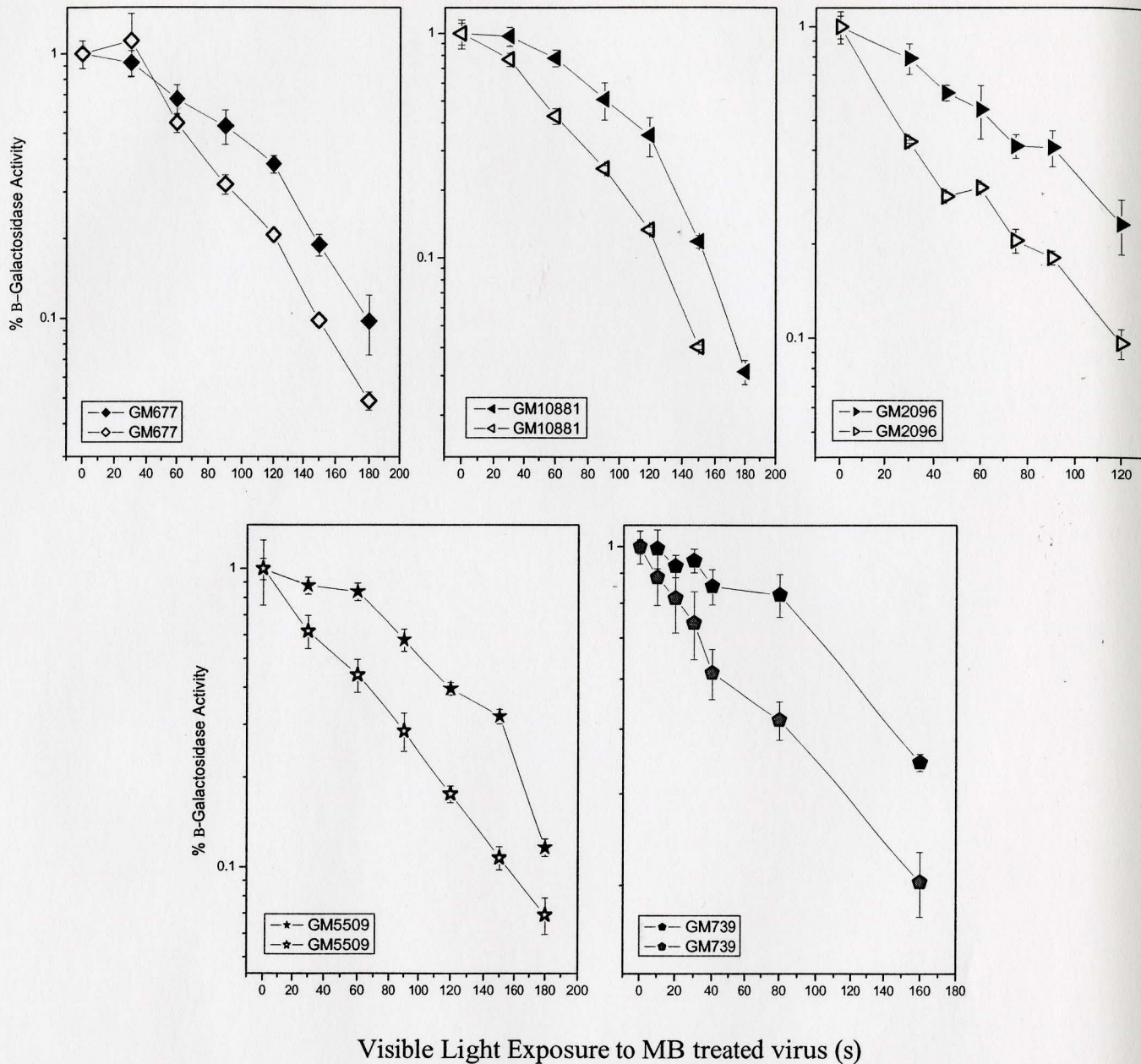
‡ significance tested by the two sampled independent t-test (p<0.05).

Visible Light Exposure to MB treated Virus (s)

Figure 9: HCR of β-galactosidase activity for MB+VL-treated Ad5HCMV*lacz* virus in UVC pre-treated normal human fibroblast cells. Cells were pre-treated with 25 J/m^2 of UVC then infected at an MOI of 40 pfu/cell with untreated or [20 μg/ml of MB + VL]-treated Ad5HCMV*lacz* and subsequently harvested 44-48 hours after infection. Results shown are for a representative experiment and include GM9503 (■), GM38A (●), NF (▲), and GM969 (▼). Each point is the average ± SE of triplicate determinations, done in one experiment. Each cell line was included with GM9503 in different experiments

Visible Light Exposure to MB treated Virus (s)

Figure 10: HCR of ß-galactosidase activity for MB+VL-treated Ad5HCMV*lacz* virus in UVC pretreated NER deficient human fibroblasts. Cells were pretreated with 25 J/m² UVC then infected at a MOI of 40 pfu/cell with untreated or [20 µg/ml of MB + VL]-treated Ad5HCMV*lacz* and subsequently harvested 44-48 hours after infection. Results shown are for a representative experiment and include GM9503 (Normal) (■), XPC- GM677 (◆), XPC -GM10881 (◀), XPC-GM2096 (▶), XPA-GM5509 (★), CSB-GM739 (●). Each point is the average ± SE of triplicate determinations, done in one experiment. Cells in each panel were assayed in the same experiment.
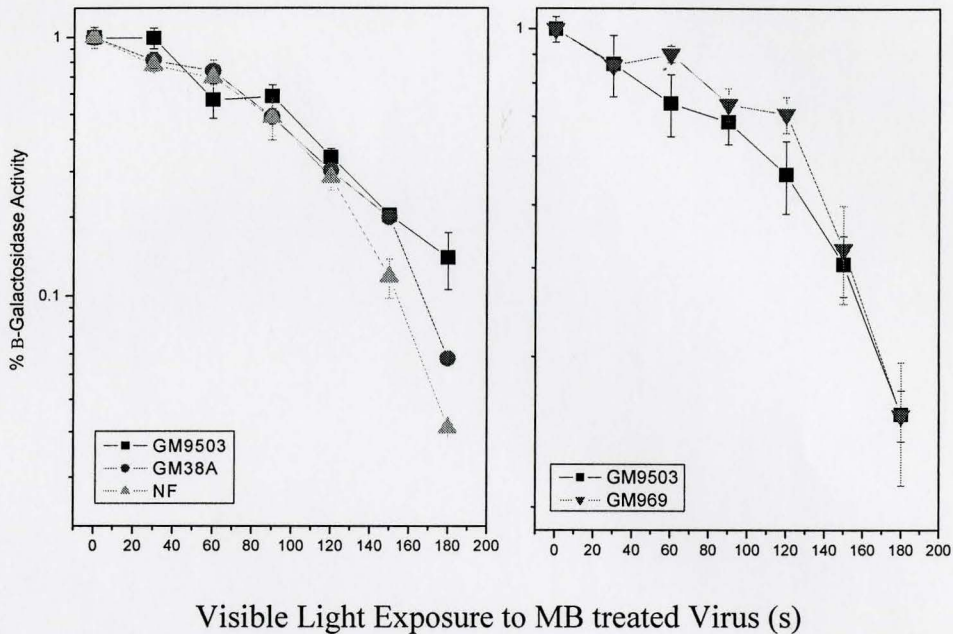
Table 7:   HCR capability for β-galactosidase activity of [20μg/ml MB + VL] treated Ad5HCMV*lacz* virus as measured by the actual D_{37} values in UVC pretreated normal and NER deficient cell lines.

Cell Line	Group	Average † Actual D_{37}	% Standard Error	# of Experiments	Actual values significantly‡ Different Compared to GM9503	(P-value)
GM9503	Normal	138.003	13.469	14	-	-
GM38A	Normal	123.405	5.882	4	No	0.500
GM969c	Normal	109.628	12.796	4	No	0.384
NF	Normal	108.300	5.325	6	No	0.273
GM677	XPC	110.393	9.530	6	No	0.404
GM10881	XPC	102.965	13.165	2	No	0.610
GM2096	XPC	115.125	7.015	4	No	0.825
GM5509	XPA	97.717	9.426	7	No	0.181
GM739	CSB	124.280	16.093	6	No	0.423

†- Mean visible light exposure to reduce reporter expression to 37% of that observed in the unexposed reporter construct in a given cell line then averaged over all experiments done under same conditions.

‡ - significance tested by the two sampled independent t-test (p<0.05).
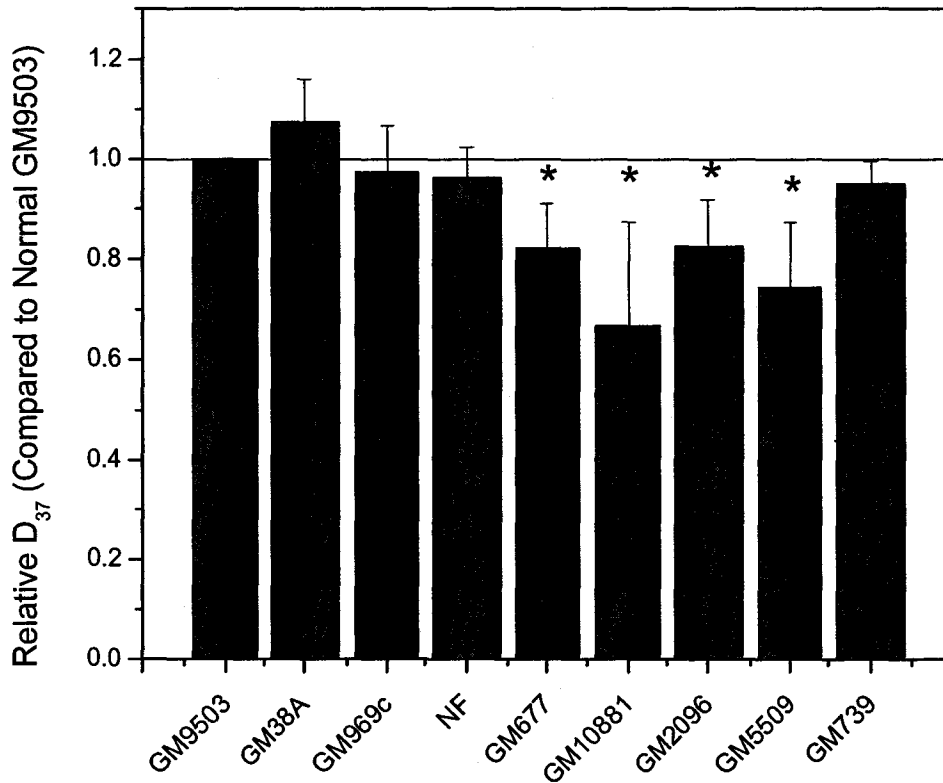
Figure 11: Relative D_{37} values obtained from HCR assays for ß-gal activity in UVC pretreated normal and NER deficient human fibroblasts. Cells were pretreated with 25 J/m^2 of UVC then infected at an MOI of 40 pfu/cell with untreated or [20 µg/ml of MB + VL]-treated Ad5HCMV*lacz* and subsequently harvested 44-48 hours after infection. Results shown are the average relative D_{37} ± SE for each cell line shown as ratio of the GM9503 of 3 – 16 independent experiments. Relative D_{37} values significantly reduced than the normal GM9503 by the 2 sample independent t-test (p<0.05) are indicated (*).

Table 8:   HCR capability for β-galactosidase activity of [20μg/ml MB + VL] treated Ad5HCMV*lacz* virus as measured by relative D_{37} values in UVC pretreated normal and NER deficient cell lines compared to the GM9503 Normal.

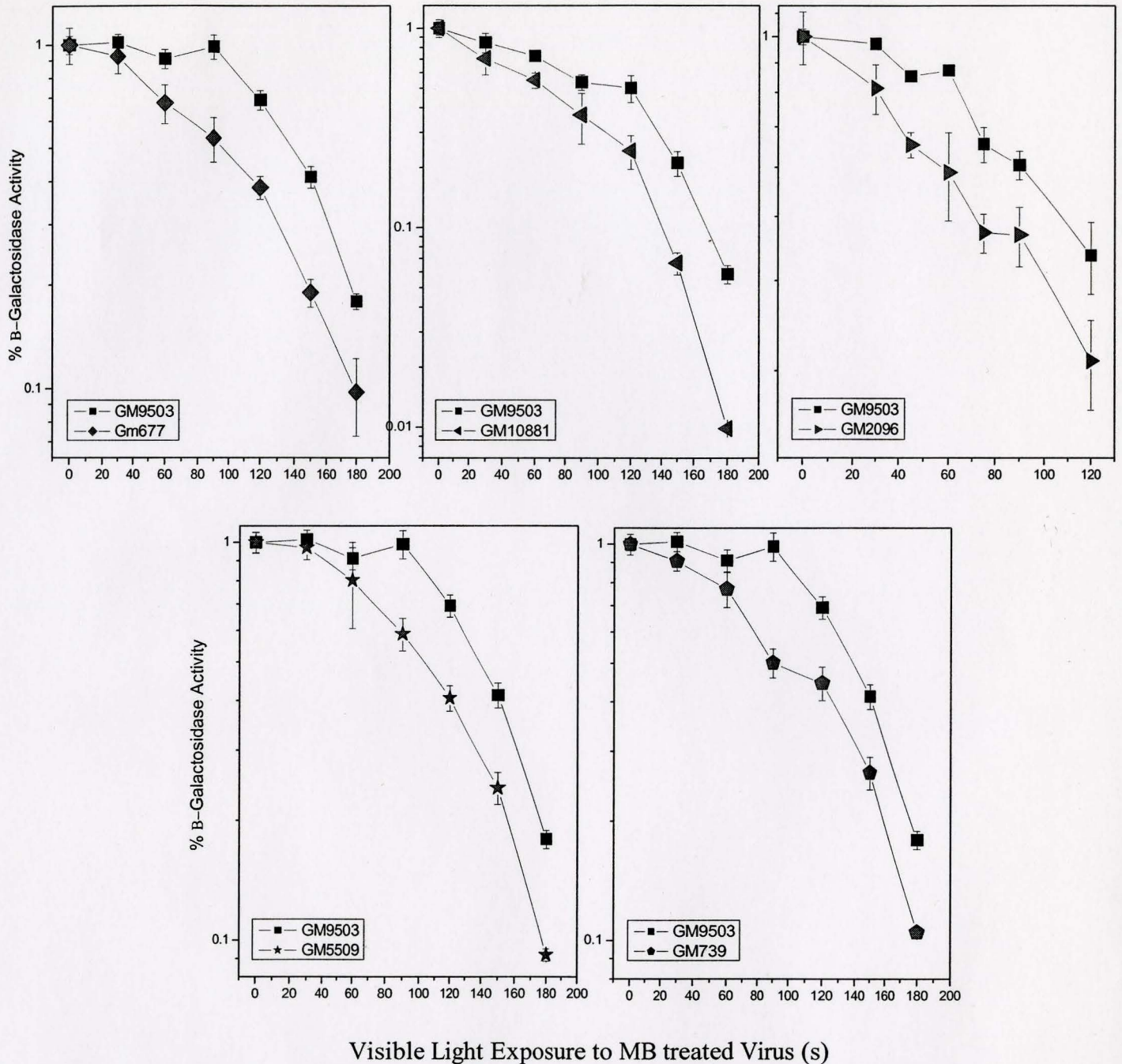| Cell Line	Group	Average[†] Relative D_{37}	% Standard Error	# of Experiments	Relative values significantly[‡] Different Compared to GM9503	(P-Value)
GM9503	Normal	1	0	15	-	-
GM38A	Normal	1.075	0.086	4	No	0.095
GM969c	Normal	0.974	0.094	4	No	0.573
NF	Normal	0.962	0.062	4	No	0.225
GM677	XPC	0.822	0.088	7	Yes	0.005
GM10881	XPC	0.667	0.206	3	Yes	0.006
GM2096	XPC	0.825	0.093	5	Yes	0.010
GM5509	XPA	0.743	0.130	6	Yes	0.014
GM739	CSB	0.950	0.045	6	No	0.097

† calculated by taking D_{37} [20μg/ml MB+VL exposed virus] for experimental pretreated cell line / D_{37} [20μg/ml MB+VL exposed virus] for pretreated GM9503 normal fibroblast within individual experiment then averaged over all experiments done under same conditions.

