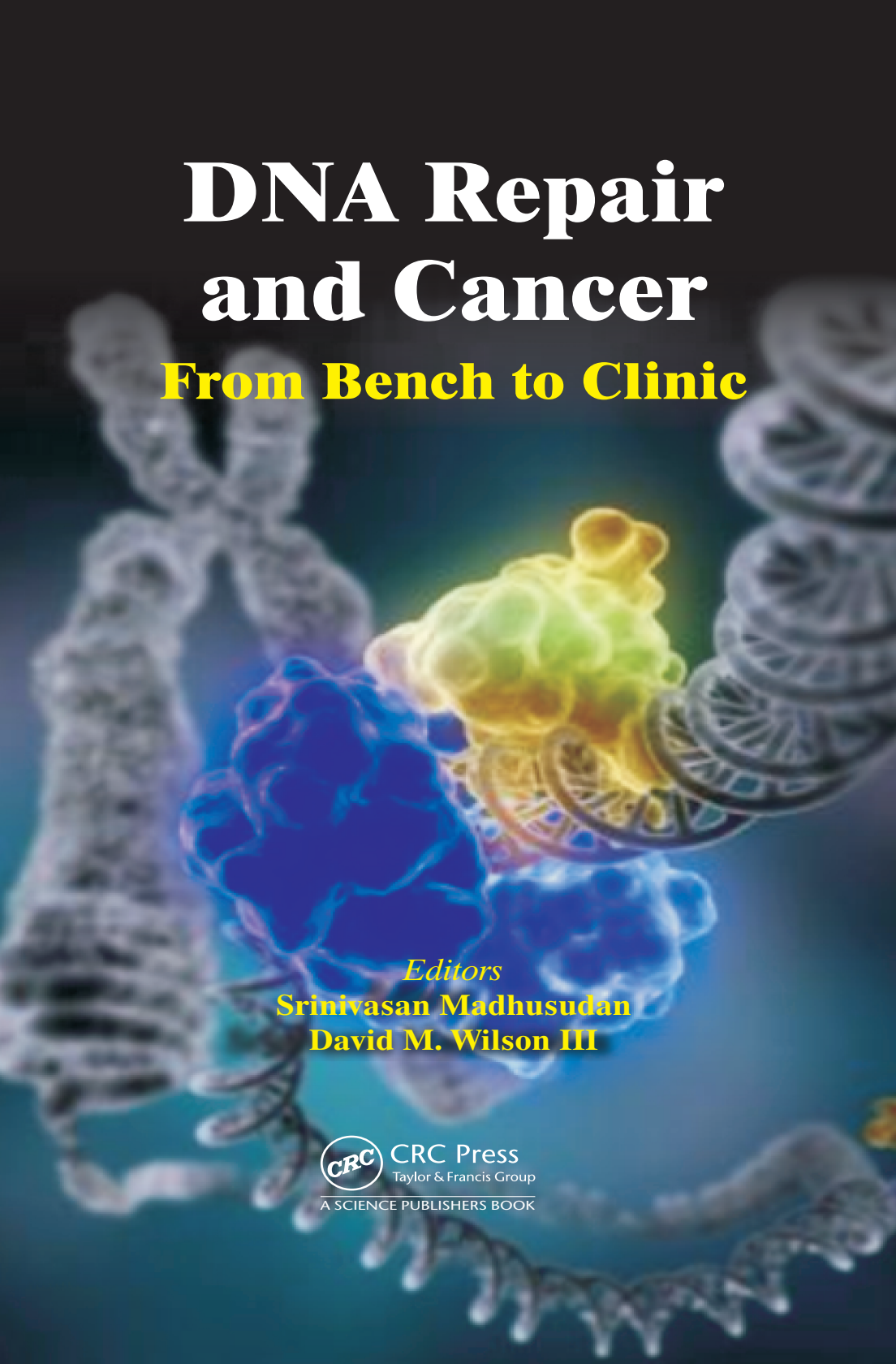
DNA Repair and Cancer

From Bench to Clinic



Editors
Srinivasan Madhusudan
David M. Wilson III

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Srinivasan Madhusudan FRCP, PhD
Nottingham
UK

David M. Wilson III PhD
Baltimore, MD
USA

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Biography

Srinivasan Madhusudan trained in Medical Oncology at the University of Oxford, England. As a Cancer Research UK Clinical Research Fellow he completed his Ph.D. work at the Weatherall Institute of Molecular Medicine, Oxford in 2006. During his fellowship, Dr. Madhusudan conducted pioneering studies targeting DNA base excision repair as a novel anti-cancer treatment strategy. In 2007, Dr. Madhusudan was appointed to the post of Clinical Associate Professor and Consultant in Medical Oncology at the University of Nottingham, England. Dr. Madhusudan specialises in the treatment of gastrointestinal cancers. As an academic oncologist, he is very active in clinical trials and has extensive experience in the investigation of novel anti-cancer therapeutic agents in patients. His pre-clinical laboratory research is focussed on the evaluation of DNA repair factors as prognostic, predictive and therapeutic targets in cancer. Dr. Madhusudan currently works on the clinical translational aspects of base excision repair. His group has identified novel biomarkers, potential drug targets and novel approaches for personalised treatment strategies targeting DNA repair in cancer.

David M. Wilson III received a Bachelor of Arts in Biology and Political Science from Bucknell University in 1989. He completed his Ph.D. work as part of the Molecular Biology Program at Loyola University of Chicago studying repair processes for alkylative and oxidative DNA damage. Dr. Wilson performed his postdoctoral training at Harvard School of Public Health, investigating the molecular mechanisms of abasic endonucleases and the base excision repair pathway. He became a Senior Biomedical Scientist at Lawrence Livermore National Laboratory in 1997 in the Biology and Biotechnology Research Program, where he continued his work on mammalian DNA repair with an emphasis on nucleases. While at Livermore, he was an adjunct faculty member in the Radiation Oncology Department at the University of California Cancer Center - Sacramento. Dr. Wilson is currently a tenured Senior Investigator at the National Institute on Aging in Baltimore in the Laboratory of Molecular Gerontology. The efforts of him and his colleagues have been instrumental in defining how specific

proteins operate mechanistically and participate in the etiology of certain hereditary disorders, including those with neurological abnormalities. Additional investigations have included evaluating the consequence of amino acid variation in DNA repair proteins on functional capacity and disease susceptibility, and identifying small molecule DNA repair inhibitors towards the development of novel therapeutics.

Foreword

Cancer is the major cause of pre-mature death and the search for treatments started long before the birth of modern science. Most treatments were ineffective and only a handful of ideas stood the test of time, such as treating cancer with Röntgen beams, chemical weapons (mustard gas), or with nucleoside analogues. Only with modern science did we understand that most if not all of these effective treatments work by introducing DNA damage.

Today, we know not only that DNA damage is an effective way of eradicating cancer cells; cancer is also caused by DNA lesions that are turned into genetic mutations that transform the normal cell into cancer. Central to understanding cancer development and treatment is the understanding of how DNA lesions are dealt with in the cell. Loss of effective DNA repair results in more genetic mutations and cancer, but may also render these cells more sensitive to DNA damaging anti-cancer agents. Information on DNA repair capacity of both normal and cancer cells can also be used to identify a therapeutic window for selective targeting.

One of the main reasons patients die from cancer is the development of resistance to DNA damaging drugs. Such resistance may arise by increasing the DNA repair capacities or altering the DNA damage response. Hence, inhibition of DNA repair may be an effective way to prevent emerging drug resistance.

There are of course many other ways DNA repair can be exploited for cancer treatment. We know that cancer cells have inherently higher levels of DNA damage, in some cases as a result of loss of one DNA repair pathway. In such circumstances, another DNA repair pathway(s) may become critical for repair and survival of the cancer cell, and could be targeted for treatments. Normal cells may be spared as their DNA repair pathways are intact and since they have an overall lower level of DNA damage.

Here, the world's opinion leaders in translational DNA repair have examined the current status on how DNA repair impacts current anti-cancer treatments and outline the opportunities to improve cancer therapy with novel inhibitors of DNA repair. This book will prove to be an invaluable resource to graduate students, as well as the perfect guidebook for basic

science researchers and clinical investigators, who are interested in understanding the mechanisms of DNA repair and the emerging strategies to eradicate tumour cells through DNA repair manipulation. I want to leave you with a quote from Dr Bruce Alberts, Editor-in-Chief of *Science*:

> "If I were the czar of cancer research, I would give a higher priority to recruiting more of our best young scientists to decipher the detailed mechanisms of both apoptosis and DNA repair, and I would give them the resources to do so"

Professor Thomas Helleday, PhD
Torsten and Ragnar Söderberg Professor of Translational Medicine
Director Science for Life Laboratory,
Division of Translational Medicine and Chemical Biology
Department of Medical Biochemistry and Biophysics,
Karolinska Institutet, Stockholm, Sweden

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Introduction

DNA: The Genetic Material

For many centuries, farmers bred different plants and animals in the hope of creating more valuable hybrids. However, since the mechanisms governing inheritance were unknown at the time, many of these essentially arbitrary breading efforts were unsuccessful. It wasn't until carefully controlled laboratory experiments were performed in the mid 1800s that the genetic mechanisms began to become understood. In particular, while a number of hypotheses were proposed to explain the transmission of hereditary traits from parents to children, it was Gregor Mendel, a little known Central European monk, who while working with pea plants unraveled the basic principles of genetics, which he would describe in 1865 from a paper subsequently entitled "Versuche über Pflanzen-Hybriden". These guiding principles—which stated that (1) inheritance of each trait is determined by "units" (now known as genes) passed on by the descendants, (2) an individual inherits one unit from each parent for each trait, (3) the trait may not show up in an individual, but can still be passed on to the next generation, and (4) the units for each trait segregate during gamete production—were not widely accepted as accurate until the early 1900s, when rediscovered and validated by other scientists in different organisms.

Around the time of Mendel's discoveries, Friedrich Miescher, in researching the composition of lymphoid cells, isolated from the nucleus (unknowingly at the time) the first crude preparation of DNA, which he referred to as nuclein (reviewed in Dahm 2008). In the late 1800s, Walther Flemming identified in the cell nucleus thread like structures that were easily stained by basophilic dyes that were subsequently termed chromosomes, meaning colored body (reviewed in Paweletz 2001). However, despite several compelling genetic studies carried out by the likes of Barbara McClintock and others, it wasn't until the 1950s, when Alfred Hershey and Martha Chase conducted the so-called "blender experiment", that DNA, and not protein, gained widespread recognition as the genetic material of bacteriophage and other organisms (Hershey and Chase 1952). In 1953, using the X-ray structural information on DNA from Rosalind Franklin and

Maurice Wilkins, James Watson and Francis Crick were able to construct an accurate model of the 3-dimensional form of DNA, consisting of the so-called double helix with little rungs (bases) connecting the two strands (Watson and Crick 1953). Through the efforts of many scientists since, DNA is now known as the blueprint of the cell, encoding the genetic instructions for the development and functioning of all known living organisms, excluding RNA viruses.

DNA Damage, Mutations and Repair

The idea that our genetic composition could be modified by external insults was recognized well before the actual discovery of DNA. Indeed, in the 1920s, Hermann Muller performed research showing that X-rays could induce mutations, although these observations were not well received at the time (reviewed in Carlson 1981). In the subsequent years, he continued to work on X-rays, as well as other mutagens like ultraviolet (UV) light and mustard gas. His studies helped pave the way for the appreciation of the biological consequences of genetic mutations and gross chromosomal structural changes.

It was in the 1950s and 60s, after DNA was established as the genetic material, that correlations between the absorption spectrum of DNA and the mutagenic wavelengths of UV radiation began to be appreciated, and when specific forms of DNA damage, such as thymine dimers, were experimentally documented (reviewed in Witkin 1966). Around the same time, Kelner and Dulbecco independently, although not deliberately, observed that bacteria exhibited differential viability after exposure to UV light that was determined by subsequent exposure to visible light (Kelner 1949; Dulbecco 1949). Soon thereafter bacterial cells were demonstrated to harbor a protein, now termed photolyase, which utilizes visible light to catalyze the resolution of covalently-linked, cytotoxic UV-induced pyrimidine dimers in DNA (reviewed in Rupert 1975). This photoreactivation process, reported in 1949, is widely considered the first evidence of "DNA repair".

The DNA Damage Response

Upon recognition around the mid 1900s that DNA was not the extremely stable molecule that it was originally thought to be, several investigators began to search for and identify different types of DNA damage induced by chemical mutagens and radiation, as well as cellular enzymes that recognize and remove specific forms of DNA damage or resolve imperfections in the genetic material. It was indeed quickly appreciated that organisms possess a range of conserved biochemical activities that have the capacity to restore

damaged DNA back to its native undamaged state in a process termed "DNA Repair". Since these early discoveries, a number of investigators have gone on to document that cells maintain multiple DNA repair pathways, which act on a subset of specific DNA lesions, and to unravel the main molecular steps of these processes, although some of the precise details remain unsolved. As is overviewed in Chapter 1 by Croteau and Bohr, the major DNA repair systems are: direct reversal (namely, O^6-methylguanine DNA methyltransferase, or MGMT), mismatch repair (MMR), base excision repair (BER), nucleotide excision repair (NER), and recombination (Table 1). All of these repair mechanisms are covered more extensively throughout the book; with Kaina and Christmann reviewing MGMT (Chapter 6), Kinsella providing a summary of MMR (Chapter 7), Kelley and colleagues describing BER (Chapter 8), and Powell and colleagues covering aspects of recombination (Chapter 14). A specialized pathway that copes with interstrand crosslinks, lesions that covalently connect the two strands of DNA, is reviewed by Brosh and colleagues in Chapter 10. Decades of research have identified well over 100 genes that are associated with the different human DNA damage responses (http://sciencepark.mdanderson.org/labs/wood/DNA_Repair_Genes.html).

In addition to the pathways that are dedicated to removing DNA modifications, cells have evolved the capacity to regulate cell cycle progression. Thus, in circumstances where DNA damage is excessive, cells can activate steps to arrest the cell cycle to permit sufficient time for accurate repair prior to DNA replication. These defense mechanisms, which involve the tumor suppressor protein p53 and the stress-activated

Table 1. Major Human DNA Repair Pathways.

Pathway	Primary DNA Substrates	Relevant Anti-cancer Agent(s)
Direct Reversal (MGMT)	O^6-methylguanine	Alkylators, such as temozolomide
Base Excision Repair (BER)	Oxidative, alkylative or spontaneous base damage; abasic sites; single-strand breaks	Ionizing radiation, radiomimetics, alkylators (monofunctional), topoisomerase inhibitors, antimetabolites
Mismatch Repair (MMR)	Mispaired nucleotides; insertion/deletion loops; certain base modifications	Alkylators, crosslinking agents, antimetabolites
Nucleotide Excision Repair (NER)	Helix-distorting base modifications	Alkylators, crosslinking agents
Recombinational Repair		
• Homologous (HR)	Double-strand breaks, collapsed replication forks	Ionizing radiation, alkylators, crosslinking agents, topoisomerase inhibitors
• Non-homologous end-joining (NHEJ)	Double-strand breaks	Ionizing radiation

kinases ATM and ATR, among others, are described in detail by Lee and colleagues (Chapter 12) and Hall and colleagues (Chapter 13). Moreover, in some situations when excessive DNA damage persists, cells can engage tolerance pathways, such as translesion DNA polymerases, which permit survival at the cost of mutagenesis. Translesion polymerases, which have the ability to bypass a potentially toxic DNA modification, are covered in detail in Chapter 11 by Yamanaka and Lloyd.

Defects in DNA Repair and Cancer

Our genetic integrity is constantly being threatened by both endogenous (namely oxygen free radicals, which are produced during mitochondrial respiration) and exogenous DNA-damaging agents, such as UV light, ionizing radiation, and a range of environmental toxins, some of which are in the food and water we consume and air we breathe. The first evidence that sub-optimal DNA repair may underlie cancer predisposition was documented in the late 1960s by James Cleaver (Cleaver 1968, 1969), who found that patients suffering from the rare genetic disorder Xeroderma Pigmentosum (XP) have a defect in DNA repair synthesis for UV light-induced base damage, a process later defined as NER. These individuals exhibit extreme UV irradiation sensitivity, displaying >100-fold increased skin cancer incidence that stems from a failure to efficiently cope with sunlight-induced DNA photoproducts. Since the discovery of XP, advances in experimental techniques and molecular genetics have aided in the identification of several cancer syndromes that arise from defects in the DNA damage response and are characterized by genomic instability (Table 2). In Chapter 3, Beckman describes how a mutator phenotype is common to cancer cells and may in fact be necessary for carcinogenesis. In Chapter 2, Vijg and colleagues discuss cellular senescence and aging as an alternative to mutagenesis, persistent cell growth and cancer.

Since many of the cancer syndromes listed in Table 2 account for a relatively small number of the total cancer cases (the so-called cancer genes), researchers have explored the hypothesis that slightly reduced DNA repair function will correlate with cancer susceptibility (the so-called susceptibility alleles), likely in a relevant exposure-dependent manner. It is now well appreciated that DNA repair composition is quite variable from individual to individual, with a large number of single nucleotide polymorphisms (SNPs) being observed, and that reduced repair capacity is associated with increased disease risk (Mohrenweiser et al. 2002). Indeed, it has been estimated that if a given DNA repair pathway is comprised of 20 genes that an individual will be variant for 5–7 genes of that pathway,

Table 2. Hereditary cancer syndromes stemming from defects in DNA damage responses. See Table 1 for pathway abbreviations.

Syndrome	**Defective gene and/or pathway**	**Primary cancer association**
Xeroderma Pigmentosum (XP)	XPA-XPG, NER*	UV light-induced skin cancer
Hereditary nonpolyposis colorectal cancer (HNPCC)	MLH1, MSH2, MSH6, PMS2, MLH3, MMR	Colorectal cancer, others
MUTYH-associated polyposis (MAP)	MUTYH, BER	Colorectal cancer
Li-Fraumeni Syndrome	p53, CHK2, checkpoint response	Various cancers
Ataxia Telangiectasia (AT)	ATM, double-strand break response	Leukemia, lymphoma
AT-like Disorder	MRE11, double-strand break response	Lymphoma
Nijmegen Breakage Syndrome (NBS)	NBS1, double-strand break response	Lymphoma
Hereditary Breast Cancer Syndrome	BRCA1/BRCA2, HR	Breast (ovarian, prostate) cancer
Ligase IV Deficiency	LIG4, NHEJ	Leukemia (?)
Werner Syndrome	WRN, many pathways	Various cancers
Bloom Syndrome	BLM, many pathways	Various cancers
Rothmund–Thomson Syndrome	RECQ4, many pathways	Osteosarcoma
Fanconi Anemia	FANC genes, interstrand crosslink repair	Leukemia, others

*There is a variant complementation group of XP (XPV) that stems from a defect in translesion DNA synthesis.

indicating that homozygous wild-type genotypes and individuals of identical genetic make-up will be rare within the population. While it is unclear whether much of the observed genetic variation has a functional consequence, it is possible that SNPs, for instance, can affect repair capacity by altering (i) the amino acid sequence of the encoded protein(s), (ii) the splicing, stability or translation of the mRNA transcript, or (iii) the promoter or epigenetic regulatory components of the genomic region, resulting in increased or decreased protein production or activity. In Chapter 9, Wei and Wang discuss the ramifications of DNA repair variation as related to cancer susceptibility, with a focus on the NER pathway. Estimation of functional DNA repair capacity in somatic cells will not only allow cancer risk estimation, but could provide prognostic and predictive information for individuals and patients.

DNA Repair in Cancer Therapeutics

It is notable that many of the therapeutic agents employed to eradicate cancer are actually DNA-damaging agents (Table 3). Indeed, besides surgical removal of the tumor, the most commonly employed strategies to kill cancer cells involve targeted ionizing radiation and a battery of systemic chemotherapeutic compounds. The concept is that rapidly dividing cells, such as cancer cells, will be particularly susceptible to the cytotoxic effects of DNA lesions that block replication and activate cell death responses, such as apoptosis. The impact of radiation-induced DNA damage in the context of cancer therapeutics is summarized by Harrison in Chapter 4. In Chapter 5, Bradshaw provides a comprehensive review on one of the most commonly employed classes of chemotherapeutic agents, DNA alkylators. The complex interactions between the tumor microenvironment and DNA damage responses, particularly as it relates to cellular responsiveness, are discussed in Chapter 17 by Bristow and colleagues.

It is clear from recent biological studies that the cellular responses induced by DNA damage in cancer cells are complex and that the ultimate fate of a cancer cell will be dictated by its ability to repair such damage. Overwhelming, unrepairable DNA damage will activate cell death responses, whereas efficient DNA repair will allow cell survival. In the clinical context, this paradigm implies that proficient DNA repair in tumors will contribute to therapeutic agent resistance and that sub-optimal repair in normal tissue could result in toxicity. Moreover, the narrow therapeutic index and the heterogeneity of patient responses to chemotherapy and

Table 3. Major classifications of anti-cancer agents. See Table 1 for relevant DNA repair response.

Agent classification	**Specific clinical examples**	**Major DNA damage intermediates**
Ionizing radiation		Base damage, single- and double-strand breaks
Alkylating agents • Monofunctional	Temozolomide, streptozotocin	Base damage, a basic sites, single-strand breaks
• Bifunctional	Nitrogen mustards (cyclophosphamide, melphalan, chlorambucil) and aziridines (thiotepa, mitomycin C)	See above, plus bulky adducts, intra/interstrand crosslinks
Topoisomerase inhibitors	Irinotecan, topotecan, camptothecin, etoposide, doxorubicin, amsacrine	Protein-DNA strand break termini, replication-dependent double-strand breaks
Antimetabolites	5-Fluorouracil, gemcitabine, thiopurines (6-thioguanine),	Base lesions, blocked DNA termini, replication-dependent double-strand breaks

radiotherapy suggests that a better understanding of the molecular basis for such differences will facilitate personalized care for cancer patients. Indeed, DNA repair-targeted, personalized therapy based on the approach of synthetic lethality is an exciting new area in cancer medicine. In Chapter 15, Curtin discusses the current status of poly(ADP-ribose) polymerase (PARP) inhibitors in clinical studies, focusing on the concept of synthetic lethality and trials involving patients with germline mutations in the *BRCA1* or *BRCA2* recombination genes. Urick and Bell review more broadly the topic of tumor genetics and personalized medicine in Chapter 16. Finally, Payne and Middleton (Chapter 18) present the current status and potential challenges of clinical trial designs that involve pharmacological inhibitors of DNA repair, which as highlighted throughout the book is an exciting new area in cancer therapeutics.

In summary, this book presents a comprehensive synopsis of DNA repair, a new frontier in anti-cancer discovery. The focus on translational applications and therapeutics will provide essential information for basic scientists, pharmaceutical investigators and clinicians interested in cancer therapy.

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CHAPTER 1

Overview of DNA Repair Pathways

Deborah L. Croteau[1,a] and *Vilhelm A. Bohr*[1,b,*]

INTRODUCTION

DNA repair deficiencies are well-known and significant risk factors for a variety of cancers, neurodegeneration and premature aging. Cells have numerous DNA repair pathways, and each system preferentially repairs a particular set of lesions. DNA repair mechanisms must balance the need to minimize mutations in somatic cells, with the need to allow for sufficient genetic diversity in germ line cells to permit organisms to evolve and thrive under pressure from diverse environmental challenges. This Chapter briefly describes the repertoire of DNA repair pathways in human cells, with particular attention to heritable defects in DNA repair functions that lead to cancer predisposition.

DNA is constantly subjected to insults that can lead to genomic instability. DNA damage can be derived from both endogenous and exogenous sources. Endogenous lesions arise from the inherent instability of DNA or as unintended byproducts of cellular respiration. For example, base mismatches can result from the spontaneous deamination of cytosine to uracil, thus creating a U:G mispair. Depurination or depyrimidination of DNA produces AP sites, which lack the instructional information encoded within the base. Additionally, reactive oxygen species (ROS) generated

[1]NIH Biomedical Research Center, 251 Bayview Boulevard, Baltimore, MD, USA 21224.
[a]Email: croteau@mail.nih.gov
[b]Email: BohrV@grc.nia.nih.gov
*Corresponding author

from mitochondrial respiration contribute significantly to endogenous damage, readily attacking DNA to cause both base and sugar modifications. Ultraviolet (UV) rays from sunlight are one of the most prevalent forms of exogenous DNA damaging agents for human skin. Other exogenous DNA damaging agents include γ-irradiation, cancer chemotherapeutics, cigarette smoke, and compounds found in some foods. Through multiple coordinated efforts, our cells fend off these potentially mutagenic or lethal DNA insults using their arsenal of DNA repair pathways.

All eukaryotes are able to efficiently repair a wide variety of DNA lesions, with each repair mechanism preferentially recognizing distinct forms of DNA damage. Furthermore, in most situations, although not all, there is a backup repair mechanism should the first line of defense fail. DNA double-strand breaks (DSBs) are among the most lethal lesions, because such damages can result in significant loss of genetic material. Homologous recombination (HR), non-homologous end joining (NHEJ) and alternative NHEJ (Alt-NHEJ) each deal with the repair of DSBs. Nucleotide excision repair (NER) is responsible for the removal of a broad spectrum of DNA lesions, including UV-induced photoproducts and other bulky DNA adducts. Mismatch repair (MMR) is responsible for correcting single nucleotide mismatches, insertions and deletions (indels), and small loops that arise as a result of DNA replication errors. Base excision repair (BER) and single-strand break repair (SSBR) are related pathways responsible for the removal of modified base lesions, such as 8-oxoguanine (8-oxoG), abasic (or AP) sites, and single-strand breaks (SSBs). DNA crosslinks can either be intrastrand or interstrand (ICLs). ICLs covalently connect bases on opposing DNA strands, while intrastrand crosslinks covalently link neighboring bases on the same strand of DNA. A variety of DNA repair mechanisms work together to catalyze the removal of DNA ICLs, whereas NER typically removes intrastrand crosslinks. Figure 1 depicts the various repair pathways, with an example of the types of DNA lesions they target. Together, these repair strategies ward off the deleterious effects of both exogenous and endogenous DNA damage.

The mitochondrial genome (mtDNA) also continuously sustains DNA damage that must be repaired. Some, but not all, of the nuclear DNA repair mechanisms are present in mitochondria, and later we will briefly discuss the differences. Persistent mtDNA and nuclear DNA damage accumulates at higher than normal levels in cells with DNA repair defects. This Chapter will discuss the implications of such defects for cell viability, as well as for cellular and organismal aging and cancer susceptibility.

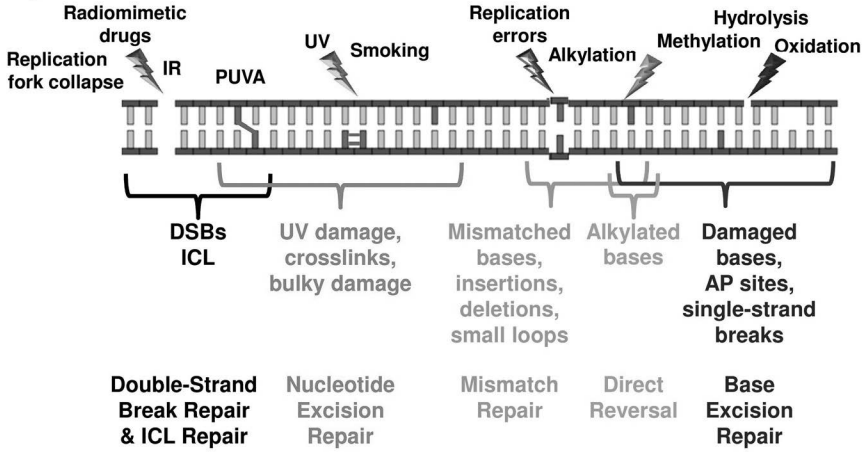

Figure 1. Overview of DNA damage and DNA repair pathways. Both endogenous and exogenous agents give rise to DNA damage. Cells possess multiple DNA repair pathways to combat DNA damage that accumulates. Some lesions are repaired by multiple pathways, as denoted by the overlapping arrows. Abbreviations: IR, ionizing radiation; PUVA, psoralen plus UVA light; UV, ultraviolet light; ICL, interstrand crosslinks; AP, apurinic or apyrimidinic site.

DOUBLE-STRAND BREAK REPAIR (DSBR)

Ionizing radiation (IR), cancer chemotherapy drugs, interrupted DNA replication and even DNA repair itself can create DSBs in the genome. IR, by virtue of its high energy particles, creates lots of damage, including both SSBs and DSBs (see Chapter 4). Chemotherapy drugs like alkylating agents, topoisomerase poisons or bleomycin cause DSBs in DNA as well (see Chapter 5). DSBs can also be created when a DNA polymerase encounters a SSB or blocking lesion at the replication fork. Additionally, the processing of multiple closely opposed lesions on the two DNA strands, such as those induced by IR, can lead to the generation of DSBs. Therefore, DSBs arise for a variety of reasons, and they are among the most deleterious lesions, because if they persist, they can block replication fork progression in proliferating cells and lead to profound genetic instability or cell death.

DSBR is divided into two major pathways: NHEJ and HR. NHEJ is further divided into classical DNA-PKcs-dependent (called NHEJ here) and Alt-NHEJ (for recent reviews see (Mladenov and Iliakis 2011; Stracker and Petrini 2011)). NHEJ and HR differ mechanistically in several important ways. First, NHEJ and Alt-NHEJ are notoriously error prone, whereas HR is considered to be an error-free mechanism. Second, NHEJ is operable in

all phases of the cell cycle, in both differentiated and replicative cells. In contrast, HR is restricted to replicating cells and to late S and G2 phases of the cell cycle when a homologous sister chromatid is available. Alt-NHEJ also shows some cell cycle bias, as it is absent in resting cells, low in G1, and up-regulated in G2 phase cells. Finally, and somewhat surprisingly, NHEJ is the preferred route of processing DSBs in human cells. Understanding why human cells have evolved to preferentially use an inherently error prone mechanism for DSBR is an active area of research.

Figure 2 shows the key proteins in NHEJ: classical (A) and Alt-NHEJ (B). Each pathway has DSB sensors, DNA end processors, DSB mediators, a DNA polymerization and ligation step. Classical NHEJ employs the DNA end binding proteins Ku70/Ku80 to signal to DNA-PKcs that a DSB is present, whereas Alt-NHEJ is proposed to use poly(ADP ribose) polymerase 1 (PARP1) and the MRN complex (MRE11, RAD50 and NBS1) to recognize the DSB. Both NHEJ pathways are fundamentally ligation events, and thus, it is necessary to make ligatable DNA ends. Therefore, a variety of DNA end processing proteins are needed, dictated by the type of termini present at the DSB ends. During Alt-NHEJ, it is thought that CtIP and MRN a DNA endonuclease, resects the DNA ends until small regions of microhomology (5–25 base pairs) are revealed between two local DSBs. Indeed, one of the defining features of Alt-NHEJ is regions of microhomology at the site of the repaired DSB. A polymerization step is sometimes necessary to fill in the missing nucleotides if end resection has occurred. Finally, a ligation step restores the continuity of the DNA strands. For ligation, NHEJ employs ligase 4 (LIG4) in complex with XRCC4/XLF, while Alt-NHEJ uses ligase 3 (LIG3) in complex with XRCC1. Human cells predominately use NHEJ for DSBR, but will employ the slower Alt-NHEJ if classical NHEJ is sufficiently compromised.

The major proteins involved in HR are shown in Fig. 2C, and this pathway is typically an error-free recombination mechanism for DSB resolution. This pathway is especially important for re-starting stalled replication forks (see Chapter 14). MRN is a multi-protein complex composed of MRE11, RAD50 and NBS1. MRE11 is a DNA binding protein with endo- and exonuclease activity. RAD50 binds to MRE11 and tethers two bound MRN complexes together on opposite DNA strands (Moreno-Herrero et al. 2005; Williams et al. 2005). NBS1 is required for downstream activation of the DNA damage response kinase, ATM (Lukas et al. 2003; Kitagawa et al. 2004). Together with CtIP, a protein that interacts with BRCA1, MRN promotes resection of the DNA ends, which then recruits RPA and RAD51 to the generated single-stranded tail. RAD51 recombinase creates filaments on the exposed 3′ single stranded DNA and conducts a homology search to eventually promote invasion of the sister chromatid duplex (Sartori et al. 2007). The invading 3′ end acts as a primer for strand

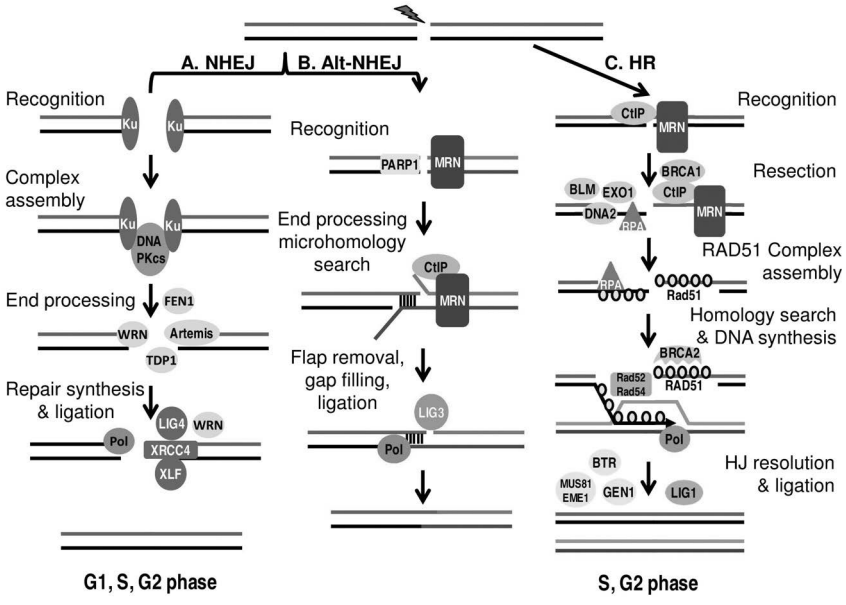

Figure 2. Schematic diagram of double-strand break repair mechanisms. (A) In non-homologous end-joining, NHEJ, double-strand breaks (DSBs) are recognized by the heterodimer Ku70/Ku80 (Ku). These DNA end binding proteins recruit the protein kinase DNA-PKcs, which in turn phosphorylates and recruits other proteins. When the DNA ends are incompatible for ligation, exo- or endonucleases are recruited to modify the ends; shown here are WRN, FEN1, Artemis and TDP1. Next, a DNA polymerase fills in any recessed ends,then LIG4 in complex with XRCC4 and XLF seals the nick. NHEJ is the predominate DSB repair pathway used in human cells and is available in G1, S and G2 of the cell cycle. (B) Alternative NHEJ, Alt-NHEJ, is employed when NHEJ is compromised. DSBs are thought to be recognized by PARP1 and/or the MRN complex. The distinguishing feature of this repair pathway is that end resectioning occurs until short stretches of homology (5–25 nucleotides) are found. The flaps are removed, DNA synthesis fills in missing nucleotides and then Ligase 3 (LIG3) seals the nick. (C) In homologous recombination (HR), the major damage recognition player is the MRN complex. Among other functions, BRCA1 activates the DNA damage response to induce cell cycle arrest following DSB formation. MRN, CtIP, EXO1, DNA2 and BLM may all function to resect the DNA and generate 3′ single-stranded tails. These tails are then bound by RPA and RAD51 filaments. RAD51 recombinase searches for homology within another homologous strand of DNA, preferentially its sister chromatid. RAD52 and RAD54 promote these processes. DNA synthesis copies the DNA off the sister chromatid, then Holliday junctions (HJ) are resolved using proteins like GEN1, Mus81-EME1 or the BTR complex, consisting of BLM, topoisomerase 3α, RMI1 and RMI2. Finally, DNA ligase, LIG1, ligates the DNA ends to restore DNA integrity. HR is only operable during S and G2 phases of the cell cycle.

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displacement DNA synthesis, ultimately leading to gene conversion with or without crossover events. If the crossovers are between sister chromatids, then the resultant products are called sister chromatid exchanges. Four-strand DNA crossovers, called double Holliday Junctions (dHJ), are resolved

by Holliday junction processing enzymes, and the ends are ligated by DNA ligase 1 (LIG1). Thus far, three alternative dHJ processing enzymatic pathways have been identified: GEN1, MUS81-EME1 (SLX1-SLX4) and BLM-Topoisomerase 3α-RMI1-RMI2 complex (Wechsler et al. 2011). Proteins that modulate the HR pathway often target RAD51 filament formation, a scenario seen for several of the RecQ helicases that destabilize the RAD51 filament, thereby inhibiting HR.

The importance of DSBR is underscored by the fact that defects in this pathway are associated with increased susceptibility to lymphoma, leukemia, breast, ovarian and colon cancers (Helleday et al. 2008). While it is essential for cells to repair DSBs, inappropriate DSBR can drive genomic instability. Specifically, if the DSBR pathway inappropriately joins two unrelated chromosomes, chromosomal translocations result. Also, recombination at telomeres can lead to chromosome end-to-end fusions.

Telomeres are the DNA-protein complexes at the ends of our linear chromosomes. They are composed of the repeat sequence TTAGGG bound by a protein complex called shelterin. These proteins bind and protect the chromosome ends from being recognized as DSBs. Thus, shelterin proteins appear to have evolved to inhibit both NHEJ and HR at telomeres. Occasionally, however, dysfunctional or unprotected telomeres will arise from natural telomere shortening, the loss of shelterin proteins or dysfunctional telomerase, which is the DNA polymerase designed for telomere elongation. Notably, loss of the shelterin protein TRF2, a double-stranded telomeric DNA binding protein, in a 53BP1 null mouse embryonic fibroblast background, leads to NHEJ-mediated telomere fusions (Smogorzewska and de Lange 2002; Celli and de Lange 2005; Rai et al. 2010). Moreover, Rai et al. found that telomere fusions due to the loss of TPP1-POT1a/b, the single-stranded telomeric DNA binding proteins, likely proceed through Alt-NHEJ, since they arise independent of LIG4 (Rai et al. 2010). These results suggest that both NHEJ pathways contribute to genomic instability by causing telomere end-to-end fusions, specifically in the absence of the protective nucleoprotein cap.

NHEJ and HR play other important roles in the cell as well. For example, NHEJ is employed to mount a normal immune response. In particular, antibody diversity depends upon V(D)J and class switch recombination, both of which utilize NHEJ to resolve genetically programmed DSBs. Furthermore, HR is a critical part of normal meiosis. Specifically, during meiotic recombination, sister chromatids align, and genetic diversity is introduced by HR between the maternal and paternal chromosomes. As might be expected, individuals with defects in DSBR have genomic instability, immunodeficiency and elevated risk for a variety of cancers (see Table 1).

Table 1. DNA repair gene defects associated with cancer predisposition.

Gene	DNA Repair Pathway	Disorder	OMIM	Cancer Predisposition
ATM	DSBR signaling	Ataxia telangiectasia	607585	leukemia
MRE11	DSBR	AT-like disorder	604391	colorectal cancer
NBS1	DSBR	Nijmegen breakage syndrome	602667	leukemia & ovarian cancer
LIG4	DSBR	Lig4 syndrome	601837	leukemia
BRCA1	DSBR-HR	hereditary breast & pancreatic cancer	113705	breast, ovarian & pancreatic
BRCA2 FANCD1	DSBR-HR	hereditary breast cancer, Fanconi anemia	600185	many
Artemis	DSBR-NHEJ	Omenn syndrome	605988	lymphoma
BLM	DSBR	Bloom syndrome	604610	many
WRN	DSBR, BER	Werner syndrome	277700	osteosarcoma & lymphoma
RECQL4	DSBR, BER	Rothmund-Thomson RAPADILINO syndrome	603780 266280	osteosarcoma & lymphoma
FA A-P	HR, ICL repair	Fanconi anemia	many	many
XP A-G	NER	Xeroderma Pigmentosum	many	skin cancer
MSH2, MLH1, MSH6, PMS1, PMS2, MLH3	MMR	Lynch syndrome, Turcot syndrome	120435 276300	bowel, endometrial, ovarian, glioblastoma multiforme
p53	damage signaling	Li-Fraumeni	191170	many
CHK2	damage signaling	Li-Fraumeni	604373	many
ATR	S-phase damage signaling	Seckle syndrome	601215	-

Online Mendalian Inheritance in Man (OMIM) identification numbers are provided for interested readers if they wish to obtain primary literature regarding any gene of interest. Abbreviations used in table are: double-strand break repair, DSBR; homologous recombination, HR; non-homologous end-joining, NHEJ; base excision repair, BER; interstrand crosslink, ICL; nucleotide excision repair, NER.

DNA damage not only provokes DNA repair, but also elicits a signal transduction cascade known as the DNA damage response (DDR). For instance, after DSB damage is detected in the cell, kinases like ATM and ATR are activated (see Chapter 13). These proteins phosphorylate a whole host of other DDR proteins to induce cell cycle delays, mobilize DNA repair proteins and promote alterations in the local chromatin structure. P53, one of the most frequently mutated genes in hereditary and sporadic cancer, is a downstream target for these kinases (see Chapter 12). Additionally, mutations in another DDR kinase, *CHK2*, are associated with the cancer syndrome Li-Fraumeni (Bell et al. 1999) (see Table 1).

NUCLEOTIDE EXCISION REPAIR (NER)

NER is one of the most versatile DNA repair systems, because this pathway does not depend on recognition of the lesion per se, but rather identifies distortions induced in the DNA structure. By utilizing this clever mechanism of DNA damage recognition, NER is able to repair a wide variety of chemically distinct DNA lesions. There are two subpathways of NER based on the mechanism used to identify the DNA damage. If a lesion is found in the bulk of the genome, then repair is mediated by general genome repair (GGR). However, if the DNA lesion is discovered by the transcription apparatus during RNA synthesis, then repair proceeds via transcription-coupled repair (TCR). After lesion recognition, the biochemical steps for removal of the target damage and restoration of the original DNA content are similar between to the two pathways (Fig. 3).

The importance of NER is underscored by the observation that individuals with a deficiency in NER can have a >10,000-fold increase in skin cancer susceptibility, elevated neurological defects and premature aging symptoms (Bradford et al. 2011). Three related but distinct diseases are associated with defects in the NER pathway: xeroderma pigmentosum (XP), Cockayne syndrome (CS) and trichothiodystrophy (TTD). XP has seven complementation groups, XP-A to G, which are associated with the core NER factors, and one complementation group, XP-V, which is defective in a translesion replicative response (see Chapter 11). Not only are XP patients at heightened risk for skin cancer, but also internal cancers. Curiously, XP-A, -B, -D and -G complementation groups are more prone to neurological dysfunction than the others, such as XPC (Bradford et al. 2011). There are two complementation groups for CS, CS-A and CS-B. Approximately 80% of CS patients have mutations in the gene encoding the CSB protein, *ERCC6* (Natale 2011). CSA and CSB are both important following DNA damage "recognition" by an RNA polymerase, and thus, are specifically associated with TCR. CS patients exhibit neurodegeneration and cachectic dwarfism, as well as photosensitivity, but do not experience an elevated risk of cancer. TTD has three complementation groups, stemming from mutations in three different protein subunits of TFIIH, namely XPB, XPD and TFB5 (Faghri et al. 2008). The majority of TTD patients carry mutations in *XPD*. Patients with TTD have brittle hair and nails due to a sulfur deficiency, ichthyotic skin, as well as physical and mental retardation. Like CS, TTD patients are not at elevated risk for skin cancer.

As indicated above, NER is responsible for the removal of sunlight induced DNA damage (e.g., photodimers) and other bulky helix-distorting DNA lesions. The NER pathway is a well characterized repair mechanism, and the basic steps as shown in Fig. 3 are: damage recognition [either as part of (A) GGR or (B) TCR], DNA unwinding, dual single-strand incision

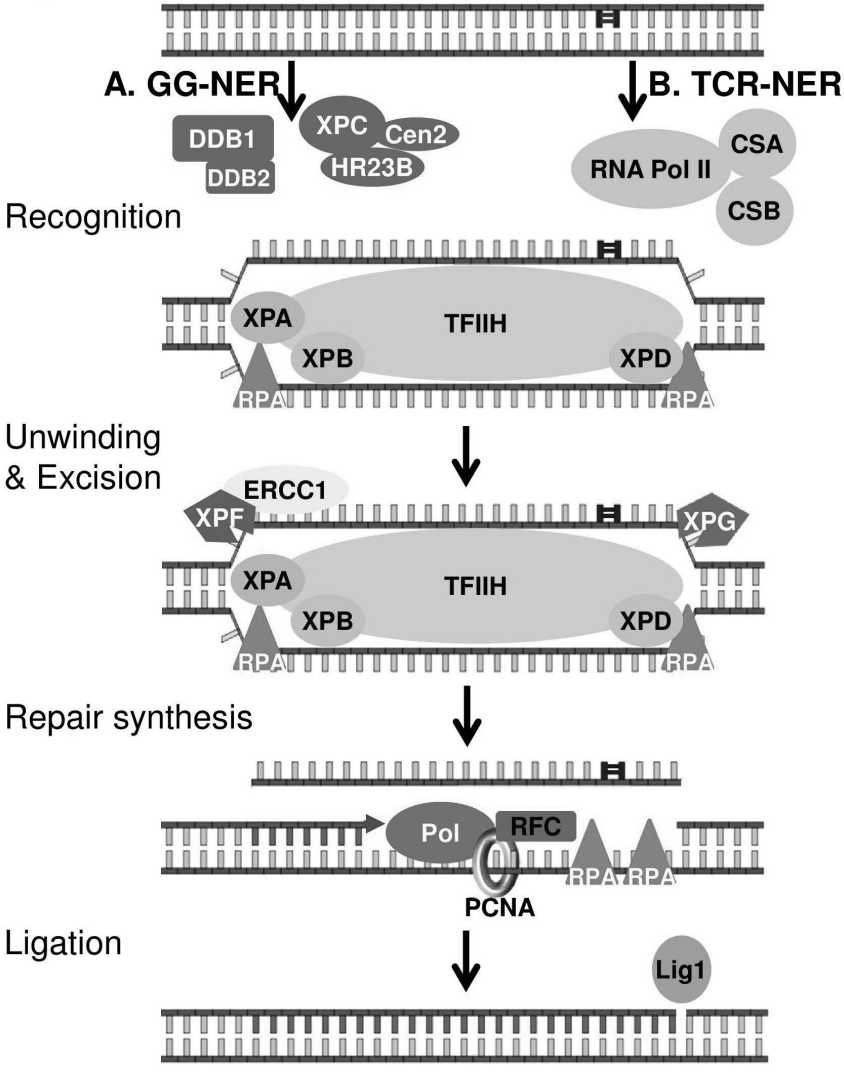

Figure 3. Schematic diagram of nucleotide excision repair. DNA damage recognition for NER is different for repair in the general genome (A. GG-NER) in comparison with actively transcribed genes (B. TCR-NER). Within the general genome pathway (A. GG-NER), XPC/HR23B/CEN or DDB1/2 recognize the damage, whereas for actively transcribed genes (B. TCR-NER), RNA polymerase II, with associated factors like CSA and CSB, is responsible for DNA damage signaling. Following recognition, the pathways converge: the transcription factor TFIIH with its associated helicases XPB and XDP are recruited along with XPA and RPA. ERCC1/XPF and XPG join the complex and are required for the 5′ and 3′ single strand incisions on either side of the damage, respectively. Repair synthesis fills in the ~30 nucleotide gap and LIG1 seals the nick.

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3′ and 5′ to the DNA lesion, repair synthesis and DNA ligation. Of note, the major RNA polymerase II transcription factor TFIIH, which contains the helicases XPB and XPD, is a core component of NER. Additionally, the structure-specific nucleases XPG and ERCC1/XPF are essential for this process. Multiple DNA polymerases have been reported to function in NER, as well as both LIG1 and LIG3/XRCC1.

MISMATCH REPAIR (MMR)

Mismatches arise in DNA due to DNA polymerase misinsertions, recombination between similar but not identical DNA sequences, and mispairing of damaged, modified or non-canonical DNA bases. Replication of genomic DNA is usually a very accurate process, in part because replicative polymerases are high fidelity enzymes, largely because they are endowed with proofreading exonuclease domains that remove incorrectly inserted nucleotides (Arana and Kunkel 2010). Occasionally though, polymerases err and introduce base-base mismatches in newly replicated DNA or slip on templates within repetitive sequences generating small indels. Polymerases can also introduce errors when copying damaged DNA templates, as the lesion may pair with more than one or an "incorrect" base, or because the damage is non-instructional (e.g., an AP site). When a non-base pairing or blocking lesion is encountered, polymerases often insert adenine (the so-called "A-rule"), a response that could be mutagenic, but may also provide a mechanism for survival as it facilitates lesion bypass and continued replication (see Chapter 11). Alternatively, mismatches can arise as a consequence of recombination, such as during meiosis or homeologous recombination. Therefore, MMR is the primary repair mechanism responsible for the recognition and excision of inappropriately paired nucleotides.

The scheme for MMR is shown in Fig. 4. DNA damage recognition and signaling is facilitated by two heterodimeric partners, MutS and MutL (Modrich 2006; Hsieh and Yamane 2008; Li 2008). The predominant MutS protein complex in human cells is MutSα, which is a heterodimer composed of MSH2 and MSH6. MutSα recognizes base-base mismatches and indels of 1–2 nucleotides. MutSβ, a second mammalian MutS complex composed of MSH2 and MSH3, recognizes indels of 2–10 nucleotides and is blind to base-base mismatches. There are also multiple MutL complexes in human cells, with MutLα, which is composed of MLH1 and PMS2, being the most prominent. MutLα acts as the matchmaker or facilitator, coordinating events in MMR. It has recently been shown to be a DNA endonuclease that introduces strand breaks in DNA upon activation by mismatch and other required proteins, MutSα and PCNA (Kadyrov et al. 2006). These strand

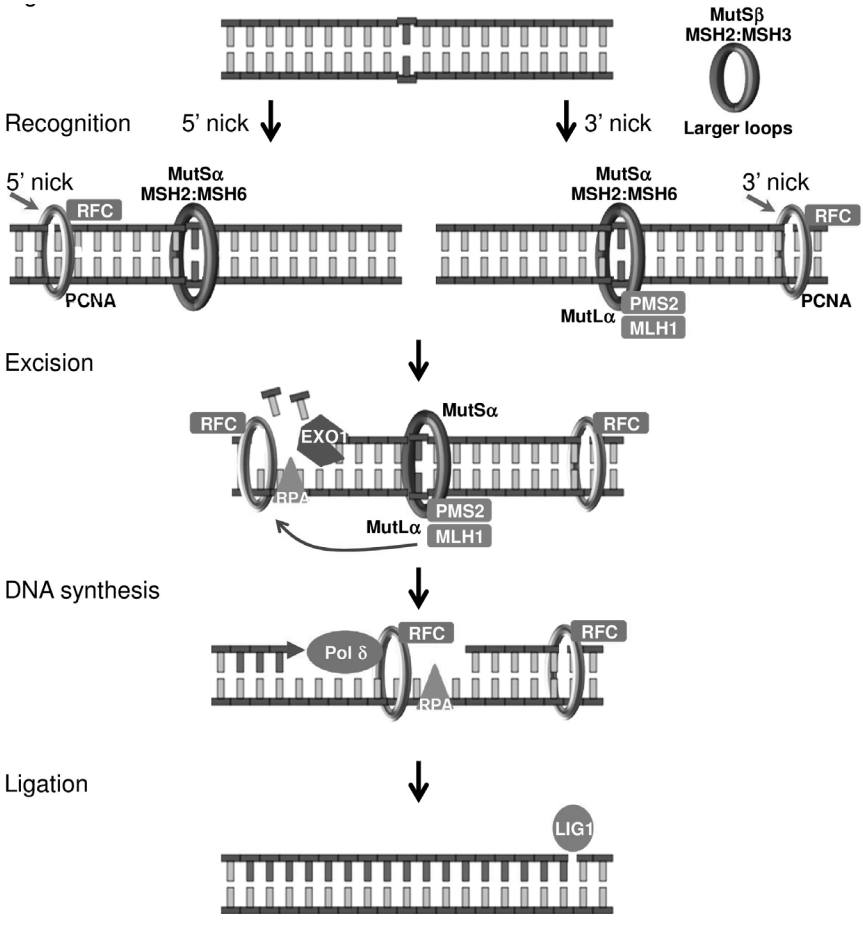

Figure 4. Schematic diagram of mismatch repair. MutSα, a complex of MSH2/MSH6, is thought to recognize base:base mismatches, whereas MutSβ, a complex of MSH2/MSH3, recognizes larger insertion and deletion loops. Once a lesion is found, MMR must determine which strand is the parental strand, and this is done by searching for a nick in one strand. Nicks are common before Okazaki fragment processing following new DNA synthesis and thus allows easy recognition of the lagging, replicating strand. Once a nick is located RFC and PCNA load. If the nick is 5′ to the damage, the exonuclease, EXO1, can directly proceed with resecting the DNA back to the mismatch. If, however, the nick is on the 3′ side of the lesion, then a cryptic endonuclease in MLH1 is activated thus allowing PCNA/RFC/EXO1 loading and exonuclease digestion. Large tracks of DNA >2kb can be excised during MMR, and a replicative DNA polymerase, like pol δ, is required to fill in the gap. LIG1 then seals the nick.

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interruptions serve as entry points for an exonuclease that removes the mismatched DNA.The roles played by the other MutL complexes, MutLβ and MutLγ, are less well understood.

One of the challenges MMR faces is to determine which DNA strand is the "parent" strand and which is the newly replicated strand, and presumably, the error containing strand. Evidence indicates that eukaryotic MMR is nick directed. When a nick is found in the vicinity of the target mispair, the EXO1 exonuclease degrades the DNA surrounding and including the mismatch. Remarkably, degradation can include large tracks of DNA ~2000 nucleotides at a time (Genschel and Modrich 2003). Once the mismatch is removed, resection is inhibited, and PCNA-dependent DNA polymerization fills in the single-stranded gap and LIG1 ligates the nick to complete repair.

As mentioned, MMR is responsible for recognizing normal mismatched bases and loops containing small indels (<10 bases). Failure to correct these types of DNA intermediates leads to elevated mutation rates in cells (a mutator phenotype), as well as expansions and contractions of repetitive DNA sequences, an outcome termed microsatellite instability. For these reasons, individuals with MMR defects are prone to develop autosomal dominant forms of colon cancer, collectively known as Lynch syndrome (Goodenberger and Lindor 2011). Microsatellite instability is also seen in sporadic colon cancer cases. Defective MMR is most frequently caused by point mutations in genes encoding the subunits of MutSα, and by silencing of *MLH1*. In addition to its cellular roles described above, MMR participates in a DNA damage signaling pathway and the apoptotic response to some types of DNA modifications (see Chapter 7). Thus, loss of MMR functionality has important implications for tumor responsiveness to chemotherapeutic drugs.

MMR is a multitasking DNA repair pathway. In addition to its role in suppression of mutations following replication, recent evidence suggests that MMR inhibits HR at DSBs and stalled replication forks (Smith et al. 2007). Interestingly, MMR can also promote mutagenesis. For example, there is evidence that MMR proteins support somatic hypermutation and class switch recombination, processes that introduce beneficial mutations to facilitate immune system adaptation (Schrader et al. 2007). Additionally, MMR can induce mutations that are not advantageous. Trinucleotide repeat expansion diseases like Huntington's, Fragile X mental retardation, mytonic muscular dystrophy and several autosomal dominant spinocerebellar ataxias arise from expansion of short repetitive DNA sequences (Lopez et al. 2010). In mouse models of some of these diseases, the MMR gene products MSH2 and MSH3 are required for expansion (Slean et al. 2008). The involvement of MMR in regulating repeat stability may originate from the capacity of repeat sequences to form stable stem-loop secondary structures either as a result of replication strand slippage or break-dependent repair synthesis. Such looped intermediates are recognized and bound by the MutSβ complex, which appears to stabilize the looped structure and fails to

promote repair. Thus, MMR plays a role in both inhibiting and promoting mutagenesis in cells, and loss of MMR function is associated with a mutator phenotype, a hallmark of cancer cells (see Chapter 3).

BASE EXCISION REPAIR (BER)

BER recognizes simple, relatively non-bulky types of base modification, sites of spontaneous base loss (AP sites) and SSBs. BER probably clears more DNA damage than any other repair mechanism each day due to the sheer volume of spontaneous hydrolysis events within DNA. For example, it is estimated that 10,000 AP sites are created per day per mammalian genome (Lindahl and Nyberg 1972). The BER system also repairs other common types of DNA damage, including oxidation and alkylation products, such as 8-oxoG, which is a biomarker of oxidative stress, and 3-methyladenine, a common product of DNA alkylation. Base lesions are typically classified as mutagenic or cytotoxic. 8-oxoG is considered mutagenic, because it has the potential to base pair with both C and A and can thereby promote G:C to T:A transversions; 3-methyladenine is cytotoxic, because it blocks the replication and transcription machinery.

Models of short-patch (SP-BER) and long-patch (LP-BER) BER are shown in Fig. 5. DNA glycosylases recognize and remove modified bases. Cells have multiple glycosylases and several of these have overlapping substrate recognition capacities, therefore creating a certain amount of redundancy in this step of BER. Following base removal, AP endonuclease 1 (APE1) incises the baseless sugar moiety, generating a strand break. Alternatively, some glycosylases, such as OGG1, possess an AP lyase function, which can incise the DNA backbone at the AP site in a mechanism distinct from that of APE1. Since some of the glycosylases leave DNA ends that are incompatible with DNA extension or ligation, such as 3′ phosphate groups, end-processing proteins such as APE1 or polynucleotide kinase 3′ phosphatase (PNKP) are required to produce the 3′ hydroxyl and 5′ phosphate termini necessary for ligation. In SP-BER, POLβ can remove the 5′-deoxyribose group after APE1 catalyzed incision, and can fill in the single nucleotide gap prior to sealing of the nick by LIG3/XRCC1. In other situations, mainly when 5′-termini are not easily handled by POLβ after strand cleavage, repair proceeds via LP-BER. In those cases, PCNA-dependent DNA strand displacement synthesis occurs, filling in several nucleotides (hence the name long patch). The 5′ flap structures that arise are then trimmed by FEN1 and the nick is ligated by LIG1.

A related sub pathway of BER, termed SSBR, is responsible for the resolution of certain SSBs. PARP1 is a high affinity strand break DNA binding protein, which is involved in damage recognition for this sub

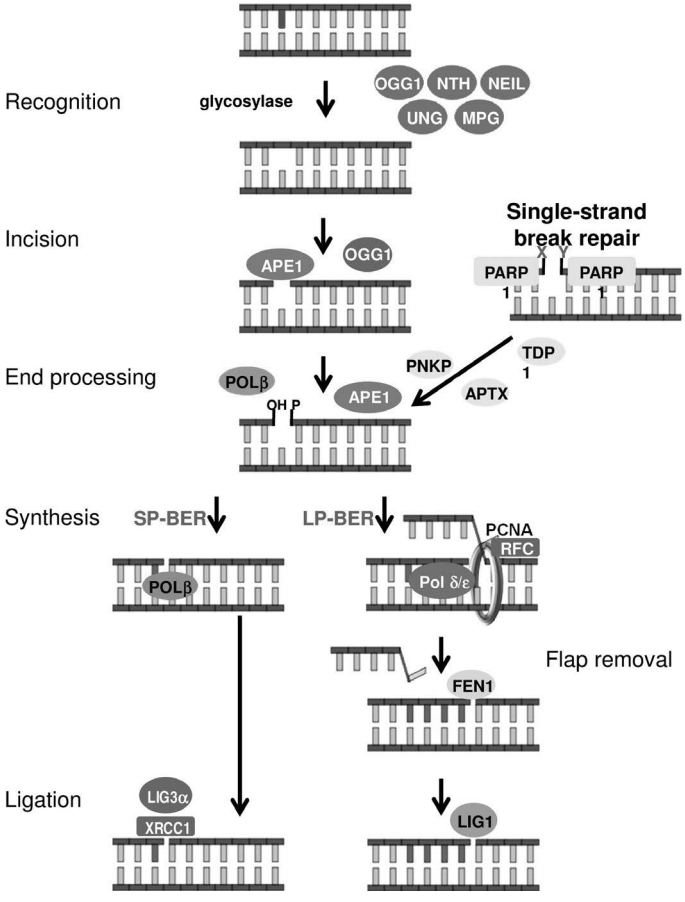

Figure 5. Schematic diagram of base excision repair. Glycosylases are a set of enzymes that remove modified bases from DNA, generating an abasic site. Some glycosylases possess DNA strand cleavage activities, like OGG1, but others do not. The endonuclease, APE1, is required to cleave the DNA backbone at the AP site and to process the ends for gap synthesis and ligation. Additional end processing proteins important at this stage are: polymerase β, (pol β), polynucleotide kinase phosphatase (PNPK), Aprataxin (APTX) and Tyrosyl-DNA phosphodiesterase I (TDP1). Following end processing, the BER pathway splits into either short patch repair (SP-BER) or long patch repair (LP-BER) depending on the number of nucleotides inserted during the repair synthesis step. POLβ does the synthesis for SP-BER and fills in the single nucleotide gap. A replicative polymerase, with the help of PCNA/RFC, typically performs strand displacement synthesis for LP-BER and replaces 2 or more nucleotides. Ligase 3α (LIG3α) and XRCC1 do the ligation for SP-BER and Ligase 1 (LIG1) functions during LP-BER. Single-strand break repair is a related but distinct repair pathway which repairs single-strand breaks with modified 5′ or 3′ ends, denoted by the X and Y in the image. In this pathway, the high affinity single-strand break binding protein poly (ADP-ribose) polymerase I, PARP1, often recognizes the ends and recruits end processing proteins to create synthesis and ligation compatible ends.

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pathway (see Chapter 15). End processing proteins, such as aprataxin (APTX) and tyrosyl-DNA phosphodiesterase 1 (TDP1), are important enzymes in certain versions of this sub pathway, namely for removing 5′ AMP groups left behind after failed ligation reactions and 3′ protein adducts stemming from trapped topoisomerase intermediates (Wilson, III, 2007). Interestingly, defects in either of these proteins lead to inherited autosomal recessive spinocerebellar ataxias (recently reviewed in (Jeppesen et al. 2011)).

Individuals with defects in BER gene products are at risk for cancer, immunodeficiency and neurodegeneration (Jeppesen et al. 2011; Wilson III et al. 2011). One of the first BER gene products found to be defective in a hereditary cancer was the MutY homolog, MUTYH (Al-Tassan et al. 2002; Jones et al. 2002; Sieber et al. 2003). MUTYH is a DNA glycosylase that excises adenine when mispaired with 8-oxoG. Mutations in *MUTYH*, in both mice and humans, give rise to genetic instability and a predisposition for colon cancer. This finding underscores the importance of suppression of mutagenesis caused by oxidation. Additionally, mutations in *POLβ* have been found in a high percentage of tumors, and the encoded variant proteins may be involved in either the initiation or progression of tumorigenesis (Starcevic et al. 2004). Uracil DNA glycosylase (UNG) is responsible for the removal of uracil from DNA, mainly after spontaneous cytosine deamination or misinsertion during DNA replication. In addition, during class switch recombination and somatic hypermutation of the immunoglobulin loci, uracils are intentionally introduced into DNA and later removed by UNG as part of a mutagenic response. Thus, not surprisingly, gene defects of *UNG* are associated with hyper IgM syndrome "type 5", which is one of a family of disorders characterized by immunodeficiency and higher than normal susceptibility to various forms of infections (Imai et al. 2003). Mutations in *UNG* specifically cause immunodeficiency and an inability to generate a diverse pool of high affinity antibodies and antibody isotypes (Imai et al. 2003). Finally, results from mouse gene knockout studies involving the core BER repair proteins APE1, POLβ, XRCC1, LIG3 and LIG1, which each result in early death or embryonic lethality, underscore the essential nature of the BER pathway (Gu et al. 1994; Sobol et al. 1996; Xanthoudakis et al. 1996; Bentley et al. 1996). Thus, BER appears to play a critical role in facilitating normal development and in protecting organisms against both endogenous and exogenous DNA damaging insults (see Chapter 8).

INTERSTRAND CROSSLINK REPAIR (ICLR)

ICLR is complex and probably the least well characterized DNA repair mechanism (Hinz 2010; Muniandy et al. 2010; Wood, 2010). This is partially due to the fact that ICLR is accomplished by the coordinated efforts of

multiple repair pathways acting sequentially or in concert with one another. In G0/G1 cells, ICLs are thought to be repaired by sequential rounds of NER. Damage recognition via NER could be mediated by either GGR or TCR, because ICLs are typically helix distorting lesions and block transcription. Additionally, the BER glycosylases MPG and NEIL1 may contribute to some ICL DNA damage recognition and/or processing. With respect to G0/G1 repair, the first round of NER unhooks the two strands and then the second NER cycle actually removes the short DNA fragment containing the ICL remnant, Fig. 6A.

An alternative pathway for ICLR occurs during S-phase (Fig. 6B), and this pathway is similarly not well characterized. As part of the process, the replication apparatus encounters the ICL and subsequently recruits the Fanconi anemia (FA) proteins (see Chapter 10). The FA proteins participate in DNA damage recognition and together with nucleases like Mus81/EME1/2, APOLLO and the MRN complex facilitate unhooking of the two DNA strands. Replication bypass may then occur to generate duplex DNA, followed by a round of NER to excise the remaining ICL remnant. Subsequently, HR appears to promote DNA replication fork re-assembly so that replication can proceed. Individuals with defects in one of the thirteen FA (or FANC) genes are hypersensitive to crosslinking agents, such as mitomycin C, and this cellular phenotype is used as a clinical diagnostic for the disorder (D'Andrea, 2010). FA patients experience bone marrow failure, developmental defects, and a predisposition to cancer (Table 1). Breast cancer is particularly prevalent in patients with *FANCD1/BRCA2* mutations (see Chapter 14). Since several DNA crosslinking agents are currently used in the clinic, careful analysis and full elucidation of the pathways for ICL resolution may have immediate impact on cancer patient care.

DIRECT REVERSAL (DR)

DR is a unique mechanism of DNA repair in that it does not require the coordinated action of multiple repair proteins. Instead, it involves a single protein, which transfers a chemical adduct from DNA onto itself. In this manner, it repairs DNA without introducing nicks or strand breaks, or involving some form of nuclease processing. However, the reaction chemistry renders the repair protein permanently inactive (so-called suicide mechanism). Perhaps the most mutagenic and carcinogenic DNA lesion repaired by DR is O^6-methylguanine (O^6meG), since this adduct mispairs with thymine (instead of cytosine) during DNA replication (Kyrtopoulos et al. 1997). The DNA repair protein that removes methyl and chloroethyl groups from the O^6 position of guanine in DNA is O^6-methylguanine DNA methyltransferase or MGMT (see Chapter 6). The

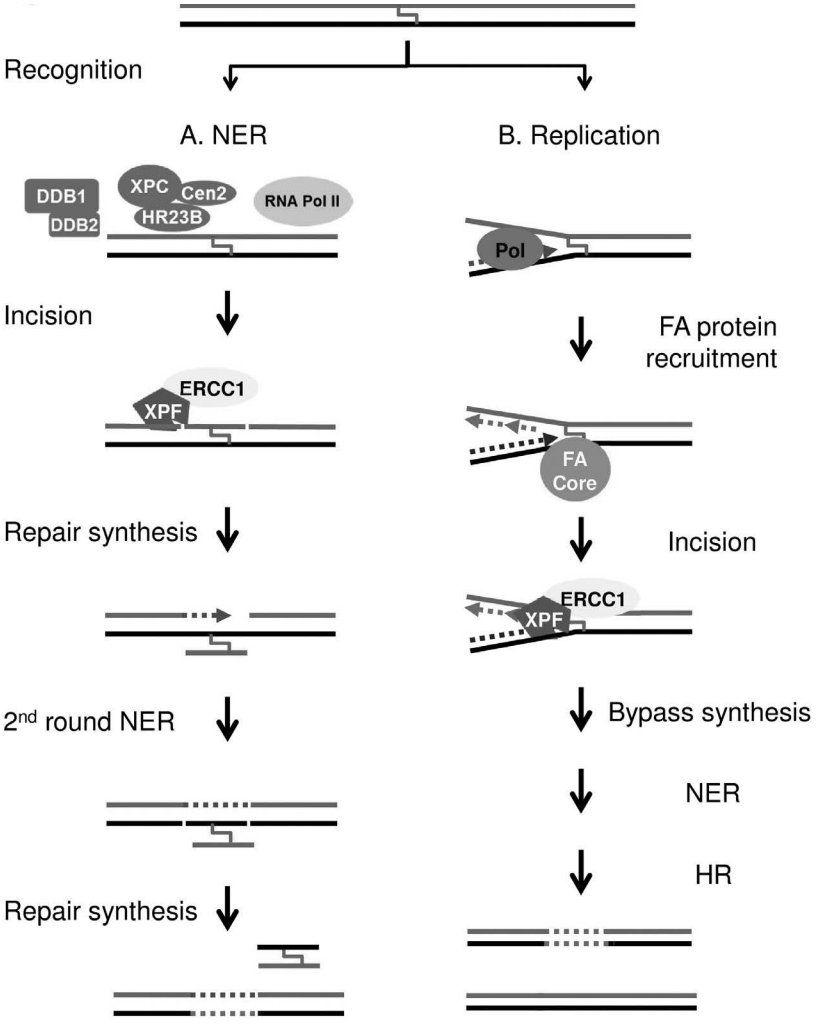

Figure 6. Schematic diagram of interstrand crosslink repair. If damage recognition is mediated by NER components (A. NER), then unhooking of the lesion is done by the NER pathway. This is then thought to be followed by a round of translesion synthesis and another round of NER to remove the short oligonucleotide-attached crosslink, a process commonly seen during the G1 phase of the cell cycle. If the DNA damage is found by the replication apparatus (B. Replication), then the Fanconi anemia (FA) proteins are recruited. This pathway is less well described, but may also involve the structure-specific endonuclease, ERCC1/XPF, to unhook the crosslink. Then, the sequential action of several DNA repair pathways might work together to complete repair. Bypass synthesis, followed by a round of NER, would remove the crosslink from DNA, leaving a gap. Finally, HR between sister chromatids would help to restore the original DNA sequence. Interstrand crosslink repair (ICLR) is still in its infancy relative to the other well characterized repair pathways and much is yet to be learned.

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mammalian AlkB homologs, ABH2 and ABH3, repair 1-methyladenine and 3-methylcytosine lesions in DNA via a mechanism that likewise involves DR (Duncan et al. 2002).

Alkylating agents are used in many chemotherapy regimens to fight brain cancers, malignant melanoma and some lymphomas (Christmann et al. 2011). Up-regulation of the expression of MGMT protects cancer cells against alkylating agents such as temozolomide and dacarbazine, to name a few, which likely contributes to a patient's chemoresistance (Christmann and Kaina 2011; Christmann et al. 2011). MMR can also mediate a response to unrepaired, replication associated O^6meG-T mismatches, leading to the eventual accumulation of DSBs (Ochs and Kaina 2000). Thus, given the relationship between DNA repair capacity and DNA damaging agent resistance, chemotherapeutic regimes that combine alkylating agent treatment with DNA repair inhibitors are underway and may improve treatment outcome (Helleday et al. 2008).

MITOCHONDRIAL DNA REPAIR

Mitochondria are the energy producing centers of the cell. These organelles possess their own mtDNA genome, which directs the expression of 22 tRNAs, 2 rRNAs and 13 polypeptides. Human mtDNA, a small circular genome of ~16.5 kB, is packaged into a protein:DNA complex called a nucleoid and is attached to the mitochondrial inner membrane (Wang and Bogenhagen 2006). There are typically multiple mtDNA genomes per nucleoid (5–10), and a range of mtDNA genomes are present per cell (hundreds to thousands) depending on the cell type. Due to the fact that mtDNA lies in close proximity to the free radical producing electron transport chain, mtDNA is in constant need of repair of oxidative damage. As with nuclear DNA, mtDNA repair is necessary to ensure the faithful replication and maintenance of the genome. The emerging theme is that mtDNA maintenance and repair are important to prevent neurodegeneration and premature aging (Jeppesen et al. 2011), so understanding how DNA damage is repaired in mitochondria is an important consideration for any new drugs under development.

All mtDNA repair and replication proteins are encoded by the nuclear genome. Interestingly, not all DNA repair pathways exist in mitochondria as compared to the nucleus Fig. 7. BER is the best characterized pathway known to be functional in mitochondria. Another DNA repair pathway that exists is an alternative form of MMR. Mitochondrial MMR is dependent on the protein YB-1 and not on the traditional MMR genes (de Souza-Pinto et al. 2009). Notably, NER, the repair pathway for the removal of bulky DNA damage such as UV photoproducts, is not present in mitochondria

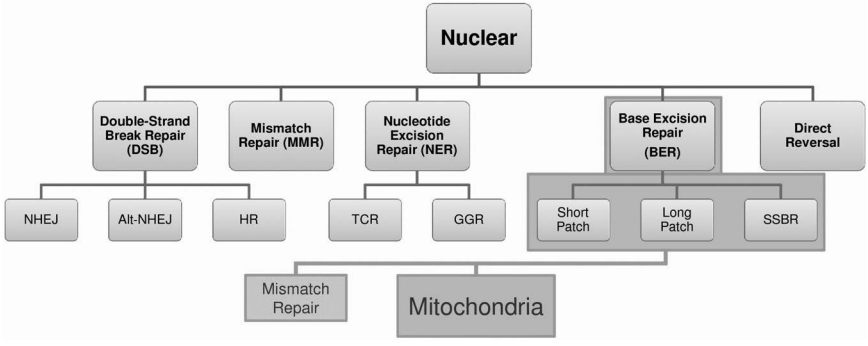

Figure 7. Comparison of repair mechanisms operable in the nucleus and mitochondria. BER is the best documented DNA repair pathway in mitochondria. An alternative form MMR exists and is not dependent upon the nuclear MMR proteins. DSBR is still controversial. No NER or mechanisms for direct reversal of DNA damage have been reported in mitochondria.

(Clayton et al. 1974). Cisplatin, a common chemotherapeutic drug that induces intra- and interstrand crosslinks, is normally repaired by NER in the nucleus. Interestingly, cisplatin damage is repaired in mitochondria by an as yet uncharacterized mechanism, since traditional NER does exist in mitochondria (LeDoux et al. 1992). Perhaps the newly identified role for BER in mediating cisplatin toxicity (Kothandapani et al. 2011) somehow plays a role in initiating the repair of cisplatin in mitochondria.

Whether there is DSBR in human mitochondria is still controversial. *In vivo* somatic cell hybrid experiments failed to detect recombination between mtDNA molecules (Zuckerman et al. 1984), whereas *in vitro* biochemical experiments using mitochondrial protein extracts and plasmids have observed HR products (Thyagarajan et al. 1996). Additional support for recombination in mitochondria has been obtained from rare individuals that are heteroplasmic for their mtDNA, harboring both maternal and paternal mtDNA genomes (Kraytsberg et al. 2004; Zsurka et al. 2005). Mitochondria are normally maternally inherited, so the identification of recombinant mtDNA genomes with both maternal and paternal mtDNA sequences suggests that recombination has occurred and is therefore possible. Nevertheless, recombinational repair following a DNA damaging insult has yet to be recorded in mitochondrial protein extracts or *in vivo*. Since there are so many mtDNA genomes within a single mitochondrial organelle, intra-organelle complementation may explain the lack of certain repair pathways. In other words, the repertoire of DNA repair pathways in mitochondria may not need to be as comprehensive as for nuclear DNA, because a single mitochondrial gene can be expressed from multiple complementing mtDNA genomes within the same mitochondria.

One of the most unexpected findings in mtDNA maintenance and repair is that mtDNA can tolerate very high levels of damage and mutations.

Specifically, mtDNA isolated from OGG1-deficient mice, in which the BER glycosylase responsible for the removal of 8-oxoG is knocked out, had a 20-fold increase in the levels of 8-oxoG, yet unstressed mice have no phenotype (de Souza-Pinto et al. 2001). Just as amazingly, mutant knock-in mice harboring an exonuclease deficient DNA POLγ, the replicative polymerase in mitochondria, exhibit a 500-fold increase in mutations and elevated deletions in their mtDNA, yet are viable (Kujoth et al. 2005;Trifunovic et al. 2004;Vermulst et al. 2008). However, these mutator mice display a variety of aging related pathologies much earlier than wild type mice and have a shortened lifespan. Thus, results from the POLγ exonuclease (proofreading)-deficient mice clearly demonstrate that mitochondrial dysfunction is directly related to aging and that preservation of mtDNA integrity is vital.

CONCLUSIONS

It is widely accepted that inherited defects in DNA repair increase cancer risk in humans and other metazoans (Table 1). Mounting evidence also suggests that DNA repair capacity is compromised in some, but not all, sporadic cancer cells (see Chapter 9). Because traditional cancer chemotherapy often employs DNA damaging agents, the success or failure of a specific therapeutic paradigms is expected to vary depending on the DNA repair capacity of the individual's cells, both normal and tumor. As a result, one might observe tumor- or region-specific susceptibility or resistance to a specific chemotherapeutic agent in different tumors of the same type. This concept is now being exploited in leading-edge oncotherapeutics. For example, defects in BRCA2 increase breast cancer susceptibility; however, BRCA2-defective cells, which are defective in HR, require efficient BER/SSBR to thrive, and therefore die in the presence of small molecule inhibitors of PARP1, an important strand break repair enzyme (Bryant et al. 2005; Helleday et al. 2008) (see Chapters 14 and 15). This approach, known as synthetic lethality, has gained significant momentum since its use as a therapy for BRCA2-mediated breast cancer, and many researchers are looking for novel small molecular inhibitors that selectively inhibit specific DNA repair pathways (see Chapter 18). Finally, there is increasing awareness that defects in mtDNA or its maintenance may lead to premature aging and neurodegeneration; thus, the effect of small molecule compounds or designer drugs on mtDNA repair could influence drug efficacy, and should be considered as novel therapeutic agents and strategies are being developed.

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CHAPTER 2

Longevity Assurance by Genome Maintenance

Paul Hasty,[1,*] *Yousin Suh*[2,a,*] and *Jan Vijg*[2,b,*]

INTRODUCTION

Organismal aging presents with a myriad of characteristics that can be categorized into two basic components: cancer and general organ decline. Cancer is an age-related disease that occurs in many, but not all, individuals in an aging population. In human or animal populations, a wide-range of cancers can be observed. These cancers may share common features, but they also have a different etiology heavily influenced by genetics and environment. No matter the etiology, all cancers have lost their ability to regulate cell growth and proliferation and no longer respect their cellular boundaries. These cells are undergoing a process of dedifferentiation and become more embryo-like. Apart from cancer, there is a wide range of examples for general organ/system decline commonly found in aging populations. These include skin and muscle atrophy, osteoporosis, arthritis, cardiovascular disease, cataracts and reduced immune function. Many of these and other symptoms are shared among aged individuals, but virtually no individual carries the entire panoply of the aging phenotype. In this respect, aging is a true stochastic process where chance and variability are the norm (Kirkwood et al. 2011). Unlike cancer, non-neoplastic symptoms

[1]Department of Molecular Medicine/Institute of Biotechnology, University of Texas Health Science Center, San Antonio, Texas 78245-3702, USA; Email: hastye@uthscsa.edu
[2]Department of Genetics, Albert Einstein College of Medicine of Yeshiva University, Bronx, NY, USA.
[a]Email: yousin.suh@einstein.yu.edu
[b]Email: jan.vijg@enstein.yu.edu
*Corresponding authors

of aging are marked not by dedifferentiation and dysregulated cellular proliferation, but instead by loss of function and reduced proliferative capacity. Therefore, cancer and non-cancer, degenerative decline are in a sense two opposing outcomes of the aging process.

Cancer and organ decline are both pleiotropic and stochastic processes highly influenced by genetics and environment and as a consequence difficult to understand. Yet, genome maintenance and in particular pathways that repair DNA damage are critical longevity assurance pathways needed to suppress cancer and the onset of aging in order to provide sufficient time for reproduction and species propagation. However, these pathways are imperfect and as a result DNA damage and mutations may accumulate with age. DNA damage is a hallmark of cancer and may be a causal factor for general aging. Thus, factors that damage DNA and pathways that respond to DNA damage may be critically important in the etiology of cancer and aging.

Even though general aging is highly stochastic and pleiotropic, it is not entirely random since there is some level of order that is genetically controlled. The best evidence for strong genetic control is a comparison of species life span. In mammals, life expectancy can range from as little as one year to over 200 years (Austad 2010). Life span scales with reproduction; the faster a species reproduces, the shorter the life span. Yet the aging process is remarkably similar between species with diverse life spans. The best-studied mammalian species are the mouse and human, with reproductive maturity occurring around 6 weeks to 13 years, and life spans ranging from 3 to over 100 years, respectively. Even with this vast range in chronological time, mice and humans display many of the same aging characteristics that occur approximately at the same point in their biological life history. So even though chronologically there can be vast differences in the onset of cancer and aging, from a biological perspective the similarities are more dominant. Since life span and cancer/aging onsets are heritable, these outcomes must be genetically controlled. In this Chapter, we will review the evidence that supports the role for genome maintenance in longevity assurance (Fig. 1). There is an accumulating body of evidence that genome maintenance is a critical component for longevity assurance, since defects in genome maintenance influence aging processes like cancer and general organ decline in both mouse and human. We discuss factors that damage DNA and the data showing that DNA damage and mutations accumulate with age. Then, we discuss the various genome maintenance pathways and human and mouse models defective for specific DNA repair pathways that exhibit premature aging phenotypes. Next, we review DNA damage responses (DDRs) and how these responses may cause cytotoxicity. Finally, we discuss humans as a model system for aging. Discussion of these topics will show that genome maintenance is critical for longevity assurance.

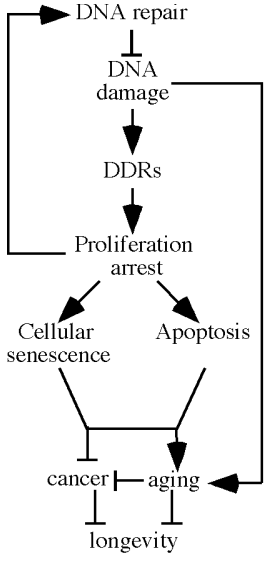

Figure 1. The influence DNA damage has on longevity assurance. A multitude of DNA repair pathways were designed to repair specific types of DNA damage. This damage, if left unrepaired may cause a mutation that has the potential to induce cancer but may also induce cellular decline in function as they accumulate with age. To facilitate repair, DNA damage may activate DNA damage responses (DDRs) to induce proliferation arrest. However, if the damage is unrepairable or overwhelming in number it may also induce cellular senescence or apoptosis. Both cellular senescence and apoptosis can inhibit cancer formation but could also accelerate aging. Thus, DNA damage may accelerate aging directly by diminishing cellular function or indirectly by inducing cellular senescence and apoptosis.

DNA DAMAGE AS A DRIVER OF AGING

As the main repository of genetic information in all five kingdoms of life, DNA is a very stable molecule. Yet, it has the tendency to undergo chemical changes at a very high rate under physiological conditions, i.e., elevated temperature in aqueous environments in the presence of oxygen. Especially through hydrolysis and oxidation, but also under the influence of exogenous factors, such as radiation and environmental mutagens, tens of thousands of chemical lesions are introduced in each cell per day (Lindahl 1993; Vijg 2007). Despite this high rate of inflicted DNA damage the actual steady state level of chemical lesions remains low due to the enormous investments made by the organism in genome maintenance systems. As described in detail in the next section, virtually all that damage is swiftly and accurately repaired through a large variety of pathways, some only working on specific lesions with others acting in a much more general way (see also Chapter 1).

While there have been numerous attempts to measure the steady state level of DNA damage in organ and tissue DNA of aging organisms, the results are inconclusive. This is due in part to a lack of assays sensitive enough to detect the very low levels of DNA damage present under normal conditions, even in tissues of very old individuals (Collins et al. 2004). To some extent, this reality may be due to the tendency of cells to undergo programmed cell death or apoptosis when experiencing high levels of DNA damage that are beyond repair capacity. Interestingly, the apoptotic response may decline with age, as has been demonstrated for rat liver after treatment with alkylating agents (Suh et al. 2002). While there are reports on increased amounts of DNA damage with age (Hamilton et al. 2001), the low steady-state levels make it difficult to predict possible functional consequences. Another reason why it remains difficult to predict the functional implications of DNA damage is the uncertainty about the types of DNA damage capable of causing adverse effects, even at very low levels. For example, while the most predominant forms of endogenous DNA damage, i.e., caused by hydrolysis, oxidation or non-enzymatic DNA methylation, are rapidly corrected, this may not be the case for minor DNA lesions. It is possible that such minor lesions, such as the rare deamination of adenines (Lindahl 1993), slowly accumulate and then have unexpectedly large biological effects at old age. They may be virtually ignored by DNA repair, because natural selection favors early survival and weakens significantly at increased age, after the age of first reproduction. How could DNA damage even at low steady-state levels be a causal factor in age-related cellular degeneration and death?

A first possibility is that DNA damage could directly interfere with transcription, thereby hampering basic cellular functions. While this may not be very likely in view of the low steady-state levels of most types of damage, a general decline in RNA synthesis in aging tissues of rodents (mostly liver) has actually been observed (van Remmen et al. 1995).

A second possible effect of persistent DNA damage, even at very low levels, is interference with DNA replication. Lesions like highly toxic interstrand crosslinks (see Chapter 10) could elicit a significant impact on cell proliferative activity, which is exemplified by mice partially defective for Ercc1. These mice lack the capacity to remove interstrand crosslinks and die within 6 months from liver failure (Weeda et al. 1997). While the number of such lesions induced from endogenous sources may be very low, their effects are significant, most likely because they interfere with liver regeneration, although an effect on transcription cannot be ruled out (Niedernhofer et al. 2006).

A third effect of increased DNA damage involves the coordinated response of most cells to acute bursts of DNA damage. Such DDRs activate

a cascade of genes leading to responses varying from cell cycle arrest and permanent cessation of mitosis (cellular senescence) to apoptosis. Frequent cell cycle arrest would adversely affect the capacity of the cell to respond to stimuli through proliferation, which would certainly affect immune responses or tissue regeneration. The same would be true for senescence, while increased apoptosis could lead to severe cell loss. Increased numbers of senescent cells (mostly in skin) and cellular atrophy have been reported for both humans and mice (Wang et al. 2009) at old age, but the numbers involved are small, and it seems unlikely that we age because we run out of cells or even out of dividing cells. Nevertheless, it seems highly likely that such cellular effects of DNA damage at least contribute to the aging phenotype.

Finally, DNA damage can lead to mutations or epimutations. Such events are consequences of errors made during repair or replication of a damaged template. They can vary from misincorporation of DNA bases, structural alterations, such as chromosomal translocations as a consequence of annealing the wrong ends of DNA double-strand breaks (DSBs), to incomplete restoration of DNA methylation or histone modification patterns. In contrast to DNA damage, DNA mutations are irreversible. Once the original template is altered there is no way for the cell to detect a change in sequence or epigenomic modification. For this reason, mutations can be predicted to accumulate with age and indeed this has shown to be the case. Studies with human or mouse lymphocytes have shown that the frequency of both point mutations at the endogenous reporter gene locus, *HPRT*, and chromosomal aberrations as detected by karyotyping significantly increase with age. This is also true for most mouse organs and tissues, as has been demonstrated using transgenic mice harboring mutational reporter genes that can be excised and recovered in *E. coli* to select for mutations. In this way, it has been shown that the frequency of spontaneous mutations at least doubles in organs such as liver and heart (Dollé et al. 1997, 2000; Dolle and Vijg 2002), as well as lung and kidney (unpublished data). The increase in mutation frequency was much steeper in the small intestine and virtually absent in brain or testes. Also the types of mutations that accumulate with age are very different from organ to organ. While in small intestine, most of the accumulating mutations were point mutations, in heart and liver, genome rearrangements were also found.

A similar mutational reporter model was constructed in *Drosophila melanogaster* and, in this organism, mutations were similarly found to accumulate with age. In this poikilotherm organism, mutations were found to accumulate much faster with age at higher temperature. Since life span in flies is inversely correlated with temperature, it is conceivable that mutations in this organism accumulate with biological rather than merely chronological age. Interestingly, the mutation frequency in fly tissues

appeared to be about three-fold higher than in the mouse, with a much higher fraction of genome rearrangements (Garcia et al. 2010).

PATHWAYS THAT MAINTAIN GENOMIC INTEGRITY AND THEIR RELATIONSHIP TO CANCER AND GENERAL AGING

There is a wide-range of pathways available to the cell for the repair of damaged DNA and the maintenance of genome integrity (see Chapter 1). The vast majority of lesions in a cell are base lesions and SSBs. There are three excision repair pathways with some overlap in function that correct these lesions: base excision repair (BER) (Barnes and Lindahl 2004; Almeida and Sobol 2007), nucleotide excision repair (NER) (de Boer and Hoeijmakers 2000) and mismatch repair (MMR) (Kolodner and Marsischky 1999). BER is prominent for repairing reactive oxygen species (ROS)-induced DNA damage (see Chapter 8). NER is most important for repairing UV light-induced lesions that distort the DNA helix and for repairing bulky adducts (see Chapter 9). MMR is critical for postreplication repair (see Chapter 7). These excision repair pathways are essential for repairing the majority of DNA lesions in a cell and promoting survival at high fidelity, i.e., preventing point mutations. As compared to base lesions and SSBs, DSBs are rare but much more severe. There are two major pathways that correct DNA DSBs: homology directed repair (HDR) and nonhomologous end joining (NHEJ). HDR utilizes the sister chromatid as a template during S/G_2 phases to ensure faithful replication (Sung et al. 2000), whereas NHEJ joins ends together without a template and functions during both G_1 and S phases (Lieber et al. 2003). HDR and NHEJ are essential for preventing chromosomal rearrangements (see Chapter 14).

In addition to the above mentioned major DNA repair pathways,there are a range of mechanisms that ensure faithful DNA replication. For example, replication fork maintenance becomes particularly specialized at telomeres where the shelterin complex is needed to prevent telomere erosion and the formation of a DSB (Xin et al. 2008). There are also structural proteins and cell cycle regulators needed for genome maintenance. All these pathways are essential for chromosomal maintenance, and defects in any of them can lead to genomic instability that enhance DDRs and possibly lead to mutations; increased mutagenesis and genomic instability is the hallmark of carcinogenesis (see Chapter 3). Many of the DNA repair mechanisms have been implicated in longevity assurance not only through suppression of tumorigenesis, but also by restraining generalized aging, presumably by regulating the cellular processes of apoptosis (cell death) and senescence (a state of arrested division).

Advanced age is the major risk factor for cancer. Even though cancer is an age-related disease,it is very different from generalized aging. Both cancer cells and aged cells exhibit increased levels of DNA damage and mutations, but with a different outcome. For cancer cells, genomic instability inactivates tumor suppressor genes and activates oncogenes, with the sum effect of increasing cellular proliferation. In addition,cancer is characterized by cellular dedifferentiation and unrestricted cellular migration. By contrast, for cells subject to generalized aging, genomic instability reduces cell function, in particular proliferation and proliferative life span. This could be the result of an accumulation of mutations that randomly inactivate genes, but also could stem from the cumulative effects of DNA damage checkpoint responses designed to suppress cancer. As a result, generalized aging is characterized by increased cellular differentiation that reduces cellular proliferation and possibly results in cellular senescence. Thus, cancer and generalized aging are different biological outcomes of age-related genomic instability.

Excision Repair Pathways

Of the excision repair pathways, NER is best known for suppressing tumors and for delaying the onset of general aging-like phenotypes. NER is essential for repairing UV light-induced lesions and is composed of two branches (Andressoo et al. 2005). The first branch is global genome-NER (GG-NER), and the second branch is transcription coupled-NER (TC-NER). GG-NER repairs DNA lesions like thymidine-thymidine dimers that distort the DNA double-strand helix. In this pathway, XPC/hHR23B recognizes the helix-distorting lesion, and then the transcription factor IIH (TFIIH) is recruited to the lesion along with the structure specific endonuclease XPG and the helicases XPB and XPD. TFIIH is a transcription factor, but its role in repair is independent of its functions in transcription initiation, and even though XPG is an endonuclease, it likely performs a structural role to stabilize the open DNA helix. XPC leaves, and XPA now identifies the damage and likely organizes the incision machinery around the damage by positioning the single strand binding protein, RPA, to the non-damaged strand to prevent reannealing. The XPF/ERCCI endonuclease assembles with XPG to cleave 5′ and 3′ of the lesion, respectively, to excise the damaged DNA within a stretch of 24–32 nucleotides. Replication machinery now fills in the gap, and DNA ligase I seals the nick. TC-NER recognizes DNA lesions that stall transcription forks that might otherwise induce apoptosis. Thus, the damage recognized by TC-NER is not necessarily the same as recognized by GG-NER. For example, cyclobutane pyrimidine dimers (CPDs) are more likely corrected by TC-NER, since they interfere with transcription but only mildly distort the DNA helix. CSA and CSB initiate TC-NER and enable

assembly of the core NER reaction that is shared with GG-NER involving TFIIH, XPG, XPA and XPF/ERCC1. Thus, there are two related pathways that repair a range of UV-induced photoproducts.

Mutations in NER genes cause a variety of pathologies that, depending on the mutant gene, range from UV light sensitivity, increased cancer predisposition and segmental aging (Andressoo et al. 2005). For many of these NER genes, mutations cause xeroderma-pigmentosum (XP, the namesake for many NER genes), a heritable disease that involves an increase in UV light sensitivity, including increased levels of cancer in areas of the skin exposed to the sun. In addition, mutating some of the NER genes cause an early aging phenotype, which is seen prominently in the genetic disorders trichothiodystrophy (TTD, a mutation in *XPD*) and Cockayne syndrome (CS, a mutation in *CSA* or *CSB*).

TTD and CS, display a segmental progeroid phenotype. TTD individuals exhibit neurological and skeletal degeneration, cachexia, and ichthyosis, along with the characteristic brittle hair and nails. A point mutation in *XPD* causes TTD. *XPD* encodes one of the two TFIIH helicases, XPB being the other. TFIIH facilitates unwinding of the DNA duplex and is essential for RNA polymerase I and II transcription initiation and for repairing DNA lesions by NER (Hoeijmakers 2001). A TTD mouse model was generated with the analogous *XPD* mutation as found in TTD patients. The phenotypes for TTD mice and humans are very similar. TTD mouse-derived cells exhibit a transcriptional defect and a 60–80% reduction in DNA repair synthesis (a measurement of NER) after exposure to UV light. TTD mice exhibit premature age-related characteristics that include osteoporosis, osteosclerosis, cachexia, gray hair and shortened life span (de Boer et al. 2002). However, unlike the patients, TTD mice showed increased susceptibility to UV or chemically-induced cancer.

The TTD phenotype could be due to either defective GG-NER/TC-NER or impaired transcription. Premature aging is not likely due solely to the NER defect, since deletion of XPA/Xpa completely ablates NER and does not accelerate aging, but instead causes increased cancer after treatment with mutagens (de Vries et al. 1995). However defective NER is likely responsible for the aging phenotype (at least in part), since deleting Xpa greatly exacerbated the aging phenotype in TTD mice (de Boer et al. 2002). This suggests that complete NER ablation in combination with defective transcription is responsible for the dramatic aging phenotype in the double mutant mice. Importantly, both the Xpd and Xpa mutations were required to measurably increase sensitivity to paraquat or ionizing radiation, suggesting that ROS-induced damage can initiate premature aging. Thus, altered XPD likely compromises both repair and transcription to cause early aging.

Unlike the situation in TTD, a defect in only TC-NER (CSA or CSB) likely causes CS. Mutations in either *CSA* or *CSB* cause CS. The exact

biochemical function of these proteins is not known, and therefore, the precise molecular defect that causes the disease is not understood. CS individuals show a segmental aging phenotype that includes cachexia, neuronal degeneration and loss of retinal cells. Similar to TTD, CS does not increase cancer predisposition. However, the CS mouse models do not exhibit as severe a phenotype as humans. As the CS mice age, they exhibit neurological abnormalities that include tremors, limb ataxia and inner ear defects. They also exhibit cachexia and retinal degeneration (van der Horst et al. 1997). Similar to TTD, deleting Xpa modestly increased cancer and greatly increased premature aging in the CSB mutant mice (Murai et al. 2001).

ERCC1/XPF endonuclease is important for both NER and DSB repair (Ahmad et al. 2008). There are few known mutations in either *ERCC1* or *XPF* in humans, but one XPF-mutant person exhibited a progeroid phenotype, with progressive liver and kidney dysfunction, cachexia, hypertension, neuronal degeneration and skin atrophy (Niedernhofer et al. 2006). Much more analysis has been done in mice. Ercc1-mutant mice die at about 3 weeks from liver and kidney abnormalities that resemble premature aging (Weeda et al. 1997). There was also a significant correlation between the liver transcriptome of Ercc1/Xpf-mutant mice and that of old mice that showed increased cell death and anti-oxidant defense, increased anabolism and reduced growth hormone/insulin-like growth factor 1 (IGF1) signaling (Niedernhofer et al. 2006). Interestingly, wild-type mice subject to chronic genotoxic stress show similar changes, suggesting that DNA damage contributes to normal aging.

Another excision repair pathway, MMR, is a multifunctional system whose principal role is to repair replication-associated lesions (see Chapter 7). It is the primary pathway responsible for correcting base mismatches and small insertion/deletions. As such, it improves replication fidelity by 50–1000-fold. This responsibility is especially important in areas composed of one, two or three nucleotide repeats. Instability within these repeats (called microsatellite instability) is diagnostic for defective MMR. Mutations in MMR genes result in hereditary nonpolyposis coli (or HNPCC) after loss of heterozygosity (LOH, 2–4% of colon cancer). In addition, ~15% of sporadic colon cancers are due to spontaneous silencing of a MMR gene (Hsieh and Yamane 2008). Even though MMR is clearly important for suppressing some tumors, there is no evidence it suppresses general aging at this time.

BER is the most important excision repair pathway for repairing ROS-induced base lesions (Holmquist 1998) and for repairing SSBs (Almeida and Sobol 2007) (see Chapter 8). Unrepaired base lesions can result in base mutations (Barnes and Lindahl 2004), while unrepaired SSBs can result in DSBs upon replication fork collapse. Since ROS-induced lesions and SSBs

are so prevalent, BER likely repairs the majority of DNA damage within a cell. Even though BER corrects the majority of DNA damage, there is little direct evidence suggesting it suppresses tumors or general aging. By this we mean that there are no examples of BER gene ablation predisposing an individual to cancer or early aging. This may be due to the presence of multiple BER pathways that utilize a variety of highly redundant proteins. In fact, BER is not a single pathway, but a conglomerate of sub-pathways that utilize multiple proteins with overlapping function (Almeida and Sobol 2007). For example, there are many glycosylases specific for certain base lesions, but they overlap in function. Thus, mutating a glycosylase often results in a minor phenotype. By contrast, deletion of any protein that is critical for the majority of the BER sub-pathways, such as the Ape1 endonuclease, is lethal to the cell. However, there are some data that suggests a marginal decline in BER results in early aging. A mutation in the protein deacetylase, Sirt6, caused a mild reduction in cell survival to oxidative stress and some signs of an early aging phenotype that include loss of subcutaneous fat, kyphosis, and greatly shortened life span (~4 weeks) (Mostoslavsky et al. 2006). However, more recently it was shown that in mammalian cells subjected to oxidative stress, Sirt6 is recruited to the sites of DNA DSBs and stimulates DSB repair through nonhomologous end joining and homologous recombination (Mao et al. 2011). This phenomenon appeared to occur through the stimulation of Parp1, which is involved in both BER and DSB repair.

DSB Repair Pathways

HDR maintains genomic stability by repairing DNA DSBs using a homologous template (West 2003; San Filippo et al. 2008). For HDR, the DSB is processed to yield single-strand DNA (ssDNA) that is coated by RAD51 to form a nucleoprotein filament that searches for and anneals to a homologous template, usually provided by the sister chromatid in somatic cells to form a joint molecule. A variety of other proteins facilitate HDR. There are mediators that enable RAD51 to replace RPA on the single stranded DNA such as BRCA2 and RAD51C. In addition, BRCA1 enables HDR by removing 53BP1 from the site of the DSB (Bouwman et al. 2010; Bunting et al. 2010). The joint molecule can be resolved in either a crossover or noncrossover plane; the former recombines sister chromatids (called sister chromatid exchange, SCE), while the latter does not. Thus, inadequate HDR leads to genomic instability caused by unrepaired DSBs (Lim and Hasty 1996). There are multiple examples of defects in HDR leading to cancer. The breast cancer susceptibility genes, Brca1 and Brca2, are critical for efficient HDR and they suppress both breast and ovarian cancer (see Chapter 14). A defect in RAD51C also results in heritable breast and ovarian cancer after

LOH (Meindl et al. 2010). Paradoxically, excessive HDR also causes genomic instability through recombination between repeats on the same chromatid or sister chromatid or through interchromosomal recombination that causes LOH, which can lead to cancer as seen with mutations in the RecQ helicase defective in Bloom's syndrome (BLM) (Luo et al. 2000). Thus, robust but managed HDR is essential for maintaining genomic integrity.

HDR is well accepted for suppressing tumors, yet only scant evidence suggests HDR regulation impacts general aging. For example, an age-dependent increase in HDR is seen in the *Drosophila* male germline (Preston et al. 2006). Yet, there is little evidence for age-related alterations in HDR in mammals, possibly because most cells are postmitotic and HDR does not function in G_0/G_1. However, the levels of nonallelic homologous recombination appear to increase with age in human blood cells, since there was an increase in rearrangements (Flores et al. 2007). It is possible these rearrangements accumulate with age due to diminished HDR regulation. Thus, age-related alterations in HDR may impact some mitotically active cells in mammals.

The most compelling evidence that HDR impacts aging comes from Brca1-mutant mice. Brca1 is well known for suppressing breast and ovarian cancer and is critical for HDR. The protein was shown to localize to RAD51 foci (Chen et al. 1999) and to negate 53BP1, prevent NHEJ and facilitate HDR (Bunting et al. 2010). Brca1-deletion is embryonic lethal due to a p53-mediated DDR that causes massive apoptosis (Xu et al. 2001). However, reducing p53 levels by half permits Brca1-mutant mice to live. These mice exhibited signs of premature aging that included decreased life span, reduced body fat deposition, osteoporosis, skin atrophy, and decreased wound healing (Cao et al. 2003). Brca1-mutant females, deficient for p53, exhibit mammary carcinoma (Xu et al. 2001). These results suggest that an HDR deficiency can cause early aging in association with p53-mediated DDRs.

NHEJ repairs both general DNA DSBs and DSBs formed during the assembly of V(D)J [Variable(Diverse)Joining] segments of antigen receptor genes; thus, NHEJ-deletion causes sensitivity to clastogenic agents and failed lymphocyte development resulting in severe combined immunodeficiency (scid) (Burma et al. 2006). Unlike HDR, NHEJ does not use a template and thus has the potential to be error prone. This is certainly the case when NHEJ repairs DSBs for V(D)J recombination (Lieber et al. 2004). However, there is little evidence that NHEJ is error prone when repairing general DSBs (Bennardo et al. 2009). NHEJ utilizes at least seven proteins in mammals: Ku70, Ku80, DNA-PK_{CS}, Artemis, Xrcc4, DNA Ligase IV (Lig4) and Xrcc4-like factor (Lieber et al. 2004). Ku70 and Ku80 form a heterodimer called Ku that binds to DNA ends (Walker et al. 2001), and together with a PI-3 kinase catalytic subunit, DNA-PK_{CS}, forms a holoenzyme referred to as DNA-PK

(DNA dependent—protein kinase). Artemis opens hairpins and processes overhangs in a complex with DNA-PK_{CS}, and these ends are ligated by the Xrcc4-Lig4 heterodimer in a complex with Xrcc4-like factor (Ahnesorg et al. 2006; Buck et al. 2006).

NHEJ functionally declines with age (Gorbunova et al. 2007) and therefore, when fully active, may extend life span by ameliorating aging (Hasty 2008). There are multiple examples that show NHEJ function declines with age. For example, as rats age, Ku levels diminish in the testis, and Ku70 or Ku80 are differentially expressed in various tissues (Um et al. 2003). As humans age, Ku DNA binding and nuclear localization is impaired in blood mononuclear cells (Frasca et al. 1999; Doria et al. 2004), and Ku70, but not Ku80, levels decline in lymphocytes (Ju et al. 2006). Similarly, Ku levels decline, and the cellular distribution of the protein is altered in human fibroblasts that approach senescence (Seluanov et al. 2007). Furthermore, NHEJ function declines in the brains of aging rats (Ren and de Ortiz 2002; Vyjayanti and Rao 2006) and in Alzheimer's disease patients (Shackelford 2006), and become less efficient and more error-prone in senescent cells (Seluanov et al. 2004). Thus, the observed decline of NHEJ, with age supports the possibility that defective NHEJ will lead to early aging.

Mice defective for some of the NHEJ genes (Ku70, Ku80, Xrcc4, DNA-PK_{CS}), exhibited an early onset of aging characteristics (Vogel et al. 1999; Espejel et al. 2004b; Chao et al. 2006; Li et al. 2007). These NHEJ-mutant mice showed early signs of osteoporosis, growth plate closure, atrophic skin, liver pathology, sepsis, cancer, and shortened life span. Multiple organs derived from these mice displayed an increase in chromosomal rearrangements (Busuttil et al. 2008). Cells derived from these mice exhibited premature replicative senescence (Lim et al. 2000), were hypersensitive to ROS (Lim et al. 2000; Li et al. 2009a), and showed early signs of age-related chromosomal abnormalities (Li et al. 2007). The early aging phenotype was not due to NHEJ's participation in V(D)J recombination or the immunodeficient phenotype (Holcomb et al. 2007). Importantly, oxidative metabolism in *ku80*$^{-/-}$ mice and cells is related to the observed DNA breaks, genomic instability and apoptosis (Karanjawala et al. 2002). Thus, ROS-induced DNA damage may be a causal factor for early aging.

Telomere Maintenance

The telomere condition may contribute indirectly to aging, since critically shortened telomeres induce cellular senescence similar to a DSB. Telomeres are higher order structures that cap and maintain chromosome ends (de Lange 2002). The telomere is composed of many TTAGGG repeats that end in a single-stranded overhang that is unable to be replicated. Therefore, a telomere-specific enzyme, telomerase, uses this end as a template to extend

and maintain telomere length. Thus, chromosomal ends are not the same as DNA DSBs. However, telomeres may erode with age resulting in a DSB (von Zglinicki et al. 2005) that is available for end joining resulting in fused chromosomes (Chin et al. 1999; Artandi et al. 2000). Similar to HDR, telomerase is important for proliferating cells, and telomerase activity is restricted to only a few cell types in an adult: germ cells and stem cells (Flores et al. 2006). Thus, changes in telomere length or structure impact proliferating cells with age. Importantly, telomerase activity is insufficient to maintain telomeres in stem cells, suggesting that tissue renewal can become compromised with age due to eroded telomeres.

The consequences of poor telomere maintenance with age are vividly demonstrated in mouse models. The RNA component (terc) of telomerase was deleted in mice, and these mice were analyzed over successive generations as the telomeres eroded. The 1st generation mice had long telomeres and did not show a phenotype. But the telomeres shortened with every successive generation and by the 3rd or 4th generation an age-related phenotype was evident in organs with proliferating cells (Lee et al. 1998). Thus, only selective age-related defects were seen including gray hair, alopecia, skin ulcerations, impaired wound healing, cancer and shortened life span (Rudolph et al. 1999). In addition, chromosomal fusions were observed. Even though only a selective phenotype was observed in mice with eroded telomeres, a more-complete and severe aging phenotype was observed in telomere-shortened mice deleted for other DNA repair proteins like Atm (Wong et al. 2003),Wrn (Chang et al. 2004; Du et al. 2004) and Ku80 (Espejel et al. 2004a). These observations suggest shortened telomeres, in combination with DNA repair defects, accelerate aging.

It is also possible that the early aging phenotype observed in NHEJ-mutant mice could be due to defective telomeres, since Ku70, Ku80 and DNA-PK_{CS} associate with telomeres (Hsu et al. 1999; d'Adda di Fagagna et al. 2001), suppress telomere fusions (Bailey et al. 1999; Hsu et al. 2000; Samper et al. 2000; Li et al. 2007), and impact telomere length maintenance (d'Adda di Fagagna et al. 2001; Espejel et al. 2002). In addition, Ku80 deficiency causes telomere loss in human somatic cells (Myung et al. 2004). Thus, Ku70, Ku80 and DNA-PK_{CS} are important for telomere maintenance as well as for DSB repair. However, the early aging phenotype observed in these NHEJ-mutant mice is not identical to that in the telomerase-mutant mice, suggesting that a telomere abnormality is not the sole contributing factor to their phenotype.

RecQ Helicases

RecQ-like DNA helicases are important for a variety of processes including DNA repair. This fact is well documented since mutations in such genes

cause Werner's syndrome (WS), Bloom's syndrome (BS), and Rothmund Thomson's syndrome in humans. Of these three disorders, WS is the best example of human premature aging, since it strongly resembles normal aging (Martin 1978; Goto et al. 1997). *WRN* is the gene mutated in WS and is a homolog to *E. coli RecQ* (Yu et al. 1996). WRN is both a 3′→5′ DNA helicase and a 3′→5′ DNA exonuclease (Huang et al. 1998). It functions in multiple DNA metabolic pathways, including replication and homologous recombination (Otterlei et al. 2006). *WRN*-mutant cells exhibit genetic instability that includes chromosomal deletions. WS individuals prematurely show atrophic skin, thin gray hair, osteoporosis, type II diabetes, cataracts, arteriosclerosis, and cancer. These aging characteristics are seen in their 2nd and 3rd decades. Interestingly, WS individuals show a different cancer spectrum than generally seen in the normal population. WS patients exhibit many more cancers of mesenchymal origin (50%), in contrast to cancers of epithelial origin (90%) typically seen in normal individuals. WS individuals often die in their fifth decade mostly from cancer or cardiovascular disease.

Mouse models for WRN do not show elevated tumor incidence or premature aging (Lombard et al. 2000), dampening enthusiasm for its role in suppressing aging. However, there is an interesting phenotype for WRN mice with eroded telomeres (Chang et al. 2004; Du et al. 2004). These mice were generated by crossing the WRN mutation into mice with the terc mutation. The WRN mice with eroded telomeres exhibit a premature aging phenotype similar to WS. These mice show premature death, hair graying, alopecia, osteoporosis, type II diabetes and cataracts. In addition, these mice exhibit increased levels of nonepithelial cancer typical of WS. Fibroblasts derived from these mice show premature replicative senescence and accumulation of DNA-damage foci and chromosomal instability. A similar exacerbation of phenotype was observed in Blm mice with eroded telomeres (Du et al. 2004). Thus, telomere dysfunction may contribute to WS and BS.

Mitochondrial DNA Damage

Mutations in mitochondrial DNA (mtDNA) have been proposed to contribute to aging (Wallace 2010). Thirteen core proteins encoded in mtDNA are important for the mitochondrial energy-generating system: oxidative phosphorylation. Somatic mutations in mtDNA may accumulate with age, diminishing mitochondrial energy production. These mutations could also impact nuclear DNA by adversely affecting oxidative phosphorylation, which may generate ROS that could accelerate mutation accumulation in nuclear DNA. The proofreading activity of the mtDNA polymerase gene product (polymerase γ, POLG) has been inactivated in the mouse germline

to study the impact that mtDNA mutations have on aging. The lack of proofreading, with the polymerization activity remaining intact, caused an increase in mtDNA errors. These mutant mice exhibited signs of premature aging including reduced lifespan, weight loss, reduced subcutaneous fat, alopecia, kyphosis, osteoporosis, anemia, reduced fertility and heart enlargement (Trifunovic et al. 2004; Kujoth et al. 2006). While this phenotype supports the notion that an accumulation of mtDNA mutations contributes to aging, several of the findings have raised controversy (Khrapko and Vijg 2009). Mice separately engineered with a conditional deficiency for mitochondrial superoxide dismutase in the connective tissue exhibit reduced superoxide anion detoxification in connective tissue mitochondria. These mice showed reduced life span and early onset of weight loss, skin atrophy, kyphosis, osteoporosis and muscle degeneration (Treiber et al. 2011). Premature cellular senescence due to increased oxidative damage may contribute to the aging phenotype, since fibroblasts derived from these animals displayed elevated levels of $p16^{INK4a}$.

Chromosomal Metabolism not Related to DNA Repair

There are molecular pathways that do not directly involve DNA repair, but are still very important for genome maintenance. These include DNA methylation proteins, certain transcription factors, the lamin proteins and the mitotic checkpoint proteins. Defects in some of these pathways cause genome instability and an early aging phenotype.

PASG is a SNF2-like putative remodeling factor that enables DNA methylation. Mice deleted for PASG exhibit global hypomethylation and show developmental growth retardation (Sun et al. 2004). In addition, these mice display a premature aging phenotype that includes graying and hair loss, reduced skin fat deposition, osteoporosis, kyphosis, cachexia, and premature death. Furthermore, mutant fibroblasts undergo premature replicative senescence. Thus, defects in DNA methylation can cause an early aging phenotype.

The nuclear receptor, TR4, is a transcription factor that influences various biological activities. TR4-mutant mice exhibit a premature aging phenotype that includes shortened life span and kyphosis. This phenotype is likely due to defective oxidative stress defense and consequent genome instability (Lee et al. 2011). Indeed, an anti-oxidant (N-acetyl cysteine) ameliorates the cellular senescent phenotype. In addition, a number of DNA repair and oxidative stress genes were upregulated in mutant mouse embryonic fibroblasts (MEFs).

Defects in the nuclear lamina, which is a dense fibrillar network inside the nucleus that provides mechanical support and regulates important cellular events such as DNA replication and cell division, can cause

segmental progeroid disorders like Hutchinson-Gilford progeria syndrome (HGPS) and Restrictive Dermopathy (RD). HGPS patients are heterozygous for a *LMNA* gene mutation that generates progerin, a protein with a 50 amino acid internal deletion. HGPS patients appear normal at birth, but display aging-like characteristics in their first decade and die at about 13 years from atherosclerosis and myocardial infarction or stroke (Gonzalez et al. 2011). An HGPS mouse model with a mutation in Lmna appears normal at birth, but by 6–8 weeks exhibits slow growth, weight loss, loss of adipose tissue, and osteolytic lesions in ribs and other bones, and death by about 28 weeks (Yang et al. 2005). ZMPSTE24 is the metalloproteinase involved in the processing of prelamin A to lamin A, and its deletion causes Restrictive Dermopathy (RD) in people, due to the generation of the mutant lamin A protein 'FC-lamina A', resulting in perinatal death. Progerin and FC-lamina A cause genome instability due to nuclear deformations and chromatin perturbations. Interestingly, defective DNA repair and premature senescence were observed in Zmpste24-deficient mice (Gonzalez et al. 2011; Krishnan et al. 2011). Progerin is also present in normal cells and appears to progressively accumulate during aging in non-HGPS cells (Gonzalez et al. 2011). Therefore, understanding how this mutant form of lamin A provokes HGPS may shed significant insight into physiological aging. Lmna-deficient mice exhibit profound nuclear architecture abnormalities and multiple histopathological defects that phenocopy an accelerated aging process. The Zmpste24 mouse model exhibits retarded growth, alopecia, micrognathia, dental abnormalities, osteolytic lesions, and osteoporosis similar to HGPS in people (Bergo et al. 2002; Pendas et al. 2002; Espada et al. 2008). Reducing levels of prelaminA can ameliorate this phenotype (Fong et al. 2004). Thus, nuclear architecture is critical for longevity assurance.

Recently, a new strain of HGPS mice was generated to test splicing-directed therapy (Osorio et al. 2011). These mice have the 1827G>T mutation that causes a splicing defect commonly found in HGPS patients. In addition, these mice contain an engineer edloxP-stop-loxP sequence that stops laminA transcription, restricting expression to only lamin C. As expected,the animals had no obvious phenotype for the first year, as measured by weight and survival. Importantly, progerin is made only after Cre-deletion. Cre-deletion removes the stop codon, such that the mutant geneis transcribed and an internal 50 amino acids is deleted that includes the ZMPSTE24 target sequence. This genetic manipulation caused an accumulation of farnesylated progerin at the nuclear envelope and structural defects in the nucleus. These mice showed typical signs of HGPS that includedprogerin accumulation, bone and cardiovascular abnormalities, and shortened life span. Therefore, this new mouse model is remarkably similar to human HGPS, both at a mechanistic and phenotypic level.

The HGPS mice were then used to develop a new antisense morpholino-based therapy that prevents abnormal Lmna splicing (Osorio et al. 2011). One morpholinobinds to the Lmna exon 10 splice donor site to limit transcription to only lamin C, while the other morpholino binds the 1827G>T mutation to prohibit alternative splicing. Both morpholinos reduced progerin accumulation along with the nuclear defects. Furthermore, the combined treatment was additive in fibroblasts. Importantly, HGPS mice treated with these morpholinos showed reduced proger in, reduced expression of p53-target genes, increased body weight and an extended life span. Thus, this technology might be beneficial for people with HGPS and warrants further clinical evaluation.

The spindle assembly checkpoint ensures faithful chromosome segregation during mitosis, and defects in this checkpoint cause aneuploidy (an abnormal number of chromosomes). Human cancer cells are commonly aneuploid and a mouse model defective for the spindle assembly checkpoint protein, Bub1, were susceptible to carcinogenesis due to aneuploidy-induced LOH (Baker et al. 2009). Another spindle checkpoint protein, BubR1, appears to influence the normal aging process (Baker et al. 2005). Complete BubR1 deletion is lethal, but mice with reduced BubR1 function exhibit premature aging. BubR1-defective mice also exhibit a short lifespan, cachectic dwarfism, kyphosis, cataracts, loss of subcutaneous fat and impaired wound healing. In addition, mice doubly haploinsufficient for the mitotic checkpoint genes Bub3 and Rae1 display early aging. MEFs derived from the Bub3/Rae1 haploinsufficient animals undergo premature cellular senescence, as indicated by the accumulation of high levels of p19, p53, p21, and p16. Interestingly, comparing BubR1 hypomorphic mice to Bub3/Rae1 haploinsufficient mice indicated that the level of aneuploidy and aging do not necessarily correlate, suggesting premature aging is linked to cellular senescence and not to aneuploidy (Baker et al. 2006). Elevated levels of p53 and p21 indicate a DDR, suggesting an accumulation of damaged DNA perhaps due to a defect in DNA repair. Thus, certain elements of the mitotic checkpoint suppress aneuploidy-induced cancer and a DDR-induced cellular senescence along with organismal aging.

Studies with several other mouse models suggest that a defect in some DDR checkpoint pathways may lead to genomic instability and premature aging. These checkpoint pathways monitor the genome for conditions that interfere with basic cellular functions like DNA replication (see Chapter 13). They halt the cell cycle to permit correction of the problem that may otherwise stall replication forks, resulting in breaks that can lead to cell death or chromosomal rearrangements. In yeast, Cdc14 plays a key role in themitotic exit; however, its exact function in mammals remains uncertain. Cdc14b-deficient mice exhibit premature signs of aging, including cataracts and kyphosis. Cells derived from these mice were defective in DNA repair

and entered premature senescence (Wei et al. 2011). In mammals, ATR is a checkpoint regulator essential for suppressing broken replication forks. ATR-mutations cause developmental defects in humans (Seckel syndrome) and embryonic lethality in mice. A conditional mutation that permits ATR deletion in adult mice revealed an appearance of age-related characteristics, including gray hair, alopecia, kyphosis, osteoporosis, thymic involution and fibrosis. ATR deletion in adults also caused acute cellular loss in tissues with proliferative cells, due to reduced populations of stem and progenitor cells that ultimately compromised tissue renewal and homeostatic capacity (Ruzankina et al. 2007). In addition, deletion of the cell cycle regulator and tumor suppressor p53 exacerbated the phenotype, suggesting an accumulation of cells with excessive DNA damage (Ruzankina et al. 2009). Thus, DDR checkpoints can suppress DNA damage that otherwise leads to age-related characteristics in tissue with mitotically active cells.

ENHANCED DDRs AND CYTOTOXICITY

Enhanced DNA damage checkpoints may have a profound impact on longevity assurance by suppressing cancer and perhaps by influencing aging. There are multiple forms of responses that are induced by many forms of DNA damage (Campisi and d'Adda di Fagagna 2007). If the problem is major and difficult to correct, these pathways may induce either cell death (apoptosis) or cellular senescence (permanent arrest from proliferation) (Hayflick 1965). DDRs are well known for suppressing tumors and occur in response to oxidative stress, DNA damage, telomere erosion and replicative stress (Reed 1999; Parrinello et al. 2003; Bartkova et al. 2005, 2006; Di Micco et al. 2006; Collado et al. 2007; Sharpless and DePinho 2007). These pathways ultimately reduce cancer-causing mutations (Busuttil et al. 2003; Sharpless and DePinho 2005; Collado and Serrano 2006).

The ATR-defective mice mentioned above suggest that defective DDRs lead to DNA damage that result in an age-related phenotype. However, it is also possible that accelerated activity of some DDRs contributes to aging by increasing apoptosis or senescence that ultimately diminishes the pool of healthy cells (Campisi 1997, 2000; Bree et al. 2002; Pelicci 2004). This process may be a particular problem in stem cells, since some of the mouse aging models show reduced haematopoietic stem cell pools (Rossi et al. 2007a), and the absence of some DDRs mitigated apoptosis in these cells (Janzen et al. 2006). Therefore, mechanisms such as apoptosis and cellular senescence are important anti-cancer strategies needed to ensure longevity that simultaneously contribute to aging later in life by depleting proliferation competent stem cell pools.

In addition to accumulated DNA damage and mutations, DDRs may be causal factors for aging. This is possible since many of the human and mouse aging models are defective for a wide range of genome maintenance pathways, but display a similar early aging phenotype (see previous section). Even though these mice accumulate various types of DNA damage, the responses are similar and have a similar biological outcome; that is, they induce either apoptosis or cellular senescence. For example, fibroblasts derived from many of these aging models exhibit premature cellular senescence that is dependent on p53 (Chin et al. 1999; Lim et al. 2000; Grillari et al. 2007). Some of these models display increased spontaneous DDRs. Importantly, the Ku80-mutant mouse model exhibits a spontaneous increase in the p53/p21 DDR (Holcomb et al. 2008). Levels of p53 were also critical for the aging phenotype in the Brca1-mutant mice (Cao et al. 2003) and the terc-mutant mice (Chin et al. 1999). Increased DDR is observed in other mouse models harboring defects in genome maintenance as well. For instance, mice defective for the mtDNA polymerase γ show elevated apoptosis (Kujoth et al. 2006), as do mice defective for Zmpste24 (Krishnan et al. 2011). In addition, PASG mutant mice exhibit premature cellular senescence, along with elevated levels of the cell cycle regulator $p16^{INK4a}$ (Sun et al. 2004). BubR1-deficient mice show high levels of the senescence-associated markers $p16^{Ink4a}$ and $p19^{Arf}$. Deletion of $p16^{Ink4a}$ in BubR1-deficient mice attenuates both cellular senescence and premature aging in skeletal muscle and fat, while inactivation of $p19^{Arf}$ exacerbates senescence and aging. Thus, BubR1-deficiency activates the Cdkn2a locus, demonstrating that $p16^{Ink4a}$ is an effector and $p19^{Arf}$ an attenuator of senescence and aging (Baker et al. 2008). In short, different defects in genome maintenance elevate DDRs that may contribute to aging.

The tumor suppressor p53 (see Chapter 12) influences the phenotype for many of the aging models; yet some evidence suggests p53 does not impact aging. p53 is a transcription factor that induces DDRs that promote apoptosis or cellular senescence (Meek 2004). Complete p53-deletion shortens life span due to increased cancer (Donehower et al. 1992), whereas p53 overexpression (from a BAC clone ensuring normal regulation) lowers cancer incidence without influencing aging (Garcia-Cao et al. 2002). Furthermore, overexpression of Arf/p53 improves cancer resistance thereby extending life span (Matheu et al. 2007). In addition, mice with reduced levels of MDM2 exhibit increased p53 and show reduced cancer without premature aging (Mendrysa et al. 2006). Thus, p53-overexpression reduced cancer without accelerating aging.

By contrast, data supports the notion that enhanced p53 responses also contribute to aging (Campisi 2000; Bree et al. 2002; Pelicci 2004). For example, p53-deletion increases replicative capacity (Harvey et al. 1993), whereas p53-overexpression decreases replicative capacity and enhances cellular

senescence (Sugrue et al. 1997). Deletion of a negative p53-regulator, e.g., MDM2 or MDM4, leads to embryonic lethality, and p53-deletion rescues this lethality (Jones et al. 1995; Montes de Oca Luna et al. 1995; Parant et al. 2001). As previously mentioned, p53 appears to play a causative role in a variety of the premature aging mouse models including Brca1, Ku70, Ku80, Terc and Zmpste24 (Chin et al. 1999; Lim et al. 2000; Cao et al. 2003; Varela et al. 2005; Holcomb et al. 2006; Li et al. 2007, 2009a). These mouse models suggest that a p53-dependent DNA damage checkpoint contributed to the age-related phenotype. Therefore, p53 activity may contribute to aging in people (van Heemst et al. 2005).

The p53 gene encodes multiple p53 isoforms (Bourdon et al. 2005; Scrable et al. 2005), and the ratio of these isoforms may contribute to the confusing p53 data with regard to aging. It is possible that the isoform ratio contributes to cancer predisposition, since variable expression is observed in breast tumors compared to normal breast tissue. Some of these isoforms may also contribute to the varied DDRs, since the ATR-intra-S phase checkpoint in response to DNA damage depends on one of these isoforms (Rohaly et al. 2005). The N-terminally truncated isoforms may contribute to aging as well, since their overexpression reduces cancer incidence and causes premature aging signs that include decreased life span and early onset of the same aging characteristics typical for many of the DNA repair deficient mice (Tyner et al. 2002; Maier et al. 2004). Full-length p53 is required to see early aging in mice that express either N-terminally truncated p53 isoform. These truncated isoforms likely associate with full-length p53 to influence its function as a p53 tetramer. For one of these mouse models, the N-terminally truncated isoform was shown to interact with full length p53 to increase its stability and enable its nuclear localization in the absence of stress (Moore et al. 2007). Additionally, another isoform was shown to stabilize p53 in the presence of Mdm2 (Yin et al. 2002). Thus, overexpression of one p53 isoform likely alters the p53 isoform ratio to change p53 function. Overexpression of the N-terminal truncated p53 isoforms appears to have reduced tissue function or regeneration (Dumble et al. 2004).

The impact of DNA damage and DDRs on an organism may be most critical for stem cells. Adult stem cells exist in most tissues, and stem cell function seems to decline with age. This decline may be intrinsic to the stem cell or it may be extrinsic to the microenvironment. There is some data to suggest that at least a part of this decline is influenced by DNA damage and the stem cells innate ability to repair and respond to DNA damage (Rando 2006). As stem cells age, accumulation of DNA damage may hinder self-renewal capacity and their ability to mediate a return to homeostasis after acute stress or injury (Rossi et al. 2007a,b). It is possible that a decline in DNA repair function contributes to an accumulation of DNA damage. Certainly, DNA repair capacity is essential for stem cell function as shown in a variety

of mouse aging models. Mice defective for NER, telomere maintenance and NHEJ exhibit reduced stem cell functional capacity that could be seen under conditions of stress. These mutant mice showed diminished reconstitution and proliferative potential and self-renewal, along with increased apoptosis and functional exhaustion (Rossi et al. 2007a). In addition, mice with a hypomorphic Lig4 mutation (Y288C) exhibit diminished DNA DSB repair that results in a progressive loss of haematopoietic stem cells and bone marrow cellularity as the mice aged, which in turn, severely impaired stem cell function in tissue culture and after transplantation (Nijnik et al. 2007). Moreover, DDRs initiated from the Ink4/Arf locus dramatically hinder the induction of pluripotent stem cells from fibroblasts (Li et al. 2009b). Thus, DNA damage and DDRs influence stem cell function.

HUMANS AS MODEL SYSTEMS TO IDENTIFY GENETIC VARIANTS OF GENOME MAINTENANCE

Although unsuitable as an experimental model, humans have proven to be an invaluable model of aging through the identification of natural mutants that cause accelerated aging. In fact, human patients were the first models for progeroid syndromes. This fact is not surprising in view of the century of clinical observations on subjects of our species. Thanks to this enormous reservoir of knowledge, clinical practitioners recognized over 100 years ago accelerated aging in a number of people suffering from life-shortening genetic defects (Martin 2005). These segmental progeroid syndromes, characterized by the premature appearance of multiple signs of normal aging, were described by the medical community well before the discovery of DNA and are, therefore, not biased towards a DNA-based hypothesis of aging. Remarkably, many of these syndromes are caused by a defect in genome maintenance. As we discussed in section 2, WS is caused by a defect in a gene that is a member of the RecQ helicase family (Yu et al. 1996). The affected gene, *WRN*, encodes a RecQ homolog whose precise biological function remains elusive, but is important for DNA transactions, probably including recombination, replication, and repair as discussed earlier. Human patients with WS prematurely exhibit signs of senescence, including atrophic skin, graying and loss of hair, osteoporosis, malignant neoplasms, diabetes, and shortened life span (Goto 1997). Furthermore, a greatly increased frequency of genomic rearrangements has been reported in peripheral blood lymphocytes from these patients (Fukuchi et al. 1989). Another progeroid syndrome, HGPS is caused by a defect in the gene *LMNA*, which through alternative splicing encodes both nuclear lamins A and C (Young et al. 2006). Nuclear lamins play a role in maintaining chromatin organization. Two related segmental progerias, CS and TTD, are

caused by defects in transcription and genome maintenance via the NER pathway and show no signs of increased cancer (de Boer and Hoeijmakers 2000). CS is an autosomal recessive disorder characterized by progressive postnatal growth failure, neurological dysfunction, and a short life span of about 12 years on average. TTD shows no predisposition to cancer, but leads to severely impaired physiological and neurological development, including retarded growth, cachexia, sensorineural hearing loss, retinal degeneration, and its hallmark features of brittle hair and nails, and scaly skin (Lehmann 2003). TTD patients have a greatly reduced lifespan, and the disease is often considered a segmental progeroid syndrome. Less striking segmental progeroid syndromes include ataxia telangiectasia, caused by a heritable mutation of the gene *ATM*, a relay system conveying DNA-damage signals to effectors (Shiloh and Kastan 2001), and Rothmund–Thomson syndrome, which like WS is based on a heritable mutation in a *RecQ*-like gene (Liu 2010). As discussed earlier, there is ample evidence that each of these genes, when defective, can also lead to aging symptoms in the mouse, sometimes when combined with other gene defects (Hasty et al. 2003).

The discoveries that human segmental progeroid syndromes are almost without exception based on heritable defects in genome maintenance and that inactivation or mutational alteration of genome maintenance genes in mice often results in the premature appearance of aging symptoms strongly support the notion that genome maintenance pathways are functioning as major longevity assurance systems. A major challenge is to demonstrate that the same genes that give rise to premature aging when inactivated in mice or humans normally act as pro-longevity genes. Normal human populations show great diversity in genotypes related to genome maintenance, and it is conceivable that DNA sequence variation at such loci is a critical factor in individual variation of longevity and healthy aging. Indeed, individual variation in some DNA repair activities among humans have been reported and found associated with differences in cancer risk (Setlow 1988; Grossman and Wei 1995), age-related disease (Ladiges et al. 2003; Hirai et al. 2005), and aging (Doria and Frasca 2001).

The hypothesis that genome maintenance is a major longevity-assurance system in humans can be directly tested through genotype-phenotype correlations of genome maintenance genes in human populations with well-defined aging-related characteristics, especially those who live to extreme old age. Classically, studies have been focused on population-based cohorts of extremely long-lived individuals. Longevity is known to have a genetic component with the estimated heritability of average life expectancy of ~25% (McGue et al. 1993; Herskind et al. 1996). Family studies on centenarians, those who age to 100 years or above, suggest that the relationship between genetics and longevity is stronger in the oldest-old adults. For example, the siblings of centenarians have a 4 times greater

probability of surviving to age 90 than the siblings of people who have an average life expectancy (Perls et al. 1998). When it comes to living 100 years, the probability is 17 times greater in male siblings of centenarians and 8 times greater in female siblings of centenarians than average lifespan of their birth cohort (Perls et al. 2002). The immediate ancestors of Jeanne Calment, the longest ever lived human being to date, who died at the age of 122 years and 164 days, were shown to have more than a 10-fold higher probability of living to 80 years or more than the control ancestors of a reference family (Robine and Allard 1998). These studies support the utility of long-lived individuals, such as centenarians, as a model system for studying genetic variations predisposing people to longevity. Therefore, genetic studies of longevity are based on the premise that they may lead to the identification of alleles that are either particularly enriched in these populations due to positive effects on extreme longevity or under-represented due to negative impact on human health.

In general, there are two strategies to test the hypothesis that alleles at loci involved in genome maintenance are associated with longevity phenotypes (Risch 2000; Carlson et al. 2004; Suh and Vijg 2005). First, in a so-called global genome approach, one would test the entire genome for regions that co-inherit with one or more aging or longevity phenotypes. A second approach is a so-called candidate approach, in which only those genes known to be involved in genome maintenance are tested. The former approach is better because it is an objective screen without bias. Both genome-wide linkage studies and genome-wide association studies (GWAS) have been performed to identify genetic markers across the genome associated with longevity-related traits. However, genome-wide studies have demonstrated that the identification of genes involved in complex phenotypes with late onset, such as longevity, is challenging. A standard linkage approach is essentially constrained due to the need to collect DNA samples from families of affected individuals, which is seldom a problem with disease phenotypes where onset is during childhood or early adulthood, but is an issue for aging phenotypes that arise later in life when family members are likely to be deceased. Because it is virtually impossible to obtain DNA samples of pedigrees to follow the segregation of extreme longevity, sibling-pair analysis has been adopted as an alternative in the study of such late-onset genetic traits. Results of a genome-wide sibling-pair study of 308 persons belonging to 137 families with exceptional longevity indicated significant evidence for linkage with a locus on chromosome 4q25 (Puca et al. 2001). A subsequent haplotype-based fine mapping study of the interval identified a marker within microsomal transfer protein (MTP), which is involved in lipoprotein synthesis, as a possible modifier of human lifespan (Geesaman et al. 2003). These results were not replicated in nonagenarian sibling pairs (Nebel et al. 2005), raising the possibility that the finding was specific for

that particular population or was an underpowered study generating a false positive result. Association-based studies are considered more effective tools than linkage studies for studying complex traits such as longevity, because they have greater statistical power to detect genes of small effect (Risch and Merikangas 1996; Long and Langley 1999). By comparing the frequency of genetic variants in cases (long-lived individuals; nonagenarians or centenarians) and unrelated controls (younger elderly individuals), association studies evaluate correlation between a genetic variation and a particular trait, in this case longevity (Fig. 2).

There was great enthusiasm for the GWA approach using single-nucleotide polymorphism (SNP) markers, the most common type of genetic variation in the human genome and the workhorse of association studies, due to the expectation that statistically powerful association studies are now feasible. However, a major criticism remained. The concern involved the assumption that there is little allelic heterogeneity within loci, and that susceptibilities for longevity are due to a small number of ancient polymorphisms that occur at high frequency in all populations, as argued in the common disease/common variant (CD/CV) hypothesis (Lander 1996; Reich and Lander 2001). However, if late-onset phenotypes such as longevity are due to large numbers of rare variants at many loci, this strategy would fail, as no single haplotype would be strongly associated with longevity and the contribution of most individual variants would be too small (Pritchard 2001). These concerns turned out to be true, even for common traits, as evident from the results of the GWAS. More than 1,000 published GWAS reported significant ($p<5 \times 10^{-8}$) associations of ~4,000 SNPs for more than 200 traits/diseases (Hindorff et al. 2009). However, each locus has a surprisingly low to modest effect. Furthermore, there is a wide gap between the population variation in disease, seemingly explained by the results of GWAS (usually less than 10%) and heritability estimates (often more than 50%) (Manolio et al. 2009).

Nevertheless, since the first wave of GWAS have generated a catalogue of common SNPs associated with the major diseases that contribute to human mortality, it was explored whether the disease susceptibility alleles are absent from the genome of long-lived individuals of nonagenarians and octogenarians. Remarkably, the frequency of a set of alleles currently known to increase the risk of coronary artery disease, cancer and type-2 diabetes (as identified by GWAS) was almost identical between long-lived individuals and younger controls (Beekman et al. 2010). These results suggest that the genome of the long-lived may harbor longevity-promoting alleles that protect against age-related diseases that contribute to population mortality, rather than the absence of alleles promoting such diseases. A standard procedure has been set for GWAS, in which accepted levels of statistical significance and credible top hits can only be obtained by a meta-analysis of several

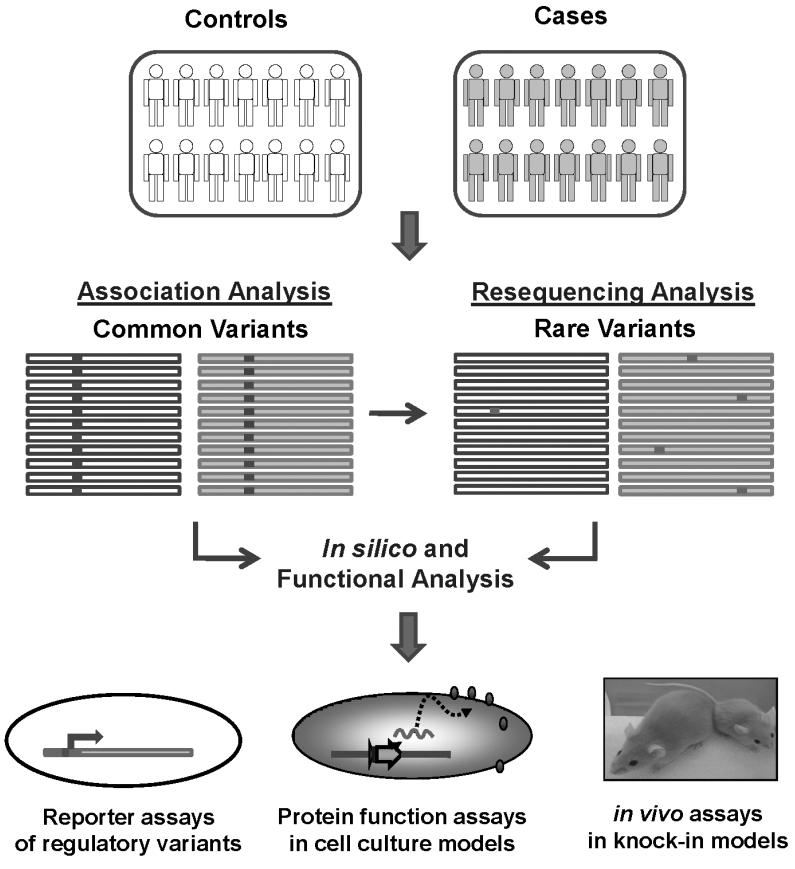

Figure 2. Discovery of functional genome maintenance variants in human longevity. A hierarchical, multidisciplinary approach will increase the chances of identifying functional variations in the genome maintenance genes that influence human longevity. Genetic association in case/control studies establishes genetic link between genome maintenance genes and human longevity. Controls are typically elderly individuals and cases are extremely long-lived individuals, such as centenarians. Genetic association established by common marker variants requires resequencing analysis to identify potentially functional variants, whereas direct resequencing of candidate genes leads to discovery of such variants. Individually rare longevity-associated variants may be enriched in cases as a group as compared to controls. *In silico* analysis predicts the outcomes of potentially functional variants and helps prioritize candidate variants for further functional analysis. Integrated multiple *in vitro* and *in vivo* assays are needed to assess the functional roles of each longevity-associated genome maintenance gene variants depending upon the nature and location of gene variants.

Color image of this figure appears in the color plate section at the end of the book.

large cohort studies, to avoid reports on false positive associations caused by multiple testing and genotyping errors that occur as a consequence of high-throughput technology (Slagboom et al. 2011). A meta-analysis of four

GWAS of survival to age 90 years or older has been performed, but has not yet reported on genome-wide significant associations (Newman et al. 2010). Recently, a modest sized GWAS, involving a discovery study of 403 nonagenarians and 1670 younger controls, followed by replication studies in a total of 4149 nonagenarians and 7592 younger controls, reported no major longevity locus other than the re-discovery of the ApoE locus (Deelen et al. 2011), a gene reproducibly associated with longevity (Schachter et al. 1994; Christensen et al. 2006). These results suggest the meta GWAS with a larger sample size and other research strategies, such as an extreme-trait design that includes rare variant discovery (Fig. 2) and an extreme-trait design that uses centenarians, may be needed to detect genetic variants contributing to longevity in humans.

Much of the speculation about the missing heritability from GWAS has focused on the possible contribution of rare variants (MAF<0.5%) (Manolio et al. 2009). Rare variants would not be strongly associated with any common alleles defined by the common marker SNPs and are not queried in most GWAS. Since extreme longevity is a rare phenotype (Perls et al. 1999), only an exhaustive and comprehensive approach will ensure that no rare but important functional SNP escapes attention. Candidate gene approaches can address this issue by focusing on identification of all possible variants, including the rare variants, in the genes or pathways that are selected based on *a priori* hypotheses about their role in the phenotype (Tabor et al. 2002). However, association studies typically leave open the question of whether the associated genetic variant is functionally important or serves only as a proxy, genetic marker co-inherited with the functional allele. To complete a genetic study with solely a statistical end point is unsatisfactory in view of the uncertainties associated with the statistical interpretation. To minimize possible errors and spurious association, an integrated approach is required to assess the functional relevance of gene variations at the molecular level and to overcome the statistical limitations. So far, this candidate approach combined with functional analysis has been successfully applied to identify functional rare variants in the insulin like growth factor-1 receptor (*IGF1R*) gene that are enriched in Ashkenazi Jewish centenarians as compared to younger controls (Suh et al. 2008; Tazearslan et al. 2011) and cause defects in IGF1R signaling, gene regulation, and cell cycle control in response to IGF-1 treatments in short-term cell culture models, supporting the role of IGF-1signaling in human longevity. In principle, these approaches can be applied to other candidate genes and pathways such as genome maintenance genes.

Coupled with the rapid advances in ultra-high-throughput sequencing technologies (Cirulli and Goldstein 2010), it is now feasible to comprehensively analyze all sequence variants in genome maintenance genes segregating with a longevity phenotype and to investigate the

functional consequences of the associated variants. Understanding the mechanisms by which longevity-associated genome maintenance gene variants contribute to human longevity will be critically important to facilitate the development of strategies to delay aging and promote health span. The complexity of aging and longevity phenotypes pose a daunting yet exciting challenge to establish functionality and causality of longevity-associated variants. A variety of experimental approaches in multiple model systems are needed to elucidate the functional consequences of gene variants in genome maintenance pathways (Fig. 2). Potential functionality of SNPs in the associated loci can be determined by *in silico* analysis, which can pave the way for further experimental studies on the impact of associated variants on gene regulation or protein function (Cooper and Shendure 2011). For regulatory variants, *in vitro* reporter assays can be utilized to characterize the variants in a promoter or enhancer region. Genotype-phenotype relationship can be directly assessed in cells derived from centenarians and controls as described for lymphoblastoid cells (Suh et al. 2008). Recently, induced pluripotent stem cells (iPSCs) generated from primary cells such as fibroblasts, present potential opportunities for human gene variant modeling to interrogate the cellular and biochemical consequences of gene variants (Zhu et al. 2011). The field is poised to make important advances. Eventually the *in vivo* roles of genome maintenance gene variants found associated with extreme longevity will have to be tested for their impact on life span. For this purpose the mouse remains the model of choice, closing the circle with the above-described premature aging phenomena in mouse models harboring defects in genome maintenance genes similar to the ones associated with human progeroid syndromes. However, in this case, the phenotype of interest in these mice would be increased life span.

SUMMARY

Genome maintenance pathways are critical for cell growth, proliferation and survival. However, for the organism as a whole, they are also longevity assurance mechanisms that allow sufficient life span for reproduction and species propagation. For mammals, some of these pathways influence cancer latency and/or the onset of multiple symptoms of aging, including skin atrophy and osteoporosis. Cancer and general aging are pleiotropic and stochastic processes, the causes of which are diverse and not limited to DNA damage and genome instability. Even so, the multitude of observations indicating that defects in genome maintenance invariably lead to cancer and aging makes it highly likely that DNA damage is a major driver in both processes. Genome maintenance involves specialized pathways to repair a large variety of genomic lesions that include base lesions, helix distorting

lesions, single strand breaks (SSBs) and double-strand breaks (DSBs). A defect in just one of these pathways could predispose an individual to a subset of cancers or symptoms of premature aging. Since these pathways are specialized, resulting phenotypes may vary and be restricted to only a subset of organs. Here we discussed genetic variation in genome maintenance and its effects on aging and life span in mice and humans. We reviewed factors that damage DNA, pathways that suppress DNA damage, the specific consequence of faulty or failed DNA damage repair and how these consequences may lead to cancer and/or general aging. Interestingly, the causative factors that increase cancer incidence often differ from those that accelerate general aging, demonstrating a unique etiology for these biological outcomes.

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CHAPTER 3

Genetic Instability of Cancer: Biological Predictions and Clinical Implications

Robert A. Beckman

INTRODUCTION

Cancer development requires multiple *oncogenic mutations* (Table 1). These mutations confer phenotypic characteristics essential for malignancy, such as limitless replicative potential, avoidance of cell death, self-sufficiency with respect to growth-stimulatory signals, insensitivity to growth inhibitory signals, induction of angiogenesis, tissue invasiveness, and metastatic potential (Hanahan and Weinberg 2000). Malignant transformation of fibroblasts may be accomplished by transfection leading to 8 genetic changes, one in each of 4 dominant oncogenes, and 2 each in 2 recessive oncogenes (Rangarajan et al. 2004). Moreover, epidemiologic studies are consistent with 2–12 rate limiting steps for oncogenesis, depending on the tumor type, and at least some of these are likely to be genetic changes (Beckman and Loeb 2005b).

Mutator mutations (Table 1) are somatic mutations within tumors that accelerate genetic change, leading to tumoral genetic instability. The *mutator hypothesis* (Table 1) states that mutator mutations play a central role in carcinogenesis by accelerating the acquisition of oncogenic mutations (Loeb et al. 1974). While the mutator hypothesis originally was limited to single base changes arising from DNA replication errors, it now

Center for Evolution and Cancer, Helen Diller Family Cancer Center, University of California at San Francisco, 1450 3rd Street, San Francisco, California 94158 USA; Email: eniac1@snip.net

Table 1. Glossary of Key Terms.

Term	Definition
Cancer cell (or malignant) lineage	An initial cell which has a full complement of oncogenic mutations for malignant transformation, and all its descendants
Efficiency of carcinogenesis	The expected number of cancer cell lineages generated by a particular carcinogenic mechanism in the time (in cell generations) it takes for a clinical cancer to arise
Fitness	The relative ability of a cell to survive in evolutionary competition with other cell types
Focused Quantitative Modeling (FQM)	A collection of methods for answering one or more focused theoretical questions in the face of sparse experimental data, with maximum generalizability
Generalizability	Ability of theoretical or experimental conclusions to be valid over a wide range of circumstances
Limiting case	An extreme theoretical case developed to prove a point about itself and all less extreme case. It does not need to be realistic
Model topology uncertainty	Uncertainty in a theoretical analysis due to lack of clarity of the correct structure (topology) of the theoretical model (for example, due to unknowns in the mechanism of carcinogenesis)
Mutator hypothesis	The hypothesis that mutator mutations play a key role in carcinogenesis
Mutator mutation	A mutation in a gene responsible for maintaining genetic stability, which then confers genetic instability
Negative clonal selection	Progressive loss of fitness due to random deleterious mutations
Oncogenic mutation	A mutation that is directly required for malignant transformation
Parameter value uncertainty	Uncertainty in a theoretical analysis due to lack of clarity in the values of key quantities (parameters)
Passenger mutation	A random mutation due to genetic instability in a gene not directly related to carcinogenesis
Premalignant lineage	A lineage with an incomplete subset of oncogenic mutations. See "cancer cell lineage"
Reduced fitness (RF) gene or locus	A gene or locus, mutation of which will reduce the fitness of the cell lineage
Sensitivity analysis	A theoretical technique of varying parameter or model topologies and observing the theoretical consequences across the range of possibilities

encompasses all possible sources of genetic instability, including disorders of checkpoint control, instability of repetitive "microsatellite" sequences, gross chromosomal instability, and DNA repair defects (Ionov et al. 1993; Fishel et al. 1993; Paulovich et al. 1997; Lengauer et al. 1998).

Several contrasting scenarios for the accumulation of oncogenic mutations have been proposed in the literature. For example, a sequential series of mutations in specific genes has been suggested for colorectal carcinogenesis (Fearon and Vogelstein 1990). Yet, the three most common mutations associated with colorectal cancer together appear in less than 7% of these cancers (Smith et al. 2002). Others have proposed a discrete series of clonal expansions, each associated with a mutation (Nowell 1976). If each clonal expansion is associated with stringent *evolutionary selection* of the most fit lineages (including extinction of those that are less fit), one might expect the resulting tumor cell population to become progressively more uniform. However, according to the mutator hypothesis, mutations are randomly accumulating throughout carcinogenesis, which does not occur in discrete orderly steps. A single tumor might contain a mutator mutation in most cells, and each cell might contain different, albeit partially overlapping, sets of oncogenic driver mutations, along with a number of random "passenger" mutations that do not directly contribute to the malignant phenotype, but comprise a source of genetic variability (Loeb et al. 2008). In addition, a single tumor or even a single tumor cell may contain several different mutator mutations and each may serve as an independent source for *generating* additional genetic variation.

These contrasting scenarios present different challenges and opportunities for cancer therapy. The mutator hypothesis, while intuitive, has been vigorously debated for several decades, due to multiple theoretical approaches and sparse experimental data. In this chapter, the arguments for and against the mutator hypothesis will be reviewed. The resolution of this debate using a new metric for carcinogenesis mechanisms, *efficiency* (Beckman and Loeb 2006) (Table 1), and a new theoretical method designed for sparse experimental data, *focused quantitative modeling (FQM)* (Beckman 2010) (Table 1), will be described. Having established the validity of the mutator hypothesis, the consequences for cancer biology and for the practice of personalized oncology will be discussed. Finally, theoretical considerations concerning treatment and prevention of cancer by manipulation of the mutation rate will be presented.

THE MUTATOR HYPOTHESIS DEBATE

In arguing in favor of the mutator hypothesis, Loeb (1991) pointed out that at normal wild type mutation rates, 10^{-11} to 10^{-9} per nucleotide base per cell division (Albertini et al. 1990; Cervantes et al. 2002), it is highly improbable that any one cell would independently accumulate 6 oncogenic mutations. However, there are several arguments against the mutator hypothesis. In preparation for the use of FQM, we will classify these arguments, as that is a

key step in FQM. The arguments will be classified according to the trend in evolutionary *fitness* (Table 1) of cellular lineages, which is assumed during carcinogenesis for each respective argument.

The *increasing fitness* argument holds that each time a cell lineage acquires an oncogenic mutation, the evolutionary fitness increases (Nowell 1976; Tomlinson et al. 1996; Fisher 1958; Cairns 1975; Bodmer 2008). The numbers of cells with this lineage then increase in comparison to wild type cells. Because of this *clonal expansion,* there is a large pool of cells with the first oncogenic mutation, effectively increasing the net rate at which cells with two oncogenic mutations can be created. This process repeats, so that each successive oncogenic mutation can be acquired more quickly than the last in at least one cell. Given this process, it can be shown that the mutator hypothesis is indeed not *necessary* to explain the observed incidence of cancer, as originally postulated by Loeb (1991). Mathematical models have matched the observed cancer incidence rate to the theoretical formation rate of cancer cells, assuming a normal wild type mutation rate and successive waves of clonal expansion following each oncogenic mutation (Moolgavkar and Knudson 1981).

The *decreasing fitness* argument highlights the fact that cell lineages with mutator mutations will more rapidly acquire random deleterious mutations. These deleterious mutations will reduce the evolutionary fitness of the lineage, increasing its probability of extinction. This effect has been termed *negative clonal selection* (Table 1). A mathematical model of this phenomenon considered in isolation suggests it is not quantitatively significant in most instances (Beckman and Loeb 2005a).

Finally, the *constant fitness* argument applies for any fitness trend during carcinogenesis, including the case where fitness remains constant until the moment of complete malignant transformation. It focuses on the fact that the mutator hypothesis requires an extra mutational step in carcinogenesis. That is, if a particular tumor type requires X oncogenic mutations for its creation from a normal wild type cell without a mutator mutation, the mutator pathway for carcinogenesis will often require X + 1 mutations: X oncogenic mutations and one mutator mutation. Since each mutation is a rare event, adding an additional mutational step may slow down carcinogenesis rather than accelerate it.

Subsequent sections will discuss previous attempts to resolve this debate, and the potentially decisive contributions of efficiency of carcinogenesis and FQM in demonstrating that mutator mutations play a critical role in most cancers.

Previous Attempts to Resolve the Mutator Hypothesis Debate

Work relevant to ascertaining the truth or falsity of the mutator hypothesis began in the 1950s several decades before the mutator hypothesis itself, and continues to this day. These efforts have encountered two difficulties: 1. The exact model or pathway of carcinogenesis is unknown, and as we will discuss below, the available experimental data can be fit with different models. This is termed *model topology uncertainty* (Beckman 2010) (Table 1). Depending on the way the question is asked, differing model topologies can lead to different conclusions. 2. Exact values of key parameters are quite uncertain, again leading to the possibility of different conclusions depending on how the question is asked. For example, the wild type mutation rate per base pair per cell generation has been variously measured from 10^{-11} to 10^{-9}, a 100 fold range (Albertini et al. 1990; Cervantes et al. 2002), and estimates of the number of cell generations before cancer begins can range 30-fold from approximately 170 to 5000 (Baca et al. 1985; Baker et al. 1995; Tomlinson et al. 2002). The product of these two parameters is important in many models of carcinogenesis, and based on the above can vary up to 3,000 fold. Efficiency of carcinogenesis and FQM are designed to reduce the sensitivity of conclusions to this *parameter value uncertainty* (Table 1) and to model topology uncertainty (Beckman 2010). Previous attempts to resolve the mutator hypothesis debate fall into two categories: modeling of cancer epidemiology data and evaluation of the mutational burden of tumors.

Modeling of Cancer Epidemiology Data

Nordling (1953) analyzed cancer incidence data from ages 25 to 74, concluding that cancer increases as the sixth power of age. In order to fit the age-incidence curve, he postulated the need for six oncogenic mutations, well before the mutator hypothesis (Loeb et al. 1974) or the existence of known experimental and molecular correlates(Hanahan and Weinberg 2000). However, the work also illustrates model topology uncertainty, as this experimental data is fit equally well by very different models, including a cluster of six cells, each requiring a single mutation (Fisher and Hollomon 1951), or alternatively two mutations, each followed by radial tumor expansion proportional to the square of time (Fisher 1958). Subsequent work incorporated the effect of latency between tumor initiation and diagnosis (Cook et al. 1969), demonstrated different power laws for age incidence curves of different cancers (ranging from the 2nd to the 12th power) (Armitage and Doll 1954; Cook et al. 1969), and predicted and confirmed the existence of recessive oncogenes through a careful statistical

analysis of the unilateral and bilateral incidence of familial and sporadic retinoblastoma (Knudson 1971; Friend et al. 1986).

Regarding the truth of the mutator hypothesis, the discussion has always been whether mutator mutations are "necessary" to explain the observed age incidence curves of cancer, assuming that the rate of generation of cancer cells is equal to the incidence of cancer (Loeb et al. 1991; Tomlinson et al. 1996, 2002). However, as discussed above, Moolgavkar and Knudson (1981) have already shown that the assumption of a mutator hypothesis is not "necessary" to match age incidence rates to the timing of generation of cancer cells, at least with their model topology and parameter values.

Nonetheless, there are three problems with this approach: 1. The uncertainty of model topology is very great. As discussed above, the original cancer incidence data were fit with three completely different models (Fisher and Holloman 1951; Nordling 1953; Fisher 1958). Conclusions about the truth or falsity of the mutator hypothesis depend on the topology of the carcinogenesis model. 2. The parameter value uncertainty, especially with respect to the wild type mutation rate and the number of cell generations to cancer, is quite significant, and also permits conclusions either pro or con with respect to the mutator hypothesis. 3. The assumption that a model should fit the rate of *cancer cell lineage* (Table 1) formation to the incidence rate of cancer is fundamentally flawed. Given that we know that many incipient cancers are eliminated by the immune system, or fail to establish a blood supply, the rate of cancer cell lineage formation should be significantly greater than the incidence rate of clinical cancers. How much greater is unknown. While it may be reasonable to assume the incidence of cancer is *proportional* to the rate of formation of cancer cell lineages, the value of such a proportionality constant remains unknown. As such, cancer models should be able to reproduce the *shape,* but not necessarily the exact *magnitude*, of the age incidence curve. Given these limitations, it does not appear that comparison of mathematical models of carcinogenesis with epidemiology data have established either the truth or the falsity of the mutator hypothesis.

Evaluation of Mutation Burden of Tumors

Using next generation sequencing techniques, tumors have been evaluated for the number of non-germline mutations they contain (Pleasance et al. 2010 a,b; Mardis et al. 2009). The question with respect to the truth or falsity of the mutator hypothesis is then: are there more mutations than expected from wild type mutation rates? Using differing assumptions, which are all within the current experimental and theoretical uncertainties, it has been argued that the mutator hypothesis is either false (Shibata and Lieber 2010) or true (Fox et al. 2010).

In addition to the uncertainty in model topology and parameter values, which complicate trying to draw conclusions from this approach, it is also important to understand that sequencing may miss rare mutations present in only a minority of cells. While deep sequencing can in principle uncover one mutation in 10^5 cells, a tumor mass may contain 10^9–10^{10} cells, so that a single cell mutation may be missed even by current deep sequencing techniques (Fox et al. 2009). Techniques for looking at mutations in a single cell at a single defined locus are available (Bielas et al. 2006), but not for use at the whole genome level; rather one has to know in advance the spot to be interrogated.

In general, therefore, examination of the mutational burden of tumors has still not conclusively proven the truth or falsity of the mutator hypothesis. Fox et al. (2010) have a somewhat stronger argument in that they compare the mutational burden of tumors to the number of mutations seen in the germline between generations (Roach et al. 2010). As we will see below, examination of *relative*, rather than *absolute*, quantities, can be more informative in the face of experimental and theoretical uncertainties.

Efficiency of Carcinogenesis

The mutator hypothesis debate has occasionally been couched as a competition between genetic instability/variation and expansion/selection of a fitter cell lineage for the "most important" mechanism of carcinogenesis (Loeb et al. 1991; Tomlinson et al. 1996; Bodmer 2008). However, a wider perspective has recently been proposed in which all genetically possible carcinogenic mechanisms are in competition, in effect a Darwinian competition among cancer evolutionary mechanisms (Beckman and Loeb 2006).

In this competition, the winners are determined by *efficiency*, which is defined as the expected number of malignant lineages initiated by the mechanism at or before the expected time (in cell generations) at which the malignancy is typically observed. The uncertainty in the number of cell generations must be addressed by *sensitivity analysis* (Table 1).

The theory of evolution itself postulates competition between all possible approaches, which appear according to their relative efficiency. Interestingly, the idea of all possible mechanisms being in play at the microscopic level and leading to observed macroscopic phenomena also has precedent in many other fields of science, including statistical mechanics, chemical reaction kinetics, and the quantum mechanical formulation of optics (Beckman 2010). Whereas in statistical mechanics or chemical reaction kinetics, multiple outcomes are in play due to random atomic and molecular motions, in carcinogenesis, multiple mechanisms are in play due to the

random order mutations can occur within a genome. However, certain mechanisms will be more effective or efficient.

The efficiency concept has six important consequences (summarized in Table 2). First, all mechanisms of carcinogenesis are in play. Second, mechanisms appear clinically in proportion to their efficiency. Third, in order to assess the importance of a mechanism, one need not determine its *absolute* efficiency; it is sufficient to determine its *relative* efficiency compared to competing mechanisms. The use of relative rather than absolute quantities is a key feature of FQM (vide infra). Fourth, there is no assumption that we must match the rate of cancer cell lineage formation to cancer incidence curves. Just as fish lay a large number of eggs, and only a few offspring survive, it is expected that the number of cancer cell lineages will far outstrip the number of clinical cancers. Logically, the most efficient mechanisms will predominate. This circumvents the debate about whether the mutator hypothesis is "necessary". Fifth, the focus of modeling of carcinogenic mechanisms shifts from *rate limiting steps* that determine when the very first cancer cell lineages will form in a stochastic process, to *ratio determining features* that determine relative efficiencies. These are easier to evaluate mathematically. In addition, some mechanisms may produce the very earliest cancer cell lineages but ultimately be less efficient when evaluated over a longer time window. In this regard, relative efficiency does depend in some instances on the estimated number of cell generations to cancer, since that determines the time window for efficiency comparisons. Finally, despite the arguments for the primacy of genetic instability or selection and expansion, they are not mutually exclusive. We will show that the most efficient carcinogenic mechanisms incorporate early onset of genetic instability followed by clonal expansion. Selection is required for expansion if the ecological niche is of a fixed size. However, it is possible in the modeling to simply allow expansion at different rates for sub-populations of differing fitness above the normal fitness, without

Table 2. Consequences of the Efficiency Formulation.

All possible carcinogenic mechanisms are in play.
A carcinogenic mechanism is observed clinically in direct proportion to its efficiency.
The importance of a carcinogenic mechanism may be determined by evaluating its *relative* efficiency compared to others.
It is not assumed that every cancer cell lineage results in clinical cancer.
Rate limiting steps are replaced by *ratio determining features* in evaluating the importance of a carcinogenic mechanism.
Genetic instability and lineage selection/expansion are not mutually exclusive. The most efficient mechanisms incorporate both.

selection except for lineages of sub-normal fitness. In this scenario, the ecological niche of the cancer continues to expand until death of the host, which seems reasonable based on clinical observations.

Focused Quantitative Modeling (FQM)

Attempts to date to resolve the mutator hypothesis debate have been hampered by sparse experimental data leading to both model topology uncertainty and parameter value uncertainty. FQM is a collection of techniques well known to mathematical modelers, applied in a concerted fashion to answer a specific question in the face of experimental and theoretical uncertainty.