‡ significance tested by the two sampled independent t-test (p<0.05).

Visible Light Exposure to MB treated Virus (s)
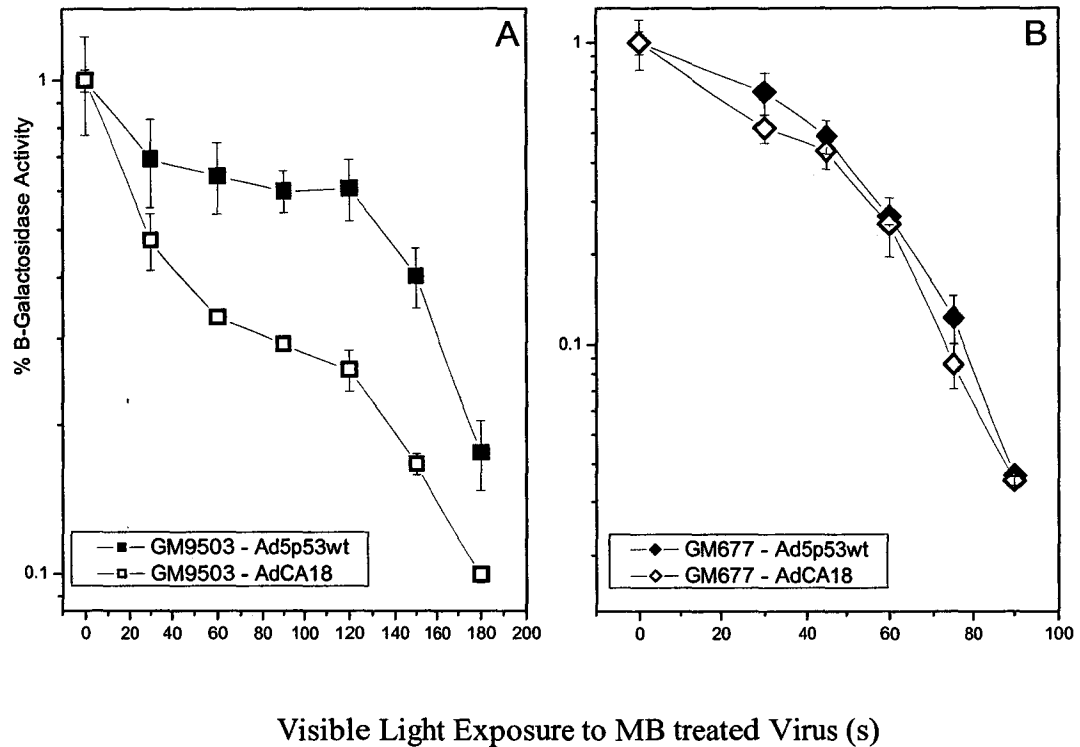
Figure 12: HCR of ß-galactosidase activity for MB+VL-treated Ad5HCMV*lacz* virus in normal human fibroblast (Panel A) and NER deficient XPC cells (Panel B) pretreated with Ad5p53wt (solid shapes) or AdCA18 (outlined shapes). Cells were pre-infected with Ad5p53wt or AdCA18 at an MOI of 200 followed by infection at an MOI of 40 pfu/cell with untreated or [20 µg/ml of MB + VL]-treated Ad5HCMV*lacz* and subsequently harvested 44-48 hours after infection. Results shown are for a representative experiments and include GM9503 (■) and GM677 (◆).   Each point is the average ± SE of triplicate determinations, done in one experiment. Cells in each panel were assayed in the same experiment.

Table 9:   HCR capability for β-galactosidase activity of [20µg/ml MB + VL] treated Ad5HCMV*lacz* virus as measured by the actual D_{37} values in normal and NER deficient cell lines pre-infected with Ad5p53wt virus compared to the same cell lines pre-infected by the AdCA18 control.

Cell Line	Group	Average Actual D_{37} †				# of Experiments	Does p53 Preinfection result in a significant increase compared to CA18 Preinfection ‡	P(Value)
		CA18	S.E.	p53	S.E.			
GM9503	Normal	65.55	15.65	78.71	15.13	4	No	0.57
NF	Normal	50.36	6.67	63.38	7.58	2	No	0.33
GM969	Normal	99.52	21.82	104.66	18.23	2	No	0.87
GM739	CSB	80.69	16.26	85.42	12.05	3	No	0.83
GM677	XPC	81.24	19.36	71.43	10.87	3	No	0.68

†- Mean visible light exposure to reduce reporter expression to 37% of that observed in the untreated reporter construct in a given cell line then averaged over all experiments done under same conditions.

‡ - significance tested by the two sampled independent t-test (p<0.05).

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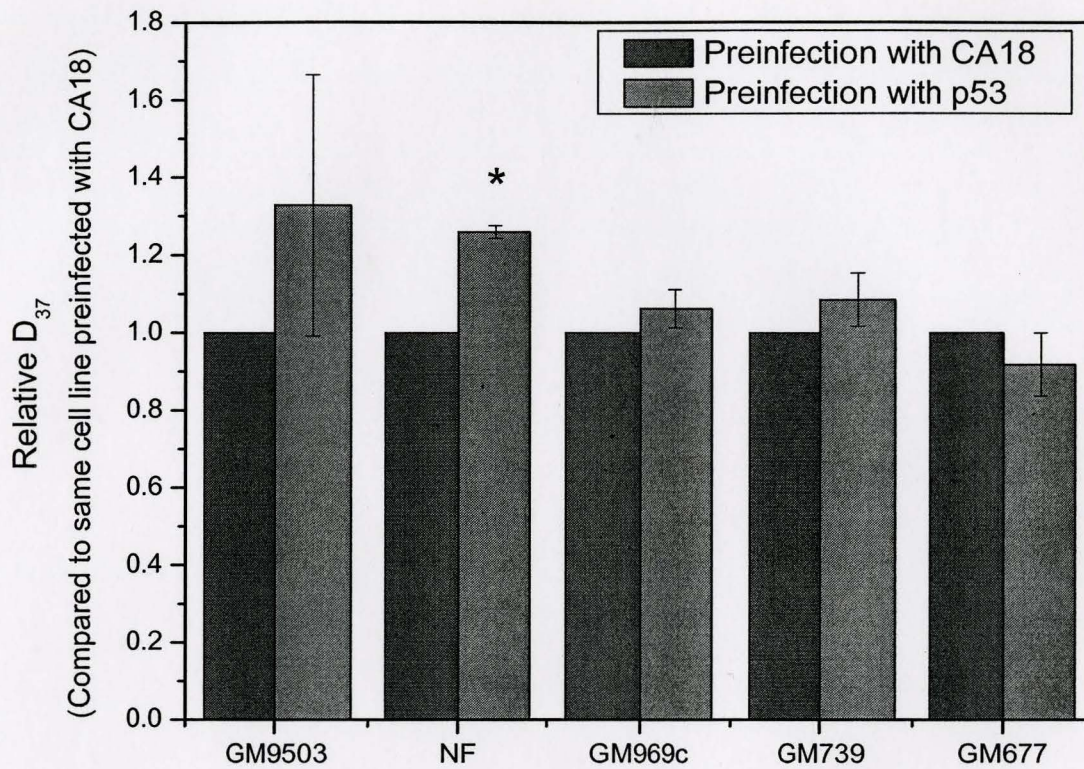
Figure 13: Relative D_{37} values obtained from HCR assays for ß-gal activity in cells pre-infected with Ad5p53wt or the AdCA18 control in normal and NER deficient fibroblasts. Cells were pre-infected at an MOI of 200 pfu/cell with Ad5HCMVp53wt or AdCA18. 24 hours later the same cells were infected at an MOI of 40 pfu/cell with untreated or [20 µg/ml of MB + VL]-treated Ad5HCMV*lacz* and subsequently harvested 44-48 hours after infection. Results shown are the average relative D_{37} ± SE for each cell line infected with p53, shown as a % increase, versus the control virus of 2 – 4 independent experiments. D_{37} values significantly increased compared to the control infected cells by the 2 sample independent t-test (p<0.05) are indicated (*).

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Table 10: HCR capability for β-galactosidase activity of [20μg/ml MB + VL] treated Ad5HGMVlacz virus as measured by the relative D_{37} values in normal and NER deficient cell lines pre-infected with Ad5p53wt virus compared to the same cell lines pre-infected by the AdCA18 control.

No UVC pretreatment following pre-infection							
Cell Line	Group	Relative Average D_{37} †			# of Experiments	Does p53 pre-infection result in a significant increase compared to CA18 pre-infection ‡	P(Value)
		CA18 §	p53	S.E.			
GM9503	Normal	1	1.33	0.34	4	No	0.37
NF	Normal	1	1.26	0.02	2	Yes	0.004
GM969	Normal	1	1.06	0.05	2	No	0.34
Average Normal	Normal	1	1.25	0.16	8	No	0.151
GM739	CSB	1	1.09	0.07	3	No	0.28
GM677	XPC	1	0.92	0.08	3	No	0.37
Effect of UVC pretreatment of CA18 infected cells							
Cell Line	Group	Relative Average D_{37} †			# of Experiments	Does UVC pretreatment result in a significant increase compared to non UVC treated CA18 pre-infected cells ‡	P(Value)
		CA18 §	UVC pretreated CA18	S.E.			
GM9503	Normal	1	1.547	0.341	4	No	0.160
NF	Normal	1	1.438	0.165	2	No	0.116
GM969	Normal	1	0.960	0.045	2	No	0.468
Average Normal	Normal	1	1.373	0.185	8	No	0.064
GM739	CSB	1	1.236	0.036	3	No	0.003
GM677	XPC	1	1.355	0.213	3	No	0.170
Effect of UVC pretreatment of p53 infected cells							
Cell Line	Group	Relative Average D_{37} †			# of Experiments	Does UVC pretreatment of p53 infected cells result in a significant increase compared to non UVC treated CA18 pre-infected cells ‡	P(Value)
		CA18 §	UVC pretreated p53	S.E.			
GM9503	Normal	1	1.564	0.211	4	Yes	0.037
NF	Normal	1	1.602	0.025	2	Yes	0.002
GM969	Normal	1	1.045	0.045	2	No	0.423
Average Normal	Normal	1	1.443	0.131	8	Yes	0.004
GM739	CSB	1	1.444	0.105	3	Yes	0.013
GM677	XPC	1	1.198	0.097	3	No	0.110

§ - the differently treated cell lines (No UVC, UVC, UVC + Ad5p53wt) are all compared to non-UVC-treated cells, infected with AdCA18.

† - Calculated by taking D_{37} [20μg/ml MB+VL exposed virus] for the differently treated cell lines / D_{37} [20μg/ml MB+VL exposed virus] of same cell lines infected with AdCA18 only, then averaged over all experiments under same conditions.

‡ - significance tested by the two sampled independent t-test (p<0.05).

Chapter 4:

Cellular survival of nucleotide excision repair proficient and deficient SV40 transformed cell lines following treatment with methylene blue + visible light

Chapter 4

Cellular survival of nucleotide excision repair proficient and deficient SV40 transformed cell lines following treatment with methylene blue + visible light.

Abstract:

Solar radiation is known to induce bulky damage in the DNA, such as cyclobutane pyrimidine dimers (CPDs), which are repaired by the nucleotide excision repair (NER) pathway.   Methylene blue (MB) acts as a photosensitizer and after excitation by visible light (VL) produces reactive oxygen species that result in oxidative damage to DNA. (MB+VL) produces predominantly 8-OxoG as well as other single base modifications in DNA that are repaired by base excision repair (BER).   The proteins involved in both BER and NER were thought to be distinct, however it has been shown that there is significant interplay between the two pathways. There are at least 9 main NER proteins, XP-A through XP-G, and, CSA and CSB, some of which are thought to be involved in BER. We have used a colony survival assay to examine the role of some of the nucleotide excision repair (NER) proteins in the cytotoxicity of MB + VL induced damage. The cell lines used were all Simian Virus 40 (SV40) transformed, and included the NER proficient: GM637 and AG02804D, and the NER deficient: XPCSV40, CSBSV40 and GM4429DXPA as XPC, CSB and XPA deficient cell lines respectively. Although un-irradiated MB is thought to be relatively inert, cell survival of treated cells decreased with increasing concentration of MB. Surprisingly, the NER deficient cell lines were more resistant to the cytotoxicity of MB than the normal cells, and this

resistance was not due to preferential uptake of the drug by normal cells. This suggests that MB alone has a cytotoxic effect on cells, and that an NER deficiency is in some way protective against this cytotoxicicity. In addition, the resistance of NER deficient cells to MB + VL treatment was also seen, suggesting some link between a deficiency in the XPC, CSB and XPA proteins and cytotoxic resistance. These results suggest some connection between a deficiency in XPC, CSB, and XPA proteins and the resistance of cells to MB alone and to MB + VL exposure.

Introduction:

Xeroderma pigmentosum (XP) is a hereditary disease that has clinical symptoms that range from sun sensitivity, cancer proneness, and in some cases neurodegeneration. Similalry, Cockayne syndrome (CS) individuals have sun sensitivity and neurodegeneration, but no elevated risk of cancer (reviewed by Lehmann, 2003, and Friedberg et al, 1995). Both of these groups of individuals have a reduced ability to repair DNA damage by the nucleotide excision repair pathway (NER) which removes bulky DNA damage induced by solar light (Hoeijmakers et al, 2000). The NER pathway can be divided into two subpathways: Transcription coupled repair (TCR) and global genome repair (GGR). TCR is responsible for the rapid removal of bulky adducts from the transcribed strand of active genes, whereas GGR removes these adducts from the genome at large. The two subpathways differ only in the initial detection step of NER, after which the mechanism is the same. Detection of the bulky adduct is facilitated by XPC and XPE proteins in GGR, whereas the CSB protein is responsible for this step in TCR.

The XPA, XPB and XPD proteins are then recruited to the site of damage and act to unwind the DNA in the vicinity of the damage. XPF and XPG then incise the DNA upstream and downstream from the damage, and an oligonucleotide containing the bulky adduct is removed. DNA polymerases then fill in the gap created and the new and old DNA are ligated together. (For review of NER and its subpathways see Hoeijmakers et al, 2000, Balagee et al, 1999).

As NER removes damage induced by solar radiation, it is surprising that CS and some XP individuals show neurodegeneration due to neuronal atrophy (Robbins et al, 1993). Neurons are not generally exposed to sunlight, so it was hypothesized that the neuronal atrophy in these patients might be due to a build-up of oxidative damage in these nerve cells, indicating that XP and CS individuals may have an impaired ability to remove oxidative damage as well (Reardon et al, 1997).

Oxidative damage occurs as a result of aerobic respiration, which is essential for mammalian life; but also creates reactive oxygen species (ROS) which damage cellular components. Damage to the DNA is the most troublesome, as any change in the integrity of the DNA can lead to carcinogenesis (as reviewed by Kawanishi et al, 2001). The main damage of ROS in DNA is the oxidation of the purine and pyrimidine bases, of those the oxidized guanine residue is the most common giving rise to the 7,8-dihydro-8-oxoguanine (8-OxoG) (Burrows et al, 1998, Neta et al, 1988, Wang et al, 1998). The mutagenic effect of this lesion is very well established, as it can pair efficiently with cytosine or adenine residues (Duarte et al, 1999).