FQM contrasts with the classical approach of systems biology (Table 3). In classical systems biology, the goal is to determine the one true model that describes the system, which can then be queried for predictions. In FQM, the goal is not to determine the one true model, but to answer a single focused question in the most *generalizable* (Table 1) fashion, which

Table 3. Comparison of classical systems biology and focused quantitative modeling.

Characteristic	**Classical Systems Biology**	**Focused Quantitative Modeling**
Goal	Identify and query one true model	Answer one focused question
Process	Converge to single model through iterative fit to experimental data	Systematically ask question of all model types in model classification system
Model characteristics	Realistic, complex	May use limiting cases, may be limited to features relevant to focused question
Output quantities	Absolute	Relative: use of ratios
Vulnerability to overfitting	High	Low: may not use fitting routines
Vulnerability to model topology uncertainty	High	Low/moderate
Vulnerability to parameter value uncertainty	High	Low/moderate
Computational complexity	High	Low/moderate
Scope	Extensive	Limited

Efficiency is defined as the expected number of malignant lineages produced by a given carcinogenic mechanism at or before the time (in cell generations) when the malignancy is typically observed. A malignant lineage is one which has a full complement of oncogenic mutations.

will be true for the greatest variety of possible true models, and to define the limits of this generalizability. Failure of generalizability is a key cause of incorrect conclusions from both theoretical and experimental studies (Beckman 2010).

In classical systems biology, the one true model is determined by an iterative process in which it is progressively refined by fitting to experimental data. However, in many relevant cases, as is true for the mutator hypothesis problem, important parameter values will be unknown. These parameters can then be "adjusted" in the model to optimize the fit. In the presence of many adjustable parameters, it may be possible to fit almost any model to the data, regardless of its truth. The great mathematician Gauss remarked: "Give me four parameters, and I will draw an elephant for you; with five I will have him raise and lower his trunk and his tail." Gauss' elephant epitomizes the problem of *overfitting* in the presence of many adjustable parameters. FQM does not attempt to rank models by fitting to experimental data in the presence of so many adjustable parameters that fitting is trivially easy for a wide variety of models. Rather, the single focused question is asked of a comprehensive family of plausible models.

In FQM, specific techniques are used to minimize the sensitivity of the results to model topology uncertainty and parameter value uncertainty. With respect to model topology uncertainty, plausible models given the experimentally known facts are classified in a relevant way considering the question at hand. In the case where the focused question was "Is the mutator hypothesis true or false", the *model classification* was performed according to the expected trend in evolutionary fitness for cellular lineages approaching malignant transformation. The arguments concerning the mutator hypothesis were divided into increasing fitness, decreasing fitness, and constant fitness arguments, as discussed above. Model classification permits a *model sensitivity analysis:* asking the focused question of a family of models that represents all classes of models. This determines the sensitivity of the conclusions to unknown model features and topologies, and hence its generalizability.

Limiting cases (Table 1) are an important tool in FQM and constitute extreme cases that prove a particular point for the less extreme, realistic cases. They can often be used to reach conclusions where the exact features of the realistic case are unknown. In the song "New York, New York", the singer boasts "If I can make it there, I can make it anywhere." If this can be shown to be true, and if the singer can make it in New York, we do not need to know the singer's current geographic location, nor evaluate his/her ability to make it in other locations, to conclude that he/she can "make it" where they are at the time.

Limiting cases may intentionally have extreme, unrealistic assumptions, a feature which may be confusing for classical systems biologists, who

are expecting to answer questions by querying true, accurate models. However, they are a very useful tactic for addressing both model topology and parameter value uncertainties. In the original decreasing fitness model, a limiting case was first created that maximized the potential importance of negative clonal selection considered in isolation (Beckman and Loeb 2005a). For example, although mutations that reduce apoptosis in response to genetic damage are very common in cancer (Komarova and Wodarz 2003), including mutations in the central apoptotic protein p53 in more than half of cancers (Levine 1997) (see Chapter 12), the possibility of reduced apoptotic sensitivity to genetic damage was not considered, maximizing the possible deleterious effects of negative clonal selection. Given that even in the extreme limiting case, negative clonal selection was not quantitatively significant, one can clearly conclude that it is not significant in realistic cases. By ignoring changes in apoptotic sensitivity to DNA damage, the mathematical treatment is simplified, and unknowns in the complex apoptotic machinery do not affect the conclusions.

Another key tactic in FQM is the calculation of *relative* rather than *absolute* quantities, such that the question of interest is evaluated based on ratios. This approach limits sensitivity to model topology uncertainties. For example, to calculate absolute carcinogenesis rates, one must know how many oncogenic mutations are required, of which kinds, how many possible genetic loci can supply these driver mutations, whether all combinations are allowed or certain ones are more or less effective, and whether or not there is a required order of appearance of these mutations, in order to generate a *combination function* that is a required component of the absolute rate calculation. However, when considering the *relative efficiency* of carcinogenic pathways with or without a mutator mutation, these unknown complexities cancel in the ratio.

Ratios also help minimize sensitivity to parameter value uncertainties. The absolute efficiency of carcinogenesis depends on the mutation rate raised to the number of required mutations. The wild type mutation rate is uncertain to within a factor of 100 (Albertini et al. 1990; Cervantes et al. 2002). For a non-mutator mechanism with 6 required oncogenic mutations, there is therefore a 10^{12} fold uncertainty in the absolute rate due to this one parameter alone. For the comparable mutator mechanism, which requires 7 mutations (6 oncogenic and 1 mutator), there is a 10^{14} fold uncertainty in the absolute efficiency. However, in taking the ratio to determine the relative efficiency, the uncertainty is reduced to 10^{2} fold.

Just as sensitivity analysis is applied by varying model topologies, it must be applied across parameter values as well. Often this is difficult to do thoroughly in classical systems biology, because calculating the output of the putative "true" model, with all its complexity, is difficult for even one set of parameter values, requiring a long simulation. If there are, for

example, 10 unknown parameters (a relatively small number for a complex biological system), there is a 10 dimensional parameter space that should be explored in detail, but it will be computationally impossible to explore this thoroughly for the complex "true" model. Using the techniques of FQM, it is often possible to capture the essential features in simpler models that lend themselves to formulas rather than long simulations. In this instance, exploration of a large number of parameter value combinations is more computationally feasible.

The advantages and disadvantages of FQM relative to classical systems biology are evident from the above. Conclusions from FQM are more robust to model topology and parameter value uncertainty, and more generalizable, particularly in the face of sparse experimental data. The models and data output are often more intuitive, allowing them to inform and enrich biological intuition. However, the scope of FQM is more limited than classical systems biology. Its output is limited to the answer to the focused question at hand, and it does determine the true model and its many details. Clearly, both methods are of importance in modeling biological phenomena.

Application to the Mutator Hypothesis Debate

The mutator hypothesis debate was resolved by evaluating the relative carcinogenic efficiency of mutator and non-mutator pathways, across a variety of models classified by the net fitness trend experienced by a cell lineage undergoing stepwise malignant transformation; i.e., across constant fitness, increasing fitness, and decreasing fitness models (Beckman and Loeb 2006; Beckman 2009). The graph of fitness versus time during carcinogenesis is termed a "fitness trajectory", and there is likely to be some random variation in fitness trajectories for individual cancers. At any given moment a cell lineage will be experiencing multiple mutations, which individually increase or decrease its fitness in the context of its environment. These mutations as a whole will have a net effect of increased, decreased, or constant fitness at any given moment. Arbitrary fitness trajectories may be constructed by splicing together intervals of the increasing, decreasing, or constant fitness models. A model sensitivity analysis is presented across models, which span all of these possibilities, and within each of these models parameter sensitivity analyses are performed. In the next section, the conclusions, which are generalizable across all the model classes and parameter values will be presented, with limitations of their generalizability where appropriate.

Key outputs of the models include the relative efficiency of mutator pathways compared to analogous non-mutator pathways. A relative efficiency of greater than 1 indicates a predominance of mutator pathways,

while a relative efficiency of less than 1 indicates a predominance of non-mutator pathways. The fraction of cancers arising by mutator pathways is given by:

$$\textit{Fraction of cancers arising by mutator pathways} = \frac{\textit{Relative efficiency of mutator pathways}}{1 + \textit{relative efficiency of mutator pathways}}$$

(equation 1).

Thus, a relative efficiency of 100 corresponds to greater than 99% of clinical cancers arising through a mutator mutation.

A second key output is $\alpha_{50\%}$, which is the degree of increased mutation rate,that, if conferred by the mutator mutation, gives the mutator pathway equal efficiency to a non-mutator pathway. An increase by at least this much in the mutation rate is required for the mutator pathway to be "worth the trouble". This parameter is compared with typical increases in the mutation rate due to known mutations in DNA polymerases *in vitro* and *in vivo* (Albertson et al. 2009; Beckman and Loeb 1993; Loeb et al. 1981; Kunkel et al. 1981, 1986). If the required degree of increase in mutation rate is equal to or less than that typically seen for known mutator mutations, mutator pathways are favored.

The models are depicted in Fig. 1. They include a *constant fitness* model in which the evolutionary fitness remains constant until the lineage achieves malignant transformation (shown for reference in all parts of the Figure); an incremental lineage expansion model (*increasing fitness*), in which an incremental increase in evolutionary fitness occurs after each oncogenic mutation (Fig. 1A); two cooperative lineage expansion models (*increasing fitness*), in which there is a sudden, cooperative fitness jump that occurs when a subset of the oncogenic mutations have been acquired, with the mutator mutation either before the fitness jump (Fig. 1B) or after it (Fig. 1C); and a negative clonal selection model (*decreasing fitness*), in which fitness progressively decreases, but cell lineages that survive this progressive decrease to acquire a full complement of oncogenic mutations increase their fitness at the instant of malignant transformation (Fig. 1D).

The constant fitness case was analyzed first (Beckman and Loeb 2006), and therefore, the increasing and decreasing fitness cases are limiting cases designed to maximize the impact of the respective fitness trends (Beckman 2009), to see if they alter the key conclusions from the constant fitness case. Thus, in the increasing fitness case, lineages with increasing fitness grow exponentially without limit rather than undergoing more limited Gompertzian growth kinetics (Laird 1964), thus maximizing the effect of lineage expansion. To further maximize the effect of lineage expansion, the process is allowed to occur continuously rather than in discrete steps after each mutation. This "tunneling" (Komarova et al. 2003) results in mosaic populations with different subsets of oncogenic mutations and further

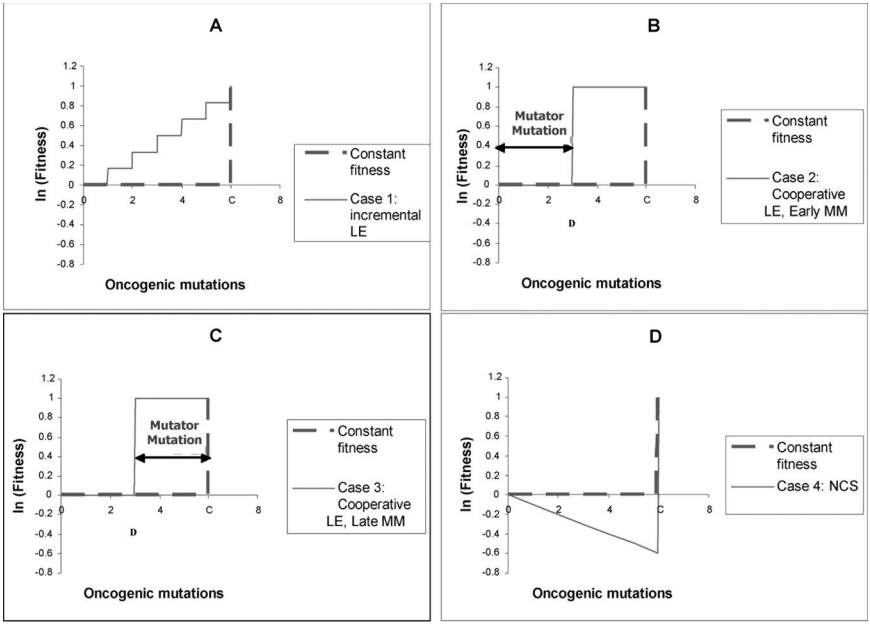

Figure 1. Representative fitness landscapes for carcinogenesis. ln R, the natural logarithm of the relative fitness advantage compared to wild type, is plotted as a function of number of oncogenic mutations for each of the four fitness landscapes considered in Beckman (2009) relative to the constant fitness case (heavy dashed lines) (Beckman and Loeb 2006). Positive and negative values of R correspond to increased and decreased fitness respectively. In this figure, it is assumed that C oncogenic mutations are required for malignant transformation, at which point the lineage acquires markedly increased fitness relative to wild type. A, Case 1: incremental lineage expansion (LE). The relative fitness increases incrementally with each oncogenic mutation. B, Case 2: cooperative lineage expansion with early mutator mutation (MM). Fitness increases suddenly and cooperatively after a predefined number, D < C, of oncogenic mutations, prior to malignant transformation after C mutations. In the mutator pathway, the mutator mutation occurs *before* the sudden increase in fitness, within the time bounded by the arrows. C, Case 3: cooperative lineage expansion (LE) with late mutator mutation (MM). As in B, except in the mutator pathway the mutator mutation occurs *after* the sudden fitness increase, within the time bounded by the arrows. D, Case 4: negative clonal selection (NCS). The lineage acquires oncogenic mutations, while the fitness continuously decreases due to accumulated random deleterious mutations. The fitness of the lineage increases only if it reaches full malignant transformation.

maximizes the potential effect of lineage expansion. It is likely more realistic than the tidy sequence of discrete alternating mutation and expansion steps. In the decreasing fitness case, mutations that decrease the probability of apoptosis in response to DNA damage are ignored, and survival, due to chance fluctuations, of lineages with decreased fitness is also ignored, maximizing the effect of negative clonal selection. Note the decreasing

fitness case differs from the analysis of negative clonal selection in isolation (Beckman and Loeb 2005a) in that, in the case depicted in Figure 1D, the acquisition of oncogenic mutations is occurring concurrently.

Key parameters in all models include the number of oncogenic mutations required for malignant transformation, the wild type mutation rate, the fold increase in the mutation rate due to a mutator mutation, the number of cell generations to cancer, the number of nucleotide loci for which single copy mutation results in a mutator mutation, and the number of nucleotide loci available for oncogenic mutations. Additional key parameters in the increasing fitness models include the fitness increment associated with each oncogenic mutation (incremental lineage expansion model) or with the cooperative fitness jump (cooperative lineage expansion models), the fraction of this fitness increase, which is due to increased proliferation (as opposed to decreased apoptosis) (all lineage expansion models), and the number of oncogenic mutations required for the fitness jump (cooperative lineage expansion models). Finally, an additional key parameter in the decreasing fitness model is the probability adjusted net number of nucleotide loci for which a single copy mutation results in a fitness decrease (termed dominant *reduced fitness* (RF) loci, Table 1). This parameter, called $N_{RFLN\text{-}D}$ (**R**educed **F**itness **L**oci **N**et—**D**ominant) is essentially a measure of the vulnerability of the cell to random genetic instability. It is probability adjusted to account for possible environmental and genetic contexts. A low number indicates a very plastic genome, a high number a very vulnerable one. As part of a limiting case, $N_{RFLN\text{-}D}$ was set at the maximum justifiable level, 10% of this level, and 1% of this level, as described in Beckman and Loeb (2005a). The true number may be much lower, as we discuss in the "Lethal Mutagenesis" section below.

Conclusions Regarding the Mutator Hypothesis

The conclusions listed in this section are only those which are general to all five models discussed above, and therefore appear to be robust across all possible fitness trajectories.

1. Mutator mechanisms are more efficient in the majority of cases. The mutator hypothesis is strongly supported.
2. The importance of mutator pathways increases dramatically with the number of oncogenic mutations required for malignant transformation. If only 2 oncogenic mutations are required, mutator pathways are not favored. This might be applicable to some pediatric tumors. If 3 or 4 oncogenic mutations are required, whether mutator pathways are favored depends on parameter values and model details. For 5 or more required oncogenic mutations, mutator pathways have large efficiency

advantages in nearly all conditions. The molecular studies of oncogenic transformation (Hanahan and Weinberg 2000; Rangarajan et al. 2004) suggest that more than five genetic changes may be required in most instances.

3. The efficiency advantage of mutator mechanisms is in the thousands to billions for typical cases. This tends to remove uncertainty due to unknown parameter values. For example, the advantage of mutator pathways is proportional to the number of dominant mutator loci, loci for which a single copy mutation results in a mutator mutation. Bodmer (2008) argues that most mutator mutations are recessive, while Loeb (response to Bodmer 2008) disagrees. Nonetheless, the calculations assumed 100 nucleotides in the entire genome that could be dominant mutator loci, and given the size of the efficiency advantage, if even one nucleotide locus in the genome is a dominant mutator locus, a sizable advantage for mutators would be sustained. As dominant mutator mutation loci in DNA polymerases are known (Albertson et al. 2009), the debate concerning the exact number of dominant mutator loci is not likely to be decisive.
4. Mutator mutations have the greatest advantage if they occur early. The only exception is models with a cooperative fitness jump occurring after 1–2 oncogenic mutations. In that case, it might be advantageous for the first mutations to be those that lead to the fitness jump.
5. Genetic instability and lineage expansion are not mutually exclusive. The most efficient carcinogenic mechanisms incorporate an early mutator mutation followed by continuous lineage expansion according to fitness level.

BIOLOGICAL PREDICTIONS

Evidence Supporting the Theory

Bielas et al. (2006) have measured the mutational burden in tumors and normal tissues at the single cell level, utilizing PCR based amplification of a genetically unselected region in intron VI of the p53 gene. Mutation of the site renders it resistant to restriction enzyme digestion. This work demonstrates that the mutational burden within tumors is several hundred-fold higher than in the surrounding normal tissues, even in the colonic epithelium in which the normal cells have a high rate of cycling. This phenomenon is unlikely to be due primarily to an increased number of cell generations given the high rate of proliferation in the normal tissue. The mutational frequency in normal tissues may be even lower, as the observed normal tissue mutation frequency is at the limit of detection due to the fidelity of the enzymes employed.

A similar several hundred-fold difference in mutation burden is seen when comparing the mutation burden in tumors subjected to whole genome sequencing (20,000–30,000 mutations per tumor, Pleasance et al. 2010a,b; Mardis et al. 2009) to the mutations that accumulate between human generations (70 mutations, Roach et al. 2010). These findings confirm the work of Bielas et al. 2006 by an independent method, and by comparing the tumors to normal tissues the experimental output is a ratio that is less sensitive to model topology and parameter value uncertainty than isolated speculative evaluations of the significance of a particular number in the tumor alone.

The mathematical analysis using efficiency and FQM predicts that tumors which require more oncogenic mutations have a greater predominance of mutator pathways. A corollary is that in inherited mutator conditions, such as hereditary non-polyposis colon cancer (HNPCC), in which there is a mutation in one copy of a mismatch repair gene (see Chapter Kinsella), allowing rapid access through loss of heterozygosity to a mutator phenotype (Ionov et al. 1993; Fishel et al. 1993), the increase in risk of developing cancer should be greater for those cancers that require more oncogenic mutations. In fact, the increase in risk is greater for colon cancer than for embryonal carcinomas, in agreement with this prediction.

Tumor Evolution Differs Quantitatively from Species Evolution

Based on the limiting case model for negative clonal selection, there is an optimum mutation rate for tumor evolution, beyond which increasing negative clonal selection leads to decreasing carcinogenic efficiency. This optimal mutation rate for tumor evolution may be derived from the model in a manner analogous to what has been done for viral evolution (Nowak and May 2000). Because the negative clonal selection model is a limiting case that maximizes the importance of negative clonal selection, the estimates of the optimal mutation rates are really lower limits. Within the range of parameter values considered in the limiting case, the *lower limit* of the optimal mutation rate is 2×10^{-10} to 4×10^{-6}, which partially overlaps the wild type mutation rate range (10^{-11} to 10^{-9}), but is generally higher.

If we assume that the wild type mutation rate has been optimized for species evolution, one can conclude that the optimal mutation rate for tumor evolution is higher than the optimal mutation rate for species evolution. Given that tumor cells are not constrained by homeostasis, a wider range of mutations may be acceptable. Presumably, if wild type species mutation rates were higher, tumors would also occur at earlier ages, limiting reproductive fitness.

The existence of an optimum mutation rate that allows evolutionary flexibility without undue negative clonal selection is evident in single cell

organisms like bacteria as well. Under cases of stringent selection, mutator strains are selected, but not extreme mutators (Loh et al. 2010).

Predicting the Level of Increased Cancer Risk from DNA Repair Syndromes

In principle, it may be possible to use the efficiency paradigm to quantitatively predict the relative increase in cancer risk associated with DNA repair syndromes (see Chapter Bohr). However, gaps in our current knowledge render this task challenging. Formulas for the relative efficiency of mutator versus non-mutator pathways for all relevant models are available (Beckman and Loeb 2006; Beckman 2009). These are derived from formulas for the "absolute" efficiency of both pathways individually (Beckman, R.A., unpublished). In order to apply this paradigm to DNA repair syndromes such as Xeroderma Pigmentosum (XP) or HNPCC, one would need to develop a relevant ratio to reduce model topology and parameter value uncertainties. However, such a ratio could easily be developed by calculating the efficiency of mutator and non-mutator pathways with the high wild type mutation rate associated with a DNA repair syndrome, and dividing it by the efficiency of the more efficient mutator pathway starting with the normal mutation rate for unaffected individuals. To calculate the relative risk for the constant fitness case, it will be important to be able to estimate the number of oncogenic mutations required for the particular malignancy (this could be estimated from the *shape* of age incidence curves and/or increasing molecular biology understanding) and the fold increase in mutation rate due to the DNA repair syndrome (may be accessible from biochemical studies of the mechanism of these syndromes). For cooperative lineage expansion models, such a calculation would be even more challenging, as it will be important to estimate the number of oncogenic mutations required for the cooperative fitness jump, perhaps again based on the underlying molecular biology. For the negative clonal selection model, $N_{RFLN\text{-}D}$ would also need to be estimated, along with the number of generations to cancer. Finally, to predict the relative risk, the model most closely reflecting the tumors likely evolutionary path needs to be identified among the different models discussed. Thus, while reproducing the relative cancer risks of DNA repair syndromes using these models is feasible in principle, more experimental data and basic understanding may be required in practice. Alternatively, we may be able to determine which model applies by comparing the observed relative risk to that predicted by the different models. This may be possible given that we are trying to calculate *relative,* rather than *absolute*, risks.

The Nature and Natural History of Premalignant Lesions and the Field from which Cancer Arises

According to the models developed, with concurrent oncogenic and/or mutator mutation acquisition and lineage expansion, there will be cell lineages with a subset of the required driver mutations, but not the full complement required for malignant transformation. These lineages may be the cells which constitute *premalignant* (Table 1) lesions. Moreover, it is likely that a cancer also includes some of these cells, which may have a greater evolutionary fitness than normal tissue. Particularly if the ecological niche of the tumor is expanding, there is no clear reason these cells should be eliminated. Indeed, in real tumors microheterogeneity is seen with respect to grade and degree of dysplasia.

A clear prediction of the modeling is that mutator mutations will occur early in carcinogenesis. One might then expect that these mutator mutations would in principle be detectable in the earliest premalignant lesions by examining mutation burden or (if technology existed) by measuring the actual mutation rate. However, a closer examination of the theoretical results suggests that this experiment would be misleading if performed on *homogenized* polyps or other premalignant lesions. Rather, to detect early mutator mutations, *single cells* would have to be examined for mutation rate.

The initial mutator mutation is a rare event and may only occur in a minority of cells at the beginning (Fig. 2). In fact, the frequency of an initial mutator mutation compared to an initial oncogenic mutation in the overall cell population is likely determined by the relative number of mutator loci compared to oncogenic loci, and is likely relatively infrequent. Thus, if a very early premalignant lesion is homogenized, the overall result may not reveal increased genetic instability.

The minority cells with an early mutator mutation will, however, accumulate subsequent oncogenic mutations more efficiently (Fig. 2). Thus, progressively advanced premalignant lesions, with larger numbers of driver mutations, will become progressively enriched for these mutator lineages. We can determine when the mutator lineages will predominate using exactly the same mathematics that revealed when mutator mechanisms predominate as a function of the number of required oncogenic mutations. Thus, lesions with 2 or less oncogenic driver mutations will likely have only a minority of cells with mutator mutations. But by the time these lesions have 5 or more oncogenic mutations, cells with underlying mutator mutations will predominate, and a homogenized later pre-malignant lesion should clearly reveal the mutator mutation. Thus, if done on homogenized lesions, the data will *appear* to indicate a "late" mutator mutation. However, if the same analysis were done at the single cell level, and if technology

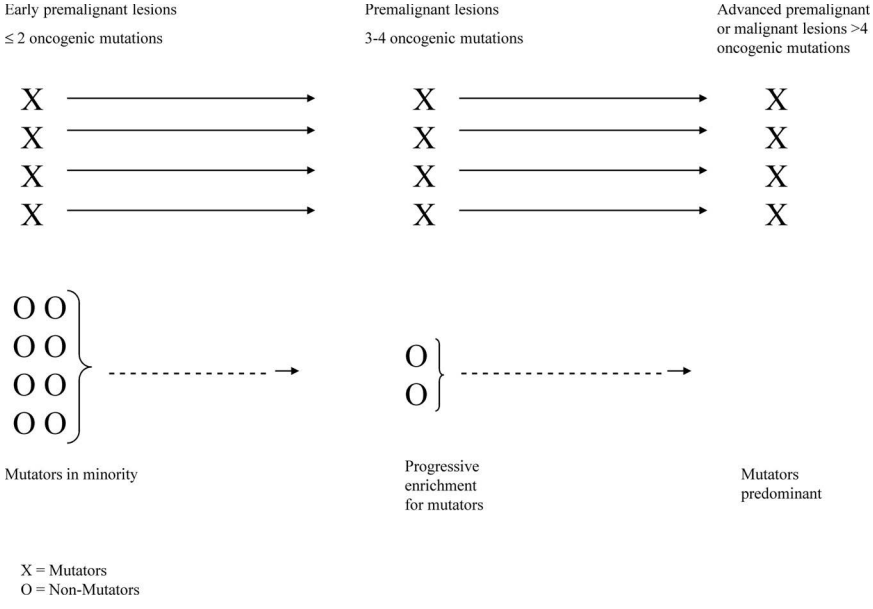

Figure 2. Progressive enrichment of mutator cells during carcinogenesis. Early in carcinogenesis, the relative number of mutator cells (X) and non-mutator cells (O) is largely determined by the relative sizes of the mutational targets presented by mutator and oncogenic loci. For cells with 0–2 oncogenic mutations, non-mutator cells will predominate, even though a minority of mutator cells exist from early in carcinogenesis. Due to the faster accumulation of oncogenic mutations in the minority mutator ancestor cells, the mutator cells will be progressively enriched in populations with more oncogenic mutations. If measurements of mutation rate and/or spectrum are averaged across a population of cells, this phenomenon will lead to the false conclusion that mutator mutations are a "late event".

were available to trace the origin of the cells in the later premalignant lesion, one would discover that the small minority of mutator cells in the early premalignant lesion were the ancestors of the larger population in the later premalignant lesion. At times, theory can inform and modify what appears to be an "obvious" interpretation of experimental data (Beckman 2010).

Clinically, efficient conversion to malignancy by mutators is evident from the comparisonof a familial colorectal cancer syndrome due to a mutator mutation, HNPCC, with a non-mutator familial colorectal cancer syndrome, familial adenomatous polyposis (FAP), which has a mutation in the adenomatous polyposis coli (APC) gene, leading to enhanced growth. Although FAP has many more polyps than HNPCC, they have similar age-incidence curves for colorectal cancer (Kohlmann and Gruber 2004; Burt and Jasperson 1998), presumably due to more efficient acquisition of the remaining oncogenic mutations required for malignant transformation in the case of the HNPCC polyps.

IMPLICATIONS FOR THERAPY

Personalized Therapy of Cancer

Personalized medicine is designed to create individualized therapy that fits an individuals' cancer like a key fits a lock (see Chapter 16). Incremental benefits in combination with traditional chemotherapy have been seen in a number of malignancies, and transformational benefits in a handful of genetically simple, "oncogene-addicted" tumors that are highly dependent on a single signaling pathway with limited ability to adapt. Cures are still rare. The key-lock metaphor and underlying strategy reflect assumptions that tumors are homogeneous and static, even though we know otherwise. Personalized medicine strategies will be even more beneficial when they are augmented to incorporate the notions of tumor heterogeneity and the dynamic nature of tumors.

Cancer was once believed to be a clonal disease, which arose through a fixed series of mutations (Nowell 1976; Fearon and Vogelstein 1990). However, the mutator hypothesis and the models of it discussed above, assume a much higher level of heterogeneity and complexity arising from multiple branching evolutionary processes in the presence of genetic instability. Subclones will have non-identical but partially overlapping sets of oncogenic driver mutations. In addition, given the genetic instability, which arose as an efficient way to acquire driver mutations, many other random *passenger mutations* (Table 1) will occur. Although these random mutations are "passenger" mutations, they may "grab the steering wheel" when the environment changes due to therapy, and create the potential for resistance (Beckman 2010). Due to the risk of resistance mutations, combination therapy may be preferred in some instances (Goldie and Coldman 1979).

The genetic heterogeneity of cancer may be underestimated by sequencing, which cannot look at the single cell level (Fox et al. 2009), or by other techniques that look at a limited number of molecular markers rather than looking genome wide. A technique exists for quantifying mutation frequency in single cells at an unselected p53 intron locus, and has revealed greater heterogeneity in tumors than in normal tissue by several hundred fold (Bielas et al. 2006).

Despite the imperfections of current techniques, evidence for the immense diversity and complexity of cancer is mounting. Mullighan et al. (2008) have documented that relapse in pediatric acute lymphocytic leukemia (ALL) is often from sub-clones that have overlapping but non-identical patterns of copy number alterations (CNA). A very complex branching phylogenetic structure and multiple subclones is revealed in ALL, both by multiplex fluorescence *in situ* hybridization (FISH) (Anderson et al.

2011) and CNA analysis (Notta et al. 2011), for both bulk tumor cells and the stem cell fraction. Deep sequencing of an immunoglobulin heavy chain locus allows identification of subclones of B chronic lymphocytic leukemia at a sensitivity of 1:5000, revealing phylogenetic substructure (Campbell et al. 2008). Divergent evolution between primary and metastatic breast cancer has been documented for chromosomal aberrations at the single cell level (Klein 2005; Klein and Holzel 2006), for microsatellite markers (Fujii et al. 1996), and by using whole genome sequencing (Shah et al. 2009). In pancreatic cancer, heterogeneity has been documented in the primary tumor, and further evolution has been observed in the metastases (Yachidi et al. 2010). It may be that like snowflakes, no two cancer cells, even from the same individual, are alike.

When attacked with targeted therapies, tumors demonstrate their dynamic nature. Acquired resistance (or pre-existing resistance) has been documented to targeted therapies such as imatinib for chronic myelogenous leukemia (Shah et al. 2007) or erlotinib for non-small cell lung cancer (Maheswaran et al. 2008). Ongoing computational simulations demonstrate that once the dynamic nature of cancer is taken into account, current personalized cancer medicine strategies are sub-optimal, even in the absence of both pre-existing resistance and increased genetic instability (Beckman, Schemman and Yeang 2012). Pathways of tumor evolution will need to be characterized, and *probabilistic* strategies based on the *risk of unobserved resistance states* developed. Stratification of therapeutic strategies based on the level of genetic instability may also be required. Hitting a moving target requires not only mapping its current position, but consideration of its speed and direction of motion (Fig. 3).

Lethal Mutagenesis

One approach to cancer therapy is to give mutagenic therapy to a patient whose tumor is already genetically unstable, in the hope of creating additional genetic instability in excess of the optimum, leading to overwhelming negative clonal selection. This approach exploits the genetic instability of tumors, and is already being attempted in the context of HIV therapy, since the HIV virus is genetically unstable (Loeb et al. 1999). Human cancer cells, with 30,000 genes, have more degrees of freedom in which to vary their genomes compared to HIV viruses, and thus may be able to survive higher mutational burdens.

Several questions arise when considering this method from a theoretical perspective. The first is: how high must the mutation rate be before a malignant cell undergoes overwhelming negative clonal selection? The optimal mutation rates for tumor evolution estimated by Beckman (2009)

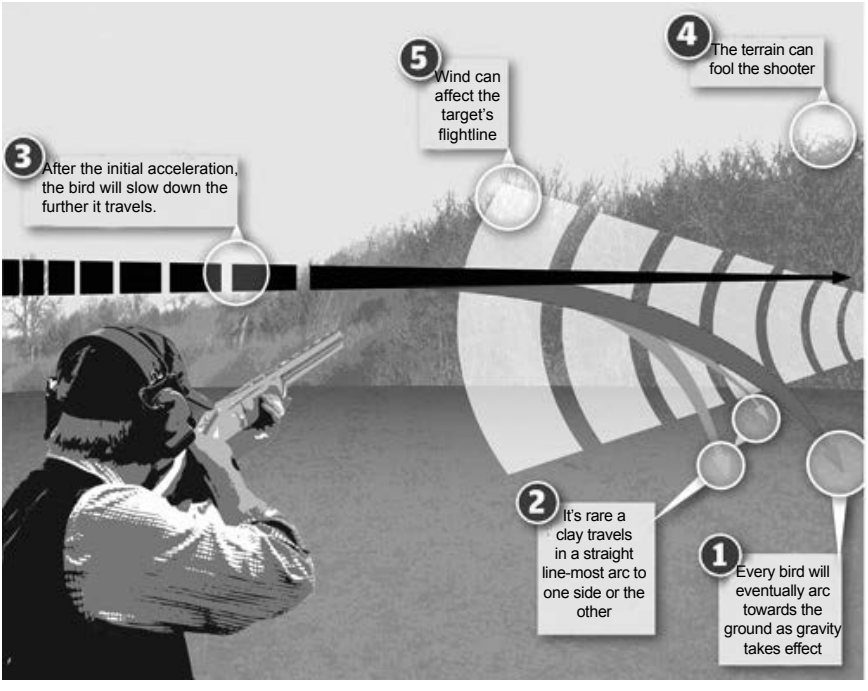

Figure 3. Hitting a moving target requires consideration of its motion.

are *lower limits, based on a limiting case maximizing negative clonal selection*. The limiting case ignores the fact that cancer cells frequently have defects in the apoptotic machinery and are thus less sensitive to genetic instability (Komarova and Wodarz 2003; Levine 1997). Moreover, the limiting case assumes that lineages with reduced fitness will become extinct with 100% probability, but in fact they have a finite probability of survival due to chance fluctuations (Beckman and Loeb 2005a). The lowest limit of the optimal mutation rate further assumes that every gene is a dominant RF gene (Table 1), of which single copy inactivation will reduce the fitness of the lineage, ignoring the presence of redundancy in cellular functions. The chance that any nucleotide in a coding exon will be a reduced fitness locus is derived from studies of enzyme inactivation as a function of amino acid substitution (Guo et al. 2004). Given these factors, it is likely that the optimal mutation rates for tumor evolution are significantly higher than the lower limits defined in Beckman (2009). Cancer cell lineages with chromosomal instability mutations are able to tolerate the loss of entire chromosomes at a rate of 10^{-2} per cell division *per chromosome*, a truly staggering rate of single copy genetic change.

Cancers probably exist in a field of dysplastic cells, which have a subset of the oncogenic mutations required for malignant transformation, as discussed above. Many of these cells may be only one mutation away from malignant transformation. What will be the rate of conversion of these cells by single step mutation to malignant cells when the mutation rate is raised so high? Will this rate of new tumor cell formation be less than or equal to the rate of destruction of these cells and the actual malignant cells by negative clonal selection? We can be hopeful in that regard, if it is really possible to push the mutation rate past the optimum, since highly dysplastic cells are likely to also be genetically unstable, having arisen from genetically unstable ancestors, as we discussed above. For example, we might predict that in a tumor that requires six oncogenic mutations, that the tumor itself and its precursors with 3, 4, or 5 oncogenic mutations, because they already have mutator mutations, might all be vulnerable to lethal mutagenesis.

How rapidly will negative clonal selection act? Can it eradicate growing tumors on a time scale sufficient to be used in settings with high tumor burden, or should it be limited to settings with low/moderate tumor burden that are less of an immediate clinical emergency? Finally, what will the effect on normal tissues be of driving the mutation rate higher? Will the benefit risk ratio be favorable for patients, even at very high mutation rates? One expects that the benefit-risk ratio might be favorable for terminal patients with no other available options. Treatment of earlier line patients should probably wait for characterization of the safety of the approach. Tumor-specific lethal mutagenesis may have a better benefit-risk ratio. For example, inhibition of secondary cell cycle checkpoints might have a selective effect in cancer cells that have defective function of the major checkpoint that depends on p53 (see Chapter 12), leading to a preferential mutagenic effect on tumor cells alone (Cliby et al. 1998).

These unknowns should be addressed clearly with intensive experimental and theoretical efforts to carefully guide the application of this potentially highly promising approach.

Prevention by Delay

Cancer is primarily a disease of the elderly and delaying its onset could lead to disproportionate reductions in cancer incidence due to competing causes of mortality. A tumor that requires 6 oncogenic mutations and one mutator mutation generates malignant lineages proportionally to both the 7th power of the mutation rate and time. A 9.5% decrease in the mutation rate would therefore halve the incidence of cancer at a fixed time. The

previous risk would occur 9.5% later. A halving of the mutation rate would double the time to cancer, effectively eliminating it from the human lifespan for many cancer types. For the example above, the risk at fixed time would be reduced by $2^7 = 128$ fold.

Compounds which reduce the mutation rate could be identified by high throughput screening. For example, bacterial strains with different intrinsic mutation rates have a characteristic survival curve when competing with each other. Strains with the optimal mutation rate win under stringent selection (Loh et al. 2010). An anti-mutator compound would potentially lead to different strains, which formally had excessively high mutation rates, winning the competition.

Anti-inflammatories might reduce mutations by reducing proliferation and hence the number of cell generations over which mutations accumulate. In addition, inhibitors of error prone DNA polymerases such as β and κ may be anti-mutagenic. The former is frequently mutated in gastric cancers (Sweasy et al. 2006), and the latter is over-expressed in small cell lung cancer (O-Wang et al. 2001).

Lifestyle and environmental changes could have a dramatic impact on cancer incidence rates, if they could be achieved.

SUMMARY

Utilizing the idea of efficiency of carcinogenesis, and the technique of FQM, designed for answering focused questions in the face of sparse experimental data, the mutator hypothesis, which proposes that genetic instability plays a key role in carcinogenesis, has been demonstrated to be strongly supported. Cancers are genetically unstable and therefore heterogeneous and dynamic. These factors need to be taken into account in treatment strategies. Current paradigms only scratch the surface of this complexity.

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CHAPTER 4

Radiation Induced DNA Damage, Repair and Therapeutics

Lynn Harrison

INTRODUCTION

The discovery of x-rays in 1895 by Wilhelm Conrad Röentgen began a new era of research and therapeutic/medical use of radiation. The "new kind of ray" or x-ray (x for unknown) was discovered during experiments on cathode rays. In 1896, the first radiograph image was captured of a hand during a public lecture by Röentgen, and the Lancet published a report on how x-rays were used to find a piece of a knife in the back of a paralyzed sailor (Hall 2000). Less than 20 years later in 1914 during World War I, Marie Curie set-up mobile radiology vans and x-rays were used for diagnostic imaging to help treat soldiers at the front. Great advances were made in the late 19th century with the discovery of radioactivity by Antoine Henri Becquerel and the isolation of radium by Pierre and Marie Curie. The use of radiation to kill or damage human tissue was demonstrated in 1897: Wilhelm Alexander Freud used x-rays to treat a hairy mole on an individual, and Henri Becquerel irradiated himself by leaving a vial of radium in his vest pocket, resulting in skin erythema and ulceration two weeks later. Over the last century, we have answered many questions about radiation, its effects on the cell and how it can be used therapeutically. This Chapter will address the present understanding about how ionizing radiation damages the DNA and how the DNA repair pathways (see Chapter 1) repair or mis-repair the damage.

Department of Molecular and Cellular Physiology, Louisiana Health Sciences Center, 1501 Kings Highway, Shreveport, LA 71130, USA; Email: lclary@lsuhsc.edu

IONIZING RADIATION

Ionizing radiation releases large amounts of energy that can be absorbed by an atom or molecule. Radiation can be directly or indirectly ionizing (Hall 2000). For direct ionization, the radiation releases enough energy to generate radicals and hence disrupt the chemical structure of the molecule. Indirect ionization is when energy from the radiation is transferred to an electron in an atom that is "hit," and this electron then gains kinetic energy. This secondary fast electron can subsequently interact with other molecules and result in further ionizations. The electron can be an orbital electron in the outer shell of an atom, and the loss of the electron results in the production of a free radical. Damage can therefore be generated by a direct mechanism where the ionization directly alters the chemical structure of a critical molecule in the cell, or by an indirect mechanism, where reactive radicals generated by the radiation cause damage to critical molecules (Hall 2000). An important example of the indirect mechanism with respect to radiotherapy is the ionization of water to generate the H_2O^+ ion and an electron. The H_2O^+ then rapidly reacts with another water molecule to produce a hydroxyl radical (OH^-) and the electron becomes hydrated. If the hydroxyl radical is situated in the hydration shell of the DNA, it can diffuse to the DNA and react to alter the chemical structure of the bases or the deoxyribose phosphate backbone.

There are two types of ionizing radiation: photon and hadron. Photon radiations include x-rays and gamma rays. X-rays are generated by abruptly stopping accelerated electrons and some of the kinetic energy is converted to x-rays, while gamma rays are due to the decay of unstable isotopes where energy is released as the isotope is changing to a more stable form. Other types of photon radiation include visible light, microwaves and radiowaves, all of which have longer wavelengths and lower energy and hence are not ionizing radiations. Hadron radiation are particles that include protons, alpha particles, neutrons and heavy charged ions such as carbon (^{12}C) and iron (^{56}Fe), and these particles can interact with the nucleus of atoms. Upon interaction with the nucleus, a fast neutron for example can produce three alpha particles from carbon, or four alpha particles from oxygen. Interaction with hydrogen during radiotherapy is more likely due to the high water content of tissue, and a neutron interacting with a hydrogen nucleus results in the release of a proton, which can cause ionization of the tissue (Hall 2000).

A major difference between hadron and photon radiation is the distance traveled within tissue and the pattern of dispersal of the ionizations along the radiation track. X-rays and gamma rays are sparsely ionizing,

deposit energy over a wide area and do not travel far into tissue. Hadron radiations are particles that are heavy and they travel in a straighter line as they penetrate deep into the tissue. The majority of energy of the particle radiations is not released until near the end of their range in the tissue, when the particles decelerate quickly and a peak of energy is released. This peak is called the Bragg peak. Past the Bragg peak there is very little energy deposited and prior to the Bragg peak some energy is lost along the track, but this is minimal compared to the point of rapid deceleration (Allen et al. 2011). This feature therefore results in hadron radiation having a therapeutic benefit over photon radiations of delivering a reduced dose to off-target tissue, which can result in organ sparing and diminished normal tissue damage. Neutrons are a hadron radiation, but are an exception with respect to dose distribution and distance traveled within the tissue. In this capacity, fast neutrons are similar to the photon radiations (Laramore 2009).

The Linear Energy Transfer (LET) of a radiation is defined as the average energy deposited in the medium per unit length of the track and has units of keV/μm. This metric determines the density of ionizations produced along the radiation track and hence the extent of damage. X-rays and gamma rays are low LET radiation and are therefore sparsely ionizing along the track. Protons are also a low LET radiation, while ^{12}C and ^{56}Fe ions and alpha particles are high LET radiation and are very densely ionizing. Fast neutrons have also been used for therapy and are a high LET radiation due to their interaction with the nuclei in atoms of biological molecules, resulting in the generation of alpha particles (Laramore 2009). High LET radiation generates a large amount of damage by the direct mechanism, while the majority of damage generated by low LET radiation is via the indirect mechanism. To compare the types of radiation, the relative biological effectiveness (RBE) can be calculated. The definition is: "The RBE of some test radiation (r) compared with x-rays is defined by the ratio D_{250}/D_r, where D_{250} and D_r are, respectively, the doses of x-rays and the test radiation required for an equal biological effect" (Hall 2000). The dose is a measure of the energy absorbed by the irradiated material and is in units of Gray, where 1 Gy is 1 Joule absorbed per kilogram. When considering an end-point, such as cell survival, the RBE increases with increasing LET up to a maximum LET of 100 keV/μm. The RBE for protons is ~1.1, but 2.5–3 for carbon ions (Allen et al. 2011). The effect of oxygen on cell killing after irradiation also changes with LET. The presence of oxygen is more important for cell killing by low LET radiation, probably due to the majority of damage being generated in critical sites in the cell by the indirect mechanism. This is very relevant to therapy as certain tumors have higher hypoxic fractions, and high LET radiation should therefore be more effective at killing these tumors.

DNA IS THE CRITICAL TARGET

When ionizing radiation interacts with a cell, protein, lipid and DNA are damaged and one of the earliest questions addressed in radiobiology was "What is the critical target for radiation within the cell?". Early studies demonstrated that amino acids in proteins were sensitive to damage by radiation: the most prone being cystine, methionine, histidine, tyrosine, threonine and serine (Kumta and Tappel 1961). Irradiation did not result in simple hydrolysis of bonds releasing peptide fragments, but caused degradation and breakdown of the amino acids and the formation of insoluble aggregates (Kumta and Tappel 1961). Hydroxyl radicals generated by gamma radiation were found to modify as well as fragment polypeptides, resulting in unfolded proteins (Wolff and Dean 1986), but the modifications and the extent of fragmentation were altered when the protein was situated in a membrane (Wolff et al. 1986). The lipid in the membrane decreased protein fragmentation for a given dose of hydroxyl radicals, indicating that the lipid was reacting with the free radicals. However, further addition of iron or copper to the system resulted in greater protein fragmentation, and it was concluded that the increased fragmentation was due to radicals generated from the lipid hydroperoxides (Dean 1987). Cellular membranes were originally considered to be a critical target for radiation (Bacq and Alexander 1961). Dr. Alper (1963) proposed that ionizing radiation resulted in two forms of damage: type N, which contributed to cell death after irradiation under anoxic conditions and was the result of primary energy deposition in the DNA, and type O, which was responsible for the radiosensitization of cells in the presence of oxygen and was due to membrane damage. Since DNA was known to be closely associated with the nuclear membrane in mammalian cells and with the cell membrane in bacteria, damage to the membrane was believed to result in death by disruption of DNA structure and function (Alper 1979, 1987). The induction of lipid peroxidation at unsaturated fatty acid residues by radiation is well established (reviewed in Leyko and Bartosz 1986), and a link with radiosensitivity was demonstrated when the cytosol of a cell line (L5178Y) was found to protect against lipid peroxidation, while the cytosol of a radiosensitive mutant of this cell line (M10) did not (Nakazawa et al. 1982). Vitamin E, a membrane soluble antioxidant, also increased the survival of irradiated mice (Malik et al. 1978), whereas vitamin E-deficient mice showed enhanced radiosensitivity (Konings and Drijver 1979). The effect of radiation on membranes is still a very relevant topic of research today (reviewed in Corre et al. 2010). Evidence indicates that radiation leads not only to lipid peroxidation or protein modification within the plasma

membrane, but disruption of the lipid bilayer, loss of barrier function, an alteration in the localization and size of lipid rafts that contain receptors and secondary messenger systems for signaling, and the production of ceramide followed by induction of apoptosis. Although some biological effects from ionizing radiation can be attributed to oxidation of proteins and lipid, the dose required to generate alterations, such as membrane permeability, occurs at a higher level than required to induce DNA damage (Kankura et al. 1969). Evidence still indicates that the most critical radiation target in the cell is DNA. In fact, lipid peroxidation has been shown to induce DNA damage (Pietronigro et al. 1977; Inouye 1984), so interventions to decrease lipid peroxidation could decrease cell killing by altering the level of DNA damage.

Early studies demonstrated that the DNA was altered by radiation. The viscosity of the DNA isolated from rat thymus and liver was found to dramatically decrease within 15 minutes of irradiation (Kuzin 1963), an effect we now know to be caused by the introduction of DNA strand breaks and damage. This was followed by an increase in viscosity within two hours of radiation treatment, and this change was different for radiosensitive and radioresistant tissues (Kuzin 1963). This latter stage alteration in viscosity we now attribute to active DNA repair processes. One of the first connections with cell survival, ability to replicate and gross chromosomal changes was described by Dr. Puck in 1958 (Puck 1958), but it wasn't until 1970 that work was published targeting alpha particles to either the cytoplasm or the nucleus of the cell. It was found that only a few alpha particles were required to damage the nucleus and cause cell death, while a dose ranging from 250–1000 Gy through the cytoplasm did not decrease the growth rate of cells (Munro 1970). This latter study does not, however, eliminate the involvement of the nuclear membrane. Further evidence in support of DNA as the critical target includes the log-linear relationship between radiation lethality and the frequency of radiation induced mutations at the hypoxanthine-guanine phosphoribosyl transferase (*HPRT*) locus in human, hamster and mouse cells (Thacker 1979; Thacker et al. 1982). In addition, using bromodeoxyuridine to specifically enhance radiation DNA damage, it was demonstrated that ionizing radiation-induced chromosomal instability was due to damage on the DNA and not damage to other cellular structures (Limoli et al. 1999). It is now established with respect to the success of radiotherapy and tumor cell killing that DNA is the critical radiation target, and enhancement of DNA damage and disruption of the DNA repair mechanisms that cope with DNA modifications within a tumor is required to enhance the therapeutic effect of cancer treatments.

TYPES OF DNA DAMAGE

High and low LET radiation introduce the same types of DNA damages, and these include modifications to guanine, thymine, adenine and cytosine, single-strand breaks (SSBs), double-strand breaks (DSBs), abasic (AP) sites, deoxyribose damage and protein-DNA crosslinks (Teoule 1987; von Sonntag 1987; Oleinick 1987; Wallace 2002). DNA radicals generated by the direct deposition of energy in DNA are predicted to form in the proportion of 30% phosphate, 28% deoxyribose, 12% guanine, 11% adenine, 10% thymidine and 9% cytidine (Bernhard 2010). In cells, the lesions identified in DNA are stable products that are produced following either the generation of DNA radicals (the direct effect) or the reaction of DNA with hydroxyl radicals (the indirect effect). Following irradiation of cells with gamma rays or carbon ions, the most prevalent base damage detected is thymidine glycol (cis and trans diastereoisomers) followed by 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyG). One of the most frequently studied lesions 8-oxo-7,8-dihydro-2′-deoxyguanosine (8oxodG), a base lesion often used as a biomarker of oxidative stress, is fourth on the list after 5-(hydroxymethyl)-2′-deoxyuridine (Pouget et al. 2002; Cadet et al. 2003). Damage at the base can destabilize the N-glycosylic bond, resulting in loss of the base and generation of an abasic site in DNA. Specific damage to the deoxyribose sugar of DNA is due to the abstraction of hydrogen atoms and frequently results in strand breakage (von Sonntag 1987). The termini of radiation-induced strand breaks are modified: all the 5′ termini and 70% of 3′ termini carry a phosphate group, while 30% of 3′ termini carry a phosphoglycolate group (Henner et al. 1983; Ward 1988). In terms of the yields of the different lesions, there is a ratio of 2.7 base damages generated per SSB (Ward 1995) for low LET radiation. This is in agreement with Monte Carlo simulations that estimated damage at 650 base damages Gy^{-1} giga basepairs $(Gbp)^{-1}$ (including AP sites) and 217 strand breaks Gy^{-1} Gbp^{-1} (Semenko and Stewart 2006). DSB induction has been measured and is introduced at 4.2–6.9 DSBs Gy^{-1} Gbp^{-1} for low LET radiation (Prise et al. 1998), indicating that the yields of damages are in the order of base damage and AP sites > SSBs >> DSBs.

Low and high LET radiation produce the same types of base damages and induction is approximately linear for both radiations over a wide dose range, although two fold less base damage is produced by high LET carbon ions compared to low LET gamma rays (Pouget et al. 2002). Even though high LET radiation is predicted to introduce DSBs more frequently and produce smaller DNA fragments than low LET radiation (reviewed by Prise 2001), studies in mammalian cells have determined that the number of DSBs induced by high and low LET radiation is similar per dose. However, the techniques to measure DSBs are thought to underestimate DSB production from high LET radiation, as the technique is not able to detect the small

DNA fragments. Löbrich et al. (1996) were able to detect a greater amount of fragments less than 200kb following irradiation of human fibroblasts with high LET radiation compared to X-rays. Work with yeast has demonstrated that the yield of DSBs does increase with LET. The smaller size of the yeast genome allows detection of smaller DNA fragments generated with higher LET radiation, and so this is likely a more accurate assessment of changes with LET (Prise 2001).

CLUSTERED DNA LESIONS: THE KILLING DAMAGE?

The same types of DNA damage introduced by ionizing radiation are also produced in the genome on a daily basis by reactive oxygen species (ROS) generated endogenously from normal cellular metabolism. In fact, any treatment that results in cellular ROS can produce the same types of DNA damage as radiation. Hydrogen peroxide is one example, yet it is not an effective tumor killing agent. To examine the lethality of DNA-damaging agents, John Ward (1987) compared the number of strand breaks produced by hydrogen peroxide, bleomycin sulfate and ionizing radiation at a dose that killed 63% of cells, which is defined as the dose needed to induce one lethal event/cell. At this dose, ionizing radiation introduced 1000 SSBs, bleomycin sulfate 150 SSBs and hydrogen peroxide 400,000 SSBs. Therefore, even though these damaging agents produce the same types of damages, fewer measurable lesions are required to kill a cell using ionizing radiation and bleomycin sulfate than hydrogen peroxide. The hypothesis put forward to explain this difference in lethality was that the spatial distribution or clustering of the DNA damage is the key factor for killing cells, because clustering of damage would result in an inability of the cell to repair the damage (Ward 1981, 1987). Hydrogen peroxide produces single lesions as it reacts with a metal ion in a Fenton reaction to produce hydroxyl radicals at sites of bound metal ions randomly distributed in the DNA (Ward 1987). Bleomycin sulfate produces DSBs and clusters of SSBs and AP sites: it intercalates into the DNA and binds metal ions such as iron, and ROS are formed by reaction of bleomycin with iron and oxygen (von Sonntag 1987). Incubating bleomycin-treated DNA with endonuclease III or putrescine to cleave at AP sites produced DSBs (Povirk et al. 1988), demonstrating that bleomycin produces SSBs and AP sites in clusters on DNA. The generation of clustered lesions, or locally multiply damaged sites (LMDS) as they were originally called, by ionizing radiation is due to the production of multiple radicals in or near the DNA as the radiation track passes through the cell. Track structure analyses provided the first evidence that multiple radicals could be generated from a single radiation track and introduce multiple lesions in the DNA (Goodhead 1994). The key is that damage is not only

produced by primary ionizations from the track itself, but the primary events lead to secondary electrons that are also ionizing. This results in spurs of ionizing events from the main track, and the outcome when modeled over the DNA structure is the formation of multiple radicals that are situated in the DNA, or generation of multiple hydroxyl radicals close enough to diffuse and react with the DNA (Goodhead 1994; Fig. 1). This scenario can occur with low LET as well as high LET radiation.

A clustered lesion (or LMDS) is defined as ≥ 2 damages within a 20 bp region (Ward 1995), and even low LET radiation that is more commonly used in the clinic is predicted to generate complex clustered lesions with ~72% containing 2 lesions, 20% with 3 lesions, 6% with 4 lesions, and the remaining 2% of the clusters consisting of 5 or more lesions (personal communication, Dr. R.D. Stewart, Purdue University, IN). The damage is predicted to be more complex with increasing LET (Nikjoo et al. 2001): Monte Carlo track simulations indicate that as the LET increases the majority of SSBs are formed

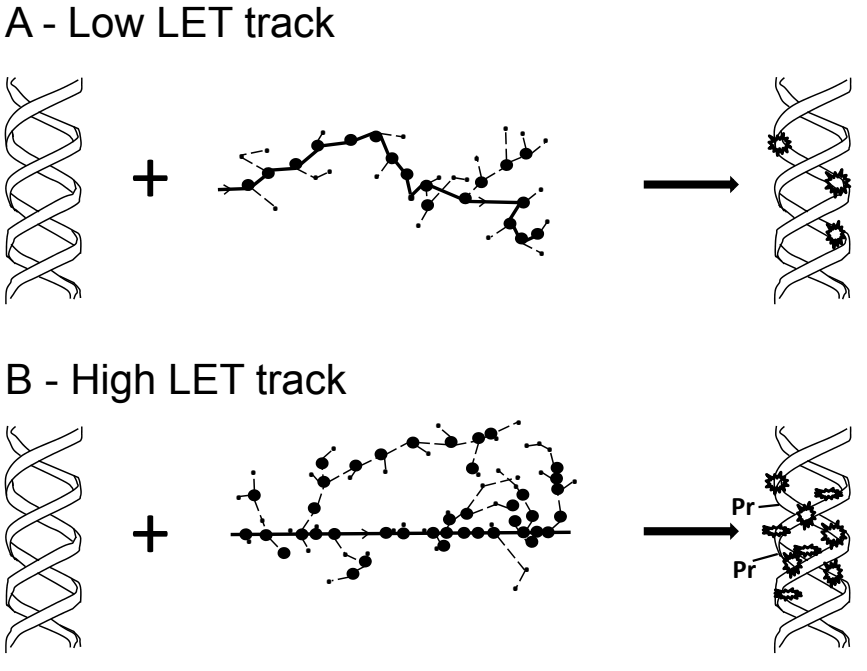

Figure 1. Low LET and High LET radiation tracks. A cartoon is shown of an example of the track structure from a low LET radiation track (A) and a high LET radiation track (B). The large dots are ionizations and the small dots are excitations. The main track is shown as a solid line and spurs of ionizations are represented as the dashed line. The explosions on the DNA represent damage. Pr—represents DNA-protein cross-links. The high LET track is much more ionizing and generates more complex damage on the DNA. This figure was re-drawn and modified from Goodhead 1994.

in a cluster with a base damage, and the frequency of DSBs with near-by base damage or additional breaks also increases (Nikjoo et al. 2001). Work by Löbrich et al. (1996) supports this latter prediction, as they demonstrated there is a shorter distance between DSBs introduced by high versus low LET radiation. The track structure analyses show that base damage and AP sites are important, as well as DSBs, for high LET radiation, even though previous studies determined that high LET radiation produces a lower yield of base damage compared to low LET radiation (Pouget et al. 2002). The base damage is still relevant as there is a greater probability that the base damage will be in close proximity with other base damages or strand breaks, and the clustering of the lesions could account for the higher RBE of high LET radiation. Evidence also suggests that radiation mutagenesis is not just due to single base damages or AP sites: hydrogen peroxide is non-mutagenic even at doses that induce single base alterations at 5×10^5 base damages/cell, and treatment of cells to induce equal base damage with alpha particles or gamma rays results in 12 times more mutagenesis from the alpha particles. Therefore, the clustered lesion has been proposed to be the probable cause of radiation mutagenesis (Ward 1995).

There are many possible permutations of clustered lesions (Fig. 2), partly because of the many stable base damages induced by radiation (Teoule 1987; Wallace 2002). This is further complicated by the possibility that damages can be on one strand only or can be situated in opposing strands. Lesions on the same strand can be separated by a few base pairs, or can be tandem lesions and thus situated immediately next to each other. Damages in opposing strands have been designated bi-stranded clusters and these are divided into two groups: non-DSB and DSB clusters. Non-DSB clusters can contain base damage, AP sites and/or SSBs. There are three categories of non-DSB clusters that have been named by the ability to detect the damage by enzymes that cleave at damaged purines, pyrimidines and AP sites, and these are designated oxypurine, oxypyrimidine and abasic site clusters. The simplest bi-stranded cluster consists of two SSBs and is a DSB, but DSBs situated near oxidative base damage or AP sites are a form of DSB cluster that are called complex DSBs.

Methods have now been developed that can detect and measure clustered lesions in DNA. Liquid chromatography-mass spectrometry has been used for lesions on the same DNA strand (for review see Box et al. 2001a). Four types of clusters with two damages on the same strand have been identified in low LET irradiated DNA: one consists of a base damage (usually an oxidized guanine) situated in the same strand immediately next to or a few nucleosides away from a SSB (Box et al. 2001b), another type is an 8oxodG in tandem with a formylamine, which is a formamido-derivative of a pyrimidine (8oxodG-dF, Box et al. 2000), and thymine-guanine and guanine-cytosine/5-methylcytosine cross-links have also been detected

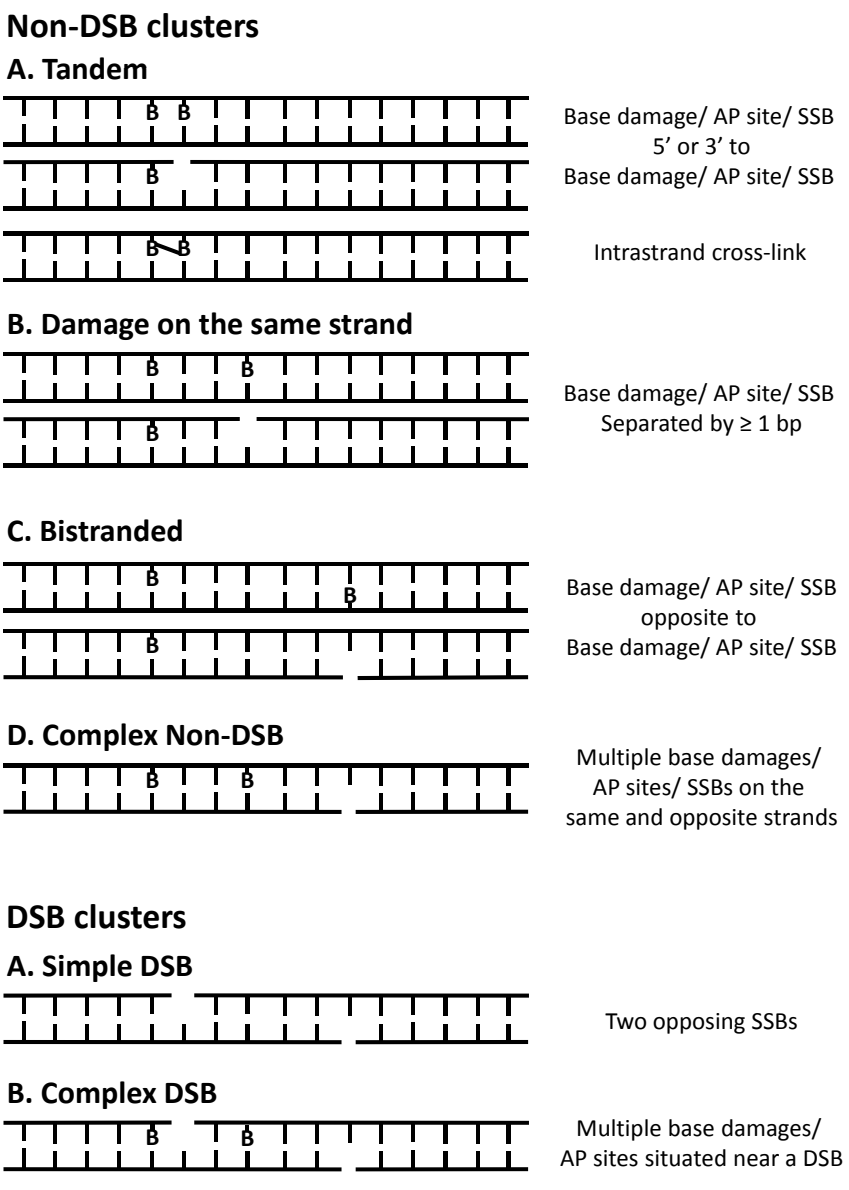

Figure 2. Types of clustered damage. There are non-DSB and DSB clusters. Non-DSB clusters can contain base damage, AP sites and SSBs situated on the same strand in tandem or separated by a few bases, or the damage can be in opposing strands. Tandem intrastrand crosslinks are also generated by ionizing radiation on the DNA. DSBs can be simple or complex. The complex DSBs are DSBs with near-by oxidative damage. B on the DNA structure represents a base damage or AP site.

(Bellon et al. 2002; Zhang and Wang 2005). The most recently identified tandem lesion consists of a SSB with an adjacent inter-strand cross-link with a cytosine on the opposite strand (Regulus et al. 2007). It is possible that lesions on the same strand could be produced from multiple radicals, but evidence indicates that tandem lesions can be produced from a single radical, where a radical intermediate on one nucleotide reacts with an adjacent nucleotide (Box et al. 2001b; Imoto et al. 2008). This mechanism is supported by the fact that 8oxodG-dF can be produced in DNA from Fenton-type reactions (Bourdat et al. 2000; Patrzyc et al. 2001), which produce single hydroxyl radicals in a random distribution pattern. This then would suggest that tandem lesions could be produced by endogenous ROS and have biological implications in un-irradiated cells.

The technique to measure bi-stranded lesions (reviewed in Sutherland et al. 2003) combines quantitative gel electrophoresis with the treatment of irradiated DNA with base excision DNA repair (BER) enzymes. Oxidative base damage and AP sites are predominantly repaired by the BER pathway and an intermediate in the repair is a SSB (see Chapter 8). Clustered base damage or AP sites in opposing strands can therefore be converted to a DSB by the repair enzymes, and this phenomenon is detected by gel electrophoresis as an increase in DNA fragmentation. The increase in fragmentation is used to quantify the specific types of clusters. The enzymes commonly used to convert clustered lesions to DSBs during the experimental analysis of irradiated DNA are from bacteria: Fpg to detect oxidized purines, endonuclease III for oxidized pyrimidines and endonuclease IV for AP sites. This technique confirmed that a variety of base damages and AP sites do exist in clustered lesions. In X-irradiated cells, 70% of the clusters were found to be non-DSB clusters and 30% were DSB clusters; there was 1 oxypurine cluster, 0.9 oxypyrimidine clusters and 0.75 abasic site clusters for each DSB cluster (Sutherland et al. 2002). Non-DSB clustered lesions have been detected and quantitated in irradiated cells (Sutherland et al. 2002) and animals (Gollapalle et al. 2007), as well as in DNA in solution (Sutherland et al. 2000a). The effect of LET on production of clustered lesions has also been measured in yeast DNA in 10 mM Tris buffer using this technique, and lesions calculated per absorbed dose and also per particle fluence (Keszenman and Sutherland 2010). Protons, oxygen, titanium and iron ions were used, with iron ions being the highest LET and protons the lowest LET radiation. The total damage yields were similar per absorbed dose with increasing LET, but the iron ions generated more damage per particle fluence, indicating a higher efficiency of damage induction by the high LET particle. With increasing LET there was an increase in the production of DSBs, but a decrease in the clusters detected by Fpg (oxypurine base damage). The abasic site clusters did not alter with

increasing LET. This indicates that DSBs form a high percentage of the damage induced by high LET radiation.

Although this technique has significantly increased our knowledge of non-DSB clusters, there are limitations to the assay. The main problem is that the repair enzymes used to convert the opposing base damage/AP sites to DSBs can be inhibited by near-by damage (Blaisdell et al. 2001, see below for a more detailed discussion), and thus, as the density of the lesions increases, the enzymes may not be able to convert the non-DSB clusters to DSBs. It is also not always possible to determine the clusters that contain an AP site with a base damage or clusters of oxidized purines with oxidized pyrimidines, as the types of clusters that can be detected are restricted by the specificity of the enzyme used to probe for the damage. As a result, there is likely an underestimation of non-DSB clusters, especially when considering damage induced by high LET radiation. Even with these limitations, this technique has proved powerful enough to detect and quantify non-DSB clustered lesions in cultured cells and human skin without irradiation (a few/Giga base pair, Bennett et al. 2005), in cells exposed to low doses of radiation (Sutherland et al. 2000b), and in mice weeks after irradiation (Gollapalle et al. 2007). Conditions expected to increase endogenous oxidative stress such as smoking (Bennett et al. 2008) or malignancy (Nowsheen et al. 2009) have also been found to elevate the levels of clustered lesions in cells, and it has been proposed that tumor-bearing mice have a chronic inflammatory condition due to the detection of elevated clustered lesions in the normal proliferating tissues of the animal (Redon et al. 2010).

Other techniques for measuring non-DSB and DSB clusters include an adaptation of the neutral comet assay where the cells are permeabilized and treated with repair enzymes (Holt and Georgakilas 2007). More recently, visualization of repair enzymes on DNA damage in irradiated cells has been used to detect and monitor the repair of clustered DNA damage (Asaithamby et al. 2011). SSBs and base damage were visualized using immunofluorescence with antibodies against XRCC1 and Ogg1 (8oxodG-DNA glycosylase), respectively, while DSBs were visualized using EGFP-tagged 53BP1. Complex lesions with overlap of all three markers comprised ~70% of the iron ion generated damage, whereas only ~18–20% of damage induced by γ rays consisted of two of the markers. This provides further evidence that clustered lesions are produced by low LET radiation, and that high LET radiation introduces more complex damage. Interestingly, hydrogen peroxide produced all three types of damage, but the markers did not overlap, demonstrating that hydrogen peroxide-induced damage is randomly distributed in DNA. This technique is a significant advancement and will likely lead to an increased understanding of the induction and repair of these complex lesions.

POTENTIAL BIOLOGICAL CONSEQUENCES

Ionizing radiation introduces a plethora of DNA damage (Wallace 2002) and some of the most studied single lesions are shown in Table 1. Removal of the majority of these lesions requires the BER pathway (see Chapter 8), which is conserved throughout evolution. There are usually multiple enzymes present in the cell to remove the same lesion, providing back-up mechanisms to eliminate the damage and maintain genetic integrity. Many of the oxidative base lesions are mutagenic, and some will block DNA replication *in vitro* as well as in cells. An example is thymine glycol, which is a strong block to DNA replication. In order to overcome this block, cells have adopted special translesion DNA polymerases (see Chapter 11). For instance, two translesion DNA polymerases are required to bypass thymine glycol: DNA polymerase κ to insert a nucleotide opposite the damage and DNA polymerase ξ to extend the replicating strand passed the lesion (Yoon et al. 2010).

As can be seen from Table 1, some oxidative base damages are also recognized by the nucleotide excision repair (NER) pathway, which mainly deals with bulky, helix-distorting DNA adducts, such as pyrimidine dimers generated by ultraviolet radiation (Reardon et al. 1997) (see Chapter 9). Another possible back-up mechanism is nucleotide incision repair. This process requires an AP endonuclease (see below) to recognize and cleave on the 5′ side of the oxidative base damage. DNA synthesis from this nick results in displacement of the downstream strand containing the 5′-damage, which is eventually cleaved as a "flap" structure before ligation completes repair (Golan et al. 2010). Endonuclease IV, an AP endonuclease from *Escherichia coli*, has been implicated in this type of repair, and nucleotide incision repair has been proposed as a mechanism for the removal of oxidative base damage from bi-stranded non-DSB clusters without the formation of DSBs (Golan et al. 2010). Evidence also suggests that Ape1 and Fen1 are involved in a nucleotide incision repair pathway in human cells for certain oxidative base damages (Gelin et al. 2010). Human cells therefore have multiple back-up repair pathways to avoid mutagenesis or a block to DNA replication.

DSB repair is essential for cell survival following ionizing radiation, and there are two major pathways: homologous recombination, which only functions in the late S and G2 phases of the cell cycle, and non-homologous end-joining (NHEJ), which repairs DSBs throughout the cell cycle (see Chapter 1). Due to the complexity of radiation-induced lesions, repair requires multiple proteins to work at the same cluster on the DNA within 20 bps of each other. Consider a non-DSB cluster: there are specific glycosylases to remove oxidized purines and pyrimidines (Table 1), the major AP endonuclease to cleave the DNA at abasic sites and remove 3′

blocking termini, as well as the enzymes required downstream of these initiating enzymes to complete repair. A further complication is that the initial steps in removal of oxidative base damage and AP sites actually introduce a SSB intermediate. It was because of this complex process and the potential for repair inhibition due to the close proximity of the DNA modifications that John Ward (1981) hypothesized that clustered lesions or LMDS would be difficult to repair/repair correctly and therefore would be more detrimental to the cell than randomly distributed single damages. Given the many different combinations of damage that can be present within a clustered lesion, experiments to understand the possible biological consequences of ionizing radiation have been difficult. The majority of the work to date has used substrates with defined synthetic lesions of oxidative damage or strand breaks in oligonucleotides or plasmids. These substrates have been employed in *in vitro* reactions with pure proteins, or bacterial, yeast and mammalian extracts, and the resulting products analyzed. The damaged plasmids have also been studied in *E.coli*, yeast and mammalian cells following transformation/transfection. This work is summarized below and in Tables 2–5.

a) Multiple Damages on the Same DNA Strand

Multiple lesions situated on the same DNA strand could inhibit/alter the ability of repair enzymes to remove the individual lesions within the cluster or could affect replication, resulting in a block to replication or enhancing mutagenesis at one or more of the lesions. Recent studies (Bergeron et al. 2010) indicate that ~50% of 8-oxopurine damage (8oxodG or 8-oxo-7,8-dihydro-2′-deoxyadenosine) generated by hydroxyl radicals in DNA is in the form of a tandem lesion (defined here as two lesions immediately next to each other). Using irradiated calf thymus DNA, it was reported that removal of 8oxodG by DNA glycosylases is less efficient when the 8oxodG is adjacent to a second lesion: under conditions where Fpg removed 95% of the single 8oxodGs, only 60% of the 8oxodG from tandem lesions was released from the DNA, while human Ogg1 released 80% of the single 8oxodGs and only ~50% of the 8oxodGs in tandem lesions (Bergeron et al. 2010). The effects on repair, replication and mutagenesis have been studied for defined lesions in tandem in synthetic substrates in oligonucleotides, circular single stranded and double stranded plasmid DNA as well. Table 2 summarizes these results for tandem lesions on the same strand. Frequently, there is enhanced mutagenicity for at least one damage in the tandem cluster, and alterations can even be introduced 5′ and 3′ within a few base pairs of the tandem lesion as in the case of the guanine-thymine cross-link (Colis et al. 2008). DNA replication studies *in vitro*, in *E.coli* and in mammalian cells, have also demonstrated that these lesions pose a problem to replication.

Table 1. Single lesions.

Lesion	Initiating Repair Enzyme		Block to DNA Polymerase *in vitro*	Base Inserted Opposite	By-Pass By Translesion Polymerase	Mutagenic	References
	Bacterial	Mammalian					
TG	EndoIII, EndoVIII, UvrABC	Neil1, Nth1, NER pathway	YES	A >>>G	DNA pol κ and ξ, DNA pol θ	POOR	Jaruga et al. 2004 Reardon et al. 1997 Yoon et al. 2010 Seki et al. 2004 Wallace 2002
5OHC	EndoIII, EndoVIII	Neil1, Neil2	NO	G > A	–	C → T C → G	Jaruga et al. 2004 Hazra et al. 2002 Wallace 2002
5OHU	EndoIII, EndoVIII	Neil1, Neil2	NO	A	–	C → T	Wallace 2002 Hazra et al. 2002
5OHMeU	EndoIII, EndoVIII	Neil1, Nth1	NO	A	–	POOR	Wallace 2002 Zhang et al. 2005
DHU	Fpg, EndoIV	Neil1, Nth1	NO	A	–	–	Wallace 2002 Jaruga et al. 2004 Golan et al. 2010 Rosenquist et al. 2003
8oxodG	Fpg	Ogg1, NER pathway	NO	C > A	DNA polη	G → T	Reardon et al. 1997 Haracska et al. 2000 Wallace 2002
FAPYG	EndoIII, Fpg, EndoVIII	Neil1	YES	–	–	NO	Rosenquist et al. 2003 Wallace 2002
8oxoA	Poor substrate (Fpg, EndoVIII)	Ogg1, Neil1	NO	T >>G	–	POOR	Wallace 2002 Grin et al. 2010

SSB (3′P, 3′PG, 5′OH)	EndoIV, ExoIII,	PNKP, Ape1, Tdp1, XPF-ERCC1	YES	–	–	YES, (Base substitutions)	Fisher et al. 2011 Weinfeld et al. 2011 Dar and Jorgensen 1995 Niawa and Moses 1981
AP site	EndoIV, ExoIII, EndoIII, Fpg, EndoVIII	Ape1, Neil1, Nth1	YES	A > G > C > T	DNA polθ DNA polι and polη	YES 1or 2 bp deletion found *in vitro*	Shibutani et al. 1997 Johnson et al. 2000 Seki et al. 2004
L (oxidized AP site)	EndoIV, ExoIII,	Ape1	YES Inserts base opposite L	A or G	*E.coli* DNA pol V	YES 1 bp deletion found *in vitro*	Kroeger et al. 2004 Berthet et al. 2001 Imoto et al. 2008

Abbreviations: 8oxodG = 8-oxo-7,8-dihydro-2′-deoxyguanine, TG = thymine glycol, DHU = dihydrouracil, AP = abasic site, 5OHC = 5-hydroxycytosine, 5OHU = 5-hydroxyuracil, 5OHMeU= 5-hydroxymethyluracil, FAPYG = 2,6-diamino-4-hydroxy-5-formamidopyrimidine, 8oxoA = 8-oxo-7,8-dihydroadenine, AP = abasic, L = 2-deoxyribonolactone (oxidized abasic site), P = phosphate, PG = phosphoglycolate, EndoIII = endonuclease III, EndoIV = endonuclease IV, Fpg = formamidopyrimidine DNA glycosylase, Ogg1 = human Ogg1, Ape1 = human major AP endonuclease, PNKP = polynucleotide kinase phosphatase, ExoIII = exonuclease III, - = not determined

Table 2. Tandem lesions on the same strand.

Tandem Lesion	Effect on Repair *in vitro*	Increased Mutagenicity Compared to Single Lesion	Increased Block to Replication Compared to Single Lesion	Possible Mechanism of Repair	Reference
8oxodG-dF	Fpg can cleave 8oxodG and dF	NO (in mammalian cells)	YES (in mammalian cells)	BER	Gentil et al. 2000 Bourdat et al. 1999
dF-8oxodG	Fpg only cleaves at 8oxodG	–	–	BER	Bourdat et al. 1999
TG-8oxodG	Decreased removal of 8oxodG by Ogg1, EndoIII can cleave at Tg	YES at 8oxodG (in *E.coli*)	YES (*in vitro* and in *E.coli*)	BER NER?	Jiang et al. 2009 Yuan et al. 2010
8oxodG-TG	Increased removal of 8oxodG by Ogg1, EndoIII can cleave at Tg	YES at 8oxodG (in *E.coli*)	YES (*in vitro* and in *E.coli*)	BER NER?	Jiang et al. 2009 Yuan et al. 2010
8oxodG-furan	Increased removal of 8oxodG by Ogg1, Decreased cleavage at furan by hApe1	–	–	BER	Malyarchuk et al. 2009
8oxodG-U or 8oxodG-AP U→AP site in cells	EndoIII and EndoIV can cleave the AP Decreased cleavage by ExoIII and Fpg at the AP	YES at 8oxodG (G→T in mammalian cells)	–	BER	Cunniffe et al. 2007 Kalam and Basu 2005
Intrastrand cross-links, G^C, G^5mC, 5mC^G, G^T	Cleaved by UvrABC	Possible 5mC^G → TT (in XPA-deficient mammalian cells) G^T results in G→T, base substitutions 5′ and 3′ to cross-link, small deletions (in mammalian cells) G^C, G→T/C (in *E.coli*)	YES G^C 80% blocked in *E.coli*, G^T blocked Klenow, G^T by-passed by yeast DNA polymerase η	NER	Bellon et al. 2002 Lee et al. 2002 Zhang and Wang 2005 Gu et al. 2006 Hong et al. 2007 Colis et al. 2008 Jiang et al. 2007

L-TG Furan-TG	EndoIII decreased cleavage at Tg Decreased cleavage by EndoIV at L and furan, Exo III and Ape1 slight decreased cleavage at L and furan, altered kinetics, Furan/Tg cleaved by UvrABC	YES at Tg in L/Tg (in *E.coli*)	YES, by-passed only under SOS conditions (L/Tg in *E.coli*)	Long patch BER NER	Imoto et al. 2008 Huang et al. 2009
DHU-DHU	Only 1 DHU removed by *E. coli*, yeast and human EndoIII and *E. coli* Endo VIII	–	–	BER	Venkhataraman et al. 2001

Abbreviations: 8oxodG = 8-oxo-7,8-dihydro-2′-deoxyguanine, dF = formylamine, TG = thymine glycol, DHU = dihydrouracil, furan = tetrahydrofuran, U = uracil, AP = abasic site, 5mC = 5 methylcytosine, L = 2-deoxyribonolactone (oxidized abasic site), EndoIII = endonuclease III, EndoIV = endonuclease IV, Fpg = formamido pyrimidine DNA glycosylase, Ogg1 = human Ogg1, Ape1 = human major AP endonuclease, ^ = intrastrand crosslink. - = not determined, BER = base excision repair, NER = nucleotide excision repair

Table 3. Bi-stranded clusters consisting of two lesions: repair initiation *in vitro*.

Target Lesion	Opposing Lesion	Pure Enzyme	Cell Extract	Cleavage at Target	Reference
DHT	DHT/U	EndoIII, Fpg		Efficient	David-Cordonnier et al. 2000 Chaudhry and Weinfeld 1995
8oxodG/AP	8oxodG	yOgg1		Efficient if >1bp apart	David-Cordonnier et al. 2001a
TG/DHT	8oxodG	EndoVIII		Efficient	Harrison et al. 1998
TG/DHT/ AP/8oxodG	SSB	EndoIII, yOgg1, Fpg		Efficient if >1bp apart	Harrison et al. 1998, 1999 David-Cordonnier et al. 2001a
TG/DHT/AP	SSB	EndoVIII		Efficient if >1bp, but less cleavage in 3′ orientation	Harrison et al. 1998
8oxodG	8oxodG/DHT/U/AP/ SSB		XRS5	Inhibited by AP or SSB, efficient cleavage opposite a base damage	David-Cordonnier et al. 2001b
8oxodG	AP/U	Ogg1, mOgg1		Efficient, except inhibited by AP site 5′ at position 1	David-Cordonnier et al. 2001b Tian et al. 2002
AP	8oxodG/DHT/AP/SSB	Ape1		Efficient when opposite base damage; inhibited by SSB or AP 5′ at position 1 and 3, but efficient if SSB or AP 3′ at position 1 and 3	Chaudhry and Weinfeld 1997 Tian et al. 2002 David-Cordonnier et al. 2002 Paap et al. 2008
AP	AP	ExoIII		Low efficiency when situated 5′ to second damage; efficient if 3′ orientation	Chaudhry and Weinfeld 1997
AP	TG	Fpg, EndoIII, EndoVIII	XRS5	Efficient	Bellon et al. 2009
AP	TG	ExoIII		Efficient > 1bp apart	Bellon et al. 2009
TG	8oxodG/AP	EndoIII		Opposite an AP site efficient > 1bp apart Opposite 8oxodG efficient unless at 5′ position 1	Bellon et al. 2009
AP	8oxodG/ 8oxoA/ DHT,	EndoIII, Fpg	XRS5	Efficient	David-Cordonnier et al. 2000

AP	AP	EndoIII, Fpg	XRS5	Fpg inhibited, EndoIII efficient if > 1bp apart, Extract inhibited if 5′ orientation	David-Cordonnier et al. 2000 Chaudhry and Weinfeld 1995
8oxodG	AP/DHT/U	yOgg1		Efficient if opposite base damage, Efficient opposite AP if > 1bp apart	David-Cordonnier et al. 2001a

Figure 3 shows the location of the 2nd damage at position 1,3 and 6, in the 5′ or 3′ orientation compared to the target lesion.

Abbreviations: 8oxodG = 8-oxo-7,8-dihydro-2′-deoxyguanine, TG = thymine glycol, DHT =5,6-dihydrothymine, U = uracil, AP = abasic site, 8oxoA = 8-oxo-7,8-dihydroadenine, EndoIII = endonuclease III, EndoIV = endonuclease IV, Fpg = formamido pyrimidine DNA glycosylase, Ogg1 = human Ogg1, mOgg1 = mouse Ogg1, yOgg1 = yeast Ogg1, Ape1 = human major AP endonuclease, ExoIII = exonuclease III,/ = or

Table 4. Bi-stranded clusters : repair and mutagenesis in prokaryotes.

Clustered Lesion	Cell Type	Assay		Biological Consequence	DSB Formation	References
		DNA Repair Only	Repair and Replication			
U opp. U	WT *E. coli*	—	√	Increase in deletions Reduced plasmid survival	√	Dianov et al. 1991 Shikazono and O'Neill 2009
	Ung⁻ E. coli	—	√		X	
U opp. U U opp. F F opp. F	WT, *uvrA⁻*, *xth⁻nfo⁻*, *uvrA⁻xth⁻nfo⁻*, *xth⁻nfo⁻ nfi⁻ E. coli*	√	—	Reduced plasmid survival when replication allowed after repair, ↑ in deletions if U ≤ 7 bp apart	√	D'souza and Harrison 2003 Harrison et al. 2006
U opp. U U opp. F	*Ung⁻ E. coli*	√	—	Result same as undamaged	X	
AP opp. AP	WT *E. coli*	—	√	Increase in deletions, point mutations, reduced plasmid survival	√	Shikazono and O'Neill 2009
AP opp. GAP						
8oxodG opp. 8oxodG	WT, *mutY⁻ E. coli*	—	√	Increase in mutations when 2nd 8oxodG at position 1, 3 and 6, peak with 2nd 8oxodG at position 3	X	Malyarchuk et al. 2003, 2004
8oxodG opp. 8oxodG	WT, *mfd⁻ E. coli*	√	—	Result same as undamaged	X	Malyarchuk et al. 2004
8oxodG opp. U						
8oxodG opp. U	WT, *fpg⁻*, *mutY⁻*, *fpg⁻mutY⁻ E. coli*	—	√	Increase in 8oxodG mutations when U at position 1, 3 and 5, peak with U at position 1	X	Pearson et al. 2004
8oxodG opp. DHT	WT, *fpg⁻*, *nth⁻*, *mutY⁻*, *fpg⁻mutY⁻*, *fpg⁻mutY⁻ nth⁻ E. coli*	—	√	Increase in 8oxodG mutations when DHT at position 1, 3 and 5	X	Shikazono et al. 2006

TG opp. U	WT *E. coli*	—	√	Reduced plasmid survival	√	Bellon et al. 2009
TG opp. 8oxodG	WT, *mutY⁻*, *fpg⁻mutY⁻ E. coli*	—	√	Increase in mutation at 8oxodG	X	
2 8oxodG opp. U	WT, *mutY⁻ E. coli*	—	√	Increase in mutation frequency	X	Eccles et al. 2010
U + 8oxodG opp. U	WT, *mutY⁻ E. coli*	—	√	Reduced plasmid survival Increase in mutation frequency	√	

X = No, √ = Yes

Table 5. Bi-stranded clusters : repair and mutagenesis in eukaryotes.

Clustered Lesion	Cell Type	Assay		Biological Consequence	DSB Formation	References
		DNA Repair Only	Repair and Replication			
U opp. U	HeLa	√	—	No loss of plasmid compared to undamaged	X	Malyarchuk and Harrison 2005
F opp. F	WT, Ku80$^{-/-}$, DNA-PKcs$^{-/-}$ mouse fibroblasts	√	—	Reduced plasmid survival; greater plasmid loss in Ku80$^{-/-}$, Increase in deletions, Apex1 involved in forming DSB	X	Malyarchuk et al. 2008 Malyarchuk et al. 2009
8oxodG + F opp. F	WT mouse fibroblasts	√	—	Loss of plasmid	√	Malyarchuk et al. 2009
F opp. F + F						
8oxodG + F opp. F + F						
8oxodG opp. F	WT mouse fibroblasts	√	—	No loss of plasmid compared to undamaged	X	
8oxodG opp. F + F						
8oxodG opp. 8oxodG	TK6 with and without overexpression of hOgg1	√	—	No loss of plasmid compared to undamaged unless hOgg1 is overexpressed	Only when hOGG1 overexpressed	Malyarchuk and Harrison unpublished data
U opp. U AP opp. AP U opp. AP	WT, *apn1*$^{-}$*apn2*$^{-}$, *rad52*$^{-}$ *Saccharomyces cerevisiae*	—	√	Reduced plasmid survival	√	Kozmin et al. 2009

5OHU opp. 8oxodG	WT, *rad52*⁻ *Saccharomyces cerevisiae*	—	√	No loss of plasmid compared to undamaged	X	Kozmin et al. 2009
8oxodG + 8oxoA opp. 5OHU + GAP +FU	*ung1*⁻*ntg1*⁻*ntg2*⁻*ogg1*⁻ *mag1*⁻, *ntg1*⁻*ntg2*⁻, *rad14*⁻*ntg1*⁻*ntg2*⁻, *rad14*⁻, *rad51*⁻, *rev3*⁻, WT *Saccharomyces cerevisiae*					
5OHU opp. 8oxodG + GAP +FU	WT *Saccharomyces cerevisiae*					
Complex DSB: AP on 5′ overhang	Normal and Artemis deficient human cells	√	—	AP decreased efficiency of repair, translesion synthesis across the AP limited deletions but increased mutagenesis, XrccIV, Ku80 and Artemis involved in repair	Not applicable	Covo et al. 2009

X = No, √ = Yes, Apex1 = mouse major AP endonuclease

Bypass can require specialized DNA polymerases such as the "Y" family of polymerases to achieve translesion synthesis (see Chapter 11). Human DNA polymerase η can bypass the G-T intrastrand cross-link *in vitro* and the majority of the time bypass is not mutagenic (Colis et al. 2008). However, a similar G-T cross-link is capable of blocking the Klenow fragment of *E.coli* DNA polymerase I *in vitro* at the G after Klenow has inserted A opposite the T. Yeast DNA polymerase η also inserts an A opposite the T, but then is able to insert either an A or G opposite the G of the cross-link to facilitate bypass (Jiang et al. 2007). Translesion synthesis of tandem lesions in the cell is therefore likely to require specialized translesion DNA polymerases and occur at the expense of sequence integrity in order to achieve survival.

Repair at the tandem lesions is often inhibited, or occurs with different reaction kinetics or reduced efficiency (Table 2). Individual oxidative base damage within the tandem lesion is generally repaired by BER enzymes, but it has been suggested that NER may remove certain tandem lesions such as 2-deoxyribonolactone adjacent to a thymine glycol (Imoto et al. 2008). An alternative pathway could involve long patch BER (see Chapter 8), where Ape1 incises at the 2-deoxyribonolactone, DNA polymerase β performs multi-nucleotide DNA synthesis and strand displacement, and Fen1 cleaves the resulting flap prior to ligation (Imoto et al. 2008). The oxidative intrastrand cross-links introduced by ionizing radiation can also be removed from DNA *in vitro* by the *E. coli* NER complex UvrABC, suggesting that NER maybe the predominant cellular pathway to remove these cross-links (Gu et al. 2006). Evidence in support of this model was the detection of 5mCG→TT mutations in NER mutant (XPA-deficient) mammalian cells after treatment with copper and hydrogen peroxide (Lee et al. 2002); these mutations presumably originated from an intrastrand cross-link between the 5-methylcytosine and guanine. The only known examples of increased removal of an oxidative lesion when it is part of a tandem cluster is incision at 8-oxodG by human Ogg1 when the lesion is situated immediately 5′ to a tetrahydrofuran (abasic site analog, Malyarchuk et al. 2009) or thymine glycol *in vitro* (Jiang et al. 2009). Jiang et al. (2009) hypothesized that the thymine glycol 3′ to 8oxodG could result in 8oxodG extruding from the helix and hence being more readily cleaved by Ogg1.

Some work has been performed on oxidative clusters with damages situated on the same strand, but not immediately next to each other. Incision at an AP site by most AP lyases (an activity commonly associated with bi-functional DNA glycosylases) and AP endonucleases was inhibited by an 8-oxo-7,8-dihydroadenine (8oxoA) or 8oxodG situated up to 5 bases away 5′ or 3′ to the AP site (Lomax et al. 2005; Cunniffe et al. 2007). *E. coli* endonuclease III, a glycosylase/AP lyase specific for oxidative pyrimidine damage, was the exception; it was not inhibited by 8-oxodG and was actually stimulated (~1.5 fold) if the 8oxoA was 3 or 5 bps away from the AP

site. Fpg, another bifunctional DNA glycosylase that like endonuclease III cleaves at AP sites, was inhibited by the 8oxoA or 8oxodG at all positions. Interestingly, the inhibitory effect was much greater for the major human AP endonuclease (Ape1) and its bacterial counterpart exonuclease III when the 8oxoA (for Ape1) or 8oxodG (for exonuclease III) was situated 5′ to the AP site (Lomax et al. 2005; Cunniffe et al. 2007). The orientation effects of the lesions may be explained by differences in the way the enzymes bind the AP site and DNA; the 8oxoA/8oxodG could disrupt the contacts that the enzyme makes with the DNA. Ape1 binds to both DNA strands around the AP site and makes contact with 3 bases 5′ and at least 2 bases 3′ to the AP site (Nguyen et al. 2000). Ape1 also introduces bending into the DNA (Mol et al. 2000). The introduction of a second lesion in the same strand could alter the ability of the protein to kink the DNA and hence affect the enzyme reaction.

The repair of two 8oxodGs situated 2 bases apart on the same strand has been examined with human cell extracts, as well as with pure enzymes, and complete repair depended on which 8oxodG was removed first. If the 5′ 8oxodG was released by Ogg1 and a SSB introduced, complete repair was not disrupted by the remaining 8oxodG. However, removal of the downstream 8oxodG, creating a 3′ SSB, resulted in reduced efficiency of repair of the SSB by DNA polymerase β. It was determined that the delay was due to Ogg1 binding to the remaining 8oxodG, preventing DNA polymerase β from repairing the SSB (Budworth et al. 2005). Further work with oxidative base damage positioned near the 3′ terminus of a SSB has demonstrated that while human endonuclease III (Nth1) and Ogg1 are unable to efficiently remove the substrate base, the human Neil1 glycosylase is able to excise the damage (Parsons et al. 2005b). Moreover, human Ape1 is able to remove an 8-oxodG positioned at the 3′-terminus of a SSB (Parsons et al. 2005a). Collectively, these repair activities are important for resolving radiation induced DNA breaks with clusters of oxidative damage, as a 3′ 8-oxodG at a SSB inhibits ligation by DNA ligase IIIα or DNA ligase I (Parsons et al. 2005a). Long patch BER, following removal of the oxidative damage at the 3′ end of a SSB by the 3′ to 5′ exonuclease activity of DNA polymerase δ, has been shown to be an alternative mechanism for repair of these 3′ blocked termini (Parsons et al. 2007).

These *in vitro* studies clearly demonstrate that attempts to bind and repair multiple lesions in the same strand will be limited, firstly by spatial constraints and access of the enzymes to the lesions when other enzymes are bound, and secondly by inhibition of repair due to repair intermediates generated at other oxidative damage in the cluster. This delayed or inhibited repair would be expected to enhance the mutagenicity of lesions, and one study in *E. coli* did find increases in the mutation frequency of clustered

lesions consisting of an 8oxodG situated within 5 bases 5′ or 3′ to a uracil on the same strand (Cunniffe et al. 2007).

b) Bi-stranded Lesions

A great deal of work has focused on bi-stranded oxidative base damage due to the possibility of converting potentially mutagenic radiation-induced lesions to potentially lethal DSBs. The key is that processing of base damage or AP sites by BER introduces a SSB intermediate. Hence two opposing base damages/AP sites could be converted to a DSB during the process of repair. This of course goes against the idea that DNA repair is always a good thing for the cell. Early work measuring DSB production did detect an increase in DSBs when mammalian cells (Ahnstrom and Bryant 1982) and bacteria (Bonura et al. 1975) were allowed time to repair radiation-induced damage. It is now recognized that this is likely the conversion of non-DSB clustered lesions to DSBs. In fact, *E. coli* mutated in three DNA glycosylases (endonuclease III and VIII, and Fpg) are more resistant to ionizing radiation and produce ~7 times less DSBs than wild-type bacteria during 8 minutes of repair at 37°C post-irradiation (Blaisdell and Wallace 2001).

Work *in vitro* using purified enzymes or nuclear extracts has greatly increased our understanding of the ability of repair enzymes to initiate/complete repair of non-DSB clusters. Early studies positioned the damage in opposing strands in plasmid DNA (Chaudhry and Weinfeld 1995), but most of the work has used double-stranded oligonucleotides. To limit complexity and to dissect the effect on repair, a majority of the analysis has examined clusters of only two damages: a target damage situated in one strand (blue strand, Fig. 3) and the second damage (pink nucleotide on white strand, Fig. 3) located on the opposing strand at position 1, 3 or 6, 5′ or 3′ to the target base damage. Table 3 summarizes the results of the majority of the *in vitro* work that focused on the first step of repair. For bacterial, yeast or mammalian DNA glycosylases, and enzymes in mammalian nuclear extracts, it is evident is that initiation of repair of the first base damage in a cluster is efficient if the opposing lesion is a base damage. However, if the opposing lesion is an AP site or SSB, then the distance separating the lesions is critical: initiation of repair dramatically improves when there is ≥ 2 bp separating the target base damage and the SSB or AP site (position 3, Fig. 3). Only certain enzymes are affected by the 5′ or 3′ orientation of the SSB or AP site with respect to the target base damage: human and mouse Ogg1 were inhibited > 7 fold when the AP site was at position 1, 5′ to the target 8oxodG compared to 1.7 fold at position 1, 3′ to the 8oxodG (David-Cordonnier et al. 2001b); *E.coli* endonuclease VIII was more inhibited by a SSB at all positions 3′ relative to 5′ of the target base damage (Harrison et al. 1998). The orientation effect with endonuclease VIII can be explained by how the

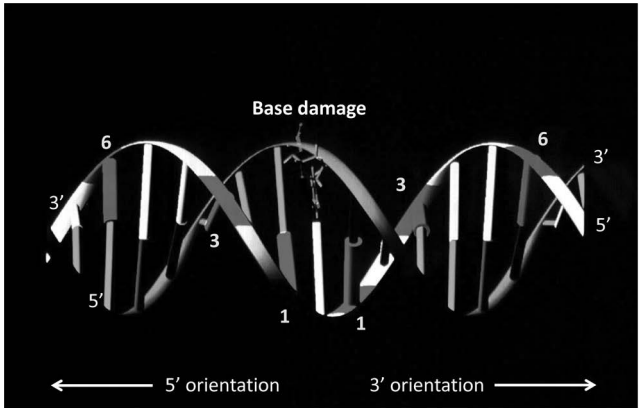

Figure 3. Positioning of damage in synthetic DNA substrates. A clustered lesion is defined as ≥ 2 damages situated within 20 bps or 1–2 helical turns of the DNA. A base damage is situated on the blue strand. The pink nucleotides on the white DNA strand are situated at position 1, 3 or 6,5′ or 3′ to the base damage. These are the positions where a second damage was placed to study closely opposed DNA lesions in synthetic defined substrates.

Color image of this figure appears in the color plate section at the end of the book.

enzyme binds the damage. Footprint analysis demonstrated endonuclease VIII binds predominantly to DNA 3′ to the target damage (Jiang et al. 1997), and an opposing 3′ SSB, especially at position 6, dramatically reduced the binding affinity of endonuclease VIII for the target damage (Harrison et al. 1998). A similar orientation effect was found for AP endonucleases: both *E.coli* exonuclease III and human Ape1 showed greater inhibition when an AP site or SSB were situated 5′ to the target AP site (Chaudhry and Weinfeld 1997). Methylation interference studies have demonstrated that Ape1 makes contact with the DNA at position 1 and 3, 5′ to the AP site (Wilson et al. 1997). Thus, in short, disruption of base damage repair at a simple non-DSB cluster is dependent on the type of opposing lesion (an AP site or SSB is more inhibitory), the distance separating the opposing lesions (inhibition decreases as the distance between the lesions increases), and whether the second lesion disrupts the binding of the enzyme to the target lesion. Evidence indicates that the repair enzyme's rate of excision, as well as binding affinity, can also be reduced by an AP site or SSB (David-Cordonnier et al. 2001c).

Since AP sites/SSBs are repair intermediates of base damage processing, it might be predicted that the formation of a DSB from two opposing base damages would be inhibited. However, opposing base damages (Chaudhry and Weinfeld 1995) or a base damage opposite a SSB (Harrison et al. 1998, 1999) can be converted to a DSB in *in vitro* biochemical repair assays. As alluded to above, DSB formation is affected by the distance between the lesions and the binding ability of the protein, as an increase

in DSBs is detected when the two damages are ≥ 2 bp apart. Having established the effects of clustered lesions on enzymes *in vitro,* the next experiments determined whether DSBs actually form in cells from clustered base damage under physiological conditions. Interestingly, in bacteria (Table 4) and mammalian cells (Table 5), opposing oxidative base damages in plasmid DNA are not readily converted to DSBs. In fact, the 8oxodG mutation frequency is increased when two opposing 8oxodGs are situated in a cluster and separated by up to 6 bp, and no DSB formation is detected (Malyarchuk et al. 2004). The increase in mutation frequency is explained by the conversion of one 8oxodG to a SSB repair intermediate, which then inhibits/slows the repair of the opposing second 8oxodG, allowing replication to occur and the insertion of an adenine instead of a cytosine opposite the 8oxodG base damage. One factor limiting DSB formation could be the physiological expression level of the protein. Overexpression of Ogg1 or Nth1 in TK6 cells does result in an increase in radiosensitization (Yang et al. 2004), and an increase in DSB production when the cells are allowed to repair for 30 minutes post irradiation (Yang et al. 2006). In recent work, our lab detected DSB formation from ~50% of clusters consisting of two opposing 8oxodG in plasmid DNA when TK6 cells were induced to overexpress Ogg1 (Malyarchuk and Harrison unpublished data). Expression of *E.coli* Fpg in Chinese hamster ovary cells was also found to lower the endogenous level of oxypurine clusters, and this was likely due to repair as there was no affect on the abasic or oxypyrimidine clusters (Paul et al. 2006).

Two opposing uracils is one type of base damage cluster that is readily converted to a DSB in bacteria (Dianov et al. 1991; D'souza and Harrison 2003; Shikazono and O'Neill 2009) and yeast (Kozmin et al. 2009). Uracils up to 7 bps apart form DSBs (D'souza and Harrison 2003). This event requires uracil DNA glycosylase, but does not require replication (Table 4). Uracils are first converted to AP sites in bacteria or yeast by uracil DNA glycosylase, and the AP sites are then converted to DSBs by the AP endonucleases in the cell. *In vitro* studies have demonstrated that two opposing AP sites can be converted to DSBs by exonuclease III or Ape1 (Chaudhry and Weinfeld 1997). Interestingly, two opposing uracils are not converted to DSBs in mammalian cells (Malyarchuk and Harrison 2005), even when uracil DNA glycosylase is overexpressed (Sage and Harrison 2011). It is possible that mammalian cells have evolved a mechanism for avoiding the production of DSBs from opposing repair intermediates. One hypothesis involves "passing the baton" (Wilson and Kunkel 2000), where the first enzyme in BER does not leave the repair intermediate until the next enzyme in the pathway is at the site of damage. This coordination prevents the release of the SSB repair intermediate and hence the formation of a DSB from opposing base damages. The enzymes in BER are known

to interact with each other, and enzyme activities are stimulated by the next enzyme in the pathway. For example, Ape1 stimulates the activity of Ogg1 (Vidal et al. 2001) and human uracil DNA glycosylase (Parikh et al. 1998); Ape1 interacts with DNA polymerase β (Bennett et al. 1997); and DNA polymerase β interacts with XRCC1 (Kubota et al. 1996; Caldecott et al. 1996) and DNA ligase I (Prasad et al. 1996). However, while evidence does indicate that there is a channeling of the intermediates through short patch BER (typically involving single-nucleotide replacement synthesis), it would appear that "passing the baton" does not occur *in vitro* for long patch (multi-nucleotide incorporation) BER (Prasad et al. 2011). Uracil is removed by short patch BER, but an oxidized AP site or a tetrahydrofuran (an AP site analog) requires long patch BER due to the 5′ blocked terminus generated after Ape1 incision. Thus, it would be predicted that two opposing furans would be converted to DSBs in mammalian cells. Indeed, this is what was found when plasmid DNA carrying two opposing furans was transfected into mouse cells (Table 5). Using siRNA it was also demonstrated that the mouse major AP endonuclease (Apex1) was involved in generating the DSBs (Malyarchuk et al. 2008).

Overexpression of Ogg1 in human cells results in DSB formation of two opposing 8oxodGs (Table 5), yet the "passing the baton" theory would predict that a DSB should not form from this cluster. However, it is possible that overexpression of a DNA glycosylase results in an imbalance of the BER pathway and decreased efficiency of channeling of repair intermediates, increasing the likelihood of DSB formation from two opposing base damages. Disruption of the channeling of repair intermediates could also occur due to the presence of near-by oxidative DNA damage: 8oxodG reduces the repair of an opposing SSB, with short patch repair proceeding if the damages are 5′ to each other, but long-patch repair occurring when the damages are 3′ to each other (Lomax et al. 2004); long patch BER is inhibited at the strand displacement and Fen1 steps by a 3′ opposing 8oxodG (Budworth et al. 2002); and an opposing dihydrothymine (Byrne et al. 2009) or thymine glycol (Budworth and Dianov 2003) reduces the repair of an AP site or a SSB by reducing the activity of DNA polymerase β and DNA ligase. From these *in vitro* studies it is clear that the protection of repair intermediates could easily break down when oxidative damage is situated in a clustered lesion, increasing the possibility of DSB formation. Once the DSB has formed, it has to be repaired by NHEJ or homologous recombination (see Chapter 1). NHEJ uses only the information at the DNA termini to facilitate direct end-joining of DSBs, and repair can be accurate as well as inaccurate. Homologous recombination employs a complementary chromosome, typically a sister chromatid, to facilitate conservative strand exchange to resolve DSBs, and is a faithful DNA repair mechanism (see Chapter 14). Work in mouse cells has implicated Ku-dependent repair,

and hence likely NHEJ, in the repair of DSBs formed from Apex1 cleavage at a cluster consisting of two furans (Fig. 4). Ku-independent DSB repair mechanisms could also be involved in repair of these "extra" DSBs, as repair products, albeit at a low level, were detected in Ku-deficient mouse cells (Malyarchuk et al. 2008).

The lesions discussed so far have been simple clusters consisting of two damages on the same strand or opposing strands. Although there is a greater probability that low LET radiation will produce simple clusters of damage, complex clusters of three and four lesions can also be generated. More complex clusters are predicted to be introduced as the LET of the radiation increases, and this is relevant to therapy, especially with the construction of more hadron radiotherapy centers. *In vitro* studies using purified proteins or cell extracts have demonstrated that the positioning of the oxidative damage in the complex cluster, as well as the types of damages in the cluster, are important factors in determining whether a DSB is formed. Besides the work described above, additional studies have found that three abasic sites situated in close proximity and 3′ to each other were readily

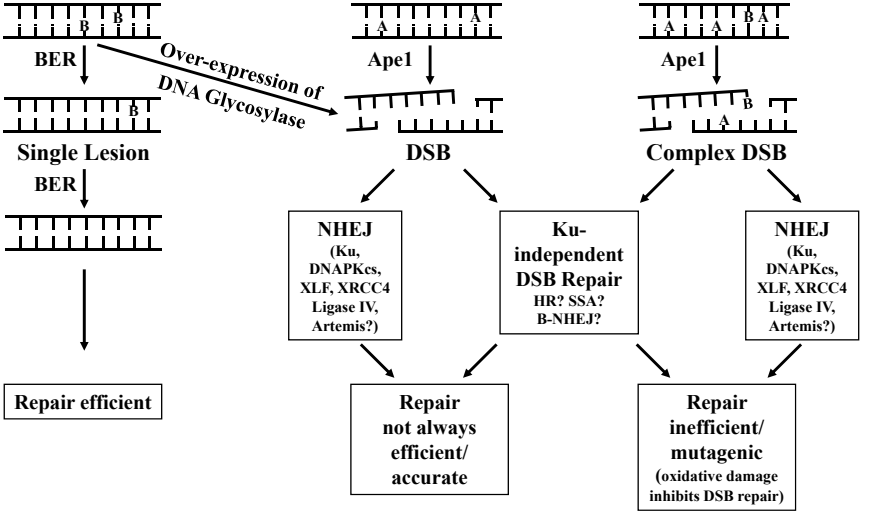

Figure 4. Summary of repair of clustered DNA damage in cells. Opposing DNA oxidative damage (B) is sequentially repaired by base excision repair (BER), so reducing the possibility of two base damages being converted to a DSB. However, replication through this clustered lesion can increase the mutation frequency of the base damage. Manipulation of BER by over-expressing the DNA glycosylase responsible for repair initiation can convert the opposing base damage to a DSB. Non-DSB clusters consisting of opposing AP sites (A) are converted to DSBs by Ape1, but the DSBs can be repaired by NHEJ. Evidence also indicates that a Ku-independent pathway can repair the DSBs, and this could be homologous recombination (HR), single strand annealing (SSA) or the back-up NHEJ mechanism (B-NHEJ). Complex DSBs formed from the processing of non-DSB clusters or from high LET radiation are more difficult to repair.

cleaved, whereas human Ape1 activity was inhibited if the lesions were 5′ to each other (Paap et al. 2008). When a cluster of three furans was incubated with Ape1, two opposing furans were cleaved, inferring DSB formation, but the two furans cleaved were the ones situated the furthest distance apart (Malyarchuk et al. 2009). Studying much more complex lesions (3 or 4 base damages and a nucleotide gap), Eot-Houllier et al. (2007) demonstrated that there is a hierarchy for lesion removal using nuclear extracts. Removal/cleavage depended on the type of lesion and the glycosylase required to process the lesion: 5-hydroxyuracil was removed more efficiently than 8oxodG, even when Ogg1 was overexpressed in the extract. Again, the physiological expression level of the repair enzyme does affect whether a damage will be processed prior to one of the other damages in the cluster. The nucleotide gap was also found to be extremely inhibitory for Ogg1 when in close proximity and opposite to an 8oxodG.

The effect on plasmid survival and mutagenicity of complex lesions has been studied in bacteria (Table 4) and yeast (Table 5). In bacteria, loss of plasmid did not occur unless the cluster contained two opposing uracils, which are readily converted to a DSB (Eccles et al. 2010). This finding agreed with the previous studies of bi-stranded two lesion clusters (Table 4). In yeast, 4 and 5 lesion clusters did not form DSBs, and there was no loss of plasmid (Eot-Houllier et al. 2007). This was likely due to the sequential (one at a time) processing of the lesions in the cluster, due to the inhibitory effects of the multiple lesions and repair intermediates, as well as the level of repair enzymes in the cell for the different individual lesions. It is also possible that these complex bi-stranded clusters may have become clusters with lesions on only one strand if repair was so slow that replication occurred. Previous studies in human cells demonstrated that AP site clusters induced by radiation slowly decreased over 14 days, and it was suggested that these repair-resistant clusters may decrease by "splitting" the bi-stranded clusters during replication (Georgakilas et al. 2004). DSB formation of clusters containing 3 or four lesions has also been examined in mouse cells in the absence of replication (Table 5): an 8oxodG reduced DSB production from two opposing furans when the 8oxodG was in tandem and 5′ to one of the furans, and this was due to reduced activity of the mouse AP endonuclease at the furan in tandem to the 8oxodG. DSB formation was not reduced when the 8oxodG was 3′ to one of the furans in the complex cluster (Malyarchuk et al. 2009). DSB formation was also found to occur in mammalian cells from a cluster consisting of three furans (Malyarchuk et al. 2009).

The formation of DSBs from these complex clusters results in the generation of a complex DSB, which typically has oxidative damage situated near the break termini (Fig. 4). It is likely that NHEJ will be required to repair these breaks. In human cells, DNA polymerase λ has

been implicated in the accurate repair of DSBs with 3′ overhangs that are partially complementary. *In vitro*, DNA polymerase λ inserts the correct nucleotide when the overhangs are aligned. When 8oxodG was situated at one terminus, DNA polymerase λ could still perform the "fill-in" reaction. However, DNA polymerase λ was inhibited when 8oxodG was present at both termini or when a thymine glycol was present on only one terminus (Zhou et al. 2008). The ligation step by DNA ligase IV/XRCC4 is also delayed *in vitro* when an 8-oxodG is within 3 bases of a 3′ terminus, or 6 bases of a 5′ terminus on oligonucleotides (Dobbs et al. 2008). In human cells, the re-joining of linear plasmid DNA with an AP site in the 5′ overhang was severely compromised, and the nuclease Artemis was necessary for efficient repair of these complex DSBs (Covo et al. 2009; Table 5). Plasmid that was recovered from these human cell studies was predominantly inaccurately repaired and contained small and large deletions as well as insertions. There was evidence from the sequence of the products that the AP site was either skipped to complete repair, generating a deletion, or was bypassed by a translesion synthesis DNA polymerase. This study in human cells clearly demonstrates that NHEJ can be compromised by oxidative damage near the DSB, and mutagenic repair is used by the cell to complete repair of these complex DSBs. Figure 4 summarizes the present knowledge of how clustered lesions are "repaired" in cells.

RELEVANCE OF CLUSTERED LESIONS TO RADIOTHERAPY

The increase in complexity of the clustered lesions with increasing LET is likely an explanation for the increase in RBE of high LET versus low LET radiation. In fact, unrepaired clustered damages in cycling cells generated from high LET radiation can lead to gross chromosomal rearrangements (Asaithamby et al. 2011). Although the majority of radiotherapy utilizes gamma and X-rays, there are at present 32 hadron therapy treatment centers in the world and 84,492 patients had been treated using hadron radiation as of the end of 2010. There are also 22 treatment centers under construction, which includes centers using carbon ions for treatment as well as protons (Jermann 2011). It is therefore important to unravel how tumor cells repair the more complex clustered lesions, especially complex DSBs, which are predicted to be a high percentage of the damage induced by high LET radiation.

Studies on radiation DNA damage and repair have progressed a long way over the past twenty years. From the studies described above, it is evident that DSBs are no longer believed to be the only potentially lethal lesions. Work has demonstrated not only the existence of radiation-induced clustered lesions, but that the biological consequences of clustered

DNA damage include inhibition of productive BER and NHEJ, enhanced mutagenesis, as well as the conversion of potentially mutagenic base or sugar damage to potentially lethal DSBs. For the single base and sugar lesions generated by both low and high LET radiation, the major repair process is seemingly BER. For DSBs that are generated from non-DSB clusters, Ku-dependent NHEJ has been implicated in the repair response (Malyarchuk et al. 2008). Inhibitors are being developed to radiosensitize tumor cells by preventing NHEJ. However, such inhibition may not be as effective at radiosensitizing cells to high LET radiation, since Ku-dependent NHEJ has already been shown to be impeded by damage induced by high LET radiation (Wang et al. 2008). Specifically, it was proposed that Ku binds to the many small DNA fragments generated by high LET radiation, reducing the probability of Ku being bound to two DNA termini in close proximity (Wang et al. 2008). Therefore, where the conversion of non-DSB clusters to DSBs is predicted to aid in the radiosensitization of cells to low and high LET radiation, inhibiting NHEJ may have a greater effect on radiosensitizing tumor cells to low LET radiation. Homologous recombination has been shown to be important for cell survival after high LET radiation as well (Zafar et al. 2010), and can repair certain types of clustered lesions: homologous recombination can repair a clustered lesion consisting of a DNA gap opposite an abasic site (Adar et al. 2009). Homologous recombination therefore could be a good target for enhancing sensitivity to high LET radiation.

Apart from inhibiting DSB repair, the studies in cells on clustered lesions suggest that manipulation of the BER pathway to generate DSBs or complex DSBs from non-DSB clusters may increase the radiosensitization of cells, and could potentially lead to the design of new complementary treatments to standard radiotherapy. The DNA glycosylase is the first step in the pathway that initiates removal of oxidative base damage. It has already been shown that overexpression of Ogg1 and Nth1 enhances the radiosensitivity of human cells and increases DSB production during initial repair of radiation damage (Yang et al. 2004). Overexpression of bacterial endonuclease III also sensitizes xrs7 cells (NHEJ-defective Chinese hamster ovary cells) to bleomycin sulfate (Harrison et al. 1992), likely by incision at AP sites opposed to a SSB or an AP site. This latter study suggests that a combination of converting non-DSB clusters to DSBs, as well as inhibiting DSB repair could improve tumor cell killing by radiation.

The AP endonuclease is pivotal to BER. This enzyme cleaves at AP sites and has been implicated in DSB production in mammalian cells from closely opposed furans, as well as opposing furans situated near-to other oxidative damage in more complex clusters (Malyarchuk et al. 2008, 2009). Work on clustered lesions would suggest that overexpression of Ape1 in tumor cells would sensitize cells to radiation. However, the majority of

studies examining the effect of Ape1 on radiation sensitivity actually show that overexpression or high expression is related to radioresistance (Naidu et al. 2010; Bobola et al. 2011), while reduction of Ape1 expression sensitizes cells to radiation and bleomycin (Fung and Demple 2011). Interestingly, reduction of Ape1 by siRNA also reduced the number of DSBs produced during the first 2 hours of repair post-irradiation (Fung and Demple 2011), indicating that Ape1 does produce DSBs from non-DSB clusters. However, the overexpression studies indicate that the "good" repair functions of Ape1 have a greater impact on cell survival following radiation. Ape1 is also known to remove 3′ blocking termini from strand breaks generated by ionizing radiation, as well as having a redox function in the cell. However, the radiosensitization caused by decreasing Ape1 was related to the DNA repair activities (Fung and Demple 2011), as resistance to radiation and bleomycin as well as DSB production post-irradiation in siRNA treated cells could be restored by Apn1; Apn1 is the major yeast AP endonuclease and does not have the redox activities of Ape1. Small molecule inhibitors of Ape1 are being developed for use with standard cancer treatments to enhance tumor cell killing (see Chapter 8).

The later stages of BER include short patch repair and long patch repair. Inhibition of the enzymes involved in completing repair of the SSB-repair intermediates would also be predicted to enhance DSB formation from non-DSB clusters. Moreover, a shift to long patch repair could "break-down" the channeling of repair intermediates, providing a greater probability that DSBs or complex DSBs will be produced by BER. Inhibition of DNA polymerase β would seem attractive and inhibition does sensitize cells to certain compounds such as MMS. However, DNA polymerase β null cells are not sensitive to ionizing radiation (Sorbol et al. 1996), and loss of DNA polymerase β in mouse cells did not promote the conversion of opposing uracils to DSBs (Sage and Harrison 2011). These latter experiments would therefore suggest that DNA polymerase β would not be a good target to enhance tumor radiosensitivity.

It is clear from all the experiments described here that understanding exactly which repair pathways are important for clustered lesion repair and knowing what each repair enzyme does in cells post-irradiation is very critical for determining the best targets for the development of complementary therapeutic strategies. What is clear, however, is that increasing certain repair activities to generate more damage from clustered lesions may need to be considered as well as the development of repair inhibitors to achieve the final outcome of enhancing tumor cell radiosensitivity.

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ABBREVIATIONS

DSB	:	double strand break
LMDS	:	locally multiply damaged sites
SSB	:	single strand break
LET	:	linear energy transfer
BER	:	base excision repair
NER	:	nucleotide excision repair
HR	:	homologous recombination
NHEJ	:	non-homologous end joining
8oxodG	:	8-oxo-7,8-dihydro-2′-deoxyguanine
TG	:	thymine glycol
DHU	:	dihydrouracil
AP	:	abasic site
5OHC	:	5-hydroxycytosine
5OHU	:	5-hydroxyuracil
5OHMeU	:	5-hydroxymethyluracil
FAPYG	:	2,6-diamino-4-hydroxy-5-formamidopyrimidine
8oxoA	:	8-oxo-7,8-dihydroadenine
AP	:	abasic
L	:	2-deoxyribonolactone (oxidized abasic site)
dF	:	formylamine
furan	:	tetrahydrofuran
U	:	uracil
5mC	:	5 methylcytosine
^	:	intrastrand crosslink
DHT	:	5,6-dihydrothymine
P	:	phosphate
PG	:	phosphoglycolate
EndoIII	:	endonuclease III
EndoIV	:	endonuclease IV
Fpg	:	formamidopyrimidine DNA glycosylase
Ogg1	:	human Ogg1
Ape1	:	human major AP endonuclease
PNKP	:	polynucleotide kinase phosphatase

ExoIII	:	exonuclease III
mOgg1	:	mouse Ogg1
yOgg1	:	yeast Ogg1

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CHAPTER 5

Chemotherapeutic Alkylating Agents in Cancer Treatment

Tracey D. Bradshaw

INTRODUCTION

The history of alkylating agent chemotherapy dates to the First World War. The chemical warfare agent sulphur mustard (mustard gas; Fig. 1), first used in September 1917, was one of the most lethal of all the poisonous chemicals used during the war. However, soldiers (and civilians) exposed to sulphur mustard developed bone marrow suppression and lymphoid aplasia (Krumbhaar and Krumbhaar 1919). These medical observations led to evaluation of sulphur mustard as an antitumour agent, and in December 1942, to secret human clinical trials of its nitrogen-based less toxic analogue—nitrogen mustard (chlormethine)—to treat patients with high white blood cell counts (lymphoid leukaemia) and lymphomas (Gilman 1963). Chlormethine became the pioneer of antineoplastic chemotherapy. Thus, alkylating agents are the oldest class of anticancer agents and remain a major cornerstone today of treatment for leukaemias, lymphomas and solid tumours.

Alkylating agents form highly reactive intermediate compounds that are able to establish covalent bonds with DNA, alkylating specific sites on purine bases; the primary alkylation site being *N7*-guanine (Fig. 2). Guanine bases in DNA possess greater negative molecular electrostatic potential than other bases (Pullman and Pullman 1981), and the atom with the most negative potential is *N7* guanine. Moreover, the electronegativity of *N7*

Centre for Biomolecular Sciences, University of Nottingham, Nottingham, NG7 2RD, UK; Email: Tracey.bradshaw@nottingham.ac.uk

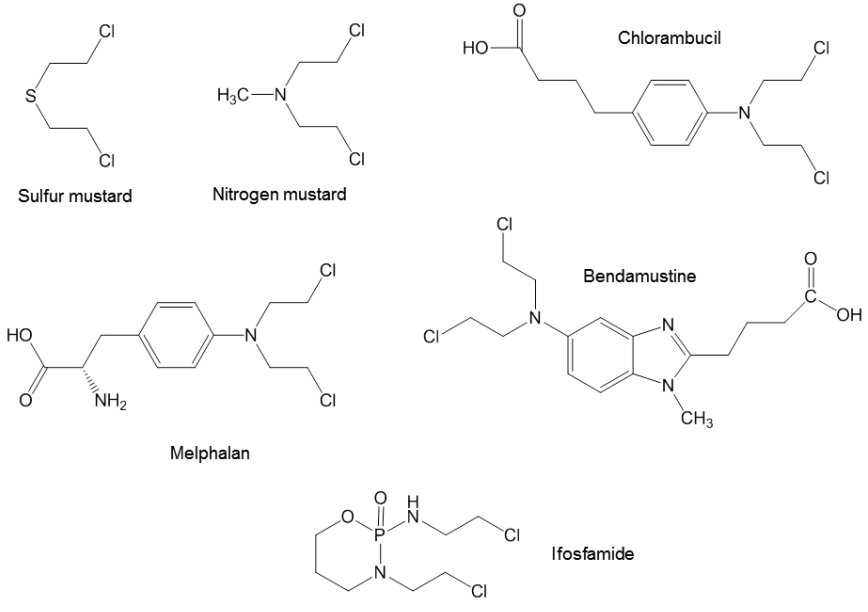

Figure 1. Chemical structures of nitrogen mustard analogues.

guanine is enhanced by neighbouring guanines, explaining why this atom is most frequently, but not exclusively, alkylated by a number of electrophilic alkylating species (Richardson et al. 1987). Alkylation can result in miscoding of DNA strands, incomplete repair of alkylated segments (leading to strand breakage or depurination), excessive cross-linking of DNA, and inhibition of strand separation at mitosis. Monofunctional alkylating agents transfer a single alkyl group and usually give rise to miscoding base modifications in DNA, strand breakage, or depurination resulting in cell death. Bifunctional alkylating agents possess two electrophilic sites typically causing intra- or inter-strand cross-linking and inhibition of mitosis with consequent cell death. Alkylating agents can also react with carboxy, sulfhydryl, amino, phosphate and hydroxyl groups of cellular components, and are potentially carcinogenic, mutagenic and teratogenic (Bautz and Freese 1960; Kyrtopoulos et al. 1997; Platzek and Bochert 1995; Pletsa et al. 1997; Van Duuren et al. 1974). Testicular and ovarian failure is a frequent long term consequence of alkylating agent chemotherapy, while acute leukaemia may arise more rarely. Mechanisms of resistance to one alkylating agent often impart resistance to other similar drugs and can be caused by increased production of nucleophilic substances (glutathione (GSH) synthesis and glutathione-S-transferase (GST)) that compete with the target DNA for alkylation. Decreased intracellular permeation of alkylating agents and

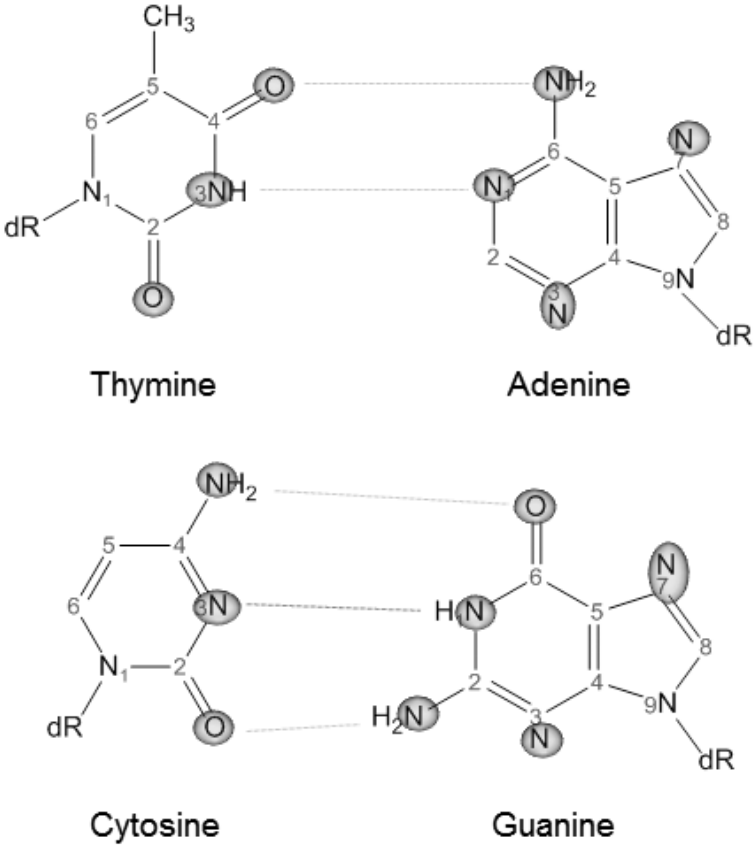

Figure 2. DNA base sites susceptible to alkylating agent attack.

increased (or disabled) activity of DNA repair processes are also common mechanisms conferring alkylating agent resistance (Hill et al. 1971; Zhang et al. 2010).

Today, several classes of alkylating agents are used to treat cancer. Within this Chapter, each class, with examples, will be considered briefly, before more detailed discussion of one particular member of the Trizene alkylating agent class—Temozolomide.

NITROGEN MUSTARDS

Nitrogen mustards are bifunctional alkylating agents. The intramolecular displacement of chlorine by the amine nitrogen atom leads to formation of the highly electrophilic aziridinium ion which attacks nucleophilic centres on DNA bases (Mann 2010). Alkylation occurs most frequently

on *N7* of guanine, but adducts also form at *O6* and *N3* of guanine, *N7*, *N3* and *N1* of adenine, and *N1* and *N3* of cytosine (Brookes and Lawley 1961; Maccubbin et al. 1989; Osborne and Lawley 1993). A further attack, following displacement of the second chlorine, comprises the second alkylation step resulting in DNA cross-links (Silverman et al. 2004; Balcome et al. 2004). It has clearly been demonstrated that nitrogen mustards form 1,3 inter-strand cross-links in the 5′ d(G-C) sequence (Rink et al. 1993; Dong et al. 1995; Bauer et al. 1997). DNA inter-strand cross-links are highly cytotoxic, and block fundamental metabolic processes, inhibit DNA replication and terminate transcription, leading to apoptosis (Masta et al. 1994) (Francisco 2008) (see Chapter 10).

Chlormethine: Chlormethine or mechlorethamine (tertiary amine methylbis(2-chloroethyl)amine; bis(2-chloroethyl)-N-methylamine (mustine or nitrogen mustard); Fig. 1) is part of chemotherapy combination regimens for treatment of Hodgkin's disease, non-Hodgkin's lymphoma (NHL), and as palliative chemotherapy in lung and breast cancers and skin lesions of mycosis fungoides (cutaneous T-cell lymphoma) (Kim et al. 2003). Adverse reactions to chlormethine therapy include bone marrow suppression—leukopaenia and thrombocytopaenia. Acquired resistance to chlormethine can emerge, a consequence of increased drug inactivation and decreased intracellular drug uptake.

Chlorambucil: Chlorambucil or Leukeran (4-[bis(2-chlorethyl)amino] benzenebutanoic acid; Fig. 1) was first synthesised in 1953 (Everett et al. 1953). Substituted on the nitrogen atom of chlorambucil is an electron-withdrawing group which reduces the nucleophilicity of the nitrogen rendering the molecule less reactive. Chlorambucil is used for treatment of chronic lymphocytic leukaemia (CLL) and primary (Waldenstrom's) macroglobulinaemia, and may be used for treatment of follicular lymphoma (Manoharan 2004). In CLL, chlorambucil maintenance chemotherapy is often required to sustain clinical remission. Indeed, chlorambucil, either alone or in combination has been accepted as treatment of choice for patients with advanced stage disease for many years. Alkylating agent chemotherapy (specifically chlorambucil or cyclophosphamide) is the mainstay treatment for B-cell CLL, alone or in combination with prednisone, frequently yielding response rates >70% (Oken et al. 2004). Successive cycles of chlorambucil may lead to its accelerated metabolism, decreasing drug bioavailability. The most common adverse reaction to chlorambucil treatment is myelosuppression; seizures are rarely reported as a further form of acute toxicity.

Melphalan: Melphalan (4-[bis(2chloroethyl)amino]-L-phenylalanine; Fig. 1) is a phenylalanine derivative of nitrogen mustard. The electron withdrawing group on the nitrogen again reduces the nucleophilicity and reactivity of

this bifunctional alkylating agent. Oral melphalan, in combination with prednisone, has been the standard treatment for multiple myeloma since 1968 (Alexanian et al. 1968), resulting in a response rate of 50%. Recently, bortezomib (Velcade) has been added to this regimen (www.cancer.net). Melphalan has also been given as treatment for breast and ovarian carcinoma, neuroblastoma, Hodgkin's disease, polycythaemia vera, and by intra-arterial regional perfusion, for malignant melanoma and soft tissue sarcoma (Pinguet et al. 2000). The adverse effects of melphalan are typical of alkylating agents and mainly haematological.

Bendamustine: The nitrogen mustard group at position 5 of the benzimidazole nucleus together with a butanoic acid residue at position 2 combines the features of an alkylating agent with those of purine and amino acid analogues (Schwanen et al. 2002) (Fig. 1). Bendamustine (4-[5-[bis-(2-chloroethyl)amino]-1-methyl-1H-benzimidazol-2-yl]butanoic acid) was first synthesized in 1963 by Ozegowski and Krebs in the former German Democratic Republic, and until 1990 it was available only in East Germany. East German investigators found that bendamustine was efficacious in treatment of CLL, Hodgkin's disease, NHL, multiple myeloma and lung cancer; this agent first received marketing approval in Germany, where it was employed as a single-agent or in combination chemotherapy for indolent NHL, multiple myeloma, and CLL (Kath et al. 2001; Ujjani and Cheson 2010).

In March 2008, Cephalon received approval from the United States Food and Drug Administration (FDA) to market bendamustine in the U.S. for treatment of CLL. Results from a phase III trial suggested that the standard initial treatment for indolent types of B-cell lymphoma should be changed as patients receiving bendamustine and rituximab lived significantly longer without disease progression, were less likely to experience major toxicities, and were more likely to experience complete responses than patients receiving standard first line chemotherapy [www.cancer.gov/clinicaltrials/results]. In October 2008, further approval was granted to market bendamustine (Treanda) for treatment of indolent B-cell NHL that progresses during or within six months of treatment with rituximab or a rituximab-containing regimen (Dennie and Kolesar 2009). Recently, a multicentre study demonstrated that a bendamustine, bortezomib and rituximab combination regimen was highly active in patients with follicular lymphoma (Fowler et al. 2011). Common adverse reactions to bendamustine are typical for alkylating agents, and include nausea, fatigue and vomiting as well as immunosuppression, anemia, and low platelet counts.

Cyclophosphamide: Cyclophosphamide (2-[bis(2-chloroethyl)amino] perhydro-1,3,2-oxazaphosphorinane 2-oxide-monohydrate; Fig. 3) is one of the most widely used cytotoxic chemotherapeutic agents administered

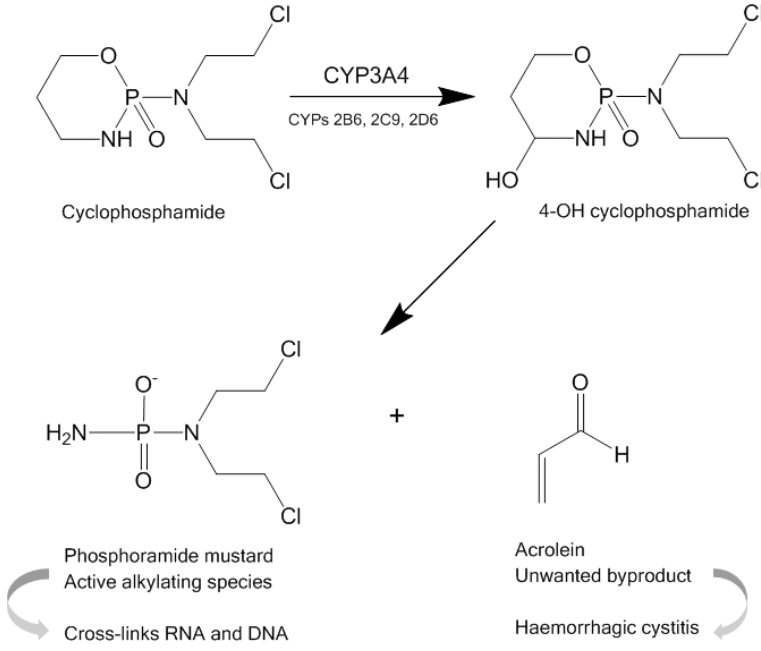

Figure 3. Activation of cyclophosphamide.

in combination or sequentially with other antineoplastic agents. It is used for treatment of Burkitt's, non-Hodgkin's and cutaneous T-cell lymphomas, acute- and chronic myeloid leukaemia (AML; CML), ALL and CLL, multiple myeloma, mycosis fungoides, thymoma, childhood malignancies (including neuroblastoma, retinoblastoma, Wilms' tumour and Ewing's sarcoma), osteosarcoma, soft tissue sarcoma and gestational trophoblastic tumours. It is also administered for treatment of solid malignancies of the bladder, brain, breast, cervix, endometrium, lung, ovary and testis (Emadi et al. 2009). In addition to treatment for cancer, cyclophosphamide is used in management of autoimmune disorders and as an immunosuppressant to control organ transplant rejection (Kanzler et al. 1997; Thone et al. 2008).

Cyclophosphamide is a prodrug requiring biotransformation by hepatic cytochrome P450 enzymes (CYPs), including CYPs 3A4, 3A5, 2C9, 2B6 and 2D6. In an initial reaction, carbon 4 of the oxazaphosphorine ring is hydroxylated producing 4-hydroxycyclo-phosphamide (4-OH-CPA). 4-OH-Cyclophosphamide breaks down by spontaneous β elimination to release the active alkylating species phosphoramide mustard and the unwanted by-product acrolein (Fig. 3) (Boddy and Yule 2000; Ohno and Ormstad 1985). Cyclophosphamide's cytotoxicity is mainly a consequence of DNA and RNA cross-links within the tumour cell. Clinically relevant

adverse reactions include myelosuppression (dose-limiting), cardiac dysfunction and haemorrhagic cystitis, caused by acrolein, which may develop after high dose or prolonged use and may be life-threatening. Because cyclophosphamide activation and elimination are dependent upon metabolic reactions, there is wide inter-individual variation and also drug interaction complications. As a consequence, numerous treatment doses and schedules exist, dependent upon disease, patient condition, concomitant therapy and response. Like all alkylating agents, cyclophosphamide possesses carcinogenic, mutagenic and teratogenic potential. Secondary malignancies have been recorded in patients, often several years after initial alkylating agent therapy.

Ifosfamide: Ifosfamide (3-(2-chloroethyl)-2-(2-chloroethylamino)perhydro-1,3,2-oxazaphosphorinane 2-oxide; Fig. 1), a structural analogue of cyclophosphamide, is used clinically for treatment of adult solid tumours including cancer of the cervix, testes, breast, ovary and lung, neuroblastoma, Ewing's sarcoma and Hodgkin's disease. Only in soft tissue sarcoma is ifosfamide a first-line treatment. Ifosfamide is also used in the treatment of paediatric solid tumours, usually combined in multi-drug regimens (Boddy and Yule 2000). As with cyclophosphamide, the mechanism of action of ifosfamide requires metabolic activation by hepatic microsomal enzymes including CYPs 3A4, 3A5, 2C9 and 2B6. CYP-mediated hydroxylation and subsequent spontaneous degradation produce the alkylating agent isophosphoramide mustard and also acrolein, which is responsible for haemorrhagic cystitis, dysuria and haematuria. Such urinary tract toxicities may be more severe with ifosfamide (compared with cyclophosphamide); thus, the uroprotective agent mesna (ethanesulfonic acid), a mercaptan which scavenges and inactivates reactive acrolein, is co-administered with ifosfamide. An additional CYP-mediated biotransformation route yields inactive ifosfamide metabolites 2- and 3-dechloroethylifosfamide and chloroacetaldehyde—a neuro and nephrotoxin (Zhou et al. 2006). As with cyclophosphamide, drug interactions must be considered when designing ifosfamide drug doses and schedules (Groninger et al. 2004).

NITROSOUREA DERIVATIVES

The anticancer activity of this class of compounds was discovered in a large scale random screening programme performed at the U.S. National Cancer Institute (NCI) in 1959; nitrosoureas possess activity against solid and non-solid tumours. Their lipophilicity allows efficient access across the blood brain barrier, providing higher cerebrospinal fluid (CSF): plasma nitrosourea ratios in comparison to other alkylating agents (McCormick and McElhinney 1990). Upon introduction into the body, nitrosoureas

rapidly undergo spontaneous hydrolysis in aqueous solution, releasing electrophilic species that are able to chloroethylate or carbamoylate DNA (Gnewuch and Sosnovsky 1997). Despite frequent use, the therapeutic efficacy of nitrosoureas is limited by emergence of resistance, which involves multiple mechanisms. *O6*-Methylguanine-DNA methyltransferase (MGMT; *O6*-alkylguanine-DNA alkyltransferase; AGT) is responsible for direct repair of alkylated *O6*-guanine (see Chapter 6), mediating nitrosourea resistance (Gerson and Trey 1988; Zhang et al. 2001). In glioma cells, overexpression of nitric oxide synthase mediated by S-nitrosoglutathione —a potent antioxidant derived from nitric oxide and GSH—confers resistance against carbamoylating lesions (Hsu et al. 2001). Neutralisation of S-nitrosoglutathione with the selective cuprous ion chelator neucoprane abolished resistance (Yang et al. 2004).

Carmustine: Carmustine (1,3-bis(2-chloroethyl)-1-nitrosourea; BCNU; Fig. 4) was the first nitrosourea extensively developed. It is used alone or in adjuvant therapy to treat brain, colon and lung cancers, Hodgkin's and NHL, melanoma, multiple myeloma, and mycosis fungoides (Barrie et al. 2005; Batts et al. 2007; Brandes et al. 2004). Both the antineoplastic and toxic effects of carmustine are caused by its active metabolites. The chloroethyl carbonium ion leads to formation of DNA cross-links during all phases of the cell cycle, resulting in cell cycle arrest and apoptosis (Lown and

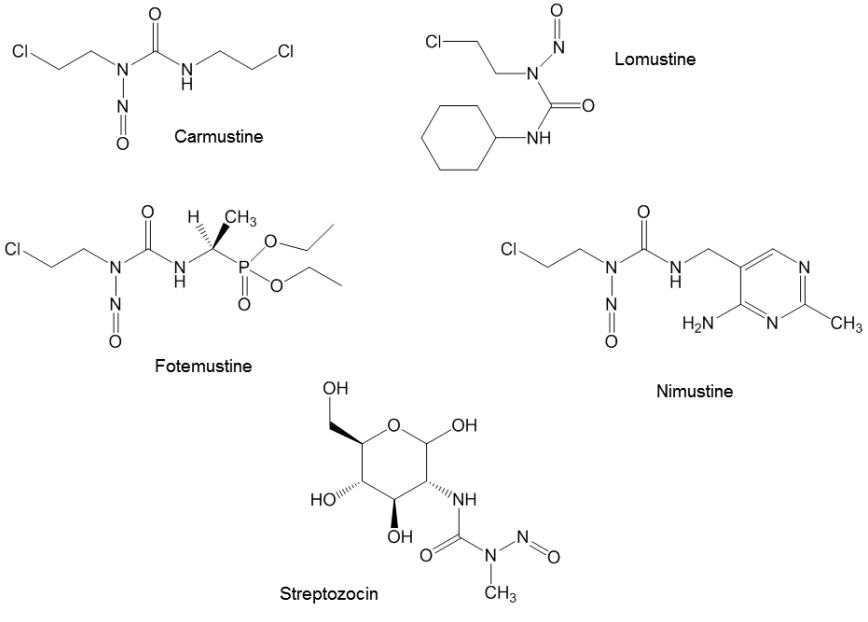

Figure 4. Chemical structures of nitrosourea derivatives.

Chauhan 1981). The most serious adverse reaction to carmustine therapy is cumulative bone marrow suppression; renal and hepatic damage, as well as pneumonitis leading to pulmonary fibrosis, may also occur (Lena et al. 1994). Novel approaches to improve carmustine efficacy have been explored, including gradual release and locally implanted carmustine-impregnated wafers that deliver high dose treatment directly to diseased areas, overcoming the blood brain barrier. Older patients with GBM have been shown to derive significant benefit from such therapy (Chaichana et al. 2011). However, this approach is not without adverse reactions including intracranial infection, cerebral oedema and brain swelling.

Resistance to carmustine is associated with MGMT expression. Combination chemotherapy of *O6*-benzylguanine (*O6*-BG; a substrate which depletes MGMT) with carmustine has been studied in clinical trials in patients with advanced soft tissue sarcoma and malignant melanoma; disappointingly, no improvement in clinical outcome was seen, whereas such treatment was associated with significant myelosuppression (Ryan et al. 2006). Although carmustine does not generally share cross-resistance with other alkylating agents, cross-resistance between carmustine and lomustine has been detected.

Lomustine: Lomustine (1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; CCNU; Fig. 4) is used for treatment of primary and metastatic brain tumours, breast and lung cancer, Hodgkin's lymphoma and melanoma. It alkylates DNA and RNA, cross-links DNA and carbamoylates DNA, resulting in inhibition of DNA and RNA synthesis and disruption of RNA processing. In addition, lomustine inhibits several enzymes by carbamoylation, including DNA repair enzymes (Lemoine et al. 1991). The most serious toxicity associated with lomustine therapy is delayed myelosuppression; pulmonary toxicity, characterised by pulmonary infiltrates and fibrosis, and reversible hepatic toxicity have also been reported (Francisco 2008). Current randomised phase II clinical trials are assessing the efficacy of bevacizumab and lomustine in patients with first recurrence glioblastoma multiforme (GBM; http://clinicaltrial.gov).

Fotemustine and Nimustine: Fotemustine ((1-[3-(2-chloroethyl)-3-nitrosoureido]ethyl)phosphonate; Fig. 4) is a third generation chloroethylnitrosourea that has significant antitumour efficacy in metastatic malignant melanoma with cerebral lesions. Adverse reactions include delayed, but reversible, neutropaenia and thrombocytopaenia (Avril et al. 2004). Nimustine (3-[(4-amino-2-methylpyrimidin-5-yl)methyl]-1-(2-chloroethyl)-1-nitrosourea; ACNU; Fig. 4) is a water soluble nitrosourea analogue, discovered in 1974 and licensed for treatment of brain tumours and brain metastases from lung and colorectal carcinomas (Sugiyama et al. 2007, 2008).

Streptozocin: Streptozocin (2-deoxy-2-[[(methylnitrosoamino)carbonyl] amino]-D-glucose; Fig. 4), a glucosamine-nitrosourea isolated from *Streptomyces achromegenes* in 1956, is an antitumour antibiotic. In 1965, Evans et al. reported that streptozocin induced rapid degranulation of islet beta cells, causing permanent diabetes mellitus. Streptozocin is therefore used alone or in combination with other antineoplastic agents to treat pancreatic endocrine (islet-cell) tumours (Kouvaraki et al. 2004). The presence of the D-glucopyranose moiety confers enhanced streptozocin uptake by pancreatic islet cells. Although streptozocin itself is unable to cross the blood brain barrier, its metabolites have been detected in CSF. *In vivo*, streptozocin spontaneously decomposes to produce reactive methylcarbonium ions which alkylate DNA and cause inter-strand DNA cross-links (Bolzan and Bianchi 2002). Like other nitrosoureas, following *in vivo* decomposition, streptozocin may carbamoylate proteins and nucleic acids. However, as streptozocin lacks a chloroethyl group, it is considered a monoalkylating agent (unlike other nitrosoureas). Also, unlike typical nitrosoureas, streptozocin causes little myelosuppression, although cumulative nephrotoxicity and occasionally hepatotoxicity may be severe (Francisco 2008).

ALKYL SULFONATES

Alkyl sulfonates are esters of alkane sulfonic acids with the general formula $R\text{-}SO_2\text{-}O\text{-}R'$.

Ethyl methanesulfonate: Ethyl methanesulfonate (EMS; $CH_3SO_3C_2H_5$; Fig. 5) is a mutagenic (teratogenic, and possibly carcinogenic) organic compound (Yang et al. 2001). The ethyl group of EMS reacts with guanine in DNA, forming *O6*-ethylguanine. During DNA replication, DNA polymerases frequently mispair *O6*-ethylguanine with thymine. Following subsequent rounds of replication, the original G:C base pair can become an A:T pair. This point mutation is often deleterious to cells.

Methyl methanesulfonate: Methyl methanesulfonate (MMS; $CH_3SO_3CH_3$; Fig. 5) is also a carcinogenic alkylating agent. It methylates DNA at *N7*-guanine and *N3*-adenine (Grzesiuk 1998), stalling DNA replication forks and inducing DNA double strand breaks (DSBs). Repair of DNA DSBs by homologous recombination (HR) (see Chapter 14) protects cells from replication-associated DSBs caused by MMS; hence, HR-deficient cells are especially vulnerable to the effects of MMS (Nikolova et al. 2010).

Busulfan: Busulfan (tetramethylene dimethanesulfonate; 1,4-butanediol dimethanesulfonate; Fig. 5) is a common alkyl sulfonate antineoplastic bifunctional alkylating agent used during management and treatment

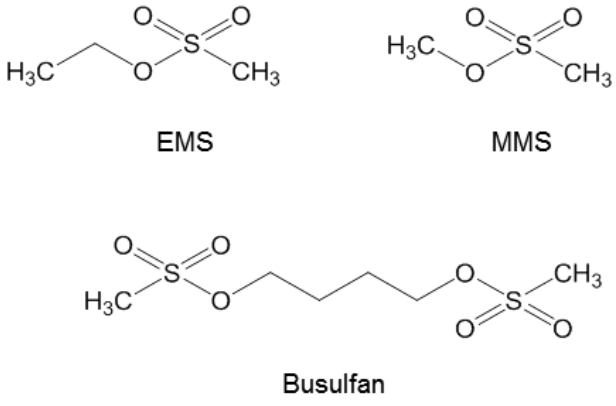

Figure 5. Chemical structures of alkylsulfonates.

of CML. Indeed, it was the mainstay of CML chemotherapy before being displaced by imatinib (glivec). As busulfan delivers a selective immunosuppressive effect on bone marrow, and is able to control tumour burden, it is used as palliative treatment for CML, providing symptomatic relief and reducing spleen size. Clinical improvement is temporary however, as resistance to these beneficial effects emerges (Francisco 2008). Busulfan, in combination with other agents, particularly cyclophosphamide, is one of the most frequently used drugs administered in high dose preparative chemotherapy regimens for myeloid leukaemia patients undergoing haematopoietic stem cell transplantation (Deeg et al. 2000; Socie et al. 2001). Currently, there is a phase II trial underway to investigate the efficacy of high dose busulfan with cyclophosphamide prior to autologous stem cell transplant in patients with multiple myeloma (www.clinicaltrials.gov). High dose thiotepa, busulfan and cyclophosphamide is an effective chemotherapeutic regime prior to haematopoietic stem cell transplantation in childhood myeloid malignancies (Worth et al. 1999). Studies have been undertaken examining the effects of busulfan treatment during the chronic phase of CML (Chabner 2006).

In aqueous conditions, busulfan hydrolyses, and the 2 methanesulfonate groups are released from the alkyl chain to rapidly produce reactive carbonium ions, which are responsible for DNA alkylation. Alkylation causes guanine-adenine intra-strand cross-links through a bimolecular nucleophilic substitution (SN2) reaction in which *N*7-guanine attacks the carbon atom adjacent to the mesylate leaving group. (Iwamoto et al. 2004). The resulting DNA strand breaks and intra-strand cross-links interrupt DNA replication and RNA transcription, and are often irreparable forcing the cell to undergo apoptosis (Karstens and Kramer 2006). Myelosuppression is the dose-limiting side effect of busulfan, manifesting as leukopaenia,

thrombocytopaenia and occasionally anaemia. Rarely, progressive interstitial pulmonary fibrosis (busulfan lung) may occur after prolonged treatment. Busulfan is potentially carcinogenic, mutagenic and teratogenic and has been associated with the development of acute leukaemias (Bishop and Wassom 1986).

HYDRAZINE DERIVATIVES

Procarbazine: Methylhydrazine procarbazine (*N*-isopropyl-α-(2-methylhydrazino)-*p*-toluamide hydrochloride; Fig. 6) was originally synthesised as a putative monoamine oxidase inhibitor, but is an antineoplastic agent with multiple sites of activity. Its mechanism of action is complex and has not been fully elucidated. After oral administration, procarbazine is oxidised by erythrocyte and hepatic P450 enzymes to azoprocarbazine with the release of H_2O_2 (which may be responsible for some of the drug's action). Azo-procarbazine can be *N*-oxidised to form isomeric methylazoxy and benzylazoxy products; the methylazoxy derivative can spontaneously give rise to a methylating species (most likely the methyldiazonium cation $CH_3N_2^+$), which directly damages DNA through alkylation (Dunn et al. 1979). Free radical intermediates may also be involved in the cytotoxicity of procarbazine (Chabner 2006; Moloney et al. 1985). This drug is used in combination chemotherapy regimens for treatment of Hodgkin's lymphoma, where it has made an important contribution to long term survival of patients with this disease (Richardson and McNamara 2011; Scholz et al. 2011; Viviani et al. 2011). Other uses include treatments for GBM, NHL and small-cell lung cancer (Francisco 2008).

Laromustine: Laromustine (cloretazine; 2-(2-chloroethyl)-N-methyl-1,2-bis(methylsulfonyl)hydrazine carboxamide; Fig. 6) is a novel antitumour sulfonylhydrazine prodrug which generates both chloroethylating (1,2-bis(methylsulfonyl)-1-(2-chloroethyl)hydrazine) and carbamoylating (1,2-bis(methylsulfonyl)-1-[(methylamino)carbonyl]hydrazine) species.

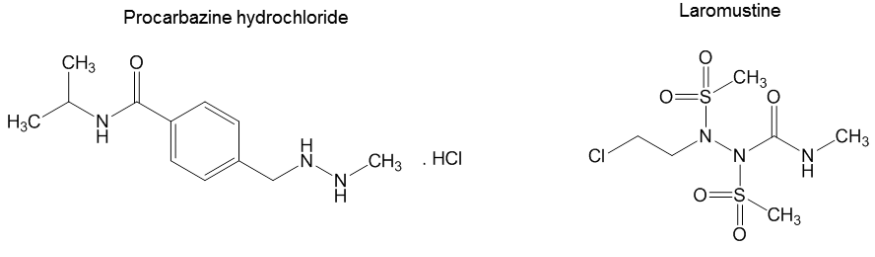

Figure 6. Chemical structures of hydrazine derivatives.

Laromustine releases chloroethylating species after entering the blood stream, and these reactive species attack the *O6* position of guanine, resulting in DNA cross-linking, strand breaks, chromosomal aberrations, and disruption of DNA synthesis. The carbamoylating species is responsible for inhibition of RNA, DNA and protein syntheses (Ishiguro et al. 2006). Intracellular metabolism of laromustine also releases methyl isocyanate, which inhibits MGMT (the enzyme responsible for direct repair of *O6*-alkyl guanine; see Chapter 6) (Pigneux 2009). Laromustine has shown efficacy in elderly patients (≥ 60 years) with previously untreated AML (Giles et al. 2009; Giles 2009; Schiller et al. 2010), a disease for which prognosis is poor and there is no standard of care.

ETHYLENE IMINES

Ethylene imine or aziridine (C_2H_5N) is a 3-membered heterocycle containing one amine group and 2 methylene groups. The bond angles in this heterocycle are approximately 60°, considerably less than the normal hydrocarbon bond angle of 109.5°, resulting in angle strain. A consequence of this bond strain is that aziridines are reactive substrates in ring opening reactions with many nucleophiles.

Thiotepa: Thiotepa (Tris(1-aziridinyl)phosphine sulphide; N,N'N'-triethylenethiophosphoramide; Fig. 7) is a trifunctional member of the ethylene imine alkylating agent family (Maanen et al. 2000). Approved by the FDA in 1959, thiotepa is a broad spectrum antitumour agent used in the treatment of breast, ovarian and bladder cancers.

Thiotepa is administered for palliation of a variety of adult neoplasms, including breast and ovarian adenocarcinoma, as well as control of intra-cavity effusions secondary to neoplastic disease (www.adienne.com). During phase 1 pharmacokinetic re-evaluation of thiotepa, 3 out of 9 ovarian cancer patients responded to treatment. All patients had advanced disease, including liver, chest wall and peritoneal cavity metastases, and had received multiple prior chemotherapy regimens (O'Dwyer et al. 1991).

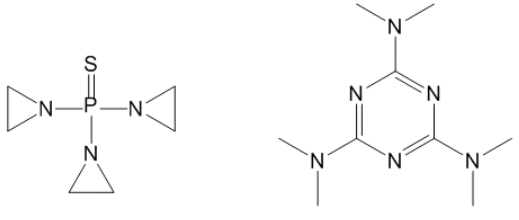

Figure 7. Chemical structures of ethyleneimines.

Long term intrapericardial treatment with thiotepa dramatically increased the quality of life of breast cancer patients, improving survival and disease natural history (Martinoni et al. 2004).

N7-Alkylation of guanine (and to a lesser extent of adenine) by thiotepa to form *N7*-aminoethylguanine and -aminoethyladenine proceeds its metabolism in the liver to triethylenephosphoramide (Gill et al. 1996). Thiotepa is also implemented with or without total body irradiation as a conditioning treatment prior to allogenic or autologous haematopoietic progenitor cell transplantation in adult and paediatric haematological diseases. Its radiomimetic action is believed to occur through release of ethylen imine radicals, which then disrupt DNA bonds through DNA alkylation, severing links between purine bases and the sugar and thereby liberating alkylated purines (www.drugs.com). Because of dose-limiting myelosuppression, thiotepa was largely replaced as a therapeutic by the nitrogen mustards (Soloway and Ford 1983). However, more recently, thiotepa has experienced renewed interest as one of the most effective anticancer drugs in high dose regimens (Huitema et al. 2002).

Altretamine: Altretamine (hexamethylmelamine Fig. 7) requires biotransformation by N-demethylation to produce reactive intermediates that covalently bind to and damage DNA (Keldsen et al. 2003). Its clinical use is in the treatment of alkylating agent-resistant ovarian cancer.

PLATINUM AGENTS

Cisplatin: The serendipitous discovery of the anticancer properties of cisplatin (cis-diamminedichloroplatinum (II); CDDP; Fig. 8) in the 1960s (Rosenberg et al. 1969) and its subsequent rapid clinical introduction resulted in major improvements in clinical outcome of patients with testicular and choriocarcinoma and has had a radical impact on treatment of other malignancies including ovarian, bladder and genitourinary cancers. Indeed, cisplatin, together with bleomycin (an antitumour antibiotic) and vinblastine (an anti-microtubular antineoplastic agent) comprises a curative therapeutic combination for non-seminomatous testicular cancer. The first patient received cisplatin in 1971, and FDA approval followed just 7 years later (Kelland 2007).

Although the platinum agents have been designated alkylating agents, they lack alkyl groups and so do not perform alkylating reactions. Cisplatin and analogs are thus more correctly classified as alkylating-like. The mechanism of action of cisplatin involves intracellular activation through aquation—the intracellular chloride ion concentration (< 100 mM) allows replacement of one chloride leaving group with water. Cisplatin may then covalently bind DNA purine bases, specifically *N7*-guanine and to a lesser

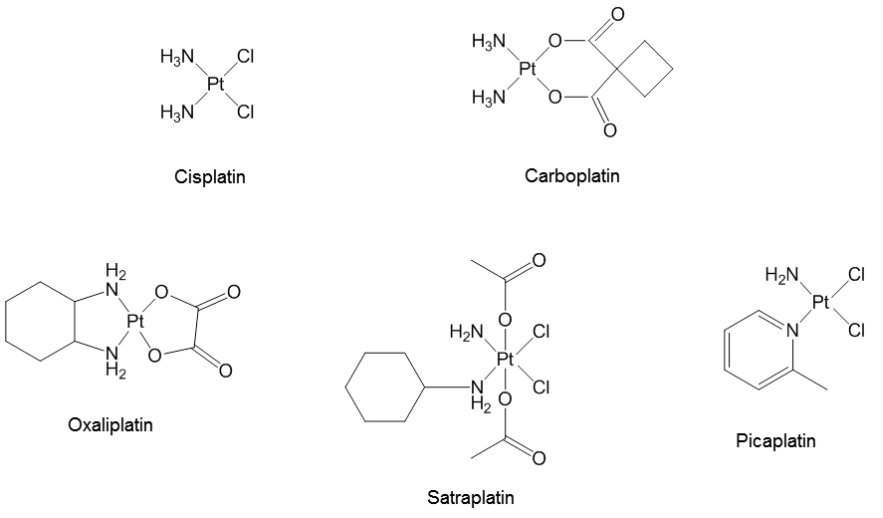

Figure 8. Chemical structures of platinum alkylating agents.

degree *N7*-adenine, to form monofunctional adducts (Olinski and Walter 1984). Cross-linking (e.g., of *N7*-PtClG) occurs following displacement of the remaining chloride group, typically by another guanine. This DNA strand crosslink ultimately inhibits DNA synthesis, triggering apoptosis. Adjacent *N7*-guanine intra-strand cross-links represent the major DNA lesion (60–65%), with adjacent adenine-guanine intra-strand cross-links comprising 20–25% of the remaining adducts. Infrequently, intra-strand guanine-guanine adducts with an unaffected base in between and monofunctional guanine adducts will form (each contributing approximately 2% of the total lesions). Inter-strand cross-links between *N7*-guanine bases on opposite strands comprise the remainder of DNA adducts (approximately 2%); interactions with cellular proteins also occur (Kelland 2007). Despite potent and therapeutic antitumour activity, the associated severe adverse reactions to cisplatin, including significant renal dysfuction and gastrointestinal toxicity, inspired development of platinum analogues with reduced kidney toxicity and improved oral bioavailability.

Carboplatin: Carboplatin (cis-diammine-[1,1-cyclobutanedicarboxylato] platinum(II); Fig. 8) was designed on the basis that a more stable leaving group than chloride might lower toxicity without impacting antitumour activity. This hypothesis was indeed correct, as carboplatin is essentially devoid of nephrotoxicity, is less toxic to the gastrointestinal tract and is less neurotoxic. In 1989, the compound was first approved for treatment of ovarian cancer. Although DNA adducts formed by carboplatin are the same as those generated by cisplatin, the rate of adduct formation is

slower. Thus, between 20- and 40-fold higher carboplatin concentrations are required for treatment efficacy (Knox et al. 1986). Randomised clinical trials showed equivalent survival rates for ovarian cancer patients receiving either cisplatin or carboplatin (Aabo et al. 1998), and a carboplatin-based therapeutic regime is now the standard of care for patients diagnosed with ovarian cancer.

Resistance to platinum therapy—intrinsic (e.g., colorectal carcinoma) or acquired (e.g., ovarian cancer), conferred by a number of mechanisms —impedes successful clinical outcomes for many patients. Preventing interaction between platinum and its target DNA are efflux proteins involved in copper transport that modulate cisplatin export (Safaei et al. 2004). Increased cytoplasmic thiol-containing species (such as GSH) mediate resistance to cisplatin and carboplatin. After platinum-DNA adducts have been generated, resistance can also be mediated through enhanced DNA repair (and removal of adducts; the converse hypersensitivity of testicular cancer cells to cisplatin is a consequence of DNA-repair deficiency) or tolerance to platinum-DNA adducts. Increased nucleotide excision repair (NER) protein, excision repair cross-complementing-1 (ERCC1), has been detected in cisplatin-resistant ovarian cancer cells (Ferry et al. 2000); ERCC1 knock down enhanced sensitivity to cisplatin (Chang et al. 2005). Clinically, enhanced ERCC1 mRNA correlates with resistance to platinum-based chemotherapy (Dabholkar et al. 1992). Increased tolerance to platinum adducts can be acquired through loss of functional mismatch repair (MMR) (see Chapter 7), or enhanced replicative bypass (see Chapter 11). The latter occurs when DNA polymerases (e.g., β, η) circumvent cisplatin-DNA adducts by translesion synthesis (Bassett et al. 2002). Loss of, or aberrant apoptotic signalling pathways, mediated through p53 (see Chapter 12) or Bcl2 family members may also confer tolerance to platinum agents. Elucidation of the mechanisms conveying resistance or tolerance to cisplatin and carboplatin inspired design of third generation platinum agents to evade resistance, or combination studies with resistance modulators to avoid resistance.