One of the common *in vitro* inducers of singlet oxygen mediated DNA damage is visible light (VL) activated methylene blue (MB) (Epe et al, 1993). MB is one of the few molecules that, when irradiated with VL, is able to excite molecular oxygen to singlet oxygen (Floyd et al, 2004). MB + VL is known to create predominantly 8-OxoG in the DNA of cell free extracts (Floyd et al, 1989) as well as in whole cells (Slamenova et al, 2002) and viruses (as reviewed by Tuite and Kelly, 1993).

8-OxoG lesions are primarily repaired by the base excision repair (BER) pathway, which is initiated by glycosylases. Glycosylases are a group of proteins that can recognize and excise oxidized bases in the DNA; the hOGG1 is one of the glycosylases that are specific for recognizing 8-OxoG. Removal of the oxidized base by a glycosylase results in the creation of an apurinic nucleotide (AP-Site), which is recognized by an apurinic endonuclease (APE1). APE 1 is able to "nick" the DNA 5' to the damaged site, and effectively remove the abasic nucleotide from the DNA. At this point DNA Polymerase ß (Pol- ß) fills in the gap created by APE1, and DNA ligase connects the old and new strands (a review of BER is provided in Slupphaug et al, 2003 and Dianov et al, 2001).

Although the BER and NER mechanisms have been described separately, there has been mounting evidence for the overlap of the two, where NER proteins have been implicated in the BER pathway (as reviewed by Rybanska and Pirsel, 2003). It has been shown by Le Page et al, that the CSB, XPD, XPB and XPG protein are involved in the BER of oxidative damage (LePage et al, 2000). Similar conclusions have been made by other groups as well, where they implicate the XPG and CSB proteins in the TCR of

oxidative damage (Cooper et al, 1997). There has also been evidence that shows the involvement of XPC in BER, where 3 of 4 XPC deficient cell lines were unable to repair oxidative damage (Runger et al, 1995). The potential mechanism of the involvement of XPC in BER was elucidated when it was shown that XPC physically and functionally interacts with DNA glycosylases (Shimizu et al, 2003). This suggests that the XPC protein detects damage in BER, which is similar to its role in NER.

In the present study, we have sought to further our knowledge of the interplay between NER and BER by investigating the role of NER proteins in the response of cells following treatment with MB + VL induced damage. We have used a colony survival assay to examine cytotoxicity following treatment of cells with MB + VL. Individual cells from various normal and NER deficient cell lines were incubated after treatment with MB + VL. The presence of a colony 5-10 days after incubation is expected only to occur if the cell is able to repair the oxidative damage induced by MB +VL. In this way, it is possible to ascertain if the NER proteins are involved in cytotoxicity after MB + VL exposure by assaying for colony survival in different NER deficient cell lines. The reports of NER deficient cell survival following oxidative damage are conflicting. It has been shown that XPA and XPC deficient cell lines do not show reduced survival following UVA treatment (Runger et al, 2000) which is known to induce oxidative damage (Runger et al, 1995). In addition, it has been shown that XPD deficient human cell lines show reduced survival following UVA treatment compared to normal, while the Chinese hamster ovary cells with a mutation analgous to a human XPD (UV51) and CSB (UV61) mutation show no reduction in cell survival following low doses of UVA

irradiation, but show a cell survival reduction following higher doses of UVA (Liu, 2003). In contrast, others have shown that XPA and CSB deficient cell lines do show reduced survival after treatment with different oxidizing agents such as paraquat (Waard et al, 2003). It appears that this discrepancy could be due to the difference in oxidizing agent used. We report here that NER deficient cell lines appear to be more resistant to MB + VL treatment compared to normal, and this resistance is not due to the preferential uptake of MB by normal cells. It is possible that this resistance could be due to the better repair of other damage caused by MB + VL in NER deficient cell lines.

Materials and Methods:

Cell lines and virus constructs:

The repair-proficient SV40 transformed fibroblasts, GM637 (Normal), and the NER deficient SV40 transformed fibroblasts, GM4429DXPA (XP12BE; XPA), XPCSV40 (XP4PA; XPC) and CSBSV40 (CS1AN; CSB) were obtained from National institute of general medical sciences (NIGMS) (Camden, NJ). The other SV40 transformed normal AG02804D was obtained from the National institute on aging (NIA) (Camden, NJ). All cell cultures were grown at 37°C in a humidified incubator in 5% CO_2 and cultured in Eagle's a-minimal essential media (a-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, Grand Island, NY).

Methylene Blue and Visible Light:

Methylene Blue (Methylthionine chloride Trihydrate) was prepared by taking 0.02g of MB (Sigma Chemicals) in powder form, and dissolving it in 20ml of PBS at 37°C to make a concentration of 1000μg/ml. The solution was filter sterilized and aliquots were frozen at -20 °C in the dark. When needed, frozen aliquots were thawed to room temperature in the dark and diluted appropriately for use. The Visible Light (VL) irradiation of cells occurred under a General Electric 1000 watt halogen lamp (GE R1000). The stage was set 83 cm distance below the bulb.

[MB] or [MB+VL] Colony Survival Assay:

Seeding

SV40 Transformed cells were seeded in a 6-well plate (Falcon, Franklin Lakes, NJ) at a density of between 400 and 1600 cells/well. The cells were left to adhere for 6-8 hours at 37°C, 5% CO_2, in a humidified incubator.

MB Addition

Between 6-8 hours after seeding, media was replaced with 1 ml of MB diluted appropriately from the frozen stock in supplemented media. The cells were further incubated overnight (15-18 hours) at 37°C, 5% CO_2, in a humidified incubator. Precautions were taken to minimize the amount of light exposure to the cells after this point, unless otherwise required.

Procedure for MB alone Colony Survival (No Visible Light)

After incubation with MB, the dilutions were aspirated and the cells were washed once with 5 ml of PBS. Subsequently, the PBS was aspirated and 5ml of supplemented a-MEM was added to each well, and the cells were left to incubate at 37 °C, 5% CO_2, in a humidified incubator for 5-10 days.

Procedure for MB+VL Colony Survival - Irradiation

After overnight incubation with MB, the dilutions were aspirated and the cells were washed once with 5 ml of PBS. After the wash, 1 ml of PBS was added to each well, and the plates were irradiated with visible light for up to 30 minutes. After irradiation, the PBS was aspirated and 5ml of supplemented a-MEM was added to each well and the cells were left to incubate at 37 °C, 5% CO_2, in a humidified incubator for 5-10 days.

Staining and analysis of Colonies

After the incubation period, colonies were stained with crystal violet prepared in 10% methanol, 27% H_2O and 63% ethanol (v/v). Colonies that contained more than 32 cells were counted and analyzed. Surviving colonies were plotted as a percentage of the number of colonies counted for the untreated control cells (average of two determinations for each dose to cells ± SE).

MB Uptake by Cells

Seeding

Cells were seeded, as described above, at a density of 7.5×10^5 cells/well in two sets of 6 well plates (Falcon, Franklin Lakes, NJ). The cells were left to incubate at 37°C, 5% CO_2, in a humidified incubator for 6-8 hours.

MB Addition

Between 6-8 hours after seeding, media in both sets of plates was replaced with 1 ml of MB diluted appropriately from the frozen stock in supplemented media. The cells were left to incubate overnight (15-18 hours) at 37°C, 5% CO_2, in a humidified incubator.

Washing, Lysis and Trypsinization

After overnight incubation with MB, the dilutions were aspirated and the cells were washed once with 5 ml of PBS. The 5 ml of PBS was aspirated, and in one set of plates 0.375 ml of lysis buffer was added (0.01% Triton X-100, 1 mM $MgCl_2$ 100 mM phosphate buffer at pH 8.3) to each well. Once the lysis buffer was added, that set of plates was left to incubate at 37°C, 5% CO_2, in a humidified incubator for 3 hours. The other set of plates had 0.5 ml of trypsin added to the wells, and the cells were allowed to trypsinize for 3-5 minutes. At that point 2 ml of supplemented a-MEM was added and the cells were resuspended. This resuspension for each well was counted by a Coulter Counter (Beckman Coulter – Z1 Coulter Particle Counter). (Any object between the range of 9.67µm – 21.66 µm was counted as a cell).

Absorbance Reading

The set of plates containing the lysed cells were removed from the incubator, and each well was agitated by pipeitting up and down within each well several times. 150 µl of lysate was removed from each well and transferred to a 96-well plate (Falcon, Franklin Lakes, NJ). Once all wells had been transferred, the 96-well plate was taken to a Safire Plate reader (Tecan Industries), and absorbance readings were taken at 662nm, which is within the optimal range of absorption for MB.

Interpretation of Uptake

The absorbance reading for each MB dilution used from one set of plates, was divided by the number of cells counted for the corresponding well on the set of plates that was counted. In this way the amount of MB taken up per cell of each cell type was inferred by absorbance.

Pooling data over many experiments

The D_{37} value of a survival curve is the dose, in this case MB or VL exposure, required to reduce the surviving fraction of a cell line to 37% of the non treated control of that same cell line. D_{37} was used as a measure of the cellular sensitivity to MB or MB+VL, such that the higher the average D_{37} for a cell line, the more resistant it was to that treatment. The actual D_{37} values of each cell line was determined from curves of each experiment and expressed as a ratio of the actual D_{37} value of an arbitrarily chosen normal.   This is the relative D_{37} value compared to that normal. The relative D_{37} of the chosen normal in each experiment would therefore always be 1, and those cell lines with

an actual D_{37} higher than the actual D_{37} of the gold standard would have a relative D_{37} greater than 1. The relative D_{37} values for each cell line were then averaged over many experiments and expressed with its % standard error.

The uptake, inferred by absorbance, of cells incubated with 10μg/ml MB was pooled in much the same way. Actual absorbance readings of cells incubated with 10μg/ml MB was determined from curves of each experiment.   These absorbance readings were expressed as a ratio of the absorbance reading of a chosen normal. This ratio is the relative uptake. Those cell lines that uptake more MB than the chosen normal would have a relative uptake value greater than one. The relative uptake values were then averaged over many experiments and expressed with its % standard error.

Results:

Clonogenic Survival of normal and NER deficient cells following treatment with MB.

The clonogenic survival of MB treated normal and NER deficient cell lines were investigated. Typical survival curves of colony forming ability for two normal and several NER deficient cell lines following increasing MB treatment are shown in figure 1. It can be seen from these curves that there are substantial differences between these cell lines when it comes to their sensitivities to MB alone. It appears that the XPCSV40 and the GM4429DXPA cell lines appear to be relatively resistant to MB, while the CSBSV40 and the two normals appear to be more sensitive to MB. The MB concentration in μg/ml required to reduce the surviving fraction to 37% of that for the non treated control was used as a measure of sensitivity to the MB. Mean D_{37} values are shown in table 1. It can

be seen that the mean D_{37} values were greater than that for the normal cells in all of the NER deficient cell lines. In order to account for the variation in D_{37} values in individual experiments, we also calculated the D_{37} values for each of the cell lines relative to the D_{37} value for the normal GM637 obtained in individual experiments. The D_{37} values relative to GM637 were then averaged over many experiments. The average relative D_{37} for each cell line compared to GM637 are shown in figure 2 and table 2. It can be seen that the three NER deficient cell lines appear to be more resistant to the MB alone compared to the normals. In fact, the XPCSV40 and the GM4429DXPA cell lines are significantly more resistant than both GM637 and AG02804D, whereas the CSBSV40 is only significantly resistant compared to the GM637. This would suggest that a NER defect in XPC, XPA, and to a lesser extent CSB, makes these cell lines more resistant to treatment with MB compared to normal.

<u>Cellular uptake of MB by normal and NER deficient cells following treatment with MB.</u>

The differences in cell sensitivity were surprising, and suggested that some confounding effect was influencing these results. It was then suggested that differential uptake of MB by cells may be the reason for this difference in cell sensitivity, and thus cellular MB uptake experiments were performed. Cells were incubated with different MB concentrations for 17 hours, at which point they were lysed, and the intracellular concentration of MB was determined. A representative curve showing absorbance per cell, which is an indication of MB uptake per cell, is shown in figure 3.   It can be seen that MB uptake is roughly linearly dependent on incubation concentration, perhaps

suggesting passive diffusion of MB. The uptake of MB however is not uniform between cell lines. It appears that as the MB incubation concentration increases, the uptake between cell lines varies to a greater degree. It can be seen that at the higher MB doses, the GM637, GM4429DXPA and the CSBSV40 uptake the most MB, whereas the AG02804D and the XPCSV40 uptake the least. This does not correlate with the differences seen in sensitivity to MB, and would suggest that the differential uptake of MB by each cell line is not the reason why there are such differences in cellular sensitivity to MB. It can also be seen from figure 3 that at the lower incubation concentrations of MB, the variation between cell lines for MB uptake is at a minimum. It was therefore of interest to determine the MB incubation concentration that lead to approximately equal uptake of MB by each cell line. Relative uptake of different cell lines incubated at 10µg/ml of MB compared to the uptake of GM637 incubated at 10 µg/ml MB is shown table 3 and figure 4. It can be seen that most of the NER deficient cell lines uptake similar amounts, within 20%, of MB compared to the GM637 normal. The GM4429DXPA cell line however, appears to uptake twice as much as the GM637, but the variability in the uptake in this cell line is greater than 90%. Moreover, the differences in uptake between any of these cell lines at 10µg/ml are not significant. This would suggest that incubation of these cell lines with 10 µg/ml of MB causes approximately equal amounts of MB to get into the cell. This is important if we want to control for the variation in uptake between cell lines.

<u>Clonogenic Survival of normal and NER deficient cells following treatment with MB and</u>

<u>VL.</u>

The clonogenic survival of normal and NER deficient cells treated with MB and then increasing amounts of VL was also investigated. As uptake of MB was potentially a confounding factor in these colony survival experiments, cell lines were first incubated with 10 µg/ml of MB, which was shown to cause approximately equal uptake of MB by each cell line (table 3, figure 4), then irradiated with VL. A typical survival curve of 10 µg/ml MB treated cells followed by VL irradiation is shown in figure 5. It can be seen that even controlling for variation of uptake between cell lines, the GM637 normal appears to be more sensitive to treatment with MB + VL than any other cell line. Mean D_{37} for each cell line is shown in table 4, while mean relative D_{37} values for each cell line compared to GM637 are shown in table 5 and figure 6. It can be seen that all of the NER deficient cell lines, XPCSV40, CSBSV40 and GM4429DXPA, are more resistant than the GM637 and AG02804D normal, however only differences compared to GM637 are significant. This would suggest that the advantage XPA, XPC and CSB deficient cells have compared to GM637 when it comes to MB induced damage, gives these cell lines the same advantage for MB + VL induced damage. However, there was no significant difference in the toxic effect of MB + VL on the NER deficient compared to the AG02804D cell line, indicating no correlation of NER deficiency with the resistance to MB + VL.