Oxaliplatin: Oxaliplatin (1R,2R-diaminocyclohexane oxalatoplatinum(II); Fig. 8) reveals an activity profile distinct from cisplatin and carboplatin in cell lines of the NCI's anticancer drug screen panel (Rixe et al. 1996). Activity is retained in certain cancer cells possessing acquired resistance to cisplatin. Intracellular accumulation of oxaliplatin is less dependent upon the copper protein transporter (Holzer et al. 2006), Moreover, MMR proteins fail to recognise oxaliplatin-DNA adducts (Fink et al. 1996), and loss of MMR, which results in tolerance to cisplatin and carboplatin adducts, does not lead to oxaliplatin resistance.

Satraplatin and Picoplatin: Satraplatin (bis-aceto-ammine-dichloro-cyclohexylamine platinum (IV); Fig. 8) was developed as an orally active platinum analogue and does indeed possess good activity when delivered orally. It retains activity in cisplatin-resistant cancer cells, where acquired resistance is a consequence of reduced platinum transport. However, satraplatin forms DNA adducts in a manner very similar to cisplatin, which can be repaired by NER (Reardon et al. 1999) (see Chapter 1).

Picoplatin (*cis*-amminedochloro, 2-methylpyridine, platinum (II) Fig. 8) was designed to provide steric bulk around the platinum centre. This design led to successful reduction in inactivation by thiol-containing species such as GSH. Picoplatin retains activity against cisplatin- and oxaliplatin-resistant cells *in vitro*, overcoming acquired resistance mechanisms of reduced transport, enhanced cytoplasmic detoxification and increased NER (Holford et al. 1998; Sharp et al. 2002). A synergistic relationship has been demonstrated for a picoplatin/paclitaxel combination. In phase II clinical trials, picoplatin has shown evidence of antitumour activity in cisplatin-sensitive ovarian cancer (Gore et al. 2002) and cisplatin-resistant small cell lung cancer (Treat et al. 2002); further trials are planned.

TRIAZENES

Triazenes are structurally unique: the 3 adjacent nitrogen atoms confer upon this class of alkylating agent versatile physicochemical properties and antitumour activity.

Dacarbazine: Dacarbazine (5-(3,3-dimethyltriazeno)imidazole-4-carboxamide; DTIC; Fig. 9) has been used for the past 3 decades, and is the only FDA-approved chemotherapeutic agent for treatment of malignant melanoma. The response rate ranges between 15% and 25%; complete responses are rare and short-lived (Middleton et al. 2000a,c). Recent trial results report improved overall survival of metastatic melanoma patients following combination dacarbazine and ipilimumab therapy (Robert et al. 2011). Dacarbazine is a cell cycle non-specific agent, which functions as an alkylating agent following hepatic bioactivation. Thereafter, its mechanism of action is similar to that of temozolomide, detailed below. Briefly, following extensive metabolism of dacarbazine by cytochrome P450 isoforms 1A1, 1A2 and 2E1 (Reid et al. 1999), its active metabolite 5-(3-methyltriazen-1-yl)-imidazole-4-carboxamide (MTIC) spontaneously decomposes to 5-aminoimidazole-4-carboxamide (AIC) and the active alkylating species – methyldiazonium ion (Fig. 9). Consequently, methyl adducts are transferred to nucleophilic DNA atoms: *N7*-guanine, *N3*-adenine and *O6*-guanine (Newlands et al. 1997). Adverse reactions to dacarbazine treatment include rare but potentially fatal hepatotoxicity.

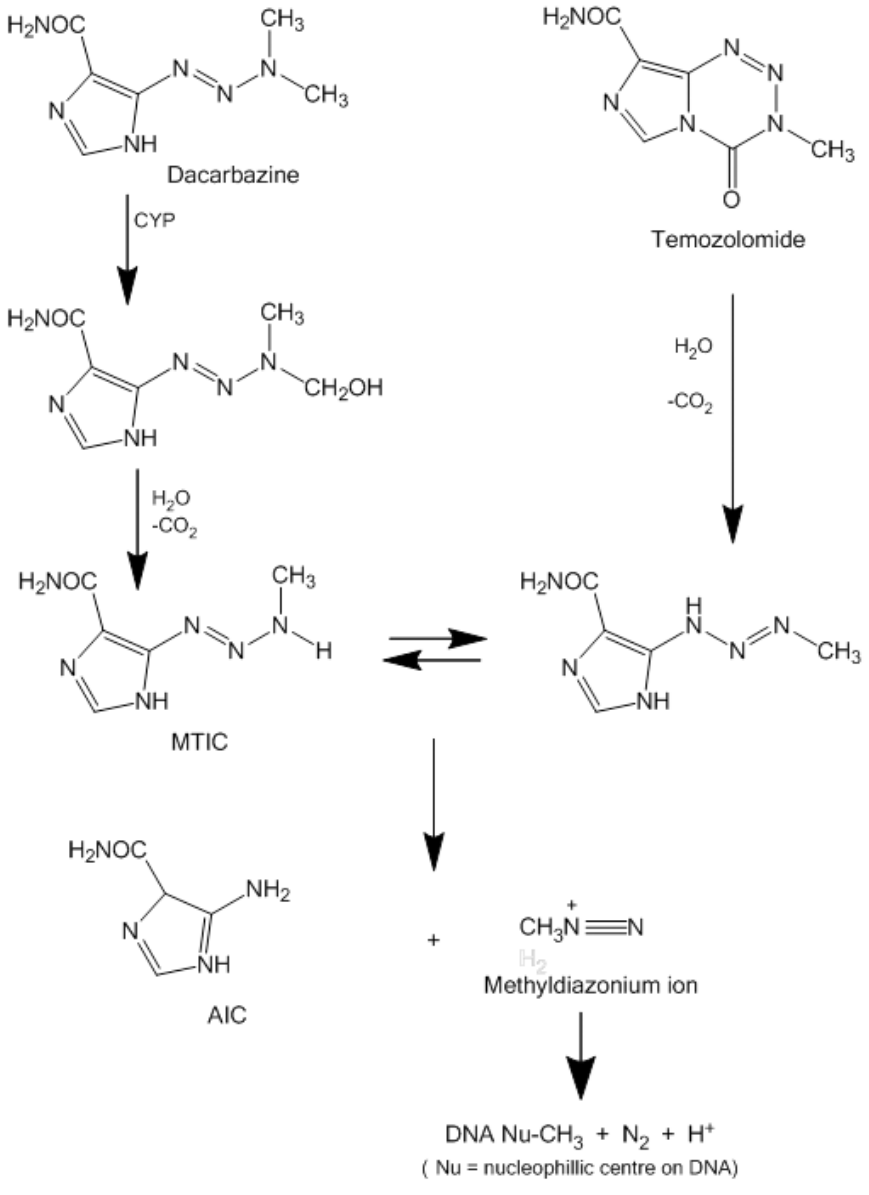

Figure 9. Chemical structures and activation of triazene alkylating agents.

Temozolomide: Temozolomide (3-methyl-4-oxoimidazo[5,1-d][1,2,3,5] tetrazine-8-carboxamide; TMZ, Fig. 9) is a monofunctional DNA alkylating imidazotetrazine licensed for treatment of refractory high grade glioma,

namely recurrent anaplastic astrocytoma and glioblastoma multiforme (GBM)(Friedman 2000; Friedman et al. 2000). TMZ, a small (194 Da) lipophilic molecule, acts as a prodrug. It is stable at acidic pH values, allowing oral administration (Newlands et al. 1992), but is labile above pH 7, with a plasma half-life of 1.8 hours at pH 7.4 (Tsang et al. 1990). Thus, TMZ is rapidly absorbed intact, but then undergoes spontaneous breakdown to form monomethyl triazene MTIC, further reacting with water to liberate 5-aminoimidazole-4-carboxamide (AIC) and the highly reactive methyldiazonium cation (Fig. 9). The active species methyldiazonium cation preferentially methylates DNA at the *N7* position of guanine, particularly in guanine rich regions (*N7*-MeG; 70%), but also methylates *N3* adenine (*N3*-MeA; 9%) and *O6* guanine positions (*O6*-MeG; 6%) (Denny et al. 1994; Tisdale 1987). There is a narrow pH window close to physiological pH at which the whole process of TMZ prodrug activation to methyl group transfer can occur, and brain tumours, which possess a more alkaline pH compared with surrounding healthy tissue, offer an environment promoting prodrug activation preferentially within tumour tissue (Rottenberg et al. 1984). Adjuvant TMZ chemotherapy, together with radiotherapy and surgery, therefore comprises the current standard of care for newly diagnosed GBM patients, and has been shown to impart significant therapeutic benefit (Stupp et al. 2009).

TMZ cytotoxicity is primarily mediated through *O6*-MeG, a carcinogenic, mutagenic and toxic lesion (Drablos et al. 2004; Wedge and Newlands 1996; Wedge et al. 1996b). Direct repair of *O6*-MeG by the suicide enzyme MGMT removes the methyl adduct, restoring guanine (see Chapter 6). However, in the absence of MGMT, unrepaired *O6*-MeG mispairs with thymine (not cytosine) during DNA replication, alerting DNA mismatch repair (MMR) (Kyrtopoulos et al. 1997; Margison and Santibanez-Koref 2002). MMR, recognising the mispaired thymine on the daughter strand, excises it, yet *O6*-MeG persists in the template strand. Therefore, futile cycles of thymine re-insertion and excision result in persistent DNA strand breaks, causing replication fork collapse (Mojas et al. 2007) (see Chapter 7). G_2/M cell cycle arrest is triggered, occurring in the second cell cycle following treatment (Cejka et al. 2003; Roos et al. 2004; Zhukovskaya et al. 1994), *via* ATR/CHK1-dependent signalling (Stojic et al. 2004); ultimately, apoptosis ensues (D'Atri et al. 1998). A favourable tumour response to TMZ therefore requires low levels of MGMT and functional MMR. Quantitatively more abundant *N7*-MeG and *N3*-MeA lesions are rapidly repaired by DNA base excision repair (BER) (see Chapter 8). *N7*-MeG appears not to be markedly cytotoxic: in contrast, *N3*-MeA lesions are lethal if not intercepted (Horton and Wilson 2007). Consequently, the most important DNA repair systems impacting TMZ cytotoxicty (and resistance) are MGMT (direct repair), MMR and BER (discussed later).

Imidazotetrazine analogues: Inherent and acquired resistance to TMZ (and other alkylating agent) therapy, a consequence of inherent or emergent MGMT expression or MMR deficiency, remains a grave clinical problem (Cahill et al. 2008; Wiewrodt et al. 2008). *In vitro* models of GBM have been developed in order to facilitate design of TMZ analogues able to evade mechanisms of resistance or tolerance to TMZ lesions (Zhang et al. 2010). SNB19M and U373M GBM cells have been transfected with the repair enzyme MGMT; their isogenic vector control partner (SNB19V and U373V) cell lines express low (56 fmol/mg) and negligible (4 fmol/mg) inherent MGMT activity, respectively. From these vector control cells, GBM cell lines possessing acquired resistance to TMZ have been generated: SNB19VR is MMR deficient through loss of hMSH6 and MGMT is upregulated in U373VR (Zhang et al. 2010).

Consistent with the mechanism of chemical decomposition of TMZ, MTIC, generated upon TMZ ring opening, was > 9-fold and > 4-fold more potent against SNB19V and U373V cells, respectively, compared with the MGMT-transfected partners. Moreover, the MGMT-transfected SNB19M and U373M GBM cell lines were 13- and 5.4-fold more resistant to TMZ than the parental SNB19V and U373V GBM cell lines, respectively.

Novel imidazotetrazine N3 analogues of TMZ (Fig. 10) have been designed to deliver alkylating species to DNA that generate lesions

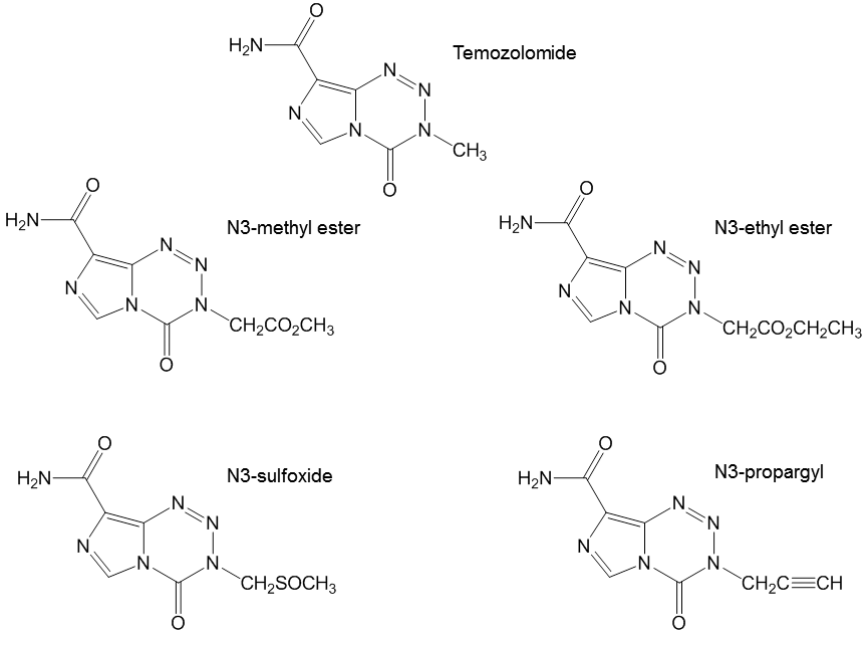

Figure 10. Chemical structures of N3-substituted temozolomide analogues.

irreparable by MGMT (Bradshaw et al. 2010; Zhang et al. 2011). N3-Methyl- and ethyl imidazotetrazine esters demonstrated a "flat" distribution of activity across V and M U373 and SNB19 GBM cell lines; similarly, their ring-opened triazene counterparts were approximately equiactive as the cyclic imidazotetrazine precursors. However, *in vivo*, imidazotetrazine esters are substrates for plasma esterases, and the corresponding imidazotetrazine carboxylic acid of the methyl ester, for example, exhibits poor activity against vector control and MGMT transfected SNB19 and U373 GBM cell lines (GI_{50} > 195 μM). Other N3-substituted TMZ analogues have been synthesised that elicit desirable *in vitro* growth inhibitory properties against GBM cell lines irrespective of MGMT expression, including N3-trifluoro, N3-chloromethyl and N3-methoxymethyl congeners. Such activity may be a consequence of DNA alkylation lesions possessing poor hydrophobic interactions, insufficient to drive molecular recognition within the active binding pocket of MGMT. Particularly potent across the GBM and MMR deficient colorectal carcinoma cell line panel (e.g., GI_{50} 5.5 μM against HCT 116 cells), and demonstrating some selectivity towards cancer cells (eg GI_{50} 54.4 μM against MRC5 fibroblasts), is the N3-sulfoxide imidazotetrazine derivative (Bradshaw et al. 2010). However, the corresponding ring opened N3-sulfoxide imidazotriazene has to date proved synthetically inaccessible.

The N3-propargyl TMZ analogue was equipotent towards MGMT-transfected GBM cells as SNB19V and U373V vector control counterparts. This imidazotetrazine molecule also inhibited growth of vector control U373VR and SNB19VR GBM cells possessing acquired resistance to TMZ (GI_{50} < 50 μM) (Bradshaw et al. 2010) and MMR deficient HCT 116 cells, irrespective of p53 proficiency. Ring-opened N3-propargyl imidazotriazene also inhibited isogenic V and M GBM cell growth with similar potency (GI_{50} values < 40 μM) Together these results imply that novel propargyl imidazotetrazine TMZ analogue ring-opens to create a DNA alkylating species which generates cytotoxic lesions that are neither repaired by MGMT nor tolerated in MMR deficient cells. Indeed, taq polymerase stop assays revealed that both N3-propargyl imidazotetrazine and N3-propargyl triazene alkylated guanine rich DNA sequences; *N-7* guanine alkylation was detected by piperidine cleavage assay. H2AX foci, inferring conversion of guanine lesions to lethal DNA double strand breaks could only be observed in vector control GBM cells following exposure to TMZ. DNA damage caused by N3-propargyl imidazotetrazine (like TMZ) initiated single strand break repair, however, γH2AX foci appeared in human GBM cells irrespective of MGMT expression and preceded cell death. Apoptosis was detected in HCT116 colorectal carcinoma cells, but in GBM cells, more resistant to apoptosis, autophagy was observed. Thus, a molecule such as N3-propargyl imidazotetrazine which delivers an alkylating species able

to escape both MGMT repair and tolerance in the absence of functional MMR may offer treatment for MGMT positive GBM and possess broader spectrum anticancer activity.

ALKYLATING AGENTS AND DNA REPAIR RESPONSES (FOCUS ON TMZ)

MGMT (*O6*-Alkylguanine DNA alkyltransferase; AGT) repairs *O6*-alkylguanine adducts in a single step, independently of any other protein or cofactor (see Chapter 6). It is a small protein (22 kDa) able to repair not only *O6*-MeG, but also guanine residues with longer *O6*-alkyl adducts, such as ethyl, chloroethyl, hydroxyethyl, n-propyl, n-butyl, and more bulky cyclic lesions conferred by benzyl or pyridyloxobutyl groups, but with diminishing efficiency as adduct size increases (Coulter et al. 2007; Fang et al. 2008; Pegg 2000). The *O6*-alkyl group is transferred from guanine to the active site cysteine residue (Cys 145 in the human protein) of MGMT in a stoichiometric, auto-inactivating reaction, thereby repairing DNA and permanently inactivating MGMT (Pegg et al. 1995). MGMT protects cells from carcinogens; however, it is also able to protect cancer cells from chemotherapeutic alkylating agents such as TMZ. Tissue expression is variable, with high protein expression in liver and lower expression in haematopoietic tissues and brain (Kaina and Christmann 2002; Margison et al. 2003). Tumour MGMT expression is immensely variable, with highest levels being found in breast, ovarian and lung tumours, while lowest activity is observed in pancreatic carcinomas, malignant melanomas and gliomas (Kaina et al. 2007).

MGMT activity has been reported to vary 300-fold in gliomas (Silber et al. 1999), where a strong positive correlation exists between MGMT activity and alkylating agent resistance *in vivo* and *in vitro* (Gerson 2002, 2004; Gerson and Willson 1995). Loss of MGMT activity is most frequently a consequence of *MGMT* promoter methylation (Esteller 2005; Esteller and Herman 2004; Middleton and Margison 2003). Gene inactivation by promoter methylation is a common epigenetic phenomenon in tumourigenesis (Esteller 2000). Enzymatic methylation, mediated by 5′-methylcytosine methyltransferase, takes place on the cytosine of CpG islands. Hypermethylation of CpG islands in the *MGMT* promoter region prevents transcription factor binding, silencing the gene (Esteller and Herman 2004; Sabharwal and Middleton 2006). *MGMT* methylation has been detected in 45%–70% of high grade gliomas (Blanc et al. 2004; Stupp et al. 2006). Clinical evidence has revealed that patients with *MGMT* promoter methylation respond better than those without promoter methylation to radiotherapy and treatment with either BCNU or TMZ (Hegi et al. 2004, 2005, 2008). The correlation between *MGMT*

promoter methylation extent and clinical response to alkylating agents means that *MGMT* promoter methylation is a good predictive marker of response to alkylating agent chemotherapy.

In the absence of *MGMT* promoter methylation, MGMT protein has a major impact on alkylating agent resistance clinically and a number of therapeutic approaches have been explored to modulate MGMT activity and enhance drug response (Verbeek et al. 2008). The potent non-toxic inhibitors of MGMT, *O6*-benzyl guanine (*O6*-BG) and *O6*-(4-bromothenyl)guanine (lomeguatrib; PaTrin-2), have been used in clinical trials to deplete MGMT before administration of alkylating agent therapy (Dolan 1997; Dolan et al. 1990; Sabharwal and Middleton 2006). *O6*-BG acts as a pseudosubstrate and binds to MGMT, covalently transferring the benzyl moiety to the active site cysteine residue (145), causing its irreversible inactivation. *O6*-BG is not incorporated into the DNA of living cells, reacting directly with both cytoplasmic and nuclear MGMT (Dolan et al. 1990). Pre-treatment of tumour cells containing high MGMT levels with *O6*-BG enhances TMZ activity *in vitro* (Zhang et al. 2010) and *in vivo*, but has little effect on tumour cells possessing low or undetectable MGMT levels (Wedge et al. 1996a).

Lomeguatrib is an orally bioavailable potent pseudosubstrate for MGMT. Covalent transfer of the bromothenyl group to the active site cysteine inactivates MGMT. Lomeguatrib has shown promising activity in sensitising a variety of human tumour xenografts to the growth-inhibitory effects of *O6*-alkylating agents, including TMZ and BCNU, at the expense of limited additional toxicity (Middleton et al. 2000b, 2002). A Phase I clinical trial combining lomeguatrib and TMZ (Ranson et al. 2006) led to a randomised Phase II study in 100 patients with metastatic melanoma (Ranson et al. 2007). In this study, the efficacy of combination treatment was similar to that of TMZ treatment alone in terms of response rates and median time to disease progression. However, the lomeguatrib schedule adopted permitted rapid recovery of tumour MGMT within 24 h. Subsequent clinical trials established pharmacodynamically effective schedule and doses of lomeguatrib, which result in complete and consistent MGMT depletion in melanoma, central nervous system, prostate and colorectal tumours (Watson et al. 2009, 2010). Moreover, significantly higher levels of O6-MeG adducts were present in peripheral blood mononuclear cell DNA following lomeguatrib/TMZ combination therapy compared with TMZ treatment alone. However, myelosuppression remains a prohibitive limiting side effect to the use of MGMT inhibitors and alkylating agent combination chemotherapy; a consequence of low MGMT expression within bone marrow (Hansen and Kelley 2000). To protect haematopoeitic cells during chemotherapy, the strategy of gene transfer of a mutant *MGMT* cDNA, encoding a protein that is resistant to inactivation, has been developed (Gerson 2004; Woolford et al. 2006; Zielske and Gerson 2002). A phase I

clinical study of such myelosuppressive gene therapy is underway in the U.S. (http://www.clinicaltrials.gov).

DNA MMR status also impacts the response of cells to alkylating agents; indeed, methylating agent cytotoxicity induced by TMZ requires functional MMR. MMR recognizes and corrects mispaired bases and insertion/deletion loops (resulting from gains or losses of short repeat units within microsatellite sequences) generated during DNA synthesis (see Chapter 7). MMR plays a critical role in correcting replicative mismatches that have escaped polymerase proofreading, and loss of MMR results in a dramatic increase in insertion/deletion mutations, particularly in repetitive sequence microsatellite DNA. Indeed, microsatellite instability (MSI) is a recognised surrogate biomarker for the loss of MMR function (Umar et al. 1998).

Studies have demonstrated that MMR is of clinical significance in several cancers particularly colorectal cancers where hereditary and sporadic MMR gene mutations are responsible for MSI. In hereditary non-polyposis colorectal cancer (HNPCC), germ line mutations in *MLH1* or *MSH2* cause microsatellite repeat replication errors to persist (Fink et al. 1998), and MMR deficiency has been observed in 15%–20% of sporadic colorectal tumours (Chai et al. 2004; Gologan and Sepulveda 2005). Somatic MMR gene mutations may be the result of epigenetic gene silencing *via* methylation of the *MLH1* promoter (Grady and Carethers 2008; Herman et al. 1998). Grady and colleagues (Grady et al. 2001) report aberrantly methylated *hMLH1* promoter DNA in the sera of 9/19 patients with microsatellite unstable colon carcinoma. MMR mutations allow microsatellite insertions/deletions that have the potential to cause inactivating frameshift mutations within tumour suppressor coding regions, genes critical to cell cycle regulation and cancer prevention. MMR-deficient cells are reported to be up to 100-fold less sensitive to methylating agents compared with their MMR proficient counterparts (Karran 2001; Stojic et al. 2004). Indeed, HCT 116 (*MLH1* mutant) and DLD1 (*MSH6* mutant) MMR deficient colon carcinoma cells are resistant to TMZ treatment (GI_{50} > 500 μM) (Zhang et al. 2011). In such cells, *O6*-MeG-thymine mispairs are not recognised, *O6*-MeG lesions are tolerated, cells continue cycling, surviving at the expense of extensive mutagenesis (Karran 2001).

BER is the major pathway involved in removal and repair of non-bulky damaged nucleotides, abasic sites and DNA single-strand breaks generated by reactive oxygen species, ionising radiation and alkylating agents (Wood et al. 2001) (see Chapter 8). *N7*- and *N3*- purines methylated by TMZ are repaired by BER. A protein key to successful DNA damage signalling and BER is poly(ADP-ribose) polymerase-1 (PARP-1) (see Chapter 15).

Constitutively expressed, but activated in response to DNA damage, PARP-1 enzyme (113 kDa), encoded by the *ADP-ribosyl transferase* (*ADPRT*) gene, modifies nuclear proteins by poly(ADP-ribosylation). In response to DNA damage, PARP-1 binds to DNA single-strand breaks (or DSBs) and cleaves β–nicotnamide adenine dinucleotide (NAD+), releasing nicotinamide and ADP-ribose. PARP-1 uses NAD+ to catalyse auto-(and other protein) poly(ADP-ribosyl)ation. Long, branched ADP-ribose polymers attract recruitment of a BER protein complex consisting of XRCC1, DNA polymerase β and DNA ligase III, and possibly other proteins, to execute repair (and serve as an energy source for ligation) (Dantzer et al. 2006; Malanga and Althaus 2005). Release of polyribosylated PARP-1 from DNA allows access to the lesion. Thus, PARP-1 facilitates efficient DNA repair and survival of cells subjected to mild genotoxic stress (Aguilar-Quesada et al. 2007). Inhibition of PARP-1 (and other PARP proteins) increases the frequency of DNA strand breaks, and accordingly, PARP-1 deficient cells are hypersensitive to carcinogenic agents (Lockett et al. 2005).

The majority of the DNA lesions generated by TMZ are *N7*-MeG and *N3*-MeA (comprising 80–85% and 8–18% of total alkyl adducts, respectively), and they are typically rapidly and efficiently repaired by BER. However, these adducts become highly cytotoxic when BER is disrupted (Sobol et al. 1996). PARP-1 inhibition enhances the cytotoxicty of base lesions normally repaired by BER, and indeed, potentiates TMZ cytotoxicity *in vitro* and *in vivo* (Curtin et al. 2004; Ratnam and Low 2007; Tentori et al. 2002). Thus, when the primary toxic TMZ lesion, *O6*-MeG, is either repaired by MGMT or tolerated following MMR disruption, *N*-Me purine base adducts become significant, and inhibition of BER may enhance TMZ therapeutic efficacy (Curtin et al. 2004). Therefore, disruption of BER by PARP inhibition may offer a means to overcome resistance that frequently develops during TMZ therapy (Tentori and Graziani 2009).

It was to test the hypothesis that PARP-1 inhibition could potentiate TMZ activity that PARP inhibitor AG 014699 first entered clinical trials in cancer patients (Drew and Plummer 2009; Plummer 2006; Plummer et al. 2006). The combination revealed increased response rates and median time to progression compared with TMZ treatment alone. ABT 888 demonstrated broad *in vivo* activity in combination with TMZ in diverse tumours (Palma et al. 2009). At least 8 PARP inhibitors are currently undergoing Phase I, II, or III clinical evaluation, either as single agent therapy (as a synthetic lethal strategy to target cancers with specific HR DNA repair defects; (McEllin et al. 2010)), or in chemotherapy combination regimens for treatment of malignant solid tumours (http://www.clinicaltrials.gov (Drew and Plummer 2009; Fong et al. 2009; Lau et al. 2009)).

CONCLUDING COMMENTS

Alkylating agents comprise the oldest class of cytotoxic antineoplastic compounds. Nevertheless, these agents remain a bastion of chemotherapy and many combination therapy regimens are used in curative, maintenance or palliative treatment of a plethora of malignancies. In the era of molecularly targeted therapy (see Chapter 16) are alkylating agent treatment endures. Indeed, as deeper understanding of tumour biology is revealed, pharmacodynamic analyses of the tumour genome and epigenome guide existing alkylating agent treatment strategies and identify patient cohorts who will derive significant benefit from alkylating agent therapy. This point is exemplified by a recent study in which patients whose mammary tumours were deficient in HR repair of DNA DSBs responded significantly better to high dose cyclophosphamide, thiotepa, carboplatin combination therapy. Identified by comparative genomic hybridisation both ER-positive and triple negative HR-deficient breast cancer patients derived a marked survival benefit from intensified DNA cross-linking alkylating agent chemotherapy (Vollebergh et al. 2011). Moreover, elucidation of molecular mechanisms conferring tumour resistance (to alkylating agents) guides treatment and shapes research programmes, as molecules delivering novel alkylating species to DNA designed to evade repair continue to be synthesised.

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CHAPTER 6

O^6-Methylguanine-DNA Methyltransferase (MGMT) in Cancer Protection and Therapy

Markus Christmann and *Bernd Kaina*[a]

INTRODUCTION

Mutagens in the environment, tobacco, food, as well as endogenous metabolic products generate highly reactive electrophilic species that alkylate cellular DNA. Among those agents are food-specific N-nitrosamines, such as N-nitrosodimethylamine (NDMA), N-nitrosodiethylamine (NDEA), N-nitrosopyrrolidine (NPYR) and N-nitrosopiperidine (NPIP), and the tobacco-specific nitrosamines 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N-nitrosonornicotine (NNN). In addition, alkylating agents are used in cancer chemotherapy due to their high cytotoxic properties (see Chapter 5). Examples of alkylating anticancer drugs are the methylating agents procarbazine, dacarbazine, streptozotocin and temozolomide(TMZ) and the chloroethylating agents carmustine (BCNU), lomustine (CCNU), nimustine (ACNU) and fotemustine.

These mutagens and chemotherapeutics react with DNA via S_N1 reactions. S_N1 reactions follow a first-order kinetics that is dependent on the formation of an electrophilic carbocation (Beranek 1990). This intermediate can covalently bind to a nucleophilic center on DNA, which can potentially be alkylated at 13 positions. The most frequent methylation lesions are

Department of Toxicology, University Medical Center Mainz, Obere Zahlbacher Str. 67, D-55131 Mainz, Germany.
[a]Email: kaina@uni-mainz.de

N7-methylguanine (N7MeG, 80–85%) and N3-methyladenine (N3MeA, 8–20%); the most critical lesion, biologically speaking, is O^6-methylguanine (O^6MeG), which represents a minor lesion (< 8%). *O*^{6}MeG lesions are repaired by the O^6-methylguanine-DNA methyltransferase (MGMT) protein. Besides *O*^{6}MeG, longer alkyl groups, such as *O*^{6}-chloroethylguanine (*O*^{6}ClG), which is produced by chloroethylating anticancer drugs, and O(6)-[4-oxo-4-(3-pyridyl)butyl]guanine (*O*^{6}pobG), a product formed via pyridyloxobutylation by the tobacco-specific nitrosamines NNN and NNK, can be repaired by MGMT.

Although MGMT protects the cell from the cytotoxic effect of alkylating agents, it is in many ways a double-edged sword. On the one hand, MGMT protects normal cells against the cytotoxic, mutagenic, genotoxic and carcinogenic effects of environmental contaminants (Margison et al. 2003). Yet on the other hand, MGMT protects tumor cells against alkylating anticancer drugs and thus counteracts anti-cancer therapy, reducing the life-span of the patient. Here, we will summarize the role of MGMT in cancer protection and therapy, and will discuss results concerning the use of MGMT inhibitors in clinical trials.

DNA DAMAGES REPAIRED BY MGMT

As mentioned above, MGMT is responsible for the removal of various alkyl-groups from the O^6-position of guanine that are induced by several N-nitroso compounds in the environment and used as anticancer drugs. The first N-nitroso compound identified in the environment was dimethylnitrosamine (DMN, also known as *N*-nitrosodimethylamine), which is the most prevalent N-nitroso compound present in the diet (Lijinsky 1999). The most important tobacco-specific nitrosamines are NNN and NNK (Hecht 1999). In the case of NNK, carbenium ions and pyridyloxobutylating agents are produced, which promote the formation of *O*^{6}MeG and *O*^{6}pobG, respectively, whereas NNN only induces *O*^{6}pobG formation (Fig. 1). Besides *O*^{6}MeG, *O*^{6}pobG can also be repaired by MGMT *in vitro* (Peterson et al. 1993; Wang et al. 1997, 1999) and *in vivo* (Thomson et al. 2003).

Exposure to alkylating compounds also occurs during anticancer therapy. Methylating and chloroethylating agents are used in the therapy of brain tumors (astrocytoma WHO grade III and glioblastoma multiforme, WHO grade IV), metastatic malignant melanoma, neuroendocrine tumours and lymphoma. The methylating drugs procarbazine (PCB, PCZ, N-Methyl hydrazine, Natulan®, Matulane®), dacarbazine (DIC, Imidazole carboxamide, dimethyl-triazeno-imidazole-carboxamide, DTIC®-Dome), streptozotocin (STZ, NSC 85998, Zanosar®) and temozolomide (TMZ, SCHS2.365, NSC 362856, Temodal®, Temodar®) act in part through the

formation of O^6MeG (Fig. 1). The chloroethylating agents carmustine (BCNU, BiCNU®), lomustine (CCNU, CeeNU®), nimustine(ACNU) and fotemustine(Muphoran®) chloroethylate the O^6-position of guanine, thereby generating O^6ClG.The adducts O^6MeG, O^6ClG and O^6pobG are the most relevant substrates for MGMT.

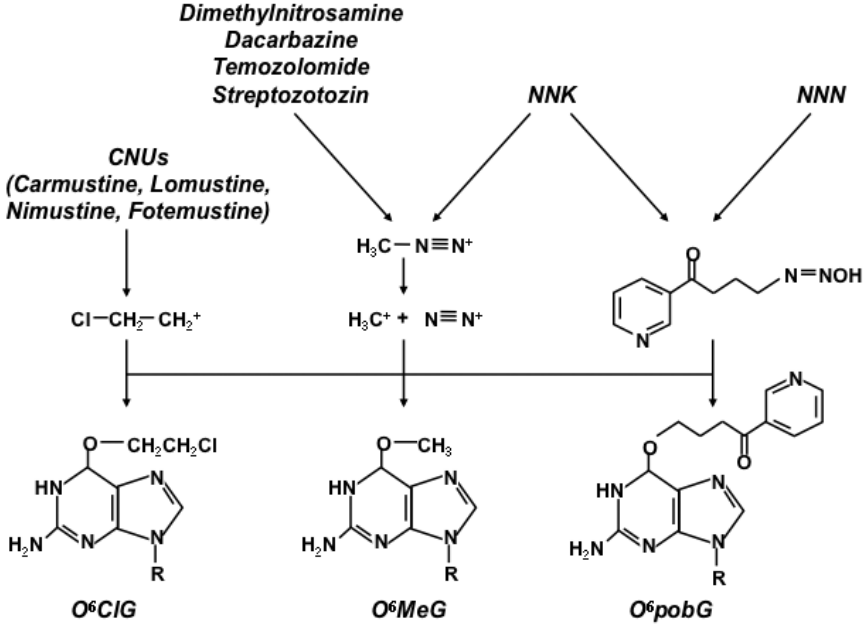


Figure 1. Formation of O^6MeG, O^6ClG and O^6pobG by anticancer drugs and tobacco specific nitrosamines.

MGMT STRUCTURE, REGULATION AND FUNCTION

The human *MGMT* gene consists of one non-coding and four coding exons, spans about 145 kb in total, and is located at chromosomal position 10q26. The gene encodes mRNA of 866 nucleotides and codes for a protein containing 207 amino acids with a molecular weight of 24 kDa. MGMT is a relatively stable protein, with a half life of >24 h (Fritz et al. 1991), and some reports showed that phosphorylation of MGMT affects its repair activity (Mullapudi et al. 2000; Srivenugopal et al. 2000).

MGMT expression is regulated via epigenetic promoter methylation (Costello et al. 1994a; Costello et al. 1994b) and transcriptional mechanisms. Methylation of the *MGMT* promoter has been shown to provoke transcriptional silencing (Harris et al. 1991; Qian et al. 1995; Qian and Brent 1997) via heterocomatinization and re-arrangement of the nucleosome

structure (Costello et al. 1994; Patel et al. 1997; Watts et al. 1997). This epigenetic silencing of *MGMT* is often observed in tumor cells and is therefore proposed to be a driving force in carcinogenesis. The mechanism leading to methylation of the *MGMT* promoter in tumor cells, however, is not fully understood. Notably, p53 appears to plays an important role since methylation of the *MGMT* promoter was observed at a higher frequency in p53 mutated lung tumors (62%) than in lung tumors harboring the p53 wild-type (wt) allel (38%) (Lai et al. 2008). One explanation could be that p53 represses the major DNA methyltransferase, DNA (cytosine-5)-methyltransferase 1 (DNMT1), a protein unlike MGMT that adds methyl groups to DNA at specific cytosine residues with CpG sequences to maintain or regulate gene expression patterns (Peterson et al. 2003; Esteve et al. 2005). In support of this hypothesis, knockdown of p53 increased the expression of DNMT1 and histone deacetylase 1 (HDAC1) leading to methylation of the *MGMT* promoter, whereas over-expression of p53 reduced the methylation of the promoter (Lai et al. 2008).

Induction of *MGMT* via transcriptional activation, which has been investigated mainly in rodent cells, has been observed upon treatment with corticosteroids, ultraviolet light, ionizing radiation and alkylating agents (Fritz et al. 1991; Grombacher and Kaina 1995; Grombacher et al. 1996). Again, p53 appears to be involved in this process, since p53 knockout mice do not show MGMT induction after whole body irradiation (Rafferty et al. 1996). The human *MGMT* promoter contains several binding sites for transcription factors, including glucocorticoid responsive elements (GRE), AP-1 and Sp1 binding sites (Harris et al. 1991). The two AP-1 binding sites can be transactivated by co-expression of c-Fos and c-Jun, and their deletion attenuates *MGMT* promoter activation (Boldogh et al. 1998). In addition, the *MGMT* transcript expression level was increased in HeLa S3 cells upon treatment with activators of protein kinase C (PKC), such as phorbol-12-myristate-13-acetate (TPA) and 1,2-diacylglycerol (DAG) (Boldogh et al. 1998). The *MGMT* promoter also harbors a NF-κB site that appears to be involved in gene regulation (Lavon et al. 2007). The important question, however, of whether MGMT is inducible in human normal and tumor tissue upon treatment with carcinogens or anticancer drugs, is still unsolved.

The MGMT-mediated repair mechanism involves a one-step reaction, in which the alkyl group at the O^6 position of guanine is transferred to a cysteine residue (Cys145) in the acceptor site of the protein (Pegg et al. 1995; Hazra et al. 1997). This process results in guanine being restored in DNA (direct reversal) and MGMT being rendered inactive, which is an irreversible process (Fig. 2). Therefore, MGMT is often referred to as a "suicide enzyme". Besides O^6MeG, longer alkyl adducts can also be repaired by MGMT, such as ethyl-, n-propyl-, n-butyl-, 2-chloroethyl-, 2-hydroxyethyl-, iso-propyl- and iso-butyl adducts. For these longer O^6G adducts, the efficiency of the

MGMT mediated alkyl group transfer decreases with increasing size (Pegg 1990). After alkyl group transfer, MGMT gets ubiquitinated and is subjected to proteasome-mediated degradation (Srivenugopal et al. 1996).

Several nucleotide polymorphisms in the human *MGMT* gene have been detected. Their function in protection against genotoxins and/or cancer predisposition, however, is still unclear (Christmann and Kaina 2012). Three of the non-synonymous polymorphisms (i.e., ones that lead to coding sequence changes), Leu84Phe, Ile143Val and Lys178Arg, may alter MGMT activity. For instance, the Ile143Val and Lys178Arg polymorphisms are in close proximity to the cysteine alkyl-residue at position 145 within the protein active site. The human Ile143Val and Lys178Arg polymorphisms did not affect the repair activity of MGMT and suppress alkylation-induced mutagenesis (Inoue et al. 2000; Ma et al. 2003). However, the Ile143Val variant was more resistant to inactivation by the MGMT pseudosubstrate O(6)-(4-bromothenyl)guanine (O^6-BTG; see more below), suggesting a possible influence of this genotype in cancer therapy with alkyating agents when they are applied concomitantly with an MGMT inhibitor (Margison et al. 2005). Another study reported that the Ile143Val and Lys178Arg variants had no impact on the repair of O^6pobG (Mijal et al. 2004), but are less sensitive to alterations in the sequence surrounding the lesion (Mijal et al. 2006). Similar to the Ile143Val and Lys178Arg polymorphisms, the Leu84Phe variant, which is not located in close proximity to the active site center (Deng et al. 1999), shows no difference in the repair of O^6MeG and O^6pobG compared to wild type MGMT (Inoue et al. 2000; Mijal et al. 2006; Fang et al. 2008). The Leu84Phe variant is near residues 98–102, which form

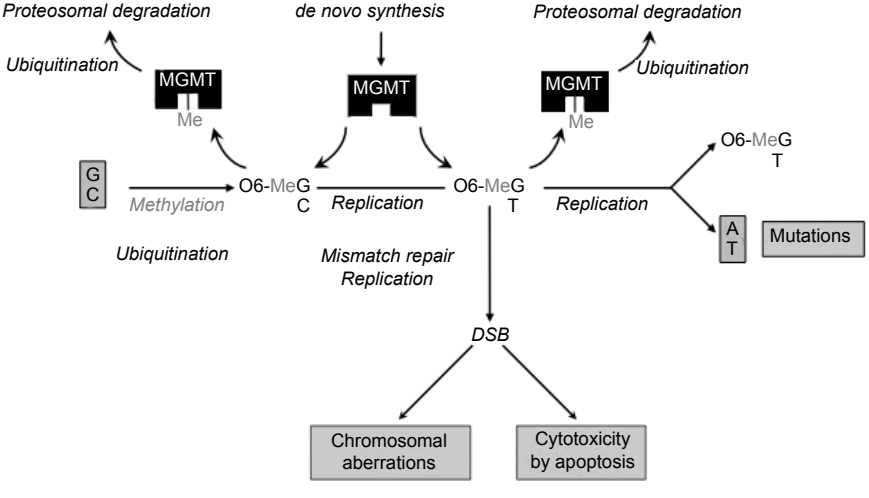


Figure 2. Mechanism of MGMT mediated repair of O^{6}MeG and consequences of abrogated repair.

a LXXLL motif that supposedly interacts with the estrogen receptor, and there are speculations that MGMT negatively affects estrogen receptor-mediated transcriptional activity and estrogen receptor-mediated cell proliferation (Teo et al. 2001). A recent meta-analysis, which assessed the impact of the MGMT polymorphisms Leu84Phe and Ile143Val in a large cohort of 13,069 cancer patients and 20,290 controls, revealed a significant association between the Leu84Phe and colorectal cancer (Zhong et al. 2010), indicating a possible impact of MGMT polymorphisms on cancer treatment and prevention.

MGMT IN DEFENSE AGAINST CANCER

Since MGMT repairs DNAlesions induced by several environmental alkylating agents, it is only natural to assume that MGMT plays an important role in protecting against cancer development, an assumption supported by several animal experiments. For example, transgenic mice over-expressing MGMT in their skin showed a reduced rate of tumor initiation (papilloma formation) and conversion of papillomas into malignant skin tumors upon exposure to N-methyl-N-nitrosourea (MNU) (Becker et al. 1996, 1997, 2003; Becker et al. 2003). In other mouse models and target systems, MGMT was shown to protect against methylation-induced liver cancer (Nakatsuru et al. 1993), lung cancer (Liu et al. 1999) and thymic lymphoma (Dumenco et al. 1993; Liu et al. 1994; Reese et al. 2001). MGMT knockout mice have no phenotype unless they are treated with alkylating O^6MeG-inducing carcinogens. This indicates, by the way, that endogenous DNA methylation by S-adenosyl methionine (SAM) does not contribute much to O^6MeG formation. In fact, SAM was shown to induce N7-methylguanine, but not O^6MeG (Rydberg and Lindahl 1982). In line with this, rat liver SAM synthetase expression in *E. coli* had no impact on the spontaneous mutation rate (Posnick and Samson 1999). MGMT knockout mice show an increased sensitivity to MNU (Sakumi et al. 1997) and the anti-cancer drugs dacarbacine (Shiraishi et al. 2000), carmustine, streptozotocin and TMZ (Glassner et al. 1999). Treatment of knockout mice with MNU also lead to increased formation of thymic lymphoma as well as lung adenoma (Sakumi et al. 1997). In the azoxymethane/dextrane sulfate sodium (AOM/DSS) model, knockout mice displayed a higher colon cancer incidence than the corresponding wild-type animals (Bugni et al. 2009; Wirtz et al. 2010). Overall, the data strongly indicate that MGMT is a powerful protector against cancers induced by monofunctional alkylating agents.

MGMT likely protects against cancers induced not only by NDMA, but also by tobacco specific N-nitrosamines. Accordingly, both NNN and

NNK have been shown to contribute to the risk of cancer development (Hecht et al. 1978, 1986; Hecht and Hoffmann 1988). For instance, it has been demonstrated that following exposure to NNK, the formation and persistence of O^6MeG are critical events in the initiation of lung tumors in A/J mice that are highly susceptible to developing lung cancer (Peterson and Hecht 1991). This observation was supported by a study using wild-type transgenic mice,which expressed human MGMT and showed a low O^6MeG adduct level in lung tissue following NNK treatment, where a significantly lower frequency of lung tumors was observed in the transgenic population (Liu et al. 1999). Another study addressed the impact of MGMT on the mutation frequency in liver and lung of MGMT deficient mice following NNK injection, and found an increase in GC -> AT transitions (the mutational signature of O^6MeG adducts, which pair preferentially with thymine when copied) in liver and lung of the mutant animals compared to the wild type counterparts (Sandercock et al. 2008).

Whereas a protective effect of MGMT against cancer formation induced by environmental mutagens has been shown in animal experiments, the correlation between MGMT and cancer development cannot be shown as easily in humans. The highest impact for MGMT would be expected in the incidence of lung cancer in smokers, due to the fact that smoke contains high levels of DMNA, NNN and NNK. Up to now, however, it is not even clear which components of tobacco (nitrosamines, benzo(a)pyrene, reactive oxygen species) are of major importance in carcinogenesis. The same is also true of food-induced cancer formation, an area in which the impact of the various food-born mutagens is still unclear. Nevertheless, two lines of evidence indicate that MGMT in humans represents an important factor in the defense against cancer formation:

1) Most studies comparing normal tissue (blood mononuclear cells, pharyngeal mucosa and lung), tissue containing pre-cancerous lesions, and fully developed cancers in smokers and non-smokers reveal an increase in MGMT activity in smokers (for review see Christmann and Kaina 2012), potentially representing a cellular protection strategy. 2) In some studies, a correlation between MGMT polymorphisms and cancer susceptibilityhas been reported (for review see Christmann and Kaina 2012). However, these data relating MGMT polymorphisms to cancer development are highly inconsistent, which is not unexpected since the end point "cancer" is affected by many variables.

It should be noted that more than 60 potentially carcinogenic and co-carcinogenic compounds are present in tobacco, and therefore the question of which DNA lesions induced by them are most important for the generation of lung and other cancers in smokers is difficult to answer. Since O^6MeG leads to GC -> AT transitions during cellular replication (Swann 1990), it would be predicted that those are the predominant mutation types

induced by N-nitrosamines in the tobacco. However, in lung cancers, GC -> TA, not GC -> AT, mutations are more frequently observed in smokers than non-smokers (Hackman et al. 2000; Pfeifer et al. 2002; Le Calvez et al. 2005; Riely et al. 2008). This finding might be seen as an indication that a significant fraction of mutations found in lung cancers of smokers are not caused by O^6MeG. Similar to O^6MeG, O^6pobG mainly produces GC ->AT transitions, but also a small number of GC -> TA transversions (Pauly et al. 2002), as exposing A/J mice to the NNK metabolite NNKOAc, which pyridyloxobutylates DNA, leads to the formation of both these mutagenic outcomes (Ronai et al. 1993). Moreover, besides O^6pobG, other products of pyridyloxobutyl modified DNA could impact the overall mutation spectrum. In conclusion, the data currently available neither proves nor disproves the role of MGMT in protection against cancer formation in humans. Therefore, additional studies in animal systems and humans are needed to elucidate the role of MGMT in protection against carcinogenesis.

MGMT IN CANCER THERAPY

Mechanism of Alkylating Agent Induced Cell Death

Contrary to the role of MGMT in cancer development, its role in cancer therapy and protection of tumor cells against alkylating anti-cancer drugs is undisputed. In the absence of MGMT, O^6MeG in the DNA leads to double-strand breaks (DSBs) that require DNA synthesis and DNA mismatch repair (MMR) to be formed (see Chapter 7) (Ochs and Kaina 2000) (Fig. 2). DSBs, in turn, trigger cell death by apoptosis (Kaina et al. 2007). Alternatively, it has been proposed that detection of the O^6MeG-T mismatch by MMR directly signals apoptosis via ATR activation (Yoshioka et al. 2006). This model, however, is disputed by experiments performed in synchronized cells which revealed that activation of the DNA damage response and cell death pathways occurs only after cells have passed through two replicative cycles (Quiros et al. 2010). This data supports the classic model whereby DSBs are formed due to the presence of unrepaired O^6MeG in the 2nd replication cycle. DSBs by themselves lead to ATR activation (Caporali et al. 2004; Stojic et al. 2004), which in turn triggers apoptosis pathways (see Chapter 13). While several downstream pathways that lead to apoptosis are mentioned herein, MGMT is the most important factor for alkylating drug resistance of tumor cells, since it repairs the critical upstream lesion. The level of resistance depends on the total amount of existing MGMT molecules prior to therapy and the rate of MGMT re-synthesis during therapy.

Correlation between MGMT Status and Clinical Response to Tumor Therapy

MGMT activity was detected in a broad range of normal tissue and tumors (for a recent compilation of data see (Christmann et al. 2011)) (Fig. 3). The activity in normal tissue is highest in the liver and lowest in the brain, while the MGMT expression in neoplastic cancer is especially high in liver, colon and ovarian cancer and is very low in brain cancer and malignant melanoma. These findings explain the relative sensitivity of gliomas to methylating and chloroethylating anti-cancer drugs (TMZ, ACNU and CCNU) and their use as first line therapeutics for these tumor groups.

In tumor cell lines, MGMT levels clearly correlate with resistance to methylating and chloroethylating agents (Pegg 1990; Dolan et al. 1991; Preuss et al. 1996). Several studies have also correlated the MGMT status with the therapeutic response of patients. Most of these trials revealed a relationship of low MGMT activity/expression and a better therapeutic response in patients with malignant gliomas on treatment with BCNU (Mineura et al. 1993; Belanich et al. 1996; Mineura et al. 1996; Jaeckle et al. 1998), fotemustine (Fabi et al. 2009) or TMZ (Friedman et al. 1998, 2000; Hegi et al. 2004, 2005; Wiewrodt et al. 2008; Stupp et al. 2009). Therefore, it is reasonable to conclude that the MGMT level of a given tumor predicts the clinical outcome of tumor therapy using monofunctional alkylating anticancer drugs.

For detecting the MGMT status, two methods are used: direct measurement of MGMT activity or methylation-specific PCR (MSP), which detects epigenetic silencing of the MGMT gene. Since direct detection

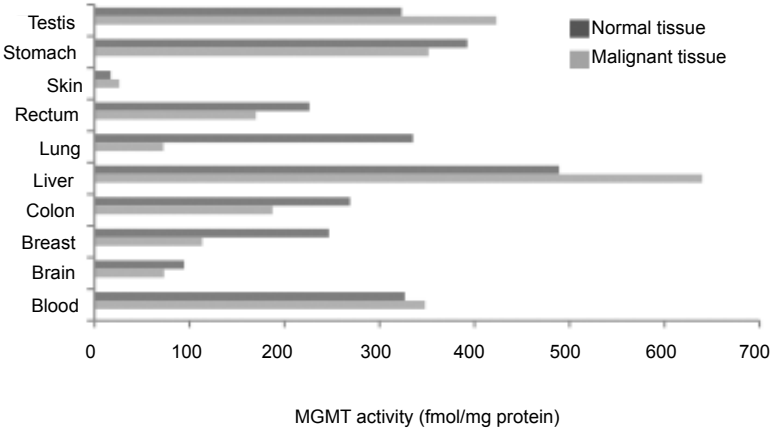


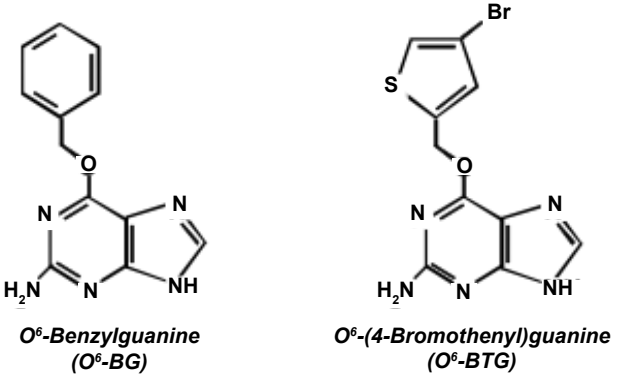
Figure 3. MGMT activity of various tumor types and corresponding normal tissues.

of MGMT activity utilizes nitrogen-frozen material and is performed measuring the transfer of a radioactively labelled substrate to the protein, MSP is predominantly used in clinics to determine MGMT status. The most frequently used primer for this assay was initially described by Herman's group (Esteller et al. 2000). It was used in a large number of studies, which showed a correlation between MGMT promoter methylation and patient survival (Esteller et al. 2000; Hegi et al. 2004, 2005; Mollemann et al. 2005; Everhard et al. 2006). Since MGMT promoter methylation correlates with the loss of MGMT protein expression and activity in the tumor (Esteller et al. 1999; Christmann et al. 2010) and, in addition, correlates with a better outcome of therapy (for review see Stupp et al. 2009), the methylation status of the MGMT promoter is currently being used to predict those patients who are likely to benefit from TMZ or combined TMZ/CCNU/ACNU chemotherapy.

MGMT Inhibition in Cancer Therapy

Since MGMT activity has a major impact on the response of tumor cells to O^6-alkylating agents, several inhibitors have been developed in order to reduce the MGMT activity and to sensitize tumor cells to O^6-alkylating anticancer drugs (for review see Kaina et al. 2010). Two of these inhibitors are currently being tested in clinical studies: O^6-benzylguanine (O^6BG) and O^6-BTG (Lomeguatrib, previously called PaTrin-2) (Fig. 4). O^6-BTG is about 53 times more potent than O^6-BG with an IC_{50} of 3.4 nM compared to 180 nM for O^6-BG (Shibata et al. 2006).

Initial preclinical experiments have shown that pre-treatment with these inhibitors increases the sensitivity of human xenografts to alkylating anticancer drugs and prolongs the life span of the xenograft bearing animals.



O⁶-Benzylguanine (O⁶-BG) ***O⁶-(4-Bromothenyl)guanine (O⁶-BTG)***

Figure 4. Chemical structure of the MGMT inhibitors O^6-BG and O^6-BTG.

In particular, pre-treatment with O^6-BG increased BCNU-induced cell death in human medulloblastoma xenografts (Friedman et al. 1992; Felker et al. 1993), malignant glioma xenografts (Friedman et al. 1992; Marathi et al. 1994; Rhines et al. 2000), and adenocarcinoma xenografts (Wan et al. 2000). The same sensitizing effect following pre-treatment with O^6-BG was also observed with TMZ in pancreatic (Kokkinakis et al. 2003), malignant glioma (Friedman et al. 2002) and neuroblastoma xenografts (Wagner et al. 2007). Pre-treatment with O^6-BTG increased TMZ-induced cell killing in human melanoma (Middleton et al. 2000a; Middleton et al. 2000b; Middleton et al. 2002) and breast carcinoma xenografts (Clemons et al. 2005).

Contrary to the preclinical studies, the clinical studies have revealed no benefits for the inhibitors in tumor therapy: a combined treatment with O^6-BG and BCNU every six weeks in 18 patients with CNS tumors failed to show any impact on the clinical outcome (Quinn et al. 2002). In another study involving 17 patients with multiple myeloma, one complete response and 3 partial responses were observed (Batts et al. 2007); in 18 patients with chemo-naive advanced melanoma, one complete response, 4 stable disease and 13 progressive disease were observed; and in 18 prior-chemotherapy patients, no responses, 3 stable and 15 progressive diseases were observed (Gajewski et al. 2005). In 12 patients with advanced soft tissue sarcoma there were no responders either (Ryan et al. 2006). It should be noted that in all these trials the response of patients receiving O^6-BG together with TMZ or BCNU was not compared with the alkylating drug only group, which makes full assessment of the data difficult.

In addition to O^6-BG, similar clinical trials have been performed using O^6-BTG; patients that received the alkylating drug and the inhibitor were compared with patients receiving only the alkylating drug. In one phase II trial, over 100 patients with metastatic melanoma were treated with TMZ alone or a combination of O^6-BTG and TMZ (Ranson et al. 2007). However, the combination with O^6-BTG was not found to significantly influence the overall response rate or the median time to disease progression. In another phase II study involving 19 patients with stage IV metastatic colorectal carcinoma, O^6-BTG and TMZ resulted in the same outcome as the group being given TMZ on its own (Khan et al. 2008).

Since tumor biopsies showed a recovery of MGMT activity within 24 h (Ranson et al. 2007), it has been suggested that the inability of O^6-BTG to enhance the clinical response to TMZ might be due to scheduling. Based on this finding, higher doses of O^6-BTG and an extended dosing period beyond that of TMZ were assessed in subsequent trials. However, treating thirty-two patients with metastatic melanoma with an extended O^6-BTG dosing schedule in combination with TMZ also showed no advantage over using TMZ alone (Kefford et al. 2009). Furthermore, this study showed that while MGMT activity was completely inactivated in PBMC and tumors biopsied

on the last day of treatment with O^6-BTG, the repair activity recovered in tumors shortly thereafter, indicating that an even more protracted dose regiment of O^6-BTG would be needed for extensive ablation of functional MGMT function (Watson et al. 2009). This observation is supported by additional studies, which showed that an O^6-BTG dose of 120 mg or 160 mg is required for complete inactivation of MGMT in prostate and colorectal cancers or in CNS tumors, respectively (Watson et al. 2010). Thus, the doses (40–80 mg) used in the phase II studies described above may have been too low.

MGMT Inhibitor Targeting

As reported above, the integration of MGMT inhibitors into clinical trials has not yet resulted in an improvement in the therapeutic efficacy of methylating or chloroethylating anticancer drugs in glioma or melanoma therapy. A contributing reason for this is that a dose reduction of the alkylating drug is necessary when combined with the inhibitor to reduce the adverse systemic side effects of the therapy. However, this dose reduction also decreases tumor cell kill. Therefore, it is highly desirable to develop strategies for selectively targeting the MGMT inhibitor to the tumor and as a result away from normal cells.

One approach is to administer the inhibitor locally, which has been done in an individual trial with a patient suffering from glioblastoma multiforme. In this trial, an Ommaya reservoir was implanted in the tumor cavity after dissection of a reccurring lesion and used to administer O^6-BG directly into the brain prior to systemic therapy with TMZ (Wiewrodt et al. 2008). No systemic or neuronal toxicity was observed due to intracranial O^6-BG administration, indicating that this approach is feasible. The effect on tumor growth is hard to assess since controls without loco-regional administration are lacking. Obviously, a clinical trial involving a larger cohort of patients is warranted.

In addition to local delivery of O^6-BG, chemical modification strategies were used for targeting MGMT inhibition to the tumor. These strategies utilize specific characteristics of tumors. In the first approach, O^6-BG was conjugated to folate, since tumor cells often exhibit high levels of expression of folate receptors. These O^6-BG-folate conjugates are effective MGMT inactivators and predominantly kill cells expressing high folate receptor levels (Nelson et al. 2004). In a second approach, O^6-BG and O^6-BTG were conjugated to ß-D-glucose, a strategy based on the finding that increased glucose consumption is a common characteristic of tumor cells (Argiles and Lopez-Soriano 1990) and that elevated glucose uptake is mediated via up-regulation of glucose transporters (Yamamoto et al. 1990). *In vitro* testing showed that a linker of 8 carbons between D-glucose and the N9 of O^6-BG

was optimal for inhibition of MGMT activity (Reinhard et al. 2001a; Reinhard et al. 2001b). It was also shown that for MGMT inactivation, linking the N9 of O^6-BG to ß-D-glucose was superior to α-D-glucose. The glucose-conjugated inhibitors inactivated MGMT in cell extracts and in living cells, with O^6-BTG-C8-ß-D-glucose being more effective than O^6-BG-C8-ß-D-glucose (Kaina et al. 2004). The conjugates were not cytotoxic in cell culture *per se* and penetrated quickly into living cells and depleted MGMT function within ~ 45 min. When given 1h prior to and again after treatment with the alkylating agent, O^6-BTG-C8-ß-D-glucose was similar to O^6-BTG in its ability to sensitize MGMT expressing Chinese hamster ovary and HeLa cells to fotemustine and TMZ toxicity in colony formation experiments (Kaina et al. 2004). These *in vitro* biochemical and cell-based studies demonstrate that the glucose conjugates are able to enter cells and inactivate MGMT, resulting in substantial potentiation of the killing effect of the O^6-alkylating agents,such as BCNU, fotemustine and TMZ. Whether the glucose conjugates allow tumor targeting is currently under investigation.

CONCLUSIONS

In summary, MGMT is a highly important protective factor, which has the ability to repair alkylation-induced DNA damage caused by several environmental mutagens and genotoxic anti-cancer drugs. Whereas the protective effect of MGMT on cancer formation is clearly demonstrated in animal experiments, convincing data on its protective role in humans is still lacking, likely due to the genetic complexity that exists as to MGMT polymorphisms, epigenetic regulation and among participants in the conducted studies. Since MGMT protects very efficiently against point mutations (Kaina et al. 1991), it is reasonable to speculate that it confers a no-adverse effect threshold in mutagenesis and carcinogenesis following exposure to genotoxicants that provoke O^6-alkylguanine formation. In contrast to the role of MGMT in human cancer protection, its role in tumor cell resistance is better established. Currently, in the clinic the MGMT status, primarily assessed by gene promoter methylation, is being used to predict the response of patients to TMZ and ACNU/CCNU-based chemotherapy. In addition, specific inhibitors are being tested in clinical trials for improved sensitization of MGMT proficient cells to alkylating anticancer drug-based therapies. Up to this point, MGMT status has been analyzed only within the scope of retrospective studies and a positive effect of MGMT inhibitors in the therapy has yet to be shown. In short, more work is clearly required to translate MGMT to the clinic, both as a predictive marker and as a direct target for pharmacological intervention.

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CHAPTER 7

DNA Mismatch Repair: Its Role in Human Carcinogenesis and as a Predictive and/or Prognostic Biomarker for Cancer Therapy

Timothy J. Kinsella

INTRODUCTION

DNA mismatch repair (MMR) is a highly conserved, but complex, DNA repair system that helps maintain genomic stability in human cells on several levels including: correcting base-base mismatches and insertion-deletion loops (IDLs) erroneously generated during DNA replication; blocking genetic recombination events between divergent DNA sequences; monitoring and correcting errors in meiosis; and mediating cell cycle delay and cell death in response to certain types of endogenous DNA damage and exogenous DNA damage from occupational and therapeutic chemical and ionizing radiation (IR) exposures (Iyer et al. 2006; Jiricny 2006; Li 2008). As such, MMR plays an essential role in the DNA damage response (DDR) pathway by removing severely damaged cells and reducing the risk of mutagenesis and carcinogenesis. However, in the absence of MMR,

Research Scholar Professor, Department of Radiation Oncology, Warren Alpert Medical School, Brown University, Physicians Office Building, Suite 130, Rhode Island Hospital, 110 Lockwood Street, Providence, RI 02903, USA; Email: tkinsella@lifespan.org

resulting from genetic and/or epigenetic alterations in the MMR genes, the persistent base-base mismatches and IDLs remaining after DNA replication result in a mutator phenotype with a 10^2–10^3 elevation of spontaneous mutations highlighted by microsatellite instability (MSI) and a significant risk of cancer (Jiricny 2006).

MMR deficiency is principally associated with the autosomal dominant Hereditary Non Polyposis Colorectal Cancer (HNPCC) or Lynch Syndrome, stemming from mutations in MMR genes such as *hMLH1*, *hMLH2*, *hMSH6*, and *hPMS2* (Lynch and de la Chapelle 2003). MMR deficiency is also associated with an increasing number of sporadic microsatellite instability high (MSI-H) solid tumors, typically related to promoter methylation of the *hMLH1* or *hMSH2* genes (Peltomaki 2003). These sporadic MSI-H cancers include several types of gastrointestinal cancers (colorectal, pancreatic, gastric, esophageal), gynaecological cancers (endometrial, ovarian), genitourinary cancers (bladder, ureter), as well as non-small cell lung (NSCL) cancers and high grade primary brain tumors, where MMR deficiency (MSI-H phenotype) is found in up to 10–20% of these common cancers. Additionally, MMR deficiency is associated with *in vitro*/*in vivo* "damage tolerance" (i.e. resistance) to multiple different classes of clinically active chemotherapy drugs (Karran 2001; Modrich 2006; Kinsella 2009) as well as to other types of DNA damage (stress), including IR (Yan et al. 2001; Brown et al. 2003; Cejka et al. 2004; Yan et al. 2009) and hypoxia (Mihaylova et al. 2003; Koshiji et al. 2005; Klein and Glazer 2010).

The clinical implications for the treatment of MMR-deficient cancers are somewhat confusing. For example, promoter hypermethylation of *hMLH1* or *hMSH2* with subsequent loss of protein expression of these key MMR proteins is found in nearly 50% of NSCL cancers occurring in non-smokers and is associated with a poor prognosis, even in early stage disease (Hsu et al. 2005). In contrast, MMR deficient colon cancers (from either genetic or epigenetic alterations) appear to have a better prognosis than MMR proficient cancers following surgery (de la Chapelle and Hampel 2010). However, analyses of the multiple clinical trials of the use of fluoropyrimidine-based adjuvant chemotherapy in MMR deficient colon and esophageal cancers found significantly less benefit in disease-free survival in comparison to a significant benefit in MMR proficient colon and esophageal cancers (Kishi et al. 2003; Ribic et al. 2003; Sargent et al. 2010). Additionally, MMR deficient endometrial and rectal cancers show reduced local control and lower pathological response rates following radiation treatment alone (Bilbao et al. 2010) or with combined 5-fluorouracil (5-FU)-radiation therapy (Choi et al. 2007), respectively. These conflicting clinical data underscore the biological complexity of MMR and its translational significance to cancer screening and cancer therapeutics.

BIOCHEMICAL MODEL OF HUMAN MMR

While the MMR pathway is best characterized genetically and biochemically in *Escherichia coli* (*E. coli*), substantial information has been generated over the last two decades on the biochemistry of human MMR (hMMR) (Kunkel and Erie 2005; Iyer et al. 2006). There are many strong similarities between hMMR and *E.coli* MMR, ranging from substrate specificity to nick-directed strand specificity and bidirectionality, underscoring the highly conserved nature of the evolution of MMR. However, hMMR is more complex (Jiricny 2006). Human MMR has two major biochemical functions: first, efficient recognition of the base-base mismatches and IDLs in the newly synthesized (daughter) DNA strand; and second, specific direction of other MMR components to allow excision, resynthesis and ligation in the daughter strand containing the erroneous genetic information. MMR has evolved to efficiently correct errors of the DNA polymerases that escape their 3′-5′ proofreading activities during DNA replicative synthesis. However, unlike other base damage repair pathways, such as Base Excision Repair (BER) or Nucleotide Excision Repair (NER) (see Chapter 1), MMR is targeted specifically to the daughter strand that carries the damage.

The MMR pathway can be divided into three sub-processes (Table 1). The first MMR sub-process involves damage recognition by the two human MutS homologs with MutSα, a heterodimer of MSH2 and MSH6, being principally involved in recognizing and binding base-base mismatches and small IDLs (1–2 nucleotides), while MutSβ, a heterodimer of MSH2 and MSH3, preferentially recognizes and binds large IDLs. Using *in vitro* assays, MMR can be directed by a strand break located either 3′ or 5′ to the mismatch. Binding of the MutS homologs triggers the second

Table 1. Functional Sublevels of MMR and the Respective Protein Complexes Involved.

Sub-level Processes	Type of Mispair	
	Base:Base 1-2 base IDL	Base:Base 2-16 base IDL
Recognition (initiation)	MutSα	MutSβ
Excision	MutLα PCNA EXO1 RPA	MutLα,β,γ PCNA EXO1 RPA
Repair Resynthesis	Polδ PCNA RPA DNA ligase	Polδ PCNA RPA DNA ligase

MMR subprocess, damage excision, which involves ATP-dependent conformational changes with the binding of the MutLα heterodimer to form a ternary complex with mismatch DNA and MutSα or MutSβ. Actually, four human MutL homologs have been identified (MLH1, MLH3, PMS1, and PMS2). MLH1 forms a heterodimer with PMS2 (MutLα) and is a necessary component for MMR processing. MLH1 also forms a heterodimer with PMS1, called MutLβ and with MLH3, called MutLγ. MutLγ plays a key role in meiosis, while no specific function for MutLβ is known at this time. The MutSα/MutLα and the MutSβ/MutLα complexes bound to the base-base mispairs and large IDLs, respectively, recruit a nuclease (EXO1) and other proteins such as PCNA and RPA to facilitate damage excision. Such excision involves sequential removal of nucleotides between an adjacent single strand break (SSB) up to and beyond the mismatch on the daughter DNA strand. EXO1 can efficiently carry out 5′ nick-directed mismatch excision, but requires the MutLα endonuclease in addition to PCNA and RFC for 3′ nick-directed excision. Finally, the third MMR sub process involves resynthesis and ligation carried out initially with a DNA polymerase (Polδ) and then sealing of the nick by a DNA ligase (DNA Ligase1). PCNA binds to both MSH2 and MLH1 and appears to be necessary for both excision and resynthesis. High Mobility Group Box 1 protein (HMGB1) may also participate in the excision step and can substitute for RPA, at least in *in vitro* MMR purified protein systems (Zhang et al. 2005).

In spite of significant progress over the last decade in better defining the genetic and biochemical aspects of hMMR, one of the fundamental questions that remains involves the mechanism(s) by which the MMR proteins/complexes coordinate communication between the two physically distinct DNA sites, i.e., between the mismatch and the strand discrimination signal, which can be separated by up to 10^3 base pairs. In both prokaryotic and eukaryotic cells, the strand discrimination signal is a strand-specific nick (or break), although the source of the nick in hMMR is not known. As mentioned, hMMR is bidirectional and there appears to be distinct excision responses based on whether the strand nick is 3′ or 5′ to the mismatch. There are three postulated models that attempt to answer (or begin to address) this fundamental question. Two of the models, the translocation model (Modrich 2006) and the "sliding clamp" model (Fishel 1998), involve signal transduction along the helix contour. The translocation model postulates that the MutS-MutL ternary complex forms at the mismatch site and then translocates in either direction, being driven by ATP hydrolysis. In the "sliding clamp" model, the MutS heterodimer binds to the mismatch in an ADP-bound state, triggering an initial conformational change that allows for an ADP⇒ATP exchange. A second conformational change allows the MutS heterodimer to form a sliding clamp. Here, ATP, not ATP hydrolysis, facilitates formation of the ternary complex with MutLα, which slides from

the mismatch to the strand nick site. Finally, the third model, the "stationary" model, postulates that following initial binding of the MutS heterodimer to the mismatch, subsequent interactions with other MMR proteins/complexes create a looping in the DNA that physically brings the two distant sites together while MutS remains bound to the mismatch. In the stationary model, MutS ATPase activity acts as a proofreader to confirm mismatch binding and initiation of downstream excision (Junop et al. 2001). To date, none of the experimental models have convincingly demonstrated where the ternary complex assembles and how the various other MMR protein complexes specifically function.

MISMATCH REPAIR LINKS TO DNA DAMAGE RESPONSE

Human MMR has been found to be an essential part of the DDR to several classes of clinically active chemotherapy drugs as well as IR, where the extent of cytotoxicity appears related to the type and duration of exposure to a particular drug class or IR, and is mediated by cell cycle alterations and activation of competing cell survival and cell death signaling pathways (Karran 2001; Jiricny 2006; Li 2008; Kinsella 2009). The drug classes include: monofunctional alkylators such as temozolomide, dacarbazine and procarbazine; bifunctional alkylators such as the platinum analogs, cisplatinum and carboplatinum; and antimetabolites such as the thiopurines, 6-thioguanine (6-TG) and 6-mercaptopurine (6-MP), fluoropyrimidines, and the halogenated thymidine analogs, iododeoxyuridine (IUdR) and bromodeoxyuridine (BUdR).

Following exposure to an experimental monofunctional alkylator such as N-methyl-N′-nitro-N-nitrosoguanidine (MNNG), MMR-proficient cells demonstrate a prolonged G_2 cell cycle delay, mediated by ATR-Chk1 activation followed by up to a 2-log cell kill in comparison to the cell cycle and cytotoxic responses in isogenic MMR deficient cells (Karran 2001; Stojic et al. 2004). It is well established that the primary cytotoxic lesion generated by monofunctional alkylators is O^6-methyl-guanine (meG), which can form mispairs with either C or T during replication; both mispairs are recognized by MutSα. If the modified base (meG) is in the template (parent) strand, MMR processing of such monofunctional alkylator damage continually regenerates meG-C and meG-T mispairs during the resynthesis and ligation step, because the newly incorporated C or T residue on the daughter strand is targeted for excision. Consequently, this "futile cycling" model results in replication fork arrest over several cell cycles following drug exposure, activating signaling pathways involved in both cell cycle arrest and an apoptotic cell death (Jiricny 2006). In contrast, MMR deficient cells demonstrate "damage tolerance" rather than true drug resistance, since in

the absence of functional MMR, cells do not process the meG-C and meG-T mispairs and survive with an increased risk of mutagenesis. Similar cellular responses are seen with the clinically active monofunctional alkylators. For example, in a human glioblastoma multiforme tumor xenograft model, loss of MMR following treatment with procarbazine was found to confer resistance (damage tolerance) to temozolomide and busulfan (Friedman et al. 1997).

MMR proficient cells are highly sensitive to the cytotoxicity (>2–3 log cell kill) of certain antimetabolites, such as 6-TG, compared to isogenic MMR deficient cells, with MMR proficient cells showing an initial prolonged G_2 arrest followed by cell death after several replication cycles (Yan et al. 2003). 6-TG is known to be incorporated into DNA in place of dGTP during the first S-phase and a small amount (≈1 per 10^4 bases) of genomic 6-TG is rapidly methylated *in situ* to 6-thiomethylguanine (6-meTG). Both 6-meTG-C and 6-meTG-T mispairs are recognized by MutSα (Swann et al. 1996). Again, MMR processing of the resulting 6-meTG-C and 6-meTG-T mispairs results in futile cycling as the 6-meTG in the parental strand is not removed. With continued futile cycling, DNA SSBs (but not double-strand breaks) result in subsequent activation of ATR and Chk1, leading to a prolonged G_2 arrest (Yan et al. 2004). Additionally, an ATM-independent activation of Chk2 occurs gradually over several cell cycles following 6-TG exposure correlating with a later increased tetraploid (4C) G_1 arrest, which blocks cells that escape from the initial G_2 arrest. MMR processing of 6-TG damage results in activation of both apoptotic (type 1) and autophagic (type 2) programmed cell death pathways using p53-dependent and p53-independent mechanisms (Yan et al. 2004; Zeng and Kinsella 2007; Zeng et al. 2007). 6-TG induced autophagy by MMR processing is positively regulated by both BNIP3 and mTOR/S6K1 activation with competing cell survival and cell death pathway processing (Zeng and Kinsella 2008, 2010). The time course and the biological components involved in the current model of the metabolic (Panel A) and futile cycling molecular processing of 6-TG damage by MMR (Panel B) are presented in Fig. 1.

MMR processing of another antimetabolite, the fluorinated pyrimidine analog 5-FU, is more complicated in that, while one 5-FU metabolite (5FdUTP) is incorporated into DNA and processed by MMR, other metabolites are incorporated into RNA (5FUTP) or bind (inhibit) thymidylate synthase (5FdUMP) and deplete dTTP pools necessary for scheduled and unscheduled DNA synthesis. Using isogenic cell lines, 5-FU treatment results in ≈1 log differential cell kill in MMR proficient cells, which are more sensitive, compared to MMR deficient cells (Meyers et al. 2001). A similar effect is reported in a human tumor xenograft model (Pocard et al. 2000). However, no differential cell cycle or cytotoxicity responses were found in isogenic MMR-proficient and -deficient cell lines following treatment with

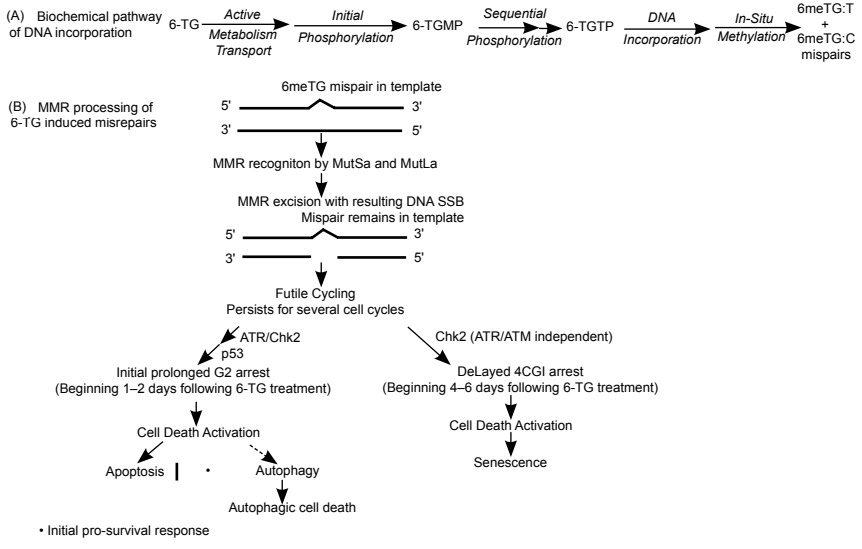


Figure 1. (A) cellular metabolism of 6-TG
(B) current experimental biology futile cycle model of MMR processing of 6-TG-induced DNA base damage in MMR^+ cells

a specific chemical inhibitor of thymidylate synthase, raltitrexed (Meyers et al. 2005), suggesting that 5-FU DNA mismatches are recognized and processed by MMR. Again, these cell cycle and cell survival responses of 5-FU treatment support the futile cycling model of MMR processing that requires multiple replicative rounds following drug exposure.

It appears that not all antimetabolites that involve MMR processing of drug-induced DNA damage show futile cycling. *In vitro* and *in vivo* data on MMR processing of the halogenated thymidine analog, IUdR, is more consistent with the direct general damage sensor model of MMR. In this model, it is postulated that the binding of MutSα and MutLα to the mispair (G:IU in the case of IUdR) directly triggers DNA damage signaling by recruiting ATR/ATRIP to the ternary complex (Wang and Qin 2003; Adamson et al. 2005). IUdR, a known radiosensitizing drug, undergoes rapid sequential intracellular phosphorylation to IdUTP, which competes directly with dTTP for DNA incorporation during the first round of replication following drug exposure (Kinsella 1996). The G:IU mispairs (but not A:IU mispairs) are recognized directly by MutSα with a similar affinity to a G:T mispair (Berry et al. 2003; Kinsella et al. 2011). However, no significant cytotoxicity nor G_2 arrest is seen in MMR proficient cells following clinically achievable IUdR drug exposures (1–10 μM) unless cells are co-treated with chemical inhibitors of ATR (caffeine) or of Chk1

(UCN-01) (Seo et al. 2006). However, MMR deficient cells when treated with IUdR retain high levels of IUdR-DNA incorporation (i.e., damage tolerant phenotype) for several cell cycles following drug exposure (Berry and Kinsella 2001). As such, IUdR-treated MMR deficient cells and tumors can be targeted for enhanced cell kill (radiosensitization) following subsequent IR treatment with sparing of MMR proficient normal cells/tissues. This potential therapeutic gain for IUdR-mediated radiosensitization of MMR deficient (damage tolerant) human cancers has been demonstrated *in vivo* using an athymic mouse model with simultaneously implanted isogenic MMR proficient and MMR deficient human colon cancer xenografts (Seo et al. 2004). A model of the cellular metabolism of IUdR (Panel A) and the general damage sensor MMR processing of IUdR-DNA mispairs (Panel B) is presented in Fig. 2.

MMR deficiency is reported to confer only a 2-fold survival advantage (damage tolerant phenotype) to cis-diammine-dichloro-platinum II (cisplatin) treatment compared to MMR proficient cells (Aebi et al. 1996; Fink et al. 1997; Papouli et al. 2004). This more modest damage tolerance to cisplatin, which causes intrastrand crosslinks, is not seen with other DNA intrastrand crosslinking drugs such as the nitrosoureas (Fiumicino et al. 2000). However, the cisplatin induced 1,2-intrastrand crosslink between the

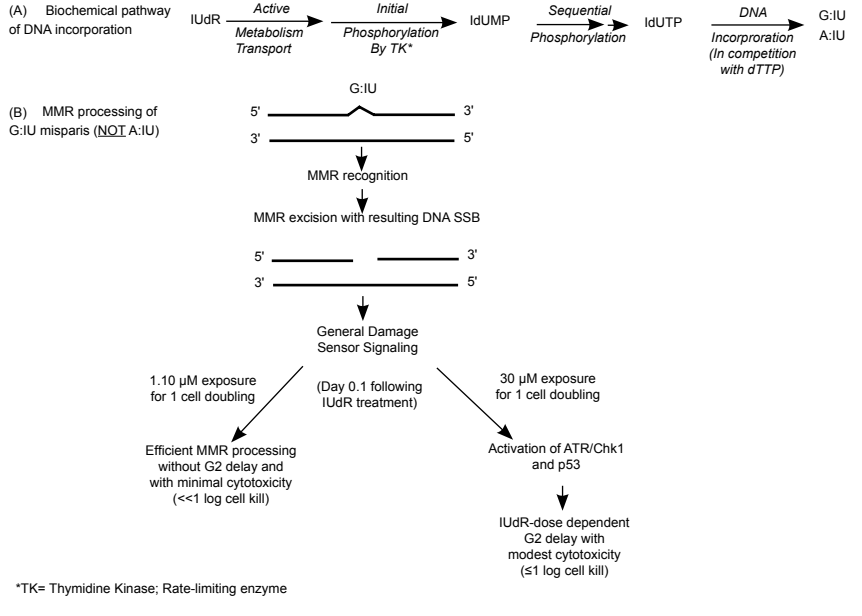
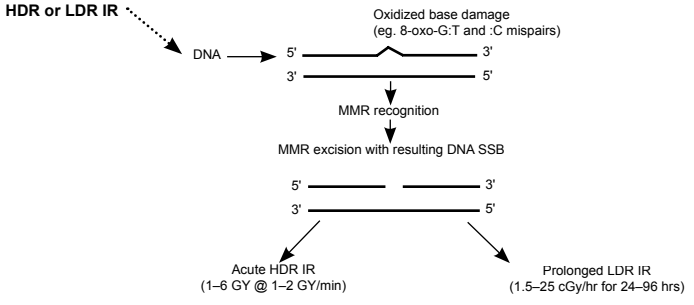


Figure 2. (A) cellular metabolism of IUdR
(B) current experimental biology general damage sensor model of MMR processing of IUdR

N^7 positions of two adjacent purines is recognized by MutSα, particularly when one of the crosslinked purines is mispaired with thymine (Yamada et al. 1997). MMR processing of these cisplatin crosslinked mispairs results in a prompt S-phase arrest followed by cell death, in contrast to the futile cycling model proposed for MMR processing of the monofunctional alkylators and thiopurines as detailed above. Thus, cisplatin damage processing by MMR appears more consistent with the general damage sensor model (Shimodaira et al. 2003).

MMR also appears to be involved in the processing of certain types of IR damage, including both high dose rate (HDR) IR and low dose rate (LDR) IR (Fritzell et al. 1997; DeWeese et al. 1998; Zeng et al. 2000; Yan et al. 2001, 2009; Brown et al. 2003; Cejka et al. 2004). Using HDR IR (1–4 Gy/min), an IR dose dependent G_2 delay occurs via a p53 independent activation of the ATM/Chk2/Cdc25A signaling pathway (Yan et al. 2001; Brown et al. 2003), as well as an IR-dose-dependent S phase delay mediated through an intra-S damage signaling pathway (Brown et al. 2003). However, MMR processing of HDR IR damage has only a modest effect on cell survival, with some studies showing MMR deficient cells to be slightly more IR-resistant (damage tolerant) (Fritzell et al. 1997; DeWeese et al. 1998), whereas other studies show little to no effect on survival (Yan et al. 2001; Franchitto et al. 2003; Cejka et al. 2004). It is postulated that IR-induced oxidative DNA damage results in 8-oxoguanine mispairs (both 8oxoG:T and 8oxoG:A), which are processed by MMR (Chang et al. 2002).

MMR processing of LDR IR (1-100 cGy/hr) damage shows a more typical cytotoxic response, with MMR proficient cells showing up to a 1 log decreased survival that is both LDR dose rate and total IR dose dependent. LDR IR cytotoxicity involves MMR activation of both apoptotic and autophagic cell death pathways (Yan et al. 2009). LDR IR treated MMR proficient cells also show more prolonged G_2 and late S phase cell cycle delays, as well as reduced HPRT gene mutation rates compared to isogenic MMR deficient cells. Moreover, during and following LDR IR, MMR proficient cells show a temporal increase in MLH1 protein levels (secondary to a reduction in MLH1 protein degradation) that correlate with a decrease in Rad51 protein levels, suggesting that the reduced MLH1 degradation (with enhanced MutLα levels) affects homologous recombination (HR) via a reduced expression of Rad51 (Yan et al. 2009). MMR is known to be involved in HR (see Chapter 14), as mismatches can be produced within the heteroduplex generated by strand exchange during bypass of unrepaired damage (Surtees et al. 2004). A summary of MMR processing of HDR IR and LDR IR damage, including the cell cycle and cytotoxic responses, is presented in Fig. 3.



A) Cell cycle	↑ G2 delay without change in G1 or S phase progression in MMR$^+$ vs. MMR$^+$ cells; *TK 2 pathway activation; p53 independent	↑G2 delay and ↑late S phase progression in MMR$^+$ vs. MMR$^+$ cells in 1st cell cycle
B) Repair protine expression	No change in MMR protein expression in MMR$^+$ vs. MMR$^+$ cells	↑MLH1 (but NOT MSH2) Levels in MMR+ cells: simultanious ↓RAD 51 levels (?↓homologous recombination)
C) Cytotoxicity	Little to no change in clonogenic survival in MMR$^+$ vs. MMR$^+$ cells	21 Log reduced clonogenic survival (IR dose rate dependence) in MMR$^+$ vs. MMR$^+$ cells; ↑HPRT mutation rate in MMR; cells
D) Cell death mechanism	Similar cell death patterns including necrosis, apoptosis, autophagy, and senescence in MMR$^+$ vs. MMR$^+$ cells	↑apoptopic and autophagic activation in MMR$^+$ cells without ↑gamma H2AX expression in MMR$^+$ vs. MMR$^+$ cells

Figure 3. Current status of experimental biology model of MMR processing of IR-induced base damage following acute HDR and prolonged LDR IR exposures.

MMR DEFICIENCY AND HUMAN CANCERS

Early Genetic Linkage and Gene Identification Studies of HNPCC

While MMR was long recognized as an important guardian of genomic stability, it was not until 1993 that MMR deficiency was linked to HNPCC as well as some sporadic colon cancers (Lynch and de la Chapelle 2003; Peltomaki 2003). The initial study found a genetic linkage of HNPCC families to the presence of a disease locus at p15–16 of chromosome 2 (Peltomaki et al. 1993). Such a linkage analysis suggested that the loss of a tumor suppressor gene at this location was responsible for HNPCC. However, subsequent genetic studies of HNPCC cancers, using available microsatellite markers, found no allelic loss as suspected, but rather, a series of insertion or deletion mutations in di- and tri-nucleotide repeats in the p15–16 region of chromosome 2 (Aaltonen et al. 1993). Additionally, these investigators found similar microsatellite alterations in the same region of chromosome 2 in some sporadic colon cancers. At the same time, two other groups found microsatellite alterations throughout the entire genome in up to 15% of sporadic colon cancers (Ionov et al. 1993; Thibodeau et al. 1993). Thus, based on these four independent studies, a strong link was suggested between the presence of MSI and HNPCC, as well as a subset of sporadic colon cancers. Later in the same year, a second locus linked to HNPCC was found at p21–23 of chromosome 3 (Lindblom et al. 1993). The

MLH1 gene was mapped to this second locus (Bronner et al. 1994), while the *MSH2* gene was mapped to the first locus on chromosome 2 (Fishel et al. 1993; Leach et al. 1993).

Since these early sentinel studies identifying two key HNPCC-linked genes, *MLH1* and *MSH2*, many other HNPCC families have been extensively screened for genetic mutations in MMR genes, with these studies collectively noting that nearly 90% of families show either *MLH1* or *MSH2* mutations, while the remaining 10% show mutations in either *MSH6* or *PMS2* (Lynch and de la Chapelle 2003). *MSH6* mutations are found in a large proportion of atypical HNPCC families, highlighted clinically by a later age of onset of colorectal and endometrial cancers and lower rates of MSI (Kolodner et al. 1999; Lynch and de la Chapelle 2003). No germline mutations in *MSH3* or *MLH3* have been identified in HNPCC families to date. Mutations in the *EXO1* gene are reported in some HNPCC families where the tumors have lost the mutated allele while retaining the wild type gene (Wu et al. 2001). While possible, there have been no reports of mutations in HNPCC families in other genes that participate in MMR, including *PCNA*, *RPA*, *RFC*, *HMGB1*, or *polymeraseδ*.

Use of Knockout Mouse Models to Better Define MMR Linkage to HNPCC-Related Cancers

Knockout mouse models of many MMR genes were developed in the mid to late 1990's to better understand the development of cancers related to MMR deficiency (Wei et al. 2002). While most single MMR gene knockouts showed MSI and increased tumorigenesis, a surprising feature of these mouse models is the lack of development of colon and endometrial cancers, as is commonly seen in HNPCC families, particularly with germline mutations in *MLH1*, *MSH2*, and *MSH6*. Rather, colonic adenomas and Non-Hodgkin's Lymphoma predominate, in addition to dermal and soft tissue tumors. Additionally, it was found that *Msh2* knockout (*Msh2*$^{-/-}$) mice develop normally and both sexes are fertile; but, these mice have reduced lifespan with 50% dying within 6 months (Reitmair et al. 1995). Homozygous *Msh6* knockouts did not show the typical features of MSI and develop tumors (lymphoma and GI tumors) later in life, similar to that seen in atypical HNPCC families (Kolodner et al. 1999). *Mlh1* knockouts show a typical MSI phenotype and a tumor spectrum similar to *Msh2* knockouts, but are infertile (Baker et al. 1996). *Pms1* knockouts exhibit limited MSI (only in mononucleotide repeats) and do not develop tumors, yet are fertile (Prolla et al. 1998), whereas *Pms2* knockouts show characteristic MSI and tumor spectrum (lymphomas and sarcomas), although males are infertile (Baker et al. 1995). Finally, *Exo1* knockouts show MSI as only mononucleotide

repeats, develop lymphomas, are infertile (both sexes), and experience a reduced lifespan (Wu et al. 2001; Wei et al. 2003).

Overall, these studies of mouse knockouts of MMR related genes support the hypothesis that MMR deficiency leads to genomic instability and increased carcinogenesis. However, these MMR knockout mice do not develop colon and endometrial cancers and show variability in the extent of MSI. Thus, the effects of MMR deficiency on carcinogenesis appear to be both tissue specific and species specific, although these discrepancies between mouse and human are poorly understood. Additionally, while MMR has an essential role in mice during meiosis and gamete formation, the effects of MMR on meiosis in humans is not well understood.

The Role of Epigenetic Silencing of MMR Gene Expression in Human Carcinogenesis

While a small percentage (≤5%) of sporadic colorectal cancers with a MSI positive phenotype show MMR gene mutations (mostly *MLH1* and *MSH2*) (Liu et al. 1995; Thibodeau et al. 1996), a majority of these sporadic MSI positive cancers show no MMR gene mutations, suggesting an alternative molecular mechanism for MSI-associated carcinogenesis. Hypermethylation of the *MLH1* promoter was first described in several sporadic MSI positive colon cancers characterized by the absence of MLH1 protein expression and no *MLH1* mutations (Kane et al. 1997). Subsequently, promoter methylation of *MLH1* was noted in up to 95% of sporadic MSI positive colorectal cancers (Cunningham et al. 1998; Herman et al. 1998). Indeed, this observation in sporadic MSI positive colorectal cancers served as one of the purposes for establishing the Bethesda 5 Panel of microsatellites to further classify MSI positive colorectal cancers into a MSI-low (MSI-L; 1 of 5 markers positive) and a MSI-high (MSI-H; ≥2 of 5 markers positive) category for further screening of possible HNPCC families (Boland et al. 1998). Initial proof-of-principle experiments to validate the association of *MLH1* promoter methylation and sporadic MSI-H colon cancers involved the use of a demethylating chemical agent, 5-aza-deoxycytidine (Herman et al. 1998; Veigl et al. 1998). Treatment of sporadic MSI-H colon cancer cell lines with this agent restored MLH1 protein expression and allowed for efficient MMR function.

It is now recognized that a MSI-positive (MSI-H) phenotype may be associated with a wide variety of non-HNPCC and non-sporadic colon cancers (Peltomaki 2003). These sporadic cancers include other GI cancers (gastric, small bowel, esophagus), gynecologic cancers (endometrial, ovarian, cervix), genitourinary cancers (bladder, ureter, prostate), breast cancer, non-small cell lung cancers and gliomas. Some of the early studies

of these non-colon cancers used different panels of microsatellite markers resulting in conflicting data. However, current immunohistochemical (IHC) staining for specific MMR protein expression has increased the sensitivity and specificity of detecting MSI and can direct subsequent PCR approaches to test for a specific MMR gene mutation or promoter methylation (Boland et al. 2008; Palomaki et al. 2009).

Association of MMR Deficiency with Mutations in Target Genes Controlling Cell Growth and Genomic Stability

While there is clear evidence to support the hypothesis that MMR genes function as tumor suppressor genes, some have argued that MMR functions primarily as a caretaker of genomic stability (Kinzler and Vogelstein 1997). It is also known that while the DNA in most solid tumors is hypomethylated at CpG dinucleotides, many CpG islands in the promoter regions of other caretaker genes involved in controlling cell growth and cell cycle checkpoint regulation can be silenced by methylation, similar to the MLH1 promoter hypermethylation in sporadic MSI-H cancers described above. As such, methylation silencing of other caretaker genes could provide a selective growth advantage for sporadic MSI-H cancers (Esteller 2002; Grady and Markowitz 2002). To test the hypothesis that promoter methylation of a key MMR gene, such as *MLH1*, could result in a mutator phenotype affecting mutations in other caretakers genes, a study of the di- and tri-nucleotide tracts within the coding region of the Type II transforming growth factor-β receptor gene, *TGF-β RII*, was conducted in a MMR-deficient sporadic colon cancer cell line. This study showed two mutational hot spots, which both result in frequent frameshift mutations with a truncated *TGF*-β *RII* gene product (Markowitz et al. 1995) suggesting that the loss of *TGF*-β *RII* function in a sporadic MMR deficient colon cancer would provide a selective growth advantage. Similar results on *TGF*-β *RII* methylation were reported from other MSI-H (MMR deficient) sporadic endometrial, gastric, cervical, and small bowel cancers, as well as high grade gliomas. Additionally, somatic frameshift mutations in mononucleotide repeat regions of several other caretaker genes involved in regulation of cell growth, including *BAX* and *IGF2-R*; involved in tumor suppression, such as *PTEN* and *APC*; or involved in other DNA repair pathways, including *MRE11* and *MED1,* have been found in MMR deficient cancer cell lines where MMR gene promoter methylation was also found (Duval and Hamelin 2002). Collectively, these data suggest that mutations in key cell growth and genomic stability genes occur more frequently in MMR deficient cells than in MMR proficient cells on a genome-wide basis and that loss-of-function mutations may contribute to the enhanced carcinogenesis of MMR deficient cells and tissues.

CLINICAL-TRANSLATIONAL ASPECTS OF DNA MISMATCH REPAIR

Mismatch Repair and the Evolving Definition of HNPCC vs. Lynch Syndrome

HNPCC or Lynch Syndrome is defined as an autosomal dominant syndrome with variable penetrance characterized by MSI and the early development (age ≤ 45 years) of colorectal cancer and a variety of non-colonic cancers, especially of gynecologic origin (principally endometrial and ovarian cancers). Almost all HNPCC/Lynch Syndrome tumors demonstrate MSI and/or abnormal (reduced) IHC staining for one or two of four MMR gene products (i.e., MLH1, MSH2, MSH6, and PMS2 proteins). While cancers with MSI account for ≈ 15–20% of all colorectal cancers, HNPCC/Lynch Syndrome accounts for 3–5% and sporadic colorectal cancers account for the remaining 12–15% (de la Chapelle and Hampel 2010). Nearly 90% of HNPCC/Lynch Syndrome colorectal and endometrial cancers have germline mutations in *MLH1* and *MSH2*, with the other 10% in *MSH6* and *PMS2*. *MSH6* mutations are associated with markedly reduced cancer risks compared to *MLH1* or *MSH2* mutations.

The initial Amsterdam Criteria for HNPCC were designed in the early 1990's to identify a suitable group of families for further study of an inherited form of colorectal cancer with MSI features. These criteria, however, did not define a specific clinical disorder. Subsequently, the Amsterdam Criteria were modified by the original Bethesda Guidelines at the United States National Cancer Institute (NCI) International Workshop on HNPCC in 1996, and further modified to the Revised Bethesda Guidelines in 2002, based on the then more sensitive testing for identifying inherited forms of colorectal cancer (Umar et al. 2004). However, even the Revised Bethesda Guidelines, which incorporate more standard testing for MSI and/or IHC analyses of MSI-H tumors, as well as genetic testing for germline mutations in *MLH1* and *MSH2* of proband individuals, led to the identification of heterogeneous groups. Such groups included some colorectal cancers associated with MMR gene mutations, some associated with hypermethylated MMR gene promoters, and others of unknown etiology, which led to confusion in clinically defining HNPCC/Lynch Syndrome. The most recent document, from the Evaluation of Genomics Applications in Practice and Prevention (EGAPP) Working Group (EWG), proposes a clinical definition of Lynch Syndrome: an individual with an identifiable MMR gene mutation, whether or not an existing colorectal cancer or other cancer is present (Palomaki et al. 2009). Such a definition allows planned analyses of clinical validity and utility of screening to be more homogenous and straightforward. The EWG is placing emphasis on the name Lynch Syndrome, as opposed to

HNPCC, because non-colorectal cancers, especially endometrial cancers, can be as common as colorectal cancers in some germline MMR gene mutation families. In this Chapter, HNPCC and Lynch Syndrome are used interchangeably.

The Clinical Relevance of MSI in Colorectal and Other Cancers

Microsatellites are short (1–6 bp), repetitive DNA sequences which are interspersed throughout the genome. It is estimated that at least 5×10^5 microsatellites occur in the human genome, both within genes and in intergenic regions. Within genes, microsatellites are commonly found in introns, but also in the promoter region, untranslated region, as well as in coding exons. Currently, there is no universally accepted definition of the minimum number of repeated nucleotides to classify as a microsatellite, but the upper range can be several hundred. A monomorphic microsatellite is one where all individuals in a population have the same number of repeat units, while a polymorphic microsatellite is defined as one where > 1% of a population shows heterozygosity for the number of repeat units. In principle, every cell of an individual should contain the same number of repeats in a microsatellite. MSI occurs when some cells (e.g., within a tumor) have one or two alleles with a different number of repeats. It is important to realize that MMR deficiency does not affect all microsatellites in the genome. Indeed, a low frequency of MSI can be found in a MMR-proficient tumor.

The repetitive nature of microsatellites results in a susceptibility to replication errors caused by slippage of DNA polymerases over tandem repeats. Normally, these replicative errors are efficiently corrected by MMR, as discussed earlier. With MMR deficiency (from genetic or epigenetic alterations), these replicative errors become fixed and the length of the microsatellite altered upon subsequent rounds of cell division. Since MMR deficiency does not affect all microsatellites in a particular tumor, it is necessary to study more that one microsatellite and to select microsatellites that are frequently associated with MMR deficiency. In the initial studies of MSI linkage to Lynch Syndrome in the early 1990's, researchers often selected microsatellite markers based on their empirical experience. At the first NCI (Bethesda) Conference in 1996 (Boland et al. 1998), a panel of 5 microsatellite markers were proposed as a standard, including 2 mononucleotide repeats (BAT25 and BAT26) and 3 dinucleotide repeats (D2S123, D5S346, and D17S250). The Bethesda 5 Panel continues to be the standard.

The most common method to assess MSI is to measure the length of a polymerase chain reaction (PCR) amplicon containing the entire microsatellite of interest. Using the Bethesda Classification of MSI (Boland et al. 1998), a MSI-H tumor was defined as ≥ 2 of 5 positive markers. However,

most MSI-H tumors will have 4 or all 5 markers positive. If only 1 of 5 markers is positive, the tumor is classified as MSI-L. Most MSI-L tumors are found to be MMR proficient with further testing (Mueller et al. 2009). A microsatellite stable (MSS) tumor shows 0 of 5 positive microsatellite markers.

While the use of the Bethesda 5 Panel is accepted and used worldwide, some studies have shown that the sensitivity of detecting MSI can be enhanced by including additional (often 3) mononucleotide markers (Palomaki et al. 2009). For example, the sensitivity of detecting MSI-H in tumors from patients with *MLH1* mutations is increased from 80% using just the Bethesda 5 Panel to 91% upon inclusion of additional mononucleotide markers; the sensitivity is increased from 84% to 87% with the use of additional mononucleotide markers in tumors from patients with *MSH2* mutations; and, the sensitivity is increased from 55% to 77% in tumors from patients with *MSH6* mutations. The lower sensitivities of detecting MSI in *MSH6* mutation patients using the Bethesda 5 Panel may be explained by the fact that many *MSH6* mutations affect more mononucleotide markers that dinucleotide markers (Plaschke et al. 2004). Overall, the 5-marker Bethesda Panel, perhaps with the addition of additional mononucleotide markers, remains a valid test for MSI with an overall specificity of 90% based on the recent EWG review (Palomaki et al. 2009). Methodological improvements in tumor tissue preparation for MSI testing, including laser capture microdissection and the number of cells tested, may also increase both the sensitivity and specificity (Muller et al. 2004).

Role of IHC Testing of MMR Protein Expression to Identify Individuals with HNPCC/Lynch Syndrome

IHC testing is often used today as an alternative screening method to detect MMR deficiency. IHC has a major advantage compared to MSI testing in that it can directly assess which MMR gene is likely to be mutated. Given the recognized MMR heterodimer pairings, IHC will show absent staining of both MSH2 and MSH6 when *MSH2* is inactivated. However, when *MSH6* is inactivated, IHC staining will be lacking for MSH6, but positive for MSH2. Similarly, when *MLH1* is inactivated, both MLH1 and PMS2 IHC staining are absent, while with mutational or epigenetic inactivation of *PMS2*, PMS2 IHC is deficient, while MLH1 IHC is positive.

Based on a recent comprehensive literature review (Palomaki et al. 2009), IHC has a sensitivity of 83% to identify patients with mutations involving *MLH1*, *MSH2*, and *MSH6*, and a specificity of 89%, quite similar to the sensitivity and specificity of MSI. However, IHC is less expensive and the necessary equipment and expertise are typically available in pathology

departments of large hospitals or readily provided by contract pathology services to smaller hospitals and clinics.

In a recent study using both MSI and IHC as screening tests in 500 patients with colorectal cancers, 64 tumors demonstrated MSI-H, while 71 tumors had abnormal IHC, of which 56 tumors were MSI-H (Hampel et al. 2008). The observed discrepancy between IHC and MSI screening in this and other studies may be explained by a higher false negative rate with MSI. This higher false negative rate may reflect the use of a lower number of microsatellite markers and/or an inadequate proportion of tumor cells in the sample, particularly seen with mucinous tumors (a characteristic histological feature of MMR-deficient tumors).

When a patient's colorectal cancer is screened for HNPCC/Lynch Syndrome and is found to be MSI-H and/or is negative for MLH1 protein expression by IHC, one can proceed to mutational analysis testing of *MLH1* to confirm the diagnosis. Alternatively, to distinguish between HNPCC/Lynch Syndrome and a sporadic form of colorectal cancer in the patient, two different molecular diagnostic tests may also be recommended. First, testing for mutations in the *BRAF* gene is recommended by some. The common somatic V600E mutation in *BRAF* is seen in up to 60% of MSI positive sporadic tumors and in up to 70% of MLH1 IHC absent sporadic tumors, but is never seen in HNPCC/Lynch Syndrome (Bessa et al. 2008; Palomaki et al. 2009). As such, detecting the *BRAF* mutation allows one to eliminate HNPCC/Lynch Syndrome from the analysis. Second, the methylation-specific PCR test can directly assess *MLH1* promoter methylation (Herman et al. 1998). A positive methylation-specific PCR test is highly suggestive of a sporadic colorectal cancer in the patient with a specificity of 80%. However, evidence of methylation of the *MLH1* promoter can also be seen in HNPCC/Lynch Syndrome (Popat et al. 2005), making mutational analysis of the *MLH1* gene from tumor or a normal tissue required to assess for a germline*MLH1* mutation.

Approaches to Identifying Individual Patients for Screening and Treatment of HNPCC/Lynch Syndrome

The Revised Bethesda Guidelines for testing colorectal cancers for MSI include the following five situations: first, a colorectal cancer diagnosed in a patient who is less than 50 years of age; second, the presence of synchronous or metachronous colorectal cancers or other HNPCC/Lynch Syndrome associated cancers, regardless of age; third, colorectal cancer with characteristic MSI-H histologic features in a patient less than 60 years of age; fourth, colorectal cancer diagnosed in one or more first degree relatives with HNPCC/Lynch Syndrome related features, with one of the cancers

being diagnosed prior to age 50 years; and fifth, colorectal cancer diagnosed in two or more first or second degree relatives with HNPCC/Lynch Syndrome related tumors, regardless of age (Umar et al. 2004). In addition to colorectal cancer, HNPCC/Lynch Syndrome related tumors include: other gastrointestinal primary tumors (stomach, pancreas, biliary tract, small bowel); gynecological tumors (endometrial, ovary); genitourinary tumors (ureter, renal pelvis); high grade brain tumors (as seen in Turcot Syndrome); and benign sebaceous gland adenomas and keratoacanthomas (as seen in Muir-Torre Syndrome) (Lynch and de la Chapelle 2003). The characteristic MSI-H histologic pattern in colorectal cancers includes the presence of abundant tumor infiltrating lymphocytes, a Crohn's-like lymphocytic reaction, mucinous differentiation and a medullary growth pattern.

If a patient with colorectal cancer fulfills one or more of these Revised Bethesda Guidelines, the optimal approach is to proceed with initial MSI and/or IHC testing, which if positive, should be followed by germline *MLH1* and *MSH2* mutational analyses. If no *MLH1* or *MSH2* mutations are found, germline mutation screening for *MSH6* and *PMS2* is next. If a MMR germline mutation is detected in the proband, then at-risk relatives (parents, siblings, children, first degree relatives) should be referred for genetic counseling and subsequently tested if they consent, as well as recommended for high-risk surveillance. If no germline MMR mutation is detected, then the patient and at-risk relatives should be counseled and recommended for high-risk surveillance, similar to genetically confirmed HNPCC/Lynch Syndrome families.

It is established that more than 80% of colorectal cancers from patients with HNPCC/Lynch Syndrome display MSI-H based on two recent comprehensive reviews (Lynch et al. 2009; Palomaki et al. 2009). This association may be increased with the use of additional mononucleotide markers, more sophisticated tumor sampling, or the additional use of IHC, as described earlier. Only a few studies have questioned whether a MSI-L tumor could be found in an HNPCC/Lynch Syndrome patient (Hampel et al. 2008; Mueller et al. 2009). In both studies, mutational analyses of MMR genes suggested that MSI-L tumors be excluded as part of the Syndrome. The same conclusion can be generally made for MSS tumors.

Recommendations for Treatment and Follow-up Surveillance for HNPCC/Lynch Syndrome Patients with Colorectal Cancer

Establishing the diagnosis of HNPCC/Lynch Syndrome in a patient with colorectal cancer is important for further management of the initial (colorectal) cancer, as well as for determining effective screening for developing second primary cancers. In HNPCC/Lynch Syndrome patients

with colon cancer, a subtotal colectomy with an ileorectal anastomosis may be the preferred surgery, as these patients have a 16% risk for developing a second primary colorectal cancer within 10 years of the initial diagnosis (Aaltonen et al. 1998). However, a segmental resection with close follow-up colonoscopic surveillance is a very reasonable treatment option for these patients, with data suggesting a better quality of life compared to patients who undergo subtotal colectomy (Vasen et al. 2007). In HNPCC/Lynch Syndrome patients with rectal cancer, a procto-colectomy with an ileal pouch-anal anastomosis or an anterior proctosigmoidectomy with primary reconstruction are the recommended surgical options (Guillem et al. 2006).

The use of 5-FU based adjuvant chemotherapy in HNPCC/Lynch Syndrome patients with colon cancer is an area of controversy. In 2003, a retrospective analysis of the impact of MMR deficiency on the effectiveness of adjuvant 5-FU based chemotherapy in Stage II and III colon cancer was published. The authors analyzed data from five large Phase 3 clinical trials (Ribic et al. 2003). Pathologic Stage II colon cancers extend through the bowel wall, but show no lymph node involvement, while pathologic Stage III patients have positive regional lymph nodes. Seventeen percent of patients entered on these trials showed MSI-H histologic features in the tumor, and these patients experienced an improved overall survival compared to similarly treated patients with MSI-L or MSS tumors. However, no benefit to the use of post-operative adjuvant 5-FU based chemotherapy was found in MSI-H tumor patients. This clinical observation on the lack of effect of 5-FU is supported by prior laboratory studies of 5-FU damage tolerance in MMR-deficient human colorectal cancer cell lines as summarized above. A more recent study, which analyzed an additional 457 patients with Stage II and III colon cancer from 4 of the 5 clinical trials included in the 2003 study, as well as patients from an additional Phase 3 trial, again found that MSI-H colon cancer patients have an improved 5-year disease free survival upon univariate analysis ($p=0.03$) (Sargent et al. 2010). Additionally, no survival benefit was found in MMR-deficient Stage II and III colon cancer patients with the use of adjuvant 5-FU based chemotherapy, in contrast to MMR-proficient colon cancer patients. Finally, a very recent study that analyzed MMR-deficient versus MMR-proficient Stage II and III colon cancers treated on two large Cancer and Leukemia Group B (CALGB) trials confirmed the improved prognosis of MMR-deficient tumors (Bertagnolli et al. 2011). Thus, MMR-deficiency in colon cancer appears to be a prognostic marker for improved survival and a predictive marker for lack of efficacy of 5-FU based adjuvant chemotherapy.

In Stage II and III rectal cancer patients with HNPCC/Lynch Syndrome (or a sporadic MMR-deficient tumor), the use of pre-operative concomitant 5-FU based chemotherapy and radiation therapy, as well as the use of post-

operative 5-FU based chemotherapy, remains the standard care (similar to MMR-proficient rectal cancers) based on the currently available, but limited, literature. One small retrospective study of 18 patients with rectal cancer treated with 5-FU chemoradiation suggested as lower pathologic response in MSI-H tumors compared to MSI-L and MSS tumors (Choi et al. 2007). Another small retrospective study of 57 rectal cancer patients treated with pre-operative 5-FU + CPT-11 (irinotecan) chemoradiation concluded the opposite, i.e., an improved complete pathological response rate in MSI-H tumors (Charara et al. 2004). Finally, a third small retrospective analysis of 17 MSI-H rectal cancer patients compared to 73 MSI-L/MSS patients suggested a significant benefit in both disease-free and overall survival in MSI-H tumor patients (Colombino et al. 2002). While much larger data bases from several randomized prospective trials in rectal cancer patients are available to further characterize the prognostic and/or predictive influence of MMR deficiency in rectal cancer patients treated with neoadjuvant 5-FU chemoradiation, no publications from these larger trials specifically addressing MMR deficient rectal cancers are currently available.

The other major issue in the management of colorectal cancer patients with HNPCC/Lynch Syndrome involves risk assessment and surveillance recommendations regarding second primary cancers. Colonoscopy is recommended every 1–2 years following initial colon-sparing surgery or a proctoscopy on a similar schedule following more extensive surgery (Guillem et al. 2006). Based on available data, the risk of a second colorectal cancer is higher (by 10–30%) with *MLH1* or *MSH2* mutations than with *MSH6* mutations. Overall, the risk of a second colorectal cancer in HNPCC/Lynch Syndrome patients appears lower in females compared to males (Quehenberger et al. 2005; Jenkins et al. 2006).

The risk of other HNPCC/Lynch Syndrome related cancers, including endometrial, ovarian, gastric, and brain cancers, is estimated to be 22% in males and 34% in females by age 70 (Jenkins et al. 2006). In general, *MLH1* mutation carriers have a higher risk of endometrial cancer, and to a lesser extent, ovarian cancer, by age 70 compared to *MSH2* or *MSH6* carriers (Quehenberger et al. 2005; Bonadona et al. 2011). While prophylactic hysterectomy and bilateral salpingo-oophorectomy reduce (eliminate) the risk of these two HNPCC/Lynch Syndrome related cancers (Schmeler et al. 2006), it is not strongly recommended, as women can be followed by surveillance studies every 1–2 years via transvaginal ultrasound, endometrial aspiration biopsies, and serum CA-125 testing (Vasen et al. 2007). Surveillance gastroduodenoscopy may be recommended on an every 1–2 year basis, particularly if there is a family history of gastric cancer (Vasen et al. 2007). There are no recommended surveillance guidelines for brain tumor screening.

Approaches and Effectiveness of Population-based Screening for HNPCC/Lynch Syndrome Using Newly Diagnosed Colorectal Cancer and Endometrial Cancer Patient Cohorts

Current evidence documents that the risk of developing colorectal cancer by age 70 in HNPCC/Lynch Syndrome patients is approximately 45% in men and 35% in women (Palomaki et al. 2009). The estimated risk of developing endometrial cancer among women with HNPCC/Lynch Syndrome may be as high as 60–65% by age 70 (Lu et al. 2005; Hampel et al. 2008). To date, a larger number of population-based studies have focused on newly diagnosed colorectal cancer populations. However, since endometrial cancer may be the sentinel cancer in HNPCC/Lynch Syndrome women, this subgroup of patients represents an additional cohort for population-based screening. In a future era of cost containment, the cost effectiveness of such population-based screening will be carefully scrutinized and balanced against the health benefits and cancer incidence reductions to these high risk HNPCC/Lynch Syndrome groups.

To date, population-based screening of newly diagnosed colorectal cancer patients has demonstrated the effectiveness and limitations of this approach. Using the Revised Bethesda Guidelines and/or Amsterdam II Criteria to identify potential HNPCC/Lynch Syndrome carriers, or MSI or IHC tumor testing, a comprehensive summary of six major studies demonstrated that these HNPCC/Lynch Syndrome carriers had *MLH1* mutations in 31% of the total population, *MSH2* mutations in 39%, *MSH6* mutations in 14%, and *PMS2* mutations in 15% (Palomaki et al. 2009). However, two studies from Finland, which were included in this comprehensive summary, showed significantly different ratios between *MLH1* and *MSH2* mutations, suggesting the influence of a founder effect in these two studies (and probably other studies), where the mutation rates of one population may not be representative of another population, even within the same country (Aaltonen et al. 1998; Salovaara et al. 2000).

Both the Revised Bethesda Guidelines and the Amsterdam II Criteria (Vasen et al. 1999) rely on the use of personal and family histories of colorectal cancer and other HNPCC/Lynch Syndrome related cancers to select patients for screening. Many studies have reported on the difficulties and inaccuracies in obtaining family histories. For example, in a screening study of over 1000 patients with newly diagnosed colorectal cancers where 23 patients were found to have HNPCC/Lynch Syndrome, only 3 of the 23 patients (13%) met the Amsterdam Criteria, and 18 of the 23 patients (78%) met the Bethesda Guidelines (Hampel et al. 2005). These data demonstrate the limitations of using the family history as a screening test for HNPCC/Lynch Syndrome. However, there are many other possible strategies to

improve the efficiency of identifying HNPCC/Lynch Syndrome in a general population, with varying specificities and cost-effectiveness.

In the recent EGAPP Working Group Report (Palomaki et al. 2009), four different strategies were compared based on the assumption of 150,000 individuals newly diagnosed with colorectal cancer (2007 U.S. incidence), of which 100,000 of these individuals agreed to the first line of testing. The testing strategies selected were as follows: Strategy 1 subjected all patients to sequencing and deletion/large rearrangement analyses for *MSH2*, followed by *MLH1*, then *MSH6*, if necessary; Strategy 2 offered initial MSI testing using at least 3 mononucleotide repeats, and only those with MSI-H had further diagnostic testing of the MMR genes as in Strategy 1; Strategy 3 offered initial IHC testing for all 4 MMR proteins (including PMS2) followed by specific MMR gene testing for appropriate individuals; and Strategy 4 was similar to Strategy 3 except that individuals with absent MLH1 IHC staining were next targeted for the V600E mutation of *BRAF*, prior to MMR gene testing. Based on the estimated screening of 100,000 newly diagnosed colorectal cancer patients, 3000 patients (3%) were expected to be found to have HNPCC/Lynch Syndrome. The number of HNPCC/Lynch Syndrome probands detected decreased from 85% in Strategy 1 to 70% in Strategy 4. However, the cost per HNPCC/Lynch Syndrome proband detected also decreased from $112,000 in Strategy 1 to $18,000 in Strategy 4. The total costs, assuming 80% of relatives accepting counseling and 20% having targeted testing according to each Strategy, ranged from $281 million for Strategy 1 to $41 million for Strategy 4. Not surprisingly, there appears to be a significant tradeoff between lower detection and lower costs and higher detection (15%) with higher (≈7X) costs.

Another group has advocated for the use of the Microsatellite Path Score (MsPath Score) as a substitute for the Revised Bethesda Guidelines to more efficiently predict MMR-deficient colorectal cancers (Jenkins et al. 2007). The pathological features evaluated in the MsPath Score include: the presence of tumor infiltrating lymphocytes; mucinous histology; poor differentiation; and any Crohn's-like reaction along with two patient specific criteria: proximal colon tumor location and an age at diagnosis of younger than 50 years. Each of these pathologic and clinical features is a strong independent predictor of MSI-H colorectal cancers. Using an optimal cutoff MsPath Score of ≥1, they reported a 93% sensitivity and 55% specificity for predicting MSI-H tumors. Thus, the MsPath Score performed well among individual patients with colorectal cancer at risk for HNPCC/Lynch Syndrome compared to using the Revised Bethesda Guidelines. A recently published population-based study using MsPath Score with a blinded pathology review of tumors from over 1,200 patients with colorectal cancer found that it accurately predicted the probability of MSI-H tumors in a general population with a sensitivity of 93% and a specificity of 64%.

However, the use of the MsPath Score was not suitable as a stand-alone screen for HNPCC/Lynch Syndrome without additional *MLH1* and *MSH2* mutation testing (Bessa et al. 2011).

Similar to the rationale articulated above to improve the accuracy and cost effectiveness of identifying HNPCC/Lynch Syndrome patients/families with colorectal cancer, screening of all women with endometrial cancer for HNPCC/Lynch Syndrome has the potential to identify a significant number of mutation carriers, but again, such screening would incur a substantial cost to the health care system. While less common than colorectal cancer, approximately 45,000 cases of endometrial cancer will be diagnosed in the United States each year, being the fourth most common cancer in women (NCI). The Amsterdam II Criteria recommend genetic testing for HNPCC/Lynch Syndrome in women with endometrial cancer who have at least two other relatives with a HNPCC/Lynch Syndrome related cancer within two successive generations, and at least one of them must have been diagnosed before age 50 years (Vasen et al. 1999, 2007). However, not all women with HNPCC/Lynch Syndrome fulfill the Amsterdam II Criteria (Hampel et al. 2005; Pinol et al. 2005).

In a recent study from British Columbia, a Markov Monte Carlo simulation model was developed to compare six scenarios for HNPCC/Lynch Syndrome testing in women with endometrial cancer (Kwon et al. 2011). The scenarios used in this simulation included: use of the Amsterdam II Criteria; age younger than 50 years with at least one first-degree relative having an HNPCC/Lynch Syndrome related cancer at any age; IHC triage of 4 MMR proteins (MLH1, MSH2, MSH6, and PMS2) if age younger than 50 years; IHC triage if age younger than 60 years; IHC triage at any age with at least one first degree relative with an HNPCC/Lynch Syndrome related cancer at any age; and finally, IHC triage of all endometrial cancers. In the model, it was assumed that women with endometrial cancer were still at risk for colorectal cancer. For those with abnormal IHC results and who were then defined as an HNPCC/Lynch Syndrome carrier by MMR gene mutational analysis, the model assumed yearly colonoscopy. The cost effectiveness of the six scenarios was compared by determining the incremental cost-effectiveness ratio (ICER), which was defined as the additional costs (in U.S. dollars) of a specific strategy divided by its health benefit (average life expectancy gain in years). The modeling study found that IHC triage of all women with endometrial cancer who have at least one first-degree relative with an HNPCC/Lynch Syndrome related cancer at any age is the most cost-effective strategy for identifying those who should be referred for genetic testing. Compared to the scenario of using IHC triage in all endometrial cancer patients, the ICER was reduced from $650,000 to $9,000 by the inclusion of one first-degree relative with an HNPCC/Lynch Syndrome related cancer. They also concluded that, if the

Amsterdam II Criteria continue to be utilized to guide genetic testing for HNPCC/Lynch Syndrome, a significant proportion (up to 70%) of women with HNPCC/Lynch Syndrome would be missed, similar to the results of prior studies (Hampel et al. 2006). This low sensitivity is contrasted to a sensitivity of 80–100% based on prior reports (Hampel et al. 2006; Lu et al. 2007) using the criteria of the proportion of women with endometrial cancer and HNPCC/Lynch Syndrome who have at least one first-degree relative with an HNPCC/Lynch Syndrome related cancer

MMR DEFICIENCY AS A POTENTIAL THERAPEUTIC TARGET

Is MMR Deficiency a Prognostic Factor for Improved Survival in Colorectal Cancer and a Predictive Factor for Lack of Response to 5-FU Based Chemotherapy?

The 15–20% of colorectal cancers that are MMR deficient are typically pathologically and clinically distinct from the 80–85% of colorectal cancers that primarily involve the chromosomal instability (CIN) pathway, in which aneuploidy and gross genomic and chromosomal changes are commonly found. As mentioned previously, a meta-analysis of available data prior to 2005 confirmed that MMR deficiency (MSI-H phenotype) is an independent prognostic factor in colorectal cancer associated with a significantly improved overall survival regardless of disease stage (combined hazard ratio = 0.65) (Popat et al. 2005). This meta-analysis also found no benefit to the use of adjuvant 5-FU based chemotherapy in Stage II and Stage III colon cancer patients (hazard ratio = 1.24). More recent studies from 2010 and 2011 confirm these two major conclusions (Sargent et al. 2010; Bertagnolli et al. 2011; Hutchins et al. 2011).

Two additional clinical caveats need to be considered when applying these two major conclusions to a patient with an MMR deficient colorectal cancer. First, some studies have suggested that colorectal cancers with the V600E mutation in *BRAF* have a worse prognosis following surgery, with or without adjuvant 5-FU based chemotherapy (Ogino et al. 2009), or following chemotherapy alone in patients with metastatic disease (Tol et al. 2009). Given the finding that up to 60% of sporadic MMR deficient (MSI-H) colorectal cancers have the V600E *BRAF* mutation, it might be expected that these patients would have a worse prognosis compared to HNPCC/Lynch Syndrome patients or sporadic MSI-H tumors without the V600E *BRAF* mutation (Loughrey et al. 2007). This hypothesis was tested in two recent studies analyzing MSI-H and V600E *BRAF* status in patients treated with adjuvant chemotherapy (French et al. 2008; Hutchins et al. 2011). The first study analyzed both MSI and *BRAF* V600E mutation status

in colon cancer tumors from patients treated with adjuvant 5-FU based chemotherapy (French et al. 2008). They found that MSI-H tumors without a *BRAF* V600E mutation had a significantly improved disease-free survival (p=0.002), whereas the presence of MSI-H and a *BRAF* V600E mutation in colon cancer showed a decreased disease-free survival, which was similar to MSS (or CIN) colon cancers. The effect of MSI-H ± a *BRAF* V600E mutation on the response to chemotherapy was not evaluated in this first study. The second, more recent study (Hutchins et al. 2011) analyzed colorectal tumor specimens for MSI, *BRAF* V600E mutation, and *KRAS* mutation from over 1900 patients entered on the Quick and Simple and Reliable (QUASAR) Trial (Gray et al. 2007), which randomly assigned curatively resected colorectal patients to 5-FU plus leucovorin chemotherapy versus observation. A majority (91%) of the study patients had Stage II colon cancer. Again, this study confirmed the strong prognostic importance of MMR deficiency on survival, which was found in both the chemotherapy and observation patient groups. The 53% of MSI-H tumor specimens with a *BRAF* V600E mutation, however, had a higher risk of recurrence both with and without chemotherapy. Thus, it appears that colorectal cancers that show both MSI-H and a *BRAF* V600E mutation (presumably sporadic MMR deficient cancers) are a worse prognostic group compared to MSI-H alone tumors. The second clinical caveat for assessing the prognostic significance of MMR deficiency in colorectal cancer involves the co-association of MSI-H and CIN or aneuploidy features in the same tumor. CIN pathological features are generally associated with a poor prognosis in colorectal cancers compared to MSI-H (Walther et al. 2008). However, the presence of CIN and MSI-H are not mutually exclusive (Trautmann et al. 2006), and at least one study suggests that the prognostic significance of MSI-H is eliminated when CIN features are also found (Sinicrope et al. 2006).

With respect to the predictive impact of MMR deficiency in determining the response to 5-FU based chemotherapy in colon cancer, the 2005 meta-analysis (Popat et al. 2005), and most subsequent studies (Jover et al. 2009; Sargent et al. 2010), found no benefit to adjuvant 5-FU based chemotherapy. However, a retrospective review of the impact of adjuvant 5-FU in MSI-H colon cancer patients entered in the National Surgical Adjuvant Breast and Bowel Project (NSABP) Trials between 1977 and 1990 showed no predictive value for MSI (Kim et al. 2007). Additionally, a small meta-analysis of the impact of MSI on the response to 5-FU based chemotherapy in patients with metastatic disease also suggested that MSI did not predict the response to chemotherapy (Des Guetz et al. 2009). Currently, this question regarding the predictive value of MSI on adjuvant 5-FU based chemotherapy is being prospectively studied in a U.S. Intergroup Trial (E5202) in patients with pathologic Stage II colon cancer where tumor tissue is assessed for MSI (both MSI-H and MSI-L vs MSS) and for loss of heterozygosity (LOH) at

chromosome 18q (as a CIN marker). Based on these prospective tumor tissue analyses, patients are stratified into low risk (MSI-H, MSS or MSI-L without 18q LOH) or high risk (MSS or MSI-L with 18q LOH) groups. The low risk patient group is observed without any adjuvant chemotherapy. The high risk group is randomized to 12 cycles of every 2-weeks administration of 5-FU, leucovorin, and oxaliplatin (FOLFOX) with or without bevacizumab(Benson 2007). No results from this ongoing trial are yet available.

Alternative Therapeutic Strategies for Targeting MMR Deficient Cancers

As reviewed previously in the Introduction and Mismatch Repair Links to DNA Damage Response sections, MMR deficiency is found in HNPCC/Lynch Syndrome related cancers and a variety of sporadic tumors, occurring either *de novo* or following cancer treatments. While MSI-H colorectal cancers have a better prognosis compared to CIN colorectal cancers, MMR deficiency in other primary tumor sites can be associated with a poor prognosis. For example, nearly 50% of non-small cell lung cancers in never smokers were reported to show MSI-H related to hypermethylation of the *MLH1* and *MSH2* promoters and were found to have a poor prognosis, even in early stage disease (Hsu et al. 2005). Additionally, MMR deficient malignant gliomas were noted to have a markedly reduced response rate and survival time compared to MMR proficient gliomas when treated with concomitant radiation therapy and temozolomide(Friedman et al. 1998). MMR deficient endometrioid endometrial cancers were reported to show a reduced pathological response rate and higher local failure following radiation therapy alone compared to MMR proficient tumors (Bilbao et al. 2010). MMR deficiency occurring during or following cancer treatment may also be associated with a poor prognosis. Somatic point mutations in *MSH6* are found in up to 30% of recurrent/progressive glioblastomas, which were not present in pre-treatment specimens. Indeed, inactivation of *MSH6* was correlated with prior or ongoing temozolomide exposure and associated with enhanced tumor regrowth and shorter survival (Cahill et al. 2007). Decreased protein expression of MLH1 following doxorubicin-based chemotherapy in breast cancer patients was also reported to correlate significantly with a reduced disease-free survival ($p=0.0025$) (Mackay et al. 2000). Finally, promoter methylation of *MLH1* in plasma DNA after cisplatin-based chemotherapy for ovarian cancer predicted a poor survival (Gifford et al. 2004).

Given the extensive pre-clinical data summarized earlier on "damage tolerance" to a wide variety of chemotherapy drugs as well as IR, and the more limited clinical data summarized above suggesting that certain

common solid cancers show *de novo* or acquired MMR deficient "damage tolerance" to chemotherapy and/or radiation therapy, a number of alternative treatment strategies to target the sporadic MMR deficient "damage tolerant" phenotype in human cancers are being pursued. Most of these treatment strategies are in pre-clinical development. It is important to point out that the *in vitro/in vivo* data demonstrating a differential response in MMR deficient cancer cells to chemotherapy and IR can be dependent on the model system used. While beyond the scope of this Chapter, the reader needs to appreciate that there exist limitations with the pre-clinical models, as well as the difficulties in translating the pre-clinical data to future clinical trials. Several recent review articles provide more detail on the potential and limitations of such translational research (Kinsella 2009; Hewish et al. 2010; Martin et al. 2010a).

One alternative therapeutic strategy to target MMR deficient tumors has been to exploit the unique sensitivity to novel combinations of chemotherapeutic agents or novel combinations of chemotherapy and radiation therapy. An intriguing example of the strategy to use a novel combination of chemotherapeutic agents involves the use of the topoisomerase inhibitor, camptothecin, and thymidine (Rodriguez et al. 2008). It had been previously shown that MMR deficient cell lines have modest increased sensitivity to either camptothecin (Jacob et al. 2001) or thymidine (Mohindra et al. 2002), although the single agent drug cytotoxicity was not reversed by correction of the MMR deficiency. However, with the use of camptothecin and thymidine, a 10–3000X enhancement of *in vitro* cytotoxicity was found compared to camptothecin alone, with a doubling of the time for *in vivo* tumor regrowth, particularly in MMR deficient cell lines that also contained an intronic frameshift mutation of *MRE11*, which is involved in HR mediated double-strand break repair (see Chapter 14). These investigators reasoned that the markedly enhanced cytotoxicity to the two-drug combination was mediated through targeting a secondary mutation (i.e., *MRE11*) associated with the MMR deficiency. They also demonstrated enhanced cytotoxicity to thymidine in HR-deficient cells in support of their hypothesis. Since the same *MRE11* frameshift mutation is found in up to 80% of MMR deficient colorectal cancers (Giannini et al. 2002),they discuss the potential clinical application of this two-drug combination in this specific group of colorectal cancers.

A second strategy for treatment of MMR deficient cancers involves the concomitant use of IUdR or its oral prodrug, IPdR, during radiation therapy. IUdR is a halogenated thymidine analog and has been recognized as an effective *in vitro/in vivo*, and potential clinical, radiation sensitizer for several decades (Kinsella 1996). The biochemical mechanisms of cellular radiosensitization are related to the generation of highly reactive free radicals by IR from IUdR-DNA incorporation, resulting in enhanced

IR-induced DNA single and double strand breaks. while also altering IR damage repair. The biochemical pathway of IUdR cellular metabolism and DNA incorporation, as well as a summary of experimental data on MMR processing of the resulting G:IU mispair are illustrated in Fig. 2. A summary of the experimental data on MMR processing of IR-induced DNA base damage (principally 8-oxo-G:T or :C mispairs) is illustrated in Figure 3. These data, as well as the limited clinical data, suggest that MMR deficient cancers show relative IR damage tolerance.

The pre-clinical rationale for using IUdR-mediated radiosensitization for MMR deficient tumors is as follows. First, MMR efficiently recognizes and removes G:IU mispairs. Consequently, MMR deficient cells do not recognize the G:IU mispairs and retain significantly higher IUdR-DNA levels compared to proliferating MMR proficient normal cells/tissues. The level of persistent IUdR-DNA incorporation is directly correlated with the extent of radiosensitzation (Berry and Kinsella 2001; Berry et al. 2003). Second, using experimental data from flow cytometry and measurements of IUdR-DNA incorporation, a probabilistic model of tumor cell kinetics for MMR deficient cancers was developed that can predict the optimal times for IR treatments during IUdR exposure to optimize IUdR-mediated tumor radiosensitization (Gurkan et al. 2007; Kinsella et al. 2011). Third, intermediate endpoints of the potential therapeutic gain for IUdR-IR treatment of MMR deficient tumors compared to dose limiting MMR proficient normal tissues (bone marrow, gastrointestinal mucosa) have been developed based on measurements of IUdR-DNA incorporation using flow cytometry and immunohistochemistry with anti-IUdR antibodies. These assays were used in an *in vivo* study which demonstrated the therapeutic gain for the IUdR + IR combined treatment in MMR deficient tumor xenografts (Seo et al. 2004). Consequently, a Phase I clinical trial of IUdR-mediated radiosensitization in MMR deficient (damage tolerant) colorectal and gynecologic cancers is being developed.

A third pre-clinical strategy for targeting MMR deficient tumors involves the concept of synthetic lethal interactions between specific MMR proteins and DNA polymerases as initially proposed by Hartwell and colleagues over a decade ago (Hartwell et al. 1997). This therapeutic strategy exploits either the interaction between MSH2 and DNA polymerase-β in repairing oxidative DNA base damage in the nucleus, or the interaction between MLH1 and DNA polymerase-γ in repairing mitochondrial DNA oxidative base damage (Martin et al. 2010b). Using a series of human MSH2 and MLH1 deficient human cancer cell lines, these investigators found that MSH2 deficiency is synthetically lethal with chemical inhibition of DNA polymerase-β, but no synthetic lethal interaction was found in MLH1 deficient cells. Conversely, they found that inhibition of DNA polymerase-γ is synthetically lethal with MLH1 deficiency, but not with MSH2 deficiency. They also demonstrated that the MSH2-DNA polymerase-β synthetic lethal

interaction correlated with enhanced 8-oxoguanine-DNA base lesion in the nucleus, while the MLH1-DNA polymerase-γ synthetic lethal interaction correlated with mitochondrial DNA accumulation of 8-oxoguanine lesions. As such, these data and other data (de Souza-Pinto et al. 2009) suggest distinct MMR pathways in the nucleus and mitochondria. More specific inhibitors of these DNA polymerases are under development (see Chapter 11).

SUMMARY

Basic research on MMR over the last two decades has significantly improved our understanding of this DNA repair pathway. While early studies focused principally on the role of MMR in mutation avoidance, more recent research, predominantly in *E. coli*, has highlighted the complexity of MMR, including its relationship to several other DNA metabolism pathways, in particular, those involved in recombination. Current work in mammalian cell systems is exploring how MMR proteins influence both meiotic and mitotic recombination events.

The causal linkage of MMR mutations with HNPCC/Lynch Syndrome has stimulated extensive translational and clinical research aimed at better defining affected individuals and family members with emphasis on effective cancer treatment and prevention strategies, as well as cost-effective population screening. It is now recognized that MMR-deficient colorectal cancers (both genetic and epigenetic variants) are clinically distinct from other types of colorectal cancer and may respond differently to treatment. MMR-deficient cancers are heterogeneous based on the underlying etiology (genetic vs epigenetic) of MMR deficiency and the secondary mutations that accompany MSI. As such, it may be necessary to target both the secondary mutations and primary MMR mutations as a therapeutic strategy. MMR deficiency is also associated with other cancer phenotypes including hypoxia and oxidative stress that may be targeted to increase the therapeutic index of MMR-deficient cancer treatment. Finally, it is recognized that MMR deficiency can result as a consequence of cancer treatment with chemotherapy and/or radiation therapy.

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CHAPTER 8

DNA Base Excision Repair Therapeutics: Summary of Targets with a focus on APE1

Melissa L. Fishel,[1] *Carlo Vascotto*[2] and *Mark R. Kelley*[3,*]

INTRODUCTION

The Base Excision Repair (BER) pathway is responsible for repairing some of DNA's most prevalent lesions—those produced both endogenously and exogenously from alkylation, oxidation, or spontaneous decomposition (e.g., deamination) of DNA bases (Izumi et al. 2003; Kelley et al. 2010; Vascotto and Fishel 2012). All forms of DNA damage alter its spatial configuration, whether the helix is distorted or not. The BER pathway is the primary repair system involved in removing single damaged bases or abasic sites. Typically this corrective action occurs prior to replication in the cell cycle; otherwise, that damage could cause nucleotide mispairing or DNA strand breaks during replication (Mitra et al. 1997). Thus, efficient repair helps to preserve genomic integrity. However, the repair process produces cytotoxic intermediates that must be resolved by completing repair swiftly

[1]Departments of Pediatrics and Pharmacology & Toxicology, Herman B Wells Center for Pediatric Research, Indiana University School of Medicine, 980 W. Walnut St. Walther Hall R3-C548, Indianapolis, IN 46202, USA.
[2]Department of Medical and Biological Sciences, University of Udine School of Medicine, P.le Kolbe 4, 33100, Udine, Italy.
[3]Betty and Earl Herr Professor in Pediatric Oncology Research, Departments of Pediatrics, Biochemistry & Molecular Biology, and Pharmacology & Toxicology, Herman B Wells Center for Pediatric Research, Indiana University School of Medicine, 980 W. Walnut St. Walther Hall R3-C528, Indianapolis, IN 46202, USA; Email: mkelley@iupui.edu
*Corresponding author

and accurately; otherwise, accumulation of these intermediates can cause cell death (Guillet and Boiteux 2002).

Programming and enhancing targeted cell death is a desirable therapeutic goal in treating cancers; therefore, studying how to modulate the expression of BER proteins is a worthy research pursuit with many potential clinical applications. Numerous inhibitors of BER proteins are in various stages of clinical and preclinical development; some show promise both as standalone and adjunctive agents. Such inhibitors also may be efficacious as secondary treatments in refractory cases (Kelley and Fishel 2008; Plummer 2010).

LESIONS THAT BER REPAIRS

Both endogenous and exogenous factors can cause DNA lesions that BER repairs. Environmental factors and normal metabolic processes inside human cells cause DNA damage at a rate of 1,000 to 1,000,000 lesions per cell per day (Lodish et al. 2004). The majority of endogenous damage is due to intrinsic instabilities of DNA and its ongoing exposure to reactive metabolites (Dianov and Parsons 2007), producing mainly chemical base modifications with the potential to corrupt genomic information (Lodish et al. 2004). Similarly, exposure of cells to exogenous reactive chemical agents, including environmental xenobiotics or anticancer drugs (see Table 1), may directly modify DNA bases, leading to BER activity on those lesions (Friedberg et al. 2004).

As seen in Table 1, the DNA modifications caused by oxidation, alkylation, deamination, and depurination/depyrimidation (abasic sites) can lead to incorrect base pairings during gene transcription and replication and subsequent mutations that affect genomic integrity. Although these particular modifications do not distort the DNA helix like other forms of DNA damage, slight deformation still occurs, which is detrimental to genomic stability. However, these subtle alterations are recognized by BER enzymes, which respond to the DNA damage (Lindahl 1993; Dianov and Parsons 2007). BER is the main pathway for removing small, non-helix-distorting base lesions from the genome; in contrast, the nucleotide excision repair (NER) pathway (see Chapters 1 and 9) repairs bulky helix-distorting lesions (Luo et al. 2010). An explanation of the most common forms of DNA base damage repaired through the BER pathway follows.

Oxidation

Human DNA is subjected continuously to the effects of reactive oxygen species (ROS) exposure, much of which is generated endogenously

Table 1. Endogenous and exogenous sources of DNA damage repaired by BER. (Zhao and Hemminki 2002; Izumi et al. 2003; Sung and Demple 2006; Chan and Dedon 2010; Kow 2002; Hosoya and Miyagawa 2009; Dedon 2010; Kelley et al. 2010; Vascotto and Fishel 2012).

Type of damage	Endogenous sources	Exogenous sources	Damage primarily manifested as	Mispairings and resultant mutations
Oxidation	Normal aerobic metabolism and respiration Inflammatory responses produce reactive oxygen species (ROS): superoxide, HOCl, H_2O_2, and NO	Platinum-based drugs, anthracyclines X-rays; gamma radiation, other radiotherapy	7,8-dihydo-8-hydroxyguanine (8-oxoG) 5-hydroxycytosine thymine glycol (Tg)	G:C to A:T transition mutation Thymine mutation Tg:Gmispair
Alkylation	S-adenosylmethionine Lipid peroxidation Metabolism by intestinal bacteria	Alkylating chemotherapeutics Methylating environmental agents (tobacco smoke, vehicle exhaust)	*N*-alkylated purines: *N*7-methylguanine and *N*3-methyadenosine (3-MeA) O^6-meG	Doesn't alter base pairs, but blocks replication G:C to A:T transition mutation (BER repairs if DR doesn't)
Deamination	Hydrolytic reactions enhanced by free radicals N-nitrosation of nucelobases by nitrous anhydride (N_2O_3) Spontaneous loss of -NH_2 group	Ionizing radiation Chemotherapeutics that produce ROS	Amino group replaced by keto group Cytosine glycol → uracil glycol → 5-OH-Ura Adenosine → hypoxanthine Guanine → xanthine	Generates transition mutations G:C to A:T A:T to G:C G:C to A:T
Abasic sites	Spontaneous depurination Endogenous ROS production	Alkylating agents	A missing purine or pyrimidine	Mutagenic if not repaired

NOTE: Irradiation from radiation therapy and radiomimetic drugs creates complex lesions that BER and other pathways repair collectively. Such lesions include closely opposed SSBs that behave like DSBs and intrastrand crosslinks.

from three main sources: (1) normal metabolic activity, (2) endogenous byproducts of oxidative phosphorylation events during mitochondrial respiration (Dawson et al. 1993), and (3) phagocytic NADPH oxidases during inflammatory responses to disease and other stressors, as well as non-phagocytic NADPH oxidases in other cell systems (Griendling et al. 2000). All these endogenous sources, as well as environmental factors such as radiation and xenobiotics, can transform oxygen to highly reactive states (Fridovich 1995). These ROS cause molecular damage, such as that to DNA, which can interfere with normal Watson-Crick pairing and be mutagenic (Kasai et al. 1991; Lindahl 1993; Dalhus et al. 2009). ROS damage can also lead to cell death (Scandalios 2005). Intentional exposure to oxidative agents via chemo- or radiotherapy produces similar lesions, although radiotherapy also produces complex DNA lesions that are repaired by the collective effort of several pathways (Hosoya and Miyagawa 2009) (see Chapter 4).

Oxidation of DNA bases occurs by one of three mechanisms: by one-electron removal, nucleophilic addition, or the insertion of singlet oxygen (Dedon 2010). Regardless of the mechanism, oxidative purine and pyrimidine derivatives are recognized and repaired by the BER system. A plethora of damage-specific glycosylases, such as 8-oxoguanine glycosylase (Ogg1), recognize and excise the different types of lesions, initiating the BER pathway.

Deamination

Endogenous deamination of DNA bases can occur spontaneously via a hydrolytic reaction, a process enhanced by the presence of free radicals (Kow 2002). Deamination replaces the amino group with a keto group, which is a hydrogen acceptor in normal Watson-Crick base pairing. Thus, the result of deamination of DNA bases is likely to be mutagenic (Wang et al. 1998), as noted in Table 1.

Interestingly, the most common product of pyrimidine deamination is uracil; the repair of this damage is initiated by a specific glycosylase, uracil DNA glycosylase, which is a member of the UNG family of enzymes. Uracil DNA glycosylase recognizes uracil in both single and double-stranded DNA, but not in RNA—an additional example of the unique specificity of BER glycosylases. Another notable mutagenic derivative that is a byproduct of deamination is 5-OH-uracil (5-OH-Ura), which is produced by exposure of DNA to ionizing radiation (IR) and other ROS-producing agents (Dizdaroglu and Bergtold 1986). This base modification is particularly harmful because the polymerases involved in BER bypass the lesion and insert an A opposite 5-OH-Ura (Purmal et al. 1994), generating G:C to A:T

transition mutations. As noted in Table 1, hypoxanthine and xanthine may also result in highly mutagenic mispairings when copied during DNA replication (Kow 2002).

Alkylation

Some of DNA's most prevalent cytotoxic and mutagenic lesions are a result of alkylation of the bases. Alkyl radicals can be inserted into all bases at vulnerable spots, which include all the exocyclic oxygens and most of the ring nitrogens. Regarding the latter, the ring nitrogens of purines are particularly susceptible (Vascotto and Fishel 2012). Non-enzymatic methylation of DNA occurs from endogenous exposure to *S*-adenosyl methionine (SAM), a compound located in the nucleus and is necessary for synthesizing certain amino acids (Lindahl 1993). Environmental toxins are also sources of alkylating agents that generate DNA alkyl lesions (Beranek 1990).

Exogenous sources of alkylation include anticancer therapeutics, particularly alkylating agents, a diverse class of cytotoxic, mutagenic, and carcinogenic compounds (Colvin 1997; Lee et al. 2010; Tell and Wilson 2010). They are classified as monofunctional or bifunctional, named for the number of reactive groups they possess. The reactive groups of these chemotherapeutic agents form covalent bonds with nucleophilic entities, including amino, phosphate, sulfhydryl, and hydroxyl groups. While monofunctional alkylating agents create mono-adducts, bifunctional alkylating agents form complex lesions, including inter- and intra-strand crosslinks, as well as DNA-protein crosslinks, which are laborious to repair (Lee et al. 2010). In addition, the highly mutagenic and cytotoxic O^6-methylguanine (O^6-meG) lesion is produced by several alkylating agents, causing a repair cascade that involves both Direct Reversal (DR) and the Mismatch Repair (MMR) pathway (see Chapters 6 and 7). O^6-meG mispairs with thymine during replication, resulting in a G:C to A:T transition mutation. This initial mispairing event triggers the DNA MMR pathway, which attempts to remove the thymine, leading to a futile cycle of nucleotide removal and synthesis that generates DNA single- and double-strand breaks (DSBs), eventually resulting in apoptosis (Fang et al. 2010).

PROTEINS OF THE BER PATHWAY

With the exception of DR, which involves a single protein termed O^6-meG DNA methyltransferase (MGMT; see Chapter 6), all repair mechanisms involve degradation of DNA and removal of the damaged nucleotide (with or without excision of the immediate surrounding area),

followed by DNA re-synthesis. The BER pathway follows this pattern of recognition, removal and re-synthesis. To accomplish this, BER employs four categories of proteins: (1) base damage recognition enzymes (with and without lyase activity), (2) endonucleases, (3) polymerases and ligases, and (4) scaffold proteins (Robertson et al. 2009). The nature of lesions involved and the phase of the cell cycle in which the damage occurs help determine the repair mechanism that is mobilized.

Overview of the BER Pathway

BER begins when an appropriate DNA glycosylase recognizes and removes an oxidized, alkylated, or deaminated base. The protein does this by catalyzing the hydrolysis of the N-glycosidic bond of the damaged nucleoside, creating an abasic (AP) site,while leaving the sugar-phosphate backbone intact. The AP site is a cytotoxic intermediate product of BER that requires processing, which is the function of an AP endonuclease or AP lyase (Hegde et al. 2010). Processing via hydrolysis of the 5′ phosphodiester bond at the AP site creates a single-strand break (SSB) with a 3′ hydroxyl primer terminus. The remaining 5′-abasic remnant is then removed, and either one or several nucleotides are replaced (depending on the BER sub-pathway chosen), using a polymerase that acts on the primer (Luo et al. 2010). Scaffold proteins, such as the non-enzymatic factor x-ray cross-complementing 1 (XRCC1), help stabilize the area during repair; they also recruit, coordinate, and stimulate other BER enzymes and perform additional functions that are still being elucidated (Sobol 2008; Hegde et al. 2010). A DNA ligase completes the repair by sealing the nick in the single-stranded DNA, restoring the phosphodiester backbone and the helix's integrity (Frosina et al. 1996; Evans et al. 2000; see Fig.1).

Short-versus Long-patch BER

The BER process can proceed along one of two sub-pathways. Short-patch (SP) BER involves replacement of a single nucleotide. Long-patch (LP) BER involves the displacement of 2 to 8 bases downstream of the AP site and resynthesis of the corresponding nucleotides, and involves DNA polymerases, ligases, and other proteins that differ from those used in the SP pathway (see Fig. 2).

The choice of sub-pathway depends on many factors: (1) the type of AP site (Luo et al. 2010), (2) the local concentration of specific BER proteins, (3) the amount of ATP present, (4) the type of DNA termini generated in the early steps of BER, and (5) the phase of the cell cycle when the damage occurs (Fortini and Dogliotti 2007). In depth studies of the BER pathway

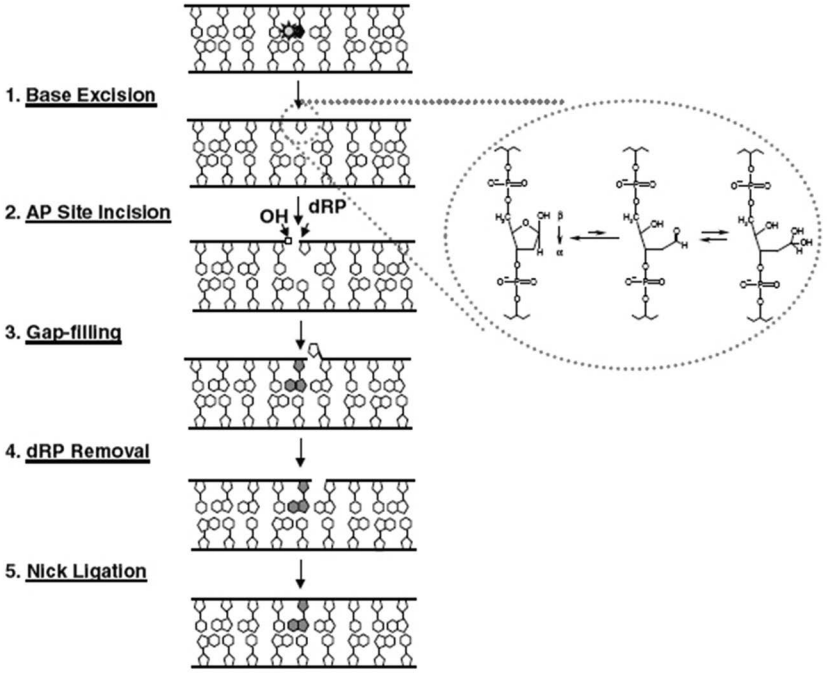

Figure 1. Overview of the BER pathway. The five major enzymatic steps of short-patch BER are shown . These steps are described in detail in the text and Fig. 2. Essentially, the damaged or incorrect base (star) is removed by a specific DNA glycosylase, the abasic site is cut by APE1 5′ to the abasic site, and the gap is filled and sealed using DNA polymerase β (Pol β) and a complex of XRCC1 and DNA ligase III (Lig III). Shown in the magnified circle is the hydrolytic 2′-deoxyribose AP site that exists primarily in a ring-closed form (left) or in one of two racemeric hemiacetal arrangements, which are in an equilibrium mixture. Reduction of the ring-closed AP site can produce a ring-opened aldehyde form (middle) (Wilson and Simeonov 2010). Reprinted with kind permission from Springer Science+Business Media: Cell. Mol. Life Sci. Small molecule inhibitors of DNA repair nuclease activities of APE1, 67, 2010, 3621–3631, Wilson, David, Fig. 1."

are providing evidence that rapid repair (likely SP-BER) operates during interphase, while lesions that are introduced or that persist during replication (S and G2 phases) take longer to repair and are likely processed via LP-BER. In addition, certain BER proteins appear to operate preferentially during the pre-replicative versus replicative phases of the cell cycle (see Table 2 (Fortini and Dogliotti 2007).

The type of damage incurred can dictate the repair pathway (Sancar et al. 2004). For example, Pol β (which predominates in SP-BER) does not recognize certain abasic sites produced by oxidative damage, and its lyase activity cannot remove related 5′ blocking termini; this inability of Pol β calls proliferating cell nuclear antigen (PCNA) and LP-BER into action. RFC loads PCNA onto the DNA strand, enabling PCNA to act as a polymerase

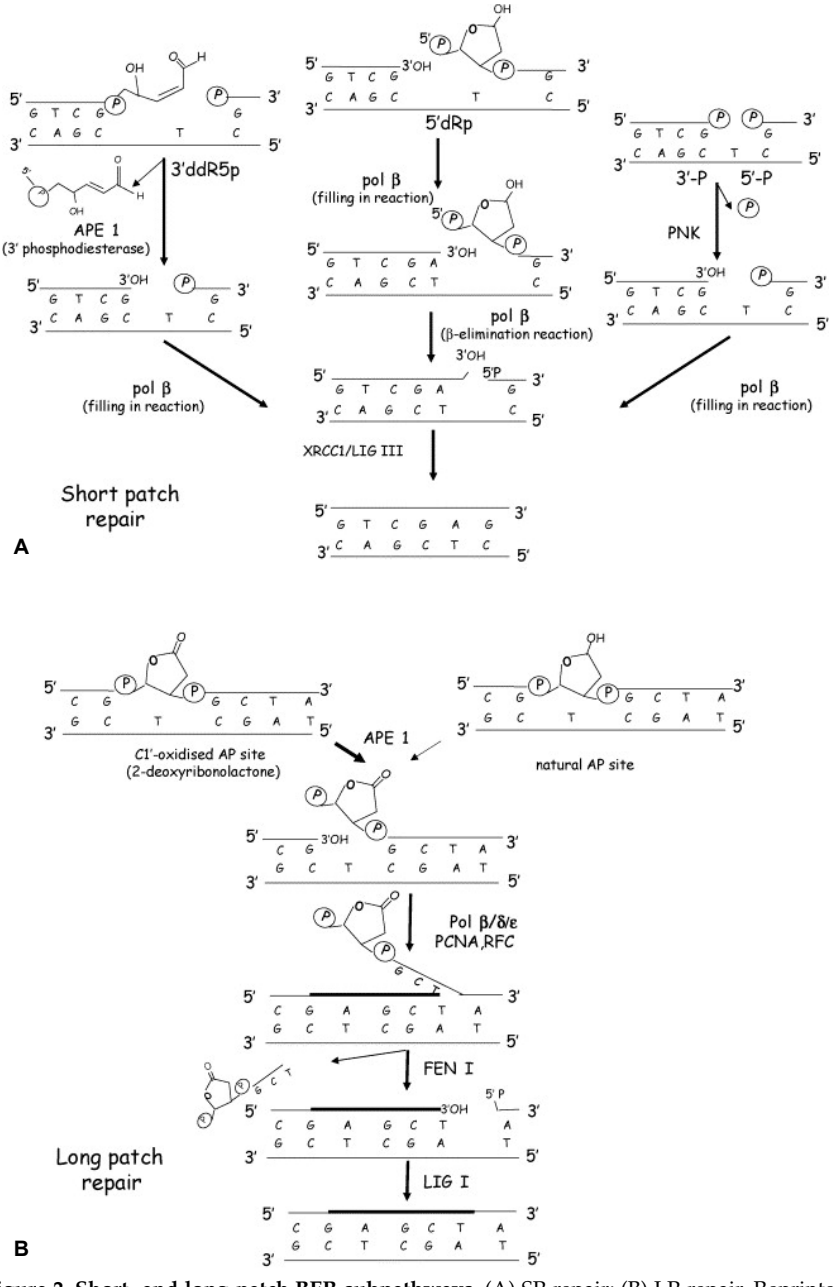

Figure 2. Short- and long-patch BER subpathways. (A) SP-repair; (B) LP-repair. Reprinted from *DNA Repair*, 6, Paola Fortini, Eugenia Dogliotti, "Base damage and single-strand break repair: Mechanisms and functional significance of SP and LP repair subpathways," 398–409 (2007) with permission from Elsevier.

Table 2. Examples of when BER proteins preferentially work.

Protein name	Function	Portion of cell cycle during which it preferentially works
UNG2	Monofunctional glycosylase	Replicative
NTH	Bifunctional glycosylase	Pre-replicative
OGG1	Bifunctional glycosylase	Pre-replicative
NEIL1	Bifunctional glycosylase	Replicative
NEIL2	Bifunctional glycosylase	All
Pol β	Polymerase	Pre-replicative
RFC	Scaffold protein	Replicative
PCNA	Sliding clamp; processivity factor	Replicative
FEN1	Endonuclease	Replicative
PARP1	Damage sensor	All

Note: These are examples, not an exhaustive list.

clamp and cofactor for Pol δ, facilitating displacement of nucleotides 3′ to the nick (producing a "flap") and DNA re-synthesis during LP-BER. Subsequently, the 5′ flap structure -specific endonuclease, FEN1, cleaves the 5′ flap created by strand-displacement repair synthesis. Ligase I (Lig I) finishes LP-BERby sealing the nick (Sancar et al. 2004).

As in LP-BER, SP-BER employs a variety of glycosylases that excise a single damaged base, and APE1 nicks the DNA backbone to further prepare the site. Then the sub-pathways diverge. In SP-BER, Pol β inserts the correct base at the 3′ hydroxyl terminus that APE1 creates. DNA Lig III, complexed with XRCC1, closes the nick to finish the repair response (Kelley et al. 2010). These steps are explained in more detail as each BER protein is discussed below (see also Fig. 2). Regardless of the sub-pathway involved, all BER is a tightly coordinated process in which the intermediates are never left "unattended." This element protects DNA from the toxic intermediates produced and putatively prevents strand displacement or other metabolic reactions (Fortini and Dogliotti 2007).

DNA GLYCOSYLASES

As mentioned earlier, damage-specific glycosylases are a unique feature of BER; 11 specific mammalian glycosylases have been identified to date (Dalhus et al. 2009). Each glycosylase recognizes a particular type of damage and removes the oxidative, alkylated or deaminated base by catalyzing cleavage of the N-glycosidic bond of the substrate nucleoside after flipping the damaged base out of the DNA helix. This enzymatic action generates an AP (abasic) site product (Lindahl 1974), which is the substrate for the next step in the repair process. As already noted, the a basic site is also a cytotoxic

repair intermediate (Lindahl 1993), which requires prompt attention so that replication is not jeopardized.

Some glycosylases are so substrate-specific that their mechanism of removal depends on what base the lesion is paired with (see Table 3). This is true for removal of 8-oxoguanine (8-oxoG) lesions. When 8-oxoG is paired with C, OGG1 recognizes and excises the lesion. However, when adenine is misincorporated opposite of 8-oxoG during DNA replication, MYH will excise adenine from the newly synthesized strand.

In addition to being damage-specific, BER glycosylases share other notable characteristics.In particular, they bind and excise damaged DNA bases in a similar manner and they coordinate with other BER enzymes. Despite the differences that glycosylases have in recognizing specific bases, they all exploit the deformability caused by the damaged base to assist with recognition. They bind to the minor groove of DNA (Fromme and Verdine 2002), "kink" or "pinch" the DNA helix at the damage site, and "flip" the damaged base out of the helix into a recognition pocket—the active site of the enzyme, configured in the right orientation for excising that base (Sancar et al. 2004; Huffman et al. 2005).

The other common attribute among BER glycosylases is the way they coordinate with enzymes that follow them in the repair process. This aspect is not yet fully understood, but because AP sites are more cytotoxic than base lesions (Lindahl 1993), most glycosylases remain bound to their a basic product until the next enzyme (typically APE1; see later) binds the substrate, continuing the repair pathway. The high affinity for the product during BER presumably increases the ability of the subsequent protein to recognize its appropriate substrate (Parikh et al. 1998). The mechanics of this "handoff and transfer" are still being explored for not only glycosylases (Vidal et al. 2001), but for all steps of the pathway (Williams and Kunkel 2011; Hegde et al. 2010). Indeed, many complexities of BER still need to be elucidated. Hegde's group has proposed that BER functions in "handoff and transfer" mode for processing exogenously induced DNA lesions, but functions primarily as a preformed complex when handling endogenous damage (Hegde et al. 2010).

Mammalian glycosylases are divided by their tertiary structure and functionality into two subclasses: monofunctional and bifunctional (Hegde et al. 2010; see Table 2). The first group is capable of only creating a free base and an AP site, by attacking the anomeric carbon of the damaged base (Dalhus et al. 2009). Bifunctional glycosylases possess that ability, but also harbor an AP lyase activity that incises the strand 3′ of the AP site, creating a 3′-damage substrate for AP endonucleases (Dodson et al. 1994; Fromme et al. 2004b). This incision activity at AP sites, and consequent nick formation, is more common when processing oxidized bases; in other types of base

damage repaired by BER, an AP endonuclease performs the function of cleaving the DNA phosphodiester backbone at the AP site (Hegde et al. 2010).

While DNA glycosylases share very little homology, they are divided into four structural subfamilies; within each subfamily are groupings based on substrate specificity and subcellular localization:

- the UNG (uracil DNA N-glycosylase) family
- the helix-hairpin-helix (HhH) family
- the helix-2 turn-helix (H2TH) family
- the AAG (alkyladenine DNA glycosylase) (Dianov and Parsons 2007)

The details for each DNA glycosylase are listed in Table 3.

Table 3. Mammalian glycosylases and their substrates.