Discussion:

It has been shown by many groups that the NER genes play a role in BER of oxidative damage (as reviewed by Rybanska and Pirsel, 2003). We have shown that several of the XP genes are involved in the BER of a MB + VL damaged reporter (Chapter 3). It was therefore of interest to determine the effect of MB + VL on cell survival in normal and NER deficient cell lines. It appears that in instances, where cells were treated with MB alone or MB + VL, NER deficient cell lines have a greater resistance to treatment than the normal cells (figure 2 and figure 6). MB that has not been excited by VL is expected to be relatively inert (as reviewed by Floyd et al, 2004). It was therefore surprising when we found that there was cytotoxicity to cellular survival of treatment with drug alone. This might suggest reactivity of the un-irradiated MB, however a more likely explanation is the activation of MB by irradiation by ambient light. Although precautions were taken to minimize the amount of VL exposure to cells in the presence of MB, it was impossible to eliminate all light from experimental conditions. It has been shown that as little as 30 seconds of 60 watt exposure of MB treated cells can cause oxidative DNA damage (Slamenova et al, 2002). Therefore it is possible that the cytotoxicity in cell survival may not be due to MB alone, but MB + ambient VL. This is further supported by the fact that there is a similar result in cell line sensitivity to MB + VL treated cells shown in figure 6. Although ambient VL may activate MB, there is still a discrepancy in the relative resistance of the cells. It was therefore suggested that there may be a difference in the MB uptake of the cells, which would facilitate more oxidative damage in those cells uptaking more MB. Looking at the

MB incubation concentrations of 10 μg/ml, uptake is not significantly different between cell lines (figure 4), whereas there are striking differences between cell survival shown in figure 2 and table 2. This would suggest that the differences in sensitivity to MB and MB + VL are not due to MB uptake and may be due to the damage spectrum induced.

A possible explanation for the significant resistance to MB alone and not to MB + VL in NER deficient compared to both normal strains may be found in analyzing the damage spectrum of MB compared to MB + VL. It is possible that the damage spectrum created by MB (+ ambient VL) and MB + VL is different, perhaps due to the length of time MB is allowed to be activated. Its possible that constant irradiation of MB under MB + VL conditions allows the activated MB to produce different types of damage, and thus changes the way the normal strains respond to the MB. This may be further confounded by polymorphisms in NER genes that differ in normals (Blakenburg, 2000).

Differences in damage spectrum may also explain why the NER deficient cell lines are more proficient than one normal for MB + VL and both normals for MB alone. It has been shown that MB + VL treatment of cells induce oxidized purines such as 7,8-dihydro-8-oxoguanine (Slamenova et al, 2002). If this oxidative damage was the cause of differences in cell sensitivity, we would expect the NER deficient cell lines to be more sensitive to MB + VL treatment than normal (Chapter 3). The fact that our results show NER deficient cells were more proficient than normal following MB alone would suggest that MB (+ ambient VL) has physiological effects on cells other than oxidative DNA damage. An explanation for this could be due to the fact that MB + (ambient) VL is known to damage more than just the DNA (as reviewed by Tuite and Kelly, 1993). It is

possible that mutations in the XP proteins somehow confers immunity to this form of non-DNA damage, and thus increases the resistance of XP cells. For example, MB + VL is known to interact with biological membranes, damaging its lipid and protein components (Valenzeno, 1987). As many XP proteins interact with the transcription machinery (reviewed in Balagee and Bohr, 1999), it is possible that transcriptional regulation by the XP proteins may affect the distribution and composition of surface proteins embedded in biological membranes, which may confer resistance to these cells. Mammalian cells have also been shown to be susceptible to lysosome rupture after MB + VL treatment (Santus et al, 1983), and NER deficient cell lines may have resistance to this type of damage as well. Another potential explanation for the differences in sensitivity can be gained from MB localization studies, where it was found that MB localized to the cytoplasm of the cell prior to VL irradiation, and only localized in the nucleus after VL irradiation (Ruck and Kollner, 1992). This suggests that the incubation of cells for 17 hours with MB causes preliminary damage in the cytoplasm, perhaps lysosmal or protein damage. Repair of this damage is not dependent on the DNA repair capacities of the cell lines, and as such some other physiological process, independent of mutations in NER proteins, may determine sensitivity.

Although all of the above are potential reasons why MB (+ ambient VL) is more toxic to normal cells compared to NER deficient cells, it is possible that the resistance of these NER deficient cells has nothing to do with the NER defect. It has been shown that SV40 transformation has the ability to cause cells normally resistant to a DNA damaging agent to become sensitive after transformation (Day, et al, 1980). This would suggest

that SV40 transformation may cause normal cells to become more sensitive to MB (+ ambient light) compared to NER deficient cells, in which the effect might be attenuated. In sum, it appears that NER deficient cells used in the present study are more resistant to MB + VL induced damage than normal. However, whether it is the NER defect or another physiological process that confers this resistance, is yet to be determined.
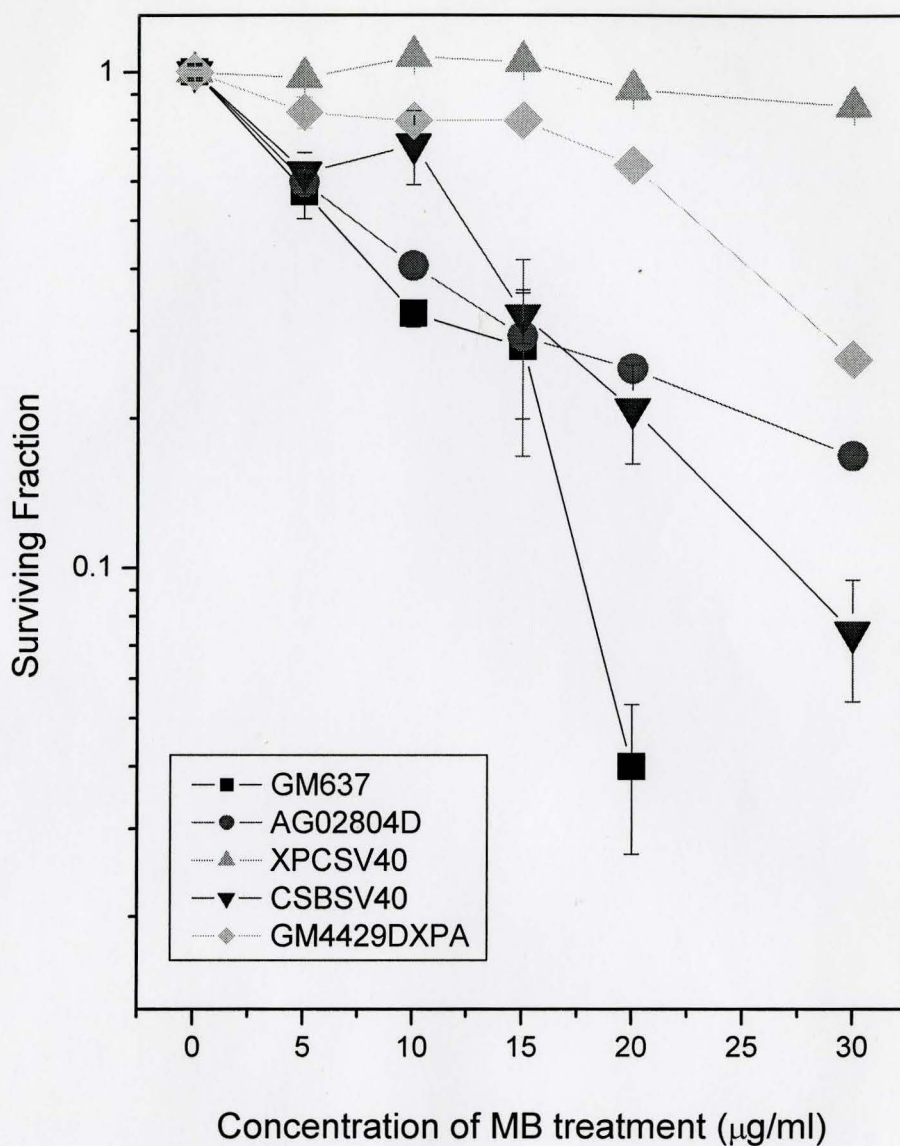
Figure 1: Clonogenic survival of SV40 transformed cells in response to MB treatment. Cells were incubated 17 hours with increasing concentrations of MB, which was subsequently washed off, and cells were harvested 5 – 10 days later. Results shown are for a representative experiment and include: GM637 (■), AG02804D (●), XPCSV40 (▲), CSBSV40 (▼), and GM4429DXPA(◆) Data is normalized to un-treated controls. Each point is the average ± SE of a single experiment performed in duplicate.

Table 1: Clonogenic survival of MB treated SV40 transformed human fibroblasts as measured by actual D_{37} values.

Cell Line¥	Group	Average † Actual D_{37}	% Standard Error	# of Experiments	Significantly different‡ compared to GM637 (P-value)		Significantly different‡ compared to AG02804D (P-value)	
GM637F	Normal	20.578	7.735	4	-	-	No	0.396
AG02804D	Normal	13.370	1.572	4	No	0.396	-	-
XPCSV40	XPC	52.540	7.490	2	No	0.063	Yes	0.00161
CSBSV40	CSB	28.083	5.998	4	No	0.472	No	0.05533
GM4429DXPA	XPA	54.657	25.184	3	No	0.198	No	0.10841

† Mean MB concentration required to reduce clonogenic survival to 37% of that observed in the un-treated control cells of the same cell line in one experiment, then averaged over all experiments done under same conditions.

‡ Significance tested by the two sampled independent t-test (p<0.05).

¥ All cell lines were SV40 transformed.
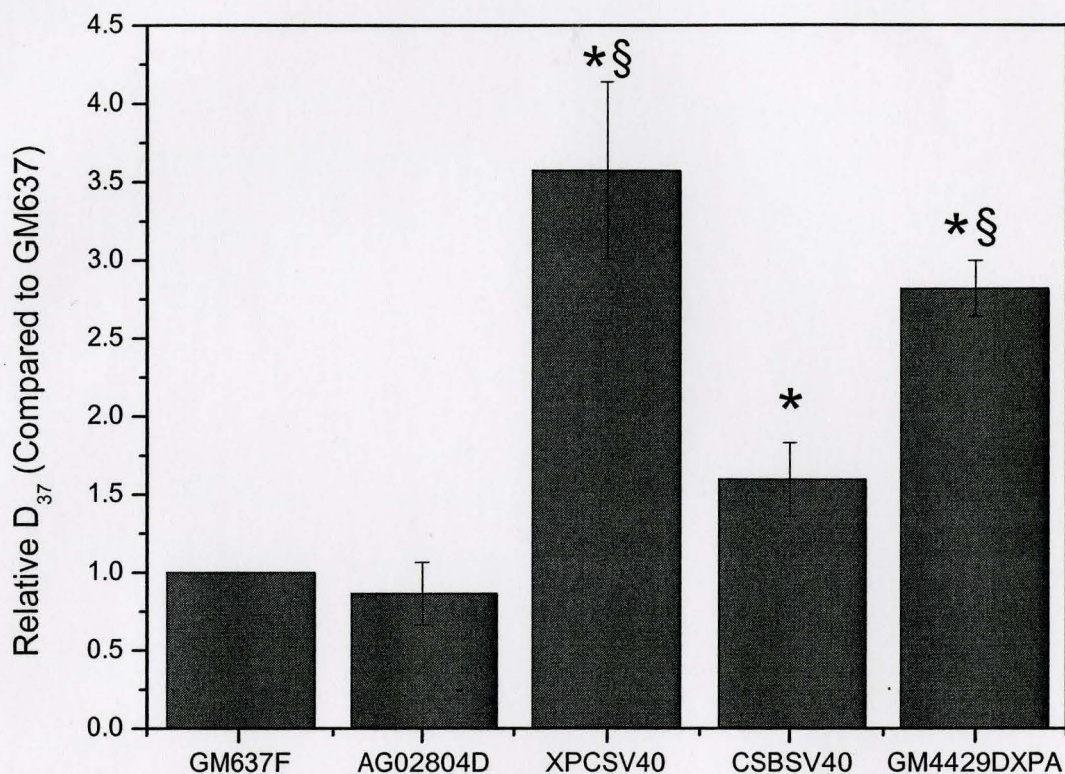
Figure 2: Relative D_{37} values obtained from clonogenic survival assays of SV40 transformed human fibroblasts treated with increasing concentrations of MB. Cells were incubated for 17 hours with MB at increasing concentrations was subsequently washed off. Cells were re-fed with fresh media and were incubated for $5 - 10$ days then harvested. Results shown are the average relative $D_{37} \pm$ SE for each cell line shown as a ratio of the GM637 of between $2 - 4$ independent experiments. Relative D_{37} values significantly increased than the normal GM637 or AG02804D by the 2 sample independent t-test (p<0.05) are indicated (*) or (§) respectively.

Table 2: Clonogenic survival of MB treated SV40 transformed human fibroblasts as measured by relative D_{37} values compared to the GM637.

Cell Line ¥	Group	Average † Relative D_{37}	% Standard Error	# of Experiments	Significantly different‡ compared to GM637 (P-value)		Significantly different‡ compared to AG02804D (P-value)	
GM637	Normal	1	0	4	-	-	No	0.524
AG02804D	Normal	0.864	0.200	4	No	0.524	-	-
XPCSV40	XPC	3.573	0.567	2	Yes	0.001	Yes	0.004
CSBSV40	CSB	1.594	0.234	4	Yes	0.044	No	0.054
GM4429DXPA	XPA	2.816	0.178	3	Yes	6.6E-05	Yes	9.31E-4

† calculated by taking D_{37} of [MB-treated] experimental cell line / D_{37} of [MB-treated] GM637 within individual experiments, then averaged over all experiments done under same conditions.
‡ significance tested by the two sampled independent t-test (p<0.05).
¥ All cell lines were SV40 transformed

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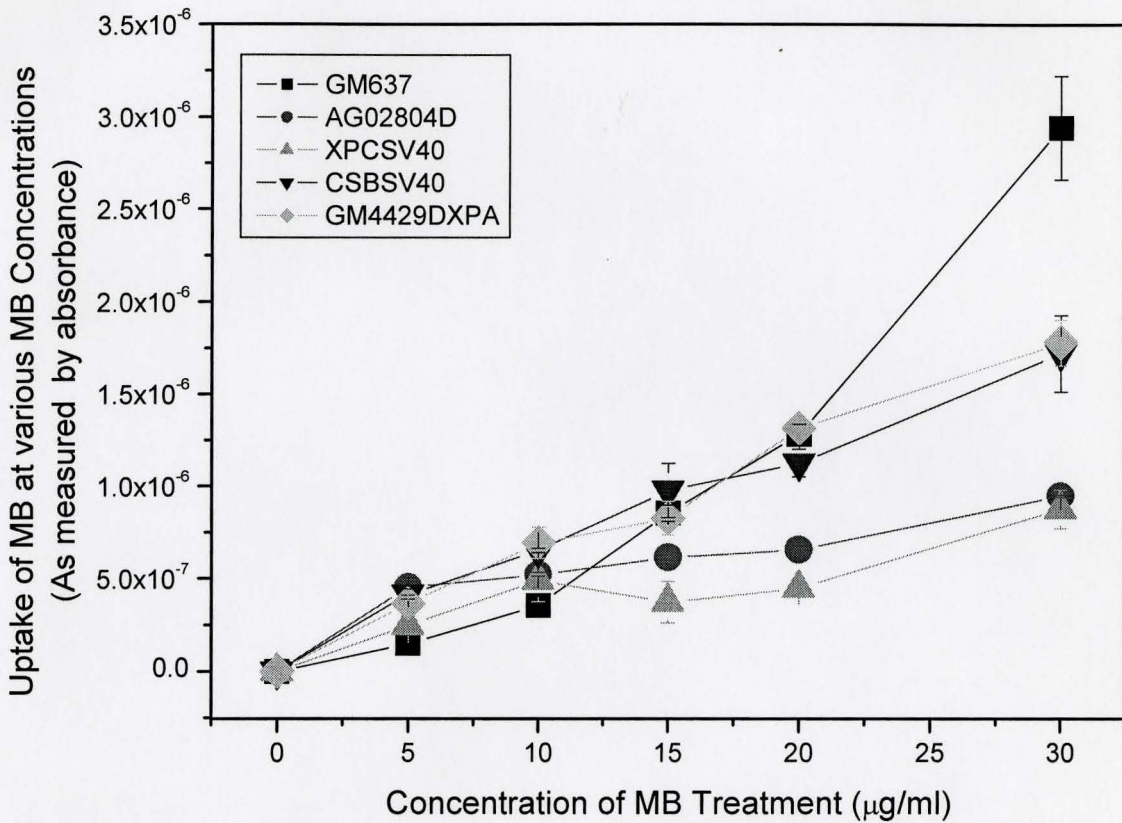
Figure 3: Uptake of MB, as measured by absorbance, of various SV40 transformed fibroblasts. Cells were incubated 17 hours with varying concentrations of MB which was subsequently washed off. Cells were lysed and absorbance readings were taken to measure amount of MB present. Results shown are for a representative experiment and include: GM637 (■), AG02804D (●), XPCSV40 (▲), CSBSV40 (▼), and GM4429DXPA(◆) Data is from one experiment, with triplet determinations.
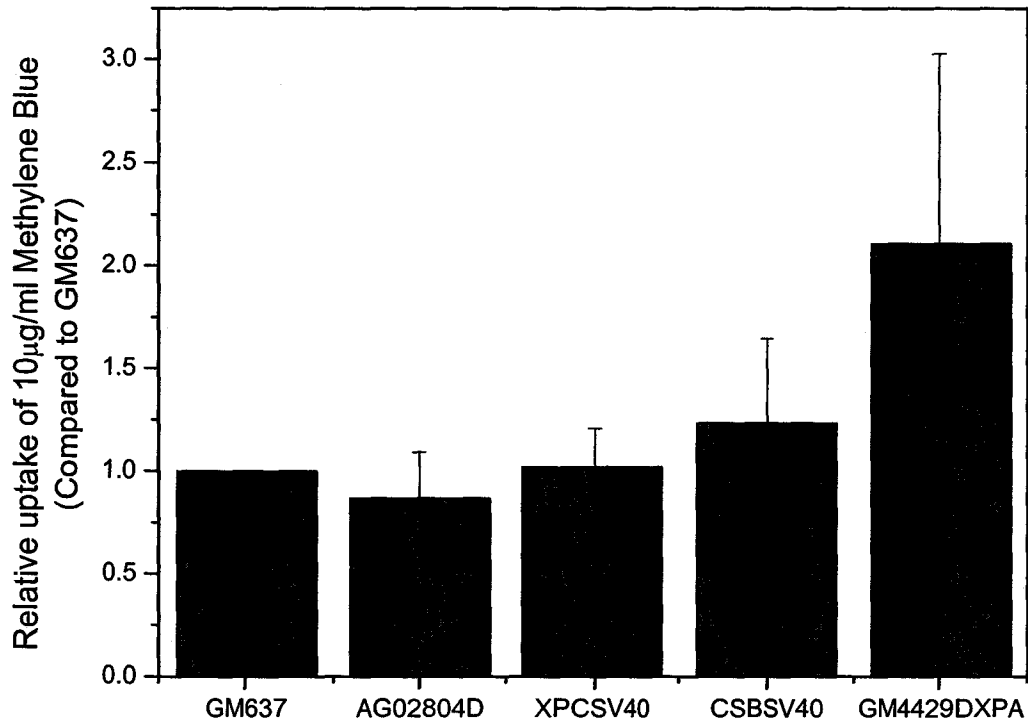
Figure 4: Relative uptake of SV40 transformed human fibroblasts, incubated for 17 hours with 10 μg/ml MB.   Cells were incubated for 17 hours with 10 μg/ml of MB which was subsequently washed off, cells were lysed and absorbance readings were taken to measure amount of MB present. Results shown are the average relative uptake ± SE for each cell line shown as a ratio of the uptake of GM637 of between 3 – 4 independent experiments. None of the relative uptake values were significantly different from one another determined by the 2 sample independent t-test (p<0.05).

Table 3: MB uptake of SV40 transformed human fibroblasts incubated overnight with 10µg/ml MB, as measured by relative uptake values compared to GM637.

Cell Line¥	Group	Average † Relative Uptake	% Standard Error	# of Experiments	Significance Test ‡
GM637	Normal	1	0	4	No cell line had a significantly different MB uptake compared to any other cell line with p-values ranging between 0.2 and 0.9
AG02804D	Normal	0.866	0.222	4	
XPCSV40	XPC	1.019	0.185	3	
CSBSV40	CSB	1.231	0.415	3	
GM4429DXPA	XPA	2.109	0.918	4	

† calculated by taking uptake of [10 µg/ml MB-treated] experimental cell line / uptake of [10 µg/ml MB-treated] GM637 within individual experiments, then averaged over all experiments done under the same conditions.

‡ significance tested by the two sampled independent t-test (p<0.05).

¥ All cell lines were SV40 transformed.
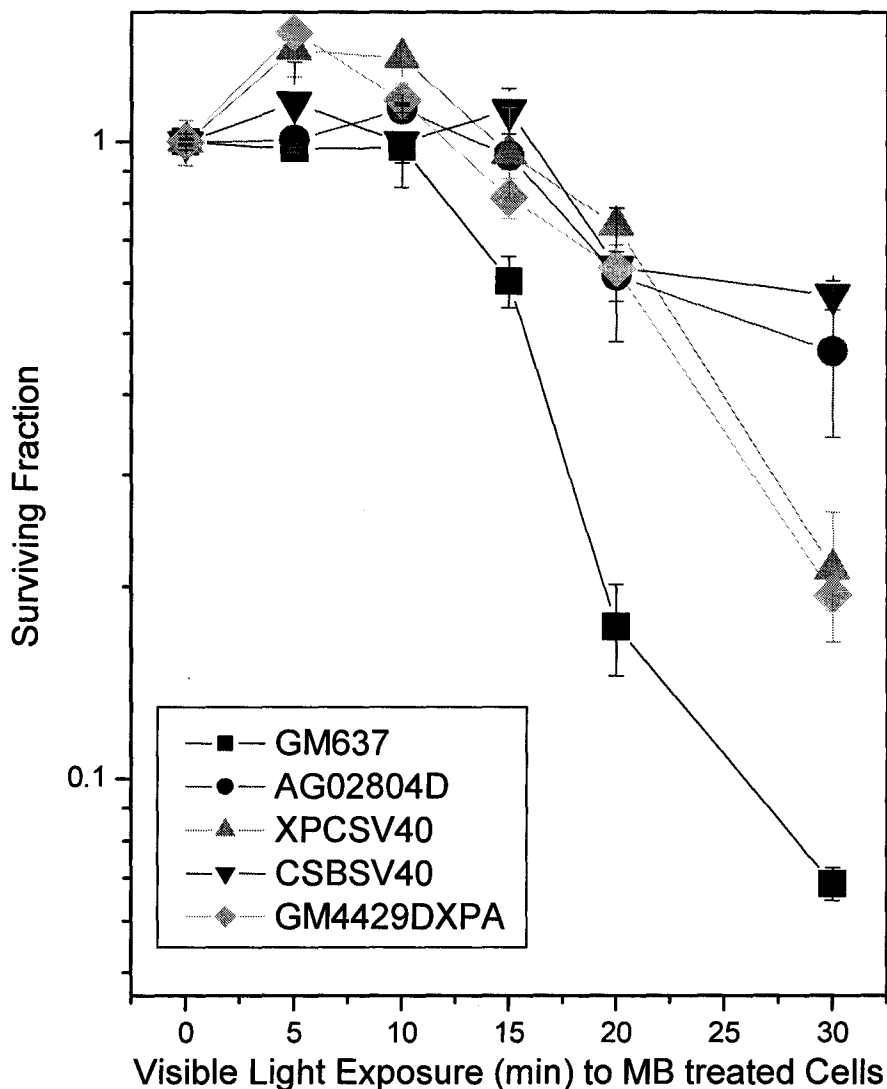
Figure 5: Clonogenic survival of SV40 transformed cells in response to MB and VL treatment. Cells were incubated 17 hours with MB at a concentration of $10\mu g/ml$, which was subsequently washed off, then irradiated with various durations of VL. Cells were re-fed with fresh media and were incubated for 5 – 10 days then harvested.   Results shown are for a representative experiment and include: GM637 (■), AG02804D (●), XPCSV40 (▲), CSBSV40 (▼), and GM4429DXPA(◆).   Data is normalized to controls that were treated only with $10\mu g/ml$ of MB. Each point is the average ± SE of a single experiment performed in duplicate.

Table 4: Clonogenic survival of MB + VL treated SV40 transformed human fibroblasts as measured by actual D_{37} values.

Cell Line¥	Group	Average † Actual D_{37}	% Standard Error	# of Experiments	Significantly different‡ compared to GM637 (P-Value)		Significantly different‡ compared to AG02804D (P-Value)	
GM637	Normal	17.037	2.203	3	-	-	No	0.410
AG02804	Normal	23.343	6.501	3	No	0.410	-	-
XPCSV40	XPCSV40	32.167	7.922	3	No	0.140	No	0.437
CSBSV40	CSBSV40	36.955	12.025	2	No	0.124	No	0.348
GM4429D	GM4429DXPA	25.030	2.316	3	No	0.067	No	0.818

† - Mean VL irradiation required to reduce clonogenic survival of 10µg/ml treated cells to 37% of that observed in the un-irradiated control cells of the same cell line in one experiment, then averaged over all experiments done under same conditions.

‡ - significance tested by the two sampled independent t-test (p<0.05).

¥ - All cell lines were SV40 transformed.

Figure 6: Relative D_{37} values obtained from Clonogenic survival assays of MB treated SV40 transformed fibroblasts irradiated with increasing amounts of VL.   Cells were incubated for 17 hours with 10µg/ml of MB which was subsequently washed off, then irradiated with increasing durations of VL.   Cells were re-fed with fresh media and were incubated for 5 – 10 days then harvested. Results shown are the average relative D_{37} ± SE for each cell line shown as a ratio of the GM637 of between 2 – 3 independent experiments. Relative D_{37} values significantly increased than the normal GM637 or AG02804D by the 2 sample independent t-test ($p<0.05$) are indicated (*) or (§) respectively.
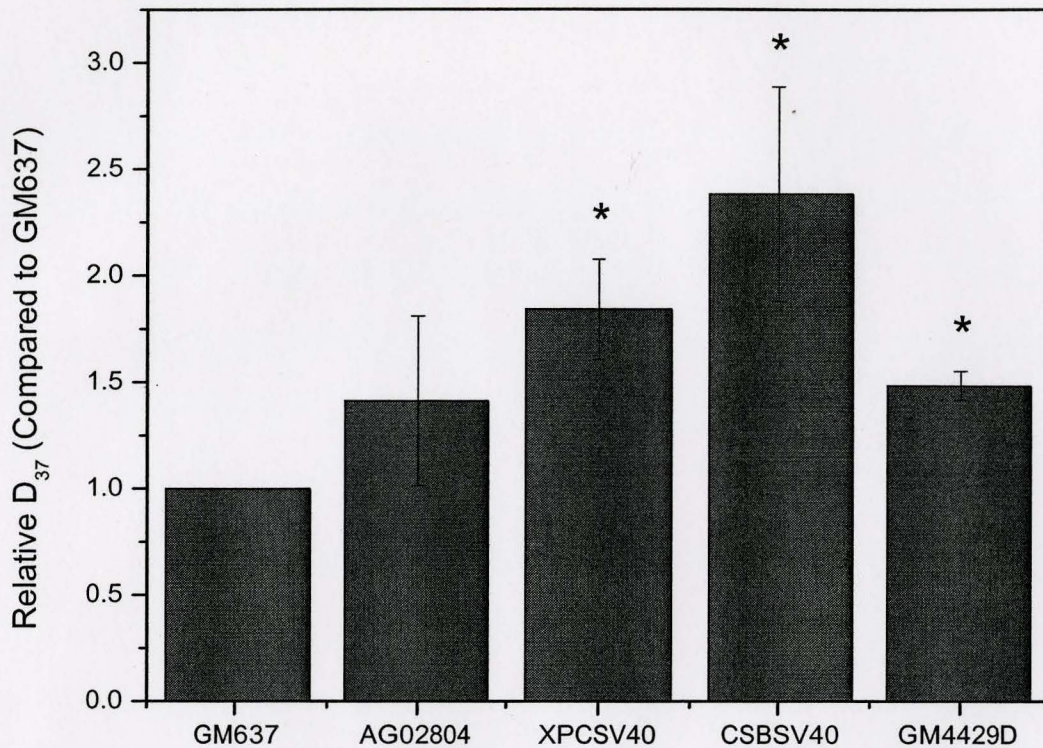
Table 5: Clonogenic survival of MB + VL treated SV40 transformed human fibroblasts as measured by relative D_{37} values compared to the GM637.

Cell Line¥	Group	Average † Relative D_{37}	% Standard Error	# of Experiments	Significantly different‡ compared to GM637 (P-value)		Significantly different ‡ compared to AG02804D (P-Value)	
GM637	Normal	1	0	3	-	-	No	0.357
AG02804	Normal	1.414	0.399	3	No	0.357	-	-
XPCSV40	XPCSV40	1.845	0.234	3	Yes	0.023	No	0.403
CSBSV40	CSBSV40	2.384	0.504	2	Yes	0.035	No	0.225
GM4429D	GM4429DXPA	1.485	0.067	3	Yes	0.002	No	0.869

† calculated by taking D_{37} of [10 µg/ml MB + VL-treated] experimental cell line / D_{37} of [10Mg/ml MB + VL -treated] GM637 within individual experiments, then averaged over all experiments done under same conditions.
‡ Significance tested by the two sampled independent t-test (p<0.05).
¥ All cell lines were SV40 transformed.

Summary & Future Directions

Summary

1.   Cells harboring compromised p53 were generally more proficient in constitutive HCR of a MB+VL treated reporter compared to normal. However these same cell lines were deficient in inducible HCR of a MB + VL treated reporter compared to normal. These results implicate p53 in inducible BER.

HCR of a MB + VL damaged reporter construct is enhanced following UVC pretreatment in normal cells ranging between 25% and 50% with p53 compromised cells showing less enhancement. p53 expression following UVC treatment correlated well with the enhanced HCR in normal cells, but not so much in p53 compromised cells, indicating that induction of BER can occur in a p53 dependent and p53 independent manner.

HCR of a MB + VL damaged reporter construct was enhanced following pre-infection of normal cells with Ad5p53wt, which expresses wild type p53 in cells. This provides further evidence of a p53 dependent enhancement of BER.

2.   SV40 transformed cells with deficient NER showed a reduction in HCR capacity of a MB + VL damaged reporter construct. In particular XPC deficient cell lines showed a substantial reduction, while cells from deficient CSB, XPA, XPD, XPG, XPF showed little or no reduction in HCR of MB + VL induced damage. This suggests an important role for the XPC protein in BER.

In particular, 3 XPC and one XPA mutant fibroblasts showed reduced constitutive and inducible BER of a MB + VL damaged reporter, further implicating the

XPC and XPA proteins in BER. It was also found that pre-UVC-treatment of normal cells caused an enhancement of HCR abilities; however the enhancement seen in XPC cell lines was lower, providing further evidence for the role of XPC in BER.

HCR of a MB + VL damaged reporter construct was enhanced following pre-infection of normal fibroblasts with Ad5p53wt, but not in the XPC deficient cell line, providing further evidence for the role of XPC in BER.