Glycosylase	Enzyme	Substrate(s)	Type	Reference
UNGs	UNG	U	Monofunctional	(Nilsen and Krokan 2001)
	TDG	U:G, T:G		(Neddermann et al. 1996)
	SMUG1	U, OHmeU		(Boorstein et al. 2001)
HhH	MBD4	U:G and T:G in CpG sites	Monofunctional	(Petronzelli et al. 2000)
	OGG1	8-oxoG:C, faPyA, faPyG	Bifunctional	(Radicella et al. 1997)
	MYH	A:8-oxoG	Monofunctional	(Slupska et al. 1999)
	NTH1	TG, DHU, faPy	Bifunctional	(Aspinwall et al. 1997)
H2TH	NEIL1	faPyA, faPyG, DHU, TG, 8-oxoG	Bifunctional	(Jaruga et al. 2004)
	NEIL2	5-OHU, DHU		(Dou et al. 2003)
	NEIL3	Unknown	Unknown	(Takao et al. 2009)
AAG	AAG	3-meA, 7-meG, hypoxanthine, many others	Monofunctional	(Chakravarti et al. 1991)

Abbreviations used:
3-meA = 3-methyladenosine; 7-meG = 7-methylguanine; 8oxoG = 8-oxoguanine; AAG = alkyladenine DNA glycosylase; DHU = 5,6 dihydro uracil; faPyA =4,6-diamino-5-formamidopyrimidine; faPyG = 2,6-diamino-4-hydroxy-5-formamidopyrimidine; HhH = helix-hairpin-helix; H2TH = helix-2 turn-helix; MBD4 = methyl-CpG binding domain 4; MYH = MutY glycosylase homologue; NTH1 = homologue of *E. coli* EndoIII; OGG1 = 8-oxoguanine glycosylase; 5-OHMeU = 5-hydroxymethyluracil; SMUG1 = single-strand-selective monofunctional uracil-DNA glycosylase; TDG = thymine-DNA glycosylase; TG = thymine glycol; UNG = uracil DNA N-glycosylase.

a. Uracil-DNA N-glycosylase

The UNG subfamily is an important group of repair enzymes that prevents mutagenesis by recognizing specific base mismatches. Although its members share limited sequence similarity, they possess a common core fold. Notably, all these enzymes vary in substrate specificity and subcellular localization. The main UNG (thus, self-named) eliminates uracil from DNA when cytosine is deaminated or dUTP is misincorporatedinto chromosomal DNA during replicative synthesis (Lindahl et al. 1977). TDG recognizes G:U and G:T mismatches and can also remove thymine when present opposite guanine. SMUG1 preferentially works on single-stranded DNA, but recognizes a broader range of oxidized pyrimidines (Masaoka et al. 2003), as well as uracil (Wibley et al. 2003).

b. Helix-hairpin-helix

The HhH group of glycosylases was named for its motif involved in non-sequence-specific DNA binding. The N-terminal domain typically has four α helices, and the C-terminal domain has six to seven α helices. The hallmark HhH structural element, followed by a highly conserved glycine/proline-rich loop (Thayer et al. 1995), provides diversity for excising a variety of oxidatively and methyl-damaged bases (Bruner et al. 2000; Fromme et al. 2004a): Mismatch-specific DNA N-glycosylase (MBD4) repairs only T:G and U:G mismatches within a CpG sequence context. OGG1 is the main enzyme responsible for excising 8-oxoG lesions generated from ROS exposure (Michaels and Miller 1992). In addition, OGG1 can catalyze the removal of formamidopyrimidine oxidative lesions (Klungland et al. 1999). MYH, the human homolog of *E. coli* MutY, removes the normal 'A' when mispaired with 8-oxoG after DNA replication. This property is unique because most glycosylases recognize only abnormal bases in DNA (Hegde et al. 2010). However, MYH's function is critical; if the 8-oxoG:Amispair is not resolved before the next round of DNA replication, a C:G to A:T transversion will occur, resulting in a permanent genetic mutation (Yang et al. 2001). NTH1 preferentially repairs oxidized pyrimidines. As with OGG1, NTH1 excises base lesions only from duplex DNA, where the undamaged strand provides the template for repair synthesis during BER (Elder and Dianov 2002).

c. Helix-2 turn-helix

The H2TH enzymes of the NEI family predominantly excise damaged purine bases, but do so by using a different molecular scaffold than the HhH glycosylases (Huffman et al. 2005). The C-terminal domain contains

the H2TH motif, characterized by α/β helices, and a zinc finger motif that fits into the minor groove of DNA (Fromme and Verdine 2002). NEIL1 and NEIL2 have greater affinity for DNA bubble or forked structures than single- or double-stranded DNA. For this reason, this class of glycosylases putatively prefers repairing oxidized bases during DNA transcription or replication (Dou et al. 2003). NEIL1 expression increases during S phase, supporting a role during DNA synthesis, whereas NEIL2 expression is independent of the cell cycle. Thus, NEIL1 appears to be preferentially involved in replication-associated repair, while NEIL2 may be involved in transcription-coupled repair (Hegde et al. 2010). There is evidence that NEIL1 interacts with Pol β and DNA Ligase IIIα (Lig IIIα) in a regulatory or coordinating function in BER (Wiederhold et al. 2004). The glycosylase activity of NEIL3 appears to be involved in repairing hydantoin and formamidopyrimidine base modifications (Takao et al. 2009).

d. Alkyladenine DNA glycosylase

AAG is an "outlier" glycosylase. It is unique in three ways: (1) It is the only glycosylase identified to date in human cells that excises alkylation-damaged bases, although other human enzymes can repair alkylation damage (Abner et al. 2001; Huffman et al. 2005). (2) AAG reportedly is able to excise at least 12 different damaged bases, quite a diverse spectrum in comparison to the other glycosylases (Berdal et al. 1998). (3) AAG has a structural topology unlike any of the other known BER glycosylases (Lau et al. 1998). Given these properties, it is likely that there is more to learn about this unique enzyme.

AP ENDONUCLEASES

When a DNA glycosylase removes a damaged DNA base, it creates an AP (abasic) site. Free radicals and alkylating agents promote the release of bases by introducing base modifications that destabilize the N-glycosylic bond, generating a better leaving group moiety (Lindahl 1993). And, as noted earlier, AP sites can also arise spontaneously. Regardless of the cause, AP sites are cytotoxic and mutagenic lesions, so must be repaired quickly to preserve cellular viability and genomic integrity (Guillet and Boiteux 2002). In the BER pathway, an AP endonuclease cleaves these sites, and 95 percent of this activity is performed by the APE1 protein (also known as Ref-1, APE1/Ref-1, or HAP1) (Demple et al. 1991; Luo et al. 2010). Using Mg^{2+} as a cofactor (Beernink et al. 2001), APE1 hydrolyzes the phosphodiester backbone immediately 5′ to the abasic site, generating a normal 3′-hydroxyl group and an abasic 5′-deoxyribose-phosphate (dRP) residue on either side

of the nucleotide gap (Myles and Sancar 1989). Repair then proceeds along one of the two sub-pathways: SP or LP BER (Fig. 2). As already noted, the choice of sub-pathway depends on the type of damage (Luo et al. 2010), the local concentration of specific BER proteins, the cell cycle stage, and other factors (Fortini and Dogliotti 2007).

While APE1's predominant repair activity is its AP endonuclease function, APE1 also possesses weak 3′-phosphodiesterase or phosphatase activity (Wilson and Barsky 2001). When a bifunctional glycosylase, such as OGG1 or NTH1, removes a damaged base and incises the DNA backbone 3′ to the AP site via its AP lyase activity, the proteinleaves behind a 3′-blocking group that APE1's 3′-phosphodiesterase activity must excise. This end cleaning step is essential, as it creates the 3′-hydroxyl end required for Pol β binding and polymerization activity (Yang et al. 2006). The 3′-repair function of APE1is also used to process 3′-oxidative strand breaks generated by ROS attack that contain phosphate or phosphoglycolate residues (Wilson and Barsky 2001). APE1 performs many additional functions within cells; those functions are discussed in depth later in this Chapter.

DNA POLYMERASES

After a damaged base is excised and then processed by APE1, the resulting DNA strand break intermediate contains a 3′-hydroxyl group to prime resynthesis, and a 5′-dRP that must be removed prior to completion of repair (Wong and Demple 2004). In SPBER, Pol β performs both DNA re-synthesis and removal of the blocking 5′-dRP residue (Burgers et al. 2001). The C-terminal domain contains the polymerase activity (Kumar et al. 1990), while the dRP lyase activity resides in the N-terminal portion of the protein (Matsumoto and Kim 1995). Some polymerases have proofreading ability (i.e., 3′ to 5′ exonuclease activity); however, Pol β does not. The error frequency of Pol β activity in mammalian cell extracts is approximately $5–10\times10^{-4}$ (Bennett et al. 2001; Zhang and Dianov 2005).

If the 5′-abasic fragment produced after incision by APE1 is refractory to the dRP lyase activity of Pol β, then Pol δ and Pol ε may intervene, promoting strand displacement synthesis and the formation of a 5′-flap. When this occurs, the repair is re-routed through the LP pathway, where 2 to 8 nucleotides are removed and replaced (Klungland and Lindahl 1997; Kovtun and McMurray 2007).

DNA LIGASES

The last step of the BER pathway is ligation and sealing of the single-stranded nick in DNA, which is performed by a DNA ligase. Two human

DNA ligases function in BER: DNA Lig I and DNA Lig III (specifically, the Lig IIIα isoform) (Tomkinson and Levin 1997). Lig III operates primarily in the realm of SP BER, while Lig I completes LP BER. However, this division of labor is not mutually exclusive (Lieberman 2008). Lig I also plays an essential role in DNA replication, where it is active in joining Okazaki fragments (Levin et al. 1997).

DNA Lig IIIα differs structurally from Lig I in that the former contains a zinc finger that serves as a nick sensor and provides extra contacts with DNA to increase the enzyme's ligation efficiency (Ellenberger and Tomkinson 2008). Lig IIIα was originally purified in complex with XRCC1 (Caldecott and Thompson 1994), a non-enzymatic factor that will be discussed in greater detail below. Further characterization revealed that a deficiency in XRCC1 reduces the level and activity of Lig IIIα by 4- to 6-fold (Caldecott et al. 1995). Moreover, XRCC1-deficient cells are deficient in SP BER (Cappelli et al. 1997). Interestingly, the XRCC1-Lig IIIα complex also plays a role in the NER pathway (Ellenberger and Tomkinson 2008).

SCAFFOLD PROTEINS INVOLVED IN BER PATHWAY

Combining the enzymes discussed thus far (glycosylase, AP endonuclease, polymerase and ligase) can reconstitute BER activity *in vitro*. However, a number of accessory proteins are involved in BER *in vivo*, and although they do not have enzymatic functions, they provide a scaffold for the "core" BER enzymes, serve as modulators of enzymatic activities, and perform additional functions.

XRCC1

XRCC1 is gaining increasing recognition as a key component of BER. Although much research has probed the functions of XRCC1, the contributions of this protein are still being elucidated. Evidence shows that XRCC1 is a molecular scaffold, facilitating the assembly of multiprotein complexes and coordinating steps during BER. XRCC1 has both stabilizing and stimulating effects on various BER proteins. For example, it is known to stabilize DNA Lig IIIα and affect its activity (Caldecott and Thompson 1994). It also may be a molecular chaperone, with regulatory influences on multiple proteins (Caldecott et al. 1996). XRCC1 interacts with many BER proteins besides Lig IIIα, including Pol β (Kubota et al. 1996), poly(ADP-ribose) polymerase 1 (PARP1)(Masson et al. 1998), APE1 (Vidal et al. 2001), OGG1 (Marsin et al. 2003), and PCNA (Thompson and West 2000; Fan et al. 2004).

XRCC1's interactions with APE1, Pol β, and Lig IIIα in SP BER suggest a coordinated effort of recruitment, sequential binding and release not unlike that of a team relay—where one teammate doesn't let go of the baton until it has been passed securely to the next teammate. The net effect accelerates the overall repair process (Vidal et al. 2001; Marsin et al. 2003). A similar synchronized process may happen when XRCC1 first interacts with OGG1. Thus, XRCC1 is thought to orchestrate all the steps of the BER pathway, as it is the only protein known to participate at every phase of BER (Marsin et al. 2003). For details of these interactions, see Table 4. Indeed, cells deficient in XRCC1 show many hallmarks of defective BER, including hypersensitivity to IR and alkylating agents, delayed SSB rejoining, and induced mutations and increased sister chromatid exchange (Thompson and West 2000). Knockout of the *XRCC1* gene results in embryonic lethality in mice (Tebbs et al. 1999), underscoring the importance of this protein.

Table 4. XRCC1 Interactions with and influences on other BER proteins. (Caldecott et al. 1996; Cappelli et al. 1997; Thompson and West 2000; Vidal et al. 2001; Fan et al. 2004)

BER Protein	XRCC1's interaction with this BER protein results in:
Pol β	• Facilitates the fit of Pol β into the kinked DNA; suppresses strand displacement • Increases its efficiency and fidelity • Acts as a "bridge" between Pol β and other repair enzymes
APE1	• Stimulates both its endonuclease activity and its 3'-dRPase activity • May modulate transcription of APE1
DNA Lig IIIα	• Stabilizes the enzyme • May modulate its activity • Lowers its ligation efficiency in SP BER
PARP1	• Negative modulator of its activity
OGG-1	• Influences it to perform as a monofunctionalglycosylase, which allows for more efficient repair without producing the toxic intermediate that would have been generated by its lyase activity • Stabilizes it at the AP site until APE1 binds
PCNA	• PCNA and XRCC1 co-localize at DNA replication foci during S phase; XRCC1 helps ensure efficient repair of SSBs in LP BER, to prevent DSB formation and replication-fork collapse

Note: Evidence exists that XRCC1 interacts with at least 8 DNA-repair-related proteins, including some in other pathways.

PARP1

PARPs are a group of enzymes that use NAD^+ to transfer ADP-ribose polymers on to specific acceptor proteins (including themselves), which modifies their properties (Hassa and Hottiger 2008; Yelamos et al. 2011). This poly(ADP)-ribosylation serves multiple purposes, including (1)

de-condensation of chromatin via histone poly(ADP)-ribosylation around the DNA damage site, enabling BER proteins to access the lesion (Rouleau et al. 2004), (2) recruitment of repair proteins to the site, (3) facilitation of DNA repair, and (4) signal transduction of downstream effectors (Drew and Plummer 2010; Yelamos et al. 2011).

PARP1, the primary polymerase of this group, is activated by binding to nicks in DNA, and is displaced only after auto-modification (Hassa et al. 2006). This mechanism appears to serve a protective function—to shield the chromosomes from degradation by unrestrained repair processing if excessive DNA damage occurs (Parsons et al. 2005). Indeed, if excessive damage is present, PARP1 can trigger p53-mediated apoptosis (Drew and Plummer 2010). Interestingly, APE1 appears to suppress the activation of PARP1 when repair is underway after oxidative DNA damage (Fishel and Kelley 2007). This may help prevent cell death and provide a negative feedback mechanism for PARP1.

PARP1's automodification may also help recruit XRCC1 and Pol β. Although PARP1 is not essential for BER activity (Allinson et al. 2003), its absence or inhibition causes DNA damage to accumulate and ultimately become DNA DSBs that must be repaired by homologous recombination (HR; see Chapters 14 and 15). Thus, PARP1 performs multiple functions: surveillance as a damage sensor, facilitator of DNA damage response and repair, and a modifier of multiple DNA repair and damage-response proteins.

PCNA

PCNA is required during LP BER as support for efficient replication by DNA Pol δ (Maga and Hubscher 2003). Besides functioning as a processivity factor during resynthesis, PCNA interacts with other BER enzymes, including the UNG and NTH1 glycosylases (Oyama et al. 2004; Ko and Bennett 2005), FEN1 (Friedrich-Heineken et al. 2005), APE1, Pol β, and DNA Lig I (Fan and Wilson 2005; Almeida and Sobol 2007). These interactions likely help coordinate an efficient repair response.

9-1-1

The 9-1-1 complex (comprised of RAD9, RAD1, HUS1) mediates multiple aspects of the DNA damage response, including DNA damage checkpoints, DNA repair, translesion synthesis, and apoptosis (Kemp and Sancar 2009). The 9-1-1 complex assists in resynthesis of nucleotides in LP BER, a process that also involves DNA Pol δ, or ε, PCNA, and possibly other factors (Klungland and Lindahl 1997). The 9-1-1 complex shares many structural

similarities with PCNA in that both of them are clamp loaders, although the two differ in their ligand protein binding mechanisms (Kemp and Sancar 2009). The 9-1-1 complex stabilizes the damage site and likely recruits damage-processing proteins to sites of stalled replication, as the complex is known to interact with and stimulate many BER proteins: MYH (Shi et al. 2001), NEIL1 (Guan et al. 2007a), TDG (Guan et al. 2007b), APE1 (Gembka et al. 2007), Pol β (Toueille et al. 2004), FEN1 (Wang et al. 2004), and Lig I (Smirnova et al. 2005).

BER PROTEINS AS TARGETS IN CANCER TREATMENT

Cells evaluate every insult to DNA in light of what will benefit the genome as a whole the most: repair, senescence, or apoptosis. Because many chemotherapeutics and radiotherapeutics kill cancer cells by damaging their DNA, efficient DNA damage response and repair mechanisms diminish the effectiveness of those treatments. Chemo- and radioresistance must be overcome, which is why anticancer regimens often employ a cocktail of drugs or a combination of chemotherapy and radiotherapy—with a goal of creating so much damage in tumors that efficient DNA repair is not feasible. Because deficiencies in or dysfunction of DNA damage response and repair are often early manifestations of tumorigenesis (Kaelin 2009) (see Chapter 1), tumors must compensate for those losses. This typically takes the form of altered levels of DNA repair proteins in the deficient pathway or a complementary pathway, a feature that often contributes to acquired or intrinsic cellular resistance to DNA-damaging agents (Bapat et al. 2009).

Resistance can be overcome when the balance is shifted from repair to apoptosis or cell death. This is the rationale behind administering multiple therapeutics concurrently or sequentially; numerous regimens are currently in clinical practice, and more are in clinical trials. But, because collateral damage and toxicities associated with treatment increase as more therapeutics are added, the scales must also be tipped in favor of selectively killing cancer cells rather than normal cells. This predicates finding therapeutics that preferentially target cancer cells. Targeted therapeutics may enable lower doses of traditional treatments to be used. Evidence of this has been seen with BER inhibitors that are already in the clinic, notably PARP1 inhibitors (Plummer 2010).

In contrast to creating a simple additive effect, targeted therapeutics often seek to create a synthetic lethality—a circumstance that capitalizes on defects in two genes that separately do not result in cell death, while their combined inhibition leads to cell death (see Chapter 14). Finding genes that cancers are "addicted" to or are deficient in, and have compensated for via certain DNA repair processes, can create a situation where synthetic lethality

can be achieved and thus be both highly effective and highly selective for tumor cell killing (Kaelin 2009; Underhill et al. 2011). Because many cancers are dysfunctional in recognizing and responding to DNA damage, many likely opportunities for creating synthetic lethality are in the realms of DNA damage response and repair. A striking example of this is in BRCA deficient cancers. The blockade of BER via a PARP1 inhibitor causes an accumulation of DNA damage that must be repaired via the HR pathway; however, because BRCA cancers are HR-deficient, PARP inhibition results in a synthetic lethality that renders those cancer cells hypersensitive to treatment (Darzynkiewicz et al. 2009; Carden et al. 2010).

Many commonly used chemotherapeutics induce DNA lesions that are repaired predominantly by the BER pathway. Such chemotherapeutics include the ones listed in Table 5. The clinical success of PARP1 inhibitors

Table 5. Chemotherapeutics that cause lesions BER normally repairs (Fawcett et al. 2005; Alexandre et al. 2007; Meynard et al. 2007; Jiang et al. 2008; Kelley and Fishel 2008; Burdak-Rothkamm and Prise 2009; American Cancer Society 2011).

Drug class	Examples	Type of damage caused
Alkylating agents	Nitrogen mustards Mechlorethamine Chlorambucil Cyclophosphamide Ifosfamide Nelphalan Nitrosoureas Streptozocin Carmustine (BCNU) Momustine Alkyl sulfonates (busulfan) Triazines Dacarbazine (DTIC) Temozolomide (TMZ) Ethylenimines Thiotepa Altretamine Procarbazine Dacarbazine	Insertion of alkyl radicals into DNA bases
Platinum drugs	Cisplatin Carboplatin Oxaliplatin	ROS (secondary effect); also, produce effects similar to that of alkylating agents
Cytotoxic antibiotics	Anthracyclines Epirubicin Daunorubicin Doxorubicin Idarubicin Bleomycin Mitomycin Methyl-lexitropsin	ROS (secondary effect)
Taxanes	Paclitaxel Docetaxel	ROS (secondary effect)

In addition, some types of damage caused by **ionizing radiation** are repaired by BER.

provides proof of concept that inhibition of DNA repair, and specifically other components of the BER pathway, may yield additional benefits in cancer treatment. Thus, by studying proteins within the BER pathway, identifying potent inhibitors of these proteins, and defining the effects of inhibiting these proteins, the administration of such inhibitors should increase the efficacy of conventional treatments for other cancers. An example of this idea that is in ongoing trials is the combination of IR + alkylating agent + a BER inhibitor.

Dose-limiting toxicity is one of the potential concerns in the development of BER inhibitors, so a number of strategies are being devised to maximize inhibitor effectiveness, while minimizing any added toxicities from the adjunctive therapy. These strategies include intermittent or alternate scheduling of inhibitors with chemotherapy, using inhibitors as single agents, and pairing those inhibitors with localized radiation treatments (Plummer 2010). A number of small-molecule inhibitors of BER proteins are in various stages of development. BER proteins being considered are FEN1, Pol β, PARP1, and APE1. Some PARP inhibitors are currently in clinical use, and clinical trials are continuing for second generation PARP inhibitors (Plummer 2010) (see Chapters 14 and 15). As FEN1 and Pol β inhibitors are in preclinical development, the longest discussion in this Chapter is devoted to APE1.

FEN1 Inhibitors

FEN1 is a structure-specific endonuclease (Williams and Kunkel 2011) that is a critical element in multiple processes related to DNA metabolism. In particular, it cleaves 5′ DNA flaps in LP BER and removes Okazaki primers during lagging strand DNA synthesis. FEN1 also has a regulatory role in recombination and a maintenance function at telomeres (Williams and Kunkel 2011). FEN1's involvement in RNA metabolism has been documented as well, including RNA primer removal during DNA replication, RNA gap endonuclease activity, and RNase H activity (Tell et al. 2010b). FEN1's efficient, accurate processing activity is critical, as any blockade in removing repair intermediates or replication primers would result in DNA that is not ligatable, delaying replication and initiating post-replicative repair events, which could endanger the integrity of the genome (Tsutakawa et al. 2011).

Cell studies demonstrate that lack of FEN1leads to hypersensitivity to alkylating agents. In addition, FEN1 is elevated in many cancers, including gastric, lung, prostate, pancreatic, breast and brain cancers (Dorjsuren et al. 2011; Tsutakawa et al. 2011). Indications of a potential synthetic lethality exist for inhibiting FEN1, although research into this area is in its early stages. For example, it is known that a RAD54B deficiency causes chromosomal

instability via dysfunction of the HR pathway. When knockdown of the *RAD54B* gene was induced in HCT116 colorectal cancer cells, the cells developed chromosomal instabilities. When FEN1 expression was simultaneously inhibited, the HCT116 cells displayed severely compromised proliferation, supporting the idea of synthetic lethality. RAD54B-proficient cells that were depleted of FEN1 were not affected (McManus et al. 2009). A few hydroxyurea-based FEN1 inhibitors are in the earliest stages of preclinical development, but new technologies to screen for other potential inhibitors show promise for finding and fast-tracking potentially useful compounds (Dorjsuren et al. 2011).

Pol β Inhibitors

Pol β, which performs polymerase activities in both SP and LP BER, has an associated lyase activity that is often rate-limiting in BER. Normally, Pol β is expressed in all tissues at low levels (Hirose et al. 1989), but is upregulated in the presence of DNA damage (Fornace et al. 1989; Cabelof et al. 2002). Pol β is often overexpressed in tumor cells, which decreases the fidelity of BER (Chan et al. 2006). In addition, Pol β mutants can interfere with normal BER processing (Chan et al. 2007). Upregulation of Pol β contributes to resistance to IR, bleomycin, monofunctional alkylating agents and cisplatin (Wong and Wilson 2005; Chan et al. 2006). Thus, modulating Pol β levels through inhibition could potentiate the damage caused by anticancer agents. Because (1) the lyase activity of Pol β is often rate-limiting in BER and (2) it may be difficult to develop an inhibitor specific to the polymerase domain that would not also inhibit polymerases involved in DNA replication, specific inhibition of the lyase activity may be more desirable than targeting polymerase activity.

One of the first Pol β inhibitors characterized inhibited both its lyase and polymerase functions (Hu et al. 2004). At present, more than 60 Pol β inhibitors have been identified to date; however, most of them either lack the potency or specificity necessary to become a drug (Barakat and Tuszynski 2011). All viable candidates are still in preclinical studies. A 2008 study by Hecht and colleagues identified four interesting Pol β inhibitors: oleanolic acid, edgeworin, betulinic acid, and stigmasterol. Each of these compounds could potentiate the cytotoxicity of bleomycin in cultured A549 cells (Gao et al. 2008). Of the four, oleanolic acid most strongly inhibited both Pol β activities in *in vitro* biochemical assays. Stigmasterol alone selectively inhibited only the lyase activity of the enzyme, but it also produced the least potentiation of bleomycin cytotoxicity. Importantly, the inhibitory effect of all four compounds appeared to affect the DNA repair process only, not scheduled replicative activity. Further mechanistic studies of Pol β's lyase activity may reveal indirect ways to selectively inhibit this activity.

Another recent development in the area of Pol β inhibitors is the preclinical studies with **NCS-666715** [4-chloro-N-(3-(4-chloroanilino)-1H-1,2,4-triazol-5-yl)-2-mercapto-5 methylbenzenesulfonamide]. NCS-666715 appears to be a potent small-molecule inhibitor of Pol β in both SP and LP BER, doing so without affecting APE1 or FEN1 activity. Preclinical studies to date show that it enhances the efficacy of TMZ for reducing the growth of colon cancer cells *in vitro* and *in vivo* (Jaiswal et al. 2009). A 2010 virtual screening process called a relaxed complex scheme has predicted new, more potent Pol β lyase activity inhibitors that may be pursued (Barakat and Tuszynski 2011).

DNA repair pathways overlap, and Pol β is a good example of an enzyme with roles in more than one pathway. Cancer cells with defects in MSH2, an MMR damage-recognition protein, have demonstrated extreme sensitivity to the Pol β inhibitor masticadienonic acid(Martin et al. 2010). Interestingly, cancer cells deficient in the MMR protein MLH1 were sensitive to inhibition of DNA pol γ, but not Pol β. These experiments demonstrate the potential for a synthetic lethal combination: protein deficiency in MMR + inhibition of Pol β.

PARP1 Inhibitors

The PARP superfamily comprises 18 known entities; two of them are activated by DNA damage (Drew and Plummer 2010). As of the writing of this Chapter, eight PARP inhibitors are in various phases of clinical trials as anticancer agents; a ninth one is in clinical trials for other applications, and some PARP1 inhibitors are already on the market (see Chapter 15) (Fig. 3). PARP1 wields its greatest influence on BER and HR, and its participation in DNA repair can confer resistance to anticancer therapy (Madhusudan and Hickson 2005; Sanchez-Perez 2006; Kinsella 2009). Conversely, reduced expression of PARP1 can induce tumor cell killing.

The success of PARP inhibitors in certain breast cancers is believed to be due to the tumor's reduced capacity for HR due to the loss or mutation of BRCA proteins. Normal cells with either no mutation or loss of only one BRCA allele have sufficient repair capacity and are not overly sensitive to PARP inhibition (Yap et al. 2011). Cancers that have lost both alleles of BRCA 1 and/or 2 have defects in the HR pathway, so DSB repair normally handled by HR is shuttled through the NHEJ pathway. Inhibition of PARP causes an excess accumulation of SSB damage, leading to recombinogenic lesions or DSBs that the HR pathway normally would repair during the S and G2 phases of the cell cyle. However, in the absence of HR, replication forks collapse, leading to increased genomic instability and ultimately cell death (Bryant et al. 2005).

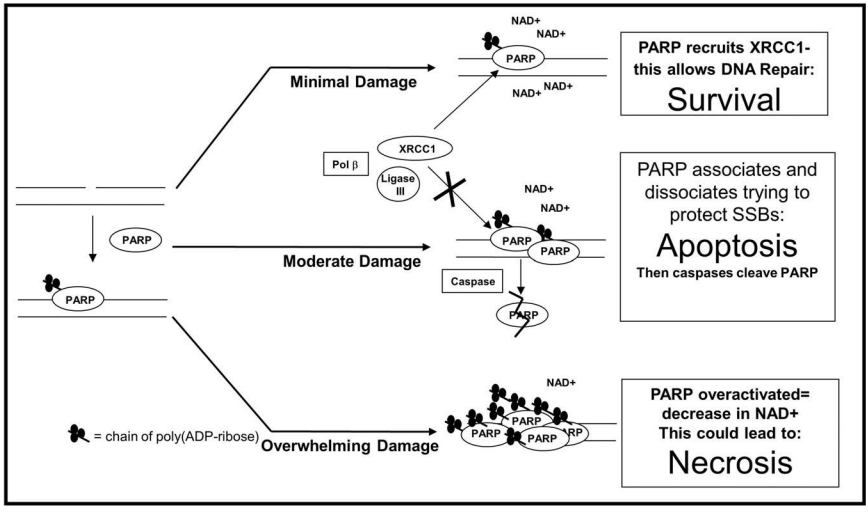

Figure 3. PARP's varying response to levels of cellular damage (Damia and D'Incalci 2007; Ratnam and Low 2007; Woodhouse et al. 2008). When there are a minimal number of SSBs, PARP1 interacts with XRCC1, Pol β and DNA Lig III to allow DNA repair. When there is moderate DNA damage, PARP1 associates and dis-associates with the DNA, trying to protect the SSB until it can be repaired. If the pathway is too overwhelmed, apoptosis is signaled and caspases come in to cleave PARP1. If there is overwhelming damage, PARP1 is overactivated and depletes NAD^+, which could lead to necrosis. Reproduced from *Future Oncology* 5(5), 713–726 (2009) with permission of Future Medicine Ltd.

PARP inhibitors have the ability to bind to PARP1 in the same way that NAD^+ does, blocking PARP1's interaction with NAD^+. Under normal circumstances, when DSBs activate PARP1, the C-terminal end of the protein synthesizes chains of poly(ADP-ribose) in a reaction driven by NAD^+ (Reed et al. 2009). When catalysis of NAD^+ by PARP1 is inhibited, downstream signaling is blocked and DNA damage overwhelms the cells, inducing apoptosis.

Because PARP1 is involved in cross-talk between multiple DNA repair pathways, not all of the anticancer potential of PARP inhibitors is considered a "BER agent" (Reed et al. 2009). However, this cross-talk feature underscores the prospective for wider applications of PARP inhibitors beyond BRCA cancers. Indeed, PARP inhibitors have already been demonstrated to exhibit synergy with alkylating agents, platinating agents, topoisomerase I poisons, and IR in a variety of tumor cell lines and animal xenograft models (Reed et al. 2009). In addition, trials are underway to determine if PARP inhibitors can sensitize tumors to chemotherapeutic agents such as TMZ, carboplatin, gemcitabine, topotecan, paclitaxel, and cyclophosphamide (Plummer 2010; Yap et al. 2011). A wide variety of tumor types are included in these trials, such as breast, ovarian, glioma, melanoma, and lung cancers.

The study and clinical application of PARP inhibition has found that cells can develop resistance to this form of treatment, a phenomenon that is common with many chemotherapeutic agents. At least two forms of acquired resistance have been documented to date (Wang and Figg 2008). Secondary mutations in *BRCA2* can create a frame shift in its open reading frame, reverting a BRCA2-deficient tumor to either wild-type or a novel functional form that is resistant to PARP inhibitors, as well as other anticancer agents. This resistance is related to the tumor cells' restored ability to form RAD51 foci in response to certain forms of DNA damage. Treating patients with proteasome inhibitors to prevent RAD51 recruitment has been proposed as a way to offset this acquired resistance.

A second form of acquired resistance to PARP inhibitors was seen in cases of P-glycoprotein (P-gp) overexpression. Hypothetically, this could be overcome by administering tariquidar, a P-gp inhibitor (Reed et al. 2009). Discovering these new forms of resistance underscores how formidable a foe cancer is, how extensive its abilities are for overcoming the cell-killing abilities of DNA-damaging agents, and how we must relentlessly uncover more information about the molecular features of tumor cells and their survival mechanisms.

Still, the combination of PARP inhibition and HR dysfunction is the most stunning example to date of successfully exploiting synthetic lethality in clinical applications. Today, third-generation PARP inhibitors are in various stages of clinical development, and more than 70 clinical trials in progress (www.clintrials.gov). The ever-broadening scope of therapeutic possibilities of PARP inhibitors as both single agents and adjunctive anticancer treatments is an exciting area of research development.

APE1: NOT ONLY A DNA REPAIR ENZYME

APE1 was first characterized as the major human apurinic/apyrimidinic endonuclease of the BER pathway in 1991 (Demple et al. 1991). One year later, Xanthoudakis and Curran described a protein that stimulated AP-1 DNA-binding activity through conserved Cys residues in Fos and Jun and named this protein Ref-1, an acronym for Redox Effector Factor-1. They also suggested that Ref-1 may represent a novel redox component of the signal transduction pathway that regulates gene expression (Xanthoudakis and Curran 1992). In fact, both the Demple and Curran group had identified the same protein, describing two separate activities on two functionally independent regions of the APE1 protein. APE1 is the only known DNA repair protein that also functions as a reduction-oxidation regulator in mammals (Tell et al. 2009). Additional functions of this unique protein have been identified, and more continues to be learned about the breadth of its influence on genomic stability.

APE1 Gene and Protein

The human gene encoding APE1 maps to chromosome 14 at location 14q11.2 (http://www.ncbi.nlm.nih.gov/). The *APE1* gene is comprised of four introns and five exons, spanning 2.64 kilobases. The 5′ flanking region (–890 bp) is located in a CpG island and lacks typical TATA and CAAT boxes, but contains TATA- and CAAT-like sequences and putative transcriptional factor recognition sites (Akiyama et al. 1994).

The APE1 protein is a 37kDa globular protein consisting of 318 amino acids, organized into three distinct portions marked by separate functionalities. The first 33 N-terminal residues of APE1 are an intrinsically disordered region involved in numerous protein-protein interactions and its RNA-binding activities (Vascotto et al. 2009b; Tell et al. 2010a). The section comprising amino acids 35–127 constitutes the redox portion of APE1. APE1's DNA repair section resides in residues ~65 through 318 (through the C-terminus) (Tell et al. 2010a; see Fig. 4).

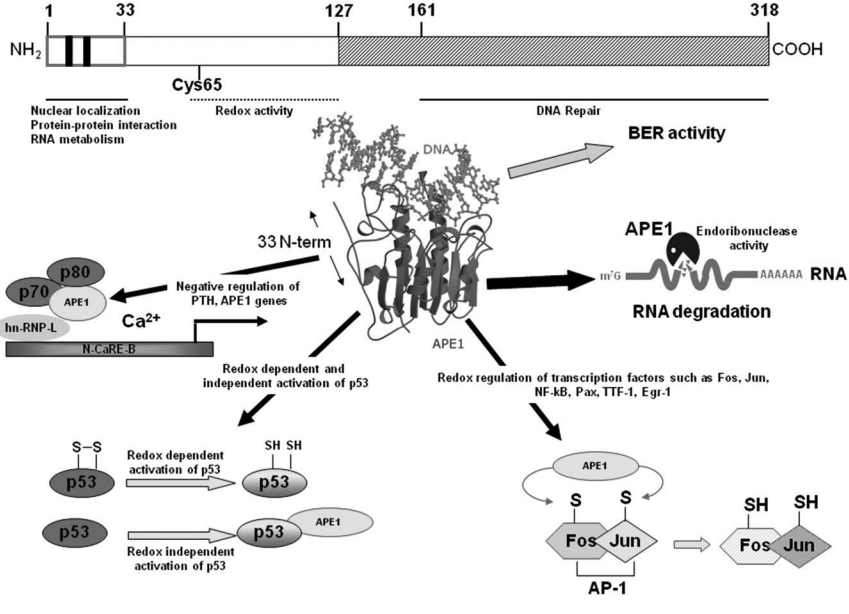

Figure 4. Cartoon of APE1 regions and functions of each region. Ribbon representation of the APE1 structure. The major functions of APE1 are illustrated; BER activity, redox-dependent and independent regulation of transcription factors, transcriptional repression of nCaRE and RNA metabolism (Tell et al. 2009). Reprinted with kind permission from Springer Science+Business Media: *Cell. Mol. Life Sci*. Understanding different functions of mammalian AP endonuclease (APE1) as a promising tool for cancer treatment , 67, 2010, 3589–3608, Tell G, Fantini D, and Quadrifoglio F., Figure 1.

Color image of this figure appears in the color plate section at the end of the book.

APE1 DNA REPAIR PROCESSES

The early part of this Chapter described two DNA repair functions of APE1: (1) it is the major human apurinic/apyrimidinic endonuclease in the BER pathway, and (2) it removes certain 3′ blocking damages, including entities produced by bifunctional glycosylases (OGG1, NTH, NEIL1 and NEIL2) and by IR. This latter functionof APE1 is possible through its 3′-phosphodiesterase and 3′-phosphatase activities (Parsons et al. 2004; Fishel and Kelley 2007; Castillo-Acosta et al. 2009). Although these activities occur at rates 200x lower than APE1's endonuclease function, they are nonetheless important repair functions. Accordingly, inhibition of these activities may have clinical significance relative to radiation and bleomycin treatments for cancer (Fishel et al. 2007b).

APE1 performs a third DNA repair function, termed nucleotide incision repair (NIR), which is considered a backup repair pathway to classic BER, because it overlaps with the repair activities of the glycosylase NTH1. APE1 nicks the DNA on the 5′ side of certain oxidatively damaged bases formed by irradiation under anoxic conditions; for example, in cell nuclei. This activity occurs at a lower pH than APE1's endonuclease activity and is modulated by the intracellular concentration of Mg^{2+} (Gros et al. 2004). Notably, the microenvironment of cancer cells is hypoxic and APE1 levels are increased in many cancers. Future studies may reveal more about APE1's NIR and other regulatory activities in such a situation.

Interestingly, optimal conditions for NIR are similar to those for APE1's 3′→5′ exonuclease activity on mismatched deoxyribonucleotides at the 3′ termini of nicked or gapped DNA. This additional APE1 repair activity has demonstrated proofreading capability (Gros et al. 2004). Inhibition of this activity could have implications in treating cancers for which nucleoside analogs such as troxacitabine or gemcitabine are used (Fishel and Kelley 2007). More functions of this versatile enzyme are likely yet to be discovered.

Redox Regulation

If APE1 repair functions are viewed as helping prevent apoptosis, APE1's reduction-oxidation functions can be considered an effort to promote cell growth (Kelley et al. 2011). ROS such as $O_2\cdot^-$, H_2O_2 and OH· can inflict oxidative damage that threatens the stability of DNA (Gros et al. 2004) and promotes loss of DNA functionality (Su et al. 2011). Oxidation of transcription factors obviates their DNA-binding capacity, rendering them unable to transcribe genes (Fan et al. 2003). Transcription factors must stay in a reduced, activated state to fold properly, bind to DNA, and thus

produce proteins the cell needs. Even sub-toxic levels of ROS can alter cellular functions significantly. Cellular redox balance is maintained by non-enzymatic systems (glutathione, α-tocopherol, L-ascorbic acid) as well as enzymatic systems (catalase, peroxidase, superoxide dismutase, APE1) (Tell et al. 2005). The former exert uniform, global influences, while the latter act on specific targets.

Overview of APE1 Redox Functions

APE1 exerts its redox regulation of transcription factors in two ways. The first, most studied method is that APE1 directly maintains transcription factors in their active, reduced state by thiol/sulfide exchanges. In this process, a reduced APE1 protein (1) recognizes an oxidized protein containing a disulfide bond, (2) binds to the protein reversibly, (3) donates a hydrogen to reduce a target Cys on the protein, and then (4) becomes oxidized in the process. How it actually performs this function and returns to its own reduced state is still not completely known, however recent studies are beginning to elucidate the mechanism (Su et al. 2011; Luo et al. 2012).Recently a second APE1 redox activity, termed "redox chaperone" activity, was discovered. In addition, a redox-independent mode of action by APE1 on transcription factor activities has been hypothesized for p53 (Jayaraman et al. 1997) and AP-1 (Ordway et al. 2003).

Direct Redox Regulation

APE1's redox function is located in the N-terminal portion of the protein. APE1 modulates gene expression directly by reducing both ubiquitous (i.e., AP-1, Egr-1, NF-κB, p53, HIF, YB-1, STAT3) and tissue-specific (i.e., Myb, PEBP-2, Pax-5 and -8, TTF-1) transcription factors (Xanthoudakis and Curran 1992; Xanthoudakis et al. 1992; Huang and Adamson 1993; Tell et al. 1998, 2009; Gaiddon et al. 1999; Nishi et al. 2002; Busso et al. 2010). APE1's redox activity also indirectly influences downstream effectors (see Fig. 5). Given the list of transcription factors that APE1 regulates, one can see the significant potential for developing a redox inhibitor of APE1. HIF-1α, p53, NF-κB, CREB, and AP-1 have been implicated in various "mission critical" aspects of cancer, including angiogenesis and tumor promotion and progression. A selective, effective APE1 redox inhibitor would prohibit those transcription factors from binding to DNA, thus stopping their tumor signaling for angiogenesis and proliferation (Fishel and Kelley 2007), as well as microenvironment signaling (Jedinak et al. 2011).

How APE1 reduces transcription factors is still being investigated (Walker et al. 1993; Georgiadis et al. 2008). Normally two Cys residues are

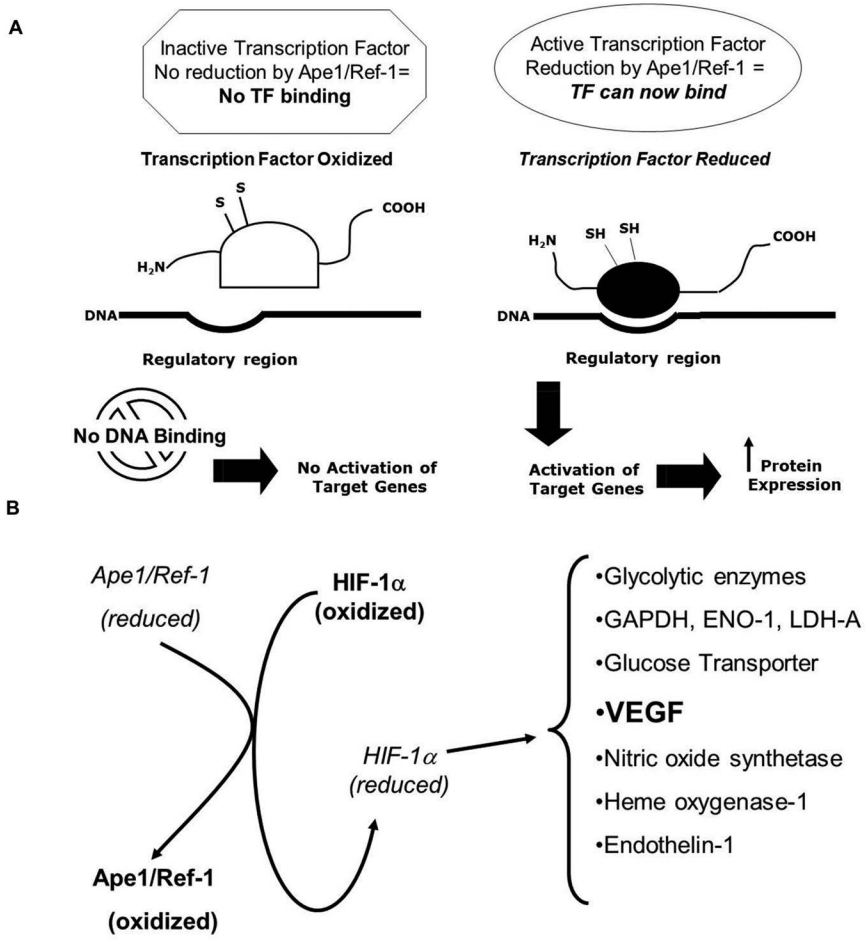

Figure 5 Redox control of transcription factors. APE1/Ref-1 redox control of transcription factors. (A) A number of transcription factors (TFs) such as AP-1(Fos/Jun), HIF-1a, NFkB, CREB, p53 and others have been shown *in vitro* and *in vivo* to be under APE1 (APE1/Ref-1) redox control. Shown here is a cartoon (adapted from (Tell et al. 2005)) of how Ape1/Ref-1converts a TF from an oxidized and inactive state to one that is reduced by APE1 and converted to a TF that can then bind to the regulatory regions of a variety of genes. This would then lead to the activation of downstream targets. (B) An example of downstream activation is shown here. APE1 converts HIF-1a from an oxidized to a reduced state allowing binding to the regulatory region of the VEGF gene, as well as others, leading to the enhancement of tumor progression, angiogenesis and vasodilation. This post-translational modification affects the binding of the TFs regardless of other post-translational modifications such as phosphorylation, acetylation, etc. Reprinted from Mol. Aspects Med. 28, Fishel M.L., Kelley M.R. The DNA base excision repair protein Ape1/Ref-1 as a therapeutic and chemopreventive target. 375–395, (2007) with permission from Elsevier.

required for athiol/disulfide exchange reaction: one acts as a nucleophile, forming a mixed disulfide bond with the target protein; the second Cys resolves the reaction, forming a disulfide bond with the redox factor, leaving the target protein in a reduced state (Su et al. 2011). To date, only one Cys residue, Cys65, has been identified as being critical for APE1's redox functions (Georgiadis et al. 2008). However, recent studies have also identified additional Cys's in APE1 that are involved in this activity (Su et al. 2011; Luo et al. 2012). More problematic is the fact that Cys65 is located near the N-terminus on the first beta strand in the fold of the β sheet in the protein's globular core. Although Cys93 (Walker et al. 1993; Georgiadis et al. 2008; Tell et al. 2009; Luo et al. 2010) and Cys99 have been implicated as interacting with Cys 65, on first glance, they are spatially too far apart and thus are seemingly in accessible. In particular, Cys65 and Cys93 are buried in APE1's core on opposite sides of the β sheet, based on the protein crystal structure (Luo et al. 2010). However, new evidence demonstrates that APE1 acquires a more open configuration, and it appears that Cys65 and Cys93 are accessible and involved in APE1 redox function as well as Cys99 (Su et al. 2011; Luo et al. 2012).

Indirect Redox Regulation through Chaperones

A novel, recently discovered APE1 redox activity is that of a "redox chaperone." In this capacity, APE1 promotes reduction of transcription factors, not by its own Cys, but by other reducing molecules such as GSH and thioredoxin. Although this activity is still being characterized, APE1 appears to mediate this chaperone function by interacting directly with the target transcription factor, but at a much lower concentration than is needed for its classic redox activity. APE1 may do this in one of three ways: (1) by recruiting the other molecules when it binds to its target transcription factor, (2) by inducing a conformational change in the target transcription factor, or (3) by forming hydrogen bonds on the thiol groups of the target transcription factor (Ando et al. 2008). Thus, through its redox and redox-chaperone activities, APE1 influences the DNA binding of numerous transcription factors involved in cell cycle control, apoptosis, cellular growth and differentiation (Tell et al. 2010a)

APE1 and RNA Metabolism

RNA possesses several intrinsic features that make it particularly susceptible to alkylative and oxidative damage: (1) it is mostly single-stranded; (2) its bases are not protected by hydrogen bonds or binding to structural proteins (such as histones), and (3) its intracellular location predisposes it to insults

(Tanaka et al. 2007; Kim et al. 2010). Damaged RNA can impair protein synthesis or cause mutagenic protein production, which can, in turn, affect the translational process, cell functions, and ultimately, cell viability (Berquist et al. 2008; Tell et al. 2010b). Cells must be able to repair RNA damage in order to create a mature mRNA transcript that is a faithful reproduction of DNA; otherwise, protein synthesis will be marred by missense, nonsense, point or frameshift mutations. Oxidative damage to RNA molecules, both those that code for proteins (mRNA) or perform translation (rRNA and tRNA), is associated with neurodegenerative diseases such as Alzheimer's disease (Moreira et al. 2008), and the effect of such damage with regard to cancer development cannot be excluded (Berquist et al. 2008).

Recent findings have shown that APE1 is involved in RNA repair and metabolism.

(1) APE1 localizes to the nucleolus by interacting with NPM1, a protein involved in rRNA maturation and ribosome biogenesis (Tell et al. 2010b).
(2) In the cytoplasm, APE1 interacts with several proteins involved in ribosome assembly and RNA maturation (Tell et al. 2010b).
(3) APE1 cleaves abasic RNA sites, performing "cleansing" and quality control functions for oxidatively damaged RNA (Kim et al. 2010; Tell et al. 2010b). Interestingly, in contrast to APE1's DNA cleaving function that requires Mg^{2+} as a cofactor, its endoribonuclease function occurs in the absence of divalent metal ions (Kim et al. 2010). Berquist et al. demonstrated that APE1 can incise complex substrates that resemble arrested replication as well as transcription intermediates (Berquist et al. 2008).
(4) APE1 also cleaves a specific coding region of the transcription factor c-myc to regulate mRNA half-life in cells (Kim et al. 2010; Tell et al. 2010b).

These newly discovered RNA-based functions of APE1 may help explain its cytoplasmic distribution and translocation in cells.

POST-TRANSLATIONAL MODIFICATION (PTM) AND REGULATION OF APE1 FUNCTIONS

PTM of a protein provides cells with a quick and often reversible way to modulate different activities of a protein. Indeed, epigenetic-related PTMs have emerged as the main mechanism for controlling signal transduction, gene expression, protein degradation, nucleoplasmic shuttling, and protein-protein interactions (Bhakat et al. 2009). APE1 activities are known to be modified by five types of PTMs: phosphorylation, S-nitrosation, acetylation, ubiquitination, and proteolytic removal of N33-terminal portion. All these

modifications take place in APE1's N terminal portion, which is also the site of action for RNA cleansing.

Phosphorylation of APE1 increases with increased alkylating damage to DNA, which may affect APE1's redox activity, and thus, gene transcription. However, the biological significance of phosphorylating APE1 is still uncertain, and it is as yet unknown if specific phosphorylated sites contribute to mutagenesis (Busso et al. 2010). A current hypothesis is that genotoxic stress leads to phosphorylation-dependent stimulation of another PTM that modulates APE1's redox activity, particularly for stress-inducible transcription factors such as AP-1, NF-κB, and p53 (Bhakat et al. 2009).

S-nitrosation may affect the cytoplasmic distribution and nuclear export of APE1 (Bhakat et al. 2009; Busso et al. 2010).

Acetylation appears to be a general mechanism by which cells regulate BER, as this occurs with BER enzymes besides APE1 (Busso et al. 2010). For example, acetylation of OGG1 enhances its glycosylase activity, whereas acetylation of NEIL1 abolishes its similar activity. In contrast, acetylation of APE1 alters its transcriptional regulatory functions (Bhakat et al. 2009).

Acetylation of APE1 has been documented on six Lys residues of the protein, which suggests that different sites may redirect APE1 activity (Fantini et al. 2010). The nCaRE (negative calcium response elements) located on the parathyroid hormone (*PTH*) gene promoter is subject to regulation by a complex comprised of nCaRE, acetylated APE1, heterogeneous ribonucleoprotein-L (hnRNP-L), and possibly other yet unidentified factors. Acetylation of APE1 by p300 HAT (histone acetyl transferase) increases APE1's ability to bind to the complex. In this conformation, acetylated APE1 functions as a co-repressor and trans-acting factor of *PTH* when intracellular Ca^{2+} levels rise (Bhakat et al. 2003). After down-regulating *PTH*, APE1 is deacetylated by HDAC1, which disengages APE1 from the complex, ensuring the temporary nature of gene suppression. What is notable about this interaction is that APE1 may be down-regulated by its own product. This may be the first example of transcriptional autoregulation in a eukaryotic DNA repair enzyme (Izumi et al. 1996; Bhakat et al. 2009).

Other examples of acetylated APE1 activity have been documented. These include the following (Bhakat et al. 2009):

- Acetylation of APE1 enhances its binding to YB-1, activating the Y-box-dependent promoter *MDR1*, which affects mRNA translation and metabolism.
- Egr-mediated activation of *PTEN* expression depends upon acetylation of APE1.

- p300-catalyzed acetylation is likely the reason why APE1 is incorporated into the hypoxia-inducible transcription complex for *VEGF* expression.

Ubiquitination produces varying results on proteins, depending on whether monoubiquitination or polyubiquitination occurs. Monoubiquitination of APE1 stabilizes it and increases its affinity for DNA; polyubiquitination of APE1 signals cell death. These activities take place almost exclusively on three Lys residues of APE1. It is likely that APE1's N-terminal region is required for these reactions, but additional mapping must be performed to confirm this conclusion. APE1 ubiquitination may occur indirectly through the activation of p53 and the transcription of other genes. Ubiquitination of APE1 may affect its functions in the nucleus, because MDM2, a protein that appears to be required for APE1 ubiquitination, is localized in this cellular compartment (Busso et al. 2010). However, the full story of APE1 ubiquitination is still to be resolved.

Proteolytic cleavage of the N-terminal portion of APE1 is a peculiar, irreversible PTM that is not yet well understood. Although the loss of the N-terminal portion of APE1 does not inhibit its endonuclease activity (Fantini et al. 2010), the formation of such a truncated protein was reported as being mediated by Granzyme A (GzmA) in natural killer cells (Fan et al. 2003). Conflicting data exist regarding which APE1 activities are affected by this proteolytic cleavage, and to what extent (Fan et al. 2003; Chattopadhyay et al. 2006). Fan proposed that GzmA abolishes APE1's redox function so that it does not interfere with GzmA-mediated cell death—similar to how PARP is cleaved by capsase during capsase-mediated apoptosis (Fan et al. 2003). While we know the N-terminal portion of APE1 is directly involved in protein-protein interactions and in RNA binding (Vascotto et al. 2009a), many questions remain concerning the utility of this PTM, the stimuli capable of inducing it, where this modification takes place, and its effect on APE1 activities. A major discrepancy in these studies is the indication that the truncated APE1 protein has a non-specific DNA nuclease activity, rather than its specific AP endonuclease function, yet numerous investigators have not observed this non-specific nuclease activity in a truncated APE1 protein (Fritz et al. 2003; Wong et al. 2007; Georgiadis et al. 2008; Li et al. 2008; Luo et al. 2008, 2010; Su et al. 2011).

For a summary of all redox-dependent and -independent functions of APE1 see Fig. 6.

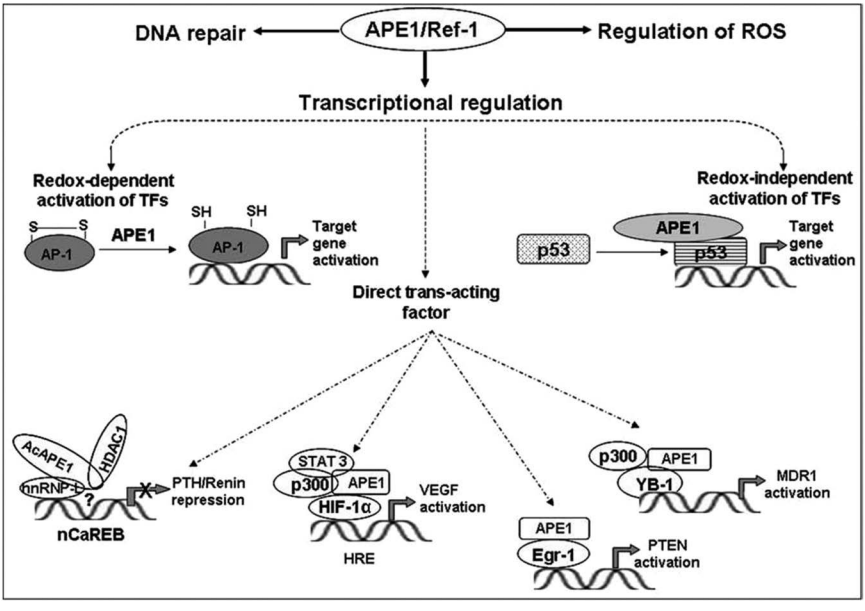

Figure 6. Redox-dependent and -independent functions of APE1. APE1 partners with a variety of different proteins in various complexes (Bhakat et al. 2009). The publisher for this copyrighted material is Mary Ann Liebert, Inc. Publishers. Reprinted with permission from Bhakat K.K., Mantha A.K., Mitra S. 2009. Transcriptional regulatory functions of mammalian AP-endonuclease (APE1/Ref-1), an essential multifunctional protein. Antioxid Redox Signal 11: 621–638.

APE1 INHIBITORS

Many aspects of APE1 make it an attractive target for clinical development of anticancer inhibitors:

- APE1 is essential for cell viability (Larsen et al. 2007; Fishel et al. 2008; Vascotto et al. 2009a; Jiang et al. 2010; Bhakat et al. 2009; Kelley et al. 2010).
- APE1 is dysregulated or upregulated in many solid cancers, including prostate, pancreatic, ovarian, cervical, germ cell tumor, rhabdomyosarcoma, and colon cancers (Hickson et al. 2004; León et al. 2005; Fishel et al. 2007a; Fishel and Kelley 2007; Abbotts and Madhusudan 2010; Luo et al. 2010).
- APE1 overexpression is associated with chemo- and radioresistance, incomplete therapeutic responses, shorter time to progression, lower

survival rates, and increased angiogenesis (Kakolyris et al. 1998; Bobola et al. 2001; Koukourakis et al. 2001; Madhusudan and Hickson 2005; Madhusudan et al. 2005; Kelley and Fishel 2008; Luo et al. 2008, 2010; Bapat et al. 2009; Simeonov et al. 2009; Kelley et al. 2011).

- APE1 levels may also have prognostic and/or predictive significance in the course of cancer treatment (Luo et al. 2008, 2010; Tell et al. 2009; Nyland et al. 2010; Kelley et al. 2012).
- Both APE1's DNA repair and redox regulatory activities affect multiple repair and signaling pathways (Luo et al. 2008, 2010; Tell et al. 2009; Zou et al. 2009; Jiang et al. 2010; Nyland et al. 2010; Kelley et al. 2012).
- In cell studies, blockade of APE1's repair functions causes an accumulation of DNA damage, potentiating the cell-killing abilities of many laboratory DNA-damaging and anticancer therapeutic agents, including methyl methanesulfonate (MMS), H_2O_2, bleomycin, TMZ, melphalan, cisplatin, IR, and gemcitabine (Wilson and Bohr 2007; Luo et al. 2008, 2010; Tell et al. 2009; Vasko et al. 2011).
- In cell studies, blockade of APE1's redox functions (1) inhibits the growth of tumor endothelial cells (Zou et al. 2009; Kelley et al. 2010), and their progenitors, and (2) inhibits differentiation of human bone marrow mesenchymal cells (Zou et al. 2009).Blockade of APE1's redox activity also causes additional antiproliferative effects (Zou and Maitra 2008; Fishel et al. 2011) and alterations to the tumor microenvironment, including downregulation of HIF1α (Kelley et al. 2010; Pietras and Ostman 2010). Inhibition of APE1's redox activity can affect expression of genes downstream of critical transcription factors involved in survival, invasion, and angiogenesis (Hickson et al. 2004; Luo et al. 2008; Fishel et al. 2010, 2011; Jiang et al. 2010; Kelley et al. 2010).

These features of the APE1 protein provide the framework and rationale for developing small-molecule inhibitors that can potentiate the effects of existing anticancer agents. Because APE1 plays such a pivotal role in BER, as well as in redox regulation of transcription factors, the search for specific APE1 inhibitors is a matter of global study.

Inhibitors of APE1 DNA Repair Function

Several inhibitors of the DNA repair activity of APE1 are in development. Currently two classes of molecules reportedly block APE1 repair functions: (1) molecules that bind to DNA to stop APE1 from performing its endonuclease activity, and (2) small molecules that bind directly to APE1 to inhibit its activity on AP sites (see Fig. 7). With one exception, all potential inhibitors of APE1's DNA repair functions are still in preclinical development and have thus far been tested only on cell lines.

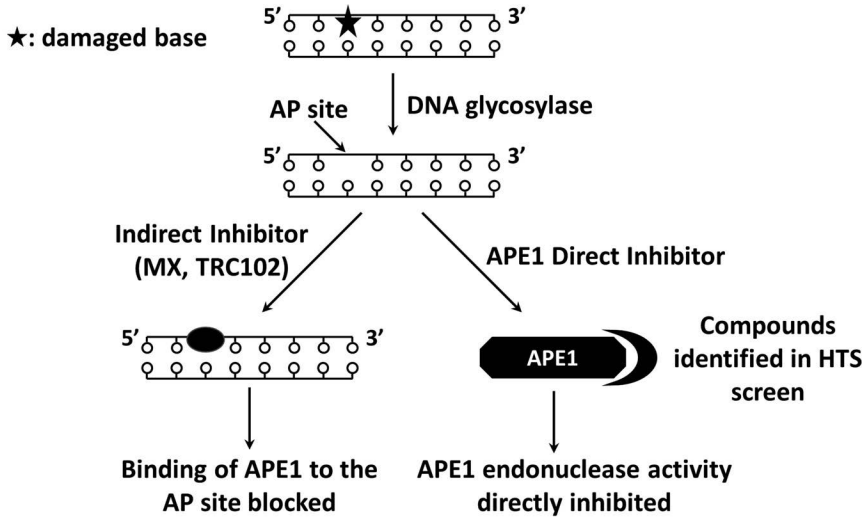

Figure 7. Modes of action for classes of APE1 repair inhibitors. Current APE1 inhibitors block the AP endonuclease activity in two ways. Inhibitors like methoxyamine (MX) bind to the AP site in the DNA blocking the downstream members of the BER pathway (left), while an APE1 direct-binding inhibitor binds to the protein and effectively blocks its ability to bind to or incise DNA at the abasic site (right). A number of compounds have been identified in high-throughput screens for APE inhibitors, but none are in clinical trials at this juncture (Vascotto and Fishel 2012). Reprinted from DNA Repair in Cancer Therapy, Vascotto C and Fishel M, Blockade of Base Excision Repair: Inhibition of Small Lesions Results in Big Consequences to Cancer Cells, Copyright 2012, with permission from Elsevier.

BER Inhibitors

Regarding the class of molecules that bind to DNA, technically they should be called BER inhibitors instead of APE1 inhibitors. An example of this is **methoxyamine** (MX, or TRC102), which binds to the aldehyde form of the AP site on DNA (see Fig. 7; (Liuzzi and Talpaert-Borle 1985; Rosa et al. 1991; Madhusudan et al. 2005)). At the time of this writing, one Phase 1 trial had been completed testing MX with pemetrexed (a folate anti-metabolite); another Phase 1 trial was in progress for testing MX combined with the alkylating agent TMZ as a regimen for patients with advanced tumors (Lowndes and Toh 2005; Tan et al. 2005). Preliminary data from the first Phase 1 trial of MX + pemetrexed showed persistence of AP sites in patients (Anthony et al. 2009). However, because MX is not specific for APE1 or AP sites, it could affect unintended targets (Horton et al. 2000).

Another class of compounds that non-specifically inhibits APE1 is arylstibonic acids. Of those, compounds 13755 and 13793 have been studied as lead compounds (Seiple et al. 2008). However, they seem to

have poor cell permeability and a high potential for toxicity, given their antimony composition. Furthermore, because their activity is similar to that of phosphate derivatives, all the arylstibonic acids are likely to inhibit additional enzymes that act upon nucleic acids (Seiple et al. 2008; Wilson and Simeonov 2010). After the initial publication, no new data have been presented to demonstrate that this class of inhibitors will have cellular or clinical relevance.

APE1 Endonuclease Inhibitors

Lucanthone, or Miracil D, is an anti-schistosome agent that inhibits the catalytic activity of topoisomerase II (TopoII). Because it has been demonstrated to enhance the tumor-killing effects of IR, it was also being studied as a potential inhibitor of APE1 DNA repair activity (Luo and Kelley 2004). Three decades ago, lucanthone was shown to sensitize HeLa cells to radiation through an unknown mechanism (Bases 1970), which is still being characterized. We now know that lucanthone (1) interacalates into DNA (Hirschberg et al. 1968), (2) causes an accumulation of AP sites (Mendez et al. 2002), and (3) potentiates the cell-killing effects of MMS and TMZ (Luo and Kelley 2004). Of note, lucanthone has no apparent effect on inhibiting APE1's redox or exonuclease activities (Luo and Kelley 2004). Whether the cellular observations arise solely from inhibition of APE1 or are due in part to lucanthone's inhibition of TopoII remains a subject for further investigation. Nevertheless, this compound may be efficacious when combined with anticancer agents that generate lesions repaired by BER.

CRT0044876 (7-nitroindole-2-carboxylic acid) is the first reported "true" APE1-specific DNA repair inhibitor (Madhusudan et al. 2005), although reports of its efficacy are conflicting (Abbotts and Madhusudan 2010). Initially, it was reported to specifically inhibit BER activity, and non-toxic levels of CRT0044876 were said to potentiate the cytotoxicity of MMS and TMZ. However, since then, lack of reproducibility of its effects has been noted and very high μM levels are required for "effectiveness", even in cell culture (Seo and Kinsella 2009). It also seems to have poor membrane permeability and aqueous solubility, which makes it ill-suited for drug delivery. Potential toxicities due to its nitro-aromatic ring are a concern as well (Wilson and Simeonov 2010). Further development of this agent and class of molecules has recently been described (Mohammed et al. 2011).

Many questions remain to be answered regarding potential APE1 DNA repair inhibitors. How should an effective APE1 repair inhibitor look and behave? Will it affect only cancer cells, or will it also cause collateral damage to healthy cells? Will it inhibit all of APE1's functions, or be able to selectively target its endonuclease activity? The former is likely, but may not

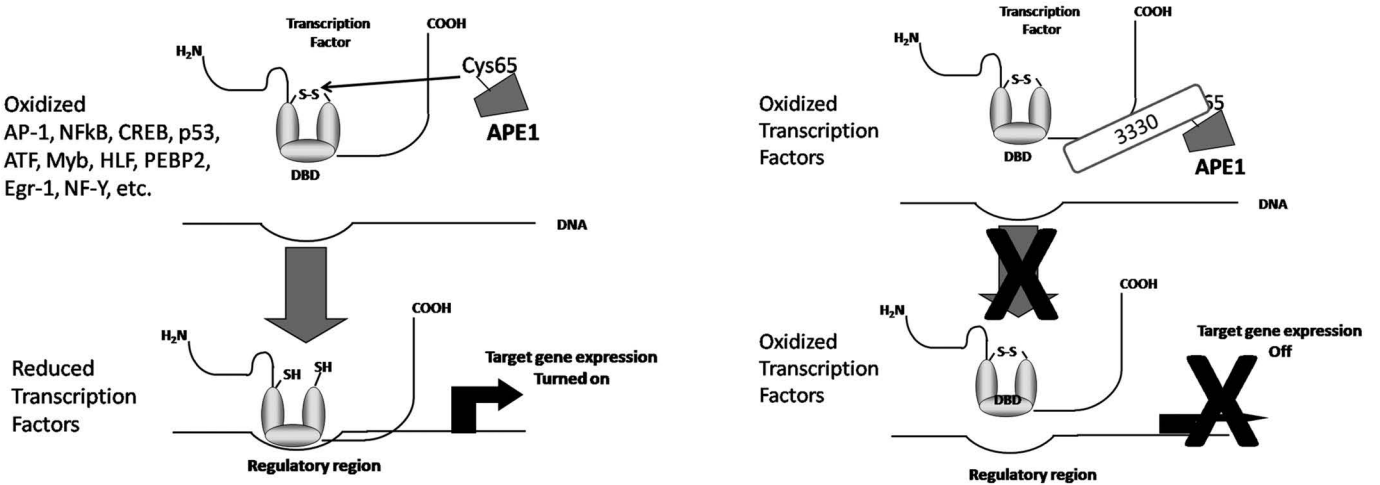

Figure 8. Small-molecule inhibition of APE1 redox signaling function. APE1 interacts with downstream transcription factors (TFs) such as NF-kB, HIF-1α, CREB, FOS, JUN, and others, converting them from oxidized to reduced states, allowing them to bind to their target promoters and switch on the transcription of genes. However, E3330 and newly discovered analogues interfere with this redox signaling by blocking the ability of APE1 to convert the oxidized TF to a reduced TF, thereby keeping the target gene transcription turned off (Su et al. 2011). The publisher for this copyrighted material is Mary Ann Liebert, Inc. Publishers. Reprinted with permission from Kelley MR, Luo M, Reed A, Su D, Delaplane S, Borch RF, Nyland II RL, Gross ML, Georgiadis M. 2011. Functional analysis of new and novel analogs of E3330 that block the redox signaling activity of the multifunctional AP endonuclease/redox signaling enzyme APE1/Ref-1. *Antioxid Redox Signal* 14: 1387–1401.

categorically be the case, if allosteric inhibitors could be developed (Wilson and Simeonov 2010). Studies are ongoing to find promising compounds.

Techniques for Screening

The search for APE1 endonuclease inhibitors has been accelerated through the use of high-throughput screens, which several research groups have pursued intensely (Madhusudan and Hickson 2005; Simeonov et al. 2009; Zawahir et al. 2009; Bapat et al. 2010). Specificity is a key concern for these screens, as a false positive can occur if a compound non-specifically binds to DNA—inhibiting APE1's ability to cut DNA, without actually inhibiting its endonuclease activity. Assays such as the fluorescent intercalator displacement (FID) assay can be used to estimate the DNA binding capacity of selected APE1 inhibitors (Tse and Boger 2004; Goodwin et al. 2006). Other studies test for persistence of AP sites as an indicator of the putative inhibitor's ability to enhance cytotoxicity with selected alkylating agents (Wilson and Simeonov 2010). Kelley's group has used a fluorescence-based high-throughput assay to screen a library of small molecule compounds for APE1 endonuclease inhibition (Bapat et al. 2010). While a number of "hits" were obtained, further work using a combination of *in silico* modeling coupled with actual screening of selected libraries and molecules has been much more fruitful, with new molecules in the sub-micromolar range identified (Kelley Laboratory, unpublished data). Zawahir et al. have also used a pharmacophore-based approach (Zawahir et al. 2009) that exploits known functional groups or interaction sites between a target of interest and a macromolecule, namely APE1 and abasicDNA, to find a molecule that disrupts that key interaction (Wolber et al. 2008).

Inhibitors of APE1 Redox Activity

While the thioredoxin and glutaredoxin/glutathione reduction-oxidation systems globally help maintain intracellular homeostasis by scavaging ROS (Holmgren 1995; Nakamura et al. 1997), APE1's redox activities distinctly differ in function and selectivity (Kelley et al. 2010). APE1 selectively reduces transcription factors that govern critical cellular functions, including stress responses to inflammation, hypoxia, and DNA damage, as well as angiogenesis (Guo et al. 2004). This regulatory activity of APE1 modulates the DNA binding activity of crucial transcription factors and also indirectly affects other "mission critical" cellular functions downstream of its effectors. Thus, inhibition of APE1's redox activity can block multiple tumor signaling pathways involved in cancer survival, growth, angiogenesis, and proliferation, as well as anti-inflammatory effects (Jedinak et al. 2011). Such

transcription factors regulated by APE1 include Egr-1, NFκB, AP-1, and HIF1α (Xie et al. 2006; Whipple and Korc 2008; Zou et al. 2009). For example, emerging evidence from studies of pancreatic cancer cells supports the hypothesis that inhibition of APE1 redox activity affects the cells' ability to respond to hypoxia, which could make the cells more sensitive to anticancer agents (Zou and Maitra 2008; Singh-Gupta et al. 2009).

Naturally-occurring APE1 Redox Inhibitors

Soy isoflavones found in soybeans may function as potential modulators of APE1 signaling (Messina et al. 2006; Raffoul et al. 2007). Genistein, daidzein, and glycitein are the main soy isoflavones; of the three, genistein is the most bioactive (Singh-Gupta et al. 2009, 2011). The effects of soy isoflavones have been tested *in vitro* and *in vivo* on APE1, NF-κB, and HIF1α, which are known to be upregulated or activated by radiation and have been implicated in radioresistance. All three proteins were inhibited *in vitro* and *in vivo*, and killing of prostate tumor cells was increased significantly when irradiation was administered concomitantly with soy isoflavones (Raffoul et al. 2007; Singh-Gupta et al. 2009; Raffoul 2007 #611). Administering soy isoflavones prior to radiation also elicited increased cell killing. In addition, pretreatment with soy isoflavones prevented phosphorylation of Src and STAT3, blocking signaling events upstream of HIF1α. Because crosstalk between APE1 and HIF1α is essential for nuclear translocation in responding to hypoxia, Singh-Gupta's group overexpressed APE1 in a series of experiments to see if the soy isoflavones inhibited APE1 directly. Indeed, direct inhibition was observed, indicating APE1-mediated regulation of the Src and STAT3 transcription factors. Interestingly, the combination of all three isoflavones produced the most favorable anticancer results; genistein alone caused lymphatic metastases (Singh-Gupta et al. 2009). Still, soy isoflavones appear to act rather non-specifically, suggesting that some of the effects attributed to APE1 inhibition may be more pleiotropic and indirect than causal (Pavese et al. 2010).

Resveratrol (3,4′,5-trihydroxystilbene), a component of red wine and grapes, is a polyphenolic compound with antioxidant properties (Kovacic and Somanathan 2010). Resveratrol is said to inhibit APE1's DNA repair and redox activities (Yang et al. 2005); however, conflicting data raise questions regarding this compound's ability to inhibit APE1 (Fishel and Kelley 2007). In addition, computer simulations of resveratrol docking on APE1 did not coincide with the region of the molecule in which the DNA repair activity resides (Kelley et al. 2011). Kelley's group was unable to elicit any APE1 redox inhibition in testing resveratrol (Kelley et al. 2011).

Engineered APE1 Redox Inhibitors

To date, the most selective and well-characterized APE1 redox inhibitor is the napthoquinone compound, **APX3330** (also known as E3330) (2E-3-[5-(2, 3 dimethoxy-6-methyl-1, 4-benzoquinolyl)]-2-nonyl-2- propenoic acid), which blocks APE1's redox function without affecting its endonuclease activity (Luo et al. 2008). APX3330 appears to act on a locally unfolded state of APE1 by increasing the level of disulfide bond formation involving Cys65 and/or Cys93, effectively stabilizing APE1 and thus decreasing its redox activity (Kelley et al. 2010; Su et al. 2011). Additional mechanistic studies using this inhibitor have recently been submitted (Luo et al. 2012).

Kelley's group has characterized APX3330 activity extensively in cancer cell lines and endothelial cells (Luo et al. 2008; Fishel et al. 2011), and is pursuing development of more potent APX3330 analogs. Recent studies have identified molecules that are 10x more potent than APX3330 and are currently being analyzed in animal models for their efficacy (Kelley, unpublished data and (Kelley 2008a,b, 2009; Kelley et al. 2011; Kelley and Fishel 2008; Kelley and Wikel 2011)).

APE1 IN NEURONAL PROTECTION

In evaluating how to modulate APE1 function for therapeutic benefit, another potential clinical application is emerging: neuroprotection. Anticancer treatments induce oxidative stress and/or cause direct damage to neuronal cell populations as well as their DNA, causing significant toxicities that are manifested as neuropathies. Evidences of peripheral (sensory) neuropathy are seen most frequently: distal paresthesia, altered proprioception, and coldness in the extremities. Cognitive impairment, also known as "chemobrain," is prevalent, and in many patients, lasts for years or indefinitely after treatment is finished. These problems are particularly prevalent after administration of platinum agents, microtubule stabilizing drugs, and IR, and limit patients' lifestyles in ways beyond that of their cancer alone (Duarte and Vasko 2011).

To mitigate treatment side effects that cause nerve damage, intense research is underway to learn how manipulation of DNA repair—specifically, altering the expression of APE1—can help restore patients' quality of life with respect to neuropathies. This is a novel approach for improving patient outcomes. Cell culture studies using primary rat dorsal root ganglia show that selectively upregulating the DNA repair component of APE1 can attenuate neurotoxicities after exposure to cisplatin (Jiang et

al. 2008). Furthermore, recent studies have demonstrated that the DNA repair activity of APE1, not its redox function, is required for the neuronal protective response to IR (Vasko et al. 2011). Additionally, APX3330 was shown to protect neuronal cultures against IR (Vasko et al. 2011). For a detailed review of all the studies that have been done to date in this area, see Chapter 13 of (Duarte and Vasko 2011). Despite the large body of evidence that points to induction of APE1 endonuclease activity as a neuroprotectant, the challenge is to determine how to spare neurons without enhancing the repair abilities of cancer cells.

CONCLUSION

The BER pathway repairs some of the most commonly occurring types of DNA damage to the genome, including oxidative and alkylative damage. Because of this, several BER proteins are attractive targets for developing repair inhibitors that can potentiate the effects of anticancer treatments. However, a close study of BER shows that it is not the simple pathway that it was once thought to be. Its complexity includes multifunctional proteins that engage in crosstalk with other pathways. This provides opportunities as well as challenges for the development of effective BER inhibitors. Four BER proteins—APE1, Pol β, FEN1, and PARP1—are being studied intensely to find inhibitors of their activities. While inhibition of the PARP proteins has shown clinical promise in the realm of synthetic lethality, development of other BER inhibitors is moving more slowly. As we characterize the molecular interactions of each BER protein more thoroughly, we draw closer to finding the best way to exploit their functions for therapeutic gains in clinical applications.

ACKNOWLEDGMENTS

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CHAPTER 9

Nucleotide Excision Repair: DNA Repair Capacity Variability and Cancer Susceptibility

Li-E Wang and *Qingyi Wei**

INTRODUCTION

DNA repair is a complicated biological process that maintains genome stability (Wood et al. 2001). Exposure to environmental carcinogens, such as tobacco smoke and ultraviolet (UV) radiation, can result in various types of DNA damage (Phillips 2002; Stokes and Comb 2008) that promote the development of diseases, including cancer. During evolution, most species have developed the necessary spectrum of DNA repair machinery to battle genomic insults from environmental hazards and maintain genomic integrity (Hoeijmakers 2001) (see Chapter 1). To date, more than 165 human DNA repair genes have been identified that can be categorized into several distinct pathways: base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), and double-strand break repair (DSBR) (Wood et al. 2005; Wood 2011).

Department of Epidemiology, Unit 1365, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030, USA.
*Corresponding author: qwei@mdanderson.org

DNA repair capacity (DRC) is defined as the host's ability to repair damage to DNA in a defined period of time, and has been used broadly to describe the readouts of experimental techniques, including phenotypic assays that measure levels of DNA damage or DNA repair gene expression (Berwick and Vineis 2000). The available DRC assays (based on mechanistic principles and the DNA damage-inducing agent) used to evaluate variation in DRC in human tissues have been comprehensively reviewed (Decordier et al. 2010; Valdiglesias et al. 2011). Relevant to the Chapter here, the observed inter-individual variation in DRC is considered an important risk factor for disease susceptibility (Berwick and Vineis 2000; Li et al. 2009; Decordier et al. 2010). Among the reported population-based studies, the host cell reactivation (HCR) and DNA adduct assays are perhaps best-known to measure DRC for removing DNA damage repaired by the NER pathway (Kennedy et al. 2005; Santella et al. 2005; Hu et al. 2007; Li et al. 2009).

Over the past 15 years, our research group has used a comprehensive approach to assess the role of DRC in cancer etiology and have performed cancer risk assessments in a series of hospital-based case-control studies. This kind of molecular epidemiological study begins with a detailed epidemiological assessment, followed by the application of specific DNA repair phenotypic assays to identify markers of genetic susceptibility to cancer. The most common strategy has employed cultured peripheral blood lymphocytes (PBLs) with benzo(a)pyrene diol epoxide (BPDE) or UV light as the test agents, which generate a range of DNA lesions repaired mainly by the NER pathway. Such cells have been used for the BPDE-induced adduct assay, the HCR-DRC assay, the reverse transcriptase polymerase chain reaction (RT-PCR) assay, and the reverse-phase protein array (RPPA). This Chapter summarizes published studies on the variation of DRC (measured by several phenotypic assays) and cancer susceptibility. While we focus herein on assays designed mainly to evaluate NER capacity and its relationship to disease risk, a similar body of work has revolved around the other DNA repair pathways. Table 1 summarizes the main DNA repair pathways and the majority of related phenotypic assays applied in population studies (Decordier et al. 2010).

HOST CELL REACTIVATION (HCR)-DNA REPAIR CAPACITY (DRC) ASSAY

Among the known DNA repair pathways, the mechanism of NER has been well characterized, and various disease models for NER deficiency have been described (see Chapters 1 and 2). NER eliminates a wide variety of DNA damage, primarily bulky DNA adducts that lead to a distortion in the DNA helix (Buschta-Hedayat et al. 1999; Wood 1999). Specifically, adducts

Table 1. Major DNA repair pathways and relevant phenotype assays.

DNA repair pathways	DNA-damaging agents	Major DNA substrates	DNA lesions	Relevant DNA repair assays
Base excision repair	Radiation, H_2O_2, Bleomycin, environmental exposure	Viable cells Cell extract	Oxidative base damage, single strand break (SSB)	Comet assay (Singh et al. 1988; Xiong et al. 2007) Gene expression profiling (Bruins et al. 2008)
Nucleotide excision repair	UV, BPDE	Viable cells Cell extract/ tissue	Photoproducts, DNA-adducts	Host cell reactivation assay (Athas et al. 1991; Wei et al. 1993) ^{32}P-labelling and immunochemical measurement of DNA-adduct removal (Yang et al. 1999; Santella and Zhang 2011) Gene expression profiling (Wei et al. 2005)
Mismatch repair				Gene expression profiling (Strom et al. 2001; Ohrling et al. 2010)
Double strand break repair	Radiation, H_2O_2, Bleomycin, environmental exposure	Viable cells	SSB, DSB	Comet assay (Olive and Banath 2006; Collins et al. 2008) Mutagen sensitivity assay (Cherry and Hsu 1983; Wu et al. 2007) Micronucleus assay (Kirsch-Volders et al. 1997; Fenech 2007) Gamma-H2AX assay (Fillingham et al. 2006) Gene expression profiling (Moeller et al. 2011)

induced by chemical carcinogens (e.g., BPDE) and photoproducts induced by UV light can be recognized and repaired by the NER pathway, which consists of a multistep process involving at least 20–30 proteins in a well-defined order (Wood 1999; Egly 2001; Friedberg 2001; Fuss and Cooper 2006). In humans, NER inactivation is associated with at least three autosomal recessive inherited disorders: xeroderma pigmentosum (XP), Cockayne syndrome (CS), and trichothiodystrophy (Sarasin and Stary 1997; de Boer and Hoeijmakers 2000). XP is an autosomal recessive disease stemming from a defect in NER, and XP patients have around a 1000-fold increased risk of developing skin cancers (Kraemer et al. 1984, 1994; Lambert et al. 1995; Sarasin and Stary 1997; van Steeg and Kraemer 1999; Norgauer et al. 2003; Cleaver 2005). The knowledge gained from studying the XP syndrome has led to many investigations into DNA repair as a biomarker for susceptibility to cancer in the general population (Berwick and Vineis 2000; Goode et al. 2002; Li et al. 2009). Indeed, a subgroup of individuals has been identified in the general population that has a suboptimal NER capacity and as a consequence is likely to be at increased cancer risk. Identification of this at-risk subpopulation is very important for cancer prevention, which aims to reduce cancer incidence in the general population (Wei et al. 1993; Grossman and Wei 1995; Li et al. 2009).

Since Athas et al. developed the HCR assay that uses a transient expression vector harboring the chloramphenicol acetyltransferase (CAT) reporter gene to measure NER of UV-induced DNA damage in human isolated and PHA-stimulated PBLs (Athas et al. 1991), several research groups have applied this *in vitro* assay in population-based studies to investigate the association between DRC and cancer susceptibility, including cancers of the skin (Wei et al. 1993, 2003; Hall et al. 1994; D'Errico et al. 1999; Landi et al. 2002; Matta et al. 2003; Landi et al. 2005), lung (Wei et al. 1996a, 2000), head and neck (Cheng et al. 1998; Ramos et al. 2004; Wang et al. 2010a), prostate (Hu et al. 2004), bladder (Lin et al. 2005) and breast (Ramos et al. 2004; Shi et al. 2004).

The HCR-DRC assay measures the efficiency of overall NER to repair transcription-blocking damage, which has been introduced exogenously on foreign DNA, after transfection into the host cells. For example, the pCMV*cat* plasmid DNA is first damaged with carcinogens, such as UV radiation and chemicals (e.g., BPDE), and then transfected into cultured, unexposed host cells as a substrate for the endogenous repair enzymes (Athas et al. 1991). A main advantage of this technique is that it measures the unaffected DRC phenotype, in that the non-replicating recombinant plasmids harboring a *CAT* reporter gene (pCMV*cat*) are damaged, but not the host cells (e.g., PHA-stimulated PBLs). Thus, the assay measures the inherent cellular DRC, reflective of the *in vivo* repair process, as the native

repair machinery remains intact when the cells are not exposed to the test carcinogen. Because a fixed time is allowed for the host cells to complete repair, the assay is representative of the true cellular NER process (Athas et al. 1991). Importantly, since the presence of only a single unrepaired bulky DNA adduct can effectively block the transcription of the reporter gene (Protic-Sabljic and Kraemer 1985; Koch et al. 1993), the HCR-DRC assay evaluates the complete process of repair. It is therefore appropriate to use this assay as described herein for measuring cellular NER capacity in human studies. Of course, changing the plasmid DNA-damaging agent would allow evaluation of a different DNA repair pathway.

The HCR-DRC assay has commonly been employed with PBLs, since these cells can be noninvasively obtained. Importantly, the repair capacity of PBLs has often been found to be reflective of an individual's overall intrinsic DRC (Grossman and Wei 1995). It has been demonstrated that both lymphocytes and skin fibroblasts from patients who have basal cell carcinoma, but not XP, have lower excision-repair rates than do individuals without cancer (Alcalay et al. 1990; Wei et al. 1994a,b, 1996a). As summarized in Table 2, we have used the CAT-HCR-DRC assay in studies of lung cancer (Wei et al. 1996a; Cheng et al. 2000; Shen et al. 2003; Wang et al. 2007a),

Table 2. HCR–DRC For risk of cancers.

Mutagen	Cancer type	Number	Risk estimate	Reference
		Case/control		
BPDE	Lung	51/56	5.70 (2.10–15.7)	(Wei et al. 1996b)
	Lung, Non-small cell	467/488	1.85 (1.42–2.42)	(Shen et al. 2003)
	Lung	764/677	1.50 (1.10–3.10)	(Spitz et al. 2003)
	SCCHN	55/61	2.20 (1.02–4.77)	(Cheng et al. 1998)
	SCCHN	744/753	1.91 (1.52–2.40)	(Wang et al. 2010a)
	Breast	69/79	3.36 (1.15–9.80)	(Shi et al. 2004)
NNK	Lung, adenocarcinoma	48/45	3.21 (1.25–8.21)	(Wang et al. 2007a)
UV	BCC	146/333	1.62 (1.07–2.45)	(Wang et al. 2007b)
	SCC	109/333	1.63 (0.95–2.79)	
	CM	312/324	2.02 (1.45–2.82)	(Wei et al. 2003)
	BCC	86/87	1.1 (0.9–1.3)	(Hall et al. 1994)
	BCC	49/68	1.2 (0.5–2.7)*	(D'Errico et al. 1999)
	SCC/BCC	280/177	3.8 (2.3–5.7)*	(Matta et al. 2003)
	Prostate	140/96	2.1 (1.2–3.9)	(Hu et al. 2004)

BPDE, benzo(a)pyrene diol epoxide; UV, ultraviolet; SCCHN, squamous cell carcinoma of head and neck; BCC, basal cell carcinoma; SCC, squamous cell carcinoma; CM, cutaneous melanoma.

*Recalculated based on the published data.

squamous cell carcinoma of the head and neck (SCCHN) (Cheng et al. 1998; Wang et al. 2010a), skin cancers (Wei et al. 2003; Wang et al. 2007b), and breast cancer (Shi et al. 2004). The results of these studies are discussed in further detail next.

Human skin is constantly exposed to carcinogenic agents, especially UV radiation, which cause various kinds of DNA damage, including bulky lesions (e.g., cyclopyrimidine dimers) that are removed effectively by the NER pathway (see Chapter 1). Following an earlier study of DRC and cancer susceptibility in 88 Maryland basal cell carcinoma (BCC) patients and 135 cancer-free dermatologic controls who had had noncancerous skin disorders (Wei et al. 1993, 1994a), we measured DRC with UV-damaged plasmids in a Texas study with 312 cutaneous melanoma (CM) cases and 324 cancer-free controls. Overall, case patients had a 19% lower mean DRC than did control subjects. Low DRC was associated with a nearly 2-fold increased risk of CM, and we observed a dose-response relationship between decreased DRC and increased CM risk. Moreover, among the patients, the subgroups that tended to have low DRC were those with blonde or red hair, blue eyes, fair skin, and poor tanning ability (Wei et al. 2003). Similar to the study of CM, in a hospital-based case-control study of 255 patients with newly diagnosed non-melanoma skin cancer (NMSC) [146 with BCC and 109 with squamous cell carcinoma (SCC)] and 333 cancer-free controls, we found a 16% reduction in DRC in the NMSC patients relative to the controls. DRC below the controls' median value was associated with a significantly increased (1.6-fold) risk for BCC (Wang et al. 2007b). These data suggest that reduced DRC is an independent risk factor for both CM and NMSC in the general population.

To simplify the HCR assay to accommodate population studies, we modified the assay to use the plasmid expression vector pCMV*luc*. This plasmid retains the human cytomegalovirus immediate promoter and enhancer (Athas et al. 1991), but harbors the luciferase (LUC) reporter gene. Unlike CAT, LUC enzyme activity can be measured by a nonradioactive method. In addition, the LUC assay can be completed much faster than the CAT assay and requires half (or fewer) of the number of viable cells. Cell culture, harvest, and transfection techniques are very similar for both assays; however, the cell extraction step is much simpler for the LUC assay than for the CAT assay. We have shown in parallel experiments that the DRC for UV damage to either the CAT or LUC plasmid DNA was highly correlated (Qiao et al. 2002), suggesting that these two strategies are comparable.

To investigate whether differences in DRC are associated with differential susceptibility to tobacco-related cancer, we used BPDE, a classic DNA-damaging carcinogen that is one of several polycyclic aromatic hydrocarbons (PAHs) found in tobacco smoke and the environment (MacLeod and Tang 1985; Cosman et al. 1992; Jernstrom and Graslund 1994),

to damage the reporter plasmids in a HCR-DRC assay. We found in a pilot study of 51 patients with lung cancer and 56 frequency-matched controls that individuals with reduced DRC had an increased risk of lung cancer (Wei et al. 1996a). We later confirmed that lower DRC was associated with a more than 2-fold increased risk of lung cancer in a larger, independent hospital-based case-control study of 316 lung cancer patients and 316 cancer-free controls (Wei et al. 2003). To investigate DRC as a biomarker for susceptibility to non-small cell lung cancer (NSCLC) and the possible interaction between DRC and tobacco smoke, we analyzed 467 newly diagnosed NSCLC patients and 488 cancer-free controls. The results showed that the overall 15.5% reduction in DRC for BPDE-induced DNA damage in the cases (7.84%) relative to the controls (9.28%) ($P < 0.001$) was associated with an approximately 2-fold increased risk of NSCLC (OR = 1.85, 95% CI = 1.42–2.42 after adjustment for age, sex, ethnicity, smoking, alcohol use, and sample storage time) (Shen et al. 2003). These results were confirmed by a series of studies using larger datasets with 764 lung cancer patients and 677 cancer-free controls (Spitz et al. 2003), and 1139 cases and 1210 controls in an expanded analysis of the same study population (Mahabir et al. 2007, 2008).

In addition to the PAHs in the smoke, tobacco-specific nitrosamines like the nicotine-derived nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), induce lung adenocarcinoma through the formation of DNA adducts. We modified the HCR assay using dimethyl sulfate to create alkylation damage in the pCMV*luc* plasmid DNA as a substitute for NNK damage. We established the damage-repair dose-response curves in both normal and NER-deficient lymphoblastoid cell lines and in PHA-stimulated PBLs, and then successfully measured the DRC in PHA-stimulated PBLs from 48 patients with lung adenocarcinoma and 45 cancer-free controls. The cases exhibited a lower mean DRC than did the controls, and a greater than 3-fold increased risk was associated with DRC levels below the control median (Wang et al. 2007a). These results suggest that suboptimal DRC for DNA alkylation damage is associated with an increased risk of lung adenocarcinoma.

To investigate the regulation of individual susceptibility to other tobacco-related cancers, we conducted a pilot study of 55 newly diagnosed SCCHN patients and 61 healthy controls using the CAT-HCR-DRC assay with BPDE as the damage-inducer. We found that the DRC of the cases was significantly lower than that of the controls (8.6% *vs*. 12.4%, $P < 0.001$). Subjects with DRC lower than the median level of the controls had a greater than 2-fold increased risk for SCCHN compared to those with higher DRC (Cheng et al. 1998). This preliminary finding was later confirmed and validated in a much larger independent study on the same population with 744 SCCHN non-Hispanic white patients and 753 age-, sex-, and ethnicity-

matched cancer-free controls (Wang et al. 2010a). In this study, patients with SCCHN had significantly lower mean DRC (8.8% ± 2.7%) than controls (10.0% ± 2.6%; $P < 0.0001$), and the association between reduced DRC and increased risk of SCCHN remained approximately 2-fold after adjustment for other covariates [adjusted odds ratio (OR) = 1.91, 95% confidence interval (CI) = 1.52–2.40]. Compared with the highest DRC quartile of controls, the increased risk was dose dependent (the second highest quartile: OR = 1.40, 95% CI = 0.99–1.98; the third quartile: OR = 1.87, 95% CI = 1.34–2.62; and the fourth quartile: OR = 2.76, 95% CI = 1.98–3.84, respectively; P for trend test < 0.0001). These findings further suggest that individuals with reduced DRC may be at increased risk of developing tobacco-related cancers including SCCHN.

In another case-control pilot study, we examined 69 previously untreated female breast cancer patients and 79 cancer-free controls frequency-matched to the cases by age and ethnicity. PBLs were used to measure DRC using the HCR assay with the *CAT* reporter gene damaged by BPDE. We found that the mean DRC level was significantly lower in breast cancer patients (10.1%) than in controls (11.1%) ($P = 0.008$). Subjects with DRC lower than the median level for the controls (11.0%) had a greater than 3-fold increased risk (OR = 3.36, 95% CI = 1.15–9.80) for breast cancer compared to those with higher DRC. These findings suggest that women with reduced DRC may be at an increased risk of developing breast cancer (Shi et al. 2004).

As summarized in Table 2, on the basis of our and other published case-control studies, we have provided sufficient evidence that a low DRC phenotype is an independent risk factor for cancers that are related to exposure to UV and tobacco smoke. Interestingly, we found that in most of these studies, cases who were younger at diagnosis, females, lighter smokers, and those who reported a family history of cancer exhibited a lower DRC compared to other cases, suggesting that these subgroups may be especially susceptible to cancer. Moreover, potential interactions between DRC and genes or environmental factors have been suggested (Wang et al. 2007b, 2010a). However, these findings need to be validated by larger, preferably prospective studies, which should provide evidence of whether the DRC phenotype is affected by disease status. Furthermore, there are some issues that still need to be resolved in terms of the suitability of PBLs as the surrogate tissue. Additional studies should be performed on the same individuals using their PBLs and relevant target cells, such as keratinocytes for NMSC, melanocytes for melanoma, and epithelial cells for lung cancer and SCCHN. It is also critical to measure variability of the tested cells to metabolize benzo(a)pyrene as well as the ability to repair the resultant DNA adducts, as oppose to the ability to repair BPDE-induced DNA adducts examined in our studies.

BPDE-INDUCED ADDUCT ASSAY

While the HCR-DRC assay measures the removal of DNA adducts on the transfected plasmid DNA, other studies have investigated the levels of genomic DNA adducts induced by *in vitro* exposure of host cells to smoking carcinogens in the laboratory (Wiencke et al. 1995; Motykiewicz et al. 2002; Kennedy et al. 2005; Santella et al. 2005) or the levels of *in vivo* DNA adducts in human tissues (Faraglia et al. 2003; Shantakumar et al. 2005; Sanyal et al. 2007). A relatively large variation has been observed in the levels of persistent *in vivo* smoking-induced DNA adducts, and this variability may reflect biologic differences in host metabolic activity and/or DRC (Wiencke 2002). BPDE-DNA adduct profiles can be considered a phenotypic marker for carcinogen metabolism and DNA repair. On the basis of this assumption, we have developed an assay that measures the *in vitro* induction of BPDE-DNA adducts in cultured PBLs as a susceptibility marker for tobacco-related cancers. Since BPDE induces formation of DNA adducts that are removed by the NER pathway (Tang et al. 1992; Friedberg 2001), measuring the levels of *in vitro* induced genomic adducts provides another tool for estimating NER capacity. While this assay provides quantitative information on the DNA adduct levels that remain in cells 67 hours after *in vitro* BPDE treatment, it does not reveal the molecular mechanisms by which the adducts were removed during this time period.

We have performed several studies and evaluated the association between levels of BPDE-induced adducts and cancer risk (Table 3) (Li et al. 1996, 2001a,b, 2007). We conducted a pilot study in the assay development phase on 21 lung cancer patients and 41 healthy frequency-matched controls (Li et al. 1996). Short-term cultured and PHA-stimulated PBLs from each subject were exposed *in vitro* to BPDE, and a ^{32}P-postlabeling method was used to measure BPDE-induced DNA adducts in the host cells. We found that the levels of the *in vitro* induced adducts in PBLs were about 100-fold higher than detected *in vivo* in PBLs (background), and that the PBLs of cancer patients tended to accumulate higher levels of BPDE-DNA adducts

Table 3. BPDE-induced adduct assay for risk of cancers.

Cancer type	Number	Risk estimate	Reference
	Case/ control		
Lung	21/41	6.40 (1.30–29.4)	(Li et al. 1996)
	221/229	2.15 (1.39–3.33)	(Li et al. 2001b)
SCCHN	91/115	2.22 (1.22–4.04)	(Li et al. 2001a)
	803/839	1.71 (1.39–2.10)	(Li et al. 2007)
Breast	158/154	2.43 (1.44–4.08)	(Kennedy et al. 2005)

SCCHN, squamous cell carcinoma of head and neck.

than did the controls. Logistic regression analysis revealed that the levels of induced adducts were an independent risk factor for lung cancer (OR = 6.4, 95% CI = 1.3–29.4) (Li et al. 1996). We confirmed these results in a larger hospital-based case-control study that included 221 patients with newly diagnosed lung cancer and 229 healthy controls. In this confirmation study, using the median adduct level of the controls as the cutoff point, we found that 64% of cases had higher levels (OR = 2.15, 95% CI = 1.39–3.33, after adjustment for age, sex, ethnicity, smoking, and alcohol use). Moreover, we observed a significant dose-response relationship between the upper quartile of levels of BPDE-induced DNA adducts and the risk of lung cancer (trend test, $P < 0.001$) (Li et al. 2001b).

In a pilot study of 91 patients with SCCHN and 115 controls, we used the BPDE-induced DNA adduct assay with short-term cultured PBLs. Again, levels of BPDE-DNA adducts were significantly higher in the cases than in the controls. Using the median level of control values as the cut-off point, we found that 66% of the cases were above this level. Logistic regression analysis indicated that the level of BPDE-induced DNA adducts was also an independent risk factor (OR = 2.22, 95% CI = 1.22-4.04) (Li et al. 2001a). To validate these findings, we performed another large, independent study that included 803 patients with SCCHN and 839 controls. We found that the mean BPDE-DNA adduct levels were significantly higher in cases (77.6 ± 111.8) than in controls (57.3 ± 98.3; $P < 0.001$). Using the median control value (29.22) as a cutoff, 63% of the cases were distributed above this level, and the elevated levels of the BPDE-DNA adducts were associated with an increased risk of SCCHN (OR = 1.71, 95% CI = 1.39–2.10, after adjustment for age, sex, ethnicity, smoking, alcohol use, and sample storage time) (Li et al. 2007).

The significant association between the levels of the *in vitro* BPDE-induced DNA adducts and risk for lung cancer and SCCHN suggests that subjects who are sensitive to BPDE-induced DNA damage may have a suboptimal ability to remove BPDE-DNA adducts and thus will be susceptible to tobacco-induced carcinogenesis. Compared with the *in vivo* adduct level, the *in vitro* BPDE-induced adduct assay is more informative, because this *in vitro* assay is independent of the individual's prior environmental exposure(s), and the levels of induced BPDE-DNA adducts detected reflect the complete genetic background that constitutes susceptibility to smoking-related disease, including cancer. Indeed, we have found that the genotypes and haplotypes of *ERCC1* and *ERCC2/XPD* genes, core genes in the NER pathway, were associated with the *in vitro* BPDE-induced DNA adduct levels (Zhao et al. 2008).

The reduced capacity for removing BPDE-DNA adducts have also been reported by Santella's research group on cell lines derived from sisters discordant for breast cancer (137 families containing 158 case patients and

154 control sisters). Immunofluorescence using a polyclonal anti-BPDE–DNA primary antibody, instead of the classical ^{32}P post-labeling method, was used to quantify BPDE–DNA adducts. The DRC was calculated as the difference between staining immediately after treatment with BPDE and that after 4 hours of repair, divided by the initial damage. The mean percent DRC was lower in breast cancer patients than in control subjects (difference = 8.6, 95% CI = 4.3–13.8, $P < 0.001$). The adjusted OR for risk of breast cancer associated with lower DRC was 2.43 (95% CI = 1.44–4.08) (Table 3). Using the highest quartile of DRC as the reference group, the adjusted OR for breast cancer risk increased from 1.23 (95% CI = 0.57–2.65) to 2.38 (95% CI = 1.17–4.86) to 2.99 (95% CI = 1.45–6.17) ($P_{trend} = 0.002$) as DRC decreased (Kennedy et al. 2005). Another study showed that the genetic polymorphism at codon 751 (rs13181) in the *XPD/ERCC2* gene may modify risk of breast cancer associated with PAH-DNA adducts and cigarette smoking (Terry et al. 2004).

DNA REPAIR GENE TRANSCRIPT LEVELS

As discussed above, our previous studies have shown that individual DRC varies widely and that suboptimal DRC is associated with the risk of developing various cancers. However, genetic determinants of the observed variation in the DRC phenotype remain largely unknown. It is conceivable that variation in the mRNA and/or protein expression levels of DNA repair genes is genetically determined. At least 165 genes have been reported to participate in various DNA repair pathways (Wood 2011), and thus, the alteration of a key gene may have an effect on DNA repair function and lead to altered cancer risk.

To investigate variation in the expression levels of DNA repair genes and its potential association with cancer risk, we developed a multiplex RT-PCR assay to measure DNA repair gene transcript levels relative to that of a ubiquitous housekeeping gene (Wei et al. 1997a). In this technique, transcripts from several repair genes of the same repair pathway, as well as the β-actin gene, were simultaneously amplified. The DNA repair transcript levels were then quantified relative to the β-actin level by computerized densitometric analysis of the multiplex RT-PCR products following gel electrophoresis. This assay has allowed us to simultaneously measure mRNA expression levels of several genes involved in MMR (Wei et al. 1995, 1997a,b), BER (Wei et al. 1995, 1997a; Liu et al. 2003), and NER (Cheng et al. 1999, 2002). However, the multiplex RT-PCR assay does not provide information on potential mRNA structural changes that may underlie any altered protein expression.

We conducted a pilot case-control study of 75 lung cancer patients and 95 controls, in which we measured the relative expression levels of five NER-related genes (*ERCC1, XPB/ERCC3, XPG/ERCC5, CSB/ERCC6*, and *XPC*) in PHA-stimulated PBLs. We found that reduced expression levels of *XPG/ERCC5* or *CSB/ERCC6* were associated with a more than 2-fold increased risk of lung cancer (Cheng et al. 2000). We conducted two pilot case-control studies of SCCHN and found that lower expression levels of several genes of both the MMR pathway (Wei et al. 1998) and the NER pathway (Cheng et al. 2002) were associated with an increased risk of SCCHN. Taken together, the results suggest that individuals with low expression levels of DNA repair genes may be at a higher risk of developing lung cancer and SCCHN.

As the technology has developed, quantitative real-time RT-PCR has been applied to measure DNA repair gene expression levels (Yang et al. 2005; Liu et al. 2007; Blomquist et al. 2009). We have conducted a pilot study of 51 prostate cancer patients and 50 age- and ethnicity-matched controls to evaluate the relative mRNA expression levels of three oxidative-damage-repair genes, human *MutM* homolog (*hMMH*), human *MutT* homolog (*hMTH*), and human *MutY* homolog (*hMYH*), with β-actin and human O(6)-methylguanine DNA methyltransferase genes (*hMGMT*) as the internal controls (Liu et al. 2003). The finding was that a high expression level of *hMTH*, but not of the other genes, was associated with a significantly increased risk of prostate cancer (odds ratio = 2.62; 95% confidence interval = 1.13–6.75). These studies implied that mRNA expression levels of DNA repair genes may serve as cancer susceptibility biomarkers. Most recently, pathway- or disease-focused PCR arrays with multiple gene panels have been developed using quantitative real-time RT-PCR technology in a 96-well or 384-well format (Wang et al. 2010b; Wu et al. 2011). Because factors such as environmental exposure, genetic polymorphisms, and variation in post-transcriptional modifications may have some effect on levels of protein expression as well as transcript levels of DNA repair genes, the data on transcript levels need to be evaluated together with information on all of these components, as well as the DRC phenotype.

REVERSE-PHASE PROTEIN ARRAY (RPPA)

Although we have shown that transcript levels of NER genes are associated with the risk of lung cancer and SCCHN, this association may be misleading, because the transcript level does not necessarily reflect the level of protein expression. The *in vitro* analysis of extracted cellular proteins may provide information on protein expression levels, modification, degradation, complex formation, activity, and localization, but the quantification of these features will be challenging. In contrast to directed experimental studies,

large scale epidemiological studies need assays that are economical and feasible for the sample size. The reverse-phase protein microarray (RPPA) assay is suitable for quantifying protein levels in a high-throughput manner. Nevertheless, this assay does not provide information about the modification of proteins, such as phosphorylation, ubiquitination, or sumoylation, which may have functional consequences. In this assay, cell lysate proteins are immobilized on a nitrocellulose-coated substrate as a dilution series with a dynamic range of at least 1000-fold for accurate quantification (Spurrier et al. 2008). Although this method assesses only one known protein per microarray, it has the ability of including multiple samples to be analyzed and compared side by side on a single array, an assay feature necessary for an epidemiological study.

To test the hypothesis that altered expression levels of NER proteins are associated with the risk of SCCHN, we obtained cultured and PHA-stimulated PBLs from 57 patients with newly diagnosed SCCHN and 63 cancer-free controls, and transfected PBLs with both damaged and undamaged plasmid DNA in parallel. We subsequently quantified NER protein levels in the cell extracts of the transfected cells using the RPPA method (Wei et al. 2005). We found that the relative NER protein levels in the 63 controls were highly correlated with each other ($P < 0.001$ for all). Compared to the controls, the cases had lower expression levels for all NER proteins, most notably XPC and XPF, which were reduced by approximately 25% ($P < 0.01$). When we used the median expression levels of the NER proteins in the controls as the cutoff value, we found a significantly increased risk of SCCHN associated with low expression of XPA (OR = 2.99, 95% CI = 1.22–7.47), XPC (OR = 2.46, 95% CI = 1.04–5.87), XPD (OR = 3.02, 95% CI = 1.18–7.76), and XPF (OR = 5.29, 95% CI = 2.01–13.9), but not ERCC1 or XPG, after adjustment for age, sex, ethnicity, smoking, alcohol use, and sample storage time. In a multivariate logistic regression model that included all covariates and NER proteins, however, only low expression of XPF remained a significant risk factor for SCCHN (OR = 11.5, 95% CI = 2.32–56.6). These results suggest that XPF may be a crucial rate-limiting factor in DNA repair and that the RPPA may be a useful tool for evaluating protein markers of DRC and susceptibility to cancer (Wei et al. 2005).

It should be noted that the summary above may reflect some bias, since some of the reports mentioned in this Chapter were pilot studies with positive findings as a part of the development of new phenotypic assays, for which we have not continued performing more assays without additional funding. For some molecular epidemiological studies that were assay-driven, any negative findings during such pilot studies were considered a failure in the assay itself, both mechanistically and methodologically, and thus were never published, or were not funded for continuation. Of course, it would be equally biased to present these negative findings, which did

not have statistical power or the chance to be peer-reviewed or validated by other investigators.

PERSPECTIVES

There are many issues in molecular epidemiological studies that use DNA repair phenotypic assays that remain unresolved. First of all, the reliability and reproducibility of any assay, either with cell lines or human samples, need to be established across multiple laboratories (Chang et al. 2006). Second, the tissue specificity needs to be known before identifying a surrogate tissue or cell line (Wiencke et al. 1995; Lee et al. 2011), although the general availability of cells is an important consideration for the feasibility of population studies, particularly longitudinal projects (Kennedy et al. 2005). Third, the optimal use of fresh or frozen tissues needs to be determined, largely due to the practical issue of assaying samples in sufficient number (Cheng et al. 2001; Hayes and Martin 2007). Fourth, the sensitivity and specificity of the assay(s) need to be established in large studies using previously identified standards (Kassie et al. 2000). Fifth, some statistical considerations, such as minimal sample size for a pilot study and the number of assays per sample, need to be resolved before embarking on a large study (Lai et al. 2003). Finally, and perhaps most importantly, the genetic basis of the DNA repair phenotypic assay ultimately needs to be elucidated.

Further development of high-throughput DRC phenotype assays, such as a microarray-based system, is necessary. As shown in Fig. 1, we are in the process of developing a risk prediction model for SCCHN, in which the relationship between genotype/phenotype of DNA repair and the outcome of SCCHN will be evaluated at 5 levels: 1. genotypes for selected single nucleotide polymorphisms (SNPs) in each of the 8 core NER genes, 2. mRNA expression levels of each gene, 3. expression levels of each NER protein, 4. DRC phenotype, and 5. cancer risk, taking into account known risk factors for SCCHN. However, this model will/may not include uncollected information on unknown exposures, SNPs of other genes, or functions of other proteins in other pathways, such as those involved in cell cycle control and apoptosis.

It is imperative to investigate whether genetic variation, in terms of SNPs, is correlated with the variability in DRC and cancer risk in the general population. Studies on genotypes and phenotypes of DNA repair as a susceptibility factor for cancer are increasing exponentially. Once these correlations are more clearly established, large-scale molecular epidemiological studies using a high-throughput genotyping platform will become feasible and efficient. As we reported, some SNPs in the NER

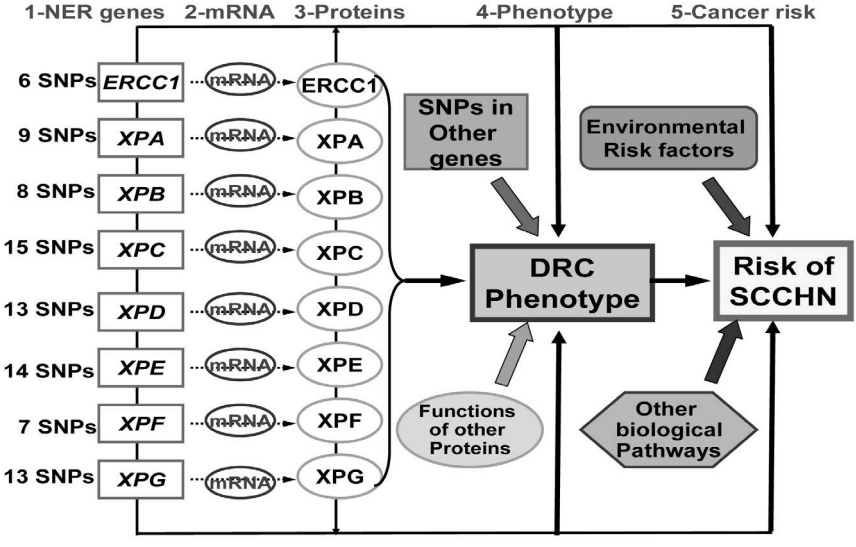

Figure 1. The relationship between genotypes and phenotypes of the nucleotide excision repair pathway and risk of SCCHN. The numbers on the left are for the common SNPs of each of the 8 core NER genes. NER—nucleotide excision repair; SNP—single nucleotide polymorphism; DRC—DNA repair capacity; SCCHN—squamous cell carcinoma of head and neck (Li et al. 2009).

genes may predict the levels of DRC (Qiao et al. 2002; Wang et al. 2007b) or the levels of *in vitro* induced DNA adducts (Zhao et al. 2008). Recent genome-wide association studies of lung cancer (Amos et al. 2008) have provided the scientific community with additional ways to evaluate cancer risk associated with genetic variation in DNA repair genes (Yu et al. 2011) and to ultimately identify the genetic determinant(s) for altered DRC. For example, we have identified that the non-synonymous SNP rs13181 in the *XPD/ERCC2* gene has the most significant association with DRC at the genome-wide level (unpublished data).

We expect that in the near future, the combination of the new high-throughput techniques such as genome-wide scans (Oefner 2002; Maresso and Broeckel 2008; Speicher et al. 2010), epigenetic profiling (Gal-Yam et al. 2008), transcriptional profiling, proteomic studies (Simpson et al. 2008) and direct sequencing will provide powerful approaches for molecular epidemiological association studies on DRC variability in predicting cancer susceptibility. While the Chapter here has focused almost exclusively on strategies to assess NER capacity and its association with cancer risk, as overviewed in Table 1, similar approaches and experimental goals are being applied to the other repair systems. We look forward to more rigorous and promising studies on the association of DRC and disease susceptibility in the near future.

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CHAPTER 10

Fanconi Anemia, Interstrand Cross-Link Repair, and Cancer

Taraswi Banerjee, Sanjay Kumar Bharti and *Robert M. Brosh, Jr.*[a,*]

INTRODUCTION

Interstrand cross-links (ICLs) that covalently bond two complementary strands of the Watson-Crick DNA double helix represent a dangerous cytotoxic form of DNA damage that effectively blocks the vital cellular processes of DNA replication and transcription (Deans and West 2011). Although DNA ICLs are well known to be induced by certain chemical agents, such as cisplatin and mitomycin C (MMC) that are conventionally used as chemotherapy drugs, it is more controversial as to the extent of naturally occurring ICLs and if these endogenous forms cause cancer or other diseases. The most prominent natural source of ICLs arise from lipid peroxidation products, such as malondialdehyde (Kozekov et al. 2003; Stone et al. 2008); however, similar to the situation for ICL-inducing agents, it is yet unclear if other forms of DNA damage, such as monoadducts, contribute to the cytotoxicity and phenotypes, such as cancer or age-related symptoms, that are associated with a cross-link repair deficiency (Deans and West 2011).

Cellular and biochemical studies provide strong support for a specialized pathway of DNA repair to remove ICLs and replace the excised sequence with the correct one (Deans and West 2011). Indeed, cellular

Laboratory of Molecular Gerontology, National Institute on Aging, NIH, NIH Biomedical Research Center, 251 Bayview Blvd, Baltimore, MD 21224, USA.
[a]Email: broshr@mail.nih.gov
*Corresponding author

deficiency of the structure-specific endonuclease ERCC1/XPF mutated in a segmental progeroid syndrome causes hypersensitivity to ICL-inducing agents (Grillari et al. 2007). Moreover, cells from patients with the hereditary disease Fanconi Anemia (FA), characterized by an elevated cancer incidence, display hypersensitivity to ICL-inducing agents as well (Kee and D'Andrea, 2010). In this Chapter, we will discuss the connections between FA, ICL repair, and cancer with a special emphasis placed on targeting the ICL response in cancer therapy by inhibition of the FA pathway.

CLINICAL SYMPTOMS AND CANCER PREDISPOSITION OF FA PATIENTS

The autosomal recessive disorder FA is characterized by multiple congenital anomalies, growth retardation, reduced fertility, progressive bone marrow failure, aplastic anemia, and high risk for the development of malignant diseases, especially acute myeloid leukemia, head and neck cancers, and epithelial tumors (Kee and D'Andrea 2010). The progressive bone marrow failure and late-developing myeloid malignant diseases are largely responsible for mortality in FA patients. The characteristic bone marrow failure that persists in children with FA is believed to be due to elevated apoptosis and subsequent failure of the hematopoietic stem cell compartment.

Fifteen genes/candidate genes have currently been identified that are implicated in the genetic manifestation of FA (Cybulski and Howlett 2011). Cells from FA patients exhibit spontaneous chromosomal instability and hypersensitivity to DNA ICL agents such as cisplatin or MMC (Kitao and Takata 2011). A chromosome breakage test using FA patient cells exposed to an ICL-inducing agent is used as a primary diagnostic tool for FA (Auerbach 2009). A number of FA mutant cell lines also display elevated spontaneous or cross-linker induced sister chromatid exchange (SCE), as well as triradial and quadriradial structures (Billardon and Moustacchi 1986; Bridge et al. 2005; Deans and West 2009; Litman et al. 2005; Suhasini et al. 2011). The pronounced chromosomal instability associated with FA and the early onset of cancers is likely to be a consequence of defective DNA repair. There has been some progress in understanding the mechanics of ICL repair, and both biochemical and cellular studies suggest that the FA pathway is highly integrated with other DNA repair factors and processing enzymes to accurately remove DNA ICLs, which are lethal due to their ability to directly block cellular DNA replication and transcription (Table 1).

Table 1. DNA Repair Proteins that Intersect with the FA Pathway and Interstrand Cross-Link Repair.

Protein	Biochemical Function	Hereditary Disease	Cancer Predisposition
BRCA1	Ubiquitin ligase; binds DNA structures	ND*	breast/ovarian cancer
HELQ	DNA helicase	ND	ND
FAN1	Binds mono-ubiquitinated FANCD2; structure-specific nuclease	ND	ND
BLM	DNA helicase; Double HJ dissolution with Top3α/RMI1/2; Strand resection to initiate HR	Bloom's syndrome	solid tumor and blood cancers
Top3α	Topoisomerase; Double HJ dissolution with BLM/RMI1/2	ND	ND
XPF/ERCC1	5' flap endonuclease	Xeroderma pigmentosum; XFE progeroid syndrome	skin cancer
MUS81/EME1	3' flap structure-specific nuclease	ND	ND
REV1	Translesion DNA polymerase	ND	ND
RAD51	DNA recombinase	ND	ND

*ND, not determined

FA PATHWAY

The FA pathway is involved in the initial recognition and unhooking of an ICL, as well as subsequent repair of the ICL-induced DNA double strand break (DSB) in conjunction with homologous recombination (HR) or translesion synthesis pathways, although the detailed molecular mechanisms and the expanded role of the FA pathway in genomic stability remain an active area of investigation (Crossan and Patel 2012; Deans and West 2011; Kee and D'Andrea 2010). The currently identified fifteen proteins of the FA pathway representing the genetic complementation groups can be classified into three major categories: core complex, ID complex, and downstream FA proteins (Fig. 1). Eight of the FA proteins (FANC A, B C, E, F, G, L, and M) constitute the core complex required for its stability and a key activation step of the FA pathway, namely the mono-ubiquitination of the FA proteins FANCD2 and FANCI; these latter two proteins constitute the so-called ID complex. Of the core complex members, FANCM hydrolyzes ATP, fueling the Holliday Junction (HJ) branch migration and fork regression that occurs when the advancing replication fork encounters an ICL lesion (Table 2). For the ID complex, FANCD2 binds DNA structures and associates with chromatin, whereas FANCI insures that FANCD2 is properly mono-

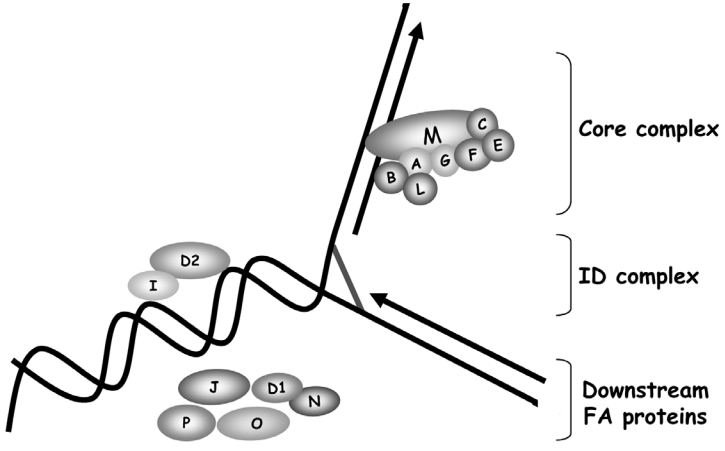

Figure 1. Proteins from the currently identified fifteen genetic complementation groups of the FA pathway. These proteins can be broadly classified into three major categories: core complex, ID complex, and downstream FA proteins. See text for details.

Table 2. Molecular Motor ATPases of the Fanconi Anemia and Bloom's Syndrome Pathways.

FA Pathway	**Protein Catalytic Functions**	**Proposed Cellular Role**
FANCM	ATP hydrolysis HJ branch migration Fork regression	Remodel stalled replication fork structures
FANCJ	ATP hydrolysis DNA helicase Strip proteins bound to DNA	Homologous recombinational repair G-quadruplex resolution
FANCO (RAD51C)	ATP hydrolysis	Homologous recombinational repair
BS Pathway	**Protein Catalytic Functions**	**Proposed Cellular Role**
BLM	ATP hydrolysis DNA helicase HJ branch migration Fork regression	Homologous recombinational repair Replication fork restart G-quadruplex resolution

ubiquitinated. Although the mechanistic link between FANCD2/I mono-ubiquitination and downstream events responsible for translesion synthesis or HR repair of the processed cross-linked DNA is still not clear, a number of downstream FA proteins have been discovered and characterized. These include: 1) FANCJ (BACH1), which unwinds DNA with a 5′ to 3′ polarity and interacts with the tumor suppressor BRCA1; 2) FANCD1 (BRCA2), which binds ssDNA and dsDNA, and stimulates RAD51 strand exchange; 3) FANCN (PALB2), which is required for stability of FANCD1; 4) FANCO (RAD51C), which binds ssDNA and hydrolyzes ATP; and 5)

FANCP (SLX4), which serves as a scaffold for structure-specific nucleases. Presumably, the recombinational repair proteins and structure-specific nucleases collaborate in replicating cells to restart a stalled replication fork or resolve recombinogenic DNA structures.

It should be emphasized that the FA proteins interact with a number of additional DNA repair factors (some of which are implicated in cancer) that are important for various aspects of DNA processing and resistance to DNA cross-linking agents (Table 1). An excellent example of crosstalk between FA and other proteins/pathways is the interaction of FA proteins with the Bloom's syndrome (BS) complex, which itself is instrumental in the suppression of cancer and chromosomal instability (discussed below) (Suhasini and Brosh, Jr. 2012). This would include the interaction of molecular motor ATPases of the FA and BS pathways (Table 2). However, it is likely that our understanding of the cast of players interacting with the FA pathway is analogous to "the tip of the iceberg" with many discoveries still to come.

DELICATE BALANCE OF DNA REPAIR CHOICE ORCHESTRATED BY THE FA PATHWAY

One of the functions of the FA pathway is to channel DSBs through the HR pathway (see Chapter 14), thereby preventing inappropriate engagement of the breaks by the error-prone nonhomologous end-joining (NHEJ) pathway (Adamo et al. 2010; Pace et al. 2010). However, the relationships between DNA repair pathways are complex, as evidenced by a recent mouse study in which it was determined that deletion of *53BP1* or *Ku* exacerbates genomic instability in FANCD2-deficient cells (Bunting et al. 2012). Nonetheless, it is generally believed that the FA pathway operates in replicating cells to facilitate a specialized pathway of HR-mediated repair in order to suppress other less faithful mechanisms such as NHEJ that might be enacted upon DSBs that arise when a replication fork encounters the DNA ICL.

FA GENES AND CANCER SUSCEPTIBILITY

Aside from their genetic linkage to FA, several of the FA genes that are classified as downstream members of the FA pathway (Fig. 1) have been identified as breast and/or ovarian cancer susceptibility genes. These would include FANCD1 (BRCA2), FANCJ (BACH1), FANCN (PALB2), and FANCO (RAD51C) (Deans and West 2011; Kee and D'Andrea, 2010). In addition, several genes that intersect with the FA pathway are also implicated in cancer susceptibility including BRCA1, XPF/ERCC1, and

BLM mutated in BS (Table 1). We will discuss next the connection between FA and BS as it provides an excellent example of the functional overlap and communication between DNA damage response factors/pathways required for the maintenance of chromosomal stability.

CONNECTIONS BETWEEN TWO HEREDITARY CANCER DISEASES, FA AND BLOOM'S SYNDROME

In addition to short stature, reduced fertility, hypersensitivity to sunlight, and immunodeficiency, BS patients are highly prone to a broad spectrum of cancers early in life (Hanada and Hickson 2007). Mutation of the *BLM* gene encoding a RecQ DNA helicase is responsible for the autosomal recessive disorder (Ellis et al. 1995). Cells from BS patients display elevated SCE (Chaganti et al. 1974) and are sensitive to ICL-inducing agents (Pichierri et al. 2004). There is strong evidence that FA proteins and BLM directly interact to preserve genomic stability. Interestingly, molecular motor ATPases of the FA pathway (FANCJ helicase, FANCM translocase, FANCO (RAD51C) ATPase) and BS pathway (BLM helicase) are intimately involved in the cross-talk between these two chromosomal instability disorders, emphasizing the direct connection of the hereditary diseases to DNA repair (Suhasini and Brosh, Jr. 2012;Vinciguerra and D'Andrea 2009) (Table 2).

The first report showed that the FA core complex and a BLM protein complex interact to preserve genomic stability (Meetei et al. 2003). FANCM was subsequently shown to serve as a bridge connecting the FA core and the BLM-TopoIIIα RMI1/2 complex (Deans and West 2009). A RMI/FANCM molecular interface was recently described that connects FANCM to the BS dissolvasome, a protein complex that is responsible for separating double HJ structures that arise during HR repair of frank DSBs or those imposed upon collision of the replication fork machinery with DNA damage such as an ICL (Hoadley et al. 2012). Additional studies have demonstrated that the FA pathway and BLM collaborate during mitosis to prevent micronucleation and chromosome abnormalities, such as those at fragile sites where sister chromatid bridging can occur (Chan et al. 2009; Naim and Rosselli 2009).

Recently, another level of cross-talk between the FA and BS pathways was discovered by our lab. We showed a physical and functional interaction between the BLM and FANCJ DNA helicases (Suhasini et al. 2011). The FANCJ-BLM interaction is important for BLM stability via a proteasome-mediated pathway and a normal response to the replication inhibitor hydroxyurea (HU). A deficiency in BLM protein levels or function in certain FA patients (such as those representing the FA-J complementation group) may contribute to chromosomal instability and cancer. On the cellular level, the coordinate action of FANCJ and BLM helicases may be important for

helping cells deal with stalled replication forks, resolving G-quadruplex DNA structures, or DSB repair (Suhasini and Brosh, Jr. 2012).

TARGETING THE ICL RESPONSE IN CANCER THERAPY BY INHIBITION OF THE BRCA-FA PATHWAY

DNA ICL agents (e.g., cisplatin, carboplatin, melphalan, MMC, chloroethylnitrosourea, nitrogen mustards, cyclophosphamides) are used for the treatment of a variety of cancers, including myeloma, ovarian, testicular, breast, bladder, and head and neck cancers (Deans and West 2011). The premise behind the treatment of tumors with DNA cross-linking agents is that the rapidly dividing and actively proliferating cancer cells are highly sensitive to ICLs, which deter replication and transcription. However, several caveats of conventional anti-cancer therapies that rely on radiotherapy or chemotherapy exist. First, certain types of cancerous tumors become resistant to the therapy by up-regulating DNA damage response pathways. For example, resistance against platinum compounds that are used for a variety of adult as well as pediatric tumors develops in cancer patients after a very positive initial response (Rabik and Dolan 2007). Second, the cytotoxicity imposed by a given drug concentration or radiation dose can be toxic to normal noncancerous cells, leading to tissue and organ decline.

To improve the outlook, cancer biologists are developing novel approaches to circumvent tumor resistance and the toxicity of anti-cancer therapies. Small molecule approaches are currently being developed to target DNA repair systems in an effort to enhance chemotherapy or radiation treatments (Helleday et al. 2008) (Fig. 2). With our improved understanding of the mechanistic basis for tumor resistance, strategies involving the use of small molecule compounds that potently and uniquely inhibit the function of a DNA repair protein are either being employed in clinical trials or optimized in model systems. The combinatorial use of small molecule inhibitors of protein function and RNA interference of key proteins in the DNA damage response represent viable approaches.

A promising area of cancer therapeutics is centered around the BRCA-FA pathway as a desirable target for anti-cancer drugs (Litman et al. 2008). The BRCA-FA DNA damage response pathway is mediated by the proteins encoded by the FA genes (Fig. 1) and the tumor suppressor genes *BRCA1* and *BRCA2* that when mutated can predispose women to breast and ovarian cancer (see Chapter 14). Like FA mutant cells, BRCA1/2 mutant cells are defective in the ICL response. Proof-of-principal that BRCA tumors defective in the DNA damage response may be targeted by small molecule DNA repair inhibitors for synthetic lethality was provided by Helleday,

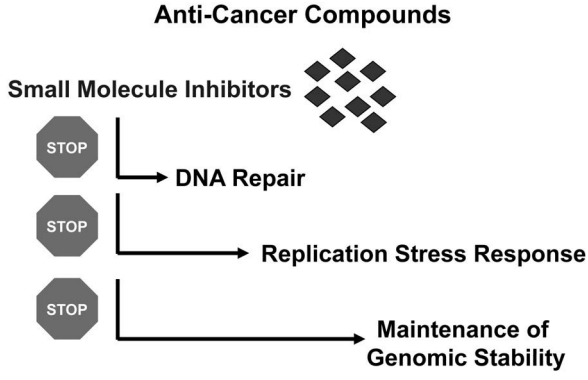

Figure 2. Small molecule screening for anti-cancer compounds. Small molecules are under investigation as an approach to target DNA repair systems in an effort to enhance chemotherapy or radiation treatments. In certain cases, tumor-specific replicative lesions may be amplified by inhibition of DNA repair pathways, leading to genomic instability and enhanced killing of cancerous cells. See text for a discussion of screens to identify inhibitors of the FA pathway that may be useful for chemotherapeutic approaches.

Ashworth, and colleagues in 2005 (Bryant et al. 2005; Farmer et al. 2005). Collectively, they showed that inhibitors of the single-stranded break (SSB) repair protein poly(ADP)-ribose polymerase (PARP)1 are synthetic lethal in BRCA1- or BRCA2- deficient cancer cells. It was proposed that the inhibition of PARP1 leads to the accumulation of SSBs, which are converted to DSBs upon encounter by replication forks in actively dividing cancer cells. The BRCA deficiency inactivates HR, which is normally elicited to help cells deal with DNA damage in an error-free manner. While the underlying mechanism for the selective killing of BRCA tumors by PARP inhibitors may be controversial (Helleday 2011), the discovery of their effectiveness has ushered in a new field of cancer therapy research (see Chapter 15).

TARGETING FA-DEFICIENT TUMORS WITH CHEMOTHERAPY DRUGS

Certain sporadic head and neck, lung, ovarian, cervical, and hematological cancers are characterized by epigenetic silencing of wild-type FA gene expression (Kennedy et al. 2007). In addition to epigenetic silencing, loss of heterozygosity from an additional mutation in an FA gene that results in loss of function of the FA pathway in heterozygous carriers may lead to increased cancer risk later in life (Hucl and Gallmeier 2011; Kennedy et al. 2007). It is estimated that 15% of all cancers harbor defects in the FA pathway (Taniguchi and D'Andrea 2006). It seems likely that a significant number of these tumors would become reliant on certain DNA repair factors to deal

with accumulation of DNA damage such as strand breaks. Therefore, an active area of study is to target DNA repair proteins for pharmacological inhibition to sensitize FA-deficient tumors to chemotherapy drugs such as DNA cross-linkers. For a nice overview of the FA pathway and cancer therapy with a particular emphasis on cell-based models, see (Andreassen and Ren 2009). We will focus our discussion on a few illustrative examples as well as some recent advances.

Pioneers of the approach to chemosensitize cancer cells to the cytotoxic effects of DNA cross-linking agents by inhibition of the FA pathway are the D'Andrea and Kern labs. Research from the D'Andrea lab showed that FA pathway-deficient fibroblasts are highly sensitive to silencing of the ATM kinase (Kennedy et al. 2007). FANCG- and FANCC-deficient pancreatic tumor lines were sensitive to a pharmacological inhibitor of ATM, raising the possibility for a novel anti-cancer treatment strategy. More recently, FA-deficient cell lines were shown to be hypersensitive to inhibition of the CHK1 kinase, either by siRNA or a pharmacological inhibitor of CHK1 kinase activity (Chen et al. 2009). Inhibition of a specific DNA repair protein by a small molecule may provide an alternative strategy for targeting FA-deficient tumors that is unique from other approaches, such as targeting the CHK1 or ATM kinase response (see Chapter 16).

The Kern lab used a high throughout screening approach to examine the effect of compounds (~40,000) on the growth of isogenic pairs of human cancer cell lines that were either deficient or proficient in FANCC or FANCG (Gallmeier et al. 2007). One particularly potent compound (80136342) exerted an effect that was distinct from that of conventional DNA cross-linking agents, since it did not induce chromosomal aberrations or increase FANCD2 monoubiquitination, H2AX phosphorylation, p53 activation, or ICL induction. It was notable, however, that 80136342 interfered with cell cycle progression, causing a G2 arrest, and exerted a greater effect on FANCC-deficient cells compared to FANCG-deficient cells. Several candidates were identified that may serve as lead compounds for therapeutic development of agents that target FA-deficient tumors. The observations that 80136342 did not cause cellular phenotypes typical of those exerted by DNA cross-linking agents or irradiation (since γ–H2AX foci were not induced) suggest that the compound may induce another form of cytotoxic stress that engages the FA pathway. It will be of interest to ascertain if one class of lesions induced by 80136342 or related compounds result in a form of replication stress, since the linkage of the FA pathway to a robust replication stress response is becoming firmly established. Moreover, it has been proposed that tumor-specific replicative lesions may be amplified by inhibition of DNA repair pathways, suggesting a promising avenue for anti-cancer therapy (Helleday 2008) (Fig. 2).

CELL-BASED SCREENING SUGGESTS EFFICACY OF NON-SPECIFIC CHEMICAL INHIBITION OF THE FA PATHWAY

Some of the latest findings in ionizing radiation (IR)- or cisplatin-induced FANCD2 foci formation screening assays suggest a diverse spectrum of pathways to sensitize cancer cells to cisplatin (Jacquemont et al. 2012). Human cells transfected with Green Fluorescent Protein (EGFP)-FANCD2 were treated with compounds from chemical libraries and exposed to IR to induce FANCD2 foci formation. Positive hits showing a significant decrease in EGFP-FANCD2 foci formation were confirmed in multiple human cell lines. The majority of the small molecules identified from the screen also showed decreased IR-induced RAD51 foci and lower homologous recombinational repair, indicating an effect that was not specific to FANCD2. The compounds identified showed pharmacological inhibition of the proteasome, cathepsin B, lysosome, CHK1, HSP90, CDK, and PKC. Among those compounds identified, inhibitors of proteasome, cathepsin B, and HSP behaved in a synergistic manner with cisplatin that was dependent on an intact FA pathway. These findings suggest that actual inhibition of the FA pathway is required for small molecule sensitization to cisplatin. Moreover, it brings to light the opportunity for the development of compounds that would render cisplatin-resistant FA pathway-proficient tumors vulnerable to cisplatin and related cross-linking agents.

XENOPUS CELL-FREE SYSTEM TO SEARCH FOR THERAPEUTIC TARGETS IN THE FA PATHWAY

Since the FA pathway is complex with numerous proteins involved at different stages, the Hoatlin lab pursued a simplified approach to screen for chemical inhibitors of the FA pathway using *Xenopus* egg extracts (Landais et al. 2009b). Many of the genes implicated in FA are conserved between *Xenopus* and human, and *Xenopus* egg extracts fully support DNA replication and the DNA damage response, allowing robust activation of the FA pathway via FANCD2 monoubiquitination. The authors therefore used the DNA-stimulated *Xenopus* FANCD2 monoubiquitination as a readout for the screen and identified DDN (2,3-dichloro-5,8-dihydroxy-1,4-naphthoquinone) as a novel and potent FA pathway inhibitor molecule. DDN was found to inhibit FANCD2-I formation in the *Xenopus* extracts as well as in human cells without disrupting the upstream FA core complex. A synergistic effect of DDN and cisplatin was observed in FA-deficient cells, raising the possibility that DDN might serve as lead compound for chemical sensitization of FA-deficient tumors and conceivably other cancer types.

Building on a discovery by the D'Andrea lab that the naturally occurring compound curcumin inhibits FANCD2 monoubiquitination and sensitizes ovarian and breast tumor cell lines to cisplatin via apoptosis (Chirnomas et al. 2006), the Hoatlin lab applied the facile *Xenopus* system to screen for chemical derivatives of curcumin for better inhibitors of the FA pathway (Landais et al. 2009a). A monoketone analog of curcumin, designated EF24, was identified that represents a new class of FA pathway inhibitors that could be developed as an anti-cancer compound. It was reported that ATM-deficient cells showed a two-fold increased sensitivity to EF24, suggesting a synthetic lethal effect that may be exploited in future studies to develop a therapy that selectively targets ATM-deficient tumors. A model proposed by the authors suggests that EF2F and related compounds inhibit the FA pathway via the inhibition of the IkB kinase complex (IKK), which is implicated in the NF-kB pathway and also interacts with the FA core complex. Further studies are required to test this proposed mechanism of action. For a recent perspective of the FA pathway as a potential therapeutic target and a predictive biomarker of chemotherapeutic response, see (Hucl and Gallmeier 2011).

DEVELOPMENT OF OTHER MODEL SYSTEMS FOR PRECLINICAL PHARMACOLOGICAL SCREENING STUDIES OF POTENTIAL ANTI-CANCER AGENTS

The use of gene-silencing or chemical inhibitors to study the functions and mechanism of the FA pathway and interacting proteins, as well as to sensitize FA-deficient tumors, has sparked interest in the development of new model systems. The Grompe lab has developed FA murine models to study bone marrow failure and tumor onset. In one study, they determined that the nitrooxide antioxidant and superoxide dismutase mimetic tempol delayed the onset of epithelial tumors in Fancd2-/- knockout mice (Zhang et al. 2008). More recently, they discovered that Fancd2-/- mice have hematopoietic defects that can be partially corrected by resveratrol (Zhang et al. 2010). These studies suggest that efforts should be pursued to develop more potent compounds that might show improved hematopoietic correction. In the meantime, resveratrol may be a candidate for clinical trials with FA patients.

As mentioned earlier, studies using FA-deficient human cell lines suggest that CHK1 inhibition may serve as a strategy for targeting FA-deficient tumors (Chen et al. 2009); however, the development of *in vivo* models that go beyond cell-based systems will be informative. It was indeed reported that a zebrafish FANCD2 knockdown model was hypersensitive to a CHK1 inhibitor (Chen et al. 2009), substantiating the synthetic lethal

relationship between the FA pathway and CHK1 inactivation. Despite the complexity of the FA pathway, the FA network is conserved in many vertebrates, including zebrafish, which could be employed for the identification of novel therapeutic compounds (Titus et al. 2006). Other model genetic organisms with conserved genes in the FA and/or other DNA repair pathways, such as *C. elegans* (O'Neil and Rose 2006) or *D. discoideum* (Zhang et al. 2009), may prove to be valuable to screen for anti-cancer drugs, as might other cell-based systems besides human, such as the DT40 chicken B cell line (Takata et al. 2009).

PERSPECTIVE

In this Chapter , we have provided a perspective on the importance of DNA ICL repair for the maintenance of chromosomal stability and suppression of cancer. Clearly, the FA pathway, in collaboration with BLM and its associated proteins as well as other DNA damage response/repair factors, plays an integral role in helping cells cope with replicational stress imposed by ICLs and probably other forms of DNA lesions. Given the importance of the FA pathway in this capacity, efforts to develop anti-cancer strategies that target the FA pathway have attracted considerable interest in the field. Small molecule inhibitors of the FA and related pathways may provide the reagents for such an approach. Future studies should bring to light if these efforts will provide new and effective strategies to combat cancer.

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CHAPTER 11

Functions of Translesion DNA Polymerases: Implications for Cancer Risk and Opportunities as Therapeutic Targets

Kinrin Yamanaka[1,2] and *R. Stephen Lloyd*[1,3,a,*]

INTRODUCTION

Genomic DNA is continuously damaged by both endogenously produced species and exogenous agents. Examples of endogenous species include byproducts of lipid peroxidation such as acrolein, *trans*-4-hydroxy-2-nonenal (HNE), and 1,N^6-ethenodeoxyadenosine (εdA). Some of these species can induce secondary lesions such as DNA–protein, DNA–peptide, and DNA–DNA cross-links (ICLs) (Stone et al. 2008; Minko et al. 2009). Exogenous agents may include environmental agents such as UV irradiation, chemical carcinogens, and therapeutic agents such as mitomycin C, platinum drugs, temozolomide, fotemustine, nucleoside analogues, nitrogen mustards, equine estrogens, and psoralen.

[1]Center for Research on Occupational and Environmental Toxicology, Oregon Health & Science University, Portland, OR 97239, USA.
[2]Department of Physiology and Pharmacology, Oregon Health & Science University, Portland, OR 97239, USA.
[3]Department of Molecular and Medical Genetics, Oregon Health & Science University, Portland, OR 97239, USA.
[a]Email: lloydst@ohsu.edu
*Corresponding author

Multiple biochemical pathways exist to either remove or tolerate DNA lesions, including direct DNA damage reversal, nucleotide excision repair (NER), mismatch repair (MMR), base excision repair (BER), non-homologous end joining (NHEJ), replication fork reversal, and homologous recombination (HR) (see Chapter 1). Because of differential cellular responses relative to the location of the damage, the magnitude of the helical distortion, and the degree of chromatin compaction, some lesions can escape the repair machineries or the timing of the repair processes can be sufficiently delayed. As a consequence, blockage to replication fork progression can occur. This interference is due to the limited ability of replicative polymerases, pol α, pol δ, and pol ε in eukaryotes, to synthesize DNA past damaged sites. Translesion synthesis (TLS) is a DNA damage tolerance mechanism that helps cells to tolerate unrepaired DNA lesions and is carried out by specialized DNA polymerases. To date, at least 11 polymerases that may potentially be involved in TLS have been identified: pol ν and pol θ from the A family, pol ζ from B family, pol β, pol λ, pol μ, and terminal deoxynucleotidyl transferase from the X family, and pol η, pol κ, pol ι, and Rev1 from the Y family (Goodman 2002; Guo et al. 2009; Waters et al. 2009; Lange et al. 2011).

Currently two models of TLS have been proposed: the polymerase-switching mechanism and a gap-filling mechanism (Guo et al. 2009; Waters et al. 2009; Lange et al. 2011). In the polymerase-switching mode, TLS takes place during active DNA replication in which fork progression could be terminated when the movement of the replicative helicase is disrupted by a DNA lesion (Suhasini and Brosh 2010). Blockage of a replicative polymerase by a DNA lesion is hypothesized to trigger polymerase switching, potentially recruiting multiple TLS polymerases to the site of replication. Subsequently, a TLS polymerase incorporates a nucleotide opposite the lesion and it either dissociates or extends the primer from opposite the lesion. In the former case, the extension step can be performed by another TLS polymerase. The identity of the polymerases that catalyze this two-step reaction is likely influenced by the substrate structure and catalytic efficiency of the polymerase, its relative abundance in the cell, and its affinity for the primer/template. The number of nucleotides incorporated by a polymerase prior to its dissociation depends on the intrinsic processivity of the polymerase, the ability of polymerase to extend the primer opposite the lesion, and the interactions of the specific polymerase with other components of the replication machinery. The re-recruitment of a replicative polymerase to the primer terminus follows, thus resuming normal DNA replication (Fig. 1A) (Kannouche et al. 2001, 2004; Bergoglio et al. 2002; Kusumoto et al. 2004; Plosky and Woodgate 2004; Guo et al. 2009; Waters et al. 2009; Lange et al. 2011). In the gap-filling mode, following replication blockage, reinitiation of DNA synthesis occurs downstream of the DNA lesion, generating a single-

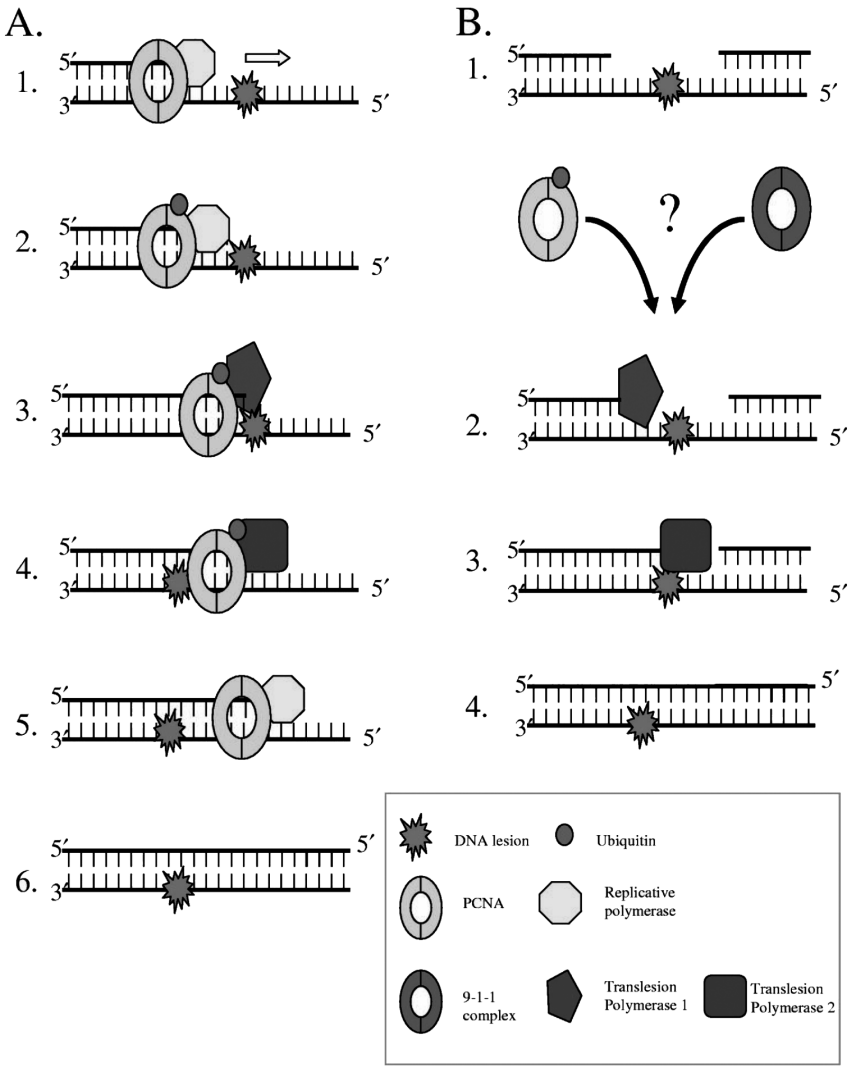

Figure 1. Two models of TLS. (A) In the polymerase-switching mode, TLS polymerases restart a blocked replication fork. (B) In the gap-filling mode, TLS polymerases fill the single-stranded gap opposite the DNA lesion. (Reprinted from (Waters et al. 2009) with permission from American Society for Microbiology).

stranded gap, and a TLS polymerase is recruited to fill-in the resulting gap (Fig. 1B). In this mode, TLS polymerases function outside the replication fork and this process may occur both during the later stages of S phase or following DNA replication in the G_1 or G_2/M phase of the cell cycle (Heller and Marians 2006; Lehmann and Fuchs 2006; Waters and Walker 2006; Guo

et al. 2009; Jansen et al. 2009; Waters et al. 2009; Lange et al. 2011). Both models are supported by strong evidence and are not necessarily exclusive. As an example, gap-filling mode is hypothesized to be utilized during the processing of ICLs (Waters et al. 2009) (see Chapter 10).

Several lines of evidence show that the recruitment of TLS polymerases to their site of action, either the site of stalled replication or a single-stranded DNA gap, is governed by multiple protein-protein interactions. Current models propose that PCNA monoubiquitination by Rad6/Rad18 ubiquitin conjugase/ligase complex signals TLS polymerases to enter their site of action (Fig. 1A, steps 2–4). Once TLS is completed, PCNA deubiquitination may occur and normal replication by replicative polymerases resumes (Fig. 1A, steps 5 and 6). In addition to PCNA, 9-1-1 complex, an alternative processivity clamp, may also be important for recruiting TLS polymerases (Fig. 1B, steps 1–3). Additionally, certain TLS polymerases, for example Rev1, can also function as scaffolds for TLS machinery and recruit other TLS polymerases to the site of the DNA lesion (Huang et al. 2006; Guo et al. 2009; Waters et al. 2009). The efficiency and accuracy of lesion bypass is both polymerase and lesion specific. Each TLS polymerase is hypothesized to be particularly proficient in the bypass of specific DNA lesions (cognate lesions), and the outcome of TLS past these lesions is generally non-mutagenic. In this case, a TLS polymerase can serve as a tumor suppressor. However, the fidelity of DNA synthesis is often compromised when TLS polymerases act on non-cognate lesions. Furthermore, these enzymes generally exhibit low-fidelity when copying non-damaged DNA, in contrast to replicative polymerases that faithfully duplicate chromosomal DNA (Arana and Kunkel 2010). Multiple factors can account for the low fidelity replication of TLS polymerases, including the lack of 3′-5′ proofreading exonuclease activity, a spacious active site, and the limited number of contacts made with the template base and incoming nucleotide (Khare and Eckert 2002; Waters et al. 2009; Arana and Kunkel 2010). Thus, TLS polymerases have dual-functions, serving as tumor-suppressors during the bypass of their cognate lesions, while promoting mutagenesis during the bypass of non-cognate lesions and the replication of non-damaged DNA. As a consequence, the dysregulation of TLS polymerases can result in carcinogenesis.

In this Chapter, the functions of each TLS polymerase and its roles in tumorigenesis and chemotherapy resistance will be discussed. The importance of the development of inhibitors targeting TLS polymerases for new combination cancer therapy and existing TLS polymerase inhibitors will be discussed at the end of the Chapter. Although polymerases belonging to X family also have known TLS activities, their primary physiological functions appear to be non-TLS activity such as in BER (see Chapter 8) and NHEJ (see Chapter 1). Therefore, our discussion focuses on polymerases

belonging to A, B, and Y family. For comprehensive analyses of inhibitor studies of viral replicative polymerases, readers are referred to the following recent review (Berdis 2008).

OVERVIEW OF THE TLS POLYMERASES

Y FAMILY POLYMERASES

DNA Polymerase Eta

General and Biochemical Properties

Pol η is a 78 kDa protein whose gene is located on chromosome 6p21.1 (Glick et al. 2001; Sweasy et al. 2006). Pol η lacks 3′-5′ proofreading exonuclease activity (Matsuda et al. 2000) and has a low processivity, generally elongating 1–8 nucleotides (Masutani et al. 2000; McCulloch et al. 2004). Pol η is a low fidelity polymerase, with the frequency of nucleotide misincorporation opposite non-damaged nucleotides being ~10^{-2} to 10^{-3} (Johnson et al. 2000c). Additionally, studies using gapped M13mp2 plasmids demonstrate that during synthesis of the target *lacZα* gene, the frequency of *lacZ* mutants generated by pol η was 34 percent (Matsuda et al. 2000) versus ≤ 1 percent measured for the high fidelity exonuclease-proficient replicative polymerases (Shcherbakova et al. 2003; Fortune et al. 2005). Additionally, pol η primarily introduces base substitutions (Zhang et al. 2000c).

In vitro, human pol η functions to bypass a variety of DNA lesions. It is particularly well-known for its ability to catalyze efficient and accurate TLS past UV-induced T-T cyclobutane pyrimidine dimers (CPDs) (Masutani et al. 1999; Zhang et al. 2000a). It has also been shown that dG-dG intrastrand cross-links induced by cisplatin or oxaliplatin are efficiently bypassed by pol η; it preferentially incorporates the correct dC opposite both the 3′ and 5′ adducted dG, but other nucleotides were also incorporated (Vaisman et al. 2000).

Additionally, pol η can bypass acrolein-derived γ-hydroxy-1,N^2-propano-2′-deoxyguanosine (γ-HOPdG) adducts and its ring-opened form; the bypass of the former lesion is error-prone with high misincorporation of dA and dG, while the bypass of the latter lesion is accurate (Minko et al. 2003). Another lesion that pol η can bypass is 7,8-dihydro-8-oxoguanine (8-oxo-dG), a common oxidative base lesion. Although this bypass was mostly accurate, pol η also incorporated incorrect nucleotides (Haracska et al. 2000b; Zhang et al. 2000a; Maga et al. 2007; McCulloch et al. 2009). Thymine glycols, another common oxidative base lesion, O^6-methyl-dG (m6G), a lesion induced by alkylating chemotherapeutic agents (see

Chapter 5), and *N*-(deoxyguanosin-8-yl)-2-acetylaminofluorene (AAF)-dG adducts can all be bypassed by pol η (Haracska et al. 2000a; Masutani et al. 2000; Kusumoto et al. 2002; Johnson et al. 2003; Seki et al. 2004). During the bypass of thymine glycols, not only correct dA, but also other nucleotides were incorporated opposite the lesion. However, the efficient extension past the lesion occurred when the correct dA was inserted opposite the lesion. Pol η inserted dC or dT opposite m6G, and dC was preferentially incorporated opposite AAF-dG. εdA, a lesion that can be produced by exposure to vinyl chloride (Nair et al. 1999), is another lesion that pol η can bypass in an error-prone manner. In addition, pol η can replicate past an abasic site (AP site). dA was preferentially incorporated opposite either εdA or an AP site (Zhang et al. 2000a; Zhou et al. 2010). Interestingly the bypass of an AP site resulted in -1 deletion when the template base 5′ to the lesion was a dT (Zhang et al. 2000a). With regard to εdA, another group, using steady-state kinetic analyses, has shown that pol η preferentially incorporated a correct dT opposite the lesion and typically extended from the correct dT primer terminus. In contrast, when analyses of TLS products included frameshift mutations, it was found that although pol η preferentially incorporated dT, incorporation of other nucleotides and a one-base deletion were also observed. Collectively, these results show that pol η catalyzes both error-free and error-prone TLS past εdA (Levine et al. 2001; Zhou et al. 2010). Finally, pol η catalyzes very inefficient and error-prone bypass of benzo[*a*]pyrene-induced (+)- and (–)-*trans*-BPDE-N^2-dG, mostly incorporating dA opposite the lesions. However, it was capable to fully bypass (+)-*trans*-BPDE-N^6-dA adducts with preferential incorporation of correct dT (Zhang et al. 2000a, 2002a; Rechkoblit et al. 2002).

Studies have shown that women treated with hormone replacement therapy consisting of the equine estrogens (equilin and equilenin) exhibit increased risk of developing cancers, including breast and endometrial cancers (Colditz et al. 1995; Grady et al. 1995). One of the major metabolites associated with such treatment is 4-hydroxyequilenin (4-OHEN), which can react with nucleobases to form lesions such as 4-OHEN-dA (Bolton et al. 1998; Shen et al. 1998; Zhang et al. 2001a; Terashima et al. 2002; Yasui et al. 2003). A study has shown that pol η can bypass 4-OHEN-dA. Although it frequently incorporated the correct dT opposite the lesion, dA was also incorporated (Yasui et al. 2006). Thus, pol η could potentially induce A to T transversions. These data correlate well with a previous study showing that A to T transversions are common at AT pairs of the *supF* reporter gene following replication of a DNA vector exposed to 4-hydroxyequilin (4-OHEQ), another metabolite that induces 4-OHEN-dA adducts (Yasui et al. 2003). Thus, pol η may be responsible for inducing these types of mutation in the cells during the bypass of the 4-OHEN-dA adduct. Pol η can also catalyze TLS past nucleoside analogues commonly used in

chemotherapy, such as cytarabine and gemcitabine (Chen et al. 2006). Despite its proficiency in the bypass of T-T CPDs, pol η is blocked by UV-induced (6-4) photoproducts (Zhang et al. 2000a) and cannot bypass a psoralen-induced ICL that models the intermediate structure during ICL repair (Zietlow et al. 2009). In addition, Pol η is inhibited by HNE-induced N^2-dG adducts (Wolfle et al. 2006), and catalyzes limited TLS past an 1,N^2-propanodeoxyguanosine (PdG) adduct when excess enzyme was used in the reactions (Minko et al. 2003).

In vivo Functions and Implications in Tumorigenesis/ Chemotherapy Resistance

The role of pol η in the processing of naturally occurring non-B-DNA structures to maintain genomic stability has been studied. When pol η was depleted from HeLa cells and the cells were transfected with plasmids containing G-rich sequences from the human c-MYC promoter region, there was an increase in the induction of DNA double-strand breaks (DSBs). Additionally, it was demonstrated that pol η may be involved in the processing of G-quadruplex DNA structures, since U2OS cells depleted of pol η showed decreased survival in response to telomestatin, a G-quadruplex ligand that stabilizes G-quadruplex structures (Betous et al. 2009).

The importance of pol η in catalyzing replication bypass of UV-induced lesions to protect cells from UV-induced carcinogenesis is evident from an autosomal recessive disease xeroderma pigmentosum variant (XP-V). Individuals with XP-V have normal function in NER (see Chapter 1), but lack high fidelity bypass of T-T CPD lesions due to defects in pol η. Thus, these individuals are at high risk of developing sunlight-induced skin cancers (Cleaver 1972; Lehmann et al. 1975; Masutani et al. 1999; Tamura et al. 2010). Multiple mutations exist in XP-V, and many of these mutations lead to truncated pol η proteins (Johnson et al. 1999a; Masutani et al. 1999; Broughton et al. 2002).

A number of studies highlight the essential role of pol η in the replication bypass of T-T CPDs. XP-V cells are sensitive to the cytotoxic and mutagenic effect of UV irradiation (Bassett et al. 2004; Chen et al. 2006). UV irradiation causes a prolonged delay in S phase, and pol η functions to prevent this event. In the presence of caffeine, an inhibitor of the cell cycle checkpoint protein ATM, the sensitivity of XP-V cells to UV irradiation was enhanced. When UV-irradiated SV40-based shuttle vector pR2 was replicated in these mutant cells, notable increases in mutant frequencies were observed, and mutations occurred at both CG and TA base pairs. When UV-damaged plasmids were replicated in UV-irradiated cells, XP-V cells exhibited high mutation rates, and large increases in transversions were observed

(Stary et al. 2003). Several studies have examined the TLS activity of pol η using a gapped plasmid-based assay. In this assay, a mixture of gapped plasmids carrying unadducted or a site-specific lesion were transfected into mammalian cells, and following gap-filling DNA synthesis, plasmids were extracted and used to transform *E. coli*. These transformants were grown on plates containing kanamycin or chloramphenicol to select for progenies of damaged or unadducted plasmids, respectively. The efficiency of TLS was determined from the ratio of kanamycin resistant to chloramphenicol resistant transformants. In order to exclude plasmids that had been repaired by non-TLS events, such as those involving DSB formation as an intermediate, the precise efficiency of TLS was determined to be the extent of plasmid repair multiplied by the fraction of TLS events out of all plasmid repair events. The accuracy of TLS was measured by assessing the mutations in the vector DNAs isolated from the kanamycin resistant transformants. Using this assay, pol η has been shown to carry out efficient and accurate TLS past a T-T CPD (Hendel et al. 2008). The accurate bypass of UV-induced lesions was also demonstrated by a study showing that the type I Burkitt's lymphoma BL2 cell lines deficient in pol η exhibited a 1.5-fold increase in UV-induced mutagenesis (Gueranger et al. 2008).

Consistent with data in human cells, a study using primary mouse fibroblasts deficient in pol η found that these cells were moderately sensitive to UV irradiation, and UV-induced mutagenesis at the *Hprt* locus was increased, with the majority of substitutions occurring at the dipyrimidine sites. Additionally, the bias of mutations on the nontranscribed strand was reduced to 2.9-fold in these cells, relative to 4.5-fold in the wild-type cells (Dumstorf et al. 2006). Furthermore, an important role of pol η in protecting cells from the cytotoxic effect of UV irradiation in the repair-deficient background has been revealed by a study showing an up to 2-fold decrease in UV sensitivity in XPA cells depleted of pol η (Ziv et al. 2009).

With regard to the ability of pol η to bypass lesions induced by chemotherapeutic agents, the function of pol η in the bypass of cisplatin-induced DNA lesions has been well-studied. Cisplatin induced elevated level of mutations at *Hprt* locus in XP-V cells, indicating the role of pol η in the accurate TLS past cisplatin-induced DNA lesions (Bassett et al. 2004). Additionally, XP-V cells were hypersensitive to a number of platinum-based drugs, not only cisplatin, but also carboplatin and oxaliplatin. These cells manifested prolonged cisplatin-induced S phase arrest (Albertella et al. 2005). XP-V cells were also sensitive to cytarabine, gemcitabine, cisplatin, and the combination of gemcitabine and cisplatin (Chen et al. 2006).

In addition to cisplatin, pol η bypasses mitomycin C-induced ICLs. By using plasmids containing both a site-specific mitomycin C-induced ICL and a firefly luciferase reporter gene, it was shown that reactivation of the luciferase activity was dramatically reduced when these plasmids were

introduced into XP-V cells. These data suggest the involvement of pol η in recombination-independent repair of the lesion. However, in this study, since the mutation frequency was only reduced from 22 percent to 15 percent relative to repair proficient cells, it was not possible to conclude that pol η catalyzed the mutagenic bypass of this lesion (Zheng et al. 2003).