3.   SV40 transformed NER deficient cells were more resistant to the cytotoxic effects of MB compared to normals. This suggests that a mutation in the XPC, XPA or CSB genes confers resistance to this treatment. This resistance may be afforded by these NER deficient cells being better able to repair non-DNA damage induced by MB, and may not reflect ability to repair oxidative damage induced by MB.

Future Directions:

The results of this study suggest that BER is inducible, and that this induction is a result of p53 dependent and p53 independent mechanisms. It has been found by other groups that Pol-ß is upregulated following DNA damage (Cabelof et al, 2002), and it is possible that the p53 independent mechanisms responsible for inducing BER may involve Pol-B. It would therefore be of value to study the role of Pol- ß enhancement of BER. This may be achieved by creating Pol-ß knockdown cells using siRNA, and conducting MB + VL HCR assays in these cells. It would also be of value to determine of pre-UVC-treatment can upregulate Pol- ß, as this may help understand why pre-UVC- treatment can enhance HCR in some p53 compromised cells. Similarly, we have implicated XPC in the BER pathway, and similar XPC knockdowns may be beneficial to corroborate this finding.

In regards to the surprising colony survival findings, it would be beneficial to assay clonogenic survival using other oxidative DNA damaging agents that produce less non-DNA damage, and are more specific for creating 8-OxoG. In this way, it would be possible to assay the BER capacities of NER deficient cell lines and not their repair abilities of lysosomal and membrane damage, which appears to be the confounding effect with MB + VL. As well, it would be of use to determine the clonogenic survival of NER deficient primary human fibroblasts, to determine if SV40 transformation is affecting the results by causing resistant cells to have a more sensitive phenotype.

References

Achanta, G. and Huang, P. (2004) Role of p53 in sensing oxidative DNA damage in response to reactive oxygen species-generating agents. *Cancer Res.* 64 6233-6239.

Addison, C.L., Hitt, M., Kunsken, D. and Graham, F.L. (1997) Comparison of the human versus murine cytomegalovirus immediate early gene promoters for transgene expression by adenoviral vectors. *J. Gen. Virol.* 78:1653-1661.

Adimoolam, S. and Ford, J.M. (2003) p53 regulation of DNA damage recognition during nucleotide excision repair. *DNA Repair* 2:947-954.

Adimoolam, S. and Ford, J.M. (2002) p53 and DNA damage-inducible expression of the Xeroderma pigmentosum group C gene. *Proc. Natl. Acad. Sci.*, 99:12985-90.

Anker, L., Ohgaki, H., Ludeke, B. I., Herrmann, H. D., Kleihues, P. and Westphal, M. (1993) p53 protein accumulation and gene mutations in human glioma cell lines. *Int. J. Cancer,* 55(6): 982-987.

Bacchetti, S. and F. L. Graham (1993) Inhibition of cell proliferation by an adenovirus vector expressing the human wild type p53 protein. *Int. J. Oncol.,* 3: 781-788.

Balajee, A.S. and Bohr, V.A. (2000) Genomic heterogeneity of nucleotide excision repair. *Gene,* 250: 15-30.

Balagee, A.S., Dianova, I. and Bohr, A. (1999) Oxidative damage-induced PCNA complex formation is efficient in Xerderma pigmentosum, group A, but reduced in Cockayne syndrome group B cells. *Nucleic Acids Res.,* 27:4476-4482.

Bar, J., Cohen-Noyman, E., Geiger, B. and Oren, M. (2004) Attenuation of the p53 response to DNA damage by high cell density. *Oncogene,* 23:2128-2137.

Barak, Y., Juven, T., Haffner, R. and Oren, M. (1993) mdm2 expression is induced by wild type p53 activity. *EMBO J.,* 12: 461-8.

Beckman, K.B. and Ames, B.N. (1998) The Free Radical Theory of Aging Matures. *Physiol. Rev.,* 78: 547-581

Bender, K., Blattner, C., Knebel, A., Iordanov, M., Herrlich, P. and Rahmsdorf, H.J. (1997) UV-induced signal transduction. *J. Photochem. Photobiol. B.* 37:1-17.

Bett, A.J., Haddara, W., Prevec, L. and Graham, F.L. (1994) An efficient and flexible system for construction of adenovirus vectors with insertions or deletions in early regions 1 and 3. *Proc. Natl. Acad. Sci. U S A.,* 91:8802-6.

Bischoff, F.Z., Strong, L.C., Yim, S.O., Pratt, D.R., Siciliano, M.J., Giovanella, B.C. and Tainsky, M.A. (1991) Tumorigenic transformation of spontaneously immortalized fibroblasts from patients with a familial cancer syndrome. *Oncogene*, 6:183-6.

Bischoff, F.Z., Yim, S.O., Pathak, S., Grant, G., Siciliano, M.J., Giovanella, B.C., Strong, L.C. and Tainsky, M.A. (1990) Spontaneous abnormalities in normal fibroblasts from patients with Li-Fraumeni cancer syndrome: aneuploidy and immortalization. *Cancer Res.*, 50:7979-84.

Blankenburg1, S., Konig, I.R., Moessner1, R., Laspe1, P., Thoms, K.M., Krueger1, U., Khan, S. G., Westphal, G., Volkenandt, M., Neumann1, C., Ziegler, A., Kraemer, K.H., Reich1, K., and Emmert, S. (2005) No association between three xeroderma pigmentosum group C and one group G gene polymorphisms and risk of cutaneous melanoma. *Eur. J. Human Genet.*, 13: 253-255.

Blumenthal, R. D., Leone, E., Goldenberg, D.M., Rodriguez, M. and Modrak, D. (2004) An *in vitro* model to optimize dose scheduling of multimodal radioimmunotherapy and chemotherapy effects of p53 expression. *Int. J. Cancer,* 108: 293-300.

Bohr, V.A., Smith, C.A., Okumoto, D.S. and Hanawalt, P.C. (1985) DNA repair in an active gene: removal of pyrimidine dimers from the DHFR gene of CHO cells is much more efficient than in the genome overall. *Cell*, 4: 359-69.

Boshart, M., Weber, F., Jahn, G., Dorsch-Hasler, K., Fleckenstein, B. and Schaffner, W. (1985) A very strong enhancer is located upstream of an immediate early gene of human cytomegalovirus. *Cell,* 41:521-30.

Cabelof, D.C., Raffoul, J.J., Yanamadala, S., Guo, Z.M. and Heydari, A.R. (2002) Induction of DNA polymerase ß-dependent base excision repair in response to oxidative stress *in vivo. Carcinogenesis,* 23:1419-1425.

Cadet, J., Delatour, T., Douki, T., Gasparutto, D., Pouget, J.P., Ravanat, J.L. and Sauvaigo, S. (1999) Hydroxyl radicals and DNA base damage. *Mutat. Res.*, 424: 9-21.

Cadet, J. and Treoule, R. (1978) Comparative study of oxidation of nucleic acid components by hydroxyl radicals, singlet oxygen and superoxide anion radicals. *Photochem. Photobiol.*, 28:661-7.

Canaani, D., Naiman, T., Teitz, T. and Berg, P. (1986) Immortalization of xeroderma pigmentosum cells by simian virus 40 DNA having a defective origin of DNA replication. *Somat. Cell. Mol. Genet.*, 12:13-20.

Cappelli, E., Degan, P., Thompson, L.H. and Frosina, G. (2000). Efficient repair of 8-oxo-7,8-dihydrodeoxyguanosine in human and hamster xeroderma pigmentosum D cells. *Biochem.,* 39:10408-10412.

Cappelli, E., Carrozzino, F., Abbondandolo, A. and Frosina, G. (1999) The DNA helicases acting in nucleotide excision reapir, XPD, CSB and XPB, are not required for PCNA-dependent repair of abasic sites. *Eur. J. Biochem.,* 259:325-330.

Chance B., Sies, H. and Boveris, A. (1979) Hydro peroxide metabolism in mammalian organs. *Physiol. Rev.,* 59:527-605.

Cleaver, J.E., Thomspon, L.H., Richardson, A.S. and States, J.C. (1999) A summary of mutation in the UV-Sensitive Disorders: Xeroderma Pigmentosum, Cockayne Syndrome, and Trichothiodystrophy. *Human Mutation,* 14:9-22.

Cooper, P.K., Nouspikel, T., Clarkson, S.G. and Leadon, S.A. (1997) Defective transcription coupled repair of oxidative base damage in Cockayne syndrome patients from XP Group G. *Science,* 275:990-993.

Cortopassi, G.A. and Wang E. (1996) There is substantial agreement among interspecies estimates of DNA repair activity. *Mech. Ageing Dev.,* 91:211-8.

Croteau, D.L., and Bohr, V.A. (1997) Repair of Oxidative Damage to Nuclear and Mitochondrial DNA in Mammalian Cells. *J. Biol. Chem.,* 272:25409-25412.

Day, R.S., Ziolkowski, C.H.J., Scudiero, D.A., Meyer, S.A., Lubiniecki, A.S., Girardi, A.J., Galloway, S.M. and Bynum, G.D. (1980) Defective repair of alkylated DNA by human tumour and SV40-transformed human cell strains. *Nature,* 288:724-727.

De Boer, J. and Hoeijmakers, H.J.H. (2000) Nucleotide excision repair and human Syndromes. *Carcinogenesis,* 21:453-460.

De Bont, R. and van Larebekel, N. (2004) Endogenous DNA damage in humans: a review of quantitative data. *Mutagenesis,* 19:169-185.

de Souza-Pinto, N.C., Harris, C. and Bohr, V.A. (2004) p53 functions in the incorporation step in DNA base excision repair in mouse liver mitochondria. *Oncogene,* 23:6559-6568.

de Waard, H., de Wit, J., Gorgels, T.G.M.F., van den Aardweg, G., Anderessoo, J.O., Vermeij, M., van Steeg, H., Hoeijmakers, J.H.J. and van der Horst, G.T.J. (2003) Cell type-specific hypersensitivity to oxidative damage in CSB and XPA mice. *DNA Repair*, 2:13-25.

Dianov, G.L., Souza-Pinto, N., Nyaga, S.G., Thybo, T., Stevnsner, T. and Bohr, V.A. (2001) Base Excision Repair in Nuclear and Mitochondrial DNA. *Nucleic. Acids Res.*, 68:285-297.

Dianov, G., Bischoff, C., Sunesen, M. and Bohr, V.A. (1999) Repair of 8-oxoguanine in DNA is deficient in Cockayne syndrome group B cells. *Nucleic Acids Res.*, 27(5):1365-8.

Dizdaroglu, M. (2005) Base-excision repair of oxidative DNA damage by DNA glycosylases. *Mutat. Res., [Epub ahead of print]*.

Donahue, B.A., Yin, S., Taylor, J.S., Reines, D. and Hanawalt, P.C. (1994) Transcript cleavage by RNA polymerase II arrested by a cyclobutane pyrimidine dimer in the DNA template. Proc. Natl. Acad. Sci. USA, 91:8502-8506.

Dorszewska, J. and Adamczewska-Goncerzewiscz, Z. (2004) Oxidative damage to DNA, p53 gene expression and p53 protein levels in the process of aging in rat brain. *Resp. Phys. & Neurobiol.* 139:227-236

de Laat, A., van Tilburg, M., van der Leun, J.C., van Vloten, W.A. and de Gruijl, F.R. (1996) Cell cycle kinetics following UVA irradiation in comparison to UVB and UVC irradiation. *Photochem. Photobiol.*, 63(4): 492-7.

Duarte, V., Muller, J.G. and Burrows, C.J. (1999) Insertion of dGMP and dAMP during *in vitro* DNA synthesis opposite an oxidized form of 7,8-dihydro-8-oxoguanine. *Nucleic Acids Res.*, 27:496-502.

Eller, M.S., Maeda, T., Magnoni, C., Atwal, D. and Gilchrest B.A. (1997) Enhancement of DNA repair in human skin cells by thymidine dinucleotides: evidence for a p53-mediated mammalian SOS response. *Proc. Natl. Acad. Sci. USA.*, 94:12627-12632.

Epe, B., Pflaum M. and Boiteux, S. (1993) DNA damage induced by photosensitizers in cellular and cell-free systems. *Mutat. Res.* 299:135-145.

Epe, B. (1991) Genotoxicity of Singlet oxygen. *Chem.Biol. Interact.*, 80:239-260.

Epe, B., Mutzel, P. and Adam, W. (1988) DNA Damage by oxygen radicals and excited state species: A comparative study using enzymatic probes *in vitro. Chem. Biol. Interact.*, 67:149-165.

Esterbauer, H., Eckl, P. and Ortner, A. (1990) Possible mutagens derived from lipids and lipid precursors. *Mutat. Res.*, 238:223-33.

Evans, M. D., Dizdaroglu, M. and Cooke, M.S. (2004) Oxidative DNA damage and disease: induction, repair and significance. *Mutat. Res.*, 567:1-61.

Fang, Y.Z., Yang, S. and Wu, G. (2002) Free radicals, antioxidants, and nutrition. *Nutrition*, 18(10): 872-9.

Farmer, G., Bargonetti, J., Zhu, H., Friedman, P., Prywes, R. and Prives, C. (1992) Wild type p53 activates transcription *in vitro. Nature*, 358: 83-86.

Fitch, M.E., I.V. Cross and J.M. Ford, (2003) p53 responsive nucleotide excision repair gene products p48 and XPC, but not p53, localize to sites of UV-irradiation-induced DNA damage, *in vivo. Carcinogenesis*, 24:843-850.

Floyd, R.A., Schneider, J.E. and Dittmer, D.P. (2004) Methylene blue photoinactivation of RNA viruses. *Antiviral Res.*, 61:141-151.

Floyd, R.A., West, M.S., Eneff, K.L. and Schneider, J.E. (1989) Methylene blue plus light mediates 8-hydroxyguanine formation in DNA. *Arch. Biochem. Biophys.*, 273:106-111.

Ford, J.M. (2005) Regulation of DNA damage recognition and nucleotide excision repair: another role for p53. *Mutat. Res.* 577:195-202.

Ford, J.M. and Hanawalt, P.C. (1995) Li-Fraumeni syndrome fibroblasts homozygous for p53 mutations are deficient in global DNA repair but exhibit normal transcription-coupled repair and enhanced UV resistance. *Proc. Natl. Acad. Sci. USA.*, 92:8876-80.

Fraga, C.G., Shigenaga, M.K., Park, J., Degan, P. and Ames, B.N. (1990) Oxidative damage to DNA during aging: 8-hydroxy-2'-deoxyguanosine in rat organ DNA and urine. *Proc. Natl. Acad. Sci. USA.*, 87:4533-4537.

Francis, M.A. and Rainbow, A.J. (1999) UV-enhanced reactivation of a UV-damaged reporter gene suggests transcription-coupled repair is UV-inducible in human cells. *Carcinogenesis*, 20:19-26.

Francis, M.A., and Rainbow.A.J. (2000) UV-enhanced expression of a reporter gene is induced at lower UV fluences in transcription-coupled repair deficient compared to normal human fibroblasts, and is absent in SV40-transformed counterparts. *Photochem. Photobiol.*, 72: 554-561,

Francis, M.A. and Rainbow, A.J. (2003) Role for retinoblastoma protein family members in UV-enhanced expression from the human cytomegalovirus immediate early promoter. *Photochem. Photobiol.*, 77: 612-6127.

Francis M.A., Bagga, P.S., Athwal, R.S. and Rainbow, A.J. (1997) Incomplete complementation of the DNA repair defect in Cockayne syndrome cells by the denV gene from bacteriophage T4 suggests a deficiency in base excision repair. *Mutat Res.*, 385:59-74.