Germane to the correlation between pol η expression and cellular response to chemotherapeutic agents, the use of pol η expression levels as a marker to predict the efficacy of cisplatin has been evaluated and the need of alternative non-platinum chemotherapy regimens has been addressed, specifically for the treatment of non-small cell lung cancer (NSCLC) patients with high pol η levels (Ceppi et al. 2009). In patients with advanced NSCLC who receive platinum-based chemotherapy, the expression of pol η predicted their survival, in which high expression correlated with poor survival. Pol η was also found to be an independent factor associated with survival of these patients. Additionally, pol η transcript levels were up-regulated in cisplatin-treated NSCLC cell lines, and NSCLC cell lines expressing a low level of pol η exhibited sensitivity to cisplatin, while cells expressing high level of pol η were refractory to cisplatin. These data indicated that high level of pol η can confer cellular resistance to cisplatin (Ceppi et al. 2009).

The expression of pol η also correlated with sensitivity of gastric cancer cell lines to oxaliplatin; cells with lower pol η expression were more sensitive to oxaliplatin (Teng et al. 2010). Additionally, pol η expression has been found to predict the response of patients with metastatic gastric cancer receiving FOLFOX (fluorouracil, leucovorin, and oxaliplatin) or XELOX (capecitabine and oxaliplatin). These data indicate that higher pol η levels correlate with a poor treatment response and shorter survival in patients treated with platinum-based drugs (Teng et al. 2010). Moreover, a cell-based study has shown that when XP-V cells were complemented with pol η, they were more refractory to gemcitabine, cisplatin, and the combination of gemcitabine and cisplatin than non-complemented XP-V cells (Chen et al. 2006). Similarly, fibroblasts depleted of pol η were more sensitive to cisplatin or gemcitabine alone, as well as the combination of cisplatin and gemcitabine, than pol η-proficient cells.

Furthermore, pol η expression is frequently dysregulated in tumors. Analyses of individual patients with NSCLC showed that pol η transcripts were highly elevated in tumor tissue compared to normal tissue (Ceppi et al. 2009). It has also been shown that pol η transcripts were downregulated in human lung, colorectal, and stomach cancers (Pan et al. 2005; Betous et al. 2009).

Pol η may be involved in TLS past other DNA lesions, such as AAF-dG adducts, since XP-V cell extracts showed defects in replication bypass of such modifications (Cordonnier et al. 1999; Broughton et al. 2002). The role of pol η in protecting cells from both spontaneous and damage-induced

mutagenesis has been studied, with emphasis on 8-oxo-dG-induced mutagenesis. In both XP-V cells and human fibroblasts depleted of pol η, the background mutant frequencies on both normal plasmids and plasmids treated with a 8-oxo-dG inducer, photoactivated methylene blue, were increased by approximately 2-fold (Lee and Pfeifer 2008).

Another function of pol η, in addition to lesion bypass, is to carry out somatic hypermutation of immunoglobulin genes. This programmed cellular process of directed mutagenesis is necessary to generate antibody diversity in the face of a foreign antigen challenge. In XP-V patients, a reduction in the mutations at A/T base pairs was observed and over 80 percent of mutations were G/C transversion at both J_H4 intron and pre-switch sequences at immunoglobulin gene locus (Delbos et al. 2005).

Phenotype of Pol η Knockout Mice

Pol η knockout mice (pol $\eta^{-/-}$) are viable and fertile. However, they are susceptible to chronic sunlight-induced skin cancer, reflective of the XP-V patient clinical phenotype. These mice are particularly susceptible to epithelial skin tumor development (Ohkumo et al. 2006). Additionally, pol $\eta^{-/-}$ mice have a dramatic reduction in mutations at A/T in the intronic sequence of the J_H4 region in germinal center B cells, and a complete lack of A/T mutations was observed in mice deficient in both the MMR protein MSH2 and pol η (Delbos et al. 2007; Masuda et al. 2007). Furthermore, in pol $\eta^{-/-}$ mice, a reduction in the mutations at A/T base pairs was observed and over 80 percent of mutations were at G/C base pairs at both J_H4 intron and pre-switch sequences at immunoglobulin gene locus (Delbos et al. 2005). These data confirm that pol η plays an important role in not only TLS, but in somatic hypermutation of immunoglobulin genes.

DNA Polymerase Kappa

General and Biochemical Properties

Pol κ is a 99 kDa protein whose gene is located on chromosome 5q13.1 (Gerlach et al. 1999, 2001; Bavoux et al. 2005). Pol κ lacks a 3′-5′ proofreading exonuclease activity and has a moderate processivity by elongating 1 to more than 25 nucleotides (Ohashi et al. 2000a; Zhang et al. 2000c; Gerlach et al. 2001; Haracska et al. 2002; Bavoux et al. 2005). Pol κ is a low fidelity enzyme, with the frequency of nucleotide misincorporation opposite non-damaged nucleotides being ~10^{-2} to 10^{-4} (Johnson et al. 2000a; Zhang et al. 2000c). Additionally, studies using gapped M13mp2 plasmids demonstrated that during synthesis of the target *lac*Zα gene, the frequency of *lacZ* mutants

generated by pol κ was 25–34 percent (Ohashi et al. 2000a). With regard to the types of mutations generated, base substitutions were the most prevalent, with the most frequent mutation being T to G transversions (Zhang et al. 2000c). Although pol κ exhibits low fidelity opposite all non-damaged bases, it most frequently misincorporates opposite a template dT (Ohashi et al. 2000b; Zhang et al. 2000c). Moreover, it can extend primers from several mismatched base pairs such as G-G, G-T and T-C, though it catalyzes the most efficient extension from canonical Watson-Crick base pairs (Zhang et al. 2000c). Furthermore, during DNA synthesis, it often generates DNA products that are one or two nucleotides shorter than what would be predicted based on the length of the template DNA (Ohashi et al. 2000b; Zhang et al. 2000b,c; Suzuki et al. 2001; Rechkoblit et al. 2002).

Pol κ has been shown to catalyze replication bypass of a wide variety of DNA lesions. It can catalyze high fidelity bypass of (+)- and (–)-*trans-anti*-BPDE-N^2-dG. In the case of bypass of (+)- and (–)-*trans-anti*-BPDE-N^2-dG, the correct dC is predominantly incorporated (Zhang et al. 2000b, 2002a; Rechkoblit et al. 2002; Sassa et al. 2011). It is intriguing that although pol κ can efficiently bypass the minor groove (+)- and (–)-*trans*-BPDE-N^2-dG DNA adducts, it is completely blocked by major groove (+)- and (–)-*trans*-BPDE-N^6-dA adducts (Rechkoblit et al. 2002). Germane to these observations, other studies have shown that pol κ could efficiently and accurately bypass acrolein-derived N^2-dG peptide cross-links, while the polymerase was strongly blocked by N^6-dA peptide cross-links; these lesions are chemically identical to N^2-dG peptide cross-links, except that they are positioned in the major, not the minor groove of DNA (Minko et al. 2008b; Yamanaka et al. 2011). Additional N^2-dG lesions that pol κ can efficiently and accurately bypass are acrolein-derived N^2-dG ICLs (Minko et al. 2008a) and the reduced ring-opened form of γ-HOPdG adducts (Wolfle et al. 2005). Pol κ also carries out accurate TLS past HNE-induced N^2-dG adducts, though with reduced efficiency (Wolfle et al. 2006). Although pol κ was inhibited by γ-HOPdG at the nucleotide insertion step, the polymerase efficiently extended the primer from dC opposite the lesion (Washington et al. 2004c). A variety of N^2-alkyl dG lesions varying in size can also be bypassed by pol κ (Choi et al. 2006b). Collectively, these data suggest that the cognate lesions of pol κ may be minor groove N^2-dG lesions. Furthermore, the polymerase catalyzes bypass of 8-oxo-dG, an AP site, and AAF-dG or *N*-(deoxyguanosin-8-yl)-2-aminofluorene (AF)-adducted guanines (AF-dG) adducts. When replicating DNAs containing 8-oxo-dG, pol κ predominantly incorporates dA opposite this lesion. Interestingly, the efficiency and specificity of nucleotides incorporated opposite an AP site were affected by the template base 5′ to the lesion. Specifically, nucleotide incorporation occurred most efficiently and further extension was enhanced when the template base 5′ to the AP site was a dT. When DNAs containing site-specific AAF-dG were replicated,

dC and dT were the most frequently incorporated nucleotides (Ohashi et al. 2000b; Zhang et al. 2000b; Gerlach et al. 2001; Suzuki et al. 2001; Rechkoblit et al. 2002; Irimia et al. 2009). Similar to the replication of non-damaged DNA, pol κ can extend a mismatched primer termini past AAF-dG (Ohashi et al. 2000b). When replicating DNAs containing AF-dG, pol κ predominantly incorporated dC and dA opposite the lesion (Suzuki et al. 2001).

The DNA lesions that block pol κ are T-T CPDs and (6-4) photoproducts, and dG-dG intrastrand cross-links induced by cisplatin (Ohashi et al. 2000b; Zhang et al. 2000b; Gerlach et al. 2001). Although pol κ cannot incorporate a nucleotide opposite a T-T CPD, it can efficiently extend from mismatched primer termini past this lesion. In particular, the polymerase catalyzes the most efficient extension when dG is opposite the 3′ T of the lesion, with the efficiency 3-fold higher than extension from a G-T mispair on non-damaged DNA. These data suggest that pol κ may play a role in mutagenic bypass of T-T CPDs (Washington et al. 2002).

A study has shown that pol κ can bypass 4-OHEN-dA. It preferentially incorporated the correct dT opposite the lesion, but also incorporated dC, inducing A to G transitions (Yasui et al. 2006). These data correlate well with a previous study showing that A to G transitions are one of the predominant base substitutions found at AT pairs of the *supF* reporter gene following replication of a DNA vector exposed to 4-OHEQ (Yasui et al. 2003). Thus, pol κ may be responsible for inducing these types of mutations in cells during the bypass of the 4-OHEN-dA adduct. It is important to mention that these findings indicate that the miscoding property of 4-OHEN-dA due to error-prone TLS may contribute significantly to the development of cancers induced by equine estrogens.

Similarly, pol κ may contribute to the generation of mutations following replication past εdA. Steady-state kinetic analyses revealed that pol κ preferentially incorporates the correct dT opposite the lesion, and also efficiently extends from a εdA:A mispair. When TLS products were analyzed for both base substitutions and frameshift mutations, it was found that one nucleotide deletions were the predominant product of pol κ-catalyzed TLS, followed by dT, dA, and dC incorporation. Overall, these results show that pol κ catalyzes both error-free and error-prone TLS past εdA (Levine et al. 2001). However, contradicting data have been presented by another group who reported that pol κ catalyzes only error-free TLS past this lesion (Zhou et al. 2010).

In vivo Functions and Implications in Tumorigenesis/ Chemotherapy Resistance

The role of pol κ in the processing of naturally occurring non-B-DNA structures has been studied. When pol κ was depleted from HeLa cells and

these cells were then transfected with plasmids containing G-rich sequences from the human c-MYC promoter region, an increase in the induction of DSBs was observed. Pol κ depletion also enhanced DSB formation in HeLa cells transfected with plasmids containing GA-rich sequences from the breakage hotspot region of the Kaposi sarcoma associated Herpes virus genome or the major break region of human *BCL-2* gene. Additionally, it was demonstrated that pol κ is involved in the processing of G-quadruplex DNA structures, since U2OS cells depleted of pol κ exhibit decreased survival in response to telomestatin. Collectively, these data show that pol κ plays an important role in genomic stability maintenance at naturally occurring unusual DNA sequences (Betous et al. 2009).

As observed *in vitro*, pol κ has been shown to be involved in TLS past various DNA lesions in cells. Using SV40-based pBS/pSB vectors, when pol κ was depleted from human fibroblasts, the efficiency of TLS past either the 5R,6S or 5S,6R thymine glycol lesion decreased to about 50 percent; additionally, replication of DNAs containing these lesions during pol κ depletion resulted in an increase in the mutagenic frequencies by more than 2-fold (Yoon et al. 2010a). These data predict a role of pol κ in efficient and accurate bypass of thymine glycols. Moreover, when gapped plasmids harboring a site-specific (+)-*trans*-BPDE-N^2-dG adduct were replicated in U2OS cells depleted of pol κ, the efficiency of TLS decreased by 2.3-fold, and mutagenic TLS decreased by 1.9-fold, with increases in deletions and insertions from 14 to 25.9 percent, respectively (Shachar et al. 2009).

Pol κ plays a role in the processing of cross-link lesions. The function of pol κ in the accurate and efficient TLS past N^2-dG ICLs was proposed based on *in vitro* observations and is supported by cellular studies reported by Minko et al. They demonstrated that human fibroblasts depleted of pol κ are moderately sensitized to a N^2-dG ICL-inducing agent mitomycin C. Furthermore, mitomycin C enhanced formation of aberrant chromosomal structures, in particular, radial structures, in these cells (Minko et al. 2008a). Studies using gapped plasmids carrying site-specific cisplatin-induced dG-dG intrastrand cross-links showed that when these plasmids were replicated in U2OS cells that had been depleted for pol κ, there was a 34 percent decrease in the efficiency of TLS and 5.7-fold decrease in mutagenic TLS (Shachar et al. 2009). This is an unexpected finding, as pol κ cannot bypass this type of cross-link *in vitro*.

Consistent with data derived from human cells, the role of pol κ in accurate TLS past B[*a*]P-induced dG adducts has been observed in mouse cells. Using a gapped plasmid containing a site-specific B[*a*]P-induced dG adduct, the efficiency of TLS was found to be reduced in pol $\kappa^{-/-}$ mouse embryonic fibroblasts (MEFs), and the incorporation of the incorrect nucleotides was increased in these cells (Avkin et al. 2004). Another group has reported similar results that mouse embryonic stem cells knocked out

for pol κ exhibit a 3-fold increase in killing following exposure to B[*a*]P and had an increase in mutation frequency by 10-fold, with G to T transversions predominating at the *Hprt* locus (Ogi et al. 2002). Additionally, pol κ has been shown to be important for cells to recover from BPDE-induced S-phase checkpoint and to overcome replication fork blocks and prevent the formation of DSBs. This study also found that pol κ is required for cellular survival after BPDE exposure, and in response to BPDE, pol κ-deficient MEFs exhibit prolonged checkpoint activation and formation of DSBs, as observed by prolonged Chk1 phosphorylation and an increased level of histone γ-H2AX. The increase in phosphorylation of ATM and Chk2 were detected in these cells as well (Bi et al. 2005). In addition to B[*a*]P, pol κ may be involved in coping with lesions induced by other types of polycyclic aromatic hydrocarbons, since both the transcript and protein levels of pol κ are upregulated when mice are exposed to 3-methylcholanthrene (Ogi et al. 2001).

Mouse embryonic stem cells and fibroblasts knocked out for pol κ exhibit elevated sensitivity to UV (Ogi et al. 2002; Schenten et al. 2002; Bi et al. 2005). Similar to the response to BPDE, pol κ was needed for cells to progress through S phase after being arrested by UV irradiation (Bi et al. 2005). The role of pol κ in the processing of DNA lesions is also evident in studies showing that pol κ transcripts can be upregulated in mice in response to several DNA-damaging agents, including UV irradiation and doxorubicin (Velasco-Miguel et al. 2003).

In addition to its role in TLS, a role for pol κ in the repair synthesis step of NER has been investigated. Several lines of evidence support this model, including the greatly diminished capability of pol κ-deficient MEFs to remove (6-4) photoproducts. This unexpected function of pol κ may explain its sensitivity to UV damage, despite its inability to bypass UV-induced lesions *in vitro* (Ogi and Lehmann 2006).

In addition to studies suppressing or knocking out pol κ, the effects of overexpression of pol κ have been investigated. In both human cells and mice, the mutation rate at the *Hprt* locus was increased upon pol κ overexpression. In a separate study when pol κ was ectopically expressed, mutation rates, DNA breaks, genetic recombination, loss of heterozygosity, and aneuploidy were enhanced. All these data suggest that dysregulated pol κ expression may perturb normally accurate chromosome duplication and thus promote mutagenic replication (Bergoglio et al. 2002; Bavoux et al. 2005).

With regard to the link between pol κ and specific types of tumors, pol κ activity may be germane to the etiology of gliomas. Recent studies have demonstrated that the expression of pol κ protein is upregulated in over 50 percent of glioma patients, and there was a significant association between pol κ expression and the advanced stage of the disease. Moreover, pol κ has

been identified as an independent prognostic factor for glioma patients, where high expression indicates poor survival (Wang et al. 2010). Pol κ has also been shown to be overexpressed in NSCLC. Since pol κ is involved in the replication bypass of adducts induced by B[*a*]P, a common constituent of cigarette smoke, pol κ expression may be dysregulated in smokers (O-Wang et al. 2001). However, data exist showing that the transcript level of pol κ is significantly down regulated in lung cancer tissues. The downregulation of pol κ transcripts was also observed in stomach and colorectal cancer tissues (Pan et al. 2005).

Phenotype of Pol κ Knockout Mice

Mice deficient in pol κ are viable and fertile (Schenten et al. 2002). However, they exhibit significantly elevated spontaneous mutations particularly in liver, kidney, and lung. Interestingly, there is a tissue-specific mutagenesis pattern observed in these mice. For instance, G:C to T:A mutations were predominant in the kidney and liver, while G:C to A:T mutations were moderately increased in the lung. Additionally, A:T to T:A mutations were moderately increased in lung and liver (Stancel et al. 2009). Finally, these mice exhibit a mutator phenotype associated with ageing and the accumulation of endogenous DNA lesions, suggesting that pol κ is involved in the tolerance of oxidative damage (Bavoux et al. 2005).

DNA Polymerase Iota

General and Biochemical Properties

Pol ι is an 80 kDa protein, whose encoding gene resides on chromosome 18q21.1 (Johnson et al. 1999b; McDonald et al. 1999; Sweasy et al. 2006). Pol ι lacks a 3′-5′ proofreading exonuclease activity and has a low processivity, only elongating 1–3 nucleotides depending on the sequence context of the template (Tissier et al. 2000b). Pol ι is a low fidelity enzyme, with a tendency to preferentially misincorporate dG opposite a template dT and is capable of extending from some mismatched bases. It also has an interesting property in preferentially utilizing dTTP (Tissier et al. 2000b). The frequency of nucleotide misincorporation opposite non-damaged bases is ~10^{1} to 10^{-6} (Johnson et al. 2000b; Haracska et al. 2001a; Washington et al. 2004a). Additionally, studies using gapped M13mp2 plasmids demonstrated that during synthesis of the target *lac*Zα gene, the frequency of *lac*Z mutants generated by pol ι was 61 percent (Bebenek et al. 2001). This value is much higher than for other Y-family polymerases.

Several DNA lesions have been shown to be substrates for pol ι-catalyzed TLS. Generally, pol ι functions at the nucleotide insertion step. Pol ι can efficiently incorporate the correct dT opposite εdA (Zhou et al. 2010). Pol ι inserts dG and dC with similar efficiency opposite 8-oxo-dG (Maga et al. 2007). In addition, pol ι can incorporate the correct dC opposite AAF-dG (Zhang et al. 2001b). Although full bypass was not achieved, pol ι can incorporate nucleotides opposite (+)- and (–)-*trans*-BPDE-N^2-dG adducts in an error-prone manner by preferential insertion of dG opposite (+)-*trans*-BPDE-N^2-dG and both dA and dG opposite (–)-*trans*-BPDE-N^2-dG. The bypass of (+)- and (–)-*trans*-BPDE-N^6-dA adducts was similarly error-prone, though dT incorporation was preferred opposite (–)-*trans*-BPDE-N^6-dA adducts (Rechkoblit et al. 2002). The polymerase also incorporated nucleotides opposite the 3′ T and 5′ T of (6-4) photoproducts and AP sites; in most sequence context, dA or dG and dT was preferentially inserted opposite the 3′ T or 5′ T of a (6-4) photoproduct, respectively, while opposite an AP site, the incorporation of either dG or dT was preferred (Johnson et al. 2000b; Tissier et al. 2000a). Pol ι can incorporate nucleotides opposite m6G as well, with dT incorporated most efficiently (Johnson et al. 2006). In contrast, pol ι catalyzed limited or no TLS past a T-T CPD (Johnson et al. 2000b; Zhang et al. 2001b), and during the limited synthesis, either dT or dG was incorporated preferentially opposite the 3′ dT (Tissier et al. 2000a). Although pol ι is generally more proficient at the nucleotide insertion than the primer extension step, the polymerase can extend primers past some lesions. For instance, pol ι has been shown to catalyze its most efficient primer extension when dT or dA is situated opposite m6G or 8-oxo-dG, respectively (Johnson et al. 2006). Furthermore, pol ι can incorporate a nucleotide opposite acrolein-induced γ-HOPdG, or its reduced ring-opened form, PdG, as well as HNE-induced N^2-dG adducts, by preferentially inserting dC or dT (Washington et al. 2004c; Wolfle et al. 2005, 2006). dC incorporation opposite some N^2-alkyl dG lesions could also be performed by pol ι (Choi and Guengerich 2006).

In vivo Functions and Implications in Tumorigenesis/ Chemotherapy Resistance

An important role of pol ι in protecting cells from oxidative DNA damage has been investigated, where human fibroblasts stably down regulated for pol ι were found to be hypersensitive to agents that induce oxidative stress, including hydrogen peroxide and menadione (Petta et al. 2008). Moreover, these cells exhibited moderate sensitivity to low dose potassium bromate treatment, another oxidizing agent. From studies using human fibroblast cell extracts that had been depleted of pol ι, it was shown that the activity of BER was reduced (Petta et al. 2008).

Pol ι may play a mutagenic role in the processing of UV-induced DNA lesions. The replication of normal and UV-damaged plasmids in human 293T cells that were depleted of pol ι was significantly decreased (Choi et al. 2006a). Fibroblasts from pol ι knockout mice were hypersensitive to UV irradiation (Ohkumo et al. 2006). A 2-fold decrease in the frequencies of total mutants and base substitutions induced by UV was observed in primary fibroblasts derived from pol ι-deficient mice. The majority of these base substitutions were at dipyrimidines on the nontranscribed strand, resulting in a 2.4-fold bias toward the nontranscribed strand. Since this strand bias was 4.5-fold in wild-type cells, pol ι is likely to contribute to the strand bias for UV-induced mutagenesis. The loss of pol ι resulted in a decrease in UV-induced mutagenesis, suggesting that mouse pol ι has a role in TLS past UV-induced lesions (Dumstorf et al. 2006).

The potential role for pol ι in modulating carcinogenesis has received support in both an animal model system and human tumor data. Pol ι may play a role in breast cancer genomic instability by catalyzing error-prone bypass of UV-induced lesions, preferentially misincorporating dT or dG opposite the 3′ T of T-T CPDs and 5′ T of (6-4) photoproducts (Tissier et al. 2000a). These data are consistent with a large increase in the frequency of T to A transversions and T to C transitions in breast cancer cells, with UV-induced mutation frequencies reduced when pol ι is immunodepleted from nuclear extracts of breast cancer cells (Yang et al. 2004). Another study showed that the mouse 129XI locus harboring a mutated *pol ι* gene conferred susceptibility to lung tumors induced by urethane, suggesting a protective role of pol ι in the prevention of lung tumor development (Lee and Matsushita 2005).

With regard to the correlation between pol ι expression and carcinogenesis, the dysregulation of pol ι expression was observed in several cancers. Pol ι is upregulated in breast cancer cells, both at the transcript and protein levels, and protein levels were rapidly increased after exposure to UV in both nonmalignant breast cells and breast cancer cells. Since breast cancer cells exhibit higher spontaneous and UV-induced mutant frequencies relative to normal breast cells in plasmid-based assays, pol ι activity may be germane to the development of breast cancers (Yang et al. 2004). Pol ι may also contribute to the etiology of some gliomas, since protein expression of pol ι is upregulated in over 25 percent of glioma patients. Furthermore, glioma patients who were positive for elevated levels of pol ι had shorter survival prognoses (Wang et al. 2010). In contrast, down regulation of pol ι transcripts were observed in other types of tumors, such as colorectal, lung, and stomach cancers (Pan et al. 2005).

Phenotype of Pol ι Knockout Mice

Pol ι knockout mice are viable and fertile. However, when exposed to chronic UV irradiation, these mice develop mesenchymal skin tumors, such as sarcomas and hemangiomas. Additionally, if there is a loss of a single allele of pol η in this genetic background, there is a concomitant increase in the incidence of epithelial skin tumor development following chronic UV exposure (Ohkumo et al. 2006). Furthermore, the presence of pol ι significantly delayed the formation of UV-induced skin tumors, since the time to UV-induced tumor formation was earlier in mice deleted for both pol ι and η versus the pol η knockout alone (Dumstorf et al. 2006).

Rev1

General and Biochemical Properties

Rev1 is a 138 kDa protein whose gene is located on chromosome 2q11.1-11.2 (Lin et al. 1999). Rev1 lacks a 3′-5′ proofreading exonuclease activity (Zhang et al. 2002b). It is a template-dependent dCMP transferase, which catalyzes efficient insertion of dC opposite dG or dU. Rev1 is a low fidelity enzyme due to its unique ability to incorporate dC opposite bases, resulting in a frequency of nucleotide misincorporation opposite non-damaged bases of ~10^{0} to 10^{-5} (Zhang et al. 2002b; Brown et al. 2010).

In vitro, Rev1 can catalyze replication bypass of a number of DNA lesions, and it primarily functions in the nucleotide incorporation step of TLS. Rev1 can insert a nucleotide dC opposite acrolein-derived γ-HOPdG (Washington et al. 2004b), an AP site, 8-oxo-dG, (+)- and (-)-*trans-anti*-BPDE-N^2-dG, εdA, and some N^2-and O^6-alkyl dG adducts. Although it can incorporate dC opposite AAF-dG, the efficiency is low. The types of lesions that block Rev1 include T-T CPDs and (6-4) photoproducts (Haracska et al. 2001b; Zhang et al. 2002b; Choi and Guengerich 2008).

In vivo Functions and Implications in Tumorigenesis/ Chemotherapy Resistance

The cellular function of Rev1 in TLS has been studied. Rev1 appears to play roles in both an early TLS pathway that allows for the progression of a stalled replication fork and late in the postreplicative gap-filling pathway for UV-induced lesions. Even though *in vitro* studies demonstrated that Rev1 was blocked at (6-4) photoproducts, it was found using gapped plasmids that TLS past a (6-4) photoproduct was reduced by more than 3-fold in Rev1-deficient MEFs. Additionally, Rev1 catalyzed mutagenic

bypass of the (6-4) photoproduct resulted in transversions of GC to TA at a dG immediately 5′ to the lesion. Furthermore, MEFs deficient in both Rev1 and the NER protein, XPC, underwent a G_2 phase arrest that was caused by accumulation of (6-4) photoproducts. Rev1 also protected cells from UV-induced lethality, since MEFs deficient for Rev1 are sensitive to UV irradiation (Jansen et al. 2009). A study using SV40-based pMTEX4 vectors to replicate in Rev1-deficient MEFs has shown that Rev1 may be involved in TLS associated with the incorporation of dA opposite a heptanone-etheno-dC adduct (Yang et al. 2009).

Rev1 activity may be associated with carcinogenesis and chemotherapy resistance. Lymphoma cells deficient in Rev1 had reduced frequency of mutants induced by cyclophosphamide (CTX, a nitrogen mustard alkylating agent). Mice transplanted with mouse lymphoma cells during cancer relapse did not respond to CTX, while tumors with decreased Rev1 continued to exhibit sensitivity to CTX. Furthermore, the majority of these tumors still responded to CTX following a third round of CTX treatment (Xie et al. 2010). A role of Rev1 in the tolerance of both spontaneous and cisplatin-induced damages has also been demonstrated by studies using ovarian carcinoma cell lines stably depleted of Rev1. Down regulation of Rev1 rendered cells less mutagenic, with a 2.9-fold decrease in the spontaneous rate of mutations being observed. Additionally, these cells were 1.5-fold more sensitive to the cytotoxic effect of cisplatin, and the frequency of cisplatin-induced mutations was 2.6-fold lower when Rev1 was depleted. Furthermore, down regulation of Rev1 decreased the cisplatin-resistant variants, suggesting that drug resistance was mediated by a mutagenic function of Rev1. At the population level, the development of acquired resistance to cisplatin was dependent on the function of Rev1 (Okuda et al. 2005). In addition, BPDE-induced mutant frequencies in primary mouse fibroblasts were reduced when Rev1 was down regulated by a ribozyme against Rev1. In these mice, a significant reduction in BPDE-induced lung tumor multiplicity was observed, and the formation of tumors was also prevented in 27 percent of these mice (Dumstorf et al. 2009). The role of Rev1 in both the spontaneous and cisplatin-induced extrachromosomal HR has been suggested, since the frequency of spontaneous and cisplatin-induced HR events was decreased when Rev1 was down regulated (Okuda et al. 2005).

Germane to the role of Rev1 in carcinogenesis, dysregulated expression of Rev1 may contribute to chemotherapy resistance. For example, ovarian carcinoma cell lines overexpressing Rev1 are resistant to the cytotoxic effect of cisplatin and exhibit an increased frequency of mutants induced by cisplatin. These cells also acquire resistance to cisplatin at a rate faster than the parental cells, indicating that Rev1 plays a role in controlling the rate of development of cisplatin resistance at the population level (Lin et

al. 2006a). Furthermore, Rev1 protein expression is increased in response to ciplatin in ovarian carcinoma cell lines (Okuda et al. 2005).

Phenotype of Rev1 Knockout Mice

The viability of Rev1 knockout mice is strain-specific; in the C57BL/6 background, mice were not viable, whereas in the 129/OLA background, they were viable. These mice also lack the C to G transversions in the nontranscribed strand during somatic hypermutation of immunoglobulin genes, and this was accompanied by significant increases in the frequency of A to T and C to A transversions and T to C transitions (Jansen et al. 2006).

B FAMILY POLYMERASE

DNA Polymerase Zeta

General and Biochemical Properties

Human pol ζ is a 350 kDa protein composed of two subunits, the catalytic subunit Rev3L and the accessory subunit Rev7. The *Rev3L* and *Rev7* genes are located on chromosome 6q21 and 1p36, respectively (Nelson et al. 1996; Sweasy et al. 2006; Gan et al. 2008). The human pol ζ has not been purified to date. Thus, the biochemical properties of human pol ζ are deduced based on studies using yeast pol ζ. Pol ζ lacks a 3′-5′ proofreading exonuclease activity and has a moderate processivity of elongating ≥3 nucleotides (Lawrence and Maher 2001). Compared to other TLS polymerases, pol ζ is a relatively high fidelity enzyme. The frequency of nucleotide misincorporation opposite non-damaged bases is ~10^{-3}–10^{-5} (Johnson et al. 2000b; Haracska et al. 2001b, 2003). Additionally, studies using gapped M13mp2 plasmids demonstrated that during synthesis of the target *lacZα* gene, the frequency of *lacZ* mutants generated by pol ζ was 10 to 12 percent. This value is much lower than for Y-family polymerases (Zhong et al. 2006).

Generally, pol ζ catalyzes inefficient nucleotide insertion opposite DNA lesions, but is proficient in extending a primer terminus positioned opposite a lesion. Although the ability of pol ζ to incorporate a nucleotide opposite 8-oxo-dG, the acrolein-mediated γ-HOPdG adduct, m6G, T-T CPD, (6-4) photoproduct, and an AP site was strongly inhibited, the polymerase can efficiently extend from the primers opposite these lesions. The extension was most efficient when dA was placed opposite 8-oxo-dG or an AP site, dC was placed opposite m6G or an acrolein-mediated γ-HOPdG adduct, or dG was placed opposite the 3′ T of a T-T CPD or a (6-4) photoproduct (Johnson et al. 2000b; Haracska et al. 2001b, 2003; Washington et al. 2004b). However,

pol ζ can fully bypass thymine glycols, preferentially incorporating the correct dA opposite the lesion and efficiently extending from this correct base pair (Johnson et al. 2003). A nitrogen mustard-like ICL is another lesion that pol ζ can accurately bypass. Additionally, it can insert dC opposite a cisplatin-induced ICL (Ho et al. 2011).

In vivo Functions and Implications in Tumorigenesis/ Chemotherapy Resistance

The function of pol ζ in TLS on a variety of DNA lesions has been demonstrated. Using gapped plasmids, depletion of Rev3L in U2OS cells was found to result in a decrease in the extent of TLS across a (+)-*trans*-BPDE-N^2-dG adduct or a cisplatin-induced intrastrand dG-dG adduct by 5.5-fold. The TLS of a (6-4) photoproduct was also reduced by 10-fold (Shachar et al. 2009). Moreover, it was demonstrated that the frequency of TLS past a thymine glycol lesion on an SV40-based pBS/pSB vector decreased about 50 percent in human fibroblasts depleted of either Rev3L or Rev7. TLS past these lesions was error-free, since knockdown of Rev3L or Rev7 increased the frequency of mutagenic TLS by more than 2-fold (Yoon et al. 2010a). The role of pol ζ in protecting cells from the cytotoxic effects of UV has been further addressed. In XP-V cells, depletion of Rev3L rendered these cells 33- to 39-fold more sensitive to UV, and in XPA cells, Rev3L knock down led to a 4 to 5-fold increase in UV sensitivity (Ziv et al. 2009). Pol ζ may also play a role in mutagenic bypass of UV-induced DNA damage, since large decreases in mutations at the 3′ nucleotide of a TT or CT dimer were observed (Gueranger et al. 2008). Furthermore, the type I Burkitt's lymphoma BL2 cell line deficient in Rev3L was 10-fold more sensitive to UV than wild-type cells (Gueranger et al. 2008), and there was a dramatic decrease in the UV-induced mutant rate in these cells (3.3 to 0.7 percent) (Gueranger et al. 2008). Double close mutations, a type of mutation that involves two point mutations spaced by one correct base, with thymidine predominantly being the correct nucleotide, were not detected in the Rev3L-deficient cells, indicating that Rev3L was responsible for introducing this type of mutation (Gueranger et al. 2008). The role of pol ζ in the processing of cisplatin-induced DNA lesions was also evident in studies using human fibroblasts (Wu et al. 2004). Cisplatin upregulated the transcripts of Rev3L, and Rev3L knockdown significantly reduced the rate at which cells acquired resistance to cisplatin; the rate of development of cellular resistance to cisplatin was decreased by approximately 3-fold. These Rev3L depleted cells were also sensitive to cisplatin and the frequency of cisplatin-induced mutants was decreased (Wu et al. 2004). Finally, besides a role in TLS, pol

ζ was shown to be important for both spontaneous and cisplatin-induced HR (Wu et al. 2004).

Consistent with data in human cells, studies in mice support the role of pol ζ in TLS past several DNA lesions. In MEFs deficient in Rev3L, the extent of TLS past a (+)-*trans*-BPDE-N^2-dG adduct, cisplatin-induced intrastrand dG-dG adduct, an AP site, 4-hydroxyequilenin-C, a (6-4) photoproduct, or an artificial lesion with a chain of 12 methylenes inserted into the DNA backbone was significantly reduced. Additionally, mutagenic TLS across a (+)-*trans*-BPDE-N^2-dG adduct, a cisplatin-induced intrastrand dG-dG adduct, a 4-hydroxyequilenin-C damage, or a (6-4) photoproduct was 6.3-fold, 7.2-fold, 3.5-fold, and 20-fold less, respectively (Shachar et al. 2009). Furthermore, Rev3L knockout mouse cells were hypersensitive to the alkylating agents temozolomide and fotemustine, and these agents increased the levels of γ-H2AX foci in these cells. O^6-benzylguanine, an agent that inactivates O^6-methylguanine-DNA methyltransferase (see Chapter 6), had no effect on temozolomide-induced cell death in these cells, but it enhanced apoptosis when cells were treated with fotemustine. These studies suggest that pol ζ may be involved in the processing of fotemustine-induced O^6-chloroethyl-dG adducts and temozolomide-induced damages other than m6G (Roos et al. 2009). Rev3L-deficient cells in a p53 null background are sensitive to several DNA-damaging agents, including UV, mitomycin C, γ-irradiation, and methyl methane sulfonate (Wittschieben et al. 2006). Furthermore, a study using SV40-based pMTEX4 vectors in Rev3L-deficient MEFs has shown that pol ζ may be involved in TLS associated with the incorporation of dA opposite a heptanone-etheno-dC adduct (Yang et al. 2009).

The contribution of pol ζ to genomic stability has been illustrated in several studies. Multiple chromosomal abnormalities not seen in Rev3L-proficient MEFs were observed in Rev3L-deficient MEFs in a $p53^{-/-}$ background. These included dicentrics, insertions, and compound isochromosome/translocation events; overall chromosomal aberrations were also increased in these cells. Among these aberrations, the largest increase was observed in the number of translocation events. Rev3L-deficient cells in a $p53^{-/-}$ background exhibit spontaneous chromosomal instability; these cells had increased numbers of fused/translocated chromosomes, chromosomes with terminal deletions, micronuclei, and rearranged marker chromosomes (Wittschieben et al. 2006). Additionally, in response to cisplatin and mitomycin C, cancer cell lines depleted of Rev3L or Rev7 had reduced survival and exhibit an increase in chromosomal aberrations (Hicks et al. 2010).

Germane to the function of pol ζ in the maintenance of genomic stability, several lines of evidence suggest that pol ζ may participate in carcinogenesis. Depletion of Rev3L sensitized cisplatin-resistant lung adenocarcinoma

cell lines and reduced mutations, suggesting that Rev3L protects cells from drug-induced cell death, but at the cost of introducing mutations in cisplatin-treated cells. These cells also exhibited increased cisplatin-induced γ-H2AX foci formation and senescence, indicating that Rev3L plays a role in the repair of cisplatin-induced DNA damage. Similar phenomena (decreased mitotic indices and increased apoptosis) were also observed in Rev3L-deficient lung adenocarcinoma cell transplants in mice treated with cisplatin. In particular, these transplants exhibited tumor regression, or at a minimum, growth stasis, and these mice survived nearly twice as long as cisplatin-treated mice bearing control lung adenocarcinoma cell transplants. A rapid reduction in lymphomas was seen in mice harboring Rev3L-deficient tumors, and these tumors exhibited enhanced sensitivity to cisplatin (Doles et al. 2010). It is interesting to note that Rev3L heterozygous cells exhibit intermediate sensitivity to temozolomide and fotemustine (Roos et al. 2009). Such data indicate that Rev3L levels dictate the extent of cellular tolerance to alkylating drugs. Rev3L also plays a role in introducing mutations in gliomas, since the frequency of cisplatin-induced mutations at the *Hprt* locus was significantly reduced in Rev3L-depleted glioma cells (Wang et al. 2009). Thus, optimal chemotherapeutic protocols may require the determination of Rev3L expression levels in tumors of patients before and after they receive treatment with an alkylating agent (see Chapter 5).

Although Rev3L is important in both MMR-proficient and -deficient malignant colon carcinoma cells, cells depend more on pol ζ-catalyzed error-prone TLS past cisplatin-induced lesions for cell survival in the absence of MMR. This was made evident in a study demonstrating that Rev3L depletion in MMR-deficient cells results in decreased cisplatin-induced mutants by 58 percent, in contrast to the 38 percent reduction seen in MMR-proficient cells. Thus, targeting pol ζ to reduce the rate of drug resistance development may be more important in tumors that are defective in MMR (Lin et al. 2006b).

The dysregulation of Rev3L may be germane to cancer etiology. Rev3L transcripts were slightly increased in lower grade gliomas (grade I and grade II), but significantly increased in higher grade gliomas (grade III and grade IV) compared with normal brain tissue, with highest expression in grade IV gliomas. As expected, glioma cells expressing high levels of Rev3L were refractory to cisplatin-induced apoptosis (Wang et al. 2009). Additionally, down regulation of Rev3 transcripts was observed in colorectal, lung, and stomach cancer tissues (Pan et al. 2005).

Pol ζ may function in protecting cells from carcinogenesis. Deletion of chromosome 6q21, including the *Rev3L* gene, has been observed in multiple cancers, such as acute lymphoblastic leukemia, T-cell lymphomas, and gastric high-grade large B-cell lymphoma (Schlegelberger et al. 1994; Takeuchi et al. 1998; Starostik et al. 2000). Additionally, the *Rev3L* gene is positioned within the fragile site of FRA6F, and there is an overlap between the 3′ region of human *Rev3L* and one of the two breakage hotspots. A wide variety of cancers, including leukemia and melanoma, have deletion breakpoints at the FRA6F fragile site in a 1.2 Mbp region at 6q21. Thus, the loss of Rev3L may have a significant consequence in carcinogenesis (Morelli et al. 2002).

Phenotype of Pol ζ Knockout Mice

Even though pol ζ is not essential for cellular survival in yeast, several initial attempts to create viable mice disrupted in the *Rev3L* gene were not successful (Bemark et al. 2000; Esposito et al. 2000; Wittschieben et al. 2000). Collectively, these investigations reported mid-gestation lethality, with retarded embryonic development, tissue disorganization, and reduced cell densities. Although these data demonstrate an essential role in embryonic genome stability, the non-redundant, critical function of pol ζ in embryogenesis can only be speculated, and may include TLS past endogenously-induced DNA damage, replication of DNA with non-canonical structures, and/or participation in recombination.

Notably, MEFs derived from even early stage embryos were not viable long term. However, the laboratory of Dr. Rick Wood was ultimately successful in creating stable MEFs from a single E10.5 $Rev3L^{-/-}$; $p53^{-/-}$ embryo (Wittschieben et al. 2006). As described above, these MEFs manifested marked chromosomal instability and displayed increased sensitivity to a variety of DNA-damaging agents (Wittschieben et al. 2006).

More recently, the Wood laboratory has been successful in creating mice where the *Rev3L* gene can be conditionally inactivated from epithelial tissues (Wittschieben et al. 2010). In mice deleted for *Rev3L* in a $Tp53^{-/-}$ background, the latency of thymic lymphomas was shortened and the incidence was higher. In addition, conditional knockout animals developed significantly more mammary tumors in both $Tp53^{+/+}$ and $Tp53^{+/-}$ backgrounds, and deletion of *Rev3L* from $Tp53^{+/-}$ mice resulted in an accelerated occurrence of these tumors. Finally, there was an increase in the number of both preneoplastic and neoplastic lesions in these mice. These data reveal that pol ζ functions to inhibit spontaneous tumor formation.

A FAMILY DNA POLYMERASES

DNA Polymerase ν

General and Biochemical Properties

Pol ν is a 102 kDa protein whose gene is located on chromosome 4p16.3. Pol ν lacks a 3′-5′ proofreading exonuclease activity and has a moderate processivity, elongating 1-100 nucleotides (Marini et al. 2003; Takata et al. 2006; Arana et al. 2007). It catalyzes efficient strand displacement on both non-damaged and damage (psoralen-induced ICL)-containing substrates (Takata et al. 2006; Zietlow et al. 2009). *In vitro*, pol ν replicates non-damaged DNA with low fidelity, primarily due to frequent misincorporation of dT opposite template dG, where the catalytic efficiency of incorporation of an incorrect dT opposite a template dG is approximately half that of the error-free reaction (Takata et al. 2006; Arana et al. 2007). The frequency of nucleotide misincorporation opposite non-damaged nucleotides is ~10^{-1} to 10^{-4} (Takata et al. 2006). Additionally, studies using gapped M13mp2 plasmids found that during synthesis of the target *lacZα* gene, the frequency of *lacZ* mutants generated by pol ν was 2.3 percent when reactions were carried out at neutral pH, yet was 18 percent when reactions were carried out at alkaline pH (Arana et al. 2007, 2008). Furthermore, the polymerase can catalyze nontemplated nucleotide addition at a blunt end (Takata et al. 2006).

Initially, pol ν was shown to carry out efficient and high fidelity TLS of template DNAs containing a thymine glycol lesion, whereas the polymerase was completely blocked by a number of other DNA modifications, including a cisplatin-induced dG-dG intrastrand cross-link, an AP site, a T-T CPD, and a (6-4) photoproduct (Takata et al. 2006). Recently, it was discovered that pol ν is able to bypass very large DNA lesions that are positioned in the DNA major groove (Yamanaka et al. 2010). Specifically, pol ν efficiently and accurately bypasses acrolein-derived DNA−peptide cross-links in which peptides are linked to N^6-dA. Additionally, pol ν bypasses an N^6-dA ICL. However, when a chemically identical DNA−peptide or DNA interstrand cross-link was located in the minor groove via an N^2-dG linkage, TLS by pol ν was completely inhibited. Thus, it is not the identity of the DNA lesion, but its location within the template that is critical for bypass by pol ν. Germane to the ability of pol ν to bypass ICLs, it was shown that pol ν can bypass an unhooked psoralen-induced ICL that models the intermediate structure generated during ICL repair in an error-free manner, albeit with low efficiency (Zietlow et al. 2009). Furthermore, it was shown that pol ν catalyzes inefficient, although accurate, bypass of εdA, and predominantly

incorporates an incorrect dA opposite (+)-*trans-anti*- BPDE-dA, though full bypass of this latter lesion was not achieved (Yamanaka et al. 2010).

In vivo Functions and Implications in Tumorigenesis/ Chemotherapy Resistance

Although cellular roles for pol ν have not been clearly identified, a function for pol ν in DNA cross-link repair via HR in human cells has been proposed (Moldovan et al. 2010). When pol ν is depleted, cells become sensitive to the cytotoxic effects of mitomycin C, cisplatin, and high doses of γ-irradiation. Indeed, the polymerase activity of pol ν is essential for conferring cellular resistance to mitomycin C (Zietlow et al. 2009; Moldovan et al. 2010). Pol ν-depleted cells also exhibit increased mitomycin C-induced chromosomal aberrations, particularly radial chromosomes, and the efficiency of HR in these cells is reduced by 50 percent. It is reasonable to hypothesize that the efficient strand displacement synthesis activity of pol ν plays an important role in HR associated with specific pathways of ICL repair. Pol ν-depleted cells are also mildly sensitive to the strand-break-inducers, camptothecin and bleomycin. Finally, hydroxyurea or ionizing irradiation increased the accumulation of DSBs in cells stably depleted of pol ν.

The role of pol ν in HR is further supported by an observation that pol ν interacts with multiple proteins involved in HR, such as RAD51, as well as proteins in the Fanconi anemia pathway, including FANCD2, FANCI, FANCA, and FANCG (see Chapters 10 and 14). Pol ν also interacts with ubiquitinated FANCD2 following mitomycin C exposure, and some of the above interactions occur exclusively in S phase, correlating with the observation that the levels of pol ν decrease as cells exit S phase (Moldovan et al. 2010). Currently, a cellular role of pol ν in TLS has not been investigated. However, studies in chicken DT40 cells show that pol ν plays roles in both HR-dependent immunoglobulin V gene conversion and TLS-dependent immunoglobulin hypermutation by catalyzing TLS past AP sites to promote diversification of immunoglobulin V genes (Kohzaki et al. 2010).

With regard to the link between pol ν and cancers, pol ν transcript levels were found to be significantly higher in breast carcinoma than in non-tumor breast tissue (Lemee et al. 2010). It is of interest to note that deletion of chromosome 4p16.3, where the *pol ν* gene is located, occurs in 50 percent of breast carcinomas (Shivapurkar et al. 1999). Since mutation frequencies generated during the replication of unadducted plasmids, UV-irradiated plasmids, or plasmids containing psoralen-induced lesions were also slightly increased when pol ν was knocked down, pol ν may function as a tumor suppressor under certain conditions (Moldovan et al. 2010). Pol ν may promote cancer progression in other situations, as suggested by

the ubiquitous expression of pol ν in several cancer cell lines (Marini et al. 2003). Currently, the creation of pol ν knockout mice is in progress. Future investigation using these mouse models will foster our understanding of the cellular functions of pol ν and its contribution to carcinogenesis.

DNA Polymerase Theta

General and Biochemical Properties

Pol θ is a > 250 kDa protein whose gene is located on chromosome 3q13.33 (Marini et al. 2003; Masuda et al. 2007). Pol θ lacks a 3′-5′ proofreading exonuclease activity, and has a moderate processivity, elongating from 1 to greater than 75 nucleotides (Seki et al. 2003; Arana et al. 2008). Pol θ possesses single-stranded DNA-dependent ATPase activity (Seki et al. 2003). It is a low fidelity enzyme that is particularly inaccurate incorporating opposite dT, with the frequency of misincorporation measured at ~10^{-2} to 10^{-3} (Seki et al. 2004). Additionally, studies using a gapped M13mp2 DNA found that during synthesis of the target *lac*Zα gene, the frequency of *lac*Z mutants generated by pol θ was 27 percent to 31 percent, similar to pol η and pol κ. It has also been shown that pol θ generates base insertion and deletion errors at a high rate, uniquely generating more addition than deletion errors. Furthermore, among the base substitutions generated by pol θ, the majority are introduced during replication of a template dA or dT (Arana et al. 2008). Pol θ potentially functions as a mismatch extender, since it is capable of extending all types of mispaired termini opposite dA or dT, and similar to pol ν, it can catalyze nontemplated addition at a blunt end (Seki et al. 2004; Masuda et al. 2007). Pol θ also possesses 5′ deoxyribose-phosphate lyase activity; it removes the deoxyribose phosphate from DNA and fills a 1 nucleotide gap, suggesting a role in single-nucleotide BER (Prasad et al. 2009). Although this enzyme contains a helicase-like domain, responsible for its ATPase activity, a DNA unwinding activity has not been detected (Seki et al. 2003).

Pol θ can bypass several DNA lesions, including an AP site and a thymine glycol, but is blocked by a number of lesions, including a cisplatin-induced dG-dG intrastrand cross-link, a T-T CPD, and a (6-4) photoproduct (Seki et al. 2004; Takata et al. 2006). During the bypass of an AP site, pol θ preferentially inserts dA opposite the lesion and then extends the primer, potentially contributing to AP site-induced mutagenesis (Seki et al. 2004). Although pol θ can not insert nucleotides opposite a (6-4) photoproduct, it can extend the primer if positioned opposite the lesion (Seki and Wood 2008). In addition, studies using a cell extract deficient in pol θ showed that the polymerase may have a role in both single-nucleotide and long-patch BER (see Chapter 8), and is important in BER of 8-oxo-dG (Yoshimura et al. 2006).

In vivo Functions and Implications in Tumorigenesis/ Chemotherapy Resistance

The role of pol θ in the cellular tolerance of DSB-inducers has been shown. Specifically, it has been demonstrated that bone marrow stromal cells derived from pol θ knockout mice are sensitive to γ-irradiation and bleomycin (Goff et al. 2009). Higgins et al. (2010) also showed that several tumor cell lines depleted of pol θ exhibit sensitivity to ionizing radiation and contain an elevated number of γ-H2AX foci. Intriguingly, minimal effect of ionizing radiation was seen with normal fibroblast cell lines, suggesting that effective radiotherapy could be achieved by modulating pol θ expression (Higgins et al. 2010).

An essential role of pol θ in the maintenance of genomic stability is highlighted by studies showing that dysregulation of pol θ expression leads to genomic instability. In human MRC5-SV cells that stably overexpress pol θ, the elongation of the replication fork is slower, and a 2- to 3-fold increase in the formation of γ-H2AX foci is observed. These cells also display altered cell cycle progression; specifically, these cells accumulate in the S and G_2/M phases. Additionally, pol θ-overexpressing cells exhibit a higher number of phosphorylated CHK2 foci, suggesting that overexpression of pol θ results in the activation of the γ-H2AX-ATM-CHK2 DNA damage checkpoint (see Chapter 13). These cells also possess elevated chromosomal abnormalities, particularly end-to-end fusions and chromatid breaks. Finally, these cells exhibit sensitivity to *N*-nitroso-*N*-methylurea and methyl methane sulfonate (Lemee et al. 2010).

The correlation between dysregulated pol θ expression and the emergence and survival of proliferating cancer cells is suggested by frequent pol θ dysregulation in tumors. In particular, pol θ transcript levels are 3- to 26-fold higher in breast carcinoma than in non-tumor breast tissue (Lemee et al. 2010). Importantly, among several other TLS polymerases investigated, including pol η, pol ι, pol κ, pol ν, Rev1, and Rev3L, this increase was the highest. There is in fact a significant association between high expression of pol θ and poor survival of patients with breast cancers. Additionally, for patients who have tumors overexpressing pol θ, there is a 4.3-fold higher risk of death than those individuals with tumors expressing normal levels of pol θ. Furthermore, a significant association between pol θ expression and a number of prognostic indicators for breast cancer, such as estrogen receptor status, has been observed. For example, there is a frequent association between pol θ overexpression and triple-negative tumors (Lemee et al. 2010). Pol θ is also overexpressed in other types of human cancers, including NSCLC, stomach and colon carcinomas. An association between high expression of pol θ and poor clinical prognosis has been found; patients expressing high levels of pol θ have a worse postoperative survival. In

particular, for lung and colon cancers, patients with high pol θ expression have significantly shorter survival based on a 5-year follow-up study, and Kaplan-Meier survival analyses show that patients with colon cancer expressing high levels of pol θ postoperatively survive for a significantly shorter period of time (Kawamura et al. 2004).

An important role of pol θ in maintaining genomic stability during cell cycle has been demonstrated in the chicken DT40 cell model. DT40 cells deficient in both the helicase and polymerase domains of the *pol* θ gene display a slower growth rate and a prolonged G_2 phase. Additionally, these cells have an elevated sub-G_1 faction, as well as elevated levels of spontaneous chromosomal breaks and sister chromatid exchanges. Consistent with *in vitro* studies, pol θ-deficient cells are hypersensitive to hydrogen peroxide, further supporting a role in BER (Yoshimura et al. 2006). A role for pol θ in generating immunoglobulin V gene diversity during an immune response has been also suggested (Kohzaki et al. 2010).

Phenotype of Pol θ Knockout Mice

Pol θ knockout mice are viable and fertile. However, their viability is severely compromised in an Atm-deficient background. Interestingly, these mice exhibit a delayed onset of thymic lymphomas. Additionally, pol θ knockout mice display an elevated frequency of micronuclei in their reticulocytes, a feature that occurs both spontaneously and as result of exposure to γ-irradiation (Shima et al. 2004; Goff et al. 2009). Furthermore, mice expressing a mutant form of pol θ, which is defective in its polymerase activity, exhibit a decrease in mutations, particularly at intrinsic C/G somatic hypermutation hotspots. In contrast to these mice, pol θ null mice exhibit a decrease in both C/G and A/T mutations in the intronic sequence of the J_H4 region in germinal center B cells, while G to C transversions are increased. These results indicate a potential role of pol θ in somatic hypermutation of immunoglobulin genes (Masuda et al. 2005, 2006, 2007). However, a recent report has shown that pol θ deficiency minimally alters the mutation spectrum in mice within the immunoglobulin loci (Martomo et al. 2008)

COOPERATIVE ACTIONS OF MULTIPLE TLS POLYMERASES IN LESION BYPASS

The bypass of some DNA lesions can involve the actions of multiple TLS polymerases. For example, TLS past CPDs has been shown to require several TLS polymerases. When gapped plasmids carrying a site-specific T-T CPD were replicated in pol η-deficient XP-V cells depleted of Rev3L, a 74 percent decrease in TLS was observed relative to cells transfected with the control

siRNA (Ziv et al. 2009). When pol ι and pol κ were simultaneously knocked down, TLS decreased by 65 percent. These results suggest that the bypass of T-T CPDs can be mediated by the cooperative action of pol ζ with pol κ and/or pol ι. Additionally, mutagenic TLS decreased by 84 percent when Rev3L was depleted in XP-V cells, and when both pol κ and pol ι were depleted, mutagenic TLS decreased by 76 percent. Similar results were obtained for accurate TLS, suggesting that these polymerases carry out both mutagenic and error-free TLS across T-T CPDs in the absence of pol η. Furthermore, the depletion of pol κ enhanced the sensitivity of XP-V cells to UV by 3.5-5-fold, suggesting that pol κ can protect XP-V cells against the cytotoxic effect of UV. In XPA cells, when either pol η or pol κ was knocked down, an up to 2-fold decrease in UV sensitivity was observed, and simultaneous depletion of both pol κ and pol η resulted in a 5 to 6-fold increase in UV sensitivity; these data suggest that both pol η and pol κ can protect repair-defective XPA cells from the cytotoxic effect of UV (Ziv et al. 2009). Similar complex cooperativity has been observed between different polymerases to bypass CPDs and (6-4) photoproducts using SV40-based vector systems (Yoon et al. 2009, 2010b). Moreover, the type I Burkitt's lymphoma BL2 cell lines deficient in pol η and pol ι are more sensitive to UV-induced killing than cells deficient in pol η alone (Gueranger et al. 2008). Furthermore, there is a significant decrease in the overall UV-induced mutagenesis in the absence of both pol η and pol ι compared to fibroblasts deficient in pol η alone, suggesting a role for pol ι in an error-prone TLS past UV-induced lesions in the absence of pol η. Since strand bias was abolished with the loss of both pol η and pol ι, it is reasonable to speculate that both polymerases play roles in generating strand bias (Dumstorf et al. 2006). Fibroblasts from mice deficient in pol η and pol ι were also very sensitive to UV irradiation (Ohkumo et al. 2006).

Double close mutations were significantly elevated in type I Burkitt's lymphoma cells deficient in pol η, pol ι, and pol η and pol ι. Other types of mutations were also observed; in the pol η and pol η/pol ι-deficient cells, an increase in misincorporation opposite the 3′ T of a TT was observed, while a significant decrease in the mutations at the 5′ TT was observed, suggesting that these polymerases may have a role in mutagenic bypass of 5′ T-T dimers (Gueranger et al. 2008). Pol η-deficient cells, with a decreased level of pol ι, have a lower overall frequency of UV-induced mutations, indicating that pol ι introduces mutations in the absence of pol η in response to UV (Wang et al. 2007).

The bypass of cisplatin-induced dG-dG intrastrand cross-links also involves multiple TLS polymerases. When gapped plasmids containing this lesion were replicated in U2OS cells depleted of both pol η and Rev3L, a dramatic 80 percent reduction in TLS was observed, and depletion of both pol κ and pol η resulted in a 78 percent decrease in TLS. In addition,

several TLS polymerases may function to cooperatively bypass (+)-*trans*-BPDE-N^2-dG, as the depletion of both pol κ and Rev3L reduced mutagenic TLS by 3.9-fold (Shachar et al. 2009).

TLS POLYMERASE INHIBITORS FOR CANCER PREVENTION AND THERAPY

Cancer cells generally have high levels of genomic instability characterized by point mutations, deletions, rearrangements, and abnormal ploidy relative to normal adjacent tissues (see Chapter 3). These differences in genomic stability cannot be adequately explained by the random accumulation of DNA damage, but rather by the indication that cancer cells have a strong mutator phenotype arising from deficiencies in DNA repair and tolerance mechanisms, cell cycle check points, and/or the dysregulation of error-prone DNA polymerases (Loeb et al. 1974; Loeb 1991; Fox and Loeb 2010; Salk et al. 2010). It is estimated that during the ~100 cell divisions which take place during the life-time of stem cell replication, as few as one or two mutant genes would be produced (Jackson and Loeb 1998). In humans, mutation rates have been calculated to be $5x10^{-11}$ mutations per base pair per replication (Drake 1999).

In contrast, the frequency of randomly generated mutations in cancer cells averages 210 $x10^{-8}$ per base pair, which is greater than 200-fold higher when compared to the matching normal tissues (Bielas et al. 2006). These studies illustrate the profound genetic instability of cancer cells. This instability is also evident by a recent study showing that the genomes of cancer cells accumulate over 20,000 mutations (Lee et al. 2010; Pleasance et al. 2010a,b). Since TLS polymerases can introduce massive numbers of mutations at the single nucleotide level and their expression is frequently dysregulated in tumors, TLS polymerases may play a causal or contributing role in genome-wide instability and carcinogenesis. Thus, inhibition of TLS polymerases may lead to a reduction in mutations and thus prevent the emergence of tumors.

Despite the continuous research efforts to combat cancers, chemotherapy resistance is still a major obstacle to successful cancer treatment. Multiple mechanisms have been implicated in drug-induced, acquired resistance, including decreased intracellular accumulation of the drug due to decreased uptake, increased efflux, or alteration in membrane lipids. These cellular adaptations can prevent apoptosis, increase the repair of DNA damage due to the upregulation of genes involved in repair, and alter cell cycle and checkpoint controls. Additional factors that may adversely affect chemotherapeutic efficacy are increased drug metabolism or compartmentalization, limiting access of the drug to sites of action (Gottesman 2002).

As mentioned above, the ability of tumor cells to acquire resistance to chemotherapeutic agents has been proposed to be caused by both enhanced replication bypass and the low fidelity of the TLS polymerases (Mamenta et al. 1994; Wu et al. 2004; Okuda et al. 2005; Lin et al. 2006a,b; Ceppi et al. 2009; Xie et al. 2010). TLS polymerases can catalyze both error-free and error-prone TLS past a number of lesions. The error-free replication bypass can enhance the capacity of preexisting tumors to tolerate the drug-induced lesions. Error-prone bypass may not only promote tumorigenesis, but may also provide an additional mutation load that results in secondary tumor initiation or the development of drug resistance. Thus, the identification of specific inhibitors of TLS polymerases may be useful as novel anticancer agents, since TLS polymerases do not only introduce mutations, but can decrease the efficacy of treatments involving DNA-damaging agents.

Although the search for compounds targeting TLS polymerases has received modest attention, a number of inhibitors are emerging. For example, 3-*O*-methylfunicone (Fig. 2A) is the most selective Y-family polymerase inhibitor identified to date. It is a natural product isolated and purified from marine fungal strains found in Australian sea salt. 3-*O*-methylfunicone is most potent against pol κ, with an IC_{50} value of 12.5 μM, in contrast to 34.3 μM and 50.1 μM against pol ι and η, respectively (Table 1). This compound is hypothesized to interact with the DNA template-primer-binding site of pol κ, rather than the dNTP substrate-binding site, since the mode of inhibition was shown to be competitive with the DNA template-primer and non-competitive with the dNTP substrate. In cell culture experiments, 3-*O*-methylfunicone was found to suppress the growth of two cancer cell lines, HCT116 and HeLa cells, with an LD_{50} value of 63.8 μM and 63.3 μM, respectively. Interestingly, 3-*O*-methylfunicone does not have an effect on cell proliferation and growth of normal human cells, such as HUVEC and HDF cells. These data suggest that this compound can be a selective anti-cancer agent with minimal toxicity to non-tumorous tissues. Additionally, 3-*O*-methylfunicone significantly enhanced HeLa cell UV-sensitivity, where the clonogenic survival was decreased by 4.3-fold upon treatment with the compound and UV irradiation. These data suggest that 3-*O*-methylfunicone inhibits the activities of Y family polymerases that cope with this specific genotoxic challenge. Whether the effects observed are due to inhibition of pol κ or another Y-family polymerase, for example pol η, needs to be determined (Mizushina et al. 2009b).

Mizushina et al. reported the discovery of a compound (1S*,4aS*,8aS*)-17-(1,4,4a,5,6,7,8,8a-octahydro-2,5,5,8a-tetramethylnaphthalen-1-yl) heptadecanoic acid, a derivative of a natural product kohamaic acid A (Fig. 2B), as a strong inhibitor of several mammalian polymerases, including pol η, pol ι, and pol κ, with IC_{50} values in the range of 7–8 μM (Table 1). Cellular studies show that this compound can inhibit the growth of HL-60 cancer

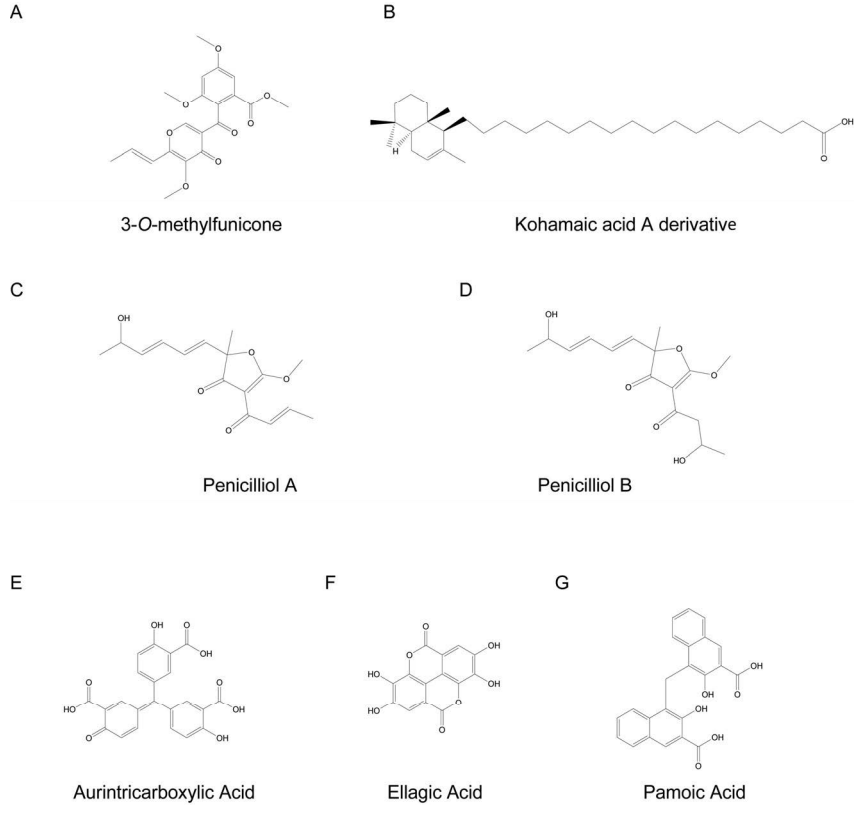

Figure 2. Structures of small molecule inhibitors of various TLS polymerases. (A) 3-*O*-methylfunicone (Mizushina et al. 2009b). (B) Kohamaic acid A derivative ((1S*,4aS*,8aS*)-17-(1,4,4a,5,6,7,8,8a-octahydro-2,5,5,8a-tetramethylnaphthalen-1-yl)heptadecanoic acid) (Mizushina et al. 2009a). (C) Penicilliol A (Kimura et al. 2009). (D) Penicilliol B (Kimura et al. 2009). (E) Aurintricarboxylic acid (Dorjsuren et al. 2009). (F) Ellagic acid (Dorjsuren et al. 2009). (G) Pamoic acid (Dorjsuren et al. 2009).

cells, demonstrating its potential as a chemotherapeutic agent (Mizushina et al. 2009a). Other natural compounds, such as penicilliols A (Fig. 2C) and B (Fig. 2D), isolated from a fungal strain derived from a sea moss, also selectively inhibit Y-family polymerases, namely pol η, pol ι, and pol κ. These inhibitors are most potent against mouse pol ι, with an IC_{50} of 19.8 μM and 32.5 μM for penicilliols A and B, respectively (Table 1). The mode of inhibition of pol ι by these compounds is non-competitive for both the DNA template-primer and the dNTP substrate (Kimura et al. 2009).

In contrast to the targeted methods used to identify natural product inhibitors as described above, a high-throughput screening procedure has been developed for the identification of small molecule inhibitors of TLS

Table 1. Summary of inhibitors of TLS polymerases[1]

inhibitor names	IC_{50} or K_d (μM)		
	pol η	pol ι	pol κ
3-*O*-methylfunicone	50.1	34.3	12.5
kohamaic acid A derivative	7.45	7.84	7.20
penicilliol A	ND	19.8	ND
penicilliol B	ND	32.5	ND
aurintricarboxylic acid	0.075	0.099	ND
ellagic acid	0.062	0.081	ND
pamoic acid	79	4.9	ND
aptamer 25	ND	ND	0.5
aptamer 32	ND	ND	0.41
aptamer 45	ND	ND	0.75

[1]ND: not determined; Kohamaic acid A derivative: (1S*,4aS*,8aS*)-17-(1,4,4a,5,6,7,8,8a-octahydro-2,5,5,8a-tetramethylnaphthalen-1-yl)heptadecanoic acid. For aptamers, values of dissociation constant (K_d) are shown instead of IC_{50}. The sequences of the aptamers are given in (Gening et al. 2006).

polymerases from large library sets (Dorjsuren et al. 2009). This assay utilizes polymerase-catalyzed strand displacement synthesis of a fluorescently-labeled oligodeoxynucleotide, in which the rhodamine fluorophore (TAMRA) is fully quenched in a duplex substrate by the quencher (BHQ-2). In the absence of an effective polymerase inhibitor, replication displaces the TAMRA-labeled reporter, resulting in a large increase in fluorescence; reactions containing inhibitors will retain the background fluorescence. With this methodology, inhibitors of pol η and pol ι have been identified using a 1536 well plate screening format (Dorjsuren et al. 2009). Aurintricarboxylic acid (Fig. 2E) and ellagic acid (Fig. 2F) inhibit these enzymes with the nanomolar potency; the former compound has an IC_{50} of 99 nM and 75 nM against pol ι and pol η, respectively, while the latter compound has an IC_{50} of 81 nM and 62 nM against pol ι and pol η, respectively. Pamoic acid (Fig. 2G) also inhibits these polymerases, though with lower potency (IC_{50} of 4.9 μM and 79 μM against pol ι and pol η, respectively) (Table 1). Although aurintricarboxylic acid and ellagic acid have improved potency relative to the previously identified compounds, they have other targets and thus do not serve as selective Y-family polymerase inhibitors (Blumenthal and Landers 1973; Bina-Stein and Tritton 1976; Benchokroun et al. 1995; Malmquist et al. 2001; Whitley et al. 2005; Cozza et al. 2006; Simeonov et al. 2009). Therefore, future studies require the development of potent compounds that selectively inhibit specific TLS polymerases.

In addition to small molecules, RNA aptamers could potentially be used to target TLS polymerases. RNA aptamers are RNA oligomers that have tight and specific binding to their targets. RNA aptamers that bind and inhibit pol κ have been described. These aptamers were originally discovered by screening a RNA library consisting of ~ $8x10^{12}$ molecules in an effort to identify aptamers that bind to and inhibit pol β. These aptamers have low selectivity as they bind to pol κ with similar affinities as to pol β; the dissociation constant of aptamer 25 is 430 nM and 500 nM for pol β and pol κ, respectively; aptamer 32 is 290 nM and 410 nM for pol β and pol κ, respectively; and aptamer 45 is 490 nM and 750 nM for pol β and pol κ, respectively (Table 1) (Gening et al. 2006).

Germane to these studies and as extensively discussed above, RNAi (siRNA or shRNA) against specific TLS polymerase used to deplete specific enzymes can modulate the cellular activities of these polymerases. The naked RNAi cannot cross the cellular membrane and enter the intracellular environment. However, recently, a novel RNAi delivery technology has been developed that couples RNAi to molecules such as pegylated immunoliposome, polyethylenimine, or myristoylated polyarginine peptides to enhance cellular uptake. With this technology, RNAi has been successfully delivered to tumors (Grzelinski et al. 2006; Medarova et al. 2007; Pardridge 2007; Wullner et al. 2009). Therefore, RNAi could be potentially used for the treatment of cancers. Additionally, gene-specific ribozymes could be employed for prevention of cancers, such as carcinogen-induced lung cancer, as demonstrated by Dumstorf and colleagues (Dumstorf et al. 2009).

In summary, TLS polymerases function to bypass drug-induced DNA lesions and can introduce mutations in the genomes during this process. Thus, inhibitors of TLS polymerases can sensitize tumor cells to DNA-damaging agents and reduce the level of drug-induced mutagenesis. In this regard, a combination therapy with a relevant DNA-damaging agent and TLS polymerase inhibitor could improve the therapeutic efficacy of chemotherapeutic agents by rendering tumor cells more susceptible to the cytotoxic effect of the drug and reducing the establishment of drug-resistant mutant cells. A strategy for such adjuvant chemotherapy would require that the agents have minimal cytotoxicity on their own, but enhanced cytotoxicity when combined. Furthermore, selective inhibitors that are toxic only to tumor cells would limit undesirable side effects. With an ongoing effort to develop highly potent and selective TLS polymerase inhibitors, the utilization of TLS polymerase inhibitors could be a novel therapy to combat cancers in the future.

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CHAPTER 12

p53 in DNA Damage, Repair and Cancer Therapeutics

Aditi Gurkar,[a] *Kiki Chu*[b] and *Sam W. Lee*[c,*]

INTRODUCTION

Since its discovery over 30 years ago, the p53 protein has emerged as a key tumor suppressor protein, and beyond doubt, a crucial player in cancer biology. p53 invokes its tumor-suppressive ability by acting as a mediator of various kinds of stress, such as DNA damage, oxidative stress, and oncogene activation (Horn and Vousden 2007). Through its activity as a transcription factor, p53 regulates the expression of various target genes to prevent tumor development, mainly by inducing cell cycle arrest and DNA repair or triggering cell death and senescence to maintain genomic stability (Kastan et al. 1991; Kuerbitz et al. 1992; Clarke et al. 1993; Lowe et al. 1993). Under mild or transient stress conditions, activated p53 targets several genes involved in cell cycle arrest and DNA repair to stop cells from proliferating and allow repair of any damaged DNA, preventing potentially oncogenic mutations from being passed on to the daughter cells. However, when stress-induced DNA damage is too severe to be reparable, p53 initiates programmed cell death/apoptosis and cellular senescence to eliminate or permanently arrest cells, respectively, which may have acquired irreparable and potentially oncogenic mutations. Relevantly, the human p53 gene

Cutaneous Biology Research Center, Massachusetts General Hospital and Harvard Medical School, 149 13th Street, Charlestown, MA 02129, USA.
[a]Email: agurkar@partners.org
[b]Email: kchu1@partners.org
[c]Email: swlee@partners.org
*Corresponding author

(*TP53*) is frequently mutated or inactivated in more than 50% of human cancers of different types (Olivier et al. 2010; Robles and Harris 2010). In addition, mice with a p53 gene (*Trp53*) deletion can develop normally, but develop cancer before the age of 6 months (Donehower et al. 1992). Thus, the importance of p53 in the inhibition of tumor development is indisputable, and consequently, p53 was assigned the title of "guardian of the genome" (Lane 1992). Furthermore, discovery and design of compounds that target the p53 pathway has provided a new approach to cancer therapy.

HISTORICAL PERSPECTIVE: FROM DISCOVERY OF p53 AS AN ONCOGENE TO A TUMOR SUPPRESSOR GENE

In 1979, scientists stumbled upon a novel 53 kDa protein, p53. This protein was found to interact with the oncogenic simian virus (SV40) large T antigen in transformed murine cells and embryonic carcinoma cells (DeLeo et al. 1979; Kress et al. 1979; Lane and Crawford 1979; Linzer and Levine 1979; Melero et al. 1979). Furthermore, p53 was later demonstrated to bind to adenovirus E1b-58 kDa tumor antigen and E6 oncoprotein of the DNA tumor virus human papillomavirus type 16 (Sarnow et al. 1982; Scheffner et al. 1990; Werness et al. 1990). Because it could associate with products of small DNA viruses and is usually more highly expressed in transformed cells, p53 was classified as an oncogene. To demonstrate the oncogenic properties of p53, a series of studies were carried out by multiple laboratories to assess the capability of p53 overexpression to promote transformation of normal cells. As anticipated, the results confirmed that overexpression of p53 in primary cells could induce immortalization and could also cooperate with other established oncogenes, such as *HRAS*, to transform normal cells (Eliyahu et al. 1984; Jenkins et al. 1984; Parada et al. 1984). More so, overexpression of p53 could augment the tumorigenic properties of established oncogenic cell lines (Eliyahu et al. 1985). However, later findings discovered that the originally identified p53 and the cDNA clones used in the overexpression experiments were a mutant form of the protein; in fact, wild-type p53 was able to suppress oncogenic transformation in embryonic fibroblasts (Finlay et al. 1989). This realization eventually shifted the view of p53 as an oncogene to a tumor suppressor gene.

Further sequence analysis of tumor-derived murine cell lines revealed that the murine *Trp53* gene was frequently mutated, and only those cell lines carrying p53 mutations possessed transforming capability (Eliyahu et al. 1988; Finlay et al. 1988; Halevy et al. 1991). From human colorectal tumor specimens, Baker and co-workers found that wild-type *TP53* alleles were frequently lost by mutations, deletions or a combination of both (Baker et al. 1989). Around the same time, two different research groups

independently demonstrated that transfection of wild-type p53 was able to suppress the transformation of primary rat embryo fibroblast by oncogenic activation (mutant p53/*HRAS*, *MYC*/*HRAS* and adenovirus E1A/*HRAS*) (Eliyahu et al. 1989; Finlay et al. 1989). Malkin and colleague later linked the inheritable human cancer predisposition disorder, Li-Fraumeni syndrome, to germline mutations of the *TP53* allele (Malkin et al. 1990). Consistently, mice with loss of p53 function (*Trp53*$^{-/-}$) are predisposed to tumor formation and develop tumors at very young age (Donehower et al. 1992). Taken together, these observations strongly validate p53 as a compelling tumor suppressor gene.

STRUCTURAL FEATURES OF p53 AND THEIR FUNCTIONS

The human *TP53* gene is located on chromosome 17p13.1 with 11 exons spanning over 20,000 bp (McBride et al. 1986). Due to alternative splicing, alternative initiation of translation, and alternative promoter usage, the *TP53* gene expresses 12 distinct p53 protein isoforms: full length wild-type p53 (FLp53), p53β, p53γ, Δ40p53, Δ40p53β, Δ40p53γ, Δ133p53, Δ133p53β, Δ133p53γ, Δ160p53, Δ160p53β and Δ160p53γ (Fig. 2) (Khoury and Bourdon 2010, 2011; Marcel et al. 2010). The 393 amino acid wild-type p53 protein consists of three major functional domains: N-terminal transactivation domain (TAD1 and TAD2, amino acid residues 1–42 and 43–92, respectively), a central DNA binding domain (DBD, amino acid residues 102–292), and a C-terminal oligomerization domain (OD, amino acid residues 319–359) (Fig. 1). Other characteristic domains include a proline-rich region with multiple copies of the PXXP sequence (amino acid residues 63–97, where X is any amino acid), three nuclear localization signals (NLS, amino acid residues 305–322, 369–375 and 379–384), and a nuclear export signal (NES, amino acid residues 339–352).

Transactivation Domain

The TAD1 of p53 is the primary domain required for its transactivation activity and interacts with various transcriptional machines to stimulate gene transcription, such as TBP, TFIIB, TFIIH, TAFII31, TAFII70, and the coactivator p300/CBP (Chen et al. 1993; Lu and Levine 1995; Thut et al. 1995; Leveillard et al. 1996; Gu et al. 1997; Lill et al. 1997). E3 ubiquitin ligase MDM2 (murine double minute 2, also as Hdm2 in humans) also binds to the FWL amino acid motif within the p53-TAD1, resulting in ubiquination of lysines in the DBD of p53, leading to p53 degradation (Honda et al. 1997). MDM2 binding to the TAD1 also prevents p53 interactions with the transcriptional machinery and p300/CBP (Chen et al. 1995). This disruption

Figure 1. Schematic representation of the functional domains of human p53.

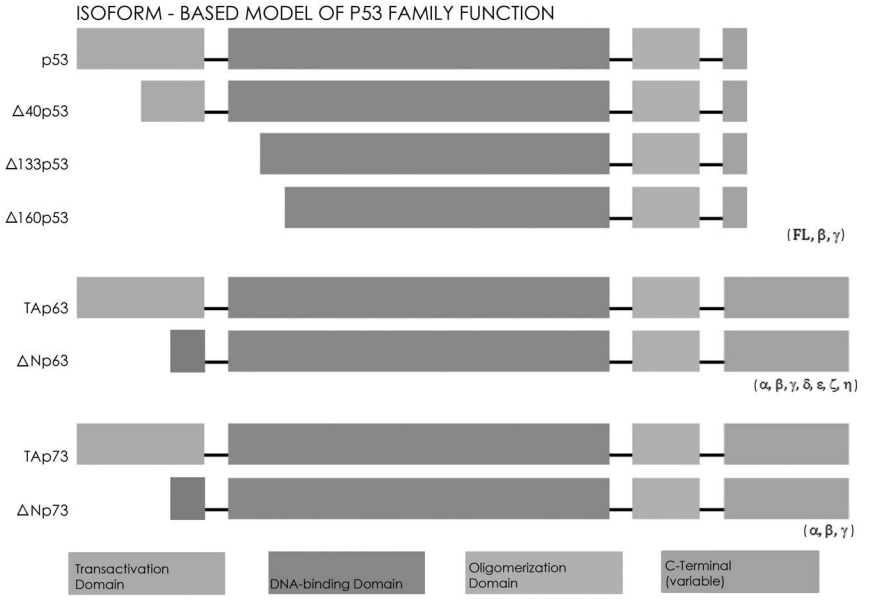

Figure 2. The p53 family of transcription factors.

Color image of this figure appears in the color plate section at the end of the book.

in interaction prevents p300/CBP-mediated acetylation of p53 and histones, and thus, chromatin remodeling for gene activation (Avantaggiati et al. 1997). Also, *MDM2* is a p53 target gene and is important for controlling p53 transcriptional activity through a negative feedback loop. Upon stress signals, the p53-MDM2 interaction is disrupted, and p53 is activated as a result of stabilization and accumulation in the nucleus. Stabilization of p53 usually results from phosphorylation of p53 in the TAD1 at multiple serine and threonine residues (as will be described later).

To determine the biological significance of the TAD1, the consequence of TAD1 deletion or mutation was investigated in mouse models. Knock-in mice containing the $p53^{QS}$ mutant (L25Q/W26S, homologue to human L22Q/W23S) resulted in embryonic lethality (Johnson et al. 2005). When mouse embryonic fibroblasts (MEFs) were derived from this mutant line, p53 was found to be highly stable due to the lack of MDM2 binding; however, it lacked the ability to activate the target genes *p21*, *MDM2*, *Perp* and *Noxa* (except *Bax*). Thus, the mutant protein was unable to induce p53-dependent cell cycle arrest, yet retained selective apoptotic responses to hypoxia. Similarly, in another transgenic mouse model where the ΔNp53 (or Δ40p53) isoform, which lacks the first 39 residues of the TAD1, was overexpressed, this protein was also found to be more stable and unable to activate *p21*, *GADD45* and *MDM2* (Maier et al. 2004). However, this transgenic mouse

model did not cause embryonic lethality like the $p53^{QS}$ mutant mouse, but instead, resulted in premature aging and growth suppression. This may be due in part to the heterotetramerization of ΔNp53 with wild-type p53, which in turn stabilizes the ΔNp53/p53 complex, leading to an imbalance in the p53 signaling network. Nevertheless, it can be concluded that the TAD1 is important for p53-mediated cell cycle arrest.

The second activation domain of p53 (TAD2) also interacts with components of the transcriptional machinery and p300/CBP. Within the TAD2 is the proline-rich domain that contains five PXXP motifs. These PXXP motifs are binding sites for SH3 domain proteins. TAD2 is also the site in which p53 interacts with corepressor deacetylase mSin3/HDAC complex, which is the major complex involved in p53 repression of genes (Murphy et al. 1999). Upon stress signals, serine and threonine residues, including Ser46, Thr55 and Thr81, in TAD2 are phosphorylated. In particular, Ser46 is involved in p53-mediated targeting of pro-apoptotic genes upon severe DNA damage. For example, a p53 mutant with a S46A substitution, which prevents phosphorylation, transcriptionally targets *MDM2* preferentially over *PTEN* following etoposide-mediated DNA damage, resulting in less apoptosis compared to wild-type p53 (Mayo et al. 2005). Conversely, a p53 mutant with a S46D substitution, which mimics a phosphorylated state of p53, preferentially targets *PTEN* over *MDM2* to promote apoptosis. In addition, Ser46 phosphorylation is required for selective induction of p53 pro-apoptotic gene *p53AIP1* (p53-regulated Apoptosis-Inducing Protein 1) in response to DNA damage (Oda et al. 2000b). Thr81 phosphorylation by JNK (NH_2-terminal kinase) is also important for p53-mediated cell cycle arrest and apoptosis in response to UV damage, as T81A mutant p53 failed to execute the response (Buschmann et al. 2001).

Unlike TAD1, the primary function of TAD2 appears to be the selective regulation of p53-mediated induction of apoptosis. For example, deletion of TAD2 or mutation of hydrophobic residues, Trp53 and Phe54 (W53Q/F54S), abrogates p53-mediated apoptosis, but only moderately impairs p53-mediated cell cycle arrest (Candau et al. 1997; Zhu et al. 1998). Furthermore, p53 mutants that lack the proline-rich domain are unable to induce apoptotic activity, demonstrating that the PXXP motifs are essential for p53-mediated apoptosis (Walker and Levine 1996; Zhu et al. 1999). More importantly, several mutations in the proline-rich domain were identified as spontaneous mutations (P85S and P89S) and as a germline mutation in Li-Fraumeni syndrome patients (P82L) (Thoresen 1992; Zhu et al. 1999). When both TADs are disrupted through quadruple mutations (L22Q/W23S/W53Q/F54S) or deletion (Δ133p53), p53 is transcriptionally inactive and is unable to induce apoptosis (Bourdon et al. 2005). Moreover, Δ133p53 dominant-negatively regulates wild-type p53. Overall, p53-TAD1 appears to act predominantly

in p53-mediated cell cycle arrest, whereas p53-TAD2 plays a greater role in p53-regulated apoptosis.

Central Core DNA-Binding Domain

The DBD of the p53 transcription factor is responsible for its sequence-specific recognition of DNA. As a tetramer, p53 binds to a consensus response element (RE): a palindromic 10 bp element defined as RRRCWWGYYY(n=0–13)RRRCWWGYYY (where R is adenine or guanine, W is a purine base, and Y is a pyrimidine) (el-Deiry et al. 1992; Funk et al. 1992). Studies with various mutations in the DBD have demonstrated its crucial role in the tumor suppressive function of p53. The DBD of p53 is its most highly conserved region, not only when p53 is compared with its homologues from *Drosophila melanogaster* and *Caenorhabditis elegans*, but also when compared to its mammalian family members, p63 and p73 (Kaelin 1999). The amino acid sequence of the DBD of p53/p63/p73 and the DNA sequences recognized by this domain have been largely preserved over billion years of evolution (Belyi and Levine 2009). Not surprisingly, more than 80% of human p53 mutations are observed in the DBD. In general, mutations in the DBD render the p53 protein inactive, i.e., unable to bind to DNA and transactivate target genes, yet the protein remains stable. Some of the most frequently mutated p53 residues in human cells ('hot spot' mutations) disrupt the p53-DNA interaction (R248 and R273) or alter the DBD structure (R175, G245, R249 and R282). The importance of these hot spot mutations in relation to cancer will be described later in the Chapter.

Oligomerization Domain

The OD is essential for active p53 to form a tetramer and bind to DNA for transactivation. The p53 tetramer is formed as a symmetric dimer of dimers, with all four subunits geometrically equivalent (Clore et al. 1994, 1995a,b; Lee et al. 1994; Jeffrey et al. 1995). Despite much focus on the active tetrameric form of p53, p53 protein also exists in monomeric and dimeric forms. However, the tetrameric p53 can bind far more tightly to its target p53-RE than the monomeric form (Weinberg et al. 2004). In addition, p53-interacting proteins, such as 14-3-3, can facilitate p53 tetramerization (Rajagopalan et al. 2008, 2010). Moreover, sometimes post-translational modifications on p53 require p53 to be in a tetrameric form (Maki 1999; Shieh et al. 1999; Itahana et al. 2009). The importance of the OD is evidenced by point mutations (L344P, R337C and R377H) that exist in this domain of Li-Fraumeni syndrome patients (DiGiammarino et al. 2002; Nakamura et al. 2007). Also, two p53 alternative splice forms that lack the OD exist: p53β and

p53γ (Bourdon et al. 2005). It has been shown that while p53β alone cannot transactivate *p21* or *Bax* promoters, p53β can interact with wild-type p53 and transactivate the *Bax* promoter, but not the *p21* promoter. The ability of p53 to homotetramerize and heterotetramerize through the OD adds another mode of regulation in which p53 exerts its activity.

The Nuclear Localization and Export Signals

The nuclear localization signal (NLS) and nuclear export signal (NES) of p53 are essential for p53 to shuttle between the nucleus and cytoplasm in response to stress signals. Under unstressed conditions, the leucine-rich NES (residues 339–352) is exposed and signals for nuclear export into the cytoplasm where p53 undergoes MDM2-mediated degradation (O'Keefe et al. 2003). This allows p53 to be maintained at a low level. Upon stress signals, the p53-MDM2 interaction is disrupted, and the bipartite NLS located in the hinge region (residues 305–322) is post-translationally modified by phosphorylation at Ser315 by CDK2/cyclin A and by acetylation at Lys320 by p300/CBP-associated factor (PCAF) (Bode and Dong 2004). These modifications result in increased DNA binding of p53 and, among many others, contribute to p53 stabilization in the nucleus by recruiting cofactors that mask the NES and inhibit p53 nuclear export. Once the p53-MDM2 feedback regulation kicks in, restoration of the MDM2 interaction with p53 will expose the NES and promote p53 nuclear export by CRM1 (exportin 1) and subsequent p53 proteosomal degradation in the cytoplasm (Gottifredi and Prives 2001). Thus, the NLS and NES of p53 are important components that contribute to the tightly regulated nuclear-cytoplasmic shuttling and subcellular localization of p53 and, as a result, to p53 signaling.

THE p53 FAMILY MEMBERS

In the late 1990s, two related orthologs of p53 were identified: p63 (Yang et al. 1998) and p73 (Kaghad et al. 1997). The p53 family of transcription factors (p53, p63, and p73) shares significant structural and functional domains: the N-terminal transactivation (TA) domain, the DBD, and the C-terminal OD (Fig. 2) (Murray-Zmijewski et al. 2006). Excluding p53, p63 and p73 also contain a sterol alpha motif (SAM) domain in the C-terminus, which is a stretch of 70 residues forming a globular five-helix structure that aids in oligomerization, interaction with other proteins, and interaction with RNA.

The human *TP63* gene was identified in 1998 on chromosome 3q27-29, consisting of 15 exons spanning over 270,000 bp (Yang et al. 1998). Like other members of the p53 family, the *TP63* gene encodes six different p63

isoforms due to alternative promoter usage (TA and ΔN) and C-terminal alternative splicing (α, β and γ) (Murray-Zmijewski et al. 2006). In addition, the p63 protein is comprised of the TA domain (amino acid residues 1–64), proline-rich domain (amino acid residues 63–127), DBD (amino acid residues 142–323), and OD (amino acid residues 353–397). Compared to the full length TAp63α, ΔNp63 lacks the N-terminal TA domain and is a dominant negative inhibitor of TAp63α and p53, (Yang et al. 1998). Accordant with the DNA damage response function associated with the p53 family, TAp63α can induce cell cycle arrest and apoptosis (Gressner et al. 2005), and its role as a suppressor of tumorigenesis and metastasis in cancer development and progression was extensively reviewed recently (Melino 2011; Nekulova et al. 2011) In addition, p63 has been shown to be important in epidermal morphogenesis and limb development in mice (Yang et al. 1998; Mills et al. 1999), and germline mutations of p63 has been found in humans that cause rare autosomal dominant developmental diseases (Celli et al. 1999).

The human *TP73* gene was identified in 1997 on chromosome 1p36.3, consisting of 15 exons spanning over 80,000 bp (Kaghad et al. 1997). The p73 protein is comprised of the same functional motifs: N-terminal TA domain (amino acid residues 1–47), proline-rich domain (amino acid residues 57–116), DBD (amino acid residues 131–312), and C-terminal OD (amino acid residues 352–390) (Scoumanne et al. 2005). In addition, the *TP73* gene gives rise to several different C-terminal isoforms (α, β, γ, δ, ε, ζ and η) and N-terminal isoforms (TA, ex2, ex2/3 and ΔN) due to alternative splicing and alternative transcriptional start sites (Murray-Zmijewski et al. 2006). Despite multiple mRNA variants (minimum of 35) that can be translated into at least 29 different p73 protein isoforms, it is still questionable whether all these variants are expressed. In fact, only about 14 different p73 isoforms have been described, but their biological functions appear to be limited. Nevertheless, since its original discovery, where p73 was mapped to a genomic region that is frequently mutated in various types of tumors, the role of p73 in cancer has been extensively studied (reviewed in Rufini et al. 2011). In general, TAp73 and ΔNp73 display opposite biological functions. TAp73 is an inducer of cell cycle arrest and apoptosis and, therefore, functions as a tumor suppressor (Rossi et al. 2004; Ramadan et al. 2005; Tomasini et al. 2008). ΔNp73 exerts a dominant negative action on TAp73 and p53, exhibiting a pro-survival role (Fillippovich et al. 2001; Stiewe et al. 2002; Zaika et al. 2002), and is often upregulated in human tumors though rarely mutated (Rufini et al. 2011). Recently, studies of various p73 mouse models have demonstrated that p73 plays an essential role in regulating neural stem cell self-renewal and maintenance in both the embryonal and adult central nervous system (Agostini et al. 2010; Fujitani et al. 2010; Talos et al. 2010; Holembowski et al. 2011).

The p53 family of transcription factors shares a high degree of homology, particularly in the DBD (55–87% homology) (Belyi and Levine 2009), suggesting that they can bind to the same DNA sequences and transactivate similar target genes. In fact, p63 and p73 can bind to p53RE and transcriptionally induce p53 target genes, such as *p21*, *Bax*, *PUMA*, and *NOXA*, in response to cellular stress, and cause cell cycle arrest or apoptosis (Harms et al. 2004; Perez et al. 2007; Smeenk et al. 2008; Noureddine et al. 2009). However, despite overlapping biological functions, each has distinct functions as described earlier. Interestingly, p63 and p73 are essential for p53-induced apoptosis in response to DNA damage (Flores et al. 2005). Furthermore, p53, p63 and p73 can regulate each other's expression though their respective promoters, as well as directly or indirectly interact with one another. The interrelationship of the p53 family members will not be described here in this Chapter, but the p53/p63/p73 network is intricately orchestrated to regulate cellular responses to various stimuli.

BIOLOGICAL FUNCTION OF p53 IN RESPONSE TO DNA DAMAGE

The primary biological role of p53 is to maintain genomic stability, and p53 exerts its task by regulating repair processes and protective mechanisms or arresting cell division and inducing cell death. To carry out its "guardian" role, p53 utilizes a wide spectrum of its abilities as a transcription factor or a regulatory protein to modulate numerous signaling pathways to ensure genome integrity (Fig. 3). In response to cellular insults that cause DNA damage, such as ionizing radiation, UV radiation, application of cytotoxic drugs or chemotherapeutic agents, or viral infection, or that involve non-DNA damaging stress, p53, which is usually kept at a very low level via its MDM2 interaction, is extensively post-translationally modified to increase its stability and nuclear accumulation, followed by subsequent activation (Fritsche et al. 1993; Appella and Anderson 2001); these post-translational modifications will be discussed in more detail later in the Chapter. Depending on the extent of the damage from genotoxic stress, p53 will determine the fate of the cell to either undergo growth arrest for DNA repair (usually when the damage is mild) or apoptosis (usually when the damage is irreparable).

CELL CYCLE ARREST AND DNA REPAIR

In response to agents that generate double strand breaks in DNA, such as ionizing irradiation (see Chapter 4), ATM (ataxia-telangiectasia mutated) protein kinase, which is a member of the phosphatidylinositol-3-kinase

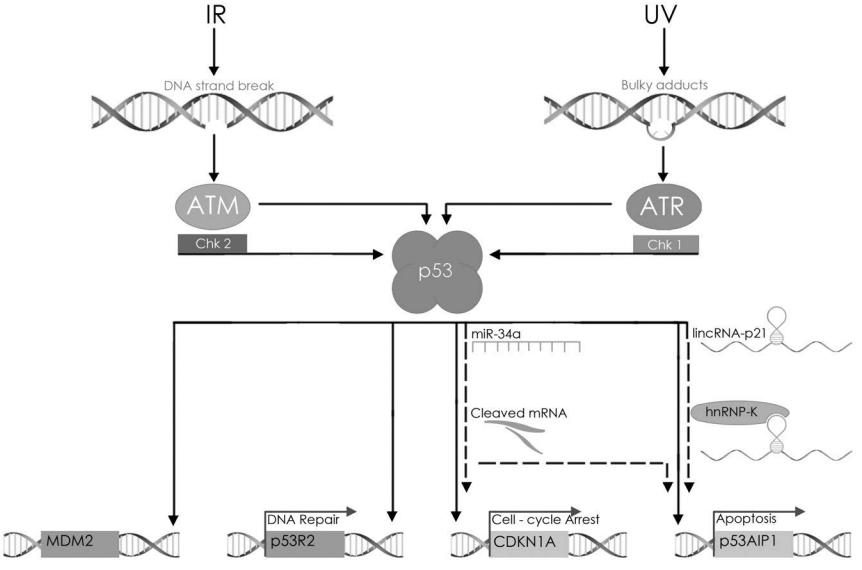

Figure 3. General cascade of p53-mediated DNA damage response.

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kinase family, is activated and directly phosphorylates Ser15 of p53. This in turn activates several other protein kinases that phosphorylate the N-terminal TA region, including Chk2 (checkpoint kinase-2), which phosphorylates Ser20 on p53 (Banin et al. 1998; Canman et al. 1998; Matsuoka et al. 1998). Similarly, when DNA damage leads to replication blockage, such as pyrimidine dimers caused by UV radiation, ATR (ATM and Rad3-related) kinase is activated and directly phosphorylates Ser15 of p53, leading to subsequent activation of Chk1 and its phosphorylation of p53 at Ser20 (Tibbetts et al. 1999; Shieh et al. 2000). These phosphorylation sites on the N-terminus of p53 are close to the region where MDM2 binds and thus, as a result, obstructs the interaction between the two proteins, allowing p53 to escape MDM2-mediated proteasomal degradation and leading to p53 stabilization and activation (Appella and Anderson 2001). Upon activation, p53 can transcriptionally target genes in cell cycle regulation and DNA repair (see Chapter 12). As a result, p53 can induce cell cycle arrest in G_1, G_2 and S phases of cell cycle (Agarwal et al. 1995). The theory is that the induction of cell cycle arrest by p53 will provide time for the cell to repair any genomic damage before entering the very crucial stages of DNA replication and mitosis. After DNA repair occurs, e.g., the nucleotide or base excision repair (BER) pathways (see Chapter 1), which are also facilitated by p53 action, cells will then re-enter the cell cycle (Zhou et al. 2001b).

Among the various p53 target genes, one of the best known downstream targets is cyclin-dependent kinase inhibitor 1A (*CDKN1A*), which encodes for $p21^{WAF1}$ (el-Deiry et al. 1993; Harper et al. 1993; Noda et al. 1994). As a primary mediator of p53-dependent G_1 arrest upon DNA damage, upregulated $p21^{WAF1}$ binds to cyclin-CDK complexes and blocks cyclin E/CDK2-mediated phosphorylation of Rb (retinoblastoma) protein, followed by release of the E2F transcription factor to induce expression of genes required for S phase progression (el-Deiry et al. 1993; Harper et al. 1993; Xiong et al. 1993; Chen et al. 1996). In human tumor-derived cells with mutated or dysfunctional p53, p53-dependent G_1 arrest fails to occur (Kuerbitz et al. 1992; Kessis et al. 1993). More so, MEFs derived from p53-null mice failed to undergo p53-dependent G_1 arrest in response to ionizing radiation (Kastan et al. 1992). Together, these observations support an important role for p53 in the G_1 arrest response to DNA damage.

In addition to DNA damage-induced G_1 arrest, p53 can block the G_2/M transition and stop cells from entering mitosis (Taylor and Stark 2001). Activated CDK1 (Cdc2) binds to cyclinB; this activated complex is a key determinant for cellular entry into mitosis. When p53 is activated in response to DNA damage, p53 regulates the cyclinB/CDK1 complex by transcriptionally targeting a subset of genes, including *$p21^{WAF1}$*, *Gadd45*, and *14-3-3*σ, leading to inactivation of the complex and blocking of the G_2/M transition. Induction of $p21^{WAF1}$ is necessary for sustained G_2 arrest in response to DNA damage (Bunz et al. 1998; Niculescu et al. 1998). Increased expression of Gadd45 (growth arrest and DNA damage inducible gene 45) upon DNA damage results in the Gadd45 protein binding to CDK1, promoting dissociation of CDK1 from the cyclinB/CDK1 complex and inhibition of its kinase activity (Wang et al. 1999; Zhan et al. 1999; Vairapandi et al. 2002). Furthermore, the scaffold protein 14-3-3σ chaperones the cyclinB/CDK1 complex from the nucleus to the cytoplasm, rendering it unable to be physically present in the nucleus to exert its cell cycle regulatory activity (Hermeking et al. 1997; Chan et al. 1999). To further enhance the cell cycle arrest, p53 can also transcriptionally repress the *CDC25C* gene, which encodes the M-phase inducer phosphatase 3 that directs dephosphorylation of cyclinB/CDK1 and triggers entry into mitosis (St Clair et al. 2004).

Once cells are arrested as a result of p53 activation, DNA repair mechanisms kick in, and it appears that p53 can additionally play a direct role in the actual repair process. A p53-inducible ribonucleotide reductase gene *p53R2* plays a critical role in supplying dNTPs for DNA repair. In response to DNA damage, *p53R2* is significantly induced in a p53-dependent manner (Tanaka et al. 2000). In addition to the Gadd45-mediated G_2 arrest in response to p53 activation, Gadd45 can interact directly with the core histones and destabilize histone-DNA complexes upon UV radiation, suggesting that Gadd45 can recognize and bind to UV-damaged chromatin

and modulate DNA accessibility for the DNA repair machinery (Carrier et al. 1999). APE1 (AP-endonuclease 1) and DNA polymerase β are two main proteins responsible for the BER process (see Chapter 8): APE1 is an endonuclease that cleaves the DNA at a damaged site and DNA polymerase β operates to insert a new nucleotide to replace the damaged (excised) one. Wild-type p53 has been shown to directly bind with APE1 and DNA polymerase β to facilitate BER, and this regulation does not require the transcriptional activity of p53, but rather the p53 protein itself (Offer et al. 2001). In summary, p53 can regulate many genes and directly interact with cellular proteins (of which only a few major ones are mentioned here) in response to DNA damage to facilitate DNA repair, including cell cycle arrest.

p53-MEDIATED APOPTOSIS: INTRINSIC AND EXTRINSIC PATHWAYS

The aforementioned DNA damage response mainly pertains to the cell survival aspect. However, when the damage is too great and the survival of the cell will compromise genomic integrity, p53 will elicit programmed cell death and drive cells toward apoptosis via two pathways: the intrinsic mitochondrial pathway and the extrinsic death receptor pathway.

For the intrinsic pathway, p53 transcriptionally activates several pro-apoptotic genes in the Bcl-2 family, such as *Bax* (Bcl-2-associated X protein) (Miyashita and Reed 1995), *Puma* (p53-upregulated modulator of apoptosis) (Nakano and Vousden 2001), *Noxa* (from the Latin word for "harm" or "damage") (Oda et al. 2000a), and *p53AIP1* (p53-regulated apoptosis-inducing protein) (Matsuda et al. 2002), as well as many other genes that encode for proteins that control mitochondrial membrane permeability and, thus, modulate the release of mitochondrial proteins that will subsequently execute apoptosis. Additionally, p53 can downregulate the expression of Bcl-2 (Miyashita et al. 1994), which would oppose apoptosis by binding and inhibiting Bax. Upon apoptotic stimuli where p53 is induced and Bcl-2 is inhibited, Bax translocates from the cytosol to the mitochondrial outer membrane and dysregulates the mitochondrial outer membrane permeability in order to induce the release of cytochrome C from the mitochondrial intermembrane space to the cytosol. The released cytochrome C then interacts with Apaf1 (apoptotic protease-activating factor-1) (Cecconi et al. 1998; Moroni et al. 2001), which is another p53-target gene that encodes a key component of the apoptosome, and activates caspase 9, initiating the downstream signaling cascade for apoptosis. Other BH3 domain-containing Bcl-2 family members, including Puma, Noxa and p53AIP1, interact with Bcl-2 to release Bax and promote the loss of mitochondrial membrane

potential, thereby inducing the release of cytochrome C. In addition to the transcription-dependent role of p53 in regulating the intrinsic mitochondrial pathway, a certain fraction of p53 can translocate to the mitochondria, where it directly interacts with Bcl-X_L/Bcl-2, displacing Bax, to induce mitochondrial membrane permeabilization and cytochrome C release (Mihara et al. 2003). p53 can also bind to the pro-apoptotic mitochondrial protein, Bak, and induce Bak oligomerization, which in turn facilitates the release of cytochrome C after mitochondrial membrane permeabilization. Furthermore, upon genotoxic stress, MDM2-mediated monoubiquitination of p53 recruits p53 to the mitochondria where the protein undergoes a rapid deubiquitination by mitochondrial HAUSP, generating stable nonubiquitylated proapoptotic p53 (Marchenko et al. 2007).

In addition to the role of p53 in controlling the mitochondrial intrinsic apoptotic pathway, p53 is an important regulator of the death receptor-mediated extrinsic pathway of apoptosis. The extrinsic apoptotic pathway is facilitated through cellular membrane-bound death receptors, including Fas/CD95 (cell-death signaling receptor) (O'Connor et al. 2000), DR4 (Liu et al. 2004) and DR5 (Takimoto and El-Deiry 2000), which are all transcriptional targets of p53 upon apoptotic stimuli. When p53 is activated in response to apoptotic stimuli (from extreme DNA damage, for example), both Fas and CD95 are transcriptionally activated. When the Fas ligand binds to the CD95 receptor, CD95 recruits several adaptor proteins, like FADD and FAF, to activate caspase 8 and caspase 10 cascade pathways that lead to cell death (Owen-Schaub et al. 1995; Bennett et al. 1998; Michalak et al. 2005). Induction of DR4 and DR5 can also trigger or induce apoptosis via the TRAIL death ligand (tumor necrosis factor-related apoptosis-inducing ligand) and the Fas ligand through caspase 8 (Sheikh et al. 1998; Liu et al. 2004). In addition, p53 may induce apoptosis via an endoplasmic reticulum-dependent pathway through transactivating *Scotin* (Bourdon et al. 2002). Scotin is a protein localized in the endoplasmic reticulum and nuclear membrane and has been shown to induce apoptosis in a caspase-dependent manner.

While p53 can exert its protective function to induce cell cycle arrest in damaged cells, it appears that cells that have undergone oncogenic transformation, like many tumor cells, are less susceptible to such protection from p53 (Crook et al. 1994). Therefore, the ability of p53 to induce apoptosis is of great importance. Consistently, there is an apparent correlation between the ability of p53 to induce apoptosis and its ability to suppress malignant transformation. For example, failure to induce p53-dependent apoptosis in mice accelerates brain tumorigenesis. Likewise, in mutant p53-R172P (the mouse equivalent of R175P in humans) mice, where the human R175P mutation demolishes the ability of p53 to initiate apoptosis, but not cell

cycle arrest, the animals developed very aggressive and fast-growing tumors, despite the late onset of tumorigenesis. Together, these observations suggest that the ability of p53 to regulate apoptosis is important for its tumor suppressor function.

p53 AND REACTIVE OXYGEN SPECIES

Recently, the role of p53 in regulating cellular reactive oxygen species (ROS) generation was examined. One of the early observations that p53 might be associated with ROS generation is the discovery of PIGs (p53-induced genes) that encode for redox-related proteins (Johnson et al. 1996; Polyak et al. 1997). Transcriptional induction of these pro-oxidant genes results in oxidative stress, followed by ROS production and consequently to apoptosis. Identification of other p53-induced pro-oxidant genes followed suit, including *p66*^{Shc}, *PUMA*, and *Bax* (Trinei et al. 2002; Macip et al. 2003; Liu et al. 2005). In addition to upregulation of pro-oxidant genes, p53 can suppress antioxidant genes, including *MnSOD* (manganese superoxide dismutase) (Drane et al. 2001; Dhar et al. 2006), to increase cellular ROS levels and elicit oxidative stress. Furthermore, monoubiquitylated p53 can directly bind to and inhibit MnSOD protein in the mitochondria to increase ROS levels and promote subsequent apoptosis (Zhao et al. 2005; Marchenko et al. 2007). However, the pro-oxidant role of p53 appears to be associated with when p53 is induced or under a hyperphysiological level following genotoxic stress. Under basal, unstressed conditions, p53 instead exerts an antioxidant role by maintaining a normal basal transcription of antioxidant genes, including sestrins and GPX1 (glutathione peroxidase-1) (Sablina et al. 2005). Under normal metabolism, a sufficient amount of ROS is generated that can cause significant damage to DNA, and normal p53 is believed to have a protective role here to eliminate such a threat and sustain genomic integrity. Interestingly, many types of cancer have elevated levels of ROS, partially due to the loss of p53's ability (mutant p53) to maintain its antioxidant role, thus contributing to tumorigenesis (Behrend et al. 2003; Klaunig and Kamendulis 2004). Targeting ROS by increasing ROS level to induce apoptosis in tumor cells may be a promising therapeutic approach. Proof of principle for this concept is provided in a recent article that demonstrates how a small molecule, Piperlongumine, can selectively kill cancer cells, and not normal cells, by enhancing further ROS accumulation in cancer cells that already possess high levels of ROS (Raj et al. 2011). Nevertheless, p53-mediated ROS generation is another mode of regulation by which p53 can contribute to DNA damage-induced apoptosis.

p53 AND NONCODING RNA

MicroRNAs (miRNAs) are small, non-coding RNAs of about 22 nucleotides in length, found in most eukaryotes, and are responsible for post-transcriptional regulation. miRNAs bind to a complementary sequence in the 3′-UTR (usually) of its target mRNA. This leads to repression of mRNA translation and/or transcript degradation and thus, gene silencing (Farh et al. 2005). Several laboratories reported around the same time that p53 is responsible for the transcription of specific miRNAs. They identified a family of miRNAs, e.g., miR-34, as direct targets of p53 that can mediate cell cycle arrest, apoptosis and senescence (Bommer et al. 2007; Chang et al. 2007; Corney et al. 2007; He et al. 2007; Raver-Shapira et al. 2007; Tarasov et al. 2007; Tazawa et al. 2007).

The miR-34 family is comprised of three processed miRNAs that are evolutionarily conserved: *miR-34a*, *miR-34b* and *miR-34c*. Both miR-34a (Bommer et al. 2007; Chang et al. 2007; He et al. 2007; Raver-Shapira et al. 2007; Tarasov et al. 2007) and miR-34b/c (Bommer et al. 2007; Chang et al. 2007; He et al. 2007), which share a common primary transcript, have a predicted p53 binding site upstream of its locus. Activation of p53 upon damage significantly induces the expression of the miR34 family members. Similar to the transcriptional regulation of its target genes, induction of miR-34 requires an intact p53-binding site, as indicated by kinetics studies and chromatin immunoprecipitation (ChIP) analysis (Bommer et al. 2007; He et al. 2007; Raver-Shapira et al. 2007). According to several studies, introduction of miR-34a in primary fibroblasts and in some cancer cell lines causes cell cycle arrest in the G_1 phase (Bommer et al. 2007; He et al. 2007). Similarly, transfection of synthetic miR-34b or miR-34c in neoplastic epithelial ovarian cells, OSN1 and OSN2, led to a significant decrease in cell proliferation and a decrease in colony formation in soft agar (Corney et al. 2007). Raver-Shapira et al. observed that inhibition of miR-34a by LNA (locked nucleic acid) prevents apoptosis in wild-type p53 cells (Raver-Shapira et al. 2007). Furthermore, it was observed that retroviral infection of miR-34a led to alteration in gene expression, affecting pathways such as DNA repair, cell cycle arrest and apoptosis (Chang et al. 2007). The miR-34 family of miRNAs directly targets genes such as CDK4, CDK6, and cyclin E (Lewis et al. 2005; Spurgers et al. 2006; Bommer et al. 2007). Recently, another novel target of miR-34a was found: c-Met. C-Met, a known proto-oncogene, is a membrane receptor that is known to promote tumor growth, invasion and metastasis. Studies indicated that miR-34a inhibits cell migration and invasion of hepatocellular carcinoma cells, HepG2, by targeting c-Met (Li et al. 2009).

Subsequent to the discovery of the miR-34 members, additional miRNAs regulated by p53 were identified: miR-107 (Yamakuchi et al. 2010),

145 (Sachdeva and Mo 2010), 192 (Georges et al. 2008), 101 (Tsuchiya et al. 2011). Angiogenesis necessary for tumor growth and invasion seems to be partly regulated by miR-107. In addition, miR-107 targets HIF1β, thereby controlling the hypoxic programming in colon cancer cells. This evidence suggests an additional tier of transcriptional regulation dependent on p53, i.e., inducing miRNAs (Yamakuchi et al. 2010). Sachdeva and colleagues reported miR-145 to be a p53 target in response to serum starvation or chemotherapeutic drugs (Sachdeva et al. 2009). In a mouse xenograft model, Ibrahim et al. demonstrated that systemic injection or local application of miR-145 suppressed c-Myc and ERK5 and increased apoptosis, thus repressing tumor growth (Ibrahim et al. 2011). miR-192 and miR-215 were also observed to be p53 targets, where overexpression of either one led to a G_1-G_2 arrest in HCT116 p53 wild-type, but not p53 null cells, by enhancing expression of p21 (Braun et al. 2008; Georges et al. 2008). These results together suggest additional p53 targets in the form of miRNAs, which help mediate its role in tumor suppression.

Recently, several large intergenic non-coding RNA (lincRNA) molecules have been identified and their function analyzed. LincRNAs are transcribed by RNA polymerases, but do not encode a protein product, and they range in size from hundreds to tens of thousands of bases. Although not well understood, some of these RNAs seem to have distinct functional roles in gene regulation (Guttman et al. 2009). Huarte and colleagues observed that a number of lincRNAs contain p53 binding sites. By performing ChIP analysis, they characterized the association of p53 at these motifs and identified certain lincRNAs as bonafide targets of p53. One such lincRNA, lincRNA-p21, controls expression of p53 target genes by interacting with heterogeneous ribonucleoprotein K (hnRNP-K), facilitating its proper localization (Huarte et al. 2010). hnRNP-K plays a role in chromatin remodeling and mRNA splicing and transcription (Bomsztyk et al. 2004). hnRNP-K has been previously found to form a repressive chromatin complex and probably blocks transcription (Kim et al. 2008). LincRNA-p21 can therefore control apoptosis by acting as a repressor in the p53 pathway through its interaction with hnRNP-K (Huarte et al. 2010). Thus, p53 modulation of lincRNAs seems to also play an important role in mediating regulation of p53 target genes. Whether one such lincRNA (or linc-RNA p21) plays a role in the regulation of p53 itself is still under investigation.

p53 REGULATION

As mentioned so far in the Chapter, p53 is a known tumor suppressor that acts as a transcription factor, controlling a number of target genes responsible for safeguarding our genome. *TP53*, the gene encoding p53, is

mutated in ~50% of human cancers. Furthermore, wild-type p53 protein expression is deregulated in many other types of cancer, thus contributing to tumor progression. To appreciate the importance of p53 during cancer progression, we first need to understand how p53 is regulated and kept in check under normal conditions.

p53 is post-translationally modified by several mechanisms that regulate its function (Fig. 4). Phosphorylation, mono- and poly-ubiquitination, sumolyation, neddylation, glutathionylation, acetylation and prolyl-isomerization are all forms of p53 modification (Appella and Anderson 2001; Bode and Dong 2004; Olsson et al. 2007; Kruse and Gu 2008; Vousden and Prives 2009). Some of these modifications are not well understood yet, while others play an essential role in the p53 regulation cascade. Phosphorylation, acetylation and ubiquitination will be further discussed in this Chapter (Fig. 5). DNA damage and other stressors lead to phosphorylation of p53, enhancing its activity. Protein kinases known to phosphorylate p53 residues include ATM, ATR (Lambert et al. 1998; Tibbetts et al. 1999), Chk1, Chk2 (Chehab et al. 2000), JNK, CK1 (Sakaguchi et al. 2000) and HIPK2 (D'Orazi et al. 2002; Hofmann et al. 2002). Phosphorylation of p53 contributes to its stabilization upon damage and also mediates selective promoter binding of its target genes to induce specific cell fates.

For example, ATM is known to phosphorylate multiple serine residues of p53: 9, 15, 20 and 46 (Saito et al. 2002). Phosphorylation of one or more of these sites can lead to disruption of MDM2 binding (Toledo and Wahl 2006) and recruitment of histone/lysine acetyltransferases (discussed below) (Polley et al. 2008; Teufel et al. 2009), thus increasing p53 protein levels upon stress. Mouse models with an S to A mutation at S15 or S20 suggest that there is redundancy in the function of these two phosphorylation sites in modulating p53 protein stability. Mice with both a S15 and S20 mutation display a stronger tissue specific pro-apoptotic phenotype (Chao et al. 2006; Toledo and Wahl 2006). In contrast, phosphorylation of S46, which is mediated by HIPK2 and DYRK2, is not required for p53 stability. Instead, this phosphorylation allows binding to and induction of specific

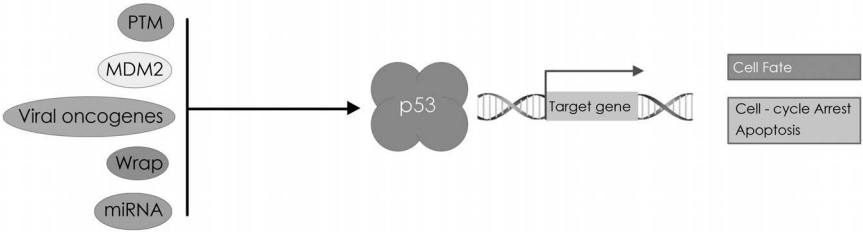

Figure 4. Regulation of p53 via different mechanisms.
Color image of this figure appears in the color plate section at the end of the book.

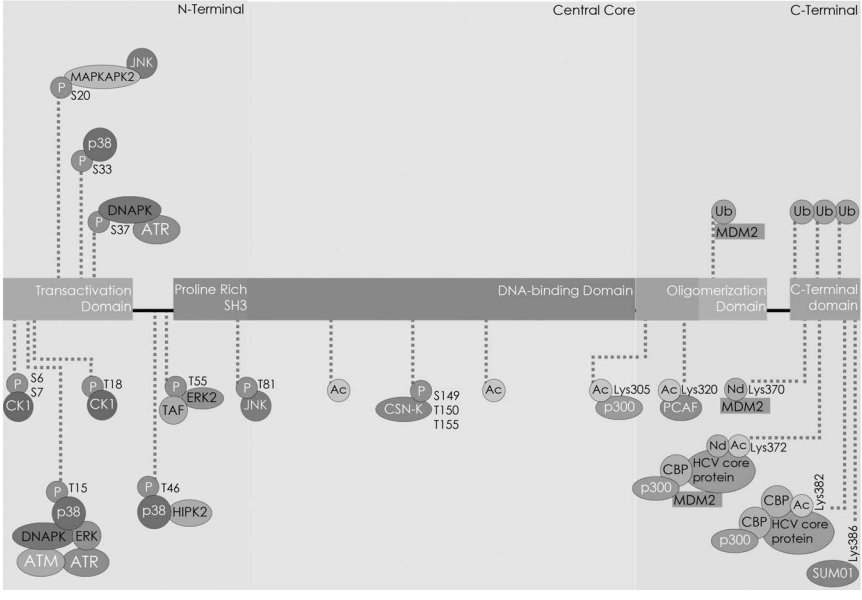

Figure 5. Post-translational modification (PTM) of human p53.

Color image of this figure appears in the color plate section at the end of the book.

pro-apoptotic genes, such as p53AIP1, but not cell cycle arrest genes such as p21 (Rinaldo et al. 2007; Taira et al. 2007). Sakaguchi and colleagues reported another site, S392, that is phosphorylated only in response to UV damage (single stranded DNA damage), but not γ-irradiation. S392 is phosphorylated by MAPK or casein kinase 2 (CK2) and promotes stabilization of the p53 tetramer (Sakaguchi et al. 1997; Kapoor and Lozano 1998). A knock-in mouse model with S389A (human S392) showed an increased predisposition to UV-induced skin cancer (Johnson and Attardi 2006). Surprisingly, S392 phosphorylation correlates with poor cancer prognosis (Matsumoto et al. 2004; Bar et al. 2009), suggesting additional functions of this phosphorylation site or its ability to stabilize mutant p53 gain-of-function tetramers. Other phosphorylation sites in p53 have been identified: S6 and S9 (Higashimoto et al. 2000) in the TAD, S315 (Blaydes et al. 2001) in the C-terminal end, and T150, T155 and S149 (Bech-Otschir et al. 2001) in the DBD, but their exact function remains to be elucidated. In addition, p53 has several serine/threonine sites that are constitutively phosphorylated during normal cell cycle progression, such as T55 and S376 (Satyamoorthy et al. 2000). These sites are dephosphorylated in response to DNA damaging agent exposures.

Acetylation of p53 at lysine residues plays an important role in its regulation. Until now, nine acetylation sites in p53 have been identified,

most of which lie in the C-terminal domain. Acetylation of p53 is known to promote stabilization of the protein by blocking those lysine sites from ubiquitination, and is also known to recruit cofactors for transcriptional activation of target genes. Several histone acetyl transferases (HATs) have been identified that acetylate p53, including p300, CREB-binding protein (CBP), p300/CBP associated factor (PCAF), MYST, TIP60, and males absent on the first (MOF) (Sykes et al. 2006; Allis et al. 2007; Kruse and Gu 2008). Acetylation of particular residues has been shown to mediate cell survival, whereas others are important for target promoter selection. In recent years, acetylation sites in the DBD have been discovered, K120 and K164, both of which having important roles in regulating p53 function. K120 acetylation is important for promoter sequence specific DNA binding of p53 (Sykes et al. 2006; Tang et al. 2006). Although redundancy is observed for acetylation of different sites, mutations of eight of the lysines inhibits p53 tumor suppression function, suggesting an indispensable role for acetylation.

Another key regulator of p53 expression is the E3 ligase, MDM family members, MDM2 (also known as HDM2) and MDM4. MDM2 interacts with the TAD of p53 and targets the protein for poly-ubiquitination and subsequent degradation (Momand et al. 1992; Oliner et al. 1993; Haupt et al. 1997; Geyer et al. 2000). MDM2 also has a nuclear export signal, and thus, upon binding p53, MDM2 mono-ubiquitinates p53 in the nucleus and exports the protein to the cytoplasm, thereby inhibiting p53 transcriptional activity (Tao and Levine 1999). Cytoplasmic localization of p53 is now believed to have a transcriptional independent role in mitochondrial dependent apoptosis (see section "p53-Mediated Apoptosis"). Recent evidence suggests that MDM2 and MDMX (an MDM2 family member) bind to promoters of certain p53 target genes, allowing them to selectively, negatively regulate p53 target gene expression (Tang et al. 2008). In normal cells, MDM2 is expressed at high levels and this keeps p53 in check. MDM2 in turn is a target gene of p53 and forms an auto-regulatory feedback loop (Juven et al. 1993; Perry et al. 1993; Barak et al. 1994; Lahav 2008). The importance of MDM2 is highlighted in MDM2 knockout mice, which are embryonic lethal, owing to high levels of p53-induced apoptosis. Double knockouts for MDM2 as well as p53 develop normally (Jones et al. 1995; Montes de Oca Luna et al. 1995), emphasizing the importance of MDM2-mediated p53 regulation.

Overexpression of MDM2 obstructs p53-mediated cell cycle arrest and apoptosis. Therefore, in response to DNA damage, stress or oncogene induction, a number of mechanisms block the MDM2-p53 interaction (Kruse and Gu 2009a; Vousden and Prives 2009). As mentioned above, upon stress such as UV or γ irradiation, several kinases, including ATM, ATR and Chk1/2, phosphorylate p53 at multiple sites. S15, T18 and S20 lie in the TAD of p53 and are phosphorylated in response to DNA damage. Phosphorylation of

these sites is known to promote p53 stabilization, leading to growth arrest and apoptosis. Although there is conflicting data on the importance of S15 phosphorylation for preventing the MDM2-p53 interaction, several studies suggest that T18 and S20 phosphorylation (sites phosphorylated after initial S15 phosphorylation) interferes with this interaction. By blocking this interaction, p53 is not ubiquitinated and degraded by MDM2, and therefore, can mediate its role as a tumor suppressor (Chehab et al. 2000; Hirao et al. 2000; Stommel and Wahl 2004). In 2008, Ofir-Rosenfeld et al. reported that MDM2 poly-ubiquitinates a ribosomal protein, RPL26, and targets it for degradation. RPL26 is known to bind p53 mRNA, stabilizing the transcript and augmenting translation. Downregulation of RPL26 by MDM2 leads to lower p53 protein levels, suggesting additional regulation of p53 via MDM2 (Ofir-Rosenfeld et al. 2008).

MDM2 is also known to form a complex with p300, an enzyme that acetylates p53 and activates it. Interaction with p300 disrupts the p53–p300 complex, thereby reducing p53 acetylation and its sequence specific DNA binding and transcriptional activity (Kobet et al. 2000). ChIP studies have shown that MDM2 is present at response element sites of certain p53 target genes, such as p21, in a p53-dependent manner. Upon DNA damage activation of p53, however, MDM2 seems to be released from such promoter sites, allowing transcription of needed target genes (Minsky and Oren 2004; Arva et al. 2005; Ohkubo et al. 2006; Tang et al. 2008). In addition, MDM2 is known to interact with histone modifying enzymes, such as HDAC1 (Ito et al. 2002) and KAP1 (Wang et al. 2005), allowing it to silence transcription through the deacetylation of histones at the promoter of target genes.

Similar to p53, MDM2 is tightly controlled in cells, so as to not have a deleterious effect on cell cycle progression or the DNA damage response. CKI (casein kinase I) phosphorylates MDM2 during the G_1 phase of the cell cycle, which in turn promotes MDM2 degradation by the E3 ligase, β-TRCP (Inuzuka et al. 2010). MDM2 is also known to self-ubiquitinate in response to DNA damage (Stommel and Wahl 2004, 2005). Whether this self-ubiquitination is important for MDM2 degradation is still unknown. Recent evidence from experiments involving mice harboring a single substitution C462A (needed for E3 ligase activity) in the ring-finger domain of MDM2 indicates that these animals are still capable of MDM2 degradation as compared to the WT. This suggests that the E3 ligase activity of MDM2 may not be important for its degradation (Itahana et al. 2007).

Stress such as damage promotes $p14^{ARF}$ upregulation, a protein that can bind to the central domain of MDM2 and block p53 degradation (Llanos et al. 2001; Sherr 2006). In addition, problems with ribosomal biogenesis due to starvation or nucleotide depletion, allows interaction of some ribosomal proteins, including RPL5 and RPL23, to MDM2. This physical association with ribosomal proteins leads to collapse of the MDM2-p53 complex. As a

result, p53 is activated and can relieve the stress through cell cycle arrest (Momand et al. 1998; Colombo et al. 2002; Zhang et al. 2003; Dai et al. 2004, 2008; Zhang and Lu 2009). As mentioned earlier, MDM2 is a transcriptional target of p53 and a feedback loop keeps both proteins in check. MDM2 is known to be amplified/overexpressed in several cancers with and without functional p53 (Momand et al. 1998). Functional p53 in cancer cells is of no consequence if high levels of MDM2 prevent its stability.

Similarly, MDM4, also known as MDMX and homologue of MDM2, negatively regulates p53. MDM4 is not known to bind to p53; instead, it exerts its inhibitory effect on p53 via dimerization with MDM2 (Finch et al. 2002; Marine et al. 2007; Kruse and Gu 2009a; Vousden and Prives 2009) Similar to MDM2, MDM4 expression is enhanced in many types of cancers (Ried et al. 1995; Tirkkonen et al. 1998; Ramos et al. 2001). Alternatively, a MDM2 protein, $p76^{MDM2}$, has been demonstrated to positively regulate p53 expression (Perry et al. 2000). *MDM2* is translated into two proteins that differ in molecular weight, $p90^{MDM2}$ and $p76^{MDM2}$. $p76^{MDM2}$ lacks amino acids in the N-terminus needed for p53 interaction. Interestingly, although a p53:$p76^{MDM2}$ interaction has not been observed, $p76^{MDM}$ modulates p53 stability and promotes its transcription (Saucedo et al. 1999). Hence, the MDM family of proteins is an important regulator of p53 function in normal and cancer cells.

In addition to MDMs, several other E3 ligases are known to poly-ubiquitinate p53: p53 induced RING-H2 domain containing protein (PIRH2) (Leng et al. 2003), constitutively photomorphogenic (COP1) (Dornan et al. 2004), carboxy terminus of Hsp70p-interacting protein (CHIP) (Esser et al. 2005), and ubiquitin-conjugating enzyme (Ubc13) (Laine et al. 2006). Male-specific lethal 2 (MSL2) (Kruse and Gu 2009b) and WW-domain containing protein 1 (WWP1) (Laine and Ronai 2007) are known to mono-ubiquitinate p53. Ubiquitination and deubiquitination (via HAUSP and USP10) (Brooks et al. 2007; Yuan et al. 2010) are important mechanisms of regulating p53 and need to be further investigated.

Regulation of p53 is also influenced by expression of viral oncogenes. Under conditions of stress, p53 is responsible for cell cycle arrest or apoptosis. This interruption of the cell cycle hinders viral replication. Therefore, viruses have developed strategies to render p53 inactive and thus hijack the control of cell cycle progression. Viral proteins known to interact with p53 are E1B 55kDa, SV40 large T antigen and HPV E6. E1B 55kDa adenoviral protein interacts strongly with p53 and inhibits the transcriptional activity of p53. It has been suggested that E1B is tethered to the transcriptional machinery, permitting recruitment of acetyl transferases or deacetylases, and affecting post-translational modifications of p53, thereby destabilizing the p53 protein (Yew et al. 1994; Liu et al. 2000b). In addition, it has been demonstrated

that E1B can lead to sequestration of p53 in the cytoplasm, thus inhibiting its transcriptional activity (Zhao and Liao 2003).

As discussed earlier, p53 was initially discovered because of its ability to bind SV40 large T antigen. p53 when bound to large T antigen is maintained in an inactive state and cannot bind to target gene promoters (Zambetti et al. 1992). Recently, Bocchetta et al. reported that SV40 large T antigen bound p53 is reprogrammed to activate cell growth by inducing growth factors such as insulin growth factor 1 (IGF1) and could thus encourage tumor growth (Bocchetta et al. 2008). In contrast, the human papilloma virus (HPV) protein E6 destabilizes p53 protein by promoting ubiquitination dependent degradation (Scheffner et al. 1990; Werness et al. 1990). E6 first forms a complex with an E3 ubiquitin ligase, E6AP, which can subsequently bind to p53 and poly-ubiquitinate it. It has also been proposed that E6 binding to p53 sequesters it in the cytoplasm either by masking the NLS or by enhancing p53 nuclear export (Talis et al. 1998). In conclusion, viral proteins are known to inactivate p53 function, block apoptosis, disrupt cell-cell adhesion and alter polarity and epithelial differentiation.

In 2009, Mahmoudi et al. demonstrated that a new gene *Wrap53* (WD40 encoding RNA antisense to p53) encodes a natural antisense RNA that binds and stabilizes the p53 transcript. Initial studies showed that Wrap53 was required for accumulation and stabilization of p53 protein. Furthermore, the authors verified that Wrap53α was necessary for p53 induction upon DNA damage (Mahmoudi et al. 2009). Recent evidence suggests that Wrap53 is overexpressed in cancer cells and its depletion can lead to apoptosis (Mahmoudi et al. 2011). Further studies to better understand the role of Wrap53 are required to fully appreciate p53 regulation.

Several lines of evidence imply miRNAs are important regulators of p53. Lodish and colleagues performed an *in silico* search for putative miRNA binding sites in the p53 3′-UTR using TargetScan and miRBase Target. They identified miR-125b, which could bind to human and zebrafish p53 mRNA and reduce p53 protein level substantially. The expression of p21 and Bax (p53 targets) was also downregulated by miR-125b, as expected. Further studies showed that miR125b regulation of p53 represses p53-mediated apoptosis in primary human lung fibroblasts. When the authors knocked down miR-125b by injecting morpholinos in zebrafish, p53 was stabilized and cells underwent apoptosis. This report was among the first that suggested negative regulation of p53 by a miRNA (Le et al. 2009).

Following this study, Hu et al. found another miRNA, miR-504, which binds the p53 transcript at two putative sites in the 3′-UTR region and negatively regulates p53 production. miR-504 reduces p53 protein accumulation, therefore suppressing its transcriptional activity after stress. Overexpression of miR-504 suppressed p53-mediated cell cycle arrest and apoptosis upon etoposide treatment. In addition, the authors demonstrated

in a xenograft model that stable ectopic expression of miR-504 in HCT116 wild-type ($p53^{+/+}$) cells promoted tumor growth *in vivo*. This data suggest an important role for miRNA regulation of p53 during cancer progression (Hu et al. 2010)

Positive miRNA regulators of p53 have also been identified. For example, miR34-a, a known miRNA activated by p53, targets silent information regulator 1 (SIRT1). SIRT1 belongs to a family of NAD^+-dependent protein deacetylases. SIRT1 is known to deacetylate histones, including H1, H3 and H4, but also deacetylates various proteins, including p53, p73, NFκB and Rb (Brooks and Gu 2009; Deng 2009). Ectopic expression of SIRT1 leads to deacetylation of p53 at K382 and attenuates its transcriptional activity upon DNA damage and oxidative stress (Luo et al. 2001; Vaziri et al. 2001). This leads to cell survival and tumor growth. miR34-a targets SIRT1 and suppresses its expression, thereby increasing p53 acetylation and the accumulation of p21 and Puma (transcriptional targets of p53), promoting cell cycle arrest and apoptosis (Yamakuchi et al. 2008). miR-29a also increases p53 activity and induces apoptosis. It does so by targeting p85α, the regulatory subunit of phosphatidylinositol-3 kinase (PI3K) and a Rho GTPase CDC42, both of which are negative regulators of p53 (Zhou et al. 2001a; Park et al. 2009).

As detailed above, regulation of p53 is a complicated and tightly controlled process. Whether mutant p53 can be regulated by one or more of these processes is still under study.

p53 MUTATIONS IN CANCER

DNA damage from endogenous (byproducts of metabolism such as ROS) or exogenous factors (UV, γ irradiation, etc.) are known to elevate expression of p53 (Lowe and Ruley 1993; Lowe et al. 1993). p53 in turn allows DNA repair or commits cells to senescence/apoptosis when the damage is too excessive and incapable of repair. Cells that have mutated/truncated/deleted p53 accumulate this damage and elude cell death, allowing tumor progression. The importance of p53 for tumor suppression is evident from the genetic mouse model of inactivated p53 ($p53^{-/-}$ mice). Although these mice develop normally, they are highly prone to cancer, with almost 60% of them developing tumors within 6 months (Donehower et al. 1992).

Interestingly, ~75% of mutations in *TP53* are single amino acid substitutions (Martin et al. 2002). Some of these mutations render p53 inactive, while others are gain-of function mutations. In addition to single amino acid substitutions, deletion, loss of the allele(s) and rearrangement of the gene have been observed in neoplasias. Normal (adjacent, non-adjacent) tissue examined from cancer patients harbors only the wild type allele,

emphasizing the role of p53 as a tumor suppressor (Soussi 2005). Almost all cancer related p53 mutations are somatic mutations with the exception of families prone to cancer, such as the Li-Fraumeni syndrome.

As mentioned earlier, p53 has three major regions: the N-terminal TAD, the sequence-specific core DBD, and the C-terminal OD. ~98% of the mutations lie in the DBD (Fig. 6), thus affecting the transcriptional activity of p53 (Hainaut and Hollstein 2000). Almost 40% of these mutations occur in six 'hot spots'—five of which encode the codon 'Arg' (Wong et al. 1999). Specific mutation spectra are seen commonly in one cancer type versus another. For example, V157F and R158L are two common mutations observed in lung cancer compared to other cancers (Shi et al. 2005). It is speculated that these mutations frequently arise due to exposure to cigarette smoke, which contains numerous carcinogens including benzo-[a]-pyrene (B[a]P). R249S is another example of a common mutation found in liver cancer and has been attributed to aflatoxin B1 containing food (Aguilar et al. 1993; Shi et al. 2005).

Mutation of p53 in its DBD gives rise to two classes of p53 alterations: 1) contact site mutants and 2) conformational mutants. Contact site mutations affect single amino acids that have direct contact with DNA (e.g., R248W and R273H) (Cho et al. 1994; Rolley et al. 1995). Conformational mutants, on the other hand, alter the structure of the protein (e.g., R249S and R175H) (Cho et al. 1994). Most of these mutations, regardless of their class, result in loss of p53 transcriptional activity. Additionally, some of the mutation types exhibit a dominant negative effect, typically by binding wild-type p53 and inhibiting its normal function (Harvey et al. 1995), or express a gain-of-function effect, promoting tumorigenesis (Wolf et al. 1984; Dittmer et al.

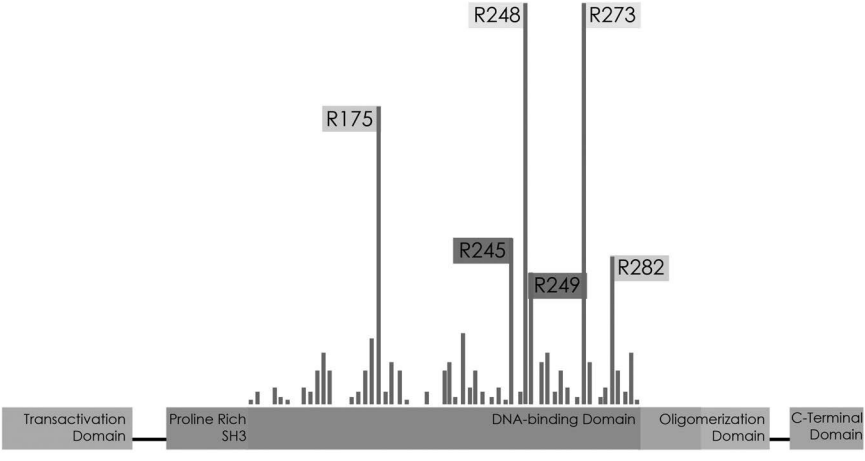

Figure 6. 'Hot spot' mutations of human p53 commonly observed in cancer.

Color image of this figure appears in the color plate section at the end of the book.

1993; Ko and Prives 1996). Some conformational mutants bind to heat shock proteins and are known to be highly stable, contributing possibly to the gain-of-function role of the mutant p53 (Hinds et al. 1987). These mutations therefore provide a selective advantage during cancer progression. For example, expression of R175H and R273H in p53 null cells increases growth in soft agar and enhances tumor progression (Dittmer et al. 1993).

Several knock-in mouse models have been generated to better understand the role of p53 hotspot mutations. Interestingly, heterozygous mutant mice for codons 172 (in humans- 175) and 270 (in humans- 273) both exhibit an increase in metastatic tumors as compared to null mice (Liu et al. 2000a; Lang et al. 2004; Olive et al. 2004). Such mice also display different tumor spectra from the null mice, suggesting that these mutations may be oncogenic in certain tissues/backgrounds. p53 mutants appear to display their dominant negative effect by inhibiting wild-type p53 function. As mentioned earlier, p53 oligomerizes to exert its effect as a transcriptional regulator. Mutant p53 forms heterodimers with wild-type p53, thus interfering with its activity (de Vries et al. 2002). *In vitro* experiments introducing exogenous mutants, as well as *in vivo* mouse models with one wild-type allele and one mutant allele, demonstrate the dominant negative effect of specific mutant p53 proteins (Liu et al. 2000a; Lang et al. 2004). Recent evidence also suggests that some p53 mutants can lead to aberrant transcription of relevant target genes, including multidrug resistance-1 (MDR1) (Chin et al. 1992), anti-apoptotic protein BAG-1 (Yang et al. 1999) and a mitochondrial transport protein Tim50 (Sankala et al. 2011), resulting in an increased growth rate and chemoresistance.

Single nucleotide polymorphisms (SNPs) have been observed in the *TP53* gene, and evidence suggests that they are important determinants of cancer predisposition, rate of response to therapy and survival (Sullivan et al. 2004). A commonly studied SNP is the substitution of arginine (CGC) to proline (CCC) at residue 72 (R72P) (Matlashewski et al. 1987). This SNP is known to play a role in apoptosis and cancer progression, and data suggests a strong correlation between the codon 72 SNP and age of disease onset, DNA damage repair capacity and survival (Pim and Banks 2004). Polymorphisms have been detected in both the intronic as well as coding sequences of p53 and in p53-related proteins p63 and p73 (Whibley et al. 2009). Large population studies to determine the role of other p53 polymorphisms in oncogenesis are underway and may potentially lead to early cancer detection and prevention.

Another modern take on p53 is that a transcription-independent role of the mutant p53 may play a role in tumor progression. Suzuki et al. noticed that p53 is an important regulator of the miRNA processing machinery. Primary miRNA (pri-miRNA) transcripts are usually transcribed by RNA polymerase II (Pol II) and further processed by Drosha (in association with a

complex that includes DGCR8, p68 and p72) to form pre-miRNA and finally miRNAs. Wild-type p53 directs the binding of pri-miRNA with Drosha to facilitate processing. This results in increased selective miRNA induction that mediates cell cycle arrest, as well as apoptosis, upon stress. These miRNAs target cell cycle regulators such as K-Ras (miR-143) and CDK6 (miR-16-1). Interestingly, although the regulation of pri-miRNA processing is independent of p53 transcriptional activity, the DBD is required for this process. Hence, wild-type p53 mediates tumor suppression independent of its transcriptional activity. However, the authors observed that introduction of mutant p53 (R175H or R273H) in HCT116 $p53^{-/-}$ cells significantly decreases the levels of these miRNAs upon damage. It does so by inhibiting the association of p68 (RNA helicase) and the Drosha complex. This data suggest another mechanism through which mutant p53 can allow cancer progression (Suzuki et al. 2009; Toledo and Bardot 2009).

Mutant p53 is known to inhibit its family members, p63 and p73. TAp63 and TAp73 are essential for preventing cancer progression as demonstrated by knock out mouse models. TAp63 is essential for inducing senescence upon DNA damage and TAp73 is important for genetic stability. There is evidence suggesting that p53 binds to p63 and p73, and inhibits their normal function, thus contributing to tumor progression.

WILD-TYPE p53: CURSE OR BOON IN CANCER

Mutations of p53 in cancer have gained a lot of interest since its discovery as a tumor suppressor. However, it is important to note that p53 is mutated in only 50% of cancers. This evidence raises a question as to whether wild-type p53 plays an important role in promoting tumorigenesis. For example, wild-type p53 plays a critical role in the survival of cells after mild DNA damage that can be easily repaired. However, if wild-type p53 can play a similar role in cancer cells, it can have a deleterious effect on cancer progression (Kim et al. 2009). Evidence from studies of radiation therapy or chemotherapy for cancer, in which wild-type p53 promotes survival, support this view (Scott et al. 2003; Bertheau et al. 2008). p53 is now known to play a role in glycolysis (Vousden and Ryan 2009) and modulating oxidative stress (as discussed above), both of which are vital for cancer cell survival. One such cancer cell viability-promoting pathway is autophagy. Tumor cells typically have limited nutrient supply. Therefore, they undergo a catabolic process of autophagy, in which they breakdown cellular organelles and long-lived proteins to provide much needed energy for survival (Mizushima and Klionsky 2007). However, deregulated autophagy (high levels) can lead to autophagy-dependent cell death (Levine and Yuan 2005). Scherz-Shouva et al. recently describe how p53 maintains the autophagic flux by post-

transcriptionally down-regulating LC3 (protein needed for autophagosome formation). In p53 null cells, the authors found that LC3 accumulates to very high levels and leads to loss of cell viability, suggesting a role of wild-type p53 in cancer cell survival (Scherz-Shouval et al. 2010). Another example is the role that p53 plays in the pentose phosphate pathway (PPP). p53 can modulate glycolysis to impede oncogenic transformation; however, p53 can also promote alternate metabolic pathways such as PPP, leading to survival of tumor cells (DeBerardinis et al. 2008). Limiting ROS and thus avoiding oxidative stress- induced apoptosis seems to depend on functional wild-type p53. Although p53-mediated regulation of ROS is important in maintaining the genome, the same antioxidant response in cancer cells could lead to chemoresistance and cancer cell viability (Gottlieb and Vousden 2010). Growing evidence, therefore, suggests that it is of utmost importance to understand the role of p53 as a tumor suppressor in normal cells, but also as a potential oncogene in tumors.

p53 AND THE MICROENVIRONMENT

Tumor microenvironment and interaction of cancer cells with the surrounding stroma has grabbed a lot of attention in recent years. Multiple studies suggest that induction of p53 not only influences self-cell fate, but also alters gene expression in the surrounding environment. It does so, apparently, by significantly altering secreted proteins—now known as the 'p53 secretome' (Khwaja et al. 2006). In an interesting study by Kiaris et al., they implanted identical MCF7, human breast cancer cells, in p53 wild-type or knockout mice and observed that tumor development was advanced in the p53 null mice. Upon further xenograft-fibroblast co-inoculation studies, it was observed that cancer cells inoculated with p53 null fibroblasts had a higher proliferation rate and reduced apoptosis (Kiaris et al. 2005). The importance of p53 in the microenvironment was also demonstrated from a study of a mouse model of prostate cancer, where loss of p53 in the mesenchymal cells led to tumor progression and invasion (Hill et al. 2005). In 2006, the Oren laboratory discovered the role of p53 in the production of a chemokine, SDF-1. In this study, they demonstrated that conditioned medium from cultured p53 null MEFs can increase invasion and metastatic properties of cancer cells (Moskovits et al. 2006). In addition, several reports suggest a role for p53 in neoangiogenesis. It has been observed that loss of wild-type p53 correlates with increased angiogenesis, leading to tumor progression. A number of p53 target genes have been reported to play a role in inhibiting angiogenesis, including the anti-angiogenic

protein thrombospondin (Tsp-1) (Dameron et al. 1994; Holmgren et al. 1998; Nishizaki et al. 1999; Narendran et al. 2003). Although the exact role, cause and consequence of stromal p53 in tumor suppression are unknown, its importance has been well demonstrated. Whether stromal p53 is maintained or lost during cancer progression will have to be further investigated in cancer patients.

REACTIVATION OF MUTANT p53: PROMISING CANCER THERAPY

As detailed above, p53 is a tumor suppressor and its activation in cancer cells may help tumor regression. Evidence for this notion comes from several animal studies in which p53 reactivation led to tumor clearance. In 2006, a mouse model of switchable p53 knock-in (carrying one wild-type copy and one that is 4-OHT dependent) showed that restoration of p53 in lymphomas led to massive apoptosis and increased survival (Martins et al. 2006). Ventura et al. developed a *Cre-loxP* based strategy to control p53 expression temporally and, in this system, demonstrated that p53 restoration led to regression of tumors without affecting normal cells. The mechanism through which p53 does so is by causing apoptosis or cell senescence (Ventura et al. 2007). In the same issue of *Nature*, Scott Lowe's group reported that restoration of p53, even briefly, in a mosaic mouse model of liver carcinoma led to tumor regression. However, they believe the reason for tumor clearance after p53 reactivation is due to senescence and not primarily apoptosis (Xue et al. 2007). Nonetheless, all these studies observed that loss of p53 activity is highly selected for during tumor progression and reactivation of the activity inhibits cancer progression.

Although restoration of p53 seems to be promising as a cancer therapeutic, p53 is not an easy druggable target. It is a transcription factor that activates or represses a number of target genes to safeguard the genome. It is not a receptor or enzyme, and hence, kinetic studies to determine if p53 is a direct target of the regulatory drug are not easy to perform. In addition, the protein forms a tetramer (McLure and Lee 1998); therefore, a small molecule/drug that binds to p53 needs to preserve its tertiary structure to maintain protein activity. p53 is inactivated in most tumors, but the mechanisms that drive the inactivation are diverse as described in earlier sections. To complicate the process, p53 is present in both normal, as well as, in cancer cells; thus activating p53 uniformly may cause non-targeted apoptosis and toxicity. While reactivating p53 in cancer is a daunting task, its benefits may be innumerable.

WILD-TYPE p53 ACTIVATION IN CANCER

Gene therapy involves the introduction of wild-type p53 via an adenoviral vector or oncolytic adenoviral expression. Gendicine and Advexin (INGN 201) are both examples of adenovirus based p53 therapy currently in clinical trials in the USA to treat head and neck cancer (Clayman et al. 1998, 1999; Han et al. 2003; Speetjens et al. 2009). Although, p53 adenoviral based therapies have shown some efficacy in impeding tumor growth, some side effects have also been associated. Mild side effects, such as fever and injection site pain, have been observed in clinical trials (Pan et al. 2009). In addition, severe toxicity was observed, but this may be an effect of apoptotic signals from tumor cells on neighboring cells (Roth 2006). In contrast to gene therapy, where a wild-type copy of p53 is introduced in a non-discriminatory fashion and is incapable of self-replication, oncolytic virotherapy uses a different method to restore p53. In this strategy, the virus can replicate only in cancer cells (anti-viral response observed in normal cells) or replication of the virus is coupled to induction of oncogenes (Crompton and Kirn 2007; Bazan-Peregrino et al. 2008). Examples of such virotherapy are Onyx-015 (E1B deleted) (Bischoff et al. 1996; Heise et al. 1997), H101 (Xu et al. 2003; Yuan et al. 2003; Lu et al. 2004; Xia et al. 2004) and KH901 (Fujimoto et al. 2006; Mace et al. 2007; Shen et al. 2007; Chang et al. 2009). The oncolytic herpes simplex virus (HSV), ONCO-Vex GM-CSF, has also been tested in clinical trials; however, long term toxicity and recurrence data are not yet available (Fujimoto et al. 2006; Mace et al. 2007). This modality of treatment sounds promising, but the right dosage and route of administration for delivery of these non-replicating adenoviral vectors has to be inspected further.

Several cancers have wild-type p53 status (multiple myeloma, chronic lymphocytic leukemia (CLL), acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), Hodgkin's, etc.), but activating this endogenous p53 has presented scientists with a few challenges. In many cancers with wild-type p53, its protein activity is abrogated by over-expression of negative regulators such as MDM2 and MDM4. As discussed earlier, animal experiments using a genetic model of low *Mdm2* expression resulted in decreased tumor formation that is dependent on p53 (Alt et al. 2003; Mendrysa et al. 2006). Also, blocking MDM2:p53 interactions to stabilize and activate p53 has been shown to sensitize cancer cells to chemotherapy and radiotherapy (Perry 2004; Guo et al. 2007; Villalonga-Planells et al. 2011). These results suggest that inactivating/inhibiting MDM2 activity/binding is an attractive treatment in wild-type p53 expressing cancer cells.

MDM2 is an E3 ligase responsible for p53 ubiquitination and degradation; therefore, accumulation of p53 using a proteosome inhibitor, Velcade, has been examined (Wang et al. 2010). In this pilot study, the

authors demonstrated that treatment of human breast cancer cell lines leads to cell cycle arrest by the induction of the p53 substrate, p21, and eventually apoptosis. However, Velcade does not target p53 directly; thus, side effects to normal cells is being examined currently. Direct inhibition of the MDM2:p53 interaction would also promote p53 stabilization. Interestingly, p53 binds to MDM2 through six amino acids ($T^{18}FSDLW^{23}$) (Picksley et al. 1994) and several classes of compounds can hinder this contact. They include benzodiazepinediones, cis-imidazolines (nutlins), sulfonamides, quiloninoles, terphenyls, spiro-oxindoles, pyrrolidine-2-ones and isoindolinones (Essmann and Schulze-Osthoff 2011).

Biacore technology, using recombinant p53, identified nutlins as p53 interactors from a screen of diverse synthetic compounds (Vassilev et al. 2004). Crystal studies revealed that nutlins fit into the p53-binding pocket of MDM2, thus inhibiting the MDM2:p53 interaction. This in turn leads to p53 accumulation and induction of p53 target genes such as p21 and puma. In a xenograft model using the human osteosarcoma cell line, SJSA-1, nutlin administration led to 90% inhibition of tumor growth compared to vehicle-control treatment. Since the discovery of nutlins, several studies demonstrating compound efficacy in various wild-type p53-exhibiting tumors have surfaced (Kojima et al. 2005; Tovar et al. 2006; Van Maerken et al. 2009). For example, in mouse xenograft studies, Nutlin-3 treatment shows considerable tumor shrinkage with low toxicity (Van Maerken et al. 2009). Similarly, Nutlin-3 has been observed to induce cell cycle arrest in normal cells (reversible), but induces apoptosis, senescence and autophagy in cancer cells (Efeyan et al. 2007; Zauli et al. 2007; Vousden and Ryan 2009). Therefore, nutlins still remain one of the most promising drugs targeting the p53 pathway.

Recently, another small molecule, NJ-26854165, has been shown to block p53 proteosomal degradation and induce apoptosis in cancer cells. Although efficacy of this drug is promising, whether this small molecule directly targets MDM2 is still debated (Kojima et al. 2010) Several other candidate molecules that block the MDM2:p53 interaction are in pre-clinical/clinical trials such as RG7112 (F.Hoffman-La Roche), MI-219 and its analogs (Shangary et al. 2008a,b; Sun et al. 2008; Mohammad et al. 2009), PXN727 and PXN822 (Priaxon).

As mentioned earlier, MDM4 regulates p53 function by forming protein heterodimers. In some cancers, p53 inactivation is observed due to overexpression of MDM4 (Danovi et al. 2004; Laurie et al. 2006; Popowicz et al. 2007). MDM4 does not have a p53-binding pocket like MDM2 (Popowicz et al. 2007); hence, drugs that target the MDM2 pocket to activate p53 fail in a setting of MDM4 overexpression. Thus, finding small molecules that target both MDM2 and MDM4 may be necessary to activate p53. Recently, SJ-172550, a small molecule that blocks the MDM4:p53 interaction was

identified (Reed et al. 2010). Further studies to examine the efficacy of nutlin-3 and SJ-172550 in conjunction are eagerly awaited.

Other pathways to activate wild-type p53 have also been explored as targets for drug therapy. In 2008, Lain et al. identified a class of small molecules, called Tenovins, that did not seem to affect p53 synthesis; instead, they protected it from MDM2 mediated degradation. Target identification studies showed that Tenovin targets two members of the NAD^+-dependent histone deacetylase family, SIRT1 and SIRT2 (Lain et al. 2008). SIRT1 is known to deacetylate L382, exposing the lysine residue for ubiquitination and facilitating degradation of p53 (Vaziri et al. 2001). Tenovins-1 and -6 inhibit the deacetylase function of SIRT1 and SIRT2, thus allowing accumulation of wild-type p53 (Lain et al. 2008). In addition, nuclear export inhibitors, which inhibit CRM1-mediated p53 export (Kudo et al. 1999), and MDM2 E3 ligase inhibitors (Yang et al. 2005; Herman et al. 2011) have been identified. RITA (reactivation of p53 and induction of tumor cell apoptosis) was found from a cell-based screen that looked at cell viability of HCT116 $p53^{+/+}$ and $p53^{-/-}$ after treatment with a compound library. Further investigation suggested that RITA binds directly to p53 and stabilizes the protein (Issaeva et al. 2004). However, recently, it has been observed that RITA can bind multiple proteins independent of p53, thus raising doubt about the mechanism of action.

MUTANT p53 REACTIVATION IN CANCER

More than 50% of cancers harbor a p53 mutation that exhibits a gain-of-function phenotype. As discussed earlier, some mutations of p53 stabilize it, leading to significant accumulation of the mutant protein. Reactivation of such mutant p53 into wild-type copies can possibly lead to better tumor clearance.

Several approaches have been examined to reactivate p53 *in vitro*: some of them cell-based and some target-based, yielding to the identification of several small molecules (Mandinova and Lee 2011). An early approach was based on the premise that stabilizing p53 in the wild type conformation could lead to induction of target genes. A monoclonal antibody, PAb421, and a synthetic peptide, p53C, which bind to the C-terminus of p53 and activate its DNA binding, were identified. However, the stability and delivery of such therapeutics to the tumors has been a dilemma (McLure and Lee 1998; Selivanova et al. 1999).

Bykov et al., in a cell-based screen using a Saos-2 ($p53^{-/-}$) H273 inducible system, monitored cell growth and identified PRIMA-1 (p53 reactivation and induction of massive apoptosis) as a mutant p53 activator. They observed that PRIMA-1 was effective on both contact site, as well as, structural p53

mutants and reactivated DNA binding capacity, thereby inducing apoptosis. In addition, they demonstrated that intratumor or intravenous injections of PRIMA-1, in a xenograft model of Saos-2 H273, reduced the tumor volume significantly (Bykov et al. 2002, 2005b). Further investigation of PRIMA-1 led to the discovery of its analog PRIMA-1^{MET} (APR-246), which exhibits better stability and efficacy in xenograft tumor models in SCID mice and syngenic hosts (Bykov et al. 2005a,b). Although there is some evidence that PRIMA-1 exerts its cytotoxic effects in a p53-independent manner, PRIMA-1 is known to form adducts with thiols in mutant p53 (Lambert et al. 2009). MIRA-1 (mutant p53-dependent induction of rapid apoptosis) and STIMA-1 (SH group-targeting compound that induces massive apoptosis) were also identified by the same group, but later shown to have poor solubility and had some toxic side effects (Bykov et al. 2005a; Zache et al. 2008).

Performing an *in vitro* screen from a library of >100,000 small molecules, Foster et al. identified a compound CP-31398 that could bind mutant p53 and change its conformation to wild-type protein (Foster et al. 1999). The mechanism of p53 stabilization by CP-31398 was independent of MDM2 and inhibiting degradation (Wang et al. 2003). The group also demonstrated that CP-31398 induced p53 target genes, such as p21, in Saos-2 H173 and R249 cell lines, and decreased tumor volume in a xenograft mouse model using A375.S2 (melanoma) and DLD-1 (carcinoma) cells (Foster et al. 1999; Demma et al. 2004; Rao et al. 2009). Further studies indicated that in APC$^{min/+}$ mice that develop intestinal tumors, treatment with CP-31398 could induce p53 and p21 and cause apoptosis (Rao et al. 2008). However, recent evidence suggests that CP-31398 does not directly bind p53 but instead binds to DNA (Rippin et al. 2002).

Another target-based approach to identify p53-interacting small molecules stemmed from the knowledge provided by the crystal structure of p53. Based on this information, the Fersht laboratory performed an *in silico* screen to identify small molecules that could bind in the crevice created by the Y220C mutation. Although this mutation is not the most frequent one observed in tumors, it is observed in quite a few patients (Petitjean et al. 2007). From this analysis, PhiKan059 was identified, which was further optimized to PhiKan083. Interaction of this small molecule with mutant p53 Y220C leads to its stabilization and activation (Boeckler et al. 2008). In a separate study, using recombinant DBD of contact mutant R273H in a gel-shift based DNA binding assay, a piperazinyl-quinazoline SCH529074 was identified. This compound reactivates mutant p53 and also prevents ubiquitination and degradation of p53 by MDM2. The authors observed SCH529074-induced p53-dependent apoptosis in several mutant cell lines tested and saw promising results in a xenograft tumor model (Demma et al. 2010). Further studies to demonstrate the efficacy of this compound and to identify the exact mechanism of p53 reactivation are required.

Other strategies to reactivate p53 include peptide-targeted therapy (Walensky et al. 2004; Bernal et al. 2007), cyclotherapy (continuous cycling cells would be weeded out but normal cells enter a reversible cell cycle arrest and can revive with minimum harm) (Sur et al. 2009), immunotherapy (exploring the use of p53 derived peptides to induce an immune response specific to cancer cells) (Bottger et al. 1996, 1997; Sangrajrang et al. 2003; Bernal et al. 2007; Speetjens et al. 2009), zinc (known to help in folding of mutant p53 to its wild type conformation) (Puca et al. 2011), indirect targeting of p53 (compounds that disrupt mitosis, damage DNA, inhibitors of dNTPs) (Sugikawa et al. 1999; Sur et al. 2009) and activating the other family members such as p63 and p73 (Davison et al. 1999; Wang et al. 2006; Kravchenko et al. 2008; Lu et al. 2008).

CONCLUSION AND FUTURE PERSPECTIVES

Since its discovery more than thirty years ago, p53 has been the most widely studied protein. Its role as 'the guardian of the genome' is indisputable. However, the role of p53 during normal cell cycle progression in response to stress/damage and in cancer is still not fully understood. The initial view of p53 only as a transcription factor has changed recently, as novel functions of this tumor suppressor protein are being identified. As the complexity of p53 grows, its importance in cancer development, progression and therapeutics increases exponentially. Although some success has been met in reactivating p53 in tumor cells, new approaches to identify small molecule activators of the p53 pathway need to be explored. Even though understanding the role, regulation and restoration of p53 is challenging, the benefits are innumerable.

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CHAPTER 13

ATM/ATR Cell Cycle Checkpoints: Mechanisms and Manipulation in Cancer Therapy

Marie Fernet,[1,2] *Sara Chiker*[1,2] and *Janet Hall*[1,2,a,*]

INTRODUCTION

The importance of the efficient detection, signaling and activation of cell cycle checkpoints, particularly following the formation of lethal DNA double-strand breaks (DSB) generated either directly for instance following exposure to ionizing radiation (IR) or indirectly during the processing of other DNA adducts or at stalled replication forks, is highlighted by the sensitivity to killing of cells in which the key players of these processes are absent or inhibited. The correct functioning of this DNA damage response (DDR) allows the cell time to repair damage or initiate cell death pathways (Fig. 1). Cancer cells often have defects in components of these damage signaling cascades or in cell cycle control, resulting in a greater dependence on the remaining functional processes. This can potentially be exploited to selectively increase the therapeutic effect of chemo- and radiotherapy in tumour cells by inhibiting the remaining intact DDR pathways and increasing cell death. Over the past decade significant effort has been directed to the development of small molecule inhibitors that target the major kinases involved in these pathways. This review will outline the roles

[1]Inserm U612, Bats 110-112, Centre Universitaire, Orsay, 91405, France.
[2]Institut Curie, Bats 110-112, Centre Universitaire, Orsay, 91405, France.
[a]Email: janet.hall@curie.fr
*Corresponding author

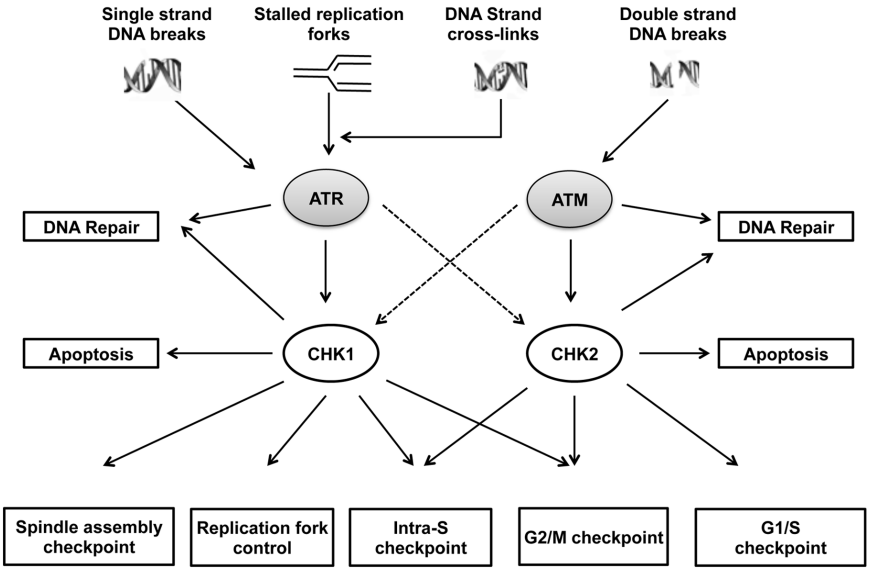

Figure 1. The roles of ATM and ATR and the checkpoint kinases Chk1 and Chk2 in the DNA damage response. Endogenous or exogenous DNA damage (single- and double-strand breaks, stalled replication forks, chemical adducts etc.) is detected by and activates the ATR and ATM kinases that in turn activate the checkpoint kinases Chk1 and Chk2. The subsequent signaling cascades can halt the cell cycle to prevent entry into S-phase (G1/S checkpoint), delay progression through S-phase (intra-S or S-phase checkpoint) or stop the cell from entering mitosis (G2/M checkpoint). Chk1 also plays a role in the control of the spindle assembly checkpoint that can delay chromosome separation in mitosis. The activated kinases can also initiate DNA repair processes specific to the nature of the DNA damage and the phase of the cell cycle in which the DNA damage occurred and the cell is arrested. If repair of the damage fails, prolonged activation of the checkpoint can trigger apoptosis through both p53-dependent and independent processes.

of two key phosphoinositide-3 kinase like kinase (PIKK) family members, Ataxia-Telangiectasia mutated (ATM) and ATM and Rad3 related (ATR), which are involved in DNA damage detection and signaling, and of the checkpoint kinases Chk1 and Chk2, which participate in cell cycle control and checkpoint activation in response to DNA damage. We will also discuss the progress made in the discovery and development of selective inhibitors for these four kinases.

ATM AND ATR: THE MASTER KINASES IN THE DDR

Ataxia-Telangiectasia (A-T) is a rare autosomal recessive disorder associated with extreme radiation sensitivity, genomic instability and predisposition to cancer (Lavin and Shiloh 1997). The defective gene in A-T is the *ATM* gene,

located on chromosome 11q22-23. It is composed of 66 exons and encodes a 13-kb mRNA, producing a protein of 3,506 amino acids with a predicted molecular mass of 350 kDa.The ATM protein is a serine/threonine kinase that has a fundamental role in the DDR through the phosphorylation of many effector proteins involved in the activation of cell cycle checkpoints, DNA repair and telomere maintenance.

The second important DDR kinase is the closely related ATR protein. The *ATR* gene is located on 3q22-24 and encodes a protein of 2644 aminoacids with a predicted molecular mass of 220 kDa (Cimprich et al. 1996). The ATR protein is essential for the viability of replicating cells, and it is activated during every cell cycle to regulate replication origin firing and repair damaged forks. Disruption of the *ATR* gene results in an accumulation of DSBs during S-phase, cell cycle arrest or apoptosis, as well as early embryonic lethality in mice (Lovejoy and Cortez 2009). No living humans have been identified that completely lack ATR function, but hypomorphic mutations in the *ATR* gene are linked to Seckel syndrome which is characterized by growth retardation and microcephaly (O'Driscoll et al. 2003).

These two PIKK family members share a conserved C-terminal kinase domain structure with other kinases in this group, including DNA PKcs (DNA-dependent protein kinase catalytic subunit), mTOR (mammalian target of rapamycin) and SMG1 (suppressor of mutagenesis in genitalia). The kinase domain is flanked on either side by two additional regions of sequence homology: the FAT (conserved sequence in FRAP, ATM, TRRAP) and the FATC (FAT C-terminal), which are involved in the regulation of PIKK kinase activity. The crystal and X-ray structure of this region determined for certain PIKKs would suggest that this tandem repeat is of functional significance for the regulation of protein kinase activity. This hypothesis is supported by the observation that mutations in these domains are associated with loss of kinase activity. The N-terminal regions of these kinases are less conserved, although ATM, ATR and DNA-PK all contain interspersed HEAT (Hungtingtin, Elongation factor 3, alpha sub-unit of PP2A and TOR1) repeats that might be regulated by phosphorylation or other post-translational modifications such as acetylation and poly(ADP) ribosylation.

Other important protein-protein interaction domains are also found in the N-terminal region (for recent reviews, readers are referred to Bhatti et al. 2011 and references therein). In addition to these structural homologies, PIKK family members make use of analogous mechanisms for their interaction and recruitment to sites of DNA damage via "adaptor" molecules. Each PIKK has a "targeting" protein that contains a conserved interaction motif: NBS1 for ATM and ATR–interacting protein (ATRIP) for ATR. These interactions mediate the functions of the PIKK (Falck et al. 2005).

ATM/ATR ACTIVATION

Although ATM and ATR are related kinases and share many substrates, they are activated in response to different DNA structures at DSBs. ATR can be activated in response to a broader range of DNA damage that involves single-stranded (SS)–DS junctions. These structures most commonly arise when the replication fork encounters a DNA lesion due to the arrest of one polymerase while the other continues (see (Chen et al. 2012) and references therein), yet they can also be generated by nucleotide excision repair or during resection of a DSB. Replication blocking causes DNA polymerases to become uncoupled from the replicative helicases, resulting in the formation of single strand breaks. These are quickly coated by the ssDNA binding protein complex, Replication Protein A (RPA) (Flynn and Zou 2011). ATRIP is indispensable for ATR activation and stabilization, as it binds directly to RPA and thereby enables the ATR-ATRIP complex to localize to sites of damage (Zou and Elledge 2003) (Fig. 2A).

Efficient ATR activation depends on the action of two mediator proteins, TopBP1 (DNA topoisomerase II binding protein I) and Claspin (Smits et al. 2010). The presence of RPA leads to the recruitment of the Rad17-RFC complex that loads the 9-1-1 (RAD9-RAD1-HUS1) checkpoint clamp to the 5′ recessed junction. TopBP1 is then recruited through interactions with phosphorylated ATRIP-ATR and phosphorylated RAD9 within the 9-1-1 complex. TopBP1's ATR-activating domain subsequently stimulates the kinase activity of ATR (Fig. 2A). Claspin, which probably also associates with active replication forks during normal replication, is then phosphorylated in a ATR-dependent manner within a short, repeated motif. Once modified, Claspin binds Chk1 and serves as a platform for ATR-dependent phosphorylation and thus Chk1 activation (Smits et al. 2010). It was shown that ATR is auto-phosphorylated on Thr1989 after exposure to hydroxyurea (HU), IR and ultraviolet (UV) light, and this phosphorylation could be a convenient biomarker of ATR activity. However, it should be noted that this phosphorylation is not essential for the ATR-CHK1 signaling axis following replication stress, but would appear to have some role in supporting cellular viability (Nam et al. 2011). The physical interactions between all these ATR partners are still being characterized. For instance Liu and colleagues have recently shown that phosphorylated Thr1989 is directly recognized by TopBP1, enabling TopBP1 to stably engage the ATR-ATRIP complex to efficiently stimulate the kinase activity and act as a scaffold for ATR-substrate interactions (Liu et al. 2011).

The ATM kinase is mainly activated in response to the formation of DNA DSBs. In the absence of DNA damage, ATM is believed to exist in an inactive homodimer, which in response to DNA damage is converted to an active monomer. The activation of ATM involves both auto-phosphorylation

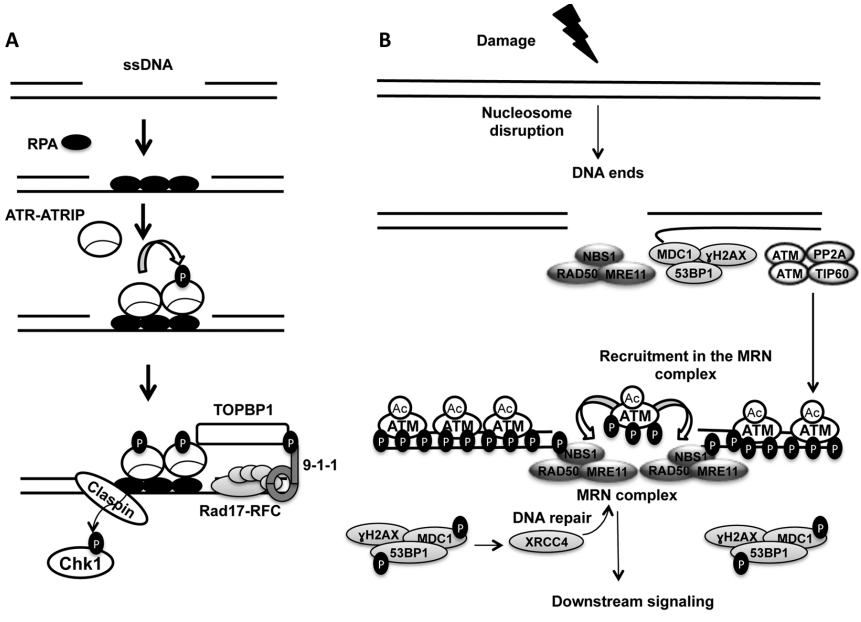

Figure 2. Molecular organization of ATR and ATM activation. (A) Model for ATR-ATRIP activation by DNA damage. Single-stranded DNA (ssDNA) and the junctions of ssDNA and dsDNA (double-stranded DNA) are the basic structural elements that trigger the ATR-ATRIP activation. (i) ssDNA is recognized and coated by RPA and (ii) RPA coated ssDNA recruits the ATR-ATRIP kinase by interacting with ATRIP, possibly contributing to its activation by promoting ATR auto-phosphorylation at Thr1989. The presence of RPA leads to the recruitment of the Rad17-RFC complex that loads the 9-1-1 (RAD9-RAD1-HUS1) checkpoint clamp to the 5′ recessed junction. TopBP1 is then recruited through interactions with phosphorylated ATRIP-ATR and phosphorylated RAD9 within the 9-1-1 complex. TopBP1's ATR-activating domain then stimulates the kinase activity of ATR. The subsequent phosphorylation of the Chk1 kinase requires the mediator Claspin that recruits Chk1 to the damage site (Xu and Leffak 2010; Liu et al. 2011). (B) Model for ATM activation. ATM is partly activated and undergoes monomerisation in the vicinity of the break, where it can phosphorylate certain substrates like p53. ATM is then recruited to the site of the break by the Mre11-Rad50-Nbs1 (MRN) complex and phosphorylates members of this complex and other downstream effectors. The activation process involves auto-phosphorylation of serines 367, 1893, 1981. The protein phosphatase PP2A is constitutively associated with ATM, presumably to ensure that it is not inappropriately activated by auto-phosphorylation. In the presence of DNA damage, PP2A dissociates from the inactive ATM dimers and loses its activity, thereby minimizing the risk of competition between phosphorylation and phosphatase activities. The acetyltransferase TIP60 is also constitutively associated with ATM, and in the presence of DNA DSBs, it becomes activated and acetylates (Ac) ATM at Lys3016. One of the earliest phosphorylation events is that of the histone H2AX and the mediator of DNA-damage checkpoint protein-1 (MDC1), which binds to γH2AX and acts as a scaffold or platform to ensure the retention of the DNA-damage recognition/repair complex on chromatin. The MRN complex binds to MDC1 through NBS1 for retention on chromatin. XRCC4, the requisite cofactor of DNA ligase 4 and non-homologous end joining (NHEJ), is detected at the break site after ATM recruitment.

events (Bakkenist and Kastan 2003) and the Mre11: Rad50: NBS1 (MRN) complex (Lee and Paull 2005) (Fig. 2B). The MRN complex is also involved in the recruitment of ATM to DNA damage sites through the formation of a molecular bridge: Mre11 and Rad50 can directly bind DNA, whilst the NBS1 protein binds ATM *via* an interaction motif located in its N-terminus (Iijima et al. 2008). The auto-phosphorylation of ATM on Ser1981 is one of the earliest events detected in response to DNA DSBs, causing the dissociation of the inactive homodimer and allowing the active ATM kinase to circulate in the cell (Bakkenist and Kastan 2003). The importance of this auto-phosphorylation for the sustained localization of ATM at DNA DSBs has been demonstrated by following the recruitment of fluorescently tagged ATM, where the ablation of the auto-phosphorylation site negatively affects the ability of ATM to phosphorylate its downstream targets after DNA damage (So et al. 2009). Two additional (auto)phosphorylation sites on ATM, Ser367 and Ser1893, may also contribute to its activation (Lavin and Kozlov 2007). A number of other phosphorylation sites have been detected in ATM, suggesting that its activity can be modulated *via* other signaling kinases; for instance, Cdk5 (Cyclin Dependent Kinase 5) has been shown to phosphorylate ATM (Tian et al. 2009). It has been reported that once ATM is activated, the MDC1/NFBD1 (mediator of DNA damage checkpoint) protein becomes hyper-phosphorylated and co-localizes with γH2AX, 53BP1, and Mre11 foci (Lavin et al. 2005) (Fig. 2B). The recruitment of MDC1 to the site of damage increases the affinity of the MRN complex (through a direct interaction between MDC1-MRN), which ultimately stimulates ATM activity and stabilizes the kinase at the site of the lesion (Bartek et al. 2004). It should be noted that as for ATR, the full details of the molecular events surrounding ATM's activation and the down-stream signaling cascades are still being unraveled. For instance the contribution of the MRN complex to the different processes is complicated by the fact that all members of the complex are substrates for ATM and other PIKKs, suggesting considerable crosstalk at DNA DSB sites. In addition, changes in chromatin structure and histone remodeling are clearly involved in both activation and signaling, and there is considerable debate about the current models, which is outside the scope of this Chapter (readers are referred to a recent review on this issue (Bhatti et al. 2011)).

ATM/ATR SIGNALING

A multitude of proteins have been identified as ATM/ATR substrates. Indeed, a recent large-scale proteomic screen of proteins phosphorylated in response to DNA damage at ATM/ATR consensus sites identified over 700 proteins. These targeted proteins have a role in numerous cellular processes related to the DDR, including DNA repair, cell death and cell cycle control,

with the serine/threonine kinases Chk1 and Chk2 being central effector proteins in these pathways (Bartek and Lukas 2003) (Fig. 3).

Chk1 and Chk2 activation occurs through distinct mechanisms: Chk1 activation is primarily downstream of ATR, whereas Chk2 is activated primarily by ATM. However, some experimental data supports the possibility that significant crosstalk takes place between the ATR-Chk1 and ATM-Chk2 signaling cascades (Stracker et al. 2009). In contrast to ATM and Chk2, ATR and Chk1 are thought to be active at low levels even during unperturbed cell cycles, particularly during S-phase, potentially explaining why they are essential in many cell types (Smith et al. 2010). Chk2 is a stable protein expressed throughout the cell cycle and appears to be largely inactive in the absence of DNA damage (Bartek and Lukas 2003), whilst Chk1 is a chromatin-associated protein with a shorter half-live that is active even in unperturbed cell cycles. Chk2-deficient mice are viable and do not have an increased risk of cancer, but Chk1-deficient mice are embryonic lethal.

THE ATR/CHK1 PATHWAY

Once an active ATR complex is assembled at a DNA lesion or stalled fork, signaling to coordinate cell cycle progression, repair, and replication begins. Activated ATR phosphorylates Chk1 on Ser317 and Ser345, leading to its activation. It would appear that the Ser317 phosphorylation is required for DNA damage responses, whereas phosphorylation at Ser345 occurs even during the unperturbed cell cycle and is required for normal cell growth (see (Wilsker et al. 2012) and references therein). Chk1 activation by ATR also requires 9-1-1 complex loading by the Rad17-RFC complex, as well as several essential mediators including Timeless (Tim/Tim1) and Tipin (Timeless-interacting protein), which enable Chk1 phosphorylation on Ser317/345 by ATR. Timeless binds to both ATR and Chk1, whereas Tipin can interact with Claspin (Smith et al. 2010). ATR also phosphorylates a number of proteins involved in the stabilization of stalled replication forks and DNA repair, such ATRIP, Rad17, Rad9, TopBP1, Claspin, H2AX, WRN, BLM, BRCA1, and FANCD2, thus linking ATR to homologous recombination repair (HRR) and DNA cross-link repair in addition to cell cycle control (see Chapters 10 and 14) (Fig. 3). In addition, some of these proteins are substrates for the activated Chk1 kinase, highlighting the complexity of the network that is involved in regulating the events in response to DNA damage. For instance, the mediator protein Clapsin interacts with Chk1 in a damage specific manner that requires the phosphorylation of Claspin on at least two sites (Ser864 and Ser895) by ATR (Cimprich and Cortez 2008), as well as Chk1 mediated phosphorylation of Claspin on Thr916 (Stracker et al. 2009).

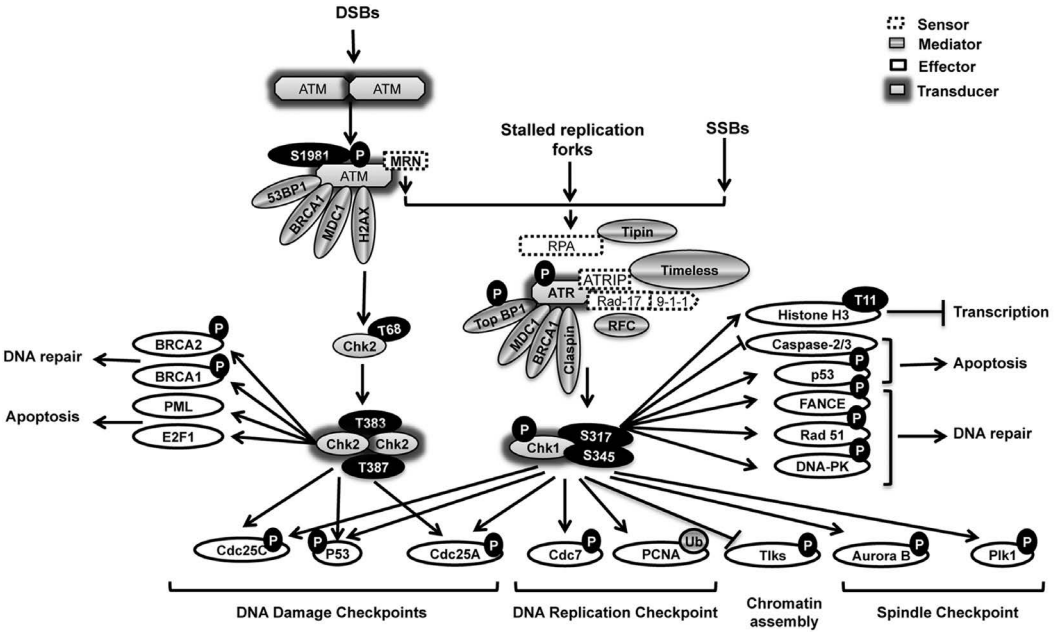

Figure 3. Chk1 and Chk2 in the DDR signaling network. DNA damage (e.g., DSBs, SSBs, and stalled replication forks) initiates ATR-mediated Chk1 and ATM-mediated Chk2 activation. In conjunction with recruited/activated sensors and mediators, ATR phosphorylates Chk1 at two canonical sites (Ser345 and Ser317), directly leading to its activation. In contrast, the activation of Chk2 involves its homodimerization and intramolecular *trans*-auto-phosphorylation (on Thr383 and Thr387) that is initiated by the phosphorylation of Thr68 by ATM. Activated Chk1 and Chk2 then phosphorylate a number of overlapping and distinct downstream effectors, which in turn are involved in cell cycle checkpoints (i.e., intra-S-phase, G2/M-phase, and G1/S-phase checkpoints), the DNA replication checkpoint, and the mitotic spindle checkpoint, as well as DNA repair, apoptosis, and transcription. Consequently, Chk1 and Chk2 are central kinases for the DDR signaling network, thereby representing particularly attractive targets in anticancer therapeutics.

Following phosphorylation of Chk1 on residues Ser317 and Ser345, Chk1 dissociates from chromatin and localizes to the cytoplasm, where a portion localizes to interphase centrosomes. Chk1 then phosphorylates Cdc25s phosphatases, p53 and many other proteins involved in the control of cell cycle progression and cell cycle checkpoints induced by DNA damage. Currently, there is another model for the activation of Chk1 in which its phosphorylation (on the C-terminal serines) blocks intramolecular interactions, uncovering the N-terminal kinase domain (Dai and Grant 2010). Interestingly, Zhang et al. have shown that ATR-mediated Chk1 activation simultaneously targets this protein kinase for polyubiquitination and proteasomal degradation (Zhang et al. 2005).

As important as activation is the inactivation of DDR effectors, especially those involved in mitigating the cell cycle checkpoints that are transient processes. Chk1 has been shown to be negatively regulated by multiple serine/threonine phosphatases. Chk1 phosphorylation at Ser317 and Ser345 and Chk1 activity following DNA damage are regulated by PP1, PP2A and Wip1 (wild-type p53-induced phosphatase 1), which are phosphatases that play important roles in the recovery from DDR checkpoints (Freeman and Monteiro 2010).

THE ATM/CHK2 PATHWAY

As discussed above, the interaction of ATM with the MRN complex initiates a highly coordinated program of further recruitment of DDR proteins, promoting amplification of the DNA damage signal. This includes MDC1 binding to phosphorylated H2AX (γH2AX) through its C-terminal BRCT domain and to ATM through its N-terminal FHA (forkhead associated) domain, bringing more ATM to the DNA damage site (Cimprich and Cortez 2008). MDC1 also recruits RNF8, an E3 ubiquitin ligase that catalyzes the ubiquitinylation of H2A and H2AX, promoting the recruitment of 53BP1 and other proteins such as RNF168, BRCA1, CtIP and BRIT1/MCPH1 to DSB sites. H2AX phosphorylation by ATM occurs on mega-base regions surrounding DSBs within seconds of DNA damage formation. γH2AX plays an important role in anchoring the above proteins to the DSB and the flanking chromatin regions (So et al. 2009). γH2AX forms foci at and near the sites of DNA breaks, colocalizing with ATM, MDC1, 53BP1, BRCA1, the MRN complex, and many other DNA damage repair proteins (Bekker-Jensen and Mailand 2010). Chk2 and ATM jointly phosphorylate the BRCA1 tumor suppressor, leading to the dissociation of Chk2 from BRCA1, an important event required for efficient repair of the DSB and survival after exposure to IR (Fig. 3).

In addition to initiating these events that will modify chromatin structure around the damage site and recruiting DNA damage repair proteins, ATM phosphorylates several other downstream effectors responsible for checkpoint controls, including Chk2. In the presence of DNA breaks, ATM directly phosphorylates Thr68 in the SQ/TQ cluster domain of the inactive monomeric Chk2. Phosphorylated monomers form dimers, allowing the autophosphorylation at Thr383 and Thr387 and other sites within the activation loop of the Chk2 kinase domain (Guo et al. 2010). These autophosphorylations increase Chk2 kinase activity, leading to the autophosphorylation at Ser516 in the C-terminal domain, which is required for the full activation of Chk2 (Ahn et al. 2000; Ahn and Prives 2002; Wu and Chen 2003). Additional phosphorylation sites have been identified, but their role in regulating Chk2 activity is presently unclear (Antoni et al. 2007). Ubiquitination of Chk2 has also been reported and is controlled by the phosphorylation of Ser379 and Ser456. While both these sites are important for Chk2 function, they differentially affect the ubiquitination and stability of Chk2. Mutation of Ser379 impairs ubiquitination, but does not alter protein stability, whereas Ser456 mutation leads to a hyper-ubiquitination and Chk2 degradation (Stracker et al. 2009). Chk2 activity is regulated by the phosphatases PP2A and Wip1. These two phosphatases dephosphorylate Thr68 and probably other sites, and bind Chk2 in a Chk2 phosphorylation-dependent manner, providing a recovery mechanism from DNA damage (Freeman et al. 2010; Freeman and Monteiro 2010).

THE CONVERGENCE OF SIGNALLING PATHWAYS

Initially it was widely speculated that the ATM/Chk2 and ATR/Chk1 pathways act in parallel, but it has become increasingly clear that an overlap in substrate specificity exists for ATM and ATR, as for Chk1 and Chk2 (Smith et al. 2010). Most ATR substrates can also be phosphorylated by ATM, and the major functions of ATR and ATM in cell cycle control are overlapping and redundant. So why is one kinase not sufficient? A simple but incomplete answer to this question is that ATM and ATR respond to different types of DNA damage: DSBs for ATM and replication stress for ATR (Cimprich and Cortez 2008). The DDR is however much more complex, as certain types of DNA damage can be converted to another, for instance, during replication. The ATM and ATR kinases clearly play a role in dealing with this dynamic situation. For instance, although ATR can respond directly and rapidly to interference with DNA replication, its activation at DSBs is dependent on ATM and is slower and predominately in S and G2 phases, as compared to the rapid activation of ATM by a DSB observed throughout the cell cycle (Jazayeri et al. 2006; Shiotani and Zou 2009).

While ATM/Chk2 and ATR/Chk1 pathways may be redundant for some functions (Fig. 3), each has specific roles as suggested by the differences between Chk1- and Chk2-deficient cells and mice. For example, deletion of a single Chk1 allele compromises G2/M checkpoint function, which is not further affected by Chk2 depletion, while Chk1 and Chk2 cooperatively affect G1/S and intra-S phase checkpoints (Niida et al. 2010). In addition, distinct roles for both proteins are suggested based on differences in their protein structure, cell cycle-dependent expression, timing of activation and stability (Bartek et al. 2001).

In addition to the convergence of signaling pathways activated by ATR and ATM, other pathways activated by DNA damage can feed into control of cell cycle progression. For instance, another effector kinase has been recently identified as a key player in some cell cycle checkpoints: the MK2 protein (MAPK kinase-2), which is activated by the Mitogen Activated Protein Kinase (MAPK) p38 (Reinhardt and Yaffe 2009; Thornton and Rincon 2009).

CELL CYCLE CHECKPOINTS

A complex regulatory network that is conserved in most higher eukaryotes controls the cell cycle at multiple levels to ensure the proper timing of cell cycle phases, and in the presence of DNA damage, to activate cell cycle checkpoints. Of the four sequential phases of the cell cycle, arguably the most important are the S-phase, when DNA replication occurs, and the M-phase, when the cell divides into two daughter cells. Separating the S and M phases are two "Gap" phases, referred to as G1 and G2, in which the cell increases in size and prepares the chromosomes for replication or prepares for entry into mitosis, respectively. Progression through the cell cycle is driven by the cyclin-dependent kinase family (CDK) family of serine/threonine kinases and their regulatory partners, the cyclins,which are small proteins expressed and degraded at specific times during the cell cycle. Cyclin D-CDK4, Cyclin D-CDK6 and Cyclin E-CDK2 drive G1 progression through the restriction point, which commits the cell to complete the cycle. S phase is initiated by Cyclin A-CDK2, and Cyclin B-CDK1 regulates progression through G2 and entry into mitosis (Fig. 4).

CDK activity is tightly regulated at multiple levels through several mechanisms. These include the abundance of the regulatory cyclin subunit, their association with the catalytic CDK subunit, activating or inhibiting phosphorylation/dephosphorylation events, and the abundance of the members of two families of CDK inhibitory proteins (CKI). CKIs include two distinct families, the INK4 (INhibitors of CDK4) family, whose four members ($p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, $p19^{INK4d}$) exclusively bind to and inhibit

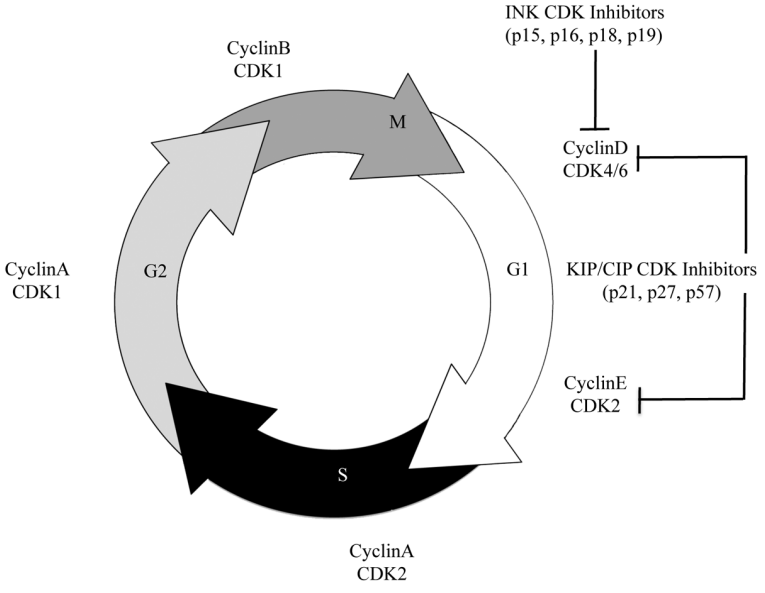

Figure 4. Cyclin-CDKs complexes and their inhibitors involved in cell cycle progression control. The cell cycle of eukaryotic cells can be divided into four successive phases: M phase (mitosis), in which the nucleus and the cytoplasm divide; S phase (DNA synthesis), in which the DNA in the nucleus is replicated, and two gap phases, G1 and G2. The transition from one phase of the cell cycle to the next is controlled by cyclin–CDK (cyclin-dependent kinase) complexes, which ensure that all phases of the cell cycle are executed in the correct order. Progression through the mitotic cycle is driven by the actions of CDKs and their activating cyclin subunits. CDK activity is suppressed through interactions with two main families of inhibitory proteins (CDK inhibitors or CDKIs): the INK4 family that exhibits selectivity for CDK4 and CDK6, and the CIP/KIP family that has a broader range of CDK inhibitory activity (Adapted from (Dehay and Kennedy 2007)).

the D-type cyclin-dependent kinases (CDK4 and CDK6), and the CIP/KIP family, whose three members ($p21^{CIP1/WAF1}$, $p27^{KIP1}$, $p57^{KIP2}$) are able to inhibit the activity of all CDKs (Fig. 4) (Malumbres and Barbacid 2005). The main post-translational modifications of CDKs are their phosphorylation by the kinase CAK (CDK activating complex) on Thr160 (Lolli and Johnson 2005) and by Wee1 and Myt1 on Thr14 and Tyr15 that inhibits the kinase activity, and dephosphorylation by the Cdc25 phosphatases of the same amino acid residues, resulting in activation of the Cyclin-CDKs complexes. Progression through each cell cycle phase and transition from one to the next are monitored by sensor mechanisms, called checkpoints. Some are active during the unperturbed normal cell cycle to maintain the correct order of events, but in the presence of DNA damage, are activated to allow cells time to properly repair the damage, thus safeguarding the integrity of the genome (Niida and Nakanishi 2006).

The regulation of cell cycle progression in response to DNA damage shares with the DNA repair processes a common signal transduction response, involving the ATM/Chk2 and ATR/Chk1 signal transduction pathways. Activated, the signal transducers phosphorylate and promote the degradation or sequestration of effector Cdc25s, specialized phosphatases that activate the CDKs. The inactivation of the Cdc25s, as well as the accumulation of the effector protein p53 (see Chapter 12), which is induced by its phosphorylation, leads to the arrest of the cell cycle progression. In addition to their role in the activation of the effector kinases Chk1 and Chk2, ATM and ATR have many other target proteins involved in the induction of cell cycle arrest that are independent of Chk1 and Chk2. The recently identified p38/MK2 pathway also seems to be important to establish certain checkpoints, with MK2 acting in parallel with Chk1 and Chk2 (Reinhardt and Yaffe 2009).

Cell cycle checkpoints exist at the G1/S and G2/M boundaries, preventing cells from replication or undergoing mitosis, respectively, in the presence of DNA damage. As DNA errors and lesions caused by endogenous or exogenous sources can be particularly harmful in S phase, multiple mechanisms, referred to as intra-S checkpoints, also exist to tightly regulate the progression of the replication fork. Cells deficient for these S-phase checkpoints are not able to stop synthesis in the presence of DNA DSBs, as is the case for cells from patients with chromosome instability syndromes such A-T or Nijmegen breakage syndrome (NBS). In contrast to these checkpoints that are directly induced by DNA damage, mitotic spindle checkpoints are activated indirectly by sensing the consequences of the damage, such as the incorrect alignment of the equatorial plane and/or impaired formation or attachment of the spindle fibers at the kinetochores.

In the following sections we will focus on the mechanisms and key players involved in the induction of the checkpoints and subsequent recovery, and give some indication of the limitations of these processes.

THE G1/S CHECKPOINT

The G1/S checkpoint is important to prevent cells damaged in G1 from entering S-phase. To achieve this, introduction of DNA damage during G1 leads to the activation of signaling cascades that inactivate the Cyclin D-CDK4/6 and Cyclin E-CDK2 complexes that regulate S-phase entry via phosphorylation of pRb, which controls pRb binding to the transcription factor E2F. This checkpoint response has two kinetically distinct components. First the initial, acute phase operates by "locking" Cyclin E-CDK2 kinase in an inactive state through the inhibition/destruction of the activating phosphatase Cdc25A. This is followed by a more delayed and sustained

G1 arrest, mediated by the stabilization of the tumour suppressor p53 protein and the activation of its target p21, which is a CKI able to bind the two Cyclin-CDK complexes (Fig. 5). Of note, both components of this checkpoint response involve the ATM/ATR and Chk1/Chk2 kinases (Bartek and Lukas 2001).

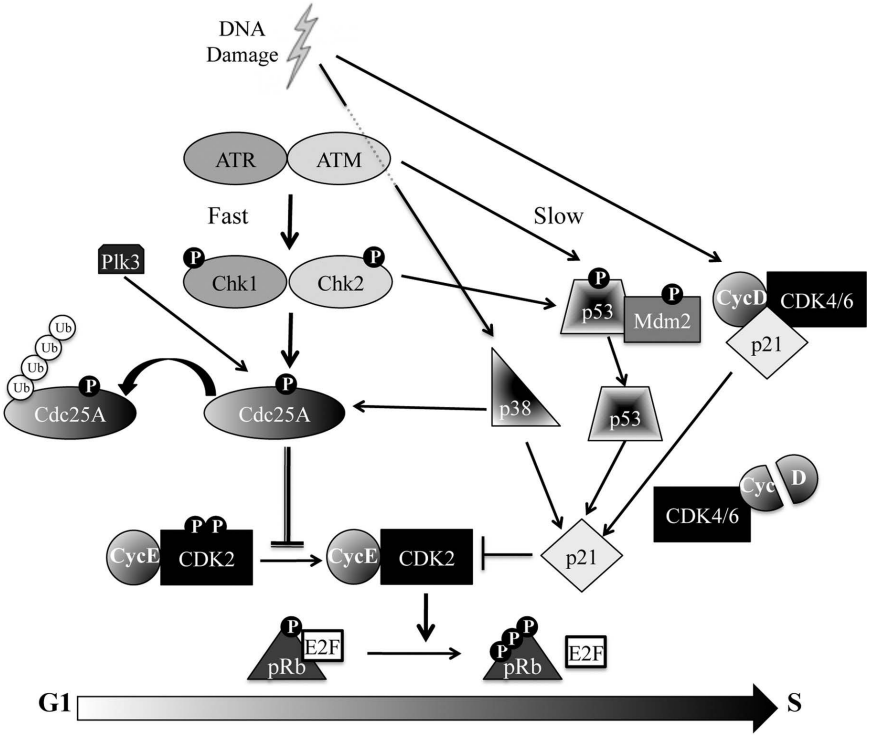

Figure 5. Molecular organisation of the G1/S checkpoint. Cells exposed to DNA damaging agents respond by activating ATM and ATR, which rapidly phosphorylate and activate the checkpoint kinases Chk2 and Chk1, respectively. In turn, these activated kinases phosphorylate Cdc25A, accelerating its degradation through the ubiquitin (Ub)-proteasome pathway. The absence of the Cdc25A phosphatase activity "locks" the CDK2 kinase in an inactive phosphorylated form, leading to G1 arrest. Another slower pathway involves the stabilization of p53 via its phosphorylation by ATM/ATR and Chk1/Chk2 that stabilizes the protein by preventing its interaction with Mdm2. Accumulation of a stable and transcriptionally active p53 protein results in the induction of a number of genes, including *P21*, which encodes a protein that binds and inhibits the Cyclin E-CDK2 complex. This pathway is important for the maintenance of the checkpoint. DNA damage also induces degradation of Cyclin D1, disruption of the Cyclin D1-CDK4/6 complex, and release of p21 that is associated with this complex, allowing p21 to then inhibit CDK2. Although less well established, p38 Map Kinase can also contribute to the induction of a G1/S checkpoint through the activation of p21 and phosphorylation of Cdc25A, which promotes its degradation. Inhibiting both CDK2 and CDK4/6 kinase complexes leads to the dephosphorylation of Rb, inhibition of the release of the E2F transcription factor and, as a consequence, inhibition of the E2F-dependent transcription of S-phase genes (see text for details).

The p53-p21 Axis

The p53 protein is a direct target of both the ATM/ATR kinases that phosphorylate Ser15 and the Chk2/Chk1 kinases that phosphorylate Ser20. These modifications of p53 lead to the disruption of its interaction with Mdm2 that mediates the ubiquitination and proteasome-mediated degradation of p53 in non-damaged cells, and consequently, the amount of p53 protein in the cell increases. Other processes influence the regulation of p53 activity (see Chapter 12), including other modifications of p53 itself and phosphorylation of Mdm2 by ATM/ATR and the Chk1/Chk2 kinases. Furthermore, Mdm2, by facilitating the nuclear export/inactivation of p53, becomes part of an inhibitory feedback loop (Meek and Hupp 2010). Once activated and stabilized, p53 affects the transcription of many genes, among them $P21^{Waf1/Cip1}$. Transcriptionally up-regulated p21 binds and inhibits the Cyclin-CDK complexes. In addition, it has been reported that p38 MAPK can directly phosphorylate and stabilize p21 and thereby contribute to the G1/S checkpoint (Thornton and Rincon 2009). Inactivation of Cyclin D-CDK4 or CDK6 inhibits the phosphorylation of the tumour suppressor protein Rb. This results in the release of E2F transcription factor, thereby inhibiting the transcriptional activation of E2F-responsive genes essential for DNA synthesis, including Cyclin E and Cyclin A, which as a consequence are unable to activate CDK2 (Fig. 5) (Musgrove et al. 2011).

Another mechanism implicated in G1 checkpoint induction is the rapid degradation of Cyclin D1 after exposure to IR. This degradation leads to the release of p21 from Cyclin D-CDK4/6 complexes that in turn permits inhibitory binding to CyclinE-CDK2. As this pathway involves transcriptional activation after post-translational modification, its full activation requires several hours and is assumed to be especially important for the maintenance of G1 arrest through the inhibition of Rb phosphorylation (Deckbar et al. 2011). At least transient inhibition of CDK2 in response to DNA damage occurs in cells lacking p53 or p21, implying the existence of another pathway that is p53-independent.

The Response via Cdc25A Degradation

A p53-independent response occurs in the first hour following the introduction of DNA damage and is mediated by the inactivation of the phosphatase Cdc25A. This inactivation is regulated by several modifications, including phosphorylation of Cdc25A by Chk1 and Chk2 (Chk2 on ser123) (Mailand et al. 2000; Boutros et al. 2006). Such phosphorylation leads to Cdc25A's nuclear exclusion and ubiquitin-mediated proteolytic degradation by the proteasome. This results in persistent inhibitory phosphorylation of CDK2 on Thr14/Tyr15 and thus inhibition of the Cyclin E-CDK2 complex.

Recent work indicates that degradation of Cdc25A is regulated by phosphorylation by the Polo-Like Kinase 3 (PLK3) (Myer et al. 2011) and that p38 MAPK can also phosphorylate Cdc25A to promote its degradation (Thornton and Rincon 2009) (Fig. 5). Although the specific target(s) for Cdc25B and Cdc25C are not clear, some data indicate that these two isoforms cooperate with Cdc25A to promote S-phase entry (Boutros et al. 2006). The Cdc25A-mediated mechanism may contribute to genomic stability by imposing a cell cycle block and preventing excessive damage accumulation before the p53-p21 pathway ensures a more sustained proliferation arrest. Deregulation of either mechanism may cause genomic instability. The over-abundance of Cdc25A found in subsets of aggressive human cancers might prevent its timely degradation in response to DNA damage, and thus, provide a growth advantage through escape from the G1-S arrest and the propagation of genetic abnormalities (Mailand et al. 2000).

The major biological mission of DNA damage checkpoints is to allow time to repair the damage so that checkpoint-arrested cells can eventually resume cell cycle progression and continue their physiological program. Until recently, however, very little was known about the molecular basis of such checkpoint termination and recovery. Currently, the only described mechanisms that promote recovery are those responsible for recovery from the G2 checkpoint (see paragraph on G2/M checkpoints). Nevertheless, we know that the G1/S checkpoint is not permanent, as cells can be released from it, and that the duration of arrest increases with dose of damaging agent.

The G1/S checkpoint is highly sensitive to the presence of DNA DSBs (Huang et al. 1996), but two aspects compromise its efficacy: at early time points after irradiation, S-phase entry is slowed but not abolished and, once activated, G1/S arrest is inefficiently maintained, allowing cells with γH2AX foci, a marker of DSBs, to escape from arrest and enter S-phase. Both routes can lead to chromosome breakage in the subsequent G2 phase (Deckbar et al. 2010). More recently, Deckbar et al. showed that the G1/S checkpoint is induced after exposure to doses of IR as low as 100 mGy, that there is a dose-dependent response, and that the early Chk2/Cdc25 process is likely to be less sensitive at inhibiting CDK activity than the late p53/p21 process (Deckbar et al. 2011).

The major role of the p53 protein in preventing entry into S-phase has important consequences for tumour treatment (see Chapter 12). Indeed, many cancer cells are deficient for p53 and fail to establish a G1 arrest in response to DNA damage, thus in these tumours, the G2 checkpoint has to be targeted for therapy.

THE INTRA-S CHECKPOINTS

Although the vast majority of cells in an adult organism are in G1 at any given time, damage occurring during S-phase can interfere with the DNA replication machinery and lead to serious genomic abnormalities. Not surprisingly, cells have therefore developed mechanisms that detect DNA damage during S-phase and transiently halt the firing of replicons still waiting to be replicated (Iliakis et al. 2003). There is a wider spectrum of DNArepair and checkpoint pathways available in S-phase compared with the other cell cycle phases, and the precise nature of the cellular response in S-phase and the ultimate fate of the responding cell depends, at least in part, on the identity and magnitude of the causative insult that threatens genome integrity.

The molecular mechanisms of S-phase progression control are the least well defined of all the cell cycle checkpoints. However, responses can involve both active replication fork slowing and suppression of the replication origin firing (Grallert and Boye 2008). When DNA synthesis is blocked, additional replication checkpoint responses are required to stabilize stalled replication forks (fork stabilization) and delay the onset of mitosis until DNA replication is complete (**S-M checkpoint**) (Branzei and Foiani 2010; Smith et al. 2010). All these checkpoint responses are closely coordinated and share some components (Bartek et al. 2004). In response to DSBs induced by IR, at least two pathways are involved: the ATM/ATR-Chk1/Chk2-Cdc25A-CDK2 pathway and the NBS1-dependent pathway, which includes the ATM/NBS1/Smc1 and the ATM/NBS1/FANCD2 pathways (Fig. 6) (Falck et al. 2002). Chk1 activation via ATR plays a dominant role in response to replication stresses (**the replication checkpoint**), but Chk1 is also required for amplification of a DSB-initiated Cdc25A signaling that is mediated by ATM/Chk2 (Conti et al. 2007; Dai and Grant 2010). Finally, we can distinguish three distinct S-phase checkpoints: the replication checkpoint and the S-M checkpoint, which both respond to problems with DNA replication and appear to be active even during unperturbed cell cycles, and the replication-independent **intra-S checkpoint**, induced by a DSB, on which we will focus next.

Defects in the intra-S checkpoint response to IR result in the inability of cells to reduce the rate of DNA replication when irradiated, a phenomenon that is known as radioresistant DNA synthesis (RDS). The assay for measuring RDS has become a "gold standard" to test whether or not any new candidate protein is required for the intra-S checkpoint. New techniques allowing the visualization of DNA replication at the single cell and single DNA molecule level will enable more detailed investigations

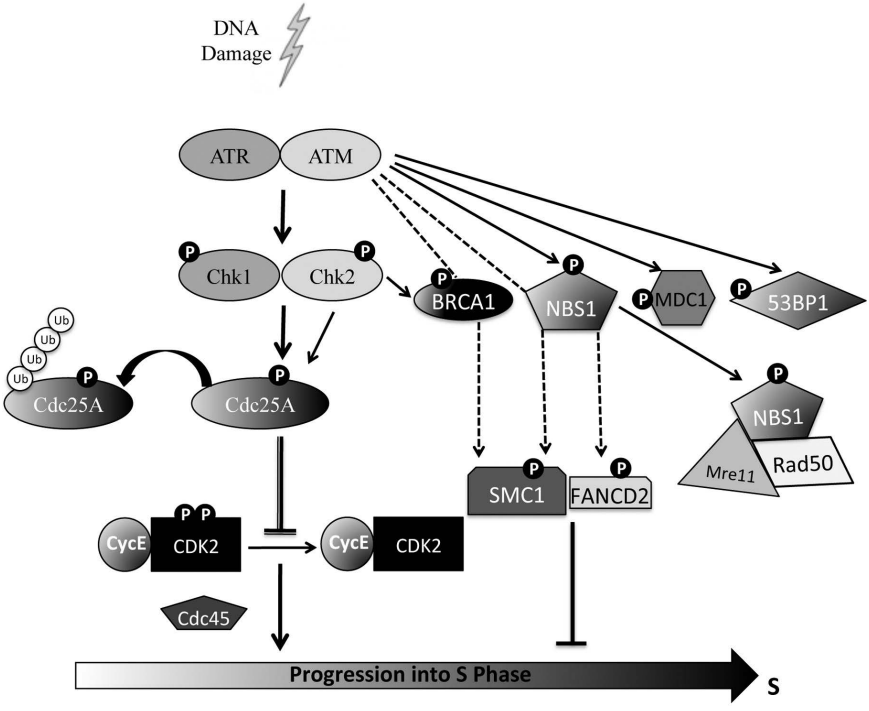

Figure 6. Molecular organisation of the intra-S checkpoint. In response to DNA damage, ATM/ATR triggers two parallel cascades that cooperate to inhibit replicative DNA synthesis. The first operates through the activation of Chk1/Chk2, Cdc25A phosphorylation and inhibition of the Cyclin E/CDK2 complex activity (see Fig. 5 and text for details), preventing the loading of Cdc45, an essential replication factor, on replication origins, and thus, causing delay in S-phase progression. A second pathway is initiated by the phosphorylation of NBS1 and involves the NBS1-dependent phosphorylation of SMC1 and FANCD2. Other targets of ATM/ATR and Chk1/Chk2, such as BRCA1, MDC1 and 53BP1, also contribute to the S-phase checkpoint, but the mechanisms remains unclear (see text for details).

into the regulation of elongation and fork velocity and their molecular effectors (Conti et al. 2007). For example, Conti et al. showed that the intra-S phase checkpoint consists of at least two main mechanisms: one operating on origin firing and the other on ongoing replication forks, with the contribution of the two mechanisms varying from organism to organism. The same checkpoint proteins have been suggested to control these two distinct mechanisms (Grallert and Boye 2008).

Given the growing number of proteins that are required for the proper activation and/or execution of the intra-S checkpoint, and thereby the prevention of RDS, the question arises as to whether all these proteins contribute to one of the two known effector branches of the checkpoint, or whether there are other downstream mechanisms that delay DNA

replication. After exposure to a genotoxic stress such as IR, ATM is activated and phosphorylates several components of the intra-S checkpoint, including Chk2. A downstream target of Chk2 is Cdc25A, whose phosphorylation leads to proteolytic degradation by the proteasome in a process similar to that described above for the G1/S checkpoint. Degradation of Cdc25A will deprive the cell of an essential activator of CDK2, either in association with Cyclin A or Cyclin E, and will block replicon firing by inhibiting Cdc45 and other cellular targets (Fig. 6). The ATM/Chk2/Cdc25/Cdc45 axis forms a rapid response system. Interference with this cascade at any step downstream of ATM results in RDS. The role of Chk1 is more complex. It was shown that RDS after disruption of Chk1 correlates with the accumulation of non-degradable, hypo-phosphorylated Cdc25A (Iliakis et al. 2003) and that phosphorylation of Chk1 is a prerequisite for the IR-induced cascade to degrade Cdc25A (Sorensen et al. 2003). Experimental evidence suggests that Chk1 and Chk2 operate through the same mechanism and have a partially redundant role in the cascade.

The NBS1-dependent branch is less well understood (Falck et al. 2002). It is initiated by the phosphorylation of NBS1 on Ser343 by ATM, an event that is required for the activation of the NBS1-Mre11-Rad50 complex. Other targets of ATM and potentially ATR have been identified as being necessary for the proper execution of the intra-S checkpoint, including SMC1 and FANCD2. Phosphorylation of Ser957 and/or Ser966 of SMC1 by ATM is required for the activation of the S-phase checkpoint in response to IR, and this modification is dependent on the phosphorylation of NBS1 by ATM (Yazdi et al. 2002). In addition, Taniguchi et al. showed that the ATM-dependent phosphorylation of FANCD2 on Ser122 is obligatory for the IR-induced S-phase checkpoint and is dependent on NBS1 and Mre11. How this phosphorylation establishes the checkpoint and if there is a functional link between the two downstream proteins SMC1 and FANCD2 remains to be established (Nakanishi et al. 2002; Taniguchi et al. 2002; Bartek et al. 2004). The mediator protein BRCA1 might also operate in the NBS1-dependent branch of the checkpoint, as its function is required for the checkpoint associated phosphorylation of SMC1. On the other hand, BRCA1, as well as the other mediator proteins, 53BP1 and MDC1, might contribute, possibly in a partly redundant manner, to the upstream events that amplify or modulate the activity of ATM and/or its interaction with the different substrates (Fig. 6) (Goldberg et al. 2003; Bartek et al. 2004). The effector mechanism through which the NBS1/SMC1/FANCD2 branch of the checkpoint inhibits DNA synthesis remains to be elucidated.

ATM and ATR are both required for maintaining replication fork stability and promoting the restart of collapsed replication forks. Furthermore, recent observations clearly show an involvement of the MRN complex activity

in these processes, in an ATM-dependent manner (Garner and Costanzo 2009).

By contrast to cells in G1 or G2, cells that experience genotoxic stress during DNA replication only delay their progression through S-phase in a transient manner, and if damage is not repaired during this delay, they exit the S-phase and arrest later after reaching the G2 checkpoint.

THE G2/M CHECKPOINTS

A large majority of cancer cells that have defective p53 fail to establish a G1 arrest in response to DNA damage, but arrest in G2 instead, showing that apart from p53, additional mechanisms exist to prevent entry into mitosis with damaged DNA (Reinhardt and Yaffe 2009). The cellular arrest that blocks progression from the G2 phase to mitosis after IR exposure was one of the first effects of radiation to be documented and investigated. It is the best defined of the cell cycle checkpoints, especially in terms of maintenance and recovery.

The G2/M checkpoint response is complex and can be mediated by a number of signaling pathways, including ATM, ATR and more recently the p38 MAPK pathway (Reinhardt and Yaffe 2009). G2 arrest is imposed by blocking activation of the mitotic Cyclin B-CDK1 complex by preventing the removal of the inhibitory Thr14/Tyr15 phosphorylation of CDK1, which was added earlier in the cell cycle by the kinases Myt1 and Wee1, respectively. This blockage is achieved, at least in large part, via the inhibition of the Cdc25 family phosphatases, which play an important role in reversing the inhibitory phosphorylation to rapidly activate the Cyclin B-CDK1 complex and trigger the onset of mitosis (Smith et al. 2010). Another pathway leading to Cyclin B-CDK1 inhibition that involves p53 has been described, but the exact function of this pathway in regulating G2/M arrest is not fully understood (Deckbar et al. 2011) (Fig. 7).

The Cdc25 Pathway

The Cdc25C phosphatase has a central role in the regulation of CDK1 activity; as a consequence, several pathways of regulation appear to converge here. Inhibition of Cdc25C is elicited by phosphorylation of Ser216 by Chk2 and Chk1. The inhibition is mediated by binding of the phosphorylated form of Cdc25C to the 14-3-3 protein, which renders Cdc25C catalytically less active and causes its sequestration in the cytoplasm, thereby blocking its interaction with Cyclin B-CDK1 (Iliakis et al. 2003). Cdc25C was the first Cdc25 phosphatase identified in the G2 checkpoint pathway, but all Cdc25s (A, B and C) shuttle between the nucleus and the cytoplasm in G2 and

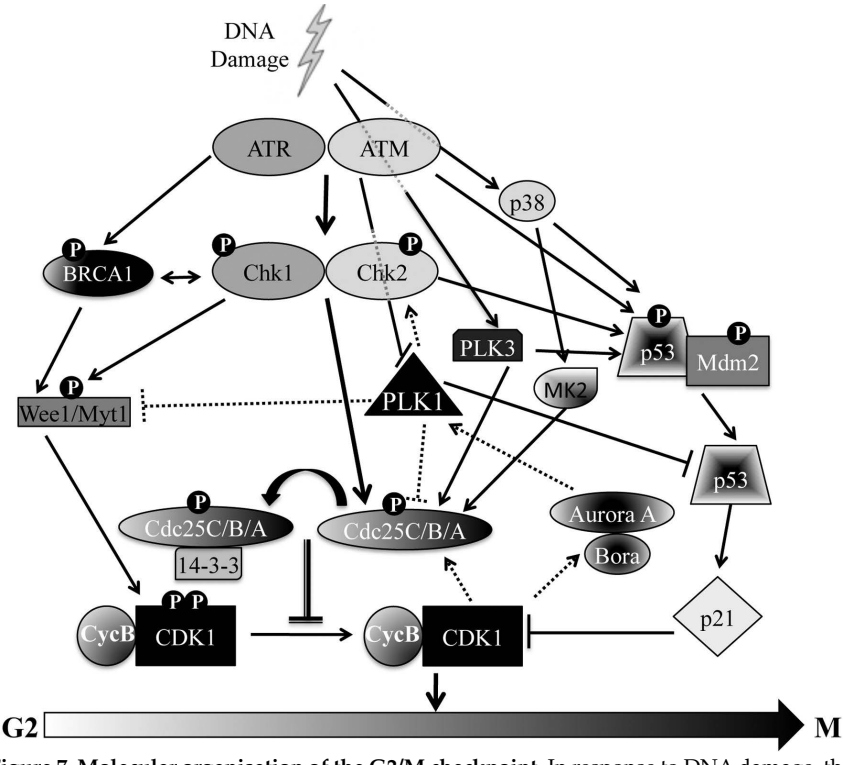

Figure 7. Molecular organisation of the G2/M checkpoint. In response to DNA damage, the G2/M checkpoints are exerted through p53-dependent and p53-independent pathways, both of which target the activation of Cyclin B-CDK1. Both responses are triggered by the activation of ATM/ATR and the Chk1 and Chk2 kinases. In the p53-independent pathway, Chk1 and Chk2 phosphorylate Cdc25s, leading to their sequestration in the cytoplasm by 14-3-3 proteins, which prevents the activation of cyclin B-CDK1 by Cdc25 and results in a G2 arrest. Further inhibition of this complex takes place through a slowly activated p53-dependent pathway. P53 is stabilized in G2 by multiple post-translational modifications, and it contributes to the inhibition of Cyclin E-CDK1 by increasing the transcription of p21 and repressing the expression of Cyclin B. The Cdc25-CDK1 pathway is also regulated by polo-like kinases PLK1 and PLK3 and the MK2 kinase.The activity of CDK1 is also directly regulated by the Wee1 and Myt1 kinases, which areregulated by PLK1 and Chk1 phosphorylation and by BRCA1.Active CDK1 is able to stimulate its own further activation by an inner feedback loop involving Cdc25s, PLK1, Wee1 and Myt1 (dottedlines).Moreover, the p38-MK2 pathway also contributes to the arrest in G2 by phosphorylation of Cdc25 and activation of p53 (see text for details).

participate in Cyclin B-CDK1 activation (Lindqvist et al. 2009). Indeed, they can all be phosphorylated and bind to 14-3-3 protein, and thus, can be degraded following DNA damage in G2 (reviewed in Boutros et al. 2006)). For example, some data show that Cdc25A is phosphorylated by Chk1, and this modification is required to prevent mitosis entry (Zhao et al. 2002). Notably, Cdc25A seems to be the only indispensable Cdc25 phosphatase for mitotic entry in mice (Ferguson et al. 2005).

Inhibition of Cyclin B-CDK1 can also be achieved by phosphorylation of Cdc25C or Cdc25B by the MK2 protein, which is a downstream effector of p38 MAPK. Although p38 is not directly phosphorylated by ATM, the activation of the p38/MK2 branch of the checkpoint has been reported to be ATM-dependent (Thornton and Rincon 2009).

When active, CDK1 activates Cdc25C, stabilizes Cdc25A, affects the localization of Cdc25B, and inhibits Wee1 and Myt1 kinases. Thus, via an inner feedback loop, CyclinB-CDK1 can stimulate its further activation by directly activating its activators and deactivating its inactivators (Lindqvist et al. 2009). DNA damage also regulates CDK1 activity by regulating Cyclin B expression levels and subcellular localization (Iliakis et al. 2003). Additional regulation of Cdc25-CDK1 pathway is driven by two proteins of the PLK family, PLK1 and PLK3. PLK1 is activated by phosphorylation in an ATM-dependent manner and promotes mitotic entry by phosphorylation of Cdc25C. Initial phosphorylation of PLK1 is conducted by Aurora-A kinase, its activation being dependent on Bora (Strebhardt 2010), which is also activated by CDK1. In addition, Wee1 and Myt1 are targets of PLK1. These PLK1-dependent modifications constitute another feedback pathway to regulate Cyclin B-CDK1 activity (Lindqvist et al. 2009; Deckbar et al. 2011). PLK3 has been shown to phosphorylate Cdc25C, contributing to its sequestration in the cytoplasm (van de Weerdt and Medema 2006).

There is evidence for an involvement of BRCA1 in the G2 checkpoint, either through ATM/ATR or possibly by directly activating CHK1(Iliakis et al. 2003). It was shown that phosphorylation of BRCA1 on Ser1423 by ATM is required for G2/M checkpoint induction (Xu et al. 2001), that BRCA1 is essential for activating Chk1, and that BRCA1 regulates the expression of Wee1 and the 14-3-3 family of proteins (Yarden et al. 2002; Yan et al. 2005). All of these mechanisms leadto G2 arrest through theregulation of CDK1 activity. In addition, BRCA1 can be phosphorylated by ATR and Chk2 (Deng 2006).

The G2 Checkpoint Maintenance

In order for cells to survive DNA damage, it is important that cell cycle arrest is not only initiated, but also maintained for the duration of time necessary for DNA repair. The existing data suggest that inactivation of a checkpoint response is an active process that requires dedicated signaling pathways.

Long term Cyclin B-CDK1 silencing for a sustained G2/M phase checkpoint requires transcriptional induction of endogenous CDK1 inhibitors (e.g., p21, Gadd45 and 14-3-3) via p53-dependent or p53-independent (e.g., via BRCA1) mechanisms that also involve Chk1.

Pathways such as those involving p53, which depend on changes in the transcription of target genes, act slowly and are mainly involved in checkpoint maintenance. Such a pathway, which involves the transactivation of CDK-inhibiting proteins, has a major role in controlling G1 arrest, but also contributes to the maintenance of the G2 checkpoint (Bunz et al. 1998). Smits et al. showed that p53-dependent activation of p21 results in the inhibition of Thr161 phosphorylation of CDK1, allowing G2 arrest to be sustained (Smits et al. 2000b). In addition, p53 can control the G2 checkpoint independently of p21 through transcriptional repression of mitotic inducers, including Cyclin B, Cdc25B and PLK1 (Medema and Macurek 2012).

The p38/MK2 pathway was shown to be critical for long-term maintenance of the G2 checkpoint through phosphorylation and, as a consequence, activation of p53 (Thornton and Rincon 2009), and also through stabilization of $p27^{Kip1}$, which can further suppress any residual CDK activity in case the DNA damage persists (Medema and Macurek 2012).

Checkpoint Recovery

When DNA lesions are successfully repaired, cells re-enter the cell cycle in a process called recovery. The G2 checkpoint switch-off requires fully activated Wip1 phosphatase and PLK1 kinase, with depletion or inhibition of PLK1 completely blocking checkpoint recovery. To be fully activated, PLK1 needs to be phosphorylated at Thr210, an event that is carried out by the Aurora-A kinase together with its cofactor Bora in late G2, initially at the centrosomes. To promote recovery, PLK1 targets the checkpoint mediator Claspin and the mitosis-inhibiting kinase Wee1 for ubiquitin/proteasome-mediated degradation, phosphorylation of Myt1 and activation of Cdc25C (and probably Cdc25B) via its phosphorylation, which promotes nuclear translocation (Bartek and Lukas 2007; Medema and Macurek 2012). An additional feedback loop is formed in which Cyclin B-CDK1 stimulates its own activation through stimulation of PLK1 activation (Lindqvist et al. 2009). In addition to these targets in or downstream of the ATR-Chk1 pathway, targets of PLK1 have been more recently described in the ATM-Chk2 pathway. Van Gut et al. showed that 53BP1 interacts with PLK1 and is phosphorylated by Cyclin B-CDK1 and PLK1, and that PLK1 phosphorylates Chk2, leading to the abrogation of their association and the inhibition of Chk2 kinase activity (van Vugt et al. 2010; van Vugt and Yaffe 2010). PLK1 also targets p53, where they physically interact, and phosphorylation of p53 by PLK1 inhibits its transactivation activity, including the induction of p21 (Ando et al. 2004; van de Weerdt and Medema 2006). In summary, multiple feedback loops affect Cyclin B-CDK1 at several levels, ranging from direct activation to enhancing the concentration of mitotic entry network

components through the regulation of transcription and specific recruitment to defined subcellular sites (reviewed in Lindqvist et al. 2009).

The activation of the PLK1-dependent pathways during checkpoint arrest counteracts the checkpoint pathways. PLK1 activity is essential but not sufficient for checkpoint recovery, indicating that additional control mechanisms exist. These pathways involved in G2 checkpoint termination act by counteracting the activity of the checkpoint kinases ATM and ATR, and engage protein phosphatases that remove phosphates from Chk1, Chk2, p53 and the damage signal amplifying histone variant H2AX (Freeman and Monteiro 2010). Multiple phosphatases participate in the inhibition of this DDR pathway, among them being PP1, PP2A and Wip1. Wip1 seems to play a central role because it specifically recognizes a p(S/T)Q motif, which is phosphorylated mostly by the ATM/ATR kinases. Among the many substrates of Wip1, p53 seems to have a special role in the G2 checkpoint recovery. Wip1 can regulate p53 by multiple mechanisms, including direct dephosphorylation, activation of Mdm2, which targets p53 for proteasomal degradation, and activation of MdmX, which directly inhibits the transcriptional activity of p53. This regulation is absolutely indispensable for recovery. Indeed, when cells depleted of Wip1 are treated with a DNA damaging agent, expression of CyclinB (as well as a number of other cell cycle regulatory proteins) decreases below the minimal level required for recovery, due to an excessive activation of p53 (Medema and Macurek 2012). P53 is also negatively regulated through direct dephosphorylation by PP1 and PP2A. Chk1 phosphorylation and activity are regulated by PP1, PP2A and Wip1, and Chk2 was found to be regulated by PP2A and Wip1 (Freeman and Monteiro 2010; Medema and Macurek 2011). In addition, PP1 can dephosphorylate BRCA1 and ATM, ATR and Chk2 phosphorylation sites.

Cell Cycle Dependence: Two Distinct G2/M Checkpoints

It is important to note that the network of interactions regulating progression through G2 after DNA damage is further complicated by the fact that the checkpoint mechanism will be determined by the phase of the cell cycle where the DNA damaging agent was used. Indeed, Xu et al. have shown, after exposure to IR, the existence of two molecularly distinct G2/M checkpoints : (1) the "immediate G2 arrest", which is triggered rapidly in cells in G2 at the time of irradiation, is ATM-dependent (for doses above 0.5 Gy), transient and dose-independent (for doses above 2 Gy) and is characterized by an abrupt reduction in the mitotic index, and (2) the "G2 accumulation", which affects cells that reach G2 after traversing S-phase and develops over many hours, is ATM-independent, dose-dependent and reflected by an accumulation of cells in G2 (Xu et al. 2002; Fernet et al. 2010).

The underlying mechanisms for either checkpoint response are not fully understood. The experimental data suggest a more stringent requirement for ATM-dependent DSB processing for rapid and efficient checkpoint activation in G2 than in S-phase, but there is a lack of understanding of the relative contribution of ATM/Chk2 and ATR/Chk1 (and p38) signaling pathways to control the G2 to M transition. Whether one kinase is able to compensate for the other, or if the role of each simply varies as a function of cell type and/or type of DNA lesion is not yet well understood (discussed in Smith et al. 2010).

Limitations of the G2/M Checkpoint

Some studies demonstrated that tumour cells can divide in the presence of unrepaired DBSs several hours after IR and G2/M checkpoint activation, suggesting that the phenomenon described in yeast and Xenopus called "adaptation" may exist in human cells. More recently, it has been shown that mitotic entry in the presence of unrepaired DSBs represents a physiological process that occurs even in non-transformed cells. While one single DSB is enough to induce the G2/M checkpoint, it is abrogated in the majority of cells when they harbor between 10–20 unrepaired DSBs. Thus, although being rapidly activated (in contrast to G1/S checkpoint), the G2/M checkpoint also has inherent insensitivity. In addition, a dose higher than 0.5–1 Gy is needed to fully initiate the G2/M checkpoint (Deckbar et al. 2011).

THE MITOTIC CHECKPOINTS

A number of checkpoint proteins, including ATM, ATR, BRCA1, Chk1, Chk2 and p53, have been detected at centrosomes, which are the organizing centers for the microtubules, and notably, there is increasing evidence that CDK1 activation needs to be coordinated with centrosomal processes to start mitosis (Golan et al. 2010). It has been shown that Cdc25B is phosphorylated by the Aurora-A kinase at the centrosome upon entry of cells into mitosis; this phosphorylation is Chk1-dependent and results in the activation of CDK1 (Cazales et al. 2005). Such events, occurring at centrosomes,are thought to be important in initiating recovery from the G2/M checkpoint and progression into mitosis. Thus, checkpoint proteins at the centrosomes could serve as points of cross-regulation for the DDR and mitotic spindle processes (Golan et al. 2010).

During unperturbed mitosis, the spindle assembly checkpoint (SAC or mitotic checkpoint) ensures that chromosome segregation is correct by preventing anaphase onset until all chromosomes are properly attached

to the spindle. Many DDR proteins, such as ATM, Chk1, Chk2, BRCA1 and BRCA2, have been reported to participate in this checkpoint as well (Yang et al. 2011a). Compared with the DNA damage responses during G2, considerably less is known about these responses during mitosis. How exactly mammalian cells respond remains a contentious issue, and the link between the SAC and the control of mitosis progression after DNA damage remains unclear. Smits et al. reported that DNA damage in mitotic cells induces a mitotic exit block via an ATM-dependent inhibition of PLK1 (Smits et al. 2000a), and more recently, the activity of PLK1 has been shown to be regulated by the phosphatase PP2A in an ATM/ATR-dependent manner (Yang et al. 2007). Moreover, inactivation of PLK1 seems to lead to an increase of cells in G2 after DNA damage in mitosis, suggesting that prometaphasic cells might revert and remain in G2 (Chow et al. 2003; Jang et al. 2007). Data from Mikhailov et al., however, has shown that extensive damage is required to delay the exit from mitosis and that this delay is not due to an ATM-mediated DNA damage checkpoint pathway, but occurs via the SAC (Mikhailov et al. 2002). In addition, DNA damage signaling has been found to be reduced in mitotic cells, with full activation only ensuing when a DSB-containing mitotic cell enters G1 (Giunta et al. 2010). In contrast, Huang et al. showed that DNA damage does not just delay mitotic exit, but blocks it, leading to mitotic catastrophe, and that in BRCA1- or Chk1-deficient cells, this DNA damage checkpoint is compromised (Huang et al. 2005). It is thus tempting to speculate that the mechanisms controlling the mitotic progression in the presence of DNA damage may be dependent on the type and level of DNA damage. More investigations are required to clarify this question, but clearly ATM/ATR and Chk1/Chk2 are implicated in these processes.

TARGETING ATM/ATR PATHWAYS IN THERAPY

As discussed above, the PIKK protein kinases ATM and ATR and their downstream effector proteins Chk1 and Chk2 control many aspects of the DDR, making them important targets for drug development for the treatment of malignant tumours. The over-arching rationale for such drug development is that the induction of DNA damage by chemotherapeutic drugs or radiotherapy in conjunction with the inhibition of DNA repair and/or cell cycle control in rapidly proliferating cells will result in increased cell death. Cancer cells often have defects in components of these signaling cascades, resulting in a greater dependence on the remaining functional processes. For instance, *TP53* is mutated in many human cancers (Olivier et al. 2010) and homologous recombination repair is often found to be defective in certain genetic backgrounds through mutations or epigenetic silencing

(Cerbinskaite et al. 2012). Such deficiencies in tumour cells can be targeted using approaches such as synthetic lethality, where a drug or combination of drugs will cause the death of the tumour cell, while sparing proficient cells (see Chapters 14 and 15). Significantly, there is emerging evidence that checkpoint kinase inhibitors will have single-agent activity in cancer cells with specific defects in DNA repair (see (Garrett and Collins 2011; Ma et al. 2011) for discussions on these issues).

SMALL MOLECULE INHIBITORS OF ATM AND ATR

As reviewed by Bolderson et al., the first evidence that ATM might be an attractive target for chemotherapy was that cells from A-T patients are exquisitely sensitive to radiation and that the nonspecific PIKK and PI3K inhibitors wortmannin and caffeine increase cellular sensitivity to radiation and chemotherapeutic drugs (Bolderson et al. 2009). Unfortunately, both compounds lack specificity, and caffeine cannot be administered at high enough doses for use in therapeutic settings. By screening a combinatorial library based around the nonspecific PI3K and DNA-PK inhibitor LY294002, a highly specific small molecule ATP competitive inhibitor of ATM, 2-morpholin-4-yl-6-thianthren-1-yl-pyran-4-one (named KU-55933), was identified (Hickson et al. 2004). The IC_{50} for KU-55933 is 12.9 ± 0.1 nmol/L. Counter-screening this molecule against other members of the PIKK family demonstrated that KU-55933 exhibits at least a 100-fold differential in selectivity, and at a single point concentration of 10 μmol/L KU-55933, did not significantly inhibit any of the 60 kinases in a commercially available test panel. This inhibitor can efficiently sensitize a number of cell types, including human melanoma cells and prostate cancer cells, to the cytotoxic effects of IR and to DNA DSB-inducing chemotherapeutic agents, such as camptothecin and etoposide (Hickson et al. 2004; Ivanov et al. 2009; Shaheen et al. 2011). An improved analogue of KU-55933 (KU-60019) with Ki and IC_{50} values half of those of KU-55933 has been reported that is 10-fold more effective than KU-55933 at blocking radiation-induced phosphorylation of key ATM targets in human glioma cells (Golding et al. 2009). Rainey et al. have also reported the development of another non-toxic rapidly reversible inhibitor of ATM function, CP466722. Using clonogenic survival assays, they showed that transient inhibition of ATM using this inhibitor was sufficient to sensitize cells to IR, suggesting that therapeutic radiosensitization may only require ATM inhibition for short periods of time (Rainey et al. 2008). There are also some indications that inhibitors of the ATM-mediated DDR may sensitize cells that are deficient in other repair pathways. For instance, Kennedy et al. showed that Fanconi anemia (FA) pathway-deficient tumour cells (see Chapter 10) were hypersensitive to the loss of ATM and that FA

complementation group G- (FANCG-) and FANCC-deficient pancreatic tumor lines were more sensitive to the ATM inhibitor KU-55933 than the isogenic corrected lines (Kennedy et al. 2007). Such observations and the findings that FA genes are disrupted in a range of cancers (Valeri et al. 2011) may open up new therapeutic options, including ATM inhibitors for specific subsets of cancers. However, to date, no ATM inhibitor appears to have entered into clinical trials.

Preclinical studies investigating the impact of ATR inhibition have been hampered until recently by the lack of ATR specific inhibitors. ATR kinase activity is also inhibited by caffeine and wortmannin but, as discussed above, both agents inhibit multiple PIKKs. The natural product, schisandrin B, which is commonly used in traditional Chinese medicine for the treatment of hepatitis and myocardial disorders, is slightly more specific for ATR, but additional studies are required to determine the clinical feasibility of using schisandrin B as a sensitizing agent for anti-cancer therapy (Nishida et al. 2009). Chen et al. (Chen et al. 2012) have recently reviewed the progress in the development of novel small molecule ATR inhibitors and the findings that two agents designed to inhibit other kinases are in fact efficient ATR inhibitors: the inhibitor NU6027, originally designed as a CDK2 inhibitor, was more efficient at inhibiting cellular ATR activity (Peasland et al. 2011), and Toledo et al. showed that NVP-BEZ235, a dual PI3K and mTOR inhibitor that is currently in phase 1/2 clinical trials for advanced solid tumours, is also very potent against ATM, ATR and the catalytic subunit of DNA-PKcs (Toledo et al. 2011). In addition, Mukherjee et al. have reported cross-inhibition of ATM and DNA-Pkcs by NVP-BEZ235 that is associated with a very high degree of radiosensitization (Mukherjee et al. 2012). As these authors discussed, this cross-reactivity may have important clinical implications in such that combining the drug with certain genotoxic chemotherapy could result in systemic toxicity and limit therapeutic gain. On the other hand, radiotherapy, like surgery, is a local treatment, and its efficacy could be significantly enhanced by the use of potent radiosensitizers, such as NVP-BEZ235, while minimizing normal tissue toxicity.

CHK1 AND CHK2 INHIBITORS

The first small molecule inhibitor used to investigate the impact of Chk1 and Chk2 inhibition was the staurosporine inhibitor **UCN-01.** Treatment with this compound led to G2/M checkpoint activation in IR-treated p53-deficient tumour cells (Graves et al. 2000), suggesting that the inhibition of these checkpoint kinases could be a promising therapeutic approach in p53-deficient tumours. UCN-01 has been tested in Phase I trials either as a single agent or in combination with a variety of chemotherapeutic agents (see Table

1 and references therein). Results indicate that UCN-01 has disappointing pharmaco-kinetic profiles and limited antitumour effects. In addition, UCN-01 inhibits several other kinases, including CDK1 and CDK2, complicating the mechanisms of action, as inhibitors of these checkpoint kinases would induce a cell cycle arrest, as opposed to the checkpoint abrogation produced by Chk1 and Chk2 inhibition. Such events may be responsible for some of the normal tissue toxicity seen in Phase I clinical trials, such as the increased myelo-suppression when used in combination with topotecan compared to topotecan alone (see (Ma et al. 2011) and references therein). However, the promising preclinical results obtained with UCN-01 spurred the pharmaceutical industry to develop and characterize more potent and kinase specific inhibitors. Most are ATP-competitive, but with varying potencies and specificities, and interestingly, are very structurally diverse. The majority that have reached Phase I or II clinical trials (XL-884, AZD-7762, PF-00477736, LY2606368 and LY2606368) are all dual Chk1/Chk2 inhibitors. Only one Chk1 inhibitor, SCH900776, has reached this stage of clinical assessment, and all of the more recently developed Chk2 specific inhibitors are still in preclinical stages of investigation (Table1).

CLINICAL TRIALS OF CHK1/CHK2 INHIBITORS

XL-884 (EXCEL-9844) is a potent ATP competitive, aminopyrazine carboxamide dual Chk1/Chk2 inhibitor that has been well characterized preclinically and was investigated in one of the first Phase I trials initiated in September 2005. Increased gemcitabine-induced Chk1 phosphorylation at Ser317, and in parallel, elevated levels of phosphorylated H2AX were found in XL-884 treated cells, suggesting that Chk1 inhibition leads to increased DNA damage and hence increased upstream signaling from ATR. No concomitant downstream degradation of Cdc25A or activation of the S-phase checkpoint was seen. Taken together, these markers indicate a greatly increased level of DNA damage resulting from the combination treatment relative to the treatment with the antimetabolite gemcitabine alone. This nucleoside analogue is incorporated into replicating DNA to produce strand breaks and also depletes the nucleotide intermediate pool through inhibition of ribonucleotide reductase, leading to an S-phase arrest that requires functional Chk1 signaling. *In vitro* studies have also shown that XL-884 is able to release the S-phase cell cycle arrest induced by gemcitabine, with the treated cells having a premature entry into mitosis and the induction of cell death through mitotic catastrophe. Furthermore, XL-884 treated cells are markedly more sensitive to the cytotoxic effects of gemcitabine, an effect seen across a panel of diverse solid tumour cell types. *In vivo* XL-884 in combination with gemcitabine was shown to result

Table 1. Properties of Chk1 and Chk2 Inhibitors in preclinical and clinical trials.

Compound	Inhibitory activity	Preclinical data	Clinical trial Phase	Combination	Tumour type	Status	Publications
UCN-01	ChK1 IC_{50} 11nM Chk2 IC_{50} 1040nM	(Courage et al. 1995) (Mack et al. 2003) (Dai et al. 2002) (Ma et al. 2012) (Tang et al. 2012) (Ma et al. 2011)	I/II I I II	Single agent Cisplatin Topotecan Topotecan	AST AST AST Ovarian cancer	Trials terminated	(Fracasso et al. 2011) (Ashwell and Zabludoff 2008)
AZD-7762 5(Astra Zeneca)	Chk1 IC_{50} 5nM Chk2 IC_{50}<10nM	(Ma et al. 2012) (Tang et al. 2012) (Ma et al. 2011) (Zabludoff et al. 2008) (McNeely et al. 2010) (Mitchell et al. 2010) (Morgan et al. 2010) (Seol et al. 2011) (Didier et al. 2012) (Aris and Pommier 2012) (Bartucci et al. 2012) (Yang et al. 2011b) (Ashwell et al. 2008)	I I I	Gem Irinotecan Gem	AST AST AST	Trials terminated	Closed awaiting publication
XL9884 (Exelixis)	Chk1 IC_{50} 2.2 nM Chk2 IC_{50} 0.07 nM	(Ashwell et al. 2008) (Matthews et al. 2007)	I	Single agent Gem	Lymphoma AST	Trials terminated further development discontinued	-
PF-00477736 (Pfizer)	Chk1 IC_{50} 0.5nM Chk2 IC_{50}47nM	(Ashwell et al. 2008) (Blasina et al. 2008) (Zhang et al. 2009)	I	Gem	AST	Trial terminated further development discontinued	-
SCH900776 (Schering Plough)	Chk1 IC_{50} 3nM Chk2 IC_{50} 1500nM	(Guzi et al. 2011)	I I	Gem Cytarabine	AST Leukemia	Trial terminated Closed	Closed awaiting publication

Table 1. contd....

Table 1. contd....

Compound	Inhibitory activity	Preclinical data	Clinical trial Phase	Combination	Tumour type	Status	Publications
LY2606368 (Eli Lilly)	Chk1 IC_{50}<1nM Chk2 IC_{50} 4.7 nM	(Lainchbury and Collins 2011)	I	Single agent	AST	Recruiting	-
LY2603618 (Eli Lilly)			I/II II	Gem Pemetrexed	Pancreatic NSCLC	Recruiting Active	-
PD321852 (Pfizer)	Chk1 IC_{50} 5nM	(Parsels et al. 2009)	Pre-clinical	-	-	-	-
CEP3891 (Cephalon)	Chk1 IC_{50} 5nM	(Sorensen et al. 2003) (Syljuasen et al. 2004)	Pre-clinical	-	-	-	-
SAR020106 (Sareum)	Chk1 IC_{50} 13.3nM Chk2 IC_{50}>10000nM	(Walton et al. 2010) (Reader et al. 2011)	Pre-clinical	-	-	-	-
VRX0466617	Chk1 IC_{50}>10000nM Chk2 Ki 11nM	(Carlessi et al. 2007)	-	-	-	-	-
PV1019	Chk1 IC_{50} 15730 nM Chk2 IC_{50} 24nM	(Jobson et al. 2009)	-	-	-	-	-
CCT241533	Chk1 IC_{50} 190 nM Chk2 IC_{50} 3nM	(Caldwell et al. 2011)	-	-	-	-	-

Based on published information and the Clinical Trials site (http://clinicaltrials.gov)
AST: Advanced solid tumours; Gem: Gemcitabine; NSCLC Non-Small Cell Lung Cancer

in tumour growth inhibition in a PANC-1 ectopic xenograft model, and in combination with daunorubicin, it caused a significant increase in median survival in a chronic myeloid leukemia model. Based on these observations, XL-884 entered into Phase I trials for leukemias and lymphomas as a single agent as well as in combination with gemcitabine, but drug development has reported to be have been discontinued (see Table 1 (Ashwell et al. 2008; Garrett and Collins 2011) and references therein).

AZD7762 is a potent and relatively selective thiophene carboxamide urea-based Chk1/Chk2 inhibitor that did not inhibit either cyclin-dependent kinases or protein kinase isoforms and abrogated the G2/M checkpoint induced by campothecin in HT-29 adenocarcinoma cells. Subsequent studies showed that the compound enhances the antitumor activity and abrogates S- and/or G2-phase checkpoints mediated by a wide panel of both antimetabolites and DNA damaging agents, including IR in various p53-deficient models and isogenic cell lines depleted for p53. These data support the hypothesis that checkpoint inhibitors specifically enhance the cytotoxicity of DNA damaging agents in a p53-deficient background (see Table 1 and references therein). Biomarker studies assessing the formation of phosphorylated Chk1 (Ser345) and H2AX have provided evidence of increased levels of DNA damage under such experimental conditions. Indeed, a molecular signature for pharmacological Chk1 inhibition has emerged from such studies, consisting of a dose dependent inhibition of gemcitabine induced Chk1 auto-phosphorylation on Ser296, inhibition of depletion of Cdc25A, decrease in pTyr15 CDK1, and increases in gamma H2AX and PARP cleavage, which are indicative of increased DNA damage and apoptosis, respectively (reviewed in Garrett and Collins 2011). In a number of xenograft models, AZD7762 overcomes topotecan-induced cell cycle arrest in a pharmacodynamic model in a dose-dependent manner and potentiates the effects of gemcitabine and irinotecan (Ashwell and Zabludoff 2008). Three phase one clinical trials are being conducted with AZD7762 (Table 1).

PF-473336 is a potent diazepinoindolone Chk1 inhibitor with a moderate selectivity over Chk2 (Table 1), but also inhibits several other tyrosine or serine/threonine kinases. Preclinical data for PF-473336 has been reported (see Table1 and references therein). *In vitro* the compound abrogates the cell cycle arrests induced by several DNA damaging agents. Chemopotentiation and radiopotentiation was shown to be p53-dependent, with cytoxicity enhanced in p53 defective tumour lines. PF-473336 also enhances the antitumour activities of docetaxel, an antimicrotubule agent that prevents depolymerisation of microtubules during mitosis and activates the mitotic checkpoint, in colon and breast cancer xenograft models, opening up the possibility for using Chk1 inhibition in combination with antimitotic agents.

Preliminary data from the phase I trial of gemcitabine in combination with PF-473336 was reported at the 2010 ASCO meeting; however, it has been reported that Pfizer has discontinued its further development (see Table 1 and references therein).

SCH900776 is a potent ATP-competitive pyrazolo[1,5-a]pyrimidine inhibitor that shows the best reported selectivity for Chk1 (compared to Chk2) to date and is effective at abrogating both the S- and G2-checkpoints caused by IR and various DNA-alkylating agents (Table 1). However, it does inhibit CDK2 activity (IC_{50} = 160nM). This off-target activity might reduce its overall effectiveness depending on dosing and scheduling, as the inhibition of CDK2 could induce cell cycle arrest and prevent checkpoint bypass in response to Chk1 inhibition. A phase I dose escalation study of SCH900776 in combination with gemcitabine in solid tumours was reported at the 2010 ASCO meeting (see (Ma et al. 2011) for details) and provided pharmacodynamic evidence of Chk1 inhibition at the clinical dose and some partial responses, including stable disease. SCH900776 has also been used in Phase I trials for acute leukemia in combination with the anti-metabolite cytarabine.

The second generation inhibitors **LY2603618** and **LY2606368** have both been through Phase I trials (Table 1). LY2606368 is being used as a single agent treatment for advanced cancers, whereas LY2603618 is being tested in Phase II trials for non-small-cell-lung-cancer in combination with pemetrexed and in Phase I and II trials for pancreatic cancer in combination with gemcitabine.

DEVELOPMENT OF CHK2 SPECIFIC INHIBITORS

The inhibitors described above are in the majority dual Chk1/Chk2 inhibitors, albeit with varying degrees of activity against the two kinases (Table 1). Thus, it is reasonable to assume that clinically effective doses are likely to inhibit both, raising the question of which preclinical and clinical responses result from Chk1 inhibition as opposed to Chk2 inhibition. This has been addressed to a certain extent by making use of specific knockdown of one or the other kinase using for instance using small interfering RNA approaches. The knockdown of *CHK1* in the presence of endogenous Chk2 is sufficient to abrogate S- and G2-checkpoints in cells with DNA damage (Zhao et al. 2002; Carrassa et al. 2004). However, *CHK2* knockdown in isogenic cell lines does not induce checkpoint bypass, nor does its knockdown synergise with *CHK1* knockdown to potentiate checkpoint bypass after exposure to antimetabolite drugs (Cho et al. 2005; Morgan et al. 2006), suggesting that it is Chk1 inhibition that enhances the cytoxicity seen. A small number of specific Chk2 inhibitors have been identified

(Table 1 and references therein), and it will be important to evaluate the pharmacological properties of these inhibitors in cell culture. In addition, it will be of great interest to address the p53 dependence of the responses, a contentious issue that remains to be fully resolved (see for instance (Antoni et al. 2007)), and to investigate their anti-proliferative effects as single agents in cancer cells with endogenous Chk2 activation before moving onto more advanced preclinical and clinical studies.

COMBINATION THERAPIES AND CHECKPOINT INHIBITORS AS SINGLE AGENTS

As highlighted above, the majority of the preclinical and subsequent clinical trials are evaluating the clinical impact of a combination of a Chk1/Chk2 inhibitor with drugs that induce replication stress and lead to an S-phase arrest, such as the antimetabolite gemcitabine. Thus, from a mechanistic basis, it would be expected that Chk1/Chk2 inhibitors could be used to potentiate the cytotoxicity of other replication stress inducing agents, such as the antimetabolite 5-flourouracil (5-FU) that is commonly used for the treatment of colon cancer and a range of DNA damaging agents that cause single-strand DNA breaks and lead to S-phase arrest. Topoisomerase I inhibitors, such as irinotecan and topotecan, which are commonly part of combination therapies for colon, ovarian and lung cancer, would fit into this latter category. Indeed, various Chk1 inhibitors have been shown to sensitise human tumour cell and xenograft models to topoisomerase I inhibitors (see (Garrett and Collins 2011) for recent review). Inhibition of topoisomerase II activity by drugs such as etoposide and doxorubicin also causes cell cycle arrest in late S and G2 phase, and there is some data suggesting that Chk1 inhibitors can potentiate the cytotoxicity of doxorubicin. Moreover, Chk1 inhibitors in combination with IR may be a therapeutic strategy. Promising results in cancer cell lines and xenograft models have been obtained, but have not yet been verified in cancer patients. As discussed above, radiotherapy can be considered a local treatment, and thus, the use of Chk1 inhibitors as radiosensitizers may increase IR efficacy whilst minimizing normal tissue toxicity. Whereas combining Chk2 inhibitors with DNA damaging agents remains controversial (see (Antoni et al. 2007; Garrett and Collins 2011)), there is some evidence that Chk2 inhibition might have therapeutic potential in combination with PARP inhibition (McCabe et al. 2006). More recent studies have shown that CCT241533, a potent and selective inhibitor that binds to the ATP pocket of Chk2, potentiates the cytotoxicity of PARP inhibitors, but not of other DNA damaging agents in a panel of tumour cell lines, including p53-deficient lines. This finding

supports earlier observations and opens new possible therapeutic avenues for such tumours (Anderson et al. 2011).

DNA repair deficient tumor cells have been shown to accumulate high levels of DNA damage. Consequently, these cells become hyper-dependent on DDR pathways, including the Chk1-kinase-mediated response, implying that DNA repair deficient tumors would exhibit increased sensitivity to Chk1 inhibitors as single agent treatments. For example, it has been shown using isogenic pairs of cell lines differing only in the FA DNA repair pathway, that FA-deficient cell lines were hypersensitive to Chk1 silencing by independent siRNAs, as well as Chk1 pharmacologic inhibition using UCN-01. In parallel, a siRNA screen designed to identify genes that are synthetically lethal with Chk1 inhibition identified factors required for FA pathway function (Chen et al. 2009). Chk1 has also emerged as the most potent hit from a siRNA screen of the protein kinome in neuroblastoma cell lines, suggesting that the kinase is potential therapeutic target specific for this disease (Cole et al. 2011).

OUTSTANDING ISSUES

There are a number of outstanding issues that will need to be resolved before the potential of Chk1 and Chk2 inhibitors can be fully realized. For instance, the optimization of their use alone or in combination will be complex, as there are many possible combinations and dose-scheduling strategies. In addition, there are questions that remain regarding the potential side-effects of kinase inhibition in normal tissues, as well as the possibility that toxicity could be exaggerated from the combination of these agents with existing chemotherapeutic agents. Chk1 inhibition is also known to cause genetic instability, and thus, the long-term risk that therapy related secondary cancer formation could be increased needs to be evaluated. Based on recent reviews of the Chk1/Chk2 patent literature (Janetka and Ashwell 2009; Lainchbury and Collins 2011), many new compounds will be entering the clinical arena. The challenge will therefore be to develop robust biomarkers to assess the efficacy of these new potential therapeutic tools and to optimize patient selection if these agents are to achieve maximum utility. In this respect, one question that still remains to be fully clarified is whether the p53 status correlates with tumour response in studies that combine DNA damage with Chk1 and/or Chk2 inhibition. Some of the conflicting reports may be related to the dosing schedules and the integrity of the p53 pathway, and clearly, this will need to be carefully assessed to resolve this issue.

CONCLUSIONS

The concept and development of DNA repair and checkpoint inhibitors is a field of intense interest and as noted above from the recent patent literature seems likely to continue to evolve. *In vitro* and *in vivo* preclinical data have shown that the inhibition of the DNA damage PIKKs ATM and ATR, or their downstream targets, the Chks, can potentiate the effects of DNA damage. However, it has to be noted that the inhibitors so far developed have not yet completed validation in clinical trials, and importantly, none have to date been assessed in combination with radiation. As discussed in this Chapter, there are a number of outstanding issues that remain to be resolved including toxicity and patient choice. The first generation Chk1 inhibitor UCN01 showed significant limiting toxicity in phase I trials, and toxicity has been associated with the second generation inhibitors, yet would appear to be drug-specific and might reflect off-target effects. It is hoped that toxicity might be reduced by the use of more specific inhibitors in combination with pharmacodynamic biomarkers to follow kinase inhibition. The findings that many tumours have lost the expression of one or more of these four kinases and that a vast majority of tumours are thought to have a defect in G1 control (Massague 2004) would suggest that ATM/ATR and the cell cycle kinase inhibitors could be applied to a wide background. Determining the molecular profile of individual tissues would allow the variation in responses to be better understood and contribute to determining the criteria necessary for the selection of appropriate patient populations that would most benefit from such targeted therapies. Clearly, over the next decade with access to technologies that will facilitate personalized medicine (see Chapter 16), many of these issues will be resolved, and the new generation potent inhibitors will then find their place in the clinic.

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CHAPTER 14

The BRCA1-BRCA2 Pathway of Homologous Recombination and the Opportunity for Synthetic Lethal Approaches to Cancer Therapy†

Laura J. Eccles, Ciara A. O'Driscoll, Alison C. Carley, Egle Balciunaite and *Simon N. Powell*[a,*]

INTRODUCTION

There are many DNA damaging agents which attack cellular DNA on a daily basis. These may be endogenous, such as cellular metabolic products, or exogenous such as ionizing- or ultraviolet-radiation. Exposure to such agents can lead to several types of DNA damage. The most deleterious of these is the double-strand break (DSB). DSBs, if left unrepaired, may lead to gross chromosomal rearrangements and ultimately cell death. Within the cell there are two main DSB repair pathways: non-homologous end joining (NHEJ) and homologous recombination (HR). NHEJ is predominantly used within G_1 of the cell cycle, with a significant contribution seen from

Department of Radiation Oncology and Molecular Biology Program, Memorial Sloan Kettering Cancer Center, 1275 York Avenue, New York, NY10065, United States.
[a]Email: powells@mskcc.org
*Corresponding author

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HR during S/G_2 when a sister chromatid becomes available for use as a template (SilvaMao et al. 2008). Due to HR utilizing a homologous template, it is typically an error-free mechanism of repair. In contrast, NHEJ involves the simple ligation of two broken ends together, without the necessity of homology and frequently with end-modification, leading to a higher probability of erroneous or mutagenic repair.

HR is a critical process in meiosis, during which time there is an increase in genetic variation through chromosomal crossover, the mechanism underlying genetic evolution. Chromosomal crossover is the exchange of genetic information between two homologous chromosomes in meiosis I, during meiosis II, the chromosomes separate into daughter nuclei, each containing a chromatid, to form gametes. Each gamete contains a complete haploid set of genetic information with each gamete having a unique combination of maternal and paternal alleles, owing to HR repair of meiosis-specific induced DSBs (Zickler and Kleckner 1999).

HR is also the predominant mechanism for repair of replication-associated DSBs (Arnaudeau et al. 2001; Saintigny et al. 2001). The DSB may be caused when the replication machinery encounters a blocking lesion, such as an inter-strand crosslink or a single strand break (SSB), formed either endogenously or from exposure to a DNA damaging agent such as cisplatin or the alkylating agent methyl methanesulfonate. Ultimately this collision leads to the replication fork stalling or collapsing, and restart of the fork is mediated by HR. In addition, when the replication machinery by-passes a parental strand lesion, a daughter strand gap (DSG) is produced. The parental strand lesion cannot be repaired by a conventional single strand repair mechanism, since there is no intact strand—resulting in the need for HR to be utilized to repair a DSG.

Defects in HR are of great clinical significance as they lead to many human diseases, including infertility, neuro-degeneration, premature aging and cancer. While there are many examples of human cancers associated with HR defects, there are very few examples of aberrant NHEJ in human cancers, illustrating the critical role of HR in the maintenance of genomic stability. Within this Chapter we will focus mainly on HR, its relevance to tumorigenesis, and particularly the BRCA1 and BRCA2 proteins, which are defective in cases of inherited breast/ovarian cancer and function in the HR pathway. In addition, we will discuss therapy based on the exploitation of synthetic lethality. After describing the concept of synthetic lethality and the techniques employed to investigate it, we review the current clinical trial data for chemotherapeutic agents that exploit tumor-specific DNA repair vulnerability.

THE HOMOLOGOUS RECOMBINATION PATHWAY

Although we now have a relatively clear picture of the mechanism of HR, the exact function of each of the proteins required is not fully understood. DSBs are detected within the cell by making a chromatin mark, which is typically a post-translational modification. This event is achieved through the coordination of the Mre11/Nbs-1/Rad50 (MRN) complex and the ATM kinase, which phosphorylates histone H2AX (Paull et al. 2000; Stiff et al. 2004). Phosphorylated H2AX (γH2AX) is then bound by the mediator of DNA damage checkpoint protein 1 (MDC1), which creates a positive feedback loop to amplify the signal through interactions with ATM and Nbs-1 (Lou et al. 2006; Chapman and Jackson 2008; Spycher et al. 2008). In order for HR to proceed, the 5′ ends of the DSB must be resected to leave 3′ single stranded overhangs (see Fig. 1). This is initiated by the MRN complex in conjunction with CtIP and BRCA1 (Yu et al. 1998; Sartori et al.

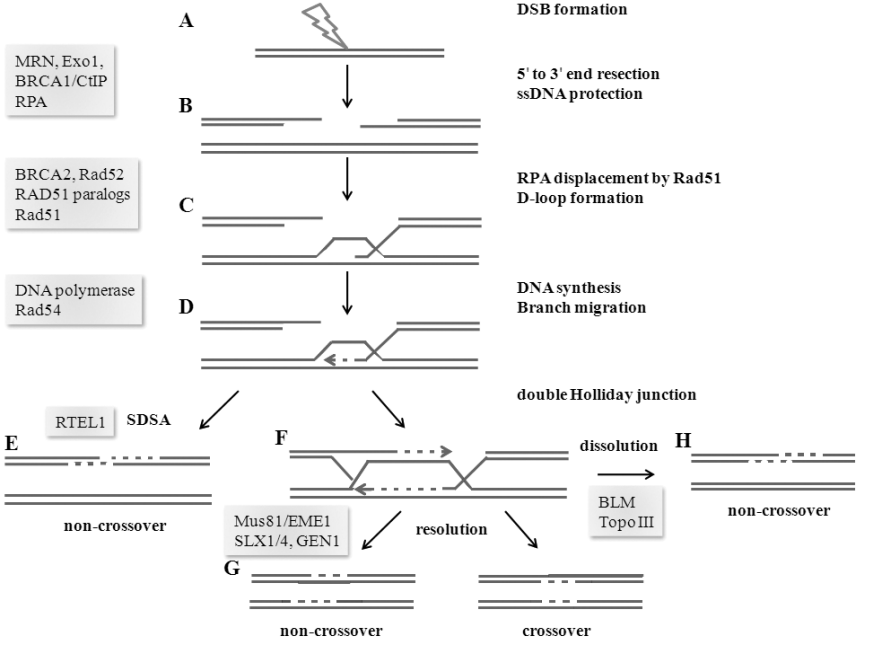

Figure 1. Mechanism of homologous recombination. Following DSB formation (A) 3′ ssDNA overhangs are created and protected (B). Rad51 filaments invade the homologous duplex forming a D-loop (C), allowing DNA synthesis to occur (D). HR can then proceed via SDSA (E) to produce non-crossover products. Alternatively, a double Holliday junction (F) may form which can be dissolved (H), also forming non-crossovers, or this structure may be resolved (G) giving either crossover or non-crossover products.

Color image of this figure appears in the color plate section at the end of the book.

2007; Williams et al. 2009). More extensive processing then occurs via the activity of helicases and exonucleases, such as BLM (Bloom's syndrome protein) and Exo1 (Mimitou and Symington 2009).

Following production of these 3′ single stranded DNA (ssDNA) regions, replication protein A (RPA) rapidly binds the exposed overhangs (Raderschall et al. 1999), melting any remaining DNA secondary structure. Whilst this step protects the exposed ssDNA from potential degradation, it also prevents binding of Rad51, a small monomeric protein that polymerises onto ssDNA that is required for strand invasion and homology search of the sister chromatid. Since RPA binds more efficiently to ssDNA than Rad51, mediator proteins are required to displace RPA and allow Rad51 binding. The mediator proteins include: BRCA2, Rad52, and the Rad51 paralogs. BRCA2 is essential for loading Rad51 monomers onto ssDNA at DSB sites and targets the Rad51 protein to the junction of the single stranded tail and the double-stranded DNA (Sharan et al. 1997; Wong et al. 1997). This process is facilitated by the Partner and Localizer of BRCA2 protein (PALB2) (Xia et al. 2006). There are five mammalian Rad51 paralogs (Rad51B, Rad51C, Rad51D, XRCC2, XRCC3) that form two distinct complexes; Rad51B-Rad51C-Rad51D-XRCC2 and Rad51C-XRCC3 (Masson et al. 2001). While the precise cellular function of these paralog proteins is unknown, there is increasing evidence suggesting that they have a role in stabilizing the Rad51 nucleoprotein and facilitating strand invasion (reviewed in Suwaki et al. 2011). Rad51-coated ssDNA invades the homologous region of the sister chromatid causing the formation of a D-loop, as shown in Fig. 1C. It is still unclear in human cells which protein is responsible for catalyzing the D-loop formation, as it has been shown that both BRCA2 and Rad52 are independently able to perform this function. BRCA2 is the better characterised of the two proteins in human cells, although it appears possible that Rad52 could act as an alternative "back-up" pathway within HR, as Rad52 and BRCA2 double-deficiency leads to severe HR defects (Feng et al. 2011). Following strand invasion, DNA synthesis can be initiated by the 3′ ssDNA using the invaded duplex DNA as a template. Once the second resected 3′ ssDNA end is captured, a Holliday junction (HJ) is formed, and branch migration of the HJ is then stimulated by Rad54 (Bugreev et al. 2006).

In a class of HR known as synthesis dependent strand annealing (SDSA), the D-loop is reversed after sufficient DNA has been synthesized, resulting in annealing of the newly synthesized strand to the other resected 3′ overhanging ssDNA (Fig. 1E). It has recently been shown that this step is dependent upon the activities of RTEL1, a helicase that was previously demonstrated to be a telomere maintenance protein (Adelman and Boulton 2010). Alternatively to SDSA, a double HJ may form, as depicted in Fig. 1F. Double HJ resolution is completed by nucleases that cleave either

symmetrically or non-symmetrically, leading to crossover or non-crossover repair products, respectively. Mus81-EME1 (Chen et al. 2001; Constantinou et al. 2002) or GEN1 and Slx1/4 (Svendsen and Harper 2010) are proteins that may be responsible for HJ resolution. The Rad51 paralog complexes have also been shown to bind HJ, suggesting a possible role for them in aiding HJ resolution, although direct binding to any of the nucleases thought to be responsible for this step has yet to be determined (reviewed in Suwaki et al. 2011). Nuclease cleavage producing a non-crossover results in the retention of DNA to the original chromatid. However, if a crossover is produced upon resolution of the HJ, there may be an exchange of genetic information, which is a common occurrence in meiosis. Despite this outcome being favorable in meiosis to increase genetic diversity, in somatic cells this may lead to loss of heterozygosity. To avoid the production of crossovers, the double HJ may be dissolved by BLM and topoisomerase III (Wu and Hickson 2003), which drive non-crossover product formation.

BRCA1 AND BRCA2 PROTEINS

BRCA1 and BRCA2 Structure

BRCA1 is a nuclear protein that consists of 1863 amino acids. BRCA1 contains an amino-terminal RING domain that imparts ubiquitin ligase activity through interaction with BARD1 (BRCA1 associated ring domain 1) (Yu and Baer 2000) (Fig. 2). In addition to enhancing E3 ligase activity, BARD1 binding also stabilizes BRCA1 protein and appears to increase the nuclear accumulation of BRCA1 by masking the nuclear export sequences of BRCA1 that flank the RING domain (Fabbro et al. 2002; Choudhury et al. 2004; Thompson et al. 2005b; Nelson and Holt 2010). The BRCA1-BARD1 heterodimer generates polyubiquitin chains at lysine 6 (K6) linkages (Wu et al. 1996; Nishikawa et al. 2004; Yu et al. 2006) that do not appear to signal protein degradation, but may instead mediate downstream signaling events. The C-terminus of BRCA1 contains a BRCA1 C-terminal (BRCT) domain, consisting of approximately 100 amino acids that is conserved in many DNA repair proteins (Koonin et al. 1996). This domain is responsible for binding phosphorylated proteins during the DNA damage response (Manke et al. 2003; Yu et al. 2003). The BRCT domain and adjacent sequences have also been implicated in transcriptional regulation and chromatin unfolding (Monteiro 2000; Ye et al. 2001). Thus, the BRCT domain contributes to the DNA repair and transcriptional functions of BRCA1, although the functional significance of transcriptional regulation in cells is not clear. The predominant cancer-associated BRCA1 mutations have been found within the RING and BRCT domains, demonstrating that both domains are important in cancer development. Interestingly, a recent

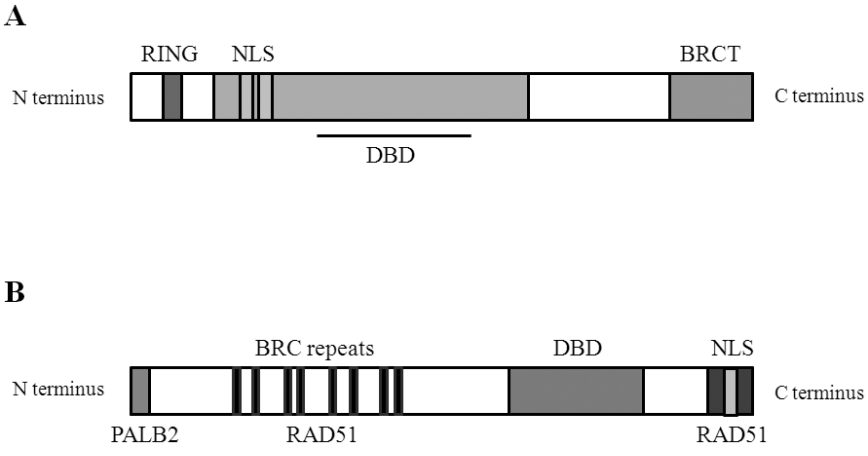

Figure 2. Schematic of BRCA1 and BRCA2 functional domains. (A) BRCA1. The BRCA1 N-terminus contains a RING domain that associates with BARD1 and a nuclear localization sequence (NLS). The central region of BRCA1 contains a DNA binding domain (DBD). The C- terminus of BRCA1 contains BRCT domains. (B) BRCA2. The N-terminus of BRCA2 binds PALB2. BRCA2 contains 8 BRC repeats that bind Rad51. The BRCA2 DBD contains a Helical domain, 3 OB folds and a Tower domain which facilitates BRCA2 binding to ssDNA, dsDNA and the DSS1 protein. The C-terminus of BRCA2 also binds Rad51.

Color image of this figure appears in the color plate section at the end of the book.

knock-in mouse with a mutant E3 ligase domain of BRCA1 was found to suppress tumor formation as well as wild-type BRCA1. BRCA1 has two nuclear localization sequences (NLS) and a DNA-binding domain (DBD) that have been mapped to a central region of the protein (amino acids 452-1092) (Zhang and Powell 2005).

The BRCA2 protein is larger in size than BRCA1 and contains 3418 amino acids. The core of the protein consists of eight repeated sequences, termed the BRC motif (Bork et al. 1996). The BRC repeats are considered the major Rad51 binding region of BRCA2 (Wong et al. 1997). An additional Rad51 binding domain resides in the C-terminal region, which also contains the NLS. The DBD of BRCA2 contains five globular domains: three oligonucleotide binding (OB) folds, which bind ssDNA and are found in many other ssDNA-binding proteins, such as RPA; a tower domain that extends from the middle of the three adjacent OB folds and binds dsDNA; and a helical domain that consists of α-helices. The ssDNA and dsDNA binding capability of BRCA2 allows it to bind the recessed ends of DSBs where the two forms of DNA meet (Yang et al. 2005). The small acidic protein DSS1 binds to the helical domain and two OB folds in order to stabilize BRCA2 (Yang et al. 2002). Recently, three independent groups have successfully purified and functionally validated the full length human BRCA2 protein (Jensen et al. 2010; Liu et al. 2010; Thorslund et al. 2010).

These studies showed that the stoichiometry of Rad51 binding to BRCA2 was 6–7 to 1, and that BRCA2, as had been predicted by genetic studies, could catalyze many steps of RPA and Rad51 function.

Multi-protein Complexes Involving BRCA1 and/or BRCA2

In comparison to the relatively limited role of BRCA2 in DNA repair, BRCA1 has more varied roles within the cell. Consistent with this, BRCA1 is found to be a component of various multi-protein complexes (Mohammad and Yaffe 2009), summarized in Table 1. A phospho-SXXF motif conserved in many DNA damage proteins acts as a binding site for the BRCT phosphopeptide binding motif within BRCA1. Such DNA damage proteins include Abraxas, BRCA1-interacting protein 1 (BRIP1; previously known as BACH1) and CtIP, and their association with BRCA1 allows for multiple complexes to form, each with distinct functions as outlined below. Historically, the first complex to be identified was the BASC complex–BRCA1-associated genome surveillance complex (Wang et al. 2000), which was the product of the proteomic technology of the time. Cellular BRCA1 was identified as part of a large multi-protein complex >2 MDa in size containing many tumor suppressors, DNA damage sensors and signal transducers. In addition to BRCA1, ATM and BLM, BASC contained multiple sub-complexes consisting of the MRN complex, the MSH2–MSH6 heterodimer, the MLH1–PMS2 heterodimer and the RFC complex containing 5 subunits. As a whole, BASC appears to be active in S and G_2 phases of the cell cycle and is triggered by DNA damage identified during replication (Wang et al. 2000). With more recent technology, three BRCA1 complexes, termed BRCA1A, BRCA1B, and BRCA1C complexes, were isolated as preformed protein assemblies in cells.

The BRCA1A complex is responsible for BRCA1 recruitment to repair foci following DNA damage (Kim et al. 2007; Liu et al. 2007b; Sobhian et al. 2007; Wang et al. 2007). The BRCA1A complex contains Abraxas, BARD1, RAP80, BRCC36, BRCC45 and MERIT40. It has been demonstrated that Abraxas is responsible for mediating the interaction between BRCA1 and RAP80, and that RAP80 is ultimately the protein responsible for BRCA1 localization at DNA damage sites (Huen et al. 2010). Both BRCC36 and BRCC45 were found to promote the E3 ubiquitin ligase activity of the BRCA1-BARD1 heterodimer within the complex (Dong et al. 2003; Chen et al. 2006). The final component of the BRCA1A complex, MERIT40, is responsible for complex stability and allows optimal targeting of BRCA1 to DSBs, as it is the mediator of RAP80 functions.

The BRCA1B complex consists of BRCA1, BRIP1 and also contains topoisomerase II binding protein 1 (TOPBP1). BRIP1 was first identified as a member of the helicase family that binds to the BRCT domain of BRCA1

(Cantor et al. 2001). All three members of the complex, BRCA1, BRIP1 and TOPBP1, are present at replication origins and facilitate DNA replication by mediating the loading of the replication licensing factor CDC45L (Van Hatten et al. 2002; Hashimoto and Takisawa 2003; Greenberg et al. 2006). In addition, this complex is vital for the optimal loading of RPA onto chromatin (Gong et al. 2010). Therefore, the BRCA1B complex is associated with replication-coupled DNA repair and checkpoint progression throughout replication.

The BRCA1C complex contains BRCA1, CtIP and the MRN complex. This complex promotes HR and inhibits NHEJ by facilitating DSB end resection (Yu et al. 1998). Therefore, this complex is hypothesised to have a role in DSB repair pathway choice (Yun and Hiom 2009). An additional protein complex contains BRCA1, PALB2 and BRCA2 and specifically functions in DSB repair (Sy et al. 2009; Zhang et al. 2009a, 2009b). PALB2 is an integral component of this complex and binds directly to both BRCA1 and BRCA2, thereby providing a physical link between the two proteins. Disruption of this link leads to severe HR defects. Loading of BRCA2 onto RPA-coated ssDNA during HR is dependent upon the function of PALB2 within this BRCA1 complex (Xia et al. 2006).

While these complexes have been identified and many investigators have examined their functional roles, it is still unknown how they physically interact/co-ordinate with other complexes to achieve faithful repair of DNA damage. It is also thought that there is a level of functional redundancy between the individual complexes (Huen et al. 2010). Future studies will hopefully elucidate the mechanisms behind the exact synchrony of the HR pathway by pin-pointing the time of assembly and disassembly of these crucial protein complexes.

BRCA1 and BRCA2 Cellular Functions

In addition to HR, BRCA1 functions encompass gene transcription, cell cycle regulation and ubiquitination. BRCA1 complexes are involved in the activation of G_1/S, S-phase, and G_2/M checkpoints. BRCA1-BARD1 activity is involved in the G_1/S checkpoint by inducing p21, a protein that functions as a regulator of cell cycle progression (Fabbro et al. 2004). The BRCA1B complex is necessary for S-phase checkpoint activation in response to stalled replication forks (Greenberg et al. 2006). BRCA1 may also function in other DNA repair pathways, such as NHEJ and single strand annealing (SSA), a variant of the HR pathway. The role of BRCA1 in NHEJ is quite controversial. Some groups reported that BRCA1 facilitates NHEJ, as cell extracts derived from Brca1-deficient mouse embryonic fibroblasts exhibit reduced end-joining activity and the addition of partially purified BRCA1, in association with MRN, complements this NHEJ deficiency

(Zhong et al. 2002). Furthermore, NHEJ has been shown to be impaired in *Brca1*$^{-/-}$ mouse embryonic fibroblasts and in the human breast cancer cell line, HCC1937, which carries a homozygous mutation in the *BRCA1* gene (Bau et al. 2004). However, others reported that BRCA1 suppresses or has no effect on NHEJ (Moynahan et al. 1999; Snouwaert et al. 1999; Wang et al. 2001). The evidence for a suppressive effect of BRCA1 was provided by the observations that BRCA1-deficient mouse embryonic stem cells have impaired HR, but proficient NHEJ (Moynahan et al. 1999), and the random plasmid integration frequency in transient BRCA1-transfected HCC1937 cells is significantly suppressed compared to that of control cells (Zhang et al. 2004). In addition, the random plasmid integration rate of BRCA1-deficient mouse embryonic stem cells is higher than that of wild-type cells, yet can be reduced to the wild-type level by reestablishing BRCA1 expression (Snouwaert et al. 1999). A lack of effect of BRCA1 on NHEJ is supported by the finding that DSB repair kinetics are similar in the BRCA1-deficient breast cancer cell line, HCC1937, and in BRCA1-proficient cell lines (Wang et al. 2001). A deletion of exon 11 in BRCA1 exhibited decreased repair via classic HR and also by SSA. This demonstrates that BRCA1 functions upstream and early in the HR response to DNA DSBs (Stark et al. 2004). BRCA1 cells generated from mutant mice have revealed several other activities of BRCA1, including its roles in spindle assembly checkpoints (Xu et al. 1999; Wang et al. 2004a), maintenance of telomere integrity (McPherson et al. 2006), and transcriptional repression of unsynapsed chromosomal regions during meiosis (Turner et al. 2004). In agreement with this, BRCA1 was shown to be required for the accumulation of TPX2, a critical factor for microtubule stability and spindle fibre assembly in human cells (Joukov et al. 2006). However, whether the effects of BRCA1 on spindle function are direct, or indirect from replication defects, is not yet resolved.

The primary function of BRCA2 is to facilitate HR through direct interaction with Rad51 (Davies et al. 2001; Pellegrini et al. 2002; West 2003). BRCA2-deficient cells have defective Rad51 filament formation and decreased HR repair of DSBs (Yuan et al. 1999). Studies in BRCA2 mutant mice have implicated BRCA2 in stabilizing stalled DNA replication forks (Lomonosov et al. 2003). In addition, a replication-specific function of BRCA2 was recently shown to protect stalled replication forks from Mre11-dependent degradation (Schlacher et al. 2011).

An important function of the BRCA1-BRCA2 pathway is its interaction with p53 function: when the BRCA-pathway is defective, p53 function is abrogated. The p53 protein plays a key role in the maintenance of genomic integrity. It works as a transcription factor that, in response to several forms of cellular stress, regulates many target genes involved in cell cycle arrest, apoptosis and/or DNA repair (Vogelstein et al. 2000). In addition to the pathway interactions that appear to be robust in both normal and cancer

cells, p53 has been reported to physically interact with BRCA2 through its C-terminal domain (CTD) (Rajagopalan et al. 2010). Over-expression of either full-length BRCA2, or its CTD, leads to a decrease in apoptosis, perhaps through a reduction in expression of p53-target genes. However, whether this interaction has significance in cells with normal levels of BRCA2 expression remains to be determined. It has also been shown that p53 physically interacts with BRCA1 (Abramovitch and Werner 2003), and it was suggested that the transcriptional activity of BRCA1 is dependent upon the cellular status of p53. Additionally, it was observed that BRCA1 is involved in p53-mediated growth suppression rather than its apoptotic function (Ongusaha et al. 2003) (see Chapter 12). Despite this protein-protein interaction being reported by multiple authors, it is not clear if this is a physiologically relevant interaction.

BRCA1 and BRCA2 Deficiency in Mice

Embryos with a loss of both functional alleles of either BRCA1 or BRCA2 die early in development at approximately day 8 or 9 due to proliferation defects (Hakem et al. 1996; Liu et al. 1996; Ludwig et al. 1997; Suzuki et al. 1997). The embryonic lethality of BRCA-deficient mice resembles the phenotype of Rad51-deficient mice. The embryonic lethality of *Brca1* mutant embryos was partially rescued in a p53-deficient background; the *BRCA1/p53* double mutants did not overlap phenotypically with the severely retarded *BRCA1* single mutant. However, the rescue of *BRCA2*-associated embryonic lethality was less clear, as one of the *BRCA2/p53* double null zygotes was indistinguishable from the wild-type, whereas the others were similar to or further developed than the most advanced *BRCA2* single mutants.

Mice models expressing truncated versions of BRCA proteins revealed some phenotypic differences. $BRCA1^{1700T/1700T}$ mice harboring a homozygous C-terminal truncating mutation that removes the second BRCT repeat show a delayed embryonic lethality, compared to the *Brca1 null* animals (Hohenstein et al. 2001). $Brca1^{Tr/Tr}$ mutants encoding BRCA1-Δ11 (exon 11 deletion) splice variant were viable, yet were found to be tumor-prone, developing predominantly lymphoid and sarcomatoid tumors with only 12 out of 92 neoplasms being mammary tumors (Ludwig et al. 2001). Embryos carrying *Brca2* mutations that did not encode any BRC repeats did not survive, whereas embryos carrying *Brca2* mutations that encoded at least three BRC repeats were partially viable (Connor et al. 1997; Friedman et al. 1998; McAllister et al. 2002). *Brca2* models that enabled survival of animals showed increased tumorigenesis in the absence of p53 mutations and a strong bias towards development of thymic lymphomas (Connor et al. 1997; Friedman et al. 1998; McAllister et al. 2002). These animal models are of limited use, however, due to the lack of mammary tumor development.

Nevertheless, a recently developed conditional knockout mouse model may show increased potential for use in cancer therapeutics (Liu et al. 2007a). This model uses a tissue-specific *p53* inactivation in a *BRCA1 null* mouse that selectively causes mammary tumors, in contrast to the high incidence of sarcomas and lymphomas that are seen in the other models.

THE BRCA-FA PATHWAY

The Fanconi Anemia (FA) pathway is predominantly responsible for repair of inter-strand crosslinks within the cell (see Chapter 10). Repair of inter-strand crosslinks requires the coordinated actions of the FA pathway, translesion synthesis polymerases, nucleotide excision repair and the HR pathway. These processes are intricately linked, as evidenced by the identification of BRCA2 as FANCD1 (Howlett et al. 2002), BRIP1 as FANCJ (Levitus et al. 2005; Litman et al. 2005) and PALB2 as FANCN (Reid et al. 2007; Xia et al. 2007), all of which were independently assigned to both the HR and FA pathways. In addition, RAD51C, a Rad51 paralog protein, has recently been identified as an FA gene from a single family in Pakistan and is therefore also termed FANCO (Vaz et al. 2010). Furthermore, Slx4, a resolvase involved in HJ resolution, has now been identified in a number of FA individuals and as such has been designated FANCP (Kim et al. 2011; Stoepker et al. 2011).

During DNA repair via the FA pathway, a core complex containing FANC-A, B, C, E, F, G, L, M forms, in conjunction with FA accessory proteins FAAP24 and FAAP100 (Ciccia et al. 2007; Ling et al. 2007). From within the core, FANCL ubiquitinates the FANCD2-FANCI subcomplex. Upon FANCD2-FANCI localization to chromatin and subsequent ubiquitination, FANCD2 is able to interact with BRCA2/FANCD1 through its C-terminus and facilitate BRCA2/FANCD1 loading onto chromatin (Wang et al. 2004b). Complementation groups FANCD1/BRCA2, FANCJ/BRIP1, FANCN/PALB2, FANCP/Slx4 and FANCO/Rad51C are all proficient in FANCD2-FANCI ubiquitination and, therefore, must function downstream of the FA core in order to complete repair.

There are direct interactions between several members of the FA-HR pathways: FANCG-FANCD1/BRCA2, FANCD2-FANCD1/BRCA2, and FANCG-XRCC3 (Hussain et al. 2003, 2004, 2006). Recently, a novel complex has been identified, consisting of FANCD1/BRCA2-FANCD2-FANCG-XRCC3 (the D1-D2-G-X3 complex) (Wilson et al. 2008), which is dependent upon FANCG phosphorylation at serine-7. Formation of this complex, however, is independent of FA core complex formation. It has recently been demonstrated, using DNA fiber analysis, that BRCA2-deficient cells are defective in protecting the nascent strand at hydroxyurea-induced stalled replication forks (Schlacher et al. 2011). In addition, it was previously

shown that XRCC3 is required for replication fork slowing on chromosomes damaged by cisplatin exposure (Henry-Mowatt et al. 2003). Taken together, these results suggest a role for the D1-D2-G-X3 complex, independent of classic HR and FA activities, in the cellular response to DNA damage that promotes stalled replication fork formation, although this has yet to be determined.

Whilst it is clear that these repair pathways act cooperatively with each other to achieve resolution of damaged DNA structures, they also have distinct functions that are not redundant; members of the HR pathway cannot substitute for a deficiency in the upstream members of the FA pathway that initiate the repair cascade, and likewise, the final steps of repair of the damaged structure is HR-dependent and cannot be performed by other FA proteins. However, the evident links between HR and FA proteins give increased potential for therapeutic manipulation (Garcia and Benitez 2008), as more targets for synthetic lethal exploitation become available (see later section for detail).

CLINICAL SYNDROMES ASSOCIATED WITH BRCA1/BRCA2 MUTATION

BRCA1 and *BRCA2* germline mutations are the most significant contributors towards hereditary breast and ovarian cancer (HBOC), accounting for 40% of recorded tumors. Defects associated with HBOC are all dominant mutations, meaning that only one mutated allele is required for disease progression. However, this is not the case with all BRCA-related diseases, and an example of disease requiring two recessive alleles is FA. While the clinical outcome of *BRCA1/2* mutation is similar, there are marked differences between the resulting tumors. This is dependent upon whether a *BRCA1* or *BRCA2* mutation was the cause of tumorigenesis. There are specific sub-types of breast cancer tumors, each having distinct clinical, biological and therapeutic implications and may be distinguished by gene profiling, histology and hormone receptor status. Basal-like is typically the most aggressive sub-type and generally hormone-receptor negative. Luminal tumors are estrogen receptor positive and express estrogen-responsive genes. BRCA1-mutated cancers are typically basal-like, and are associated with a higher frequency of *TP53* mutation in comparison to BRCA2 (Holstege et al. 2010a). In contrast, BRCA2-mutated tumors are predominantly luminal (Bane et al. 2007). Using array comparative genomic hybridization (aCGH) technology, which analyses gains and losses of DNA within the genome, it was determined that there are differences in the profiles of BRCA1- vs. BRCA2-mutated tumors (Holstege et al. 2010b). The risk of male breast cancer is increased in both BRCA1 and BRCA2 mutation

carriers, with a specific increased risk in BRCA2 mutation carriers (Bane et al. 2007; Tai et al. 2007). Indeed the overall range of cancers observed in BRCA2 mutation carriers is broader than those observed in BRCA1 mutation carriers, including prostate and pancreatic cancer.

Although it is seen in the majority of cases, loss of heterozygosity (LOH) is not necessarily required for cancer to develop in BRCA-mutation carriers. LOH is described as the loss of the normal/functional allele of a gene; in the case of BRCA-mutations, tumorigenesis may occur while the normal allele is still present. This was displayed in a study of pancreatic cancers caused via the *BRCA2* 999del5 mutation, observed in a small study of pancreatic cancers of Icelandic origin (Thorlacius et al. 1996; Skoulidis et al. 2010). Despite the strong contribution from *BRCA1/2* mutations, there is a considerable proportion of HBOC that must be attributable to mutations in other genes. As yet, it is unknown why *BRCA1/2* mutations cause cancer only in selective tissue types.

Abraxas mediates the interaction between BRCA1 and RAP80, with RAP80 responsible for complex localization to DNA damage sites. Disruption of either Abraxas or RAP80 would potentially lead to a decrease in HR through decreased levels of BRCA1 at DNA DSBs. However, it was found that mutations in either of the corresponding genes were low penetrance and did not significantly contribute to the development of HBOC (Novak et al. 2009). Several genes with intermediate-penetrance relating to HR proteins have been suggested, such as *ATM*, *CHK2*, and *PALB2* (Shuen and Foulkes 2011). ATM functions as a checkpoint kinase that phosphorylates both p53 and BRCA1 (see Chapter 13). Mutations in this protein result in a slight increase (~ 2-fold) in the risk of breast cancer in individuals without *BRCA1* or *BRCA2* mutations (Thompson et al. 2005a; Renwick et al. 2006). A specific mutation within *CHK2*, 1100delC, has been associated with a 2–3-fold increased risk of breast cancer (Weischer et al. 2007), and other mutations in this gene have now been identified (Campeau et al. 2008). Mutation of PALB2, the BRCA2 interacting protein, confers a 2–4-fold increased risk of breast cancer, which is further increased in individuals with a family history of HBOC (Rahman et al. 2007; Byrnes et al. 2008). However, in comparison to BRCA1 and BRCA2, mutations in *PALB2* leading to HBOC are rare (Hellebrand et al. 2011). NBS1 is a member of the MRN complex which is involved in DSB recognition. *NBS1* 657del5 was identified as a significant contributor to breast cancer tumorigenesis, but only within Polish and Byelorussian populations (Steffen et al. 2006; Bogdanova et al. 2008). Mutations in *BRIP1* have been identified in a number of patients presenting with early onset breast and ovarian tumors (Cantor et al. 2004). The coding sequence changes observed in these patients led to a defective helicase activity of the BRIP1 protein, resulting in decreased HR function. BARD1, responsible for BRCA1 E3 ubiquitin ligase functions *in vivo*, has

been proposed as a high penetrance gene in breast cancer carcinogenesis and a number of allele variants have been discovered, each with varying degrees of significance (Ghimenti et al. 2002; Ishitobi et al. 2003; Huo et al. 2007). In addition, three non-synonymous single nucleotide polymorphisms have been identified in *BARD1* that have a low-penetrance effect on breast cancer risk (Huo et al. 2007).

Alteration of *TP53* is the most common mutation found in sporadic human cancers, and its role in tumorigenesis associated with or without *BRCA* mutations is reviewed in more detail elsewhere (Roy et al. 2012). Recently, germline *TP53* mutations have been identified, where a patient exhibiting early onset breast and ovarian tumors was found to be mutation negative in *BRCA1/2* and further investigation led to discovery of *TP53* mutation (Janavicius et al. 2011). This may suggest that the incidence of p53 mutation within human cancers has thus far been underestimated, as it is normally presumed to be a sporadic mutation, rather than inherited. Despite a wealth of information discovered over the past several years, the mutations in genes responsible for 25–30% of HBOCs are still unknown.

In addition to HBOC, BRCA1 may have a significant impact on the progression of certain lymphomas; 32% of primary acute myeloid leukemia (AML) tumors and 75% of therapy-related AML tumors are associated with decreased BRCA1 expression (Scardocci et al. 2006). This is not limited solely to BRCA1 mutations, as BRCA2 truncations are over-represented in non-hodgkins lymphoma tumors. In addition, abnormalities in the MRN complex and mutations in both ATM and CHK2 are all associated with leukemias and lymphomas (reviewed in Friedenson 2007).

SYNTHETIC LETHALITY

Background of Synthetic Lethality

Recent advances in cancer research have led to promising clinical results using the concept of synthetic lethality. Two genes are said to be "synthetic lethal" if mutation of either gene alone is compatible with viability, but simultaneous mutation of both genes causes death (Kaelin 2005) (Fig. 3). The idea of synthetic lethality was first observed by Calvin Bridges in the early 20th century through his study with *Drosophila melanogaster*. His observations showed that certain non-allelic genes were lethal only in combination, even though the homozygous parents were viable (Nijman et al. 2010). The term synthetic lethal was later coined by Dobzhansky (1946) through his work with *Drosophila pseudoobscura*. His studies involved recombining particular genes, which were individually viable when on separate chromosomes; however, once on the same chromosome, led to a recessive lethal effect. Although first identified in *Drosophila*, most synthetic lethal relationships

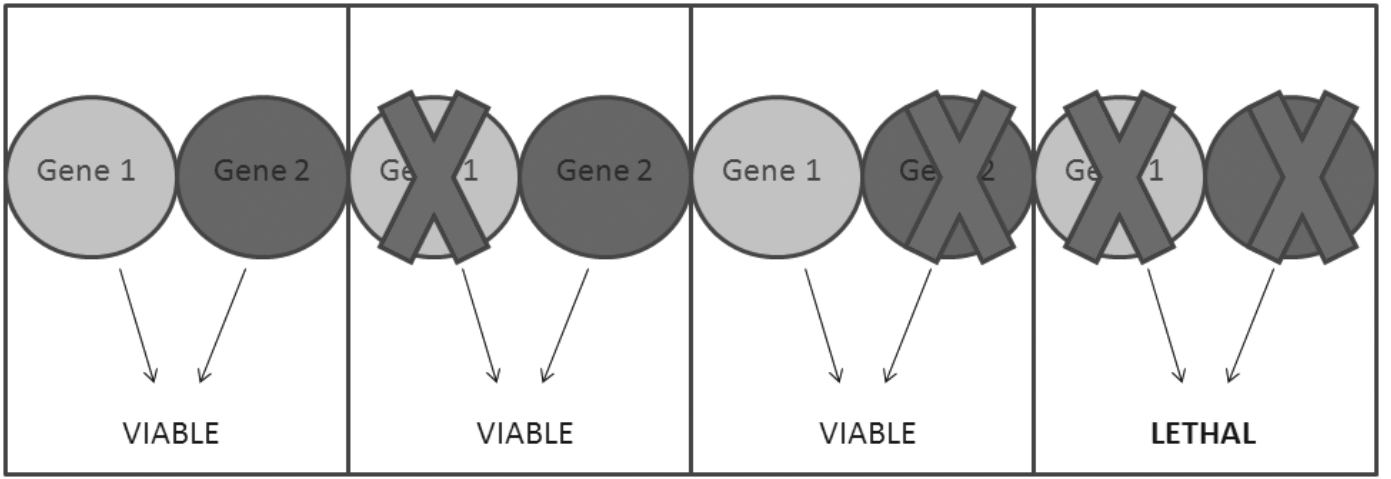

Figure 3. Schematic illustration of a synthetic lethal relationship. Two genes are synthetically lethal when inactivation of both results in cellular death. Inactivation of either gene individually does not affect cell viability.

have been examined in *Saccharomyces cerevisiae*, *Escherichia coli*, *Caenorhabditis elegans* and, more recently, in mammalian cell lines.

Yeast is an ideal model organism to study the relationship of synthetic lethality due to its genome compactness and its short non-coding regions. These features make gene prediction in yeast less complex than in other eukaryotes and greatly simplify functional analyses (Ooi et al. 2006). The *Saccharomyces* genome-deletion project was undertaken to create a library of deletion or substitution mutations. This worldwide collaboration has led to the collection of yeast knockout (YKO) mutants and has become a valuable source for identifying synthetically lethal relationships. Synthetic genetic array (SGA) analysis, which was developed by Tong and Boone in 2006, is another approach to study synthetic lethal relationships. This is an efficient approach for the systematic construction of double mutants and enables a global analysis of synthetic lethal genetic interactions. An alternative to SGA is a technique called synthetic-lethality analysis by microarray (SLAM). In this technique, a particular mutation is introduced into the haploid YKO pool by direct integrative transformation and double mutants are then analyzed by microarray (Zhao et al. 2011). Synthetic lethal analyses in lower eukaryotic model organisms have provided invaluable information for the examination of subsequent mammalian screens.

There are two ways in which synthetic lethal relationships can occur, depending on whether the genes act in the same pathway (within-pathway interaction) or in separate pathways (between-pathway interaction). The within-pathway interaction is where genes A and B belong to the same pathway and the function of this pathway is reduced by single gene mutations, but rendered below the viability threshold by mutation of both A and B (Fig. 4A). Between-pathway synthetic lethality assumes that genes A and B act in parallel pathways that can compensate for defects in the other (Lippert et al. 2010) (Fig. 4B). There are also the rare events of one gene becoming essential for survival when a second gene is over-expressed; this phenomenon is known as synthetic dosage lethality. Dosages suppression screens involve identification of wild-type genes that, at increased copy number or when over expressed, complement the phenotype caused by a reference gene mutation (Kroll et al. 1996).

Synthetic Lethality in Humans

Although synthetic lethality as an approach to cancer therapy was proposed more than a decade ago (Hartwell et al. 1997), only recently has this approach reached the clinic (Brough et al. 2011). Tumor suppressor genes should theoretically be excellent targets for synthetic lethality based anticancer therapy. Inhibiting a gene with a synthetic lethal relationship with a tumor suppressor that is dysfunctional in a tumor cell will help selectively kill

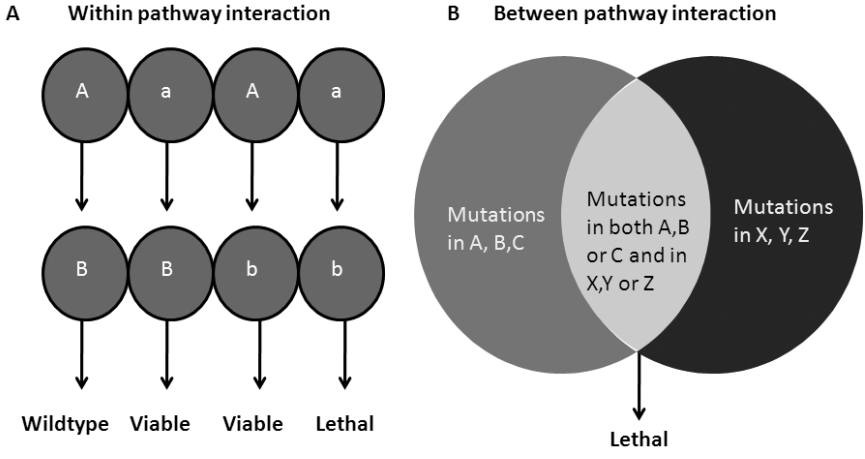

Figure 4: Within and between pathway interaction. (A) A within pathway interaction between genes A and B occurs when the double mutant A and B is lethal in comparison to single A or B mutations. (B**)** A between pathway interaction occurs when two genes working in separate pathways both contribute to an essential process. When either gene 'A', 'B' or 'C', or gene 'X', 'Y' or 'Z' is mutated, the organism or cell remains viable. However, the combination of these mutations ('A', 'B' or 'C' with 'X', 'Y' or 'Z') results in death.

cancer cells and preserve normal cells. Due to the selective lethal effect on cancer cells harboring specific genetic alterations, it is expected that targeting synthetic lethal genes would provide wider therapeutic windows compared with cytotoxic chemotherapeutics (Mizuarai and Kotani 2010). Side effects like hair loss, nausea and immuno-suppression are harrowing for the patient undergoing treatments such as chemotherapy. Over the last several decades, more emphasis has been placed on identifying therapies that lack these side effects and are more specific to the target. Using anticancer drugs that exploit synthetic lethality interactions promises specificity in targeting cancer cells that have genetic mutations not found in normal cells, known also as conditional genetics. One clinical example of this is the use of PARP inhibitors on BRCA1 and BRCA2 (see later section). The following section will discuss the various strategies that have been developed to evaluate and target potential synthetic lethal interactions.

Methodology for Synthetic Lethality Studies

Until recently, synthetic lethality screens were limited to using chemical compounds; however, with the discovery of RNA interference (RNAi), it has now become possible to systematically identify synthetic lethal interactions in human cells (Whitehurst et al. 2007; Turner et al. 2008; Nijman et al. 2010). Synthetic lethal screens in mammalian cells can be broadly categorized

under forward and reverse genetics. Forward genetic studies begin with a phenotype of interest and use the variability in a collection of cancer cell lines to identify a particular gene of interest. This type of screen involves using non-isogenic cell lines, which are cells that are not derived from a single progenitor, but instead are categorized by whether they have a mutation of interest or not (Rehman et al. 2010). In forward genetics based studies, non-isogenic cell lines need to be monitored carefully, as their genetic backgrounds vary greatly. This can be seen as advantageous in that it represents a larger faction of patients representing the true genetic diversity. However, it is necessary to use a large number of these cell lines due to additional genetic alterations occurring within them. It is also important to compare these cell lines directly with isogenic cell lines to ensure that the genetic variation being analyzed is a direct contributor to synthetic lethality.

In contrast, reverse genetics screening requires introducing a single specific genetic alteration to generate an isogenic cell line pair. Isogenic cell lines are derived from a single progenitor and are most frequently used for synthetic lethality screens (Bommi-Reddy et al. 2008). However, there are limitations to using isogenic cell lines, where the single genetic variation may not be the only difference between the cells. This is also known as "genetic drift" and this problem can be especially acute when the mutation of interest results in a defect in DNA repair or other genome maintenance factors (Chan and Giaccia 2011). Isogenic cell line pairs do not exist for every gene of interest, and when they do exist, they may not be derived from the same species or cell type to that of the tumor of interest (Chang et al. 2011). It is also necessary to use several isogenic cell line pairs for a particular gene of interest. This eliminates the chance that these interactions are just sporadic interactions. Sometimes a particular isogenic cell line may have varying characteristics in relation to other functions, including cell cycle distribution or proliferation.

Both isogenic and non-isogenic cell lines have been successfully employed using RNAi-mediated approaches. RNAi is a powerful and widely used gene silencing strategy for studying gene function in mammalian cells (Wu et al. 2007). Its main components are miRNA (micro RNA), shRNA (short hairpin RNA) and siRNA (small interfering RNA). Most synthetic lethal interactions in mammalian cells have been identified through large-scale screening with RNAi, typically targeting 500 to 20,000 genes (Schlabach et al. 2008). Screening of RNAi based libraries can identify genes that are involved in a specific pathway and can provide a better understanding of the basic biology behind genetic interactions. RNAi allows investigation into long term knockdown effects on cancer related phenotypes, which include viability, apoptosis and cell cycle. This

approach can help to discover genetic interactions that may lead to potential therapeutic targets upon further analysis.

There are several RNAi based approaches used to study synthetic lethality. One such method uses a siRNA library to achieve sufficient gene-silencing *in vivo*, which can be difficult to achieve for all targets. siRNA is used to perform high-throughput screening that enables knockdown of individual genes in single well assays (Mizuarai and Kotani 2010). To avoid the limitation of screening individual vectors in a single well, several groups have developed a more practical screen using pools of shRNA clones (Ngo et al. 2006; Silva et al. 2005).

Pooled shRNA assays use a single dish that has been infected with large numbers of shRNA viral vectors. The abundance of individual shRNA's is monitored over time, and each vector is amplified by PCR across a specific sequence (bar-code), which is unique to the individual shRNA vector (Brummelkamp et al. 2002). These sequences are hybridized to a microarray which contains probes that correspond to the barcodes in the library of shRNA's (Fig. 5). The barcode technique allows for the rapid identification of individual shRNA vectors from a large pool of shRNA vectors that produce a specific phenotype (Mullenders et al. 2009). Isogenic cells are infected with pooled shRNA libraries and these populations are divided in two. One of these two populations acts as a reference, while the second is subjected to the selected treatment. Isolation of the shRNA's from each of the populations, and then labeling the barcode identifiers with different fluorescent dyes and hybridizing to DNA microarrays, will reveal the relative differences between the control and the treated cells. Knockdown of a specific gene using a shRNA vector leads to three outcomes after treatment: 1- Cells remain unaffected. 2-Cells become more sensitive to treatment. 3- Cells acquire resistance to the treatment.

The variability in knockdown efficiency and the fact that RNAi typically results in 70–90% inhibition of expression highlights a weakness in this tool (Nijman et al. 2010), since normal function of a protein can be observed at only 10–30% of the usual protein level. Another problem with RNAi is that it can produce false positive results, leading to many off-targets being included in subsequent analyses. Off-target effects can lead to toxicity of the cell or inefficiency of drug introduction (Dua et al. 2011), but they are not usually reproduced with a second siRNA targeting a different region of the gene. The screening method must therefore be developed further, employing at least duplicates for each gene to eliminate the possibility of false positives and to determine the possible side effects from hitting off-target genes. More robust and powerful screening techniques are also required to characterize knockdown libraries and to validate potential hits. There is no doubt that RNAi screening methodology will become substantially more sophisticated in years to come.

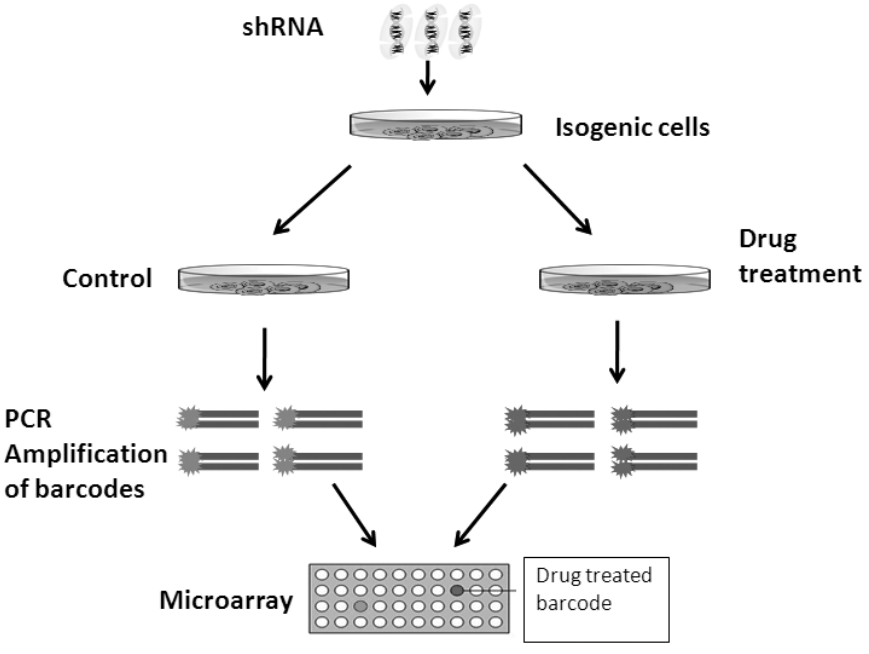

Figure 5. shRNA bar-coding screen. Plasmids encoding shRNA and barcode sequences are transfected into isogenic cells. Each cell type is then sub-divided and one is treated with a specific drug, the other is a control. When gene-specific lethality is observed, DNA is extracted from the untreated sample and the barcode is recovered through PCR amplification. The abundance of barcodes is determined through microarray hybridization and shRNA in groups is identified.

Color image of this figure appears in the color plate section at the end of the book.

Small Chemical Compound Libraries

Screening small compound libraries has identified many new drugs for cancer treatment (Chan and Giaccia 2011). There are several similarities between screening siRNA/shRNA libraries and small molecule compound libraries. For drug-induced synthetic lethality, small compound libraries require isogenic cell lines of the target gene of interest and a functional readout for drug efficacy. Identifying a target molecule by screening libraries can potentially recruit the compound into clinical trials. However, a component of screening compound libraries is that any hit needs a detailed work-up of how the drug may be working, which could lead to the identification of yet additional, new genetic targets. PARP inhibitors are one of the first classes of small-molecule compounds to be identified that interact in a synthetically lethal manner with mutations in DNA repair genes (Chan and Giaccia 2011).

RNAi Libraries used in Conjunction with a Chemical Inhibitor

RNAi screening in combination with a chemical compound is another approach to investigating synthetic lethal therapies between genes. Following successful identification of synthetically lethal interactions, RNAi libraries of genes that interact with synthetic lethal genes are tested with a targeting drug designed against already identified synthetic lethal genes. An expected outcome of chemical inhibitor screens is the identification of gene products that are targets of currently available compounds, revealing novel combinational therapeutic regimens (Whitehurst et al. 2007). The most documented example of this is the PARP and BRCA1 interaction, which will be described in detail later. However, there is a disadvantage to using this method of screening; focusing on the known function of the target gene may be misleading, as other independent functions of the gene may be responsible for the phenotype (Chan and Giaccia 2011).

Biomarkers

Biomarkers are indicators for a particular biological state before or after treatment. RAD51 foci formation is a biomarker for HR activity (Rehman et al. 2010). In response to IR or camptothecin, RAD51 localizes to discrete foci in the nucleus, which can be visualized. Localization of RAD51 is dependent on functional BRCA1 and other HR proteins, and thus, cells that are defective in RAD51 foci implies a defective HR pathway and sensitivity to PARP inhibition. Biomarker assays combined with high-throughput mutation screening could be an alternative method for identifying the impact of inhibitors on the HR pathway (Yang et al. 2011). By using biomarkers in these studies, a defined subgroup of patients who respond favorably to inhibitors, such as those against PARP, can be defined. Biomarker results will also limit the use of specific agents where a therapeutic effect is unlikely. Such biomarkers need to be validated through clinical trials, and further investigations of using this type of approach in the clinical setting needs to be determined.

SYNTHETIC LETHAL RELATIONSHIP BETWEEN POLY(ADP-RIBOSE) POLYMERASE AND BRCA PROTEINS

The synthetic lethal relationship between Poly (ADP-Ribose) Polymerase (PARP) and the BRCA proteins provides a proof of principle example that synthetic lethality can be utilized for cancer treatment. PARP depletion has been shown to be lethal to cells lacking BRCA1 or BRCA2 (Bryant et al. 2005; Farmer et al. 2005). In cancer patients carrying *BRCA* mutations,

only tumor cells are BRCA deficient (usually heterozygous $BRCA^{mut/-}$), while normal cells are proficient in HR, containing one functional and one nonfunctional copy of *BRCA1* or *BRCA2*. In this scenario, inhibition of PARP is being exploited to selectively kill tumors in patients with *BRCA* mutations (Anders et al. 2010; Annunziata and O'Shaughnessy 2010; Aly and Ganesan 2011; Yap et al. 2011). As described below, PARP inhibitors are currently in clinical trials and are likely to have applications beyond cancer patients with BRCA mutations (see Chapter 15).

PARP Proteins

The PARP family consists of 17 proteins, each containing a conserved catalytic domain which is responsible for poly (ADP-ribose) polymerization (Krishnakumar and Kraus 2010). This activity generates long chains of poly (ADP) ribose on target proteins, a process known as PARylation. PARP proteins are important in several cellular processes, including genomic stability, DNA repair, cell cycle progression, and apoptosis (Krishnakumar and Kraus 2010). Only six of these proteins (PARP1, PARP2, PARP3, PARP4, TNKS1/PARP5 and TNKS2/PARP6) have been confirmed to have true PARP activity (Hassa and Hottiger 2008). Other PARP family members have roles in adding a single ADP-ribose to target proteins rather than generating chains, with the exception of PARP9 and PARP13, which are believed to lack ADP-ribose transfer activity entirely (Krishnakumar and Kraus 2010).

The sub-cellular localization and functions of many of the PARPs are unknown; however, PARP1 and 2 have been shown to be involved in SSB repair (SSBR) (Krishnakumar and Kraus 2010). PARP1 is a nuclear protein that associates with SSBs, and subsequently recruits and PARylates target proteins such as XRCC1 (Masson et al. 1998; El-Khamisy et al. 2003; Woodhouse and Dianov 2008; Krishnakumar and Kraus 2010). PARP2 has also been shown to localize to the nucleus and plays a role in SSBR, though to a lesser extent compared to PARP1 (Schreiber et al. 2002; Yelamos et al. 2008). PARP1 and 2 also facilitate HR and fork restart by promoting MRE11, RPA, and RAD51 recruitment to collapsed replication forks (Bryant et al. 2009). Their role in HR is believed to be specific to fork restart and not general DSB repair, since PARP depletion does not affect gene targeting in embryonic stem cells or HR repair of restriction enzyme induced DSBs (Yang et al. 2004; Sugimura et al. 2008). Mouse knockouts of PARP1 or PARP2 are viable and fertile and do not develop early onset tumors (Wang et al. 1995). However, PARP1 knockout mice and normal cells treated with PARP inhibitors do exhibit defective SSBR, increased HR and increased sister chromatid exchange, suggesting that HR may be vital to repairing lesions in PARP deficient cells (Molinete et al. 1993; Lindahl et al. 1995; de Murcia et al. 1997; Wang et al. 1997; D'Amours et al. 1999; Bryant et al. 2005).

Mechanism of BRCA/PARP Synthetic Lethality

The current model for the synthetic lethality relationship between PARP and HR hinges on PARP's role in the repair of SSBs (Fig. 6) (Helleday et al. 2005; Ashworth 2008b); however, recent evidence suggests that the interaction is more complicated. PARP inhibition leads to the accumulation of SSBs, which stall replication forks and cause the formation of DSBs (Bryant et al. 2005; Farmer et al. 2005; Saleh-Gohari et al. 2005). These DSBs are normally repaired by the HR pathway and therefore cannot be repaired effectively in BRCA-deficient cells (Saleh-Gohari et al. 2005). Thus, PARP inhibition in HR-deficient cells results in synthetic lethality, as the resulting DNA damage induces apoptosis or other forms of cell death. While this model focuses on PARP's role in SSBR, its function in HR-mediated restart of stalled replication forks (Bryant et al. 2009) as well as alternative pathways of NHEJ (Wang et al. 2006) may contribute to the sensitivity of BRCA-deficient cells to PARP inhibitors (Helleday 2011). Another mechanism has been suggested in which PARP inhibitors trap PARP on a SSB intermediate (Strom et al. 2011), which may then be converted to a more toxic lesion during replication (Helleday 2011). PARP proteins are also involved in DNA methylation, transcription, chromatin modification, and cell death pathways, and these functions may contribute to the sensitization of BRCA-deficient cells as well (Krishnakumar and Kraus 2010).

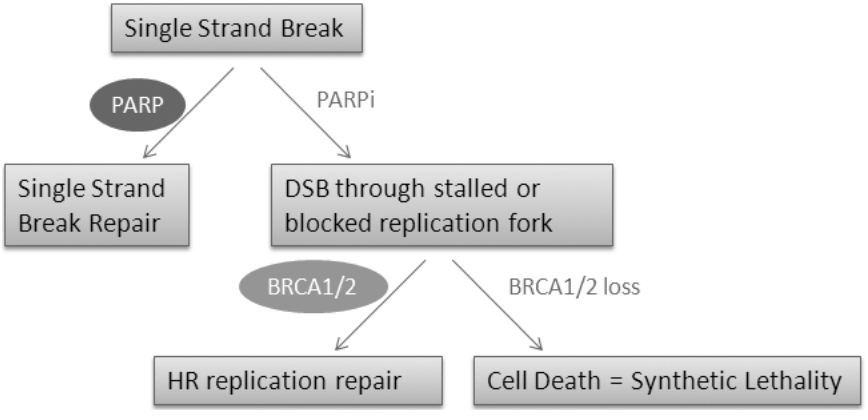

Figure 6. Model for synthetic lethality between PARP and BRCA proteins. PARP inhibitors prevent PARP from promoting SSBR and cause PARP to remain bound at SSBs. When replication forks encounter these lesions they are converted to DSBs, which are repaired by HR in wild-type cells. In cells lacking BRCA1 or BRCA2, the breaks are unable to be repaired by HR, and the inability to repair the DNA triggers cell death.

Preclinical Studies

Several *in vitro* studies have confirmed that inhibition of PARP in a BRCA-deficient background results in synthetic lethality (Bryant et al. 2005; Farmer et al. 2005). Initial reports showed that the Chinese hamster BRCA2-deficient cell line V-C8 was hypersensitive to low concentrations of PARP inhibitors (NU1025 and AG14361) as compared to wild-type cells; this effect was lost with BRCA2 complementation, demonstrating its specificity for BRCA2 (Bryant et al. 2005). *Brca1*$^{-/-}$ and *Brca2*$^{-/-}$ mouse embryonic stem cells were also identified as being sensitive to PARP inhibitors (KU0058684 and KU0058948), which induced chromosomal aberrations that led to cell cycle arrest and apoptosis, supporting the hypothesis that PARP inhibitors cause the accumulation of DSBs in BRCA-deficient cells (Farmer et al. 2005). Experiments in mice showed that BRCA2-deficient xenografts had reduced tumor growth when treated with PARP inhibitors, while BRCA2 complemented xenografts treated with PARP inhibitors saw no reduction in growth (Bryant et al. 2005). Additionally, the human breast cancer cell lines MCF7 (p53 WT) and MDA-MB-231 (p53 mutant) were very sensitive to the PARP inhibitor NU1025 when BRCA2 was specifically depleted by siRNA, demonstrating that this effect is independent of p53 status (Bryant et al. 2005). Though PARP inhibitors block both PARP1 and PARP2, experiments using siRNA depletion of PARP1 or PARP2, each with simultaneous BRCA2 depletion, suggested that PARP1 (and not PARP2) was responsible for the synthetic lethal effect (Bryant et al. 2005). Interestingly, siRNA depletion of PARP1 was not as effective for synthetic lethality as the PARP inhibitors, perhaps due to incomplete siRNA depletion (Bryant et al. 2005). Alternatively, it has been suggested that inhibiting PARP activity without inhibiting its DNA binding causes inactive PARP to accumulate at DNA breaks, resulting in a more toxic lesion (Bryant et al. 2005).

Sensitivity to PARP inhibitors has also been demonstrated in cells deficient in other HR genes, including Rad51, Rad54, DSS1, RPA1, NBS1, ATR, ATM, CHK1, CHK2, FANCD2, FANCA, and FANCC (McCabe et al. 2006). This supports the hypothesis that BRCA mutant cells are sensitive to PARP inhibitors specifically because of the HR deficiency and suggests that PARP inhibitors may be used for a broader class of tumors with HR defects, or showing "BRCAness," as will be discussed in more detail below. These studies demonstrate that PARP inhibition is a promising approach to selectively target HR-defective tumors, and has led to clinical studies using PARP inhibitors in cancer patients carrying BRCA mutations (see Chapter 15).

PARP INHIBITORS AND CLINICAL TRIALS

Currently, many PARP inhibitors are in early phase clinical trials. Thus far, Olaparib (AZD2281, AstraZeneca) has been one of the most extensively studied (Anders et al. 2010). A phase I trial of Olaparib enrolled a cohort of patients enriched for BRCA-mutation carriers with advanced tumors (received a range of doses from 10mg daily for 2 of every 3 weeks up to 600 mg twice daily) and later enrolled an additional cohort only of BRCA mutation carriers (receiving 200 mg twice daily) (Fong et al. 2009). Olaparib was found to have few of the adverse effects seen with traditional chemotherapy, but did cause fatigue and gastrointestinal symptoms. BRCA mutation carriers and non-mutation carriers did not show differences in their side effect profiles. Pharmocodynamic studies confirmed PARP inhibition in patient samples and substantial anti-tumor activity, and PARP inhibition was found in BRCA1/2 mutation patients with ovarian, breast, and prostate cancer (Fong et al. 2009). Interestingly, Olaparib's anti-tumor activity was correlated with platinum sensitivity, suggesting that PARP inhibitors and platinum treatment may have similar mechanisms of activity and resistance (Fong et al. 2010). Since platinum sensitivity may also indicate HR defects, these observations support the hypothesis that PARP inhibitors target HR defective cells specifically.

Phase 2 studies of Olaparib in patients with advanced breast (54 patients) (Tutt et al. 2009) and ovarian (56 patients) (Audeh et al. 2009) cancers with confirmed *BRCA1* or *BRCA2* mutations and recurrent, advanced disease also showed promising results. Patients were given the pharmacodynamically active dose (100mg twice daily) of Olaparib or the established maximum tolerated dose (400mg twice daily). These studies confirmed the favorable side effect profile of Olaparib, and though both doses showed clinical activity, the 400mg dose twice daily appeared more effective. The response rate was 41% for breast cancer patients in the 400mg cohort (22% in the 100mg cohort) with a median progression free survival of 5.7 months (3.8 months for 100mg), and for the ovarian cancer patients the response rate was 57.6% and 5.8 months at 400mg (16.7% and 1.9 months at 100mg). Though these responses are impressive, they are still low compared to other targeted therapies (Audeh et al. 2009; Tutt et al. 2009).

These trials and others provide clinical validation of PARP inhibitor use in patients with germline *BRCA* mutations. There are several ongoing clinical trials of PARP inhibitors as single agents in patients with *BRCA* mutations, which are listed at the www.clinicaltrials.gov website. Many of these inhibitors show promising results in the treatment of BRCA mutated cancers, yet highlight the need to identify and improve upon methods to determine which patients will most likely respond to PARP inhibition.

PARP INHIBITION AND APPLICATIONS BEYOND BRCA MUTATION CARRIERS: TNBC

Triple negative breast cancer (TNBC) lacks expression of the estrogen and progesterone receptors and lacks over-expression or amplification of the HER2 oncogene, and therefore is non-responsive to clinical treatments targeting either the estrogen receptor, estrogen production or the Her2-protein. TNBCs are aggressive cancers associated with a poor prognosis (Anders et al. 2010). TNBC, basal-like, and BRCA-mutated breast cancers share many molecular and pathologic characteristics, and TNBCs often have HR defects (Anders et al. 2010). Further, TNBC in patients without *BRCA* mutations may have epigenetic silencing of *BRCA1* by promoter methylation or have other defects elsewhere in the HR pathway (Wei et al. 2008; Annunziata and O'Shaughnessy 2010). Because of this "BRCAness" of TNBC, researchers are investigating the use of PARP inhibitors in their treatment. Preclinical studies established that basal-like breast cancer and TNBC cell lines are sensitive to PARP inhibitors, which may be due to defective HR or SSBR (Alli et al. 2009). A phase II study treated TNBC patients with gemcitabine and carboplatin with or without the PARP inhibitor iniparib: patients receiving iniparib displayed significantly-improved progression free and overall survival (O'Shaughnessy et al. 2011).

CHALLENGES TO USE OF PARP INHIBITORS: RESISTANCE

PARP inhibitors have displayed effectiveness in cancers that are difficult to treat, but this is often limited to less than a year of disease control (Anders et al. 2010). A few mechanisms for resistance to PARP inhibitors have therefore been suggested. One potential mechanism for the development of resistance involves upregulation of the multidrug resistance efflux pumps (Rottenberg et al. 2008). Genetically engineered mouse models deficient in BRCA1 were used to study long term treatment with the PARP inhibitor AZD2281 (Rottenberg et al. 2008). This treatment inhibited tumor growth and increased survival, but the mice developed resistance after long term use, which was frequently caused by up-regulation of the *Abcb1a/b* genes. These genes encode the P-glycoprotein efflux pumps, i.e., drug efflux transporters involved in multidrug resistance. The acquired resistance to PARP inhibitors in mice could be reversed by using a P-glycoprotein inhibitor (tariquidar) (Rottenberg et al. 2008). As another resistance mechanism, restoration of BRCA2 function caused resistance to PARP inhibitors (Edwards et al.

2008). The Capan-1 pancreatic cancer cell line containing the truncating frameshift mutation 6174delT in BRCA2 was treated with a PARP inhibitor until resistant clones were found. These cells had mutations restoring the open reading frame and the C-terminus of BRCA2 and were competent in HR. Interestingly, these mutations may have been caused by the use of error-prone repair due to BRCA2 deficiency (Edwards et al. 2008). Several other studies also found that secondary mutations in BRCA1 or BRCA2, generating a partially functional gene and thereby likely restoring HR function, can cause resistance to PARP inhibitors and platinum therapy (Ashworth 2008a; Sakai et al. 2008; Swisher et al. 2008; Wang and Figg 2008).

SYNTHETIC LETHAL RELATIONSHIP OF BRCA2 AND RAD52

Another promising example of synthetic lethality in DNA repair that may be utilized in cancer treatment is between the BRCA2 and Rad52 proteins. Since yeast lack the *BRCA2* gene, Rad52 performs the essential mediator function that BRCA2 performs in humans and other vertebrates (Sung 1997; Liu and Heyer 2011). Like BRCA2 in humans, yeast Rad52 promotes Rad51 filament formation on RPA coated single-stranded DNA and strand exchange through Rad51 (Sung 1997). Rad52 knockouts in mice show little phenotype and no sensitivity to agents that induce DSBs, and vertebrate Rad52 is not essential for Rad51 function (Rijkers et al. 1998; Yamaguchi-Iwai et al. 1998). Since BRCA2 is believed to perform these essential functions of Rad52 in humans, the role of human Rad52 has remained unclear.

A recent study demonstrated that in BRCA2-deficient human cancer cell lines, depletion of Rad52 reduced cell survival and proliferation, signifying a synthetic lethal relationship between the two proteins (Feng et al. 2011). Rad52 functions independently of BRCA2, since its localization to damage was not affected by the presence of the BRCA2 protein (Feng et al. 2011). Additionally, Rad52 was necessary for Rad51-mediated HR in BRCA2-deficient cells (Feng et al. 2011). These results suggest that Rad52 provides a backup HR pathway in human cells: while BRCA2 is present, Rad52 has little effect on HR and viability, whereas in BRCA2-deficient cells, Rad52 is important for viability and for HR (Feng et al. 2011). These observations indicate that targeting Rad52 could cause BRCA2-deficient tumor cells to stop proliferating with no or little effect on normal cells. Due to this relationship, Rad52 is now a potential target in cancer therapy, like PARP, but since it uses a completely different mechanism for synthetic lethality, inhibition of Rad52 should be synergistic with PARP-inhibition.

OTHER EXAMPLES OF SYNTHETICALLY LETHAL RELATIONSHIPS

MMR and DNA Polymerases

Synthetic lethal interactions have been proposed to exist between mismatch repair (MMR) proteins and DNA repair polymerases, suggesting another useful approach in cancer treatment (Hartwell et al. 1997; Martin et al. 2010, 2011). Germline mutations in the *MSH2* and *MLH1* MMR genes are associated with hereditary nonpolyposis colorectal carcinomas (Jacob and Praz 2002), and sporadic colorectal cancer (Bettstetter et al. 2007). MMR corrects mispaired nucleotides, which can arise during replication or recombination, and also contributes to the repair of oxidative DNA damage (see Chapter 7). The DNA repair polymerases Pol β and Pol γ are involved in nuclear and mitochondrial base excision repair, respectively, a pathway that corrects oxidative damage to bases or nucleotides (see Chapter 8). Martin and colleagues reported that depletion of Pol β in MSH2 mutated cells and depletion of Pol γ in MLH1 mutated cells caused increased oxidative DNA damage and reduced clonogenic survival (Martin et al. 2010). Interestingly, Pol β and Pol γ expression levels were found to be elevated in MSH2 and MLH1 cells, respectively (Martin et al. 2010). In another report, Martin and colleagues also showed that PTEN-induced putative kinase 1 (PINK1) and other mitochondrial kinases had similar synthetic lethal relationships with MLH1 and MSH2 using high throughput RNA interference screens (Martin et al. 2011). These authors propose that these relationships exist due to an inability to repair oxidative DNA damage when the polymerases or kinases are depleted in MMR-deficient cells (Martin et al. 2010, 2011). Such results suggest that inhibitors targeting Pol β or Pol γ could be useful in treating cancers with defects in MSH2 or MLH1.