Francis M.A., Bagga, P., Athawal, R. and Rainbow, A.J. (2000) Partial complementation of the DNA repair defects cells from Xeroderma Pigmentosum groups A, C, D and F by the denV gene from bactriophage T4. *Photochem. Photobiol.* 72:365-373.

Friedberg, E. C. (2001). How nucleotide excision repair protects against cancer. *Nature Reviews: Cancer*, 1:22-33.

Friedberg, E. C., Walker, G. C. and Siede, W. (1995) DNA repair and mutagenesis, *ASM Press*, Washington DC.

Gainer, A. L., Korbutt, G. S., Rajotte, R.V., Warnock, G.L. and Elliott, J.F. (1996) Successful Biolistic Transformations of Mouse Pancreatic Islets while preserving cellular function 1. *Transplantation*, 61:1567-1571.

Gallego, M.P. and Sarasin, A. (2003) Transcription-coupled repair of 8-oxoguanine in human cells and its deficiency in some DNA repair diseases. *Biochimie*, 85: 1073–1082.

Ganesan A.K. and Hanawalt P.C. (1994) Removal of cyclobutane pyrimidine dimers from a UV-irradiated shuttle vector introduced into human cells. *Somat. Cell Mol. Genet.*, 20:233-42.

Ginsberg, D., Mechta, F., Yaniv, M. and Oren, M. (1991) Wild type p53 can down-modulate the activity of various promoters. *Proc. Natl. Acad. Sci. USA.*, 88: 9979-9983.

Gollahon, L.S., Kraus, E., Wu, T.A., Yim, S.O., Strong, L.C., Shay, J.W. and Tainsky, M.A. (1998) Telomerase activity during spontaneous immortaliztion of Li-Fraumeni syndrome skin fibroblasts. *Oncogene*, 17:709-717.

Graham, F.L. and Prevec, L. (1991) Manipulation of adenovirus vectors. In Gene Transfer and Expression Protocols. *The Humana Press Inc.*, 7:109-128.

Guyton, K.Z. and Kensler, T.W. (1993) Oxidative mechanisms in carcinogenesis. *Br. Med. Bull.*, 49:523-44.

Hanawalt, P.C. (2002) Subpathways of nucleotide excision repair and their regulation. *Oncogene*, 21:8949-8956

Hanukolgu, I., Rapoport, R., Weiner, L. and Sklan, D. (1993) Electron leakage from the Mitochondrial NADPH-adrenodoxin reductase-adrenodoxin-PH450scc (cholesterol side chain cleavage) system. *Arch. Biochem.Biophy.*, 305:489-498.

Hazra, T.K., Hill, J.W., Izumi, T. and Mitra, S. (2001) Multiple DNA glycosylases for repair of 8-oxoguanine and their potential *in vivo* functions. *Prog. Nucleic. Acid. Res. Mol. Biol.*, 68:193-205.

Henle E.S. and Linn, S. (1997) Formation, Prevention, and Repair of DNA Damage by Iron/Hydrogen Peroxide. *The American Society for Biochemistry and Molecular Biology, Inc.*, 272:19095-19098

Hess R.D. and Brandner, G. (1997) DNA-damage-inducible p53 activity in SV40-transformed cell. *Oncogene*, 15:2501-2504.

Hoeijmakers, J.H., Odijk, H. and Westerveld, A. (1987) Differences between rodent and human cell lines in the amount of integrated.DNA after transfection. *Exp. Cell. Res.*, 169:111-9.

Hollstein, M., Sidransky, D., Vogelstein, B. and Harris, C.C. (1991) p53 mutations in human cancers. *Science*, 253:49-53.

Hsai, K.T., Millar, M.R., King, S., Selfridge, J., Redhead, N.J., Melton D.W. and Saunders P.T.K. (2003) DNA repair gene Ercc1 is essential for normal spermatogensis and oogenesis and for functional integrity of germ cell DNA in the mouse. *Development*, 130:369-378.

Hussein, M.R. (2005) Ultraviolet radiation and skin cancer: molecular mechanisms. *J. Cutan. Pathol.*, 32:191-205.

Hwang, B. J., Ford, J.M., Hanawalt, P.C. and Chu, G. (1998) Expression of the *p48* xeroderma pigmentosum gene is p53-dependent and is involved in global genomic repair. *Proc. Natl. Acad. Sci. USA*, 96:424-428.

Ide, H., Kow, Y.W. and Wallace, S.S. (1985) Thymine glycols and urea residues in M13 DNA constitute replicative blocks *in vitro*. *Nucleic. Acids. Res.*, 13: 8035-52.

Inoue, S. and Kawanishi, S. (1987) Hydroxyl radical production and human DNA damage induced by ferric nitrilotriacetate and hydrogen peroxide. *Cancer. Res.*, 47:6522-7.

Jaiswal, M., Lipiniski, L.J., Bohr, V.A. and Mazur, S.J. (1998) Efficient *in vitro* repair of 7-hydro-8-oxodeoxyguanosine by human cell extracts: involvement of multiple pathways. *Nucleic. Acids Res.*, 26:2184-2191.

Johnson, P., Gray, D., Mowat, M. and Benchimoli, S. (1991) Expression of wild type p53 is not compatible with continued growth of p53-negative tumor cells. *Mol. Cell. Bio.*, 11:1-11.

Kao, J.Y., Goljer, I., Phan, T.A. and Bolton, P.H. (1993) Characterization of the effects of a thymine glycol residue on the structure, dynamics, and stability of duplex DNA by NMR. *J Biol. Chem.*, 268: 17787-93.

Kawanishi, S., Hiraku, Y. and Oikawa, S. (2001) Mechanism of guanine-specific DNA damage by oxidative stress and its role in carcinogenesis and aging. *Mutat. Res.*, 488:65-76.

Khan, S.G., Metter, E.J., Tarone, R.E., Bohr, V.A., Grossman, L., Heayati, M., Bale, S.J., Emmert, S. and Kraemer, K.H. (2000) A new Xeroderma pigmentosum group C poly(AT) insertion/deletion polymorphisms. *Carcinogenesis*, 21:1821-1825.

Kim, S.Y., Kim, E.J. and Park, J.W. (2002) Control of singlet oxygen-induced Oxidative Damage in Escherichia coli. *J. Biochem. Mol. Bio.*, 35:353-357.

Kielbassa, C., Roza, L., and Epe, B. (1997) Wavelength dependence of oxidative DNA damage induced by UV and visible light. *Carcinogenesis*, 18: 811-816.

Klaunig, J. E. and Kamendulis, L.M. (2004) The Role of Oxidative Stress in Carcinogenesis. *Annu. Rev. Pharmacol. Toxicol.*, 44:239-467.

Kohli, M. and Jorgensen, T.J. (1999) The influence of SV40 Immortalization of Human Fibroblasts on p53-Dependent Radiation Responses. *Biochem. Biophy. Res. Comm.*, 257:168-176.

Kovacic, P. and Jacintho, J. D. (2001) Mechanisms of Carcinogenesis: Focus on Oxidative Stress and Electron Transfer. *Curr. Med. Chem.*, 8: 773-796.

Krahn, J. M., Beard, W.A., Miller, H., Grollman, A.P. and Wilson, S.H. (2003) Structure of DNA Polymerase with the mutagenic DNA lesion 8-oxodeoxyguanine reveals structural insights into its coding potential. *Structure*, 11: 121-127,

Kraemer, K.H. (1997) Sunlight and skin cancer: another link revealed. *Proc. Natl. Acad. Sci.* USA, 94: 11-14.

Krokan, H.E., Standal, R. and Slupphaug, G. (1997) DNA glycosylases in the base excision repair of DNA. *Biochem*, 325: 1-16.

Lai, C.M., Lai, Y.K.Y. and Rakoczy, P.E. (2002) Adenovirus and Adeno-associated virus vectors. *DNA Cell Biology*, 21:895-913.

Lan, J., Li, W., Zhang, G., Sun, F.Y., Nagayama, T., O'horo, C. and Chen, J. (2003) Inducible repair of oxidative DNA lesions in the rat brain after transient focal ischemia and reperfusion. *J. Cereb. Blood. Flow. Metab.*, 23:1324-1339.

Lane, D.P. and Crawford, L.V. (1979) T antigen is bound to a host protein in SV40-transformed cells. *Nature*, 278: 261-263.

Larsen, E., Kwon, K., Coin, F., Egly, J.M. and Klungland, A. (2004) Transcription activities at 8-oxoG lesion in DNA. *DNA Repair*, 1457-1468.

Latonen, L., Taya, Y. and Laiho, M. (2001) UV-radiation induces dose-dependent regulation of p53 response and modulates p53-HDM2 interaction in human fibroblasts. *Oncogene*, 20:6784-6793.

Lay, M.A.,Lai, Y.K.Y. and Rakoczy, E. (2002) Adenovirus and Adeno-associated virus Vectors. *DNA Cell Biology*, 21:895-913.

Leadon, S.A. and Cooper, P.K. (1993) Preferential repair of ionizing radiation-induced damage in the transcribe strand of an active human gene is defective in Cockayne syndrome. *Proc. Natl. Acad. Sci.* USA, 90:10499-10503.

Le, X.C., Xing, J.Z., Lee, J., Leadon, S.A. and Weinfeld M. (1998) Inducible repair of thymine glycol detected by an ultrasensitive assay for DNA damage. *Science*, 280:1066-9.

Lee, D.F., Drouin, R., Pitsikas, P. and Rainbow, A.J. (2004) Detection of an involvement of the human mismatch repair genes hMLH1 and hMSH2 in nucleotide excision repair is dependent on UVC fluence to cells. *Cancer Res.*, 64:3865-70.

Lehmann, A.R. (2003) DNA repair-deficient diseases, Xeroderma pigmentosum, Cockayne syndrome and trichothiodystrorphy. *Biochemie*, 85:1101-1111.

Le Page, F., Klungland, A., Barnes, D.E., Sarasin, A., and Boiteux, S. (2000a) Transcription coupled repair of 8-oxoguanine in murine cells: The Ogg1 protein is required for repair in nontranscribed sequences but not in transcribed sequences. *Proc. Natl. Acad. Sci. USA.,* 97: 8397-8402.

Le Page, F., Kwoh, E.E., Avrutskaya, A., Gentil, A., Leadon, S.A., Sarasin, A. and Cooper, P.K. (2000b) Transcription-coupled repair of 8-oxoguanine: Requirement for XPG, TFIIh, and CSB and Implications for Cocayne Syndrome. *Cell,* 101:159-171.

Li, F.P. and Fraumeni, J.F. Jr. (1969) Soft-tissue sarcomas, breast cancer, and other neoplasms. A familial syndrome. *Ann. Intern. Med.,* 71(4):747-52.

Li1, W., Luo, Y., Zhang, F., Signore, A.P., Gobbel, G.T., Simon, R.P., and Chen, J. (2005) Ischemic preconditioning in the rat brain enhances the repair of endogenous oxidative DNA damage by activating the base-excision repair pathway. *J. Cereb. Blood Flow Metab.,* In Press.

Liu, L. (2003) Host cell reactivation of a UV-damaged reporter gene in un-irradiated and pre-uv-irradiated rodent cells. MSc. Thesis, McMaster University

Lipinski, L.J., Hoehr, N., Mazur, S.J., Dianov, G.L., Senturker, S., Dizdaroglu, M. and Bohr, V.A. (1999) Repair of oxidative DNA base lesions induced by fluorescent light is defective in Xeroderma pigmentosum group A cells. *Nucleic. Acids Res.,* 27:3153-3158.

Ljungman, M., Zhang, F., Chen, F., Rainbow, A.J. and McKay, B.C. (1999) Inhibition of RNA polymerase II as a trigger for the p53 response. *Oncogene,* 18:583-592.

Loft, S. and Poulsen, H.E. (1996) Cancer risk and oxidative DNA damage in man. *J. Mol. Med.,* 74:297-312.

Malkin , D. (2001) The role of p53 in human cancer. *Journal of Neuro-Oncology* 51: 231-243.

Manos M.M. and Gluzman, Y. (1984) Simian virus 40 large T-antigen point mutants that are defective in viral DNA replication but competent in oncogenic transformation. *Mol. Cell. Biol.,* 4:1125-1133

Mantovani, F. and Banks, L. (1999) The interaction between p53 and papillomaviruses. *Semin. Can. Biol.,* 9:387-95.

Marnett, L.J. (2000) Oxyradicals and DNA damage. *Carcinogenesis,* 21:361-370.

Matsumura, Y. and Ananthaswamy, H.N. (2002) Short-term and long-term cellular and molecular events following UV irradiation of skin: implications for molecular medicine. *Expert Rev. Mol. Med.,* 1-22.

McKay, B.C., and Ljungman, M. (1999) Role of p53 in recovery of transcription and protection against apoptosis Induced by Ultraviolet Light. *Neoplasia,* 1: 276-284.

McKay, B.C., Francis, M.A, and Rainbow, A.J. (1997) Wild type p53 is required for heat shock and ultraviolet light enhanced repair of a UV-damaged reporter gene. *Carcinogenesis,* 18:245-9.

McKay, B. C. Winrow, C. and Rainbow, A. J. (1997) Capacity of UV-irradiated human fibroblasts to support adenovirus DNA synthesis correlates with transcription-coupled repair and is reduced in SV40-transformed cells and cells expressing mutant p53. *Photochem. Photobiol.* 66:659-664.

McKay, B.C., Becerril, C., and Ljungman, M. (2001) p53 plays a protective role against UV- and cisplatin-induced apoptosis in transcription coupled repair proficient fibroblasts. *Oncogene,* 20: 6805-6808.

Mellon, I., Spivak, G. and Hanawalt, P.C. (1987) Selective removal of transcription-blocking DNA damage from the transcribed strand of the mammalian DHFR gene. *Cell.,* 51:241-9.

Mellon, I., Bohr, V.A., Smith, C.A. and Hanawalt, P.C. (1986) Preferential DNA repair of an active gene in human cells. *Proc. Natl. Acad. Sci. U S A.,* 83: 8878-82.

Mellon, I., and Hanawalt, P.C. (1989) Induction of the Escherichia coli lactose operon selectively increases repair of its transcribed DNA strand. *Nature,* 342: 95-8.

Mietz, J.A., Unger, T., Huibregtse, J.M. and .Howley, P.M. (1992) The transcriptional transactivation function of wild type p53 is inhibited by SV40 large T-antigen and by HPV-16 E6 oncoprotein. *The EMBO Journal,* 11:5013-5020.

Miller, D.M., Buettner, G.R. and Aust S.D. (1990) Transition metals as catalysts of "autoxidation" reactions. *Free Radic. Biol. Med.,* 8:95-108.

Moan, J., Pettersen, E.O. and Christensen, T. (1979) The mechanism of photodynamic inactivation of human cells *in vitro* in the presence of haematoporphyrin. *Br. J. Cancer,* 39:398-407.

Modesitt, S. C., Ramirez, P., Zu, Z., Bodurka-Bevers, D., Gershenson, D., and Wolf, J.K. (2001) *In vitro* and *in vivo* Adenovirus-mediated *p53* and *p16* Tumor Suppressor Therapy in Ovarian Cancer. *Clinical Cancer Research*, 7: 1765-1772.

Moshe, Oren. (1999) Minireview regulation of the p53 tumor suppressor protein. *J. Biol. Chem.*, Vol. 274, Issue 51, 36031-36034.

Nouspikel, T., Lalle, P., Leadon, S.A., Cooper, P.K. and Clarkson, S.G. (1997) A common mutational pattern in Cockayne syndrome patients from Xeroderma pigmentosum group G: implications for a second XPG function. *Proc. Natl. Acad. Sci. U S A.*, 94:3116-21.