53BP1 and BRCA1

In contrast to synthetic lethality, a "synthetic viability" interaction has been suggested to exist between BRCA1 and 53BP1 (Aly and Ganesan 2011). Loss of BRCA1 results in a severe proliferation defect; BRCA1 homozygous null mice exhibit early embryonic lethality, and embryonic stem cells from these mice are not viable (Hakem et al. 1996; Liu et al. 1996). Thus, in order for BRCA1 null cancer cells to proliferate they must acquire additional mutations. Several groups recently reported that loss of 53BP1 allows BRCA1-mutated cells to proliferate (Cao et al. 2009; Bouwman et al. 2010; Bunting et al. 2010). 53BP1 is important in the DNA damage response; it localizes to break sites with γH2AX and BRCA1 and its loss is

associated with radiation sensitivity. 53BP1 has also been suggested to be important in determining whether DSBs are repaired by NHEJ or HR (Xie et al. 2007).

53BP1 deletion in a BRCA1-deficient background ($BRCA1^{-/\Delta 11}$) reduces cellular senescence and cell death profiles of mouse embryonic fibroblasts and allows mice to age normally (Cao et al. 2009). Loss of 53BP1 also restores HR function of $BRCA1^{-\Delta 11}$ cells, and permits embryonic stem cells to proliferate (Bunting et al. 2010). 53BP1 deletion in a BRCA1-deficient background reverses many of the phenotypes associated with BRCA1 deletion in cells: it reduces sensitivity to PARP inhibition and alleviates spontaneous DNA damage, chromosomal abnormalities and checkpoint activation (Bouwman et al. 2010). $BRCA1^{-/\Delta 11}$ cells are deficient in end resection during recombinational repair, and 53BP1 loss was also found to restore this defect. These findings led to the hypothesis that BRCA1 is needed to overcome 53BP1 end resection inhibition for HR to occur (Bunting et al. 2010). Therefore, through regulation of end resection, BRCA1 and 53BP1 are likely important in repair pathway choice between HR and NHEJ (Bunting et al. 2010; Aly and Ganesan 2011). Interestingly, reduced 53BP1 expression was seen in TNBC and BRCA-associated breast cancers, implicating mutations in 53BP1 as a possible contributing factor to resistance to PARP inhibitors and platinum agents (Aly and Ganesan 2011).

Helicases

Helicases are of vital importance during HR, since they unwind the DNA helix and increase the availability of the DNA template for strand exchange. HR is exclusively used to repair replication-associated strand-breaks. Since cancer cells are rapidly dividing, they accumulate many replication-associated DSBs; therefore, helicases could be a potentially suitable target for manipulation in cancer treatment. A number of studies have investigated links between various helicases, many of which have an associated cancer phenotype. The BLM helicase, defective in the genetic disorder Bloom syndrome, has been shown to be synthetically lethal with Mus81 in *Drosophila* (Trowbridge et al. 2007) and to be closely associated with Mus81 in human cells (Zhang et al. 2005). Another model organism, *C. elegans*, was used to demonstrate synthetic lethality between BRIP1/FANCJ and RTEL, a helicase necessary for DNA repair and HR suppression (Barber et al. 2008). Alternatively, as deficiencies in helicases lead to inefficient HR, it is possible that PARP inhibitors could have a specific effect in tumors that arise in this setting. The WRN helicase is involved in aiding transcription, DNA repair and replication, all of which are typical of helicases. Mutations in WRN are responsible for Werner syndrome, a rare autosomal recessive disorder characterized by premature aging, yet less strongly associated

with cancer predisposition. Flap endonuclease-1 (FEN-1) is essential for DNA replication and also has a role in the base excision repair pathway. It has been demonstrated that WRN and FEN-1 physically interact in human cells and are synthetically lethal in *Drosophila* (reviewed in Aggarwal and Brosh 2009).

Chk1 in FA

An estimated 15% of all tumors have defects within the FA pathway (Taniguchi and D'Andrea 2006) (see Chapter 10). Tumors deficient in the FA pathway require activation of the Chk1-dependent G_2/M checkpoint to repair DNA before mitosis (Kennedy et al. 2007). Therefore, Chk1 is a candidate for synthetic lethal exploitation of FA-deficient cancer cells, whilst leaving normal cells unaffected. A number of Chk1-targeting inhibitors are in clinical trials (Sha et al. 2007; Zabludoff et al. 2008) and have been shown to increase the efficacy of other DNA damaging agents through abrogation of the G_2/M checkpoint (see Chapter 13). This is an example of where synthetic lethality could be exploited in sporadic tumors, as opposed to the strategy that employs PARP inhibitors with germline mutation BRCA deficiencies (see above).

Redox Proteins

The hypoxic environment of most solid tumors can also be used to target cancer cells by conditional synthetic lethality (see Chapter 17). Conditional synthetic lethality manipulates temporary situations (like hypoxia) rather than fixed mutations. Tumor hypoxia has recently been shown to decrease the expression of HR proteins, such as Rad51 (Bindra et al. 2004; Chan et al. 2008), and may therefore respond to the use of PARP inhibitors, similar to the situation involving impaired DNA helicase function. In addition to PARP inhibitors, the use of conditional synthetic lethality by inducing hypoxia can also be exploited by redox-specific agents. Many important cellular processes are balanced redox reactions, such as respiration, where electrons are transferred from one molecule to another. Hypoxia leads to specific redox changes within the cell and numerous clinical trials are investigating the effects of novel redox drugs as single agents or in combinational therapy (reviewed in Wondrak 2009).

FUTURE DIRECTIONS

Synthetic lethality is a valuable concept to understand the function of genes, to determine the mechanistic actions of drugs and their targets,

and most importantly to uncover novel approaches to cancer therapy. The discovery of PARP inhibitors in targeting cells with impaired HR function has demonstrated real clinical benefits, inspiring the search for a new class of anti-cancer therapies. Through the combination of powerful genetic tools, such as RNAi, and drug screens, synthetic lethality provides a new and exciting area of both basic and applied clinical cancer research.

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CHAPTER 15

PARP and PARP Inhibitor Therapeutics

Nicola J. Curtin

INTRODUCTION

The human genome is continuously subject to a variety of genotoxic insults from endogenous sources, such as DNA replication errors, spontaneous deamination of cytosine and oxygen radicals formed as part of normal metabolism, as well as exogenous sources, such as chemical mutagens in the diet or atmosphere and ultraviolet radiation. Therefore, all living organisms have, of necessity, evolved a variety of unique and overlapping or complementary pathways to signal the damage to either cell cycle checkpoints and DNA repair or, if the damage is too great, then to cell death pathways. These DNA damage detection and repair pathways essentially behave as tumor suppressors and their defects can enable tumorigenesis in the presence of ongoing genotoxic stress. There are several pathways of DNA repair that are responsible for the repair of different types of lesions (see Chapter 1). There are pathways that repair damage on one strand of the DNA: the base excision repair/single strand break repair (BER/SSBR) pathway corrects damaged bases and single strand nicks (see Chapter 8), and the nucleotide excision repair (NER) pathway removes more bulky damage (see Chapter 9). DNA double strand breaks (DSBs) are repaired primarily by

Professor of Experimental Cancer Therapeutics, Newcastle University, Northern Institute for Cancer Research, Paul O'Gorman Building, Medical School, Framlington Place, Newcastle upon Tyne, NE2 4HH, UK.
Email: nicola.curtin@ncl.ac.uk

non-homologous end joining (NHEJ) or homologous recombination (HR), depending on the cell cycle phase (HR requires a complimentary sister chromatid to act as template so, by definition can only take place during late S/G2) (see Chapter 14). DNA mismatch repair (MMR) is involved in the detection and repair of base mismatches, insertions and deletions that arise due to errors during replication (see Chapter 7).

Cancer cells are characterised by genomic instability (see Chapter 3), which has been classified as an "enabling characteristic" (Hanahan and Weinberg 2011). One cause of this instability is an imbalance of DNA damage signalling and repair. For example, inherited defects in MMR predispose carriers to hereditary non-polyposis colon cancer (Bronner et al. 1994) and inherited defects in BRCA1 and BRCA2, which are important in HR, predispose carriers to breast and ovarian cancer (O'Donovan and Livingston 2010). Although DNA repair defects lead to tumorigenesis, paradoxically, the sustained replication of a cancer cell in the presence of genotoxic stress requires some intact DNA repair systems. Thus, defects in one repair pathway may be compensated by upregulation of a complementary pathway to which the cancer cells become 'addicted' for survival. The upregulated pathway(s) imparts a mechanism of resistance to anticancer DNA damaging therapy, therefore targeting such pathways may selectively sensitise cancer cells. In addition, these upregulated DNA damage signalling and repair pathways to which cancer cells are addicted may represent the cancer's "Achilles' heel". For example, a specific inhibitor of this pathway could potentially lead to a selective anti-tumor effect by preventing the repair of intrinsic DNA damage. Poly(ADP-ribose) polymerase (PARP), an important enzyme in the recognition and repair of DNA strand breaks, is upregulated in many cancers, which causes resistance to certain DNA damaging anticancer therapies. The protein is also in the first line of defence against oxidative DNA damage, which is increased in cancer cells, and its complementary pathway is HR, which is frequently lost in cancer (see Chapter 14). These important functions make PARP an excellent target for anticancer therapy.

PARP catalyses the formation of ADP-ribose polymers from NAD^+. The therapeutic potential of PARP inhibitors (PARPi) as chemosensitisers for cancer therapy was first described in 1980 (Durkacz et al. 1980). Early studies showed that PARPi delayed DNA repair and increased cytotoxicity after treatment with DNA methylating agents, topoisomerase I poisons and ionising radiation (Curtin 2005 and references therein). However, it took more than 20 years before the first clinical trial of a PARPi was initiated in 2003 (Plummer et al. 2008). In the eight years since then there has been further stimulus to the development of PARPi for cancer therapy, namely the identification of synthetic lethality of single agent PARPi in BRCA mutant tumours (Bryant et al. 2005; Farmer et al. 2005). As a result, there

are now 9 PARPi undergoing clinical evaluation. This Chapter describes the studies leading to the discovery of PARP and its function, the development of targeted inhibitors, and the pre-clinical studies that led to the initiation of clinical trials and the emerging data from these trials.

DISCOVERY OF PARP-1 AND ITS FUNCTION

The earliest indication that there was a chemotherapy-activated NAD^+-consuming enzyme was made in the 1950s, when it was discovered that cells treated with an alkylating agent had impaired glycolysis (Roitt 1956). However, at that time, DNA had only recently been discovered, and DNA damage had not been recognised as the cytotoxic lesion induced by alkylating agents. It took a further 10 years of research for the ADP-ribose polymers and the enzyme responsible for their synthesis to be discovered (Chambon et al. 1963; Sugimura et al. 1967). The enzyme was originally called ADP ribosyl transferase or ADP ribosyl synthetase (ADPRT or ADPRS). In the 1990s, Josiane Menissier-de Murcia coined the name PARP (de Murcia and Menissier de Murcia 1994), and although there have recently been suggestions that a more scientific nomenclature be adopted (Hottiger et al. 2010), it is likely that the term, PARP, will persist.

PARP-1, the major enzyme of the PARP superfamily comprised of 17 paralogs (see below), is highly conserved across plant as well as animal species, and a 50 amino acid sequence (residues 859–908) in the catalytic domain is so highly conserved (>90% across various species), that it was termed the "PARP signature" (de Murcia and Menissier de Murcia 1994). The critical role of PARP-1 in DNA repair was initially elucidated using the benzamide inhibitors (Shall 1975; Durkacz et al. 1980) and later by genetic means. It was somewhat surprising that the three groups that generated PARP-1 knockout mice found them to be viable and fertile (Wang et al. 1995; Mennissier-de Murcia 1997; Masutani et al. 1999). Later studies showed that cells from PARP-1 knockout mice were able to generate ADP ribose polymers (Shieh et al. 1998). This led to the discovery of a second DNA damage-activated PARP enzyme, and this enzyme has been termed PARP-2, with the original enzyme now being termed PARP-1.

PARP-1 and PARP-2 share extensive sequence homology, particularly in the catalytic domain, where there is 43% identity and the PARP signature (residues 401–450) (Ame et al. 1999). The superfamily of 17 PARP enzymes was identified using this PARP signature. Of these enzymes, PARP-1,-2, -3, V-PARP and tankyrase-1 and -2 are the only bona fide polymerases, while the others have mono ADP-ribosyl transferase activity or at present no known function (Schreiber et al. 2006). PARP-1 and PARP-2 have somewhat overlapping roles in DNA repair (Yelamos et al. 2008), with perhaps some

differing preferences for specific types of DNA breaks. PARP-2 knockout mice are also viable and fertile, but knockout of both PARP-1 and PARP-2 confers embryonic lethality around day 7. PARP-3 was originally thought to be involved with centrioles and S-phase entry (Augustin et al. 2003); however new evidence shows that PARP-3 co-operates with PARP-1 in the response to DNA DSBs (Boehler et al. 2011). V-PARP is part of the large ribonuclear protein complexes, known as vaults, which are involved in drug resistance and nucleo-cytoplasmic transport (Berger et al. 2009). Tankyrase-1 and -2 play an important role in telomere maintenance (Hsiao and Smith 2008). Tankyrase-1 knockout mice are small but viable, as are tankyrase-2 knockout mice; however the knockout of both genes results in embryonic lethality at day 10 (Chiang 2008). Since most PARPi act via an interaction with the catalytic site, it is likely that they inhibit other PARPs to a certain degree. However, the extent to which this non-specificity interferes with normal cellular function is unknown, and most research has focused on the inhibition of PARP-1 activation by DNA damage.

STRUCTURE AND FUNCTION OF DNA DAMAGE ACTIVATED PARPs

PARP-1 (EC. 2.4.2.30) is the most intensively studied PARP. It is a 113 KDa multidomain protein, 1014 amino acids long, and encoded by the *parp-1* gene located on chromosome 1q41-42. It is constitutively expressed in a basally active state, but its activity is stimulated 100–400x by DNA breaks. There are 3 zinc fingers (F1, F2 and F3): F1 and F2 are located in the DNA binding domain, adjacent to the nuclear localisation sequence (NLS), and F3 is located in the C domain between the NLS and BRCT domain. Proteins with a BRCT sequence are generally involved in DNA damage recognition and/or repair. The catalytic domain is at the C-terminus with amino acid residues critical for NAD^+ binding and polymer formation (Fig. 1 A). The nicotinamide moiety of NAD^+ forms hydrogen bond interactions with Ser904 and Gly863, and π-π-interactions with Tyr904 and Glu988 are critical for glycosidic bond formation in the growing chain (Ruf et al. 1998). F2 has the greatest affinity for DNA breaks; F1 also recognises breaks, and although its interaction with DNA is weaker than that of F2, F1 plays a more pronounced role in stimulating the catalytic activity of PARP-1 in response to DNA damage (Eustermann et al. 2011; Langelier et al. 2011). F3 also has a role in catalytic activation (Langelier et al. 2008).

Upon binding to DNA, PARP-1 catalyses the cleavage of the glycosylic bond between the C-1′ atom of ribose and the nicotinamide in NAD^+, releasing nicotinamide. At the same time, PARP-1 catalyses the formation of a new glycosylic bond with a glutamic acid, aspartic acid or lysine

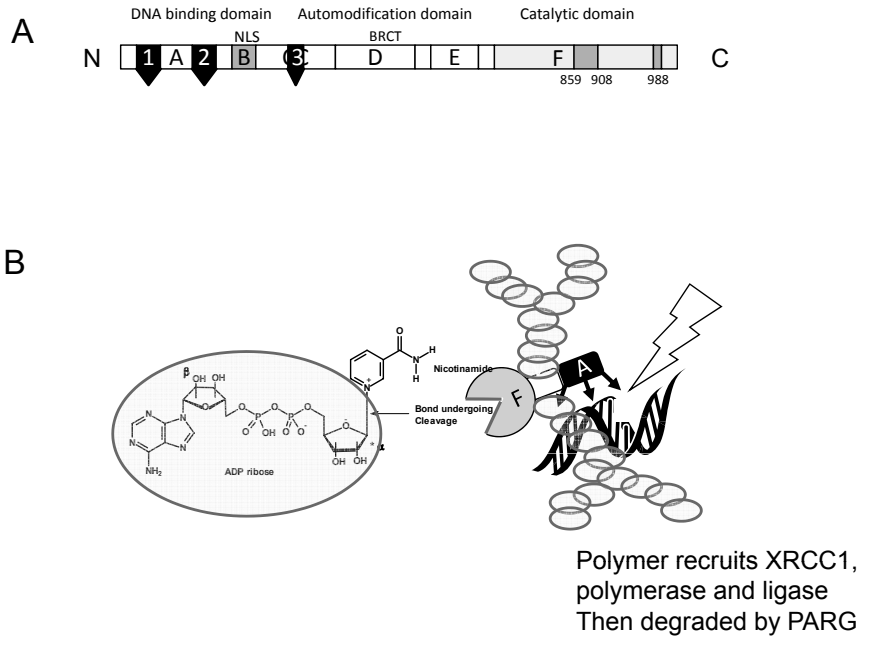

Figure 1. Structure and Catalytic activity of PARP-1.

- **A.** Linear domain structure of PARP-1. Two of the 3 zinc fingers, F1 and F2 (1, 2 in black), are in the DNA binding domain (A); the nuclear localisation sequence (NLS) is in the B domain; and the third zinc finger F3 (3 in black) is located in the C domain. There is a BRCT domain in the D domain. The catalytic domain (F) is at the C-terminus, with amino acid residues critical for NAD^+ binding and polymer formation shaded.
- **B.** On binding to damaged DNA, mostly by F2 and F1, PARP-1 cleaves NAD^+, releasing nicotinamide, and the ADP-ribose moiety is covalently attached to an acceptor protein, such as PARP-1 itself and histones. These modifications loosen the chromatin and recruit the scaffold protein, XRCC1, which in turn recruits DNA polymerase and ligase to fill in and re-seal the gap. The polymers are degraded by poly ADP-ribose glycohydrolase (PARG), releasing unmodified PARP-1 to bind other DNA breaks.

residue on an acceptor protein, and subsequently, on the growing ADP-ribose chain, in a progressive fashion, to form long linear and branched homopolymers of ADP-ribose; i.e., poly(ADP-ribose) or PAR (Fig. 1B). The major acceptor proteins are PARP-1 itself and histone H1 at the site of the break. These long and branching polymers are highly negatively charged, and not only aid the relaxation of chromatin by negating the positive charge on histones and causing their electrostatic repulsion from the negatively charged DNA, but also serve to recruit other proteins that associate with the ADP-ribose polymer (Althaus et al. 1994). These recruited partners include other chromatin-modifying proteins (Rouleau et al. 2010; Krishnakumar and Kraus 2010; Ahel et al. 2009) and proteins intimately involved in DNA BER/SSBR. PARP-1 has mostly been associated with SSBR, and its binding

to DNA breaks and synthesis of ADP-ribose polymers has been shown to be necessary to recruit XRCC1, a BER scaffold protein (ElKhamisy et al. 2003). Depending on whether short patch or long patch BER is needed (see Chapter 8), XRCC1 recruits DNA pol β or δ/ε, and ligase III or ligase I (with Fen-1), to replace the missing nucleotide(s) and reseal the nick (Sukhanova et al. 2010; Petermann et al. 2003). PARP-1 acts as a catalytic dimer (Mendoza-Alvarez and Alvarez-Gonzalez 1993), and it protects 7 nucleotides on either side of the break in DNA footprinting studies (Menissier-de Murcia 1989). It is tempting to speculate that these observations mean that the two PARP-1 molecules sit on either side of the break and poly(ADP-ribosyl)ate each other. Automodification of PARP-1 causes it to dissociate from the DNA, terminating its catalytic activity. Poly(ADP-ribose) glycohydrolase (PARG) (Davidovic et al. 2001) degrades the (ADP-ribose) polymers, restoring the catalytic activity of the PARP-1 enzyme and allowing re-association of the histones with DNA. The degradation of PAR to ADP-ribose units by PARG, combined with the pyrophosphate liberated by the action of DNA polβ at the site of the break, might provide a local source of ATP to allow completion of the repair process by DNA ligase III, under conditions of ATP shortage (Oei and Ziegler 2000). The cycle of PARP-1 binding to DNA breaks, recruitment of repair proteins and polymer degradation is measured in a few minutes. This rapid NAD^+ consumption, synthesis and turnover of the polymer imposes a high energy cost to the cell, underlying the fundamental importance of the pathway.

PARP-1 clearly also has a role in the repair of DNA DSBs, and there are several conflicting hypotheses regarding the mechanism. In *in vitro* studies, blunt-ended DSBs are the most powerful activators of PARP-1 (Benjamin and Gill 1980; d'Silva et al. 1999). PARP-1 is required for the recruitment of the DSB-sensors, MRE11 and NBS1, at the site of the DSB (Hiance et al. 2008). The cellular requirement for PARP-1 activity was demonstrated by the observation that induced over-expression of the PARP-1 DNA binding domain, which acts as a dominant-negative inhibitor, blocked rejoining of DSBs after 60 Gy ionising radiation (Rudat et al. 2001). A major DSB repair pathway is NHEJ, in which the DNA-PK holoenzyme, consisting of the Ku heterodimers and the catalytic subunit DNA-PKcs, are key components. PARP-1 interacts with and poly(ADP-ribosyl)ates the Ku and catalytic subunits of the DNA-PK complex (Ruscetti et al. 1998). Consistent with this observation are reports that PARP-1 co-operates with DNA-PK in the repair of ionising radiation-induced DSBs, based on studies using PARP and DNA-PK inhibitors in cells lacking either PARP-1 or DNA-PKcs (Mitchell et al. 2009). Alternatively, PARP-1, XRCC1 and Ligase III may be responsible for a backup pathway of NHEJ (a.k.a., alt-NHEJ) and compete with Ku for the DNA ends (Wang et al. 2005, 2006). These authors suggest that in irradiated cells the high affinity of Ku for DNA ends, and the recruitment

of PARP-1 to other damages such as SSBs, limits the DSB repair by the secondary PARP-1 dependent backup NHEJ pathway. In cell-free studies, a PARP-1 dependent, DNA-PK-independent DSB repair mechanism, requiring PARP-1 in a DNA end synapsis step, in complex with XRCC1 and ligase III, was observed, with a further requirement for polynucleotide kinase/phosphatase (PNKP) for processing of 5-OH termini (Audebert et al. 2004, 2006). An alternative hypothesis, based on the observation that PARPi radiosensitisation predominates in S-phase, suggests that the effect is due to unrepaired SSBs stalling replication forks that are repaired/reactivated by PARP-1, rather than the direct repair of DSBs (Nöel et al. 2006; Yang et al. 2004).

Less is known about PARP-2, transcribed by the *Parp-2* gene located at 14q11.2. It is a 62 KDa protein, which has a nuclear localisation signal, but no zinc fingers. The catalytic domain has 69% similarity to that of PARP-1. PARP-2 is also activated by DNA damage, with a preference for gaps and flaps, and can form homodimers or heterodimers with PARP-1 at the site of the break; the enzymes can also poly(ADP-ribosyl)ate each other (Menissier-de Murcia et al. 2003). In response to DNA damage, PARP-2 is responsible for 5–10% of the total polymer formation, mainly on core histones (reviewed in Yelamos et al. 2008). Additionally, PARP-2 interacts with other SSBR/BER proteins, such as XRCC1, DNA polβ and ligase III. PARP-1 binding and activation at DNA breaks is more rapid and transient than PARP-2, and XRCC1 recruitment appears to be more dependent on PARP-1 activation. To date, there is no evidence for a role of PARP-2 in DNA DSB repair.

PARP-3 has recently been described as having a role in DNA DSB repair. The protein is rapidly recruited to DNA damage sites in the presence or absence of a PARPi, and thus, recruitment is not dependent on PARP-1 activity. Depletion of PARP-3 by shRNA results in increased spontaneous DSB formation and a significant delay in the repair of radiation-induced DSBs. Loss of PARP-3 alone, either through genetic knockdown in cells or following homozygous deletion in mice, does not result in hypersensitivity to ionising radiation, but in combination with knockdown or deletion of PARP-1, it further increases radiosensitivity (Boehler et al. 2011).

RATIONALE FOR, AND EARLY DEVELOPMENT OF, PARP INHIBITORS

The early identification of a role for PARP-1 in DNA repair and the enhancement of DNA alkylating agent and radiation-induced cytotoxicity by the first PARPi, 3-aminobenzamide (3AB), provided the initial stimulus to develop PARP inhibitors as chemo- and radiosensitisers for cancer therapy (Durkacz et al. 1980). Observations that PARP activity is higher in tumours

compared to surrounding normal tissues (Hirai et al. 1983; Nomura et al. 2000; Zaremba et al. 2011) indicated that PARP activity may not only contribute to chemo- and radio-resistance, but that tumours may be more dependent on PARP activity. Thus, it was hypothesized that PARPi may have tumour-selective chemo- and radiosensitisation properties. Another potential role of PARP-1 in cancer is its ability to act as a transcriptional co-activator of NF-κB (Hassa and Hottiger 2002; Veuger et al. 2009). NF-κB is a stress inducible transcription complex that triggers expression of genes involved in the immune response and inhibits apoptosis, enhancing cell survival. NF-κB is activated by ionising radiation and topoisomerase II poisons, and inhibition of NF-κB by IκBα overexpression potentiates the cytotoxicity of these therapies (Wang 1996). NF-κB activity is elevated in numerous malignancies, and this increased activity also correlates with progression of these tumours (Rayet and Gelinas, 1999) due to proliferation stimulation and synthesis of pro-inflammatory mediators—critical components of tumour growth (Coussens and Werb 2002). Such evidence implicates PARP-1 in inflammatory processes that can lead to tumour progression. Recent studies point to gender differences in PARP activity (Zaremba et al. 2011), and it is conceivable that these may be implicated in the sex differences in the development of some cancers lacking an obvious hormonal component, e.g., hepatocellular carcinoma.

The observation that nicotinamide, the by-product of the PARP reaction, was a weak PARP inhibitor led to the design of further analogues; indeed virtually all PARPi contain a nicotinamide pharmacophore (Fig. 2). The benzamides, and in particular 3AB, were the first generation inhibitors and provided useful tools to explore PARP function and lead molecules for further inhibitor development. Although the K_i for the benzamides was in the 10–20 μM range, the concentrations needed for chemo- and radiopotentiation were 3–10 mM. At these concentrations, the benzamides can inhibit glucose metabolism and *de novo* purine biosynthesis (Milam and Cleaver 1984; Milam et al. 1986), complicating the interpretation of some of the data obtained with these inhibitors. However, the consistent demonstration of potentiation of ionising radiation and DNA methylating agent cytotoxicity by 3AB has been vindicated by molecular studies using antisense strategies (Ding and Smulson 1994), trans-dominant inhibition of PARP-1 (Molinete et al. 1993; Kupper et al. 1995) and disruption of the gene encoding PARP-1 (Menissier-deMurcia et al. 1997; Masutani et al. 1999), which are associated with delayed DNA repair and hypersensitivity to alkylating agents and ionising radiation (Masutani et al. 2000; Trucco et al. 1998). Studies using the benzamides in combination with other cytotoxic agents, e.g., cisplatin, 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and etoposide, show a degree of inconsistency in terms of cytotoxic potentiation (Boike et al. 1990; Alaoui-Jamali et al. 1994), possibly due to

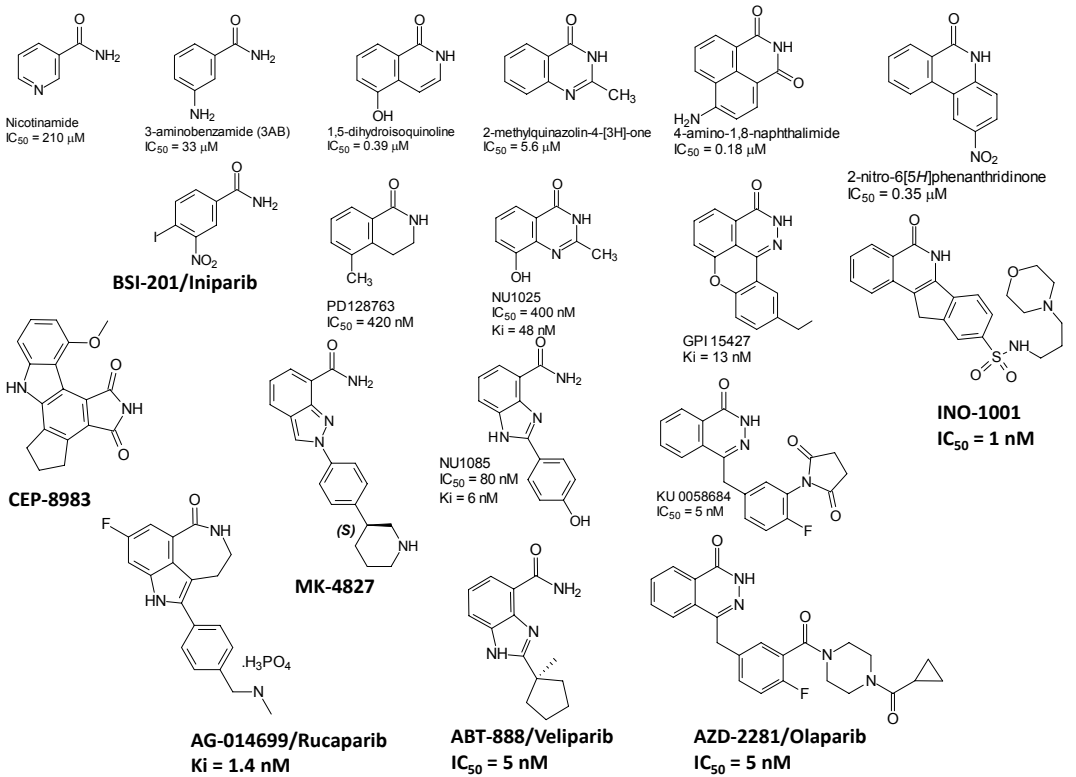

Figure 2. Structure and activities of PARP inhibitors. The development of PARP inhibitors (PARPi) largely initiated around the by-product of the PARP reaction, nicotinamide, and subsequently advanced to compounds such as 3AB, PD128763, NU1025 and the chemical leads identified by Banasik et al. (1992) to compounds that have entered clinical trial (in bold).

the non-specific effects of the benzamides or to cell specific differences in repair mechanisms.

In the 1990s, academic and industrial groups developed more potent inhibitors based on structure-activity relationships (SARs). These studies identified 3,4-dihydro-5-methylisoquinolin-1(2*H*)-one, PD128763 (Suto et al. 1991; Arundel-Suto et al. 1991), and 8-hydroxy-2-methylquinazolin-4-[3*H*]-one, NU1025 (Griffin et al. 1995), as being around 50-fold more potent than 3AB in enzyme inhibition studies; 50–100 μM of PD128763 or NU1025 gave superior chemopotentiation compared to 1–5 mM 3AB. At the same time, a groundbreaking "analogue by catalogue" approach, screening 170 compounds that in some degree resembled nicotinamide, identified several very potent PARP inhibitors, such as quinazolinones, quinazoline diones, dihydroxyisoquinolinones, phenanthridinones and phthalazinones (Banasik et al. 1992). These compounds have formed the basis for further drug development, for example the phenanthridinone PJ34 (Jagtap and Szabo 2005). Continued development of this inhibitor led to INO-1001, which entered clinical trial in 2005 (Morrow 2009). Development of the phthalazinones resulted in potent inhibitors, such as KU 58684, with an IC_{50} of 5 nM (Menear et al. 2008), and ultimately AZ2281 (olaparib) and the tetracyclic phthalazinone, GPI 15427, with an IC_{50} of 31 nM (Tentori et al. 2003). Other potent PARP inhibitors based on the structurally unique pyrrolocarbazole lactams that incorporate a heavily substituted arylamide core, such as CEP-6800 (IC50 = 27 nM) and CEP 9722 (K_i = 20 nM), have been developed (Miknyoczki 2003, 2007). The above studies indicate that constraining the carboxamide group into a heterocyclic ring, and thus restricting the rotational freedom of the amide group, improved potency.

An alternative approach of constraining the carboxamide through an intramolecular hydrogen bond, such as in the 2-substituted benzimidazole-4-carboxamides, also resulted in highly potent compounds. For example, the K_i of 2-(4-hydroxyphenyl)benzimidazole-4-carboxamide, NU1085, was 6 nM, demonstrating good chemosensitising activity at a concentration of only 10 μM in cell culture (Delaney et al. 2000). Further development of benzimidazole carboxamides resulted in ABT-888 (Veliparib), which has a K_i of 5 nM (Donawho et al. 2007). The indazole carboxamide, MK4827, was also developed from a series of heterocyclic benzamides related to the benzimidazole carboxamides (Jones et al. 2009). Mechanism-based, irreversible benzamide and isoquinolinone inhibitors with substitutions that mimic the adenine oxonium ion and bioreductively activated prodrugs have been developed as well (Watson et al. 1998; Parveen 1999).

An important step in the development of PARPi was solving the crystal structure of the PARP catalytic domain (Ruf et al. 1996). Crystallization of PD128763, 4-amino-naphthalimide and NU1025 in the NAD^+ binding site of chicken PARP (87% homologous with human) demonstrated that

the carboxamide group made several important hydrogen bonds to the protein: the oxygen with Ser904-O and the Gly863-N, and the nitrogen with Gly863-O. These studies confirmed the prediction from a SAR study that restriction of the carboxamide, rather than allowing free rotation as in 3AB, improved the compound-protein interaction (Ruf et al. 1998). Methylation of the free carboxamide NH group abrogated PARP inhibition confirming the critical hydrogen bonding (Calabrese et al. 2003). Further crystallographic analysis of the binding of the benzimidazole, NU1085, demonstrated a pocket occupied by the pendant 2-phenyl ring that could accommodate further substitutions to improve potency and solubility (White et al. 2000). Based on crystallographic data, further inhibitors were therefore developed in which the carboxamide group was held in a favourable orientation by incorporation of a non-planar 7-membered ring, which was thought to improve the interaction by moving the lactam carbonyl and NH closer to Ser904 and Gly863 (Canan-Koch et al. 2002; Skalitzki et al. 2003; Calabrese et al. 2003, 2004). The benzimidazole/indole core of this analog series and the NH group participated in π-π-type interactions with tyrosine residues (Tyr907, Tyr889, and Tyr896) in the active site pocket and in hydrogen bonding with the important catalytic Glu988 residue (via an ordered water molecule), respectively. These tricyclic lactam indoles and benzamidazoles include AG14361 and later, AG-014699, which was the first PARPi to enter clinical trial for the treatment of cancer (Plummer et al. 2008). Subsequently, the co-crystallization of an indolinedione inhibitor within the catalytic domain of human PARP-1 demonstrated the same Ser904 and Gly863 interactions, and in addition, the 4-phenyltetrahydropyridyl group was pushed into a hydrophobic pocket comprised of Leu769, Arg878, Ile879 and Pro881 with the nitrogen of this group hydrogen bonding with Asp766 (Hattori et al. 2004).

Virtually all PARPi have been developed to interact in a NAD+ competitive manner at the active site, but 2 inhibitors, 4-iodo,3-nitrobenzamide (BSI-201) and 6-nitroso-1,2-benzpyrone (BSI-401), were reported to disrupt the zinc fingers of PARP-1 and hence prevent binding to DNA (Mendeleyev 1995; Buki 1991). More recent research has called into question the ability of BSI-201 to inhibit PARP activity (Ji et al. 2011; Patel et al. 2012), and the compound has been reported to have other targets, namely GAPDH (Bauer et al. 2002).

CHEMOSENSITISATION—DNA METHYLATING AGENTS

The pioneering paper demonstrating that 3AB increased the cytotoxicity of DNA methylating agents in murine leukaemia cells (Durkacz et al. 1980) was the initial stimulus to develop PARPi for cancer therapy. Monofunctional

DNA methylating agents are the most potent activators of PARP-1 (and -2), and most PARPi development has included their evaluation as chemosensitisers of DNA methylating agents. Dacarbazine (DTIC) and temozolomide (TMZ) are the most commonly used anticancer DNA methylating agents (see Chapter 5), but their use is largely restricted to the treatment of brain tumours and melanoma. These drugs methylate the DNA at the O^6- and N^7-position of guanine and the N^3 position of adenine (Denny et al. 1994). In many cells, the major cytotoxic lesion is O^6-methyl guanine, even though it only accounts for 5% of the total lesions. The other, much more numerous, N-methylpurines are primarily repaired by SSBR/BER. Evidence that chemosensitisation of DNA methylating agents by PARPi is due to inhibition of PARP-1 and -2 rather than an off-target effect comes from the observation that genetic depletion of these enzymes also results in hypersensitivity. Specifically, PARP-1 knockout mice and cells derived from them are hypersensitive to DNA methylating agents (Menissier de Murcia et al. 1997; Masutani et al. 2000), and PARP-2 knockout cells show reduced delay in the repair of DNA strand breaks induced by the alkylator N-nitroso-N-methylurea (Schreiber et al. 2002). Stable knockdown of PARP-1 in melanomas increases sensitivity to TMZ, which can further be increased by co-treatment with a PARPi, implicating PARP-2 (or other PARPs) in the response to this alkylating drug (Tentori et al. 2008, 2010).

Early studies showed that 50–100 μM of PD128763 or NU1025 were at least as effective in increasing TMZ-induced DNA strand breakage and cytotoxicity (4- to 7-fold) as 1 mM benzamide or 5 mM 3AB (Boulton et al. 1995). NU1025 enhanced cytotoxicity to a similar extent when it was added after removal of the methylating agent as it did when added concurrently, confirming that PARP inhibition during the repair phase alone was sufficient for potentiation (Bowman et al. 1998). NU1025 (200 μM) and NU1085 (10 μM) potentiated the cell killing effects of TMZ in a panel of 12 human lung, colon, breast and ovarian tumour cell lines (representing some of the commonest human malignancies), and potentiation was independent of tissue of origin or p53 status (Delaney et al. 2000). The enhancement of TMZ-induced DNA damage and/or cytotoxicity in a variety of cell lines has been demonstrated with the PARPi CEP 6800 (Miknyoczki et al. 2003), GPI 15427 (Tentori et al. 2003) and a series of potent benzimidazoles and tricyclic lactam indoles (Calabrese et al. 2003). A panel of phthalazinone PARPi increased the cytotoxicity of MMS, another DNA methylating agent, with the most active inhibitors causing an 18-fold enhancement (Loh et al. 2005; Cockroft et al. 2006). Further pre-clinical studies with the clinical candidate AZD2281 (olaparib) demonstrated enhancement of the efficacy of TMZ in the SW620 cell model (Menear et al. 2008). Interestingly, potentiation of TMZ by ABT-888 (Veliparib) was shown to be associated with S-phase accumulation of DNA DSBs, suggesting that TMZ-induced SSBs are elevated

by the inhibitor leading to the formation of more profoundly cytotoxic DSBs upon replication (Liu et al. 2008).

The major clinical use of TMZ is for brain tumours and melanoma (see Chapter 5). NU1025 lacks the solubility and potency required for extensive pre-clinical evaluation, but intracranial injection of NU1025 was found to increase the TMZ-induced survival of mice with brain lymphomas (Tentori et al. 2002a). Pre-clinical studies of TMZ in combination with CEP-6800 (30 mg/kg) caused the complete regression of U251MG (human glioblastoma) tumours (Miknyoczki et al. 2003). GPI 15427, at a dose of 40 mg/kg i.v., enhanced the antitumour activity of TMZ in mice bearing intracranial melanomas, gliomas and lymphomas, and increased the antimetastatic effect of TMZ against B16 melanoma (Tentori et al. 2003). PARPi show impressive antitumour activity with TMZ in a variety of different models, not just brain tumours and melanomas. This suggests that PARPi may have the potential to change the current limited clinical use of TMZ. For instance, ABT-888 (Veliparib), a PARP inhibitor currently undergoing clinical evaluation, enhanced the antitumour activity of TMZ in a variety of tumour models (murine melanoma, rat glioma and orthotopic human breast and prostate xenografts), models of metastasis to bone and brain and spontaneous lung metastases, as well as sub-cutaneous xenografts of pancreatic, ovarian and non small cell lung cancer (NSCLC) (Donawho et al. 2007; Palma JP et al. 2009). The only studies to be conducted to date with pre-clinical models of paediatric cancers demonstrate that AG-014699 (rucaparib) enhances the antitumour activity of TMZ against neuroblastoma and medulloblastoma xenografts (Daniel et al. 2009, 2010).

Loss of DNA MMR, which confers tolerance to O^6-methyl guanine and hence TMZ resistance in laboratory and clinical studies (Liu et al. 1996, Friedman et al. 1998), is associated with the development colon and ovarian cancer (Hernam et al. 1998) (see Chapter 7). 3AB, PD128763 and NU1025 enhance TMZ cytotoxicity in MMR-deficient cell lines (Wedge et al. 1999; Tentori et al. 1999), and in a study using matched pairs of MMR-proficient and -deficient cells, AG14361 completely overcame MMR deficiency-mediated TMZ resistance (Curtin et al. 2004). Sensitisation of TMZ by ABT-888 was also more effective in MMR defective leukaemia cells (Horton et al. 2009). GPI 15427, which sensitised both MMR-proficient and -deficient cell lines to TMZ *in vitro*, only caused significant enhancement of the antitumour activity of TMZ against a MMR-defective xenograft (Tentori et al. 2006). Similarly, INO-1001 enhanced TMZ-induced tumour growth delay to a greater extent in MMR defective glioma xenografts (Cheng et al. 2005). Thus, in tumours lacking MMR, PARP inhibition, in combination with TMZ, may represent a potentially selective therapeutic approach. In contrast to these findings, AG14361 appeared to be more effective against MMR-proficient colon cancer xenografts. These studies found that AG14361

increased the antitumour activity of TMZ against MMR defective LoVo xenografts by 3-fold, whereas in mice bearing SW620 xenografts, the combination of AG14361 and TMZ caused complete tumour regression (Calabrese et al. 2004). The clinical candidate from this series, AG-014447, of which the phosphate salt is AG-014699 (rucaparib), also caused the complete regression of SW620 xenografts (Thomas et al. 2007). However, since inhibition of PARP activity failed to increase the cytotoxicity of TMZ in SW620 cells, the *in vivo* sensitisation was thought to be due to an effect on the tumour micro-environment (see below).

TOPOISOMERASE I POISONS

Topoisomerase I poisons are used more widely than DTIC or TMZ in cancer therapy and may represent a greater potential application for PARPi as chemosensitisers. Topoisomerase I relieves the torsional strain generated by transcription and replication by catalyzing the breakage of one strand of DNA, unwinding it by one turn and religating it. Topoisomerase I poisons, such as camptothecin and its clinical derivatives (irinotecan and topotecan), stabilize the topoisomerase-DNA cleavable complex at the point of breakage, preventing religation and converting these essential enzymes into cellular poisons. Since the number of frozen complexes/SSBs determine the level of cytotoxicity, cells with more active topoisomerase I will experience the greatest cytotoxicity with topoisomerase poisons. The cytotoxicity is thought to result from the collision between the replication fork and the covalent topoisomerase I-DNA complex, which produces a stalled replication fork with a protein-associated SSB and a non-protein associated DSB (Pommier et al. 1994). PARP-1 co-localises with topoisomerase I throughout the cell cycle, thereby modulating its activity. However, upon DNA damage, automodification of PARP-1 disrupts this association and hence control over topoisomerase I activity (Yung et al. 2004).

An early investigation in L1210 cells revealed that 3AB enhanced camptothecin cytotoxicity (Mattern et al. 1987). PARP activity was stimulated by camptothecin in L1210 cells and inhibition of PARP with NU1025 increased both camptothecin-induced DNA breaks and cytotoxicity to a similar extent, suggesting the two events were related (Bowman et al. 2001). NU1025 and NU1085 enhanced the cytotoxicity of topotecan up to 5-fold in a panel of human breast, colon, lung and ovarian cancer cell lines, again with no evidence of p53 dependence or tissue specificity (Delaney et al. 2000). Subsequently more potent and specific PARPi have been shown to enhance various topoisomerase I poisons. For example, AG14361 potentiated topotecan-induced growth inhibition in LoVo, SW620 (human colon cancer) and A549 human lung cancer cells (Calabrese et al. 2004). These observations

with PARPi have been supported by genetic manipulation studies: PARP-1 null mice are also hypersensitive to topoisomerase I poisons, with 64% of null mice dying within 2 weeks of exposure to CPT-11 (140 mg/kg), while all of the wild-type mice were still alive 8 weeks later (Burkle et al. 2000). The early studies with 3AB that suggested PARP inhibition augmented the cytotoxicity of topoisomerase II poisons (Mattern et al. 1987) has not been replicated with the more potent and specific inhibitors (Bowman et al. 2001).

There are several hypotheses concerning the mechanism by which PARPi potentiate topoisomerase I poisons that include a PARP-mediated modulation of topoisomerase I activity or repair of topoisomerase I DNA lesions. In support of the proposed modulation of topoisomerase I activity, PARP-1 poly(ADP-ribosylates) topoisomerase I, down-regulating its activity *in vitro* and in intact cells (Ferro and Olivier 1984; Krupitza et al. 1989). The cytotoxicity of topoisomerase I poisons is dependent on the generation of DNA breaks by topoisomerase I activity. If inhibition of PARP leads to increased topoisomerase I activity, this will lead to increased DNA breakage and sensitivity to topoisomerase I poisons. In support of this idea is the observation that topoisomerase I was activated 800% upon association with PARP-1, but in response to DNA damage, poly(ADP-ribosylation) of topoisomerase I, or binding to automodified PARP-1, inhibited topoisomerase I activity, which could be re-activated by the PARPi benzamide (Bauer et al. 2001; Yung et al. 2004). Consistent with PARP negatively regulating topoisomerase I activity, the more potent PARPi, CEP 6800, increased DNA breakage and G2 arrest in HT29 (human colon cancer) cells treated with camptothecin, which traps topoisomerase I on DNA during strand cleavage (Miknyoczki et al. 2003).

An alternative hypothesis is that PARP is involved in the repair of topoisomerase I associated DNA damage. In support of this hypothesis was the observation that AG14361 retarded the repair of camptothecin-induced DNA breaks, while there was no difference in topoisomerase I activity in nuclear extracts from AG14361-treated cells compared to the controls (Smith et al. 2005). EM9 cells. which lack the BER scaffold protein XRCC1, are also hypersensitive to camptothecin (Caldecott and Jeggo 1991), raising the possibility that there may be a role for BER in the repair of topoisomerase I poison-induced DNA damage. A model by which XRCC1 recruits tyrosyl DNA phosphodiesterase (TDP-1), which removes topoisomerase I that is covalently attached to DNA, has been proposed (Plo et al. 2003). PARP-1 could participate in this process by recruiting XRCC1 to the site of the topoisomerase I -associated DNA break. Consistent with this model is the recent demonstration that camptothecin-induced DNA breaks are increased by ABT-888, and by inactivation of TDP-1. In this study, ABT-888 did not increase the topoisomerase I-DNA cleavable complex formation, indicating

that topoisomerase I activity was not affected (Zhang et al. 2011). Other studies have found that poly(ADP-ribosyl)ated PARP-1 and PARP-2, but not the unmodified enzymes, block the formation of topoisomerase I-DNA covalent complexes, inhibit DNA cleavage by topoisomerase I, and accelerate the removal of camptothecin-stabilized topoisomerase I-DNA complexes (Malanga and Althaus 2004). In antitumour activity studies, CEP-6800 caused a 60% enhancement of irinotecan-induced delay in the growth of human colon cancer xenografts (Miknyoczki et al. 2003), AG14361 increased the irinotecan-induced tumour growth delay by 2 to 3-fold in a human colon cancer model (Calabrese et al. 2004), and GPI 15427 enhanced irinotecan antitumour activity (Tentori et al. 2006) confirming the *in vitro* data.

PLATINUM AGENTS

There are sporadic reports of chemosensitisation of platinum agents by PARPi, but to date there is no supporting evidence of hypersensitivity to platinum agents by genetic knockdown of PARP-1. Cisplatin and carboplatin induce DNA intra and inter-strand cross links that are repaired by NER and HR pathways. Cisplatin-mediated DNA damage has been shown to activate PARP-1 (Zhu et al. 2010). Conflicting results have been reported using the benzamide PARPi: the inhibitors enhanced cisplatin cytotoxicity in ovarian and cervical cancer cells (Boike et al. 1990; Nguewa et al. 2006), but not in mammary carcinoma cells (Alaoui-Jamali et al. 1994). In addition, some of the more potent inhibitors failed to potentiate cisplatin in a panel of ovarian cancer cell lines (Bernges and Zeller 1996). A mechanism of resistance to cisplatin cytotoxicity is the loss of the DNA MMR pathway; however, the potent and specific PARPi, AG14361, failed to potentiate MMR competent or defective cells (Curtin et al. 2004). These conflicting data have been somewhat reconciled by the observation that the potentiation of cisplatin by PARPi is cell-line dependent (Guggenheim et al. 2008). This raises the possibility that the cell-line dependency of potentiation of cisplatin cytotoxicity by PARPi reflects the molecular pharmacology of the cell. In particular, cells lacking HR function are hypersensitive to cisplatin and to PARPi (see later). Defects in HR function observed in tumours are the BRCA1 or BRCA2 mutations associated with breast and ovarian cancer (see Chapter 14). Synergistic cytotoxicity of AZD2281 (olaparib) in combination with cisplatin was observed in BRCA2 defective cells, but not with BRCA functional cells, suggesting the interaction between the inhibitor and the chemotherapeutic might be associated with HR status (Evers et al. 2008). It is possible that the ovarian cancer cell lines in which the benzamide PARPi enhanced cisplatin cytotoxicity were HR defective. In contrast, PARP

inhibition protected cells and xenografts lacking retinoblastoma tumour suppressor protein (pRB) from cisplatin cytotoxicity (Liu et al. 2009).

PARPi have shown greater activity with platinum drugs in the *in vivo* setting. However, this may be due to a potential effect of PARPi on tumour vasculature, promoting drug delivery (see later). For example, CEP-6800 failed to enhance cisplatin cytotoxicity in Calu-6 (human NSCLC) cells *in vitro*, but caused a 35% enhancement of cisplatin-induced reduction in the growth of Calu-6 xenografts (Miknyoczki et al. 2003). Similarly, BGP-15 enhanced the antitumour activity of cisplatin in xenograft models (Racz et al. 2002), but did not potentiate cisplatin cytotoxicity *in vitro*. ABT-888 increased the antitumour activity of cisplatin and carboplatin against MX-1 mammary xenografts (Donawho et al. 2007), and AG-014699 enhanced carboplatin-induced tumour growth delay in mice bearing Capan-1 pancreatic tumours (Drew 2011). Capan-1 cells have mutant BRCA2 and MX-1 cells lack both BRCA1 and BRCA2, implying that the observed synergy could be at the level of HR status or drug delivery. Subcutaneous xenografts may not reflect tumour growth *in situ*, so the finding that AZD2281 increased the survival of mice genetically engineered to develop BRCA-defective mammary tumours (Rottenberg et al. 2008), together with the *in vitro* data with HR-defective cells, indicates that the combination of PARPi and platinum agents is likely to be more effective than either drug alone against HR-defective tumours in the clinical setting.

IONISING RADIATION

Some of the earliest studies with PARPi demonstrated radiosensitisation (Ben-Hur et al. 1985; Kelland et al. 1988), and these studies have been substantiated by the finding that PARP-1 or PARP-2 knockout mice and cells are sensitive to ionising radiation (Shall et al. 2000; Masutani et al. 1999; Menissier-de Murcia et al. 2003). Radiotherapy, which is one of the commonest and effective treatments for cancer (see Chapter 4), causes a plethora of DNA damages, including base damage and single and DSBs, many of which depend on PARP activity for repair. DSBs are the most cytotoxic lesions, and radiosensitisation by PARPi may be due to inhibition of SSBR/BER, resulting in the persistence of SSBs that are converted to DSBs on collision with the replication fork. This hypothesis was supported by the early demonstration that the PARPi 4-amino-1,8-naphthalimide preferentially sensitised S-phase cells to ionising radiation and that following radiation exposure there was an accumulation of DSBs in PARP inhibited cells (Banasik et al. 1992). This compound caused a concentration-dependent radiosensitisation in a panel of human and rodent cell lines with enhancement ratios of 1.3 to 1.5 (Schlicker 1999). The PARPi

E7016 (formerly GPI 21016) caused the persistence of ionising radiation-induced DNA DSBs and sensitised human glioblastoma and pancreatic cell lines to ionising radiation with a dose-enhancement ratio of 1.4 to 1.7 (Russo et al. 2009). Similar levels of radiosensitisation have been observed with INO-1001 (Brock et al. 2004). PARPi also enhanced sensitivity to low dose ionising radiation (0.05–0.3 Gy) in exponentially growing, but not confluent, cultures of T98G human glioma cells (Suto et al. 1991). These data have been confirmed by more recent studies using AZD2281 (aka KU-00059436, Olaparib) with four human glioma cell lines (T98G, U373-MG, UVW, and U87-MG), in which radiosensitisation was observed in S-phase cells and accompanied by an increase in replication-associated γH2AX foci, which are markers of stalled replication forks/replication-associated DSBs (Dungey et al. 2008).

In contrast, other studies have indicated that PARPi can radiosensitise growth arrested cells. This is important because radioresistance has been attributed to the growth-arrested hypoxic fraction within a tumour that can re-populate the tumour after radiotherapy (Weichselbaum and Little 1982; Barendsen et al. 2001). *In vitro* models of potentially lethal damage (PLD) recovery are an attempt to mimic this situation in the laboratory. In this PLD recovery model, the survival of growth-arrested cells is assessed after a recovery period, in comparison with the survival of cells without the recovery period. PD128763 blocked PLD recovery and approximately doubled X-ray-induced cell killing in both proliferating and stationary cultures (Arundel-Suto et al. 1991). In similar studies, NU1025 retarded the rejoining of ionising radiation-induced DNA strand breaks, prevented recovery from PLD, and enhanced the cytotoxicity of γ-irradiation in proliferating L1210 cells (Bowman et al. 1998). More recently, the potent PARPi, AG14361, caused a more marked sensitisation of growth-arrested cells (Calabrese et al. 2004). The radiosensitisation of growth arrested cells provides further support for the role of PARP-1 in the repair of DSBs by NHEJ, which is the major functional DSB repair pathway in the absence of replication. Early studies in CHO cells demonstrating radiosensitisation and inhibition of radiation-induced DSB repair by NU1025, and additive effects with the DNA-PK inhibitor wortmanin (Boulton et al. 1999), have been followed up using cell lines lacking DNA-PK or PARP-1. These studies, which employed more potent and specific PARP and DNA-PK inhibitors, showed that radiosensitivity in PARP-1 deficient cells was increased by the DNA-PK inhibitor, NU7026, and that inhibition of PARP by AG14361 increased the radiosensitivity of DNA-PKcs-deficient cells. In these studies, inhibition or loss of PARP-1 had at least an additive effect on the delay in DSB repair in DNA-PK defective or inhibited cells. The finding that inhibition of PARP-1 blocked DNA-PK activity, and vice versa, in cells proficient in both enzymes suggested either loss of mutual stimulation or

competition of the two enzymes for the DNA break (Veuger et al. 2003, 2004). However, in these studies very high doses of irradiation were used (75 Gy). Further investigations using more physiologically-relevant doses (2 Gy, 10 Gy) revealed that the repair of DNA DSBs in cells lacking PARP-1 or DNA-PKcs was slower than in repair competent cells, and could not be impaired further by inhibition of the other enzyme, and that the use of DNA-PK and PARP inhibitors together was no more effective than either alone (Mitchell et al. 2009). These observations suggested that both PARP-1 and DNA-PKcs had equivalent roles in DNA DSB repair and that they co-operated in the same pathway. It was hypothesised that poly(ADP-ribosylation) might be necessary for the recruitment of Ku and DNA-PKcs.

Alternative *in vitro* models of clinical radiotherapy involve the measurement of recovery from sub-lethal damage. In this case, the dose of radiation that would cause PLD is divided into multiple doses, separated by a recovery period with or without a PARPi. Cell survival following exposure to short pulses of ionising radiation separated by a short time interval (1–60 seconds) fluctuated with the duration of the interval between doses of radiation. This oscillatory response was not seen in PARP-1$^{-/-}$ cells and it was largely abolished in PARP-proficient cells by 3AB, 4-amino-1,8-naphthalimide or 2-nitro-6[5H]phenanthridinone (Fernet et al. 2000). In contrast, the DNA-PK inhibitor, wortmannin, did not affect the oscillatory response, suggesting that PARP, but not DNA-PK, plays a major role in the early radiation response. It was proposed that the rapid poly(ADP-ribosylation) of target proteins, or recruitment of repair proteins to sites of initial DNA damage, affected the induction of, or response to, DNA damage by the second dose of irradiation. Studies using 3AB and NU1025 revealed that the most profound radiosensitisation is observed with low doses (0.05 to 0.4 Gy) (Chalmers 2004).

Early studies investigating the *in vivo* radiosensitisation by nicotinamide and the benzamide PARPi were complicated by their effect on haemodynamics (Horsman 1995). However, *in vivo* radiosensitisation has also been demonstrated with the second and third-generation PARPi. PD128763 caused a 3-fold enhancement of X-ray-induced tumour growth delay in mice bearing SCC7 sarcomas. In addition, this PARPi enhanced the X-ray activity against RIF-1 and KHT sarcomas (Leopold et al. 1992). However, PD128763 was found to cause acute and profound hypothermia, an indication of hypotension, which suggests that altered blood flow may be a contributory factor in the observed radiosensitisation. PD128763 caused hypothermia to a similar extent and duration in PARP-1$^{+/+}$ and PARP-1$^{-/-}$ mice, but in parallel studies, AG014361 did not cause hypothermia in any mouse, suggesting that the effect was not PARP-1 mediated (Calabrese et al. 2004). AG14361 doubled the antitumour effect of fractionated X-rays in mice bearing human colon cancer xenografts. In other studies, GPI 15427

significantly enhanced the radiation-induced inhibition of growth of human head and neck cancer xenografts (Khan et al. 2010), and ABT-888 was found to have *in vivo* radiosensitising properties against colon and lung cancer xenografts (Donawho et al. 2007; Albert et al. 2007). In chemoradiation studies, E7016 had greater than additive effects in combination with TMZ and ionising radiation against glioma xenografts (Russo et al. 2009). The scheduling of the radiotherapy and PARPi may be important because following a single high radiation dose (6 Gy), ABT-888 increased the growth delay of PC3 prostate cancer xenografts but not Du-145 xenografts (Barreto-Andrade et al. 2011).

VASOACTIVITY OF PARPi

As described above, *in vivo* studies with nicotinamide and the benzamide PARPi revealed a significant vasoactive effect, causing profound hypotension. This observation has been exploited clinically in the ARCON trial, where nicotinamide and carbogen breathing are used to increase tumour perfusion and oxygenation to improve radiotherapy response (Kaanders et al. 2002). Although AG014361 did not cause hypothermia in mice, suggesting it did not affect normal blood flow, it did have a marked and immediate effect on the tumour vasculature. In general, tumours are poorly and erratically perfused because of the high interstitial pressure within the tumour and, when combined with disordered tumour vasculature, this leads to blood vessels periodically failing. The result is that the blood supply shuts down intermittently causing different areas of the tumour to have periods of hypoxia (see Chapter 17). Studies using fluorescent dyes administered intravenously to mice revealed that both AG014361 and AG-014699 reduce the number of blood vessels shutting down and thus improve drug flow. This observation was confirmed using tumours grown in a dorsal window, allowing the effect of AG-014699 to be measured in real-time by following the diffusion of fluorescent labels (Calabrese et al. 2004; Ali et al. 2009). The effect of AG-014699 was also demonstrated *ex vivo*, where it was found to be a more potent relaxer of pre-constricted rat arteries than the common anti-hypertensive drug, hydralazine (Ali et al. 2009). These findings have obvious implications not only for the reduction of the radio-resistant hypoxic fraction, but in the improvement of drug delivery. Furthermore, since hypoxia induces HIF1α, which promotes the malignant phenotype and angiogenesis, PARPi may ameliorate some of these undesirable characteristics.

The studies on tumour blood flow were initiated because of the curious observation that AG14361 did not enhance TMZ sensitivity in SW620 cells, but did cause a profound enhancement of the antitumour activity of TMZ

against SW620 xenografts, implying a greater delivery of TMZ to the tumour (Calabrese et al. 2004). Similar studies found that CEP-6800 did not enhance cisplatin cytotoxicity in Calu-6 (human NSCLC) cells *in vitro*, but caused a 35% enhancement of the growth reduction induced by cisplatin in Calu-6 xenografts (Miknyoczki et al. 2003), consistent with the view that PARPi may alter the microdistribution and improve drug delivery.

PROTECTION FROM NORMAL TISSUE TOXICITY

The other reason that PARPi may be beneficial in chemotherapy is the potential for normal tissue protection. In normal quiescent tissues, DNA SSBR does not have to be completed rapidly before the lesion collides with the advancing replication fork. Reactive oxygen species (ROS), formed as normal metabolic by-products, can be cytotoxic in quiescent tissues by hyperactivating PARP-1, which leads to catastrophic NAD depletion during ADP-ribose polymer formation. This triggers Apoptosis Inducing Factor (AIF) release from mitochondria, initiating caspase-independent apoptosis. Inhibition of PARP will therefore prevent this cascade of events that culminate in cell death. There is abundant evidence for the protective effects of PARPi against a variety of insults: ischemia-reperfusion injury (e.g., stroke and heart attack), chronic and acute inflammation (e.g., arthritis, asthma, septic shock, diabetes) (reviewed in Jagtap and Szabo 2002, Tentori et al. 2002b). Pertinent to anticancer chemotherapy are the dose-limiting cardiotoxicity of doxorubicin and the renal toxicity of cisplatin, both of which are thought to be due to ROS formation rather than DNA adduct formation (Mimnaugh et al. 1986; Mukhopadhyay et al. 2009). Several studies show that doxorubicin-induced cardiotoxicity is due to PARP hyperactivation and it can be ameliorated by PARP inhibition (Joseph et al. 2004; Pacher et al. 2002; Szenczi et al. 2005). Similarly, both 3AB and the PARPi, BGP-15, have a protective effect against cisplatin-induced kidney damage (Racz et al. 2002; Chan et al. 1988).

SYNTHETIC LETHALITY OF PARPi

The discovery that PARPi are synthetically lethal to cells lacking the HR DNA repair pathway is the most exciting aspect of PARPi research, as it demonstrates the possibility for truly tumour-selective cancer therapy with minimal or no side-effects. The term "synthetic lethality" was originally coined by the geneticists in the 1940s to describe the concept where mutation of two genes independently does not impair cell viability but mutation of both genes together results in cell death. The application of this concept to cancer therapy came somewhat later with the idea that molecular defects in

cancer cells may be responsible for selective tumour killing by some agents, and that these molecular determinants of sensitivity could be identified in model systems (Hartwell et al. 1997). This principle probably underlies the efficacy of conventional chemotherapy. For example, defects in the tumour suppressor pRB and its associated pathway are common in cancer and lead to uncontrolled entry into S-phase and consequent activation of topoisomerase II. An increase in topoisomerase II activity is predicted to result in increased vulnerability to topoisomerase II poisons, such as doxorubicin and etoposide, both effective agents in cancer therapy. This has been confirmed by studies linking loss of pRB pathway function to sensitivity to topoisomerase II poisons (Dolma et al. 2003).

The idea that there might be synthetic lethal interactions with PARPi came from the observation that loss or inhibition of PARP-1 results in a high level of sister chromatid exchange, indicating a hyperrecombinogenic phenotype. As described above, endogenous and environmental factors generate a high level of SSBs in DNA; these relatively non-toxic lesions are repaired by mechanisms that include PARP-dependent SSBR. Failure to repair SSBs leads to the generation of the much more cytotoxic stalled replication forks and replication-associated single-ended DSBs. Failure to repair DSBs can have a number of consequences, including gross chromosomal rearrangements and eventually cell death. HR repairs DSBs in an error-free process through the alignment of homologous sequences of DNA using the sister chromatid as a template (Helleday 2010) (see Chapter 14). DSBs are detected by ATM and ATR kinases that phosphorylate numerous protein substrates, including H2AX, NBS1, BRCA1 and FANCD2, CHK1, CHK2 and p53, to set off a cascade of repair and checkpoint signaling (Fig. 3). Resection of the broken ends by the RAD50-MRE11-NBS1 (MRN) complex, mediated by BRCA1, exposes the 3′ ends on either side of the DSB. The single stranded DNA is rapidly coated by RPA (replication protein A). Then, BRCA2, probably with some assistance from BRCA1 and PALB2, replaces RPA with RAD51. This allows the 3′ strand from the broken chromatid to invade the complementary sequence of the homologous chromatid forming a displacement loop (D-loop). A DNA polymerase subsequently extends the invading strand using the duplex DNA as a template. The second end of the break may be captured by the D-loop, which acts as a template for the extension of this strand; the cross-shaped structures formed are known as Holliday junctions (HJs). RAD54 promotes branch migration and the HJs can be resolved by the Blooms syndrome or Werner syndrome helicases (BLM or WRN) (Sung et al. 2006; Opresko et al. 2009), possibly in cooperation with the GEN1 nuclease (Wechsler et al. 2011). The function of the entire repair pathway can be affected if one or more genes involved in the process is rendered defective.

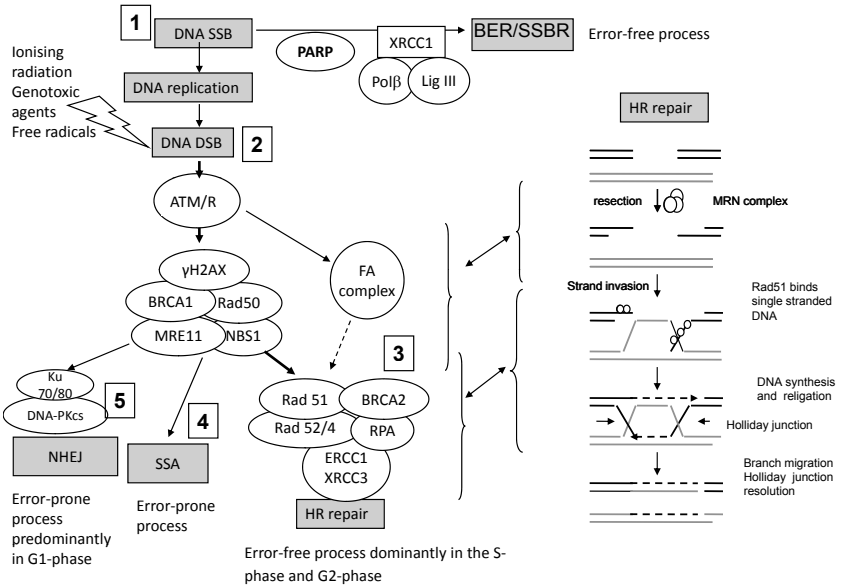

Figure 3. Complementary Roles of BER/SSBR and HR in maintaining genomic stability under conditions of genomic stress. Left side: Endogenously-induced or cytotoxic agent-induced, DNA single strand breaks (SSBs) **1** are repaired by error-free PARP-1-dependent BER/SSBR to promote survival. If SSBs persist they will cause replication fork stalling and replication-associated double strand breaks (DSBs) **2**. These are preferentially repaired by error-free homologous recombination (HR) **3** to promote cell survival in replicating cells. HR is a complex process involving a multitude of proteins, only a few of which are illustrated here (see Chapter 14). When HR is defective, DSBs persist or are repaired by error-prone single strand anealing (SSA) **4** or non-homologous end joining (NHEJ) **5**, resulting in cell death.

Line drawing: HR at the DNA level. The DSB is resected by endonucleases, including the MRN complex. The resultant single-stranded DNA is initially coated by RPA, then through the action of BRCA2, the RPA is replaced by RAD51. This allows invasion into the homologous sequence, forming a D-loop and crossovers—Holliday Junctions. Using the homologous sequences as a template, the missing DNA is filled in and the branches migrate until complete. The Holliday junctions are then resolved by resolvases/helicases.

PARP-1 does not co-localise with RAD51 foci, which mark sites of HR, and RAD51 foci form normally in PARP-1$^{-/-}$ cells, indicating that PARP-1 does not play a direct role in HR. However, RAD51 foci were increased in PARP-1$^{-/-}$ cells, confirming hyperrecombination activity in the absence of PARP-1 (Schultz et al. 2003). These studies implied that the PARP-1-dependent pathway and the HR pathway were complementary and that loss of one of these pathways could lead to a hyperdependence on the other (Fig. 3). This hypothesis was followed up by the Helleday group, in collaboration with the Newcastle PARPi development group, who confirmed that Chinese hamster cells with mutations in the HR genes, BRCA2, or the RAD51 homologues, XRCC2 or XRCC3, were hypersensitive to a panel of PARPi, including 3AB, NU1025 and AG14361. Furthermore, AG14361 was selectively cytotoxic to human breast cancer cells in which BRCA2 was depleted by siRNA, whether they were p53 wild type or mutant. This PARPi also caused the regression of BRCA2-mutant xenografts (Bryant et al. 2005). Parallel independent studies in the Ashworth lab demonstrated that another potent PARPi, KU0058948, was selectively toxic to BRCA1 and 2 defective mouse embryonic stem (ES) cells, as well as tumours derived from them, and that KU0058948 caused a substantial increase in chromosomal aberrations (Farmer et al. 2005). Both groups noted that inhibition of PARP led to an increase in γH2AX foci, indicative of an increase in DSBs/stalled replication forks, in both HR competent and deficient cells, but that there was a corresponding increase in RAD51 foci only in the HR competent cells. The proposed mechanism of cytotoxicity was that when PARP is inhibited endogenously-generated DNA SSBs go unrepaired, leading to stalled replication forks and replication-associated DSB formation. In repair-competent cells, these lesions are resolved and replication is re-started by the process of HR. However, in BRCA1/2 defective cells, the lesions persist or are repaired by error-prone pathways such as NHEJ or single-strand annealing (SSA), and the cell dies. In support of this latter hypothesis is the recent observation that in HR defective cells, the synthetic lethality of PARPi is dependent on functional NHEJ. Mutation, knockdown or inhibition of DNA-PKcs, a direct participant in NHEJ, reversed the genomic instability and cytotoxicity caused by ABT-888 in cells lacking BRCA1, BRCA2 or ATM (Patel et al. 2011). In addition, loss of 53BP1, which promotes NHEJ, partially restores HR function in BRCA1 mutant cells and can rescue them from DNA damaging agent and PARPi sensitivity (Bouwman et al. 2010; Bunting et al. 2010).

These initial demonstrations of the synthetic lethality of PARPi in BRCA-defective cells caused significant excitement because of the association of BRCA1 and 2 mutations with hereditary breast and ovarian cancer syndromes (Venkitaraman 2002) (see Chapter 14). Carriers of BRCA1/2 mutations have one functional allele, and can therefore conduct

HR repair in normal tissues, but tumour development is dependent on somatic inactivation of the second allele rendering them defective in HR (Welcsh and King 2001). Thus, inhibition of PARP, whilst being non-toxic to normal tissues, could be selectively toxic to at least a sub-set of tumours. These observations probably led to the explosion in the number of PARPi undergoing clinical trial since 2005.

However, caution should always be exercised in extrapolating data gained from studies conducted in genetically manipulated rodent cells to the human condition. Just because hamster and mouse cells, where the BRCA status had been altered by mutagenesis or genetic manipulation, were hypersensitive to PARPi, it does not necessarily follow that human cancers, which have evolved to survive despite a defect in BRCA1/2, will be equally sensitive. Indeed, BRCA2 defective human pancreatic cancer Capan-1 cells were insensitive to NU1025 (Gallmeier and Kern 2005), and other studies indicate that human *BRCA1*-positive, *BRCA1* heterozygous, and *BRCA1*-negative breast cancer cell lines did not exhibit differential sensitivity to three different PARPi (3-aminobenzamide, NU1025, and AG14361) (deSoto et al. 2006). In contrast to these data, a recent study using a panel of nine human cancer cell line, including the Capan-1 cells and the same breast cancer cell lines used in the negative study above, did show that the PARPi AG-014699 was selectively toxic to cell lines and xenograft models with defective BRCA 1 and 2 (Drew et al. 2011). In this study, a model of sporadic breast cancer BRCA1 loss was also investigated; the UACC3199 breast cancer cell line, which has epigenetically silenced BRCA1 due to promoter methylation, was also sensitive to PARP inhibition, suggesting a wider role for these agents in non-BRCA mutated cancers.

RESISTANCE TO PARP INHIBITORS

As with all chemotherapeutic agents, including targeted agents, acquired resistance to PARPi has been observed. In two independent laboratories, pre-clinical studies demonstrate that secondary mutations in either mutated BRCA1 or BRCA2 can restore their function and hence HR function. For example, Capan-1 cells carry a 6174delT frame shift mutation in BRCA 2, making them HR defective, as demonstrated by an inability to form RAD51 foci and exquisite sensitivity to PARPi and platinum agents. Following *in vitro* selection, PARPi-resistant clones were both highly resistant to the drug (over 1,000-fold) and cross resistant to cisplatin. Interestingly, these resistant clones acquired the ability to form RAD51 foci, indicating re-acquisition of HR function. DNA sequencing of PARPi-resistant clones revealed new BRCA2 isoforms as a result of an intragenic deletion of the c.6174delT mutation, and thus restoration of the open reading frame

(Edwards et al. 2008; Sakai et al. 2008). Similar findings were demonstrated for BRCA1 mutant cells (Swisher et al. 2008).

Although this phenomenon has not so far been demonstrated clinically, such secondary mutations have been observed in platinum resistant tumours. As described above, fully functional NHEJ seems to be required for PARPi sensitivity in HR defective cells. Loss of 53BP1 or DNA-PK in BRCA1 mutant cells can partially restore HR function and can rescue DNA damaging agent and PARPi sensitivity (Bouwman et al. 2010; Bunting et al. 2010; Patel et al. 2010). Loss of 53BP1 appears to be relatively common in triple negative and BRCA1 mutant breast cancer samples (Bouwman et al. 2010), but it is too early to determine if these mechanisms will develop clinically in response to PARPi therapy.

In another interesting study, resistance to ABT-888 and TMZ together was investigated in HCT116 cells. Three clones were isolated after prolonged exposure to TMZ and ABT-888. These clones all had consistent down regulation of PARP-1 (about 20-fold), but a >2-fold upregulation of a number of genes associated with HR: RAD51, BRCA1, BRCA2, BARD1, BLM FANCG and CHK2. In the resistant cells, γH2AX foci disappeared more rapidly following exposure to TMZ, and these cells were also cross-resistant to ionising radiation. At the protein level, PARP-1 loss and RAD51 increase were confirmed; Ku and DNA-PK protein levels were similar in parental and resistant clones, indicating that the increased repair was due to HR rather than NHEJ (Liu et al. 2009). Thus, increased HR activity seems to be a mechanism of PARPi resistance.

ROLE OF PARP BEYOND BRCA

It is clear that mutations in (or epigenetic silencing of) BRCA1/2 are just the "tip of the iceberg" when it comes to PARPi-exploitable HR defects in cancer. HR is the principal error-free DNA DSB repair mechanism and is a complex process involving several proteins that include damage signalling and checkpoint kinases (e.g., ATR and CHK1), the FANC and Rad51 homologues, MRE11/RAD50/NBS1 (MRN) and many other components, some of which remain to be identified (Fig. 3) (see Chapters 10, 13 and 14). Most importantly, HR is defective in a wide variety of cancers, not just BRCA-associated breast and ovarian cancer, due to defects in any one of the above-mentioned genes (Kennedy and D'Andrea 2006). PARPi may be synthetically lethal in sporadic cancers that bear somatic mutations or epigenetic silencing in the various components of the HR pathway. Indeed, recent studies show that KU0058684 and KU0058948 had single agent activity in cells defective in several of these proteins (McCabe et al. 2006) and that AG-014699 has single agent activity in cells and xenografts with

BRCA1 promoter methylation (Drew et al. 2011). Other proteins, such as EMSY, are also implicated, as they regulate the activity of other components of the pathway (Cousineau and Belmaaza 2011). Using a siRNA screen, loss of ATR, XRCC1, PCNA, DDB1 and RAD51, all of which have known functions in DNA repair (although not necessarily HR), were identified as being synthetically lethal with PARP inhibition. Perhaps more surprising was the finding that silencing of PTEN and CDK5, not normally associated with HR, was also synthetically lethal with PARP inhibition (Lord et al. 2008; Turner 2008). These novel observations have been validated by investigations showing that CDK5 is important in checkpoint signalling and that PTEN controls RAD51 function (Shen et al. 2007). *PTEN* is one of the most commonly mutated tumour suppressors in human cancer and its deficiency was targeted successfully by the PARPi, olaparib (Mendes-Pereira et al. 2009).

Interestingly, hypoxia can lead to the reduced expression of proteins involved in HR (Bindra et al. 2005, Chan et al. 2008), suggesting that hypoxic cells may be sensitive to single agent PARPi. Recent studies show that more γH2AX and 53BP1 foci, as a consequence of altered DNA replication firing, and S phase-specific cell killing were seen in hypoxic cells treated with ABT-888 relative to cells in normoxic conditions. Moreover, in xenografts of mice treated with a PARPi, both increased γH2AX and cleaved caspase-3 expression were seen in the hypoxic sub-regions, indicating increased DNA damage and apoptosis. These regions were RAD51-deficient, indicating compromised HR (Chan et al. 2010). Such data raise the enticing prospect that PARPi may have broader clinical utility beyond tumours with synthetically lethal mutations, as all solid tumours contain hypoxic regions. Indeed, this demonstration of "contextual synthetic lethality" is particularly appealing because hypoxia drives genetic instability, metastasis and chemotherapy-resistance as well as the radioresistance described above (see Chapter 17).

Recent data suggest that an HR defect may be "engineered" using another targeted agent. Following the discovery that CDK1 phosphorylates BRCA1 enabling it to form repair foci, the combination of AG-014699 and a CDK1 inhibitor was investigated in lung cancer cells and xenografts, as well as genetically induced lung tumours in mice. In all cases, CDK1 inhibition potentiated cell killing by AG-014699. Interestingly, this phenomenon appeared to be tumour-directed, as there was no appreciable body weight loss in the mice, indicating a lack of systemic toxicity (Johnson et al. 2011). The therapeutic scope for the synthetic lethality of PARPi is potentially much wider than implied by the term "BRCAness", which focuses on phenotypic characteristics in breast and ovarian cancers.

PREDICTIVE BIOMARKER FOR HR DEFECTS

Given the relatively common loss of HR function in cancer, it is clear that many cancer patients outside of the BRCA mutation carrier population could benefit from PARPi therapy. However, the multiple potential causes of defective HR poses something of a challenge as to the identification of HR defective cancers. Sequencing for mutations in individual genes known to be involved in HR is likely to be costly and time-consuming as well as limited to the genes positively identified as having a role in HR. Moreover, it will not detect epigenetic silencing events. Aberrant DNA methylation is a frequent epigenetic event leading to silencing of genes in cancer, and, in the context of HR, inactivation of the BRCA1 promoter has been demonstrated in up to 30% of sporadic ovarian cancers and breast cancers (Esteller et al. 2000). A similar level of FANCF gene methylation has also been reported in primary ovarian tumours (Taniguchi et al. 2003).

One method that would detect epigenetic silencing is to employ gene expression arrays. Attempts have been made to this approach to identify a *BRCA*-like profile in tumours by comparing gene expression profiles of *BRCA1/2* mutated cancers with sporadic epithelial ovarian cancers (Jazaeri et al. 2002). DNA microarrays have the potential to identify HR defects, with the advantages of this approach being that DNA is more stable than RNA or proteins and is readily available from paraffin blocks. Recently, Konstantinopoulos et al. (2010) developed a BRCAness profile (comprised of 60 genes) using a publicly available microarray dataset that included 61 patients with epithelial ovarian cancers (sporadic/BRCA germline mutant). In a further 70 patients enriched for sporadic disease, the BRCAness profile was found to correlate with responsiveness to platinum and was predictive of improved outcome.

Immunohistochemistry (IHC) analysis on formalin fixed paraffin embedded blocks may ultimately prove a useful tool to identify HR defects, although the number of proteins that would potentially need to be measured is somewhat daunting. Nevertheless, in triple negative breast cancers, which closely resemble the BRCA1 mutant ("BRCAness") phenotype, expression of several DNA repair proteins, or products of their activity, including XPF, FANCD2, PAR, MLH1, PARP1, pMK2, p53 and Ki67, could be assessed by IHC to stratify patients into recurrence risk categories (Alexander et al. 2010). An investigation using Capan-1 cells compared with an HR competent pancreatic cancer cell line, coupled with studies in the BRCA2 mutant and PARPi-resistant revertant clone, has led some to suggest that high levels of PARP activity is characteristic of an HR defect (Gottipati et al. 2010) and could be a potential biomarker to identify HR defective tumours. The

likelihood that these assays, performed in a limited number of cell lines, will be validated in tumour samples or primary cultures may be slim, as PARP activity is related to proliferation (Wein et al. 1993), and in a clinical study, PARP activity in human melanoma tumour biopsies was found to be highly variable, consistent with tumour heterogeneity (Plummer et al. 2005).

An alternative screening approach that would capture all defects, genetic or epigenetic, is to assess HR function in viable cells. In cell line studies, investigators have assessed competent HR by co-transfecting a plasmid containing two incomplete copies of the green fluorescent protein (GFP) gene with a restriction enzyme that cuts the plasmid. In a cell with functional HR, full length wild-type GFP will be produced following recombination, while cells without effective HR will not exhibit green fluorescence (Pierce et al. 1999). However, this assay may be of limited use with clinical material, as primary cultures/tumour samples are difficult to transfect. Another option is to use immunofluorescence microscopy to study RAD51 foci, which accumulate at sites of HR, after *ex vivo* DNA damage. RAD51 foci (along with BRCA1 and FANCD2 foci) were measured in a small study of 7 fresh breast tumour biopsies, following *ex vivo* irradiation (Willers et al. 2009). Four of the tumours did not exhibit an increase in focus formation, and three of these potentially HR defective tumours were triple negative breast cancers, a phenotype that has been associated with BRCA1 deficiency. A similar approach has been undertaken in a study of RAD51 focus formation in 25 primary ovarian cancer cultures; failure to form foci correlated with *ex vivo* sensitivity to AG-014699 with a negative predictive value of 100% and positive predictive value of 93%. In this study, a 50–60% incidence of HR deficiency in sporadic ovarian cancers was reported (Mukhopadhyay et al. 2010). It is important to note that these samples were not from BRCA1/2 mutation carriers and reveal a sizeable population of HR defective tumours that could potentially benefit from PARPi therapy. There is emerging clinical evidence to confirm this idea, with the observation that responses to the PARPi, olaparib, were achieved in a substantial proportion of patients with high grade serous ovarian cancer without a BRCA-defective background (Gelmon et al. 2010). In a study in leukemic cell lines and primary cultures developed from AML patients, increased γH2AX foci and decreased RAD51 foci formation were seen in PARPi-sensitive cells compared to PARPi resistant cells (Gaymes et al. 2009), supporting the hypothesis that γH2AX/RAD51 foci can be a potential biomarker for HR defects and PARPi sensitivity irrespective of tumour types.

Clearly the performance of assays on fresh tissue following *ex vivo* DNA damage has technical limitations. The alternative is to investigate the induction of HR activity after damaging the tumour *in situ*, i.e., post chemotherapy. In a prospective study, measurement of foci of 4 HR related proteins, BRCA1, γH2AX, conjugated ubiquitin and RAD51, in formalin

fixed pre- and post-treatment biopsies from 60 primary breast cancer patients revealed that BRCA1 positive baseline foci and baseline or chemotherapy induced RAD51 foci inversely correlated with response to chemotherapy (neo-adjuvant epirubicin and cyclophosphamide) (Asakawa et al. 2010). A similar study measured RAD51 foci in geminin-staining (proliferating) cells in core biopsies obtained 24 hours after neo-adjuvant anthracycline chemotherapy in 68 patients with sporadic breast cancers (Graeser et al. 2010). The investigators found that a low RAD51 score correlated with high histological grade, triple negative breast cancers and was a strong predictor for a pathologic complete response to chemotherapy. Although a PARPi was not used in either of these studies, the data do support the hypothesis that functional HR predicts for poor responses to DNA damaging agents.

Evaluation of HR function in circulating tumour cells (CTCs) may prove useful in the future to identify patients suitable for PARPi therapy. Induction of γH2AX after treatment with DNA damaging agents has been demonstrated in CTCs (Yap et al. 2011), and similar studies evaluating RAD51 foci formation are ongoing (Wang et al. 2010). These studies are in their infancy, but such a minimally invasive technique that would permit longitudinal studies would clearly be valuable as tumour surrogates. To summarise, although various approaches to predict HR defects continue to be developed, such assays will require further evaluation and validation within prospective clinical trials before they can be adopted as predictive tools directing treatment decisions in patients. Although cumbersome, the functional assays currently represent the most reliable way to identify HR defects, particularly in light of the recent results showing that even in BRCA1 mutant tumours co-incident loss of DNA-PK or 53BP1 can restore HR function and PARPi resistance.

CLINICAL EVALUATION OF PARPi

Based on the pre-clinical data summarised above, the clinical development of PARPi has been on two fronts i) as chemosensitisers in combination with conventional chemotherapy and ii) as single agents in selected populations, because of the exciting promise of synthetic lethality to HR-defective tumours. There are currently nine PARPi undergoing clinical investigation, some of which have been characterized for their pharmacodynamic (PD) effects and pharmacokinetics (PK) (Table 1). Such PD measurements of PAR formation and/or measurement of DNA damage have been performed in surrogate normal tissues as well as on tumour material.

The first of these trials, i.e., of i.v. AG-014699 (rucaparib) in combination with TMZ, was initiated in 2003, prompted by the complete tumour regression observed pre-clinically with this drug combination (Calabrese

Table 1. PARP inhibitors in clinical development.

Agent/Company Date entered trial	Route	Single agent/ combination	Disease	Clinical status
AG-014699/Rucaparib Pfizer/Clovis 2003	Intravenous	Various combinations Single agent	Solid tumours Melanoma	Phase I/II ongoing
KU59436/AZD2281 Olaparib AstraZeneca 2005	Oral	Single agent Various combinations	Various	Phase I complete Several Phase II
ABT888 Veliparib 2006	Oral	Single agent Various combinations	Solid and lymphoblastoid	Ph 0/I complete Several Phase II
BSI-201/ iniparib BiPar/Sanofi 2006	Intravenous	Gem-carbo/ TMZ combinations	TNBC	Phase II complete Phase III
INO-1001 Inotek/ Genentek 2003/6	Intravenous	TMZ combinations	Melanoma, GBM	Phase II
MK4827 Merck 2008	Oral	Single agent	Solid BRCA ovarian	Phase I
CEP-9722 Cephalon 2009	Oral	TMZ combinations	Solid tumours	Phase I
GPI 21016/E7016 MGI Pharma 2010	Oral	TMZ combinations	Solid tumours	Phase I
BMN763 Biomarin 2011	Oral	Single agent Combinations	Solid tumours	Phase I

et al. 2004; Thomas et al. 2007). In the pre-clinical studies, the efficacious dose inhibited PARP activity ≥ 50% for 24 hr. The Phase I trial involved a Phase 0 component, where PK and PD assays were performed, in addition to safety evaluation, following a single dose of AG-014699 (1, 2, 4, 8, 12 or 18 mg/m^2) approximately 1 week prior to administering the combination of AG-014699 at the same dose daily for 5 days with TMZ. Inhibition of PARP activity by >50% for 24 hr in surrogate normal tissues (peripheral blood mononuclear cells (PBMCs)) was the target PARP-inhibitory dose (PID) in this study (Plummer et al. 2008). This figure was based not only on the pre-clinical studies, but also on previous investigations measuring day-to-day variation in PARP activity in healthy volunteers and melanoma patients treated with TMZ (Plummer et al. 2005). Because this was a first in class trial and because of concerns regarding potential toxicities based on the toxicities seen with TMZ in combination with other chemosensitisers (Ranson et al. 2006, 2007), AG-014699 was escalated cautiously (from 1 mg/m^2 to 12 mg/m^2) with half the recommended maximum dose of TMZ

(100 mg/m^2) until the PID was achieved. PARP inhibition was seen at all doses without any serious adverse events; PID was estimated at 12 mg/m^2 based on 74% to 97% inhibition of PARP activity in PBMCs. DNA breaks as well as PARP inhibition were assessed in PBMCs taken during the first cycle of the combination. These studies demonstrated that AG-014699 showed linear PK with no interaction with TMZ, that TMZ did not affect AG-014699 PD, and that, as well as a dose-dependent increase in PARP inhibition, there was a corresponding dose-dependent increase in DNA breakage. No toxicities were observed at the PID in combination with 50% TMZ, so in the second half of the study, TMZ was escalated in combination with 12 mg/m^2 AG-014699 in patients with metastatic melanoma who consented to pre- and post treatment biopsies. In this study, it proved possible to give full dose TMZ with the PID. However, increasing the PARPi dose further by 50% to 18 mg/m^2/day did cause dose limiting myelosuppression. AG-014699 was detected in tumour samples, and mean PARP inhibition in tumour biopsies taken 4–6 hours after administration of AG-014699 was 92% (range 46–97%). The recommended phase II dose was 200 mg/m^2 of TMZ with 12 mg/m^2 of AG-014699.

Following the success of the Phase 0 element of the AG-014699 trial, velaparib (ABT-888) was investigated in an innovative purely phase 0 trial, the first such study in oncology (Kummar et al. 2009). The primary study endpoint was inhibition of PARP activity after a single dose of velaparib, with significant inhibition being observed at the 25 and 50 mg doses. This proof-of-concept Phase 0 model has the potential to accelerate drug development in oncology. There is an extensive clinical trial program associated with this agent, with 32 ongoing trials of velaparib in combination with cytotoxins in a variety of solid and haematological malignancies.

AG-014699 was taken into a Phase II study in combination with TMZ, at the Phase I recommended dose and schedule in patients with metastatic melanoma. In this study, enhanced TMZ-induced myelosuppression was observed. However, the regimen was well tolerated following a 25% dose reduction of TMZ, and despite the dose-reduction, there was an increase in the response rate and median time to progression compared to TMZ alone (Plummer et al. 2006). In other studies of PARPi/alkylating agent combinations, dose-limiting myelosuppression has also been observed. Phase I studies with INO-1001 at 100, 200 and 400 mg/m^2 indicated myelosuppression and elevated liver enzymes in combination with TMZ (Bedikian et al. 2009). Disappointingly, Phase I studies of olaparib with a similar alkylating agent, dacarbazine, in patients with advanced melanoma was found to be myelosuppresive with the maximum tolerated dose being 100 mg of olaparib with 600 mg/m^2 of dacarbazine, and there was no observed clinical benefit over dacarbazine alone (Khan et al. 2011).

Combinations of PARPi with other chemotherapy agents are under investigation. ABT-888 was investigated in Phase I trials with topotecan, a topoisomerase I poison, in patients with refractory solid tumours and lymphomas. In this study, myelosuppression was observed with the standard dose of topotecan, necessitating dose reductions. The maximum tolerated dose (MTD) was topotecan at 0.6 mg/m^2/day and ABT-888 at 10 mg BID, on days 1–5 of a 21-day cycle. The PK of ABT-888 was not affected by topotecan, and PD assays in PBMCs showed >50% reduction in PARP activity in most patients and increased levels of γH2AX were seen in both PBMCs and circulating tumour cells. In 3 patients with pre-and post-treatment biopsies, there was a >75% reduction in PARP activity (Kummar 2011). In a Phase I study of olaparib in combination with topotecan, the dose-limiting toxicities were neutropenia and thrombocytopenia, which was dose-related. In this study, topotecan affected olaparib PK, reducing Cmax and AUC by 20%. The most common adverse events (AEs) included fatigue and gastrointestinal events, and the MTD was topotecan at 1.0 mg/m^2/day for 3 days plus olaparib at 100 mg bid.

BSI-201/Iniparib showed good activity without an increase in toxicity when combined with gemcitabine and carboplatin in a randomized phase II trial in 123 patients with triple-negative breast cancer, which included those who had received prior chemotherapy for metastatic disease (O'Shaughnessy et al. 2011). Iniparib increased the overall response rate (from 32% to 52%), progression-free survival (from 3.6 to 5.9 months) and overall survival (from 7.7 to 12.3 months). Preclinical studies indicate synergy between PARPi and platinum therapy in HR defective cancer. Triple-negative breast cancers are believed to share the molecular characteristics of *BRCA1*-associated cancers, such as a high degree of genomic instability, implying an impaired ability to repair DNA damage. HR defects are commonly seen in triple-negative breast cancer and, as well as BRCA1 mutations, defects include BRCA1 promoter methylation, overexpression of de-regulators including ID4 and HMG, and aberrations in MRE11, ATM and PALB2 (Alexander et al. 2010; Alli et al. 2009). This study of iniparib was presumably conducted in triple-negative breast cancer patients because of their likely HR defects. The promising results led to the first PARPi phase III study that enrolled over 500 patients. However, this phase III study did not meet the pre-specified criteria for significance for overall survival and progression-free survival, although patients who had received 1–2 prior chemotherapy regimens appeared to benefit (Guha, 2011). The negative results of the phase III study are disappointing, but since the mechanism of action of iniparib is not clearly understood (see earlier), these data may not represent a 'class effect'. In particular, as described above, iniparib has a unique mode of action and has also been shown to inhibit GAPDH (Bauer et al. 2002). More recently, doubt has been cast on its ability to inhibit PARP, as a dose and time-dependent

inhibition of PARP formation was demonstrated with veliparib, olaparib and MK-4827, but not with iniparib (Ji et al. 2011). In this study, γ-H2AX induction occurred with all agents, including iniparib, suggesting other mechanisms of action for iniparib besides PARP inhibition.

Olaparib was the first PARPi to go into Phase I clinical trial as a single agent. In this study of initially 60 patients, the olaparib dose was escalated from 10 mg daily for 2 out of every 3 weeks to 600 mg twice daily. Based on the exciting pre-clinical demonstration of synthetic lethality of PARPi in BRCA mutant cancers, the dose of 200 mg twice daily was chosen for further study in a select cohort of 23 patients with BRCA mutations, 19 of whom had BRCA-associated tumors, including breast, ovarian, and prostate cancers. In this group, 9 had partial responses according to the NCI response evaluation criteria (RECIST) (Fong et al. 2009). On the basis of these promising preliminary data, two multicenter, international phase II studies of olaparib in BRCA1 or BRCA2 carrier patients with breast or ovarian cancers, who were refractory to standard chemotherapeutic regimens, were conducted. A clear dose response effect was seen with an overall response rate of 41% in the 27 breast cancer patients treated with 400 mg olaparib twice daily for 28 days compared with 22% in the 27 patients recieving 100 mg olaparib on the same schedule (Tutt et al. 2009). The median time to progression was 5.7 months in the 400 mg group and 3.8 months in the 100 mg group. The common adverse effects were mild and included fatigue, nausea and vomiting. A similar dose effect was observed in ovarian cancer, where an overall response rate of 33% was observed in the 400 mg group, and a response rate of 12.5% was observed in the 100 mg group (Audeh et al. 2010). In further studies of ovarian cancer patients with confirmed BRCA mutations or a strong family history, there was a complete or partial response in 40% of patients as measured by RECIST and/or tumour markers (CA125). Response to olaparib correlated with platinum sensitivity, with the clinical benefit rate being 69% in the platinum sensitive group, 45% in the platinum resistant and only 23% in the platinum refractory group (Fong et al. 2010). These proof-of-concept studies confirmed that BRCA1 or BRCA2 mutational status serves as a predictive marker for PARPi, but that platinum resistance may indicate secondary BRCA mutations or other events restoring HR function to confer PARPi resistance. Clearly larger randomised trials are required to confirm these data.

TOXICITY ISSUES

Myelosuppression seems to be a common outcome in trials of PARPi in combination with conventional chemotherapy, particularly where a continuous dosing schedule is employed. Currently, the mechanism is

unclear, but there may be parallels with the pre-clinical data demonstrating that the MTD of PARPi in combination treatments is much less than when employed as a single agent. For example, in mice, the MTD of AG-014699 in combination with TMZ on a single 5-day schedule is 1 mg/kg, but as a single agent, repeated cycles of 25 mg/kg daily x5 (once a day for 5 days) every 21 days as well as daily x10 is completely non-toxic (Drew et al. 2011; Thomas et al. 2007). Continuous dosing with a PARPi will result in prolonged DNA repair inhibition that may paradoxically result in secondary cancers. Disruption of PARP-1 caused a high incidence (49%) of aggressive brain tumours in p53 null mice that showed typical features of human cerebellar medulloblastomas, implicating PARP-1 in tumour suppression (Rouleau et al. 2010; Tong et al. 2003). However, there is wide inter-individual variability in PARP activity in humans, thus potentially limiting toxicity to subpopulations only (Zaremba et al. 2011).

CONCLUSIONS

Genomic instability in cancer may result from the loss of some DNA damage signalling and repair pathways, with the upregulation of other compensatory pathways. These upregulated pathways make good targets for cancer therapy because a) the cell may be dependent on them for survival and b) they may be responsible for chemotherapy and radiotherapy resistance. In the case of "a", inhibition of this pathway may result in single agent activity specifically in the cancer cell, where repair of endogenously generated DNA damage is inhibited. In the case of "b", inhibition may lead to tumor-selective chemo- and radiosensitisation. PARP-1 is the first identified, most abundant and best characterised member of the PARP superfamily of enzymes that catalyses the cleavage of NAD^+, releasing nicotinamide and synthesising elongating polymers of ADP-ribose. PARP-1, PARP-2 and PARP-3 are specifically involved in DNA strand break repair. Most PARPi are catalytic inhibitors and contain the nicotinamide pharmacophore. Because of the structural similarity in their catalytic domains, most PARPi inhibit more than just PARP-1. PARPi increase the anticancer activity of TMZ, topoisomerase I poisons and ionising radiation in *in vitro* and *in vivo* tumour models, results that have been validated by genetic knockdown of PARP-1 and PARP-2. Most importantly, PARPi alone selectively kill cancer cells that lack HR function, without affecting repair competent cells. This potential to selectively target certain tumours without serious side-effects has led to a surge of interest in the pharmaceutical industry, and currently there are 9 inhibitors under investigation clinically. Pre-clinical studies indicate that more profound and sustained PARP inhibition is required for single agent activity compared to chemo- or radiosensitisation, reflecting the

dependence on generation of DNA damage endogenously. Intense dosing schedules to give profound and sustained PARP inhibition are toxic in pre-clinical chemosensitisation studies. Clinical data are emerging to show that PARPi have good anticancer activity in BRCA1 and BRCA2 patients with breast, ovarian and prostate cancer with only mild toxicities. When the "safe dose" determined in single agent studies are used in combination with cytotoxic chemotherapy, unsurprisingly, toxicities are encountered. HR is a complex and multi-component pathway and preclinical data indicates that PARPi will be useful in tumours lacking any one of a number of these key proteins. Identification of these potentially PARPi-responsive tumours is the next challenge. Gene expression signatures and assays of HR function can fulfil this objective, but the existing approaches currently lack the necessary specificity or are too cumbersome to become routine clinical practice. Resistance to single agent PARPi therapy may develop through mechanisms that restore HR function, and resistance to combination chemotherapy may develop by upregulation of HR. Finally, the vasoactivity seen with some of the inhibitors remains to be confirmed as a "class effect" using other inhibitors and tested clinically.

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CHAPTER 16

Tumor Genetics and Personalized Medicine

Mary Ellen Urick and *Daphne W. Bell*[a,*]

INTRODUCTION

There are several defined "hallmarks" or acquired functional attributes shared by cancer cells; namely, their ability to propagate their own growth signals, insensitivity to anti-growth signals, ability to evade apoptosis, capacity for unlimited replication, acquired angiogenic properties, and acquired invasive and metastatic potential (Hanahan and Weinberg 2000), as well as two recently added hallmarks, i.e., ability to avoid immune destruction and reprogram energy metabolism (Hanahan and Weinberg 2011). Furthermore, interactions between cancer cells and surrounding normal cells are believed to create supportive "tumor microenvironments" (Hanahan and Weinberg 2011), which add an additional level of complexity to the quest for cancer therapeutics. It is now well established that alterations in the tumor genome, epigenome, transcriptome, proteome, and metabolome, as well as the tumor microenvironment, all underlie the pathogenesis of human cancer (reviewed in Kolch and Pitt 2010; Brower 2011; Gilbertson 2011; Nagrath et al. 2011; Stratton 2011).

Although the first indication of a genetic basis for cancer was observed in 1890 (reviewed in Balmain 2001), it was not until 1976 that the first human protooncogene was cloned (Stehelin et al. 1976). That landmark

National Human Genome Research Institute, National Institutes of Health, Building 50, MSC-8000, Bethesda, MD 20892, USA.
[a]Email: belldaph@mail.nih.gov
*Corresponding author

discovery was followed, in 1982, by the first description of a somatically mutated human cancer gene (Parada et al. 1982; Reddy et al. 1982; Tabin et al. 1982; Taparowsky et al. 1982). In the 30 years that have followed, at least 457 cancer genes have been identified (Forbes et al. 2008). These cellular cancer genes are so-called oncogenes or tumor suppressor genes, and are protein-encoding. In recent years, a seminal discovery by Croce and colleagues revealed the existence of micro-RNAs, non-coding RNAs that can serve to regulate the activity of cellular genes (Calin et al. 2002). Many micro-RNAs target cellular cancer genes and are, themselves, dysregulated in cancer (Calin et al. 2002, 2005, 2007). Over the course of the next decade, comprehensive catalogues of somatic genomic alterations will be assembled for the most common and deadly forms of sporadic human cancer. These efforts are unprecedented and have been made possible by the recent revolution in sequencing technologies that permits faster and cheaper sequencing than ever before. The overarching goal of these efforts is to identify "actionable" targets that can ultimately be utilized to improve the clinical management of cancer patients. Clinically actionable targets include genomic alterations that serve to molecularly classify tumor subtypes, genomic alterations that are predictive of outcome, and genomic alterations that represent an "Achilles' heel" and thus can be leveraged for targeted, or individualized, therapy.

The concept of individualized therapy for cancer was suggested as early as 1976 by Peter Nowell (Nowell 1976). Nowell proposed that during tumor progression, genetic instability within the tumor cell population results in the appearance of rare tumor cells that have acquired new mutations; if a mutation confers a selective advantage, the cell in which it occurs will eventually become the dominant clone within a heterogeneous tumor cell population (Nowell 1976). Based on the resulting heterogeneity of tumors, Nowell proposed that individualized therapy might be required for cancer treatment but would likely be complicated by the outgrowth of pre-dominant subpopulations of tumor cells that are drug resistant (Nowell 1976). Within the past decade, these predictions have come to fruition. Individualized therapy, or personalized medicine, has become a reality for certain cancers. However, just as Nowell forecasted, the success of personalized medicine is usually confounded by primary or secondary drug resistance, which in many instances is explained by the appearance of tumor cells with newly acquired mutations or the expansion of pre-existing subpopulations of tumor cells.

The purpose of this Chapter is to overview some of the most significant advances in personalized medicine for cancer and to look forward to what may come within the next decade. We will provide examples of early seminal

advances in this field that resulted from drugging kinases and we will also dedicate equal attention to an emerging field that relies on synthetic lethal interactions to exploit undruggable targets. Because of the scope of this book, as well as the integral role that DNA repair pathways play as barriers to tumorigenesis (Bartek et al. 2007), discussions of DNA repair proteins have been incorporated as they relate to each section (see also Chapter 1).

DRUGGABLE TARGETS IN CANCER THERAPY

Targeting Kinases

Tyrosine kinases are enzymes that phosphorylate tyrosine residues on substrate proteins and, hence, serve to modulate the activity of downstream signal transduction pathways. They exist as both receptor and non-receptor tyrosine kinases. The activity of tyrosine kinases is normally highly regulated (Blume-Jensen and Hunter 2001). In contrast, their activity is often deregulated in cancer cells; among the 90 tyrosine kinases, at least 25 (28%) are currently annotated as consensus cancer genes (http://www.sanger.ac.uk/genetics/CGP/Census/).

The observation that tyrosine kinases are activated in many human tumors (Bishop 1987), coupled with the fact that their activity is ATP-dependent, pointed to their potential to be "druggable" targets through the use of (i) ATP-competitive small molecule inhibitors, which are organic compounds that function as ATP-mimetics and competitively block the ATP-binding site on the intracellular domain of both receptor- and non-receptor tyrosine kinases, or (ii) monoclonal antibodies directed against the extracellular domains of receptor tyrosine kinases (Glossmann et al. 1981; Graziani et al. 1982; Traxler et al. 2001). This concept ushered in the era of personalized medicine based upon tumor genetics and genomics. There are now at least twelve kinase inhibitors approved by the United States Food and Drug Administration (FDA) for specific indications in oncology (Table 1) (Chang et al. 2011). Others are currently being evaluated in clinical trials.

One of the cellular properties that determines the effectiveness of a small molecule inhibitor or monoclonal antibody is whether or not the survival of the cancer cell depends on the activity of the targeted kinase, a phenomenon known as "oncogene addiction" (Weinstein and Joe 2008). In the following sections, we will present historical paradigmatic examples of druggable kinases, discuss recent exciting results in the treatment of deadly cancers, and highlight promising new drugs that are currently in clinical development and exemplify the concept embodied by personalized medicine.

Table 1. US FDA-approved targeted therapies for cancer treatment.

Drug	Tradename	Indication for which the drug is approved
Small molecule inhibitors		
Imatinib mesylate	Gleevec®	Newly diagnosed adult and pediatric patients with Philadelphia chromosome–positive chronic myeloid leukemia (Ph+ CML) in the chronic phase (CP)
		Patients with Ph+ CML in blast crisis (BC), accelerated phase (AP), or CP after failure of interferon-alpha therapy
		Adult patients with relapsed or refractory Ph+ acute lymphoblastic leukemia (ALL)
		Adult patients with myelodysplastic/ myeloproliferative diseases associated with platelet-derived growth factor receptor (PDGFR) gene rearrangements
		Adult patients with aggressive systemic mastocytosis without the D816V c-Kit mutation or with c-Kit mutational status unknown
		Adult patients with hypereosinophilic syndrome and/or chronic eosinophilic leukemia who have the FIP1L1-PDGFRα fusion kinase and for patients with HES and/or CEL who are FIP1L1-PDGFRα fusion kinase negative or unknown
		Adult patients with unresectable, recurrent, and/or metastatic dermatofibrosarcoma protuberans (DFSP)
		Patients with Kit (CD117)–positive gastrointestinal stromal tumors (GIST) that are cancerous, cannot be surgically removed, and/or have spread to other parts of the body
		Adult patients after surgery who have had their Kit (CD117)–positive GISTs completely removed. Approval is based on survival without a return of cancer (recurrence-free survival) with a median follow-up of 14 months. Clinical benefit has not been demonstrated by a long-term effect on recurrence-free survival or survival.
Dasatinib	Sprycel®	Patients with newly diagnosed Ph+ CML in CP or Ph+ CML with resistance/intolerance to prior therapy that included imatinib
		Patients with Ph+ ALL with resistance/intolerance to prior therapy
Nilotinib	Tasigna®	Patients with newly diagnosed Ph+ CML in CP or Ph+ CML in CP or AP with resistance/intolerance to prior therapy that included imatinib
Lapatinib ditosylate (in combination with capecitabine)	Tykerb®	Advanced or metastatic human epidermal growth factor receptor 2 –positive (HER2+) breast cancer patients who have received prior therapy including an anthracycline, a taxane, and trastuzumab

Table 1. contd....

Table 1. contd....

Drug	Tradename	Indication for which the drug is approved
Small molecule inhibitors		
Lapatinib ditosylate (in combination with letrazole)	Tykerb®	Hormone-positive and HER2+ advanced breast cancer in postmenopausal women for whom hormonal therapy is indicated
Gefitinib	Iressa®	Locally advanced or metastatic non-small cell lung cancer (NSCLC) in patients included in the Iressa Access Plan
Erlotinib	Tarceva®	Patients with locally advanced or metastatic NSCLC that is unresponsive to standard treatment or that has not progressed after four cycles of platinum-based first-line chemotherapy
Erlotinib in combination with gemcitabine	Tarceva®	Patients with locally advanced, unresectable or metastatic pancreatic cancer
Temsirolimus	Torisel®	Patients with advanced renal cell carcinoma
Everolimus	Afinitor®	Patients with unresectable, locally advanced, or metastatic pancreatic neuroendocrine tumors
		Patients with advanced renal cell carcinoma that is unresponsive to standard treatment
		Unresectable subependymal giant cell astrocytoma in patients who have tuberous sclerosis that cannot be surgically removed
Romidepsin	Istodax®	Cutaneous T-cell lymphoma in patients previously treated with chemotherapy
Vorinostat	Zolinza®	Cutaneous T-cell lymphoma in patients previously treated with two systemic therapies
Veurafenib	Zelboraf®	Patients with metastatic or unresectable melanoma expressing BRAFV600E
Crizotinib	Xalkori®	Patients with locally advanced or metastatic ALK-rearranged NSCLC
Monoclonal antibodies		
Trastuzumab in combination with other drugs	Herceptin®	Patients with node-positive, HER2+ breast cancer
		Patients with HER2+ metastatic gastric or gastroesophageal junction adenocarcinoma
Cetuximab	Erbitux®	Epidermal growth factor receptor (EGFR)-expressing, recurrent metastatic colon cancer in patients previously treated with chemotherapy
		Recurrent or metastatic squamous cell carcinomas of the head and neck in patients previously treated with chemotherapy
Cetuximab in combination with radiation therapy		Patients with locally or regionally advanced squamous cell carcinomas of the head and neck Cetuximab in combination with platinum-based therapy plus 5-fluorouracil
		First-line therapy for patients with recurrent or metastatic squamous cell carcinomas of the head and neck
Panitumumab	Vectibix®	EGFR-expressing, metastatic colorectal cancer in patients previously treated with chemotherapy

Table 1. contd....

Table 1. contd....

Drug	Tradename	Indication for which the drug is approved
Retinoids		
Bexarotene	Targretin®	Skin problems caused by cutaneous T-cell lymphoma in previously treated patients
Altiretinoin	Panretin®	Cutaneous lesions in patients with AIDS-related Kaposi sarcoma
Tretinoin	Vesanoid®	Certain patients with acute promyelocytic leukemia
Proteasome inhibitor		
Bortezomid	Velcade®	Patients with multiple myeloma
		Mantle cell lymphoma in patients who have already received at least one other form of treatment
Antifolates		
Pralatrexate	Folotyn®	Patients with chemotherapy resistant, or recurrent peripheral T-cell lymphoma
Antiangiogenics		
Bevacizumab	Avastin®	Glioblastoma in patients whose disease progressed following prior treatment
		Patients with metatstatic colorectal cancer
Bevacizumab in combination with paclitaxel	Avastin®	Patients with metastatic HER2-negative breast cancer that is chemotherapy naïve (June 29, 2011: FDA recommended withdrawing this approval)
Bevacizumab in combination with carboplatin and paclitaxel	Avastin®	Patients with unresectable locally advanced, metastatic, or recurrent NSCLC
Bevacizumab in combination with interferon alfa	Avastin®	Patients with metastatic renal cancer
Sorafenib	Nexavar®	Patients with advanced renal cell carcinoma
		Patients with unresectable hepatocellular carcinoma
Sunitinib	Sutent®	Patients with imatinib-resistant GIST; or GIST patients who cannot take imatinib
		Patients with unresectable, locally advanced, or metastatic pancreatic neuroendocrine tumors
		Patients with metastatic renal cell carcinoma
Pazopanib	Votrient®	Patients with advanced renal cell carcinoma
Drugs that work to target the immune system		
Rituximab	Rituxan®	Patients with certain types of CD20+ B-cell non-Hodgkin lymphoma
Rituximab in combination with other drugs	Rituxan®	Patients with CLL
Alemtuzumab	Campath®	Patients with B-cell CLL
Ofatumumab	Arzerra®	Patients with CLL that is unresponsive to other chemotherapy

Source: http://www.cancer.gov/cancertopics/factsheet/Therapy/targeted

Targeting the Human Epidermal Growth Factor Receptor 2 (HER2/ERBB2) Kinase

Trastuzumab (anti-HER2 mAb; Herceptin; Genentech), a humanized monoclonal antibody that binds the extracellular domain of HER2/ERBB2 (Carter et al. 1992), was the first kinase inhibitor approved for the treatment of cancer. It was approved in 1998 as first-line therapy in combination with paclitaxel chemotherapy for the treatment of metastatic breast cancers that overexpress HER2 (HER2+), and as a single agent following one or more chemotherapy regimens (Cobleigh et al. 1999; Slamon et al. 2001). The rationale that led to the clinical development of therapies targeting the HER2/ERBB2 kinase stemmed from initial observations that the *HER2* proto-oncogene is amplified and/or over-expressed in about one-third of breast cancers (King et al. 1985; Slamon et al. 1987, 1989). Furthermore, for breast cancer patients, *HER2* amplification was a significant independent predictor of time to relapse and overall survival (Slamon et al. 1987, 1989).

Preclinical studies of trastuzumab showed that it had antitumor activity in HER2+ breast cancer cell lines, and that it enhanced the antitumor activity of paclitaxel and doxorubicin in xenografts established from HER2+ breast tumors (Baselga et al. 1998). Consequently, trastuzumab was taken into clinical trials for HER2+ breast cancers. A phase I trial of trastuzumab in HER2+ metastatic breast cancer showed the antibody to be effective in the first-line setting with a response rate of 26% as a single-agent therapy (Vogel et al. 2002). In phase II trials, a subset of HER2+ metastatic breast cancer patients who had relapsed after receiving chemotherapy, were responsive to trastuzumab (Baselga et al. 1996; Cobleigh et al. 1999). It was later shown that trastuzumab combined with chemotherapy was superior to chemotherapy alone for the treatment of HER2+ metastatic breast cancer (Slamon et al. 2001; Marty et al. 2005). Trastuzumab was also found to be effective when administered as an adjuvant therapy for early-stage, or operable, breast cancer (reviewed in Baselga et al. 2006). Therefore, in 2006, the United States FDA approved trastuzumab as part of a treatment regimen containing doxorubicin, cyclophosphamide, and paclitaxel for the adjuvant treatment of women with node-positive, HER2+ breast cancer (Table 1).

An understanding of the precise mechanism(s) of anti-tumor activity of trastuzumab remains elusive, but several possible mechanisms have been suggested. For example, it has been shown that trastuzumab causes antibody-dependent cellular cytotoxicity (ADCC) through CD16 signaling in human breast cancer cell lines (Cooley et al. 1999). ADCC was also shown in patients with primary operable HER2+ breast cancer after single-agent treatment with trastuzumab (Gennari et al. 2004). In addition, trastuzumab has been shown to prevent HER2 shedding, which is the release

of the HER2 extracellular domain and formation of the truncated HER2 fragments known as p95HER2 that are believed to be oncogenic (Molina et al. 2001; Pederson et al. 2009). Other proposed mechanisms of anti-tumor activity include down-regulation of HER2 (Cuello et al. 2001), inhibition of angiogenesis (Izumi et al. 2002), cell cycle perturbation (Mayfield et al. 2001), and inhibition of downstream phosphatidylinositol 3-kinase (PI3K) signaling through phosphatase and tensin homolog (PTEN) activation (Nagata et al. 2004).

The clinical development of trastuzumab provided a new therapeutic option for women with HER2+ breast cancer. Nonetheless, the efficacy of this drug was limited by both primary and secondary (acquired) resistance. In fact, the reported response rates for trastuzumab are 15–26% for single agent therapy (Cobleigh et al. 1999; Vogel et al. 2002; Baselga et al. 2005) and 40–78% when combined with chemotherapy (Slamon et al. 2001; Jahanzeb et al. 2002; Seidman et al. 2008; Infante et al. 2009). Uncovering the molecular mechanisms that lead to resistance could help to avoid overtreatment, and also provide a foundation for the development of alternative targeted therapies to overcome trastuzumab resistance.

One mechanism that may contribute to primary trastuzumab resistance in human breast tumors is the presence of high levels of p95HER2 expression, which has been associated with poor clinical outcome as well as trastuzumab resistance (Scaltriti et al. 2007; Sperinde et al. 2010). Importantly, however, breast cancer cell lines and tumor xenografts expressing p95HER2 were sensitive to lapatinib (Tykerb; GlaxoSmithKline), a small molecule inhibitor of HER2/ERBB2 that binds within the intracellular domain of p95HER2 and full-length HER2 (Scaltriti et al. 2007). This finding raises the possibility that lapatinib might be effective in overcoming trastuzumab resistance that is driven by p95HER2. Lapatinib is currently approved in the United States for use as a combination therapy with capecitabine for the treatment of HER2+ metastatic breast cancer patients who have received prior therapy, including an anthracycline, a taxane, and trastuzumab (Table 1) (Ryan et al. 2008).

Other mechanisms implicated in primary resistance to trastuzumab, and to lapatinib, include activation of the PI3K pathway either by mutational activation of *PIK3CA* or inactivation of *PTEN* (Nagata et al. 2004; Berns et al. 2007; Eichhorn et al. 2008). Accordingly, in preclinical studies, a dual inhibitor of PI3K/mammalian target of rapamycin (mTOR), known as NVP-BEZ235, reversed the lapatinib resistance of cells with PI3K pathway alterations (Eichhorn et al. 2008). Other proposed mechanisms of trastuzumab resistance include p27 loss, or signaling through other growth factor receptors (Bedard et al. 2009). Recently, it was shown that cyclin E amplification/over-expression correlates with reduced clinical benefit and lower overall survival of HER2+ breast cancer patients that had been treated with trastuzumab (Scaltriti et al. 2011a). Additionally,

resistance to trastuzumab could be induced *in vitro* and *in vivo* by cyclin E over-expression, which suggests that cyclin-dependent kinase 2 (CDK2) inhibitors may be useful in treating patients with HER2+ tumors and cyclin E over-expression (Scaltriti et al. 2011a). Other potential therapeutic targets for trastuzumab-refractory HER2+ breast cancer include heat shock protein 90 (Hsp90) (Scaltriti et al. 2011b), nonreceptor tyrosine kinase c-SRC (SRC) (Zhang et al. 2011), histone deacetylases (HDACs) (Huang et al. 2011), and inhibition of glycolysis (Zhao et al. 2011).

Targeting the v-abl Abelson Murine Leukemia Viral Oncogene Homolog 1 (ABL) Kinase

Imatinib mesylate (STI-571, Gleevec; Novartis) was the first small molecule kinase inhibitor to receive the United States FDA approval for use in oncology. It was approved in 2001 for the treatment of Philadelphia-chromosome-positive (Ph+) chronic myelogenous leukemia (CML), which is driven by a specific translocation between chromosomes 9 and 22 (Nowell and Hungerford 1960; Rowley 1973; Prakash and Yunis 1984). The (9;22)(q34;q11) translocation results in the production of the Breakpoint Cluster Region (BCR)-ABL fusion protein, which encodes a constitutively active form of the ABL tyrosine kinase (de Klein et al. 1982; Groffen et al. 1984; Konopka et al. 1985; Ben-Neriah et al. 1986). Early *in vivo* experiments demonstrated a causal relationship between the BCR-ABL fusion protein and the development of leukemia (Daley et al. 1990; Elefanty et al. 1990; Heisterkamp et al. 1990; Kelliher et al. 1990; Lugo et al. 1990).

Imatinib is a small molecule that was rationally designed to target the ABL kinase. In addition to its activity against ABL, imatinib has activity against the platelet-derived growth factor receptor (PDGFR) and v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (Kit) kinases (Buchdunger et al. 1995, 1996; reviewed in Druker and Lydon 2000). Imatinib preferentially targets the ATP-binding site of inactive BCR-ABL (Nagar et al. 2002; Schindler et al. 2000), thus stabilizing an inactive form of the kinase. Binding of imatinib to ABL results in inhibition of downstream signaling through the PI3K and mitogen-activated protein kinase (MAPK) survival pathways (Kawauchi et al. 2003; Steelman et al. 2004). Imatinib treatment of CML cells has also been shown to induce autophagy, although it is unclear whether this represents a cell survival mechanism or type II cell death (Bellodi et al. 2009; Can et al. 2011; Ertmer et al. 2007). In early preclinical studies, imatinib inhibited the proliferation and tumor-forming capabilities of BCR-ABL positive cells, and inhibited colony formation of committed progenitor cells within blood or bone marrow samples taken from Ph+ CML patients (Druker et al. 1996).

The anti-tumorigenic observations in preclinical studies led to the expectation that imatinib might be clinically effective in the treatment of Ph+ CML. These expectations held true. The ensuing clinical trials of imatinib showed that it had substantial clinical activity against Ph+ CML and Ph+ acute lymphoblastic leukemia (ALL) (Druker et al. 2001a,b). In the United States, imatinib is now the standard treatment for CML in any of the three diagnostic phases: chronic phase, accelerated phase, and blast crisis (the final phase of evolution of the disease) (Cohen et al. 2002, 2005; Dagher et al. 2002). Imatinib is also effective in the treatment of chemotherapy-naive, pediatric CML in early chronic phase (Druker et al. 2001a). In addition, imatinib has been approved as a first line therapy for unresectable or metastatic Kit-mutated gastrointestinal stromal tumors (GISTs) (Cohen et al. 2009), and as an adjuvant therapy for the treatment of Kit-mutated GISTs (Table 1) (Cohen et al. 2010). The clinical development of imatinib caused a massive paradigm shift in oncology, towards personalized medicine targeting specific driver genes in tumor cells, and revolutionized the treatment options for patients with imatinib-sensitive tumors.

Targeting Imatinib-Resistant CML

Despite the clinical success of imatinib in treating Ph+ CML, a significant number of imatinib-responsive CML patients subsequently developed drug resistance (reviewed in Corbin et al. 2011). Unraveling the underlying mechanism of imatinib resistance was facilitated by the ease with which leukemia cells can be sampled for molecular characterization. By modeling imatinib resistance *in vitro*, and by resequencing the ABL gene from imatinib-resistant CML cells, it was discovered that resistance can be caused by the appearance of secondary mutations in the kinase domain of the BCR-ABL fusion protein that result in altered imatinib binding (Barthe et al. 2001; Gorre et al. 2001; Branford et al. 2002; Shah et al. 2002; Azam et al. 2003). Other mechanisms of imatinib resistance included BCR-ABL amplification and overexpression (Gorre et al. 2001), defective drug transport/drug sequestration (Gambacorti-Passerini et al. 2003), activation of downstream effector proteins including the v-yes-1 Yamaguchi sarcoma viral related oncogene homolog (LYN) kinase (Donato et al. 2003), and insensitivity of human CML stem cells to imatinib (Corbin et al. 2011).

Once the mechanisms of imatinib resistance were understood, it was possible to move forward with rational approaches to try to overcome resistance. In the most straightforward approach, for cases of CML in which resistance was driven by genomic amplification of BCR-ABL, simply increasing the dose of imatinib was effective in restoring drug sensitivity (Kantarjian et al. 2003; Jabbour et al. 2009). However, alternative approaches,

in the form of new inhibitors, were needed to overcome resistance caused by the acquisition of secondary mutations within the kinase domain of BCR-ABL. Two drugs, dasatinib and nilotinib, proved to be effective in overcoming clinical resistance to imatinib in molecularly defined subsets of CML. Nilotinib binds the same ATP-binding site of the inactive BCR-ABL protein, but with a better topological fit than imatinib (Weisberg et al. 2005). Dasatinib binds the active conformation of BCR-ABL (Tokarski et al. 2006; Vajpai et al. 2008).

In 2007, nilotinib received accelerated United States FDA approval for the treatment of adults with Ph+ CML in chronic phase or accelerated phase that displayed resistance or intolerance to imatinib (Table 1). Dasatinib was granted full approval in 2006 for the treatment of adults with all diagnostic phases of Ph+ CML, and adults with Ph+ ALL, who displayed resistance or intolerance towards prior imatinib therapy (Brave et al. 2008). Notably, in 2010, both nilotinib and dasatinib received accelerated approval for the treatment of newly diagnosed cases of adult Ph+ CML in chronic phase as a result of phase III clinical trials, which showed that dasatinib and nilotinib were superior to imatinib for the initial treatment of these cancers (Kantarjian et al. 2010; Saglio et al. 2010). However, it should be pointed out that neither nilotinib nor dasatinib is capable of inhibiting the activity of the BCR-ABL^{T315I} mutant, a so-called gatekeeper mutation that is often associated with imatinib resistance. Acquired resistance to nilotinib and dasatinib, which may arise from the emergence of the BCR-ABL^{T315I} mutant, is problematic (von Bubnoff et al. 2006; Soverini et al. 2007; Baranska et al. 2008; Mahon et al. 2008). Thus, novel therapeutic agents that can inhibit the BCR-ABL^{T315I} mutant may be effective alone or in combination with nilotinib, dasatinib, or imatinib (O'Hare et al. 2008; Eide et al. 2011). Some of these novel drugs are currently being evaluated in clinical trials (clinicaltrials.gov identifiers NCT00827138, NCT01207440, NCT00660920).

Other second-generation inhibitors and therapeutic strategies are being explored for the treatment of imatinib-resistant CML (reviewed in Fullmer et al. 2011). These efforts include the exploration of additional small molecule inhibitors targeting the ABL kinase, as well as various combination treatments. Although results from previous clinical studies of combination treatment with imatinib were discouraging (reviewed in Deininger et al. 2005), it has recently been demonstrated that exposure of imatinib-resistant CML cells to antitelomerase treatments in combination with imatinib caused either cell death or antiproliferative effects (Deville et al. 2011). Deville et al. therefore suggested that combining antitelomerase agents with imatinib at the onset of treatment may be a better therapeutic regimen for some patients with CML. In addition, a recent large-scale RNAi screen performed in CML cells identified the Wnt/Ca^{2+}/nuclear factor of activated T-cells (NFAT) pathway as being synthetic lethal with imatinib

treatment (Gregory et al. 2010); inhibition of components of this pathway increased the imatinib sensitivity of CML cells. These findings raise the possibility that combining imatinib treatment with NFAT inhibition might improve the clinical efficacy of imatinib (Gregory et al. 2010).

Targeting the Epidermal Growth Factor Receptor (EGFR) Kinase

Unlike hematological malignancies, most solid tumors are not defined by a signature genetic abnormality. However, early observations that (i) EGFR is over expressed in a significant fraction of many epithelial malignancies (reviewed in Salomon et al. 1995), (ii) EGFR overexpression transformed NIH3T3 cells (Di Fiore et al. 1987b) and correlated with poor prognosis in certain cancers (Nicholson et al. 2001), and (iii) a monoclonal antibody directed against EGFR resulted in decreased proliferation of cultured tumor cells and induced xenograft cell death in athymic mice (Masui et al. 1984), lead to the expectation that therapeutically targeting EGFR might lead to clinical responses in a large number of cancer patients with a variety of malignancies. Gefitinib (Iressa; ZD1839, AstraZeneca) (Wakeling et al. 2002) and erlotinib (CP-358774, OSI-774, Tarceva, Genentech) (Moyer et al. 1997), two reversible, ATP-competitive small molecule inhibitors of EGFR, showed encouraging preclinical activity (Moyer et al. 1997; Pollack et al. 1999; Barker et al. 2001; Ciardiello et al. 2001; Wakeling et al. 2002; Grunwald and Hidalgo 2003). Early studies of gefitinib and erlotinib activity *in vitro* and in mouse xenograft models showed that gefitinib decreased vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and transforming growth factor alpha (TGFα) production (Ciardell et al. 2001), while erlotinib inhibited EGFR autophosphorylation, and induced G1 arrest by inhibiting phosphorylation of pRb (Moyer et al. 1997). In addition, both drugs were shown to induce apoptosis (Moyer et al. 1997; Ciardello et al. 2000).

Both gefitinib and erlotinib were advanced into clinical trials. Phase I trials of gefitinib in patients with solid tumors showed that the drug was well-tolerated and led to objective radiographic responses in some non-small cell lung cancer (NSCLC) patients (Herbst et al. 2002; Ranson et al. 2002; Nakagawa et al. 2003). However, two subsequent phase II trials of gefitinib monotherapy for the treatment of chemorefractory NSCLC were, overall, disappointing; only 10–18% of patients had objective clinical responses (Fukuoka et al. 2003; Kris et al. 2003). Nonetheless, a subset of patients had dramatic clinical responses, often times referred to as "Lazarus responses". Consequently, the United States FDA approved gefitinib as a third-line therapy for NSCLC in 2003, under the expanded access program. Interestingly, gefitinib-responsiveness was statistically higher among

women than men, among East-Asians than other ethnic groups, among non-smokers than smokers, and among bronchioloalveolar adenocarcinomas than among other histotypes of NSCLC.

In 2004, two landmark studies demonstrated that somatic *EGFR* mutations in NSCLC accounted for the clinical responsiveness of NSCLC patients to gefitinib (Lynch et al. 2004; Paez et al. 2004). Lynch et al. showed that activating *EGFR* mutations that clustered near the ATP binding site of the encoded protein were present in the tumors of eight out of nine responders, but were not present in tumors of non-responders ($P<0.001$) (Lynch et al. 2004). In an independent study, Paez et al. searched for somatic mutations among a number of tyrosine kinases in NSCLCs and showed that a subset of NSCLCs had somatic *EGFR* mutations and that some of the same demographic characteristics that correlated with clinical responses to gefitinib also correlated with the presence of tumor-associated *EGFR* mutations (Paez et al. 2004). Together, these two studies provided a molecular explanation for the basis of clinical responsiveness of a subset of NSCLC patients to gefitinib. Mechanistically, the *EGFR* mutations associated with TKI sensitivity lead to hyperactivation of the receptor; alter downstream signal transduction mediated by AKT, signal transducer and activator of transcription 3/5 (STAT3/5) and extracellular signal-regulated kinase (ERK1/2); and generate pro-survival and anti-apoptotic signals upon which the tumor cells are dependent (Ono et al. 2004; Sordella et al. 2004; Han et al. 2005; Mukohara et al. 2005). EGFR inhibitors inhibit the altered signaling on which *EGFR*-mutant cells are dependent, thus leading to selective cell death (Sordella et al. 2004).

In 2005, a phase III trial, known as ISEL (Iressa Survival Evaluation in Lung cancer), reported that there was no significant increase in survival of patients treated with gefitinib versus placebo for second-line therapy in chemorefractory NSCLC patients (Thatcher et al. 2005). Consequently, the United States FDA restricted the use of gefitinib to patients participating in a clinical trial or to patients who had previously been administered the drug and continued to benefit from treatment. Importantly, however, when only the subset of East Asian patients within the ISEL trial was considered, gefitinib treatment was associated with a survival benefit (Thatcher et al. 2005). Molecular analysis of tumor material collected within the ISEL trial showed that although *EGFR* mutation was predictive of clinical response to gefitinib, increased *EGFR* copy number was predictive of a survival benefit accompanying gefitinib (Hirsch et al. 2006).

A recent meta-analysis of seven clinical trials that employed gefitinib as monotherapy in the treatment of Japanese cases of *EGFR*-mutant NSCLC revealed an overall response rate of 76.4% (95% confidence interval (95% CI), 69.5–83.2), a median progression-free survival interval of 9.7 months (95% CI, 8.2–11.1), a median overall survival of 24.3 months (95% CI, 19.8–28.2),

and a 1-year overall survival rate of 76.7% (95% CI, 69.8–83.6) (Morita et al. 2009). Of the 148 *EGFR* mutation-positive NSCLC patients included in the meta-analysis, 87 received gefitinib as first-line therapy, whereas 61 received systemic chemotherapy as first-line treatment, followed by gefitinib. The response rate was significantly higher for the first-line gefitinib group than for the first-line chemotherapy group (79.3% versus 24.6%; $P< 0.001$). Likewise, progression-free survival was significantly longer in the first-line gefitinib group than in the first-line chemotherapy group (median of 10.7 versus 6.0 months; $P< 0.001$). In contrast, there was no significant difference in overall survival between the two groups of patients (median of 27.7 versus 25.7 months, respectively). Three randomized, prospective phase III trials have confirmed that gefitinib is superior to chemotherapy as a first-line treatment for *EGFR*-mutant NSCLC, resulting in significantly longer progression-free survival (Mok et al. 2009; Maemondo et al. 2010; Mitsudomi et al. 2010).

The clinical history of erlotinib (CP-358774, OSI-774, Tarceva) is similar to that of gefitinib. Following on from a phase I trial of erlotinib (Hidalgo et al. 2001), a phase II trial was initiated in NSCLC patients who had received prior treatment with platinum-based chemotherapy (Perez-Soler et al. 2004). The objective response rate to erlotinib was 12.3% (95% CI, 5.1% to 23.7%). It was subsequently shown that *EGFR* mutations also correlated with the clinical responses of NSCLC patients to erlotinib (Pao et al. 2004). In contrast to the analogous ISEL trial of gefitinib, a phase III trial of erlotinib, known as BR.21, demonstrated a survival advantage with a median overall survival of 6.7 months in the erlotinib group versus 4.7 months in the placebo group ($P<001$) (Shepherd et al. 2005). Based on those results, the United States FDA approved erlotinib for second- and third-line treatment of NSCLC in 2004. Erlotinib has also received FDA approval as a combination therapy for the treatment of pancreatic cancer based on the results of a phase III trial of erlotinib in combination with gemcitabine in patients with unresectable, locally advanced, or metastatic pancreatic cancer (Table 1). The erlotinib/gemcitabine arm of the trial showed prolonged overall survival and prolonged time to progression compared to patients who received placebo/gemcitabine (Moore et al. 2007).

It is currently estimated that around 70% of *EGFR*-mutant NSCLC patients exhibit tumor regression after treatment with EGFR inhibitors (reviewed in Workman and Clarke 2011). The clinical experience with EGFR inhibitors highlights two important points relating to personalized medicine for solid tumors. First, it is critical to define the molecular alteration that predicts response (or lack of response) to a targeted therapy; and second, the most meaningful clinical trials of targeted therapies are those that are enriched for patients most likely to respond.

Although the development of EGFR inhibitors provided new therapeutic options for a subset of NSCLC patients, an unfortunate reality is that up to 30% of *EGFR*-mutant NSCLC patients do not respond to EGFR inhibitors and the vast majority of responders eventually relapse (reviewed in Workman and Clarke 2011). Primary resistance of NSCLC to gefitinib and erlotinib in the presence of a sensitizing *EGFR* mutation has been associated with the presence of a concomitant *KRAS* mutation or the $EGFR^{T790M}$ gatekeeper mutation (Shih et al. 2005). The molecular mechanisms of acquired EGFR inhibitor resistance have been uncovered for some NSCLC patients, but still remain unexplained in others. Acquired clinical resistance to gefitinib and erlotinib in NSCLC patients has been associated with the appearance of secondary mutations in *EGFR*, *EGFR* amplification, amplification of *MET*, mutational activation of *PIK3CA*,which encodes the catalytic subunit of PI3K, and/or *CTNNB1* mutation (Kobayashi et al. 2005; Pao et al. 2005; Sequist et al. 2011). Although most secondary *EGFR* mutations are accounted for by the $EGFR^{T790M}$ gatekeeper mutation (Sequist et al. 2011), other rare mutations in *EGFR* that confer resistance have also been described (Balak et al. 2006; Costa et al. 2007, 2008; Bean et al. 2008). In some cases the acquisition of the $EGFR^{T790M}$ mutation is accompanied by amplification of the $EGFR^{T790M}$-allele (Sequist et al. 2011).

Acquired resistance to EGFR inhibitors can be transient. Serial biopsies from a small number of patients showed that after the administration of the inhibitor was stopped, the genetic mechanisms of resistance were lost and subsequent sensitivity to EGFR inhibitors was restored (Sequist et al. 2011). Some resistant tumors, however, undergo an epithelial-to-mesenchymal transition, and others actually convert from NSCLC to SCLC (Sequist et al. 2011); patients displaying the latter phenotypic change had concomitant sensitivity to treatments typically used for SCLC (Sequist et al. 2011).

Although we will not discuss this topic in detail, TKIs targeting EGFR have exhibited some success in tumors other than NSCLC. In 2006, cetuximab (Erbitux), a chimeric monoclonal antibody that targets EGFR, received United States FDA approval for the treatment of recurrent or metastatic squamous cell carcinomas of the head and neck in patients previously treated with chemotherapy, and for use in combination with radiation therapy for the treatment of locally or regionally advanced squamous cell carcinoma of the head and neck (Table 1) (http://www.cancer.gov/cancertopics/druginfo/fda-cetuximab#Anchor-Hea-5647). On November 7, 2011, cetuximab was granted further FDA approval for use in combination with platinum-based therapy plus 5-fluorouracil as first-line treatment of patients with recurrent or metastatic squamous cell carcinomas of the head and neck (Table 1) (http://www.cancer.gov/cancertopics/druginfo/fda-cetuximab#Anchor-Hea-5647). Cetuximab has also shown clinical activity in patients with advanced or metastatic colorectal cancer

either alone or in combination with chemotherapy (Cunningham et al. 2004; Cartwright et al. 2008) and was approved by the United States FDA in 2004 in combination with irinotecan for treatment of patients with EGFR-expressing colorectal carcinoma that is refractory to irinotecan, or as single-agent treatment of colorectal cancer in patients who do not respond to irinotecan (Table 1) (http://www.cancer.gov/cancertopics/druginfo/fda-cetuximab#Anchor-Hea-5647). In colorectal cancer, the presence of a *KRAS* mutation correlates with lack of clinical benefit from cetuximab (Karapetis et al. 2008; Van Cutsem et al. 2009). In contrast, high levels of expression of the EGFR ligands epiregulin and amphiregulin in metastatic colorectal tumors correlates with a significantly increased likelihood of disease control by cetuximab (Khambata-Ford et al. 2007; Jacobs et al. 2009; Baker et al. 2011). Panitumumab (Vectibix, Amgen, Inc.), a human monoclonal antibody against EGFR, received United States FDA approval in 2006 for the treatment of "patients with EGFR-expressing, metastatic colorectal carcinoma with disease progression on or following fluoropyrimidine-, oxaliplatin-, and irinotecan-containing chemotherapy regimens" (Table 1) (Giusti et al. 2007). Like cetumaxib, colorectal cancer patients with *KRAS*-mutant tumors derive no clinical benefit from treatment with panitumumab (Amado et al. 2008).

Targeting the Anaplastic Lymphoma Kinase (ALK)

Within the past five years ALK has emerged as a new target for cancer therapy. Somatic mutations of *ALK*, primarily structural rearrangements that lead to the expression of a constitutively active ALK kinase in the form of oncogenic ALK fusion proteins, have been found in NSCLC (Soda et al. 2007; Choi et al. 2008), anaplastic large cell lymphomas and other large B-cell lymphomas (Rimokh et al. 1989; Arber et al. 1996), esophageal squamous cell carcinoma (Du et al. 2007), inflammatory myofibroblastic tumors (Griffin et al. 1999), breast cancer, and colorectal cancer (Lin et al. 2009). ALK fusion proteins have been shown to signal through MAPK (Turner et al. 2003), AKT, ERK (Li et al. 2011b), STAT3 (Zamo et al. 2002; Zhang et al. 2002; Li et al. 2011b), and/or STAT5 (Nieborowska-Skorska et al. 2001). Crizotinib (PF-02341066, Xalkori; Pfizer), an ATP-competitive inhibitor of the ALK and MET kinases (Rodig et al. 2010), was shown to effectively inhibit phosphorylation of ALK, AKT, ERK1/2, and STAT3 in cancer cell lines expressing ALK fusion proteins (McDermott et al. 2008).

Fueled by the discovery of transforming Echinoderm Microtubule-associated protein-Like 4 (*EML4*)-*ALK* rearrangements in NSCLC (Soda et al. 2007, 2008), crizotinib was taken into clinical trials for this malignancy (Kwak et al. 2010). Dramatically, just three years after the original description of *ALK* translocations in NSCLC, a phase II, genotype-directed clinical trial

was completed and showed that a substantial fraction of patients with ALK-positive tumors derived clinical benefit from crizotinib (Kwak et al. 2010). Of the 82 patients with *ALK*-rearranged advanced NSCLC within the trial, 57% of patients had measurable responses and an additional 33% of patients had stable disease (Kwak et al. 2010). In an anecdotal case report, a patient with NSCLC that lacked an *ALK* rearrangement, but had somatic amplification of the MET oncogene, the original target of crizotinib (Zou et al. 2007), exhibited a clinical response to this drug (Ou et al. 2010). Crizotinib received accelerated United States FDA approval for the treatment of locally advanced or metastatic *ALK*-rearranged NSCLC on August 26, 2011 (Table 1) (http://www.cancer.gov/cancertopics/druginfo/fda-crizotinib).

Insights into possible mechanisms of crizotinib resistance have come from both *in vitro* and *in vivo* studies. *In vitro*, the establishment and characterization of drug resistant NSCLC cell line(s) demonstrated two distinct molecular mechanisms of resistance: the appearance of a secondary mutation (ALK^{L1196M}) at the gatekeeper residue of ALK kinase domain and genomic amplification of *EML4-ALK* (Katayama et al. 2011). *In vivo,* acquired clinical resistance to crizotinib in a case of EML4-ALK-positive NSCLC was associated with the appearance of two secondary mutations, ALK^{C1156Y} and the ALK^{L1196M}gatekeeper mutation, within the rearranged form of ALK, in independent subclones of the resistant tumor (Choi et al. 2010). It has been speculated that the ALK^{C1156Y} mutation might interfere allosterically with crizotinib binding (Choi et al. 2010). Uncovering the molecular mechanisms of clinical crizotinib resistance is important because a number of additional ALK inhibitors have now been developed (Katayama et al. 2011; Lovly et al. 2011; Okamoto et al. 2011; Sakamoto et al. 2011), and several of these inhibitors (CH5424802, NVP-TAE684, AP26113, X-376, and X-396) are capable of inhibiting the activity of the ALK^{C1156Y} and ALK^{L1196M} mutants *in vitro* (Katayama et al 2011; Sakamoto et al. 2011; Lovly et al 2011). Whether these new ALK inhibitors will be clinically effective in overcoming crizotinib resistance remains to be determined.

Targeting the V-RAF Murine Sarcoma Viral Oncogene Homolog B1 (BRAF) Kinase

The historical development of BRAF inhibitors for the treatment of malignant melanoma highlights some of the challenges that may be encountered in the process of translating molecular findings into clinical applications. The *RAF* gene family encodes three cytoplasmic serine/threonine kinases known as ARAF, BRAF and CRAF. In 2002, Davies et al. discovered that one of these genes, *BRAF*, was somatically mutated in two-thirds of malignant melanomas (Davies et al. 2002). Importantly, a single mutant, $BRAF^{V600E}$,

accounted for 80% of all the *BRAF* mutations in this disease and caused constitutive activation of the BRAF kinase (Davies et al. 2002). Subsequent studies confirmed the role of mutant BRAF in driving malignant melanomas via activation of the RAF-MAP kinase kinase (MEK)/ERK signaling pathway, leading to the expectation that BRAF inhibitors would be clinically effective in treating this deadly disease (Karasarides et al. 2004; Sharma et al. 2005). However, clinical trials of sorafinib, a multikinase inhibitor, in patients with malignant melanoma were disappointing, because little, if any, clinical activity was observed (Eisen et al. 2006; McDermott et al. 2008; Hauschild et al. 2009). This lack of clinical efficacy was subsequently attributed to the lack of specificity of sorafinib for BRAF (reviewed in Shepherd et al. 2010).

In the past four years, the clinical activity of BRAF-specific inhibitors in the treatment of melanoma has generated a tremendous amount of excitement. In 2008, Tsai and colleagues reported the development of PLX4720, an inhibitor that was not only selective for BRAF, but specifically targeted the $BRAF^{V600E}$ mutant (Tsai et al. 2008). Preclinical studies of PLX4720 in xenograft models resulted in significant delays in tumor growth, as well as tumor regression (Tsai et al. 2008). An analogue of PLX4720 known as vemurafenib (PLX4032; Plexxikon) caused significant reductions in cell proliferation, inhibition of MEK and ERK phosphorylation, and an increase in apoptosis in melanoma cell lines expressing $BRAF^{V600E}$ (Sala et al. 2008; Yang et al. 2010). In a phase I trial of vemurafenib, complete or partial tumor regression was observed in 81% of metastatic melanoma patients with a somatic $BRAF^{V600}$ mutation (Flaherty et al. 2010). Although clinical resistance to vemurafenib developed within two to 18 months (Ribas and Flaherty 2011), the responses to this drug were unprecedented in melanoma therapy. Consequently, vemurafenib was taken into a phase II trial for previously treated $BRAF^{V600E}$-mutant metastatic melanoma, and a response rate of 52% has been observed (Ribas et al. 2011).

Very recently the results were reported of a phase III trial that compared vemurafenib to dacarbazine among patients with metastatic melanoma and no previous treatment (Chapman et al. 2011b). At the time of this trial, dacarbazine was the sole chemotherapeutic agent approved by the United States FDA for the treatment of metastatic melanoma. In a dramatic turn of events, the trial was stopped early, because the significantly greater efficacy of vemurafenib compared with dacarbazine raised ethical issues about denying vemurafenib to the patients in the control group. The interim analysis of the trial reported a relative reduction of 63% in the risk of death ($P<0.001$) and 74% in the risk of either death or disease progression ($P<0.001$) associated with vemurafenib as compared with dacarbazine (Chapman et al. 2011b). Almost half (48%) of patients treated with vemurafenib had a confirmed objective response, compared to just 5% treated with dacarbazine

($P<0.001$). In August 2011, the United States FDA approved vemurafenib (Zelboraf, Genentech) for the treatment of patients with metastatic or unresectable melanoma expressing $BRAF^{V600E}$ (Table 1) (http://www.cancer.gov/cancertopics/druginfo/fda-vemurafenib).

Although treating *BRAF* mutant melanoma patients with $BRAF^{V600E}$ inhibitors resulted in initial responses in the majority of patients, 19% of patients showed intrinsic resistance, and most patients who initially responded eventually relapsed (Garber 2009; Flaherty et al. 2010; Smalley and Sondak 2010; Ribas and Flaherty 2011). Interestingly, secondary mutations at the so-called "gatekeeper" residue of BRAF have not been observed as a mechanism of resistance (Flaherty et al. 2010; Nazarian et al. 2010). A recent study published by Paraiso et al. focused on the question of why some patients are initially resistant to BRAF inhibitors (Paraiso et al. 2011). They found that loss of PTEN expression, leading to the suppression of apoptosis, contributed to PLX4720 resistance in $BRAF^{V600E}$-mutant melanoma cell lines. Additionally, they found loss of PTEN in 10–27% of melanomas they examined. This is one of four non-mutually exclusive mechanisms believed to underlie resistance to vemurafenib, some of which were identified by comparing pre-treatment and post-relapse biopsies from patients (reviewed in Aplin et al. 2011). These mechanisms include reactivation of MEK signaling, amplification of cyclin D1, or other alterations in the ERK1/2-regulated cell cycle events, as well as changes in chromatin remodeling that allow cells to enter a "drug-tolerant" state until secondary mutations can enable permanent resistance (Sharma et al. 2010).

With the recent revolution in DNA sequencing technologies, the genomes of many human cancers, both common and rare, are now being decoded at a rapid pace. This effort has led to a number of exciting advances in the field of cancer genomics. Among these advances is the very recent discovery that 100% of hairy cell leukemias (HCLs) harbor the somatic $BRAF^{V600E}$ mutation (Tiacci et al. 2011). *In vitro*, HCL cells cultured with PLX4720 demonstrated decreased phosphorylation of MEK and ERK, downstream targets of BRAF. Many HCLs can be treated effectively with interferon or purine analogues (Golomb 2011). Nonetheless, as Tiacci et al. pointed out, the identification of *BRAF* mutations in HCL might ultimately have clinical utility for the treatment of patients who do not respond to standard treatments, have undergone relapse, or display unacceptable toxicity to standard treatments (Tiacci et al. 2011).

Another approach to leverage mutant BRAF for personalized treatment involves targeting downstream effector proteins that are inappropriately activated by mutant BRAF signaling. The MEK1/2 kinases are mutant BRAF substrates and therefore represent potential therapeutic targets. Accordingly, enhanced and selective sensitivity to MEK inhibitors is observed in *BRAF*-mutant cancer cell lines (Solit et al. 2006). Although these data depict an

example of rationally targeting a pathway that is oncogenically activated, it is important to note that the response rates of patients with *BRAF*-mutant melanoma to MEK inhibitors was lower than the response rates to RAF inhibitors, and MEK inhibitors also adversely affect normal tissue (Ribas and Flaherty 2011). *In vitro* models of drug resistance have revealed that resistance of *BRAF*-mutant melanoma cells to MEK inhibitors is associated with acquired mutations within *MEK1* (Emery et al. 2009). A study utilizing human colorectal cancer cells further supported these results by showing that MEK-inhibitor resistant cells harbor a *MEK1* mutation (Wang et al. 2011a).

Targeting MAP Kinase Kinase (MEK)

The MEK/ERK pathway is a critical signal-transduction cascade that is activated by many growth factor receptors. The pathway mediates a number of cellular processes, including proliferation, differentiation, apoptosis, transformation and survival, and is frequently altered in human cancer (reviewed in Montagut and Settleman 2009; Trujillo 2011).

Currently, a number of novel MEK inhibitors are being tested in clinical trials, either alone or in combination with other agents (clinicaltrials.gov). Because preclinical data indicated that enhanced sensitivity to MEK inhibitors is conferred by mutations in *BRAF, RAS,* or *RAF* (Davies et al. 2002; Solit et al. 2006; Garon et al. 2010), some trials evaluating MEK inhibitors are recruiting patients with mutant *KRAS* (clinicaltrials.gov identifiers NCT01229150, NCT01085331, NCT01362296) or mutant *BRAF* (clinicaltrials.gov identifiers NCT01037127, NCT01166126, NCT00888134) and a number of other trials aim to retrospectively assess molecular correlates of response (clinicaltrials.gov).

Selumetinib (ARRY-142886, AZD6244; AstraZeneca) is one of several MEK1/2 inhibitors in clinical development. A phase II study of selumetinib in patients with advanced hepatocellular carcinoma indicated minimal single-agent activity; no radiographic responses were seen among 17 evaluable patients although it was noted that *RAS* and *RAF* mutations are relatively uncommon in this cancer type (O'Neil et al. 2011). In contrast, a phase II study of selumetinib in patients with metastatic biliary cancers has shown promising results; 12% of patients had an objective response and 68% experienced stable disease (Bekaii-Saab et al. 2011). Although there was no molecular correlate of response, the presence of low levels of phospho-ERK was associated with lack of response within this trial.In a phase II clinical trial that stratifies patients with advanced lung and thymic malignancies for targeted therapy, the presence of *KRAS*, *BRAF*, *HRAS*, or *NRAF* gene mutations is being used to stratify patients for the evaluation of selumetinib

(clinicaltrials.gov identifier NCT01306045). In addition, other phase II trials are determining the efficacy of (i) selumetinib in combination with an mTOR inhibitor in *BRAF* mutant, treatment naive unresectable stage IV melanoma (clinicaltrials.gov identifier NCT01166126), (ii) single agent selumetinib in cancers other than melanoma with *BRAF* mutations (clinicaltrials.gov identifier NCT00888134), and (iii) selumetinib in combination with erlotinib in NSCLC patients with mutant versus wildtype *KRAS* (clinicaltrials.gov identifier NCT01229150).

Targeting the Phosphoinositide 3-Kinase (PI3K) Pathway

The PI3K signal transduction pathway promotes cell survival and growth and is speculated to be one of the crucial core pathways for cancer development (Wong et al. 2010). The PI3K pathway is inappropriately activated in a large number of cancers, principally through somatic mutations or copy number alterations in the *PIK3CA*, *PIK3R1*, *AKT*, and *PTEN* genes or by activation of upstream receptor tyrosine kinases (RTKs) (Engelman 2009; Rudd et al. 2011; Urick et al. 2011). Preclinical data has indicated that PI3K pathway inhibitors may be effective as single agents in cancers that exhibit *HER2*-amplification, *PIK3CA* mutation, or phospho-AKT (She et al. 2008; Dan et al. 2010; O'Brien et al. 2010; Weigelt et al. 2011), whereas RTK or RAS/RAF oncogene addicted cancers may be sensitive to concurrent PI3K and MEK inhibition (Sos et al. 2009a). In fact, *KRAS/BRAF* mutations were shown to correlate with inefficacy of PI3K inhibitors (Dan et al. 2010). Furthermore, activation of the PI3K pathway is believed to be a mechanism of acquired resistance to inhibitors targeting EGFR (Engelman et al. 2007), as well as resistance to chemotherapy (Jin et al. 2003; Lee et al. 2004; Nagata et al. 2004) and to ionizing radiation (Gupta et al. 2001).

Little doubt remains that the PI3K pathway is an important therapeutic target for many human cancers. Consequently, numerous inhibitors of the PI3K pathway are in clinical development, including dual PI3K/mTOR inhibitors, PI3K inhibitors, AKT inhibitors, and mTOR inhibitors (reviewed in Engelman 2009). The clinical development of PI3K pathway inhibitors is complex and has been reviewed in detail elsewhere (Engelman 2009). As noted by Engelman, the general approaches that are being applied to evaluate the efficacy of such pharmacological inhibitors are either to test them against a large number of cancers to identify those in which there is the most anti-tumor activity, or to perform genotype-directed trials based on preclinical findings that point to molecular correlates of activity (Engelman 2009). Presently, the clinical impact of PI3K pathway inhibitors remains to be completely elucidated.

Interestingly, and related to the emphasis of this book, several lines of evidence suggest a clinically relevant link between the PI3K pathway

and DNA repair. For example, studies have indicated that PI3K inhibitors may be used as sensitizers to DNA-damaging agents, such as ionizing radiation or chemotherapy. In glioblastoma cell lines and stem cells, the mechanism of chemosensitization caused by the PI3K inhibitor PI-103 was attributed to the cross-inhibition of the DNA-dependent protein kinase, DNA-PK (Westhoff et al. 2009), a protein complex involved in resolving DNA double-strand breaks (see Chapter 14). Several other PI3K inhibitors, such as LY294002 (Rosenzweig et al. 1997), wortmannin (Hashimoto et al. 2003), ZSTK474 (Kong et al. 2009), NVP-BEZ235 (Kong et al. 2009), and GSK2126458 (Knight et al. 2010) have also been shown to inhibit DNA-PK. Some of these inhibitors exhibit chemo- and/or radio-sensitizing effects (Rosenzweig et al. 1997; Hashimoto et al. 2003; Konstantinidou et al. 2009; Dubrovska et al. 2010; Anzai et al. 2011). The cross-inhibition of DNA-PK by PI3K inhibitors is believed to be due to the structural similarity between members of the PI3K family and DNA-PK (Hartley et al. 1995).

The inhibition of DNA repair following treatment with PI3K inhibitors might occur not only through off-target effects, but also because the PI3K pathway may directly regulate DNA repair responses. For example, it has been shown that AKT1 regulates the activation of DNA-PK in irradiated cells (Toulany et al. 2008). Specifically, treatment of cells with an AKT pathway inhibitor, or *AKT1*-siRNA, inhibited the phosphorylation of DNA-PK and DNA repair as measured by γH2AX foci following radiation treatment. Furthermore, it has recently been shown that RNAi knockdown of the catalytic subunit of PI3K resulted in reduced DNA repair as measured by γH2AX foci quantitation and doxorubicin-induced apoptosis (Westhoff et al. 2009). Taken together, these data indicate that PI3K inhibitors may be of clinical utility as sensitizers to DNA-damaging agents, in addition to their potential efficacy as single agent cancer therapies.

UNDRUGGABLE TARGETS IN CANCER THERAPY

As we have previously described in this Chapter, the earliest examples of personalized medicine have directly targeted "druggable" proteins, i.e., the products of oncogenes, to shut off their activity in cancer cells. In contrast, tumor suppressor genes sustain loss-of-function mutations or downregulation in cancer cells. Consequently, their altered protein products cannot be targeted pharmacologically. In the past few years, however, a new paradigm has emerged in cancer therapy. This new approach utilizes so-called synthetic lethal interactions to *indirectly* target the effects of tumor suppressor gene loss in cancer cells. Figure 1 indicates some of the undruggable and druggable targets in the DNA double strand break response pathway. We invite the reader to refer to this figure throughout the remainder of this Chapter.

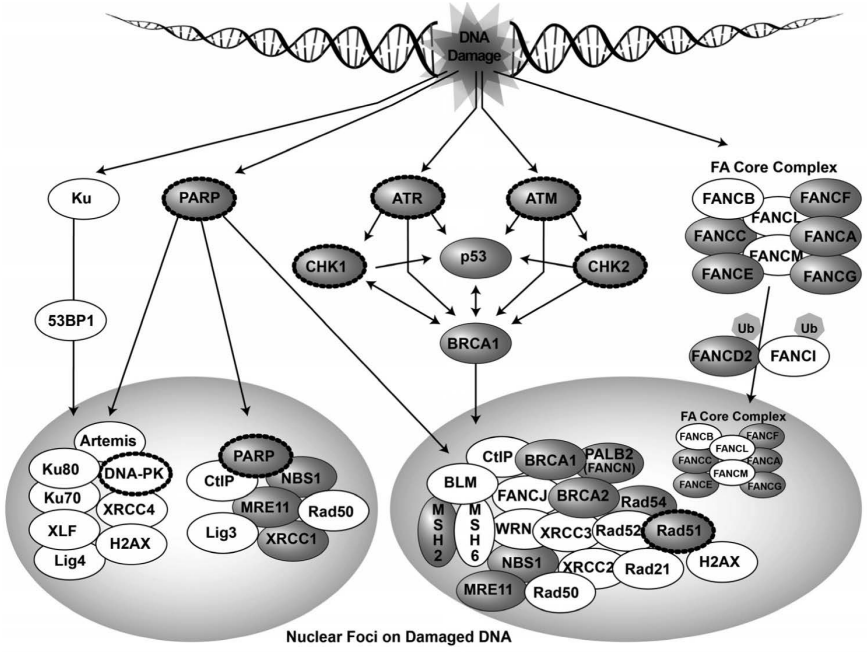

Figure 1. A simplified schematic depicting key proteins involved in double strand DNA break repair that are discussed in this Chapter. Dark grey shading of individual proteins indicates undruggable targets that have synthetic lethal partners. Outer black rings indicate druggable targets that have direct inhibitors in preclinical or clinical development.

SYNTHETIC LETHAL INTERACTIONS

Synthetic lethality refers to the phenomenon of cell death, or lethality, that results from the co-occurrence of two mutations, neither of which is lethal by itself (reviewed in Hartwell et al. 1997). In tumorigenesis, there is selective pressure for tumor cells to retain mutations that confer a survival advantage and to preserve the integrity of a synthetic lethal gene or pathway. In a clinical setting, this means that therapeutically targeting the synthetic lethal partners of so-called "undruggable" targets can be used to exploit loss-of function mutations in cancer therapy. For example, tumor cells that have one defective DNA repair pathway are forced to rely on an alternate intact repair pathway(s) to maintain genomic viability (Shaheen et al. 2011). By pharmacologically inhibiting the DNA repair pathway upon which cancer cells have become reliant, a synthetic lethal situation is created that leads to selective cytotoxicity of cancer cells, while leaving non-tumor cells intact. The utility of synthetic lethality in the clinic is exemplified by the use of poly(ADP-ribose) polymerase (PARP) inhibitors to treat molecularly-

defined subsets of cancer patients whose tumors have an impaired DNA damage response resulting from defects in homologous recombination, for example, patients with BRCA-deficient tumors (Fong et al. 2009; Tutt et al. 2010).

Targeting "BRCA-ness"

The proteins encoded by the breast cancer 1 (*BRCA1*) and breast cancer 2 (*BRCA2*) genes, are required for the repair of double-strand breaks by homologous recombination (Moynahan et al. 1999, 2001) (see Chapter 14). *BRCA1* and *BRCA2* are highly penetrant cancer susceptibility genes (Hall et al. 1990; Miki et al. 1994; Wooster et al. 1994). Individuals who carry a monoallelic germline mutation in *BRCA1* or *BRCA2* are at increased risk of developing breast and ovarian cancer (Hall et al. 1990; Miki et al. 1994; Wooster et al. 1994). Tumors that arise in carriers of germline mutations in one allele of either *BRCA1* or *BRCA2* have somatic mutations or loss of the second allele and thus are deficient in homologous recombination. Carriers of biallelic germline mutations in *BRCA2* have Fanconi anemia D1 (FANCD1) (Howlett et al. 2002); these children have a higher risk of developing acute leukemia at a younger age than children in other Fanconi anemia (FA) complementation groups (Wagner et al. 2004).

BRCA-deficient tumor cells rely on other repair mechanisms, such as base excision repair or non-homologous end joining, to respond to DNA breaks. The PARP-1 protein is required for base excision repair and also plays a role in non-homologous end joining (Audebert et al. 2006; Wang et al. 2006). Early preclinical studies showed that BRCA1- or BRCA2-deficient cells were particularly sensitive to PARP inhibitors (Bryant et al. 2005; Farmer et al. 2005). This finding was later confirmed by *in vivo* studies using a mouse model of BRCA1-associated mammary cancer, which exhibited high sensitivity to olaparib (AZD2281, Astra Zeneca), a small molecule inhibitor of PARP (Rottenberg et al. 2008).

A number of PARP inhibitors have been developed and several have entered clinical trials (see Chapter 15). Based on preclinical observations that PARP sensitivity correlated with defective homologous recombination, most clinical trials of PARP inhibitors have been of selected subsets of cancer patients who are most likely to respond (O'Shaughnessy et al. 2011). Among these cohorts are so-called triple-negative breast cancer patients, defined as patients whose tumors are estrogen receptor (ER)-negative, progesterone receptor (PR)-negative, and HER2-negative (reviewed in Rastelli et al. 2010; Pal et al. 2011). Triple-negative breast tumors are a particularly aggressive form of breast cancer that account for 15–20% of all breast cancers. They typically have defective homologous recombination resulting from mutations in the genes that encode the BRCA proteins, or

in ataxia-telangiectasia mutated (*ATM*), partner and localizer of BRCA2 (*PALB2*), or the *MRE11-RAD50-NBS1* genes that encode the DNA damage sensor complex (Bartkova et al. 2008; Tommiska et al. 2008; Heikkinen et al. 2009).

Iniparib (BSI-201, Sanofi-Aventis) is a small molecule inhibitor of PARP that is administered intravenously in a clinical setting. Results of a recent phase II clinical trial demonstrated an increased clinical benefit to metastatic triple-negative breast cancer patients who received iniparib in combination with gemcitabine/carboplatin compared with patients who received gemcitabine/carboplatin alone: patients whose treatment included iniparib showed increased "clinical benefit" rate (56% versus 34%, $P = 0.01$), increased overall rate of response (52% versus 32%, P=0.02), longer median progression-free survival (median 5.9 months versus 3.6 months, $P = 0.01$), and longer median overall survival (median 12.3 months versus 7.7 months, $P = 0.01$) (O'Shaughnessy et al. 2011). A phase III trial (clinicaltrials.gov identifier NCT00938652) of iniparib with or without gemcitabine/carboplatin for triple-negative metastatic breast cancer patients has completed accrual and is ongoing (reviewed in Liang and Tan 2010).

Olaparib (AZD2281, Astra Zeneca) is an orally administered PARP inhibitor that in preclinical studies induced synthetic lethality in *BRCA2*-deficient mouse mammary epithelial tumor cell lines, either alone or when combined with cisplatin (Evers et al. 2008). In a recent phase I trial, in a cohort of patients enriched for BRCA-associated tumors, the drug was found to have few adverse side-effects and led to clinical benefit exclusively in patients whose tumors were associated with a mutation in *BRCA1* or *BRCA2* (Fong et al. 2009); of 19 BRCA-associated patients evaluated for response, 12 (63%) had a clinical benefit, evidenced by a decline in tumor-marker levels, a measurable response, or disease stabilization (Fong et al. 2009). A phase II genotype-directed trial of single-agent olaparib, administered to recurrent ovarian cancer patients who carried a germline mutation in *BRCA1* or *BRCA2* and who had failed at least one line of prior therapy, also reported encouraging results (Audeh et al. 2010). The objective tumor response rate observed in that study was 33% (11 of 33 patients) (95% CI, 20–51) for patients who received the maximal tolerated dose (400 mg twice daily) compared to 13% (3 of 24 patients) (95% CI, 4–31) who received a lower dose (100 mg twice daily) (Audeh et al. 2010). Similarly, the results of a phase II trial of olaparib for the treatment of advanced breast cancer patients with *BRCA*-deficient tumors, and who had failed prior chemotherapy, showed an objective response rate of 41% (11 of 27) (95% CI, 25–59) for patients administered 400 mg twice daily, and 22% (6 of 27) (95% CI, 11–41) for patients administered 100 mg twice daily (Tutt et al. 2010).

The original preclinical studies described by Farmer et al. led them to formulate a model which predicted that PARP sensitivity relied not on the

existence of mutations specifically in *BRCA1* or *BRCA2*, but rather on the existence of defective DNA repair by homologous recombination (Farmer et al. 2005). This premise has been borne out in subsequent preclinical studies, which confirmed that mutations in several other genes that function within the homologous recombination pathway also confer sensitivity to PARP inhibitors in preclinical studies (McCabe et al. 2006; Buisson et al. 2010). The synthetic lethal targets uncovered in these studies were RAD51, RAD54, DSS1, replication protein A1 (RPA1), Nijmegen breakage syndrome protein (NBS1), AT and Rad3-related protein (ATR), ATM, CHK1, CHK2, FANCD2, FANCA, FANCC, and PALB2 (McCabe et al. 2006; Buisson et al. 2010). In addition, an unrelated synthetic lethal siRNA screen identified CDK5, a protein that phosphorylates ATM within the homologous recombination pathway, as a synthetic lethal partner of the PARP inhibitor KU0058948 (Turner et al. 2008).

The constantly evolving genetic makeup of tumors appears to be responsible for acquired resistance to PARP inhibitors. For example, it has been found that secondary mutations in *BRCA1* or *BRCA2,* which restore the correct reading frame of these genes, can result in acquired resistance to platinum-based chemotherapy and rescue PARP inhibitor sensitivity (Edwards et al. 2008; Sakai et al. 2008; Swisher et al. 2008). Additionally, recent studies have indicated that loss of p53-binding protein 1 (53BP1) can rescue the DNA repair defect of *BRCA1* mutated cells and can revert their hypersensitivity to DNA-damaging agents (Edwards et al. 2008; Sakai et al. 2008; Swisher et al. 2008). Since that study also reported reduced 53BP1 expression in some triple-negative and BRCA-mutated breast cancers, loss of 53BP1 function may be another mechanism of resistance to PARP inhibitors or platinum drugs.

Targeting Phosphatase and Tensin Homolog (PTEN) Deficiency

The *PTEN* tumor suppressor gene is one of the most frequently mutated genes in human cancer. Somatic alterations of *PTEN* are present at high frequency in many sporadic cancers, including those of the endometrium, prostate, skin, and brain (Forbes et al. 2008). In addition, germline *PTEN* mutations cause Cowden's syndrome and Bannayan-Ruvalcaba-Riley syndrome (Nelen et al. 1996; Liaw et al. 1997; Marsh et al. 1997). PTEN is perhaps best known for its role as a phosphatase that antagonizes AKT activation, downstream of PI3K. However, within the last decade it has been established that PTEN also functions within the homologous recombination pathway, in response to DNA damage, through the transcriptional regulation of RAD51 (Shen et al. 2007). PTEN-null cells lack RAD51 foci formation (Dedes et al. 2010) and exhibit spontaneous double-strand breaks (Shen et al. 2007). Accordingly, recent preclinical studies have shown that

PTEN-deficient tumor cells are sensitive to PARP inhibition, both *in vitro* and *in vivo* (Mendes-Pereira et al. 2009; Dedes et al. 2010; McEllin et al. 2010). PTEN-deficient glioma cells are also hypersensitive to the alkylating agent *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG), a temozolomide analog, which induces replication-associated DNA double-strand breaks (McEllin et al. 2010). Together, these observations have led to the prediction that patients with PTEN-deficient tumors might respond clinically to targeted therapy using PARP inhibitors and, in the case of glioblastomas, to DNA alkylating agents such as temozolomide (McEllin et al. 2010). This prediction awaits the results of genotype-directed clinical trials. Although no current trials, that we are aware of, stratify patients according to *PTEN* status and/or expression before treatment with a PARP inhibitor or temozolomide, several aim to determine *PTEN* status as a secondary outcome measure for patients with glioma or glioblastoma following temozolomide alone or combined with other treatments including olaparib (clinicaltrials.gov identifiers NCT00553150, NCT00486603, NCT00895960, NCT00887146, NCT01308632, NCT01390571).

A recent case report described a positive response for a PTEN-deficient, presumed BRCA-intact, endometrial cancer patient to olaparib (Forster et al. 2011). This result is both encouraging and exciting, given the prominent role of *PTEN* mutations in sporadic human cancer, and the fact that PTEN deficiency has been associated with poor response to certain tyrosine kinase inhibitors that target upstream proteins. For example, loss of PTEN has been associated with clinical resistance to trastuzumab, a monoclonal antibody that targets HER2, in breast cancer (Nagata et al. 2004); clinical resistance to cetuximab, a monoclonal antibody that targets EGFR, in colorectal cancer (Frattini et al. 2007); and clinical resistance to EGFR inhibitors (gefitinib and erlotinib) in glioblastoma patients (Mellinghoff et al. 2005). In addition, laboratory studies have shown that PTEN loss correlates with resistance of melanoma cells to a BRAF inhibitor (PLX4720) *in vitro* (Paraiso et al. 2011), and resistance of lung cancer cells to erlotinib (Sos et al. 2009b).

Targeting p53-Deficiency

The p53 tumor suppressor protein plays key roles in activating DNA repair proteins, initiating apoptosis, and controlling the cell cycle (see Chapter 12). The gene is somatically mutated in ~30% (range 6% to 47%) of human cancers (Petitjean et al. 2007), and germline *TP53* mutations cause Li-Fraumeni syndrome (Malkin et al. 1990), a rare high penetrance cancer syndrome that predisposes carriers to the development of multiple cancers (Li and Fraumeni 1969). Because *TP53* is mutated so frequently in human cancer, it is an attractive target for rationally designed therapies. Several

strategies have been used to devise therapies targeting p53-deficient cells. These strategies include (i) attempts to restore p53 function in tumors that have amplified the p53-regulating murine double minute 2 (MDM2) ubiquitin ligase through the development of molecules that disrupt the p53-MDM2 protein-protein interaction (Dudkina and Lindsley 2007), (ii) the development of molecules that restore normal function to mutant p53 (Di Cintio et al. 2010), and (iii) searches for synthetic lethal interacting partners of p53 (Sur et al. 2009). The development of molecules that restore p53 function or that block the MDM2-p53 interaction has recently been reviewed in detail elsewhere (Lauria et al. 2010, Vu and Vassilev 2011). Here we will focus on the emerging body of literature that has pointed to members of the ATM/ATR-mediated DNA damage response and the downstream G2/M checkpoint (see Chapter 13) as synthetic lethal partners of p53.

ATM/ATR Inhibition and p53-Deficiency: Phosphorylated p53 binds directly to DNA breaks (Al Rashid et al. 2005), and p53-deficiency leads cells to rely on ATM- and ATR-mediated signaling to respond to DNA damage (Reinhardt et al. 2007). This phenomenon sets up a potential synthetic lethal relationship between p53-deficiency and inhibition of the ATM or ATR pathway, and *in vitro* studies have confirmed an increased sensitivity of p53-deficient cells to ATR inhibition (Nghiem et al. 2001). The synthetic lethal interactions between p53 and ATR have also been shown using knockout mice (Ruzankina et al. 2009). This synthetic lethality may be enhanced when combined with exposure to a DNA-damaging agent. For example, selective killing of p53- or ATM-deficient cancer cells was achieved using an ATR inhibitor in combination with various DNA-damaging agents (Reaper et al. 2011). Likewise, colorectal cancer cells that have inactivated p53 exhibit enhanced cisplatin sensitivity following ATR inhibition (Sangster-Guity et al. 2011). Similar to ATR, the suppression of ATM sensitizes p53-deficient tumors to genotoxic chemotherapy, whereas suppression of ATM or CHK2 in the presence of functional p53 was actually shown to protect tumors from DNA damage-induced cell death (Jiang et al. 2009).

CHK1 Inhibition and p53-Deficiency: Targeting proteins downstream of ATM/ATR (Fig. 1) also appears to be effective in inducing death of p53-deficient cancer cells. For example, inhibition of CHK1 in combination with DNA-damaging agents has shown selectivity for p53-deficient cells. In particular, a recent study reported that, following DNA damage induced by chemotherapy, targeted small-molecule inhibition of CHK1 is synthetic lethal with mutant p53 (Blasina et al. 2008). Laboratory results also show that inhibition of CHK1 can selectively sensitize cancer cells lacking p53 to γ-radiation (Busby et al. 2000). Specifically, UCN-01, a non-selective inhibitor of CHK1, which also inhibits CHK2, Wee1, and AKT, preferentially sensitizes p53-deficient cancer cells to γ-radiation, ionizing radiation, and

cisplatin (Wang et al. 1996; Petersen et al. 2010; De Witt Hamer et al. 2011). UCN-01 is also a potent inhibitor of MAPKAP kinase 2 (MK2), a kinase that is activated independent of CHK1 and is involved in the G2/M and S phase checkpoints. Depletion of MK2 caused selective killing of p53-deficient cells, but not p53-wildtype cells (Reinhardt et al. 2007). Although a number of phase I and II clinical trials of UCN-01 have been completed or are ongoing (clinicaltrials.gov), we found no trials that considered the *p53* status of the patients' tumors.

Wee1 Inhibition and p53-Deficiency: The Wee1 kinase serves as a gatekeeper that regulates G2 arrest and functions downstream of CHK1. Wee1 mRNA, protein and gene expression have been assessed in various studies, and both increased (Masaki et al. 2003; Iorns et al. 2009; Mir et al. 2010) and decreased (Backert et al. 1999; Butz et al. 2010) expression of Wee1 has been reported for a number of cancer types, as well as upregulated protein expression in breast cancer stem cells (Wang et al. 2011b). It was also reported that lack of Wee1 expression may be a prognostic indicator for NSCLC patients, because reduced expression correlated with a higher recurrence rate and poorer prognosis (Yoshida et al. 2004). Preclinical data suggests that p53-deficient cancer cells are sensitive to Wee1 inhibitors in combination with DNA-damaging cancer therapy (Hirai et al. 2009, 2010; De Witt Hamer et al. 2011). Furthermore, one potent Wee1 inhibitor, MK-1775, has been shown to synergize with gemcitabine and lead to regression of p53-deficient pancreatic cancer xenografts (Rajeshkumar et al. 2011). Other inhibitors of Wee1 with varying selectivity include PD0166285, PD0407824, Wee1 inhibitor II, and 4-(2-phenyl)-9-hydroxypyrrolo[3,4-c]carbazole-1,3-(2H,6H)-dione (PHCD). Along with the potential usefulness of Wee1 kinase inhibitors in patients with p53-deficient tumors, other preclinical evidence suggests tumor-cell specific efficacy of Wee1 inhibitors, either alone or in combination with DNA-damaging agents, in breast cancer and glioblastoma cells regardless of *p53* status (Mir et al. 2010; Murrow et al. 2010).

Phase II trials of the Wee1 inhibitor MK-1775 in combination with chemotherapy for p53-mutated ovarian cancer patients are ongoing (clinicaltrials.gov identifiers NCT01164995, NCT01357161). Efforts to identify biomarkers for Wee1 inhibitor clinical response are also underway. For example, one published report used xenograft models to identify a p53 context-specific mRNA gene signature that specifically changes after treatment with gemcitabine and MK-1775 that could be used to dictate dosing in clinical settings (Mizuarai et al. 2009). Moreover, a recent patent has been filed that claims a set of biomarkers can be used to identify patients with dysfunctional or aberrant p53 that will respond to a Wee1 inhibitor (WO2011027800). With the combined efforts of these preclinical and clinical trials, the therapeutic validity of Wee1 inhibitors will hopefully be determined in the near future.

G2/M Checkpoint Inhibition and p53-Deficiency: Inhibitors of other proteins involved in the G2/M checkpoint have been shown to be potential therapeutic agents for patients with p53-deficient tumors. For example, increased sensitivity of p53 mutant or null cells as compared to p53 wild type cells, in culture or in nude mice bearing xenografts, was observed following treatment with the specific CDK2 inhibitor NU6102, as well as the polo-like kinase 1 (PLK1)-selective inhibitor GSK461364A (Sur et al. 2009; Degenhardt et al. 2010; Thomas et al. 2011). These results are consistent with *in vitro* studies showing that CDK2 is required for p53-independent G2/M checkpoint activation (Chung and Bunz 2010) and that depletion of PLK1 is cytotoxic to p53-defective cells, but not to p53 wildtype cells (Guan et al. 2005). To our knowledge, no clinical trials of PLK1 or CDK inhibitors to date have stratified patients according to *p53* status.

The data reviewed above in total suggest that *TP53* mutations may be used clinically to identify patients that might respond to inhibitors of the ATM/ATR damage response pathway, and/or the downstream G2/M checkpoint. Furthermore, evidence suggests that response in patients with p53-deficient tumors may be particularly enhanced when inhibitors of DNA damage response kinases are combined with DNA damaging agents.

Targeting Fanconi Anemia (FA)-Deficiency through DNA Damage Kinase Inhibition

The potential use of inhibitors of DNA damage response kinases, such as ATM or CHK1, as personalized medicine for patients with *TP53* mutation may not be the only clinical utility of these drugs. Preclinical data has also shown that tumor cells with defects in the FA pathway (see Chapter 10) are hypersensitive to the ATM inhibitor KU-55933 as compared to isogenic corrected cells (Kennedy et al. 2007). Furthermore, FA-deficient cell lines are hypersensitive to CHK1 siRNA depletion, and the specific CHK1 inhibitor Gö6976 displays synthetic lethality with siRNA depletion of FANCA, FANCC, FANCD2, FANCD1, FANCF, FANCE, or FANCG (Chen et al. 2009). A synergistic effect of combining ATM and CHK1 inhibition was likewise observed in FANCF-deficient ovarian cancer cells but was not observed in isogenic corrected cells (Chen et al. 2009). Importantly, because the FANC genes have been associated with a number of different cancer types, these results suggest that ATM inhibitors as well as CHK1 inhibitors alone or in combination could potentially have broad uses as targeted therapies (Peng and Lin 2011). These data, combined with the p53-targeted data presented above, provide a compelling case that personalized medicine using DNA damage response kinase inhibitors may be a potent anti-cancer therapy in specific genotypic and treatment contexts.

Targeting O^6-Methylguanine-DNA Methyltransferase (MGMT)

As described above, rationally targeting DNA repair pathways in the clinic has led to success in some cases, most notably the use of PARP inhibitors for BRCA-deficient patients. This effectiveness is largely due to the specificity of PARP inhibitors for *BRCA* mutated cancer cells while leaving normal cells unharmed. Unfortunately, not all agents targeting DNA repair have had the specificity for tumor cells that PARP inhibitors have displayed. Prime examples are drugs that target MGMT, a DNA repair protein that reverses the alkylation of guanine bases in DNA (reviewed in Rodriguez-Paredes and Esteller 2011) (see Chapter 6). The O^6 position of guanine is the site of action of alkylating agents such as carmustine (BCNU), nemustine (ACNU), procarbazine, dacarbazine, and temozolomide (Rodriguez-Paredes and Esteller 2011). Preclinical studies showed that coadministering MGMT inhibitors and alkylating agents could enhance the cytotoxic effects and circumvent resistance of anti-cancer alkylating drugs (Dolan et al. 1991; Baer et al. 1993; Wedge and Newlands 1996; Wedge et al. 1996). However, the mechanism by which MGMT repairs alkylated DNA bases results in irreversible inactivation of this "suicide protein". Thus, normal cells are depleted of MGMT activity if the inhibitor is not cancer cell specific (Kaina et al. 2010). A lack of specificity towards tumor cells has in fact been observed in clinical trials of MGMT inhibitors, resulting in little therapeutic efficacy likely due to the dose reduction required to alleviate systemic toxicity (Kaina et al. 2010). Consequently, it has been suggested that local administration of MGMT inhibitors to sites of tumors might decrease systemic toxicity, but the benefits of this approach are inconclusive (Koch et al. 2007).

Following the observation that methylation of the *MGMT* promoter in tumors was associated with longer survival for glioblastoma patients treated with combined radiotherapy and temozolomide, the field of epigenetics has been used to refine the concept of using MGMT inhibitors and alkylating agents together (Hegi et al. 2005). The idea is that targeting tumors with methylated and epigenetically silenced *MGMT* could lead to cancer cell specific toxicity of alkylating agents, a feature that is crucial for successful personalized medicine. Published results testify that glioma patients with methylated *MGMT* benefit from temozolomide (Hegi et al. 2004, 2005; Paz et al. 2004) or BCNU treatment (Esteller et al. 2000). Additionally, a recent report described two consecutive patients with metastatic colorectal cancer that had decreased expression of MGMT (Shacham-Shmueli et al. 2011). Both patients responded to single agent temozolomide with impressive clinical response and partial tumor regression. Results such as these support the use of *MGMT* methylation status to stratify cancer patients into clinical trials that evaluate alkylating agents. In fact, as of August 2011, a number of clinical trials do stratify patients according to *MGMT* status, and a trial

to determine an accurate method to establish *MGMT* methylation as a way to minimize unnecessary treatment of glioblastoma patients who are unlikely to respond to alkylating agents is currently recruiting patients (NCT01345370). It should be noted that cell death due to unrepaired alkylation of guanine occurs after activation of the mismatch repair (MMR) system (see Chapter 7), so cells deficient in MMR are resistant to alkylating agents even in the absence of MGMT (Weller et al. 2010). This fact may suggest that stratification of patients by the mutational status of genes involved in MMR could further improve the efficacy of alkylating agents in patients with methylated *MGMT* promoters.

Preclinical data suggests that methylation of other DNA repair genes may be prognostic indicators of response to anti-cancer therapies: *MLH1* methylation in ovarian cancer may predict response to cisplatin, carboplatin, temozolomide, and epirubicin (Plumb et al. 2000); *WRN* methylation in colorectal tumors may predict response to irinotecan (Agrelo et al. 2006); and *BRCA1* methylation may predict response to PARP inhibitors in breast or ovarian cancer patients (Veeck et al. 2010). Future clinical trials will hopefully ascertain whether the methylation of these genes is in fact useful in a clinical setting.

RESISTANCE TO TARGETED THERAPIES: CHALLENGES AND OPPORTUNITIES

As we have noted throughout this Chapter, the clinical successes of targeted therapies are generally tempered by the subsequent acquisition of drug resistance. This phenomenon is not unexpected given the dynamic nature of cancer genomes and the nonclonal nature of tumor cell populations. In fact, the clonal evolution of tumor cells led Nowell to propose, as long ago as 1976, that individualized approaches may be required for cancer therapy (Nowell 1976). As we have seen in the past decade, in which personalized medicine for cancer has taken center stage, this prediction has held true. Primary resistance to a targeted therapy reflects the fact that the majority of tumor cells harbor a mutation or other alteration that confers resistance, whereas secondary resistance may result from the outgrowth of drug-resistant tumor cells that represent a minority subpopulation of the original tumor, the acquisition of secondary mutations, or the activation of the targeted signal transduction pathway by an alternative mechanism. Relapse may also occur if cells enter a "drug tolerant state" that enables them to survive until treatments are terminated (Sharma et al. 2010). With this in mind, the clinical development of targeted therapies should be accompanied by *in vitro* modeling of drug resistance, as well as molecular analyses of tumor recurrences in order to rapidly identify possible mechanisms of resistance

and work towards overcoming this phenomenon using rationally based approaches, including the development of second generation inhibitors. A paradigm for how this can be achieved is provided by the example of imatinib resistance in CML patients, as discussed earlier in this Chapter.

THE FUTURE OF PERSONALIZED MEDICINE FOR CANCER

As we eluded to earlier in this Chapter, large national and international efforts to systematically generate catalogues of all the somatic mutations present in the most lethal and prevalent forms of cancer are underway. These initiatives include studies conducted under the purview of The Cancer Genome Atlas (TCGA), The International Cancer Genome Consortium (Hudson et al. 2010), the Therapeutically Applicable Research to Generate Effective Treatments (TARGET) initiative, The Sanger Center Welcome Trust, and the St. Jude's Hospital-Washington University Pediatric Cancer Genome Project, among others. Already, exomic and genomic analyses by these groups and many others have shed important new insights into the mutational landscapes of a number of common and deadly tumors, including brain cancers (The Cancer Genome Atlas Research Network 2008; Parsons et al. 2008, 2011; Yan et al. 2009), leukemias (Ley et al. 2008, 2010; Mardis et al. 2009; Mullighan et al. 2009a,b; Walter et al. 2009; Dickinson et al. 2011; Puente et al. 2011; Yan et al. 2011), breast cancer (Sjoblom et al. 2006; Wood et al. 2007; Shah et al. 2009b; Stephens et al. 2009), pancreatic cancer (Jones et al. 2008, 2009; Campbell et al. 2010), colorectal cancer (Sjoblom et al. 2006; Wood et al. 2007; Dalgliesh et al. 2010; Varela et al. 2011), serous ovarian cancer (The Cancer Genome Atlas Research Network 2011), prostate cancers (Taylor et al. 2010; Berger et al. 2011), melanomas (Harbour et al. 2010; Pleasance et al. 2010; Wei et al. 2011), head and neck cancers (Agrawal et al. 2011), lung cancers (Weir et al. 2007), bladder cancer (Gui et al. 2011), and hepatitis C virus associated hepatocellular carcinomas (Li et al. 2011a), as well as rarer tumor types, such as clear cell renal cancer (Dalgliesh et al. 2010; Varela et al. 2011), hairy cell leukemia (Tiacci et al. 2011), multiple myeloma (Chapman et al. 2011a), soft tissue sarcomas (Barretina et al. 2010), non-Hodgkin lymphoma (Morin et al. 2011), pancreatic neuroendocrine tumors (Jiao et al. 2011), and ovarian granulosa cell tumors (Shah et al. 2009a).

The ultimate goal of comprehensive resequencing efforts is to uncover "actionable" targets, including druggable proteins and pathways. Once catalogues of somatic mutations are in hand, the challenge for members of the larger research community will be to understand the biological relevance of the mutations; some mutations will be biologically meaningful mutations

(drivers), but many are likely to be bystander mutations (passengers) with no relevance to tumorigenesis. Among the driver mutations the quest will be to determine which of these are actionable within a clinical setting—some may serve as prognostic indictors, some may be valuable as diagnostic classifiers (Parsons et al. 2008; Shah et al. 2009a; Watanabe et al. 2009), some may be predictive of drug response (Soda et al. 2007; Kwak et al. 2010), and others predictive of drug resistance (Sequist et al. 2011).

It is expected that the future of personalized medicine in oncology will ultimately incorporate next generation sequencing of tumor exomes and/or genomes into the clinical setting, in order to personalize the clinical management of each individual patient. Although there are anecdotal examples of success stories, as discussed below (Villarroel et al. 2011), there are still many hurdles that have to be crossed before this approach becomes mainstream. For example, interpreting next generation sequencing data is challenging, and it is not clear how such information would best be conveyed to a pathologist or physician. To this end, the Ontario Institute for Cancer Research (Canada), in partnership with Cancer Care Ontario, has launched the Genomics Pathway Strategy, under the auspices of their High Impact Clinical Trials program. The Genomics Pathway Strategy aims to determine how to incorporate sequencing technologies into clinical practice in oncology, with a rapid turnaround time. A subsequent validation trial, the Clinical Genomics Assessment Trial, is planned.

A recent case report serves as an elegant example of personalized medicine in action and has provided the first evidence that *PALB2* mutations correlate with clinical response to DNA-damaging agents (Villarroel et al. 2011). Villarroel et al. described the personalized clinical management of a patient with familial pancreatic cancer enrolled in NCI clinical trial NCT00276744. The objective of this trial was to determine the feasibility of individualized treatment of patients with advanced pancreatic cancer. Xenografts of each patient's tumor were established and treated with a panel of ten drugs; the drug(s) that invoked the most dramatic response in the xenograft model was then administered to the patient. In a remarkable case report of one patient enrolled in this trial, treatment with mitomycin C (MMC) followed by cisplatin, both identified in the corresponding xenograft model, resulted in a long-lasting (36+ month) response. This outcome was astounding considering that patients with metastatic pancreatic cancer have a 5-year relative survival of less than 2% (SEER statistics: http://seer.cancer.gov/). Companion whole exome sequencing of the patient's tumor revealed that it harbored biallelic, inactivating mutations in *PALB2*. Molecularly, the loss of proper PALB2 function caused an impaired BRCA1-BRCA2 interaction and mechanistically explained the MMC and cisplatin

sensitivities (reviewed in Foulkes 2006). Even though this type of diagnostic care is at present too costly and time-consuming to be used routinely, it is a wonderful example of the potential of personalized medicine in oncology.

The dynamic state of cancer genomes and the emergence of new clonal outgrowths as a result of changing selective pressures, including treatment, require that tumor cells be assessed not only at diagnosis, but throughout the course of treatment. To meet the challenge of obtaining serial biopsies, many new approaches to non-invasively sample tumor cells, or tumor DNA, are in development. Among these approaches are methodologies that aim to collect circulating tumor cells present within the blood of metastatic cancer patients, as well as genetic tests that utilize free circulating tumor DNA present within the blood stream. Both approaches have been successful in very defined settings (Nagrath et al. 2007; Maheswaran et al. 2008; Leary et al. 2010). For example, Leary and colleagues recently used next generation sequencing to uncover somatic rearrangements within the genomes of a small number of cancer patients (Leary et al. 2010). They then developed a PCR based assay, specific for the rearrangements, and used it quantify tumor burden throughout the course of treatment.

In closing, we have provided some pivotal examples that exemplify the expectation that targeted therapies will be more effective than many of the blunt therapies historically used as cancer treatment. The use of genetic information gleaned from preclinical studies has already positively altered the outcome of some clinical trials. Precisely when and how comprehensive sequencing of cancer genomes will be incorporated into routine clinical practice remains to be seen. However, the advances that have taken place in cancer genomics over the past few years, and that are still to come, will without doubt catalyze revolutionary change in the practice of oncology.

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CHAPTER 17

The Tumor Microenvironment and DNA Repair: Implications for Cancer Progression and Treatment

Norman Chan,[1] *Shaqil Kassam*[1] and *Robert G. Bristow*[2,*]

INTRODUCTION

This Chapter will outline the known impact of the tumor microenvironment on DNA repair and the implications for cancer progression and treatment. In particular, the effect of tumor hypoxia on the expression and function of the DNA repair pathways will be discussed with emphasis on how these changes may modify the effectiveness of cancer therapies.

ACUTE AND CHRONIC HYPOXIA

The microenvironment of solid tumors differs greatly from that of normal tissues. Tumors contain regions of hypoxia (a decreased level of oxygen) and increased interstitial fluid pressure (IFP), as well as decreased pH and

[1]Princess Margaret Hospital (University Health Network) and Departments of Medical Biophysics and Radiation Oncology, University of Toronto, Toronto, ON, M5G 2M9, Canada.
[2]Radiation Medicine Program, Princess Margaret Hospital, 610 University Avenue, Toronto, Ontario Canada, M5G 2M9; Email: Rob.Bristow@rmp.uhn.on.ca
*Corresponding author

nutrient delivery (Harris 2002; Brown and Wilson 2004). Clinically relevant levels of hypoxia are detected in 50–60% of all human tumors (Ljungkvist et al. 2006) and are associated with both therapy resistance and decreased disease-free survival in many cancer types (Birner et al. 2000, 2001a,b; Aebersold et al. 2001; Bos et al. 2001; Kaanders et al. 2002c; Schindl et al. 2002; Nordsmark et al. 2005; Fyles et al. 2006; Vergis et al. 2008). The link between hypoxia and poor prognosis holds true for patients treated with both radiotherapy and surgery. This suggests hypoxia is driving biological effects beyond local tumor resistance and may also drive tumor progression and/or metastasis.

Both acute and chronic hypoxia can exist in human tumors (Harris 2002; Brown and Wilson 2004). The abnormal vasculature of tumors that arises as a result of unregulated angiogenesis is probably the most important contributor to both acute and chronic hypoxia in the majority of solid tumors. Tumor blood vessels are often chaotic, leaky, unevenly distributed and are generally of poor quality. Chronic hypoxia, or potentially anoxia (a complete lack of oxygen), develops in solid tumors because of abnormally long intravascular transit times (i.e., erythrocytes release oxygen early during their passage through the tumor, leading to intravascular hypoxia) and the irregular distribution of tumor blood vessels (i.e., limited diffusion of oxygen through the tumor interstitium leads to increasing cellular hypoxia at distances greater than 150 μm from the vessels). Acute hypoxia arises because of transient changes in blood flow and can be due to temporary occlusions of blood vessels, possibly aggravated by elevated IFP (Harris 2002; Brown and Wilson 2004). This opening and closing of tumor blood vessels can expose tumor cells to repeated cycles of hypoxia and re-oxygenation (termed "cycling hypoxia"). Therefore, tumor hypoxia can be both spatially and temporally heterogeneous within rapidly changing gradients of oxygenation and nutrients (Fig. 1).

Despite similar biophysical levels of oxygen, acute and chronic hypoxia may lead to a very different biology within the tumor. For example, acute hypoxia and subsequent re-oxygenation cycles in rapidly proliferating tumors may lead to the formation of reactive oxygen species (ROS) causing DNA breaks (Hammond et al. 2003). These cycles of fluctuating oxygenation can also prime the tumor vessel endothelium to resist radiation-induced apoptosis (Martinive et al. 2006). By contrast, those cancer cells exposed to chronic hypoxia could have slower cell cycle transit times, which lead to decreased translation of cellular proteins in a manner distinct from acute hypoxia/re-oxygenation (Koritzinsky et al. 2006).

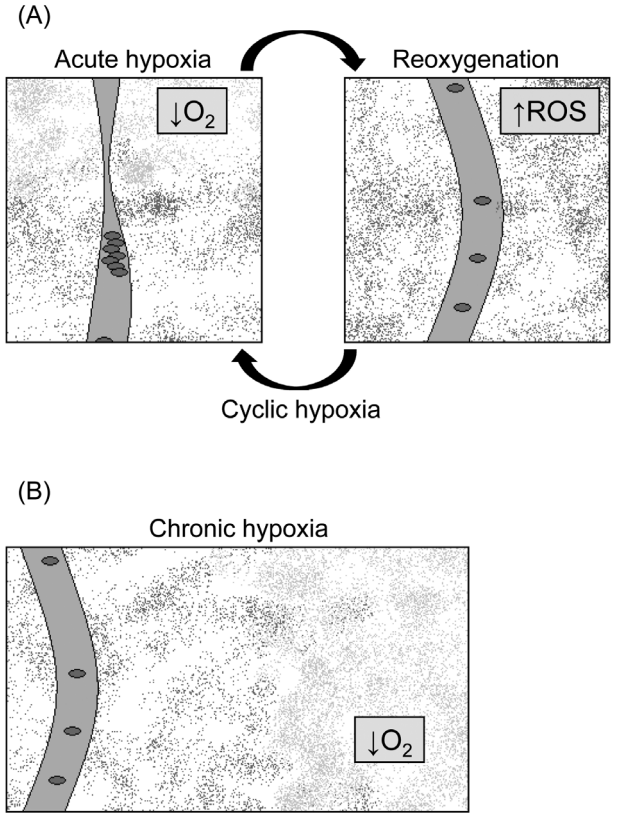

Figure 1. Regions of acute and chronic hypoxia can develop within solid tumors. A) Acute hypoxia occurs as a result of sudden and temporary changes in blood flow (e.g., collapsed blood vessels). Subsequent reoxygenation leads to cyclic hypoxia and the generation of reactive oxygen species (ROS). B) Solid tumors can often have uneven distribution of blood vessels leading to chronic or diffusion-limited hypoxia in cells >150 μm from blood vessels near the diffusion limit of oxygen.

Color image of this figure appears in the color plate section at the end of the book.

HYPOXIA AND CANCER PROGRESSION

Cancer progression includes altered oncogenesis and tumor suppression, leading to cell mutation and clonal selection within a background of increased angiogenesis. Preclinical data have demonstrated that hypoxia can lead to the selection of aggressive cancer cells with suppressed DNA repair, driving a mutator phenotype and genetic instability (see Chapters

1 and 3). These hypoxic cells manifest a loss of heterozygosity (LOH), copy number alterations (CNA) and aneuploidy (Cairns et al. 2003; Subarsky and Hill 2003; Cairns and Hill 2004; Bindra and Glazer 2005). In many cancer patients, the presence of hypoxia in their primary tumors at diagnosis is associated with local recurrence and metastases, regardless of whether the initial treatment is with surgery or radiotherapy (Vergis et al. 2008). These data implicate hypoxia as a determinant of both local and systemic tumor aggression in many cancers (Fig. 2).

Laboratory studies have proven that exposure of rodent tumor cells to hypoxia *ex vivo* or *in vivo* can increase experimental and spontaneous metastases (Young et al. 1988; Cairns et al. 2001; Cairns and Hill 2004). Cairns et al. demonstrated that tumor-bearing mice exposed to cyclic

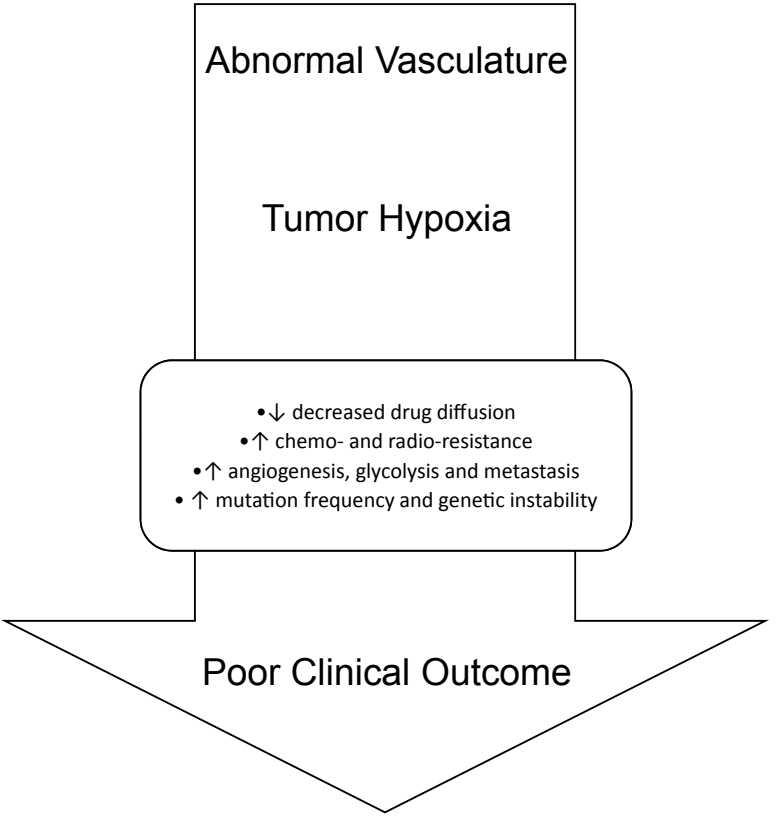

Figure 2. Solid tumors are prone to develop abnormal blood vasculature leading to regions of acute and chronic hypoxia. The hypoxic response may include multiple changes such as decreased drug diffusion as well as increased therapeutic resistance, angiogenesis, glycolysis, metastasis, and genetic instability. These changes can all contribute to poor clinical outcome.

hypoxia developed more spontaneous lung metastases than the control or chronic hypoxia treatmented animals (Cairns et al. 2001). The basis of hypoxia-induced metastases has been associated with up-regulation of genes involved in the metastatic cascade, including plasminogen activator urokinase receptor, the chemokine receptor CXCR4, osteopontin, lysyl oxidase (LOX), interleukin 8 (IL8), vascular endothelial growth factor (VEGF) and LAMP3 (Rofstad 2000; Erler et al. 2006; Chan and Giaccia 2007; Mujcic et al. 2009).

Hypoxia has been associated with increased genetic mutation frequencies. Using defined genetic models, Graeber and colleagues first observed that hypoxia could select for mutant p53-expressing cells that had reduced stress-induced apoptosis and an increased cell-survival advantage compared to wild-type p53-expressing cells (Graeber et al. 1996). Multiple research groups have reported that tumor cells grown *in vivo* have increased rates of mutagenesis compared to growth *in vitro* (under typical oxygenation conditions of 20%) using several different reporter systems (Reynolds et al. 1996; Li et al. 2001; Papp-Szabo et al. 2005). In contrast, Banath et al. reported that hypoxic subpopulations of V79 Chinese hamster xenografts did not have increased spontaneous or radiation induced mutations at the hypoxanthine phosphoribosyltransferase (HPRT) locus (Banath et al. 2005). This may suggest that V79 cells are not a good model to assess HPRT mutations or that the link between hypoxia and a mutator phenotype could be model-dependent.

HYPOXIA ALTERS TRANSCRIPTION AND TRANSLATION

In rapidly proliferating tumor tissues, metabolic need may exceed metabolic supply. The hypoxic response leads to a series of changes in protein expression to protect against cell death stemming from inadequate oxygen and nutrient delivery (Semenza 1999). The level of normal tissue oxygenation ranges from approximately 2.5% to 12.5% O_2 (pO_2 range of 20 to 100 mmHg), but altered gene expression, biology and radioresistance are consistently reported at hypoxic pO_2 levels of less than 10 mmHg (Vaupel 2004; Chan et al. 2009).

Hypoxia inducible factor 1α (HIF-1α) is the transcription factor responsible for mediating most cellular responses to hypoxia (Semenza 2000). HIF-1α is constitutively transcribed and translated, but is quickly degraded in the presence of oxygen by the ubiquitin-proteasomal pathway (Salceda and Caro 1997; Semenza 1999). The poly-ubiquitination of HIF-1α is mediated by the von Hippel-Lindau E3-ubiquitin ligase complex that binds to the oxygen-dependent degradation domain of HIF-1α (Maxwell et al. 1999; Cockman et al. 2000). Upon stabilization by hypoxia, HIF-1α

forms a heterodimer with HIF-1β (ARNT) at hypoxic responsive elements in the promoters of hypoxia-responsive genes. This action induces the transcription of a wide range of genes, including genes involved in angiogenesis (VEGF), glycolysis (glucose transporter 1 [GLUT-1]) and low pH (carbonic anhydrase IX [CAIX]) to facilitate cell growth and survival (Semenza 1999).

There are also HIF-1α transcriptional-independent effects on gene expression within the hypoxic response. Recent work has demonstrated that HIF-1α induces a cell cycle arrest by functionally counteracting Myc, resulting in the depression of $p21^{waf}$, a key cyclin-dependent kinase inhibitor that controls the G_1 checkpoint (Koshiji et al. 2004). The HIF-1α antagonism is caused by displacing Myc binding from $p21^{waf}$ and does not require HIF-1α-transcriptional or DNA binding activity. In this case, the HIF-1α/Myc pathway can regulate a new array of hypoxia-responsive genes independent of direct HIF-1α transcriptional activation. Furthermore, hypoxia can alter the relative expression of activating and repressing E2F transcriptional factors that regulate the expression of the recombinational repair and checkpoint proteins, BRCA1, RAD51 and retinoblastoma, in a HIF-1α-independent manner (Bindra et al. 2005; Bindra and Glazer 2007b).

Severe hypoxia or anoxia can also lead to a rapid, but reversible, suppression of protein synthesis, and is thought to be a means of energy conservation during times of hypoxic cell stress (Koritzinsky et al. 2005, 2006; Wouters et al. 2005). There are two distinct pathways leading to hypoxia-mediated translational inhibition (van den Beucken et al. 2006; Wouters and Koritzinsky 2008). The first is rapid, HIF-1α-independent and mediated by the unfolded protein response. This results in eukaryotic initiation factor 2α (eIF2α) phosphorylation by the endoplasmic reticulum kinase, PERK, leading to the inhibition of mRNA translational initiation. The second, more chronic pathway, is a delayed response that is only activated after prolonged hypoxia and is associated with disruption of the mRNA cap-binding complex, eIF4F, resulting in the inhibition of the transcript recruitment step of mRNA translation. The upstream signaling that leads to eIF4F disruption is unclear and may have both HIF-1α-dependent and -independent components.

DETECTION OF TUMOR HYPOXIA

Tumor hypoxia can be evaluated using a variety of techniques, including direct measurement with oxygen electrode probes, immunohistochemical (IHC) assessment of intrinsic and extrinsic hypoxic biomarkers, and noninvasive radiologic imaging techniques. Determination of the hypoxic fraction of a tumor could lead to improved prognostication and prediction

of response to conventional treatments like surgery, radiotherapy or chemotherapy. In addition, measurements of hypoxia could be used to classify patients according to their suitability for direct hypoxic cell cytotoxins, such as tirapazamine, or molecular inhibitors of pathways activated by hypoxia to counteract tumor progression.

The intrinsic biomarkers of hypoxia include the IHC detection of HIF-1α, VEGF, CAIX and GLUT-1. Additionally, measurement of the plasma levels of the noncollageneous matrix protein, osteopontin, can be used. Osteopontin levels have been associated with decreased survival for cancers of the prostate, lung, and head and neck (Ramankulov et al. 2007; Isa et al. 2009; Snitcovsky et al. 2009). Although intrinsic IHC markers provide information about hypoxia with regards to the tissue architecture, they do not measure absolute oxygen concentrations. CAIX and GLUT-1 have been suggested to be markers of chronic hypoxia, because long exposure to low levels of oxygen is required for up-regulated expression. However, no individual intrinsic marker can differentiate between acute and chronic hypoxia. Furthermore, many intrinsic hypoxia markers, such as CAIX and GLUT-1, are HIF-1α-dependent, which can be problematic as the HIF-1α pathway can be activated by factors other than hypoxia. The most well known case is von Hippel-Lindau (VHL) disease, where loss of VHL leads to constitutive activation of HIF-1α (Latif et al. 1993).

Extrinsic hypoxic biomarkers represent the detection of cellular adducts caused by irreversible hypoxic bioreduction of 2-nitroimidazole drugs (e.g., pimonidazole and EF5) at low pO_2 levels (Ljungkvist et al. 2006). Using antibodies to the bound and reduced biomarkers allows for the assessment of hypoxia using enzyme-linked immunosorbent assay (ELISA), IHC and flow cytometric approaches. The sequential administration of two extrinsic markers within 2–3 h of each other allows for the determination of chronic vs. acute hypoxia; colocalization of the two markers indicates regions of chronic hypoxia, while mismatched staining indicates regions of acute hypoxia (Ljungkvist et al. 2006).

Noninvasive techniques for imaging tumor hypoxia include the use of radiolabeled 2- nitroimidazoles imaged with positron emission tomography (PET; ^{18}F-FMISO, ^{18}F-FAZA, ^{18}F-EF5 and ^{60}Cu-ATSM) or single photon emission computed tomography (SPECT; ^{123}IAZA) to achieve clinically useful signal-to-noise ratios (Tatum et al. 2006). Additionally, functional computed tomography (CT) and blood oxygen-level dependent (BOLD) magnetic resonance imaging (MRI) can provide information about the tumor microenvironment (perfusion, vascular permeability, extracellular volume and hypoxia) as well as detailed anatomical information (Henderson et al. 2003; Buckley et al. 2004; Ljungkvist et al. 2006).

Overall, there is often a poor correlation between different hypoxia markers (i.e., HIF-1α vs. pimonidazole), as many of these markers can be

affected by factors other than hypoxia (Janssen et al. 2002; Hutchison et al. 2004). However, for the study of DNA repair and hypoxia, the use of fluorescent nitroimidazole-based probes allows for correlations between hypoxia and DNA repair protein expression *in situ*.

HYPOXIA AND RADIORESISTANCE

The relative level of oxygen at the time of irradiation determines the efficacy of radiotherapy by limiting the type and number of lethal DNA lesions (e.g., DNA double strand breaks [DSBs]) through altered radiochemistry (see Chapter 4). At partial pressures of oxygen (pO_2) below 10 mmHg, tumor cells can acquire radiobiologic hypoxia, whereby they are up to two to three times more radioresistant than aerobic cells (representing the oxygen enhancement ratio [OER]) (Palcic et al. 1982; Palcic and Skarsgard 1984; Astor 1986). Radiation-mediated DNA damage results from ionizations in or very close to the DNA that produce a radical near the DNA. In the presence of oxygen, the radical may undergo oxidation which fixes, or makes permanent, the damage. Furthermore, in contrast to DNA DSBs, ionizing radiation under anoxic conditions can cause a relatively large increase in the number of DNA protein cross-links (DPCs) compared to aerobic conditions (Zhang et al. 1995). Although re-oxygenation during fractionated radiotherapy is thought to partially offset the hypoxic radioresistance, intratumoral hypoxia is still viewed as an important cause of radiotherapy failure.

HYPOXIA AND CHEMOTHERAPY

Relatively little is known about the repair of DNA damage induced by cytotoxic drugs in a hypoxic milieu, as opposed to that induced by radiation therapy. Overall, mounting evidence suggests that hypoxia confers resistance to many clinically relevant chemotherapeutics (Table 1). This resistance may be mediated by (i) decreased drug action in the absence of O_2 (e.g., bleomycin); (ii) decreased effect of cell cycle dependent agents in poorly proliferating hypoxic cells; (iii) altered pH gradients (leading to altered activity of alkylating agents and antimetabolites); (iv) induction of gene amplification (e.g., methotrexate resistance) and (v) an overall decrease in drug diffusion/delivery to those tumor cells distant from functional vasculature (Minchinton and Tannock 2006).

It is becoming more evident that hypoxic resistance to chemotherapy is a multifactorial problem dependent on the chemotherapeutic in question, the type of tumor, and the degree of hypoxia involved. Intra-tumoral hypoxia causes molecular, cellular, and extracellular changes that are thought to play a role in chemoresistance (Trédan et al. 2007; Rohwer and Cramer 2011). A

Table 1. The effect of hypoxia on DNA damage induction.

DNA Damaging Agentz	Effect of Hypoxia
(i) Ionizing Radiation:	
Single strand breaks	Decreased
Double strand breaks	Decreased
Damaged Bases	Decreased
DNA-DNA Crosslinks	Decreased
DNA-Protein Crosslinks	Increased
(ii) Chemotherapeutics:	
5-Fluorouracil (single base damage)	Decreased
Bleomycin (DSBs)	Decreased
Carboplatin (ICLs)	Variable
Cisplatin (ICLs)	Variable
Etoposide (DSBs)	Decreased
Gemcitabine (DNA intercalation)	Decreased
Ifosfamide (ICLs)	Decreased
Irinotecan (DSBs)	Decreased
Oxaliplatin (ICLs)	Decreased
(iii) Bioreductive Drugs	
Mitomycin C (ICLs)	Variable
Porifiromycin (ICLs)	Increased
Tirapazamine (ICLs)	Increased
PR-104 (ICLs)	Increased
SSBs: single strand breaks DSBs: double strand breaks ICLs: interstrand crosslinks	

practical explanation for chemoresistance is thought to be due to the tenuous and limited blood flow to hypoxic regions of tumors, which results in poor delivery and a diffusion limited supply of cytotoxic agents to hypoxic cells (Durand 2001). Moreover, a diffusion limited supply of oxygen decreases the efficacy of some chemotherapeutics, such as bleomycin, adriamycin, and etoposide, which exert their effect by oxygen radical intermediaries (Teicher 1994). Hypoxia is also known to increase the sustainability and hence the functional activity of HIF-1α, which acts as a transcription regulator for many genes that can facilitate chemoresistance (Liu et al. 2007; Ebbesen et al. 2009). HIF-1α is known to be a transcriptional activator for GLUT-1, which facilitates anaerobic respiration (glycolsis) in hypoxic low nutrient conditions, and CAIX, which helps to maintain a physiological neutral intracellular compartment at the expense of an acidic extracellular compartment (Ebbesen et al. 2009). This acidic extracellular compartment protonates weakly basic chemotherapeutics thereby preventing cellular uptake through the cell membrane (Tannock and Rotin 1989; Gerweck et al. 2006), and as such, confers chemoresistance to drugs like mitoxantrone, doxorubicin, vincrisitine, and vinblastine (Trédan et al. 2007). Another transcriptional role of HIF-1α is its ability to increase the expression of drug efflux pumps, such as P-gp (Liu et al. 2007), which actively expel chemotherapeutics such as doxorubicin from the intracellular compartment

into the acidic extracellular compartment, where protonation prevents re-uptake into the cell (Mellor and Callaghan 2011). Finally, HIF-1α has been implicated in the suppression of apoptosis by shifting the balance of pro-apoptotic signals in favour of anti-apoptotic signals, allowing cells to survive DNA-damaging agent insults that would normally be catastrophic (Erler et al. 2004; Liu et al. 2007).

Most cytotoxic drugs exert their tumoricidal effect by causing DNA damage, such as inter/intrastrand DNA cross-links, single strand and double strand DNA breaks, as well as single base damage; all of which are repaired by separate but overlapping DNA repair pathways (see Chapter 1). It has been reported that hypoxia confers chemoresistance to DNA damage induced in hypoxic conditions by cisplatin, etoposide, bleomycin, 4-OOH-ifosfamide, carboplatin, paclitaxel, gemcitabine, oxaliplatin, irinotecan, and mitomycin-C (Koch et al. 2003). Although these findings are intriguing, the inter-experimental variability in hypoxic conditions, cell lines interrogated, and chemotherapeutics used do not allow general conclusions to be drawn, and further experiments are needed to elucidate the role of hypoxia and the repair of chemotherapy induced DNA damage.

Tumor hypoxia has also been viewed as a potential target for chemotherapy and there has been much interest in the development of hypoxia-targeting drugs such as tirapazamine, AQ4N and PR-104 (Brown and Wilson 2004; Tredan et al. 2009; Guise et al. 2010).

INCREASING TUMOR OXYGENATION

Multiple strategies for overcoming tumor hypoxia have been studied to hopefully circumvent hypoxia-associated radioresistance (Overgaard and Horsman 1996). For example, hyperbaric oxygen (HBO) therapy has been used to increase tumor oxygenation. HBO therapy involves inhalation of 100% O_2 at a pressure of at least 1.5 atmospheres absolute (150 kPa). This effectively causes O_2 to dissolve in blood plasma for delivery to tissues independent of haemoglobin (Mayer et al. 2005). Conflicting reports as to the benefit of HBO treatment with radiotherapy have been published for carcinomas of the cervix, with an early report showing a significant benefit (Watson et al. 1978), whereas a subsequent study showed no therapeutic benefit with increased morbidity (Dische et al. 1999). A similar study in bladder carcinomas showed that HBO and radiotherapy was no better than the radiosensitizer misonidazole plus radiotherapy (Hoskin et al. 1999). A recent review of 19 randomized HBO clinical trials concludes that there is reduced mortality for head and neck cancers at 1 and 5 years after therapy and improved local tumor control at 3 months (Bennett et al. 2008). While there maybe some benefit of HBO for certain tumors, early clinical trials

had practical difficulties, namely with the simultaneous application of HBO and radiotherapy (Haffty et al. 1999a,b). Furthermore, increased normal tissue side effects have been noted and partially negate the increased tumor control (Dische 1991; Bennett et al. 2008).

Another approach is a schedule of accelerated radiotherapy to overcome tumor cell proliferation, carbogen (95% O_2, 5% CO_2) to overcome diffusion-limited hypoxia, and nicotinamide (a vasoactive agent) to minimize capillary bed shutdown and thereby reduce perfusion-related acute hypoxia (Rojas 1992; Kaanders et al. 2002a). Phase II trials for this combined approach, called ARCON, have been completed for treatment of advanced bladder (Hoskin et al. 2009) and head and neck cancers (Kaanders et al. 2002b). Historic comparisons with other studies indicate good local and regional control of both cancer types after ARCON radiotherapy with an acceptable level of normal tissue side effects.

An alternative approach has attempted to increase the haemoglobin levels and thus the O_2 carrying capacity of blood in the hopes of increasing tumor oxygenation. Red blood cell transfusions can quickly increase the O_2 capacity of blood, but this effect is transient and carries inherent risks including exposure to infectious agents and transfusion-related acute lung injury (Barbara 2004; Looney et al. 2004). Several phase III trials of head and neck cancer patients have been conducted to determine the benefit of adding erythropoietin (EPO, a cytokine that stimulates red blood cell production) to radiotherapy (Henke et al. 2003; Machtay et al. 2007). However, results from these trials were generally considered negative and a meta-analysis of 5 randomized controlled trials indicated that radiotherapy plus EPO has a negative influence on outcome compared to radiotherapy alone (Lambin et al. 2009). The reasons for the poor outcome are unclear, but may be a result of increased cardiovascular (thrombotic) events due to stimulation of tumor cells with EPO receptors.

All of these approaches are based on the concept that hypoxic tumor cells are 2–3 fold more radioresistant than aerobic cells (Palcic et al. 1982; Palcic and Skarsgard 1984; Astor 1986). However, our recent results indicate that not all hypoxic cells will respond to ionizing radiation in the same manner as will be discussed later.

HYPOXIC DOSE PAINTING

As hypoxic tumor cells are radioresistant, it seems reasonable to simply deliver more radiation dose to hypoxic sub-regions of human tumors. With the advent of intensity modulated radiotherapy (IMRT) and non-invasive hypoxic imaging techniques, this approach is being studied to counteract hypoxic radioresistance (Lee et al. 2008; Lin et al. 2008). The feasibility of

such an approach has been demonstrated in a pilot study in which hypoxic tumor regions were identified by ^{18}F-FMISO PET imaging and given a 20% increased dose with IMRT, while keeping the organs at risk at the same tolerance levels (Lee et al. 2008). However, serial ^{18}F-FMISO PET hypoxia scans separated by 3 days revealed significant temporal heterogeneity in the regions that were hypoxic (Lin et al. 2008). Thus, in order to fully utilize this approach with fractionated radiotherapy, daily PET imaging prior to each fraction of radiation would be required; but unfortunately, this is currently not logistically feasible. Furthermore, if hypoxic cells have differential biology, radiosensitivity and/or DNA repair, these approaches will not be sufficient to predict clinical ionizing radiation dosing regimens with accuracy.

HYPOXIC CELL CYTOTOXINS

The development of drugs designed to exploit tumor hypoxia has focused on prodrugs that are activated by metabolic reduction under hypoxic conditions to form cell cytotoxins uniquely in hypoxic tumor cells (Brown and Wilson 2004). The first generation of these drugs were quinine bioreductive drugs such as mitomycin-C and porfiromycin (Rockwell et al. 1982; Haffty et al. 2005). However, there has been difficulty in evaluating mitomycin-C clinically as a hypoxic cytotoxin due to its significant activity under aerobic conditions. Preclinically, the mitomycin-C analogue porfiromycin has demonstrated an improved bioreductive profile and less systemic toxicity than mitomycin-C (Durand and Olive 1992). Unfortunately, a prospective randomized trial with porfiromycin showed no advantage compared with mitomycin-C, and subsequently, interest in this compound has faded (Haffty et al. 2005).

Currently *N*-oxides, such as tirapazamine, are the best studied prodrugs (Zeman et al. 1986; Rischin et al. 2005; Evans et al. 2008). Under hypoxic conditions, tirapazamine undergoes intracellular one-electron reduction to a radical anion that is converted to either a highly toxic hydroxyl radical or to an oxidizing radical by the elimination of water (Baker et al. 1988; Anderson et al. 2003; Zagorevskii et al. 2003). These radicals cause DNA SSBs, base damage and DPCs, which can stall and collapse replication forks, which in turn are resolved by the homologous recombination (HR) pathway (see Chapter 14). Consequently, HR-deficient cells are more sensitive to tirapazamine (Evans et al. 2008). In preclinical studies, tirapazamine is 15- to 200-fold more cytotoxic under hypoxia compared to aerobic conditions (Zeman et al. 1986). The randomized phase II and III tirapazamine studies completed to date have shown mixed tumor responses, while frequently having increased normal tissue toxicity (von Pawel et al. 2000; Rischin et al.

2005; Williamson et al. 2005). However, a recent study using ^{18}F-FMISO PET imaging to detect tumor hypoxia has provided the first clinical evidence that tirapazamine can be efficacious in patients with hypoxic tumors. Specifically, there was a striking improvement in locoregional control in patients with hypoxic tumors treated with a tirapazamine-containing regimen (Rischin et al. 2006). Focus has now shifted to newer *N*-oxides, such as AQ4N, which shows improved tumor penetration in preclinical and phase I clinical studies (McKeown et al. 2007; Albertella et al. 2008; Williams et al. 2009).

Recently, a new class of hypoxia-activated prodrugs has been identified. These dinitrobenzamide mustards (DNBM) contain a latent nitrogen mustard moiety, which becomes activated when either of the nitro groups is reduced to the corresponding hydroxylamine or amine. This results in the selective generation of reactive nitrogen mustard metabolites that induce DNA cross-linking in hypoxic cells (Helsby et al. 2003). PR-104, a novel DNBM currently in clinical trials, has shown great promise in preclinical studies and holds several advantages over other bioreductive drugs such as tirapazamine. First, its activation is confined to lower oxygen concentrations allowing for greater specificity, and second, its activated metabolites are able to diffuse locally in tumor tissue, providing an efficient bystander effect. Whether this compound can also preferentially sensitize repair-deficient cells is as yet unknown.

Overall, there has been limited success with existing strategies in dealing with the problem posed by hypoxia-mediated therapeutic resistance. This may be a result of improper identification of patients with hypoxic tumors that would benefit the most from these treatments due to altered gene expression, oxygenation and DNA repair.

HYPOXIA AND DNA REPAIR

In addition to altered oncogenesis and angiogenesis, hypoxia can modify cell cycle checkpoints and DNA repair. DNA damage-associated checkpoints normally protect against carcinogenesis by allowing for reduced cell proliferation as a means to repair potentially mutagenic or lethal DNA damage (see Chapters 12 and 13). Severe hypoxia or anoxia can lead to an S phase arrest in the absence of DNA damage, while subsequent re-oxygenation increases ROS production and DNA damage to trigger a CHK2-dependent G_2 arrest (Hammond et al. 2002, 2003; Freiberg et al. 2006; Bencokova et al. 2009).

Hypoxia can also alter the expression and function of DNA repair proteins (Mihaylova et al. 2003; Bindra and Glazer 2007b; Chan et al. 2008). Residual or misrepaired DNA breaks can be carcinogenic, potentially

leading to chromosomal deletions, translocations and rearrangements in the affected cell (Bristow and Harrington 2005).

HYPOXIA AND MISMATCH REPAIR

DNA mismatch repair (MMR) is responsible for recognizing and repairing insertion/deletion loops and mis-incorporated bases that arise as a result of replication errors that escape the proofreading function of DNA polymerases (Harfe and Jinks-Robertson 2000; Hsieh and Yamane 2008) (see Chapter 7). In eukaryotes, MMR begins with the recognition of the mismatch by the MutS complex. MutS then works in concert with the MutL complex to facilitate endonucleolytic cleavage by a third MMR protein, PMS2. Single strand exonucleases resect the DNA allowing for DNA polymerase δ (POLD) and Ligase I to repair the resulting DNA gap (Hsieh and Yamane 2008). Consequently, loss of functional MMR leads to microsatellite instability (MSI) and a mutator phenotype (Wu et al. 2000; Umar et al. 2004).

Suppression of the MMR pathway by hypoxia has been previously documented with specific down-regulation of the MMR proteins MLH1, MSH2 and MSH6, leading to genomic instability (Mihaylova et al. 2003; Koshiji et al. 2005; Shahrzad et al. 2005; Rodriguez-Jimenez et al. 2008). Several mechanisms for the decreased gene expression have been proposed. Koshiji et al. reported that the altered expression of MSH2 was associated with hypoxic up-regulation of HIF-1α transcription factor, which displaced c-MYC from the *msh2* promoter in a p53-dependent manner (Koshiji et al. 2005). Nakamura and colleagues suggested that hypoxia-induced DEC1/2 may impair MMR function through repression of MLH1 expression, possibly via a histone deacetylase-mediated mechanism in cancer cells (Nakamura et al. 2008). Other work has demonstrated that the repression of MLH1 and MSH2 occurs via a HIF-1α-independent shift in occupancy from activating c-MYC/MAX to repressive MAD1/MAX and MNT/MAX complexes at the proximal promoters of both genes (Bindra and Glazer 2007a).

Based on data from germline or somatic loss of MMR gene expression, MMR-deficient hypoxic cells would be expected to be more sensitive to topoisomerase poisons such as camptothecin and etoposide (Jacob et al. 2001), as well as to certain alkyating agents such as 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (Aquilina et al. 1998) and mitomycin-C (Fiumicino et al. 2000; Helleday et al. 2008). Conversely, MMR defects can also impart resistance to many common chemotherapeutic agents, including DNA minor grove binders (Fedier et al. 2003), antimetabolites such as 6-thioguanine (Aebi et al. 1997), certain alkylating agents such as temozolomide (Pegg 1990), and certain platinum compounds such as cisplatin (Drummond et al. 1996). Therefore, the functional effects of hypoxia on MMR gene expression

and consequences for tumor cell radiosensitivity and chemosensitivity requires further study, as this may direct individualized cancer therapy.

HYPOXIA AND NUCLEOTIDE EXCISION REPAIR

Nucleotide excision repair (NER) is an important DNA repair pathway responsible for the removal of helix-distorting DNA adducts, including ultraviolet (UV)-induced cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4PPs) (see Chapter 9). Failure to properly deal with these bulky DNA lesions is the underlying cause of sunlight-induced skin cancer (Melnikova and Ananthaswamy 2005).

There are two NER sub-pathways, global genomic NER (GG-NER) and transcription-coupled NER (see Chapter 1). GG-NER removes bulky DNA adducts from anywhere in the genome, whereas transcription-coupled NER removes DNA damage exclusively from the transcribed strand of active genes (Gillet and Scharer 2006; Fousteri and Mullenders 2008). GG-NER is initiated by the UV-DDB1/UV-DDB2 and XPC/HR23B heterodimers, which bind to the damaged site when they recognize the helical distortion caused by the bulky adduct. The damage is repaired by a process of strand unwinding, strand incision, lesion excision and finally gap filling (Gillet and Scharer 2006). Transcription-coupled NER only differs from GG-NER in the way the lesion is recognized. Instead of recognizing the helical distortion, transcription-coupled NER is triggered by blockage of RNA polymerase II at the damaged site along the transcribed strand (Fousteri and Mullenders 2008).

Very little is known about the effect of hypoxia on NER. Rezvani et al. has recently reported that HIF-1α transcriptionally regulates the expression of two NER proteins, XPC and XPD, in keratinocytes by binding to hypoxia responsive elements within their promoters (Rezvani et al. 2010). Additionally, the NER protein RAD23B has been reported to be down-regulated under hypoxia through a mechanism involving HIF-1α-dependent activation of miR-373 (Crosby et al. 2009). Two contradictory reports have been published using a host cell reactivation (HCR: NER-dependent repair of a UV-damaged plasmid) reporter assay to measure functional NER. Yuan et al. first showed that hypoxia combined with low pH (24 h x 0% O_2 + pH 6.5) decreased HCR of a UV-damaged plasmid encoding the luciferase gene (Yuan et al. 2000). A more recent study found increased repair under conditions of hypoxia (12–24 h x 1% O_2) or hypoxia + low pH (pH 6.5) of a UV-damaged adenovirus harboring a *lacZ* reporter gene (Dregoesc and Rainbow 2009).

Recently, two novel assays has been developed to evaluate the kinetics of NER (Rouget et al. 2008; Orelli et al. 2010). Both assays detect UV-induced

DNA photoproducts by staining with antibodies against CPDs or 6-4PPs; however, one method uses flow cytometry based detection (Rouget et al. 2008), while the other uses immunofluorescent microscopy (Orelli et al. 2010). Notably, these techniques can be adapted to measure NER as a function of the cell cycle and have demonstrated that the checkpoint kinase ATR is required for NER exclusively during S phase (Auclair et al. 2008). This may have implications under hypoxic conditions as hypoxia has been shown to activate ATR (Hammond et al. 2003), as well as its partner protein ATM, which is defective in the radiosensitive disorder ataxia telangiectasia (Bencokova et al. 2009) (see Chapter 13). This may explain the increased NER-based HCR reported by Dregosec and Rainbow (Dregoesc and Rainbow 2009).

HYPOXIA AND DOUBLE STRAND BREAK REPAIR

Perhaps the most critical DNA lesions are DSBs, which are primarily repaired by the HR or nonhomologous end-joining (NHEJ) pathways in mammalian cells (Rothkamm et al. 2003; Helleday et al. 2007) (see Chapter 14). DSBs are initially sensed by the MRE11–RAD50–NBS1 (MRN) complex. This leads to activation and recruitment of the ATM and DNA-dependent protein kinase catalytic subunit (DNA-PKcs) kinases, and then phosphorylation of the histone variant H2AX (termed γH2AX) around the site of the break. Subsequently, a number of DNA damage sensing proteins, such as mediator of DNA damage checkpoint 1 (MDC1) and p53-binding protein 1 (53BP1), and DNA DSB repair proteins involved in HR and NHEJ are recruited within the first 1–6 h of damage recognition to repair the DSB.

HR is a template-guided, error-free repair pathway, predominantly operating in the S and G_2 phases of the cell cycle, as it requires a repair template from a sister chromatid or chromosome (Rodrigue et al. 2006; Helleday et al. 2007; Ip et al. 2008). Free DNA ends formed at the site of a DSB are first processed to produce single stranded DNA (ssDNA) with a 3′-hydroxyl overhang through the action of the MRN complex, along with the CtIP, Exo1, BLM and BRCA1 proteins (Ira et al. 2003; Lengsfeld et al. 2007; Mimitou and Symington 2008; Yun and Hiom 2009). Replication protein A (RPA) binds and stabilizes the resulting single-stranded DNA (Sung and Klein 2006). RAD51 displaces RPA to form the nucleoprotein filament with the assistance of BRCA2 (Sharan et al. 1997; Wong et al. 1997; Pellegrini et al. 2002). This nucleoprotein filament subsequently searches for homologous DNA, and when found, facilitates the formation of a D-loop intermediate in which the 3′-end initiates DNA synthesis using the duplex DNA as a template (Weterings and van Gent 2004). The joint DNA molecule (termed a

Holliday junction) can be resolved by the Holiday junction resolvase, GEN1 (Ip et al. 2008), or the BLM-topoisomerase IIIα (Wu and Hickson 2003) or Mus81-Eme1 (Chen et al. 2001; Constantinou et al. 2002) complexes.

In contrast, NHEJ is predominant in G_1, but can act throughout the cell cycle as it does not require a homologous template for repair. NHEJ mediates repair by directly re-joining DNA strands. It involves recognition of the DSB, processing of non-complementary or damaged DNA ends, and the subsequent ligation of DNA termini. Processing of DNA ends can lead to loss or gain of nucleotides, rendering NHEJ less accurate than HR (Weterings and van Gent 2004; Helleday et al. 2007).

Several groups have reported that the expression and function of HR repair proteins, including RAD51, BRCA2 and BRCA1, are compromised under hypoxic conditions (Fig. 3) (Bindra et al. 2005; Meng et al. 2005; Chan et al. 2008). Given the relationship between HR and the cell cycle, it was an important observation that decreased HR gene expression was independent of p53, HIF-1α and cell cycle distribution (Bindra et al. 2005; Meng et al. 2005; Chan et al. 2008). Data pertaining to the function of the NHEJ pathway is more mixed, with reports suggesting it is either unchanged (Meng et al. 2005) or possibly up-regulated (Um et al. 2004) by hypoxia.

An initial model of hypoxia-induced transcriptional repression of HR genes was proposed by Bindra and colleagues (Bindra et al. 2005; Bindra and Glazer 2007b), who showed that the hypoxic down-regulation of RAD51 and BRCA1 is associated with a switch from E2F-based transcriptional activation to that of repression. However, RNA and protein expression of HR genes can be discordant under hypoxia (Meng et al. 2005). Another model invokes translational repression as the basis for decreased HR protein expression (Chan et al. 2008; Wouters and Koritzinsky 2008). Under hypoxia, this translational suppression is controlled through at least two distinct pathways: first, by PERK mediated phosphorylation of eIF2α, which is required for the recruitment of aminoacylated tRNA, and second, by disruption of the mRNA cap-binding complex, eIF4F (Wouters et al. 2004). Nevertheless, specific studies comparing the exact role of transcription and translation in mediating differential protein expression within the MMR, NER and DSB repair pathways in hypoxic versus oxic cells has not yet been reported. Additionally, HIF-1α-dependent activation of miR-210 has been shown to down-regulate the HR protein RAD52 (Crosby et al. 2009).

HR-defective cells are known to be more sensitive to mitomycin-C and cisplatin, suggesting that hypoxia would result in a similar sensitivity. Indeed, Chan and colleagues observed that HR defective hypoxic cells are sensitive to ionizing radiation, cisplatin and mitomycin-C, but not taxanes (Chan et al. 2008). Some studies suggest that hypoxic tumor cells with HR defects may also be more sensitive to etoposide (Treszezamsky et al. 2007). Additionally, HR-defective cells are more sensitive to inhibition of PARP1

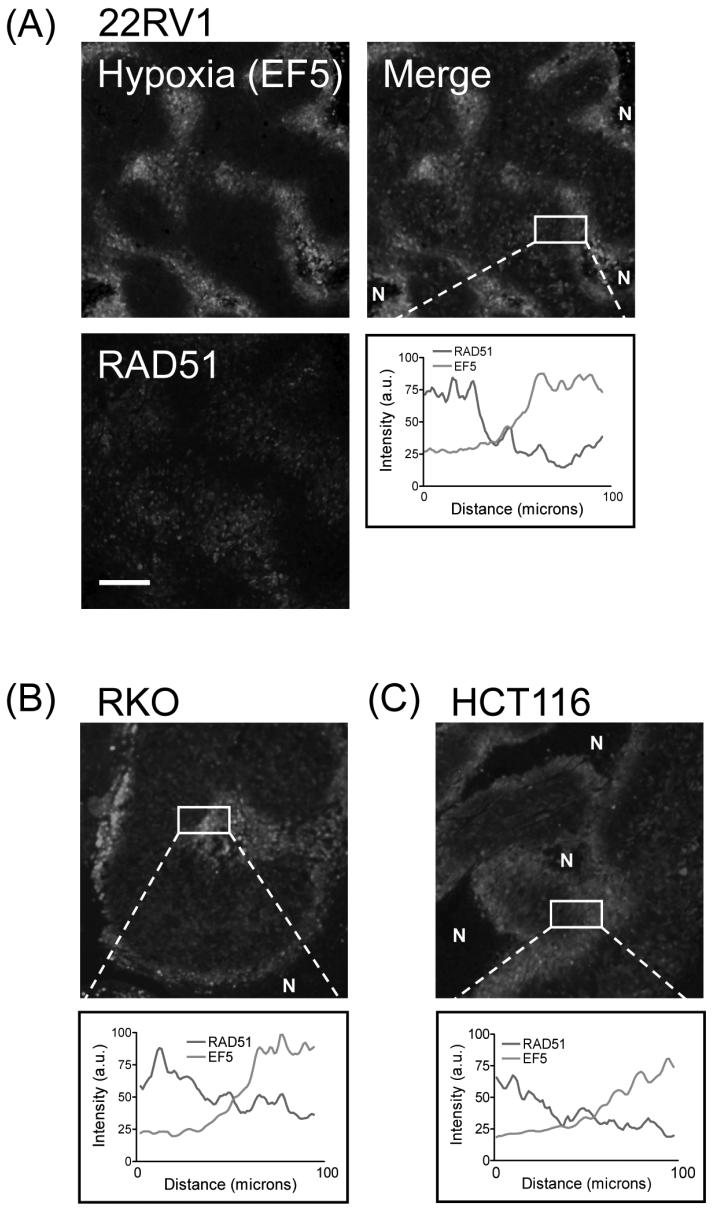

Figure 3. Hypoxia and RAD51 protein expression is inversely correlated *in vivo*. Representative images from (A) 22RV1, (B) RKO and (C) HCT116 xenografts stained for hypoxia (EF5-green) and RAD51 (red). Line intensity profile shows inverse association between the hypoxic marker EF5 and the HR protein RAD51. Scale bar represents 100 microns. N denotes necrotic regions.

Color image of this figure appears in the color plate section at the end of the book.

due to synthetic lethality (Bryant et al. 2005; Farmer et al. 2005; Hegan et al. 2010), and this special case in relation to hypoxic cells is discussed in detail below.

Data pertaining to the function of the NHEJ pathway indicate that it is either unchanged (Meng et al. 2005) or possibly up-regulated (Um et al. 2004) by hypoxia. NHEJ is the primary repair pathway for DNA DSBs caused by ionizing radiation and cells deficient in NHEJ are exquisitely sensitive to ionizing radiation. However, Chan et al. determined that cells pretreated with hypoxia are only moderately sensitive to ionizing radiation, consistent with an HR defect, but not necessarily a NHEJ defect (Chan et al. 2008). The functional capacity of NHEJ under hypoxia warrants further study.

Fanconi Anemia (FA) is a genetic disease often resulting in cancer and is caused by a defect in a cluster of proteins involved in the resolution of stalled replication forks (see Chapter 10). Kuhnert and colleagues have recently reported that FANCD2-deficient fibroblasts are hypersensitive to radiation under hypoxic conditions (Kuhnert et al. 2009). This may explain the discrepancy between the clinical and cellular radiosensitivity of FA patients and further studies are needed to clarify FA protein expression and function under hypoxic conditions.

TARGETING HYPOXIA-INDUCED REPAIR DEFECTS: "CONTEXTUAL SYNTHETIC LETHALITY"

Synthetic lethality is the concept that mutation in two genes leads to death, whereas mutation of either gene alone is compatible with viability (Kaelin 2005). For example, cells with defects in the HR pathway can be preferentially sensitized to inhibitors of the SSB repair protein PARP1 (Bryant et al. 2005; Farmer et al. 2005; McCabe et al. 2006; Fong et al. 2009; Kuhnert et al. 2009; Annunziata and O'Shaughnessy 2010; Plummer 2010; Redon et al. 2010). Tumor cells exposed to chronic hypoxia leading to an HR defect have increased sensitivity to PARP inhibitors (Chan and Bristow 2010; Hegan et al. 2010). The use of PARP inhibitors to target hypoxic tumor cells is an example of "contextual synthetic lethality", where a hypoxia-induced repair defect is targeted by inhibiting or disrupting the backup (or compensatory) pathway. Inhibition of PARP1 results in the accumulation of SSBs, leading to collapsed replication forks in S-phase as the cell attempts to undergo DNA replication. The collapsed replication forks can be resolved through HR using BRCA2-dependent pathways. Therefore, cells with defects in both SSB repair and HR undergo synthetic lethality, but cells with either defect alone survive (Fig. 4). This concept has already shown promise in clinical trials using PARP inhibitors specifically for BRCA1/2-deficient tumors (Fong et al. 2009, 2010) (see Chapter 15).

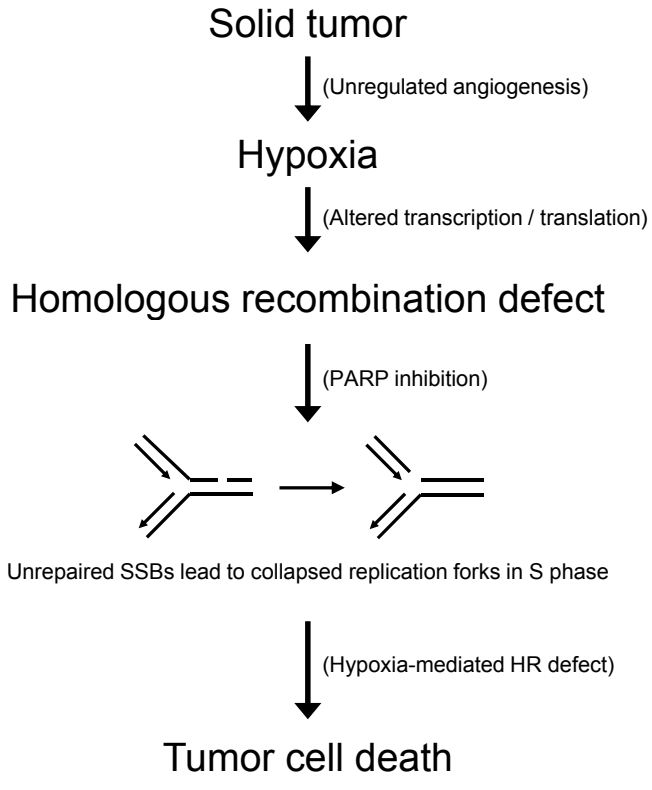

Figure 4. Model for hypoxia-mediated contextual synthetic lethality with PARP inhibition. Solid tumors have substandard vasculature leading to gradients of moderate to severe hypoxia. Hypoxia also decreases HR capacity by altering the transcription and translation of HR genes. PARP inhibition results in unrepaired single strand breaks (SSBs) which collapse replication forks in S phase. These collapsed replication forks are lethal to tumor cells with hypoxia-induced HR defects.

This approach has significant therapeutic potential as highly potent and selective PARP inhibitors have already shown clinical effectiveness in treating BRCA-deficient tumors (Fong et al. 2009). It therefore seems reasonable to take advantage of deficiencies in DNA repair to kill hypoxic cells, which have been shown to acquire a repair-deficient and mutator phenotype. This would still preserve the therapeutic ratio as very few normal tissues contain hypoxic cells.

A caveat to this approach is the requirement for proliferation, as PARP inhibitors mediate their toxicity by inducing collapsed replication forks (Bryant et al. 2005). It has been previously demonstrated that tumor cells can have hypoxia-mediated decreases in DNA repair protein expression at moderate levels of hypoxia that still allow for proliferation (Chan et al.

2008). Therefore, hypoxic tumor cells at an intermediate distance from the blood vessels would theoretically still be sensitive to a contextual synthetic lethality approach. This is a testable hypothesis using bromodeoxyuridine staining to detect proliferating cells, EF5 staining to detect hypoxic cells, RAD51 staining to detect HR-deficient cells and γH2AX staining to detect DNA damage/cell death.

Recently, deficiency in the MMR proteins MSH2 and MLH1 was shown to be synthetically lethal with disruption of the DNA polymerases β (POLB) and γ (POLG), respectively (Martin et al. 2010). As discussed earlier, both of these MMR proteins are known to be down-regulated by hypoxia, and therefore, inhibition of POLB or POLG may show contextual synthetic lethality with hypoxia. At the moment, clinically useful inhibitors of POLB or POLG are not yet available, but given the strong inhibition of MSH2 and MLH1 by hypoxia, this is a concept that warrants further study. Another example of potential contextual synthetic lethality is the observation that the FA pathway can be compromised under hypoxic conditions (Kuhnert et al. 2009), and FA defective cells are more sensitive to ATM inhibitors (Kennedy et al. 2007). Table 2 summarizes known hypoxia-induced DNA repair defects and agents that may potentially have synthetic lethality or increased efficacy under hypoxic conditions.

Table 2. Summary of hypoxia-mediated DNA repair defects and chemotherapeutic agents with theoretic potential for increased efficacy.

Hypoxia-mediated DNA repair defect	Class of chemotherapeutic agent with potential for increased efficacy	Example
Homologous recombination	Bifunctional alkylators	MMC, cisplatin
	Monofunctional alkylators	Temozolomide
	Ionizing radiation	Gamma irradiation
	Radiomimetics	Bleomycin
	Topoisomerase inhibitors	Etoposide
	PARP inhibitors	ABT-888, AZD2281
Base excision repair	Ionizing radiation	Gamma irradiation
	Monofunctional alkylators	MMS, Temozolomide
Mismatch repair	Topoisomerase inhibitors	Etoposide, camptothecin
	Bifunctional alkylators	MMC, CCNU
	DNA polymerase inhibitors	POLB and POLG inhibitors

HYPOXIA AND "CONTEXTUAL LOSS OF HETEROZYGOSITY"

We propose that tumor hypoxia may drive malignant progression and possibly carcinogenesis through a model of "contextual LOH" for DNA repair genes. Instead of an inactivating mutation, contextual LOH could

occur by hypoxia-mediated loss of expression/function of one allele of a DNA repair gene in which the other allele is already inactivated by genetic deletion, mutation or hypermethylation. If the gene in question is a tumor suppressor gene involved in DNA damage checkpoint control (e.g., ATM, ATR, Rb, p53 or MDM2) or a critical DNA repair protein (e.g., PARP1, DNA-PKcs, BRCA1 or BRCA2), malignant transformation or progression may result. In fact, we have documented monoallelic losses for a number of DSB and SSB repair genes in prostate cancer, a tumor in which hypoxia is known to be a negative predictive factor (Chan et al. 2007; Vergis et al. 2008; Ishkanian et al. 2009). This model could also be tested for colorectal cancer, where regions of hypoxia have been documented in normal mucosa, benign adenoma and carcinomas (Greijer et al. 2008). Germline mutations in *mlh1* or *msh2*, two genes known to be suppressed by hypoxia, are linked to hereditary nonpolyposis colorectal cancer (HNPCC) (Jacob and Praz 2002). Furthermore, accumulation of K-*ras* mutations (a common alteration in colorectal cancer) has been correlated with hypoxia-induced decreases in MSH2 expression (Shahrzad et al. 2005). Thus, it is conceivable that colorectal cells with only one normal allele of *mlh1* or *msh2* could have further reduced functional protein expression under hypoxic conditions. This could ultimately drive genetic instability, carcinogenesis and tumor progression. A similar biology could underlie hypoxic modification of NER status and UV- or carcinogen-induced skin cancers (Bedogni and Powell 2006; Michaylira and Nakagawa 2006; Cannito et al. 2008; Simiantonaki et al. 2008). This hypothesis will require testing of the effect of hypoxia on carcinogenesis and tumor progression using isogenic models which are wild type, heterozygous or homozygous-null for DNA repair gene expression and function.

CONCLUSIONS AND IMPLICATIONS FOR CANCER THERAPY

Moderate to severe hypoxia is predominately found only in solid tumors. The limited normal tissues with regions of significant hypoxia, as measured with staining with the hypoxic marker EF5, are the epidermis and portions of some sebaceous glands and hair follicles (Evans et al. 2006). The prolonged hypoxia found in solid tumors can lead to decreased DNA repair protein expression and function. These findings may allow for novel strategies that target repair defects and chronic hypoxia. The therapeutic ratio is favoured in this approach, given that aerobic normal tissues would be repair-proficient. In addition, hypoxic malignant tissues may have decreased cell cycle checkpoint control following DNA damage, which can augment the cytotoxic effects of the repair-deficiency (Choudhury et al. 2006). Furthermore, many late-reacting normal tissues are non-proliferating, and

because the cells are in G_1 phase, use NHEJ to repair DSB DNA damage. Conversely, due to an increased fraction of cells in the S and G_2 phases, HR may be the preferred DNA DSB repair pathway in malignant cells. Targeting HR may therefore allow for relatively selective killing of tumor cells. This strategic approach could include the use of DNA cross-linking agents, such as mitomycin-C and cisplatin, or molecular targeted agents, such as PARP inhibitors; all of which can preferentially sensitize HR-deficient cells (Table 2) (Bristow et al. 2007).

Beyond HR, it is now known that hypoxia can also suppress the MMR pathway (Mihaylova et al. 2003). MMR defects can render tumors sensitive to certain topoisomerase poisons, such as camptothecin and etoposide (Jacob et al. 2001), as well as to certain alkyating agents, such as 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (Aquilina et al. 1998) and mitomycin-C (Fiumicino et al. 2000). Conversely, MMR defects can impart resistance to many common chemotherapeutic agents, including DNA minor groove binders (Fedier et al. 2003), antimetabolites such as 6-thioguanine (Aebi et al. 1997), certain alkylating agents such as temozolomide (Pegg 1990), and certain platinum compounds such as cisplatin (Drummond et al. 1996).

Fractionated radiotherapy is a widely used and relatively successful cancer therapy and is thought to maintain the therapeutic ratio by differential cellular repair between malignant and normal tissues (Bristow et al. 2007). Traditionally, hypoxic cells have been documented as being extremely radioresistant. Oxygen enhances the biological effects of radiation by interacting with and "fixing" DNA damage, following the indirect effect of ionizing radiation in the vicinity of DNA (Brown and Wilson 2004). Acute anoxia generally decreases ionizing radiation-induced DNA DSBs by 2–3 fold, reflecting the OER for this O_2 level. It is believed that this resistance is partially overcome by the process of re-oxygenation during fractionated radiotherapy, leading to improved tumor control. Fractionation allows the initial treatments to destroy the radiation-sensitive, well-oxygenated tumor cells. This allows the hypoxic cells to be resupplied with oxygen, rendering them sensitive once again. Furthermore, these hypoxic cells may be DNA repair-deficient and re-oxygenation would render them even more radiosensitive than tumor cells exposed to a continuous supply of oxygen. Thus, while acutely hypoxic tumor subregions may be highly resistant to treatment, chronically hypoxic tumor subregions may in fact be relatively sensitive to ionizing radiation and treatments targeting hypoxia-mediated repair defects, as compared to acutely anoxic cells (Fig. 5).

A prerequisite for the use of novel therapies or predictors of outcome based on preclinical studies is the ability to predict the fraction of repair-deficient hypoxic cells in solid tumors. One strategy using xenografts could involve using a serial injection of two different hypoxic markers, such as pimonidazole and EF5, in combination with markers of proliferation

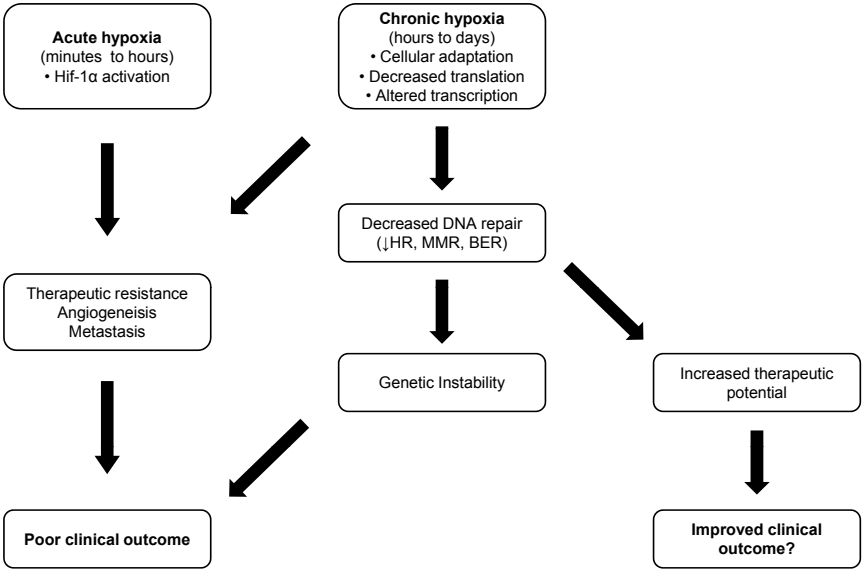

Figure 5. Acute hypoxia can lead to therapeutic resistance, angiogenesis and metastasis resulting in poor clinical outcome. Chronic hypoxia may also contribute to therapeutic resistance and decreased DNA repair leading to genetic instability and poor clinical outcome. However decreased DNA repair may potentially be vulnerable to targeted therapies such as PARP inhibitors to improve clinical outcome.

(e.g., bromodeoxyuridine) and blood vessels as described by Ljungkvist et al. (Ljungkvist et al. 2007). Intratumoral regions that are matched for the two hypoxic markers are chronically hypoxic, and those mismatched for staining are acutely hypoxic. Simultaneous staining for DNA repair proteins (e.g., RAD51) would allow correlation of the hypoxic status to DNA repair protein expression. The relative repair of DNA DSBs could then be tracked as a function of acute and chronic hypoxia following DNA damage and could be correlated to tumor radio- and chemoresponse. If proven, this concept will be clinically feasible when innovative, non-invasive imaging techniques are developed to track both acute and chronic hypoxia during treatment to allow effective intervention with novel therapies, including the use of synthetically lethal approaches.

In summary, the current literature has shown that hypoxic tumor cells can have suppression of the HR, NER and MMR pathways. However, the impact of hypoxia on the NHEJ and base excision repair pathways still requires additional study. Further understanding of the contextual synthetic lethality to these and other DNA damage signaling and repair pathways could define new approaches to chemoprevention and selection of best agents to individualize cancer therapy (see Chapter 16).

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CHAPTER 18

Early Phase Clinical Trials Using DNA Repair Inhibitors: Lessons Learnt

Miranda Payne and *Mark Middleton*[a,*]

INTRODUCTION

A greater understanding of DNA repair pathways offers the tantalizing prospect of the development of novel agents designed to inhibit these repair processes. Cells are reliant on specific DNA repair pathways for their recovery from chemotherapy and radiotherapy-induced DNA damage (see Chapter 1). Hence inhibition of DNA repair should improve the efficacy of current therapies and offer the potential to exploit the genetic differences between normal and malignant cells. It is becoming clear that the successful transition from a promising pre-clinical DNA repair inhibitor to mainstream clinical usage is a process fraught with complexity. In this Chapter, we examine the questions that some recent early phase trials of DNA repair inhibitors have attempted to answer. The limitations inherent in the design of the trials are discussed. We analyze the results, dissect the lessons learnt and address some of the pitfalls in their interpretation. Finally, we offer guidelines for the future design of early phase clinical trials of DNA repair inhibitors.

Oxford Cancer Centre, Churchill Hospital, Oxford, OX3 7LJ, United Kingdom.
[a]Email: mark.middleton@oncology.ox.ac.uk
*Corresponding author

DNA REPAIR INHIBITORS: WHAT'S 'ENOUGH' INHIBITION?

There is increasing interest in agents theoretically capable of inhibiting human DNA repair pathways. Clinical trial design must address their most effective mode of deployment. In this section, we use two therapeutic examples to demonstrate the two roles of DNA repair inhibitors in cancer therapy.

DNA repair inhibitors are increasingly administered in conjunction with the triazene chemotherapeutic agent, temozolomide. In this context, the DNA repair inhibitor is used with the intention of circumventing the cell's resistance to the chemotherapy, which generates a spectrum of cytotoxic DNA lesions. Early phase clinical trials of these combination regimens are thus helping unravel the relationship between anti-cancer DNA damaging agents and DNA repair inhibitors. An alternative mechanism engages the idea that tumour proliferation is driven by particular oncogenic mutations, which cause overactivation of signalling pathways. In this context, it is intended that the DNA repair inhibitor will specifically target the activated pathway, which is necessary for cancer cell survival. Besides combinatorial therapies involving temozolomide, this Chapter also discusses the BRAF inhibitor vemurafenib as an example of a DNA repair inhibitor used in monotherapy.

Temozolomide and Pathways Implicated in its Resistance

Temozolomide is an oral alkyating agent, structurally related to dacarbazine and currently licensed for the treatment of malignant melanoma, anaplastic astrocytomas and malignant gliomas (UK-2011) (see Chapter 5). It spontaneously hydrolyzes to MTIC (methyltriazen-1-yl imidazole-4-carboxamide) (Denny et al. 1994), resulting in a reactive methylating compound capable of methylating DNA at nucleophilic sites. Although constituting just over 5% of the total adducts formed, it is the methylation of guanine at the O^6-position, to produce O^6-methylguanine (MeG), which appears to be responsible for the anti-cancer activity of temozolomide (Middleton and Margison 2003). During subsequent replication, O^6-MeG is incorrectly paired with thymine. The error is detected by the cell's mismatch repair system, but instead of excising the methylated guanine, the thymine opposite is excised and then reincorporated as part of a futile cycle of failed repair. After two rounds of DNA replication (Karran and Bignami 1994), this futile repair ultimately causes apoptotic cell death (D'Atri et al. 1998). Failure to engage apoptosis potentially reduces the efficacy of temozolomide (Hickman and Samson 1999). At least three DNA repair pathways are involved in the repair of damage caused by temozolomide, and hence, are also implicated in resistance to the drug.

O^6-methylguanine-DNA methyltransferase (MGMT)

O^6-methylguanine-DNA methyltransferase (MGMT) is a 'suicide' protein capable of irreversibly accepting the methyl group from DNA onto an internal cysteine residue within its active site (see Chapter 6). This MGMT-mediated resistance reverses the DNA damage caused by temozolomide, yet results in the ubiquitylation and subsequent degradation of the protein (Xu-Welliver and Pegg 2002). Hence, existing cellular levels of MGMT, and the rate of its re-synthesis following alkylating damage, should determine the capacity to repair toxic O^6-MeG adducts. At a cellular level, MGMT depletion increases sensitivity, whilst transfection with MGMT cDNA increases cellular resistance, to temozolomide.

In reality, the relationship between MGMT levels and clinical response to O^6-alkylating agents, such as temozolomide, is highly complex. In some tumours, MGMT expression has been shown to correlate with temozolomide sensitivity. For instance, MGMT is often very low in gliomas (Silber et al. 1999; Pollack et al. 2006), a tumour type that often responds to treatment with temozolomide. Despite this, there is evidence suggesting that the time to tumour progression after treatment may be similar regardless of initial glioma MGMT levels (Silber et al. 1999). In malignant melanoma, the significance of tumour MGMT is even less clear. Significant variability in MGMT levels within metastases from the same patient can exist, even within a single lesion (Egyhazi et al. 1995), and there is no consistent correlation between tumour MGMT levels and sensitivity to O^6-alkylating agents, probably due to the dominance of alternative mechanisms of resistance (see later). Hence, caution must be exercised in the interpretation of pre-clinical studies involving temozolomide and MGMT status, and the design of early phase clinical trials of DNA repair inhibitors must not be over-reliant on results obtained *in vitro*.

At present, the methylation status of the MGMT gene (*mgmt*) promoter is the only molecular marker linked to the sensitivity of a tumour to a specific treatment. *Mgmt* promoter hyper-methylation appears critical for gene silencing (Danam et al. 1999) and has been shown to correlate with loss of MGMT expression in several human tumours (Esteller et al. 1999). In one study of 206 patients with glioblastoma multiforme (GBM), *mgmt* promoter hyper-methylation was shown to be an independent favourable prognostic factor (Hegi et al. 2005). Despite demonstrating an association between promoter hyper-methylation and a statistically significant increase in survival following treatment with radiotherapy plus temozolomide, the study did not explicitly correlate the methylation status of the gene promoter region with cellular MGMT protein expression (Hegi et al. 2005). Nor has the methylation status of the *mgmt* promoter been shown to be of value

in predicting response to temozolomide therapy in patients with other malignancies (Martini et al. 2008; Martinez and Esteller 2010).

DNA base excision repair

Although the formation of O^6-MeG correlates with the cytotoxicity of temozolomide, the majority of methylation actually occurs at sites other than the O^6-position. Resistance to temozolomide may arise from repair of these alternative DNA lesions, >80% of which are *N*-methylated bases. The majority of temozolomide-induced base adducts are repaired by the base excision repair (BER) pathway, a DNA repair pathway involved in the repair of single strand breaks (SSBs) (see Chapter 8). Poly(ADP-ribose) polymerase (PARP, or namely PARP1) cooperates with the BER pathway, detecting DNA damage caused by methylating agents (see Chapter 15). PARP activation and consequent poly(ADP)ribosylation is a normal cellular response to DNA damage. Inhibition of PARP causes accumulation of SSBs and a greater dependence on alternative repair pathways, such as homology-directed repair (reviewed in Chen 2011) (see Chapter 14).

DNA Mismatch Repair

An intact DNA mismatch repair pathway, which is primarily involved in correcting errors made during DNA replication (see Chapter 7), is required for the toxicity of temozolomide (Karran and Bignami 1994). If DNA mismatch repair is dysfunctional, the cell tolerates the alkylation caused by temozolomide and apoptosis is not triggered (Humbert et al. 1999; D'Atri et al. 1986). This appears to correlate with the clinical response to temozolomide. In one study in which tumour samples from astrocytomas were analysed for mismatch repair proteins, patients whose tumours expressed higher levels of mismatch repair proteins were more likely to respond to temozolomide (Friedman et al. 1998).

DNA Repair Inhibitors Used in Combination with Temozolomide

Resistance to chemotherapy often arises due to the action of DNA repair pathways. It would therefore be hoped that inhibition of DNA repair may circumvent these resistance mechanisms and increase the efficacy of chemotherapy. In the design of an early phase clinical trial in which a cytotoxic agent is to be combined with a DNA repair inhibitor, there are two key strategic themes. The optimum scheduling of the regimen is dealt with later in this Chapter. However, the trial design must first address whether the DNA repair inhibitor is merely an adjunct to traditional full-

dose cytotoxic therapy. Alternatively, the inhibitor can form the backbone of therapy, with any additional full-dose chemotherapy administered only if side effects permit.

MGMT inhibitors

Lomeguatrib (6-(4-bromo-2-thienyl) methoxy purin-2-amine) is an orally available, pseudosubstrate inactivator of MGMT. *In vitro*, pre-treatment with an MGMT inhibitor, such as lomeguatrib, results in an increase in the anti-tumour effects of O^6-alkylating agents. For instance, treatment of ovarian cancer cell lines with lomeguatrib has been demonstrated to produce both a reduction in cellular MGMT and an increase in sensitivity to temozolomide (Barvaux et al. 2004). Translating these encouraging pre-clinical results into valid clinical data is proving more problematic.

A first step in the early phase trial design is to establish the safest, most efficacious dose of the DNA repair inhibitor. In the context of regimens in which a DNA repair inhibitor is to be paired with a chemotherapeutic agent, determining the most efficacious dose of an individual component can be problematic. For instance, the combination of lomeguatrib with temozolomide has been studied in a series of Phase I (Ranson et al. 2006; Kefford et al. 2009) and Phase II (Khan et al. 2008; Watson et al. 2009) trials, the results of which demonstrate the interdependence of the two drugs and hence the complexities inherent in selecting the 'ideal' dose of either. In one Phase I trial (Kefford et al. 2009), the dose of lomeguatrib was selected based on a previous Phase II trial (Ranson et al. 2007) with the aim of abolishing tumour MGMT activity. Patients received ten days treatment with lomeguatrib and five days of temozolomide, either at a dose of 75 mg/m^2 or 100 mg/m^2. The higher dose was associated with significantly greater myelosuppresion and a further planned dose escalation never happened. Indeed, the longer the duration of treatment with lomeguatrib, the less temozolomide was administered (Kefford et al. 2009). Within this study, the converse analysis was not undertaken, i.e., starting patients with the highest planned dose of temozolomide (125 mg/m^2) and assessing the tolerability and efficacy of various doses of lomeguatrib. This demonstrates another of the difficulties of early phase trial design for novel DNA repair inhibitors. Often the potential variations in both drug dose and duration are so numerous, and the eligible patients so sparse, that it is unfeasible to design a trial in which the many alternatives are directly paired. Instead, realistic trial design must inevitably rely on the composite picture that emerges from a series of smaller studies. In approaching the design of early phase clinical trials in which DNA repair inhibitors are to be paired with cytotoxic agents, this dilemma is central. Should the chemotherapy be

administered at its 'standard' dose, with the DNA repair inhibitor titrated cautiously upwards? Or is the priority to achieve the maximum possible inhibition of DNA repair processes, adding the chemotherapy only if side-effects present? The answer must be that it is optimal, but not necessarily feasible, to design a complementary pair of trials such that each approach can be assessed on merit.

PARP inhibitors

Inhibition of the BER pathway may sensitize tumour cells to the cytotoxic effects of temozolomide. For instance, inhibition of PARP increases the cytotoxicity of temozolomide in tumour cell lines and augments its anti-neoplastic effects in mouse xenograft models (Calabrese et al. 2003). In one *in vitro* study, mismatch repair deficient human leukaemic and colon cancer cell lines were exposed to temozolomide alone, and to the combination of temozolomide and a PARP inhibitor. The cytotoxicity of the single agent temzolomide was confirmed. Concomitant PARP inhibition markedly potentiated the cytotoxicity of temozolomide (Tentori et al. 2001). In a more recent paper, systemic administration of a PARP inhibitor significantly increased temozolomide's anti-tumour activity in intra-cranial melanoma, glioma and lymphoma human xenografts (Tentori et al. 2003a).

Susceptibility to temozolomide in otherwise resistant cells may be restored by combined administration with a PARP inhibitor. This effect is preserved even in temozolomide-resistant slowly dividing melanoma cell lines (Tentori et al. 2003b; Wedge et al. 1996) and in mismatch repair deficient cell lines. Ideally an understanding of the potential mechanisms of resistance to temozolomide should inform the design of early phase clinical trials in which chemotherapy is to be combined with a DNA repair inhibitor. In reality, translating a theoretical understanding of intracellular pathways into an evidence-based trial protocol often requires far greater scientific knowledge than is currently available. In the Phase I trial of the PARP inhibitor AG014699, escalating doses of the inhibitor were combined with a five day course of temozolomide with the stated aim of establishing the PARP inhibitory dose (PID) (Plummer et al. 2008), arbitrarily defined as >90% inhibition. In fact, the necessary degree of PARP inhibition required to prevent BER, or a PARP-specific DNA damage response for that matter, remains unknown (Plummer et al. 2008).

Whilst the augmentation of the anti-tumour effects of O^6-alkylating agents by PARP inhibitors is the therapeutic intention, concomitant augmentation of haematological toxicity can complicate trial design. In the Phase I trial of the PARP inhibitor olaparib in combination with dacarbazine (Khan et al. 2011), a significantly lower dose of DNA repair inhibitor was selected than

had previously been validated in a trial of olaparib monotherapy (Fong et al. 2009) with the specific aim of minimizing myelosuppression. This caution in trial design can be justified by the salutary results that emerged in the Phase II trial of temozolomide in combination with AG014699 (Plummer et al. 2006). Myelotoxicity far exceeded expectations, illustrating the difficulties inherent in transferring an apparently successful Phase I regimen to the more widely applied Phase II clinical trial.

BRAF Inhibitors

The mitogen-activated protein kinase (MAPK) pathway is an intracellular signalling pathway central to the regulation of cell growth, differentiation and division. V-RAF murine sarcoma viral oncogene homologue B1 (BRAF) kinase plays a critical role within the MAPK signalling pathway. BRAF is a RAS activated serine/threonine kinase commonly mutated in solid tumours, particularly malignant melanoma (>80%) (Platz et al. 2008). For instance, the substitution of glutamic acid by valine at position 600 (V600E) is observed in up to half of metastatic melanomas and causes constitutive BRAF activation, increasing its kinase activity and hence downstream signal transduction (Platz et al. 2008). *In vitro* studies examining the response of melanoma cell lines to BRAF inhibition were encouraging (Calipel et al. 2003) and led to the development of novel therapies designed to inhibit BRAF (BRAFi) in humans, offering the prospect of improved clinical outcomes.

Early phase clinical trials of BRAFi have been promising (reviewed in Arkenau et al. 2011). Vemurafenib (PLX4032) is a reversible ATP-competitive BRAFi with particular potency for the BRAF allele containing the V600E mutation. In a Phase I trial, the recommended dose for the subsequent Phase II trial was defined as the highest dose at which no more than one out of six patients experienced dose-limiting side effects. A twice daily regime was used and was shown to expose patients to relatively constant levels of the drug. Tumour biopsies taken from patients with $BRAF^{V600E}$ after 15 days of therapy demonstrated changes consistent with inhibition of the downstream MAP kinase pathway, and overall, 81% of these patients experienced a partial or complete response to therapy. No patients with melanomas without the $BRAF^{V600E}$ mutation responded (Flaherty et al. 2010). Progression-free survival was significantly improved. The trial was designed to achieve the maximum kinase inhibition possible, within the constraints of patient tolerance. This is a valid approach to the design of early phase clinical trials, particularly with single-agent regimes which do not need to take into consideration the risks of overlapping toxicity profiles.

Understandably, interest is now focussing on whether agents which inhibit BRAF, or proteins downstream in this signalling pathway, can be combined with conventional chemotherapies, potentially even with 'full-

dose' cytotoxics. Phase II trials are underway assessing these agents in combination with dacarbazine or docetaxel in selected patient groups, and it will be revealing to establish the tolerability and efficacy of these resistance modulating agents in the presence of myelosuppressive chemotherapy.

WHAT'S THE OPTIMUM SCHEDULE?

Once a DNA repair inhibitor has been validated in pre-clinical trials, careful thought must be given to the design of the schedule to be evaluated in the early phase clinical trial. This requires an interpretation of the presumed mechanism of action of the agent, in order that its effects may be optimized. Alterations in scheduling can have a profound affect on clinical results.

We return to the example that involves the combination of temozolomide with the MGMT inhibitor lomeguatrib. An initial Phase I established a well tolerated regime of five consecutive days of combined therapy (Ranson et al. 2006). However, when this method was replicated in a randomized Phase II trial involving patients who had already progressed on temozolomide, no benefit from the addition of lomeguatrib was demonstrated (Ranson et al. 2007). It emerged that after lomeguatrib dosing is finished, the recovery of MGMT levels is rapid. In a regime involving five consecutive days of combined lomeguatrib and temozolomide, MGMT levels were recovering sufficiently to allow for the repair of the O^6-MeG lesions. This was happening prior to the completion of the two rounds of DNA replication required for O^6-MeG to cause apoptosis.

This mechanistic explanation provided the rationale for a further Phase I study. which attempted to clarify the optimal scheduling of lomeguatrib in relation to temozolomide. Either a five day course of lomeguatrib or an extended ten- or 14-day course was administered alongside the five days of treatment with temozolomide. The longer the duration of lomeguatrib treatment, the greater the myelosuppression, which limited the total amount of temozolomide that could be administered (625 mg/m^2 with five days of lomeguatrib fell to 375 mg/m^2 with ten days of lomeguatrib) (Kefford et al. 2009). Regardless, the longer duration of lomeguatrib was associated with persistence of DNA damage in the peripheral blood mononuclear cells (PBMCs). In theory, this inhibitor treatment strategy should minimize the chances for the repair of temozolomide-induced damage and hence improve the efficacy of the chemotherapy. In reality, this trial demonstrated no clinical advantage when lomeguatrib was added to temozolomide. Such a finding may reflect the lower total dose of temozolomide administered to patients on the combination regimes, as opposed to those on monotherapy. It is feasible there may be no further therapeutic gain to achieve, as the increase in damage in bone marrow equates to, or outweighs, that produced in the

tumour. Alternatively, the compensatory DNA repair pathways discussed earlier may circumvent much of the damage wrought by temozolomide.

Although the combination of lomeguatrib and temozolomide has been evaluated in both melanoma and colorectal cancers, the results have remained disappointing (Ranson et al. 2006, 2007; Khan et al. 2008). Nevertheless, these trials provide useful lessons in the design of early phase clinical trials. Firstly, encouraging pre-clinical data (Barvaux et al. 2004) may be difficult to translate into a successful early phase clinical trial (Ranson et al. 2006). This disappointment may follow even if the pharmacokinetic and pharmacodynamic data imply the regime is achieving its intended target effects (Kefford et al. 2009). Secondly, an understanding of the mechanism of action of any drug used, and the application of that knowledge, will ultimately permit a far more rational trial design. If a chemotherapy agent, such as temozolomide, is reliant on two rounds of DNA replication to induce cell death, then a regime in which a DNA repair inhibitor is administered only synchronously is flawed from the outset.

THE TUMOUR: ARE BIOPSIES REALLY NECESSARY?

There is increasing drive to tailor a patient's anti-cancer treatments according to the molecular profile of their cancer (see Chapter 16). Clinical trial design must therefore reconcile the need to include adequate numbers of patients to generate meaningful data, with the ambition of selecting therapy according to both tumour biology and patient characteristics.

The design of early phase clinical trials of DNA repair inhibitors requires an approach which differs from the design of large Phase III trials. Establishing the maximum tolerated dose of the agent, and ensuring patient safety, is paramount. The tumour biopsy has two critical roles in the design of early phase clinical trials. Pre-treatment, it can help define the study population. During, or after, therapy, it can provide valuable information on the damage the treatment has caused. Early phase trials often offer the first opportunity to assess the *in vivo* effects of the drug in humans. The biopsy is the most direct method of establishing those effects. However, tumour biopsies can be technically problematic for the clinician and unpleasant for the patient, with many cancers remaining inaccessible unless considerable clinical risk is taken. Attempts to use surrogate markers to predict the response to or assess the effects of DNA repair inhibitors have been largely unrewarding.

One example is the analysis of O^6-MeG levels in PBMCs as a measure of MGMT inactivation. This Chapter has already described why the MGMT status of both malignant and normal tissue can act as a prognostic marker for tumour resistance to alkylating agents, such as temzolomide, in specific

cancers. However, obtaining tissue for analysis is often problematic and can complicate the design of early phase clinical trials, both practically and ethically. Much interest was therefore generated by the possibility of using O^6-MeG levels in PBMCs as a peripheral marker of MGMT inactivation, with the hope that this would correlate with clinical response. Unfortunately, despite the clinical convenience, MGMT levels in PBMCs do not consistently correlate with therapeutic outcome (Watson et al. 2009).

Similarly, in a Phase I trial of lomeguatrib, it was hoped that the use of PBMC MGMT levels would provide evidence in support of PBMC MGMT as a surrogate marker for the effects of lomeguatrib. Indeed, MGMT levels in PBMCs were preferentially used for defining an MGMT-depleting dose (MDD) of lomeguatrib for subsequent clinical trials, as opposed to the more usual Maximum Tolerated Dose (MTD) (Ranson et al. 2006). However, during the Phase II trial, it became evident that the MDD of lomeguatrib did not achieve the intended DNA repair inhibition. Residual MGMT activity could be detected in melanoma biopsies taken from patients shortly after completing their treatment and the lomeguatrib dose was consequently doubled. Hence, there is currently little evidence in support of the use of surrogate markers for the response to DNA repair inhibitors.

Indeed, there is strong evidence in favour of a biopsy-driven trial design. The analysis of somatic mutations in melanoma tumour biopsies in the early phase clinical trial of vemurafenib (discussed earlier) proved critical in validating the mechanism of action of the drug. Whilst 81% of patients with $BRAF^{V600E}$ mutation responded to treatment with vemurafenib, no patients without the mutation responded (Flaherty et al. 2010). Additionally, in seven patients, the tumours were re-biopsied after 15 days of treatment and the effects of the drug, i.e., down-stream in the MAPK signalling pathway, were apparent. These results starkly emphasise the need, not only for biopsy-driven early phase clinical trials, but for biopsy-driven therapy in melanoma. The appropriate categorization of melanomas, according to their tumour biology, must be a driving force in the future care of these patients. This is further emphasised by the fact that mutant-selective BRAFi have been reported to induce paradoxical activation of the MAP kinase pathway (Hatzivassiliou et al. 2010; Poulikakos et al. 2010). This poses the theoretical risk that, if there is failure to establish the genetic profile of the tumour, the therapy may actually exacerbate the cancer.

So should the design of early phase trials of DNA repair inhibitors place even greater reliance on the assessment of tumour biopsies? If the use a PARP inhibitor is proposed, would it be valid to select only those patients whose tumours express high levels of PARP? There is indeed increasing interest in defining the molecular biology of the tumour in more detail and in using this information in clinical trial design. However, considerable caution is urged with this approach. Many of the documented 'mechanisms

of action' of DNA repair proteins, and their inhibitors, at best represent gross over-simplifications and at worst may simply be wrong. Whilst potential targets may have a documented association with malignancy, we have few for which direct evidence of oncogenicity exists. For instance, although PARP appears to participate in BER, additional roles in the repair of DNA double strand breaks (DSBs) or cell cycle control have been proposed. Results from the various PARP 'inhibitors', which have previously been used in clinical trials, vary to a degree that is sufficient to imply their actions cannot be identical (reviewed in Calvert and Azzariti 2011). We would argue that our understanding of the DNA repair pathways and their complex interactions remains inadequate for trial design to be dictated solely by results from tumour biopsies.

THE PATIENT: WHOM TO INCLUDE?

The ultimate aim is that cancer treatment is individualized for each patient. Increasingly, clinical trials are defining their patient population with greater specificity. The hope is that these enriched patient populations will reveal the potentially modest gains that many DNA repair inhibitors are likely to offer.

We have already established that somatic mutations within the tumour are increasingly critical in the design of early phase clinical trials. Thus, in the very near future, the choice of treatment for melanoma is likely to be dominated by the BRAF status of the tumour, at the very least. However, there is growing evidence that patient factors are also important. We have described earlier the role of PARP in the BER pathway. Inhibition of PARP places a greater reliance on alternative DNA repair pathways, including homologous recombination (HR). The DNA repair protein BRCA2 is essential for HR (Yang et al. 2002), amongst other roles (Powell et al. 2002). Hence, it was predicted that patients known to be deficient in BRCA2 would prove hypersensitive to inhibitors of PARP. This has been shown to be the case and is being clinically exploited (Amir et al. 2010), the so-called 'synthetic lethal' approach to cancer therapy (see Chapters 14 and 15). To complicate the matter, it has recently been described that BRCA2 deficiency is associated with hyperactivation of PARP (Gottipati et al. 2010). An extrapolation of this would be the development of a method to allow screening of patients to assess the functionality of their HR pathway. Indeed, Gottipati et al. proposed that poly(ADP-ribose) polymers formed in PARP deficient cells offers a putative biomarker for defective HR (Gottipati et al. 2010). Many other mutations are likely to exist that result in defective repair of DSBs and therefore may potentially exhibit hypersensitivity to PARP inhibitors.

Is our understanding of DNA repair pathways sufficient to permit trial inclusion criteria, which place stringent restraints on patient selection? This Chapter has already described how hyper-methylation of the *mgmt* promoter is predictive of response to temozolomide therapy in patients with GBM (reviewed in Martinez and Esteller 2010). However, the relationship between these two correlates remains poorly defined and is unlikely to provide a comprehensive explanation for the clinical response. There remains the very real possibility that promoter hyper-methylation is merely a biomarker, rather than a direct causative factor in the sensitivity of GBMs to temozolomide. Clinicians need to be wary of seizing upon plausible mechanistic models and giving them undue influence in trial design. For instance, our understanding of why patients deficient in BRCA2 may be sensitive to PARP inhibition continues to evolve (Gottipati et al. 2010). If we anticipate clinical outcomes too stringently, rather than being open to novel interations, trial design risks becoming too restrictive to reveal 'off-target' effects important in determining clinical response. Although over 80% of malignant melanomas contain alteration of the BRAF gene (Platz et al. 2008), there is mounting evidence that co-existing mutations also affect tumour phenotype (Gopal et al. 2010; Sondergaard et al. 2010). A recent study demonstrated that, when combined with the ^{V600E}BRAF mutation, mutation of the PTEN tumour suppressor gene attenuates the survival function of the former. The proposal is that clinical response may be diminished in tumours bearing alteration of both genes (Xing et al. 2011).

Biomarkers with true predictive power would clearly be useful in directing patient therapy. However, when designing early phase clinical trials, we urge caution in placing over-reliance on the value of 'predictive markers' until their role in clinical practise has been validated. Heterogenity of clinical outcome is likely to represent the complex interactions within the spectrum of underlying genetic alterations, and there is much to be learnt before we should permit early phase trials of DNA repair inhibitors to become excessively focussed.

CONCLUSION

In summary, we have aimed to examine some of the critical issues that relate to the design of early phase clinical trials of DNA repair inhibitors. Reviewing the recent literature, certain broad themes are emerging. A general understanding of the mechanisms of action and potential pathways for repair of damage must underlie the trial design involving an anti-cancer DNA damaging agent and/or a relevant DNA repair inhibitor. Pre-clinical trials have often been poor predictors of successful clinical trials, although emerging technologies, such as syngeneic tumour models, may improve

this approach. There remains no consensus on whether a combination regime should aim for maximum cytotoxic therapy or maximum DNA repair inhibition, and hence, it is still ideal to examine both strategies. The tumour biopsy, both before and after treatment, remains pivotal in the interpretation of trial results. Despite attempts to replace them with more readily accessible surrogate tissues or cells (e.g., PBMCs), it is the molecular biology of the biopsies that has provided the most valuable data on *who* is responding and *how* they are responding. Only with a greater emphasis on biopsy-driven study design will we move closer to the ultimate goal of treatment tailored to both the patient and the cancer.

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Color Plate Section

Chapter 1

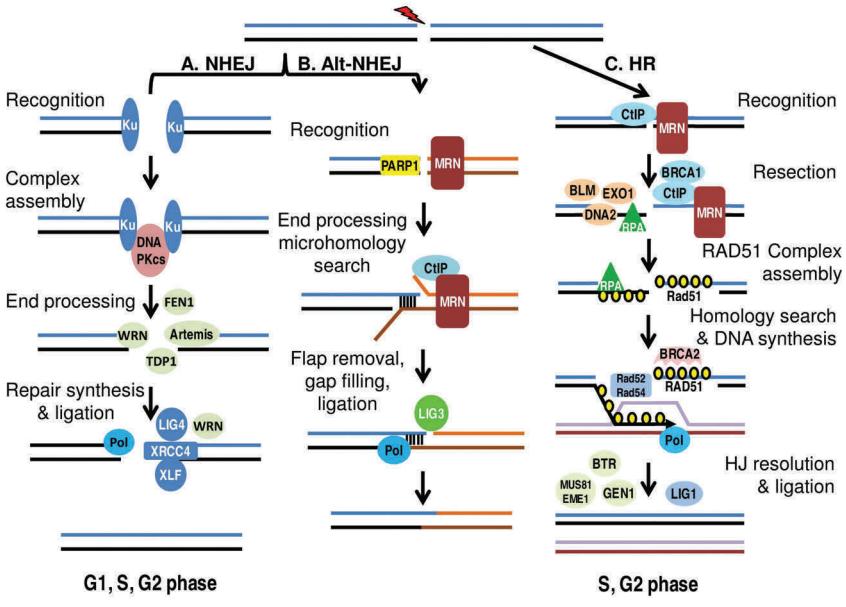

Figure 2. Schematic diagram of double-strand break repair mechanisms. (A) In non-homologous end-joining, NHEJ, double-strand breaks (DSBs) are recognized by the heterodimer Ku70/Ku80 (Ku). These DNA end binding proteins recruit the protein kinase DNA-PKcs, which in turn phosphorylates and recruits other proteins. When the DNA ends are incompatible for ligation, exo- or endonucleases are recruited to modify the ends; shown here are WRN, FEN1, Artemis and TDP1. Next, a DNA polymerase fills in any recessed ends,then LIG4 in complex with XRCC4 and XLF seals the nick. NHEJ is the predominate DSB repair pathway used in human cells and is available in G1, S and G2 of the cell cycle. (B) Alternative NHEJ, Alt-NHEJ, is employed when NHEJ is compromised. DSBs are thought to be recognized by PARP1 and/or the MRN complex. The distinguishing feature of this repair pathway is that end resectioning occurs until short stretches of homology (5–25 nucleotides) are found. The flaps are removed, DNA synthesis fills in missing nucleotides and then Ligase 3 (LIG3) seals the nick. (C) In homologous recombination (HR), the major damage recognition player is the MRN complex. Among other functions, BRCA1 activates the DNA damage response to induce cell cycle arrest following DSB formation. MRN, CtIP, EXO1, DNA2 and BLM may all function to resect the DNA and generate 3′ single-stranded tails. These tails are then bound by RPA and RAD51 filaments. RAD51 recombinase searches for homology within another homologous strand of DNA, preferentially its sister chromatid. RAD52 and RAD54 promote these processes. DNA synthesis copies the DNA off the sister chromatid, then Holliday junctions (HJ) are resolved using proteins like GEN1, Mus81-EME1 or the BTR complex, consisting of BLM, topoisomerase 3α, RMI1 and RMI2. Finally, DNA ligase, LIG1, ligates the DNA ends to restore DNA integrity. HR is only operable during S and G2 phases of the cell cycle.

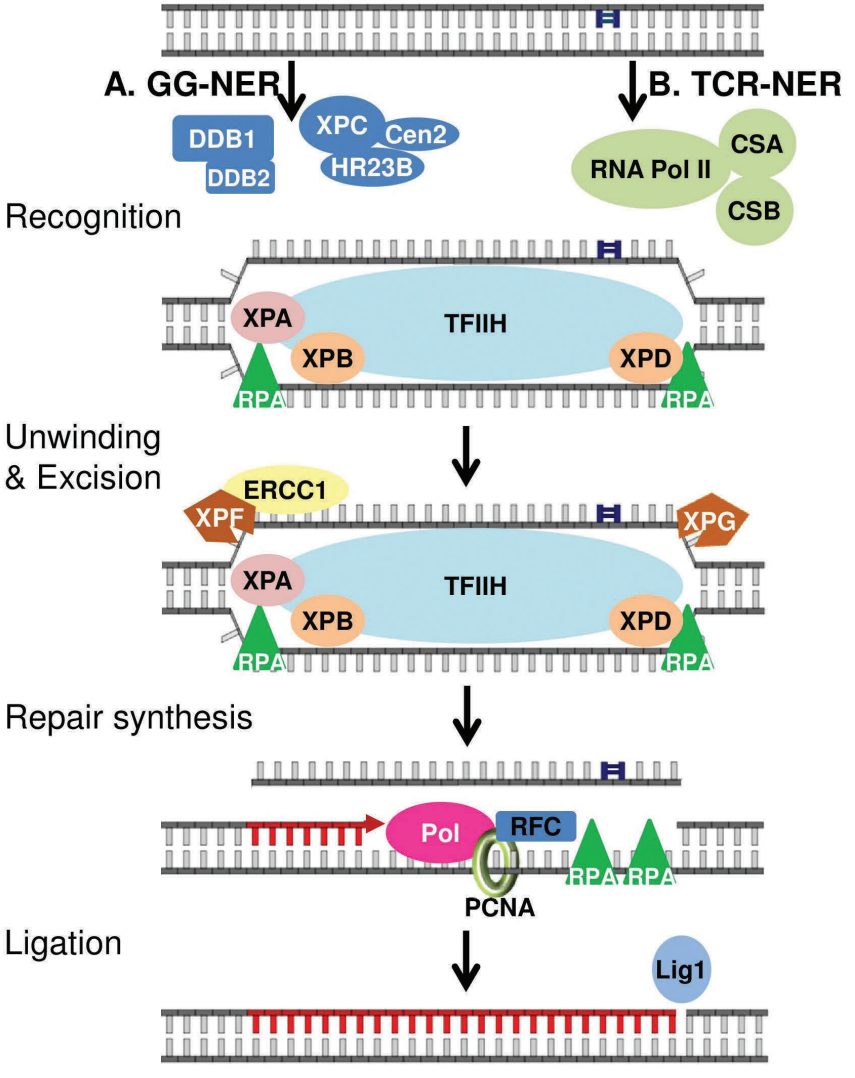

Figure 3. Schematic diagram of nucleotide excision repair. DNA damage recognition for NER is different for repair in the general genome (A. GG-NER) in comparison with actively transcribed genes (B. TCR-NER). Within the general genome pathway (A. GG-NER), XPC/HR23B/CEN or DDB1/2 recognize the damage, whereas for actively transcribed genes (B. TCR-NER), RNA polymerase II, with associated factors like CSA and CSB, is responsible for DNA damage signaling. Following recognition, the pathways converge: the transcription factor TFIIH with its associated helicases XPB and XDP are recruited along with XPA and RPA. ERCC1/XPF and XPG join the complex and are required for the 5′ and 3′ single strand incisions on either side of the damage, respectively. Repair synthesis fills in the ~30 nucleotide gap and LIG1 seals the nick.

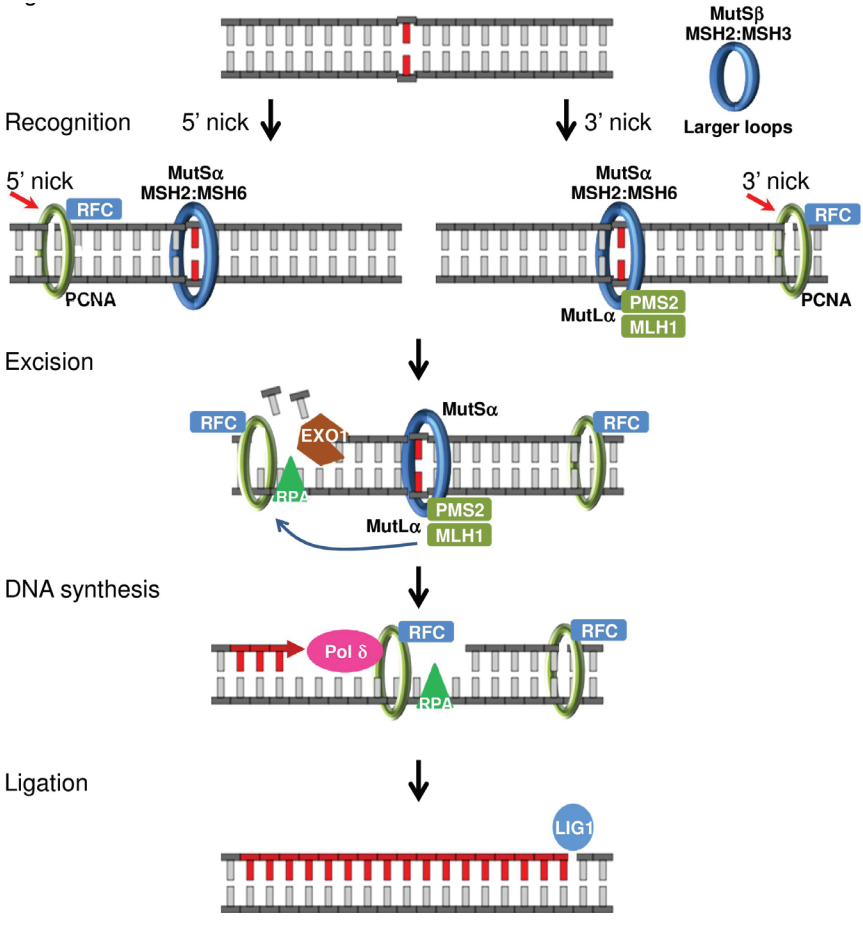

Figure 4. Schematic diagram of mismatch repair. MutSα, a complex of MSH2/MSH6, is thought to recognize base:base mismatches, whereas MutSβ, a complex of MSH2/MSH3, recognizes larger insertion and deletion loops. Once a lesion is found, MMR must determine which strand is the parental strand, and this is done by searching for a nick in one strand. Nicks are common before Okazaki fragment processing following new DNA synthesis and thus allows easy recognition of the lagging, replicating strand. Once a nick is located RFC and PCNA load. If the nick is 5′ to the damage, the exonuclease, EXO1, can directly proceed with resecting the DNA back to the mismatch. If, however, the nick is on the 3′ side of the lesion, then a cryptic endonuclease in MLH1 is activated thus allowing PCNA/RFC/EXO1 loading and exonuclease digestion. Large tracks of DNA >2kb can be excised during MMR, and a replicative DNA polymerase, like pol δ, is required to fill in the gap. LIG1 then seals the nick.

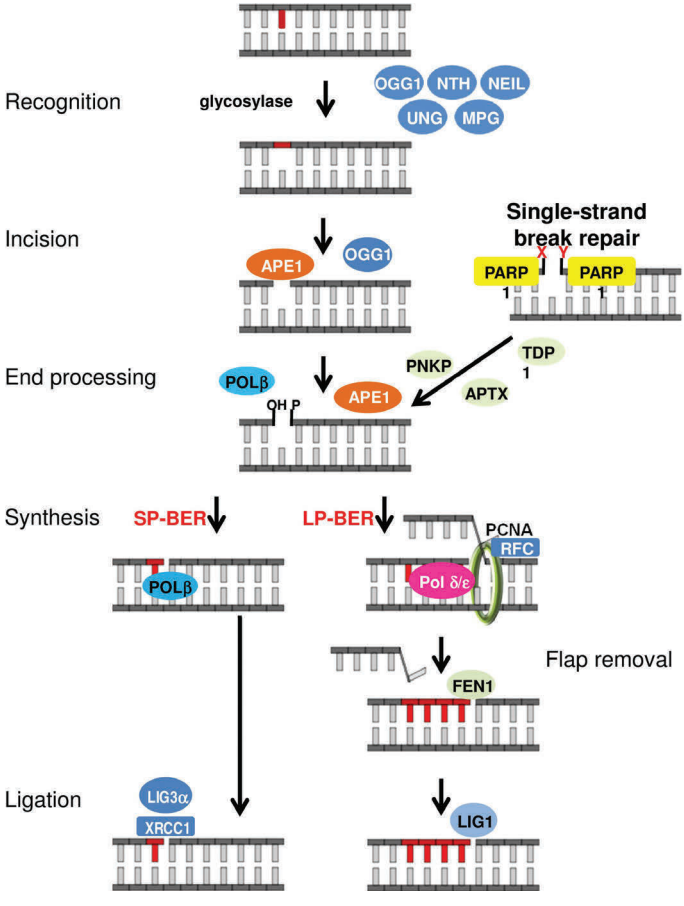

Figure 5. Schematic diagram of base excision repair. Glycosylases are a set of enzymes that remove modified bases from DNA, generating an abasic site. Some glycosylases possess DNA strand cleavage activities, like OGG1, but others do not. The endonuclease, APE1, is required to cleave the DNA backbone at the AP site and to process the ends for gap synthesis and ligation. Additional end processing proteins important at this stage are: polymerase β, (pol β), polynucleotide kinase phosphatase (PNPK), Aprataxin (APTX) and Tyrosyl-DNA phosphodiesterase I (TDP1). Following end processing, the BER pathway splits into either short patch repair (SP-BER) or long patch repair (LP-BER) depending on the number of nucleotides inserted during the repair synthesis step. POLβ does the synthesis for SP-BER and fills in the single nucleotide gap. A replicative polymerase, with the help of PCNA/RFC, typically performs strand displacement synthesis for LP-BER and replaces 2 or more nucleotides. Ligase 3α (LIG3α) and XRCC1 do the ligation for SP-BER and Ligase 1 (LIG1) functions during LP-BER. Single-strand break repair is a related but distinct repair pathway which repairs single-strand breaks with modified 5′ or 3′ ends, denoted by the X and Y in the image. In this pathway, the high affinity single-strand break binding protein poly (ADP-ribose) polymerase I, PARP1, often recognizes the ends and recruits end processing proteins to create synthesis and ligation compatible ends.

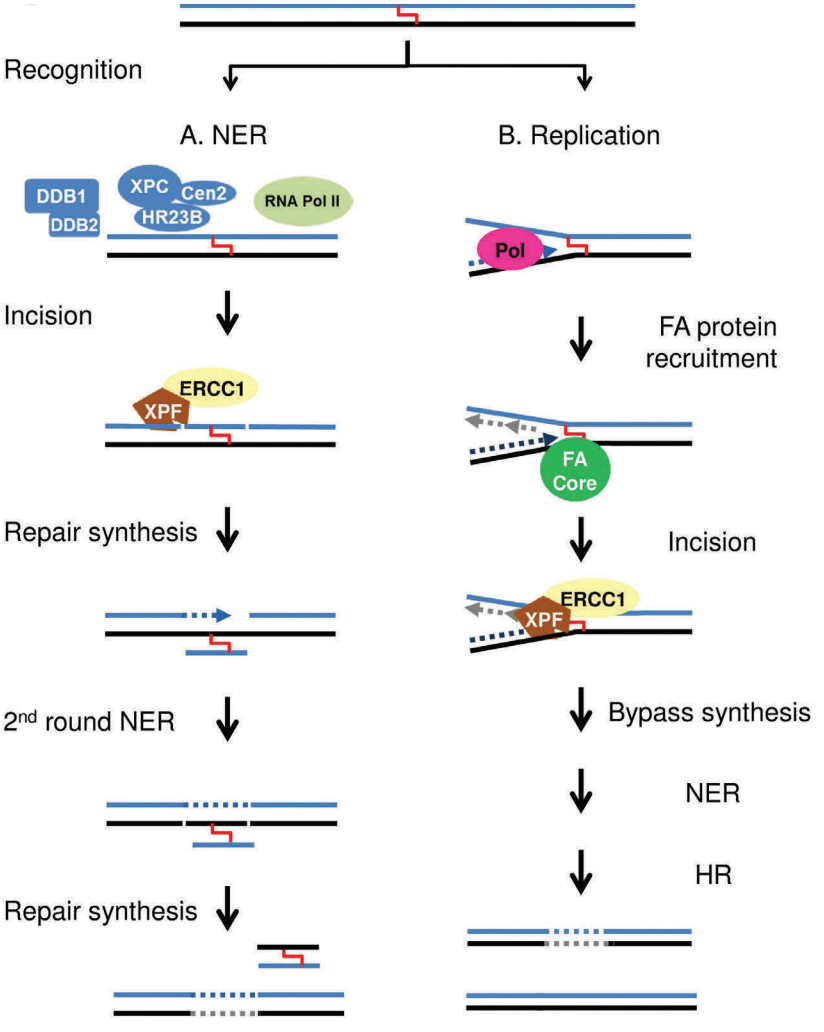

Figure 6. Schematic diagram of interstrand crosslink repair. If damage recognition is mediated by NER components (A. NER), then unhooking of the lesion is done by the NER pathway. This is then thought to be followed by a round of translesion synthesis and another round of NER to remove the short oligonucleotide-attached crosslink, a process commonly seen during the G1 phase of the cell cycle. If the DNA damage is found by the replication apparatus (B. Replication), then the Fanconi anemia (FA) proteins are recruited. This pathway is less well described, but may also involve the structure-specific endonuclease, ERCC1/XPF, to unhook the crosslink. Then, the sequential action of several DNA repair pathways might work together to complete repair. Bypass synthesis, followed by a round of NER, would remove the crosslink from DNA, leaving a gap. Finally, HR between sister chromatids would help to restore the original DNA sequence. Interstrand crosslink repair (ICLR) is still in its infancy relative to the other well characterized repair pathways and much is yet to be learned.

Chapter 2

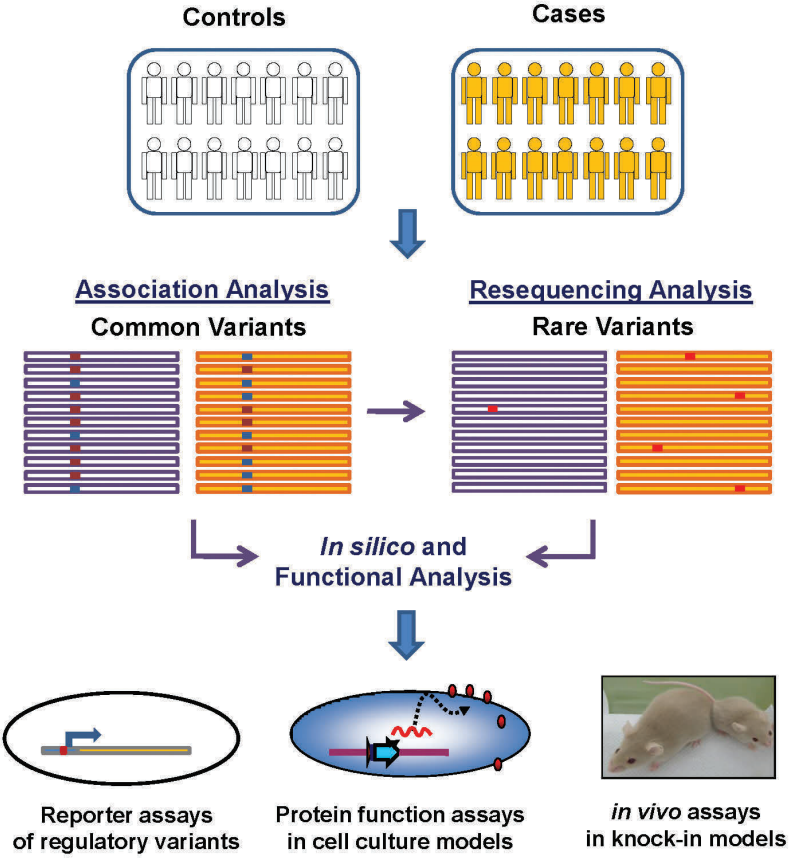

Figure 2. Discovery of functional genome maintenance variants in human longevity. A hierarchical, multidisciplinary approach will increase the chances of identifying functional variations in the genome maintenance genes that influence human longevity. Genetic association in case/control studies establishes genetic link between genome maintenance genes and human longevity. Controls are typically elderly individuals and cases are extremely long-lived individuals, such as centenarians. Genetic association established by common marker variants requires resequencing analysis to identify potentially functional variants, whereas direct resequencing of candidate genes leads to discovery of such variants. Individually rare longevity-associated variants may be enriched in cases as a group as compared to controls. *In silico* analysis predicts the outcomes of potentially functional variants and helps prioritize candidate variants for further functional analysis. Integrated multiple *in vitro* and *in vivo* assays are needed to assess the functional roles of each longevity-associated genome maintenance gene variants depending upon the nature and location of gene variants.

Chapter 4

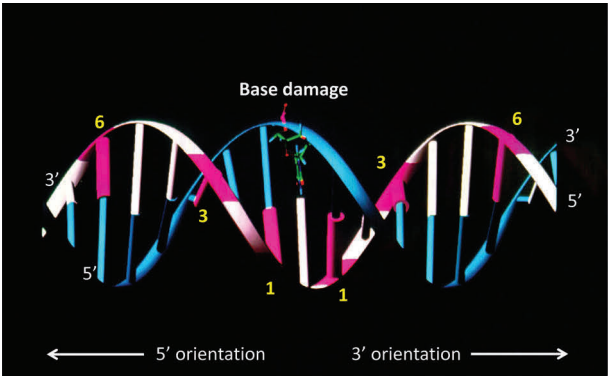

Figure 3. Positioning of damage in synthetic DNA substrates. A clustered lesion is defined as ≥ 2 damages situated within 20 bps or 1 -2 helical turns of the DNA. A base damage is situated on the blue strand. The pink nucleotides on the white DNA strand are situated at position 1, 3 or 6 5′ or 3′ to the base damage. These are the positions where a second damage was placed to study closely opposed DNA lesions in synthetic defined substrates.

Chapter 8

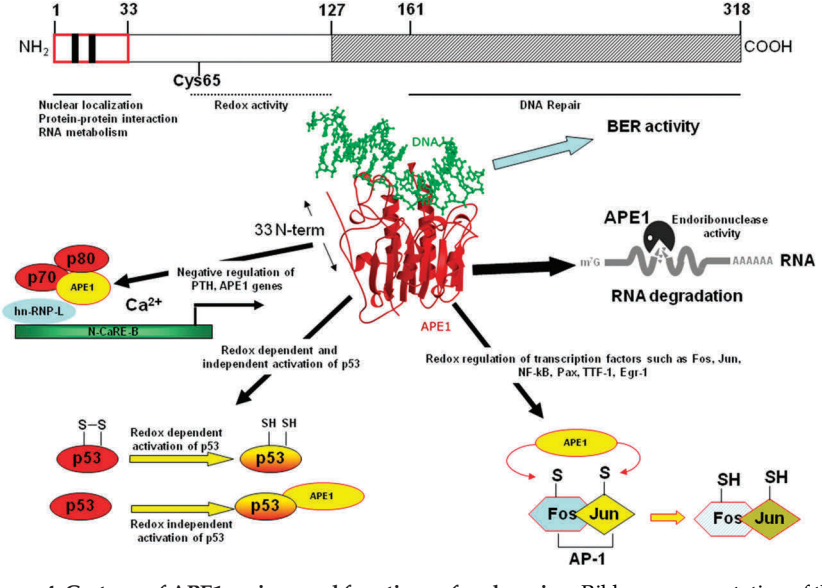

Figure 4. Cartoon of APE1 regions and functions of each region. Ribbon representation of the APE1 structure. The major functions of APE1 are illustrated; BER activity, redox-dependent and independent regulation of transcription factors, transcriptional repression of nCaRE and RNA metabolism (Tell et al. 2009). Reprinted with kind permission from Springer Science+Business Media: *Cell. Mol. Life Sci*. Understanding different functions of mammalian AP endonuclease (APE1) as a promising tool for cancer treatment , 67, 2010, 3589–3608, Tell G, FantiniD, and Quadrifoglio F., Figure 1.

Chapter 12

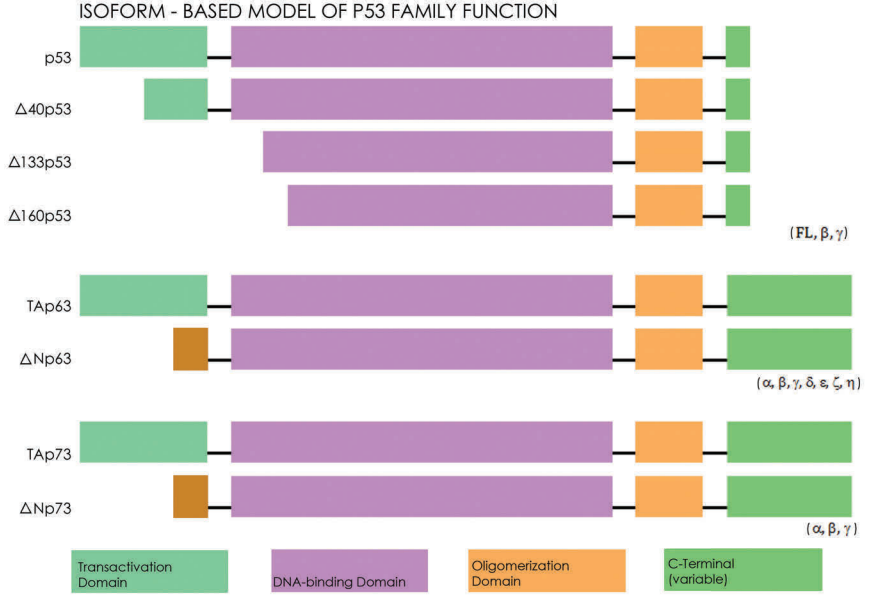

Figure 2. The p53 family of transcription factors.

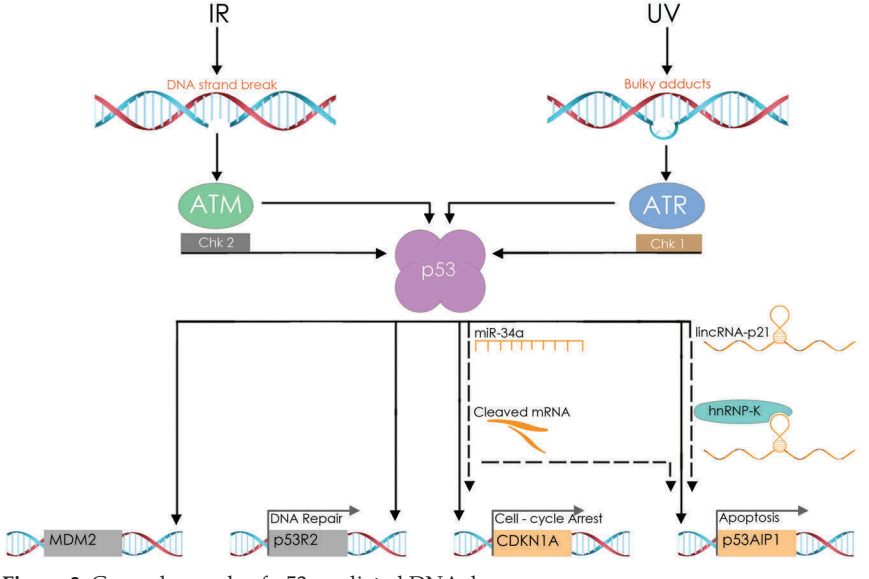

Figure 3. General cascade of p53-mediated DNA damage response.

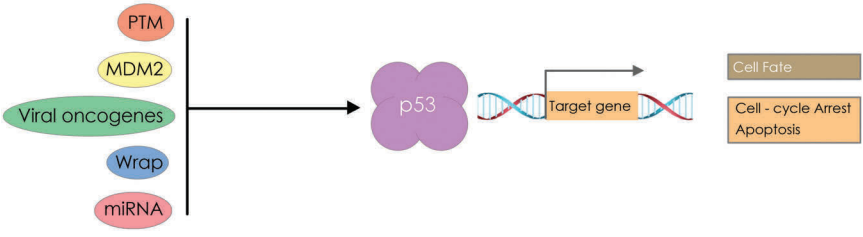

Figure 4. Regulation of p53 via different mechanisms.

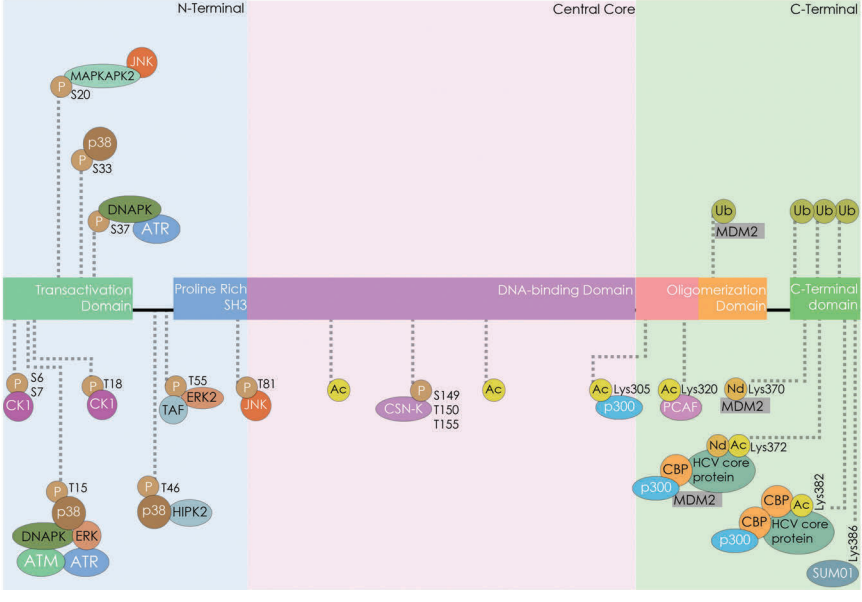

Figure 5. Post-translational modification (PTM) of human p53.

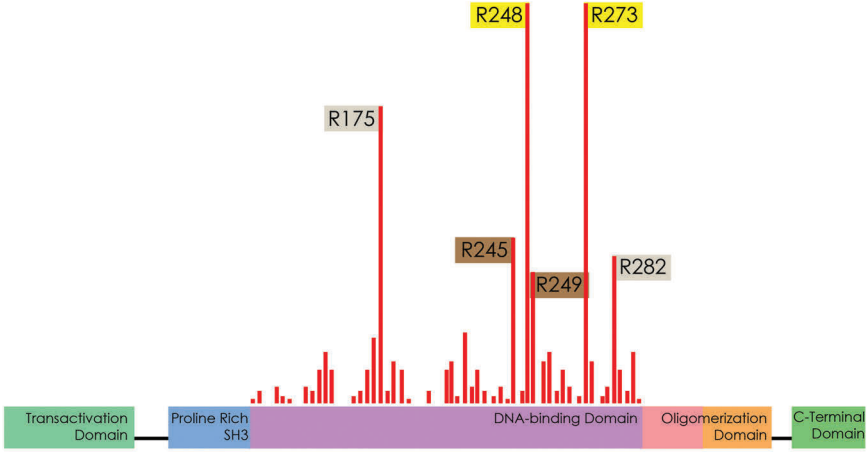

Figure 6. 'Hot-spot' mutations of human p53 commonly observed in cancer.

Chapter 14

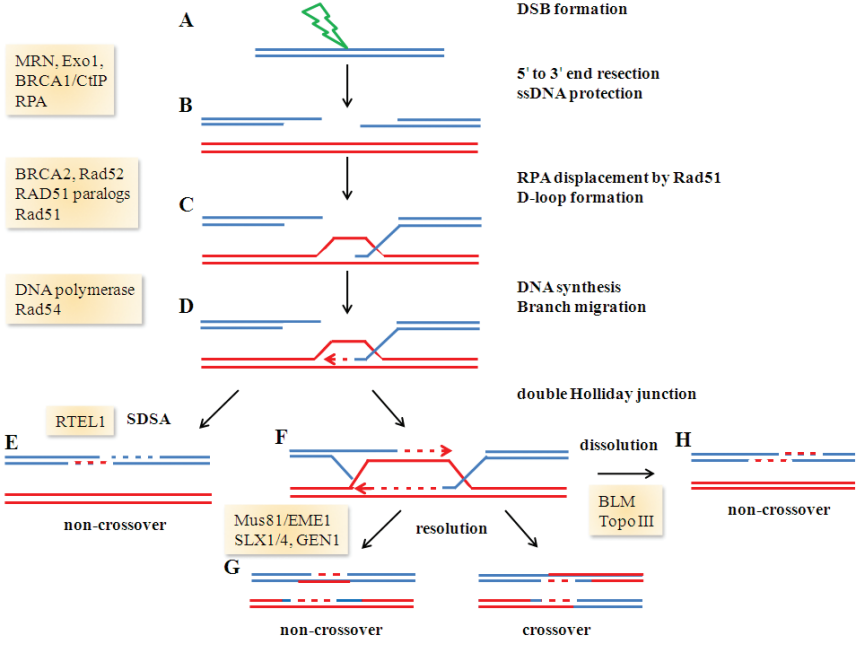

Figure 1. Mechanism of homologous recombination. Following DSB formation (A), 3′ ssDNA overhangs are created and protected (B). Rad51 filaments invade the homologous duplex forming a D-loop (C), allowing DNA synthesis to occur (D). HR can then proceed via SDSA (E) to produce non-crossover products. Alternatively, a double Holliday junction (F) may form which can be dissolved (H), also forming non-crossovers, or this structure may be resolved (G) giving either crossover or non-crossover products.

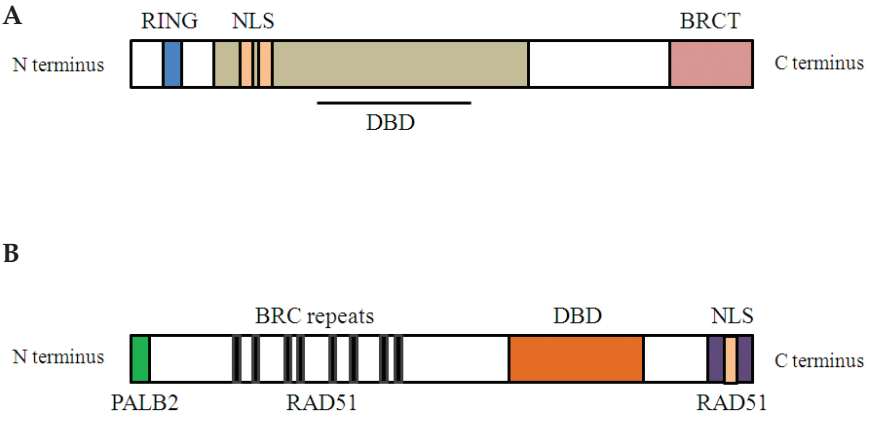

Figure 2. Schematic of BRCA1 and BRCA2 functional domains. (A) BRCA1. The BRCA1 N-terminus contains a RING domain that associates with BARD1 and a nuclear localization sequence (NLS). The central region of BRCA1 contains a DNA binding domain (DBD). The C- terminus of BRCA1 contains BRCT domains. (B) BRCA2. The N-terminus of BRCA2 binds PALB2. BRCA2 contains 8 BRC repeats that bind Rad51. The BRCA2 DBD contains a Helical domain, 3 OB folds and a Tower domain which facilitates BRCA2 binding to ssDNA, dsDNA and the DSS1 protein. The C-terminus of BRCA2 also binds Rad51.

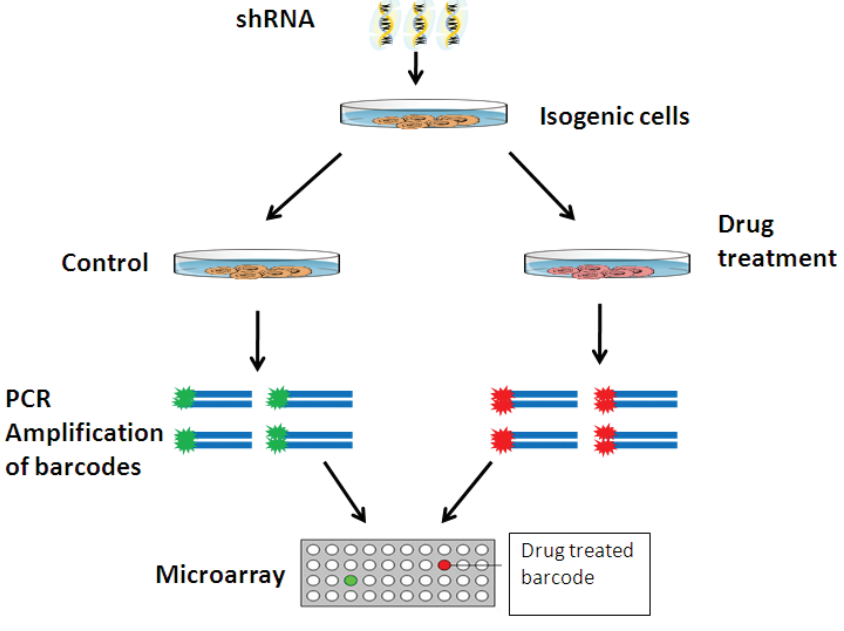

Figure 5. shRNA bar-coding screen. Plasmids encoding shRNA and barcode sequences are transfected into isogenic cells. Each cell type is then sub-divided and one is treated with a specific drug, the other is a control. When gene-specific lethality is observed, DNA is extracted from the untreated sample and the barcode is recovered through PCR amplification. The abundance of barcodes is determined through microarray hybridization and shRNA in groups is identified.

Chapter 17

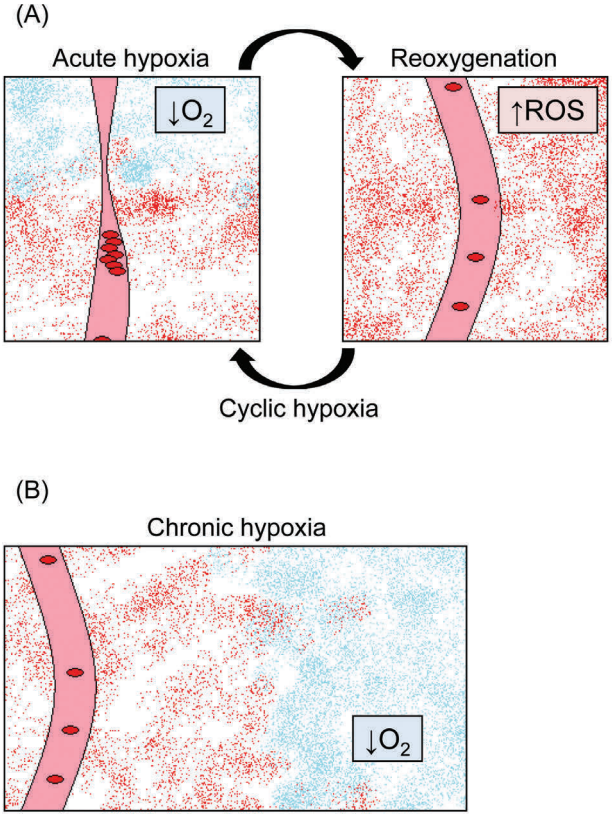

Figure 1. Regions of acute and chronic hypoxia can develop within solid tumors. A) Acute hypoxia occurs as a result of sudden and temporary changes in blood flow (e.g., collapsed blood vessels). Subsequent reoxygenation leads to cyclic hypoxia and the generation of reactive oxygen species (ROS). B) Solid tumors can often have uneven distribution of blood vessels leading to chronic or diffusion-limited hypoxia in cells >150 μm from blood vessels near the diffusion limit of oxygen.

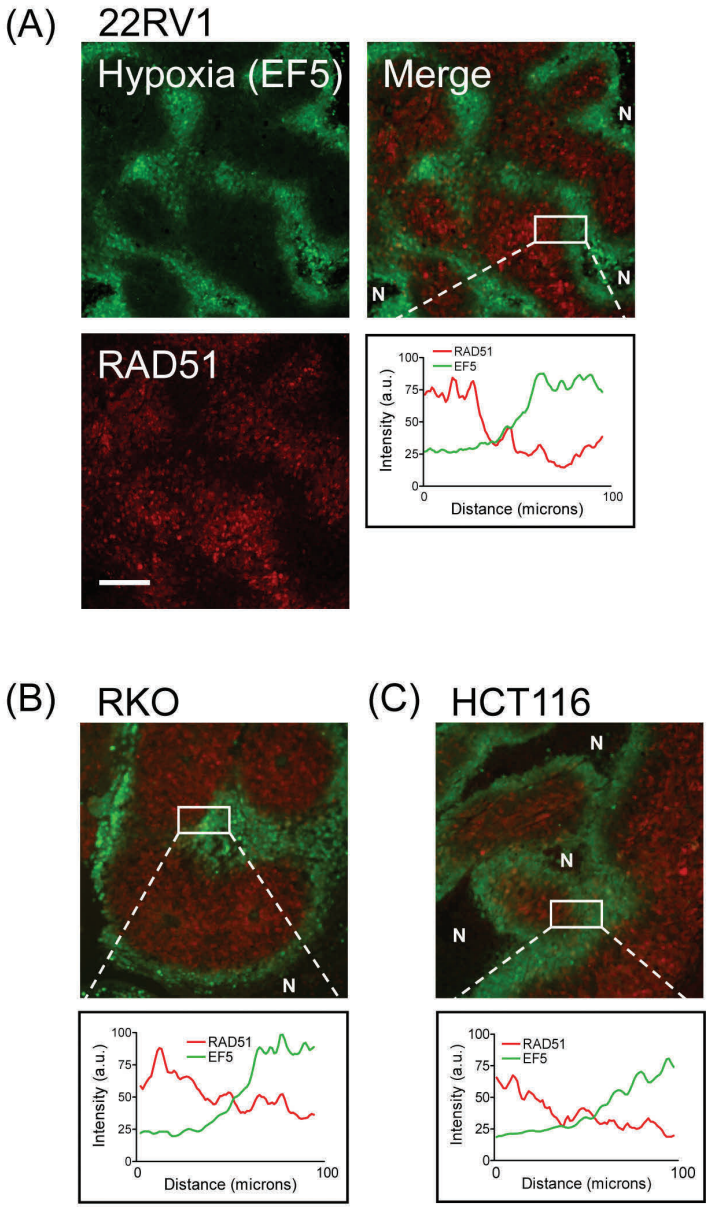

Figure 3. Hypoxia and RAD51 protein expression is inversely correlated *in vivo*. Representative images from (A) 22RV1, (B) RKO and (C) HCT116 xenografts stained for hypoxia (EF5 - green) and RAD51 (red). Line intensity profile shows inverse association between the hypoxic marker EF5 and the HR protein RAD51. Scale bar represents 100 microns. N denotes necrotic regions.

DNA Repair is a rapidly advancing field in biology. Dr. Madhusudan and Dr. Wilson have brought together renowned experts to produce this exciting volume focused on DNA Repair and its relationship to cancer. DNA repair systems represent a major defense mechanism against DNA damaging environmental and intracellular damaging agents, such as sunlight, ionizing radiation and reactive oxygen species. Defects in DNA repair may predispose to cancer and likely influence the aging process. Since many approaches to eradicate cancer involve direct DNA-damaging agents, such as ionizing radiation and a battery of chemotherapeutics, strategic targeting of DNA repair mechanisms in cancer cells has emerged as an attractive approach to improve the efficacy of current treatment paradigms. The book provides a comprehensive overview of biochemical, pre-clinical and clinical aspects of the six major DNA Repair pathways operating in Man. In eighteen chapters, internationally recognized research scientists and clinical investigators review in detail (i) the molecular mechanisms of the various DNA repair systems, as well as the complementary cell cycle control components that prevent unmonitored cell growth, (ii) the role of DNA repair and DNA damage responses in cancer susceptibility and their relationship to aging, (iii) commonly used strategies to eliminate cancer cells, namely the employment of DNA-damaging agents, (iv) the concept of targeting DNA repair systems either through inhibition or the phenomenon of “synthetic lethality” to selectively kill cancer cells, (v) progress towards individualized medicine in the treatment of cancer, and (vi) the current picture of clinical efforts that engage DNA repair targeting strategies. 'DNA Repair and Cancer: From Bench to Clinic' will provide essential information to scientists, pharmaceutical investigators and clinicians interested in cancer therapy.

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