Offer, H., Erez, N., Tang, X., Milyavsky, M., Goldfinger, N. and Rotter, V. (2002) The onset of p53-dependent DNA repair or apoptosis is determined by the level of accumulated damaged DNA. *Carcinogenesis*, 23:1025-1032.

Offer, H., Zurer, I., Banfalvi, G., Reha'k, M., Falcovitz, A., Milyavsky, M., Goldfinger and N., Rotter, V. (2001) p53 modulates base excision repair activity in a cell cycle-specific manner after genotoxic stress. *Cancer Res.*, 61:88-96.

O' Neil, F.J., Hu, Y., Chen, T., and Carny, H. (1997) Identification of p53 unbound to T-antigen in human cells transformed by simian virus 40 T-antigen. *Oncogene*, 14:955-965.

Oren, M. (1999) Regulation of the p53 Tumor Suppressor Protein. *J. Biol. Chem.*, 274: 36031-36034.

Osterod, M., Larsen, E., LePage, F., Hengstler, J.G., van der Horst, G.T.J., Boiteux, S., Klungland, A. and Epe, B. (2002) A global DNA repair mechanism involving the Cockayne syndrome B (CSB) gene product can prevent the in-vivo accumulation of endogenous oxidative DNA base damage. *Oncogene*, 21:8232-8239.

Patterson, M.S., Madsen, S.J. and Wilson, B.C. (1990) Experimental tests of the feasibility of singlet oxygen luminescence monitoring *in vivo* during photodynamic therapy. *J. Photochem. Photobiol. B.*, 5:69-84.

Phoa, N. and Epe, B. (2002) Influence of nitric oxide on the generation and repair of oxidative DNA damage in mammalian cells. *Carcinogenesis*, 23:469-475.

Pipas, J.M. and Levine, A.J. (2001) Role of T antigen interactions with p53 in tumorigenesis. *Seminars in Cancer Biology*, 11:23-30.

Pitot, H.C., Goldsworthy, T. and Moran, S. (1981) The natural history of carcinogenesis: implications of experimental carcinogenesis in the genesis of human cancer. *J. Supramol. Struct. Cel. Biochem.*, 17:133-46

Pryor, W.A. (1986) Oxy-radicals and related species: their formation, lifetimes, and reactions. *Annu. Rev. Physiol.*, 48:657-67.

Qiao, Y., Spitz, M.R., Shen, H., Guo, Z., Shete, S., Hedayati, M., Grossman, L., Mohrenweiser, H. and Wei Q. (2002) Modulation of repair of ultraviolet damage in the host-cell reactivation assay by polymorphic XPC and XPD/ERCC2 genotypes. *Carcinogenesis*, 23:295-9.

Rainbow, A.J., Pitsikas, P., Caney, C., Boszko, I., McKay, B.C. and Francis, M.A. 2005. Reactivation of UV-damaged viruses and reporter genes in mammalian cells. In: Regen Drouin, Evelene Sage and Mahmoud Roubhia (Volume Eds.). From DNA photolesions to mutations, skin cancer and cell death. Donat-P. Hader, Giulio Jori (Series Eds.). Comprehensive Series in Photosciences. Amsterdam: Elsevier Science

Rainbow, A.J., McKay, B.C. and Francis, M.A. (2000) Recombinant adenoviruses as expression vectors and as probes for DNA repair in human cells. *Gene Therapy and Molecular Biology*, 5:87-100.

Reardon, J.T., Bessho, T., Kung, H.C., Bolton, P.H. and Sancar, A. (1997) *In vitro* repair of oxidative DNA damage by human nucleotide excision repair system: Possible explanation for neurodegeneration in Xeroderma pigmentosum patients. *Proc. Natl. Acad. Sci. U S A*. 94:9463-8

Reihsaus, E., Kohler, M., Kraiss, S., Oren, M. and Montenarh, M. (1990) Regulation of the level of the oncoprotein p53 in non-transformed and transformed cells. *Oncogene*, 5:137-145.

Reiter, R.J. (1998) Oxidative damage in the central nervous system: protection by melatonin. *Prog. Neurobiol.*, 56:359-384.

Renzing, J. and Lane, D.P. (1995) p53-dependent growth arrest following calcium phosphate-mediated transfection of murine fibroblasts. *Oncogene*, 10:1865-8.

Robbins, J,H,, Brumback, R.A. and Moshell, A.N. (1993) Clinically asymptomatic xeroderma pigmentosum neurological disease in an adult: evidence for a neurodegeneration in later life caused by defective DNA repair. *Eur. Neurol.*, 33(3):188-190.

Ruck, A., Kollner, T., Dietrich, A., Strauss, W. and Schneckenburger, H. (1992) Fluorescence formation during photodynamic therapy in the nucleus of cells incubated with cationic and anionic water-soluble photosensitizers. *J. Photochem. Photobiol. B.*, 12:403-412.

Runger, T.M., Moller, K., Jung, T. and Dekant, B. (2000) DNA damage formation, DNA repair, and survival after exposure of DNA repair-proficient and nucleotide excision repair-deficient human lymphoblasts to UVA1 and UVB. *Int. J. Radiat. Biol.*, 76:789-797.

Runger, T.M., Epe, B. and Moller, K. (1995) Repair of Ultraviolet B and Singlet Oxygen-Induced DNA damage in Xeroderma Pigmentosum Cells. *J. Invest. Dermatol.*, 104:68-73.

Rybanska, I. and Pirsel, M. (2003) Involvment of the nucleotide excision repair proteins in the removal of oxidative DAN base damage in mammalian cells. *Neoplasma*, 50:389-395.

Rykhlevskaya, A.I.1 and Kuznetsova, S.A.1. (2000) Mono- and Bifunctional DNA Glycosylases Involved in Repairing Oxidatively Damaged DNA. *Molecular Biology*, 34:860-874.

Sakumi, K., Furuichi, M., Tsuzuki, T., Kakuma, T., Kawabata, S., Maki, H. and Sekiguchi, M. (1993) Cloning and expression of cDNA for a human enzyme that hydrolyzes 8-oxo-dGTP, a mutagenic substrate for DNA synthesis. *Biol. Chem.*, 268:23524-30.

Salomoni, P. and Pandolfi, P.P. (2002) p53 de-ubiquitination: at the edge between life and death. *Nat. Cell Bio.*, 4:152-153.

Sancar, A. (1995) DNA Repair in Humans. *Annu Rev. Genetics*, 29: 69-105.

Santus, R., Kohen, C., Kohen, E., Reyftmann, J.P., Morliere, P., Dubertret, L. and Tocci, P.M. (1983) Premeation of lysosomal membranes in the course of photosensitization with methylene blue and hematoporphyrin:study by cellular microspectrofluorometry. *Photochem. Photobiol.*, 38:71-77.

Seluanov, A., Gorbunava, V., Falcovitz, A., Sigal, A., Milyavasky, M., Zurer, I., Shohat, G., Goldfinger, N. and Rotter, V. (2001) Change of the death pathway in senescent human fibroblasts in response to DNA damage is caused by an inability to stabilize p53. *Mol. Cell Biol.*, 21:1552-1564

Sengupta, S. and Harris, C.C. (2005) Traffic Cop at the crossroads of DNA Repair and recombination. *Nat. Rev. Mol. Cell Bio.*, 6:44-55.

Seo, Y.R. and Jung, H.J. (2004) The potential roles of p53 tumor suppressor in nucleotide excision repair (NER) and base excision repair (BER). *Exp. Mol. Med.*, 36:505-509.

Seo, Y.R., Fishel, M.L., Amundson, S., Kelly, M.R. and Smith, M.L. (2002) Implication of p53 in base excision repair: *in vivo* evidence. *Oncogene*, 21:731-737.

Sheppard, H.M., Corneillie, S.I., Espiritu, C., Gatti, A. and Liu, X. (1999) New Insights into the Mechanism of inhibition of p53 by simian virus 40 large T antigen. *Mol. Cell Biol.*, 19:2746-2753.

Sheih, S.Y., Ikeda, M., Taya, Y. and Prives, C. (1997) DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2. *Cell.* 91:325-34.

Shimizu, Y., Iwai, S., Hanaoka, F., and Sugasawa, K. (2003) Xeroderma Pigmentosum group C protein interacts physically and functionally with thymin DNA glycosylase. *The EMBO Journal*, 22:164-173.

Siliciano, J.D., Canman, C.E., Taya, Y., Sakaguchi, K., Appella, E. and Kastan, M.B. (1997) DNA damage induces phosphorylation of the amino terminus of p53. *Genes Dev.*, 11: 3471-81.

Sinha, R.P. and Häder, D. (2002) UV-induced DNA damage and repair: a review . *Photochem. Photobiol.*, 225-236.

Sladek, T.L., Laffin, J., Lehman, J.M. and Jacobberger, J.W. (2000) A subset of cells expressing SV40 large T antigen contain elevated p53 levles and have an altered cell cycle phenotype. *Cell Prolif.*, 33:115-125

Slamenova, D., Kuboskova, K., Horvathova, E., and Robichova, S. (2002) Rosemary-stimulated reduction of DNA strand breaks and FPG-sensitive sites in mammalian cells treated with H_2O_2 or visible light-excited Methylene Blue. *Cancer Letters*, 177:145-153.

Slupphaug, G., Kavli, B. and Krokan, H.E. (2003) The interacting pathways for prevention and repair of oxidative DNA damage. *Mutat. Res.*, 531: 231-251.

Srivastava, S., Katayose, D., Tong, Y.A., Craig, C.R., McLeod, D.G., Moul, J.W., Cowan, K.H.AND Seth, P. (1995) Recombinant adenovirus vector expressing wild-type p53 is a potent inhibitor of prostate cancer cell proliferation. *Urology*, 46: 843-848.

Steenken, S. and Jovanovic, S.V. (1997) How easily oxidizable is DNA? One-electron reduction potentials of adenosine and guanosine radicals in aqueous aolution. *J. Am. Chem. Soc.*, 119: 617-618.

Stewart, A.K., Lassam, N.J., Quirt, I.C., Bailey, D.J., Rotstein, L.E., Krajden, M., Dessureault, S., Gallinger, S., Cappe, D., Wan, Y., Addison, C.L., Moen, R.C., Gauldie, J. and Graham, F.L. (1999) Adenovector-mediated gene delivery of interleukin-2 in metastatic breast cancer and melanoma: results of a phase 1 clinical trial. *Gene Therapy.*, 6:350-63.

Sugasawa, K., Ng, J.M., Masutani, C., Iwai, S., van der Spek, P.J., Eker, A.P., Hanaoka, F., Bootsma, D. and Hoeijmakers, J.H. (1998) Xeroderma pigmentosum group C protein complex is the initiator of global genome nucleotide excision repair. *Mol. Cell*, 2: 223-32.

Tang, J. and Chu, G. (2002) Xeroderma pigmentosum complementation group E and UV-damaged DNA-binding protein. *DNA Repair (Amst).*, 1:601-16.

Therrien, J.P., Drouin, R., Baril, C. and Drobetsky, E.A. (1999) Human cells compromised for p53 function exhibit defective global and transcription-coupled nucleotide excision repair, whereas cells compromised for pRb function are defective only in global repair. *Proc. Natl. Acad. Sci. U S A.*, 96: 15038-43.

Tomasevic, G., Kamme, F. and Wieloch, T. (1998) Changes in proliferation cell nuclear antigen, a protein involved in DNA repair, in vulnerable hippocampal neurons following global cerebral ischemia. *Molecular Brain Research*, 60:168-176.

Tong, Z., Singh, G., Rainbow, A.J. (2000) The role of the p53 Tumor Suppressor in the response of human cells to photofrin-mediated photodynamic therapy. *Photochem. Photobiol.*, 71:201-210.

Tuite, E.M. and Kelly, J.M. (1993) Photochemical interactions of methylene blue and analogues with DNA and other biological substrates. *J. Photochem. Photobiol. B*, 21:103-124.

Tuo, J., Jaruga, P., Rodriguez, H., Bohr, V., and Dizdaroglu, M. (2003) Primary fibroblasts of Cockayne syndrome patients are defective in cellular repair of 8-hydroxyguanine and 8-hydroxyadenine resulting from oxidative stress. *FASEB J.*, 17: 668-674.

Varley, J.M. (2003) Germline TP53 mutations and Li-Fraumeni Syndrome. *Hum. Mutat.*, 21:313-320.

Valenzeno, P. (1987) Photomodification of biological membranes with emphasis on singlet oxygen mechanisms. *Photochem. Photobiol.*, 46:147-160.

Valko, M., Izakovic, M., Mazur, M., Rhodes., C.J. and Telser, J. (2004) Role of oxygen radicals in DNA damage and cancer incidence. *Mol. Cell. Biochem.*, 266:37-56.

von Zglinicki, T., Saretzki, G., Docke, W. and Lotze C. (1995) Mild hyperoxia shortens telomeres and inhibits proliferation of fibroblasts: a model for senescence. *Exp. Cell Res.*, 220:186-93.

Wallace, S.S. (2002) Biological consequences of free radical-damaged DNA bases. *Free Radical Biology & Medicine*, 33:1–14.

Wan, F.Y., Yang, L., Zhong, Y.G., Zhu, W., Wang, Y.N. and Zhang, G.J. (2002) Enhancement of lysosomal osmotic sensitivity Induced by the Photooxidation of membrane thiol groups. *Photochem. Photobiol.*, 75:134-139.

Wang, C. I. and Taylor, J.S. (1991) Site-specific effect of thymine dimer formation on DNA tract bending and its biological implications, *Proc. Natl. Acad. Sci. U. S. A.*, 88, 9072-9076.

Wang, X.W., Yeh, H., Schaeffer, L., Roy, R., Moncollin, V., Egly, J.M., Wang, Z., Freidberg, E.C., Evans, M.K, and Taffe, B.G. (1995) p53 modulation of TFIIH-associated nucleotide excision repair activity. *Nat. Genet.*, 10:188-95.

Wang, D., Kreutzer, D.A. and Essigmann, J.M. (1998) Mutagenicity and repair of oxidative DNA damage: insights from studies using defined lesions. *Mutat. Research*, 400: 99-115.

Weinstein, I.B., Gattoni-Celli, S., Kirschmeier, P., Lambert, M., Hsiao, W., Backer, J. and Jeffrey, A. (1984) Multistage carcinogenesis involves multiple genes and multiple mechanisms. *Cell Physiol. Suppl.*, 3:127-37.

Wong, P.K. and Floyd, R.A. (1994) Photochemical synthesis of 8-hydroxyguanine nucleosides. *Methods Enzymol.*, 234:59-65.

Wood, R.D. (1996) DNA repair in eukaryotes. *Annu. Rev. Biochem.*, 65:135-167.

Wood, M.L., Dizdaroglu, M., Gajewski, E. and Essigmann, J.M. (1990) Mechanistic studies of ionizing radiation and oxidative mutagenesis: genetic effects of a single 8-hydroxyguanine (7-hydro-8-oxoguanine) residue inserted at a unique site in a viral genome. *Biochemistry*, 29: 7024-32.

Zhao, R., Gish, K., Murphy, M., Yin, Y., Notterman, D., Hoffman, W.H., Tom, E., Mack, D.H. and Levine, A.J. (2000) The transcriptional program following p53 activation. *Cold Spring Harb. Symp. Quant. Biol.*, 65:475-82.

Zhou, J., Ahn, J., Wilson, S.H. and Prives,(2001) A role for p53 in base excision repair. *C. EMBO J.*, 20:914-23.

Zurer, I., Hofseth, L.J., Cohen, Y., Xu-Welliver, M., Hussain, S.P., Harris, C.C. and Rotter, V. (2004) The role of p53 in base excision repair following genotoxic stress. *Carcinogenesis,* 25:11-19